ASCORBATE TRANSPORT IN CORONARY ARTERY

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by

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ABSTRACT

Vitamin C (ascorbate or Asc) is an essential vitamin for humans. The transport of oxidized ascorbate occurs via Na^+-dependent vitamin C transporters (SVCT1/SVCT2). Ascorbate is a powerful antioxidant that may be beneficial in scavenging the reactive oxygen species associated with cardiovascular diseases. The objectives of this thesis were: to identify the SVCT isoform(s) expressed in pig coronary artery smooth muscle and endothelium, to determine if preloading cultured pig coronary artery smooth muscle cells with ascorbate protects them against oxidative stress, and to overexpress SVCT2 in these cells to see if an increase in ascorbate reserve helps protect the cells even more.

Pig coronary artery smooth muscle tissue and cells cultured from the same tissue express SVCT2 and not SVCT1. Cultured pig coronary artery endothelial express SVCT2, however the limited amount of fresh endothelium isolated, restricted us from determining the isoform present in the fresh tissue.

Ascorbate preloading (200 μM overnight) did not decrease the damage caused by hydrogen peroxide as measured by oxidation of dichlorodihydrofluorescein diacetate or mitochondrial reductase activity.

The mRNA and ^{14}C-Asc uptake was marginally greater in pig coronary artery smooth muscle cells stably transfected with a linear pcDNA3.1SVCT2 plasmid than mock transfected controls. The ^{14}C-Asc uptake was 1.5 times greater than mock transfected cells after 60 min. A new SVCT2 plasmid, that contained SVCT2 coding region only, did not

show greater ^{14}C-Asc accumulation compared to the plasmid that had the entire SVCT2 cDNA in transiently transfected HEK293T cells. This thesis is a beginning towards further study on the molecular and physiological role ascorbate plays in the coronary artery.

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LIST OF ABBREVIATIONS

Asc	ascorbic Acid or vitamin C
bp	base pair
CAT	catalase
cDNA	complementary DNA
DHA	dehydroascorbic acid
DTT	1-4-dithiothreitol
EDTA	(ethylenedinitrilo)-tetracetic acid ethylene glycol
G3PDH	glyceraldhyde 3-phosphate dehydrogenase
G6PDH	glucose 6-phosphate dehydrogenase
GLUT	glucose facilitative transporter
GPx	glutathione peroxidase
GSH	glutathione
HEK293T	human embryonic kidney tumour cells
HEPES	4-(2-hydroxyethyl-1-piperazine ethane sulfonate)
LDH	lactate dehydrogenase
MTT	3-(4,5-dimethylthiazzol-2-yl)-2,5-diphenyl tetrazolium bromide
OPA	one-phor-all buffer
PCEC	pig coronary artery endothelial cells
PCSMC	pig coronary artery smooth muscle cells
ROS	reactive oxygen species
RT	reverse transcription
SGLT	sodium dependent glucose transporter
SVCT1	sodium dependent vitamin C transporter 1
SVCT2	sodium dependent vitamin C transporter 2
SOD	superoxide dismutase
Tris	Tris (hydroxymethyl) aminomethane

1.0 INTRODUCTION

Ascorbic acid (Asc or vitamin C) is an antioxidant that may play a major role in the prevention of arterial damage by scavenging reactive oxygen species (ROS) that impair arterial function. Objectives of this thesis were to determine the molecular identity of the vitamin C transporters expressed in smooth muscle and endothelium of pig coronary arteries, to determine if vitamin C loaded cells are protected better under oxidative stress and to assess if overexpression of vitamin C transporters would enable the cell to be protected even better against oxidative stress. The Introduction focuses on coronary artery function, the effects of ROS on this artery, metabolism and cellular transport of vitamin C and the role of vitamin C in cardiovascular diseases.

1.1 Coronary artery structure and function

The coronary arteries supply the heart with oxygen and nutrients (87). The left and right coronary artery arise from the base of the aorta and run anteriorly on either side of the pulmonary artery (57). The left anterior descending coronary artery proceeds anteriorly toward the cardiac apex. There are three layers that compose the arterial wall: intima, media and adventitia (57). The intima is made up of a single layer of endothelial cells, which serve as a barrier between the circulating blood and the underlying tissues and regulate artery tone and function (88). The internal lamina separates the media from the intima. Smooth muscle cells, which are involved in maintaining vascular tone, and extracellular matrix protein are

the main components of the media. The external elastic lamina divides the media from the adventia. Adventitia, which is the most external layer, provides mainly a mechanical support for the artery (57).

1.1.2 *Endothelium*

Endothelium is essential to maintaining proper arterial function (61). Structurally, the endothelial layer is only one cell layer thick however, the cells are adjoined tightly to prevent large molecules from invading the sub-endothelial space (29). Endothelial cells are able to metabolize anti-thrombotic molecules that are either released into the circulating blood or remain on the endothelial cell surface (26; 29). They are also able to release signalling molecules such as nitric oxide and endothelin, which modulate the tone of the vascular smooth muscle, causing vasodilation or vasoconstriction, respectively (26; 87). Since endothelial cells contain 17X greater catalase activity than smooth muscle cells (30) and are an effective barrier for large molecules, they may play an important role in protecting smooth muscle cells from circulating ROS.

1.1.3 *Smooth Muscle*

Smooth muscle cells are responsible for maintaining vascular tone as well as synthesizing many molecules. Agonists released from the endothelial cells or from local nerve terminals cause a response by the smooth muscle cells, changing the diameter of the vessel through contraction and relaxation of the tissue (29). The ability of the smooth muscle cells to contract and relax is essential in maintaining vascular tone. Moreover, smooth muscle cells synthesize many extracellular matrix molecules such as collagen, elastin and

proteoglycans. They are also able to produce many vasoactive and inflammatory mediators, such as cytokines (29).

1.2 Reactive Oxygen Species

Free radicals are characteristically defined as molecules with unpaired electrons that are capable of independent existence. Chemically, they are highly reactive and have short half lives (94). Some of the ROS are loosely grouped with the 'free radical' although they do not necessarily have unpaired electrons (23). In fact, they may be cationic, anionic or neutral however, they are highly reactive and some are able to diffuse across membranes (87).

The most common source of ROS in a metabolically active cell is the mitochondria (8; 9). During aerobic respiration, mitochondria can produce superoxide anions with a 1-electron reduction of molecular oxygen (94). Other cellular sources of the superoxide ion include: NAD(P)H oxidase, lipooxygenase, hypoxanthine/xanthine oxidase and cyclooxygenase (10).

Superoxide can react rapidly with itself without any catalyst, however, this reaction is increased nearly ten thousand fold with the action of superoxide dismutase (24). The products of this dismutation reaction are hydrogen peroxide and water. Hydrogen peroxide can be either broken down by catalase or by undergoing Fenton reactions, i.e., react with metal ions to form the hydroxyl radical. The byproducts of the action of catalase are water and molecular oxygen. During the Fenton reaction, superoxide reduces Fe^{3+} to Fe^{2+} which

releases the iron from its storage sites. Fe^{2+} is then able to react with hydrogen peroxide to yield the hydroxyl radical (9; 10; 47)(Figure 1).

Our defence systems against ROS include both antioxidants synthesized by our body as well as antioxidants ingested from our diet (25). Enzymatic defence mechanisms against ROS synthesized in vivo include superoxide dismutase, catalase, glutathione peroxidase and thioredoxin peroxidase (24). Some of the antioxidants obtained from our diet include vitamin C, α-tocopherol, β-carotene (25). These antioxidants are essential in maintaining a balance between ROS formation and reduction in order to avoid the damage of cellular components.

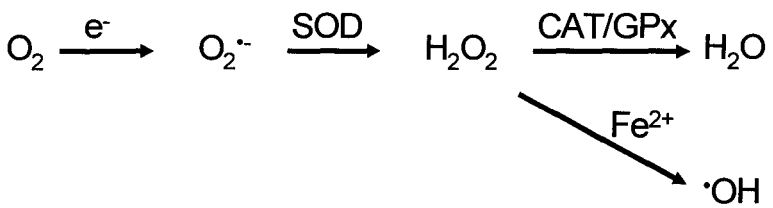

Figure 1. ROS production A schematic diagram showing how ROS are formed. ($O_2^{\bullet-}$ superoxide, SOD superoxide dismutase, H_2O_2 hydrogen peroxide, CAT catalase, GPx glutathione peroxidase, • OH hydroxyl radical).

1.2.1 *Importance of ROS in physiology of endothelium and vascular smooth muscle*

Approximately 5 % of the oxygen consumed by tissues is converted into ROS and under physiological conditions, the antioxidant capacity of cells is sufficient to detoxify the ROS without injury to the cell (87). In fact, ROS are not only responsible for causing cellular damage, but also can play an important role in a number of signalling mechanisms (42). For instance, hydrogen peroxide, which is not as reactive as other ROS, can act as an endothelium derived hyperpolarizing factor (at concentration in the 10- 25 μM range) in human coronary arterioles, mouse small mesenteric arteries and dog left circumflex coronary artery (24; 58; 59; 65). In vascular smooth muscle cells, platelet derived growth factor (PDGF) increased intracellular concentration of hydrogen peroxide which led to an increase in mitogen-activated protein kinase stimulation and DNA synthesis. This mitogenic response was hindered by blocking the PDGF activated production of hydrogen peroxide with catalase (84). In fact, overexpression of catalase inhibits proliferation and can cause apoptosis in vascular smooth muscle cells (12).

1.2.2 *Damage caused by ROS to coronary arteries*

During oxidative stress or in some pathological conditions, the level of ROS increases beyond the antioxidant capabilities of the cell, thereby causing oxidation, cleavage, cross-linking and modification of lipids, proteins, carbohydrates and DNA (68). These can eventually lead to the activation of apoptotic or necrotic pathways (94). In the cardiovascular system, excessive production of ROS may play a role in atherosclerosis, myocardial infarction and ischemia-reperfusion (21; 87). Myocardial ischemia/reperfusion, a brief

period of decreased or lack of blood flow followed by reflow, is common during clinical procedures such as angioplasty and coronary bypass surgery (95) and is accompanied by an excessive ROS production (14). Moreover, studies conducted on rabbits also showed a significant increase in production of ROS, a decrease in reduced glutathione and an increase in oxidized glutathione following balloon injury of arterial rings (82). The pathological damage after such injuries is connected with the damage of endothelial cells, which are thought to protect underlying smooth muscle against ROS. In pig de-endothelialized coronary artery rings, hydrogen peroxide pretreatment irreversibly impaired contractility through damage to the sarcoplasmic reticulum calcium pump (32-34). In addition, a decrease in cell viability as well as an increase in DNA fragmentation was observed following the generation of hydrogen peroxide by the glucose oxidase enzymatic system (52). This evidence implies that damage caused by ROS in the coronary artery affects its cellular function and vascular tone.

1.3 Vitamin C Structure and Function

1.3.1 *Structure of Vitamin C*

Vitamin C (Asc), is a six carbon α-ketolactone derived from D-glucose (Figure 2)(51). The chemical name of vitamin C is 2.3-didehydro-L-threohexano-1, 4 lactone and it is also known as hexuronic acid. It exists as two enantiomers: L-ascorbate and D-isoascorbate due to the asymmetric carbon 5 (44). Two ionizable enolic hydrogen atoms give the compound two characteristic pK_a values (pK_{a1} at hydroxyl group at carbon 3 = 4.17 and

pK_{a2} at hydroxyl group at carbon 2 = 11.57) (44). Vitamin C is readily oxidized to dehydroascorbaic acid (DHA), which may be reduced back to Vitamin C. If reduction does not occur, DHA is broken down to 2,3-diketo-1-gulonic acid which can no longer be reduced back to DHA or Asc (60; 63) (Figure 2).

1.3.2 *Functions of Vitamin C*

The biological functions of vitamin C are primarily based on its ability to be reversibly oxidized (44)(Figure 3), therefore playing an important role in many enzymatic reactions by providing electrons directly or indirectly to enzymes that require prosthetic metal ions in a reduced form (51). This is evident in the synthesis of neurotransmitters and the posttranslational hydroxylation of proline and lysine residues during the formation of collagen (44; 51). Due to the ability to donate electrons, Asc is effective in scavenging ROS, and is readily reduced back by glutathione and NADPH (5; 63) (Figure 3). Asc has been shown to quench ROS including the hydroxyl and superoxide radicals as well as reactive peroxides such as hydrogen peroxide (44). The plasma membrane is protected by Asc scavenging peroxyl radicals in the extracellular fluids before they are able to oxidize the lipids of the plasma membrane (25) and reducing the oxidized form of α-tocopherol (vitamin E), a lipid soluble antioxidant which exists in the plasma membrane (36). Therefore, Asc may be the most effective water-soluble dietary antioxidant (64).

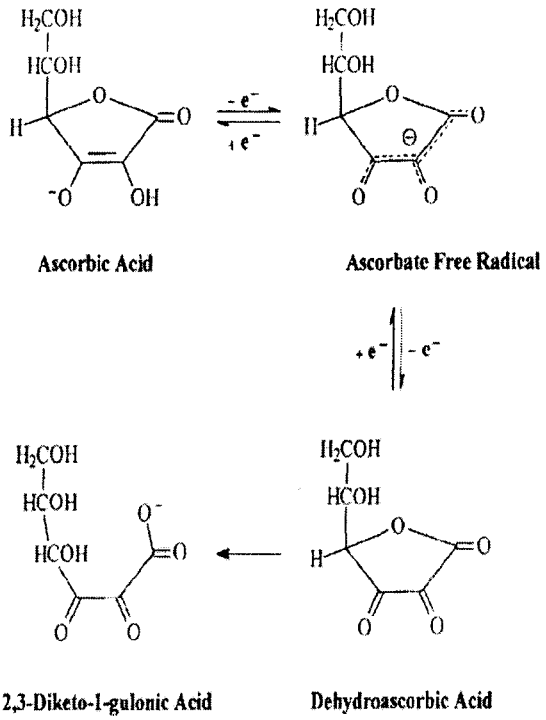

Figure 2: Structure of Ascorbic Acid. Ascorbic acid can reversibly lose one electron forming the ascorbate free radical or two electrons forming dehydroascorbic acid. Once dehydroascorbic acid is broken down to 2,3-diketo-1-gulonic acid, it can not be reduced back to ascorbic acid. Figure is take from May, J (1999)(60).

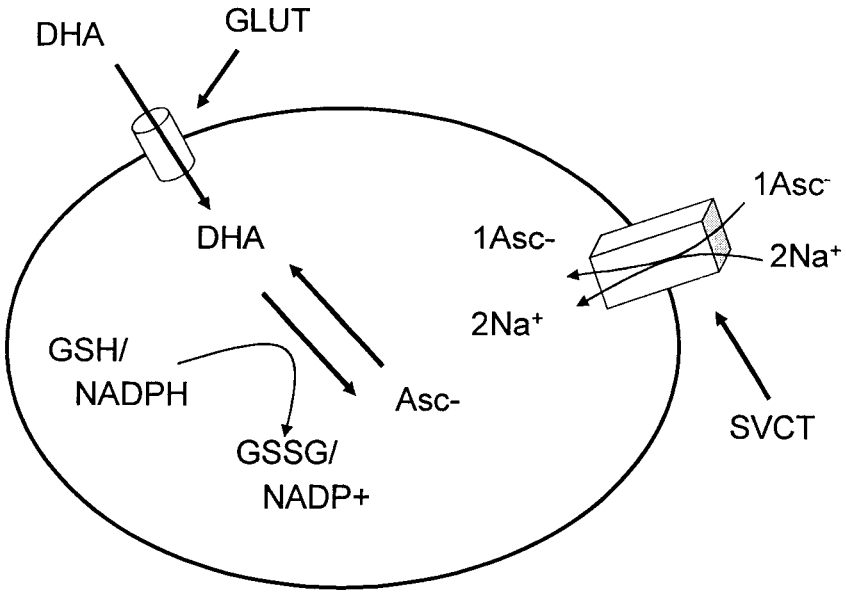

Figure 3: Transport and Recycling of Ascorbate. Asc is transported into the cell via SVCT (sodium dependent vitamin C transporter), while the oxidized form, dehydroascorbic acid (DHA) enters by GLUT (facilitative glucose transporter). DHA can be reduced back to Asc by glutathione or NADPH dependent enzymes (GSH or NADPH, respectively).

1.4 Vitamin C transport

Higher vertebrates synthesize vitamin C in the liver although humans, guinea pigs and bats have lost their ability to synthesize vitamin C due to a mutation in the L-gulono-gamma-lactone oxidase gene (71). However, in these animals, vitamin C is present in nearly all tissues, but it must be obtained from the diet. Asc levels in the plasma usually range from 50 to 100 μM (45; 60), but in some tissues Asc can reach even millimolar levels (41; 76; 92).

At physiological pH, ascorbate exists as a monoanion; as a result it cannot simply diffuse across the membrane (92). DHA is a neutral molecule at physiological pH but does not cross the membranes on its own because it is hydrophilic (53). Therefore these two forms of Vitamin C require specialized and distinct mechanisms of transportation across cell membranes.

1.4.1 *The Transport of Asc*

Earlier studies showed that the transport of Asc is sodium dependent (67; 73; 80; 93). Currently we know that this transport occurs via sodium dependent transporters (SVCT) for which at least two identified isoforms, SVCT1 and SVCT2, have been reported.

1.4.1.1 *SVCT1*

Rat SVCT1 has been identified as a membrane bound 604 amino acid protein, showing Na^+-dependent ascorbic acid transport activity (86). Human SVCT1 (hSVCT1) was previously identified as YSPL3 (nucleobase transporter-like protein), but the function of this YSPL3 had not been determined (39; 53). hSVCT1 cDNA (2306 bp) has an open reading frame of 1797 bp (nucleotides 47-1844), including the termination codon, that encodes a 598 amino acid polypeptide, which has a predicted molecular mass of 64 823 Da (17; 53; 90). The gene encoding hSVCT1 (SCL23A2) has been mapped to the long arm of chromosome 5 in band 5q31.2-31.3 (83; 90). Hydropathy studies predict that the protein has 12 transmembrane domains. The amino acid sequence contains sites for phosphorylation by protein kinase A (1 site) and protein kinase C (5 sites) and sites for N-glycosylation (3 sites) (17). Glycosylation has been shown in hSVCT1 transfected COS-1 cells (54).

SVCT1 is mainly confined to the epithelial tissues of kidney, intestine and liver as determined by *in situ* hybridization (86). Northern blot analysis also identified SVCT1 in the colon, ovary and prostate with a weaker signal evident in the pancreas (90). SVCT1 expression may be directed to the brush-border surfaces of epithelial cells as judged from its kinetic characteristics (53). Both isoforms of SVCT have a high affinity for ascorbate, but with respect to SVCT2, SVCT1 has a lower affinity. However, tissues expressing SVCT1 may have a higher maximum velocity of Asc transport (17; 53; 54). K_m for L-ascorbate ranges from 90 to 252 μM in different studies (17; 90). SVCT1 has been shown to be specific for L-ascorbate in uptake and voltage clamping studies by competing L-ascorbate uptake with other compounds, such as D-isoascorbate, DHA, biotin or thiamine (89; 90). ^{14}C-L ascorbate uptake or currents associated with ascorbate transport were not blocked when these compounds were included in the uptake solution, indicating a specificity for L-ascorbate (89; 90).

As the uptake of ascorbate is sodium dependant, with a Hill coefficient of 2, replacing Na^+ with other anions abolishes the ascorbate accumulation (89; 90). Furthermore, the uptake of ascorbate is inhibited by compounds such as sulfinpyrazone and 4,4'-diisothiocyanatostilbene 2,2'-disulfonate (7). Although the regulation of hSVCT1 has not yet been well studied, there is evidence that protein kinase C may be crucial for this process. Incubating oocytes expressing hSVCT1 with the protein kinase C stimulator (phorbol 12-myristate 13-acetate) results in a 50 % decrease in Asc uptake (17). In addition, phorbol 12-myristate 13-acetate in hSVCT1 transiently transfected COS-1 cells also decreases the Asc

transport (54). However, Western blot and confocal microscopy analyses revealed that the treatment led to a redistribution of the transporter from the cell surface to intracellular membranes. This suggests that protein kinase C may be regulating the trafficking of hSVCT1 to the plasma membrane (54).

1.4.1.2 *SVCT2*

Rat brain SVCT2 cDNA encodes a 592 amino-acid protein that shares 65% amino-acid homology with rat SVCT1 (86). Similar to hSVCT1, hSVCT2 was determined to be identical to a previously identified nucleobase-like transporter (YSPL2), that had no previously known function (39; 53). The hSVCT2 cDNA (4238 bp) has a single open reading frame of 1950 bp. There is a non-coding region of 393 bp on the 5' end and 1892 bp on the 3' end (74). The resulting protein is composed of 650 amino acids with a relative molecular mass of 70 337 Da (74). The amino acid sequence of hSVCT2 is 95% identical to the rat homolog, but only 65% identical to the hSVCT1 sequence (22; 74). SLC23A1, the gene coding for hSVCT2, has been mapped to the short arm of chormosome 20 at position 20qp12.2-12.3 (83). It has been predicted, based on hydropathy analysis, that SVCT2 has 12 transmembrane domains with both the N and C termini facing the intracellular side (74). There are two possible sites for N-glycosylation between transmembrane domains 3 and 4, and five potential sites for protein kinase C-dependent phosphorylation in the putative intracellular domains (74). Unlike hSVCT1, there are no protein kinase A sites found, which may indicate different methods of regulation of these two isoforms (53). Similar to hSVCT1, glycosylation of hSVCT2 has been demonstrated in transfected COS-1 cells, although

possibly to a lesser extent (54).

The tissue distribution of hSVCT2 is more widespread than hSVCT1. In Northern blot analysis, mRNA for hSVCT2 has been detected in most tissues tested, including heart, brain, placenta, liver, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes, except skeletal muscle and lung (74; 90). Similar kinetic and substrate specificity studies performed using hSVCT1 have also been examined with hSVCT2. Like hSVCT1, hSVCT2 is specific for L-ascorbate and is Na^+ dependent, requiring two Na^+ molecules for every one Asc molecule transported (41). The two transporters do differ in their kinetics, with hSVCT2 having a higher affinity for Asc, but possibly a lower rate of uptake (17; 54). For example, when expressed in *Xenopus* oocytes, hSVCT1 was calculated to have a K_m of 252 μM and a V_{max} of 15.8 pmol/min/oocyte while hSVCT2 had a K_m of 21.3 μM and a V_{max} of 0.2 pmol/min/5 oocytes (17). However, it is possible that the differences in velocity are due to different levels of protein expression and not a greater rate of transport. In the studies by Daruwala *et al.* (1999) and Liang *et al.* (2002) it was found that incubating the cells with phorbol 12-myristate 13-acetate decreased the Asc uptake. However, different from hSVCT1 expressing cells, there did not appear to be a redistribution of hSVCT2 in the COS-1 cells following confocal microscopy analysis (54). Therefore, protein kinase C does seem to play a role in redistribution of the transporter to intracellular membranes, but is able to regulate hSVCT2 by decreasing the transport efficiency.

1.4.2 *Dehydroascorbic acid transport*

DHA is similar in structure to glucose, therefore it is not surprising that DHA uptake is mediated by glucose transporters (40). Na^+-dependent glucose transporters (SGLT1 and SGLT2) are found in the intestine and kidney and are responsible for efficient uptake of dietary glucose and minimal urinary loss (11). Since glucose is metabolised quickly within cells, there is always a concentration gradient across the plasma membrane and active transport is not necessary. Therefore, throughout the rest of the body, glucose is taken up by cells through facilitative glucose transporters (11). Currently, there are 7 proposed isoforms of facilitative glucose transporters (GLUT 1-7), but the function of GLUT6 and GLUT7 has not yet been determined (11). The oxidized form of Asc, DHA, is transported by a Na^+ independent pathway, which rules out the active transporters. DHA enters cells through facilitative glucose transporters (GLUT1, GLUT3, and GLUT4) (91). Once inside the cell, DHA is rapidly reduced back into Asc by glutathione or NADPH dependent enzymes thus decreasing the intracellular concentration of DHA (25; 60). Therefore, there is typically a concentration gradient favouring the transport of DHA into the cell from the plasma or interstitial fluid (91). Although a concentration gradient exists, glucose can inhibit the transport of DHA by competing for binding sites to the transporter (40; 91). However, during oxidative stress it is thought that this pathway of Asc accumulation in cells would play a minimal role due to the decreased availability of reduced glutathione or NADPH. Therefore, the conversion of DHA to Asc would be slower and the concentration gradient for DHA would not be as great.

1.5 Vitamin C and cardiovascular disease

Cardiovascular diseases, including arteriosclerosis, are a major cause of strokes, myocardial infarction, and heart failure (72). Such pathological situations can develop if a balance between ROS and antioxidants is disturbed, with ROS exceeding the endogenous antioxidant capabilities of a cell (87; 94). ROS damage that can lead to cardiovascular disease may include lipid peroxidation, endothelial dysfunction, cell proliferation and apoptosis (6; 70; 94). Many studies have been performed to determine if Asc, as an antioxidant, plays a role in preventing cardiovascular damage (21; 43; 49; 69; 70; 75; 77; 81). In general, Asc has been shown to be a much more effective antioxidant than α-tocopherol in preventing lipid peroxidation induced by aqueous peroxyl radicals because it appears to reduce ROS before they are able to diffuse and oxidize the plasma lipids (25). Vitamin C, in concentrations similar to those found in human plasma, is able to decrease the amount of homocysteine-induced oxidation of low density lipoproteins isolated from fresh human plasma (2). Moreover, in human vascular smooth muscle cells, Asc (100 μM) was able to protect against apoptosis induced by oxidized low density lipoproteins (81). However, it is disputed that excess Asc supplementation is not always useful in cardiovascular diseases (49; 85).

Asc is also known to enhance the bioavailability of nitric oxide, which is synthesized and released by endothelium to cause smooth muscle relaxation (95). Therefore, any disturbance in this signalling molecule would greatly affect vascular tone. Asc enhances the bioavailability of nitric oxide by either stabilizing tetrahydrobiopterin, a cofactor in nitric

oxide synthesis in endothelial cells, or scavenging superoxide, which may in turn react with nitric oxide to produce peroxynitrite (38; 95).

During the formation of atherosclerotic plaques, vascular smooth muscle cells undergo dedifferentiation from a contractile phenotype to a synthetic phenotype (3). During this change, the amount of extracellular matrix that is secreted increases and the smooth muscle cells undergo increased proliferation (43). Incubating vascular smooth muscle cells with Asc (0.5 to 2 mM) decreases both ^{3}H-thymidine incorporation as well as cell number (43). In rat vascular smooth muscle cells, Asc stimulated the production of smooth muscle-specific myosin heavy chain-1 and calponin 1, which are characteristic proteins of differentiated smooth muscle cells (3). Similarly, in *in vivo* studies where Asc was administered orally to balloon injured rats, smooth muscle-specific myosin heavy chain-1 and calponin expression was also greater than control rats (3). A human *in vivo* study also suggests that vitamin C, possibly in conjunction with vitamin E or provitamin A carotenoids, may decrease carotid artery wall thickness, although more extensive, long-term studies are needed (49). These results strongly suggest that Asc may slow the initiation or development of cardiovascular diseases.

1.6 Objectives of this study

The first objective of this thesis is to determine the presence of SVCT isoform(s) in endothelial and smooth muscle cells from the pig coronary artery. Epithelial cells in kidney and intestine express SVCT1 and most other cell types express SVCT2. Therefore it is of

interest to determine whether endothelial cells that line the artery would express SVCT1 or SVCT2. One would expect the smooth muscle cells to express SVCT2.

In vitro and *in vivo* animal studies strongly suggest that Asc is a valuable antioxidant against oxidative stress and that it may help smooth muscle cells maintain a healthy state (3; 4; 38; 81). However, in epidemiological studies the data are a little more controversial. Some studies show that there is an inverse correlation with Asc plasma levels and cardiovascular disease risk (27; 69), while other studies do not (75; 85). It is possible that a more local increase in Asc may be beneficial to the cells at risk of injury during ischemia/reperfusion or angioplasty rather than an overall increase in plasma Asc levels. Therefore, the second and third aims of my study are to demonstrate that Asc loaded pig coronary artery smooth muscle cells do protect the cell against oxidative stress and to determine if increased Asc levels in pig coronary artery smooth muscle cells caused by the overexpression of SVCT2 are even more beneficial. If successful, our lab has future plans for this project for *in vivo* animal studies where SVCT2 would be introduced locally to cells through the use of adneovirus during balloon angioplasty.

2.0 MATERIALS AND METHODS

2.1 *Cell Isolation and Cultures*

Pig hearts were obtained from Maple Leaf Foods (Burlington, Ontario) and the right descending coronary artery was dissected out to isolate smooth muscle and endothelial cells. Smooth muscle cells (PCSMC) were isolated by collagenase/elastase digestion, as previously described (31; 78). After the second passage, a large number of cells were aliquoted and stored in liquid nitrogen until further use. Typically, cells were thawed and used after passage 4 for experiments as well as RNA isolation. Endothelial cells (PCEC) were cultured as previously described (31). Briefly, cells were dislodged from the inside of the artery using sterile cotton swabs and plated in 6 well plates. The cells were allowed to grow to confluence and were frozen after the third passage. Cells were typically used after passage 5 for RNA isolation.

Characteristics of the PCEC and PCSMC used here have described earlier (20; 79). In Western blots, the lysates from PCEC react positively to anti-von Willebrand factor and anti-endothelial NO synthase, but negatively to anti-smooth muscle α-actin. Conversely, the lysates from PCSMC react positively to anti-smooth muscle α-actin, but not to anti-von Willebrand factor or anti-endothelial NO synthase. In addition, immunocytochemical staining of PCSMC showed intense and uniform expression of smooth muscle specific α-actin. No cells were observed that did not show this reaction.

Human embryonic kidney cells (HEK293T) cells were a gift from Mike Zhu, Ohio

State University, Columbus Ohio.

All cells were cultured in Dulbecco's modified Eagle's medium (Gibco 12800-017) supplemented with: 2 mM HEPES (4-(2-hydroxyethyl-1-poperazine ethane sulfonate)) pH 7.4, glutamine (2 mM), gentamicin (50 mg/l), amphotericin B (0.125 mg/l), and 10% fetal calf serum (FCS) (CanSera, Rexdale, Ontario). Cell isolation, culturing and transfections were carried out by Sue Samson.

2.2 *RNA Isolation*

Total cellular RNA from fresh tissue or cultured cells was obtained using the TRIzol RNA extraction reagent (Invitrogen, Burlington, Ontario). The amount of RNA isolated was estimated by measuring the absorbance at 260 nm using a Beckman DU 640 Spectrophotometer (Beckman Coulter Mississauga, Ontario).

PolyA$^+$ RNA was isolated using the Oligotex direct mRNA Midi/Mini Isolation kit (Qiagen, Mississauga, Ontario). Typically, 5-6 confluent plates of smooth muscle or endothelial cells or 2 plates of HEK293T cells were used to obtain 50 µl of polyA$^+$ RNA.

2.3 *Reverse Transcription (RT)*

Total cellular RNA (approximately 5 µg RNA) or polyA$^+$ RNA (5-7 µl) was DNase treated with 1 unit DNase (MBI Fermentas, Burlington, Ontario) for 15 min at room temperature. DNase was inactivated with the addition of 2.5 mM (ethylenedinitrilo)-tetraacetic acid ethylene glycol (EDTA), and incubation at 65 °C for 10 min. Subsequently,

cDNA was synthesized using the Thermoscript RT-PCR systems kit (Invitrogen, Burlington, Ontario). PolyA$^+$ RNA was then primed with 5 μM oligo(dT) for 10 min at 65 °C. The reverse transcription reaction was carried out at 60 °C for 1 hour and contained: 15 units ThermoScript RT, 1X cDNA synthesis buffer, 80 units RNaseOUT, 1 mM dNTP, and 5 mM 1-4-dithiothreitol (DTT). Following the 1 hour incubation, the mixture was heated to 85 °C for 5 min to inactivate the enzymes. RNaseH (2 units) was added to the synthesized cDNA for 20 min at 37 °C. The cDNA was aliquoted and kept at -20 °C until further use.

2.4 *Determination of SVCT Isoforms in PCEC and PCSMC*

Pig kidney cDNA was used along with six primers, three upstream and three downstream, based on the human SVCT1 sequence (accession # AF17091), for PCR amplification. A 579 bp PCR product was obtained using primers numbered 444 upstream and 446 downstream

hSVCT1up 444 5'-GTTAGTCAGCTCATCGGCACCATCT-3'

hSVCT1dn 446 5'-TCAGGACATAGCAGAGCAGCCACAC-3'

corresponding to the sequence at 266 and 845 of the hSVCT1 sequence of the cDNA, respectively. The primers were designed using the software Primer Designer. The primers were then screened for a lack of identity with other sequences in the Genbank. The PCR reaction contained 2 μl of the pig kidney cDNA, 1 X PCR buffer (Applied Biosystems, California) , 200 μM each of dATP, dCTP, dGTP, dTTP, 5 mM $MgCl_2$, 0.5 units of Amplitaq (Applied Biosystems, California) and 1 μM of each primer in a total volume of 20

µl. The reaction was carried out at a denaturing temperature of 94 °C for 5 min, annealing temperature of 64 °C for 1 min and an extension temperature of 72 °C for 7 min for a total of 30 cycles. Once the conditions were set, a large scale PCR was performed to obtain enough PCR product to be sent for sequencing. The conditions were identical to the above, however the total reaction volume was 1 ml. The product was precipitated overnight (10% of a 3M sodium acetate solution pH 5.5 and 2.5 times the volume of 95% ethanol), redissolved and run on a 3% agarose gel with a 100 bp DNA ladder (MBI Fermentas, Burlington, Ontario). The gel was stained with ethidium bromide, and the 579 bp band was cut and purified using the QIAquick Gel Extraction kit (Qiagen, Mississauga, Ontario). It was sent for sequencing at the MOBIX facility at McMaster University. More specific primers matching 100% of the pig SVCT1 fragment obtained were designed for further RT-PCR with endothelial and smooth muscle cDNA.

pSVCT1up 5'-AACTGGAGCCTGCCTCTGAACA-3'

pSVCT1down 5'-CTCACCAGCAGCTTGGAAGACA-3'

Primers based on the pig SVCT2 (accession #AF058320) sequence were designed.

pSVCT2up 5'-TCCGGTGGTGACCAATGGAG-3' (102-122 bp of pig SVCT2 cDNA)

pSVCT2down 5'-GATTGTGCCGCTGAAGCAGG-3' (331-351 pig SVCT2 cDNA)

The PCR products from these two sets of primers would result in 200 and 249 bp fragments for pSVCT1 and pSVCT2, respectively. A 672 bp G3PDH product obtained from the following primers was used as a positive control. An equivalent amount of DNase treated RNA was also amplified under the same conditions for pSVCT1 and pSVCT2 as a negative

control.

G3PDHup 5'-CACGGTCAAGGCTGAGAAC-3' (241-259 upstream)

G3PDHdown 5'-CGACCTGGTCCTCGGTGTA-3' (894-913 downstream)

The PCR reaction mixture for pig SVCT1 and G3PDH contained the following in a total volume of 20 µl: 1 µM of each primer, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 1 unit of Amplitaq (Applied Biosystems, California), 1X PCR buffer and 2 µl of cDNA. PCR reaction mixture for pig SVCT2 was identical except for the $MgCl_2$ concentration, which was 1.25 mM. PCR conditions were: denaturation at 94°C for 60 sec; annealing at 61°C (G3PDH and SVCT2), or 63°C (pig SVCT1 primers) for 60 sec and extension at 72°C for 60 sec for 35 cycles. Gel loading dye (6X) was added to PCR products and they were then analyzed by gel electrophoresis using 7.5% polyacrylamide gels and a 100 bp DNA ladder (MBI Fermentas, Burlington, Ontario). Gels were stained with ethidium bromide and visualized under UV light using Kodak 1D Image Analysis Software.

The following negative controls were carried out for all the RT-PCR experiments: (a) use RNA as a template without RT, (b) use water as a template. An additional control that was not done would have been for any contamination in the reagents used for the RT reaction. Positive controls included DNase treated RNA as a template, kidney RNA and G3PDH which should be expressed in all samples.

2.5 *Cell Viability Assays*

2.5.1 *Glucose 6-Phosphate Dehydrogenase Release*

The release of glucose 6-phosphate dehydrogenase (G6PDH), a cytosolic enzyme,

was measured using the Vybrant™ Cytotoxicity Assay Kit (V-23111) from Invitrogen (Burlington, Ontario). PCSMC were plated on 96 well plates. The outside rows and columns were left blank to be used as control buffer blanks. On the day of the experiment, the growth media was removed and the cells were washed 3X with 200 µl Na^+-HEPES buffer (290-300 mOsm), which contained in mM: 134 NaCl, 5.4 KCl, 10 glucose, 0.8 $MgSO_4$, 20 HEPES and 1.8 $CaCl_2$. 50 µl of Na^+-HEPES buffer, containing different concentrations of H_2O_2 or 1% TritonX100 or buffer alone was added to each well. The plate was incubated for 30 min at 37 °C. After 30 min, 50 µl of the resazurin reaction mixture (prepared exactly as described in kit) was added and fluorescence readings (excitation 530 nm, emission 580 nm, gain set at 60) on a CytoFluor (Applied Biosystems, California) were taken immediately, then every 5 min for 30 min. When the plate was not being read, it was wrapped in foil to protect the dye from light.

2.5.2 *Lactate Dehydrogenase Assay*

Lactate dehydrogenase (LDH) leakage from PCSMC was measured using the Cytotoxicity Detection Kit from Roche (Laval, Quebec). Cells were plated in a 24 well plate and used within 5-7 days after plating. The growth media was removed and the cells were washed 2 times with 500 µl Na^+-HEPES buffer, and 300 µl of Na^+-HEPES buffer with or without H_2O_2 or 1% TritonX100 was added to each well. Additional solutions were kept in microcentrifuge tubes to be used as blanks. After a 30 min incubation at 37 °C, 10 µl of 1000 U/ml catalase (1 unit will decompose 1 µmole of H_2O_2 /min at pH 7.0 at 25 °C Sigma, Mississauga, Ontario) was added to each well. Another 10 µl of catalase was added after 2

min to ensure all H_2O_2 was broken down. The plate was put on ice and 200 µl of the supernatant was transferred to microcentrifuge tubes and spun at 250 g for 10 min. Following centrifugation, the tubes were placed on ice and 100 µl of the supernatant was transferred to a 96 well plate, including the buffer controls. 100 µl of the reaction mixture (containing the dye and a catalyst), prepared according to the kit instructions, was added to each well. The increase in the amount of formed formazan was measured at 492 nm, every 5 min for a total of 30 min.

2.5.3 *Intracellular oxidant stress measured by oxidation of dichlorodihydrofluorescein*

To measure the reactive oxygen levels in PCSMC, cells plated in 35 mm plates were loaded with 0 or 200 µM Asc overnight. The following morning, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Invitrogen, Burlington, Ontario) was added to the plates to a final concentration of 10 µM. The loading was carried out for 40 min at 37 °C in the incubator (48). The extracellular dye was washed out by rinsing cells 3 times with Na^+-HEPES buffer (with or without Asc). 2 ml of Na^+-HEPES buffer (with or without Asc) was added to the plate. It was then incubated for a another 20 min at 37 °C to allow for deesterification of the dye. The cells were then rinsed 2 times with Asc free Na^+-HEPES buffer (to remove extracellular Asc in the loaded cells) and 2 ml of Na^+-HEPES buffer was added. They were then visualized using an Axiovert LSM 510 confocal microscope with excitation set at 488 nm and emission at 525 nm. Once the background was adjusted, H_2O_2 was added to the plate to a final concentration of 1 mM. Pictures were taken immediately and then every 5 min for 30 min. The change in

fluorescence was determined by counting the number of fluorescent cells at time 0 (just before H_2O_2 addition) and at each time point after H_2O_2 was added. The counting was carried out in a blind fashion.

2.5.4 *Mitochondrial Reductase Activity*

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Mississauga, Ontario) assay is based on the ability of mitochondrial reductase to reduce MTT into a formazan pigment (28; 66). PCSMC were plated in 96 well plates and used when confluent, 5-7 days later. Growth media was removed and cells were washed 2 times with 250 µl Na^+-HEPES buffer. 100 µl of Na^+-HEPES with or without H_2O_2 or 1% TritonX100 was added to the wells. The plate was incubated at 37 °C for 30 min. After incubation, 10 µl of 1000 U/ml catalase was added to all wells. About 1-2 min later, another 10 µl of catalase was added. MTT (10 µl), prepared as 5 mg/ml in Na^+-HEPES buffer, was added to all wells and the plate covered with foil and placed in the incubator at 37 °C for 2 or 4 hrs, as specified in the results. After the incubation with MTT, the crystals formed in the cells were dissolved by adding 100 µl of 10% SDS and 0.01 N HCl. The crystals were left to dissolve overnight, rocking gently and covered in foil. The following morning, the plate was read on an ELISA reader at a wavelength of 540 nm. In some experiments, alternating columns of PCSMC were loaded with 200 µM Asc the night before the experiment for about 18 hrs. In these experiments, the loading was carried out by another person so the MTT assay was a blind study.

2.6 *Transfections*

2.6.1 *Transient Transfection of HEK293T cells*

HEK293T cells were split ½ and plated on 10 cm plates. 24 hrs later, they were transfected using Lipofectamine 2000 (L2000)(Invitrogen, Burlington, Ontario). For five-10 cm plates, 270 µl of L2000 was added to 15 ml of DMEM (serum and antibiotic free) and left for 5 min. In another tube, 90 µg of DNA was added to the same serum and antibiotic free DMEM. The diluted DNA was added to the L2000 mixture and left for 20 min. 3 ml of the DNA/L2000 mixture was added to each plate. The transfected cells were used 48 hrs following transfection.

2.6.2 *Stable Transfection of PCSMC*

Primary pig coronary artery smooth muscle cells were isolated as described above. Cells were passaged two times and plated on 10 cm plates in antibiotic free DMEM with 10% FCS. Transfection was carried out using the transfection reagent FuGENE 6 (Roche, Laval, Quebec) in a 3:2 ratio with DNA (pcDNA3.1SVCT2 circular or linear). The transfection was carried out according to the manufacturer's directions using 27 µl FuGENE6 and 18 µg DNA per plate. Transfected cells were selected by the addition of 150 µg/ml geneticin (Invitrogen, Burlington, Ontario) 48 hrs following the transfection. The medium was changed every 3-4 days, at which time new geneticin was added. Three weeks later, the geneticin concentration was reduced to 100 µg/ml. The cells were grown to confluence, passaged again and allowed to grow to confluence. They were then frozen in aliquots and stored under nitrogen until further use. The cells were thawed and used in

passage 5 for uptake experiments and RNA isolation.

For PCSMC transfection with the linear pcDNA3.1SVCT2 plasmid, the plasmid was cut using ScaI (Amersham, Piscataway N.J.). Refer to Appendix III for a map showing the cut site. The restriction digest contained 60 μg pcDNA3.1SVCT2, 5 μl of 10X buffer H (supplied with ScaI, Amersham), 300 units of ScaI, in a total volume of 50 μl. The reaction was incubated at 37 °C for 1.5 hrs. ScaI was inactivated by heating to 85 °C for 30 min. The DNA was then precipitated (Na^+-acetate, ethanol precipitation) overnight. The transfection was carried out as described above.

2.7 *^{14}C-Ascorbate Uptake*

2.7.1 *^{14}C-Ascorbate Uptake in PCSMC*

Asc uptake was carried out using ^{14}C-ascorbate as previously described (41). Transfected PCSMC cells were plated on 60 mm plates and grown to confluence (typically 2 weeks). ^{14}C-Ascorbate uptake was performed in Na^+-HEPES buffer (same composition as described above). Tissue culture dishes (60 mm) were placed in a shaking (30 rpm) water bath at 37 °C. Dishes were washed twice with 37 °C Na^+-HEPES buffer. ^{14}C-Ascorbate uptake solution (2 ml) that contained 10 μM ^{14}C-ascorbate and 1 mM DTT (to maintain ascorbate in its reduced form) in Na^+-HEPES buffer, was added to the plate and incubated for 12 min. After 12 min, the plate was washed 6 times with an ice-cold sucrose-Tris (hydroxymethyl) aminomethane-HCl solution (320 mM sucrose, 10 mM Tris-HCl, pH 7.3). Cells were then scraped from the plate in 1 ml of water. 100 μl was saved for protein

estimation and the remaining 900 µl was placed in a vial, and 5 ml of Ready Safe cocktail (Beckman Coulter, Mississauga Ontario) was added, for scintillation counting using a Beckman LS6800 scintillation counter. Incubation times with ^{14}C-Asc varied as outlined in the Results.

2.7.2 *^{14}C-Ascorbate Uptake in HEK293T Cells*

Two 10 cm plates of transfected HEK293T cell were rinsed and harvested in 2 ml of Na-HEPES buffer. The uptake solution contained 1 mM DTT and ^{14}C-Asc so that there was a final concentration of 10 µM when added to the cells. The incubation began when 100 µl of cells was added to 100 µl of the uptake solution in a test tube. The uptake was carried out at 37 °C for the times indicated in the Results section. The reaction was stopped by adding 5 ml of Sucrose Tris (same as above) and pouring onto a glass fibre filter (gf/c Whatman, Clifton, N.J.) on a Beckman Coulter manifold. The filter was washed two more times with an additional 5 ml each time. The filters were removed and placed into a scintillation vial. Ready safe scintillation cocktail was added (5 ml) and the samples were counted for ^{14}C. 100 µl of each sample of cells was saved for protein estimation.

2.8 *Construction of pcDNA3.1SVCT2 coding region*

2.8.1 *PCR of the SVCT2 coding region*

The coding region of SVCT2 was excised from pSPORTSVCT2 (Appendix IV), which we received as a gift from Puttur D. Prasad, Medical College of Georgia, Augusta,

Georgia (74). The initial strategy was to obtain a PCR fragment with restriction sites (EcoRI and HindIII at the 5' and 3' end, respectively) at each end of the fragment (restriction sites in bold).

EcoRISVCT2up 5'-**GAATTCGAATTC**CAAAGGCTATCCCGTGGC-3'

HindIIISVCT2dn 5'-**AAGCTTAAGCTT**GTTTCTCTTAATGATGGGTA-3'

The PCR reaction mixture contained the following components: 200 μM each of dATP, dCTP, dGTP and dTTP, 10 ng pSPORTSVCT2, 1 μM of each primer, 1 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Burlington, Ontario), 1X Platinum Taq High Fidelity Buffer, and an additional 4 mM $MgSO_4$. The PCR conditions were: denaturation at 94 °C for 2 min, annealing at 52 °C for 30 sec, extension 68 °C for 2 min for 5 cycles, followed by denaturation 94 °C for 30 sec, annealing 72 °C for 30 sec, extension 68 °C for 2 min for an additional 10 cycles. A small amount of the PCR product (1 μl) was run on a 1% agarose gel with a 1 kb ladder (MBI Fermentas, Burlington, Ontario) to confirm the size and to quantify the product. The remaining product was purified using a MiniElute PCR Purification kit (Qiagen, Mississauga, Ontario) and eluted in 30 μl sterile dH_2O.

2.8.2 *Restriction endonuclease digestion*

The PCR product was digested with HindIII in a reaction containing 20 μl of the PCR product, 1X one-phor-all buffer (OPA buffer) (Amersham, Piscatawa, N.J.) and 20 units HindIII to a total volume of 50 μl. The reaction was carried out at 37 °C for 1.5 hrs. The product was further digested with EcoRI (20 units) in 2X OPA for 1.5 hrs at 37 °C. The enzymes were inactivated by heating to 85 °C for 30 min.

The pcDNA3.1+ vector was digested in a similar manner, however following the HindIII digestion, the plasmid was run on a 1% agarose gel, stained with ethidium bromide, cut with a clean blade and purified using QIAquick Gel Extraction kit (Qiagen, Mississauga, Ontario) to remove any uncut plasmid. It was then digested with EcoRI in 2X OPA.

2.8.3 *Dephosphorylation of vector DNA*

Following digestion, the pcDNA3.1+ plasmid was dephosphorylated using Calf Intestine Alkaline Phosphatase (MBI Fermentas, Burlington, Ontario), by adding 1 unit directly to the digestion mixture. The reaction was incubated at 37 °C for 1 hr. The enzyme was inactivated by heating at 85 °C for 15 min.

2.8.4 *Ligation reaction*

Ligation of the PCR insert (SVCT2 coding region) and the vector (pcDNA3.1+) was done with T4 DNA ligase (MBI Fermentas, Burlington, Ontario) as instructed in the manufacturer's directions. Briefly, the reaction mixture contained 2 μl of 10X ligation buffer, 5 units of T4 DNA ligase, different ratios of vector:insert (1:0, 1:1, 1:2, 1:3), T4 DNA ligase to a total volume of 20 μl, which was incubated at 17 °C overnight. T4 DNA ligase was inactivated by heating the ligation reaction to 65 °C for 10 min.

2.8.5 *Bacterial transformations*

XL2 Blue Ultracompetent Cells (Stratagene, Cedar Creek, TX) were used for transformation of pcDNA3.1SVCT2 coding region. The cells were thawed on ice and β-mercaptoethanol was added to a final concentration of 25 mM. The cells were gently transferred to 15 ml polypropylene tubes (50 μl/tube). Then, 5-10 μl of the ligation product

was added to the cells and was left on ice for 1 hr. The cells were heat-shocked for 30 sec at 42 °C and placed back on ice for 2 min. 200 µl SOC medium (2% trypticase peptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM $MgSO_4$, 20 mM glucose) was added to each tube and incubated at 37 °C for 1 hr in a shaker at 250 rpm. The cells were then diluted with SOC and spread with a sterile spreader on LB agar (85.6 mM NaCl, 0.5 % yeast extract, 1 % trypticase peptone, 1.5 % agar) plates containing 100 µg/ml ampicillin. The plates were incubated at 37 °C overnight. Competent cells were also transformed with pcDNA3.1 that had not undergone restriction digest as a positive control. Cells were also transformed with pcDNA3.1 that was cut with both EcoRI and HindIII and self ligated as a negative control.

2.8.6 *Isolation of Plasmid DNA*

Clones were picked from the LB agar plates using toothpicks and placed in 3 ml LB (85.6 mM NaCl, 0.5% yeast extract, 1% trypticase peptone) containing 100 µg/ml ampicillin. The clones were allowed to grow overnight at 37 °C in a shaker at 250 rpm. 12-18 hrs later, plasmid DNA was isolated using an QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario). Briefly, cells were harvested by centrifugation, resuspended, then lysed in NaOH-SDS buffer and neutralized with acidic potassium acetate buffer. The resulting mixture was passed through a column provided in the kit and the plasmid DNA bound to the column allowing the genomic DNA, cell debris and proteins to pass through. The plasmid DNA was washed and eluted from the filter. The resulting DNA was sent to MOBIX (McMaster University, Hamilton, Ontario) for sequencing using a T7 primer (supplied by MOBIX) to determine if the insert was present. Alternatively, the plasmid DNA was digested with

EcoRI (similar to the above digestion) and run on a 1% agarose gel against the vector alone and a 1 kb DNA ladder to determine if the insert was present.

2.9 *Construction of pCRSVCT2 coding region using TA cloning (Invitrogen)*

2.9.1 *PCR of SVCT2 coding region*

The coding region of SVCT2 was amplified from pSPORTSVCT2 using the PCR primers:

SVCT2TAup 5'-CAAAGGCTATCCCGTGGC-3'

SVCT2TAdn 5'-GTTTCTCTTAATGATGGGTA-3'

The upstream primer starts 10 bases upstream from the start site and continues for 7 bases after the start. The downstream primer corresponds to 9 bases upstream from the stop site to 6 bases downstream. The PCR reaction mixtures contained the following: 200 μM each of dATP, dCTP, dGTP and dTTP, 10 ng pSPORTSVCT2, 1μM of each primer, 1 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Burlington, Ontario), 1X Platinum Taq DNA Polymerase High Fidelity Buffer, and an additional 3 mM $MgSO_4$. The PCR conditions were: denaturation at 94 °C for 4 min, annealing at 55 °C for 30 sec, extension 68 °C for 2 min for 20 cycles. A small amount of the PCR product was run on a 1% agarose gel with a 1 kb DNA ladder (MBI Fermentas, Burlington, Ontario) to verify the product was the expected 1969 bp, and to quantify the amount of product obtained.

2.9.2 *Ligation reaction for pCR3.1*

The SVCT2 coding region obtained by PCR was ligated into the pCR3.1 vector supplied in the Eukaryotic TA Expression Bidirectional Kit (Invitrogen, Burlington, Ontario). The ligation reaction followed the suggested 1:0 (negative control) 1:1 and 1:2 vector to insert molar ratios. Therefore, 0, 23 and 46 ng of the PCR product was ligated with 60 ng of vector. In addition to the vector and the insert, the ligation reaction contained: 1 µl of 10X ligation buffer, 4 units T4 DNA ligase and sterile water to a final volume of 10 µl. The ligation was carried out at 15 °C overnight.

2.9.3 *Bacterial Transformation*

The transformation was carried out following the procedure supplied with the Eukaryotic TA Expression Kit. Briefly, One Shot TOP10F' (Invitrogen, Burlington, Ontario) competent cells were thawed on ice and transferred to a 15 ml polypropylene tubes. 2 µl of each ligation reaction, as well as vector that had not been ligated, was added to one 50 µl aliquot of competent cells. The tubes were incubated on ice for 30 min, followed by a 30 sec heat shock at 42 °C. 250 µl of SOC medium was added to each tube and they were then placed in a 37 °C shaking (250 rpm) incubator for 1 hr. The cells were then diluted in SOC medium and spread on LB agar plates containing 25 µg/ml kanamycin (Sigma, Oakville, Ontario). The plates were put into a 37 °C incubator overnight. 20 clones were picked and placed into separate tubes containing 3 ml of LB with 25 µg/ml kanamycin and incubated overnight at 37 °C and shaking at 250 rpm. The following day, the plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario) as described

above. The clones were cut with HindIII to determine if the insert was present in the clones picked. The restriction digestion reaction contained 2 µl of plasmid DNA, 1X OPA buffer, and 20 units HindIII. The reaction was carried out at 37 °C for 1.5 hrs, followed by heat inactivation for 30 min at 85 °C. 10 µl of the product was loaded onto a 1% agarose gel with a 1 kb DNA ladder. To ensure the insert was in the proper orientation, clones were cut with 20 units XhoI (New England Biolabs, Beverly, MA) and 2X OPA buffer (Amersham, Piscataway, NJ) producing two fragments of 1471 and 5558 bp. If the insert was in the reverse direction, the fragments expected would be 580 and 6449 bp. The 4 clones that contained the insert in the proper orientation were sent to MOBIX (McMaster University, Hamilton, Ontario) for sequencing (for primer sequences used in sequencing, see Appendix VII).

2.9.4 *Isolation of Plasmid DNA for Cell Transfections*

One clone obtained from the TA cloning (pcr3.1SVCT2) was grown in 1000 ml of LB containing 25 µg/ml kanamycin overnight at 37 °C in a shaker at 250 rpm. Plasmid DNA was extracted as described earlier, however an EndoFree Plasmid Purification Kit (Qiagen, Mississauga, Ontario) was used. The eluted DNA was precipitated with isopropanol and washed with 75 % ethanol. The resulting pellet was resuspended in EndoFree TE (Tris-EDTA) buffer, aliquoted and reprecipitated by Na^+-acetate/ethanol precipitation. A small aliquot was saved to estimate the DNA concentration by agarose gel electrophoresis. The DNA was stored at -20 °C as a pellet until further use.

2.10 *RT-PCR with Transfected PCSMC*

Overexpression of SVCT2 was detected by Co-RT-PCR using polyA mRNA from transfected PCSMC. The primers that were used to detect SVCT2 and G3PDH previously (section 2.4) were also used to detect overexpresion of SVCT2 in stably transfected PCSMC. The reaction mixture contained the following in a total volume of 20 µl: 1 µM of each (SVCT2, G3PDH, or SVCT2 and G3PDH) primer, 1.25 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 1 unit of Amplitaq (Applied Biosystems, California), 1X PCR buffer and 2 µl of cDNA. The PCR conditions were: denaturation at 94°C for 60 sec; annealing at 61°C for 60 sec and extension at 72°C for 60 sec for 25-35 cycles. Gel loading dye (6X) was added to PCR products and they were then analyzed by gel electrophoresis using 7.5% polyacrylamide gels and a 100 bp DNA ladder (MBI Fermentas, Burlington, Ontario). Gels were stained with ethidium bromide and visualized under UV light using Kodak 1D Image Analysis Software.

2.11 *Protein Estimation*

Protein estimation was carried out using the Bradford Reagent (BioRad, Mississauga, Ontario). Bovine serum albumin standards were used to construct a standard curve. Protein concentration was interpolated from this standard curve.

2.12 *Data Analysis*

Values are given as mean ± SEM of (n) replicates. Where applicable, student's t-test

was used and values of $p < 0.05$ were considered statistically significant. FigP software (Biosoft Corporation, Ancaster, Ontario) was used to construct graphs. Maps of the plasmids used throughout this thesis were constructed using Clone Manager 5 Version 5.03 (Science and Educational Software).

3.0 RESULTS

3.1 *AIM I: identification of SVCT isoform(s) in pig coronary artery smooth muscle and endothelial cells*

SVCT1 is mainly confined to the epithelial tissue of the intestine, kidney and liver, whereas SVCT2 is widely expressed in almost all tissues studied, except for the lung and skeletal muscle (86). The isoform expressed in coronary artery smooth muscle and endothelium has not yet been identified. Therefore, we set out to determine which SVCT isoform(s) exist in these two tissues.

3.1.1 *Identification of the pig SVCT1 sequence*

Since the sequence for pig SVCT1 was not known, the first step of this aim was to design primers based on the hSVCT1 sequence to obtain a PCR fragment for pig SVCT1. SVCT1 is known to be abundantly expressed in kidney tissue, therefore RNA was isolated from pig kidney. We designed three upstream primers and three downstream primers based on the hSVCT1 sequence (accession # AF17091). The upstream primers correspond to 250-274 (primer 443), 266-290 (primer 444) and 256-280 (primer 445) of the hSVCT1 sequence and the downstream primers are complementary to the hSVCT1 sequence from 821-845 (primer 446), 836-860 (primer 447) and 835-861 (primer 448). After optimization of PCR parameters, a PCR product from kidney cDNA, with the expected size of 579 bp, was obtained with 444 upstream and 446 downstream primers in 5 mM $MgCl_2$ and an annealing temperature of 64 °C (Figure 4).

The amplified cDNA for pig SVCT1 was sent to MOBIX (McMaster University, Hamilton, Ontario) for sequencing using the same primers that were used for PCR (primers 444, 446). Alignment between the PCR product and the hSVCT1 sequences (16) revealed that the pig SVCT1cDNA fragment was 92 % identical to hSVCT1 cDNA (Appendix I). When the pig cDNA sequence was translated, it was 97 % identical to the corresponding hSVCT1 protein (Appendix II). The pig SVCT1 sequence was deposited into Genbank as accession number AY353718.

3.1.2 *SVCT expression in cultured endothelial and smooth muscle cells*

Cultured endothelial and smooth muscle cells from the pig left descending coronary artery were used for RNA isolation after passage 5 and 4, respectively and cDNA was subsequently synthesized. Pig SVCT1 primers were designed to match 100 % of the pig SVCT1 sequence, which would give a product of 200 bp and pig SVCT2 primers were designed based on the previously known sequence (accession # AF058320), to give a product of 250 bp. After optimization of PCR, gels such as shown in Figure 5 were obtained. Figure 5 shows that PCEC and PCSMC mainly express mRNA for SVCT2 (lanes 9 and 18, respectively), which differs from kidney as it expresses both SVCT1 and SVCT2 (lanes 2 and 3, respectively). The intron and exon structure of SVCT1 and SVCT2 is not known for pig, therefore primers flanking introns could not be designed. Hence we conducted DNase treatment with no reverse transcription as a negative controls to avoid interference in PCR by genomic DNA. G3PDH (679 bp) was used as a positive control. In two PARS, a faint

band for SVCT1 was seen using PCEC cDNA, however it was not consistently present and was very faint compared with SVCT2 (not shown).

3.1.3 *SVCT expression in fresh tissue*

Fresh endothelial and smooth muscle tissue was isolated from the similar artery that PCEC and PCSMC were cultured from. Figure 6 shows that SVCT2 is present in the smooth muscle (lane 10), however there was no band detected for SVCT1 (lane 9). This is consistent with the results from the cultured cells. On three separate tissue isolations, no band for SVCT1 or SVCT2 was seen for the endothelial tissue following 35 cycles of amplification (Figure 6, lanes 14-16). A faint band for G3PDH was detected following 25 plus an additional 25 cycles of PCR, however there was still no band visible for either SVCT1 or SVCT2 in PCEC (not shown).

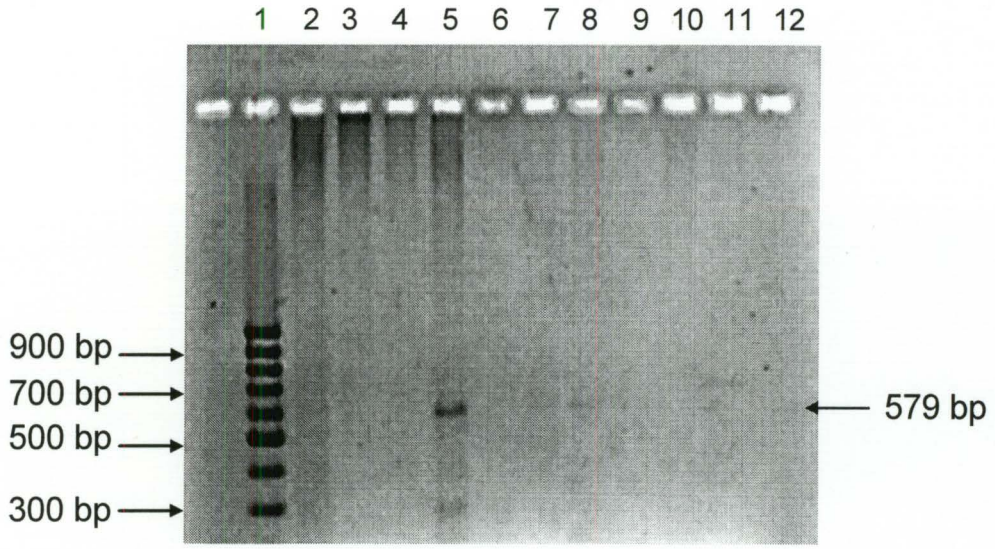

Figure 4: PCR fragment of SVCT1 in pig kidney. Three different upstream primers and three different downstream hSVCT1 primers were used in different combinations to obtain a pig SVCT1 fragment. For primer locations on hSVCT1, see RESULTS. Lane 5 (primers 444,446) contained PCR product of the expected size of 579 bp. The band was purified and sequenced (Appendix I and II). Lane 1, 100 bp marker. Lane 2 primers 443,446; lane 3 primers 443,447; lane 4 primers 443,480; lane 5 primers 444,446; lane 6 primers 444,447, lane 7 primers 444,448; lane 8 primers 445,446; lane 9 primers 445,447; lane 10 primers 445,448.

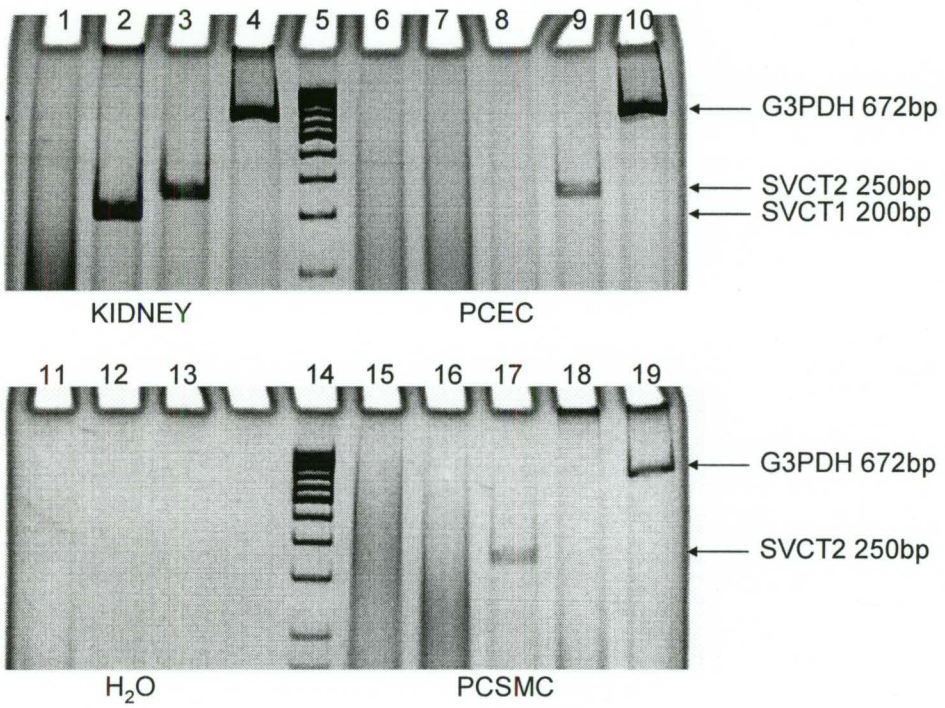

Figure 5: SVCT expression in endothelial (PCEC) and smooth muscle cells (PCSMC) cultured from the pig coronary artery following RT-PCR. SVCT2 was detected in PCEC and PCSMC (lanes 9 and 17, respectively). SVCT1 was not detected in either PCEC (lane 8) or PCSMC (lane 18). Pig kidney (lanes 2 and 3) was used as a positive control as it possesses both SVCT1 and SVCT2. G3PDH was used as a positive control (lanes 4,10,20). DNase treated RNA was used as a negative control (SVCT1 primers lanes 1,6,15 SVCT2 primers lanes 7,16). H_2O was also used as a negative control (SVCT1, SVCT2, G3PDH primers for lanes 11-13, respectively). Lanes 5 and 14 contain a 100 bp marker.

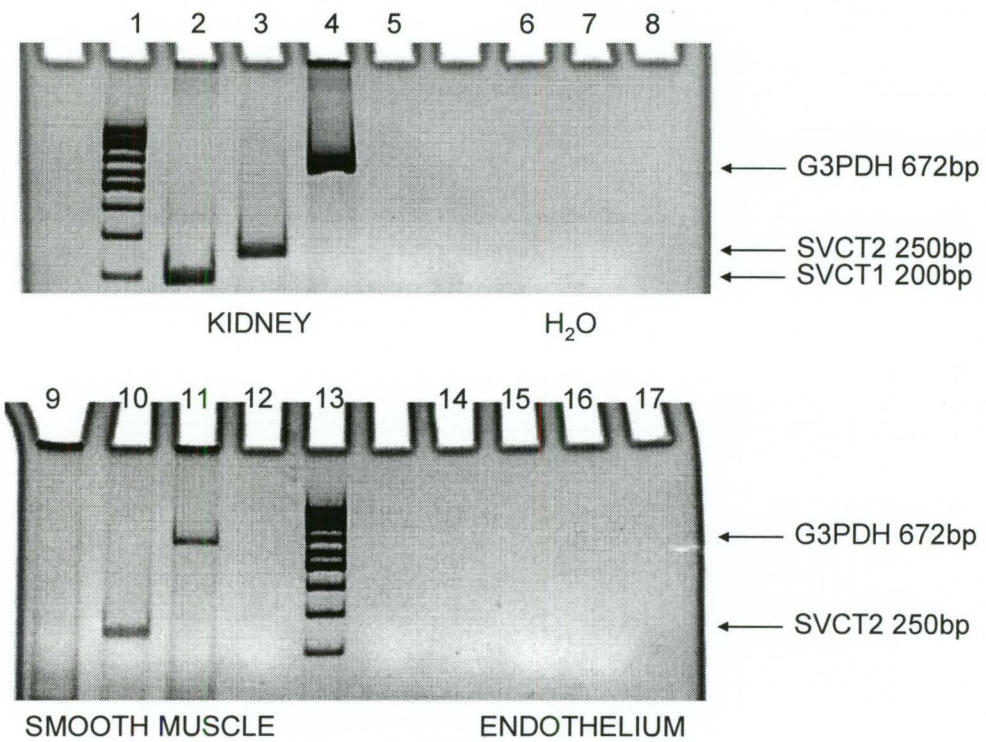

Figure 6: SVCT expression in fresh endothelial and smooth muscle tissue from the pig coronary artery. SVCT2 was detected in smooth muscle (lane 10), but not SVCT1 (lane 9). RT-PCR with endothelium did not show SVCT1, SVCT2 or G3PDH (lanes 14-16, respectively). Kidney tissue was used as a positive control and showed both SVCT1 and SVCT2 (lanes 2 and 3). G3PDH was also used as a positive control (lanes 4, 11 and 16). G3PDH primers were included with DNase treated RNA as a negative control (lanes 5, 12, and 17). Lanes 6-8 contain PCR with H_2O as the template for a negative control with primers for SVCT1, SVCT2, and G3PDH (lanes 6-8, respectively). Lanes 1 and 13 contain a 100 bp ladder.

3.2 *AIM II: effect of Asc loading on ROS exposure of PCSMC*

ROS are known to oxidize proteins, lipids and DNA which will affect the function of cells and may lead to diseases such as atherosclerosis (21; 87). However, Asc is an effective antioxidant and may prevent oxidation of cellular components, thereby decreasing the risk of developing diseases associated with ROS. The hypothesis that Asc loaded PCSMC would decrease the damage caused by hydrogen peroxide was tested.

3.2.1 Measurement of cell viability

3.2.1.1 *Glucose-6-phosphate dehydrogenase release*

Membrane permeability is a common measure of cell damage. Therefore, the first attempt to measure cell viability was glucose-6-phosphate dehydrogenase (G6PDH) release using the Vybrant™ Cytotoxicity Assay Kit (Invitrogen, Burlington, Ontario). The assay detects G6PDH, a cytosolic enzyme, through a 2-step enzymatic process that leads to the reduction of resazurin into red-fluorescent resorufin. The amount of resorufin produced is proportional to the amount of G6PDH released from the cells or the number of damaged cells (Vybant™ Cytotoxicity Kit User Manual). In this assay, the hydrogen peroxide treatment continued after the addition of the kit reagents (resazurin, diaphorase, glucose 6-phosphate and $NADP^+$). As time progressed, the amount of fluorescence detected should have increased with increasing concentrations of hydrogen peroxide. However, the amount of fluorescence decreased with time, even in the absence of cells (data not shown). The assay uses the G6PDH that is released from the cells to convert glucose 6-phosphate to 6-

phosphogluconate, which in turn generates NADPH from $NADP^+$. The presence of hydrogen peroxide possibly oxidized the NADPH before it was able to reduce resazurin into resorufin, which would explain why there was a decrease in fluorescence with increasing concentrations of hydrogen peroxide. The possibility of adding catalase before the assay should have been considered.

3.2.1.2 *Lactate Dehydrogenase Release*

Lactate dehydrogenase (LDH) leakage from PCSMC was measured using the Cytotoxicity Detection Kit from Roche (Laval, Quebec). In this method, the amount of LDH lost from the cells is measured by a 2 step electron transfer causing a reduction in a yellow tetrazolium salt INT (2-[4-idophenyl]-3-4-nitrophenyl]-5-phenyltetrazolium chloride) to a red formazan salt. The increase in the red formazan salt is proportional to the amount of LDH released into the supernatant. The amount of LDH released from the cells was calculated as % cytotoxicity by the following equation:

% cytotoxicity = 100*((sample - control)/(triton treated cells - control))

Since all of the enzyme would be released in the presence of the detergent TritonX100, this activity was taken as 100%. Following 30 min treatment with 30 to 1000 μM hydrogen peroxide, only a maximum of 36 % cytotoxicity was observed. Thus this assay was not sensitive enough and the number of replicates one is able to do per experiment would not be sufficient once the cells were loaded with Asc.

3.2.1.3 *Oxidation of dichlorodihydrofluorescein*

Dichlorodihydrofluorescein is an intracellular dye that measures the amount of oxidative stress to which a cell is exposed by fluorescing when oxidized. PCSMC preloaded with 0 or 200 μM Asc were loaded with DCHF and then exposed to hydrogen peroxide. The increase in the number of green cells from time 0 to 10 min following treatment with hydrogen peroxide was determined. Figure 8 shows representative pictures of control (A) and Asc loaded (B) before and after © and D, respectively) after 10 min of exposure to 1 mM hydrogen peroxide. The difference between the increase in the number of green cells between the two groups was not significantly different (Figure 8, E).

3.2.1.4 *Mitochondrial Reductase Activity*

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Mississauga, Ontario) assay is based on the ability of mitochondrial reductase to reduce MTT into a formazan pigment (28; 66). The catalytic reactions required to form the formazan pigment are coupled to NADPH- and NADH-dependent redox systems. The reaction converts a yellow MTT solution to purple/blue formazan crystals. The greater the amount of crystals formed correlates to greater mitochondrial activity. Treatment with hydrogen peroxide should damage the mitochondria, therefore decrease ability of the mitochondria to produce formazan crystals. After the hydrogen peroxide incubation, catalase was added to the wells to ensure that any remaining hydrogen peroxide was broken down, so it would not interfere with the NADPH coupled reactions.

A four hour incubation with MTT was determined to be optimum to give the greatest difference from the control (Figure 9). The data are represented as a percent of control (0 μM hydrogen peroxide). There was very little difference of hydrogen peroxide treatment with concentrations below 30 μM compared to the control (data not shown). Asc preloaded cells showed an increase in MTT activity compared with control cells after treatment with hydrogen peroxide (Figure 10). The increase in MTT activity at both 300 μM and 1000 μM hydrogen peroxide for the Asc loaded cells were significantly different ($p < 0.05$). However, when this experiment was repeated, Asc provided no protection from hydrogen peroxide, i.e. the MTT activity for Asc preloaded cells was less than for the control cells.

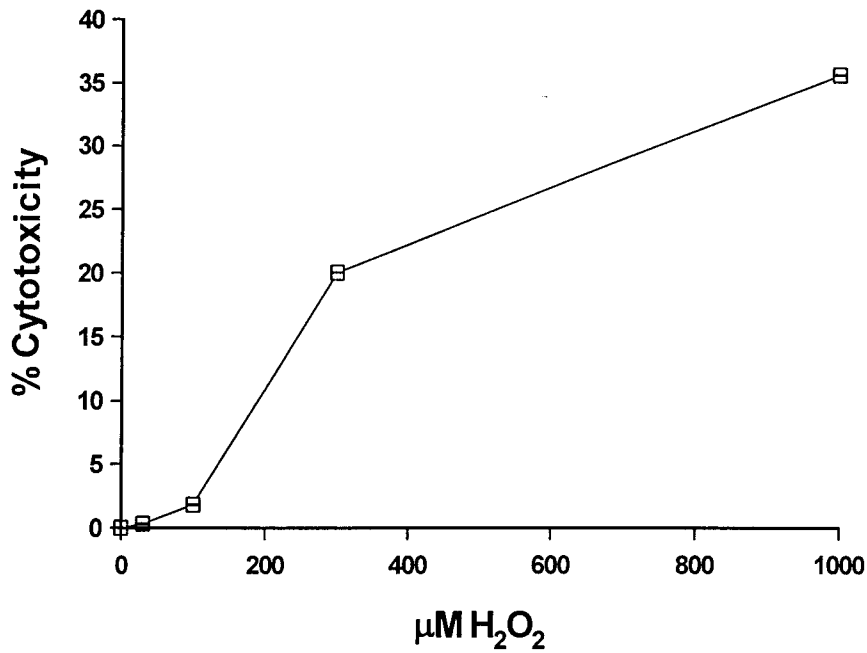

Figure 7: LDH leak assay for the effect of hydrogen peroxide on PCSMC. Following 30 min treatment with H_2O_2 (30, 100, 300, 1000 μM), the amount of LDH released from the cells was measured. % cytotoxicity calculated by 100%*(sample - control)/(triton treated cells - control).

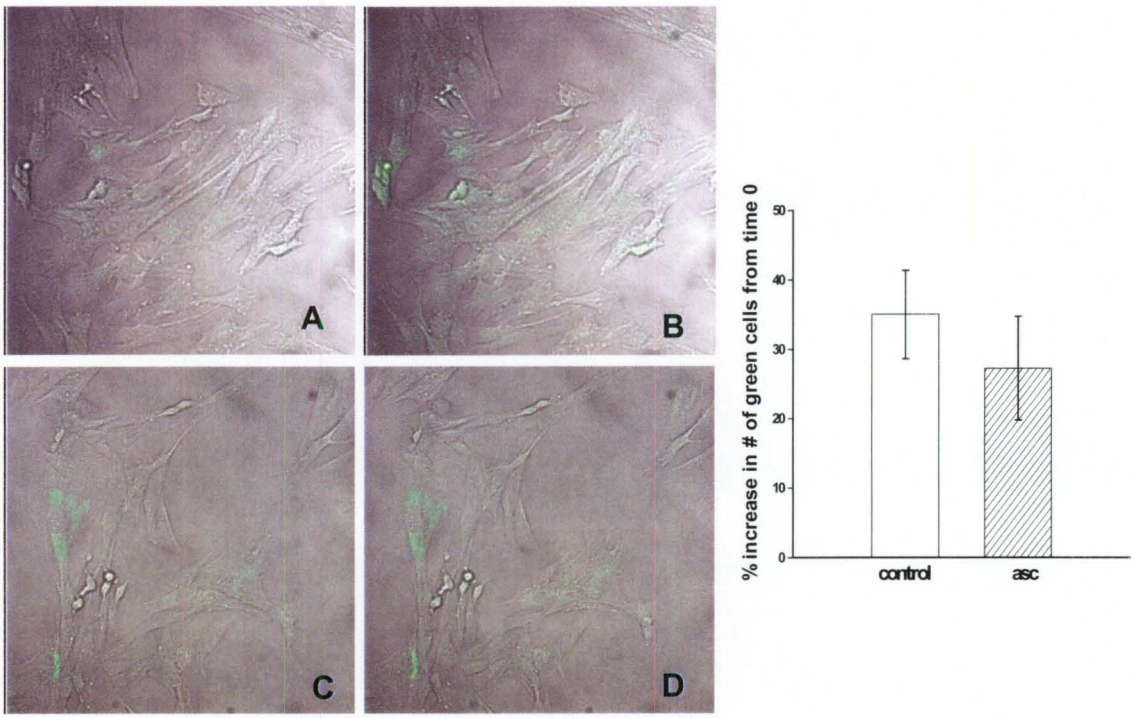

Figure 8: Oxidation of dichlorodihydrofluorescein to determine oxidant stress following treatment with hydrogen peroxide. Panels A-D are representative overlay images of control and Asc loaded PCSMC. A. PSCMC control cells at time 0. B. PCSMC control cells 10 min after the addition of 1 mM hydrogen peroxide. C. Asc loaded PCSMC at time 0. D. Asc loaded cells after 10 min with 1 mM hydrogen peroxide. E. Summary of experiments showing the increase in the number of green cells from time 0. The values are mean ± SEM from 8 control and 6 Asc plates. Control = change from A to B, Asc = change from C to D. The difference between control and asc loaded cells is not significant ($p>0.05$).

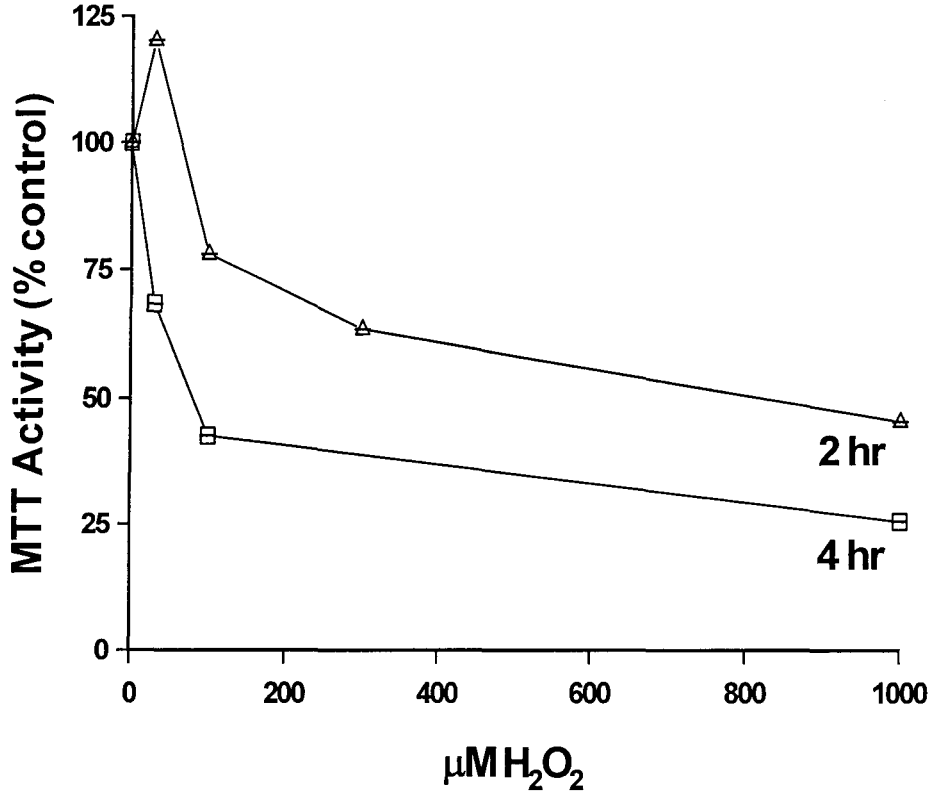

Figure 9: MTT activity assay for the effect of hydrogen peroxide on PCSMC. After 30 min treatment with H_2O_2 (30, 100, 300, 1000 μM), cells were incubated for either 2 hrs or 4 hrs with MTT. The 4 hr incubation gave the greatest difference from control and was chosen for further experiments. % control = 100* (treatment - triton/control - triton).

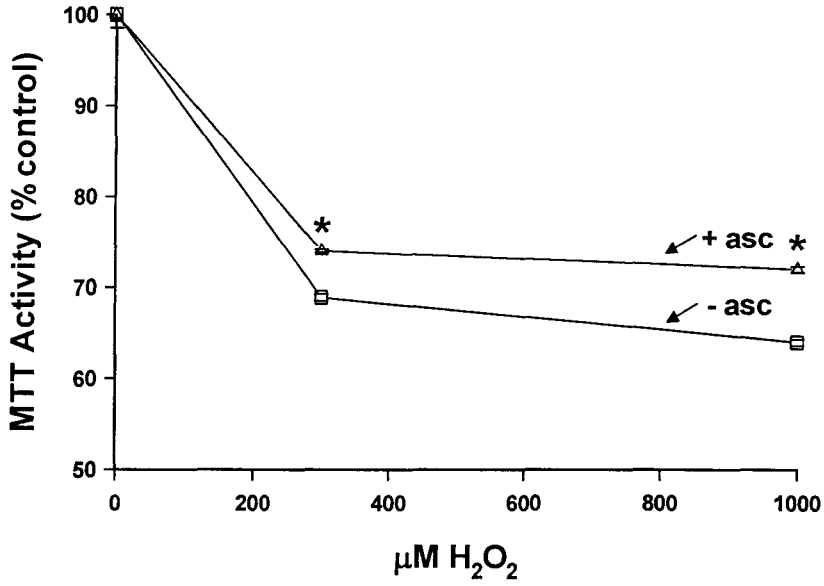

Figure 10: MTT activity of Asc loaded and Asc free cells following hydrogen peroxide treatment. Asc loaded cells (200 μM overnight loading) had significantly greater MTT activity after 30 min treatment with hydrogen peroxide at both 300 and 1000 μM ($p<0.05$). Data shown are mean ± SEM of 11 replicates. Although the results are significantly different, repeating the experiment did not give consistent results.

3.3 *AIM III: Overexpression of SVCT2 in PCSMC*

The current literature that looked at the effect of Asc on ROS damage has been in cells with endogenous SVCT2 expression and uptake (15; 18; 19; 62). Therefore, the third aim was to determine if overexpression of SVCT2 in PCSMC, thereby possibly increasing the Asc in the cells, would increase the protection of Asc against ROS damage.

3.3.1 *Overexpression of pcDNA3.1SVCT2 in HEK293T cells*

Since HEK293T cells do not have any endogenous Na^+-dependent Asc uptake, they were used to determine if the pcDNA3.1SVCT2 plasmid (Appendix III), that was previously constructed in our lab from pSPORTSVCT2 (Appendix IV), would increase the Asc accumulation. The sulfinpyrazone sensitive ^{14}C-Asc uptake of transiently transfected HEK293T cells, after 2 min, was over 5 times greater than the mock transfected cells and after 12 min, 12 times greater than mock transfected cells (Figure 11). Sulfinpyrazone, an anion channel blocker, is known to block Asc uptake (41). Therefore, uptake was performed in the presence and absence of 1 mM sulfinpyrazone to determine the amount of non-specific ^{14}C uptake. The ^{14}C-Asc uptake values represented in all of the graphs was calculated by subtracting the non-specific ^{14}C accumulation, when sulfinpyrazone was present, from the ^{14}C uptake when sulfinpyrazone was absent (i.e. sulfinpyrazone sensitive uptake).

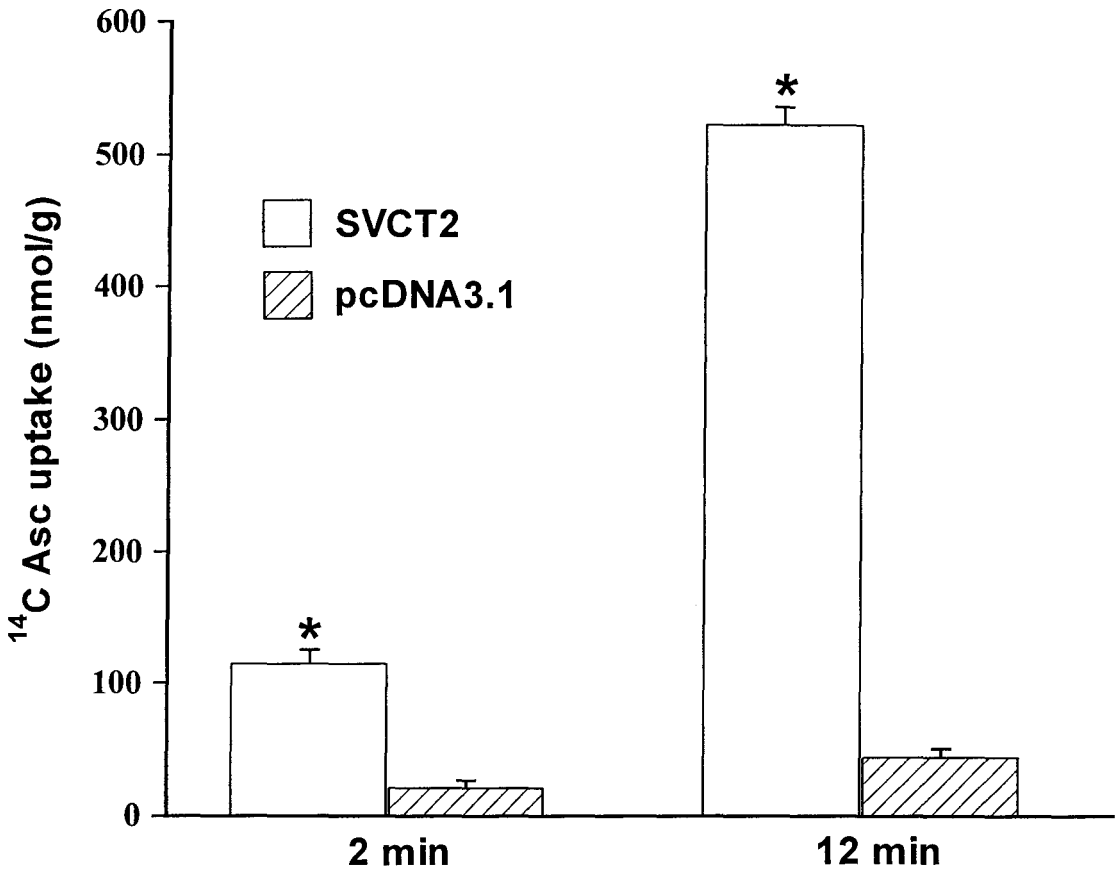

Figure 11: Sulfinpyrazone sensitive ^{14}C-Asc uptake in transiently transfected HEK293T cells. HEK293T cells were transfected with pcDNA3.1SVCT2 or mock transfected with pcDNA3.1. The data shows sulfinpyrazone sensitive uptake, calculated from the values of ^{14}C uptake without sulfinpyrazone - ^{14}C uptake with 1 mM sulfinpyrazone. Values are mean ± SEM of 6 replicates. ^{14}C-Asc uptake at both 2 and 12 min was significantly greater in the SVCT2 transfected cells compared to the mock transfected cells ($p < 0.05$).

3.3.2 *Overexpression of pcDNA3.1SVCT2 in PCSMC*

PCSMC have been successfully transfected with green fluorescent protein using FuGENE6 in our lab. Therefore, FuGENE6 was chosen as the transfection reagent to introduce pcDNA3.1SVCT2 into PCSMC. Geneticin was used to select the cells with expression of the plasmid since the pcDNA3.1 vector has antibiotic resistance for geneticin. Figure 12 shows that PCSMC transfected with pcDNA3.1SVCT2 do not exhibit more sulfinpyrazone sensitive ^{14}C-Asc accumulation than the mock (pcDNA3.1+) transfected cells after 12 min ($p > 0.05$). In a separate experiment, the pcDNA3.1SVCT2 transfected cells had almost 3 times more Na^+-dependent ^{14}C-Asc uptake than the mock transfected cells after 15 min (data not shown). However, this result is not consistent with mRNA expression shown in Figure 14, where the mRNA expression of SVCT2 in pcDNA3.1SVCT2 (circular plasmid) was not greater than mock or untransfected PCSMC.

3.3.3 *Transfection of PCSMC with linear pcDNA3.1SVCT2*

The initial transfection of PCSMC with pcDNA3.1SVCT2 was not successful in increasing the ^{14}C-Asc accumulation, possibly because the plasmid was circular when introduced into the cells. When the plasmid is circular, it is able to incorporate into the genomic DNA at any position. It is possible that a population of the PCSMC transfected with pcDNA3.1SVCT2 that were selected had incorporated the plasmid in the middle coding region of the SVCT2 gene. Therefore, they were still able to express the resistance to geneticin, but not necessarily the SVCT2 gene. To overcome this possibility, pcDNA3.1SVCT2 was cut with the restriction enzyme ScaI to make the plasmid linear in a

non-essential region (Appendix III). The sulfinpyrazone sensitive ^{14}C-Asc uptake showed a statistically significant increase in Asc accumulation over the mock transfected cells at 2, 12 and 60 min (Figure 13) ($p < 0.05$).

To confirm that the increase in ^{14}C-Asc uptake is a result of overexpression of SVCT, Co-RT-PCR was utilized. PolyA$^+$ mRNA was isolated from PCSMC transfected with either pcDNA3.1SVCT2, linear pcDNA3.1SVCT2, pcDNA3.1 alone, or untransfected cells. SVCT2 primers which will amplify both the human and pig SVCT2 sequence were used along with G3PDH as a standard. Figure 14 shows that the mRNA expression of the pcDNA3.1SVCT2 (circular) is similar to the mock and untransfected PCSMC (Figure 14 lanes 7, 10 and 13, respectively). However, the PCSMC transfected with the linear pcDNA3.1SVCT2 show a much higher level of SVCT2 mRNA expression after 30 cycles of amplification. In another PCR, the SVCT2 band did not appear for the circular pcDNA3.1SVCT2, pcDNA3.1 or untransfected cells after 25 cycles, but a strong band for SVCT2 was present with the linear pcDNA3.1SVCT2 transfected cells. In addition, PCR was performed using primers that specifically recognize the hSVCT2 sequence (primers 478, 487 refer to Appendix VII for sequences). A slightly darker band was seen in the cDNA from PCSMC that were transfected with the linear pcDNA3.1SVCT2 plasmid compared to the cDNA from the PCSMC that were transfected with the circular pcDNA3.1SVCT2 plasmid (data not shown). RNA was also PCR amplified for, without the RT step, using SVCT2 primers to ensure that the results obtained were not due to genomic DNA contamination. Following 35 amplification cycles, no bands were detected.

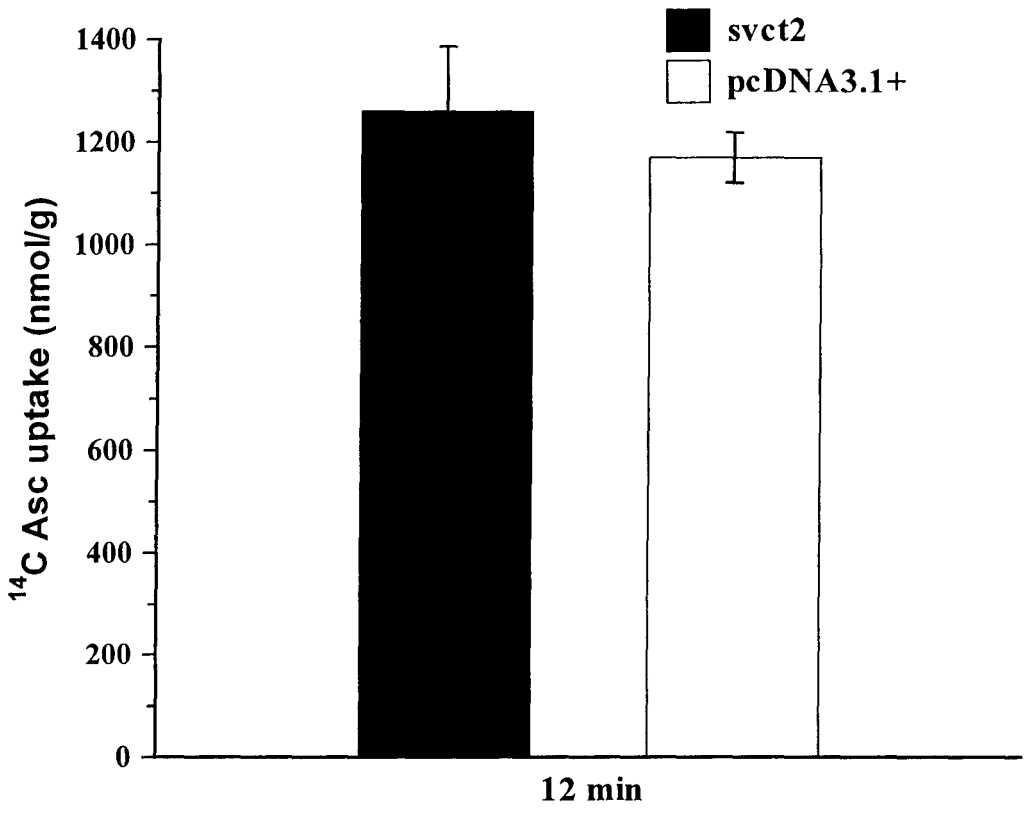

Figure 12: Sulfinpyrazone sensitive ^{14}C Asc in stably transfected PCSMC with circular SVCT2 or pcDNA3.1 (mock transfection) vectors. The data shown represent 12 min ^{14}C-Asc uptake values with 1 mM sulfinpyrazone subtracted from the ^{14}C-Asc uptake without sulfinpyrazone to obtain sulfinpyrazone sensitive uptake. The SVCT2 transfected cells did not differ significantly from the mock transfected cells ($p>0.05$). Data are mean ± SEM of 6 (SVCT2) and 4 (pcDNA3.1) replicates.

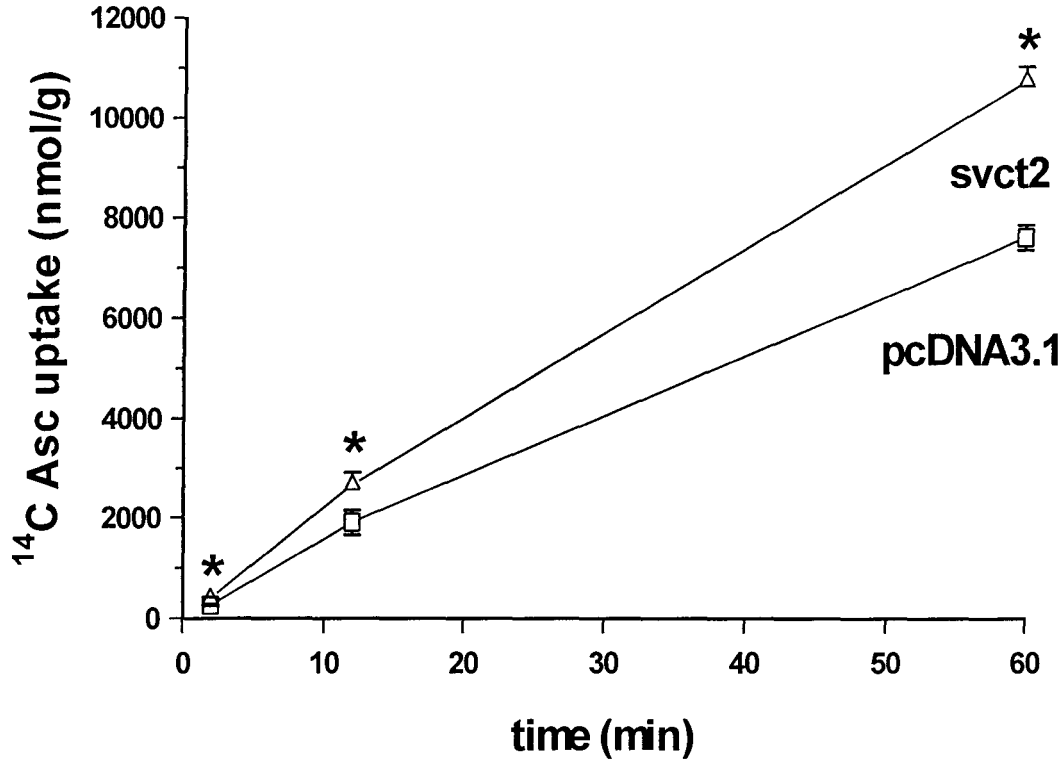

Figure 13: Sulfinpyrazone sensitive ^{14}C-Asc uptake in stably transfected PCSMC with linear SVCT2 or pcDNA3.1 (mock) plasmids. SVCT2 and pcDNA3.1 plasmids were linearized by digesting them with the restriction enzyme ScaI and were used to transfect PCSMC. ^{14}C-Asc uptake is represented as sulfinpyrazone sensitive (^{14}C uptake in the presence of 1 mM sulfinpyrazone was subtracted from the sulfinpyrazone free uptake). ^{14}C-Asc uptake values for SVCT2 transfected PCSMC are significantly different at 2, 12 and 60 min ($p<0.05$). Data are mean ± SEM of 8 (2 min) or 6 (12 and 60 min) replicates.

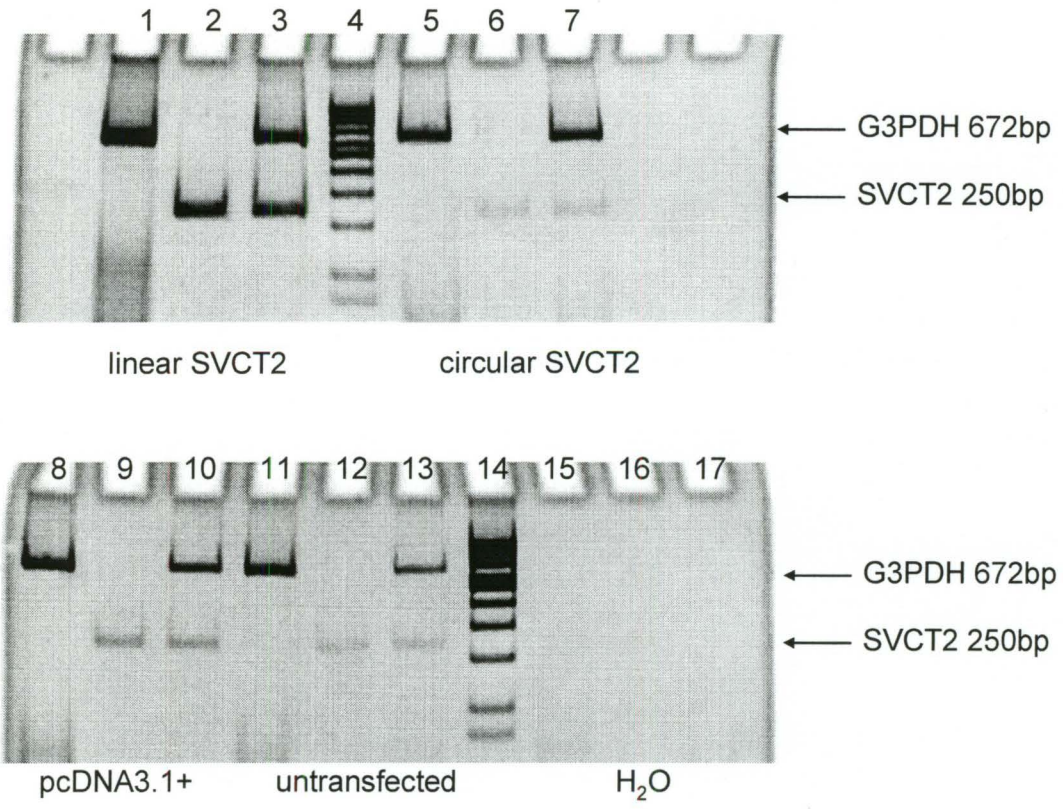

Figure 14: RT-Co-PCR from polyA mRNA of stably transfected PCSMC. Following 30 cycles of PCR, PCSMC transfected with linear SVCT2 showed greater mRNA expression for SVCT2 than with circular, mock or untransfected cells (lanes 2,6,9,12, respectively). Lanes 1,5,8 and 11 contain G3PDH product. Lanes 3,7,10 and 13 represent co-PCR with G3PDH and SVCT2. Lanes 15-17 are negative controls (G3PDH, SVCT2 and G3PDH+SVCT2 primers, respectively with no template). Lanes 4 and 14 contain a 100 bp marker. To verify that the results were not due to genomic DNA contamination, RNA was used as a template with SVCT2 primers. No bands were seen after 35 amplification cycles (not shown).

3.3.4 *Creation and expression of a new SVCT2 plasmid*

Although the mRNA expression in PCSMC transfected with the linear pcDNA3.1SVCT2 was increased compared to control cells, the increase in ^{14}C-Asc uptake was only marginal. There is a possibility that the length of the SVCT2 gene is hindering the transcription or translation into a functional protein, since SVCT2 has a long untranslated 3'-end. Also, the translation signals and polyadenylation sites in the new plasmid may be stronger than in SVCT2. Therefore, we decided to design a new plasmid that contained only the coding region of SVCT2. Our initial strategy was to isolate the coding region of SVCT2 with primers that contained restriction sites on either end (Appendix V). Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Burlington, Ontario) was used in the PCR reactions due to its high fidelity and processivity. The resulting PCR product was cut with the corresponding restriction enzymes (EcoRI and HindIII) and ligated into pcDNA3.1+, which had also been digested with the same restriction enzymes. Unfortunately, the clones that were selected did not contain the insert. This was the case after numerous attempts, so we decided to use the TA Bidirectional Cloning Kit (Invitrogen, Burlington, Ontario).

The TA cloning kit takes advantage of Taq polymerase activity, which adds a single deoxyadenosine (A) to the 3' end of duplex molecules. High fidelity enzymes such as Vent do not possess this activity and therefore cannot be used with this kit. Platinum Taq DNA Polymerase is a combination of enzymes including recombinant Taq DNA polymerase, *Pyrococcus species* GB-D polymerase and PlatinumTaq antibody. The GB-D polymerase possesses a proofreading ability which enhances Taq polymerase fidelity while maintaining

the nontemplate dependent activity of Taq polymerase so that the PCR product will contain the single deoxyadenosine on the 3' end. The linearized vector that is supplied with the kit (pCR3.1) has single 3' deoxythymidine (T) residues. Therefore, efficient ligation is achieved directly from the PCR product and no restriction digests are necessary. The same primers as above, without the restriction sites, were used to obtain a 1969 bp product, which was ligated into pCR3.1 (Appendix VI). The pCR3.1 vector has antibiotic resistance to both ampicillin and kanamycin. Since the ligation was performed immediately following PCR, and no purification of the PCR product was carried out, kanamycin was used as the selection antibiotic in the LB agar plates. pSPORTVCT2 contains the ampicillin resistance gene, but not kanamycin, therefore, including kanamycin in the LB agar plates ensures that clones picked would not be pSPORTSVCT2. Once the clones were selected, they were digested with HindIII to determine if the insert was present. The clones that contained the insert were digested with XhoI, which cut the plasmid in two places (once inside the SVCT2 coding region and once outside) producing two fragments of 1471 and 5558 bp, if the SVCT2 coding region was in the correct orientation. Figure 15 shows that 4 of the 17 clones that contained the insert had it in the proper orientation. 8 upstream and 5 downstream overlapping primers were designed from the hSVCT1 cDNA sequence. These 13 primers were used to sequence the 4 clones that had the SVCT2 insert in the correct orientation. The results obtained from sequencing were aligned with the SVCT2 cDNA and only clone "P" did not contain any mutations (see Appendix VII for the primer sequences and the alignment of "P"). The new plasmid is referred to as pCRSVCT2.

pCRSVCT2, pcDNASVCT2 and pCR (control) were transiently expressed in HEK293T cells to determine if the new plasmid increased the ^{14}C-Asc uptake compared with the old plasmid (pcDNA3.1SVCT2). Figure 16 demonstrates that the sulfinpyrazone sensitive ^{14}C-Asc uptake is significantly greater with the pCRSVCT2 and pcDNASVCT2 compared to the mock (pCR) transfected cells, however there is no increase in uptake between the new and old plasmids.

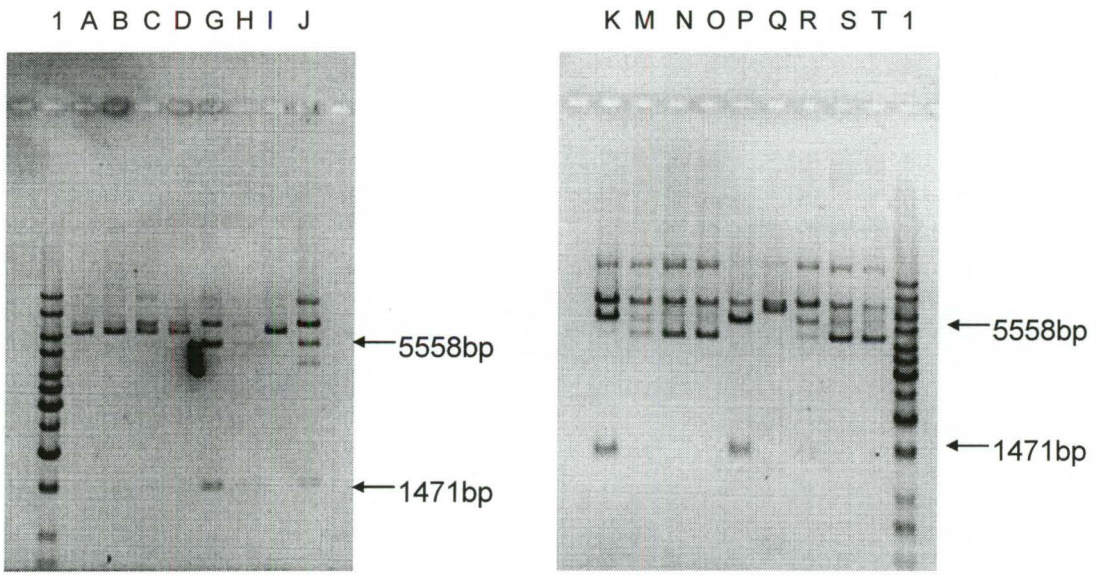

Figure 15: Selection of clones containing the SVCT2 coding region in the proper orientation. Three of the 20 picked clones did not have the SVCT2 coding region (not shown). The 17 remaining clones were cut with XhoI. The expected sizes of the fragments after the digestion were 1471 and 5558 bp if the insert was in the vector in the proper orientation, and 580 and 6449 bp if it was in the reverse direction (see Appendix VI for a map of pCRSVCT2). Clones G,J,K and P were sequenced. P was the only clone that did not have any mutations (see Appendix VII for alignment of "P" with cDNA of hSVCT2).

Lanes 1 contain a 1 kb DNA ladder.

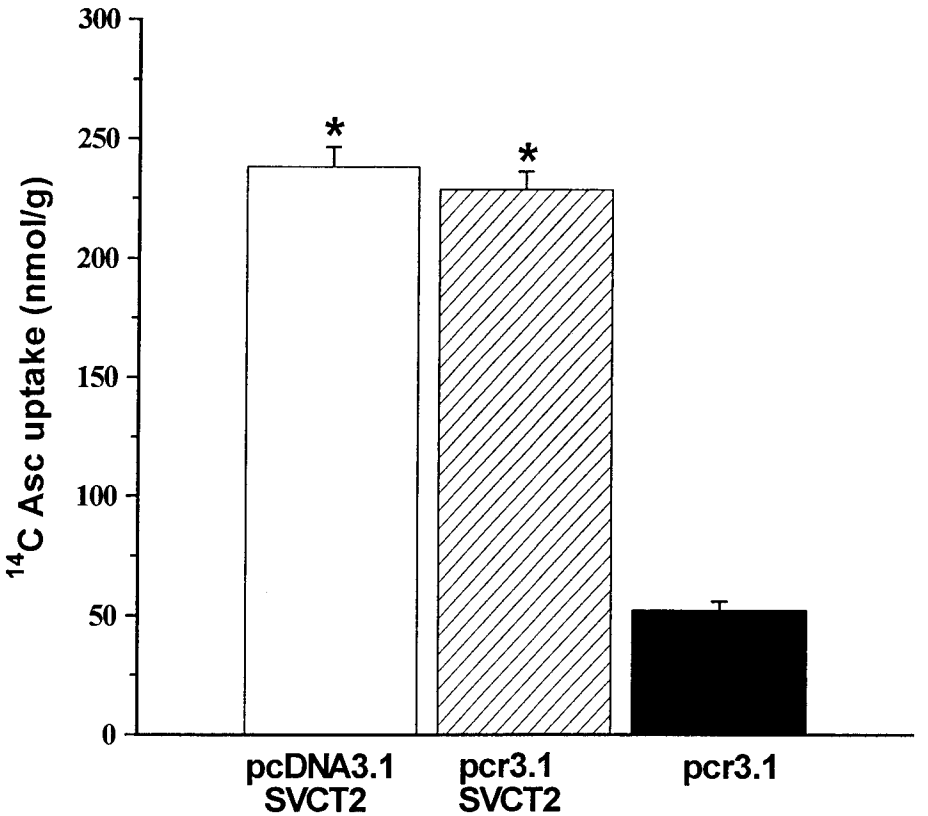

Figure 16: Sulfinpyrazone sensitive ^{14}C-Asc uptake in transiently transfected HEK293T cells. HEK293T cells were transfected with 2.5 μg DNA per 60 mm plate with either pcDNA3.1SVCT2 (entire gene), pCR3.1SVCT2 (coding region only) or pCR3.1 (mock transfection). Data shown are the sulfinpyrazone sensitive (sulfinpyrazone containing uptake subtracted from sulfinpyrazone free uptake) ^{14}C-Asc uptake after 12 min. Uptake in both pcDNA3.1SVCT2 and pCR3.1SVCT2 transfected cells are significantly different from pCR3.1 transfected cells ($p<0.05$). Data are mean ± SEM of 4 replicates.

4.0 DISCUSSION

Results indicate that fresh smooth muscle tissue from the pig coronary artery as well as cells cultured from this tissue express SVCT2. In addition, endothelial cells cultured from the coronary artery also express SVCT2. However, which isoform(s) present in the fresh tissue was inconclusive. The first part of the Discussion will compare the RT-PCR observations with Asc uptake values in the literature. The Results also show that hydrogen peroxide increases the oxidant stress and decreases mitochondrial reductase activity in PCSMC. However, the protective effect of Asc preloading on PCSMC treated with hydrogen peroxide is minimal. Our hypothesis that cells overexpressing SVCT2, and subsequently loaded with Asc, would provide greater protection against ROS could not be tested due to difficulties stably overexpressing functional SVCT2 in PCSMC. These results will be compared to previously published papers, and suggestions will be made to explain the observations.

4.1 Vascular smooth muscle and endothelial expression of SVCT

SVCT2 is a high affinity vitamin C transporter that is widely expressed in nearly all tissues studied (53). The distribution of SVCT1, which has a lower affinity for Asc than SVCT2, but possibly a greater rate of transport, is mainly confined to the epithelial cells of the intestine, kidney and liver (89; 90). Although the vascular endothelium has been said to represent a population of squamous epithelial cells (26), in this study, we found only SVCT2

to be expressed in endothelial cells cultured from the pig coronary artery. Unfortunately, due to the small amount of fresh endothelial tissue we were able to isolate from pig coronary arteries, this result could not be confirmed in the fresh tissue. It is likely that SVCT1 is expressed only in tissues that are exposed to an abundance of Asc, where a greater rate of uptake is more important than a high affinity for Asc. However, it is possible that the cultured endothelial cells differ from the endothelium in the artery. SVCT2 was also found in the pig coronary smooth muscle tissue as well as in cells cultured from this tissue. The expression of SVCT2 in PCEC and PCSMC is consistent with the ^{14}C-Asc uptake values reported for these cells (41). For PCEC, the K_m for Asc was found to be 27 ± 3 μM and 22 ± 2 μM for PCSMC. The K_m values from PCEC and PCSMC also correspond well to published values for human umbilical vein cells (19) and *Xenopus laevis* oocytes expressing hSVCT2 (17).

4.2 Protection of Ascorbate against ROS

A lot of evidence supports the idea that damage to proteins, lipids and DNA initiated by excessive ROS occurs in many cardiovascular diseases (21; 87). It has been suggested that increasing antioxidant concentrations in the plasma or cells would help to scavenge the excess ROS, thereby decreasing the damage and possibly preventing or slowing down the progression of diseases. Asc has been proposed to play a role in decreasing damage caused by ROS due to its ability to donate electrons and be reduced back to Asc (60; 64). We hypothesized that preloading cells with 200 μM Asc overnight would protect the cells against the harm caused by exposure to hydrogen peroxide. No significant difference between Asc

loaded and control cells was observed when exposed to hydrogen peroxide in either the level of oxidation of dichlorodihydrofluorescein or the mitochondrial reductase activity.

The combination of pro-oxidant and antioxidant properties of Asc may decrease the beneficial effects of Asc. Asc can act as a pro-oxidant at high concentrations where free metal ions exist (13; 35) since Asc is able to reduce transition metal ions. This occurs via the Fenton reaction and results in generation of hydroxyl radicals which could have devastating effects on the cells (13). A study by Ek *et al* (1995) included Desferal, an iron chelator, in cells that were preloaded with Asc to see if removing the free metal ions would increase the beneficial effects of Asc following treatment with glucose oxidase, a hydrogen peroxide generator. Desferal did not increase the protection Asc provided against the hydrogen peroxide. An extensive review by Carr and Frei (1999) examined a large number of studies to determine if Asc has a pro-oxidant effect under physiological conditions. According to this review, 38 studies demonstrated a decrease in oxidative damage of DNA, lipids or proteins, 14 showed no change and 6 showed an increase in oxidative damage following Asc supplementation. Although the role of free transition metals cannot be ruled out in the present study, it does not seem to be a likely cause of the minimal protection Asc offered on PCSMC.

Another possible reason that Asc did not show a protective effect on the cells treated with hydrogen peroxide could be that the extracellular Asc had to be removed to prevent extracellular reduction of hydrogen peroxide. During this step as well as the incubation period with hydrogen peroxide Asc may have been released from the cells due to the

concentration gradient. PCSMC are known to have a basal efflux of 5-20 % in the first 5 min and 25-35 % more in the next 55 min (41). In the future, it may be beneficial to have an extracellular non-hydrolyzable Asc compound such as ascorbate 2-phosphate, which will not react with hydrogen peroxide, to prevent efflux of the intracellular Asc.

4.3 Overexpression of SVCT2

There is much debate over the level of benefit Asc exerts during oxidative stress. Many in vitro and in vivo studies showed Asc protection against oxidative stress (2; 19; 37; 55), however studies also suggest that Asc plays a minimal role (18; 46; 50). During oxidative stress, the availability of glutathione and other molecules able to regenerate Asc from DHA is decreased (37). Therefore, we hypothesized that increasing the antioxidant reserve by increasing the Asc uptake may be more beneficial to cells during oxidative stress, specifically looking at pig coronary artery smooth muscle cells.

There are limited reports of transiently transfected cells with SVCT2 (54; 56; 74; 96) and only one study reported a stable SVCT2 transfected cell line (56). Rajan *et al* (1999) expressed SVCT2 in human retinal pigment epithelial (HRPE) cells where the SVCT2 gene was under the control of the T7 promoter. In this case, the HRPE cells were infected with a virus carrying the gene for T7 RNA polymerase. They obtained 8 fold higher Asc uptake compared to the mock transfected cells. COS-1 cells showed a 2 fold increase in the rate of Asc uptake 48 hrs post transfection with SVCT2 compared to untransfected cells (54). We were able to increase the amount of ^{14}C-Asc accumulation by nearly 12 times after 12 min in

transiently transfected HEK293T cells. However, the uptake seen in transfected HEK293T cells was still very low compared to the level of uptake in untransfected PCSMC. When the same plasmid was used to transfect PCSMC, we did not see an increase in mRNA expression or ^{14}C-Asc uptake unless the plasmid was linearized prior to transfection. Linearization of the plasmid before introduction into the cells decreased the probability that the plasmid would be incorporated into the genomic DNA in the SVCT2 coding region or other regions required for expression in mammalian cells (Invitrogen pcDNA3.1 manual). In the cells transfected with the circular pcDNA3.1SVCT2, it is possible that the plasmid DNA was integrated into the genomic DNA in a position allowing transcription of the antibiotic resistance but not complete transcription of SVCT2. Lutsenko *et al* (2004) developed a stable HEK293T cell line that was able to accumulate 2.5 mM Asc after 10 min, compared with less than 1 mM Asc after 60 min for untransfected cells, although the values were not standardized to protein concentration or cell number.

The increase in SVCT2 mRNA observed in the smooth muscle cells overexpressing SVCT2 was 350 % over the control. However, the increase in ^{14}C-Asc uptake was only 50 %. We were unable to determine if the increase in mRNA produced a substantial increase in expressed protein because SVCT2 antibodies are not well established. Until very recently (56) there were no published reports using commercially available SVCT2 antibodies.

One reason why we did not obtain a great increase in ^{14}C-Asc accumulation in the PCSMC stable cell line is that the long 3' untranslated region may have been interfering with efficient transcription or translation in the PCSMC. Therefore, we created a new plasmid

containing only the coding region of SVCT2 and transiently expressed it in HEK293T cells. Unfortunately, in this cell line, the ^{14}C uptake values were similar for the new vector (pCRSVCT2) and the old vector (pcDNA3.1SVCT2), however, this may differ in PCSMC as transfection efficiency and plasmid expression may vary in different cell lines. In all of the transfection attempts, one cannot rule out the possibility that the processing and trafficking of SVCT2 was not efficient, thereby accounting for transfection difficulties (1).

In addition to processing of the protein, regulation by protein kinase C may have prevented increased ^{14}C uptake. Five protein kinase C phosphorylation sites have been suggested for SVCT2, based on the amino acid sequence (74). Liang *et al* (2002) found that a PKC activator, PMA (phorbol 12-myristate 13 acetate) caused a dose and time dependent decrease in Asc transport, due to a change in maximum velocity. Western blot and confocal microscopy analysis verified that the change in uptake was not due to a decrease in protein expression. They concluded that the decrease in uptake by PMA was associated with a reduced catalytic transport efficiency and not a decrease in binding affinity. Phosphorylation status of the SVCT2 protein in PCSMC is not known.

4.4 Summary and Future Directions

We established that SVCT2 mRNA is expressed in smooth muscle and cells cultured from this pig coronary artery tissue. Although the isoform expressed in fresh endothelial tissue could not be determined, cells cultured from this tissue express SVCT2. Asc preloading had little to no effect on preventing damage caused by hydrogen peroxide on

PCSMC. Overexpression experiments were only marginally successful.

The most pertinent future work is to determine why transfected PCSMC did not show a large increase in ^{14}C-Asc uptake, whether it is due to protein expression, processing and trafficking of the protein or regulation of SVCT2 by PCSMC. However, this could not be done at this time due to a lack of suitable antibodies. If this issue is sorted out, one could continue the cell viability assays to see if an increase in Asc reserve would reduce or increase the damage caused by ROS. If increasing Asc uptake is beneficial to PCSMC, this work could then be continued by introducing SVCT2 into arteries via adenovirus during angioplasty in animal models, which would pave the way for Asc therapies during cardiovascular surgeries.

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6.0 APPENDICES

Appendix I: Alignment of hSVCT1 cDNA with pig SVCT1 PCR fragment. A 579 bp PCR product was gel purified and then sequenced at MOBIX (McMaster University). Primers used for sequencing were hSVCT1 444 upstream (5'-GTTAGTCAGCTCATCGGCACCATCT -3' corresponding to 234 from the start site of hSDVCT1 cDNA) and 446 downstream (5'-TCAGGACATAGCAGAGCAGCCACAC-'3 corresponding to 813 from the start site of hSVCT1 cDNA). The sequence obtained showed 92% identity to the similar section of hSVCT1. The alignment was done with the Multalin version 5.4.1 software (16). Differences between the sequences obtained from MOBIX and hSVCT1 are bolded. The start site for hSVCT1 is bolded and underlined.

```
           1                                                      50
444        .......... .......... .......... .......... ..........
446        .......... .......... .......... .......... ..........
svct1      CTCAGGAACT GCTCAAACCT GTGCCCCAAA GATGAGGGCC CAAGAGGACC
Consensus  .......... .......... .......... .......... ..........

           51                                                     100
444        .......... .......... .......... .......... ..........
446        .......... .......... .......... .......... ..........
svct1      TCGAGGGCCG GGCACAGCAT GAAACCACCA GGGACCCCTC GACCCCGCTA
Consensus  .......... .......... .......... .......... ..........

           101                                                    150
444        .......... .......... .......... .......... ..........
446        .......... .......... .......... .......... ..........
svct1      CCCACAGAGC CTAAGTTTGA CATGTTGTAC AAGATCGAGG ACGTGCCACC
Consensus  .......... .......... .......... .......... ..........

           151                                                    200
444        .......... .......... .......... .......... ..........
446        .......... .......... .......... .......... ..........
```

```
    svct1  TTGGTACCTG TGCATCCTGC TGGGCTTCCA GCACTACCTG ACATGCTTCA
Consensus  .......... .......... .......... .......... ..........

           201                                                  250
     444   .......... .......... .......... .......... ..........
     446   .......... .......... .......... .......... ..........
    svct1  GTGGTACCAT CGCCGTGCCC TTCCTGCTGG CTGAGGCGCT GTGTGTGGGC
Consensus  .......... .......... .......... .......... ..........

           251                                                  300
     444   .......... .......... .......... .......... ...CCTGTGT
     446   .......... .......... ..AAGTCATN GGCACCATCT TCACCTGTGT
    svct1  CACGACCAGC ACATGGTTAG TCAGCTCATC GGCACCATCT TCACGTGCGT
Consensus  .......... .......... ..a..tcat. ggcaccatct tcaCcTGtGT

           301                                                  350
     444   GGGCATCACC ACCCTCATCC AGACCACACT GGGCATCCGG CTGCCGCTGT
     446   GGGCATCACC ACCCTCATCC AGACCACACT GGGCATCCGG CTGCCGCTGT
    svct1  GGGCATCACC ACTCTCATCC AGACCACCGT GGGCATCCGG CTGCCGCTGT
Consensus  GGGCATCACC ACcCTCATCC AGACCACacT GGGCATCCGG CTGCCGCTGT

           351                                                  400
     444   TCCAGGCCAG CGCCTTTGCA TTTCTGGTCC CAGCCAAGTC CATCCTGGCA
     446   TCCAGGCCAG CGCCTTTGCA TTTCTGGTCC CAGCCAAGTC CATCCTGGCA
    svct1  TCCAGGCCAG TGCCTTTGCA TTTCTGGTTC CAGCCAAAGC CATACTGGCT
Consensus  TCCAGGCCAG cGCCTTTGCA TTTCTGGTcC CAGCCAAgtC CATcCTGGCa

           401                                                  450
     444   CTGGAGAGAT GGAAATGCCC CCCGGAAGAG GAG.ATCTAC GGTAACTGGA
     446   CTGGAGAGAT GGAAATGCCC CCCGGAAGAG GAGNATATAC GGTAACTGGA
    svct1  CTGGAGAGAT GGAAATGCCC CCCGGAAGAG GAG.ATCTAC GGTAACTGGA
Consensus  CTGGAGAGAT GGAAATGCCC CCCGGAAGAG GAG.ATcTAC GGTAACTGGA

           451                                                  500
     444   GCCTGCCTCT GAACACCTCT CACGTCTGGC ATCCACGCAT GCGAGAGGTC
     446   GCCTGCCTCT GAACACCTCT CACGTCTGGC ATCCACGCAT GCGAGAGGTC
    svct1  GTCTGCCCCT GAACACCTCT CATATTTGGC ACCCACGGAT ACGGGAGGTC
Consensus  GcCTGCCtCT GAACACCTCT CAcgTcTGGC AtCCACGcAT gCGaGAGGTC

           501                                                  550
     444   CAGGGTGCAA TCATGGTGTC CAGCATGGTG GAGGTGGTGA TTGGGCTGAC
     446   CAGGGTGCAA TCATGGTGTC CAGCATGGTG GAGGTGGTGA TTGGGCTGAC
    svct1  CAGGGTGCAA TCATGGTGTC CAGCGTGGTG GAGGTGGTGA TTGGCCTGCT
Consensus  CAGGGTGCAA TCATGGTGTC CAGCaTGGTG GAGGTGGTGA TTGGgCTGac

           551                                                  600
     444   GGGGCTGCCT GGGGCCCTGC TCAGCTACAT TGGGCCTCTT ACAGTCACCC
     446   GGGGCTGCNT GGGGCCCTGC TCAGCTACAT TGGGCNTCTT ACAGTCACCC
    svct1  GGGGCTGCCT GGGGCCCTGC TCAACTACAT TGGGCCTCTC ACAGTCACCC
Consensus  GGGGCTGCcT GGGGCCCTGC TCAgCTACAT TGGGCcTCTt ACAGTCACCC

           601                                                  650
     444   CCACCGTCTC CCTCATTGGC CTCTCTGTCT TCCAAGCTGC TGGTGACAGA
     446   CCACCGTCTC CCTCATTGGC CTCTCTGTCT TCCAAGCTGC TGGTGACAGA
```

```
    svct1  CCACTGTCTC CCTCATTGGC CTTTCTGTCT TCCAAGCTGC TGGCGACCGA
Consensus  CCACcGTCTC CCTCATTGGC CTcTCTGTCT TCCAAGCTGC TGGtGACaGA

           651                                                 700
     444   GCTGGCTCCC ACTGGGGCAT CTCAGCTTGC TCCATTCTCC TGATCATCCT
     446   GCTGGCTCCC ACTGGGGCAT CTCAGCTTGC TCCATTCTCC TGATCATCCT
    svct1  GCTGGCTCCC ACTGGGGCAT CTCAGCTTGC TCCATTCTCC TGATCATCCT
Consensus  GCTGGCTCCC ACTGGGGCAT CTCAGCTTGC TCCATTCTCC TGATCATCCT

           701                                                 750
     444   GTTCTCCCAA TACCTGCGAA ACCTCACGTT CCTGCTGCCT GCCTACCGCT
     446   GTTCTCCCAA NACCTGCGAA ACCTCACGTT CCTGCTGCCT GCCTACCGCT
    svct1  CTTCTCCCAG TACCTGCGCA ACCTCACCTT CCTGCTGCCT GTCTACCGCT
Consensus  gTTCTCCCAa tACCTGCGaA ACCTCACgTT CCTGCTGCCT GcCTACCGCT

           751                                                 800
     444   GGGGCAAGGG CGTCACTCTC TTCCGCGTCC AGATCTTCAA GATGTTTCCT
     446   GGGGCAAGGG CGTCACTCTC TTCCGCGTCC AGATCTTCAA GANGTTTCCT
    svct1  GGGGCAAGGG CCTCACTCTC CTCCGCATCC AGATCTTCAA AATGTTTCCT
Consensus  GGGGCAAGGG CgTCACTCTC tTCCGCgTCC AGATCTTCAA gAtGTTTCCT

           801                                                 850
     444   ATCGTGCTAG CCATCATGAC CGTGTGGCTG CTCTG..... .....
     446   ATCGTGCTAG CCATCA.... ..... ..... .....
    svct1  ATCATGCTGG CCATCATGAC CGTGTGGCTG CTCTGCTATG TCCTGACCTT
Consensus  ATCgTGCTaG CCATCAtgac cgtgtggctg ctctg..... .....

           851                                                 900
     444   ..... ..... ..... ..... ..... ..... .....
     446   ..... ..... ..... ..... ..... ..... .....
    svct1  GACAGACGTG CTGCCCACAG ACCCAAAAGC CTATGGCTTC CAGGCACGAA
Consensus  ..... ..... ..... ..... ..... ..... .....

           901                                                 950
     444   ..... ..... ..... ..... ..... ..... .....
     446   ..... ..... ..... ..... ..... ..... .....
    svct1  CCGATGCCCG TGGTGACATC ATGGCTATTG CACCCTGGAT CCGCATCCCC
Consensus  ..... ..... ..... ..... ..... ..... .....

           951                                                1000
     444   ..... ..... ..... ..... ..... ..... .....
     446   ..... ..... ..... ..... ..... ..... .....
    svct1  TACCCCTGTC AGTGGGGCCT GCCCACGGTG ACTGCGGCTG CTGTCCTGGG
Consensus  ..... ..... ..... ..... ..... ..... .....

           1001                                               1050
     444   ..... ..... ..... ..... ..... ..... .....
     446   ..... ..... ..... ..... ..... ..... .....
    svct1  AATGTTCAGC GCCACTCTGG CAGGCATCAT TGAGTCCATC GGAGATTACT
Consensus  ..... ..... ..... ..... ..... ..... .....

           1051                                               1100
     444   ..... ..... ..... ..... ..... ..... .....
     446   ..... ..... ..... ..... ..... ..... .....
    svct1  ACGCCTGTGC CCGCCTGGCT GGTGCACCAC CCCCTCCAGT ACATGCTATC
```

```
Consensus  .......... .......... .......... .......... ..........

           1101                 1121
      444  .......... .......... .
      446  .......... .......... .
    svct1  AACAGGGGCA TCTTCACCGA A
Consensus  .......... .......... .
```

Appendix II: Alignment of hSVCT1 protein (translated from Genbank accession # AF170911) with pig SVCT1 protein (translated from cDNA sequences obtained from PCR product). The hSVCT1 and pigSVCT1 share 97 % identity for the corresponding section of the protein. Differences between the sequences are in bold.

```
                1                                                   50
   446protein   .......... .......... .......... .......... ..........
   444protein   .......... .......... .......... .......... ..........
hsvct1protein   MRAQEDLEGR AQHETTRDPS TPLPTEPKFD MLYKIEDVPP WYLCILLGFQ
     Consensus  .......... .......... .......... .......... ..........

                51                                                  100
   446protein   .......... .......... .......... .......... ..........
   444protein   .......... .......... .......... ........CV GITTLIQTTL
hsvct1protein   HYLTCFSGTI AVPFLLAEAL CVGHDQHMVS QLIGTIFTCV GITTLIQTTV
     Consensus  .......... .......... .......... ........cv gittliqtt.

                101                                                 150
   446protein   .......... .......... .......... ....IYGNWS LPLNTSHVWH
   444protein   GIRLPLFQAS AFAFLVPAKS ILALERWKCP PEEEIYGNWS LPLNTSHVWH
hsvct1protein   GIRLPLFQAS AFAFLVPAKA ILALERWKCP PEEEIYGNWS LPLNTSHIWH
     Consensus  GiRlplfqAs afaflvPaks ilaleRwkcp PeeeIYGNWS LPLNTSH!WH

                151                                                 200
   446protein   PRMREVQGAI MVSSMVEVVI GLTGL.GALL SYIG.LTVTP TVSLIGLSVF
   444protein   PRMREVQGAI MVSSMVEVVI GLTGLPGALL SYIGPLTVTP TVSLIGLSVF
hsvct1protein   PRIREVQGAI MVSSVVEVVI GLLGLPGALL NYIGPLTVTP TVSLIGLSVF
     Consensus  PRmREVQGAI MVSSmVEVVI GLtGLpGALL sYIGpLTVTP TVSLIGLSVF

                201                                                 250
   446protein   QAAGDRAGSH WGISACSILL IILFSQ.LRN LTFLLPAYRW GKGVTLFRVQ
   444protein   QAAGDRAGSH WGISACSILL IILFSQYLRN LTFLLPAYRW GKGVTLFRVQ
hsvct1protein   QAAGDRAGSH WGISACSILL IILFSQYLRN LTFLLPVYRW GKGLTLLRIQ
     Consensus  QAAGDRAGSH WGISACSILL IILFSQyLRN LTFLLPaYRW GKGvTLfR!Q

                251                                                 300
   446protein   IFK.FPIVLA I......... .......... .......... ..........
   444protein   IFKMFPIVLA IMTVWLLC.. .......... .......... ..........
hsvct1protein   IFKMFPIMLA IMTVWLLCYV LTLTDVLPTD PKAYGFQART DARGDIMAIA
     Consensus  IFKmFPIvLA Imtvwllc.. .......... .......... ..........

                301                                                 350
   446protein   .......... .......... .......... .......... ..........
   444protein   .......... .......... .......... .......... ..........
hsvct1protein   PWIRIPYPCQ WGLPTVTAAA VLGMFSATLA GIIESIGDYY ACARLAGAPP
     Consensus  .......... .......... .......... .......... ..........

                351                                                 400
```

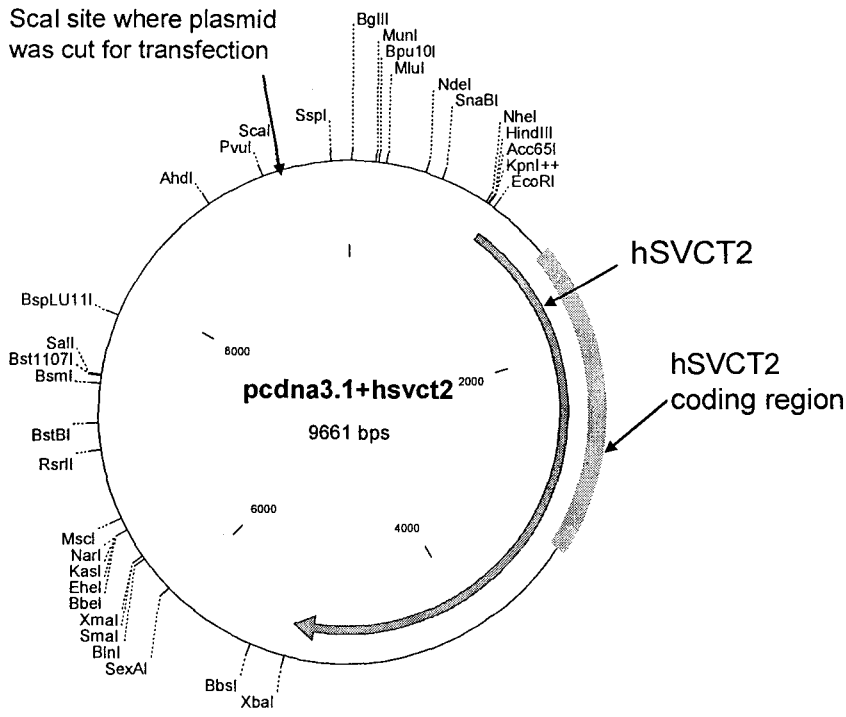
```
446protein   .......... .......... .......... .......... ..........
444protein   .......... .......... .......... .......... ..........
hsvct1protein PPVHAINRGI FTEGICCIIA GLLGTGNGST SSSPNIGVLG ITKVGSRRVV
Consensus    .......... .......... .......... .......... ..........

             401                                                 450
446protein   .......... .......... .......... .......... ..........
444protein   .......... .......... .......... .......... ..........
hsvct1protein QYGAAIMLVL GTIGKFTALF ASLPDPILGG MFCTLFGMIT AVGLSNLQFV
Consensus    .......... .......... .......... .......... ..........

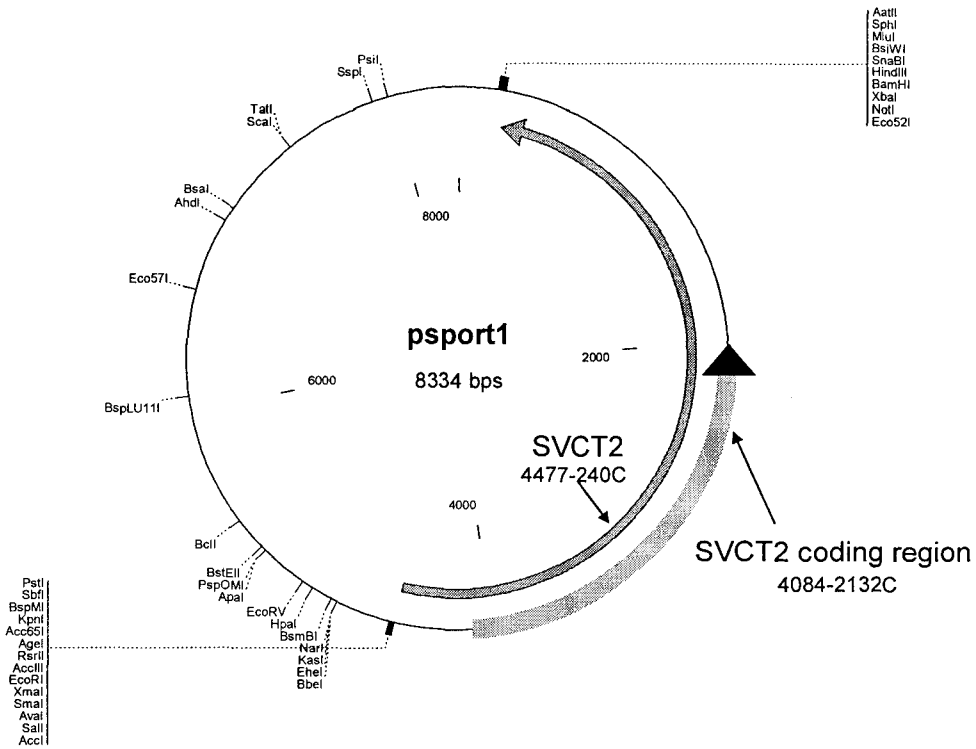
             451                                                 500
446protein   .......... .......... .......... .......... ..........
444protein   .......... .......... .......... .......... ..........
hsvct1protein DMNSSRNLFV LGFSMFFGLT LPNYLESNPG AINTGILEVD QILIVLLTTE
Consensus    .......... .......... .......... .......... ..........

             501                                                 550
446protein   .......... .......... .......... .......... ..........
444protein   .......... .......... .......... .......... ..........
hsvct1protein MFVGGCLAFI LDNTVPGSPE ERGLIQWKAG AHANSDMSSS LKSYDFPIGM
Consensus    .......... .......... .......... .......... ..........

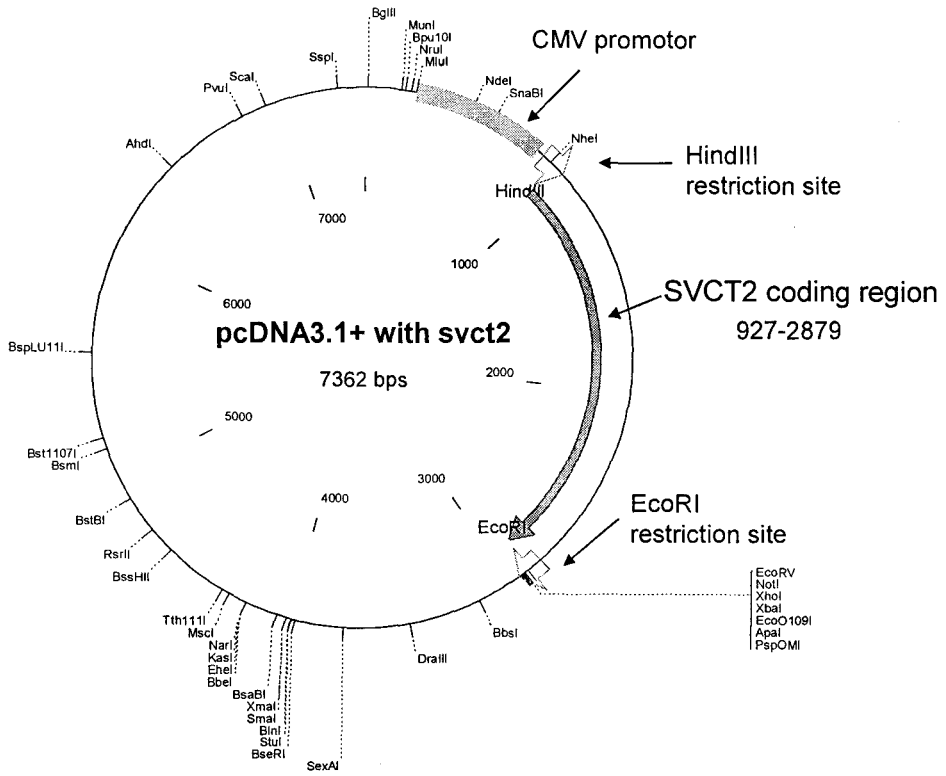
             551                                               598
446protein   .......... .......... .......... .......... ........
444protein   .......... .......... .......... .......... ........
hsvct1protein GIVKRITFLK YIPICPVFKG FSSSSKDQIA IPEDTPENTE TASVCTKV
Consensus    .......... .......... .......... .......... ........
```



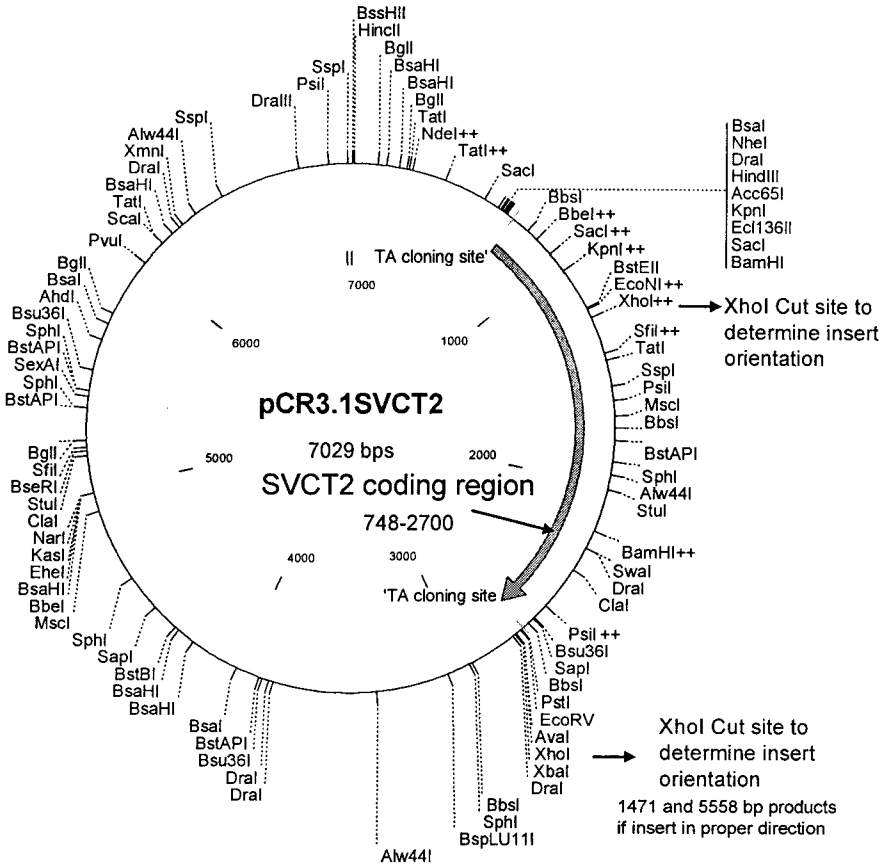
Appendix III Map of pcDNA3.1SVCT2. SVCT2 gene (973-5210) and SVCT2 coding region are shown. The restriction site for ScaI was used to cut the plasmid for transfection of PCSMC with the linear form. The plasmid was used for both linear and circular stable transfections of PCSMC. The plasmid has an antibiotic resistance gene for Neomycin (Geneticin, Invitogen, Burlington, Ontario) (not shown).



Appendix IV: Map of pSPORTSVCT2. pSPORTSVCT2 was received as a gift from Putter D. Prasad, Medical College of Georgia, Augusta, Georgia (Rajan 1999). The hSVCT2 gene starts from 4477-240C and the hSVCT2 coding region is from 4084-2131C. Primers were designed 10 bases upstream from the initiation site and 6 bases downstream from the termination site of the SVCT2 coding region for subcloning into pcDNA3.1 or pCR3.1.



Appendix V: Cloning strategy and map of pcDNA3.1SVCT2 (coding region only). The SVCT2 coding region PCR product (obtained from pSPORT1 plasmid, see Appendix IV), which included restriction sites for EcoRI and HindIII on the 5' and 3' ends, respectively was planned to be ligated into the pcDNA3.1 plasmid that had been cut with EcoRI and HindIII. Ligation of the insert and vector was unsuccessful.



Appendix VI: Cloning strategy and map of pCR3.1SVCT2. SVCT2 coding region is from 748 to 2700 between the two TA cloning sites. Restriction sites for XhoI were used to determine proper orientation of the SVCT2 insert. If the insert is in the proper direction, XhoI restriction digestion will give two fragments of 1471 and 5558 bp. If the insert is in the reverse direction, the digestion will give two products of 580 and 6449 bp. pCR3.1 has a resistance gene for neomycin (Geneticin, Invitrogen, Burlington, Ontario)(not shown).

Appendix VII: Alignment of hSVCT2 coding region with sequence from the pcr3.1SVCT2 clone sequenced by MOBIX (McMaster University). The alignment was done using the Multalin version 5.4.1 program (16). The start (95 bp) and end (2049 bp) of the hSVCT2 coding region (full) are bolded and underlined. After the analysis of the alignment, the PCR product matched 100% to the coding region of hSVCT2 (accession #AF058320). Primers used for seque.ncing were as follows (all positions are relative to initiation site):

477–hSVCT2 10u	5'-GTTTCTCTTAATGATGGGTATTG-3'
478-hSVCT2 318u	5'-GCTACAGCACTACCTGACAT-3'
479-hSVCT2 577u	5'-TCAGTTGCCAATGGAACAGC-3'
480-hSVCT2 811u	5'-GGCATTGCCATGCTGACA-3'
481-hSVCT2 1134u	5'-CGGTGTCATCGGCATGCTCA-3'
482-hSVCT2 1371u	5'-CGTGATACAGTGCGGAGCAG-3'
483 hSVCT2 1645u	5'-GTGTTGAACGTCCTTCTCAC-3'
484 hSVCT2 1894u	5'-GGCCTCAGGAAGAGCGACAA-3'
485 hSVCT2 1930d	5'-CATCTGAACTCCGGCTGTTG-3'
486 hSVCT2 1458d	5'-CACAGGATCCGGAAGGGA-3'
487 hSVCT2 1064d	5'-ACGCCTTGCCTGGCATCTGT-3'
488 hSVCT2 693d	5'-GAGGCCGATGACTACTTCTA-3'
489 hSVCT2 250d	5'-GGTCCAGACTGCCAGTGCTA-3'

```
      1                                                        50
  477 .......... .......... .......... .......... ..........
  488 .......... .......... .......... .......... ..........
  478 .......... .......... .......... .......... ..........
  480 .......... .......... .......... .......... ..........
  486 .......... .......... .......... .......... ..........
  481 .......... .......... .......... .......... ..........
 full TAGAAGAGCC GGCTGATCCT GGGCTCCTAG CTTGAATAAG CCTTCACTTC
  485 .......... .......... .......... .......... ..........
  482 .......... .......... .......... .......... ..........
  487 .......... .......... .......... .......... ..........
  489 .......... .......... .......... .......... ..........
```

```
Consensus  .......... .......... .......... .......... ..........

           51                                                  100
       477 .......... .......... .......... .......... ..........
       488 .......... .......... .......... .......... ..........
       478 .......... .......... .......... .......... ..........
       480 .......... .......... .......... .......... ..........
       486 .......... .......... .......... .......... ..........
       481 .......... .......... .......... .......... ..........
      full CAGCTGCTCT CCCCAACGGC TGTGTAAACT ACTCGTTTCT CTTAATGATG
       485 .......... .......... .......... .......... ..........
       482 .......... .......... .......... .......... ..........
       487 .......... .......... .......... .......... ..........
       489 .......... .......... .......... ..TTGTTTCT CTTAATGATG
Consensus  .......... .......... .......... .......... ..........

           101                                                 150
       477 .......... .......... .......... .......... .......AAC
       488 .......... .......... .......... .......... ..........
       478 .......... .......... .......... .......... ..........
       480 .......... .......... .......... .......... ..........
       486 .......... .......... .......... .......... ..........
       481 .......... .......... .......... .......... ..........
      full GGTATTGGTA AGAATACCAC ATCCAAATCA ATGGAGGCTG GAAGTTCAAC
       485 .......... .......... .......... .......... ..........
       482 .......... .......... .......... .......... ..........
       487 .......... .......... .......... .......... ..........
       489 GGTATTGGTA AGAATACCAC ATCCAAATCA ATGGAGGCTG GAAGTTCAAC
Consensus  .......... .......... .......... .......... ..........

           151                                                 200
       477 AGAAGGCAAA TACGAAGACG AGGCAAAGCA CCCAGCTTTC TTCACTCTTC
       488 .......... .......... .......... .......... ..........
       478 .......... .......... .......... .......... ..........
       480 .......... .......... .......... .......... ..........
       486 .......... .......... .......... .......... ..........
       481 .......... .......... .......... .......... ..........
      full AGAAGGCAAA TACGAAGACG AGGCAAAGCA CCCAGCTTTC TTCACTCTTC
       485 .......... .......... .......... .......... ..........
       482 .......... .......... .......... .......... ..........
       487 .......... .......... .......... .......... ..........
       489 AGAAGGCAAA TACGAAGACG AGGCAAAGCA CCCAGCTTTC TTCACTCTTC
Consensus  .......... .......... .......... .......... ..........

           201                                                 250
       477 CGGTGGTGAT AAATGGAGGC GCCACCTCCA GCGGTGAGCA GGACAATGAG
       488 .......... .......... .......... .......... ..........
       478 .......... .......... .......... .......... ..........
       480 .......... .......... .......... .......... ..........
       486 .......... .......... .......... .......... ..........
       481 .......... .......... .......... .......... ..........
      full CGGTGGTGAT AAATGGAGGC GCCACCTCCA GCGGTGAGCA GGACAATGAG
       485 .......... .......... .......... .......... ..........
       482 .......... .......... .......... .......... ..........
       487 .......... .......... .......... .......... ..........
```

```
      489  CGGTGGTGAT AAATGGAGGC GCCACCTCCA GCGGTGAGCA GGACAATGAG
Consensus  .......... .......... .......... .......... ..........

           251                                                  300
      477  GACACTGAGC TCATGGCGAT CTACACTACG GAAAACGGCA TTGCAGAAAA
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  GACACTGAGC TCATGGCGAT CTACACTACG GAAAACGGCA TTGCAGAAAA
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  GACACTGAGC TCATGGCGAT CTACACTACG GAAAACGGCA TTGCAGAAAA
Consensus  .......... .......... .......... .......... ..........

           301                                                  350
      477  GAGCTCTCTC GCTGAGACCC TGGATAGCAC TGGCAGTCTG GACCCCCAGC
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  GAGCTCTCTC GCTGAGACCC TGGATAGCAC TGGCAGTCTG GACCCCCAGC
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  GAGCT..... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           351                                                  400
      477  GATCAGACAT GATTTATACC ATAGAAGATG TTCCTCCCTG GTACCTGTGT
      488  .......... .....ATACC ATAGAAGATG GTCCTCCCTG GTACCTGTGT
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  GATCAGACAT GATTTATACC ATAGAAGATG TTCCTCCCTG GTACCTGTGT
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           401                                                  450
      477  ATATTTCTGG GGCTACAGCA CTACCTGACA TGCTTCAGCG GCACGATCGC
      488  ATATTTCTGG GGCTACAGCA CTACCTGACA TGCTTCAGCG GCACGATCGC
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  ATATTTCTGG GGCTACAGCA CTACCTGACA TGCTTCAGCG GCACGATCGC
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
```

```
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           451                                                  500
      477  AGTGCCCTTC CTGTTGGCTG ATGCCATGTG TGTGGGGTAC GACCAGTGGG
      488  AGTGCCCTTC CTGTTGGCTG ATGCCATGTG TGTGGGGTAC GACCAGTGGG
      478  .......... .TGTTGGCTG ATGCCATGTG TGTGGGGTAC GACCAGTGGG
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  AGTGCCCTTC CTGTTGGCTG ATGCCATGTG TGTGGGGTAC GACCAGTGGG
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           501                                                  550
      477  CCACCAGCCA GCTCATTGGG ACCATTTTCT TCTGTGTGGG AATCACTACT
      488  CCACCAGCCA GCTCATTGGG ACCATTTTCT TCTGTGTGGG AATCACTACT
      478  CCACCAGCCA GCTCATTGGG ACCATTTTCT TCTGTGTGGG AATCACTACT
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  CCACCAGCCA GCTCATTGGG ACCATTTTCT TCTGTGTGGG AATCACTACT
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           551                                                  600
      477  TTGCTACAGA CAACGTTTGG ATGCAGGTTA CCCCTGTTTC AGGCCAGTGC
      488  TTGCTACAGA CAACGTTTGG ATGCAGGTTA CCCCTGTTTC AGGCCAGTGC
      478  TTGCTACAGA CAACGTTTGG ATGCAGGTTA CCCCTGTTTC AGGCCAGTGC
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  TTGCTACAGA CAACGTTTGG ATGCAGGTTA CCCCTGTTTC AGGCCAGTGC
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           601                                                  650
      477  TTTTGCATTT TTGGCCCCTG CTCGAGCCAT CCTGTCTTTA GATAAATGGA
      488  TTTTGCATTT TTGGCCCCTG CTCGAGCCAT CCTGTCTTTA GATAAATGGA
      478  TTTTGCATTT TTGG...... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  .......... .......... .......... .......... ..........
     full  TTTTGCATTT TTGGCCCCTG CTCGAGCCAT CCTGTCTTTA GATAAATGGA
      485  .......... .......... .......... .......... ..........
```

```
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            651                                                  700
       477  AATGTAACAC CACAGATGTT TCAGTTGCCA ATGGAACAGC AGAGCTGTTG
       488  AATGTAACAC CACAGATGTT TCAGTTGCCA ATGGAACAGC AGAGCTGTTG
       478  .......... .......... .......... .......... ..........
       480  .......... .......... .......... .......... ..........
       486  .......... .......... .......... .......... ..........
       481  .......... .......... .......... .......... ..........
      full  AATGTAACAC CACAGATGTT TCAGTTGCCA ATGGAACAGC AGAGCTGTTG
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            701                                                  750
       477  CACACAGAAC ACATCTGGTA TCCCCGGATC CGAGAGATCC AGGGGGCCAT
       488  CACACAGAAC ACATCTGGTA TCCCCGGATC CGAGAG.... ..........
       478  .......... .......... .......... .......... ..........
       480  .......... .......... .......... .......... ..........
       486  .......... .......... .....GGATC CGAGAGATCC AGGGGGCCAT
       481  .......... .......... .......... .......... ..........
      full  CACACAGAAC ACATCTGGTA TCCCCGGATC CGAGAGATCC AGGGGGCCAT
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            751                                                  800
       477  CATCATGTCC TCACTGATAG AAGTAGTCAT CGGCCTCCTC GGCCTGCCTG
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  .......... .......... .......... .......... ..........
       486  CATCATGTCC TCACTGATAG AAGTAGTCAT CGGCCTCCTC GGCCTGCCTG
       481  .......... .......... .......... .......... ..........
      full  CATCATGTCC TCACTGATAG AAGTAGTCAT CGGCCTCCTC GGCCTGCCTG
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            801                                                  850
       477  GGGCTCTACT GAAGTACATC GGTCCCTTGA CCATTACACC CACGGTGGCC
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  .......... .......... .......... .......... ..........
       486  GGGCTCTACT GAAGTACATC GGTCCCTTGA CCATTACACC CACGGTGGCC
       481  .......... .......... .......... .......... ..........
      full  GGGCTCTACT GAAGTACATC GGTCCCTTGA CCATTACACC CACGGTGGCC
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      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           851                                                  900
      477  CTAATTGGCC TCTCTGGTTT CCAGGCAGCG GGGGAGAGAG CCGGGAAGCA
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  CTAATTGGCC TCTCTGGTTT CCAGGCAGCG GGGGAGAGAG CCGGGAAGCA
      481  .......... .......... .......... .......... ..........
     full  CTAATTGGCC TCTCTGGTTT CCAGGCAGCG GGGGAGAGAG CCGGGAAGCA
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           901                                                  950
      477  CTGGGGCATT GCCATGCTGA CAATATTCCT AGTATTACTG GTTTCTCAAT
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  CTGGGGCATT GCCATGCTGA CAATATTCCT AGTATTACTG TTTTCTCAAT
      481  .......... .......... .......... .......... ..........
     full  CTGGGGCATT GCCATGCTGA CAATATTCCT AGTATTACTG TTTTCTCAAT
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           951                                                 1000
      477  ACG....... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .....AGAAA TGTTAAATTT CCTCTCCCGA TTTATAAATC CAAGAAAGGA
      486  ACGCCAGAAA TGTTAAATTT CCTCTCCCGA TTTATAAATC CAAGAAAGGA
      481  .......... .......... .......... .......... ..........
     full  ACGCCAGAAA TGTTAAATTT CCTCTCCCGA TTTATAAATC CAAGAAAGGA
      485  .......... .......... .......... .......... ..........
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... ...ATAAATC CAAGAAAGGA
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1001                                                1050
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  TGGACTGCGT ACAAGTTACA GCTGTTCAAA ATGTTCCCTA TCATCCTGGC
      486  TGGACTGCGT ACAAGTTACA GCTGTTCAAA ATGTTCCCTA TCATCCTGGC
      481  .......... .......... .......... .......... ..........
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      full  TGGACTGCGT ACAAGTTACA GCTGTTCAAA ATGTTCCCTA TCATCCTGGC
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  TGGACTGCGT ACAAGT.ACA GCTGTTCAAA ATGTTCCCTA TCATCCTGGC
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1051                                                1100
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  CATCCTGGTA TCCTGGCTGC TCTGCTTCAT CTTCACGGTG ACAGATGTCT
       486  CATCCTGGTA TCCTGGCTGC TCTGCTTCAT CTTCACGGTG ACAGATGTCT
       481  .......... .......... .......... .......... ..........
      full  CATCCTGGTA TCCTGGCTGC TCTGCTTCAT CTTCACGGTG ACAGATGTCT
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  CATCCTGGTA TCCTGGCTGC TCTGCTTCAT CTTCACGGTG ACAGATGTCT
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1101                                                1150
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  TCCCTCCCGA CAGCACAAAG TATGGCTTCT ATGCTCGCAC AGATGCCAGG
       486  TCCCTCCCGA CAGCACAAAG TATGGCTTCT ATGCTCGCAC AGATGCCAGG
       481  .......... .......... .......... .......... ..........
      full  TCCCTCCCGA CAGCACAAAG TATGGCTTCT ATGCTCGCAC AGATGCCAGG
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  TCCCTCCCGA CAGCACAAAG TA........ .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1151                                                1200
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  CAAGGCGTGC TTCTGGTAGC CCCGTGGTTT AAGGTTCCAT ACCCATTTCA
       486  CAAGGCGTGC TTCTGGTAGC CCCGTGGTTT AAGGTTCCAT ACCCATTTCA
       481  .......... .......... .......... .......... ..........
      full  CAAGGCGTGC TTCTGGTAGC CCCGTGGTTT AAGGTTCCAT ACCCATTTCA
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1201                                                1250
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  GTGGGGACTG CCCACCGTGT CTGCGGCCGG TGTCATCGGC ATGCTCAGTG
       486  GTGGGGACTG CCCACCGTGT CTGCGGCCGG TGTCATCGGC ATGCTCAGTG
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       481  .......... .......... .......... .......... ..........
      full  GTGGGGACTG CCCACCGTGT CTGCGGCCGG TGTCATCGGC ATGCTCAGTG
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1251                                                1300
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  CCGTGGTCGC CAGCATCATC GAGTCTATTG GTGACTACTA CGCCTGTGCA
       486  CCGTGGTCGC CAGCATCATC GAGTCTATTG GTGACTACTA CGCCTGTGCA
       481  .......... .....TCATC GAGTCTATTG GTGACTACTA CGCCTGTGCA
      full  CCGTGGTCGC CAGCATCATC GAGTCTATTG GTGACTACTA CGCCTGTGCA
       485  .......... .......... .......... .......... ..........
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1301                                                1350
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  CGGCTGTCCT GTGCCCCACC CCCCCCCATC CACGCAATAA ACAGGGGAAT
       486  CGGCTGTCCT GTGCCCCACC CCCCCCCATC CACGCAATAA ACAGGGGAAT
       481  CGGCTGTCCT GTGCCCCACC CCCCCCCATC CACGCAATAA ACAGGGGAAT
      full  CGGCTGTCCT GTGCCCCACC CCCCCCCATC CACGCAATAA ACAGGGGAAT
       485  .......... .......... .......ATC CACGCAATAA ACAGGGGAAT
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1351                                                1400
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  TTTCGTGGAA GGCCTCTCCT GTGTTCTTGA TGGCATATTT GGTACTGGGA
       486  TTTCGTGGAA GGCCTCTCCT GTGTTCTTGA TGGCATATTT GGTACTGGGA
       481  TTTCGTGGAA GGCCTCTCCT GTGTTCTTGA TGGCATATTT GGTACTGGGA
      full  TTTCGTGGAA GGCCTCTCCT GTGTTCTTGA TGGCATATTT GGTACTGGGA
       485  TTTCGTGGAA GGCCTCTCCT GTGTTCTTGA TGGCATATTT GGTACTGGGA
       482  .......... .......... .......... .......... ..........
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1401                                                1450
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  ATGGCTCTAC TTCATCCAGT CCCAACATTG GAGTTTTGGG AATTACAAAG
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      486  ATGGCTCTAC TTCATCCAGT CCCAACATTG GAGTTTTGGG AATTACAAAG
      481  ATGGCTCTAC TTCATCCAGT CCCAACATTG GAGTTTTGGG AATTACAAAG
     full  ATGGCTCTAC TTCATCCAGT CCCAACATTG GAGTTTTGGG AATTACAAAG
      485  ATGGCTCTAC TTCATCCAGT CCCAACATTG GAGTTTTGGG AATTACAAAG
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1451                                                1500
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  GTCGGCAGCC GCCGCGTGAT ACAGTGCGGA GCAGCCCTCA TGCTCGCTCT
      486  GTCGGCAGCC GCCGCGTGAT ACAGTGCGGA GCAGCCCTCA TGCTCGCTCT
      481  GTCGGCAGCC GCCGCGTGAT ACAGTGCGGA GCAGCCCTCA TGCTCGCTCT
     full  GTCGGCAGCC GCCGCGTGAT ACAGTGCGGA GCAGCCCTCA TGCTCGCTCT
      485  GTCGGCAGCC GCCGCGTGAT ACAGTGCGGA GCAGCCCTCA TGCTCGCTCT
      482  .......... .......... .......... .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1501                                                1550
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  GGGCATGATC GGGAAGTTCA GCGCCCTCTT TGCGTCCCTT CCGGATCCTG
      486  GGGCATGATC GGGAAGTTCA GCGCC..... .......... ..........
      481  GGGCATGATC GGGAAGTTCA GCGCCCTCTT TGCGTCCCTT CCGGATCCTG
     full  GGGCATGATC GGGAAGTTCA GCGCCCTCTT TGCGTCCCTT CCGGATCCTG
      485  GGGCATGATC GGGAAGTTCA GCGCCCTCTT TGCGTCCCTT CCGGATCCTG
      482  .......... GGGAAGTTCA GCGCCCTCTT TGCGTCCCTT CCGGATCCTG
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1551                                                1600
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  TGCTGGGAGC CCCTGTT... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  TGCTGGGAGC CC.TGTTCTG CACGCTCTTT GGAATGATCA CAGCTGTTGG
     full  TGCTGGGAGC CC.TGTTCTG CACGCTCTTT GGAATGATCA CAGCTGTTGG
      485  TGCTGGGAGC CC.TGTTCTG CACGCTCTTT GGAATGATCA CAGCTGTTGG
      482  TGCTGGGAGC CC.TGTTCTG CACGCTCTTT GGAATGATCA CAGCTGTTGG
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1601                                                1650
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
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       480  .......... .......... .......... .......... ..........
       486  .......... .......... .......... .......... ..........
       481  CCTCTCTAAC CTGCAGTTCA TTGATTTAAA TTCTTCCCGG AACCTCTTTG
      full  CCTCTCTAAC CTGCAGTTCA TTGATTTAAA TTCTTCCCGG AACCTCTTTG
       485  CCTCTCTAAC CTGCAGTTCA TTGATTTAAA TTCTTCCCGG AACCTCTTTG
       482  CCTCTCTAAC CTGCAGTTCA TTGATTTAAA TTCTTCCCGG AACCTCTTTG
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1651                                                1700
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  .......... .......... .......... .......... ..........
       486  .......... .......... .......... .......... ..........
       481  TGCTTGGATT TTCGATCTTC TTTGGGCTCG TCCTTCCAAG TTACCTCAGA
      full  TGCTTGGATT TTCGATCTTC TTTGGGCTCG TCCTTCCAAG TTACCTCAGA
       485  TGCTTGGATT TTCGATCTTC TTTGGGCTCG TCCTTCCAAG TTACCTCAGA
       482  TGCTTGGATT TTCGATCTTC TTTGGGCTCG TCCTTCCAAG TTACCTCAGA
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1701                                                1750
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  .......... .......... .......... .......... ..........
       486  .......... .......... .......... .......... ..........
       481  CAGAACCCTC TGGTCACAGG GATAACAGGA ATCGATCAAG TGTTGAACGT
      full  CAGAACCCTC TGGTCACAGG GATAACAGGA ATCGATCAAG TGTTGAACGT
       485  CAGAACCCTC TGGTCACAGG GATAACAGGA ATCGATCAAG TGTTGAACGT
       482  CAGAACCCTC TGGTCACAGG GATAACAGGA ATCGATCAAG TGTTGAACGT
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1751                                                1800
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
       478  .......... .......... .......... .......... ..........
       480  .......... .......... .......... .......... ..........
       486  .......... .......... .......... .......... ..........
       481  CCTTCTCACA ACTGCTATGT TTGTAGGGGG CTGTGTGGCT TTTATCCTGG
      full  CCTTCTCACA ACTGCTATGT TTGTAGGGGG CTGTGTGGCT TTTATCCTGG
       485  CCTTCTCACA ACTGCTATGT TTGTAGGGGG CTGTGTGGCT TTTATCCTGG
       482  CCTTCTCACA ACTGCTATGT TTGTAGGGGG CTGTGTGGCT TTTATCCTGG
       487  .......... .......... .......... .......... ..........
       489  .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... ..........

            1801                                                1850
       477  .......... .......... .......... .......... ..........
       488  .......... .......... .......... .......... ..........
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      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  ATAACACCAT CCCAGGCACT CCAGAGGAAA GAGGAATCCG GAAATGGAAG
     full  ATAACACCAT CCCAGGCACT CCAGAGGAAA GAGGAATCCG GAAATGGAAG
      485  ATAACACCAT CCCAGGCACT CCAGAGGAAA GAGGAATCCG GAAATGGAAG
      482  ATAACACCAT CCCAGGCACT CCAGAGGAAA GAGGAATCCG GAAATGGAAG
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1851                                                1900
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  AAGGGTGTGG GCAAAGGGAA CAAATCACTC GACGGCATGG AGTCGTACAA
     full  AAGGGTGTGG GCAAAGGGAA CAAATCACTC GACGGCATGG AGTCGTACAA
      485  AAGGGTGTGG GCAAAGGGAA CAAATCACTC GACGGCATGG AGTCGTACAA
      482  AAGGGTGTGG GCAAAGGGAA CAAATCACTC GACGGCATGG AGTCGTACAA
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1901                                                1950
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  TTTGCCATTT GG.CATGAAC ATTATAAAAA AATACAGATG CTTCAGCTAC
     full  TTTGCCATTT GG.CATGAAC ATTATAAAAA AATACAGATG CTTCAGCTAC
      485  TTTGCCATTT GG.CATGAAC ATTATAAAAA AATACAGATG CTTCAGCTAC
      482  TTTGCCATTT GGGCATGAAC ATTATAAAAA AATACAGATG CTTCAGCTAC
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           1951                                                2000
      477  .......... .......... .......... .......... ..........
      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  TTACCCATCA GCCCAACCTT TGTGGGCTAC ACATGGAAAG GCCTCAGGAA
     full  TTACCCATCA GCCCAACCTT TGTGGGCTAC ACATGGAAAG GCCTCAGGAA
      485  TTACCCATCA GCCCAACCTT TGTGGGCTAC ACA....... ..........
      482  TTACCCATCA GCCCAACCTT TGTGGGCTAC ACATGGAAAG GCCTCAGGAA
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........

           2001                                                2050
      477  .......... .......... .......... .......... ..........
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      488  .......... .......... .......... .......... ..........
      478  .......... .......... .......... .......... ..........
      480  .......... .......... .......... .......... ..........
      486  .......... .......... .......... .......... ..........
      481  GAGCGACANC AGCCGGAGTT CAGATGAAGA CTCCCAGGCC ACGGGATAGC
     full  GAGCGACAAC AGCCGGAGTT CAGATGAAGA CTCCCAGGCC ACGGGATAGC
      485  .......... .......... .......... .......... ..........
      482  GAGCGACAAC AGCCGGAGTT CA........ .......... ..........
      487  .......... .......... .......... .......... ..........
      489  .......... .......... .......... .......... ..........
Consensus  .......... .......... .......... .......... ..........
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