Regulation of SLC19A3 thiamin transporter by HIF-1α

By

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor that regulates gene expression in response to hypoxia, including SLC19A3, thiamine transporter 2, a facilitative carrier that mediates intracellular uptake of thiamin across the plasma membrane. Thiamin plays a significant role in energy production, which is required for myocardial contraction. In addition, thiamin deficiency (TD) induces heart complications that lead to heart failure as observed in type 2 diabetics, who are prone to be susceptible to ischemic complications. These studies suggested that HIF-1 enhances the expression of the thiamine transporter expression in H9c2 cardiomyocytes.

Our initial findings led us to hypothesize that the HIF-1α-SLC19A3 signaling axis could be a cardioprotective therapeutic target against myocardial ischemia-reperfusion injury. Our results indicate that thiamin administration minimized infarction size when compared to control groups. However, further studies are required to elucidate the mechanistic potential of thiamin in treatment and prevention of myocardial ischemia-reperfusion injury.

Acknowledgments

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List of Abbreviations

CVD	cardiovascular disease
GLUT-1	glucose transporter isoform 1
GLUT-3	glucose transporter isoform 3
HIF	hypoxia inducible factor
Hsp70	heat shock protein 70
α-KGDH	alpha ketoglutarate dehydrogenase
LAD	left anterior descending coronary artery
MI	myocardial infarction
STEMI	ST-segment elevation myocardial infarction
PDHC	pyruvate dehydrogenase complexes
PDK	pyruvate dehydrogenase kinase
SLC19A2	solute carrier family 19A member 2
SLC19A3	solute carrier family 19A member 3

TD	thiamin deficiency
THTR1	thiamin transporter 1
THTR2	thiamin transporter 2
TK	transketolase
RDA	recommends daily allowance
VHL	von Hippel-Lindau

1. INTRODUCTION

1.1 Background

Cardiovascular disease (CVD) has remained the leading cause of mortality and morbidity worldwide for more than a decade and is projected to increase in the near future. According to World Health Organization statistical report 2015, among 17.5 million people died from CVD, an estimated 7.4 million deaths were due to ischemic heart disease. During ischemia, hypoxia-inducible factor-1 (HIF-1) plays a critical role in the molecular pathway in response to low oxygen availability by transcriptionally influences the expression of a number of genes involved in myocardial energy metabolism and cell survival. It is well established that HIF-1 is a key component responsible for up-regulation of glucose transporters; GLUT-1 and GLUT- 3 and glycolytic enzymes including hexokinase 1 and 2, pyruvate kinase and lactate dehydrogenase leading to metabolic switch from aerobic to anaerobic respiration. Thus, glucose metabolism through glycolysis and lactate oxidation becomes a critical metabolic pathway for ATP production in the ischemic heart. In contrast, approximately 60 to 70 % of ATP production in the normal heart generates from fatty acid because it requires high demand for energy to sustain the cardiac function and ATP production from fatty acid oxidation is sufficient for high energy demand of the heart. Therefore, a shift in metabolic status, such as β-oxidation to glycolysis during ischemia contributes to cardiac inefficiency and an imbalance between energetic demand and supply. During prolonged ischemia, lactate accumulation and ATP depletion causes

irreversible myocardial necrosis known as infarction. Recently, thiamin has been reported to provide cytoprotective effects against prolonged hypoxia-induced apoptosis in neonatal rat cardiomyocytes by elevating the anti-apoptotic protein Heat Shock protein 70 (Hsp70) (Shin, Choi et al. 2004). Interestingly, SLC19A3, a gene encoded thiamine transporter 2, has been observed to be induced by HIF-1 in breast cancer cell line (Sweet and Zastre 2013). This suggests that the HIF-SLC19A3 signaling pathway could play an important role in response to hypoxic stress; however, this adaptive mechanism has not been investigated in the ischemic heart whether it undergoes similar molecular adaptation as in hypoxic cancer cells. Myocardial cell death is not limited to ischemic injury but restoration of coronary blood flow from surgical treatment for acute myocardial infarction causes another concern so called reperfusion injury. Although patient outcomes after ischemic- reperfusion injury have improved due to advances in surgical procedures and devices for ameliorating coronary artery blockage, identifying novel signaling pathways for development of molecular therapeutic agents for improving outcomes has remained unsolved. Thus, adjunctive pharmaceutical approaches still need to be developed to improve short-term and long-term outcomes of patient undergoing primary coronary intervention or thrombolytic therapy, in particular diabetic patients, who have a higher risk of myocardial infarction and contributes to a significant decrease in life expectancy compared to non-diabetic patients (Orlander, Goff et al. 1994).

1.2 Objective

The aim of the current study was to determine whether HIF-1 stabilization leads to changes in expression of SLC19A2 and SLC19A3 and transporter protein levels in H9c2 cardiomyocytes. Second aim was to investigate whether supplementation of thiamin would offer cardioprotection against ischemic-reperfusion injury *in vivo* study.

2. REVIEW OF LITERATURE

2.1 Thiamin

2.1.1 Introduction of thiamin

Thiamin or vitamin B1 is an indispensable exogenous compound required for maintaining the function of several enzymes in living organism. Mammals are incapable of synthesizing and storing thiamin, thus exogenous source of thiamin is daily required. The recommended daily allowance (RDA) is based on energy expenditure. Typically, 0.33 mg of thiamine is required for every 1000 kcal of total energy consumption. For the average healthy adult, it is recommended to consume 1.0 -1.5 mg/day on a regular basis (Rucker 2001). Daily intake less than 0.16 mg for 1,000 kcal was reported to develop signs and symptoms of thiamin deficiency.

2.1.2 Thiamin deficiency (TD)

According to World Health Organization, thiamin deficiency (TD) is defined as *a clinical syndrome that arise*s *insidiously as a result of a severe, prolonged deficiency of thiamin in the diet.* TD is categorized into four major types; Dry Beriberi, Wet Beriberi; cardiovascular beriberi, Wernicke-Korsakoff Encephalopathy (most common), and Leigh's syndrome (rare disease); Subacute Necrotizing Encephalomyopathy. The most common manifestation of thiamine is Wernicke-Korsakoff syndrome as a consequence of chronic alcohol consumption.

Despite the fact that cardiac beriberi is rare in industrialized countries, it contributes to fatal outcome (Attas, Hanley et al. 1978) (Yang, Acharya et al. 2012). Cardiac beriberi is characterized by ventricular failure, sinus rhythm, high cardiac output, oedema, low activity of thiamin-dependent enzyme; transketolase and accumulations of serum lactate and pyruvate (Rucker 2001). Acute severe cardiac TD has been observed in patients with congestive heart failure (CHF) with left ventricular systolic dysfunction and lactic acidosis, resulting in attenuated ATP production and thus levels in cardiomyocytes (Bakker and Leunissen 1995). Improvement in circulating levels of thiamin were observed after twelve hours of high dose thiamin administration (100 mg), which offers a potential therapeutic mechanism for ischemia reperfusion injury (Essa, Velez et al. 2011). Previously, a clinical trial conducted in diabetic patients (n=26; type 1 diabetes mellitus, n=48; type 2 diabetes mellitus, n=20; healthy control group) indicated that plasma thiamin concentration in type 1 and type 2 diabetes mellitus (DM) were significantly declined by approximately 76% and 75% respectively when compared to non-diabetic control group. The reduction in thiamin plasma levels was correlated with increase in renal clearance in both type 1 and type 2 diabetes (Thornalley, Babaei-Jadidi et al. 2007). The underlying mechanism was proposed by an experimental study conducted in human proximal tubular epithelial primary cell lines (hPTECs) and maintained under high glucose condition (26 mmol/l incubated for 5 days). The mRNA and protein levels of both thiamin transporters, THTR1 and THTR2, were found to be profoundly reduced in proximal tubules, possibly leading to impaired renal reabsorption of thiamin under hyperglycemia (Larkin, Zhang et al. 2012).

Recent studies have reported that one–third of CHF patients (33%) had significantly lower erythrocyte TPP levels compared to control group (n=100). Interestingly, approximately 40% of those TD patients were found in New York Heart Association functional Classification heart

failure (class III/IV. Recently, systemic reviews and meta-analysis of studies in heart failure patients concluded that patients with heart failure are more prone to develop TD than those with non-heart failure. Further and more significantly that thiamin supplementation in these patients, resulted in positively improving left ventricular end systolic and diastolic function. However, the correlation between TD and heart complication requires further investigation in a large-scale clinical trial (Jain, Mehta et al. 2015). To help understand the molecular mechanism by which thiamin deficiency propagates damage to the cardiomyocyte, Sinleton et al have reported that TD leads to impairment of thiamin-dependent mitochondrial enzymes, resulting in depletion of ATP production, impartment in oxidative metabolism, accumulation of lactate, mitochondria dysfunction and eventually apoptosis (Singleton and Martin 2001). These findings were also observed in a model of isolated rat cardiomyocytes cultured in thiamin free media (DMEM, 10% BSA), resulting in significant reduction of ATP production, contractility after 8-10 days and death after 12-16 days of thiamin depletion. Thiamin pyrophosphate (TPP) concentrations were found to decline and determined to display 4-5 days biological half-life. Contractile dysfunction in cardiomyocytes was overcome with Thiamin (10 μM) supplementation (Zangen and Shainberg 1997).

To further explain the mechanism by which TD disrupts the normal cellular process for regulating energy, studies in a rodent model of TD demonstrated a reduction in resting oxygen consumption and an increase in plasma lactate in rats fed TD diet (35 days) compared to those in thiamin control diets. Additionally, glutathione peroxidase and catalase activity in heart tissue samples were increased. Data suggested that TD disturbed redox balance resulting in apoptosis in cardiomyocytes (Gioda, de Oliveira Barreto et al. 2010).

2.1.3 Thiamin Transporters and mitochondrial thiamin pyrophosphate transporter

Transporters play an essential role in intracellular thiamin homeostasis. There are two human thiamin transporters that have been well established; thiamine transporter 1 (THTR1) encoded by SLC19A2 and thiamine transporters 2 (THTR2) encoded by SLC19A3. Both genes are members of a superfamily 19A of solute carrier protein which consist of three members, SLC19A1, SLC19A2 and SLC19A3 (Eudy, Spiegelstein et al. 2000) (Rajgopal, Edmondnson et al. 2001).

SLC19A2 is a thiamin transporter gene encoding a 497 amino acids 12 putative transmembrane domain. *In vitro* studies in a mouse neuroblastoma cell line, indicated high-affinity for SLC19A2 for thiamin (Km ~ 35 nM) (Bettendorff 1995). SLC19A2 is highly expressed in skeleton muscle and in the heart (Figure 2.1A) (Dutta, Huang et al. 1999). Furthermore, a study indicated that the transport mechanism of thiamin in over expressing hTHTR1 in HeLa cells showed a saturable process with a Kt of 2.5 ± 0.6 μM for thiamin (Dutta, Huang et al. 1999).

SLC19A3 encodes another high-affinity thiamin transporter 2 (THTR2) containing 496-amino acid with predicted molecular weight at 56 kDa. Studies conducted in Caco-2 cells expressing hTHTR-2 indicated an apparent Km of 27.1± 7.9 nM (Said, Balamurugan et al. 2004). SLC 19A3 is expressed ubiquitously in placenta, kidney and liver (Figure 2.1B) (Rajgopal, Edmondnson et al. 2001).

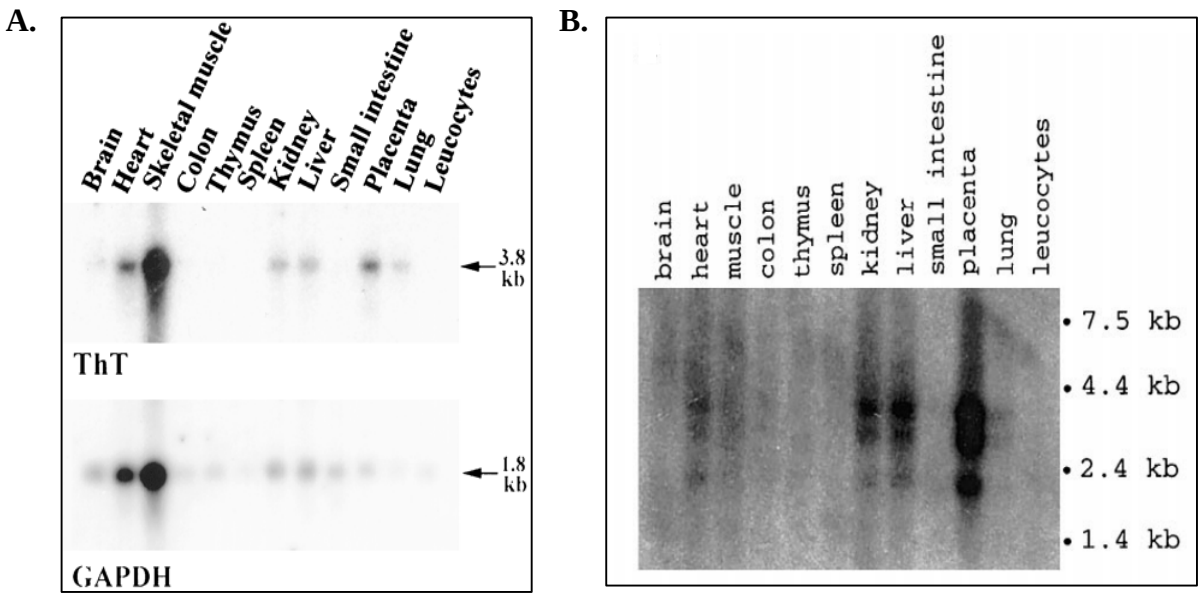


Figure 2.1 Northern blot analysis of thiamin transporter gene expression in human tissue samples (A) THTR1 (Dutta, Huang et al. 1999) and (B) THTR2 (Eudy, Spiegelstein et al. 2000)

SLC25A19 is a six-transmembrane-helix protein encoded 320 amino acid mitochondrial TPP transporter (MTPPT) that specifically facilitates transport of TPP across the inner mitochondrial membrane. Study in mouse liver mitochondria indicated Km = 6.79±0.53 µM. (Said, Balamurugan et al. 2004). However, the role of these transporters in the myocardium is unknown. Taken together, these transporters play a critical role for thiamin-mediated control of energy regulation.

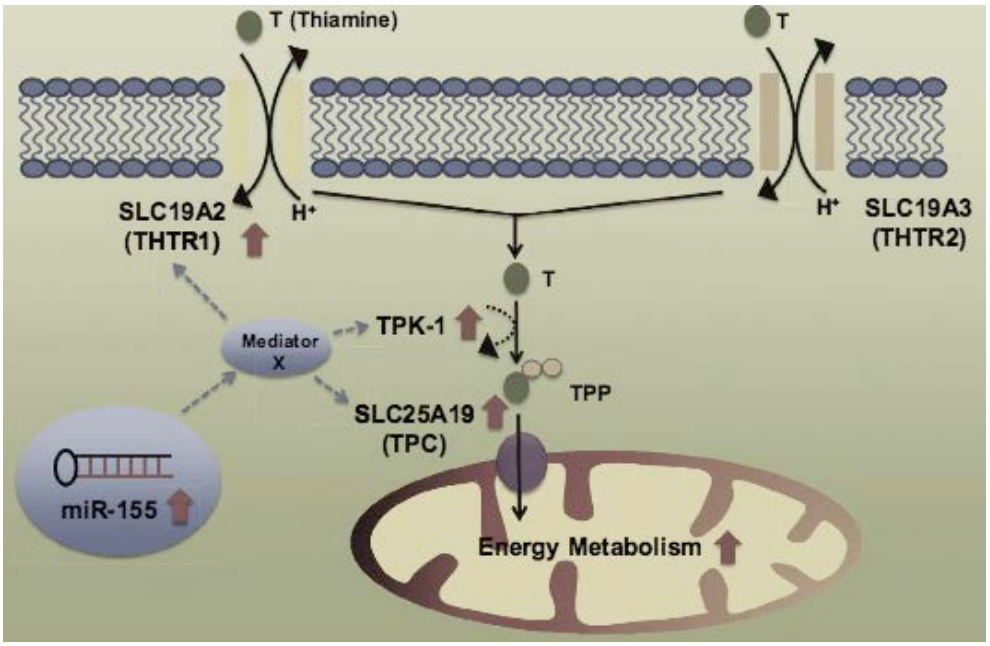


Figure 2.2 Transportation of thiamin required SLC19A2, SLC19A3 and SLC25A19 (Kim, Rhee et al. 2015)

2.1.4 Transporter deficiency and diseases

Mutations in SLC19A2 causes an autosomal recessive disorder so called Thiamin-responsive megaloblastic anemia syndrome (TRMA) characterized by megaloblastic anemia, non-type -1 diabetes mellitus and sensorineural deafness (Labay, Raz et al. 1999). This disease starts developing during infancy and in adolescence. Molecular pathology of TRMA is related to a change or defect of thiamin transportation via THTR1 in erythrocytes leading to low levels of intracellular thiamin and leads to apoptosis. Typically, thiamin at pharmacological doses (25-75 mg/day) improves anemia and diabetes in TMRA patients (Diaz, Banikazemi et al. 1999) (Neufeld, Fleming et al. 2001). Interestingly, although the active thiamin transporter in TMRA patients is dysfunctional, serum thiamin levels are at normal levels and patients don't display signs nor symptoms of thiamine deficiency. This is explained by a compensatory mechanism by another transporter system that exists and has a low affinity non-saturatable mechanism, which has not been fully investigated.

The human mutation in SLC19A3 leads to an impairment of intracellular thiamine transportation into cells resulting in brain dysfunction in terms of recurrent episodes of encephalopathy, so called biotin-thiamine-responsive basal ganglia disease (BBGD), which is usually existent during childhood (Tabarki, Al-Shafi et al. 2013). The lack of treatment with biotin (5-10 mg/kg/day) or thiamine (300 mg to 900 mg orally) can potentially result in the patient entering into a coma or death (Tabarki, Al-Shafi et al. 2013). In regards to the effects of mutations in the mitochondrial TPP transporter, SLC25A19, complications in the brain called the Amish lethal microcephaly (MCPHA), results in abnormal brain development and α-ketoglutaric aciduria. In these patients, data suggests a reduction of alpha-ketoglutarase complex activity was correlated with reduction of TPP transportation into mitochondria (Lindhurst, Fiermonte et al. 2006).

2.1.5 Regulation of thiamin transporters

Several studies have reported that intracellular thiamin uptake has been regulated by factors such as extracellular thiamin levels, high glucose levels (26 mmol/l), chronic alcohol exposure and hypoxic conditions in various cells. Studies conducted in human intestinal cells (CaCo cells) indicated that SLC19A3 mRNA expression was significantly increased (~2.5 fold) in thiamin deficient medium and this upregulation was found to be associated with SP1 transcriptional factor (Nabokina, Subramanian et al. 2013). Similar to *in vitro* studies in human-derived renal epithelial cells (HEK-293 cells) under a thiamin-deficient medium for 96 hours shown that SLC19A2 and SLC19A3 and protein level were increased significantly suggested that both genes were mediated by extracellular thiamin levels (Ashokkumar, Vaziri et al. 2006). Another recent study has suggested that SLC19A3 gene but not SLC19A2 mRNA level was increased in breast cancer cell lines under hypoxia (1% O_2) and chemical induced hypoxia (DFO 250 μM) (Sweet, Paul et al. 2010). In vitro studies in human primary proximal kidney tubule epithelial cells showed that

SLC19A3 mRNA and thiamin transporter 2 protein expression were decreased under high glucose condition (26 mmol/l) as well as SLC19A2 mRNA and thiamin transporter 1 protein level (Larkin, Zhang et al. 2012).

2.1.6 Biochemical functions

Thiamin pyrophosphate (TPP), an active form of thiamin, plays an important role in cellular production of energy. TPP functions as a cofactor; a non-protein chemical compound, for TPP-dependent enzymes involved in energy metabolism for glucose, and amino acids oxidation. (e.g. pyruvate dehydrogenase complex (PDHC), α-ketoglutarate dehydrogenase complex, branch-chain α-ketoacid acids dehydrogenase) and cytosolic enzyme, transketolase in the pentose phosphate pathway.

Pyruvate dehydrogenase complexes

PDHC is a mitochondrial enzyme that connects glycolysis and the citric acid cycle by catalyzing oxidative decarboxylation of pyruvic acid to acetyl CoA, which takes place within the matrix of mitochondria. Therefore, PDH plays a critical role in glucose oxidation in aerobic respiration to produce ATP (Martin, Rosenthal et al. 2005). TPP is involved in the first step of decarboxylation of pyruvate due to its contains acidic carbon atoms in a thiazole ring, which ionize to a carbanion ion, a free electron pair which acts as a nucleophile, and subsequently attack to the carbonyl group of pyruvate followed by decarboxylation, which catalyzed by pyruvate dehydrogenase component E1 (Berg, Tymoczko et al. 2002).

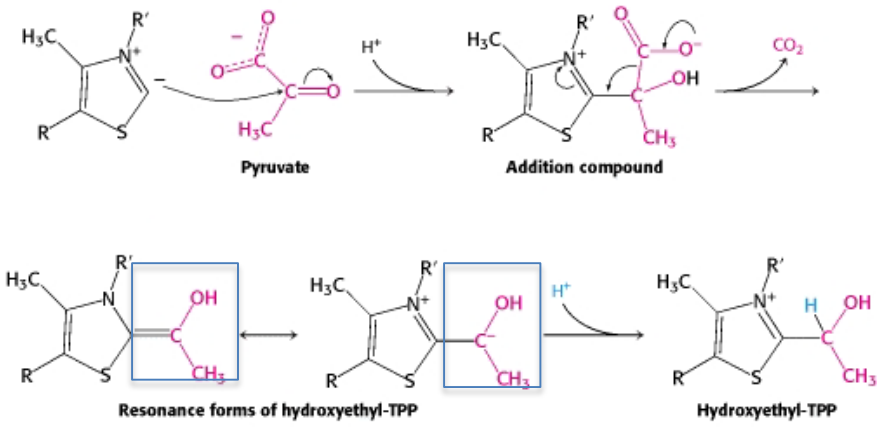


Figure 2.3 Step I reaction of pyruvate decarboxylation by TPP and PDC-EI (modified from (Berg, Tymoczko et al. 2002)

The regulation of pyruvate dehydrogenase is controlled by phosphorylation at PDH-E1α subunit by pyruvate dehydrogenase kinase 1(PDK-1) at Ser232, Ser293 and Ser300. The phosphorylation of these sites leads to an inactivation of PDH (Rardin, Wiley et al. 2009). However, inhibition of PDH can be reversed by pyruvate dehydrogenase phosphatase. It has been known that a decrease in the activity of pyruvate dehydrogenase is reported in diabetic patients. Seymour et al. has reported that total PDH activity is reduced in activity due to the reduction of the active form of the PDH fraction, which could affect mitochondria bioenergetics leading to failing in ATP production in diabetic heart thus contributing to the subsequent heart failure (Seymour and Chatham 1997). Nevertheless, thiamin supplementation in a model of diabetic animals and *in vitro* studies using cardiac fibroblast under high glucose helps improve PDH inhibition by diabetes (Kohda, Umeki et al. 2010).

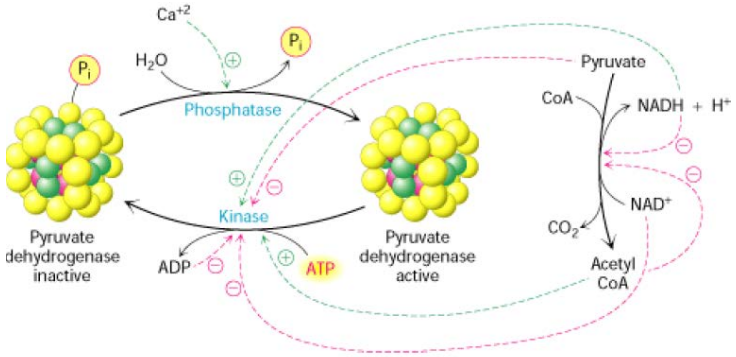


Figure 2.4 Factors mediated regulation of PDH (Berg, Tymoczko et al. 2002)

The pentose phosphate pathways; Transketolase

This pathway consists of two phases: oxidative and non-oxidative phases. Products from both phases are NADPH dependent, a powerful reducing agent involved in regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSH), and ribose 5-phosphate; RNA and DNA components. Transketolase is a thiamin diphosphate dependent enzyme which catalyzes the conversion of ribose 5– phosphate to glyceraldehyde-3 phosphate and thus links the Pentose phosphate pathway to glycolysis. Therefore, TPP functions as a carbanion that attacks the carbonyl group on substrates by the same mechanism as found in the pyruvate decarboxylase reaction (Berg, Tymoczko et al. 2002).

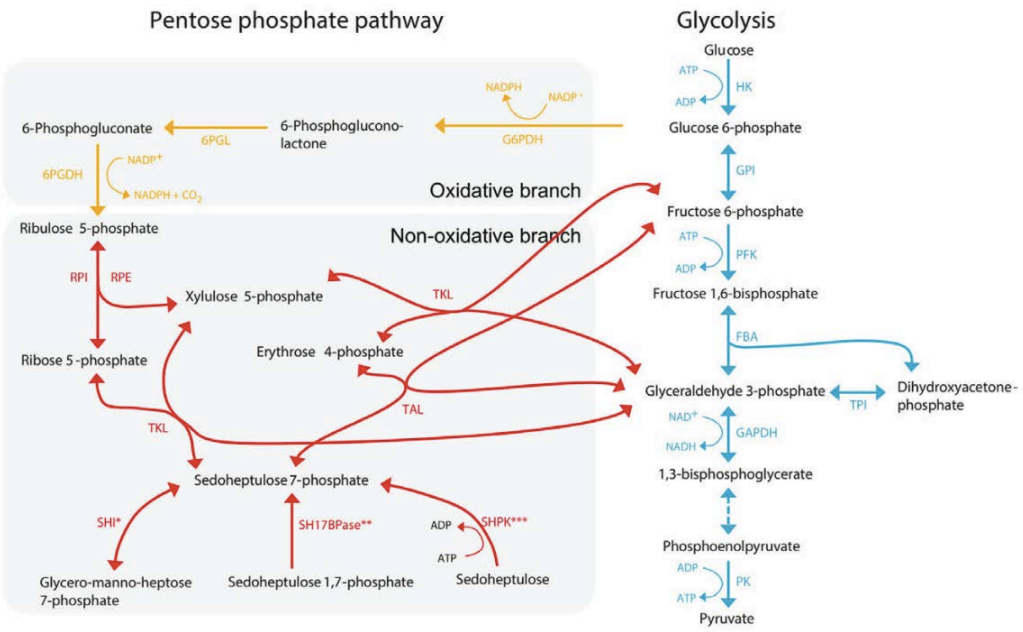


Figure 2.5 The pentose phosphate pathway demonstrates Transketolase, a TPP-dependent enzyme that links the pentose phosphate pathway to glycolysis via Fructose 6-phosphate and Glyceraldehyde 3-phosphate (Stincone, Prigione et al. 2014)

2.1.7 Pharmacokinetics

Absorption

Intestinal absorption of thiamin takes places in the jejunum. Since thiamin molecules contain a positive charge, transporter mechanisms are required. There are two transporters involved in the intestinal cellular uptake of thiamin; transporter 1 encoded by SLC19A2 and transporter 2 encoded by SLC19A3 (Eudy, Spiegelstein et al. 2000) (Rajgopal, Edmondnson et al. 2001). At low doses (<1.5 µM), thiamin is taken up via a saturable active transport system, while at higher concentrations, it undergoes passive diffusion (Rindi 1984). TMP and TPP in the intestine

are hydrolyzed to a free thiamine by intestinal phosphatases before absorption (e.g. TMPase and TPPase) (Kohlmeier 2003). Furthermore, TMP is also taken up the by reduced folate carrier (RFC-1) encoded by SLC19A1 (Zhao, Gao et al. 2002).

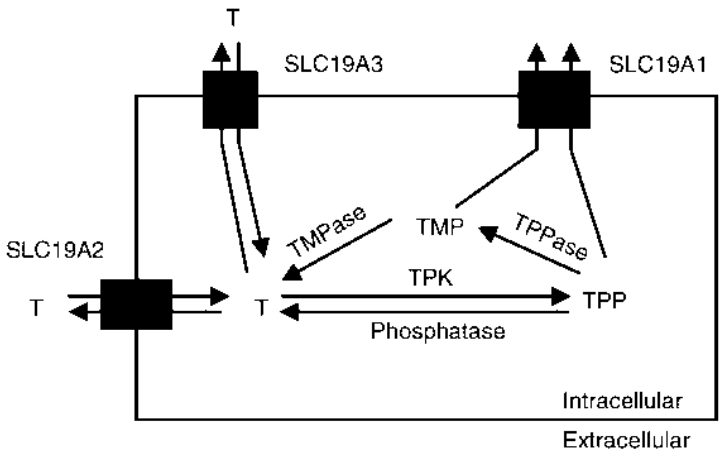


Figure 2.6 Transportation of thiamin and its derivatives via thiamin transporters (Zhao and Goldman 2013)

Distribution, disposition and excretions

After entering blood circulation, thiamin is taken up into erythrocytes via a saturable high affinity transporter. Thiamin is immediately phosphorylated to TPP by thiamin pyrophosphokinase 1 (TPK1) (Rindi G et al, 2000). Total human plasma (free + protein bound) thiamin concentration is very low approximately 10 -20 nM (Gangolf, Czerniecki et al. 2010), while TPP concentrations in erythrocytes is approximately 75% of blood thiamin content. (Manzetti, Zhang et al. 2014). Normal plasma concentration of thiamin in human is 80-150 nmol/L (Essa, Velez et al. 2011). There has been a report that total thiamin concentrations in an adult human are approximately 30 mg, with a biological half-life of 9.5–18.5 days in tissue. Thiamin deficiency may develop approximately 15 days after restricted intake. (Manzetti, Zhang et al. 2014) Further the urinary excretion of thiamin varies from 16.3 – 72.2 mg during 24 hours.

The major form of TPP is found ubiquitously in animal tissues (~80% of total thiamine level) (Leenheer, Lambert et al. 2000). Thiamin is found in high concentrations in skeletal muscle, heart, liver, kidneys and brain (Singleton and Martin 2001). Previously, tissue distribution of thiamin and phosphate esters was determined by HPLC methods in a variety of human biopsies. TPP was found predominantly in blood and heart auricles compared to other organs (Gangolf, Czerniecki et al. 2010).

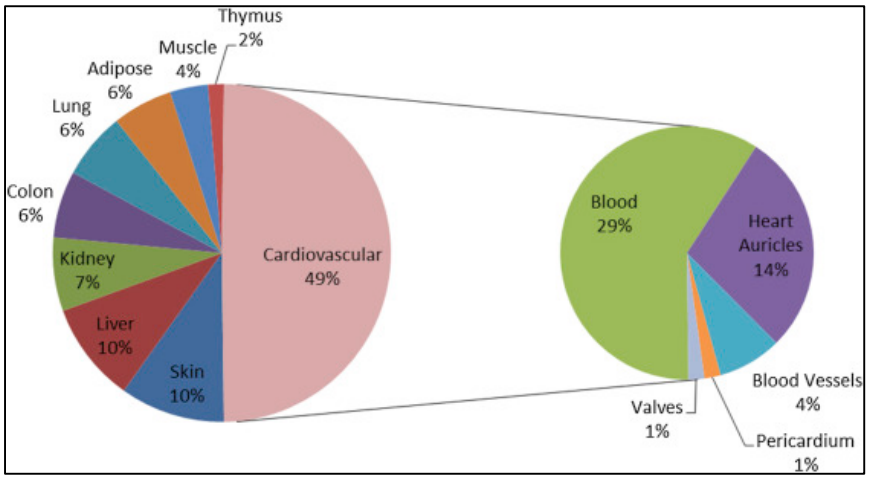


Figure 2.7 Distribution of total body thiamine in major organs in human (Jain, Mehta et al. 2015)

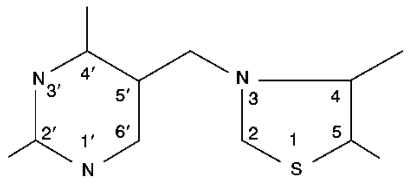
2.1.8 Chemical properties

The most common commercial forms of thiamin are hydrochloride salt. It is a white crystalline substance with a molecular weight of 337.28. Thiamin hydrochloride is freely soluble in water (1 g/ml) and slightly soluble in 95% alcohol (1g/100 ml) but more soluble in methanol. (O'Neil 2001) The thiamin aqueous solution is stable to heat and oxidation in acidic solutions between pH 2.0 to 5.5. At this condition, thiamine can be sterilized at 110 °C. It is rapidly degraded under the influence of heat if the pH of solution is higher than pH 5.5. It is oxidized to thiochrome

by oxidizing agent (e.g. potassium ferricyanade), alkaline solution (pH >5.5) and ultraviolet light (Rucker 2001) (Leenheer, Lambert et al. 2000).

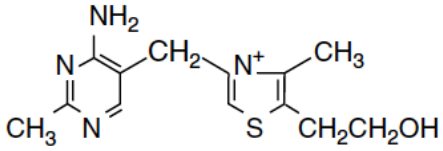
The chemical structure of thiamine consists of a pyrimidine (2, 5-dimethyl-6-aminopyrimidine) and a thiazole moieties (4-methyl-5-hydroxy ethyl thiazole) linked by a methylene bridge. An amino group on C4 of the pyrimidine ring, a quaternary nitrogen, and alkyl group (phosphorylated site) at C5 of the thiazole ring are required for its physiological function (Berdanier and Berdanier 2015). The structures of thiamin and phosphate esters are shown in figure 2.8.

A.

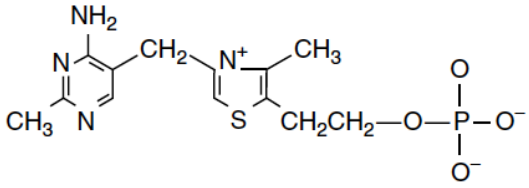


Pyrimidine ring **Thiazole ring**

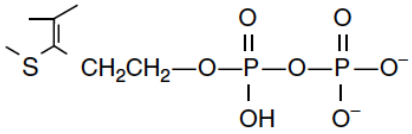
B.



C.



D.



E.

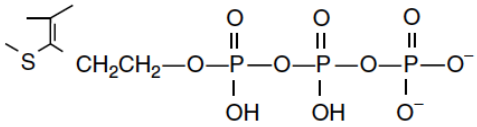


Figure 2.8 Structure of thiamin and its derivatives (A) thiamin backbone (B) thiamin (C)thiamin monophosphate (D) thiamin diphosphate (E) thiamin triphosphate

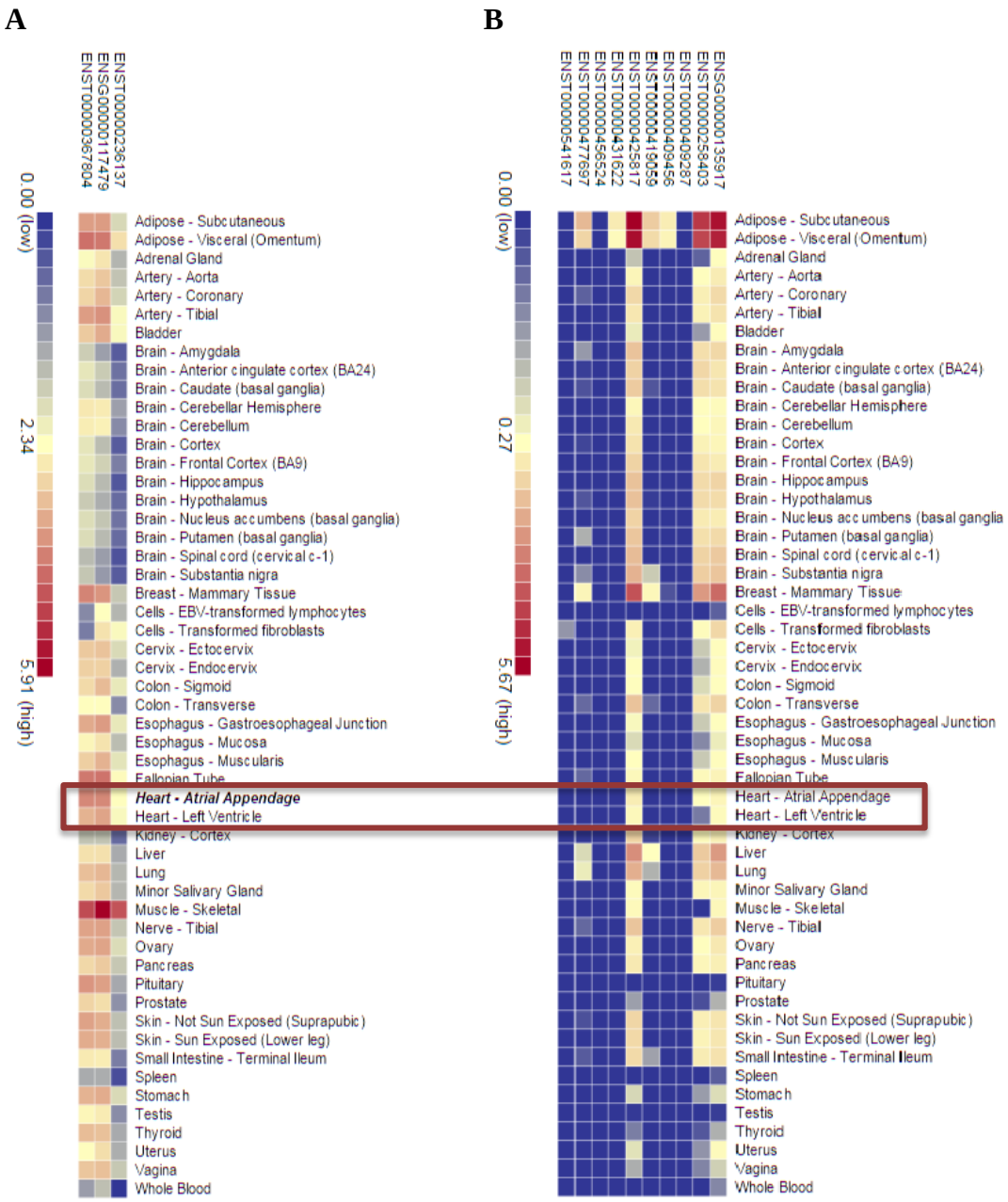


Figure 2.9 Heat maps shows relative expressions of thiamin transporter genes in human by tissue types (A) SLC19A2 and (B) SLC19A3. Both genes are expressed at moderate levels in the human heart. The UCSF Pharmacogenomics of Membrane Transporters project database is supported by NIH/NIGMS (U01 GM61390)

2.2 Hypoxia Inducible Factors (HIFs)

2.2.1 Introduction to HIF-1

Hypoxia is defined as a reduction in oxygen tension and is involved in several pathophysiologies of cardiovascular disease, cerebrovascular disease and cancer (Semenza 2000).

The hypoxia inducible factor (HIF) is a heterodimeric transcriptional factor composed of alpha and beta subunits. There are three isoforms of alpha subunits; HIF-1α, HIF-2α and HIF-3α that have been identified. It has been determined that HIF-1α and HIF-2α play key roles as transcriptional regulators in response to low level of oxygen tension. They have greatly similar amino acid sequences, but differ in tissue distribution pattern. HIF-1α is ubiquitously expressed in most tissues and has a general role in multiple physiological responses to hypoxia, whereas HIF-2α has specifically tissue distribution particularly in the lung, endothelium, and carotid body (Ke and Costa 2006).

The structure of both HIF subunits contains the basic-helix-loop-helix (bHLH) domain, Per-Aryl hydrocarbon nuclear translocator (ARNT) -Sim (PAS) domain and transcriptional activation domains (TADs). Additionally, HIF-α subunit contains the oxygen-dependent degradation domain (ODDD), two transcriptional activation domains (TADs) located at the N and C terminal and the transcriptional inhibitory domain (ID) (Lisy and Peet 2008).

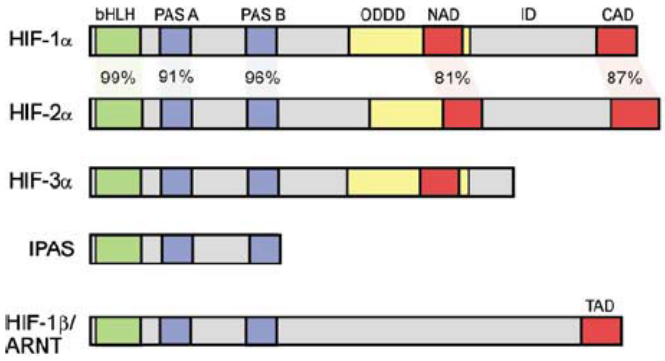


Figure 2.10 Structure of HIF family (Lisy and Peet 2008)

The activity of HIF-1α is regulated by cellular oxygen availability by two independent mechanisms. (Hirsila, Koivunen et al. 2005) Under normoxia, HIF-1α is rapidly degraded by the ubiquitin-mediated proteolysis pathway; therefore, its biological half-life is short approximately 5-8 mins (Edurne Berra et al., 2001). Degradation of HIF-1α is regulated by hydroxylation of its proline residues at P406 and P562 located on the oxygen dependent degradation (ODD) site by the oxygen-dependent prolyl hydroxylases (PHDs; PDH 1, 2 or 3 isoforms). Hydroxylation recognizes the binding of von Hippel-Lindau (pVHL) tumor suppressor protein to HIF-1α resulting in ubiquitination at the 26S proteasome. On the contrary, HIF-1α subunit is stabilized at low levels of cellular oxygen (<5%) due to a decrease in PHD function (Bing-Hua Jiang et al., 1997). PHD functionally relies on molecular oxygen, Fe^{2+} and 2-oxoglutarate (Ivan, Haberberger et al. 2002). Under low oxygen tension, HIF-1α translocates into the nucleus and dimerizes with HIF-1β resulting in the complex binding to the hypoxia response elements (HREs) on the promoter region of its target gene. However, transactivation by coactivators; Creb-Binding Protein (CBP) and p300 at NAD or CAD is required for transcriptional activity of HIF-1. Under normoxia, hydroxylation at asparagine 803 residues in C-TAD by factor inhibiting HIF-1 (FIH) prevents the binding of co-activators, leading to inhibition of HIF-1α transactivation function (Mahon, Hirota et al. 2001, Lando, Peet et al. 2002). Like PHDs, FIH enzyme activity also depends on molecular oxygen, Fe^{2+} and 2-oxoglutarate. Therefore, under hypoxia, FIH activity is impaired leading to the binding of co-activators to CAD which driving HIF-1 transcriptional activity.

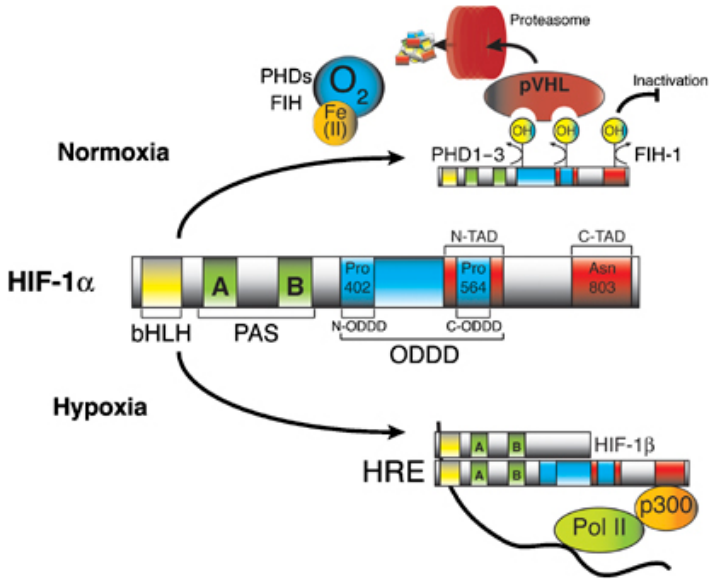


Figure 2.11 the structure and the regulation of HIF-1 (Weidemann and Johnson 2008)

2.2.2 HIF-1 α target gene

HIF-1 is a master transcriptional activator that induces a broad range of genes involved in angiogenesis, metabolic homeostasis (e.g. glucose metabolism, ATP production) and cell survival. The intensity in expression of HIF-1 target genes varies from tissue type, duration of hypoxia exposure (acute, intermittent and chronic) and severity of oxygen deprivation.

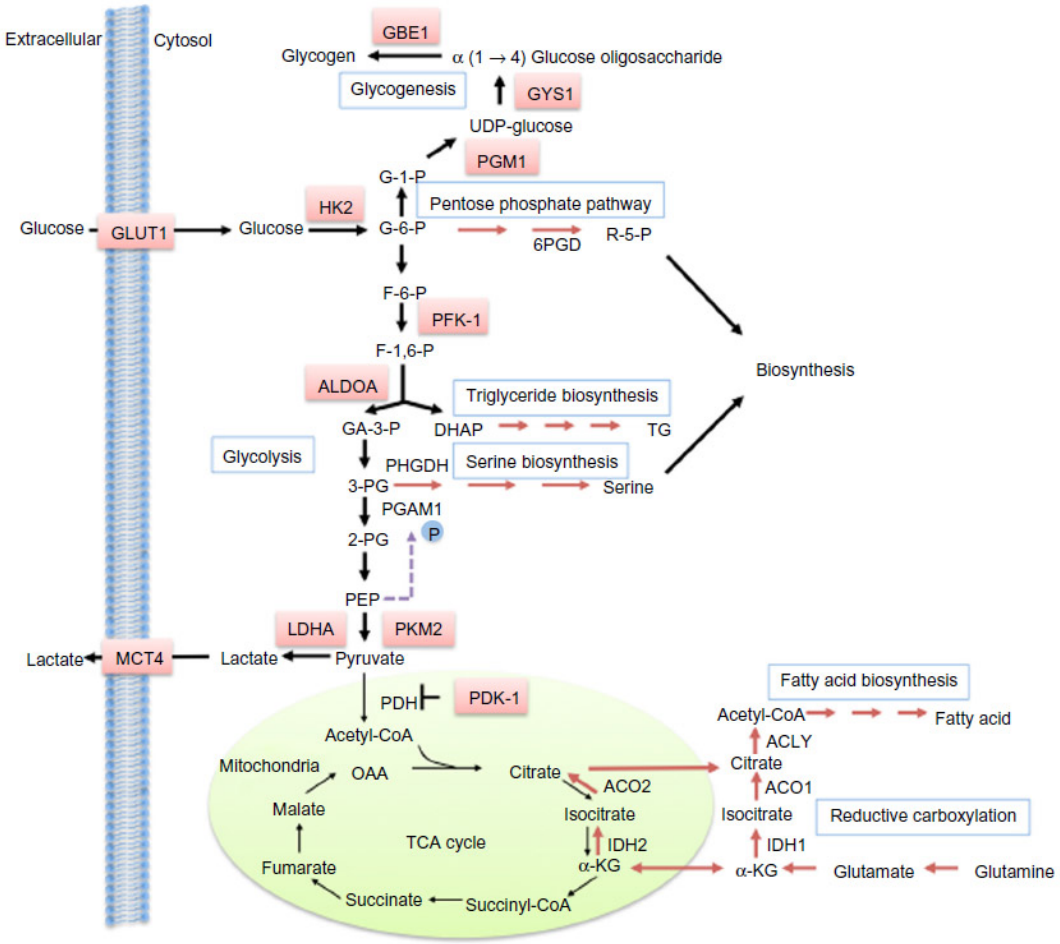


Figure 2.12 Summary of HIF-1 target genes (Ming Yang,1 Huizhong Su,1 Tomoyoshi Soga,2 Kamil R Kranc,3 Patrick J Pollard1Prolyl hydroxylase domain enzymes: important regulators of cancer metabolism)

Gene	Protein	Cells/ Hypoxic conditions	Reference
SLC2A1	Glucose transporter 1	Rat1-*ras* and Rat1 cells/ hypoxia (0.2% oxygen) for 6 h.	(Chen, Pore et al. 2001)
HK2	hexokinase 2	Human pulmonary cells (A549 cells) / DFO 200 μM, 0% O2 induction occurred at 6 h.	(Riddle, Ahmad et al. 2000)
LDHA	Lactate dehydrogenase A	Hep 3B cells / CoCl2, DFO or 1% O2 for 4 h (5'-RCGTG-3')	(Semenza, Jiang et al. 1996)
PDK1	Pyruvate dehydrogenase kinase 1	human lymphoma cells (P493-6) , 0.1% - 0.5% O2 , 22-48 h CoCl2 (100 μM)	(Kim, Tchernyshyov et al. 2006)
PDK3	Pyruvate dehydrogenase kinase 3	HeLa cells, 1% O2 for 16 and 24 h	(Lu, Lin et al. 2008)
COX4-1 and COX4-2	Cytochrome c oxidase	Hep3B cells (liver), HeLa cells (uterus), Hct116 cells (colon) and A594 cells (lung)/ Hypoxia (1% O2) for 24 h or CoCl2 , DFO and DMOG for 24 h	(Fukuda, Zhang et al. 2007)

Table 2.1 Example of selected HIF-1 target genes

2.2.3 HIF-1α and cardioprotection

HIF-1 mediates a favorable role in protection of cardiovascular disease including myocardial ischemia and paradoxically HIF-1 also mediates apoptosis in prolonged hypoxia. (Semenza 2000). Previous studies reported protective effects of cardiac specific HIF-1α overexpression in rodent models of diabetes (STZ-induced type I DM,) by preventing the reduction of regulated glucose transporter 1 (GLUT-1) and Hexokinase II (HK-II) mRNA expression (glycolytic enzyme) in diabetic rodent models. In these studies the investigators also observed improved cardiac ATP production (Xue, Cai et al. 2010), when compared to diabetic rodents.

HIF-1 has a role as an endogenous cardioprotection during acute ischemia (Garcia-Dorado, Rodriguez-Sinovas et al. 2014). Over three decades, it has been known that ischemic preconditioning (IP), a technique that induces a brief episodes of ischemia prior to I/R surgery, protects the heart against ischemia-reperfusion injury by limiting the infarction size due to an increase in tolerance of the heart against I/R damage (Murry, Jennings et al. 1986) (Das and Das 2008). Activation of HIF-1 by various mechanisms including pharmacological agents, PHD2 siRNA and gene therapy that mimics preconditioning phenomenon prior to ischemia– reperfusion (I/R) surgery have been demonstrated to protect against cardiac ischemia-reperfusion injury in animal models (Semenza 2001, Eckle, Kohler et al. 2008, Tekin, Dursun et al. 2010).

2.2.4 Induction of hypoxia inducible factor in *in vitro* models

Divalent transition metal, cobalt, has been well established for mimicking hypoxia induced HIF-1α in *vitro* model at an effective concentration 100 μM (Fukuda, Zhang et al. 2007) (Kim, Tchernyshyov et al. 2006)). The mechanism of action for HIF-1 stabilization by $CoCl_2$ has been suggested to be due to its ability to bind to the iron-binding site on the proline hydroxylases, which

leads to the inhibition of pVHL binding to HIF-1α. $CoCl_2$ has been observed to also be a potent inhibitor of FIH (Hirsila, Koivunen et al. 2005) (Hirsila, Koivunen et al. 2005).

Another compound that has been observed to stabilize HIF-1 is the non-specific prolylhydroxylase inhibitor dimethyloxalylglycine (DMOG), analog of α-ketoglutarate (Zhdanov, Okkelman et al. 2015, Mayorga, Kiedrowski et al. 2016). The utilization of this compound in rodent models to induced HIF-1 varies from 40 to 100 mg/kg (Poynter, Manukyan et al. 2011, Zhang, Ma et al. 2016) and 1 mM in H9c2 cardiomyocyte cells (Mayorga, Kiedrowski et al. 2016).

2.3 Ischemic-Reperfusion injury

2.3.1 Introduction to myocardial ischemia and reperfusion injury

Myocardial ischemia is a condition that coronary blood flow is restricted concomitant with lack of oxygen supply to certain area distal to narrowed coronary artery. Once oxygen demand is not sufficient with oxygen supply (i.e. during physical exertion, emotional stress), patient can develop to complications called Angina pectoris (also known as stable angina), which is referred to typically chest pain or pain can possible be spread to left arm, shoulder and neck. Furthermore, acute blockaded of thrombus can lead to myocardial infarction. According to AACCF/AHA guideline for the management of ST-Elevation myocardial infarction (2013), reperfusion therapy including either using fibrinolytic therapy or percutaneous coronary intervention (PCI) is indicated for myocardial infarction with ST-Elevation patients. PCI is first line therapy and is recommended to be performed in a timely manner prior to experiencing symptoms or EKG confirmation of ST elevation in order to prevent irreversible cells damage, limit infarct size and improve the left ventricular function. However, re-introducing blood flow to the ischemic heart so called reperfusion results in unfavorable effects to the ischemic heart. Possible pathophysiological mechanisms as a consequence of I/R injury are shown in figure 2.13.

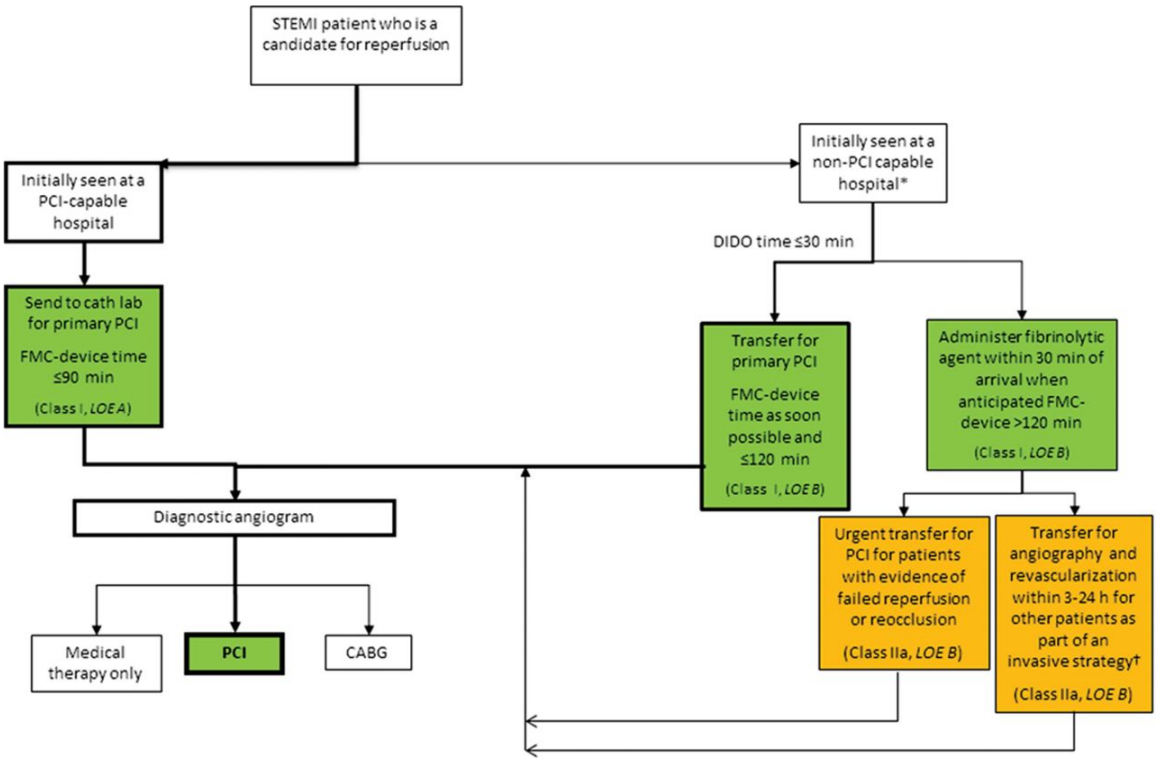


Figure 2.13 Timeline for treatment of ST-elevation according to AACCF/AHA guideline (2013)

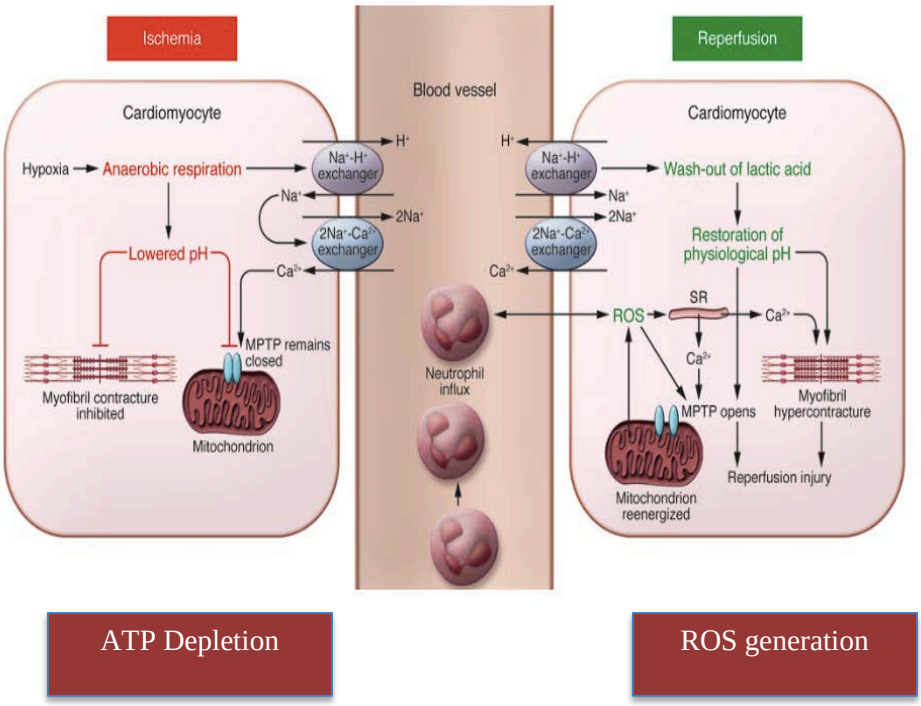


Figure 2.14 Molecular mechanisms of ischemic and reperfusion injury (modified from (Hausenloy and Yellon 2013))

In brief, it has been known that metabolic switch from aerobic to anaerobic glycolysis respiration is considered as an ischemic phenotype of hypoxic cardiomyocytes in response to low level of O_2 during lack of blood supply. Oxidative phosphorylation and citric acid cycle are inhibited due to insufficient oxygen levels leading to accumulation and activation of Hypoxia-inducible factors (HIF), which transcriptionally regulates glucose transporters (GLUT-1 and GLUT-3) genes as well as several glycolytic enzymes as shown in table 2.1. Hypoxic cells utilize glucose as a main energy source for ATP production via glycolysis as a result of accumulation of lactic acid (Depre, Vanoverschelde et al. 1999). Disruption of ionic as a consequence of ischemia is explained by activation of the Na^+/H^+ exchanger to efflux H+ to extracellular fluid in exchange to influx of Na^+. Then, Na^+/Ca^{2+} exchange is stimulated to transport Na^+ in exchange to Ca^{2+} resulting in intracellular Ca^{2+} overload, which can activate enzymes such as phospholipases,

nucleases, and proteases to damage protein resulting in irreversible necrosis. Moreover, accumulation of hypoxanthine was reported during ischemia, which can be converted to xanthine by xanthine oxidase which results in the development of more reactive oxygen species (ROS) during reperfusion. ROS has been known as the primary contributor to induce myocardial necrosis during reperfusion. ROS generation results from several mechanisms including reactivation of oxidative phosphorylation and electron transport chain and conversion of hypoxanthine to xanthine by xanthine oxidase. The consequences of excessive ROS are lipid peroxidation, induction of Ca^{2+} release from damaged sarcoplasmic reticulum leading to intracellular calcium overload. Hypercontraction of the myocardium triggered by calcium overload results in arrhythmia during initial phases of reperfusion. Rapid reduction of the cellular pH triggers opening of mitochondrial permeability transition pore (MPTP) located in inner membrane leading to an increase in the mitochondria permeability, allowing macromolecules into the mitochondria and mitochondria swelling which propagates cytochrome c release and the activation of program cell death (Yellon and Hausenloy 2007, Hausenloy and Yellon 2013).

Therapeutic interventions

Recent studies in cardiovascular research have been focused on therapeutic strategies for preventing cardiovascular events after restoration of blood flow. Unfortunately, there has been a limitation of effective pharmacologic therapies that prevent myocardial ischemia-reperfusion injury and mostly fail to translate to the clinical setting. Cyclosporine is an example of a pharmaceutical therapeutic strategy that successfully translated protective effects from animal model studies to phase 2 clinical trials (Piot, Croisille et al. 2008). Only mechanical therapeutic intervention including induction of brief episodes of ischemia so called myocardial conditioning and therapeutic hypothermia (recommend in AACCF/AHA guideline: evidence level B) have been

well established to offer significant reduction in myocardial infarction size both in experimental animal models (Murry, Jennings et al. 1986) and in phase 2 clinical trials (Hausenloy and Yellon 2013, Ovize, Thibault et al. 2013). Recently, a novel therapeutic intervention for prevention of reperfusion injury has been introduced to modify the myocardial energy substrate metabolism (Jaswal, Keung et al. 2011). Several pharmaceutical compounds such as L-carnitine and dichloroacetate (DCA), pyruvate dehydrogenase kinase (PDK) inhibitor, have been shown to protect against I/R injury in vivo mice model by either stimulation of glucose oxidation via pyruvate oxidation by pyruvate dehydrogenase (PDH) or inhibit fatty acid oxidation during reperfusion period (Ussher, Wang et al. 2012).

(4) Stimulation of glucose oxidation by Pharmaceutical dichloroacetate

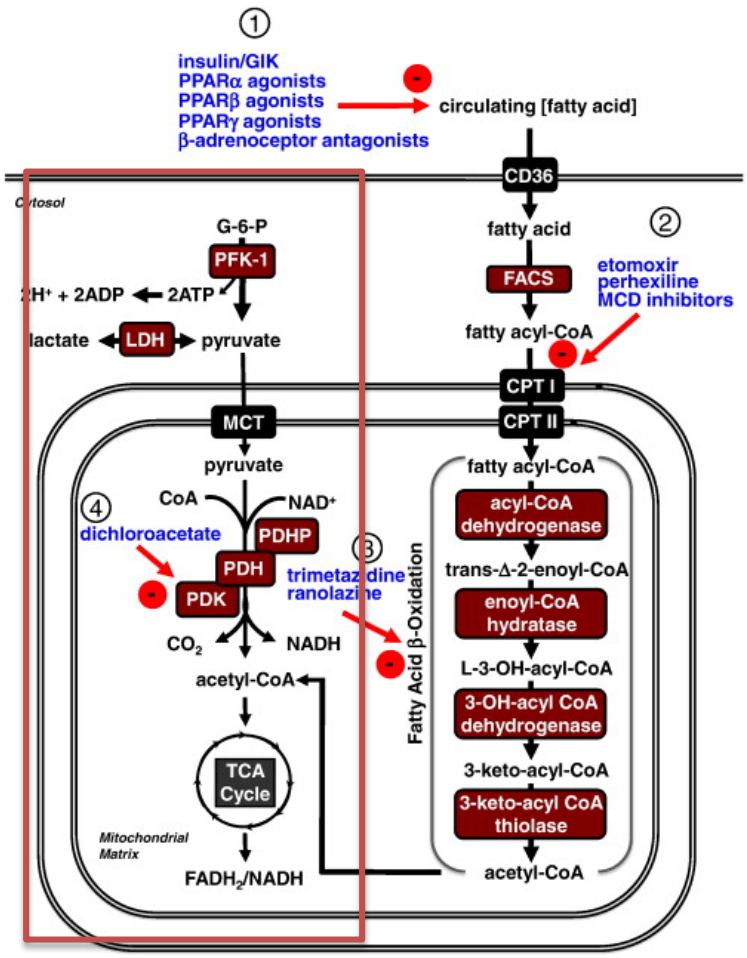


Figure 2.15 Molecular mechanisms of pharmaceutical compounds that modulate energy metabolism (modified from (Jaswal, Keung et al. 2011)

2.3.2 Thiamin and ischemic-reperfusion injury

Recently thiamin has been observed to display cardio-protective effects against ischemic-reperfusion injury in *in vitro* and in several animal models of myocardial ischemia. However, the molecular mechanism underlying its protection is not well understood**.** *In vivo* animal models i.e., dog models (n=14), post-administration of thiamine pyrophosphate (TPP, 150 mg/kg via central line 15, 45 min after 60 minutes of LAD ligation) has been observed to offer cardioprotective effects by improving systemic hemodynamic results (Larrieu, Yazdanfar et al. 1987). In rodent models of myocardial infarction, 2 hours pretreatment with thiamin (200 mg/kg) enhanced

resistance to ischemia-induced cell death determined by an increase in the relative volume of protection zone (Vinogradov, Shneider et al. 1991). Administration of thiamin (60 mg/kg i.p.) 30 minutes or 1% thiamin in drinking water 7 days before middle cerebral artery occlusion (30 min occlusion, reperfusion 3 days) in rats was reported to attenuate the reactive oxygen species-induced reductions in PDH and ketoglutarate activities and reduced the middle cerebral artery occlusion-induced size of infarct. (Sheline and Wei 2006)

To extend the cardio-protective emphasis conferred by thiamin against ischemia reperfusion injury into diabetes, benfotiamine, a synthetic S-acyl derivative of thiamin, (70 mg/kg daily in drinking water for 4 weeks) has been observed to offer protection against myocardial ischemia (permanent ligation of LAD for 2 weeks) in type 1 diabetes mellitus murine model. This report observed that the cardioprotective signaling of benfotiamine induced G6PD and transketolase enzyme activity, which ameliorates advanced glycation end-product (AGE) and induced the pro-survival VEGF receptor-2/Akt/Pim-1 signaling pathway (Katare, Caporali et al. 2010).

2.3.3 Cardiac ischemic/reperfusion surgery in murine model

Cardiac surgical procedure

Temporary occlusion of the left anterior descending (LAD) coronary artery followed by reperfusion is a surgical procedure that is extensively used to mimic pathophysiology and underlying mechanisms of myocardial ischemia-reperfusion (IR) injury in murine model (Bohl, Medway et al. 2009). Methods for myocardial ischemia–reperfusion have been detailed in Michael et al, 1995 (Michael, Entman et al. 1995) (Tarnavski 2009). The model requires that the duration of ischemia to be between 30 to 60 minutes resulting in the development of an injury (myocardial infarction) with 20 to 65% area at risk (AAR) and this duration of ischemia was improved survival

rates of mice during the surgery (Michael, Entman et al. 1995). In addition they observed that the size of the infarction is proportional to the duration of ischemia. For example, the extension in time of the vascular occlusion from 30 to 60 minutes significantly increased the infarction size (IS) from 18% to 69%. However, the size of the infarction was not dependent upon extended time of reperfusion from 120 to 240 minutes in murine model (Redel, Jazbutyte et al. 2008). Therefore, these investigators offered that the optimal time points for observing up-regulation of molecular signaling for development of ischemic injury was 30 minutes of ischemia followed by 120 minutes of reperfusion.

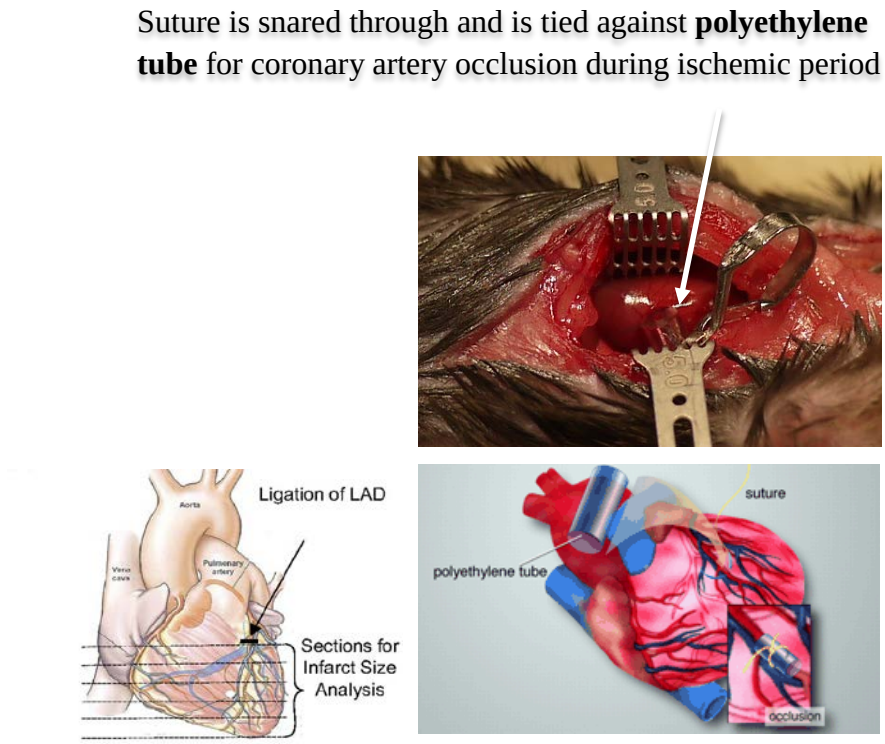


Figure 2.16 Schematic diagram of Left Anterior Descending coronary artery occlusion (modified from (Klocke, Tian et al. 2007)

Staining methods

To assess the histopathology of the heart following the myocardial IR surgery we

completed an Evan blue dye and 2, 3, 5-triphenyltetrazolium chloride (TTC) stain to assess the areas at risk (AAR), the necrotic and the perfused regions of the heart (Michael, Entman et al. 1995). Evans blue dye has been used widely for viability assay to differentiate between perfused and non-perfused myocardium. The area at risk (AAR) and infarct area (IS) remains unstained by Evan blue dye. TTC is used for staining heart slices by separating between areas of survival and areas of necrosis. This is accomplished by imaging viable myocardium that has been reduced to a deep red color when active mitochondrial dehydrogenases and reductases reduce the 1,3,5-triphenyltetrasolium or triphynyl formazan compound. Further the infarcted area remains unstained and thus appears pale white (Steffen Bohl et al., 2009).

Assessment of area at risk (AAR) and infarct area (IS)

Area at risk (AAR) is defined as the area located distal to and supplied by the occluded coronary artery (Management of Myocardial Reperfusion Injury). Infarct area (IS) is defined as after double staining with Evan's blue dye and TTC. Images usually are taken with a digital camera and the AAR and IS can be quantified by ImageJ. There are two methods to calculate AAR and IS; area-based and length-based method. Area-based method is used to quantify infarct area and total area of LV myocardium. Measurement of infarct size is usually expressed as a percentage of area at risk. The area at risk is expressed as a ratio to totally area of each heart slice (Takagawa, Zhang et al. 2007).

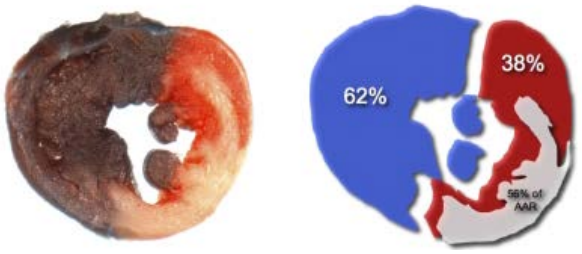


Figure 2.17 Assessment of perfused (blue), area at risk (red) and necrotic area (white) (modified from (Klocke, Tian et al. 2007, Bohl, Medway et al. 2009)

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell culture reagents

Dulbecco's modified Eagle's medium (DMEM) (containing 4,500 mg/L or 2.5 mM glucose and 4 mM L-glutamine, 1 mM sodium pyruvate and 1500 mg/L sodium bicarbonate), Trypsin-Versene Mixture and Penicillin/Streptomycin (P/S) were purchased from Lonza (Walkersville, MD). Fetal Bovine Serum (FBS) was from Hyclone (Logan, UT).

3.1.2 Pharmaceutical compounds and hypoxia mimic reagents

S-Benzoylthiamine o-monophosphate was purchased from MP Biomedicals (LLC, France). Thiamin Hydrochloride was from EMD Milipore (Billerica, Massachusetts). Thiamin pyrophosphate chloride was obtained from Tokyo Chemical Industry Co, Ltd (Tokyo, Japan). Cobalt (II) chloride hexahydrate, Thiamin pyrophosphate (pharmaceutical secondary standard grade) and Thiamin-(4-methyl-13C-thiazol-5-yl-^{13}C3) hydrochloride were from Sigma-Aldrich (St Louis, MO). Dimethyloxalylglycine (DMOG) was obtained from Frontier Scientific (Logan, UT).

3.1.3 Primers and qRT-PCR reagents

Qiazol® lysis reagent was purchased from Qiagen Sciences (MD, USA). Vesso® cDNA synthesis kit and SYBR Green were from Thermo Scientific (Rockford, IL). All primers used in this study were purchased from Integrated DNA Technologies as given in Table 3.1.

Primers	**Oligonucleotides Sequence (5'-3')**	**Range**	**Tm**
Slc19a2 NM_001276455.1	Sense 5'-GGGCCTTGGCTTGTGTATTA-3 Antisense 5'-CTGTGCAGAGTCCTTGCTTG-3'	20 bp 20 bp	55.2 56.2
Slc19a3 NM_001108228.1	Sense 5'-GCTCAGCATCTGGGTGTGCTATACT-3' Antisense 5'-TCTATGCCGAACACCAGGGCATAA-3'	24 bp 24 bp	60.4 60.3
Slc19a3 NM_030556.2	Sense 5'-AGAAGCGACTCAATGGAGGA-3' Antisense 5'-GGTGGGAAGATTCACTGG AA-3'	20 bp 20 bp	55.7 54.5
β-actin NM_031144.3	Sense 5'-TTGCTGACAGGATGCAGAAGGAGA-3' Antisense 5'-ACTCCTGCTTGCTGATCCACATCT-3'	24 bp 24 bp	60.2 60.3

Table 3.1 List of primers

3.1.4 Antibodies and Western blot reagents

SLC19A3 Polyclonal Antibody, Anti-rabbit was obtained from Proteintech (Rosemont, IL). Pyruvate Dehydrogenase Antibody, Anti-rabbit IgG and Protease Inhibitor Cocktail (100X) were

from Cell Signaling Technologies (Danvers, MA). HIF-1α rabbit monoclonal IgG was purchased from Santa Cruz Biotechnology. Anti-alpha tubulin was from DSHB (University of Iowa, IW). DYKDDDDK tag (D6W5B) Rabbit mAb was from Cell Signaling (Danvers, Mass.). Pierce® BCA Protein Assay kit, 0.5M EDTA solution and Halt Protease Inhibitor cocktail (100X) were from Thermo Scientific (Rockford, IL). Luminata® Forte Western HRP Substrate was obtained from Millipore (Billerica, MA). Blotting-Grade Blocker (nonfat dry milk), 40% Acrylamide/Bis Solution (29:1), Nitrocellulose membranes (0.45 µm) and Precision Plus Protein Standards were purchased from BioRad (Hercules, California). Anti-alpha-tubulin mouse was obtained from DSHB (Iowa City, IO). Bovine Serum Albumin (BSA) was purchased from EMD Chemical Inc. (Gibbstown, NJ). Cell lysis buffer (10x) was from Cell Signaling.

3.1.5 Cell line

H9c2 cardiomyocyte cells were purchased from American Type Culture Collection (Manassas, VA) embryonic rat heart-derived cells that were used in this experiment an *in vitro* model are representative of ventricular cardiac muscles.

3.1.6 Plasmid and viral construction system

SLC19a3 siRNA/shRNA/RNAi lentivirus (Rat) pooled was from Applied Biological Materials (ABM) Inc. (BC, Canada), SLC19A3-adenovirus #VH846121 was from Vigene Biosciences (Rockville, MD) 5HRE/GFP plasmid #46926 was 5HRE/GFP was a gift from Martin Brown & Thomas Foster (Addgene plasmid # 46926) HRE-luciferase (plasmid#26731) was from Addgene. Polybrene was from Santa Cruz Biotechnology (Dallas, Texas).

3.1.7 Fluorescent dye

Tetramethylrhodamine ethyl esterperchlorate (TMRE) was obtained from Biotium (Hayward, CA).

3.1.8 Instrumentations

BD Accuri C6 (BD Biosciences), ChemiDoc XRS HQ (Bio-Rad), iQ5 real-time PCR detection system (Bio-Rad), NanoDrop 2000c UV-Vis Spectrophotometer (Themo Scientific), PCR Thermal Cyclers (Bio-Rad).

3.1.9 Software

BD Accuri (BD Biosciences), ImageJ 1.49 software (NIH), Quantity-one analysis software (Bio-Rad), GraphPad Prism 6

3.2 Methods

3.2.1 Cell culture procedure

H9c2 cells were grown according to manufacturer instruction. Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, 4.5 g/L glucose) supplemented with 10% Fetal Bovine Serum (FBS) and 5% Penicillin/Streptomycin (P/S) in 75 cm^2 tissue culture flasks in a 37 ° C humidified incubator at atmospheric O_2 levels with 5% CO_2. Cells were routinely passaged at around 80–90% confluence and medium was changed every 2-3 days. The passage number used did not exceed more than 10 passages.

3.2.2 Stabilization of HIF-1 in cell culture

Chemical hypoxia: For HIF-1α activation, cells were incubated under chemical hypoxia using either dimethyloxalylglycine (DMOG) at a final concentration of 1 mM or cobalt (II) chloride ($CoCl_2$) at a final concentration of 400 µM in 21% oxygen (normoxia), 5% CO_2 under 37 °C humidified atmospheric incubator. The incubation time with DMOG or $CoCl_2$ was 24, 48, 72 and 96 hours of exposure.

3.2.3 The quantitative real-time polymerase chain reaction (qRT-PCR)

3.2.3.1 Primer Designing

Primers were designed using IDT's Primerquest® tool (http://www.idtdna.com/Primerquest/Home/Index) and were selected based on primer length, the terminal nucleotide, GC content and Tm as previous described. (Dieffenbach, Lowe et al. 1993) All designed oligonucleotide primers were verified specificity and efficiency of amplification by using Basic Local Alignment Search Tool® (http://blast.ncbi.nlm.nih.gov/Blast.cgifrom).

3.2.3.2 RNA isolation

Total RNA was purified from H9c2 cells using Qiazol® lysis reagent. The extraction was performed according to the manufacturer's protocol with minor modification. After purification, RNA concentrations were quantified using a NanoDrop 2000c UV-Vis Spectrophotometer. The ratio of absorbance at 260/280 was used to determine RNA purity at a ratio of 1.8-2. Extracted RNA of each treatment condition was stored at -80°C before cDNA synthesis.

3.2.3.3 Generating cDNA by polymerase chain reaction (PCR)

Complementary DNAs were generated according to manufacture's manual. RNAs template at a concentration of 1 μg were added to a master mix of Verso® Reverse Transcriptase cDNA synthesis kit and was amplified using PCR Thermal Cyclers (Bio-Rad) at 42 °C for 30 minutes. cDNAs of each treatment condition were kept at 4°C before quantification of mRNAs.

3.2.3.4 Quantification of mRNA by Real-time PCR

The PCR reaction was carried out by iQ5 real-time PCR detection system (Bio-rad). A 25 uL of final volume of master mix containing SYBR Green, forward primer, reverse primer, nuclease-free water and cDNA (final concentration of 250 ng/reaction for SLC19A3) were prepared according to manufacturer protocol. The amplification condition is given in table 3.2.

Cycles	Step	Temperature	Time
1 (1X) Step	Initial Denaturation	95.0 °C	15 minutes
2 (40X) Step 1 Step 2	Denaturation Annealing Extension	95.0 °C 58 °C 72 °C	15 seconds 30 seconds 30 seconds
3(81X) Step 1		55.0 °C – 95.0 °C	10 seconds
Increase set point temperature after cycle 2 by 0.5 °C			

Table 3.2 Real-time PCR amplification condition

3.2.3.5 Calculation of relative gene expression

Threshold cycle (C_t) of SLC19A2 and SLC19A3 mRNA copy number for each sample was normalized by β-actin C_t (housekeeping gene) as follows.

$$\mathbf{\Delta\ Ct} = \boldsymbol{Ct\ of\ target\ gene} - \mathbf{Ct\ \ of\ \beta\ actin\ gene}$$

The relative copy number ($\Delta\Delta C_t$) for each sample was calculated as follow.

$$\mathbf{\Delta\Delta Ct} = \mathbf{\Delta Ct\ of\ treatment\ group} - \mathbf{\Delta Ct\ of\ control\ group}$$

$$\boldsymbol{The\ fold\ change\ from\ control} = \mathbf{2}^{-\boldsymbol{\Delta\Delta Ct}}$$

3.3 Western blotting

To investigate the effect of thiamin and benfotiamine on the extent of pyruvate dehydrogenase protein under HIF-1α stabilization, H9c2 cells were seeded in 100 mm^2 plates and were treated with DMOG (1 mM) for 24, 48, 72 or 96 hours. Cells were washed twice in cold phosphate-buffered saline (PBS) and were collected and lysed in cell lysis buffer containing Protease Inhibitor Cocktail. Following sonication, supernatant was collected by centrifugation at 12,000 rpm at 4 °C for 20 minutes. Protein concentrations were established with the use of the Pierce® BCA Protein Assay kit was used for protein quantification according to manufacturer protocol. Concentrations were based upon a bovine serum albumin standard curve established in lysis buffer and used to establish equal protein concentrations. Thirty to forty micrograms of protein lysate were resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel. The gels were subsequently transferred to nitrocellulose membranes overnight at 4 degrees Celsius. Afterward, the membranes were washed three times with Tris-buffered saline-Tween 20 (TBS-T) for 5 minutes each and then were blocked in TBS-T containing 5% (w/v) nonfat dry milk and immunoblotted with different dilutions of primary antibodies overnight at 4 °C. The SLC19A3 antibody was diluted at 1:500 dilution in 1% Blotting Grade Blocker in TBS-T and pyruvate dehydrogenase antibody was diluted at 1:1000 dilutions in 5% Bovine Serum Albumin (BSA) in TBS-T. The membranes were washed in TBS-T three times for 5 minutes each followed by incubating in secondary anti-rabbit HRP antibody for 1 hour. Immunoreactive bands were visualized using chemiluminescence substrate that were captured by a CCD-camera (ChemiDoc XRS HQ, Biorad). In order to verify equal protein loading, the membranes were stripped using freshly prepared stripping buffer and were incubated at 50 °C for 10 minutes. After which, the membranes were washed extensively in TBS-T three times for 5 min each and were further incubated with anti-mouse α-tubulin antibody overnight. Densitometry

of the blots was analyzed by Quantity-one analysis software. Intensity of band was quantified by ImageJ 1.49 software (http://rsb.info.nih.gov/ij/index.html). Intensity of target protein band was normalized to of intensity α-tubulin band from each treatment using formula as follows.

$$\boldsymbol{Normalized\ target\ protein} = \frac{\boldsymbol{Intensity\ of\ target\ protein\ band}}{\boldsymbol{Intensity\ of\ \alpha\ tubulin\ band}}$$

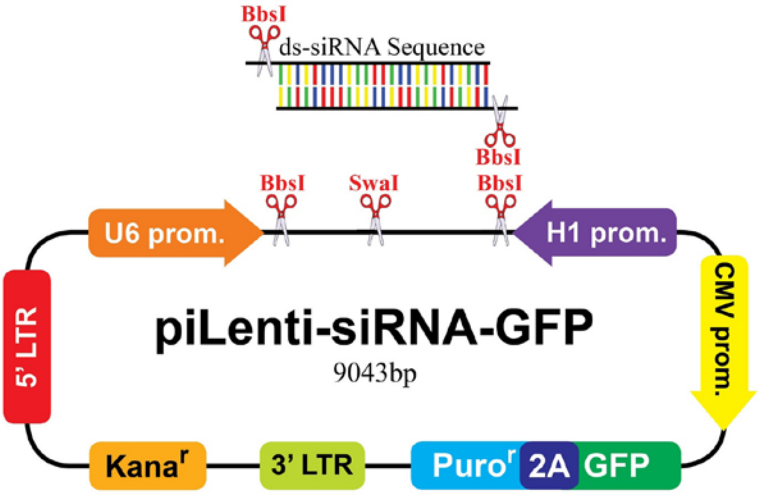
The fold over control for each replicate was calculated in Excel spreadsheets using formula as follows.

$$\boldsymbol{Fold\ change} = \frac{\boldsymbol{Normalized\ target\ protein\ of\ treatment\ group}}{\boldsymbol{Normalized\ target\ protein\ of\ control\ group}}$$

3.4 SLC 19A3 knockdown H9C2 cardiomyocytes

3.4.1 Construction of SLC19A3 siRNA/shRNA/RNAi pooled lentiviral system

SLC19A3 knockdown cells were constructed by using a ready-to use siRNA/shRNA/RNAi pooled (Rat) piLentivirus system with GFP reporter and puromycin selection marker (Applied Biological Materials (ABM) Inc. (BC, Canada). Vector detail and target sequence according to manufacturer's data sheet were demonstrated in figure 3.1 and table 3.3 .



(Illustrator provided by Applied Biological Materials)

267	CTTCACCATTTGCTACCTGTTTCTCTTGT
640	CCATGTACCGTCCTCAAGGAAGCTCACAA
954	ATTCTCAGTGGGCTACATAAAGGTCGACT
114	GCAGTTAACCTGAGCTTAGAACGTTATGC

Table 3.3 Target sequence of siRNA/shRNA/RNAi pooled (Rat) piLentivirus system (Sequences provided by Applied Biological Materials)

3.4.2 Lentiviral Infection in H9c2 cells

H9c2 cells were infected with 100 µL of SLC 19A3 siRNA/shRNA/RNAi pooled (Rat) lentivirus. Polybrene (8 µg/ml), a cationic polymer, was used for increasing transfection efficiency. Cells were closely monitored for 24 hours. The medium was changed, and cells that had incorporated the lentiviral gene were purified by 1 µg/ml puromycin treatment and maintained in puromycin-containing medium for 5 days. The cells were then maintained in regular media. To determine successful knockdown of SLC19A3 by lentivirus system, GFP reporter was detected in fluorescent microscopy (EVOS™ FL cell Imaging System, ThermoFisher) under GFP mode: 470 nm excitation, 525 nm emissions and western blotting of SLC19A3 were conducted compared to control H9c2 cells.

3.4 Measurement of mitochondrial membrane potential ($\Delta\psi$) by Flow cytometry

H9c2 cells were seeded at a density of $5x10^5$ in multiple 6-well plates and allowed to grow for 24 hours. On the next day, cells were pretreated with benfotiamine (50 µM or 100 µM) or thiamine hydrochloride (50 µM or 100 µM) for 2 hours. Afterward, DMOG at a final concentration of 1 mM was added and further incubated for 24, 48 or 72 hours. After completing a designed

incubation period, cells were treated with tetramethylrhodamine ethyl ester (TMRE) at a final concentration of 500 nM for 30 minutes in the dark at 37 °C humidified incubator. TMRE is cell a positive charge fluorescent dye that accumulates into space of polarized mitochondria in inverse proportion to $\Delta\psi m$ according to the Nernst equation. Sample preparation was conducted according to in-house protocol. Cells were washed with PBS three times, harvested and resuspended in 300 µL of complete DMEM to further analysis. Cell population was collected at 20,000 events and each individual cell was analyzed by BD Accuri C6 Flow cytometry using emission filter at 585/40 nm (FL-2). Mean fluorescent intensity of TMRE was automatically calculated by the BD Accuri 6 software.

3.5 SLC19A3 over-expressed H9c2 cardiomyocytes

3.5.1 Amplification of adenovirus

Amplification of adenovirus was conducted according to manufacturer protocol. HEK 293 cells were plated at a density of 10^6 cells in 10 cm^2 dish. Cells were allowed to attach and grow for 24 hours. On the day of infection viral stock solution at 50 µL and 100 µL were added into each dish and incubated for 72 hours. After 72 hours of infection, cells displayed cytopathic effects such as morphology change to round shape and form cluster. Approximately 95% of cells were detached from dish bottom after 72 hours infection. Cells then were harvest according to manufacturer protocol and were stored at 4 °C. MOI of virus was calculated by following formula.

$$MOI = PFU/cell$$

$$\frac{\text{VP}}{\text{PFU}} = \ 20{:}\,1 \text{ to } 50{:}\,1$$

According to product certification of analysis, titer of premade adenovirus is 1.93 x 10^{11} vp/ml or 9.65 x 10^9 PFU/ml. Desired MOI that we used for amplification was 500 to 1000. Therefore, the amount of viral stock solution was calculated by 10^6 cells x 500 MOI = 5 x 10^8 PFU

desired. Then the volume of viral stock solution required was 5 x 10^8 PFU / 9.65 x 10^9 PFU/ml = 51.8 µL for 500 MOI to 100 µL for 1000 MOI.

3.5.2 Infection of adenovirus

H9C2 cells were seeded in 6-multiple well plates and allowed to grow for 70% confluence. On the day of adenoviral infection, three different concentrations of viral stock solution (1, 5 and 10 µL) were added in to new medium and additionally incubated for 72 hours. After 72 hours post infection immunostain with DYKDDDDK tag (flag tag) antibody for fluorescence microscopy. H9c2 cells were plated and allowed to grow at 70% confluence in 10 cm^2 dishes. On the day of transfection, three different concentrations of viral stock solutions (10, 20 and 30 µL) were added into each dish. Cells were harvest after 72-hour post-transfection. Western blot analyses of cells lysates were perform with DYKDDDDK tag (flag tag) antibody.

3.6 Ischemic/ Reperfusion (I/R) surgery in mice

Experimental procedures and protocols were reviewed and approved by Institution of Animal Care and Use Committee (IACUC), Auburn University. C57BL/6 male mice (n=3) and cardiac specific HIF-1 knockout male mice (n=3) at the age of 8 week-old (weight range between 25-30 g) were randomized into thiamin treatment and control group. Thiamin (40 mg/kg body weight i.p.) was administered for 6 consecutive days. On the day of surgery, mice were anaesthetized with sodium pentobarbital (50 mg/kg body weight i.p.) in sterile 0.9% normal saline and were given additional dosing as needed (30 mg/kg body weight) during the surgery. The mice were placed in the supine position on a warming pad and heating lamp to maintain body temperatures at 37°C. Electrocardiography (EKG) was instantaneously monitored from limb leads from lead 1 recording to evaluate changes in heart rate and EKG complexes (Biopac, Santa Barbara, CA). A tracheotomy was performed and mice were intubated with a polyethylene tube

and mechanically ventilated (Kent Scientific, Torrington, CT) at a rate of 120 breaths/minute (BPM), 0.15 ml tidal volume with O_2. Following a left thoracotomy, the pericardium was dissected and the proximal LAD was tied with 6-0 silk suture mounted on a tapped needle (BV-1, Ethicon). LAD coronary artery occlusion was maintained for 30 minutes. Myocardial ischemia was observed by discoloration of the left ventricle or a pale ventricle distal to the ligation site. After 30 minutes of occlusion, ligature was removed, followed by 120 minutes of reperfusion. After 120 minutes, Evans blue dye was injected and then heart was immediately removed and was cross-section into 4 (~ 1.5 mm) transverse pieces and processed for TTC stain. Heart slices were incubated in 1% of TTC solution at 37 °C for 15 minutes. Heart slices were photographed by a digital camera. TTC-stained region for each heart slice was determined using ImageJ. Ratios of area at risk to total area of the slices and ratio of infarct area to area at risk were calculated and expressed as a percentage.

4. RESULTS

4.1 Identification of SLC19A2 and SLC19A3 mRNA expression in H9c2 cells

Little is known about the expression pattern of thiamin transporter genes and no information regarding regulation and intracellular signaling pathways that influences expression of these genes in cardiomyocytes. To investigate the expression pattern and the effect of hypoxia-inducible factor 1 (HIF-1) on SLC19A2 and SLC19A3 mRNA expression, qRT-PCR experiments were conducted. H9c2 cells were incubated in a medium containing a final concentration of $CoCl_2$ (400 μM) for 24, 48 and 72 hours in normoxic condition (21% O_2). $CoCl_2$ at 100 to 1000 μM has been extensively used for mimicking hypoxic condition *in vitro* model**.** Additionally, our previous study demonstrated that H9c2 cells treated with $CoCl_2$ (400 μM) for 18 hours resulting in a significant increase in HIF-1α protein level detected by western blotting. (Data not shown)

We demonstrated that both transporter genes were expressed in H9c2 cells. The relative mRNA level of SLC19A3 was slightly increased to 3.645 fold (SEM 1.06, n=2) at 72 hours under $CoCl_2$ treatment but was not statistically significant different change from control (P=0.355). In opposition, SLC19A2 mRNA level was not observed any significant change from control over 24 to 72 hours under $CoCl_2$ treatment.

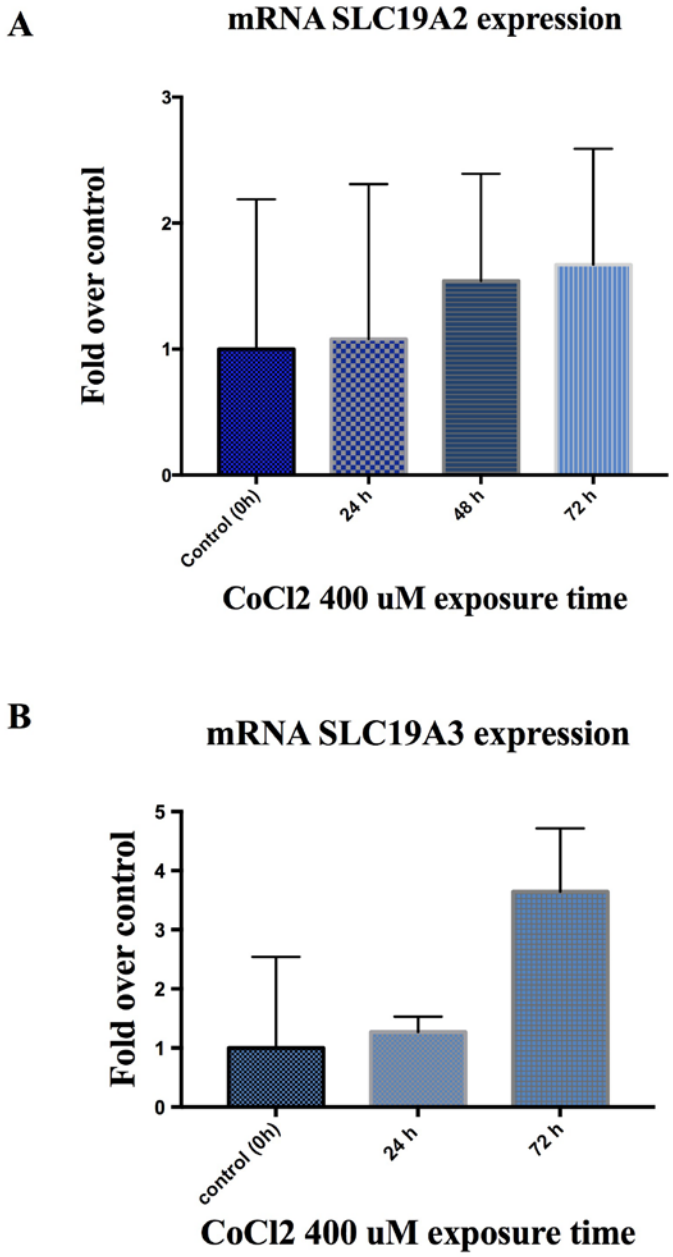
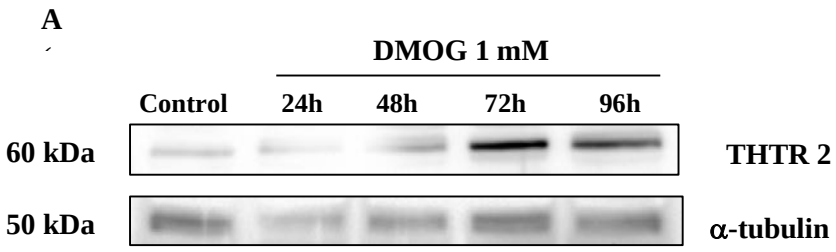


Figure 4.1 The effect of $CoCl_2$ (400 µM) for 24, 48 and 72 hours on SLC19A2 (Figure 4.1A) and SLC19A3 (Figure 4.1B) expression. Fold difference was normalized with β-actin and is compared with control group. Data were analyzed by ANOVA with post hoc t-tests (Tukey's multiple comparisons test). Results are presented as mean of biological triplicates (n=3) for SLC19A2 data and biological duplicates (n=2) for SLC19A3 data with *error bars* representing SEM.

4.2 Identification of THTR2 transporter protein expression in H9c2 cells

To investigate the effect of HIF-1 stabilization on thiamin transporter 2, product of SLC19A3, we selected an alternative chemical-induced hypoxia agent, dimethyloxalylglycine (DMOG**),** which is well established to inhibit proline hydroxylase (PHD) leading to stabilization of HIF-1α in normal oxygen levels, but has been shown not to induce apoptosis when compared to $CoCl_2$. We, therefore, increased exposure time up to 96 hours to observe the maximum protein expression result of thiamin transporter 2 (THTR2). H9c2 cells were incubated with DMOG (1mM) for 24, 48, 72 and 96 hours. The protein levels were measured and standardized by a BCA(Thermo-Fisher) based upon a bovine serum albumin standard curve assay. Proteins were resolved by SDS-Page analysis followed by immunoblot analysis. Densitometry analysis of immunobot bands of THTR2 suggested that treatment with DMOG markedly induces THTR2 at 3.46 fold (SEM 0.19, n=3, p=0.0034) and 3.91 fold (SEM 0.10, n=3, p<0.001) only after 72 and 96 hours respectively of DMOG treatment when compared to control group.



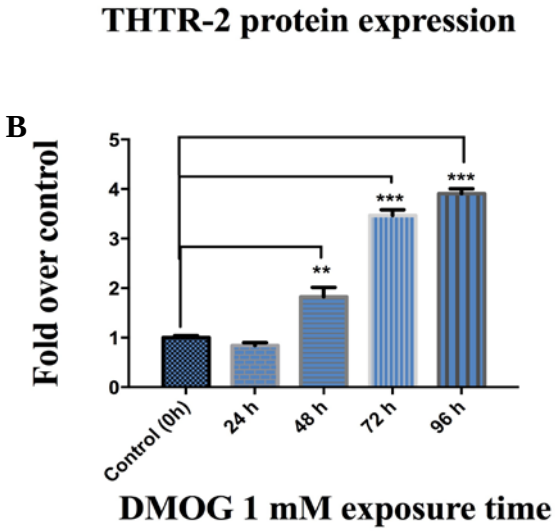


Figure 4.2 The effect of DMOG 1 mM on THTR2 protein expression. Fold difference was normalized with α-tubulin and is compared with control group. Data were analyzed by ANOVA with post hoc t-tests (Tukey's multiple comparisons test). Results are presented as mean of biological triplicates (n=3) with *error bars* representing SEM. ****P<0.001*; ***P<0.05* Representative image of three blots is shown on Figure 4.2A.

4.3 HIF-1 protein was stabilized under DMOG treatment.

To further validate HIF-1α stabilization by DMOG treatment over the time cause of experiment, H9c2 cells were incubated with DMOG (1mM) for 24, 48 and 72 hours and HIF-1 protein was quantified by western blotting. Preliminary data suggested that HIF-1 protein were stabilized over the time course of experiment up to 72 hours.

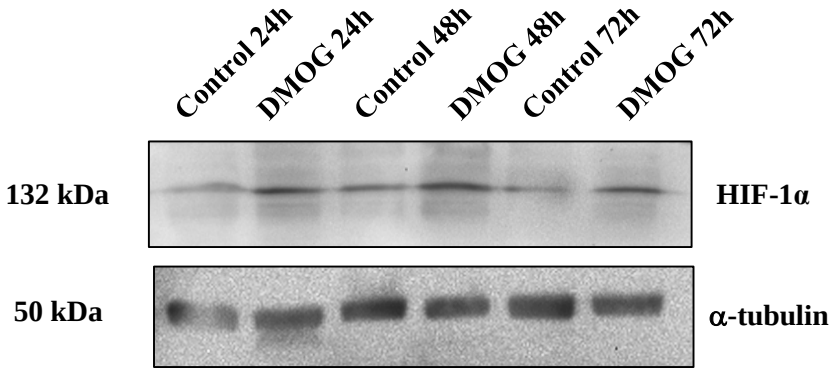


Figure 4.3 The effect of DMOG on HIF-1 α protein expression.

4.4 Thiamin transporter 2 protein levels are not altered by reduction of substrate levels in a medium over the time up to 96 hours.

The effect of thiamin depletion on THTR2 expression was investigated to determine whether thiamin transporter protein expression levels may be influenced by thiamin depletion in media over 24 to 96 hours. To test this theory, H9c2 cells were grown in normal media (DMEM) containing 12 μM thiamin under normoxia for 24, 48, 72 and 96 hours. The preliminary result suggests that there was no change in THTR 2 protein levels throughout the course of the study.

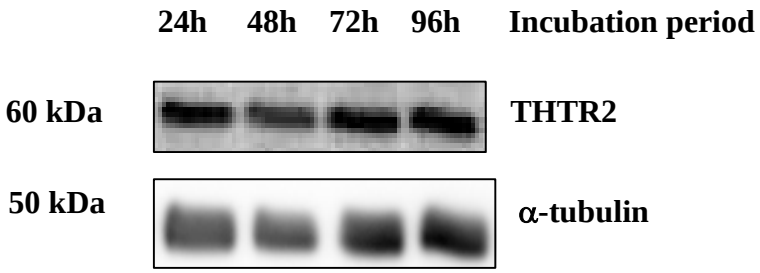


Figure 4.4 The effect of thiamin depletion in media on THTR2 level under normoxia. One experiment for preliminary data was conducted.

4.5 Pyruvate dehydrogenase (PDH) protein level was not altered under HIF-1α stabilization in H9c2 cell.

To investigate the effect of HIF-1 on pyruvate dehydrogenase (PDH) protein level, H9c2 cells were treated with DMOG (1mM) for 24, 48, 72 and 96 hours. Pyruvate Dehydrogenase antibody detects endogenous levels of E1 α1 and α2 subunits of pyruvate dehydrogenase complex. The result showed only a modest induction of PDH protein level that were not statistically significant from control during 24 to 48 hours and a small reduction of PDH protein after 48 to 96 hours of DMOG treatment.

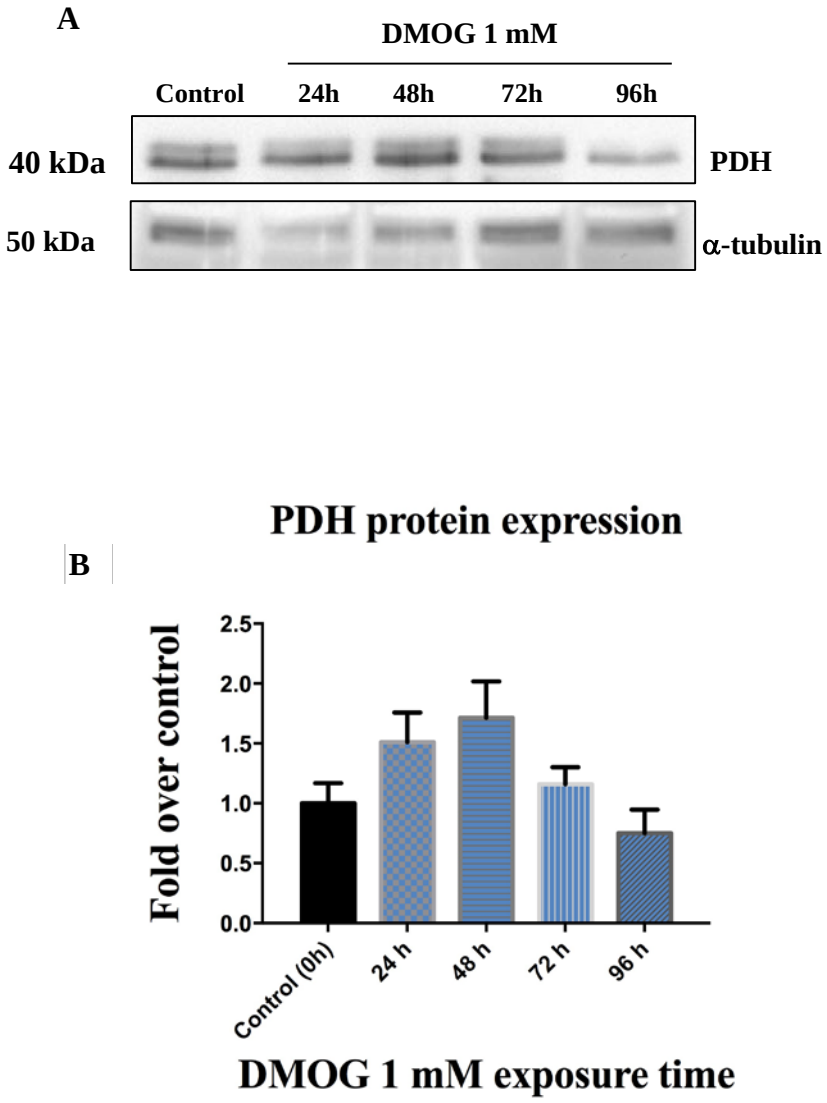
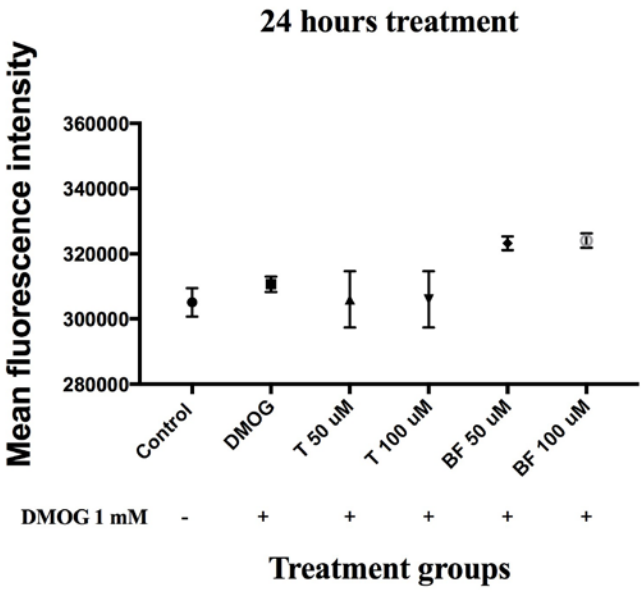


Figure 4.5 the effect of DMOG (1mM) on PDH protein expression. Fold difference was normalized with α-tubulin and is compared with control group. Data were analyzed by ANOVA with post hoc t-tests (Tukey's multiple comparisons test). Results are presented as mean of biological triplicates (n=3) with *error bars* representing SEM.

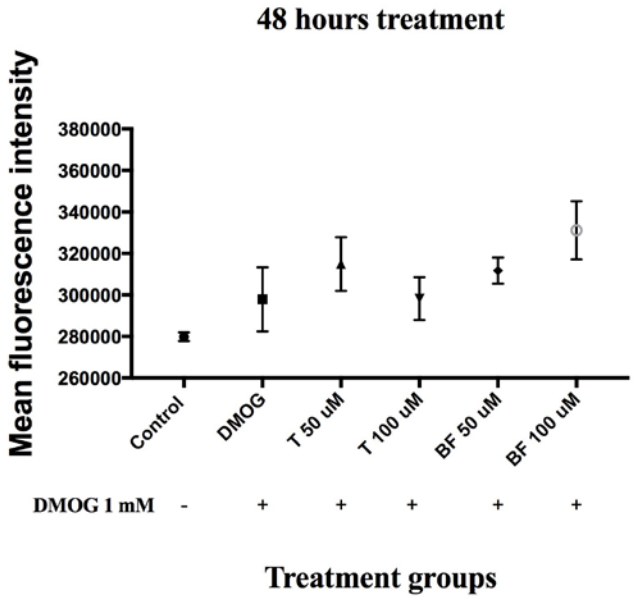
4.6 Benfotiamin increases mitochondrial membrane potential (ΔΨm) under 72 hours HIF-1 activation in H9c2 cells

Mitochondrial membrane potential is an indicator for detemining mitochondrial function. Change in O_2 consumption and ATP production in mitrocondria due to the shift from oxidative phosphorylation to glycolysis during HIF-1 stabilization may supress mitochondrial function. To study the effect of thiamin and benfotiamine 50 and 100 uM on changes in mitochondrial membrane potential (ΔΨm), H9c2 cells were pretreated with benfotiamine (50 µM or 100 µM) or thiamine hydrochloride (50 µM or 100 µM) for 2 hours followed by DMOG (1 mM) for 24, 48 or 72 hours. Mitochondrial membrane potential (ΔΨm) was assessed by tetramethylrhodamine ethyl esterperchlorate (TMRE), a lipohillic cationic dye penetrated into active (polarized) mitochondrial matrix space in inverse proportion to ΔΨ. (Perry, Norman et al. 2011) The more negative of ΔΨm, the more intensity of probe. The fluorescence intensity were quantified by Flow cytometry at λ Ex 549 and λEm 574 nm. These observations indicate that Benfotiamine increased mitochondrial membrane potential (ΔΨm) determined by a significant change in mean fluorescence intensity with a dose-dependent manner in the presence of DMOG for 72 hours vs control normoxia (P= 0.0134, N=3 at 50 µM and P=0.0026, N=3 at 100 µM). Futhermore, mitochondria membrane poteintial (ΔΨm) was significant increased in benfothiamin plus DMOG groups vs DMOG alone under 72 hours. (P= 0.0095, N=3 at 50 µM and P=0.0019, N=3 at 100 µM).

A.



B.



C.

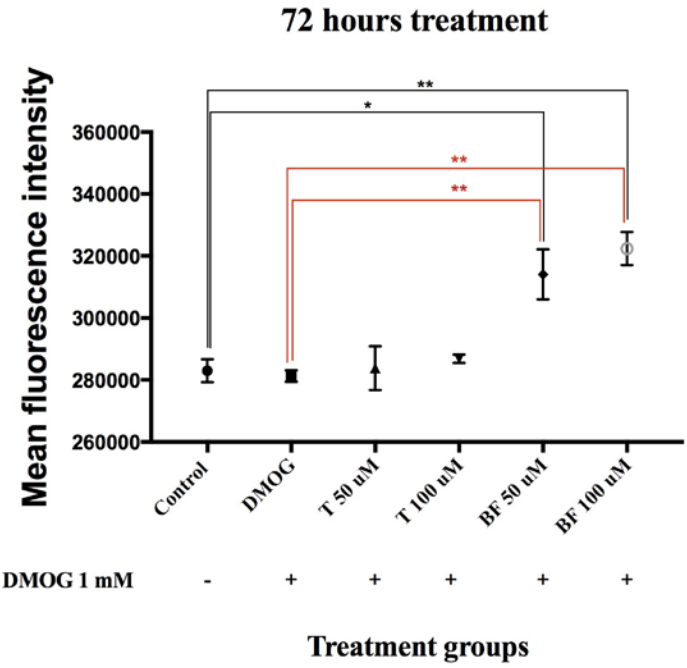


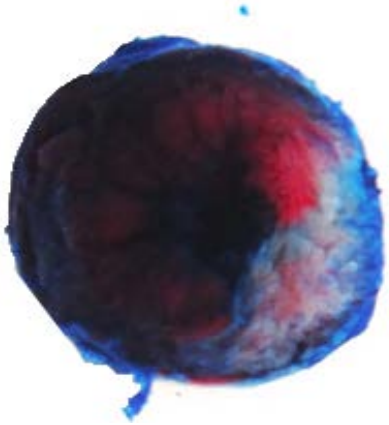
Figure 4.6 Quantification of mitochondria membrane potential (ΔΨm) in H9c2 cells (20,000 events/group) by flow cytometry. Average TMRE fluorescence intensity of H9c2

cells treated with thiamin or Benfotiamin 50 uM or 100 uM in the precense of DMOG (1 mM) or control (A) DMOG 24 hours treatment (B) DMOG 48 hours treatment and (C) DMOG 72 hours treatment. Data were analyzed by ANOVA with post hoc t-tests (Tukey's multiple comparisons test). Results are presented as mean of biological triplicates (n=3) with *error bars* representing SEM. *$P<0.05$, **$P<0.001$

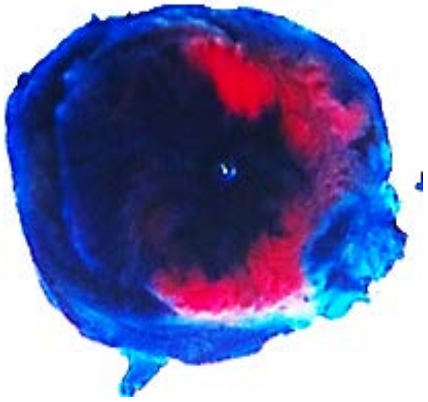
4.7 Myocardial Ischemic/Reperfusion surgery

To determine the effect of thiamin pretreatment on infarct size from ischemic and reperfusion injury, study was conducted in mice model of acute coronary artery occlusion. WT and cardiac specific HIF-1 knockout mice were pretreated with thiamin 40 mg/kg, i.p. multiple doses for consecutive 6 days and subjected to ischemia (30 minutes) followed by reperfusion (120 minutes). Efficacy of thiamin against ischemic-reperfusion damage was determined by assessment of infarct size (IS) expressed as % of area at risk (AAR) compared between thiamin treatment versus control. Infarct size was visualized by TTC and Evan's blue staining. The AAR expressed a percentage of total area of left ventricle. The result suggested that the mean % of AAR was similar (P= 0.2591) in all groups accounting for WT + thiamin 36.88 % (SEM 4.56, n=3), WT 46.00 % (SEM 3.57, n=4), HIF-KO+ thiamin 51.85 % (SEM 5.03, n=4) and HIF-KO 40.90% (SEM 6.39, n=4). We observed % infarct area reduced from 31.02% (SEM 2.22, n=4) in WT control group to 15.05 % (SEM 3.31, n=3) in WT thiamin treatment group. However, statistical analysis performed using two-tails t-test showed non-significant differences in average % necrosis in both group (P=0.0698).

A. WT mice

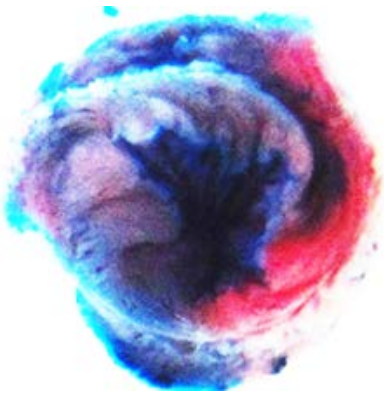


I/R

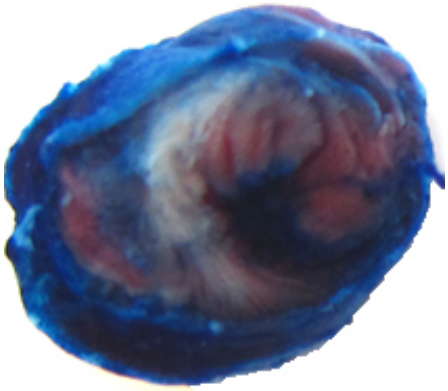


I/R + thiamin

B. HIF-1α Knockout mice



I/R



I/R + thiamin

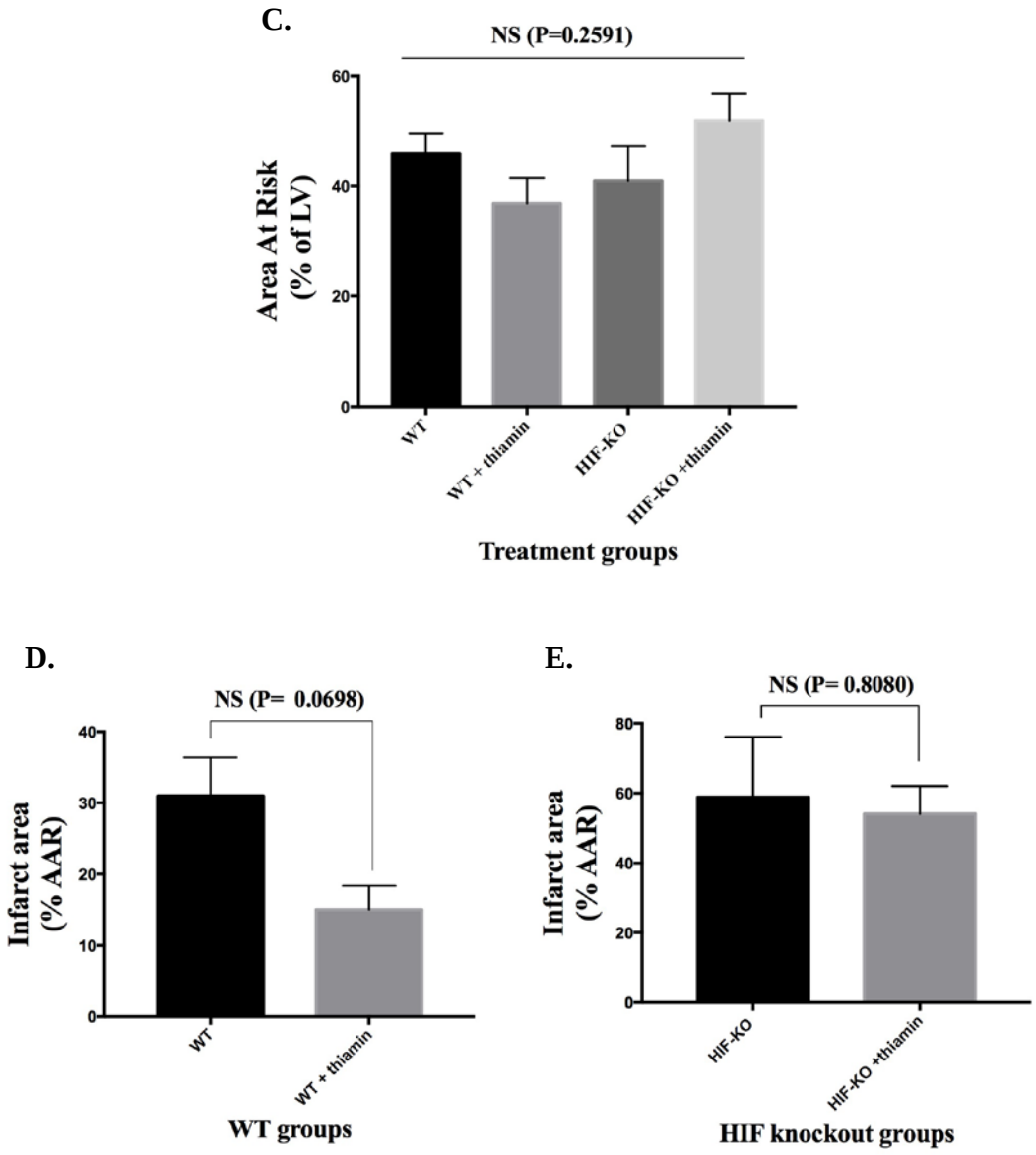


Figure 4.7 Representative images illustrating infarcted myocardial tissue (white), Area at risk (AAR) tissue (red) and viable tissue (blue) in (A) WT mice and (B) HIF-1 knockout mice. The AAR is expressed as the percentage of left ventricle. The significant difference in AAR was determined by ANOVA. The infarct size is expressed as the percentage of the AAR. The significant difference in infarct size between thiamin treatment and control groups was determined by two-tails t-test in (C) Wild type mice and (D) HIF-1 knockout mice. The data are expressed as the mean ± SEM (n=4)

Additional experiments

4.8 Verification of RNAi lentiviral vector for knockdown of SLC19A3 in H9c2 cells

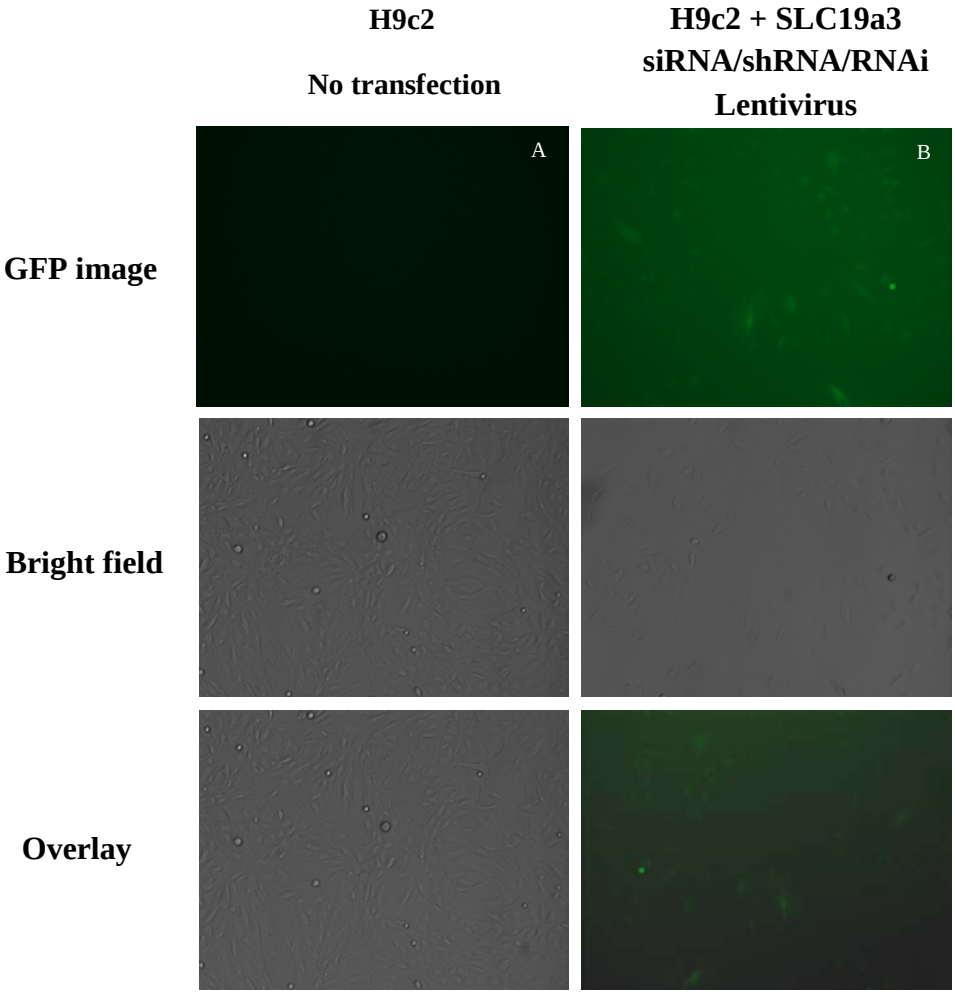


Figure 4.8 (A) H9c2 cells under bright field microscope (B) H9C2 cells transfected with SLC19a3 siRNA/shRNA/RNAi lentivirus pooled 100 uL Top; GFP image of H9c2 cells Middle; Bright field Bottom; Overlay image. Images were collected using fluorescent microscopy (EVOS™ FL cell Imaging System, ThermoFisher) with 20X objective under GFP mode: 470 nm excitation, 525 nm emissions. (n=3).

4.8 Verification of recombinant adenovirus vector overexpress of SLC19A3 in H9c2 cells.

We infected constructed recombinant Adenovirus ORF with C terminal Flag and His tag of SLC19A3 in order to transiently overexpress SLC19A3 in H9c2 cells. Difference concentration of amplified adenoviral stock solutions at 10 µL, 20 µL or 30 µL was added into H9c2 cells. Cells were harvest after 72 hours post-transfection, cells lysates were immunoblotted with DYKDDDDK tag.

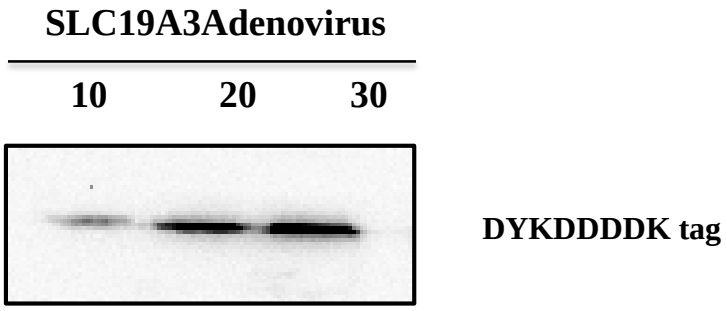


Figure 4.9 Western blot analysis of overexpress thiamin transporter 2 protein with C terminal Flag tag. Protein was detected with DYKDDDDK tag which binds to the same epitope with an anti-flag tag® M2 antibody (n=1).

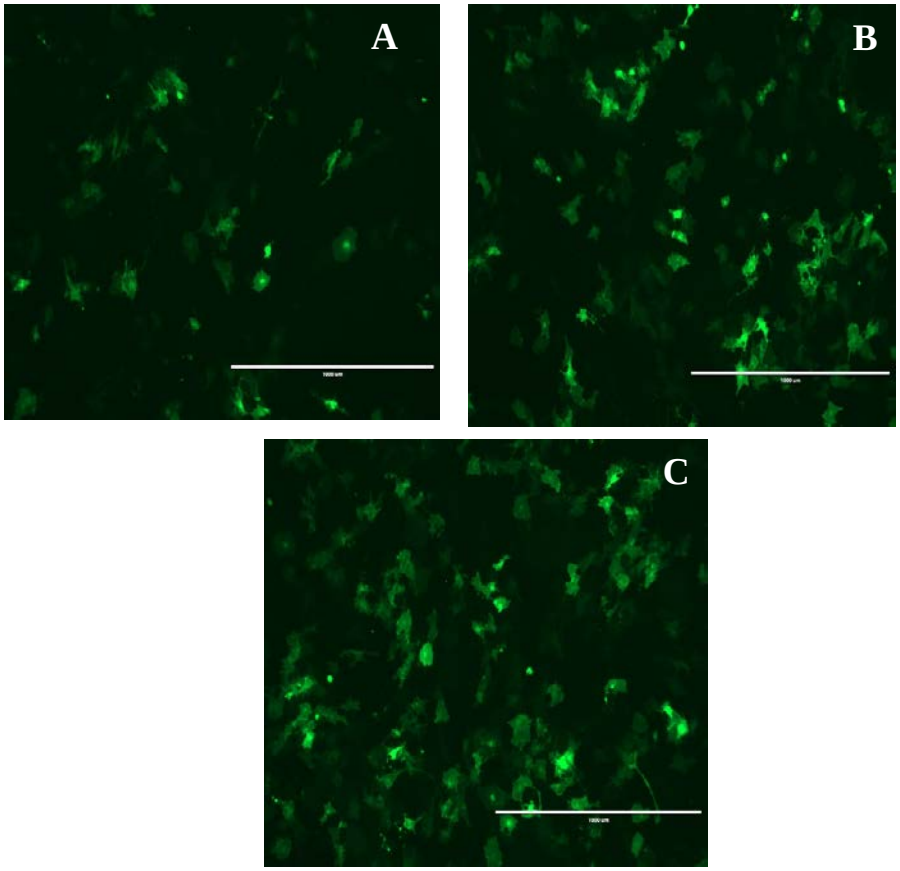


Figure 4.10 Fluorescent intensity of H9c2 cells infected SLC19A3-adenovirus with Flag and His tag at C-terminal at three difference concentrations of adenovirus stock solution (A) 10 µL (B) 20 µL (C) 30 µL Images were obtained by fluorescent microscopy (EVOS ™ FL cell Imaging System, ThermoFisher) with 10X objective under GFP mode: 470 nm excitation and 525 nm emission.

5. DISCUSSIONS, CONCLUSION AND FUTURE DIRECTION

Discussion

Few studies have been evaluated the functional characteristics of thiamin transporters and its role in the failing heart. Uptake of thiamin into cells relies upon transporter mechanism encoded by SLC19A2 and SLC19A3. Both thiamin transporter genes were reported in the human heart but the significance of the regulation of these genes has not been elucidated (Dutta, Huang et al. 1999) (Eudy, Spiegelstein et al. 2000). However, SLC19A3 was found to be expressed at high levels in the hypoxic environment in breast cancer cells in response to a high rate of glycolytic metabolism (Sweet, Paul et al. 2010). Our study indicated that both SLC19A2 and SLC19A3 were found to be expressed in H9c2, cardiomyocytes. Additionally, without statistically significant difference from the normoxic (control) group, SLC19A3 mRNA levels were observed an elevated trend (3.645-fold) in response to chronically inducible hypoxia induced by $CoCl_2$. In contrast, previous report shows that SLC19A3 mRNA level and intracellular thiamin uptake were increased markedly in response to hypoxia (1% O_2) in breast cancer cell lines cultured under chronic hypoxia as induced by DFO-induced hypoxia (Sweet, Paul et al. 2010). DFO is an iron chelator and alters HIF-1 activity by inhibiting proline 2-hydroxylase (PHD2). We, therefore, investigated whether thiamin transporter 2, THTR2, a product of SLC19A3, would also respond to HIF-1 stabilization under DMOG treatment.

We observed that THTR2 protein was increased significantly after 72 to 96 hours of DMOG exposure. Additionally, HIF-1 protein was detected under DMOG treatment over the time course of experiment up to 72 hours. The result confirmed that HIF-1 was stabilized over the time course of DMOG treatment. Therefore, an increase in transporter protein level could be a consequence of HIF-1 stabilization in H9c2 cells under chemical-induced hypoxia. Although, a significant difference in SLC19A3 mRNA level was not observed, thiamin transporter 2 was increased under HIF-1 stabilization might be due to posttranslational modification. Therefore, further studies are required to validate the effect of HIF-1 on SLC19A3 mRNA expression.

Another possibility that could influence THTR2 induction could be level of thiamin as a substrate of THTR2. Several studies have shown that thiamin deficiency or depletion of thiamin in cell culture medium resulted in up-regulated SLC19A3 mRNA levels in various cells such as CaCo cells, HEK 293 cells and neuroblastoma cells (Nabokina and Said 2004, Ashokkumar, Vaziri et al. 2006, Sweet and Zastre 2013). In this study, we contradicted these findings by our observation validated that increase in THTR2 protein level was not a consequence of thiamin depletion in media. The preliminary data shows that THTR2 protein levels were not altered by reduction in substrate levels in the medium. Based on our findings, we observed that DMOG (1mM) increases HIF-1α protein levels over the time course of experiment. Furthermore, there was no change in THTR2 expression occurred over the course of the experiment and thus we conclude that HIF-1 influences the up regulation of THTR2 in H9c2 cells.

It is well established that pyruvate dehydrogenase I (PDK-1) is a downstream target gene of HIF-1(Kim, Tchernyshyov et al. 2006). The up-regulation of PDK1 gene during hypoxia leads to an increase in phosphorylated pyruvate dehydrogenase (PDH) at the E1-α subunit. Impairment

of PDH enzyme function to convert pyruvate to Acetyl-CoA causes the TCA cycle shut down due to the limitation of Acetyl-CoA leading to cardiac metabolic remodeling resulting in elevating lactate production and low glucose oxidation (Kim, Tchernyshyov et al. 2006, Solaini, Baracca et al. 2010). Since, PDH requires TPP as a cofactor for its function, the up regulation of THTR2 could be an adaptive mechanism of cells to restore PDH impairment under HIF-1 activation. Since, we did not observe significant change in pyruvate dehydrogenase protein levels under HIF-1 activation in H9c2 cells, we suggest that an increase in THTR2 protein expression was not correlated with PDH protein levels.

In this study, we also investigated the effect of thiamin and benfotiamine on mitochondrial membrane potential ($\Delta\Psi m$) under HIF-1 activation. We observed significant induction in $\Delta\Psi m$ in H9c2 cells treated with benfotiamine 50 uM and 100 uM in the presence of DMOG for 72 hours compared to normoxic control. Interestingly, we also observed significant change in $\Delta\Psi m$ in H9c2 cells treated with DMOG alone compared to DMOG plus benfothiamine for 72 hours. Mitochondrial membrane potential ($\Delta\Psi m$) is one of the indicaters to determine mitochondia bioenergetic (Brand and Nicholls 2011). In normoxic condition; mitochondria maintain ATP production via oxidative phosphorylation and electron transport chain. ATP production from electron transport chain is also important for maintain physiological function of mitochondria and stabilizes a proton gradient across the inner membrane of mitochondria. However, it has been well establish that under hypoxia-induced HIF-1, oxidative phosphorylation and electron transport chain are inhibited by several cellular mechanisms, which affect mitochondria fusion resulting in mitochondria depolarization and impair mitochondria metabolism (Solaini, Baracca et al. 2010) (Semenza 2011). Our finding suggested a protective role of benfothiamine on mitochondia function under chronic HIF-1 activation. Similarity to previous study shown the pretective effect

of benfothiamin (150 uM) pretreated in HL-1 cardiomyocytes for 48 hours prior to serum starvation (8 hours) followed by 0.2% O_2 (18 hours) in normal and high glucose levels indicated benfothiamine activated pro-survival pathways via VEGFR2-Akt-Pim1 in hypoxia but not shown effect under normoxia and normal glucose conditions (Katare, Caporali et al. 2010). Taken together with our finding, chronic HIF-1 activation may require for the function of benfothiamin in mitochondria bioenergetic to compensate imparment of ATP production. However, the relevent underlying mechanism of benfothiamin on mitochondria energy production under chronic hypoxia need to be futher investigated. Nevertheless, we did not observed any change in $\Delta\Psi m$ under thiamin treatment groups in the presence of DMOG at any time points. This might be due to we used low dose of thiamin was used in this study. Effective dose of thiamin need to be investigated under pathological condition such as chronic hypoxia.

Thiamin diphosphate (TPP) is an essential cofactor for two important mitochondrial enzymes; pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase, which is required for glucose oxidation and mitochondrial synthesis of ATP in the normal heart (Depre, Vanoverschelde et al. 1999). Conversely, in pathological conditions such as myocardial ischemia, glucose utilization via glycolysis becomes an important energy source for the heart due to metabolic switch by HIF-1 mediated activation of glycolytic enzymes and glucose transporters (Depre, Vanoverschelde et al. 1999). By taking advantage of HIF-1 stabilization during myocardial hypoxia/ischemia based on our findings from our *in vitro* studies, we postulated that administration of thiamin at a pharmaceutical dose before ischemia- reperfusion injury could lead to protective effects against ischemia reperfusion induced cell damage as determined by the limitation of infarction or necrosis area. Therapeutic potential of thiamin was observed in the rodent model of cerebral ischemic-reperfusion. Thiamin given at 60 mg/kg i.p. single dose 30 minutes before

surgery or at 1% thiamin in drinking water for 7 days was previously shown to display similar protective effects by attenuating the reactive oxygen species-induced reductions in pyruvate dehydrogenase (PDH) and ketoglutarate activities which improves infarct area (Sheline and Wei 2006). In our study, we observed that administration of thiamin 40 mg/kg i.p. for 6 days prior to ischemic-reperfusion surgery in WT mice reduced infarct size by 15.97% reduction compared to the control group. Although the mean difference of % infarct area was not statistically significant amongst the control and treatment groups in WT mice (P= 0.0698), it may be due to a limited number of animals and thus increased numbers may be needed to improve the significance of our findings.

In HIF-KO mice, we found infarct size greater than 50% in both thiamin treatment and control group. A large infarct could lead to significant cardiac change or remodeling however, based on our study design, we did not measure cardiac functional parameters for this experiment. There is a contradictory report that benfotiamine, a lipid-soluble thiamin prodrug (70 mg/kg body weight, administration in drinking water for 2 weeks, n=5), protects the diabetic heart from ischemic damage in a model of myocardial infarction by improvement in 14 day-post-MI survival rate and cardiac functions in both normal control and diabetic mice via activation of the pentose phosphate pathway enzyme resulting in increased NADPH levels (Katare, Caporali et al. 2010). Therefore, therapeutic potentials of thiamin may have greater beneficial effects in a diabetic model under ischemia due to impairment of enzyme associated with glucose utilization. Although our study did not identify the biological mechanisms that account for the protective effects of thiamin, we proposed possible mechanisms that should be further investigated in future studies. It has been shown in several *in vivo* studies that stimulation of glucose oxidation rate rather than fatty acid oxidation during initial phase of reperfusion leads to beneficial outcomes against ischemic-

reperfusion induced myocardial cell damage (McVeigh and Lopaschuk 1990, Broderick, Quinney et al. 1993). Thiamin has been reported to improve PDH activity *in vivo* models of diabetes by reduction of PDK-1 activity leading to improvement of PDH function (Kohda, Umeki et al. 2010). Interestingly, a recent study reported that thiamin at high doses reduced PDH phosphorylation by PDK-1 in SK-N-BE cells and Panc-1 cells (Hanberry, Berger et al. 2014). For all reasons, the proposed mechanism underling the protective effect of thiamin could due to an improvement of PDH function during reperfusion and subsequently leads to an increase in the rate of glucose utilization via glucose oxidation.

Conclusions

Our *in vitro* findings indicated that thiamin transporter 2 (THTR2), which facilitates intracellular uptake of thiamin, is mediated by the HIF-1 transcription factor in H9c2 cardiomyocyte cells. Thiamin (40 mg/kg) supplementation prior to I/R surgery did not improve the infarction size in ischemia-reperfusion murine models when compared to control I/R treated mice. Further studies are required to fully elucidate the mechanistic potential of thiamin in treatment and prevent of myocardial ischemic-reperfusion injury.

Future direction

Little is known regarding significant of thiamin transporter 2 genes and protein in the hypoxic heart, We have recently created a stable knock down and transient overexpression of SLC19A3 in H9c2 cells to further investigate the significance of this gene to understand the significance of intracellular thiamin levels under hypoxic and normoxic conditions. We, as well, are developing liquid chromatography mass spectrometry techniques for determination of intracellular thiamin and its active phosphorylated form, TPP to investigate the correlation between upregulated transporters and intracellular level of both forms.

In animal models of I/R injury, we will investigate whether the transporters play an important role in the heart by measuring SLC19A2 and SLC19A3 mRNA levels as well as THTR1 and THTR2 protein levels and functional uptake under hypoxia or HIF-1 stabilization. Further we will verify that SLC19A3 as a target gene of HIF-1 expression of SLC19A3 in vitro by validating our preliminary luciferase analysis and identifying the specific HRE region on the SLC19A3 promoter and in DMOG treated mice by chromatin immunoprecipitation assays. Most significantly we will explore the significance of the HIF-1- SLC19A3 signaling in a diabetic model that is associated with HIF-1 activation or impairment of HIF-1 activity.

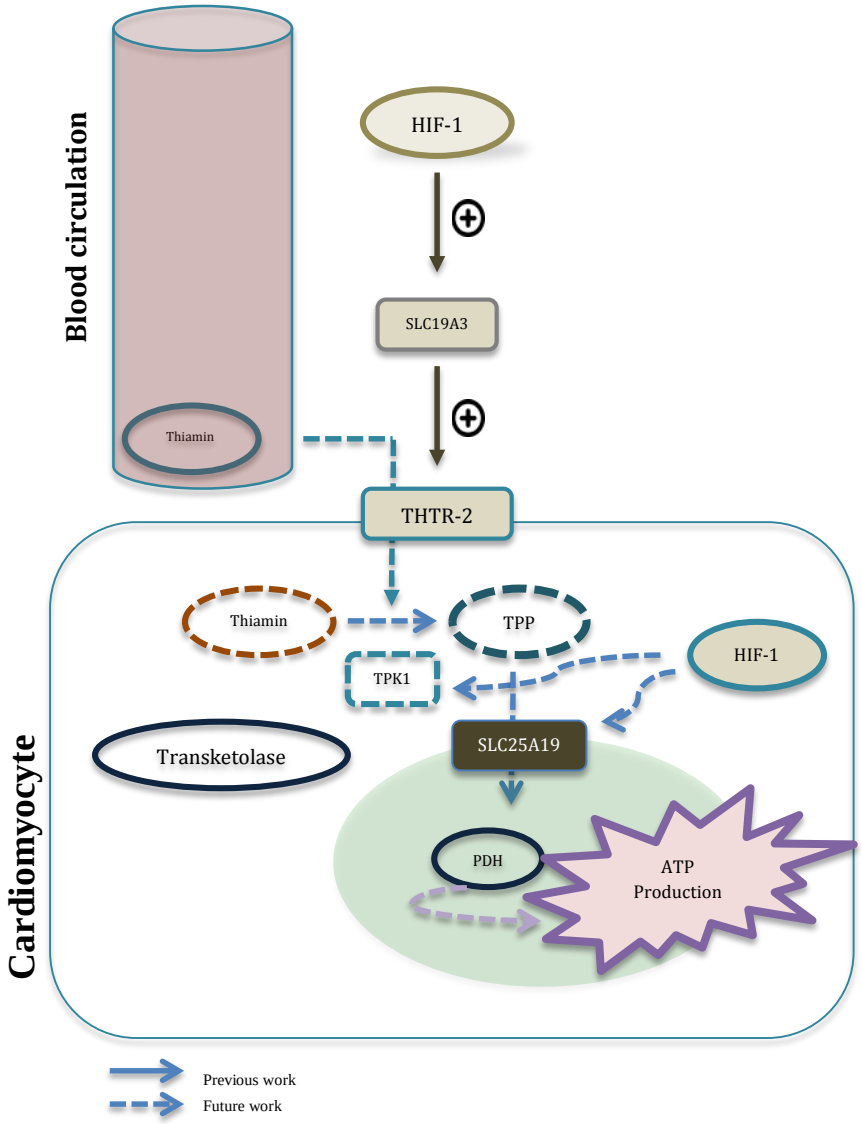


Figure 5.1 Summary diagram for HIF-1-SLC19A3 thiamin transporter

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APPENDIX I: ADDITIONAL EXPERIMENTS

Development and determination of intracellular thiamin and Thiamine-(4-methyl-13C-thiazol-5-yl-^{13}C3) hydrochloride in H9c2 cells by liquid chromatography mass spectrometry (LC-MS/MS)

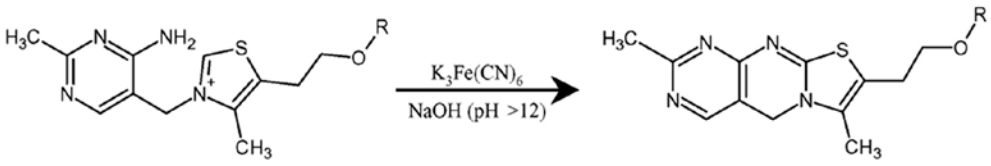
Introduction

Thiamin is an essential compound required for maintaining mitochondrial cellular energy production in human body. Unlike lipophilic compounds, the structure of thiamin contains a positive charge; therefore, an uptake of thiamine is dependent upon on the saturable transporter-mediated process. Thiamin from exogenous sources is rapidly absorbed via two specific transporters belonging to the solute carrier (SLC) superfamily 19, which then transports thiamin across the intestinal lumen to systemic circulation and distributes to target organs. After the uptake process, thiamin is biochemically converted to the active form, TPP which then influences energy regulation in the cell. Therefore, transporters play an important role to determine pharmacokinetics and pharmacodynamics profiles of thiamin.

Recently, we found that thiamin transporter 2 encoded by SLC19A3 is increased in response to Hypoxia-inducible factor 1, a transcriptional factor that regulated several genes in response to low level of oxygen, in H9c2 cells, cardiomyocytes. In order to better understand the role of thiamin transporter 2 in the hypoxic heart, development of a quantification method to detect intracellular thiamin and its active metabolite, TPP. The rational for measuring intracellular thiamin status could tell us more about the relationship between thiamin, TPP and metabolic status inside hypoxic cells. Thiamin and TPP potential can be used as biomarkers for disease detection

associated with thiamin deficiency. More importantly the up-regulation of these transporters could be a drug target for delivery of drug to prevent impairment of cell function in the future.

The challenge is that simultaneous validation methods for detection of thiamin and its phosphates; thiamin monophosphate (TMP) and diphosphate (TPP) from biological samples has been limited. For example, conventional methods to quantify thiamin including Reverse phase HPLC with fluorescence detection of thiochromes has been widely used. However these techniques measure by indirect detecting methods and require pre-or post-column derivatization of thiamin and its phosphate ester by oxidizing agents such as potassium ferricyanide ($K_3Fe(CN)_6$) to thiochromes which can be detected by fluorometry at excitation 366 nm/emission 435 nm (Batifoulier, Verny et al. 2005) as showed in Figure 1.



Thiamin and its derivatives Thiochromes

Figure 1 Oxidation of thiamin to thiochromes (modified form (Lu and Frank 2008))

The limitation of thiochrome quantification is due to the conversion of thiamin and/or TPP to thiochrome by chemical oxidation may not be completed leading to misinterpretation the actual level of both substances. Therefore, the second part of my Master's thesis was to develop an

analytical method to detect intracellular thiamine and thiamine pyrophosphate (TPP) during the same analytical measurement in H9c2 cells without derivatization process in under normal physiological and pathological conditions such as hypoxia or transporter deficiency.

Analyses/Internal Standard (IS)	Samples	Extraction Methods	Method of detection	Mobile phases	Limit/Calibration curves	Highlight
TDP/ Thiazole-methyl-D3 (deuterium labelled internal standard) (Puts, de Groot et al. 2015)	Human Whole blood	70% Perchloric acid ($HClO_4$) for deproteinization	RPUHPLC-MS/MS Zorbax Eclipse Plus C18, 1.8 µm (Agilent Technologies)	Mobile phase A; 98% water + 4.8 g/L ammonium carbonate (50 mmol/L) Mobile phase B; 2% methanol (pH 9.5).	LOD= 2.8 nmol/l LOQ= 9.4 nmol/l	Measurement of underivatized form of TPP, active form without using ion paring reagent *Only one publication measure TPP level with internal standard using LC-MS/MS with validated parameters including sensitivity, imprecision, linearity, recovery and limit of quantification
Thiamin, TMP and TPP/ Pyrithiamine (internal standard) (Basiri, Sutton et al. 2016)	HEK-293 Cells	15% TCA, 72% TCA deproteinization, isopropyl Ether to remove TCA	RPHPLC with fluorescence (ex: 375 nm, emission; 435) Phenomenex Kinetex EVO C18 column; 5 uM	Mobile phase A; 25 mM Na2HPO4 and tetrabutylammonium hydroxide in water-methanol (90:10) Mobile phase B; 70% methanol	LOD = 1.0 nM LOQ = 5.0 nM CS= 1.0-4000 nM	Ion paring reagent; tetrabutylammonium hydroxide to improve the separation Precolumn derivatization by K3Fe(CN)6
Thiamin, TMP and TPP / cabutamind (internal standard) (Chen, Shu et al. 2014)	Mouse plasma	Methanol+ 0.1% formic acid Evaporation under heated Nitrogen gas	HPLC-MS/MS Phenomenex, Synergi 4 uM (reverse phase chromatography)	Mobile phase A; water + 0.01% Trifluoroacetic acid (v/v) Mobile phase B; 0.01% Trifluoroacetic acid (v/v)	N/A	Using LC-MSMS method to determine thiamin and TPP. **No method validation parameters were reported**.
Thiamin (Zastre, Hanberry et al. 2013)	Human Breast cancer cells /Tissue sample	Methanol/CHCl3/H2O	HPLC-MS/MS Zorbax HILIC plus 3.5 uM (hydrophilic interaction normal phase column)	Mobile phase A; ammonium formate in ACN/H2O (50:50 v/v) Mobile phase B; ammonium formate in ACN/H2O (90:10 v/v)	CS= 0.1-1000 nM	Precolumn derivatization by K3Fe(CN)6

Table 1 Summary of quantification of thiamine and its phosphate metabolites methods in previous literatures

Methods:

Preparation of thiamin and internal standard stock solution and working solution

Thiamin and Thiamin-(4-methyl-13C-thiazol-5-yl-^{13}C3) hydrochloride (internal standard) stock solutions were prepared daily prior to the sample analysis. Thiamin and the internal standard stock solutions were weighed and prepared in 0.01M HCl (pH =2) and were diluted to a final concentration of 1,000 ng/ml. The stock solutions were kept on ice during all sample preparation process.

Cells sample preparations

Extraction method I

H9c2 cells were cultured as previously described in the methods and materials section and were maintained to 80% to 90% confluence in 150 mm dish. Cells were washed three times with PBS and detached by using trypsin. Total cell count was performed using TC20™ Automated Cell Counter, Life Science. Cell suspensions were centrifuged at 1000 x g for 5 minutes at 4 °C to separate cell pellets. The supernatant was then discarded and the pellet was washed with PBS and spun down again at 1,000 x g for 5 minutes. PBS washes wee then repeated for an additional 5 times. Followed by discarding the PBS and resuspending the cell pellet in 500 μL of 0.01M HCL followed by sonication for 30 seconds. Five microliters (5 uL) of each aliquot was processed for protein measurement by bichonic acid protein assay (Pierce). Cell sample (100 μL) was treated with 10 uL of concentrated HCl (12N) to precipitate protein. The precipitated sample was spiked with 5uL of internal standard (1,000 ng/ml) stock solution. After thoroughly vortexing, the cell

sample was centrifuged at 14,000 x g at 4°C for 20 minutes and an aliquot 100 μL was transferred to glass vial for LC-MS/MS analysis.

Preparation of Calibration standard curve solution

Thiamin standard curve solutions consisted of 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, 7.81, 3.91and 1.95 ng/ml from a thiamin, 1000 ng/ml stock solution in 0.01M HCl. A total of 10 calibrators were used for this experiment and these calibration standard solutions were treated the same way as the cell sample. One hundred (100 uL) of each standard concentration solution was added with 10 L of concentrated HCl (12N) and was spiked with 5uL of internal standard (1000 ng/ml) stock solution. After thoroughly vortexing, calibrators were centrifuged at 14,000 x *g* at 4°C for 20 min and an aliquot 100 μL from each tube was transferred to glass vials for LC-MS/MS analysis.

Chromatographic conditions

Column	Acquity UPLC BEH HILIC 1.7 mm 2.10 x 100 mm (Waters Co., MA)
Mobile Phase (v/v)	A = 0.1% formic acid and 20 mM Ammonium formate in water B= Acetonitrile
Flow rate	0.5 mL/min
Column oven temperature	40 °C
Run time	2.5 min

Mass Spectrometric conditions

Parameters	Conditions
System	6460 MS system (Agilent Technologies)
Mode	Positive ESI
Nebulizer	45 psi
Gas temperature	300 °C
Gas flow	10 L/min
Transition	265.2>122.1 (thiamin) 269.2>122.1 (Thiamin-(4-methyl-13C-thiazol-5-yl-13C3)

Results

Part I: Method validation

Recovery

The extraction technique in this study was performed by protein precipitation using 12N HCl. Recovery was evaluated by comparing the detector response of extracted thiamin in blank H9c2 cells spiked with at three concentrations of quality control samples (QCs) with detector response obtained from unextracted samples (thiamin 10 ng/ml; low QC, 100 ng/ml; middle QC and 500 ng/ml; HQC in 0.01 M HCl aqueous solution). In preliminary data, we tested one replicate per concentration of each QC sample spiked with internal standard measured by LC-MS/MS in triplicate. The preliminary data for recovery is presented in Table 1.

Run date	Quality control sample	Un-extracted thiamin peak area response	Un-extracted IS	Extracted thiamin in blank cell peak area response	Extracted blank cell IS peak area response	Extracted blank cell + thiamin peak area response	Extracted blank cell IS peak area response
5/2/2016	100 mg/ml	327435	55806	25163	95452	593941	104428
	Peak ratio	5.8674		0.2636		5.6875	
	% Recovery	$\frac{\mathbf{5.6875 - 0.2636}}{\mathbf{5.8674}} \times \mathbf{100} = \mathbf{92.31}\%$					
6/2/2016	10 mg/ml	3895.67	2149	3226	4682	142436.33	4209.67
	Peak ratio	1.812781139		0.689021786		33.83553726	
	% Recovery	$\frac{\mathbf{33.8 - 0.68}}{\mathbf{1.812}} \times \mathbf{100} = \mathbf{1,827.81}\ \%$					
	100 mg/ml	26010	3181	3226	4682	42226.33	4472.33

	Peak ratio	8.17		0.689021786		9.44	
	% Recovery	$\frac{\mathbf{9.44 - 0.68}}{\mathbf{8.17}} \times \mathbf{100} = \mathbf{107.22}\%$					
	500 mg/ml	88269.67	2562	3226	4682	150816.33	5096.67
	Peak ratio	34.45		0.689021786		29.59	
	% Recovery	$\frac{\mathbf{29.59 - 0.68}}{\mathbf{34.45}} \times \mathbf{100} = \mathbf{83.92}\%$					

Table 2 Recovery of thiamin and internal standard

Linearity of calibration standard curve

The standard thiamin samples with concentrations range 1.90 ng/ml to 1000 ng/ml (ten points, n=1 curve) were used to determine linearity of calibration standard curves. Preliminary data showed that the standard curve was linear over the range with r 2 > 0.998. A linear regression equation was demonstrated in Figure 2.

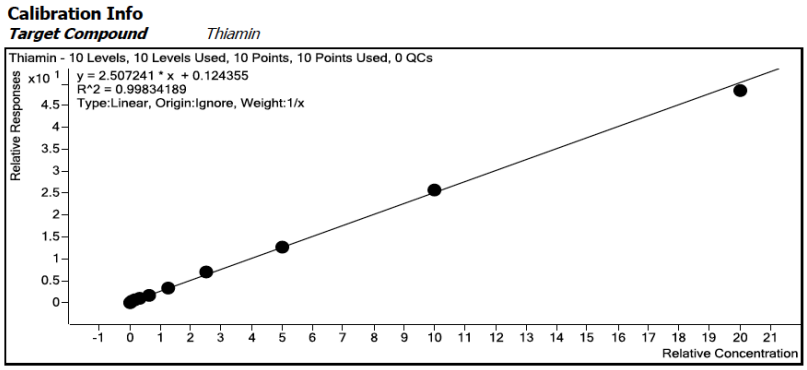


Figure 2 Linearity of calibration standard curve (n=1)

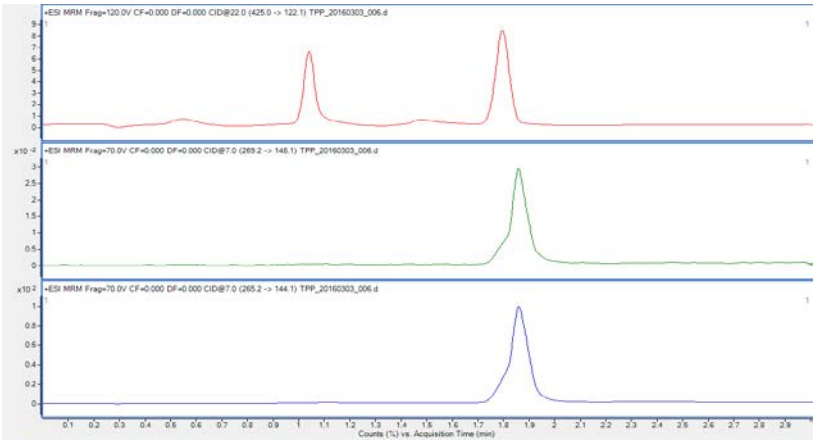


Figure 3 Peak asymmetry shape of TPP under chromatographic condition and MS condition as thiamin

Part II: Preliminary data for determination of thiamin in wildtype H9c2 and SLC19A3 knockdown H9c2 cells (n=1)

Run date	**Thiamin calibration standards (ng/ml)**										**Slope**	**Intercept**	**r2**
5/3/2016	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	CS9	CS10	**2.507241**	**0.124355**	**0.9983**
Actual value	967.97	514.06	250.47	135.94	66.65	32.55	16.49	8.98	3.59	1.33			
Nominal value	1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.8	3.90	1.95			
% Nominal value	96.80	102.81	100.19	108.75	106.64	104.15	105.59	114.96	92.01	68.11*			
% Acceptance criteria	85-115	85-115	85-115	85-115	85-115	85-115	85-115	85-115	85-115	80-120			

Run date	**Backed calculate thiamin concentration (ng/ml)**	
5/3/2016	WT H9c2	A3 KD H9c2
Mean (ng/ml)*	**4.4122**	**11.4052**
SD	0.0141	0.1838
Cell number (cells/ml)	456,500	570,000
Protein concentration (ug/ml)	**659.08**	**1093.7**
Normalized concentration (ng/mg protein)	**6.69**	**10.43**

Discussion

The challenges exist when attempting to develop the analytical method to detect parent and metabolites from the same biological sample in the same analytical run. Due to the difference between chemical properties of thiamin and TPP, our preliminary data indicated that simultaneous determination of thiamin and TPP was not achieved in this study. After extraction of mass spectral peaks of TPP, we observed asymmetric peak shapes possibly due to a high ionization of TPP when measured in the same chromatography and ionization mass spectrometer conditions as thiamin (Figure 3). The possible reason is due to the differences between physiochemical properties of thiamin and TPP. To handle this challenge, stable isotope label of thiamin pyrophosphate may be used for correcting this variation. However, this internal standard is very expensive, thus, similar chemical properties of other compounds could be replaced such as compounds that contains two phosphate molecules in their structures such as chloroquine diphosphate, which exhibit similar chemical properties (i.e. solubility in water 50 mg/ml) to thiamin pyrophosphate.

Another challenge was the extraction method. Previously, we used methanol and chloroform for the cell sample preparation process in order to extract thiamin and TPP from H9c2 cells and separate them from other substances such as protein that could interfere with the detection method. However, this method yielded low % accuracy, possibly due to loss of thiamin and TPP possibly during aqueous phase separation (data not shown). We, therefore, selected an alternative extraction method by using 37% HCl for protein precipitation followed by centrifugation to separate both components in liquid layer. The recovery of this extraction method still has to be conducted to evaluate the variability and to be assure that thiamin and TPP fully release from cells and will not be degraded by concentrated HCl. Additional studies will be conducted to compare

between other organic solvents (e.g. perchloric acid or trichloroacetic acid) to optimize the suitable extraction method for both thiamin and TPP.

We have validated the linearity of the calibration standard curve in this study. Preliminary data from our calibration curve displayed linearity of the line with goodness of fit ($r^2 > 0.998$, 1/x weight). The calibration range can be therefore be used to quantify thiamin in H9c2 cells according to preliminary studies. However, a minimum of 6 replicates is required to be performed to further evaluate % accuracy and % precision.

Preliminary data for quantification of thiamin in H9c2 cells and in thiamin transporter knockdown cells were conducted using calibration standard range from method validation study. The data showed that thiamin levels in both type of cells were within the calibration range limit. The lowest level of thiamin was detected at 4.4122 ng/ml in wild type H9c2 cells and at 11.4052 ng/ml in thiamin transporter knockdown cells. Interestingly, we observed higher intracellular thiamin level in SLC19A3 knockdown cell at 10.43 ng/ml protein vs WT H9c2 at 6.69 ng/ml protein. The reason that could explain this observation possibly due to the adaptation of transporter knockdown cell to uptake thiamin via the other transporters such as thiamin transporter 1 to compensate deficiency in function of transporter 2. However, biological replications and further expression analysis of transporters will be required to better explain this phenomenon. Future studies will be conducted to further evaluate method validation parameters including % accuracy, % precision and stability study in biological matrix and stock solution in order to use this LC-MS/MS method to determine thiamin levels in H9c2 cells and heart tissue samples.