Cysteine Dependence of Insulin-like Growth Factor Effects on Redox and Methylation Status

Thesis Presented

by

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to

The Bouvé Graduate School of Health Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmaceutical Sciences with Specialization in Pharmacology

NORTHEASTERN UNIVERSITY

BOSTON, MASSACHUSETTS

August 20th, 2012
Northeastern University

The Graduate School of Bouvé College of Health Sciences

Thesis title: Cysteine Dependence of Insulin-like Growth Factor Effects on Redox and Methylation Status

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Program: Master of Science in Pharmaceutical Sciences with a Specialization in Pharmacology

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List of Abbreviations

AD: Alzheimer’s disease
AdoCbl: adenosylcobalamin
BSO: buthionine sulfoximine
Cbl: cobalamin
Cbl(I): cob(I)alamin
Cbl(II): cob(II)alamin
CBS: cystathionine β-synthase
Cys: cysteine
CGL: cystathionine γ-lyase
CNCbl: cyanocobalamin
CSF: cerebrospinal fluid
EAAT: excitatory amino acid transporter
FBS: fetal bovine serum
GCS: γ-glutamylcysteine synthetase
GSCbl: glutathionylcobalamin
GSH: reduced glutathione
GSSG: oxidized glutathione
GR: glutathione reductase
HBSS: Hank’s balanced salt solution
HCY: homocysteine
IGF-1: insulin-like growth factor-1
Km: Michaelis constant
MAPK: mitogen-activated protein kinase
MeCbl: methylcob(III)alamin
MethylTHF: 5-methyltetrahydrofolate
MS: methionine synthase
NAC: N-acetyl-cysteine
OHCbl: hydroxyocobalamin
PDK1: Phospholipid-dependent kinase 1
ROS: reactive oxygen species
SAM: S-adenosylmethionine
SAH: S-adenosylhomocysteine
SO$_3$Cbl: sulfitocobalamin
SOD: superoxide dismutase
Acknowledgement

First, I would like to thank my advisor, Dr. Deth for assigning me this wonderful project which gives me great opportunities to learn how these small molecules lead to big outcomes. His enthusiasm for science always inspires me in my own academic research. I could not have finished this thesis without his immeasurable guidance and support.

I am sincerely grateful to the rest of my thesis committee members, Dr. Loring, Dr. Gatley and Dr. Tucker for their tremendous efforts to improve my thesis research.

I would like to thank Dr. Gatley for offering me precious opportunities which have amazing impacts on my academic and career development.

I would like to thank Nate for teaching me various techniques from cell culture to HPLC operation, for his great patience in explaining science, for constantly providing encouragement and for revising my thesis many times until the end. If write down all great help Nate has given to me, the length of my thesis will be doubled.

I would like to thank Malav and Jayni for their warm friendship.

I would like to specially thank Yifei, for his endless love and support. Thanks him for taking good care of me and encouraging me to be a better me.
Abstract

Methionine synthase serves as a bridge between the methionine cycle and the dopamine-stimulated phospholipid methylation cycle. Cobalamin is the co-factor of methionine synthase to catalyze the conversion of homocysteine to methionine. Oxidized cobalamin halts methionine synthase activity, increasing homocysteine diversion to the transsulfuration pathway leading to formation of cysteine which can then be converted to the primary intracellular antioxidant glutathione.

Methionine synthase is a multi-domain enzyme and its linker domain termed ‘cap’ is responsible for protecting cobalamin from dissociation and oxidation. In human brain, the cap domain of methionine synthase can be alternatively spliced in response to oxidative stress. Spliced methionine synthase requires GSCbl to produce MeCbl and reactivate the enzyme. OHCbl can be rapidly and irreversibly converted to GSCbl in the presence of glutathione. The human brain is especially vulnerable to oxidative stress. Neurodegenerative diseases are associated with cobalamin deficiency, especially MeCbl and AdoCbl which are cofactors directly utilized by cellular enzymes.

Cysteine is the rate-limiting precursor for GSH synthesis. In neurons, activity of the transsulfuration pathway is limited, and extracellular uptake upon cysteine transporter EAAT3 is the predominant way for neuronal cysteine uptake. Insulin growth factor IGF-1 increases cellular surface expression of EAAT3 by activating the PI3 kinase pathway, which consequently stimulates cellular cysteine uptake.

Using SH-SY5Y cells as a model for mature neurons, my studies show that GSCbl is one of the most abundant intracellular cobalamin derivatives in SH-SY5Y cells.
IGF-1 shifts the cellular environment to a more reduced state mainly by increasing intracellular levels of GSH and GSCbl. Intracellular levels of AdoCbl, MeCbl and GSCbl increase or decrease synchronously, proving that GSCbl is the precursor of AdoCbl and MeCbl synthesis. These findings may contribute to treatment of neurodegenerative diseases which are closely related to oxidative stress and cobalamin deficiency.

In addition, using serum-free media with low cysteine level for neuroblastoma cell culture, I showed that cysteine level in cell culture media affects intracellular cobalamin and thiol status. Therefore, optimization of cell culture conditions to better mimic CSF in the brain may bring great advantages for in vitro neuronal research.

Overall, this thesis improves our understanding of mechanisms by which neuronal cells defend against oxidative stress and by which IGF-1 regulates redox and thiol status. This improved understanding may be beneficial for both neurodegenerative diseases treatment and in vitro neuronal research.
Introduction

A. Statement of the Problem

This thesis investigates how IGF-1 regulates cellular cobalamin (Cbl) composition and thiol status and how this affects the redox and methylation status in SH-SY5Y neuroblastoma cells.

Insulin-like growth factor 1 (IGF-1) belongs to the insulin-like growth factor family and it has broad effects on cellular growth and development. Its role in brain development has been well examined (Ye et al., 2002). Research in our lab shows that IGF-1 increases cysteine uptake in SH-SY5Y cells by activating the PI-3 kinase signaling pathway and promoting EAAT3 cellular expression. As cysteine is the rate-limiting precursor of glutathione (GSH) synthesis and GSH is a thiol antioxidant and the primary determinant for cellular redox status, IGF-1 may influence cellular redox status.

Studies have shown that IGF-1 is capable of activating methionine synthase (MS) (Waly et al., 2004). MS can convert homocysteine (HCY) to methionine, which is further converted to the universal methyl donor S-adenosylmethionine (SAM). By this way, IGF-1 may potentially influence cellular methylation status. Cbl is known as vitamin B12. Its reduced form cob(I)alamin (Cbl(I)) serves as a sensor to cellular redox status by regulating MS activity. In addition, many Cbl derivatives play roles in regulating both cellular redox and methylation status.

As cellular redox and methylation status both depend upon the network of all these molecules, effects of IGF-1 on Cbl and thiol content will be investigated, with the
goal of revealing potential mechanisms employed by IGF-1 to affect cellular redox and methylation status.

B. Production of Reactive Oxygen Species

Life on earth has evolved into two major categories of life forms: photosynthetic plants that trap solar energy to drive thermodynamically unfavorable reactions to produce reduced carbonaceous compounds, and the rest of us, who consume these compounds and utilize energy released in thermodynamically favorable reactions. For humans and other aerobic organisms, the latter reactions are accompanied by returning electrons to the molecule with particularly high electron affinity, molecular oxygen (McCord, 2000). Electrons in most molecules associate as pairs with anti-parallel spins. Oxygen itself, however, contains two unpaired electrons and each resides in a different π* anti-bonding orbital. This unconventional distribution of electrons requires that molecular oxygen be reduced by accepting electrons from other molecules one at a time (Halliwell and Gutteridge, 1984). This single electron transfer is slow and results in the production of chemically reactive oxygen-containing molecules, or reactive oxygen species (ROS). Primary ROS molecules include superoxide, hydrogen peroxide, hydroxyl radical and nitric oxide (Nordberg and Arnér, 2001). The mitochondrial respiratory chain is the major source of intracellular ROS generation. Approximately, 1% to 2% of total electron flux through the chain is leaked to oxygen, producing primarily superoxide (McCord, 2000; Turrens, 2003).
C. Oxidative Stress and Antioxidant Defenses

ROS play important roles in the regulation of intracellular signaling and the maintenance of cellular homeostasis. ROS are produced during normal oxidative metabolism, as part of the immune response to infection, and by exposure to toxic substances or heavy metals (Carpenter et al., 2002). The term “oxidative stress” describes the cumulative production of ROS via either exogenous or endogenous insults (Valko et al., 2006). Oxidative stress may cause cellular damage including lipid peroxidation, carbohydrate chain breaks, DNA mutation, and protein dysfunction (Devasagayam et al., 2004). Oxidative stress affects all human cells and the particular nature of the cell determines the specific type of damage and loss of cellular function that will ensue. For neurons particularly, oxidative stress can lead to a decrease in cognitive abilities such as memory, learning and attention (Dröge and Schipper, 2007).

Antioxidants with the ability to neutralize oxidative effects are necessary to counterbalance these oxidative risks. Normally cells defend themselves against oxidative damage via several strategies. The first level of defense is to stop ROS formation, by means such as metal chelation, which can restrict DNA fragmentation and lipid peroxidation through the binding of metal ions. Interception is the second level of defense, which not only deactivates the damaging species but also transfers them from more deleterious target sites to less sensitive compartments of the cells. If damage is sustained despite the cell’s defense mechanisms, the cell may recruit multiple enzymes to initiate repair mechanism (Sies, 1997). In addition to these mechanisms, cells can restrict ROS formation by restricting electron transport through the respiratory chain. This can be
accomplished, for example, by glutathionylation of Complex I, which is accompanied by a decrease in aerobic production of ATP (Hurd et al., 2008).

D. Oxidative Stress and Antioxidants in the Human Brain

All aerobic organisms are subject to oxidative stress, but the human brain is said to be especially vulnerable (Halliwell, 1992). The adult human brain accounts for only a few percent of the body’s mass, but about 20% of the basal oxygen consumption (Dringen, 2000). High oxygen consumption increases the probability of ROS formation. Many brain areas such as caudate nucleus, putamen, globus pallidus and substantia nigra have high amounts of iron, which can catalyze free radical reactions to convert less reactive to more reactive species (Halliwell, 2006). Neuronal membrane lipids are rich in unsaturated fatty acid side-chains, especially DHA residues which are primary targets for lipid peroxidation capable of damaging brain (Halliwell and Gutteridge, 1997).

Antioxidant defenses in the brain can be both enzymatic and non-enzymatic. Non-enzymatic antioxidants include ascorbate (vitamin C), $\alpha$-tocopherol (vitamin E) and coenzyme Q. Superoxide dismutases which are present in all parts of the nervous system can remove superoxide radicals, one radical is oxidized to molecular oxygen and the other reduced to hydrogen peroxide (Halliwell, 2001; Liochev and Fridovich, 2005). The hydrogen peroxide can be further eliminated by glutathione peroxidases (GPx), a family of selenium-containing enzymes. GPx reduces hydrogen peroxide to water utilizing GSH (Brigelius-Flohé, 1999).

$$\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$$

GSSG is reduced back to GSH by glutathione reductase (GR) (Fig 1).
E. Glutathione

GSH, a tripeptide composed of cysteine, glutamate and glycine, is the most abundant thiol present in human cells with intracellular concentrations reaching up to 10 mM (Meister, 1988). In eukaryotic cells, about 90% of cellular GSH is stored in the cytoplasm and the remaining 10% is contained in the mitochondria and endoplasmic reticulum (Meredith and Reed, 1982; Macsween, 1989; Hwang et al., 1992). GSH has many vital functions, including detoxification of xenobiotics, maintenance of essential thiol status, acting as a reservoir of cysteine and a modulator of critical cellular processes such as DNA synthesis and immune function (Meister, 1988; DeLeve and Kaplowitz, 1991; Hutter et al., 1997). The predominant role of GSH, however, is as a cellular antioxidant. GSH is mainly present in its reduced form but in the event of oxidative stress it is oxidized to GSSG, a molecule containing two glutathione molecules linked by a disulfide bond. Accumulated GSSG is reduced back to GSH by GR using NADPH as an electron donor, or exported out of the cell (Fig 1). The GSSG/2GSH couple serves as an important indicator of cellular redox environment (Lu, 1999).
Figure 1: Antioxidant function of GSH. In the cytoplasm GSH peroxidase removes H$_2$O$_2$ by coupling its reduction to H$_2$O with the oxidation of GSH. GSSG is reduced back to GSH by GR at the expense of NADPH. GSSG can also react with a protein sulfhydryl (PSH) or be exported out of the cell. Organic peroxides can be reduced by either GSH peroxidases or GSH S-transferase (Lu, 1999).

F. **Glutathione Synthesis**

Synthesis of GSH from precursor amino acids occurs in all mammalian cells. The liver is the major organ for GSH synthesis and export into the plasma (Bray and Taylor, 1993). GSH synthesis involves two ATP-dependent enzymatic steps:

\[
\text{glutamate + cysteine + ATP} \xrightarrow{\gamma\text{-glutamyl cysteine synthetase}} \gamma\text{-glutamyl-L-cysteine + ADP + Pi} \\
\gamma\text{-glutamyl-L-cysteine + glycine + ATP} \xrightarrow{\text{glutathione synthetase}} \text{GSH + ADP + Pi}
\]

The first step of GSH synthesis is rate-limiting and catalyzed by the enzyme $\gamma$-glutamyl cysteine synthetase (GCS), which is subject to feedback competitive inhibition by GSH. In human cells, the apparent $K_m$ values of GCS for its substrates glutamate and
cysteine are 1.8 and 0.1 mM respectively (Misra and Griffith, 1998). In healthy cells, the concentration of glutamate is normally many fold higher than its Km value 1.8 mM while the cysteine level is lower than 0.1 mM (Lu, 1999). Therefore, the intracellular cysteine availability is the critical rate-limiting factor for GSH synthesis.

G. Cysteine Uptake

Cysteine contains a thiol side chain which contributes to its nucleophilic properties and its' ability to undergo redox reactions. Extracellular uptake is the major way for cells to acquire cysteine. Many types of cells including astrocytes are also able to take up cystine, the oxidized dithiol derivative of cysteine, through system $X_c^-$. System $X_c^-$ is a Na$^+$-independent cystine/glutamate antiporter and is able to transport cystine into cells in a 1:1 exchange with glutamate (Maher et al., 2008). Once cystine enters the cell, it is rapidly reduced to cysteine. However, system $X_c^-$ doesn’t play a role in neuronal cysteine uptake so cysteine sources in neurons are highly dependent on extracellular uptake of cysteine or cysteine precursors such as CysGly and N-acetylcysteine (NAC) (Dringen, 2000).

Uptake of extracellular cysteine in cells is accomplished by specific transport proteins, including system $X_{A,G}^-$ (the EAAT family of transporters), system ASC, system L and system A (Shanker and Aschner, 2001). The contributions of these uptake systems vary among different cell types. For neurons particularly, EAATs (Excitatory Amino Acid Transporters) play major roles and up to 90% of the total neuronal cysteine uptake is mediated by the EAAT family of proteins (Shanker et al., 2001). EAATs are Na$^+$-dependent transporters and are named for their abilities to transport glutamate and aspartate (Shigeri et al., 2004). The human EAAT family is composed of five subtypes,
which are the glutamate aspartate transporter (also known as GLAST or EAAT1), glutamate transporter-1 (also known as GLT-1 or EAAT2), excitatory amino acid carrier 1 (also known as EAAC1 or EAAT3), EAAT4 and EAAT5. EAAT1 and EAAT2 are predominantly localized on glial cells. EAAT4 and EAAT5 are expressed on Purkinje cells and photoreceptor cells respectively (Amara and Fontana, 2002). Only EAAT3 is capable of importing cysteine into mature human neurons (Waly et al., 2012).

H. IGF-1 Regulation of EAAT3

EAAT3 is a transporter of glutamate, aspartate and cysteine present at the postsynaptic neurons. During human fetal brain development EAAT3 is expressed earlier than other EAAT transporters, indicating its critical role in the developmental effects of excitatory amino acids (Sims and Robinson, 1999). Although in the adult brain it is expressed at a relatively low level and makes only a minor contribution to glutamate transport, EAAT3 plays an important role in neuroprotective mechanisms against oxidative damage by importing the GSH precursor cysteine into neurons (Nieoullon et al., 2006). Unlike other glutamate transporters present only at the synapse, EAAT3 is diffusely distributed across the entire neuronal membrane. Under basal conditions, approximately 20% of EAAT3 is localized at the cell surface while the majority is located in cytoplasmic vesicles (Conti et al., 1998; Kugler and Schmitt, 1999; Yang and Kilberg, 2002). Translocation of EAAT3 from the intracytoplasmic pool to cellular surface can be triggered through activation of at least three different signaling mechanisms: Akt/PI3K activation, PKC activation and MAP kinase activation (Himi et al., 2003; Sheldon et al., 2006; Watabe et al., 2008).
IGF-1, a polypeptide hormone, preferably binds to the IGF-1 receptor (IGF-1R) which is a tyrosine kinase mediated signaling by transferring a phosphate group from ATP to protein tyrosine residues, and is one of the most potent activators of the Akt/PI3K signaling pathway (Marín-García, 2010). Once IGF-1 binds to IGF-1R, tyrosine residues present in the intracellular domain of the receptor are phosphorylated. The phosphatidylinositol 3-kinase (PI3K) is activated by complexing with phosphorylated residues, and PI3K then converts PIP2 to PIP3. PIP3 binds to phosphoinositide dependent protein kinase 1 (PDK1), followed by the phosphorylation and activation of Akt (Góra-Kupilas and Jośko, 2005; Rexhepaj et al., 2007; Nguyen et al., 2010). As a result of Akt activation, cellular surface expression of EAAT3 is increased (Nieoullon et al., 2006) (Fig 2).

Figure 2: Proposed mechanism for NTGF regulation of EAAT3 and redox in neuronal cells. Binding of neurotrophic growth factors to receptor tyrosine kinases causes activation of PI3 kinase. PI3 kinase phosphorylates PIP2 to PIP3, an activator of PDK1. PDK1 phosphorylates Akt. Akt activation leads to increased surface expression of EAAT3, and increased cysteine uptake. Increased intracellular cysteine leads to increased GSH synthesis and a shift in the redox state to a more reducing potential (N. Hodgson, unpublished results).
I. Transsulfuration Pathway

In response to oxidative stress, neurons can increase the conversion of intracellular HCY to cysteine to augment its availability for GSH synthesis. This serves to augment EAAT3-mediated cysteine uptake. The thiol amino acid HCY is formed by the methionine cycle and is first converted to cystathionine and then to cysteine through a metabolic pathway known as the transsulfuration pathway (Fig 3). Two pyridoxal 5’-phosphate (PLP)-dependent enzymes are involved, which are cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). CBS first catalyzes the condensation of HCY and serine to form cystathionine in an irreversible manner, determining the unidirectional flow in the transsulfuration sequence (Finkelstein, 1998). Cystathionine is subsequently hydrolyzed by CGL, giving rise to α-ketobutyrate, ammonia and cysteine (Stipanuk, 2004).
Figure 3: Redox and methylation pathways in neurons. Red text highlights the transsulfuration pathway, ending with the formation of GSH. Blue text features dopamine-stimulated phospholipid methylation, utilizing methionine synthase. The orange text shows cysteine uptake into neurons via the EAAT3 transporter. Regeneration of methylcobalamin through the addition of SAM and glutathionylcobalamin is shown in green.

CBS is a tetrameric enzyme with a redox sensitive heme group. The oxidation of the iron in the heme group from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state is associated with higher CBS activity. Interestingly, enzymatic activity is negatively regulated by its carboxy-terminal domain and this inhibition can be relieved by the binding of the methyl donor SAM. This indicates that the transsulfuration pathway is normally inhibited unless the intracellular level of SAM is high enough (Banerjee et al., 2003). Low expression of CGL in neurons and glial cells suggests that the transsulfuration pathway is not as active in the central nervous system as in peripheral tissues (Spector et al., 1980; Mattson and Shea, 2003; Vitvitsky et al., 2006). Restriction of the transsulfuration pathway may be a potential mechanism to keep neurons in a moderate level of oxidative stress to restraining neuronal division and avoiding loss of saved information content (i.e. synaptic
connectivity) (Jellinger, 2010). On the other hand, activity of CGL in the liver is more than 100-fold higher than it in the brain, consistent with the fact that liver is one of the organs with highest content of GSH (Finkelstein, 1990).

J. Methionine Cycle

Besides being converted to cysteine through the transsulfuration pathway, HCY can receive a methyl group from 5-methyltetrahydrofolate (methylTHF) to form methionine, a sulfur-containing essential amino acid. Methionine is continuously converted to SAM in the presence of ATP. SAM is a universal methyl donor to some 209 methyltransferases in the human, and is essential for DNA methylation, a crucial regulator of gene expression. Thus the cellular availability of SAM is a regulator of many methylation reactions (Martin and Zhang, 2007). S-adenosylhomocysteine (SAH), a product of methylation reactions, can be hydrolyzed back to HCY (Fig 3). Since conversion between SAH and HCY is reversible, inhibition of MS activity makes more HCY available for SAH formation. SAH is a potent inhibitor of all methylation reactions because of its particularly high affinity for methyltransferases. As such, MS activity exerts very broad influence over methylation levels through its influence on the methionine cycle (Bandarian et al., 2002).

K. Methionine Synthase

Methionine synthase serves as a bridge between the methionine cycle and the dopamine-stimulated phospholipid methylation cycle. It is the only enzyme that catalyzes the liberation of tetrahydrofolate (THF) from 5-methyltetrahydrofolate (methylTHF), which is a critical metabolite for protein and nucleic acid biosynthesis (Wolthers et al., 2007). The modular organization of MS has allowed dissection of this large enzyme into
five functional domains. The first two N-terminal domains bind HCY and methylTHF, followed by the cap domain, the Cbl binding domain and the C-terminal SAM binding domain (Bandarian et al., 2002) (Fig 4).

![Figure 4: Structure of methionine synthase](image)

The enzyme consists of five domains.

The cap domain, a four-helix bundle unit, shields the upper face of the Cbl binding domain and protects the cofactor against oxidation, thereby sensing variations in the intracellular oxidative state.

L. Cobalamin and Its Role in Methionine Synthase

Cobalamin, also called vitamin B12, is a water-soluble vitamin which humans must acquire through the dietary route, although a portion of B12 can also arise from intestinal microbes, some of which have the capacity to synthesis it. Vitamin B12 deficiency can result in many diseases such as developmental delay, pernicious anemia and neurological disorders (Oh and Brown, 2003). It has two active forms,
adenosylcobalamin (AdoCbl) and MeCbl which are cofactors for the mitochondrial enzyme methylmalonyl-CoA mutase and MS respectively (Pezacka et al., 1992).

Cbl contains a reduced tetrapyrrole, named a corrin ring, and the cobalt atom in the corrin ring can exist in different oxidation states. In its Cbl (I) state, it normally abstracts a methyl group from methylTHF to form MeCbl which is subsequently utilized to convert HCY to methionine. Cbl(I) contains a pair of electrons in the $dz^2$ orbital oriented perpendicularly to the plane of the corrin ring which makes it an excellent nucleophile (Finkelstein, 1990). Thus in the presence of ROS it is easily oxidized by giving away a single electron, giving rise to Cbl(II). In Cbl (II) state, MS activity is temporarily restricted and this, in turn, promotes HCY diversion toward GSH synthesis (Deth et al., 2008). In addition, a low level of methylTHF may prolong the duration of the Cbl (I) state, giving rise to the increased possibility of Cbl inactivation. Therefore, Cbl serves as a redox sensor and plays an important role in cellular response to oxidative stress.
Materials and Methods

A. Cell Culture

SH-SY5Y human neuroblastoma cells were purchased commercially from ATCC®. Cells were grown as monolayers in 10 cm standard tissue culture dishes, containing 10 mL of alpha-modified Minimum Essential Medium (α-MEM) supplemented with 1% penicillin-streptomycin-fungizone (antibiotics) and 10% fetal bovine serum (FBS). Cultured cells were maintained in an incubator chamber with 5% CO₂ at 37°C. For cell passage, confluent cells were detached with 1.5 mL trypsin-EDTA solution. Cells were resuspended in 10 mL fresh medium and seeded at low density in 10 cm standard tissue culture dishes. For most experiments, cells were plated and incubated for 24 hours then media was switched to low serum media (1%) or low cysteine (20 or 200 µM) serum-free media for an additional 24 hours prior to use. For cell lines, experiments utilized cells between 5-30 passages, with no significant differences in results.

Human embryonic kidney HEK293 cells and human hepatocellular carcinoma Hep G2 cells were purchased commercially from ATCC®. Cells were maintained and passaged under the same conditions as SH-SY5Y cells. For experiments, cells were plated and incubated in standard culture media (10% FBS contained media) for 48 hours prior to use.
B. Lowry Protein Assay

Protein concentrations were determined by using the modified Lowry method for protein quantification using bovine serum albumin (BSA) as the standard (LOWRY et al., 1951).

C. Growth Factor and Inhibitors Treatment

SH-SY5Y human neuroblastoma cells were plated in 10 cm² petri dishes for cobalamin and thiol experiments. Cells were grown in normal media (10% FBS) for 24 hours, after which media were replaced by low-serum media (1% FBS) or low-cysteine media (20 or 200 µM) with 1% N-2 supplement for another 24 hours totaling 48 hours before the assay. Confluent cells were pretreated with compounds as shown in the table.

Table 1: Summary of compounds used to treat SH-SY5Y cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Treatment time</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>10 nM</td>
<td>1 or 24 hrs</td>
<td>Cobalamin Measurement</td>
</tr>
<tr>
<td>IGF-1</td>
<td>10 nM</td>
<td>2 or 24 hrs</td>
<td>Thiol Measurement</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>100 nM</td>
<td>1 or 24 hrs</td>
<td>Cobalamin Measurement</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>100 nM</td>
<td>2 or 24 hrs</td>
<td>Thiol Measurement</td>
</tr>
<tr>
<td>NAC</td>
<td>100 mM</td>
<td>24 hrs</td>
<td>Cobalamin Measurement</td>
</tr>
<tr>
<td>BSO</td>
<td>10 mM</td>
<td>24 hrs</td>
<td>Cobalamin Measurement</td>
</tr>
<tr>
<td>PD98059</td>
<td>10 µM</td>
<td>1 hr</td>
<td>Cobalamin Measurement</td>
</tr>
</tbody>
</table>

D. Cobalamin Extraction

Cbl extraction was performed under dim-red light due to the light sensitivity of Cbl. Confluent cells in 10 cm standard tissue culture dishes were washed with 10 mL of Phosphate Buffered Saline (PBS) three times. For cell lysis, cells were treated with 1.0 ml of 50mM Tris buffer, containing 1% Triton X-100 for 10 min at room temperature.
100 μL of cell lysate was taken from each culture dish for protein assay. Cell lysate left in each dish was aliquoted into two microcentrifuge tubes. To extract Cbls, 0.68 mL of ice-cold absolute ethanol was added into each vial and incubated for 10 min at room temperature. Protein precipitates were removed by centrifugation at 10600 rpm for 3 min at 20°C. The resulting supernatant was taken to dryness in a Speed Vac at a medium drying rate. Enriched residue was re-suspended with 0.3 mL PBS and passed through a syringe driven filter (0.22 μm). Cbl extracts were ready for identification by HPLC. This protocol was developed by Jacobsen et, al (Hannibal et al., 2008).

E. HPLC Cobalamin Quantification

100 μL of cbl sample was added to a conical micro autosampler vial, blown with nitrogen, capped and kept at 4°C in the autosampler cooling tray. The cooling tray was covered by aluminum foil paper to avoid Cbl degradation. 10 uL of sample was injected onto an Agilent Eclipse XDB-C8 (3 x 150mm; 3.5 μm) and Agilent Eclipse XDB-C8 (4.6 x 12.5mm; 5 μm) guard column by the autosampler. Samples were eluted using the following step gradient: 0-2 min 0% B, 2-14 min 17% B, 14-19 min 30% B, 24-31 min 58% B, 31-32 min 100% B, then equilibrate column with 0% B for 2 min at a flow rate of 0.6 mL/min. Mobile phase A contains 0.1% acetic acid/acetate buffer titrated to pH 3.5 with NH₄OH. Mobile phase B is acetonitrile containing 0.1% acetic acid. Cbls were measured using the ESA CoulArray with BDD analytical cell model 5040 electrochemical detector at an operating potential of 1500 mV. Samples were normalized against protein content.
F. Thiol Isolation

Treatments were carried out using either 6-well cell culture plates or 10 cm standard round dishes. Confluent cells were pretreated in culture media as indicated for individual experiments. After treatments, media was aspirated and the cells were washed 2x with 1 mL of ice-cold HBSS. HBSS was aspirated from the cells and 0.5 mL ice cold dH2O was added to each well. Cells were scraped from the dish and suspended in dH2O. 100 μL of homogenate was used to determine protein content using a modified Lowry Protein Assay. The cell suspension was sonicated for 15 seconds on ice and an equal volume of 0.4 N PCA solution was added. Sonicates were then spun at 13,000 RPM on a tabletop microcentrifuge for 60 min. 100 μL of sample was added to a conical micro autosampler vial, blown with nitrogen, capped and kept at 4°C in the autosampler cooling tray. Samples were normalized against protein content.

G. HPLC Thiol Quantification

100 μL of thiol sample was added to a conical micro autosampler vial, blown with nitrogen, capped and kept at 4°C in the autosampler cooling tray. 10 uL of sample was injected onto an Agilent Eclipse XDB-C8 (3 x 150mm; 3.5 μm) and Agilent Eclipse XDB-C8 (4.6 x 12.5mm; 5 μm) guard column by the autosampler. Samples were eluted using the following step gradient: 0-9 min 0% B, 9-19 min 50% B, 19-30 min 50% B, then, the column was equilibrated column with 5% B for 12 min at a flow rate of 0.6 mL/min. Mobile phase A consists of 25 mM Sodium phosphate, 1.4 mM 1-Octanesulfonic acid in dH2O, adjusted to pH 2.65 with phosphoric acid. Mobile phase B consists of 50% Acetonitrile, 50% dH2O, 25 mM Sodium phosphate, 1.4 mM 1-Octanesulfonic acid in dH2O, adjusted to pH 2.65 with phosphoric acid. Thiols were
measured using the ESA CoulArray with BDD analytical cell model 5040 electrochemical detector at an operating potential of 1500 mV. Samples were normalized against protein content.

H. **Statistical Methods**

Statistical analyses were carried out using Graph Pad Prism® version 5.01. The Student’s t-test for independent means was used to test for significant differences between control and experimental groups. Data were expressed as mean ± standard error of the mean (SEM). Best-fit values, including correlation coefficients, were calculated using non-linear and linear regression models.
Results

A. Validation of Intracellular Cobalamin Quantification

Running and isolation conditions were optimized for electrochemical HPLC detection of cellular Cbls from cellular lysates. Column loads of $10^{-5}$ M OHCbl, GSCbl, SO$_3$Cbl, CNCbl, AdoCbl and MeCbl were used to optimize the running conditions for resolution and quantification. Figure 5 shows a prepared cell lysate spiked with $10^{-5}$ M of each Cbl measured.

![Typical chromatogram showing the measurement of intracellular Cbls. OHCbl, GSCbl, SO$_3$Cbl, CNCbl, AdoCbl and MeCbl are resolved in a single HPLC separation and measured by electrochemical detection.](image)

Figure 6 shows a sample standard curve for each Cbl form ranging from $10^{-7}$-$10^{-5}$ M. The $R^2$ value for each trend line is above 0.99, showing the accuracy of the detection method.
Figure 6: Typical standard curve of measured Cbls. Standard curves for OHCbl, GSCbl, SO3Cbl, CNCbl, AdoCbl and MeCbl are shown.

B. Validation of Intracellular Thiol and Thioether Quantification

Running and isolation conditions were optimized for electrochemical HPLC detection of cellular thiols and thioethers from SH-SY5Y cell lysates. Column loads of 1-200 ng cysteine, cystine, cystathionine, GSH, HCY, methionine, GSSG, homocystine, SAH and SAM were used to optimize the running conditions for resolution and quantification. Figure 7 shows a prepared cell lysate spiked with 10 µg/mL of each thiol or thioether measured.
Figure 7: Typical chromatogram showing the measurement of intracellular thiols. Cysteine, cystine, cystathionine, GSH, HCY, methionine, GSSG, homocystine, SAH and SAM are resolved in a single HPLC separation and measured by electrochemical detection.

Figure 8 shows a sample standard curve for each thiol or thioether ranging from 25-200 ng. The $R^2$ value for each trend line is above 0.99, showing the accuracy of the detection method.

Figure 8: Typical standard curve of measured thiols and thioethers. Standard curves for cysteine, cystine, cystathionine, GSH, HCY, methionine, GSSG, homocystine, SAH and SAM.
C. Specific Aim 1: Evaluate the influence of insulin-like growth factor on cobalamin status in cultured SH-SY5Y cells.

Studies in our lab have shown that neurotrophic growth factor, especially IGF-1, can increase cellular cysteine uptake by activating PI3 kinase signaling pathway and promoting cellular surface expression of EAAT3, a cysteine transporter capable of regulating the intracellular level of cysteine (Waly et al., 2012) (N. Hodgson, unpublished results). Cysteine is the rate-limiting precursor for the synthesis of the primary cellular antioxidant GSH. Augmentation of cysteine uptake by IGF-1 could change cellular redox status. Cbl serves as a sensor of cellular redox status. To test whether IGF-1 influenced Cbl status, intracellular levels of Cbl were measured in IGF-1 treated SH-SY5Y cells.

First, the composition of the intracellular Cbl pool was evaluated in cells cultured under normal serum conditions (10% FBS) (Fig. 9).

Figure 9: Cobalamin content in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y human neuroblastoma cell line. Cells were grown in alpha MEM with 10% FBS. n=4.
To diminish background stimulation from growth factors contained in FBS, cells were first plated under normal serum conditions (10% FBS) to reach confluence. Complete confluence was required to arrest cell division and increase reproducibility. After 24 hours, cells were switched to low serum medium (1% FBS) for another 24 hours prior to Cbl extraction experiment. Extracted Cbls were immediately measured by HPLC (Fig 10).

Figure 10: Effect of growth factors in FBS on cobalamin content in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y human neuroblastoma cell line. Cells were grown in alpha MEM with 10% or 1% FBS. n=4. Asterisks (*) show a significant difference (p<0.05) from 10% FBS group.

Low serum growth media reduced intracellular levels of GSCbl, SO₃Cbl, AdoCbl, MeCbl and increased level of OHCbl. The level of CNCbl was unchanged. AdoCbl was increased by 70% and MeCbl by 55%. AdoCbl and MeCbl are the two active forms of intracellular Cbl, these data suggest that multiple growth factors in FBS are associated with a dynamic change in the intracellular composition of Cbls. Therefore, to measure the
effect of IGF-1 on Cbl content, SH-SY5Y cells cultured in low serum medium were used as a control group for the following experiment.

After 24-hour growth in 10% FBS containing medium and 24-hour maintenance in 1% FBS containing medium, SH-SY5Y cells were pretreated with 10 nM IGF-1 for 24 hours. Then intracellular Cbl was isolated and measured (Fig 11).

![Graph showing the effect of IGF-1 on cobalamin content in SH-SY5Y cells.](image)

**Figure 11: Effect of IGF-1 on cobalamin content in SH-SY5Y cells.** Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y human neuroblastoma cell line. Cells were treated with IGF-1 (10 nM) for 24 hours prior to measurement. n=4. Asterisks (*) show a significant difference (p<0.05) from control.

24 hour treatment with IGF-1 increased intracellular levels of GSCbl and AdoCbl. GSCbl can be produced by binding of OHCbl to GSH. Previous studies in our lab showed that IGF-1 increased cysteine uptake and cellular level of GSH by activating the PI3 kinase signaling pathway (N. Hodgson, unpublished results). The 75% increase in GSCbl by IGF-1 indicates the involvement of the PI-3 kinase pathway.
To investigate such a role of the PI3 kinase signaling pathway, cells were treated with IGF-1 (10 nM), or IGF-1 and the PI3 kinase inhibitor wortmannin (100 nM) for 24 hours under low serum conditions (Fig 12).

**Figure 12: Effect of PI3 kinase signaling inhibition on IGF-1 mediated cobalamin content in SH-SY5Y cells.** Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y human neuroblastoma cell line. Cells were treated with IGF-1 (10 nM) or IGF-1 (10 nM) and wortmannin (100 nM) combined for 24 hours prior to measurement. n=4. Asterisks (*) show a significant difference (p<0.05) from control.

The PI3 kinase inhibitor wortmannin diminished IGF-1 associated increases in the intracellular levels of both GSCbl and AdoCbl. IGF-1 increased the AdoCbl level by 76% (Fig 11) and the stimulation was completely blocked by treatment with wortmannin (Fig 12). For GSCbl, only 65% of IGF-1 stimulation was blocked by PI3 kinase inhibition, suggesting that beside the PI3 kinase pathway, other signaling pathways might be involved in Cbl content regulation.
IGF-1 is capable of stimulating the Mitogen-activated protein (MAP) kinase pathway (Kim et al., 1997a). To examine whether the MAP kinase pathway is involved in Cbl level regulation, cells were treated with MEK1 inhibitor PD98059 10 µM for 1 hour, followed by Cbl extraction and measurement (Fig 13).

![Figure 13: Effect of MAP kinase signaling inhibition on cobalamin content in SH-SY5Y cells.](image)

MEK1 inhibitor PD98059 reduced intracellular levels of GSCbl, indicating the involvement of MAP kinase signaling pathway in augmentation of GSCbl. A greater-than two-thirds reduction of CNCbl by PD98059 was observed. CNCbl is the stable form of vitamin B12 in the cell culture media, it can be taken up by cells and then converted to other forms of Cbl (Reizenstein, 1967). In a previous experiment, 24-hour 10 nM IGF-1 treatment didn’t change the level of CNCbl (Fig 11). Thus, while it is possible that the
MAP kinase signaling pathway may regulate Cbl cellular transport, this effect does not appeared to be mediated by IGF-1 stimulated cysteine uptake.

IGF-1 stimulation increased the intracellular level of GSCbl, which is produced by OHCbl binding to GSH (Fig 11). To further investigate the relationship between intracellular level of GSH and Cbl status, 1 mM buthionine sulfoximine (BSO) was used to treat SH-SY5Y cells for 24 hours (Fig 14). BSO is an inhibitor of GCS, the enzyme that catalyzes the first step of GSH synthesis.

Figure 14: Effect of GSH synthesis inhibition on cobalamin content in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y human neuroblastoma cell line. Cells were grown in α MEM with 10% FBS. Cells were treated with BSO (1 mM) for 24 hours prior to measurement. n=4. Asterisks (*) show a significant difference (p<0.05) from control.

GCS inhibition by BSO decreased intracellular levels of GSCbl, CNCbl and AdoCbl, but increased levels of OHCbl and MeCbl. OHCbl is the oxidized form of Cbl and is formed from Cbl (II) in the presence of superoxide (Waly et al., 2004). The
increases in OHCbl and decrease in GSCbl are signs of accumulated oxidative stress suffered by the cells. This oxidative stress is created by the decrease in GSH synthesis, caused by BSO. Interestingly, GSH inhibition increased the MeCbl level by roughly twofold. MeCbl is the cofactor of MS. A potential explanation for the increase in MeCbl could be that such a high concentration (1 mM) and longtime treatment (24 hours) of BSO created high levels of oxidative stress which caused Cbl to be in its inactive Cbl (II) state, leading to inhibition of MS. The decrease in MS activity would cause a decrease in its utilization of MeCbl which in turn could result in the large accumulation of MeCbl.

Figure 15: Effect of N-acetylcysteine on cobalamin content in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y human neuroblastoma cell line. Cells were treated with NAC (100 mM) for 24 hours prior to measurement. n=4. Asterisks (*) show a significant difference (p<0.05) from control.
Figure 16: Effect of low cysteine growth conditions on Cbls in SH-SY5Y cells. Electrochemical HPLC isolation and detection of Cbls in the SH-SY5Y human neuroblastoma cell line. Cells were grown for 24 hours in alpha MEM (570 µM cysteine) with 10% FBS or alpha MEM (200 µM) with 1% N-2 or alpha MEM (20 µM cysteine). Cells were grown in media with 570 µM cysteine were selected as control group. N=4. Asterisks (*) show a significant difference (p<0.05) from control.

Effects of extracellular cysteine level on Cbl content were examined (Fig 15, 16). NAC is an alternative source of cysteine, which can be taken up by cells for GSH synthesis. Interestingly, NAC increased the intracellular level of MeCbl rather than GSCbl. NAC stimulated intracellular GSH synthesis by improving cysteine availability, which may result in an increased level of GSH, at first. Formed GSCbl was later converted to MeCbl, so the net level of GSCbl remained unchanged. If this is the case, it supports the idea that GSCbl is the precursor of MeCbl (Pezacka et al., 1990). On the other hand, when the extracellular cysteine level dropped, intracellular levels of GSCbl, AdoCbl and MeCbl decreased. An increased level of OHCbl may result from oxidative stress accumulation caused by shortage of GSH.
D. Specific Aim 2: Investigate differences in the intracellular levels of cobalamin among cultured SH-SY5Y, HEK-293 and HepG2 cells.

The brain is a closed system which is surrounded by cerebrospinal fluid (CSF) and shielded by the blood-brain barrier. The brain consumes 20% of the total amount of oxygen consumed by the whole body at a rate that is about 10-fold higher than other organs. However, the level of primary antioxidant GSH in the CSF is 12-fold lower than in plasma and the level of the GSH synthesis precursor cysteine is 10-fold lower (Castagna et al., 1995). These facts indicate that a highly effective antioxidant system exists in the brain to maintain its normal function by fully utilizing limited antioxidant sources.

By functioning as a redox sensor, Cbl plays a critical role in the antioxidant system and the composition of the cobalamin pool in the brain may be unique and indicative of a unique antioxidant system in the brain. In opposition, the liver is one of organs with the highest amounts of GSH (Lu, 1999). A large pool of Cbl is present in the kidney which influences Cbl distribution over the body (Scott et al., 1984). To investigate differences of Cbl status among these three organs, SH-SY5Y cells were used as a model for mature neurons, while HEK-293 and HepG2 cells were selected to represent the kidney and the liver respectively. All three cell lines were seeded and maintained in alpha MEM with 10% FBS under the same conditions. After 48 hours, Cbl extraction and measurement by HPLC were conducted (Fig 17).
Figure 17: Cobalamin content in SH-SY5Y, HEK293 and HepG2 cells. Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y, HEK293 and HepG2 cell lines. n=4. Asterisks (*) show a significant difference (p<0.05) from the SH-SY5Y cell group.

The intracellular level of CNCbl in HepG2 cells was highest among the three cell lines. As CNCbl is the storage form of Cbl, this suggests that there is a large pool for cobalamin storage in the liver. This is consistent with the literature, which reports that in the human body, more than 50% of Cbl is stored in the liver (Finkelstein, 1990). The level of GSCbl in SH-SY5Y and HepG2 cells was 3-fold higher than it in HEK293 cells. This suggests that more Cbl is diverted to engage in cellular antioxidant defense by reacting with antioxidant GSH in both the brain and liver than in the kidney. The reverse pattern of OHCbl in three cell lines further reinforces this observation. Higher level of MeCbl in HEK293 cells may indicate more active methionine production in the kidney, but more studies are required to support this idea.
Figure 18: Cobalamin content in SH-SY5Y, HEK293 and HepG2 cells. Electrochemical HPLC isolation and detection of intracellular Cbls in the SH-SY5Y, HEK293 and HepG2 cell lines. n=4.

The representation of these data in a pie chart clearly shows that the overall pattern of Cbl composition in SH-SY5Y neuronal cells appears to be more similar to that of HepG2 cells than HEK293 cells (Fig 18). Although the literature suggests that the intracellular level of GSH in liver hepatocytes is 50-fold higher than in neurons (Sun et al., 2006), similarities between hepatocytes and neurons in both GSCbl level and Cbl composition pattern, suggest that the involvement of Cbl in the cellular antioxidant systems is similar. In the liver, the high content of GSH is capable of dealing with the oxidative stress present in those cells. However, in neurons which contain low levels of GSH compared to hepatocytes, an effective antioxidant system may depend on higher levels of selenium, or other antioxidant enzymes or cofactors. Notably, one shortcoming
of these data is that the Cbl levels may reflect cell culture conditions, which are different from the physiological environment.

E. **Specific Aim 3: Evaluate the influence of IGF-1 on thiol status in cultured SH-SY5Y cells.**

Previous studies have shown that IGF-1 promotes the conversion of Cbl to its active form, which is involved in cellular antioxidant systems and consequently increases the cellular capacity against oxidative stress. The link between oxidative stress and impaired DNA methylation has been identified in many neurodegenerative diseases such as Alzheimer’s disease (Pogribny and Beland, 2009). As described in the model of neuronal redox and methylation pathways built up by our lab (Fig 3), intracellular thiols and thioethers play essential roles in this linkage. On the one hand, thiol antioxidants like GSH function as critical molecules to protect neurons from oxidative damage. On the other hand, thiol-containing compounds like SAM serve as primary methyl donors. Ultimately, intracellular thiol status is closely connected to cellular redox as well as methylation status.

Cysteine availability is limited in the human brain. The level of cysteine in CSF is more than 100-fold lower than in plasma (Castagna et al., 1995). To create more “brain-like” cell culture conditions, SH-SY5Y cells were first plated in alpha MEM with 10% FBS to reach confluence. After 24 hours, cells were switched to one of three media and maintained for another 24 hours: The first medium contained 10% FBS and 570 µM cysteine (standard concentration of cysteine used for previous experiments). The second medium contained 1% N-2 and 200 µM cysteine. The third medium contained 1% N-2
and 20 µM cysteine. Intracellular thiols were isolated and measured by HPLC (Fig 19, 20).

![Graph](image-url)

**Figure 19: Effect of low cysteine growth conditions on thiols and thioethers involved in the transsulfuration pathway in SH-SY5Y cells.** Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were grown for 24 hours in alpha MEM (570 µM cysteine) with 10% FBS or alpha MEM (200 µM) with 1% N-2 or alpha MEM (20 µM cysteine). Cells grown in media with 570 µM cysteine were selected as the control group. n=4. Asterisks (*) show a significant difference (p<0.05) from control.

The reduction of extracellular cysteine decreased the intracellular level of GSH, consistent with cysteine being the rate-limiting precursor of GSH. The increase of GSSG under low cysteine conditions may be a sign of increased oxidative stress in these cells. Cystathionine is the metabolic intermediate between HCY and cysteine in the transsulfuration pathway (Fig 3). A negative correlation between extracellular cysteine level and intracellular cystathionine level suggests that transsulfuration pathway is
activated to generate more cysteine in response to limited cysteine availability. The intracellular level of cysteine tended to decrease when the extracellular level of cysteine dropped, but this was not statistically significant. Part of the reason may be compensation from the transsulfuration pathway. Because this pathway is supposed to be largely inactive in the brain, this may be a limiting factor of this thesis. In future studies an inhibitor of one of the enzymes involved in transsulfuration might be useful.

Figure 20: Effect of low cysteine growth conditions on thiols and thioethers involved in the methionine cycle in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were grown for 24 hours in alpha MEM (570 µM cysteine) with 10% FBS or alpha MEM (200 µM) with 1% N-2 or alpha MEM (20 µM cysteine). Cells were grown in media with 570 µM cysteine were selected as the control group. N=4. Asterisks (*) show a significant difference (p<0.05) from control.

Homocysteine is the oxidized dithiol of HCY. Its increase along with the decrease in the cysteine level indicates the accumulation of intracellular oxidative stress. When the cysteine concentration in the media was dropped to 200 µM, the level of methionine was
remarkably reduced. This may be a sign that more HCY was diverted to the transsulfuration pathway and that ROS accumulation limited GSCbl and MeCbl availability, causing an inhibition of MS activity. This inhibition would explain the reduction in methionine levels. Interestingly, cells grown in the 200 µM cysteine condition, showed decreased levels of SAH while cells grown in the 20 µM cysteine condition showed increased levels. While the 200 µM cysteine condition could cause the cells mild oxidative stress, the 20 µM cysteine condition might cause much more extreme oxidative stress. Under mild oxidative stress the transsulfuration pathway might not be activated. SAH could accumulate under moderate oxidative stress, as MS activity is decreased and HCY accumulates, the conversion of SAH to HCY is reversible. The more extreme oxidative stress caused by the 20 µM cysteine condition may activate the transsulfuration pathway, decreasing the levels of HCY, and in turn SAH.

To further examine the effect of IGF-1 on cellular redox status under low cysteine conditions, SH-SY5Y cells were plated in normal media with 10% FBS for 24 hours and then switched to media with different concentrations of cysteine (570 or 200 or 20µM) for another 24 hour maintenance. Cells were then treated with 10 nM IGF-1 or 10 nM IGF-1 and 100 nM wortmannin combined for 24 hours, followed by thiol isolation and measurement by HPLC. As GSH/GSSG is the most abundant thiol redox buffer in cells, the ratio of GSH/GSSG was calculated to indicate cellular redox status (Fig 21).
Figure 21: Effect of IGF-1 on GSH/GSSG ratio under low cysteine culturing conditions in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular GSH and GSSG in the SH-SY5Y human neuroblastoma cell line. Cells were grown for 24 hours in alpha MEM (570 µM cysteine) with 10% FBS or alpha MEM (200 µM) with 1% N-2 or alpha MEM (20 µM cysteine). Cells were treated with IGF-1 (10 nM) or IGF-1 (10 nM) and wortmannin (100 nM) combined for 24 hours prior to measurement. N=4. Asterisks (*) show a significant difference (p<0.05) from cell cultured with 570 µM cysteine.

The GSH/GSSG ratio decreased with the reduction of extracellular cysteine, suggesting that cell culture condition is capable of influencing redox status inside of cells. IGF-1 diminished the cellular oxidative stress associated with the low extracellular cysteine level. IGF-1 is capable of increasing cellular cysteine uptake. Such an effect of IGF-1 can be blocked by PI3 kinase inhibitor wortmannin. Notably, when cells cultured in 20 µM cysteine contained media were treated with wortmannin, GSH level was too low to be detected by HPLC, indicating a critical role for PI3 kinase under this “brain-like” condition.
Discussion

A. IGF-1 Effects on Cellular Redox Status

IGF-1 belongs to the family of insulin-like growth factors which have broad effects over fetal and postnatal growth and development in mammals. IGF-1 normally circulates at high concentrations in the human bloodstream (150-400ng/ml in plasma) (Clemmons, 2007). Dysfunctional regulation of IGF-1 levels has been associated with many diseases, such as AD and many cancers (Renehan et al., 2000, 2004; Carro et al., 2002). IGF-1 has been identified as an important regulator in cellular growth responses to oxidative stress (Delafontaine and Ku, 1997). Studies by Meng et al. showed that ROS generation is capable of triggering IGF-1 signaling response in vascular smooth muscle cells (Meng et al., 2008). Azar and his colleagues found that transactivation of the IGF-1 receptor mediates downstream effects of hydrogen peroxide (Azar et al., 2006, 2007). IGF-1 has been also suggested to regulate activity of endothelial nitric oxide synthase, which catalyzes the production of nitric oxide (Repetto et al., 2005; Pu et al., 2008).

We that IGF-1 is capable of shifting the cellular environment to a more reduced state by promoting the synthesis of the antioxidant GSH. This activity was found to be PI3 kinase-dependent. Binding of IGF-1 to IGF-1R stimulates autophosphorylation of tyrosine residues on the receptor (e.g., Y1131, Y1135 and Y1136), and phosphorylation of these tyrosine residues activates the receptor to continuously phosphorylate the downstream substrate, insulin receptor substrate 1 (IRS1). Phosphorylation of IRS1 promotes binding and activation of the p85 and p110 subunits of PI3K, followed by the phosphorylation of phosphatidylinositol 4, 5 diphosphate (PIP2) to form
phosphatidylinositol 3, 4, 5 triphosphate (PIP3). Formation of PIP3 allows binding of the plekstrin homology (PH) domain of protein kinase B/Akt (PKB/Akt), leading to its phosphorylation by phospholipid-dependent kinases 1 and 2 (PDK-1/2). Activated PKB/Akt has many downstream substrates, which are involved in various physiological functions (Vardatsikos et al., 2009). Phosphorylation of one important substrate, mammalian target of rapamycin (mTOR), stimulates cellular surface expression of EAAT3 by increasing its membrane insertion without inducing its endocytosis (Almilaji et al., 2012). Studies by our lab have shown that EAAT3 functions as a cysteine transporter (Himi et al., 2003), and increased surface expression of the EAAT3 can import more extracellular cysteine, which is the rate-limiting precursor for GSH synthesis (N. Hodgson, unpublished results). Accordingly, more GSH is synthesized after IGF-1 stimulation, which protects cells from oxidative stress and alters cellular redox status.

Figure 22: Schematic representation of IGF-1 signaling of PKB activation and physiological role (Vardatsikos et al., 2009).

IGF-1 is not a just simple compound used to treat cells in experiments, but rather
it is an important component of the strategy cells use to avoid oxidative damage. Studies show that when vascular smooth muscle cells are treated with hydrogen peroxide, a very reactive ROS, synthesis of IGF-1 is upregulated (Delafontaine et al., 2004). Other studies found that overproduction of ROS enhanced expression of IGF-1R (Du et al., 1996). These studies suggest that IGF-1 plays a central role in the complicated cellular anti-oxidative defense system.

B. Cobalamin and Neurodegenerative Disease

Cbl plays an important role in brain development and function. Cbl has two physiologically active forms, which are AdoCbl and MeCbl. MeCbl is cofactor for the enzyme MS which catalyzes conversion of HCY to methionine and thereby promotes activity of the methionine cycle to produce SAM. SAM is a universal methyl donor which contributes to more than 200 methylation reactions, giving rise to broad effects over biological activities. For example, SAM-dependent methylation reactions can convert N-acetyl-serotonin to melatonin, which functions as an internal synchronizer for the timing of multiple physiological events (Bottiglieri et al., 1994). An alternative methylation pathway in which betaine-homocysteine-methyltransferase methylates homocysteine to methionine is active in many organs, such as liver and kidney, but this pathway is not detected in brain (Sunden et al., 1997). This makes methylation reactions in the brain highly dependent on MS and any Cbl shortage in the brain may develop into a “hypomethylation state”, leading to abnormal brain function and/or abnormal neurodevelopment. The other active form of cobalamin, AdoCbl, is the co-activator for mitochondrial methylmalonyl-CoA-mutase. AdoCbl deficiency may cause enzyme dysfunction, resulting in conversion of methylmalonyl-CoA to MMA, which is a
Many studies have shown that there is a relationship between Cbl deficiency and neurodegenerative diseases, including AD, autism, mood disorder and others (Prodan et al., 2009; Pineles et al., 2010; Tufan et al., 2012). Folate deficiencies, reductions in the plasma levels of SAM and increases in SAH are also associated with these diseases and oxidative stress could connect all of these factors. As long as we keep inhaling oxygen and exhaling carbon dioxide to survive, our body will always be fighting oxidative stress. The molecules and enzymes mentioned in this thesis are all involved in this battle. Because the human brain is more sensitive to oxidative stress, once the balance is lost, the brain inevitably becomes the victim, leading to various neurodegenerative diseases.

The big story of this thesis is the model, built up by our lab, to explain how compounds and enzymes work together to protect neurons, as well as to regulate their activity (Fig 3). The thesis, which mainly focuses on the role of Cbl in this neuronal antioxidant neuronal network, is just one of the chapters in this story (green text in Fig 3). Our studies found that the conversion of different forms of Cbl is dynamic and subject to the cellular environment. Intracellular levels of GSH are positively correlated to the GSCbl level. IGF-1 promotes formation of the active forms of Cbl, which are AdoCbl and GSCbl. Each cell type has its unique Cbl composition pattern, but a similarity can be found between the SH-SY5Y cell line and the HepG2 cell line.

C. **Role of GSCbl**

GSCbl was first identified as a product of H$_2$OCbl$^+$ bound to GSH (Wagner and
Bernhauer, 1964). Despite being identified in 1964, the X-ray crystal structure was not elucidated until 2010 (Hannibal et al., 2010). All Cbls share the common structure of a cobalt atom located in the center of a corrin ring. This position is maintained by the coordination of four equatorial donor nitrogens, which form a ring. Additionally, the cobalt atom binds to the fifth nitrogen, part of a 5, 6-dimethylbenzimidazole moiety at the lower (α) axial position. The upper (β) axial position can be bound by various ligands, giving rise to a large number of Cbl derivatives, such as AdoCbl and MeCbl (23, a). Jacobsen and co-workers proved that the glutathionyl moiety in GSCbl is bound to the cobalt through the sulfur atom, with a Co-S bond distance of 2.295 Å (Fig 23, b).

**Figure 23:** (a) chemical structure of cobalamin. Two axial sites (upper = β, lower = α) are along with the corrin ring (Xia et al., 2004). (b) Thermal ellipsoid plot of GSCbl. The Cbl complex is colored green (C), red (O), blue (N), and cyan (P). The cobalt is shown as a gray sphere (Hannibal et al., 2010).

Although GSCbl is unlike AdoCbl or MeCbl, both of which directly function as coenzymes, research by Jacobsen and others suggested that GSCbl is a naturally occurring form of Cbl in mammalian cells which could serve as a precursor in the
biosynthesis of AdoCbl and MeCbl (Wagner and Bernhauer, 1964; Amagasaki et al., 1990; Pezacka et al., 1990). Rosenblatt et al. discovered that dietary Cbls are processed by the MMACHC (methylmalonic aciduria combined with homocystinuria type C) gene product (Lerner-Ellis et al., 2006, 2009; Morel et al., 2006). One main strategy employed by MMACHC is to remove alkyl groups in dietary Cbls via nucleophilic attack of GSH, giving rise to GSCbl and Cbl (I). GSCbl may directly bind to MS or methylmalonyl-coenzyme A mutase and be reduced to Cbl (I) which later reacts with either SAM or ATP to form MeCbl and AdoCbl respectively (Xia et al., 2004; Hannibal et al., 2009; Kim et al., 2009). Brasch et al. supported this idea by demonstrating that once H2OCbl+ is formed upon removal of a β-axial ligand, it can be rapidly and irreversibly converted to GSCbl (Xia et al., 2004).

Accordingly, studies in our lab found that in human brain, the cap domain of MS can be alternatively spliced, apparently as a response to oxidative stress, especially in aging (Muratore, 2010). Cap domain is capable of covering Cbl from dissociation, and when the structure of MS is intact, Cbl (II) is likely to remain continuously bound to MS. Bound Cbl(II) is reduced to Cbl(I) by methionine synthase reductase, which can be consequently converted to MeCbl by SAM (Fig 24, left panel). However, when a portion of the cap domain is missing in alternatively spliced forms of MS, Cbl (II) may more easily dissociate from MS. Thus a different mode of reactivation may be required. Alternatively-spliced MS exhibits GSH dependence, suggesting its requirement for GSCbl to reduce Cbl(II). Dissociated Cbl(II) is likely converted to OHCbl by environmental ROS. For spliced MS, reactivation requires conversion of OHCbl to GSCbl by GSH, followed by SAM-dependent methylation of GSCbl to MeCbl (Fig 24,
To investigate the physiological role of GSCbl, McCaddon and colleagues proposed that GSCbl could have therapeutic effects on neurodegenerative diseases, like AD. They suggested that these disorders are associated with a “functional” Cbl deficiency (McCaddon et al., 2002; Birch et al., 2009). “Functional Cbl” refers to the co-enzyme forms of Cbl, such as MeCbl or AdoCbl rather than dietary Cbls. GSCbl is the precursor for “functional Cbl” synthesis, so improving “functional Cbl deficiency” could be achieved by increased level of GSCbl. However, more studies are required on GSCbl administration to determine its absorption and stability. My findings, which show that IGF-1 mediated cysteine uptake increases the intracellular levels of GSCbl, suggest that an alternative way to increase GSCbl levels in the brain may be to increase intracellular cysteine or decrease oxidative stress. By doing so a “functional Cbl deficiency” could be treated. This treatment may have some potential benefits for neurological diseases.
D. SH-SY5Y cell and Neurons

The SH-SY5Y cell line is a subline of SK-N-SH cells, which were originally cloned from a metastatic neuroblastoma found in the bone marrow of a female patient (Biedler et al., 1973). In this thesis, the SH-SY5Y neuroblastoma cell line was selected as a model for mature neurons because of its many advantages, including expression of neuronal marker enzyme (tyrosine and dopamine-β-hydroxylases) activity, neuronal transmitter uptake (norepinephrine) and nerve growth factor receptor expression (Ciccarone et al., 1989). Most importantly, SH-SY5Y cells display intact IGF-1 signaling pathways, especially the MAP kinase and PI3-kinase pathways, from functional receptors to downstream signaling molecules (Mattsson et al., 1990; Kim et al., 1997a, 1997b; Kurihara et al., 2000).

However, SH-SY5Y cells can’t fully represent neurons. One big difference is that mature neurons are differentiated and do not divide, while SH-SY5Y cells are continuously dividing. Oxidative damage may be less in dividing SH-SY5Y cells because cell division may diminish the accumulation of damage. It is uncertain whether the antioxidant mechanism in dividing cells is as same as it in non-dividing cells. Another point is that in vitro culturing conditions can’t replicate the complex in vivo environment. In the brain, the cysteine level is low in the CSF. However, glial cells release GSH which is broken down to cysteine for neurons to utilize. In my experiments SH-SY5Y cells were cultured alone, without this localized cysteine resource. There are many limitations to my results, but it is possible to optimize the cell culture conditions, especially culture media, to closely mimic the CSF in the brain.

E. Future Studies
In my thesis research, serum-free media with a 1% N-2 supplement and defined concentrations of cysteine (20 or 200 μM) were designed to better mimic the CSF in brain compared to standard culture media (10% FBS and 570 μM cysteine contained media). FBS is widely used as a growth supplement because of its rich content of growth factors as well as other compounds required for cell division and maintenance. However, FBS is a poorly defined medium supplement and variations in composition among batches may be considerable, giving rise to unpredictable factors in experiments (Mattsson et al., 1990; Bjare, 1992). N-2 supplement is a chemically defined supplement specifically recommended for neuroblastoma and neuron (Bunt-Milam, 1984). N-2 supplement includes five main components (Tab. 2). Transferrin is essential for cellular iron transport. Insulin is responsible for cellular glucose uptake. Selenite serves as selenium source utilized by intracellular selenium-dependent enzymes (Gstraunthaler, 2003). Therefore, culturing cells in media supplemented with N-2 rather than FBS diminishes uncontrollable factors.

Table 2: Composition of N-2 supplement (Bottenstein, JE, 1985).

<table>
<thead>
<tr>
<th>Components</th>
<th>Molecular weight</th>
<th>Concentration (mg/L)</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Transferrin</td>
<td>10000</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Insulin Recombinant</td>
<td>5808.7</td>
<td>5</td>
<td>0.861</td>
</tr>
<tr>
<td>Progesterone</td>
<td>314.47</td>
<td>0.0063</td>
<td>0.02</td>
</tr>
<tr>
<td>Putrescine</td>
<td>161</td>
<td>16.11</td>
<td>100.1</td>
</tr>
<tr>
<td>Selenite</td>
<td>173</td>
<td>0.0052</td>
<td>0.0301</td>
</tr>
</tbody>
</table>

Although serum-free media have been used since the 1980s, use of serum-free media with low cysteine levels for neuroblastoma cell culture has not been documented in the literature, to the best of our knowledge. Cysteine concentration in standard culture
media for SH-SY5Y cells is 570 µM. The purpose of decreasing the cysteine level in culture media was to create more “brain-like” conditions, as the cysteine level in CSF can be as low as 2 µM (Castagna et al., 1995). My studies showed that intracellular levels of GSCbl and GSH were decreased under low cysteine culture conditions. Because of the critical physiological roles of GSCbl as well GSH, once their intracellular availabilities change, metabolic pathways in which they are involved may accordingly change. Thus to accurately reflect brain metabolism and activity, limitation of extracellular cysteine availability for in vitro neuron culture is a reasonable strategy. More efforts could be made to refine other factors in media, such as growth factors, in order to build up a more complete “brain-like” condition for neuron culture. For future studies Cbl and thiol levels will be measured in a postmortem brain sample to learn more about Cbl, as well as thiol, status in the brain and thereby improve our understanding of how well in vitro neuronal cell lines represent the in vivo organ, in terms of Cbl and thiol status.
Summary and Conclusions

In this thesis, we examined the effects of IGF-1 on Cbl and thiol content in SH-SY5Y cells, and investigated its influence on cellular redox and methylation status in a cysteine-dependent manner. The main conclusions of this work include: (1) IGF-1 increased intracellular levels of AdoCbl by activating the PI3 kinase signaling pathway, and increased intracellular levels of GSCbl by activating both the PI3 kinase and MAP kinase pathways. (2) The Cbl composition pattern in SH-SY5Y cells is more similar to HepG2 cells than HEK293 cells. (3) Decreased extracellular cysteine decreased the GSH/GSSG ratio. (4) IGF-1 increased the ratio of GSH/GSSG in SH-SY5Y cells through the PI3 kinase signaling pathway. Together, these findings illustrate a potentially significant role for IGF-1 in regulating redox status, especially in neurons, although further studies in differentiated neurons are needed to confirm these findings.
References


