AN ABSTRACT OF THE DISSERTATION OF


Title: SLC23A1, the Gene Encoding Sodium-Dependent Vitamin C Transport Protein 1 (SVCT1): Regulation of Transcription and its Functional Consequences

Abstract approved:

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Tory M. Hagen

Vitamin C is an essential component of the human diet. Uptake of vitamin C in the cell is regulated by the function of sodium-dependent vitamin C transporters (SVCTs). SVCT1 (encoded by the SLC23A1 gene) is expressed in the colon, liver and kidney, where it plays a vital role in determining vitamin C levels in the body. Despite the importance of SVCT1 in vitamin C homeostasis, little is known about its genetic regulation. We isolated a 1239-base-pair fragment of the SLC23A1 5’-flanking sequence from HepG2 cells for the construction of a reporter gene assay. Deletion analysis attributed all promoter activity in this sequence to a small 135-bp region of the SLC23A1 promoter, where basal transcription of SLC23A1 required the binding of Hepatic Nuclear Factor 1 (HNF-1).

We then sought to determine if regulation of SLC23A1 could occur through HNF-1 dependent pathways. Results showed that neither oxidants nor thiol
appreciably altered gene expression. Only insulin and the dithiol compound, \((R)-\alpha\)-Lipoic Acid (LA), significantly elevated reporter gene expression over controls. LA did not elicit its effects by acting as a redox modulator, but through signaling pathways associated with the insulin signaling pathway. These results suggest a critical role for insulin-mediated signal transduction pathways in SLC23A1/SVCT1 regulation.

To determine if a loss of SLC23A1 transcription results in changes of vitamin C homeostasis, we looked at vitamin C transport in an aging model. Ascorbic acid concentrations in hepatocytes from old Fischer 344 rats were 68% lower than cells from young animals. This was associated with a 45% decline in SVCT1 mRNA with age, with no significant changes in SVCT2 mRNA levels. This loss in SVCT1 transcription corresponded to a functional decline in sodium-dependent vitamin C uptake in older animals. LA treatment reversed some of the changes in the SLC23A1 transcription, suggesting that this would be an effective therapy for increasing vitamin C uptake in old animals.

Thus, the data outlined in this dissertation suggests that vitamin C transport via SVCT1 gene transcription is primarily governed by the induction of HNF-1 and insulin signaling, which may have dramatic effects on vitamin C transport in aging.
SLC23A1, the Gene Encoding Sodium-Dependent Vitamin C Transport Protein 1 (SVCT1): Regulation of Transcription and its Functional Consequences

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Alexander J. Michels, Author
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SLC23A1, the Gene Encoding Sodium-Dependent Vitamin C Transport Protein 1 (SVCT1): Regulation of Transcription and its Functional Consequences

Chapter 1

General Introduction
1.1 Background and Significance

The Role of Vitamin C in Human Health

Vitamin C is essential for proper development (1-3) and normal cell function in vertebrate species (4,5). Failure to reach adequate plasma and tissue levels of vitamin C leads to severe declines in biosynthetic processes in the cell, and its deficiency in humans is characterized by a severe debilitating disease known as scurvy (5). If left untreated, vitamin C deficiency leads to loss of bone mass, inadequate collagen formation, hemorrhaging, declines in cellular energy status, impaired neurotransmitter production and, eventually death (6). Low levels of vitamin C in the human diet are needed to prevent the onset of scurvy (10 mg/day), but saturation of plasma and tissues occurs at much higher levels of consumption (200 mg/day). Higher vitamin C intake is associated with decreased risk of chronic disease conditions, most notably cardiovascular disease, cancer, diabetes, cataract formation, and macular degeneration (4,7). Thus, maintaining vitamin C levels is an integral part of human health and the prevention of disease.

For centuries, fresh fruits and vegetables were known to prevent scurvy (8), but Vitamin C was not isolated as the anti-scorbutic factor until 1929. Initially characterized by its ability to inhibit biological oxidation reactions (9), the chemical structure and properties of vitamin C were determined shortly thereafter. The reduced form of vitamin C is known as ascorbic acid (2,3-didehydro-L-threo-hexano-1,4
lactone) (Figure 1.1, left), a water soluble molecule that at neutral pH is ionized at the C-3 hydroxyl group, forming ascorbate. The unique structure of ascorbic acid is key to its biological activity (10), giving it the ability to act as a water soluble electron donor that develops a resonance-stabilized radical after a one-electron oxidation (ascorbyl radical; Figure 1.1, middle). The ascorbyl radical has low reactivity with biological molecules, and can be further oxidized to dehydroascorbic acid (DHA; Figure 1.1, right). A variety of mechanisms exist in the cell that reduce DHA or ascorbyl radical back to the fully reduced ascorbic acid by the expense of NADH or NADPH (11-13). Since any of the oxidized states of ascorbic acid are eventually reduced to active form, the name ‘vitamin C’ refers to the combination of all three molecules in biological systems. However, only the reduced form, ascorbic acid, contains all known biological activity (5).

The reduction potential of ascorbic acid is relatively high (-280 mV), allowing it to participate in a large number of reduction/oxidation (redox) reactions (14). Ascorbic acid is known to be a specific electron donor to eight human enzymes (5) and is implicated in the function of several other enzymatic reactions (15). Vitamin C will recycle enzyme cofactors such as tetrahydrobiopterin, maintaining them in a reduced state (16) to allow full catalytic activity of partner enzymes (17). Depending on the cell type, Vitamin C will also activate the MAP kinase signaling pathway (18-21) and influence redox-sensitive transcription (22). Vitamin C may also play a yet undefined role in mRNA stability in the cell (23).
Figure 1.1 The structure of the reduced and oxidized forms of vitamin C. Ascorbic acid (left) predominantly exists at physiological pH as the ascorbate anion. After one-electron oxidation, ascorbyl radical is produced (also known as ascorbate free radical; center) that forms a resonance-stabilized intermediate. This intermediate may either undergo reduction back to ascorbic acid or be oxidized to dehydroascorbic acid (DHA; right). DHA may be reduced either to ascorbic acid or the ascorbyl radical. Alternatively, DHA may be hydrolyzed, which effects rapid metabolism and loss from the cell.
Most importantly, ascorbic acid is a potent cellular antioxidant that reacts with reactive oxygen species (ROS), although its reactivity with nitrogen species is limited (14,24,25). ROS can be produced endogenously through normal metabolism of the cell (26), but can also come from exogenous sources. The reaction of oxidants with cellular constituents results in the accumulation of damage, impaired function, and death (27,28). Thus, ascorbic acid functions to prevent the accumulation of damage by intercepting oxidants before they participate in harmful cell reactions, at the expense of its own redox state. This function has been demonstrated in vitro and in vivo (24,25).

The oxidation of ascorbic acid results in the formation of a relatively benign free radical species. Ascorbyl radical has low reactivity with cell components, and may participate in dismutation reactions or undergo enzymatic reduction back to ascorbic acid (14,24), resulting in the safe detoxification of oxidant species. Since ascorbic acid may also recycle other cellular antioxidants such α-tocopherol (vitamin E) and glutathione (GSH) (10,13), it may play an integral role in antioxidant defenses in the cell. The maintenance of this ‘antioxidant network’ is critical for human health and the prevention of chronic disease states.

Due to its extreme importance for life, many animal species synthesize ascorbic acid from six-carbon sugars through the activity of L-gulonolactone oxidase. Humans, other primates, guinea pigs, teleost fish and certain species of fruit bat have lost this ability (28). Thus, vitamin C is only a true ‘vitamin’ to a select few species, and in others must simply be considered an essential nutrient. All animals have recycling mechanisms that efficiently reduce oxidized forms of vitamin C back to
ascorbic acid; however, irrevocable loss of vitamin C occurs through the spontaneous degradation of DHA (10) and the continuous renal excretion of ascorbic acid (29). Thus, species that cannot synthesize ascorbic acid are completely dependent on a constant supply of vitamin C from the diet to maintain healthy levels of ascorbic acid in tissues and plasma (30,31) to prevent disease (4,5). Intake of fresh fruits and vegetables in the diet is enough to counteract vitamin C loss as long as the mechanisms that govern its absorption remain intact.

*The Role of Vitamin C Transport*

A significant gap in knowledge for understanding the role of vitamin C in health and disease is the dearth of information on mechanisms involved in its tissue distribution and homeostasis. As mentioned earlier, vitamin C is only bioavailable from one or two sites: from intestinal dietary absorption and/or from hepatic synthesis. Thus, all species depend on transport of vitamin C across cell membranes for redistribution into other tissues, and the maintenance of plasma vitamin C status. Though all forms of vitamin C are water soluble, its large size and ionic charge prevents it from readily diffusing across cell membranes. Therefore, protein-mediated transport mechanisms for vitamin C are required for maintenance of cellular vitamin C status (32). Plasma concentrations of ascorbic acid are normally tightly controlled, and kept within a maximum range of 70-90 µM. Transport mechanisms present in the intestine, liver and kidney maintain ascorbic acid within this range by limiting or enhancing absorption or increasing clearance from the body (30). Steady-state tissue
concentrations of ascorbic acid are much higher than the plasma (typically 1-5 mM). Because plasma levels are at least 10-fold lower than in most cells, vitamin C uptake mechanisms into all tissues work against this concentration gradient. Plasma levels of vitamin C ultimately determine the concentration available for all tissues and organs (30,31). However, few studies have examined the precise control mechanisms involved in whole-body vitamin C status. It is also unknown how vitamin C distribution systems react to exogenous stresses, disease states, global genetic dysregulation, or simply respond to situations when vitamin C availability is limiting. Further elucidation of this system is vital for any progress to be made furthering the role of vitamin C in health and disease.

Two known mechanisms exist for vitamin C transport in tissues. Each transport system is specific towards vitamin C in a particular oxidation state, and do not recognize any other form of the vitamin (32). Fully oxidized vitamin C (DHA) is transported across cell membranes using glucose transporter (GLUT) proteins (33). Alternatively, the reduced and active form of vitamin C is transported by a sodium-dependent transport process that specifically recognizes ascorbic acid as its sole substrate. These so-called Sodium-Dependent Vitamin C Transport (SVCT) proteins (34) are relatively newly discovered members of the SLC superfamily of solute carriers. Although both modes of vitamin C accumulation do occur in vivo, only the SVCT system is considered to be active under normal physiological conditions. However, because both routes of vitamin C transport may contribute to cellular and tissue ascorbic acid status, each will be discussed in more detail below.
DHA Transport via Glucose Transport (GLUT) Proteins

As mentioned above, DHA is considered a source of vitamin C because of the efficient reduction of intracellular DHA to ascorbic acid. DHA uptake was the first specific mode of vitamin C transport characterized in the cell, and occurs through pre-existing glucose uptake mechanisms. GLUT family of transport proteins, specifically GLUT1, GLUT3, and GLUT4, all recognize DHA due to its structural similarity to preferred substrates (33,35). Therefore, facilitative transport of DHA occurs in a similar manner to that of glucose, driving transport down a concentration gradient into cells. Intracellular reduction mechanisms maintain this concentration gradient by converting DHA to ascorbic acid rapidly, causing ascorbic acid to appear in the cell within seconds after DHA administration (13). Though the exact mechanisms for this activity are not entirely clear (11,13,36,37), high influx of DHA overwhelms these systems, resulting in DHA efflux and degradation (32). However, this phenomenon is only observed with pharmacological doses of DHA. Under normal conditions, endogenously produced DHA is handled easily by these recycling mechanisms, preserving ascorbic acid levels at the expense of NADH/NADPH.

Despite substantial evidence for GLUT-mediated DHA transport in vitro, the physiological relevance of this system is debatable. GLUT proteins do not recognize ascorbic acid (32,33), and thus this system requires a significant expense of intracellular reducing equivalents to maintain ascorbic acid levels. DHA is produced only at exceedingly low rates under normal conditions, as it is not usually detected in
biological solutions. High quantities of exogenously provided DHA can be cytotoxic (38,39). In order for this to be a physiologically relevant mode of vitamin C uptake, cellular oxidation systems would need to exist to oxidize ascorbic acid in response to cellular demands. However, even in this scenario it must be noted that the GLUT family of proteins transport glucose, which is in exceedingly high quantities relative to plasma DHA levels. Several studies have shown that DHA transport is inhibited in the presence of glucose, and further affected by insulin signaling (32). Therefore, DHA transport may only exist as an important scavenging mechanism for vitamin C, increasing intracellular ascorbic acid levels and protecting vulnerable cells from injury in times of oxidative stress.

Despite these limitations, DHA uptake may be an important mechanism in several cell types, especially those that have lost the ability to transport ascorbic acid directly, such as neutrophils (40,41) or erythrocytes (42). During an inflammatory response, DHA is produced and accumulates in circulating white blood cells (40), buffering against oxidative stress by raising intracellular ascorbic acid levels. Pharmacological use of DHA prevents injury from oxidative stress insults by loading vitamin C into cells rapidly (43). However, in light of these limited physiological roles of DHA transport, the contribution of DHA uptake to whole-body vitamin C homeostasis is thought to be relatively insignificant.

Ascorbate uptake via Sodium-Dependent Vitamin C Transporters (SVCTs)
Direct transport of ascorbic acid occurs in most tissues. Ascorbic acid is taken up into the cells from the plasma against its cellular concentration gradient. This otherwise unfavorable reaction is thermodynamically allowed because of the inwardly directed sodium gradient across cell membranes generated by the sodium/potassium-ATPase. Even before the discovery of the SVCT proteins (below), sodium-dependent ascorbic acid transport was already recognized in many cell types, and especially well-characterized in the intestine and kidney (32, 44, 45). Plasma vitamin C status is maintained by the regulation of vitamin C uptake in these organs (32), emphasizing the role of sodium-dependent vitamin C transport in vitamin C homeostasis.

Expression cloning from functional cDNA libraries led to the identification of two SVCT proteins based on their predicted function. SVCT1 is encoded by the gene SLC23A1, while SVCT2 is encoded by SLC23A2. Two other members of the SLC23 family have been identified (SLC23A3 and SLC23A4) but are not found in vivo and have no known ascorbic acid transport capacity. Thus, the combined contribution of SVCT1/SLC23A1 and SVCT2/SLC23A2 accounts for all of the observed ascorbic acid transport activity in tissues (46).

The physiological presence of SVCT1 and SVCT2 has been established by immunohistological staining and detection of specific mRNAs for these proteins (34, 46, 47). SVCT1 and SVCT2 in humans have a similar size and function, sharing 66% amino acid identity (48-50). Each protein has 12 transmembrane spanning motifs and several putative sites for post-translational modification (34, 47, 51, 52). SVCT1 and
SVCT2 will only recognize ascorbate and sodium ions, and both are specifically required for transport activity (34,47). Kinetically, SVCT1 has a low affinity ($K_m$ between 65-237 µM) for its substrate as compared to SVCT2 ($K_m$ between 8-62 µM). However, SVCT1 has a significantly higher capacity ($V_{max}$) for vitamin C transport than its homolog, suggesting that SVCT1 may be involved in bulk dietary absorption and plasma vitamin C homeostasis (46,47,51). Alternatively, SVCT2 kinetic analysis reveals that maximal velocity is reached when ascorbic acid levels are near the saturation point of the plasma (70-90 µM). This indicates that SVCT2 may function primarily as a means to transport vitamin C from the plasma into tissues.

This potential delineation in the functional roles of these two transporters, however, is complicated by evidence that some cells contain both transport proteins. Because this ultimately plays a role in the regulation of systemic vitamin C levels, the general tissue distribution of SVCT1 and 2 will now be discussed.

The presence of SVCT2 has been noted in almost every tissue and cell type examined, and is especially prevalent in tissues with high metabolic activity or increased need for protection from oxidative stress. This includes the central nervous system, eyes, adrenal gland, bone, and skeletal muscle (34,53,54). On the other hand, SVCT1 is expressed primarily in epithelial tissues, such as the intestine, kidney, lung and liver; it has also been found in the pancreas, thymus, testes and ovaries (34). SVCT1 is typically targeted to the apical side of polarized epithelium (55), suggesting its involvement in dietary vitamin C uptake in the intestine and reabsorption in the
proximal tubule in the kidney. This select distribution again suggests that SVCT1 may be involved in systemic regulation of vitamin C status, controlling both the uptake and excretory mechanisms involved in maintaining ascorbic acid homeostasis.

Importance of the SLC23 Family on Vitamin C Status

SLC23 (Solute carrier family 23) members A1 and A2 are the HUGO designations for the genes encoding SVCT1 and SVCT2. These genes have been cloned in humans, rodents and other organisms (46,48-50,56). The functional expression of the gene appears to be well conserved between human, mouse, rat, dog and pig as homology across mRNA transcripts for the SLC23 gene products in these species is high (46). SLC23A1 has been mapped to human chromosome 5q31.2, while SLC23A2 exists on chromosome 20p12.2 (57). Functional comparisons between the two genes show that despite high homology in their exon sequences, there are vast differences in their introns and non-coding regions (58). SLC23A2 is by far the larger of the two genes, stretching 158,398 base pairs across 17 exons, while SLC23A1 is only 16,096 base pairs and 15 exons in length (57,58).

Evidence for the importance of the SVCT system to overall health is underscored by the phenotype of SLC23 knockout mice. SLC23A2(-/-) mice survive development until birth but perish shortly thereafter as a result of cerebral hemorrhaging and respiratory failure (3). Tissue levels of vitamin C in these mice are uniformly low. The lethal effects of this knockout are not surprising, given that
SVCT2 plays a prominent role in maternal supplementation of vitamin C to the fetus (2,59).

The recently developed SLC23A1(-/-) mouse has not been completely characterized, but it appears to develop normally in utero. These knockout mice suffer from a 40-70% depression in plasma vitamin C levels, resulting in decreased availability of ascorbic acid to tissues (60). This loss is despite the presence of endogenous vitamin C synthesis. Further work reveals the lower plasma vitamin C levels observed in this mouse strain result from increased renal clearance of the vitamin. Without ascorbic acid synthesis, it is predicted that a loss in SLC23A1 gene expression would prevent uptake from the diet and increase renal excretion, leading to severe declines in plasma ascorbic acid status (60). In turn, a loss of plasma vitamin C lowers the availability of vitamin C to all cells and tissues. Thus, especially for humans, understanding the functional regulation of SLC23A1/SVCT1 is necessary for control of whole-body vitamin C status.

**Functional Regulation of SLC23A1**

A functional promoter analysis has only been performed on SLC23A2 (61) and potential pathways for its transcriptional regulation have been delineated (1,62-64). Direct comparison of the 5′-flanking regions of SLC23A1 and SLC23A2 show poor conservation of nucleotide sequences, suggesting divergent modes of transcriptional regulation (58). The limited studies that have been performed on SLC23A1 (65,66)
suggest the signal transduction pathways involved in SLC23A1 transcription are not the same as SLC23A2. These two genes may thus be regulated at the transcription level by entirely different mechanisms.

The human SLC23A1 promoter has only been partially analyzed by Erichsen et al. (67). No rigorous analysis of the regulatory mechanisms involved in SLC23A1 transcription was performed by this group. However, some putative transcription factor binding sites were identified in the first 200 base pairs of the gene by computational analysis of the DNA sequence. Both classical CAAT and TATA boxes were present, along with two AP-1 sites and one GATA binding site (67) (Figure 1.2). However, more precise analysis using nuclease-protection footprinting shows that the transcriptional start site of the gene overlaps with both of the AP-1 sites and the CAAT box. These latter results strongly suggest that these theoretical transcription factor binding sites may have nothing to do with the functional regulation of SLC23A1 gene expression. Computational analysis has also revealed numerous other putative transcription factor binding sites within the SLC23A1 promoter, but without knowing a functional domain for promoter activity, the number of theoretical DNA-binding elements and the proteins involved in transcriptional control of this gene are impossible to define with the currently available information.

Sequence comparison of the SLC23A1 gene between species (rat, mouse, human, chimpanzee and dog) revealed several conserved DNA binding regions in the 5′-flanking region (Figure 1.2). However, identification of these elements and the
Figure 1.2 Diagrammatic representation of the SLC23A1 promoter.
Sequence analysis of the first 1200 base pairs in the SLC23A1 promoter revealed several sites of homology between mammalian species. No significant homology was observed in sequences beyond this area. For the sake of reference, the human transcription start site was labeled +1 and each base in the un-transcribed region was represented by negative numbers. In homologous regions, several transcription factor binding sites were identified by computational analysis. These DNA sequences, along with those proposed by Erichsen et al. (67), are displayed as black bars at the location of the core binding motif. Overlapping sequences were separated to denote their individual contributions to the promoter. Names of the individual transcription factors are presented under the location of the appropriate binding site.
potential transcription factors that control them is based on computational DNA sequence analysis, and there is no molecular data regarding function of the 5’-flanking region of the SLC23A1 gene. To determine if vitamin C or any other regulatory compounds play a role in vitamin C transport and metabolism, functional characterization of these promoter regions must be performed.

Relationship of SVCT1 to Vitamin C Levels in Human Health

Despite the noted work using SLC23A1(-/-) transgenic mice, no comparable work analyzing the function of SVCT1/SLC23A1 on absorptive capacity of vitamin C in humans has been undertaken. Genetic analysis of SLC23A1 in humans shows several sites for non-synonymous single-nucleotide polymorphisms (SNPs) that may impact SVCT1 protein function or transcriptional regulation (58,67). However, no functional roles of these mutations have been determined. Preliminary data in mice show that SVCT1 is necessary for maintenance of vitamin C levels (60), but extrapolating this to the functional regulation of SLC23A1 and vitamin C transport is difficult. There are several instances, however, where human pathophysologies display a loss of vitamin C bioavailability and declines in plasma levels, and may be instructive as to the regulatory pathways involved in SLC23A1 expression. One of the most notable instances where a loss of vitamin C homeostasis is observed is during aging. The elderly display significant declines in the ability to absorb vitamin C from the diet (68,69) a phenomenon which is also evident in animal models of aging. A telling meta-analysis which combined data from 36 studies illustrates this point (69).
Here, Brubacher et al. (69) examined the relationship between plasma levels and intake of vitamin C, analyzing data from studies recruiting both young and elderly subjects. They concluded that plasma vitamin C levels in aged subjects were only half the levels seen in younger adults at any given dose below 200 mg/day. Moreover, the data suggested that significantly more vitamin C was needed to maintain plasma saturation in older people (69). This suggests that there is a significant age-related loss in vitamin C bioavailability, which would implicate declines in SVCT1 function because this transporter regulates plasma ascorbic acid homeostasis.

Dietary factors may also play a large role in the regulation of vitamin C uptake through the regulation of SLC23A1 or SVCT1. Because of its relationship to SVCT1 function, vitamin C is one of the dietary components considered likely to impact SVCT1 regulation. However, only one study on this regulatory relationship has been performed. Caco2 cells were treated with up to 15 mM of ascorbic acid and monitored for effects on SVCT1 mRNA. Results show that SVCT1 transcription declined 77% after 24 hours of incubation (65). No follow-up analysis into these regulatory pathways was performed, so little can be discerned regarding the mechanisms of ascorbic acid regulation of its own transporter. There is also cursory evidence that glucose (70) and insulin regulate ascorbic acid uptake (71), but the exact molecular mechanisms of this relationship remain a mystery. Any analysis of SLC23A1 must examine the effect these dietary factors, especially ascorbic acid itself, have on the regulation of vitamin C transport.
1.2 Dissertation Hypotheses and Aims

Despite substantial evidence that vitamin C levels are important in the maintenance of human health, no attempts have been made to understand the functional regulation of its transport in relation to vitamin C bioavailability and plasma homeostasis. Because optimal vitamin C levels will not only prevent scurvy, but also potentially lower oxidative injury and the risk for diseases related to increased oxidative damage, understanding the regulation of vitamin C transport provides important information for vitamin C supplementation in health and disease. This knowledge may be particularly important to design chemoprotective strategies to maintain optimal health in the elderly where vitamin C bioavailability becomes limiting. However, without specific analysis of vitamin C transport proteins at the genetic level, and correlations with functional characteristics of ascorbic acid transport, little other work on the subject can be accomplished.

Thus, the major aims of this dissertation were to analyze the promoter of the SLC23A1 gene and provide the first-ever look at the functional regulation behind its transcription. Specifically, we sought to determine one or more transcription factors that are involved in the regulation of this gene. Furthermore, a cursory examination of the potential signal transduction pathways involved in transcriptional control of SLC23A1 was surveyed in order to focus future work on the upstream sensory mechanisms associated with SVCT1-dependent vitamin C transport. Lastly, potential age-related changes to SLC23A1 expression were examined to determine its
involvement in limiting vitamin C bioavailability with age. The specific aims of this dissertation are as follows:

1. **To analyze the functional characteristics of the SLC23A1 promoter and identify transcription factor(s) involved in its expression.** To address this aim we performed a computational and functional analysis of the 5’-flanking region of the SLC23A1 gene and determined areas of functional consequence for its transcription in a human cell line (Chapter 2).

2. **To identify endogenous transcription factors that regulate SLC23A1 expression, and delineate cell-signaling pathways that may be involved in its expression.** Since nothing is known about the regulation of SLC23A1, we chose a broad spectrum approach, analyzing likely pathways involved in its transcription, determining which factors, if any, could alter gene expression (Chapter 3).

3. **To determine the extent of the age-related changes in vitamin C status and its relationship to SLC23A1 function.** Using an animal model for aging that shows age-related declines in vitamin C status, we determined if there were concomitant declines in age-related uptake similar to that seen in humans. Furthermore, the contribution of sodium-dependent vitamin C transport on this process was characterized (Chapter 4).

The data outlined in this dissertation elucidates the molecular and regulatory characteristics of SLC23A1 and how it relates to vitamin C homeostasis. Here we
show that SVCT1 expression is regulated by HNF-1, a transcription factor known to govern glucose handling and metabolism. Vitamin C transport via SVCT1 gene transcription is primarily governed by the carbohydrate nature of ascorbic acid and not redox-dependent mechanisms. The importance of this novel pathway for vitamin C transport regulation is underscored by the decline of SLC23A1 transcription in older animals, and its impact on vitamin C uptake during the aging process.
Chapter 2

Hepatocyte Nuclear Factor 1 (HNF-1) is Essential for Transcription of SLC23A1, the Gene Encoding Sodium-Dependent Vitamin C Transporter Protein 1 (SVCT1)

Alexander J. Michels and Tory M. Hagen
2.1 Abstract

Vitamin C is an essential component of the human diet, required in many cellular processes including the defense against reactive oxygen species (ROS). Uptake of vitamin C is regulated partly by the function of sodium-dependent vitamin C transporters (SVCTs). SVCT1 is expressed in the colon, liver and kidney, organs that together regulate vitamin C homeostasis. Thus, SVCT1 plays a vital role in whole-body vitamin status. Despite the importance of SLC23A1 on vitamin C status, little is known about the molecular and cellular regulation of this protein. The purpose of the present study is to investigate the transcriptional regulation of human SLC23A1, the gene that encodes for SVCT1. A 1239 base pair fragment of the SLC23A1 5’-flanking sequence from HepG2 cells was cloned and tested for promoter functionality. Robust transcriptional activity was seen in this sequence, nearly 25-fold above the control vector. Deletion analysis attributed high promoter activity to a small 135 base pair region proximal to the transcriptional start site, thus defining the minimal promoter region of SLC23A1. While several transcription factor binding sites were identified in this sequence, firefly luciferase reporter constructs showed that basal transcription required the binding of Hepatic Nuclear Factor 1 (HNF-1) to its cognate sequence. Mutation in the HNF-1 binding site resulted in complete loss of luciferase expression, even in context of the whole promoter. Taken together, this suggests that HNF-1 is required for basal SVCT1 transcription in the liver, and represents a novel mechanism for the regulation of vitamin C transport.
2.2 Introduction

Maintenance of vitamin C levels is essential to normal cell function as ascorbic acid participates in a wide range of biosynthetic reactions and contributes to antioxidant defenses in tissue and plasma (4,5,25). Primates and certain other species have lost the ability to synthesize vitamin C de novo (28) and are at risk for developing scurvy with inadequate diet (5,29,31). Even in species that synthesize ascorbic acid, it now appears that its dietary availability significantly contributes to tissue vitamin C status. Regardless of the source of vitamin C, accumulation into tissues and clearance from the plasma are governed by proteins that facilitate vitamin C transport across membranes (32). Thus, it is clear that transport mechanisms play a significant role in overall vitamin C bioavailability, tissue distribution, and metabolism throughout mammalian life (30,31,72).

Physiological ascorbic acid uptake has been ascribed to a group of Sodium-Dependent Vitamin C Transport proteins (SVCT), which mediate direct ascorbic acid absorption from the diet or uptake into tissues from the plasma (34,48,49,51). To date, only two functional proteins of the SVCT family have been identified (SVCT1 and SVCT2), which are the products of distinct genes, SLC23A1 and SLC23A2, respectively (46). While SVCT2 is widely expressed in many tissues (34,48,49,63,73), SVCT1 is limited to a few organs, notably the epithelial cells of the liver, kidney, intestine, and pancreas (34,73). SVCT1 is normally regulated to the apical side of cells (55,74), indicating a different functional role in vitamin C homeostasis from SVCT2.
Kinetic characteristics of SVCT1 show it to be a high capacity, low affinity carrier (48,50,55,73,75) involved in bulk ascorbic acid absorption from the diet and renal reuptake. Thus, SVCT1 activity effectively regulates plasma vitamin C levels and availability for other tissues.

The essential role of SVCT2/SLC23A2 in prenatal development has been established (3) and some of the regulatory pathways for SVCT2 elucidated (1,62,76-79). Few comparable studies have been performed for SVCT1. The SLC23A1(-/-) mouse dramatically shows the importance of SVCT1 on vitamin C homeostasis. These animals display 50-70% lower plasma vitamin C concentrations, resulting in depressed ascorbic acid levels in tissues compared to their heterozygous littermates (60). Vitamin C loss is due to its increased renal excretion and the loss of ‘tight’ control mechanisms in the kidney and liver.

Despite the overall importance of SVCT1 to vitamin C status little is known about the regulation of this protein, especially at the molecular level. In particular, factors that regulate the transcription of SLC23A1 are poorly characterized (58,67), and significantly limit further molecular studies to elucidate the role of SVCT1 on vitamin C homeostasis.

In the present study, we examined the transcriptional regulatory elements responsible for expression of human SLC23A1. Using promoter deletion constructs, we show that a 135-base-pair proximal segment of the 5’-flanking region is critical for
transcription. Additionally, site-directed mutagenesis of reporter gene constructs transfected in HepG2 human hepatocarcinoma cells reveal a critical role of Hepatic Nuclear Factor 1 (HNF-1) in SLC23A1 transcription. Because HNF-1 is regulated partly in an insulin-dependent manner, these results suggest that bulk vitamin C absorption and cellular distribution may be regulated more by dietary factors that influence carbohydrate metabolism than reduction/oxidation-dependent mechanisms.
2.3 Materials and Methods

Materials Used

Cell culture materials were obtained from American Type Culture Collection (ATCC; Manassas, VA) or Sigma-Aldrich (St. Louis, MO). Materials for DNA manipulations were obtained from New England Biolabs (Ipswich, MA). All other chemicals were reagent grade or the highest quality available from Sigma Aldrich, or as noted.

Cell Culture

HepG2 cells, a hepatocellular carcinoma cell line, were obtained from ATCC. Cells were maintained in Eagle’s Modified Minimum Essential Medium (MEM) with 2 mM L-glutamine and Earle's balanced salts solution (BSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, with the addition of 10% fetal bovine serum (MEM and FBS, ATCC). Cells were cultured at 37°C and 5% CO₂, and passaged as needed upon reaching 80-90% confluence.

Creation of a Human SLC23A1 Reporter Gene Construct

The reporter gene, pSLC23A1-Luc, was constructed using a pGL3-Basic (Promega) reporter gene template. Briefly, genomic DNA isolated from HepG2 cells was amplified using PCR amplification of the ‘Full Length’ forward and reverse primers (Table 2.1) to generate a 1239 base pair fragment. This PCR product was
### Table 2.1 Sequence of primers used in PCR reactions for construct creation

<table>
<thead>
<tr>
<th>Fragment Name</th>
<th>Sequence (5’-3’)</th>
<th>Position(^a)</th>
<th>Fragment Size(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Length</td>
<td>ACTTTGGAAATTGGGAGCAGTA</td>
<td>-1224 to -1202</td>
<td>1239</td>
</tr>
<tr>
<td>Δ1</td>
<td>ACAGCCACCCAGAAGTAGGTAG</td>
<td>-1042 to -1020</td>
<td>1067</td>
</tr>
<tr>
<td>Δ2</td>
<td>TTGGTTTCTGCAACCTTGCTAT</td>
<td>-531 to -509</td>
<td>556</td>
</tr>
<tr>
<td>Δ3</td>
<td>GCTCTCTCTTGGAACACGAAGAA</td>
<td>-215 to -193</td>
<td>240</td>
</tr>
<tr>
<td>Δ4</td>
<td>GGTGGCTTTTACTGCCCCTAGTG</td>
<td>-145 to -123</td>
<td>160</td>
</tr>
<tr>
<td>Δ5</td>
<td>TGGTTACAGATTTACTTTGAAC</td>
<td>-53 to -33</td>
<td>80</td>
</tr>
<tr>
<td>Δ6</td>
<td>AAAGTCCTCCTCCTT</td>
<td>-15 to +1</td>
<td>40</td>
</tr>
<tr>
<td>Reverse(^c)</td>
<td>AAGAGGGGATGACTTGACAAAG</td>
<td>+25 to +3</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Position is the region of primer annealing in relation to the transcriptional start site set as +1 (25 bases upstream from the 3’ end of the sequence).

\(^b\) Fragment Size is the total length of fragment amplified in base pairs.

\(^c\) Downstream primer used for the generation of each construct.
isolated by agarose gel purification and then cloned into the pCR-II TOPO vector (Invitrogen) using manufacturer’s protocols. After sequencing to confirm identity, the DNA fragment was removed by restriction enzyme digestion and cloned into the Hind III and Xho I sites of pGL3-basic (Figure 2.1). For the creation of deletion constructs, identical procedures were used as described above with alterations made only in the forward primer (the same reverse primer was used in all constructs), resulting in smaller PCR fragments generated. Primers used for the generation of each of these constructs are detailed in Table 2.1.

Site-Directed Mutagenesis

Mutations in transcription factor binding sites were made using the Quik-Change II Site-Directed Mutagenesis kit (Stratagene). Primer sequences were designed that contained the putative transcription factor binding sites, but incorporated two base pair mutations that would remove the consensus sequence for the identified transcription factor (Table 2.2). Annealing, elongation, and digestion steps were performed as per the manufacturer’s instructions. Clones containing the mutations were determined by direct sequencing of the SLC23A1-containing vectors to ensure no spontaneous mutations occurred during this process.

DNA Sequence Analysis

Subcloned DNA fragments and site-directed mutations were confirmed by DNA sequencing using an ABI automated DNA sequencer (CGRB; Oregon State University). Sequence searches were performed using the BLAST, the TRANSFAC,
Figure 2.1 Diagrammatic representation of the SLC23A1 reporter vector. As described in Materials and Methods, all promoter fragments used in this study were inserted A) upstream of a firefly luciferase gene in a pGL3-Basic vector backbone. Indicated fragments of the SLC23A1 genomic sequence were ligated into the pGL3-Basic vector at the Hind III and Xho I sites present on this vector (“Insert Site”). B) Schematic representation of the Full Length 1239 base pair construct. The transcriptional start site was designated +1. Shaded areas refer to DNA from the SLC23A1 genomic sequence. All generated constructs retain the transcription start sequence (+1 to +25), but differ in amount of upstream DNA sequence.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Location (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF1</td>
<td>GGCATTGTCAAAGTAAGACTGTAAATCAGATGCCC</td>
<td>-46</td>
</tr>
<tr>
<td>Tal1β</td>
<td>CAAAGTAAATCTGTAACCAATGCCCAGCTCCGGATTGC</td>
<td>-56</td>
</tr>
<tr>
<td>TATA Box</td>
<td>TTAACTCTCACTAGGGCAGCAGAAGCCACCCAAAGGCGAATTC</td>
<td>-125</td>
</tr>
</tbody>
</table>

Underlined portion refers to changes from wild-type sequence to remove transcription factor binding.

“Location” is the location first altered base in relation to the transcriptional start site set as +1 (25 bases upstream from the 3’ end of the sequence).
and the TFSEARCH databases. Transcription factor binding sites were identified with publicly available computer algorithms such as Match (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) or the various components of Genomatix software package (http://www.genomatix.de).

**Transient Transfection of Cells**

HepG2 cells were grown to approximately 40-50% confluence before transient transfection on 6-well plates. Transient transfection was performed using the Effectene reagent from Qiagen as per manufacturer’s instructions. Briefly, 0.8 µg of firefly luciferase plasmid and 50 ng of a constitutively expressed *Renilla* Luciferase plasmid (pRL-CMV; Promega) were complexed with 10 µl Effectene and 6.8 µl Enhancer reagent in a final volume of 100 µl. Immediately before the addition of transfection complexes to cells, 2 mL of normal growth medium (MEM and FBS, above) was added to the reaction. Cells were left in the presence of Effectene complexes for 18-72 hours.

**Luciferase Assay Procedure**

Transiently transfected HepG2 cells were harvested using the Passive Lysis Buffer included in the Dual Luciferase Assay Kit (Promega). Cells were flash frozen and thawed twice before 20 µl of each cell extract was placed in a 96-well non-binding assay plate (Corning) and firefly and *Renilla* luciferase levels measured as described in the manufacturer’s instructions. Because *Renilla* luciferase values are indicative of the total number of cells transfected per experiment, luciferase data is represented as the
ratio of firefly to *Renilla* luciferase expression in Relative Light Units, and then normalized to the indicated control plasmid as a fold change or percent variation.

*Nuclear Extract Preparation*

HepG2 cells were harvested from 75 cm² flasks by incubation with Trypsin-EDTA (ATCC) until cells were liberated. Trypsin was diluted by the addition of ice-cold PBS containing Protease and Phosphatase Inhibitor cocktails (Sigma), and the cells were pelleted by centrifugation at 500xg. After removal of the supernatant, cells were lysed by the procedure of Dignam et al. (80) and cytosolic and nuclear fractions obtained. Proteins were stored in aliquots at -80°C until use.

*Electrophoretic Mobility Shift Assay (EMSA)*

A pair of oligonucleotides encoding the human SLC23A1 promoter sequence near the HNF-1 binding site was synthesized. The top strand of this sequence is as follows: 5′-GGCATTGTTCAAAGTAAATCTGTAACCAGATGCCAGCTCCG-3′, (HNF-1 site underlined). Each oligonucleotide was labeled with \(\gamma^{32}\text{P}\)ATP (Amersham) by T4 polynucleotide kinase (New England Biolabs). The labeled oligonucleotides were separated from free ATP on Sephadex G50 (Amersham) columns and then annealed by heating to 95°C for 5 minutes followed by slow cooling to room temperature. Unlabeled probes were also created for competitive binding studies, at a stock concentration of 3 mM (3 μM working concentration). The HNF-1 consensus binding sequence was encoded by the oligonucleotide: 5′-TAGGTTAATAATAATTACATTACCATTA-3′ along with its reverse sequence complement.
(HNF-1 recognition site is underlined). The human SLC23A1 sequence containing a mutation in the HNF-1 site (mutation underlined) was encoded by 5'-GGCATTGTCAAAGTGATGTACCAGATGCCAGCTCCG-3' and was synthesized along with its reverse sequence complement.

EMSA binding assays were carried out on ice in 20 µl (total volume) reactions containing 11 mM Hepes (pH 7.9), 10% glycerol, 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, and 0.5 µg of poly(dI-dC). This mixture was incubated at room temperature for 10 minutes with or without competitive inhibitors (1 µl) and with or without nuclear extract from HepG2 cells (5 µg protein). This was followed by the addition of 50,000 cpm (approximately 30 fmoles) of the above labeled oligonucleotides and incubated at room temperature for another 20 minutes. For supershift assays, 2 µg of anti-HNF-1 antibody (Santa Cruz; sc-8986) was incubated in the binding mixture for the last 10 minutes. The mixture was electrophoresed on a 5% polyacrylamide gel in 0.5× TBE buffer as a running buffer. After electrophoresis, the gel was then dried, and the [³²P]-DNA pattern on the gel was analyzed by overnight exposure to autoradiography film (Amersham).

**Statistics**

Data are represented as the arithmetic mean ± standard error of the mean of a minimum of three separate experiments. Statistical differences between two experimental conditions were evaluated using the two-tailed Student t-test with a p value of <0.05 considered significant.
2.4 Results

Creation of human SLC23A1 reporter gene constructs

Using the published genomic sequence for human chromosome 5, we constructed primers for the 5’ regulatory region of SLC23A1, the gene encoding SVCT1 (as described in Materials and Methods). These primers amplified a 1239-base-pair DNA fragment from genomic DNA derived from HepG2 cells. Sequence analysis confirmed the fragment’s identity. This segment of the SLC23A1 promoter contains the transcriptional start site, which is 25 base pairs upstream from the 3’ end, as identified by alignment with the full-length SVCT1 mRNA sequence (GenBank no. NM_005847).

To determine if this gene fragment actively regulated SLC23A1 gene expression, the sequence was ligated into a pGL3-Basic luciferase expression vector (Figure 2.1a). This vector was chosen because it lacks any endogenous promoter elements and allows for low baseline firefly luciferase expression in the absence of DNA inserts. Because of this, firefly luciferase expression could be safely ascribed to have come entirely through the SLC23A1 promoter DNA inserted into the vector (Figure 2.1a). After transient transfection into HepG2 cells, firefly luciferase expression was detected only in cells with the SLC23A1-containing construct, whereas those cells transfected with empty vector alone showed no firefly luciferase activity above background levels. Luciferase activity was observed between 18 and 72 hours after transfection (data not shown) and reached a maximum after 48 hours. Maximal
reporter gene activity was approximately 25±3-fold higher than the empty pGL3-Basic vector. This suggests that the 1239-base-pair fragment of the SLC23A1 5’-flanking region is transcriptionally active, and the extent of promoter activity can be gauged directly from the measurement of luciferase activity in transiently transfected HepG2 cells.

**Determination of Promoter Activity**

In order to establish which part of the 1239-base-pair sequence (now designated as the ‘full length’ construct) was essential for transcriptional activity, a series of deletion constructs were synthesized by PCR. Inserted into the pGL3 backbone, these vectors contained progressively smaller regions of the full length SLC23A1 DNA, as described in Table 2.2. These constructs, designated Δ1-Δ6, all contained a preserved proximal element near the transcription start site (Figure 2.1a, b). The fragments were cotransfected into HepG2 cells along with a vector expressing Renilla luciferase (pRL-CMV) that served as a control for efficiency of transfection.

As promoter size increased (Figure 2.2, left), we analyzed luciferase production (Figure 2.2, right) to determine the minimal promoter region necessary for the expression of SLC23A1. Results show that constructs containing either of the two smallest sized fragments (Δ5 or Δ6) had no appreciable firefly luciferase activity over that seen in the empty vector. In contrast, construct Δ4 exhibited a nearly 56-fold (±3.7) increase in activity over the empty vector. Interestingly, activity of this fragment also displayed a significant (p<0.001) 2-fold increase of luciferase activity
Figure 2.2 Functional analysis of the human SLC23A1 promoter in HepG2 cells.
Fragment size of the SLC23A1 promoter placed in the pGL3-Basic vector is expressed in relation to transcription start site (+1) and displayed on the left. For example, the full length construct (‘Full’) contains 1239 base pairs of the genomic sequence stretching from the positions -1214 to +25 bp. After transient transfection into HepG2 cells, luciferase production of the various constructs was measured and shown on the right. Firefly luciferase activity is normalized to the simultaneously expressed Renilla luciferase and expressed relative to the pGL3-Basic control vector without DNA inserted. The full length construct and Δ1, Δ2, Δ3 and Δ4 showed substantial activity over the empty vector (p<0.001). Deletions to the Δ3 fragment showed no change in activity from the full length vector (p>0.5). The Δ4 construct showed a significant increase of activity (p<0.01) compared to the full length sequence. Deletion constructs Δ5 and Δ6 showed no appreciable change from pGL3-Basic control vector alone (p>0.5). This suggests the minimal promoter region for SLC23A1 lies in the Δ4 construct, with a possible repressive motif between Δ3 and Δ4.
over that of the full-length sequence. This enhanced activity was not observed in the larger Δ3, Δ2, or Δ1 constructs where similar levels of gene expression to the full length construct were observed. These results indicate that a repressive motif lying between Δ3 and Δ4 constructs may have been identified. However, as the minimal promoter element needed for SLC23A1 expression is clearly contained within the Δ4 fragment of the SLC23A1 5’-flanking region, we focused on this region for the remainder of the study.

**SLC23A1 Promoter Analysis**

Comparing published DNA sequences of the SLC23A1 gene from a number of diverse species (e.g. rat, dog, mouse, chimpanzee and human) reveals a high homology for the SLC23A1 5’-flanking region proximal to the transcription start site. This highly conserved region is found in the core DNA sequence located between the Δ4 and Δ6 SLC23A1 constructs and to some degree between the Δ3 and Δ4 constructs. These highly conserved areas may harbor regulatory elements that govern SLC23A1 expression. Using Genomatix and Match software to identify potential transcription factor binding elements, multiple sites of potential regulation were noted. We focused our analysis on the region between the Δ4 and Δ5 constructs because this region was necessary for maximal luciferase activity. Interestingly, the conserved elements in this region of SLC23A1 were few and included binding sites for Hepatocyte Nuclear Factor 1 (HNF-1), Tal1β, as well as a TATA box (Figure 2.3). Of these three sites, the HNF-1 was represented on both Δ3 and Δ4 fragments. Since the last HNF-1 binding
Figure 2.3 Analysis of the SLC23A1 promoter. Sequence analysis of the Δ4 fragment of the SLC23A1 5’-flanking region reveals several putative regulatory elements. Diagrammatic representations of these elements are shown in black, and designated by name and starting position of the site relative to the transcription start (+1). The full length of each element is about 15-20 base pairs long on average. DNA sequences included in the SLC23A1 reporter gene constructs from this region (Δ4-Δ6) are represented below the depictions of the transcription factor binding sites. Between the Δ4 and Δ5 fragment, only two full consensus sites for the TATA Box and Tal1β and one partial binding site for HNF-1 are present.
sequence was prematurely terminated in the Δ3 sequence, the only fully functional HNF-1 binding site lay in the Δ4 promoter construct.

To determine if any of these putative transcription factor binding sites play an active role in basal transcription of SLC23A1, the Δ4 vector was modified at specific locations via site-directed mutagenesis. Each altered vector construct contained a one or two base-pair substitution that effectively abolished any potential for a transcription factor to recognize and bind to the particular DNA element. The alterations were conservative, resulting in no deletions of the overall sequence and maintained relative position of all adjacent promoter elements. These vectors were transiently transfected into HepG2 cells and the effect of specific mutations on gene expression was analyzed by monitoring luciferase activity. Mutations in either the Tal1β site or the TATA box did not diminish luciferase reporter gene expression vs. wild-type constructs; however, mutating the HNF-1 site resulted in a 50-fold loss of transcription (Figure 2.4). This decline was so complete that mutating this site essentially abrogated all activity over that seen for the empty vector. Thus, we conclude that the HNF-1 binding region may be essential for basal expression for SLC23A1.

To further evaluate HNF-1 as a regulatory factor for SLC23A1, the HNF-1 binding sequence in the full length vector was altered by site-directed mutagenesis, as described above for the Δ4 fragment. After transient transfection into HepG2 cells, expression of the HNF-1 altered vector displayed luciferase activity that was less than 5% of that seen for the wild-type vector (Figure 2.5). This loss in expression was
Figure 2.4 The HNF-1 binding site is essential for basal transcription in the SLC23A1 minimal promoter. Transcription factor binding sites of interest were altered by site-directed mutagenesis of the Δ4 construct, representing the minimal promoter for SLC23A1. Altered vectors were transiently transfected into HepG2 cells and luciferase assayed as previously described. Results show that only the HNF-1 binding site mutant showed any changes from control, displaying nearly 50-fold less luciferase activity than control (p<0.0001). The activity of the HNF-1 mutant was indistinguishable from the pGL3-Basic control vector (p>0.5).
Figure 2.5. The HNF-1 binding site is essential to SLC23A1 expression in context of the full length promoter. Reporter gene constructs containing the full length (1239 bp) SLC23A1 sequence containing or lacking a functional HNF-1 binding site were transiently transfected into HepG2 cells and luciferase assayed as previously described. Firefly luciferase activity of the altered vector was normalized to Renilla luciferase activity, and expressed as percent decrease from unaltered vector. Results show a highly significant decline (p<0.0001) in the HNF1 mutant from the unaltered vector, which was no longer significantly (p>0.5) different from pGL3-Basic controls.
similar in magnitude to the HNF-1 mutant constructs for the Δ4 core sequence and was indistinguishable from the empty pGL3-Basic vector. This suggests that even in the context of the entire promoter, an intact HNF-1 site between the positions -40 and -57 bp relative to the transcriptional start site is absolutely necessary for full SLC23A1 activity.

**Association of HNF-1 with the SLC23A1 Promoter**

To further define the role of the HNF-1 binding site and, by analogy, the importance of HNF-1 in transcriptional control of SLC23A1, electromobility shift assays (EMSA) were performed to gauge the extent of transcription factor binding. A double-stranded segment of the HNF-1 site of SLC23A1 was used for this analysis. Incubation of this radiolabeled promoter sequence with HepG2 nuclear extracts (Figure 2.6; Lane 2) resulted in the appearance of a single, strong band near the top of the gel, indicating a shift in the free probe and the formation of a protein-DNA complex. Addition of HNF-1-specific antibodies caused a super-shift in the probe complex to the degree that no band remained in its initial position (Figure 2.6; Lane 3). This suggests that the entire shifted portion of radiolabeled DNA was bound to HNF-1. As a control for specificity, competition assays with the HNF-1 consensus sequence resulted in the complete loss of the shifted band. These data suggest that HNF-1 binds to its respective cognate recognition element in the SLC23A1 promoter, indicating that this transcription factor and its DNA recognition sequence may play an essential role in regulating human SVCT1 transcription. Although diminished gel shift was observed
Figure 2.6 HNF-1 binding to the SLC23A1 promoter by EMSA. A radiolabeled portion of the SLC23A1 promoter corresponding to the HNF-1 binding site was incubated with (Lane 2-5) or without (Lane 1) nuclear extracts from HepG2 cells. Probe shift was detected upon binding of extract to probe (Lane 2) and supershifts generated upon addition of anti-HNF1 antibodies (HNF1ab; Lane 3). Specificity of the binding reaction was determined with the addition of 200-fold molar excess of the HNF-1 consensus binding site (HNFcp; Lane 4) or the probe containing the HNF-1 mutant sequence (Lane 5) was added as a competitive inhibitor.
when the SLC23A1 HNF-1 mutant sequence was used as a competitive inhibitor, this may be due to the large excess of mutant DNA used in this reaction.
2.5 Discussion

SLC23A1 is expressed in organs that regulate the dietary availability (55) and renal reabsorption (81) of vitamin C, and contributes greatly to plasma vitamin C status (60). Thus, the regulation of SLC23A1 is a critical part of understanding vitamin C homeostasis. This study is the first to characterize the 5’-flanking region of human SLC23A1 with respect to putative transcription factor binding domains and control of basal transcription. In this study, we successfully constructed a human SLC23A1 reporter gene assay, and demonstrated its functionality in HepG2 cells. We determined that only a small region of the SLC23A1 gene was absolutely necessary for the production of SVCT1 transcripts, though other areas may elicit further levels control. Most importantly, this data reveals the involvement of Hepatic Nuclear Factor 1 (HNF-1) as an absolute requirement for the transcription of SLC23A1. This was confirmed using gel mobility shift assays that showed HNF-1 binds to its cognate DNA binding sequence within the SLC23A1 promoter. These results thus provide us with a novel view as to the regulation of SVCT1-mediated vitamin C transport. Because HNF-1 regulates cellular glucose metabolism, its genetic regulation of SLC23A1 suggests that vitamin C bioavailability is modulated more by glucose and other dietary factors than in a redox-dependent manner.

One of the novel findings presented in this study is the identification of HNF-1 as the transcription factor essential for SLC23A1 gene regulation. Therefore, it would be instructive to discuss some of the biochemical and biophysical properties of this
protein. HNF-1 is a POU domain transcription factor that was first identified in the liver and named for its involvement in hepatic gene expression (82,83). HNF-1 actually exists as two isoforms, HNF-1α and HNF-1β, which display a large degree of functional homology (84). HNF-1 proteins are expressed in the intestine, kidney, skin, lung, pancreas, testis and ovary as well as the liver (82). Interestingly, these are the same tissues that express SVCT1 (34,51). This co-localization of the two proteins suggests that HNF-1 may be involved in both the basal regulation and tissue-specificity of SVCT1.

The functions of HNF-1 proteins are numerous. HNF-1 is a key transcriptional regulator for glucose metabolism genes, and the loss HNF-1 leads to “Maturity Onset Diabetes of the Young” (MODY) that is characterized by loss of insulin secretion and response to plasma glucose levels (85,86). HNF-1 regulates the production of many secretory proteins, including insulin (83), and also the regulation of many members of the solute carrier family (87-89). Our data shows that SLC23A1/SVCT1 can be added to this growing list of genes under the functional regulation of the HNF-1 transport pathway.

Regulation of HNF-1 protein levels and binding to target genes is mediated by a complex web of transcription factors, cell signaling pathways, and regulation by dietary signals (88). Transcription of HNF-1 is the first critical step in its regulation. HNF-1 transcription is activated directly or indirectly by HNF-4α (Figure 2.7), a member of the nuclear receptor family which also has critical roles in the coordination
Figure 2.7 HNF-4α and HNF-1 regulation. HNF-4α is the major regulatory factor for the production of HNF-1. HNF-4α transcription is controlled by multiple pathways, including by factors that can partner with HNF-1. Post-translational modification of HNF-4α mediates much of its control over target genes in the cell, and determines its activity on HNF-1 production (see text). HNF-4α in its active form binds to the HNF-1α promoter in concert with HNF-1 proteins to increase HNF-1α transcription, or can bind to the HNF-6 promoter to indirectly increase HNF-1β transcription. HNF-1 proteins alone can participate in regulatory events that govern HNF-4α along with multiple other transcription factors.
of glucose metabolism genes in many tissues (90-92). HNF-4α mediates a large number of upstream signals that affect target gene transcription, including HNF-1 expression (93-97). HNF-4α activity is modulated by a diverse set of regulatory factors (92,98-102), including the regulation by HNF-1 proteins. Indeed HNF-1 and HNF-4α participate in regulatory feedforward and feedback mechanisms to regulate gene expression (88,94,103,104). Therefore, any future studies on HNF-1 regulation of SLC23A1 must address the contribution of HNF-4α.

The insulin signaling pathway has been postulated to be a major control point for the regulation of HNF proteins (88). The activation of the Akt kinase, an integral part of the insulin signaling pathway, increases the transcription of HNF-1β, which can be modulated by retinoic acid (105). Furthermore, HNF-1α is an accessory protein required for the regulation of glucose-6-phosphatase by insulin (106) and is upregulated after Akt stimulation by fructose-2,6-bisphosphate (107). Additionally, ceramide-induced down-regulation of Phosphatidylinositol-3 Kinase (PI3K), a master control kinase in the insulin signaling pathway, leads to a directed loss of HNF-1 in a rat liver cell line, including the loss of glutathione-S-transferase expression (108). Lastly HNF-4α is heavily influenced by hormone signaling, and activation of HNF-4α gene transcription by insulin has been reported (99,101). In total, this provides evidence that HNF-1 is regulated by insulin and the insulin signaling pathway. Because vitamin C transport activity may also be related to glucose levels (70), future work will determine if insulin can increase SLC23A1 gene through HNF-1 mediated gene expression.
As HNF-1 usually acts as an accessory protein with other transcription factors and dimerization partners (Figure 2.8), we considered the possibility that HNF-1 binds in conjunction with other DNA binding proteins on the SLC23A1 promoter. Disruption of adjacent binding sites by site-direct mutagenesis revealed several locations in the SLC23A1 sequence that could influence gene expression. Mutation of the SOX5, GATA1 and CAAT box sites show significant increases in luciferase expression, suggesting binding at these sites might antagonize HNF-1 activity (data not shown). Additionally, mutation in AP1-2 and HNF4 sites caused a 50% loss in luciferase activity. Because AP-1 (109) and HNF-4α (94) both have been shown to coordinately regulate HNF-1-dependent genes, these transcription factors may act to synergistically activate SLC23A1 under certain conditions. However, it must be stressed that in the absence of HNF-1 binding, there is no appreciable activity of the SLC23A1 promoter despite the presence of other intact transcription factor binding sites. HNF-1 is an obligate member of the SVCT1 regulatory pathway.

Although our analysis focused on the regulation of HNF-1 in the Δ4 fragment of the SLC23A1 promoter, we also noted a possible repressive element between the Δ3 the Δ4 sequences. However, attempts at defining a repressive element by altering transcription factor binding sites in this region failed to identify any active repressive motif (data no shown). Regulation of this site may be specific to humans and not conserved between species. Several transcription factor binding sites that are present in this region are unique to the human promoter, and many have the capacity to directly repress gene transcription. Additionally, more complex regulation could be occurring
Figure 2.8 Regulation of HNF1 DNA binding and transcription. HNF-1 (α or β) dimerizes before binding to its dimerization cofactor, DCoH. This mediates the binding of other protein cofactors (p300/CBP) which mediate histone deacetylase activity on native chromatin structures. After DNA unwinding, HNF-1 and partner proteins bind to target sequences in the presence of co-activators or other transcription factors that modulate its activity (see text). HNF-1 activity can also be regulated by phosphorylation and the activation of upstream signaling pathways directly, or through DCoH.
through tertiary structure of DNA in this area. Regulatory motifs such as the formation of hairpin structures would go undetected by a directed mutagenesis approach, as employed in our study. Although computational analysis of this domain showed no thermodynamically favorable higher-order structures, further analysis into these sequences is warranted to determine the interaction of these elements with the core HNF-1 binding that is necessary for the regulation of SLC23A1.

The regulation of SLC23A1 by HNF-1 family members is a novel mechanism for the regulation of SVCT1 and vitamin C transport by glucose metabolism. While evidence points to a functional regulation of SLC23A2/SVCT2 by oxidative stress mechanisms (1,51,64,66,110), there is only cursory evidence for redox control of SLC23A1. High doses of ascorbic acid have been shown to decrease SVCT1 mRNA production in Caco2 cells (65), but our data presented here do not support a role for vitamin C to directly regulate SVCT1 expression. Rather, we propose that the functional regulation of SLC23A1 depends on pathways that regulate carbohydrate metabolism. The links between glucose handling, insulin signaling, and vitamin C homeostasis have been postulated for decades (70,71), but its role in vitamin C transport has not heretofore been examined. Thus, our results may provide a fresh new look into the correlations between vitamin C transport and glucose handling in many tissues, to shape our understanding of the global regulation of vitamin C homeostasis.

In summary, the data presented here elucidates a previously unrecognized regulatory pathway for SLC23A1 and vitamin C uptake. The absolute requirement of
HNF-1 in the transcriptional activation of SLC23A1 suggests that the regulation of vitamin C transport is similar to that of other carbohydrates, and may not be functionally related to the antioxidant activity of ascorbic acid. Although future studies will have to determine which of the multiple signal transduction pathways leading into HNF-1 can regulates the transcription of SLC23A1, there are indications for the involvement of insulin signaling in this process. Thus, like the regulation of DHA uptake by the GLUT family, active ascorbic acid accumulation by SVCT1 may be influenced more by diet and insulin sensitivity than oxidative stress mechanisms.
Chapter 3

R-\(\alpha\)-Lipoic Acid Treatment Induces the Transcription of Sodium-Dependent Vitamin C Transporter 1 (SVCT1) in HepG2 Cells.

Alexander J. Michels and Tory M. Hagen

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3.1 Abstract

SLC23A1, the gene encoding the first member of the Sodium-Dependent Vitamin C Transport Protein family (SVCT1), requires Hepatocyte Nuclear Factor 1 (HNF-1) as an obligate transcription factor for its basal expression (see Chapter 2). However, it is not known whether cellular redox conditions or dietary effectors further influence transcription of this gene. To this end, a number of compounds that either alter cellular antioxidant redox status or modulate glucose metabolism, which could hypothetically affect SLC23A1 gene expression, were provided to HepG2 cells transiently transfected with SLC23A1-luciferase reporter constructs. Results showed that neither oxidants nor cellular glutathione status affected SLC23A1 gene expression. Notably, vitamin C did not influence transporter gene activity except modestly (47% increase) at supraphysiological levels (25 mM). Of all the agents tested, only insulin (85% increase) and the dithiol compound, \((R)-\alpha\text{-Lipoic Acid} (LA; 176\% increase)\), significantly elevated SLC23A1-mediated luciferase expression over controls. Further experiments showed that LA did not elicit its effects by acting as a redox modulator or by its inducement of Nrf2-dependent transcription. As insulin and LA are both compounds which affect signaling pathways associated with glucose metabolism, these results suggest that that SLC23A1 transcription can be induced via glucose-regulated mechanisms. Indeed, LA-induced SLC23A1 transcription was blocked by either LY294002 (5 µM) or Triciribine (20 µM), inhibitors of phosphatidylinositol-3 kinase (PI3K) or Akt, respectively. These results suggest a critical role for insulin-mediated signal transduction pathways in SLC23A1/SVCT1
regulation. Furthermore, our data support the hypothesis that LA may be an effective inducer of SVCT1 transport in times of need.
3.2 Introduction

Ascorbic acid is both an essential enzyme cofactor and a powerful cellular antioxidant (4). Humans are one of a few species that have lost the ability to synthesize vitamin C (15,111) and constantly need a dietary source of the vitamin to prevent scurvy, reduce the risk of cardiovascular disease, and maintain normal cellular antioxidant balance (4,5,29). Both vitamin C bioavailability and tissue uptake are governed by protein-mediated transport mechanisms (47). While early work suggested that dehydroascorbic acid (DHA), the oxidized form of the vitamin, is the preferred substrate for vitamin C transport, the physiological relevance of DHA uptake is now debatable, owing to its highly unstable nature (10) and its non-specific accumulation through glucose transport proteins (33,35). Alternatively, direct transport of ascorbic acid occurs in all vertebrate species studied and is mediated by Sodium-Dependent Vitamin C Transporters (SVCT) (34,48,57). Since loss of SVCT proteins causes severe depletion of plasma and tissue vitamin C levels (3,60), it is now recognized that these transporters are the primary route for physiological vitamin C uptake in tissues.

Currently, two functional members of the SVCT family, SVCT1 and SVCT2, have been identified (34,48). These proteins are encoded by the SLC23A1 and SLC23A2 genes, respectively. SLC23A1 maps to human chromosome 5q31.2-31.3, while SLC23A2 is found on chromosome 20p12.2-12.3 (57). SVCT1 and SVCT2 exhibit 66% sequence identity but differ widely in their tissue distribution (34) and functional characteristics (32,47,51). SVCT2 is expressed in almost every cell type
studied and thus represents the predominant ascorbic acid transport protein (34,51). In contrast, SVCT1 is localized to a discrete subset of epithelial and parenchymal cells of the intestine, kidney, and liver (34). This expression pattern, along with its subcellular localization suggests that SVCT1 is vital for vitamin C uptake from the diet and in regulating plasma vitamin C homeostasis. Recently, SLC23A1(-/-) mice were developed but have not yet been completely characterized (60). It is known, however, that these mice cannot maintain normal levels of plasma vitamin C when compared to their heterozygous littermates. This is attributed to increased renal excretion of the vitamin and potentially less intestinal absorption through the diet. Diminished vitamin C occurs despite endogenous vitamin C synthesis in the livers of these animals. Thus, the regulation of SLC23A1 on the genetic level may ultimately determine tissue vitamin C distribution in the body.

Previously, we showed that the Hepatocyte Nuclear Factor 1 (HNF-1) transcription factor family binds to its cognate DNA recognition sequence in the 5’-flanking region of the SLC23A1 gene in HepG2 cells (Chapter 2). Because HNF-1 tissue distribution also coincides with SVCT1 expression patterns (34,51,55,81-84,94,103), our data strongly suggest that this transcription factor may principally regulate SLC23A1 transcription. HNF-1 proteins regulate the transcription of many secretory proteins in hepatic and extra-hepatic tissues (83,84), such as for insulin production in the pancreas (112,113). Mutations in HNF-1 cause “Maturity Onset Diabetes of the Young (MODY)” (86,114) due to a loss of sensitivity to plasma glucose levels and impaired insulin sensitivity.
The signal transduction pathway(s) that ultimately influence HNF-1-mediated gene expression are not completely understood. Thus, the precise dietary and metabolic signals that regulate SLC23A1 transcription and affect SVCT1 activity are not known. What has been discerned suggests that a complex network of interdependent pathways affect overall cellular HNF-1 levels (83,84,94,115) and/or HNF-1/DNA binding characteristics (83,84,88,106,109,115,116). Interestingly, both signal transduction pathways affected by cellular energy status and oxidative stress (e.g. phosphoinositide-dependent signaling, mitogen-activated protein kinases) ultimately influence HNF-1-mediated gene expression (106,108,117,118). This suggests that HNF-1 proteins may ultimately be a general transcriptional mediator for an extremely diverse set of stimuli that sense both nutrient and oxidative stresses (99,119,120), which may be involved in the coordinate expression of SLC23A1.

Because multiple stimuli influence HNF-1-mediated transcription, this study focused on the contributions of three areas of regulation of SLC23A1, which include i) the contribution of vitamin C toward transcription of its own transport protein; ii) the effect of cellular redox status to which vitamin C can contribute; and iii) the signaling pathways regulating carbohydrate metabolism that are specifically involved in regulating HNF-1 transcriptional activity. Because multiple effectors ultimately are involved in either of these pathways, a “top-down” approach was instituted where cells transfected with SLC23A1-luciferase reporter gene constructs were treated with agents that influence glucose metabolism, cause oxidative stress, or modulate cellular
antioxidant status. Herein, we show that providing physiological levels of ascorbic acid does not influence the transcription of its transport gene. Moreover, compounds affecting cellular glutathione levels do not modulate SLC23A1-mediated gene transcription, though increases in oxidative stress may play a role in SVCT1 production. Only insulin (+85%), and the insulin mimetic (R)-α-lipoic acid (LA; +176%) positively induced SLC23A1 reporter activity. Because LA affects a number of signaling pathways (22,121), further work revealed that its role, in this instance, was related to insulin signaling rather than redox regulation of transcription. These results suggest that HNF-1-mediated SVCT1 gene expression and hence regulation of bulk tissue vitamin C homeostasis, is dependent on the vitamin’s nature as a dietary carbohydrate rather than its antioxidant properties.
3.3 Materials and Methods

Materials and Chemicals Used

Cell culture materials were obtained from ATCC (Manassas, VA) or Sigma-Aldrich (St. Louis, MO). Ascorbic Acid (sodium salt), Insulin, Hydrogen Peroxide, Glutathione Ethyl Ester, Buthionine Sulfoximine (BSO), Pyrrolidine Dithiocarbamate (PDTC), and Anethole Dithiolethione (ADT) were obtained from Sigma-Aldrich. Inhibitors Mifepristone, ERK inhibitor (FR180204), p38 MAPK inhibitor (SB203580) JNK inhibitor (SP600125), PI3-Kinase inhibitor (LY294002) and PKB/Akt inhibitor (Triciribine) were obtained from Calbiochem (San Diego, CA). Dexamethasone was also obtained from Calbiochem. All other chemicals were reagent grade or the highest quality available from Sigma-Aldrich, or as noted.

Antibodies

Rabbit Anti-HNF-1 antibody (sc-8986) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse Anti-β-Actin antibody (A5316) was purchased from Sigma Aldrich. Appropriate secondary HRP-conjugates were purchased from Cell Signaling Technologies (Danvers, MA).

Cell Culture

HepG2 cells, a human hepatocellular carcinoma cell line, were obtained from ATCC. Cells were maintained in Eagle’s Modified Minimum Essential Medium with 2 mM L-glutamine and Earle's Balanced Salts Solution and further adjusted to contain
1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. Fetal bovine serum (10%) (MEM and FBS, ATCC) was also provided as a supplement. Cells were cultured at 37°C and 5% CO₂, and passaged as needed upon reaching 80-90% confluence.

Administration of Lipoic Acid and Other Effectors

Cells were treated with the indicated concentrations of the selected compounds no earlier than 6 hours after medium change. Insulin (5 ng/mL final) and Mifepristone (20 µM final) were added directly to the cell culture medium. (R)-α-Lipoic Acid (a kind gift from Dr. Hans Tritschler, Viatris) was made fresh in 1000x stock concentrations in dimethylformamide (DMF) immediately before delivery to the cell culture medium. FR180204 (1 mM stock), SB203580 (1 mM stock), SP600125 (100 nM stock), LY294002 (5 mM stock) and Triciribine (20 mM stock) were also prepared in DMF before addition to cells. ADT (100 mM stock), PDTC (100 mM stock) and Dexamethasone (1 mM stock), were also added to DMF before cell treatment. In all preparations the total DMF added was maintained at a constant 0.1% (vol/vol), and activity was compared to control cells treated with DMF alone.

Transient Transfection of HepG2 Cells

The full length reporter gene, pSLC23A1-Luc was constructed using the luciferase reporter vector pGL3-basic (Promega) as described previously (Chapter 2). This gene contains a 1239-base-pair fragment of the human SLC23A1 5’-flanking sequence and was previously determined to contain an active promoter for SLC23A1
gene expression (Chapter 2). HepG2 cells were grown to approximately 40-50% confluence before transfection on 6-well plates using the Effectene reagent (Qiagen) as per manufacturer’s instructions. Briefly, 0.8 μg of the pGL3 vector with or without the SLC23A1 promoter and 50 ng of a constitutively expressed Renilla Luciferase plasmid (pRL-CMV; Promega) were complexed with 10 μl Effectene and 6.8 μl Enhancer reagent in a final volume of 100 μl. Immediately before the transfection complexes were placed upon cells, 2 mL of normal growth medium (MEM and FBS, above) was added to the reaction. Cells were left in the presence of Effectene complexes for a minimum of 18 hours before medium was changed.

Luciferase Assay Procedure

Transiently transfected HepG2 cells were harvested using Passive Lysis Buffer included in the Dual Luciferase Assay Kit (Promega) a total of 48 hours after transfection. Cells were flash frozen and thawed twice before 20 μl of each cell extract was placed in a 96-well assay plate with a non-binding surface (Corning) and firefly and Renilla luciferase levels measured as described in the manufacturer’s instructions. The constitutively expressed Renilla luciferase is taken as a measure of transfection efficiency of the cells, thus luciferase data are represented as the ratio of firefly to Renilla luciferase expression in Relative Light Units, and normalized to the indicated control plasmid and/or treatment group.
qPCR Analysis

HepG2 cells were plated on 6-well plates and treated with 100 μM LA as described. At various time points, cells were harvested and RNA was isolated by the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. qPCR reactions were performed as described (122), except using the following amplification primers:

SVCT1-Forward: 5’-CTGCACTCTCTTTGGCATGA-3’;
SVCT1-Reverse: 5’-CTCCCTGGCACTGTTTGTC-3’;
β Actin-Forward: 5’-ACCAGATCAATGTTTGAGACCT-3’;
β Actin-Reverse: 5’-AAGGAAGGCTGGAAGAGTGC-3’.

SVCT1 and β–actin levels were quantified relative to standard curves containing known amounts of the full-length cDNAs, and expressed as a ratio of SVCT1 to β–actin.

Nuclear Extract Preparation

Cells were harvested from 75-cm² flasks by incubation with Trypsin-EDTA (ATCC) until cells were liberated. Trypsin was diluted by the addition of ice-cold PBS containing Protease and Phosphatase Inhibitor cocktails (Sigma-Aldrich), and the cells were pelleted. After removal of the supernatant, cells were lysed by the procedure of Dignam et al. (80) and cytosolic and nuclear fractions obtained. Proteins were stored in aliquots at -80°C until use.
**Western Blotting**

Nuclear proteins were incubated in 4X Laemmli buffer (250 mM Tris/HCl, pH 6.8, 8%, w/v, SDS, 40% glycerol, 0.02% Bromophenol Blue and 4%, v/v, 2-mercaptoethanol) at 95°C for 5 minutes to allow for protein denaturation. Soluble proteins (30 µg) were loaded on a 10% ReadyGel (BioRad) and separated by SDS-PAGE using a Mini Protean II apparatus at 100V. Proteins were transferred to PVDF membranes using a semi-dry electroblot transfer system. After blocking with 5% milk in PBS containing 0.1% Tween-20, membranes were washed and probed with anti-HNF-1 (Santa Cruz) and anti-β-actin (Sigma) antibodies and imaged after HRP-conjugated secondary antibodies were added. Images were obtained after the addition of chemiluminescence detection reagents (Santa Cruz) and exposure to autoradiography film. Relative densities of the bands were digitally quantified by using Image-J analysis software (http://rsb.info.nih.gov/ij/).

**Statistics**

Data are represented as the arithmetic mean ± standard error of the mean of a minimum of three separate experiments, except as noted on Table 1.1. Statistical differences between two experimental conditions were evaluated using the two-tailed Student t-test with a p-value of <0.05 considered significant. Statistical differences between series of samples were evaluated using ANOVA and Tukey post-hoc analysis. A p-value of <0.05 was considered significant.
3.4 Results

**Determination of effectors of SLC23A1 regulation**

HepG2 cells transiently transfected with a previously developed SLC23A1 reporter construct were used to examine potential effectors of gene expression. This reporter gene contains the human 5’-flanking region of the SLC23A1 gene inserted upstream of the gene for firefly luciferase (Chapter 2). Thus, by adding various effectors that may theoretically influence SLC23A1 reporter activity into the cell medium, a ready screening assay was developed. In keeping with the purported roles for vitamin C or HNF-1-mediated glucose handling, agents that affected cellular antioxidant levels, redox status, or glucose metabolism were primarily examined. As the pathways affecting both cell redox environment and glucose homeostasis are complex, this “top-down” approach was designed to gauge the influence of these general mechanisms on SLC23A1 gene transcription and was not intended to be a thorough examination of any particular pathway. The results of this analysis are presented in Table 3.1, and will be discussed in more detail below.

_Vitamin C does not regulate SVCT1 Transcription_

To begin an examination of how environmental stimuli influence reporter activity, we determined whether exogenous vitamin C levels affected expression of SVCT1. After transient transfection of the SLC23A1 reporter vector, HepG2 cells were treated with increasing concentrations of vitamin C and luciferase activity monitored over time. Results show that supplying vitamin C at doses considered
physiologically relevant did not significantly affect SVCT1 reporter gene activity (Table 3.1 and Figure 3.1). Even when supra-physiological levels of ascorbic acid were administered to cells (25 mM), SLC23A1 activity only increased an insignificant 47±13% over vehicle-treated controls after 24 hours. Shorter incubations with ascorbic acid did not change the overall result. While these results are in contrast to a report where millimolar amounts of ascorbic acid incubation resulted in a significant loss of SVCT1 mRNA in Caco2 cells (65), our results clearly show that ascorbic acid does not influence transcription of its transport gene in hepatocytes.

Because vitamin C transport could be hypothetically induced by changes in the cellular pro- vs. antioxidant environment rather than by intracellular ascorbic acid concentrations, experiments were undertaken where H$_2$O$_2$ at levels known to cause acute oxidative stress were supplied to HepG2 cells. As shown in Table 3.1, 0.5 mM hydrogen peroxide generated only a modest (p>0.10) 16% decline in SLC23A1 gene activity. These results indicate that a pro-oxidant cellular environment per se was insufficient to affect SVCT1 expression.

Vitamin C antioxidant activity has been implicated in the regulation of members of the MAP kinase family, specifically inducing p38 and ERK (18,19,21). Theoretically, signals through either of these pathways could influence SLC23A1 transcription. However, incubation of cells with the p38 MAPK inhibitor (SB203580) or the ERK inhibitor (FR180204) did not significantly affect luciferase expression in transfected HepG2 cells (Table 3.1). Taken together, this indicates that vitamin C
Figure 3.1 Ascorbic acid does not increase transcription of SLC23A1. HepG2 cells were transiently transfected with a SLC23A1 reporter gene. Ascorbic acid (100 µM – 25 mM) was provided in the cell culture medium and samples were harvested 24 hours later and analyzed for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity, and expressed as the percent increase from cells supplemented with vehicle alone. Results show that no significant increase on luciferase activity by ascorbic acid administration was noted.
<table>
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<tr>
<th>Compound and Dose</th>
<th>Functional Properties</th>
<th>Luciferase Activity b</th>
<th>p-value c</th>
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<tr>
<td>Ascorbic Acid (100 µM)</td>
<td>Antioxidant/Carbohydrate</td>
<td>109.0 ± 4.1%</td>
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<td>Ascorbic Acid (5 mM)</td>
<td>Antioxidant/Carbohydrate</td>
<td>134.7 ± 16.6%</td>
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<tr>
<td>Ascorbic Acid (25 mM)</td>
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<td>147.0 ± 13.0%</td>
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<td>H₂O₂ (500 µM)</td>
<td>Oxidant/Cell Signaling Agent</td>
<td>86.0% (n=2)</td>
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<td>Insulin (5 ng/mL)</td>
<td>Peptide Hormone</td>
<td>184.8 ± 25.0%</td>
<td>p&lt;0.05</td>
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<tr>
<td>LY294002 (5 µM)</td>
<td>PI3-Kinase Inhibitor</td>
<td>45.9 ± 19.6%</td>
<td>p&lt;0.005</td>
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<td>Dexamethasone (1 µM)</td>
<td>Glucocorticoid</td>
<td>111.0% (n=2)</td>
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</tr>
<tr>
<td>Mifepristone (20 µM)</td>
<td>Glucocorticoid Inhibitor</td>
<td>97.5% (n=2)</td>
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<td>p38 inhibitor (1 µM)</td>
<td>MAP Kinase Inhibitor</td>
<td>70.0% (n=2)</td>
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<tr>
<td>ERK Inhibitor (1 µM)</td>
<td>MAP Kinase Inhibitor</td>
<td>77.0% (n=2)</td>
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<tr>
<td>Lipoic Acid (100 µM)</td>
<td>Antioxidant/Dithiol/Phase II</td>
<td>276.0 ± 46%</td>
<td>p&lt;0.02</td>
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<td>Glutathione-Ethylester (100 µM)</td>
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<td>98.9 ± 3.1%</td>
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<td>PDTC (100 µM)</td>
<td>Antioxidant/Dithiol</td>
<td>85.0% (n=2)</td>
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<td>ADT (100 µM)</td>
<td>Dithiol/Phase II</td>
<td>102.3% (n=2)</td>
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</table>

*a* All compounds were administered to HepG2 cells as described in Materials and Methods or described in the text. Ascorbic acid supplementation was performed for 24 hours (see text); all other compounds were given for a total of 12 hours.

*b* Luciferase activity as a percentage of unstimulated HepG2 cells transiently transfected with the SLC23A1 reporter gene activity.

*c* Reported if only significantly different from control. p-values greater than 0.05 were not considered statistically significant.
levels, the generation of oxidative stress, and signaling through p38 or ERK have only minor effects on SLC23A1 transcription.

**SVCT1 gene expression is induced with effectors of insulin-dependent signaling pathways**

Because the SLC23A1 gene is under HNF-1 control (see Chapter 2) and regulation of this transcription factor is strongly influenced by insulin-mediated signaling pathways, we hypothesized that SLC23A1 expression may also be insulin dependent. HepG2 cells containing the SLC23A1 reporter plasmid were treated with insulin (5 ng/mL for 12 hrs) and luciferase expression assessed. Results showed that insulin induced SLC23A1 expression by approximately 85% over controls. This stimulation was significant (p<0.05), but modest relative to other directly mediated insulin-dependent genes (120,123). To further define whether insulin-influenced signaling pathways were involved in SLC23A1 expression, we treated cells with pharmacological inhibitors of phosphatidylinositol-3 kinase (PI3K), as this is a principal mediator of insulin-dependent signaling pathways in hepatocytes (119,124). After a 12-hour incubation with LY294002 (5 µM), HepG2 cells showed a significant (p<0.005) 54% inhibition in luciferase production, suggesting that insulin-regulated signaling through PI3-K strongly regulates the baseline expression of SLC23A1.

To gauge whether other pathways regulating glucose metabolism not directly mediated by insulin also stimulated reporter gene activity, the glucocorticoid effector, dexamethasone (1 µM), or Mifepristone (20 µM) an inhibitor of glucocorticoid
receptor activity was provided to HepG2 cells with the luciferase reporter gene. Results showed that neither stimulation nor inhibition of glucocorticoid-mediated signaling pathways affected SLC23A1 transcription. Taken together, these results indicate that SVCT1 may be moderately influenced by insulin or insulin-dependent signal transduction pathways.

**(R)-α-lipoic acid (LA) as an inducer of SLC23A1**

To further define the influence of insulin-mediated signal transduction pathways on SLC23A1 reporter gene activity, (R)-α-lipoic acid (LA) was provided to cells. LA has been used clinically for over 50 years to improve glucose transport and utilization in patients with Type II diabetes and as a prophylactic against polyneuropathies associated with this syndrome (121). At the cellular level, LA acts as an insulin mimetic by inducing the autophosphorylation of the insulin receptor. This results in an LA-induced activation of signaling pathways primarily driven through PI3K and Akt kinase (125-129). To determine whether LA elicited expression of SVCT1 at doses consistent with its plasma levels reached following an oral dose (121), transiently transfected HepG2 cells containing the SLC23A1 reporter gene were treated with increasing concentrations of LA in the cell medium and luciferase activity monitored 12 hours after stimulation (Figure 3.2). At all doses given, LA increased SVCT1 activity; however, elevation in luciferase activity did not reach significance until cells were incubated with LA concentrations at or above 100 µM. At the 100 µM dose, LA induced a significant 176 ± 46% increase in luciferase activity after 12 hours (Figure 3.3). Reporter activity actually increased in a dose-dependent manner and
Figure 3.2 LA stimulation of SVCT1 reporter gene activity is dose-dependent. Transiently transfected HepG2 cells were treated with various concentrations of LA (0, 1, 10, 50, 100 and 250 µM) and harvested after 12 hours. Luciferase activity was measured as described, and reported as a ratio of firefly to Renilla luciferase, using unstimulated cells as a control. The results show a linear dose-response curve for the effect of LA, though significant changes (p<0.05) from control were only observed at doses of 100 µM or greater.
Figure 3.3 LA treatment of HepG2 stimulates SLC23A1 transcription. HepG2 cells were transiently transfected as described with the SLC23A1 reporter gene. 100 µM LA was provided in the cell culture medium and samples were harvested at the indicated times, and analyzed for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity, and expressed as a percent increase from cells supplemented with vehicle alone. Results show a significant increase (p<0.05; ANOVA) in reporter gene activity 12 and 18 hours after LA stimulation.
maximal stimulation was achieved when cells were provided 250 µM LA. Higher LA concentrations beyond this amount did not further stimulate luciferase production (data not shown), suggesting a plateau for the effect of LA on SLC23A1 transcription had been achieved. Considering that plasma LA concentrations have been reported to peak between 60 to 200 µM following an oral dose (121), these results indicate that LA induced SVCT1 transcription may be pharmacologically relevant.

Because LA was the most potent inducer of SVCT1 gene expression discovered in this initial screen, further experiments were undertaken to discern its mode of action on SLC23A1 gene transcription.

To confirm that increased luciferase activity represented enhanced SLC23A1 promoter activity and not a direct interaction between firefly luciferase and LA, SVCT1 mRNA levels were measured in HepG2 cells after LA stimulation. Using qPCR analysis, elevated SVCT1 message levels were evident as early as 6 hours after LA stimulation and became significantly higher (p<0.0005) at 24 hours (Figure 3.4). Interestingly, no changes were observed in SVCT2 mRNA under the same conditions (data not shown), indicating that LA specifically induces SLC23A1 gene transcription and does not act as a general inducer of vitamin C transport. Thus, these overall results suggest that LA immediately increases the promoter activity and message abundance of SVCT1 in HepG2 cells.
Figure 3.4 LA administration increases in SVCT1 mRNA content in HepG2 cells. HepG2 cells were stimulated with 100 µM LA and harvested at the indicated time points. Total RNA was isolated, and quantified by reverse transcription qPCR. RNA Copy number was determined in relation to a SVCT1 cDNA standard curve, and expressed as a ratio of SVCT1 to β-actin levels. Results show a significant (p<0.0005) increase in SVCT1 mRNA 24 hours after LA treatment.
**LA increases HNF-1 levels in HepG2 cells**

The binding of HNF-1 to its cognate site on the SLC23A1 promoter is critical to SVCT1 transcription (Chapter 2). Since LA stimulates multiple cell-signaling pathways, and this may in turn lead to increased HNF-1 production, we sought to determine if the effect of LA on SVCT1 was due to increased HNF-1 nuclear levels. HepG2 cells were supplemented with 100 μM LA and harvested after 2, 6, or 12 hours. Nuclear extracts were isolated and HNF-1 levels were determined by western blot analysis (Figure 3.5). Results show increases in nuclear HNF-1 within 6 hours of LA stimulation, a statistically significant (p<0.005) increase of 200%. Slightly elevated levels of HNF-1 persisted in cells until the 12 hour time point, though this was no longer significantly higher (p>0.2) than HNF-1 levels observed in vehicle treated control cells. Together, this suggests that HNF-1 regulation is the mechanism by which LA elicits an effect on SLC23A1 transcription.

**LA stimulation of SVCT1 is independent of its thiol antioxidant activity**

LA is known to be a direct-acting antioxidant *in vitro*, and also forms a powerful redox couple that modulates gene transcription through the Nrf2 and/or AP-1 transcriptional regulatory pathways (22,122,130). Even though hydrogen peroxide failed to stimulate reporter gene activity, it is possible that LA may induce SVCT1 gene transcription through thiol redox-associated signaling pathways. Experiments were thus performed where cellular thiol status was manipulated and SVCT1 gene expression determined.
Figure 3.5 LA increases nuclear HNF-1 levels in HepG2 cells. HepG2 cells were treated with 100 µM LA for the indicated times and nuclear extracts were obtained as described. Proteins were run on SDS-PAGE and transferred to PVDF membranes. Western blot analysis showing relative HNF-1 and β-actin levels with LA stimulation are shown on top. HNF-1 is present in HepG2 nuclear extracts as a single band at 79kDa. Quantification of western blot data by densitometry is shown on the bottom, where HNF-1 levels were normalized to β-actin and expressed as a ratio between the two, using unstimulated cells as a control. The results show a significant (p<0.005) increase in nuclear HNF-1 6 hours after LA administration.
Because LA particularly affects the cellular thiol redox environment that is principally governed by the reduced to oxidized glutathione ratio, we examined whether providing thiol antioxidants could induce SLC23A1 reporter gene, similar to LA. First, HepG2 cells transfected with the reporter gene were incubated with glutathione ethylester (GSH-EE; 100 µM) or the dithiol antioxidant pyrrolidine dithiocarbonate (PDTC) (100 µM). Neither treatment increased gene expression beyond that seen in vehicle-treated cells (Table 3.1). Additionally, treatment with buthionine sulfoximine, an inhibitor of glutathione synthesis, had no effect on reporter gene activity (data not shown). Taken together, this suggests that LA is not acting in a thiol antioxidant capacity to induce SVCT1 transcription.

In addition to direct thiol redox modification, LA is a potent inducer of Nrf2-mediated detoxification gene expression (122,130). Since PDTC also stimulates Nrf2-mediated gene expression through MAPK induction (131), this would suggest the effect of LA is independent of a Nrf2-based mechanism. To confirm this, anethole dithiolethione (ADT; 100 µM), a specific activator of Nrf2-mediated transcriptional pathways was provided to HepG2 cells to determine its effects on SLC23A1 gene transcription. Similar to PDTC, our results show that ADT did not raise luciferase activity over controls (Table 3.1). These results strongly suggest that LA is not affecting SLC23A1 transcription through an Nrf2- or thiol redox-mediated mechanism. These interesting results, along with the data showing that vitamin C did not alter reporter activity, indicate that LA induces SVCT1 transcription as a
modulator of the insulin signaling pathway and not as an effector of cellular redox status.

Dependence of PI3-Kinase/Akt Pathway for LA activity on SVCT1

Previous data in our laboratory (127) and from others (125,126,128) showed that LA stimulates PI3K and Akt, two key serine/threonine kinases in the insulin signaling pathway. Because LA also increases nuclear HNF-1 levels and incubation with PI3K inhibitors reduces SLC23A1 transcriptional activity, we hypothesized that inhibition of the PI3K/Akt pathway might result in a loss of LA-mediated SLC23A1 reporter gene expression. Transiently transfected HepG2 cells were pre-treated with the PI3K inhibitor, LY294002, for 2 hours prior to treatment with 100 µM LA and luciferase activity was monitored over time (Figure 3.6a). Results confirmed our earlier data as they showed LY294002 alone decreased the baseline reporter gene activity over time. When cells were pretreated with LY294002 before LA administration, no LA-dependent increase of SLC23A1 luciferase activity was observed. In fact, LY294002 pretreatment completely abrogated LA-mediated increases in luciferase gene expression and was only 58±4% (p<0.001) of activity seen in vehicle treated cells 12 hours after LA and LY294002 treatment. This suggests that LA cannot overcome the chemical inhibition of PI3K.

To further explore whether LA mediated its effects through a PI3K-dependent mechanism, additional experiments were performed where Akt, a kinase “downstream” of PI3K, was pharmacologically inhibited before LA treatment. When
Figure 3.6 LY294002 and Triciribine inhibit the effect of LA on SVCT1 transcription. HepG2 cells were transiently transfected with the SVCT1 reporter gene as described. Cells were pre-treated with A) 5 μM LY294002 or B) 20 μM Triciribine or vehicle 2 hours before 100 μM LA administration. Luciferase activity was monitored over time, and expressed in relation to DMF-treated cells. Closed circles refer to luciferase activity in cells pre-treated with DMF and stimulated with LA (Panel A). Open circles represent cells that received both inhibitor and LA treatments. As a reference, cells stimulated only with inhibitor are represented with triangles and a dashed line. Results show that pre-treatment with either inhibitor blocked the ability of LA to stimulate luciferase activity in these cells.
cells were pre-treated with Triciribine (20 µM), an inhibitor for Akt activity, a similar effect was observed in luciferase production as seen with LY294002 (Figure 3.6b). Triciribine administration equally prevented the induction of LA-stimulated luciferase activity and resulted in a significant decline in the baseline expression of luciferase (54±14% of untreated cells after 12 hours) in the absence of LA. Together, this suggests that the PI3K/Akt pathway plays an essential role in SVCT1 transcription and mediates the effect of LA on SVCT1 transcription.
3.5 Discussion

The findings of the present study delineate the general signal transduction pathways that ultimately lead to HNF-1-mediated SLC23A1 gene expression. Moreover, the data suggest that the signaling pathways involved in the expression of SVCT1 are independent of many known regulators of its sister protein SVCT2. Specifically, SLC23A1 regulation in HepG2 cells is independent of the presence of vitamin C. Furthermore, thiol antioxidant status, as shown by GSH-EE and PDTC treatment, or modulating cellular oxidant levels by hydrogen peroxide addition, did not affect gene expression. Only insulin or effectors of the insulin signaling pathways induced SLC23A1 reporter gene activity. This was confirmed by using an insulin mimetic, R-α-Lipoic Acid (LA), which strongly increased SLC23A1 gene expression. Thus, we have demonstrated SVCT1 expression is not heavily influenced by its role in maintaining antioxidant status. Rather, carbohydrate metabolism appears to be the main regulatory influence on SVCT1-mediated vitamin C transport.

Our data shown in Chapter 2 indicates that transcriptional control of SLC23A1 is regulated through HNF-1. Because HNF-1 primarily controls carbohydrate metabolism and has undefined roles in redox regulation, it was necessary to take a broad spectrum approach to examine the signaling pathways most likely to regulate the transcription of this gene. However, the regulation of HNF-1 activity by post-translational modifications, especially phosphorylation events, is emerging as a critical control mechanism behind its function. Dimerization factors for HNF-1 independently
coordinate its activity by recruiting protein kinases to the HNF-1 complex (115,117). This leads to HNF-1 phosphorylation and activation of HNF-1-bound promoter elements (117). However, the extent of this regulation is not well understood. Phosphorylation events via PI3K and Akt are associated with increases in HNF-1 levels and activity (105,107,108). Under some circumstances, direct phosphorylation of HNF-1 leads to a repression of its DNA binding activity and increased degradation, though likely through the action of protein kinase C (132). Additionally, phosphorylation events modulate transcription of the HNF-1 gene to modulate HNF-1 levels. HNF-4α is a primary regulator of HNF-1 transcription, and phosphorylation may increase or decrease HNF-4α activity (88,95,96,99,118), which is reflected in the HNF-1 protein levels. Thus, protein kinase activity heavily influences HNF-1 gene expression, and may be an important regulator of SLC23A1.

Along these lines, we found that insulin stimulation leads to consistent increases in the transcriptional activity of SLC23A1. HNF-1 is responsible for the production of insulin in the pancreas by regulation of insulin gene transcription, insulin release from the beta islet cells, and it believed to mediate the effect of insulin in other cell types (106). However, the exact modes of HNF-1 regulation by upstream signals, including insulin, are not well defined. Our data shows that chemical inhibition of PI3K and Akt leads to a loss of SLC23A1 reporter gene activity is HepG2 cells. Because both of these kinases are regulated by insulin, we must conclude that the HNF-1 dependent regulation of SLC23A1 is, in part or in whole, dependent on the insulin signaling pathway.
LA is a dithiol compound found naturally in plants and animals and functions as a cofactor in mitochondrial energy metabolism (121,133,134). LA has powerful redox activity, and its reduced form may reduce other cellular antioxidants (22). The treatment of unbound LA in cell culture, animals, and humans promotes glucose metabolism by the induction of the insulin signaling pathway (121,135-138). This represents a pharmacological stimulation of signal transduction pathways that is not seen with endogenous LA synthesis. Although the direct mechanism of action of LA on the insulin receptor or downstream proteins is not entirely clear, there is clear evidence that LA activates both the insulin receptor and insulin receptor substrate proteins (125-127,129,139,140). Therefore, there is supporting evidence that LA is an insulin mimetic, and our data supports this role in the regulation of SLC23A1 transcription.

We also have supporting data that physiologically relevant quantities of LA were sufficient to induce the SLC23A1 reporter gene activity and increase SVCT1 mRNA. However, LA activation was prevented when HepG2 cells were preincubated with either LY294002 or Triciribine. This links the cellular action of LA to the PI3K/Akt pathways, which are, in turn, regulated through insulin signaling. Moreover, nuclear levels of HNF-1 rise within 6 hours after LA administration. This confirms that insulin signaling results in the up-regulation of HNF-1 regulatory pathways. However, the mechanism by which LA acts to increase HNF-1 is not entirely clear, nor is it understood whether LA coordinately regulates SLC23A1 transcription through
other pathways that may synergistically regulate transcription. LA is known to be a potent thiol redox modulator and induces a variety of redox sensitive transcription factors such as Nrf2 (122) or AP-1 (22). Thus LA may indirectly influence HNF-1 through thiol redox mechanism. However, our data using antioxidants, oxidants or classic Nrf2 inducers show that these effectors do not influence SLC23A1 gene activity, which indicated little to no influence of redox modulation in SVCT1 regulation. Thus, we conclude that the direct effect of LA on insulin signaling is the most important mechanism for HNF-1 regulation of this gene.

The redox and carbohydrate nature of vitamin C appears to independently regulate the two isoforms of the SVCT family. We show that insulin signaling dominates the genetic regulation of SLC23A1, but redox mechanisms appear to heavily influence regulation of SLC23A2 (64,66,110,141-144). In tissues that express both SVCT1 and SVCT2, such as the liver, we could expect functional declines in either protein could lead to a loss in vitamin C homeostasis. Aging is marked by declines in vitamin C levels, but is also a period of heightened oxidative stress and increases in insulin resistance (145-147). In aging, a loss of vitamin C could theoretically be due to a change in either SVCT protein. Thus, it would be worthwhile in an aging model to determine the relative importance of SLC23A1 gene regulation by insulin to SLC23A2 regulation via redox changes in the cell. Similarly, patients with diabetes show increases in oxidative stress, impaired glucose handling, and decline in vitamin C homeostasis (147-152). However, the disposition of the SLC23 genes is equally unknown in diabetics. Future work will need to focus on determining
what role, if any, the changes in insulin sensitivity seen in aging or diabetes affects the roles of SVCT1 or SVCT2 and the overall outcome on vitamin C homeostasis.

In summary, our data support our previous conclusions that functional regulation of SLC23A1 is through HNF-1-mediated carbohydrate metabolism. We have also confirmed that insulin signaling regulates SLC23A1/SVCT1. Moreover, LA appears to stimulate SLC23A1 gene activity by acting as an insulin mimetic and not be its redox modulating properties. This shows that regulation of SLC23A1 is highly distinct from the redox-sensitive nature of SLC23A2/SVCT2. Future work must focus on the functional regulation of these two genes and their associated proteins in ascorbic acid uptake.
Chapter 4

Age-Related Decline of Sodium-Dependent Ascorbic Acid Transport in Isolated Rat Hepatocytes.

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4.1 Abstract

This study investigated whether the age-related decline in hepatic ascorbic acid level is a functional consequence of a loss of sodium-dependent vitamin C transport (SVCT) proteins. Ascorbic acid concentrations in freshly isolated hepatocytes from old (24-26 months) Fischer 344 rats were 68% lower than in cells from young animals (3-5 months). Subsequent analysis of SVCT messenger RNA (mRNA) levels shows that one isoform of this transport protein, SVCT1, declines 45% with age, but no significant changes were observed in SVCT2 mRNA levels. This corresponded to an age-related loss in SVCT1/SLC23A1 transcription, as measured by luciferase reporter gene assay. To determine if this loss in SVCT1 levels resulted in a functional decline in vitamin C uptake, we measured the accumulation of ascorbic acid following vitamin C supplementation in hepatocytes isolated from both age groups. Old animals showed a significant 66% decline (p<0.001) in ascorbic acid transport when compared to young animals, though no changes were observed in dehydroascorbic acid transport (DHA). Using both HPLC and $^{14}$C measurements of ascorbic acid uptake into cells, we determined that ascorbic acid transport was primarily a sodium-dependent process, implicating the activity of SVCT proteins. Administration of lipoic acid to cells from old animals reversed the age-related declines in SLC23A1 transcription, suggesting that this would be an effective therapy for increasing vitamin C uptake in the elderly. These results show that losses in SVCT1 contribute to lowered vitamin C transport seen in aging, which may account for much of the loss in plasma and tissue ascorbic acid content.
4.2 Introduction

Ascorbic acid participates in a number of reactions required for normal cell function and is one of the most important cellular antioxidants (4,5,25). Many studies show that tissue ascorbic acid levels decline significantly in different mammalian species with age, including animals that synthesize the compound. Loss of ascorbic acid in tissues is consequent to nearly system-wide declines in plasma vitamin C status in old animals (153). Concomitant with lower age-related ascorbic acid status, hepatic glutathione levels also decline (154-156). However, the loss of glutathione is much less severe than loss of ascorbic acid, suggesting that ascorbic acid is preferentially lost with age. This argues for ascorbic acid-specific mechanisms that account for the decreased level of this antioxidant in tissues, and not simply increases in oxidant production. A meta-analysis by Brubacher et al. recently concluded that lower plasma vitamin C status in the elderly stemmed from diminished dietary absorption of the vitamin (69). This suggests an age-related decline in vitamin C uptake into cells is the main regulatory factor accounting for the age-related declines in ascorbic acid levels.

Vitamin C accumulation occurs through two independent transport mechanisms (32). Dehydroascorbic acid (DHA) is accumulated through facilitated transport by glucose transport proteins. Intracellular DHA is rapidly reduced to ascorbic acid, thus contributing to overall cellular vitamin C status. Since DHA production in cells and plasma is considered to be minimal, however, this is not considered a pathway of normal vitamin C accumulation in the liver. Ascorbic acid
accumulates directly through an active transport mechanism via sodium-dependent vitamin C transport proteins (SVCTs). Two isoforms of SVCT have been identified (SVCT1 and 2) and isolated from rat, human, and other species (34,47,48,51). Whereas SVCT1, the lower-affinity, higher-capacity carrier, is predominant in the liver, intestine, and kidney, SVCT 2 is found in most tissues of the body. The tissue distribution and kinetic properties of SVCT1 suggest that it may be involved in whole-body vitamin C status. Indeed, SVCT1-knockout mice show deficits in their ability to maintain plasma vitamin C status, which affects tissue accumulation of the vitamin (60). In the liver, both SVCT1 and SVCT2 are expressed although the relative importance of either protein towards hepatic vitamin C homeostasis is unclear. The contribution of SVCT1 or SVCT2 to age-related changes in vitamin C status is also unknown.

In the present study, we investigated whether age-related declines in ascorbic acid status in Fischer 344 rats were a functional consequence of a loss of either SVCT1 or SVCT2. We present evidence for a decline in SVCT1 mRNA and a loss of SLC23A1 transcriptional activity in cells from old rats when compared to cells from young animals, suggesting a marked age-related decline in sodium-dependent ascorbic acid uptake. This loss of ascorbic acid transport can only partially be overcome by providing increased vitamin C to cells. However, the supplementation of R-α-Lipoic Acid (LA) stimulated the transcriptional activity of SLC23A1, suggesting an increase in SVCT1 levels. This suggests that dietary factors which increase SVCT1 expression
may also improve both ascorbic acid concentrations and help maintain normal vitamin C status in the elderly.
4.3 Materials and Methods

Chemicals

L-Ascorbic acid (ACS reagent), dibutyl phthalate, heparin (sodium salt), diethyltriaminepentaacetic acid (DTPA), dimethylformamide (DMF), 2-deoxy-D-glucose, ethidium bromide, and dehydroascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO). Perchloric acid was from Fisher Scientific (Hampton, NH). L-[carboxy-\(^{14}\)C] ascorbic acid (17 mCi/mmol) was obtained from Amersham (Piscataway, NJ). Cell culture materials were obtained from American Type Culture Connection (Manassas, VA) or Sigma-Aldrich (St. Louis, MO). All materials for DNA use were obtained from New England Biolabs (Ipswich, MA) unless otherwise noted. R-\(\alpha\)-Lipoic Acid (LA) was a kind gift from Dr. Hans Tritschler, Viatris. All other reagents were reagent grade or better. Double distilled/deionized water was used throughout.

Plasmid Constructs

The SLC23A1 plasmid used in this study was designed previously (Chapter 2). This plasmid contains the minimal promoter region of the human SLC23A1 sequence, and shares high sequence homology to the rat SLC23A1 sequence. The backbone for this plasmid is the pGL3-Basic vector from Promega (Madison, WI), which contains a firefly luciferase gene.
**Animals**

Both young (3-5 months) and old (24-26 months) rats (Fischer 344, virgin male, outbred albino) were obtained from the National Institute on Aging animal colonies and acclimatized at the Oregon State University animal facilities at least 1 week prior to experimentation. All rats were fed Purina standard laboratory chow diet for rodents and water *ad libitum* throughout.

**Hepatocyte Isolation**

Before liver isolation, rats were anesthetized with diethyl ether and a midline incision made in the abdomen. Heparin (0.2 % w/v) was injected in the iliac vein and the portal vein cannulated. After liver perfusion was established, rats were sacrificed by cutting through the diaphragm, followed by cutting the vena cava.

Liver tissue was dispersed into single cells by collagenase perfusion as described (154,157,158). The cell suspension was washed three times with 150 mL of Krebs-Henseleit medium supplemented with 2 mM glucose and 7 mM glutamate (pH 7.4) to remove nonparenchymal cells. Cells were kept in suspension in a round-bottom flask rotating at 50 rpm at room temperature until used in experiments.

Cell number was assessed using a hemocytometer and viability was determined by trypan blue (0.2% [w/v]) exclusion. Viability was usually > 85% for cells from both young and old rats.
Transient Transfection of Primary Hepatocytes

Isolated hepatocytes (1.5 x 10^7) were placed on collagen coated 6-well plates, and maintained at 37°C and 5% CO₂ for a minimum of 48 hours in Williams E medium containing 5% FBS, 2 mM L-glutamine, 100 ng/ml insulin, and 1 µM dexamethasone. Transient transfection was carried out using the Effectene reagent from Qiagen as per manufacturer’s instructions. Briefly, 1 µg of firefly luciferase-containing plasmid with or without the human SLC23A1 minimal promoter (above) and 100 ng of a constitutively expressed Renilla Luciferase plasmid (pRL-CMV; Promega) were complexed with 10 µl Effectene and 8.8 µl of Enhancer in a total volume of 100µl. Immediately before the transfection complexes were placed upon cells, 2 mL of normal growth medium (MEM and FBS, above) was added to the reaction. Cells were left in the presence of Effectene complexes for 24 hours before harvesting.

When noted, 0.1% in DMF with or without 100 µM R-α-Lipoic Acid in was administered to cells in culture 12 hours before harvesting for luciferase activity. Total time of transfection remained constant at 24 hours.

Luciferase Assay Procedure

Transiently transfected primary hepatocytes were harvested using the Passive Lysis Buffer included in the Dual Luciferase Assay Kit (Promega). Cells were flash frozen and thawed twice before 20 µl of each cell extract was placed in a 96-well non-binding assay plate (Corning) and firefly and Renilla luciferase levels measured as
described in the manufacturer’s instructions. Luciferase data is represented as the ratio of firefly to *Renilla* luciferase expression in Relative Light Units, and then normalized to the pGL3-Basic vector.

*Ascorbic Acid Analysis*

Ascorbic acid analysis was performed as previously described (25). Acid soluble cell extracts were thawed on ice and 20 μl was added to 6 μl of 2.58 M KH₂PO₄ buffer (pH 9.8), and brought to a final volume of 100 μl using mobile phase. Samples were placed in a chilled 4ºC autosampler for analysis. Ascorbic acid was separated by high performance liquid chromatography using a LC-8 column (Supelco) with an isocratic solvent delivery system and sodium acetate/methanol/water (0.3%/7.5%/92% [w/v]) as mobile phase. Ascorbic acid was detected using an applied potential of +0.6 V by a LC 4B (Bioanalytical Systems, West Lafayette, IN) electrochemical detector (ECD). The peak corresponding to ascorbic acid was integrated by using HP Chemstation software (Hewlett-Packard, Mountain View, CA) and quantified relative to authentic ascorbic acid.

*SVCT mRNA RT-PCR Analysis*

To quantify the mRNA levels of both SVCT1 and SVCT2 in the cell, total RNA was isolated from liver tissue homogenates using the SV Total RNA Isolation System (Promega) and stored at -80°C in nuclease-free water until used. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on the isolated
RNA samples (1 µg/tube) in a single-step reaction using Ready-to-go™ RT-PCR Beads (Amersham Pharmacia Biotech). The following primer sequences were used:

SVCT1-forward: 5’-TCGGGAGGTCCAGGGTGCAATCA-3’
SVCT1-reverse: 5’-TGCCCCATCGGTAAACAGGCAACA-3’
SVCT2-forward: 5’-GTGCTAAAAGCTGCCTCTGGTC-3’
SVCT2-reverse: 5’-TTCTCTGCAATGCCATTCTCTG-3’
β-Actin-forward: 5’-CCAAGGCCAACCGCGAGAAGATGAC-3’
β-Actin-reverse: 5’-AGGGTACATGGTGGTGCCGCCAGAC-3’

The cDNA transcript was created by incubation of the reaction mixture at 42°C for 30 minutes. PCR was performed by multiple cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. This was followed by a final elongation step of 72°C for 10 minutes in a GeneAmp 2400 (Perkin Elmer) thermocycler. The linear range of product formation was determined by cycle controls. For SVCT1, 22 cycles were used; SVCT2 24 cycles; β-Actin 20 cycles. PCR products were separated on a 2.0% agarose gel and visualized by ethidium bromide staining using a FluorChem Imager (Alpha Innotech) and bands were analyzed using Scion Image analysis software (Frederick, MD).

**Ascorbic Acid Uptake Assay**

Freshly isolated hepatocytes were suspended in Chelex-treated Krebs-Henseleit medium (pH 7.4) at a concentration of 2 x 10^6 cells/ml. ascorbic acid stock solution (10 mM; final concentration) was freshly prepared in Chelex-treated Krebs-Henseleit
medium (pH 7.4) and kept on ice until use. Cells were incubated at 37°C following ascorbic acid addition. At various times following ascorbic acid addition, cell aliquots (0.5 mL) were removed and added to 600 μl of dibutyl phthalate layered over 300 μl of 10% (v/v) perchloric acid (PCA) that contained 10 mM DTPA (an iron chelator) (159). The tube was immediately centrifuged for 1 minute (15,000 rpm, IEC Micromax centrifuge), allowing cells to pass to the acid layer, thus excluding any extracellular ascorbic acid and non-viable cells. All but the bottom PCA layer was removed by aspiration, which was subsequently frozen at -80°C until analysis.

For experiments where radioactivity was used, a stock solution containing L-[14C]-ascorbic acid mixed with unlabeled ascorbic acid (specific final activity, 0.333 mCi / mmol) was employed. Cells were treated as described above, and 100 μl aliquots were kept from the Krebs-Henseleit medium (top layer) and PCA layer (bottom layer) for analysis of extracellular and intracellular ascorbic acid, respectively. Samples were mixed with 5 ml scintillation fluid (Beta Blend, ICN) and quantified by liquid scintillation counting using a Beckman LS 6500 scintillation counter.

For studies examining the relative contribution of ascorbic acid and DHA uptake on ascorbic acid accumulation, the same procedures were used as described above, except with changes to the Krebs-Henseleit medium. For studies involving the inhibition of DHA uptake, the Krebs-Henseleit medium contained 7.1 mM 2-Deoxy-D-Glucose, a competitive inhibitor of GLUT function. For studies examining the sodium-dependent nature of ascorbic acid uptake a sodium-free Krebs-Henseleit
medium was used, containing equimolar amounts of choline chloride in place of sodium chloride and potassium bicarbonate instead of sodium bicarbonate. All glassware was washed prior to use with concentrated hydrochloric acid and double distilled water to remove extra sodium from detergents.

For experiments requiring the use of boiled cells, tubes containing cell suspensions were placed in a boiling water bath for 15 minutes. The boiled cells were resuspended in Krebs-Henseleit medium at the appropriate concentration before ascorbic acid was added.

**Protein Analysis**

Total protein content was measured in cells by the procedure of Lowry *et al.* as modified by Peterson (160) using a reagent kit from Sigma. On average, cells from older rats contained more protein per million cells than cells from young rats, but this difference was not statistically significant (p>0.10).

**Statistics**

Data are expressed as the arithmetic mean ± standard error of the mean. The numbers of samples were a minimum of three separate animals per experimental condition. Statistical differences between two experimental conditions were evaluated using the two-tailed Student’s t-test with a p-value of < 0.05 considered significant. Differences in multiple experimental conditions were evaluated using repeated-
measures ANOVA analysis followed by a Tukey-Kramer post-hoc analysis with a
significance value ($\alpha$) of 0.5
4.4 Results

Age-related changes in hepatic ascorbic acid status correspond with a loss of SVCT1 transcription

To determine the age-related differences in basal hepatocellular ascorbic acid levels, acid extracts of freshly isolated hepatocytes from both old and young rats were analyzed for ascorbic acid using HPLC and electrochemical detection. Hepatocellular ascorbic acid levels from young rats were 1.76 ± 0.05 nmoles/mg protein. In contrast, ascorbic acid levels in cells from old rats were 0.53 ± 0.07 nmoles/mg protein, a significant (P<0.005) 68% decline (Figure 4.1). These results are in agreement with our previously published data, as well as the work by others, and suggest that cells from old rats cannot maintain normal ascorbic acid status despite their ability to synthesize this molecule.

Since SVCT proteins are thought to be the major determinants for vitamin C homeostasis through the modulation of ascorbic acid uptake, we sought to determine whether an age-related decline in ascorbic acid levels were a result of the loss of one or both isoforms of SVCT. SVCT mRNA levels were determined from total RNA isolates of liver homogenates using RT-PCR. Using primers specific for SVCT1 and SVCT2 single band PCR products of 270 and 305 bp, respectively, were observed which corresponded to the predicted sizes based on published mRNA sequences. Results show that there was a significant age-related decline in SVCT1 mRNA levels (Figure 4.2a). Expressed as a ratio of SVCT1:β-actin mRNA levels to correct for RNA
Figure 4.1 Age-related loss of hepatocellular ascorbic acid content. Hepatocytes were isolated from young and old rats and ascorbic acid levels were assessed by HPLC. Cells from old rats have significantly (P<0.0005) lower ascorbic acid levels than cells isolated from young rats.
Figure 4.2 Age-related declines in SVCT mRNA levels. Reverse transcription-polymerase chain reaction (RT-PCR) amplification products of total RNA isolated from the liver of young and old animals. A) SVCT1-specific primers showing a single 270 bp DNA product. B) SVCT2-specific primers showing a single 305 bp DNA product. Semi-quantification of the gel data is shown in relation to β-actin levels. Results show only for SVCT1 mRNA a significant change from young control animals (p<0.01). SVCT2 mRNA levels were not significantly affected with age.
integrity and content, SVCT1 message levels declined by 45%, a significant loss (p<0.01) with age. In contrast, SVCT2 mRNA levels showed no significant age-related change (p>0.3; Figure 4.2b). These results suggest that a preferential loss in SVCT1 levels may occur with age.

To determine if the loss of SVCT1 mRNA in cells from old animals was due to a decline in transcription of the SLC23A1 gene, rather than an age-related change in SVCT1 mRNA stability, we made use of our previously developed (Chapter 2) reporter gene assay. Primary hepatocytes from young and old animals were placed on collagen coated plates and maintained in culture as described in Materials and Methods. When cells from young and old animals were transiently transfected with the luciferase reporter gene containing the minimal promoter for SLC23A1, a 62% decline in luciferase activity was observed in the hepatocytes from old animals when compared to young (Figure 4.3), a significant change with age (p<0.03). Together, this suggests that aging results in a decreased transcription factor binding to the SVCT1 promoter, leading to a loss of SVCT1 mRNA and in SVCT1 protein levels that may have a functional consequence on vitamin C uptake in the cell.

**Age-related declines in ascorbic acid uptake in isolated hepatocytes**

To determine if losses of SVCT1 transcription resulted in a loss of steady-state ascorbic acid levels through an impaired ability to transport ascorbic acid from the exogenous milieu, cells were incubated with 100 μM ascorbic acid and uptake was monitored over time. For cells from young rats, ascorbic acid increased at a linear rate.
Figure 4.3 Age-related changes in SLC23A1 transcription. Hepatocytes isolated from young and old rats were placed into primary culture and transiently transfected with the minimal promoter of the SLC23A1 luciferase construct. After 24 hours, cells were harvested and assayed for luciferase activity, and displayed as fold control of pGL3-Basic empty vector. Results show a significant decline (p<0.05) in luciferase production in cells isolated from old cells when compared to cells from young animals, suggesting a loss in SLC23A1 gene expression with age.
for up to 20 minutes, where a steady-state level of $3.57 \pm 0.22$ nmoles/mg protein was reached (Figure 4.4a). A similar saturable mode of ascorbic acid uptake was seen in cells from old rats. For these cells, a linear rate of ascorbic acid uptake was observed following 15 minutes after ascorbic acid addition (Figure 4.4a). However, two major differences in transport characteristics were noted between cells from young and old rats. First, a significant age-related decline ($p<0.005$) in the rate of ascorbic acid uptake was observed. Whereas cells isolated from young rats showed a linear rate of increase of $0.108 \pm 0.018$ nmoles ascorbic acid/mg protein/min, cells from older rats showed a rate of $0.036 \pm 0.007$ nmoles ascorbic acid/mg protein/min, a 66% decline. These results suggest that ascorbic acid uptake mechanisms may be altered during the aging process.

After ascorbic acid uptake reached saturation, ascorbic acid levels remained markedly lower in hepatocytes from old compared to young rats. Steady-state ascorbic acid levels were $1.21 \pm 0.20$ nmoles/mg protein 20 minutes after ascorbic acid addition, a 66% decline from that seen in young rats. The extent of this age-associated decline was correlated to the significant loss of SVCT1 gene expression.

Rat hepatocytes have the ability to synthesize ascorbic acid, which could influence the results for ascorbic acid uptake (Figure 4.4a). Although the ascorbic acid biosynthetic capacity of isolated rat hepatocytes does not change with age, we wished to factor out endogenous ascorbic acid synthesis in our cell model. Hepatocytes were incubated with 100 $\mu$M L-[carboxy-$^{14}$C]-ascorbic acid and radiolabel uptake was
Figure 4.4 The rate of hepatocellular ascorbic acid uptake declines with age. Hepatocytes isolated from young (●) and old (○) rats were exposed to A) 100 μM ascorbic acid or B) 100 μM L-[\(^{14}\)C]-ascorbic acid and incubated at 37°C. At the times indicated aliquots were withdrawn and acid extracts were analyzed for ascorbic acid as described. C) The initial rate of ascorbic acid uptake into hepatocytes from old rats was significantly (P<0.005) lower than that seen in cells from young rats.
monitored over a 1-hour time-course. Cellular incorporation of radiolabel was evident in hepatocytes from young and old rats, and amount of accumulation (Figure 4.4b) and rates of uptake (Figure 4.4c) were similar to those seen with unlabeled ascorbic acid. The authenticity of radiolabel as genuine ascorbic acid, the acid soluble fraction of $^{14}$C-ascorbic acid-treated cells was analyzed by HPLC (Figure 4.5a), the column eluate fractions collected every 30 seconds, and the amount of $^{14}$C content determined by scintillation counting (Figure 4.5b). The results showed that over 95% of the $^{14}$C radiolabel incorporated into cells co-eluted with ascorbic acid, confirming that the age-related decline in radiolabel uptake was due to loss of ascorbic acid transport.

**Functional characteristics of ascorbic acid transport in hepatocytes**

Because DHA uptake may contribute to intracellular ascorbic acid accumulation, we sought to determine the relative contribution of GLUT transport to the age-related declines in ascorbic acid status. Freshly isolated hepatocytes from young and old rats were incubated with 100 $\mu$M DHA. Cells from both young and old animals rapidly accumulated intracellular ascorbic acid as a result of DHA uptake (Figure 4.6a). However, steady-state ascorbic acid levels in cells after DHA administration were the same, regardless of age. This suggests that there are no age-related changes in the ability of the liver to take up DHA from the extracellular medium, confirming data from other studies that report no age-related changes in GLUT levels (153).
Figure 4.5 Radioactive ascorbic acid is taken up from medium. 100 μM L-[\textsuperscript{14}C]-ascorbic acid (0.5 μCi) was incubated with isolated hepatocytes and assayed for ascorbic acid content. The ascorbic acid peak A) on the HPLC/ECD chromatogram co-elutes with B) >95% of counts seen in scintillation detection, suggesting radiolabeled ascorbic acid accurately reflected changes in cellular ascorbic acid uptake.
Figure 4.6 DHA uptake does not contribute to age-related changes in ascorbic acid accumulation. A) Hepatocytes isolated young (●) and old (○) rats were exposed to 100 µM DHA. Intracellular ascorbic acid was measured by HPLC coupled electrochemical detection. No significant changes in ascorbic acid accumulation were detected. B) Hepatocytes isolated from young rats were incubated with 100 µM L-[14C]-ascorbic acid in Krebs-Henseleit medium containing (○) or lacking (●) 2-deoxy-D-glucose. At specific time points, cells were collected and analyzed for 14C content. Results show no changes in the rate or accumulation of ascorbic acid in the presence of the glucose analog.
To further rule out the contribution of DHA uptake, cells from young rats were incubated with 100 µM 14C-labeled ascorbic acid in the presence of 7.0 mM 2-deoxy-D-Glucose (2-DG), a competitive inhibitor of glucose transport. Addition of 2-DG did not significantly (P>0.6) alter the rate of ascorbic acid uptake from cells isolated from young animals without inhibitor (Figure 4.6b). Additionally, 2-DG supplementation had no effect on the accumulation of ascorbic acid in old rat hepatocytes (data not shown). Thus, DHA production and GLUT transport do not appear to play a role in ascorbic acid accumulation in the liver.

Declines in SVCT1 suggest a loss in direct ascorbic acid uptake by a sodium-dependent process. To confirm this, cells from young and old rats were incubated with 100 µM 14C-labeled ascorbic acid in sodium-free Krebs-Henseleit medium (pH 7.4). Under these conditions, the rate of ascorbic acid accumulation was approximately 74% lower than cells from young animals in sodium-containing medium (Figure 4.7a). A similar decrease in ascorbic acid uptake under sodium-free medium was seen in cells isolated from old animals (Figure 4.7b). This suggests that transporter-driven ascorbic acid uptake was primarily through the SVCT, the specific contributions of which were determined by the differences in ascorbic acid accumulation between the sodium-free and sodium-containing medium (Figures 4.7c,d).

To determine whether aging results in altered catalytic properties of the sodium-dependent transport proteins, hepatocytes were incubated with increasing amounts of radiolabeled ascorbic acid (100 - 500 µM). In cells from both young and
Figure 4.7 Sodium-dependent nature of ascorbic acid transport. Hepatocytes isolated from A) young and B) old rats were incubated with 100 μM L-[14C]-ascorbic acid in Krebs-Henseleit medium containing (●) or lacking (○) sodium. At specific time points, cells were collected and analyzed for 14C content. Sodium-dependent uptake in cells from C) young and D) old rats, corrected for non-sodium-dependent ascorbic acid accumulation. Results show that a majority of ascorbic acid accumulation in cells is via a sodium-dependent process.
old rats, higher exogenous ascorbic acid resulted in increased ascorbic acid uptake, but cells from old rats displayed a decline in the linear rate of increase (Figure 4.8a) and steady-state ascorbic acid level (Figure 4.8b) than cells from young rats. Eadie-Hofstee transformation of the data (Figure 4.8c,d) shows an age-related decline in both the apparent maximum velocity ($V_{\text{MAX}}$) and the dissociation constant of ascorbic acid transport systems ($K_T$) with age. This suggests a loss in a high-capacity, low-affinity transport process with age, which is consistent with a decline in SVCT1. Thus it is apparent that the age-related decline in the transcriptional regulation of SLC23A1 has functional consequences on vitamin C transport characteristics in hepatocytes.

*R-$\alpha$-Lipoic acid reverses the age-related changes in SVCT1 transcription*

Since ascorbic acid does not affect the transcription of SLC23A1 and alone cannot completely reverse the age-related changes in vitamin C levels, other routes of modulating SVCT1 status may be necessary to improve vitamin C transport in hepatocytes. Previous results have shown that the SLC23A1 gene is under the influence of HNF-1-mediated transcription (Chapter 2) that is influenced by the administration of R-$\alpha$-Lipoic acid (LA; Chapter 3). Additionally, we have shown that feeding old rats LA for two weeks results in a reversal of the age-related losses in vitamin C status in many tissues, especially the liver (154,158,161). Thus we hypothesized that LA may affect SLC23A1 gene transcription in the aging animal.

Primary cultured hepatocytes from young and old animals were transiently transfected with the SLC23A1 minimal promoter. Cells were then provided with
Figure 4.8 Dose-dependent characteristics of ascorbic acid uptake. Hepatocytes from old rats were incubated with increasing concentrations of L-[14C]-ascorbic acid. The A) rate of 14C uptake and steady-state B) 14C-ascorbic acid levels in old rats are displayed. As a reference, the appropriate values from cells from young rats given 100 µM ascorbic acid is represented as a dashed line. C) Eadie-Hofstee plots of the rate of ascorbic acid accumulation in cells from (●) young and (○) rats. Results show significant alterations in the affinity and capacity for ascorbic acid transport in hepatocytes from old animals.
100μM LA for 12 hours and luciferase expression determined as a measure of SLC23A1 promoter activity. Results show that cells from old rats showed LA-dependent increases in reporter gene activity (Figure 4.9). LA caused a significant 85% increase (p<0.05) in the SLC23A1 gene activity in cells only from old animals, restoring luciferase expression to the level of young control cells (p>0.15). This suggests that LA may stimulate SVCT1 production in old animals to compensate for age-related declines in vitamin C transport.
Figure 4.9 Age-related changes in SLC23A1 transcription are reversed with LA. Hepatocytes isolated from young and old rats were transiently transfected with the SLC23A1 reporter gene construct. Cells were provided with or without 100 μM LA for 12 hours before cells were harvested as assayed for luciferase activity. Results show a significant decline increase in luciferase production only in the cells from old animals (p<0.05).
4.5 Discussion

Antioxidant levels in many tissues decrease during the aging process (153,154,156,158,161-163). This may be due, in part, to increased antioxidant utilization because of the heightened pro-oxidant environment in aged tissues. Lower synthetic capacity, as is the case for glutathione (122,164), may also contribute to diminished antioxidant status. However, there appears to be no uniform age-related decline in cellular antioxidants. Glutathione, one of the major cellular antioxidants, does not decline to the same degree as ascorbic acid, and vitamin E levels do not change with age (154,165,166). These observations suggest a lack of a common utilization pathway for cellular antioxidants. The decline in ascorbic acid observed in these experiments suggests a unique mechanism for its loss during the aging process, one not necessarily related to its oxidant-mediated usage. Because aging does not alter the rate of ascorbic acid synthesis in rats (158), our results suggest that loss of vitamin C transport may be involved in age-related changes in vitamin C status. Moreover, recent studies show that dietary absorption of ascorbic acid decline in the elderly, which adversely affects optimal plasma vitamin C levels (69). Since plasma vitamin C is strongly dependent on vitamin C transport and the normal functioning of SVCT1 (60), age-associated loss of absorptive capacity for the vitamin provides a rationale to suggest that diminished SVCT1 levels may also underlie the decline in cellular levels of this antioxidant. Our data thus presents a plausible mechanism explaining the age-dependent decline in SVCT1 levels and lower circulating vitamin C levels seen in the elderly.
Ideally, the losses in SVCT1 mRNA we observed should be confirmed by measuring age-related changes in SVCT1 and SVCT2 protein content. While antibodies to these proteins are commercially available, we were unable to demonstrate cross-reactivity of these antibodies with rat proteins. Thus it remains to be established whether lower SVCT1 mRNA levels translate to lower protein content with age, though the decrease in transport activity suggests a trend for its decline. However, we have demonstrated that age-related declines in ascorbic acid uptake were attributed to the loss of a high-capacity, low-affinity, sodium-dependent transport system in rat hepatocytes. The lack of age-related declined in GLUT-mediated DHA uptake or a significant contribution of GLUT activity to ascorbic acid uptake, suggests the DHA uptake pathway is not playing a significant role in the age-related loss of ascorbic acid transport. Thus, we conclude that the loss of SLC23A1 transcription results in functional declines in SVCT1-mediated transport, but the extent of which will need to be determined in future studies.

The uptake of ascorbic acid in rat hepatocytes appears to be a combination of SVCT1- and SVCT2-mediated transport processes. Our data suggests a preferential decline in the transcription of SVCT1, the high-capacity, low-affinity ascorbic acid carrier, with age. Although the precise mechanism behind the loss of SLC23A1 gene expression was not explored in the present study, previous work in our laboratory (Chapter 2 and 3) suggests the involvement of HNF-1 and its regulation by insulin signaling. Metabolic changes that affect carbohydrate metabolism and insulin signaling are frequent in the elderly (138,146,149,152,167,168), thus a decline in
SVCT1 mRNA argues for a loss in HNF-1/DNA binding or to lower HNF-1 levels per se. To date, no age-related changes in hepatic HNF-1 levels have been documented, though HNF-4α, the major regulatory transcriptional regulator for HNF-1, declines significantly with age (169,170). Because HNF-4α mediates many of the upstream signals from regulatory pathways to HNF-1 and is involved in the insulin signaling pathway (99), this would suggest an inability of insulin to sustain SVCT1 expression in older animals. Other reports show similar age-related declines in insulin-dependent PI3-kinase and Akt activity (127,146,171-173) that would suggest a functional loss in the HNF-1/insulin signaling axis. Further work in this area will be necessary in order to fully explore whether this is the root-cause for decline in SVCT1 transcription with age.

The age-associated loss of insulin-mediated signaling and HNF-1 regulation may be further exemplified by our studies using LA to induce SLC23A1 transcription. LA is known to act as an insulin mimetic, inducing the activation of the PI3K/Akt pathway (125,129,135,139,150,174-177). Our previous data (Chapter 3) suggest that LA works through this pathway to increase the abundance of nuclear HNF-1 in HepG2 cells, which subsequently increases SLC23A1 transcription. This in vitro data has been augmented in this study by feeding rats LA (0.2% [v/v] in the chow) for 2 weeks prior to monitoring ascorbic acid status. In previously published data, we show that LA restores tissue vitamin C status, which otherwise declines with age (154,158,161,162). Because SVCT1 is directly involved in the control of plasma ascorbic acid levels, LA may act through HNF-1 expressing organs (e.g. the liver, intestine and kidney) to
increase plasma ascorbic acid through increased dietary and renal absorption, which provides additional vitamin C to all tissues of the body. The fact that LA administration did not increase levels of vitamin C in cells from young rats is not unusual. Previous studies in which LA was supplemented to young and old rats, reversed the age-dependent declines in tissue GSH levels, but had no or limited effects in young animals (127,154,158,161,162,178-180). This may be due to differential genetic regulation during aging that is ‘reset’ by LA administration.

A possible alternative mechanism for both the age-associated declines of ascorbic acid levels and the loss of SVCT1 mRNA is through increased pro-oxidant conditions in aging tissues. Old age is marked by a shift to a more pro-oxidant environment due to mitochondrial decay, redox-active metal accumulation, and buildup of advanced glycation end-products (164,181-183). Thus, steady-state ascorbic acid levels may fall due to heightened utilization for scavenging reactive oxygen species (ROS). Additionally, it is possible that increased ROS production could lead to a down-regulation of the SLC23A1 gene, and concomitant declines in SVCT1. This could be through redox-sensitive pathways that have been previously established for HNF-4α (184), HNF-1 (185), or any of the possible redox-sensitive transcription factors present on their promoters. However, we observed that cellular ascorbic acid utilization in cells from both young and old animals did not change significantly over time in the absence of exogenous ascorbic acid (data not shown), arguing against a significant increase in oxidant production in cells from old rats in these experiments. Additionally, our previous results suggest that altering the redox environment will not
significantly alter SLC23A1 transcription (Chapter 3). Although SVCT2 is the predominant redox-sensitive vitamin C transport protein, levels of SVCT2 mRNA do not change with age. Together, we conclude that the regulation of ascorbic acid transport in aging is associated with the changes in glucose metabolism rather than redox environment.

In conclusion, our results show that SVCT1-regulated vitamin C uptake declines with age. Increasing plasma ascorbic acid levels in older individuals may reverse some of the age-associated decline in tissue ascorbic acid concentrations, but decreased bioavailability and increased renal excretion would be hallmarks of this condition. To increase steady-state plasma ascorbic acid levels, or to restore ‘tight’ control that is lost during genetic dysfunction, additional measures such as the supplementation with lipoic acid, may be necessary to provide adequate levels of this antioxidant during aging.
Chapter 5

General Conclusion
5.1 General Conclusions

The importance of vitamin C to human health cannot be overstated. The versatile nature of ascorbic acid allows it to play numerous roles in the cell: enzyme cosubstrate, redox scavenger, and cellular antioxidant (4,5,10,13). Through these functions, ascorbic acid prevents disease and the accumulation of oxidative damage. However, in the course of evolution, humans have lost the ability to synthesize ascorbic acid. Without constant dietary supplementation, vitamin C levels in plasma and tissues will fall to dangerous levels within weeks (5,30). Beyond the obvious signs of deficiency, vitamin C plays additional roles in maintaining optimal health status. Saturating plasma and tissues with vitamin C maintains antioxidant defenses and reduces the risk of many chronic diseases (4). Although many of the mechanisms by which vitamin C elicits these responses are known at a molecular level, much more work is still needed in order to understand the roles and regulation of vitamin C in the body as a whole.

Public awareness of the importance of dietary vitamin C has steadily increased along with literature reports of its effectiveness in human trials. The RDA for vitamin C has also increased over the years, reflecting the growing evidence for its importance in health, but there is debate over the ‘optimum’ dietary intake of vitamin C. Although recent pharmacokinetic studies have shown that vitamin C absorption and tissue saturation peaks with doses of approximately 200 mg/day (30), we know very little about the regulatory processes involved in vitamin C bioavailability. Along these lines,
it is unknown whether the bioavailability of vitamin C changes when genetic
regulation of SVCT1 protein expression is altered, such as through changes in chronic
health status. As discussed in Chapters 2-4, the physiological roles of the SVCT
transport proteins are clearly implicated in maintenance of plasma and tissue vitamin C
status. Yet, the transcriptional, translational, and post-translational regulation of these
transporters has been explored at only rudimentary levels. Without further
investigation into the role(s) these two proteins play in vitamin C homeostasis, our
ability to determine optimal vitamin C intake for any individual is severely limited.

To effectively understand the regulation of these transport proteins, it is
necessary to first examine control on a genetic level. This dissertation directly
addressed the transcriptional regulation of SLC23A1, the gene that encodes for
SVCT1, and the consequences behind the loss of this vitamin C transport protein. The
large array of putative elements defined on the human SLC23A1 promoter presented a
challenge in understanding the discrete regions of its functional regulation. Therefore,
we constructed a SLC23A1 reporter vector that mimicked endogenous transcription of
this gene, a major achievement for all future studies on its transcriptional control.
Furthermore, our results with this construct show that the expression of SLC23A1 in
HepG2 cells is absolutely dependent on an intact binding site for Hepatic Nuclear
Factor 1 (HNF-1). Mutations that prevented binding by other putative transcription
factors show no other site on the SLC23A1 promoter was necessary for transcription
as long as the HNF-1 site remained. As a result of this study, we now define SVCT1 as
one of an ever-growing family of proteins under the control of HNF-1 transcriptional regulation.

Based on this finding, a new paradigm for understanding vitamin C transport has been established. As demonstrated in Chapter 3, Vitamin C is transported not for its antioxidant activity, but as a dietary carbohydrate under the influence of glucose metabolic pathways. Thus, factors that alter the availability and utilization of carbohydrate sources, and glucose in particular, play a much greater role in maintaining systemic vitamin C homeostasis. To this end, LA was a powerful stimulator of SLC23A1 transcription in HepG2 cells and this effect was independent of its antioxidant activity or alterations of thiol redox state. Rather, stimulation of the insulin signaling pathway was necessary to mediate all the effects of LA on SLC23A1.

Future investigations into SLC23A1/SVCT1

Future work should focus on the precise mechanism by which LA and insulin regulate SLC23A1. While this dissertation did not specifically seek to answer this question, our data strongly imply that insulin-mediated signal transduction ultimately directs HNF-1 dependent transcriptional regulation of SLC23A1. The connections between insulin, HNF-1 and SLC23A1 could be multifold (Figure 5.1). For instance, both PI3K and Akt have a multitude of effects on the cell, including the phosphorylation of transcription factors such as forkhead (FKHR). FHKR binds directly to the HNF-4α protein and prevents it from binding to both the HNF-1α and
Figure 5.1 Possible routes of insulin regulation of SLC23A1. HNF-1 activity may be modulated by several routes by insulin signaling. Insulin stimulates PI3K and Akt (left), which in turn will phosphorylate FKHR and promote dissociation from HNF-4α. Once released, FKHR is exported from the nucleus and degraded, while HNF-4α binds to HNF-1 promoters, increasing protein levels. Insulin also stimulates MAPK Kinase (MKK3; center) which activated Mirk. Mirk binds to DCoH, Dimerization Cofactor of HNF-1. Mirk phosphorylates HNF-1, stimulating DNA binding and transcription of genes such as SLC23A1, increasing SVCT1 protein levels. Alternatively, insulin may also regulate other transcription factors that HNF-1 coactivates on the SLC23A1 promoter (right). Unknown signaling cascades may also affect SVCT1 protein post-translational modifications, affecting transport activity.
HNF-6 promoters, the latter of which regulates HNF-1β levels. Activation of Akt by insulin specifically targets FKHR for phosphorylation, which forces its dissociation from HNF-4α, allowing transcription of HNF-1 (99). Thus, insulin ultimately signals for an increase in HNF-1 protein levels, which fits with the LA-induced elevation in HNF-1 following LA stimulation.

There are additional roles by which insulin and LA regulate HNF-1 activity. A new and relatively unexplored role of insulin signaling is through DCoH, the Dimerization Cofactor for HNF-1. The signals that regulate DCoH activity are largely unknown, and it is unclear whether DCoH may be under the influence of insulin. However, it is known that insulin stimulates MAPK Kinase 3 (MKK3) which leads to binding of DCoH by the protein kinase Mirk also known as Dyrk 1B. Mirk is an arginine-directed serine/threonine kinase that functionally overlaps with Akt. Mirk has demonstrated the ability to phosphorylate HNF-1 which leads to increased HNF-1-mediated gene transcription (117). Mirk regulation of SLC23A1 is completely unknown, but represents a synergistic pathway for insulin stimulation of HNF-1 activity. Future studies are needed to determine which path is ultimately relevant to the function of SVCT1.

Since HNF-1 does not act upon target genes alone, the direct activation by upstream signaling pathways also allows synergistic binding of neighboring transcription factors. Our analysis of the SLC23A1 promoter (Chapter 2) revealed many additional putative binding sites as well as a repressive motif on the reporter
gene that have not been fully explored. The interaction of all these sites with HNF-1 is necessary to understanding the regulatory events that comprise SLC23A1 signaling. Only with future studies examining all of these binding regions in conjunction with HNF-1 can a precise understanding of SLC23A1 regulation on a global level be achieved.

Transcriptional regulation of SLC23A1 through HNF-1-dependent mechanisms increase SVCT1 mRNA, but the extrapolation of this data to protein levels must be confirmed. While transcription is certainly an important regulatory point for SVCT1, the intracellular distribution, either on membrane vesicles or the cell surface is equally important (55,74,186). However, little is known about the mechanisms of redistribution of SVCT1, its half-life, or post-translational modifications that may influence its activity. Insulin may play a role in this process as it does for the GLUT proteins, but more work on SVCT1 is necessary. The relationship between SVCT1 gene regulation and protein levels must be explored before we can fully understand its control of vitamin C homeostasis.

If the insulin-regulated SLC23A1 transcription ultimately increases SVCT1 protein activity, then there are clear differences in how the two SVCT transporters are regulated. SVCT2 is present in most tissues of the body and involved in the uptake of vitamin C from the plasma. Recent studies into the regulation of SVCT2 show that its activity is sensitive to cellular antioxidant levels, and the induction of oxidative stress. SVCT1, on the other hand, is situated in the intestine, liver, and kidney, and mediates
the uptake and clearance of vitamin C in the plasma. By doing so, SVCT1 regulates the availability of vitamin C for all tissues. Based on our data, we conclude that dietary factors greatly influence the levels of SVCT1 to transport vitamin C in times of plenty, flooding the plasma with ascorbic acid. SVCT2 would then transport vitamin C into tissues that require it the most, either during increased oxidant production or for biosynthetic reactions. Thus, the two proteins, through divergent evolution, have functionally separated the roles of vitamin C as a dietary carbohydrate and an antioxidant. Understanding these divisions allow us to formulate the proper approach to treating a functional loss in vitamin C transport.

This functional division is observed best in our work in Chapter 4, which addresses the age-related changes in vitamin C status. Aging is characterized by an increased production of reactive oxygen species, and a decreased ability to combat stresses of all types. However, aging is also marked by increased insulin resistance and impaired glucose metabolism. The selective loss of SVCT1 transcription over SVCT2 reflects these changes in insulin signaling, which appears to dominate over that of increased oxidant production and SVCT2 stimulation. Thus for vitamin C, antioxidant defenses may fail with age not because of increased demand, but decreased supply. Since vitamin C absorption declines on a genetic level, increasing vitamin C supplementation would be only partially effective. This is consistent with Brubacher et al., who indicated that a significant increase in vitamin C intake would be necessary in order to maintain plasma vitamin C saturation in the elderly (69). An alternative strategy would therefore be necessary to improve insulin sensitivity in older people.
Regardless of the treatment used, improving vitamin C status requires attention to SLC23A1 and SVCT1. Compounds like LA that address insulin signaling may provide an effective means of restoring SLC23A1 regulation and vitamin C uptake.

The declines in vitamin C homeostasis and SLC23A1 regulation observed in aging may also extend to other chronic diseases, especially those that exhibit alterations in the insulin signaling pathway. The best example of this is diabetes mellitus. Declines in insulin sensitivity observed in diabetics coincide with an increase of oxidative stress and a loss of vitamin C in the plasma. Although there are associations with a loss in vitamin C transport in diabetic animal models (71), and general relationships between glucose and vitamin C transport have been postulated (70), no functional links with SVCT1 have been established. With these same associations made in aging, impaired insulin signaling is likely a causal factor for declines in vitamin C homeostasis in diabetes or other conditions marked by insulin resistance.

In summary, this dissertation has fulfilled the goal of performing a functional characterization of the SLC23A1 promoter. Furthermore, we have determined consequences in this loss of vitamin C transport regulation in aging, and determined potential strategies to maintain these systems through insulin signaling.
Bibliography


