HYPOTONIC SHOCK INDUCED

ASCORBATE RELEASE

CHARACTERIZATION OF HYPOTONIC SHOCK INDUCED ASCORBATE RELEASE FROM PIG CORONARY ARTERY ENDOTHELIAL CELLS

by

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ABSTRACT

Ascorbate (Asc) is a key antioxidant in preventing cardiovascular dysfunction during diseases exacerbated by altered shear stress. According to the literature endothelial responses to hypotonic shock share some characteristics with those induced by shear stress. Thus to study the physiological responses of endothelium to shear stress, the characterization of the Asc release by pig coronary artery endothelial cells in response to hypotonic shock was performed.

The pig coronary artery endothelial cells that had been loaded with ^{14}C Asc and ^{3}H deoxyglucose, were exposed to buffers of varying osmolality for different time periods and the release of ^{14}C Asc and ^{3}H deoxyglucose was examined. Based on various parameters like relative release of ^{14}C Asc and ^{3}H deoxyglucose, their rate of release and protein loss, it was decided to use buffer of 67 percent osmolality for 2 min for these characterization studies. The Asc release was authentic and not a result of membrane damage.

The hypotonic shock induced Asc release was not due to endogenously released ATP. The inhibition of ATP induced release by anion channel inhibitors niflumic acid and NPPB was complete but only partial in case of hypotonic shock induced release. The release was not inhibited under nominally Ca^{2+} free conditions. Additive release by hypotonic shock and ATP or hypotonic shock and Ca^{2+} ionophore A23187 suggests that there are two independent Asc release pathways.

Asc release by two different mechanisms may help endothelial cells deal with stressful conditions efficiently and preserve endothelial function.

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LIST OF ABBREVIATIONS

9-AC	anthracene-9-carboxylic acid
ADP	adenosine 5`-diphosphate
ANOVA	analysis of variance
Asc	ascorbate
ATP	adenosine 5`-triphosphate
BAECs	bovine aortic endothelial cells
BAPTA	1,2-bis (2-aminophenoxy) ethane-N, N, N`, N`-tetraacetic acid
BAPTA-AM	1,2-bis (2-aminophenoxy) ethane-N, N, N`, N`-tetraacetic acid tetrakis (acetoxymethyl ester)
$[Ca^{2+}]_i$	intracellular calcium
CaCCs	calcium activated chloride channels
CFTR	cystis fibrosis transmembrane conductance regulator
DAG	diacylglycerol
DHAA	dehydroascorbic acid
DIDS	4,4'-diisothiocyanostilbene-2, 2'-disulfonic acid
DMEM	dulbecco's modified eagle's medium
DOG	deoxyglucose
EDTA	ethylene diamine tetra acetic acid
ET-1	endothelin-1
GLUT	glucose transporter
cGMP	cyclic guanosine monophosphate
GSH	glutathione, reduced form
HEPES	N-2 hydroxyethylepiperazine-N`-2-ethanesulfonic acid

HPLC	high performance liquid chromatography
HTS	hypotonic shock
IAA-94	indanyloxyacetic acid –94
IC_{50}	half-maximal inhibitory concentration
IP_3	inositol 1,4,5-trisphosphate
MAPK	mitogen activated protein kinase
MLCK	myosin light chain kinase
MW	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NO	nitric oxide
eNOS	endothelial nitric oxide synthase
NPPB	5-nitro- 2-(3-phenylpropylamino) benzoic acid
PCECs	pig coronary artery endothelial cells
PCSMCs	pig coronary artery smooth muscle cells
PGI_2	prostaglandin I_2
PIP_2	phosphatidyl inositol 4, 5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
ROS	reactive oxygen species
SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum calcium ATPase
SITS	4-acetamido-4'-isothiocyanatostilbene-2, 2'-disulfonic acid

SMCs	smooth muscle cells
SOD	superoxide dismutase
SVCT	sodium-dependent vitamin C transporter
UTP	uridine 5`-triphosphate
VRACs	volume regulated anion channels

1.0 INTRODUCTION

Human derived endothelial cells have mechanisms to regenerate ascorbate from its oxidized forms in order to combat stressful conditions efficiently (67). The main objective of this study is to characterize the ascorbate (Asc) release by cultured pig coronary artery endothelial cells (PCECs) in response to hypotonic shock (HTS), in addition to relating this release to the one caused by extracellular ATP. Since this release is unique to endothelial cells and specific for ascorbate, the introduction focuses on endothelial functions, followed by the chemistry, functions, and transport of ascorbate.

1.1 Cardiovascular system and Homeostasis

The circulatory system comprises of the cardiovascular and lymphatic systems. The cardiovascular system is composed of the heart, vessels and blood (and all the cellular components with in the blood) while the lymphatic system consists of lymphatic microvessels (capillaries) and larger lymph vessels. The circulatory system plays an integral role in the movement of blood through out the body. It is through this extensive network of arteries, veins and capillaries that cellular function (growth and development), absorption of essential nutrients (such as vitamins and minerals) and removal of metabolic waste-products i.e. vascular homeostasis are maintained. Hereby I will focus on cardiac blood supply via the coronary arteries since these arteries, as the major blood supplier to the heart are crucial towards the maintenance of vascular homeostasis.

1.1.1 Coronary arteries: Structure and Function

The cardiac vessel consists of the left and right coronary arteries. The main function of the coronary arteries is to supply oxygen and nutrients to cardiac muscle. The left main coronary artery passes between the left atrium and the pulmonary trunk to reach the atrioventricular groove, where it divides into the left anterior descending coronary artery and the circumflex artery(57). The former and its branches supply blood to the apical wall of the left ventricle, right and left bundle branches and the papillary muscle of the left ventricle. The right main coronary artery gives rise to the posterior descending artery and nourishes the apical half of the ventricular septum(57). The coronary arteries run on the surface of the heart and are embedded in the sub-epicardial fat and ventricular tissue.

The internal structure of an artery can provide an idea about the strategic position of endothelium between the flowing blood and smooth muscle cells (SMCs). An artery is made up of three layers of tissues: *tunica-adventitia*, *tunica-media* and *tunica-intima*(57). The outermost layer *tunica-adventitia* is composed up of SMCs, fibroblasts, matrix, blood vessels and nerves. The *tunica-media* is made up of multiple layers of SMCs and the secreted extra cellular matrix. It is due to this thickest middle layer that the walls of the arteries can stretch or expand without tearing in response to pressure changes. The thinnest layer *tunica-intima* consists of a monolayer of endothelial cells mounted on a basement membrane. Below this are a sub-endothelial fibro-elastic tissue layer and an organized layer of elastic lamina that provides flexibility and stability for endothelial cells.

1.2 The Endothelium

The nature of the endothelium (i.e. their direct contact with the blood) suggests their critical role in all aspects of vascular homeostasis. In a person of body weight 70kg, the endothelium as the inner lining of cardiovascular system covers an area of approximately $700m^2$ and weighs about 1-1.5 kg^2 (118). It is important to note that throughout different tissues of the body, as well as different locations in an organ the endothelium exhibits physiological and anatomical variations.

1.2.1 Structural features of the Endothelium

An endothelial cell has three distinct cell surfaces: luminal, abluminal and lateral. Luminal surface, an interface between flowing blood and endothelial cell has multiple receptors and adhesion molecules, which stimulate second messengers, triggering cellular structural and functional changes. The abluminal surface rests upon self-secreted sub-endothelium. The lateral surfaces bridge the adjacent endothelial cells via three major types of intercellular junctions: *tight junctions, gap junctions and adherens junctions.* Gap junctions allow an exchange of ions and molecules between adjacent cells and are thus a means of communication between endothelial cells. Tight junctions and adherens junctions together provide adhesive connections between cells and thus strengthen the endothelial barrier(23).

An important rod-shaped secretory organelle unique to endothelial cells is Weibel-Palade Bodies that stores von-Willebrand factor. This organelle is used as a marker to differentiate between endothelial and SMCs. Endothelial cells have caveolae, omega-

shaped invaginations of cell membrane which have been found to sequester several isoforms of adenylate cyclase, tyrosine kinases and endothelial nitric oxide synthase (eNOS). Their role in the regulation of vascular tone is still largely debated.

1.2.2 Functional features of the Endothelium

Endothelium significantly contributes to a variety of cardiovascular functions, which include regulation of permeability, blood pressure, coagulation, transmigration of leukocytes, immuno-responses, vessel growth and repair, exchange of oxygen, nutrients and metabolites. Discussed in detail below, are the roles of the endothelium in the regulation of vascular tone and the responses against external stressors (shear stress and oxidative stress).

1.2.2.1 Regulation of the vascular tone

Vascular tone is a parameter that defines optimal blood flow by alternating between vasoconstriction and vasorelaxation in response to the factors secreted by endothelial cells and other factors present in the blood stream. In SMCs, intracellular calcium concentration ($[Ca^{2+}]i$) is the primary mechanism of regulating vascular tone. Furthermore, $[Ca^{2+}]i$ in the endothelium mediates the release of vasoactive factors towards the SMCs. The following paragraph describes the mechanisms that cause an increase or decrease in $[Ca^{2+}]i$.

During the resting stage of a SMC, the extracellular concentration of Ca^{2+} is approximately 1mM while the intracellular concentration is 0.1μM. Extracellular calcium

ions can enter the vascular SMCs by voltage operated calcium channels and receptor-operated calcium channels in the plasma membrane(74). The sarcoplasmic reticulum also has a high Ca^{2+} concentration relative to the cytosol. When G-protein coupled receptors are bound by a ligand like acetylcholine, they activate phosphoinositide-specific phospholipase C (PLC), which hydrolyzes phosphatidylinositol biphosphate (PIP_2) into two intracellular mediators – inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG)(74). IP_3 binds to the IP_3 gated Ca^{2+} release channels in the sarcoplasmic reticulum membrane. Calcium released from the sarcoplasmic reticulum increases the $[Ca^{2+}]i$. Increase in $[Ca^{2+}]i$ activates myosin light chain kinase (MLCK). The subsequent phosphorylation of MLC allows myosin to interact with actin thus initiating contraction(122). The $[Ca^{2+}]i$ is lowered by active transport of Ca^{2+} out of the cell across the plasma membrane or into the sarcoplasmic reticulum. Plasma membrane Ca^{2+} ATPases and Na^+/Ca^{2+} exchangers pump Ca^{2+} out of the cell to keep resting concentration of cytosolic Ca^{2+} low. SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) pumps restore Ca^{2+} ions from the cytosol into the lumen of the sarcoplasmic reticulum. The fall in $[Ca^{2+}]i$ causes dephosphorylation of MLC by myosin phosphatase thus instigates relaxation (74; 77).

1.2.2.1.1 Role of endothelial factors in the regulation of vascular tone

Endothelium plays a dual role in regulating the vascular tone by secreting various vasoconstricting and vasorelaxing factors(61). The vasorelaxing factors secreted by the endothelium include nitric oxide (NO), prostacyclin (PGI_2), bradykinin and endothelium

derived hyperpolarizing factor. The vasoconstricting factors include endothelin-1 (ET-1), superoxide anions, endothelium-derived constricting factor (poorly characterized), locally produced angiotensin II and thromboxane (61).

Vasorelaxation factor NO: Nitric oxide, also well known as endothelium derived relaxing factor is the main vasorelaxing factor secreted by endothelial cells(115). It is synthesized by eNOS, a heme containing oxygenase from L-arginine by oxidation of guanidine-nitogen terminal(15).

L-arginine $\xrightarrow{\text{eNOS}}$ **L-citrulline + NO**

eNOS although is primarily present in endothelial cells, it has also been identified in platelets (79). NO is synthesized by the endothelial cells in response to physical stimuli (such as shear stress and changes in arterial pressure) or chemical messengers (such as acetylcholine, arachidonic acid and catecholamines). It diffuses to the underlying smooth muscle cells (SMCs) and activates soluble guanylyl cyclase on binding to the heme group of that enzyme. This in turn leads to the accumulation of the second messenger cyclic guanosine mono-phosphate (cGMP) which in turn activates cGMP-dependent protein kinase; in case of SMCs this leads to relaxation as a result of subsequent decrease in $[Ca^{2+}]i$ (10).

In addition, endothelial cells release NO into the blood stream. NO before being inactivated by oxyhaemoglobin can inhibit leukocyte adhesion to endothelial cells(115). Regulation of vascular tone by NO holds significance as its dysregulation has been

detected in various diseases like atherosclerosis, hypercholesterolemia, ischemia-reperfusion, diabetes and hypertension (15; 16).

Vasorelaxation factor PGI_2: Prostacyclin otherwise known as prostaglandin I_2, is a derivative of arachidonic acid that exerts its vasorelaxing effects in response to shear stress (107). Decreased levels of PGI_2 have been observed in atherosclerosis (107). NO and PGI_2 synergistically inhibits platelet aggregation thus preventing clot formation (34; 73; 92).

Vasoconstriction factor ET-1: Endothelin-1 is a potent vasoconstrictor polypeptide secreted by endothelial cells and is reported to be associated with an increase in $[Ca^{2+}]i$ (50). ET-1 also stimulates the release of arachidonic acid via the activation of cytosolic phospholipase A_2 (PLA_2). This in turn results in the synthesis of PGI_2 (109).

1.2.2.1.2 Role of P2Y receptors in the regulation of vascular tone

In healthy vasculature, nucleotides (such as ATP) released from the platelets act on the endothelium stimulating release of NO and PGI_2 by acting at PLC-coupled P2Y receptors found on endothelial cells and causing vasorelaxation of the blood vessel (49; 88). There are 6 cloned and functionally defined subtypes of P2Y receptors that exhibit varying affinities to different agonists (93). Endothelial cells from different origins vary in their pattern of P2Y receptor expression. Cultured bovine aortic endothelial cells (BAECs) express both P2Y1 and P2Y2 receptors(76; 129). The principal endogenous

agonist for P2Y1 is ADP and for P2Y2 are ATP and UTP. In the adrenal medulla microvasculature, endothelial cells are exposed to very high levels of ATP. Here only P2Y2 receptors are found(91). The brain endothelium has P2Y2 receptors coupled to PLC and elevated $[Ca^{2+}]i$ and also P2Y1 which stimulate an increase in $[Ca^{2+}]i$ in the absence of a detectable increase in IP_3 levels(2; 29).

It has been shown that both P2Y1 and P2Y2 receptors are coupled to NOS activation in BAECs(13). Thus P2Y receptors are central to the regulation of vascular tone via NO and PGI_2 production.

1.2.2.2 Adaptive responses to shear stress

Shear stress is a frictional force generated by blood flowing past the surface of endothelial cells and is measured in dynes/cm^2(21). It causes cell-deformation but the direction of deformation differs from that produced by blood pressure as the force acts in a single direction. Also note that only the endothelial cells are exposed to shear stress and not the SMCs. Endothelial cells, as an adaptive measure respond to shear stress by vasodilation as an increase in the vessel diameter decreases shear stress. Endothelial responsiveness to shear stress plays a central role in normal vascular physiology through regulation of vascular tone and is involved in the etiology of particular abnormalities particularly atherosclerosis. Early atherosclerotic lesions develop in regions where shear stress is multidirectional and disturbed. On the other hand, arterial regions exposed to unidirectional shear stress remain largely spared of early atherosclerosis(53; 54).

In humans, normal shear stress magnitudes vary from negative values to approximately 50 dynes/cm^2 (53). In hypertensive individuals this may exceed upto 100 dynes/cm^2. The level of shear stress required to detach the endothelium is about 400 dynes/cm^2. The effects of acute and chronic shear stress have been studied extensively *in vitro*. Acute shear stress refers to conditions where endothelial cells are not previously exposed to this force with a time frame measured in seconds to hours(22). Acute shear stress may mimic in part the *in vivo* responses of endothelium. Chronic shear stress refers to conditions in which endothelial cells are exposed to shear stress for several days to weeks. Chronic shear stress model approximates the *in vivo* conditions. Extensive information is available about acute responses to shear stress but less is known about chronic responses because of the difficulty in maintaining cultured cells for long periods and also it is difficult to examine shear stress *in vivo*.

1.2.2.2.1 Responses to acute shear stress

Research has shown that the most rapid response to shear stress is activation of K^+ channels within milliseconds in BAECs after application of shear stress(85). This in turn results in membrane hyperpolarization(78). The increase in membrane potential induces Ca^{2+} intake via a calcium / phosphatidylinositol / hyperpolarization activated calcium permeable channel. In some cases, in addition to K^+ channels shear stress also activates Cl^- channels. Due to this the hyperpolarization reverses to depolarization. This modulates Ca^{2+} entry into the cells and attenuates cellular functions that rely on Ca^{2+} influx (NO release and vasorelaxation). The nucleotides (such as ATP) released by endothelial cells

in response to shear stress activate G protein coupled receptors, which stimulates PLC (21). Currently the mechanism stimulating PLC activation (in 1 min- 1 hour) in response to shear stress is unclear. IP_3 diffuses rapidly to the cytosol from the cell membrane and releases Ca^{2+} to the cytosol from the intracellular Ca^{2+} sequestering compartment. Most investigators have been unable to find any significant increases in bulk cytosolic Ca^{2+} in endothelial cells that were exposed to shear stress in the absence of Ca^{2+} mobilizing nucleotides like ATP(24; 72). It may be possible that shear stress causes changes in local Ca^{2+} concentration in the vicinity of the cell membrane without causing any increase in the bulk cytosolic Ca^{2+} concentration. DAG can either be cleaved to release arachidonic acid that is subsequently converted to PGI_2 or activates phosphokinase C (PKC). PKC mediates MLCK activation and phosphorylation of junctional proteins, which facilitate cytoskeletal reorganization. In addition, phospholipase A_2 (PLA_2) is also activated with the release of arachidonic acid from membrane phospholipids(7). Ca^{2+} mobilizing agonists stimulate PLC and PLA_2 activity in endothelial cells with consequent IP_3, DAG and arachidonic acid release and production of the two most well known vaso-relaxing agents NO and PGI_2. Thapsigargin, which depletes $[Ca^{2+}]i$ stores profoundly inhibits agonist, but not shear stress stimulated NO and PGI_2 production(62). Such findings suggest that acute NO and PGI_2 release in response to shear stress is not due to Ca^{2+} mobilization from intracellular stores (SERCA pumps). Nevertheless, as shear stress induced NO and PGI_2 release is inhibited by chelators of extracellular Ca^{2+} it seems that their release is partly dependent on Ca^{2+} influx(132).

As an adaptive response to unidirectional shear stress (1-6 hours), cytoskeletal rearrangement takes place. This aids the cells to align in the direction of blood flow(126). Reorganization of cytoskeletal components requires initial actin depolymerization and then repolymerization. Along with redistribution of integrins and focal contacts takes place. This cytoskeletal organization can be inhibited by chelators of intra- and extracellular Ca^{2+} and by tyrosine kinase inhibitors thus suggesting a role of $[Ca^{2+}]i$ and integrin-mediated signaling in the process(63; 75). Integrins can also signal mitogen activated protein kinase (MAPK) activation, which plays a role in endothelial-differentiation. Shear stress also activates transcription of eNOS(132) and manganese superoxide dismutase (Mn SOD) (108). eNOS plays a role in vasorelaxation as said before and Mn SOD aids in combating oxidative stress (discussed later). It remains to be established whether cytoskeletal rearrangement and integrin signaling is central to the activation of signal cascades during acute shear stress.

1.2.2.2.2 Responses to chronic shear stress

Cell realignment and flattening are sustained with prolonged shear stress. In response to chronic shear stress, cells hypertrophy, density of actin microfilaments and focal adhesions increases (86). In renal microvascular endothelial cells, there is a dramatic increase in clathrin-coated pits and vesicles (86). In aortic endothelial cells exposed to chronic shear stress, there is forty-fold greater density of Weibel-Palade Bodies(119). In addition to structural reorganization, shear stress also stimulates cells adhesion to the substratum. Endothelial cells cultured for nine days under arterial levels

of shear stress (15 dyne/cm^2) adhere more firmly than cells cultured for the same time under less shear stress (1 dyne/cm^2)(55). Chronic shear stress results in the reduction of ET-1 gene expression. Enhanced NO generation stimulated by shear stress may reduce ET-1 expression in an auto regulatory fashion (52; 55).

These changes suggest that cells in the presence of chronic shear stress achieve a greater degree of differentiation thus raising the possibility that shear stress may be the stimuli for promoting endothelial cells differentiation *in vivo* (94). These findings in endothelial cells exposed to acute shear stress cannot be extrapolated simply to chronic shear stress conditions. Differences in these responses may help to unravel the mechanisms whereby shear stress elicits responses in endothelial cells. It is possible that acute shear stress responses may resemble *in vivo* responses of the proliferating endothelium whereas responses observed in cells adapted to chronic shear stress may more closely reflect those of the differentiated endothelium.

1.2.2.3 Protective responses against oxidative stress

The normal life span of a human endothelial cell is approximately 30 years(114). Therefore any damage caused to the endothelium will have long-term effects. Oxidative stress is a condition where production of reactive oxygen species (ROS) overrides the scavenging effects of antioxidants resulting in steady state level of oxidative damage. ROS are a range of free and non-free radicals, oxygen derived molecules that display moderate to high reactivity towards various cellular constituents (27). It is estimated that approximately five percent of the oxygen consumed by tissues is transformed into

ROS(5). The most familiar ROS include superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). Superoxide anion is the one electron reduction product of molecular oxygen (O_2) and is a byproduct of metabolic reactions where in the absence of electron acceptors, stray electrons are taken up by oxygen (27). Endothelial cells may release superoxide anions, generated by a variety of enzymes including NAD(P)H oxidase, xanthine oxidase and eNOS itself. H_2O_2 is a product of two-electron reduction of oxygen, being the protonated form of $O_2^{-\bullet}$. The enzymatic reduction of superoxide anion into H_2O_2 is catalyzed by the enzyme SOD of which several forms are known as copper/zinc (Cu/Zn) SOD, manganese (Mn) SOD, iron (Fe) containing SOD and are localized within or on the surface of endothelial cells(1; 46). H_2O_2 is further metabolized into water and oxygen by catalase or glutathione (GSH) peroxidase. PCECs have a higher specific activity of catalase than PCSMCs thus can metabolize H_2O_2 faster and protect the underlying SMCs(32; 33). The reduction of superoxide and H_2O_2 with metals like Cu/Fe may generate the highly reactive hydroxyl radical, which damages cells by initiating lipid peroxidation and causing DNA strand breaks(45).

The superoxide anion reacts with NO to form peroxynitrite ($ONOO^-$). This blunts the vasorelaxation caused by NO (35). Peroxynitrite can react with a number of compounds such as deoxyribose sugars, sulfydryl groups, cellular lipids and also with tyrosine residues in proteins and peptides, generating 3-nitrotyrosine(4; 39). Increased nitrotyrosine levels have been linked with atherosclerosis.

Peroxynitrite also damages intracellular Ca^{2+} stores (SERCA pumps) (31; 100). This damage is more severe in PCSMCs than PCECs. This is due to expression of ROS

resistant SERCA isoform in PCECs. This shows that PCSMCs are more susceptible to damage caused by ROS than PCECs(32; 33; 100).

Antioxidants can scavenge superoxide and protects the relaxant vascular tone exhibited by NO. Of the aqueous antioxidants, ascorbate is the most powerful electron donor and is the first plasma antioxidant sacrificed upon exposure to oxidative stress. Another important antioxidant is vitamin E (α- tocopherol). The efficient function of vitamin E depends on its regeneration following oxidation to tocopheryl radical. A direct electron transfer with vitamin C (ascorbate) achieves this (87). ROS produced by endothelial cells may have a wide range of functions separate from their oxidizing effects. The dismutated form of superoxide anion is H_2O_2, which relaxes SMCs(44; 127). Thus ROS may act as signaling molecules in short term but excess ROS over long term may affect pliability of the arteries (121).

There is a growing interest in combating the diseases caused by oxidative stress using anti-oxidants like Vitamin C and E. A role for oxidative stress has been postulated in many conditions including atherosclerosis, inflammatory conditions, certain cancers and the process of aging as in these conditions increased amounts of free radicals that were observed in body fluids(3; 38; 96). Hypertension is also found to be associated with increased superoxide anion and H_2O_2 production as well as decreased antioxidant capacity(48; 110). Oxidative stress has also been accompanied with decreased SOD and GSH peroxidase activity. Transgenic mice that overexpress GSH peroxidase are less susceptible whereas GSH peroxidase knockout mice are more susceptible to ischaemia-reperfusion myocardial injury as compared to their wild counterparts(133). Clinical trials

have proved that combined treatments with vitamins C and E may suppress free radical production in neutrophils.

1.3 Anion channels of the endothelium

The anion channels are also termed as chloride channels because of their predominant permeability to chloride ions in addition to other anions. Three classes of chloride channels have been described in endothelial cells (83). These are: volume regulated anion channels (VRACs) or volume regulated chloride channels; calcium activated chloride channels (CaCCs); and high-conductance chloride channels which may neither be regulated by Ca^{2+} nor by cell volume but by voltage or cAMP(83). The cAMP regulated cystic fibrosis transmembrane regulator (CFTR) channels are not expressed in healthy endothelial cells. As this study specifically focuses on VRACs, I will describe them first followed by CaCCs and CFTR.

1.3.1 Volume regulated anion channels

These channels are modulated by cell swelling, hyperpolarization, intracellular pH, and $[Ca^{2+}]i$. These channels are not only permeable to Cl^- but also to amino acids and organic osmolytes thus they have a broader permeability range than other anion channels. These channels are also known as volume sensitive organic osmolyte anion channels and volume regulated chloride channels(104; 105). The anion permeability sequence is $SCN^- > I^- > NO^{3-} > Br^- > Cl^- > HCO^{3-} > F^-$ > gluconate >glycine > taurine >

aspartate, glutamate(82). It is not certain that VRACs that pass organic osmolytes like taurine are identical to those that pass Cl^-.

VRACs activity is dependent on cytosolic ATP concentrations but not on ATP hydrolysis suggesting a non-hydrolytic binding of ATP to the channel(47). Changes in $[Ca^{2+}]i$ is not required for the activation of VRACs. These channels may play a role in endothelial cell signal transduction during shear stress as they can counteract the depolarizing action of other channels and contribute to stabilization of the inwardly driving force for Ca^{2+}.

The pharmacology of VRACs is quite extensively studied and should be interpreted with caution, as most blockers are poorly selective (see table 1). The molecular nature of the VRACs is still unknown and the five putative candidates are P-glycoprotein, pI_{Cln}, phospholemman, ClC-2 and more recently ClC-3(80).

1.3.2 Calcium activated chloride channels

CaCCs are mainly expressed in endothelial and many excitable cells including neurons and SMCs (81). These channels require ATP, $[Ca^{2+}]i$ and stronger depolarization to get activated. The anion permeation profile is $SCN^- > I^- > Cl^-$> gluconate. Niflumic acid, DIDS, SITS, 9AC are potent but not very selective blockers of CaCC. The molecular nature of CaCC is not yet resolved. Putative candidates are endothelial adhesion protein bCLCA2, bovine bCLCA1, 2, murine mCLCA1-4, pig pCLCA1 and human hCLCA1-4 proteins(80).

1.3.3 Cystic fibrosis transmembrane conductance regulator

CFTRs are mainly expressed in the epithelium and the heart. These are by far the most intensively investigated Cl^- channels (80). The anion permeability sequence is $Br^- > Cl^- > I^- > F^-$. A functional role for CFTR in endothelial cell biology is not clear but it could be involved in trans-endothelial transport and act as a regulator of other channels. It has been associated with ATP transport(80).

Blocker	***VRACs***	***CaCCs***	***CFTR***
DIDS	10-50	100-200	>200
SITS	>200	100-200	100-200
NPPB	5-10	10-50	>200
9-AC	>200	50-100	-
IAA-94	100-200	-	>200
Niflumic Acid	100-200	5-10	-
Tamoxifen	1-5	-	-

Table 1: IC_{50} (half-maximal inhibitory concentration, in μM) values of various anion channel blockers (19)

1.4 Vitamin C: Chemistry and Metabolism

The asymmetrical nature of carbon-5 of ascorbic acid makes two enantiomeric forms possible: L- and D-ascorbic acid. L-ascorbic acid is the naturally occurring and biologically active form. At physiological pH, > 99 percent of L-ascorbic acid gets ionized to L-ascorbate. This in turn can donate a hydrogen atom to produce ascorbyl free radical or semidehydroascorbate. The ascorbyl free radical can donate a second electron to form the two-electron oxidation product of ascorbate, dehydroascorbic acid (DHAA) (Fig 3 in appendix).

Alternatively, the ascorbyl free radical may be enzymatically reduced back to ascorbate by NADH dependent semidehydroascorbate reductase or the NADPH dependent selenoenzyme, thioredoxin reductase. DHAA can be reduced back to ascorbate by the GSH dependent enzyme, glutaredoxin or thioredoxin reductase(17). If not recycled to ascorbate, DHAA is irreversibly hydrolyzed to 2,3 diketo-L-gulonic acid, which does not function as an anti-oxidant like ascorbate. Diketogulonic acid on further degradation produces oxalic acid, L-threonic acid, xylose, xylonic acid and lyxonic acid (97).

1.4.1 Functions of Ascorbate

Ascorbate plays an important role in a number of physiological responses:

1. Synthesis of collagen (donates electrons to proline hydroxylase, lysine hydroxylase).
2. Modulating energy metabolism (required for carnitine biosynthesis and transport of fatty acids across inner mitochondrial membrane)

3. Metabolism of cholesterol to bile acids
4. Dietary iron absorption
5. Maintaining normal endothelial function (enhances activity of eNOS by keeping its cofactor tetrahydrobiopterin in reduced (active) form)(30; 43)
6. An effective antioxidant

Ascorbate is an effective antioxidant as it can be generated from its oxidized forms (ascorbyl free radical and DHAA). Other properties of ascorbate that make it a strong physiological antioxidant are the low one-electron reduction potentials of ascorbate and its one electron oxidation product, ascorbyl free radical(14). These low reduction potentials enable it to react spontaneously with and reduce the most physiologically relevant radicals and oxidants. Ascorbate readily scavenges ROS such as superoxide anion, singlet oxygen, reactive nitrogen species such as NO_2 and N_2O_4 and hypochlorous acid thereby protecting biological macromolecules such as proteins, lipids and DNA from oxidative damage(14).

The ascorbyl free radical is quite stable and unreactive as compared to ascorbate. It is neither strongly oxidizing nor strongly reducing and does not react with molecular oxygen to form more reactive peroxyl radical. Instead, it readily forms ascorbate or DHAA. DHAA is then reduced back to ascorbate by GSH, glutaredoxin or thioredoxin reductase or is rapidly and irreversibly hydrolyzed.

1.4.2 Transport and uptake of Ascorbate and Dehydroascorbic acid

Ascorbic acid is found at a concentration of 30-60 μM in the plasma of unsupplemented individuals. These concentrations can be doubled with oral supplements(59; 99). Plasma ascorbate concentrations are maintained by intestinal absorption(69; 103), renal excretion(65; 95), slow release from circulating erythrocytes(70) and recycling of DHAA.

Most tissues take up and retain ascorbic acid in its reduced form i.e. ascorbate. In human white blood corpuscles, concentrations are as high as 6-8mM(6; 58; 125). Cellular retention of ascorbate is due to low permeability of ascorbate across membranes. It is taken up via one or more high affinity, energy and sodium dependent vitamin C transporters (SVCT 1 and 2). The cDNA for these transporters have been cloned from rat(111) and human cDNA libraries(20; 123). SVCT1 is expressed mainly on the epithelial surface of the intestine and kidneys, and in the liver where as SVCT2 expression has been found in most other tissues. This distribution suggests that SVCT1 is expressed in tissues with slow uptake of ascorbate whereas SVCT2 is expressed in tissues where there is a need to take up ascorbate with high affinity from extracellular fluids.

DHAA is taken into cells by facilitated diffusion via the glucose transporters, GLUT1, GLUT3 and to some extent GLUT4(116; 128). Intracellularly, DHAA is rapidly reduced to ascorbate through reduction by either GSH or NADPH dependent mechanisms. Since ascorbate is not a substrate for the glucose transporter, it is trapped inside the cells(42). This process may also contribute to a large gradient between the plasma and the cellular ascorbate concentrations.

Cultured human umbilical vein endothelial cells take up ascorbate rapidly against a concentration gradient(26). BAECs take up ascorbate very slowly due to the lack of sodium dependent ascorbate transporters in them(64). Both of the above-mentioned cell types rapidly take up DHAA by facilitated diffusion by the low affinity glucose transporter followed by intracellular reduction to ascorbate(26).

Endothelial cells in culture can accumulate reduced ascorbate to a concentration of 3-8mM(26; 64). Although intracellular ascorbate concentrations in freshly prepared endothelial cells have not been reported, the capacity of these cells for ascorbate uptake and recycling supports the notion that they can use ascorbate as an antioxidant.

1.4.3 Ascorbate recycling

Endothelial cells, as a barrier between bloodstream and the underlying tissues are exposed to oxidative stress from both compartments. The ability of endothelial cells to regenerate ascorbate from its oxidized forms (ascorbyl free radical and DHAA) may be important not only for the ascorbate economy of the cell but it also may determine the amount of ascorbate available for passage across to the endothelium(67). Human umbilical vein endothelial cells have the mechanisms to recycle ascorbate from ascorbyl free radical and DHAA(67). In these cells recycling of ascorbate from ascorbyl free radical is probably the most important mechanism in the absence of severe oxidative stress. Under severe oxidative stress these cells recycle ascorbate from DHAA as a line of defense against loss of ascorbate. Human neutrophils and mononuclear phagocytes can accumulate ascorbate when incubated under conditions of oxidative stress(56; 117; 124;

125). Recently Nualart *et al* (84) used co-cultures to prove that DHAA released from one type of cells may be recycled by another. It was observed that superoxide produced by activated cells (HeLa-60 neutrophils and normal human neutrophils) oxidized extracellular ascorbate to DHAA (84). This DHAA generated can be transported intracellularly and converted to ascorbate by any cell present in the immediate area, as glucose transporters are present in all cells and tissues.

1.4.4 Ascorbate release

Hepatocytes have also been known to take up and reduce DHAA and release ascorbate(112). In rat luteal cells, PGF 2α increases the oxidation and release of ascorbate(89). Osmotic swelling has been shown to cause ascorbate release in rat cerebral astrocytes(101).

Ascorbate is released from pig coronary artery endothelial cells (PCECs) upon challenge with ATP, UTP, Ca^{2+} ionophore A23187 and the SERCA pump inhibitor, cyclopiazonic acid. This release has also been observed in human coronary artery endothelial cells. The release was not a result of increased cell permeability. The release was increased with the application of ATPγS (non-hydrolyzable analog) thus indicating that it is a purinergic receptor mediated phenomena. As the release was not increased on applying adenosine, ADP or UDP, it may occur through a pathway involving P2Y2 or P2Y4 receptors. The cessation of release with the application of ATP-receptor inhibitor suramin suggested that P2Y2 receptor activation is the most likely pathway for this

release. This should be considered as tentative since affinities for ligands between species may differ and subtypes of P2Y receptors in pig are not yet fully classified.

The acceleration of the release by A23187 suggests that it is a Ca^{2+} dependent process. This was confirmed by using Ca^{2+} free conditions and the Ca^{2+} chelator BAPTA. The cessation of release on using inositol specific phospholipase C inhibitor U73122 indicates that the ATP induced release of ascorbate from PCECs by increasing $[Ca^{2+}]i$ is via an IP_3 selective PLC. To say in short ATP induced ascorbate release by increasing $[Ca^{2+}]i$ by an IP_3 mediated pathway.

ATP induced release was inhibited by anion channel inhibitors (niflumic acid, NPPB, sulfinpyrazone and IAA-94). But this could not be used to rule out a role played by SVCT (Na^+-Asc symporter), which was also inhibited by these agents. This release could occur through CaCCs as these channels allow large ions to pass through (81).

1.5 Problem definition

PCECs release ascorbate (Asc) on application of extracellular ATP. HTS would be rarely applied *in vivo* to endothelial cells since plasma osmolarity is strictly controlled in a narrow range. However as evident from the literature HTS induced endothelial cell responses such as anion channel activation(21), NO release(51), cell hypertrophy(98) and cytoskeletal rearrangement(21) share some characteristics with those induced by shear stress. **Thus HTS induced Asc release studies can be used as a model to study the shear stress inducible physiological responses in endothelial cells.**

The two main objectives of the present study are:

Firstly, the characterization of HTS stimulated Asc release involving:

1. Optimization of the time-interval and the osmolality strength for future experiments
2. Correlation, if any in the amount of Asc loaded and released
3. Confirmation of the authenticity of released Asc
4. Role of ATP in Asc release
5. Role of Ca^{2+} in Asc release
6. Role of the anion channels in Asc release

Secondly to conclude whether the Asc release pathways elicited by ATP or HTS are linked or are separate.

2.0 METHODS

2.1 Materials

Adenosine 5'- triphosphate (ATP), A23187, Indanyloxyacetic acid - 94 (IAA-94), Niflumic acid, 5-nitro-2- (3-phenylpropylamino) benzoic acid (NPPB), Saponin, Sulfinpyrazone, and Suramin were purchased from Sigma-Aldrich Canada. The radioisotopes ^{14}C Ascorbate and ^{3}H deoxyglucose were purchased from Amersham-Pharmacia Canada.

2.2 Cells culture

PCECs were cultured after being taken from the innermost layer of coronary arteries using a sterile cotton swab as described previously(32). Briefly, the cells were plated into 6 well plates in Dulbecco's modified Eagle's medium (DMEM) (Gibco 12800-017) supplemented with 0.5 mM HEPES pH 7.4, 2 mM glutamine, 50 mg/L gentamicin, 0.125 mg/L amphotericin and 10% fetal bovine serum (CanSera). Once the cells grew to confluence, they were removed by trypsinization (0.25% trypsin, 1 mM EDTA in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution, Gibco) for 4 min at 37°C and replated. At the third passage cells were frozen into aliquots. Confluent cells from passage 4 were split into 6 to 60mm petri dishes and used on day 7 of growth.

The cultured PCECs have been characterized previously(32). These cells react positively to anti-endothelial NO synthase and anti-Von Willebrand factor. A negative reaction with anti-smooth muscle α - actin confirmed that these are not SMCs present in the immediate vicinity of endothelial cells. The cultured cells have also been shown to

differ from the SMCs in expressing a different isoform of the sarco/endoplasmic reticulum Ca^{2+} pump (SERCA3), and being rich in catalase(33).

2.3 Loading cells with ^{14}C-Ascorbate and ^{3}H-Deoxyglucose

Cells were loaded with ^{14}C-Ascorbate (Asc) and ^{3}H-deoxyglucose (DOG) 18h before use in an experiment. For ^{14}C, the Carbon at position 1 is radiolabelled (Fig 1 in appendix) and for ^{3}H, 2H at position 2 and one at 6 are radiolabelled (Fig 2 in appendix). In initial experiments, several samples containing known amounts of ^{14}C and ^{3}H were used for scintillation counting. Based on these experiments the following formulae were derived $^{14}C = {}^{14}C$ cpm - 0.001065 * ^{3}H dpm and $^{3}H = {}^{3}H$ dpm -1.64 * ^{14}C cpm. These formulae were used subsequently to determine the amounts of ^{14}C and ^{3}H in the experimental samples. Typically final concentration of Asc was adjusted to 200μM including 20μM of ^{14}C-Asc. Preliminary results obtained in our laboratory indicated that loading PCECs with 1μM DOG did not affect their growth or ascorbate release but 100μM could be toxic. Thus the final concentration of ^{3}H-DOG was adjusted to 1μM.

2.4 Experimental procedure

Petri dishes (60mm) containing cells were washed twice with Na^{+}-HEPES buffer pre-warmed to 37°C and containing (in mM) NaCl 134; KCl 5.4; Glucose 10; $MgSO_4$ 0.8; HEPES 20 and $CaCl_2$ 1.8, pH 7.3. To prepare nominally Ca^{2+} free buffer, $CaCl_2$ was replaced by 2.6 mM $MgSO_4$. These solutions have been reported to be 290-300 mOsm. Cells after two washings with this buffer were placed in 1 mL of ascorbate free normal or

hypotonic (4:1/3:1/2:1 made by mixing corresponding parts of normal buffer and water) Na^+-HEPES buffer (with or without additives) in a shaking water bath (25rpm) at 37°C. After 2 or 5 min, 0.9 mL of the release solution was removed to a vial and the rest was discarded. 1mL of ice-cold water was added to the cells and the cells were scraped from the plates and removed to another vial. An aliquot of 0.1mL of the release solution was removed from these vials for protein estimation and the rest was used for scintillation counting.

The above procedure was modified for experiments involving the anion channels blocking agents. For that, the washing buffer contained the same concentration of these agents as was in the release solution.

The protein estimation was carried out with Bradford reagent purchased from BioRad using bovine serum albumin as standard (12). The concentration of the protein was estimated by intrapolation of an external standard curve.

2.5 High performance liquid chromatography assay

Some modifications were introduced in the above procedure for HPLC assay(130). The release solutions contained 10μM dexferoxamine to prevent ascorbate oxidation. After saving 0.1 mL for protein estimation, cold 8.5 percent meta-phosphoric acid was added to the release samples up to a final concentration 0.85 percent. The samples were vortexed immediately and placed on ice. Later 0.2 mL of this sample was saved and stored at -80°C to be analyzed later by HPLC (Waters 450, Mississauga Canada). The rest was used for scintillation counting. Ascorbate was quantified by HPLC

using a Waters M460 amperometer detector. Assay sensitivity was 2pmol ascorbate. Concentration of ascorbate was determined by intrapolation of an external standard curve(102).

2.6 Ethidium bromide staining

Cells were cultured as described before but on coverglass mounted on a petridish. They were washed twice with Na^+-HEPES buffer prewarmed to 37°C and then placed in the same buffer (normal or hypotonic) containing ethidium bromide (20mg /mL) and saponin (0.6mg/mL). After incubation for 2 min at 37°C, cells were washed three times with normal buffer without ethidium bromide and viewed under an LSM 510 confocal microscope.

The total number of cells observed in a field was counted along with the number of stained cells. From this the percentage of cells stained was calculated. The data obtained were subjected to one way ANOVA ($P>0.05$) followed by a Tukey-Kramer multiple comparisons statistical test.

2.7 Data analysis

Ascorbate release was calculated as percent of total uptake and was corrected for cells dislodged from the plate during the course of an experiment. Increase in ascorbate release over basal was calculated using the following formula: 100* (mean release as percent of total uptake - mean basal release as percent of total uptake) / mean basal release as percent of total uptake. Values are presented as mean ± SEM of specified

number of replicates. One-way ANOVA was used to test the null hypothesis and a P value of < 0.05 was used to negate it. This was followed by the Tukey-Kramer multiple comparisons statistical test to find the degree of significant difference between various groups. Each experiment was replicated 3-5 times, analyzed separately and one representative experiment closest to the mean result was selected and presented in this thesis.

3.0 RESULTS

It was hypothesized that the HTS induced ^{14}C-Asc release may vary with time and with the degree of hypotonicity. Therefore, the PCECs were subjected to buffers of varying osmolality for different time periods. Thus 4:1 (80 percent osmolality), 3:1 (75 percent osmolality) and 2:1 (67 percent osmolality) hypotonic buffers were prepared by mixing the corresponding proportions of normal isotonic buffer: water and the release was examined for 2 and 5 min as described in the methods.

3.1 Characterization of hypotonic shock induced ascorbate release

As evident from Fig 1A, a small amount of ^{14}C-Asc release (basal release) was observed even in the absence of HTS. Also observed from Fig 1A that ^{14}C-Asc release values for 4:1 HTS were not significantly different from the corresponding basal values for the release in either 2 or 5 min ($P>0.05$). Hence 4:1 was eliminated as an effective form of HTS for these characterization studies. Upon further inter-group comparisons, it was found that there was a significant difference in 3:1 and 2:1 HTS release values when compared to their respective basal values for 2 and 5 minutes ($p<0.05$). The difference was not found to be significant ($P>0.05$) when the 3:1 HTS release values for 2 and 5 min were compared with each other. This was also true for the 2:1 HTS. ***Thus ^{14}C-Asc release studies using either 3:1 or 2:1 HTS buffers for 2 or 5 min prove to yield promising results.***

PCECs were loaded with ^{14}C-Asc and ^{3}H-DOG. The main reason for choosing DOG as a control is that upon entering the cells it gets converted to the anion ^{3}H-DOG 6

phosphate(18). Thus Asc and DOG 6-phosphate are anions of similar molecular weights and therefore, DOG can be used to prove the specificity of the release pathway for Asc.

Fig 1B compares the ^{3}H-DOG release at different values of HTS over 2 and 5 min as percent of total ^{3}H loaded into the cells. On comparing the DOG release values calculated as percent of total uptake for 2 min, a significant increase in DOG release was observed when PCECs were stimulated with 2:1 HTS as compared to basal release DOG values ($P<0.05$). In contrast, the difference was not significant for 3:1 and 4:1 HTS caused DOG release with respect to basal release ($P>0.05$). For 5 min, a significant difference was observed when release caused by HTS (4:1/3:1/2:1) was compared to its basal release value ($P<0.05$). On comparing 4:1 vs 4:1 and 3:1 vs 3:1 HTS release values for 2 and 5 min, no significant difference was observed ($P>0.05$). This lack of significance is most likely due to high basal release value for 2 min as compared to 5 min. The possible explanation for this can be insufficient washings of cells during the experiment (see methods). ***Thus using ^{3}H-DOG release values as criteria, either 4:1 or 3:1 HTS for 2 or 5 min prove to yield promising results for further studies.***

The loss of protein (cells) in the efflux solution during the course of the experiment was calculated as percent of total protein (Fig 1C). This loss may originate from cells dislodging from the plate, cell damage due to HTS during the course of the experiment or protein remnants from fetal bovine serum in the culture media. On comparing various groups, it was found that the loss of protein in the release solution did not depend on release times or the strength of HTS. This proves that the increase in Asc release due to HTS is not an artifact.

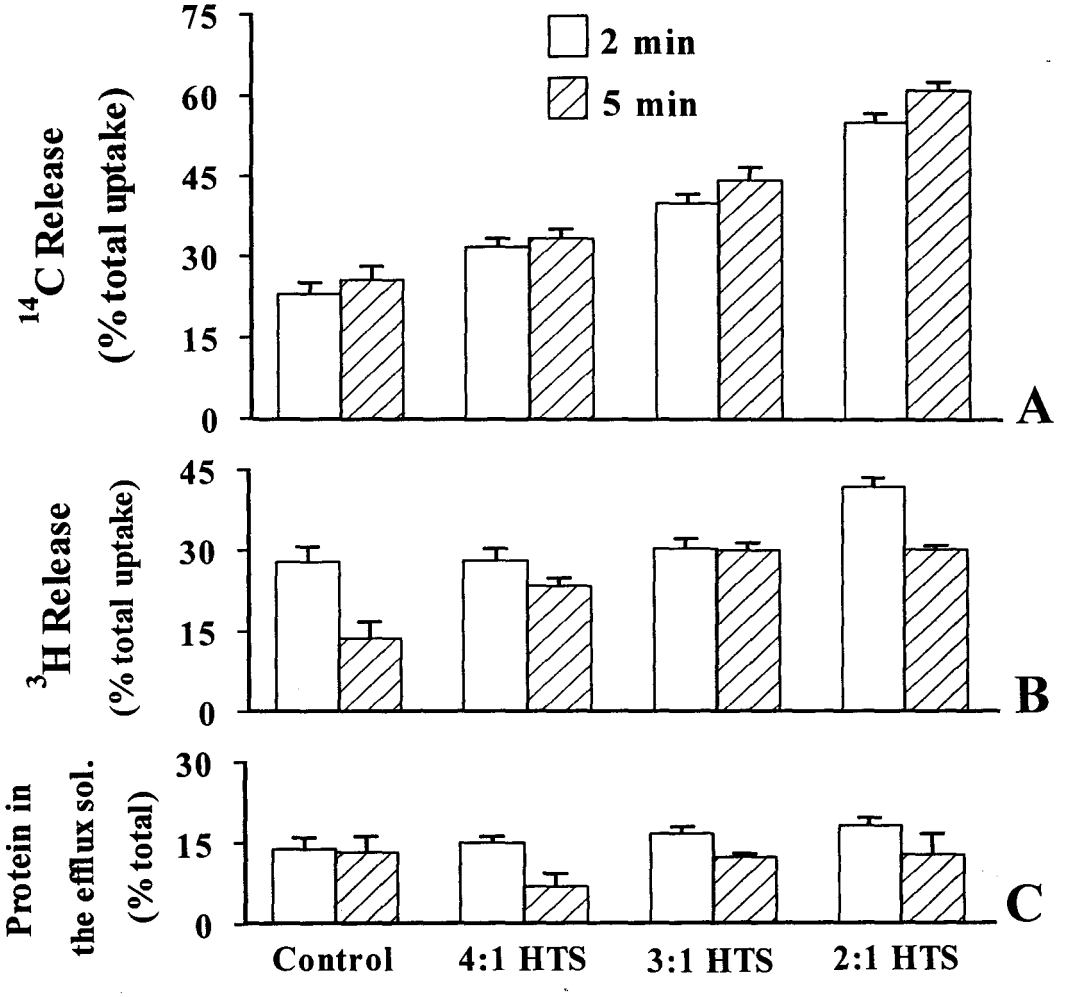

75
60
45
30
15
0
2 min
5 min
^{14}C Release
(% total uptake)
A
45
30
15
0
^{3}H Release
(% total uptake)
B
30
15
0
Protein in
the efflux sol.
(% total)
C
Control
4:1 HTS
3:1 HTS
2:1 HTS

Figure 1. Comparison of the effects of time and strength of hypotonicity on ^{14}C-Asc release

PCECs were loaded with ^{14}C Asc and ^{3}H DOG for 18h before being used for HTS induced Asc release studies. Different concentrations of hypotonic buffer were made up of corresponding parts of normal osmotic strength release buffer and water i.e. 4:1, 3:1 and 2:1. The release was examined for 2 and 5 min in addition to protein loss in the release solution. Procedural details for loading, washing and release-determination are given in the methods.

Fig 1A. ^{14}C release calculated as percent of total uptake.

Values are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test are given in Table I in the Appendix. No significant difference was observed when ^{14}C-Asc release caused by 4:1 HTS in 2 and 5 min was compared to its respective basal value ($P>0.05$). The differences were found to be significant on comparing release by 3:1 and 2:1 HTS to its respective basal value for both time plots ($P<0.05$). The experiments were replicated 4 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

Fig 1B. ^{3}H DOG release calculated as percent of total uptake.

Values are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons

statistical test are given in Table II in the Appendix. Significant difference was observed when DOG release caused by 2:1 HTS in 2 min was compared to its basal value ($P<0.05$), which was not the case for 3:1 and 4:1 HTS ($P>0.05$). Significant differences were observed when basal value was compared to all HTS conditions at 5 min ($P<0.05$). The above experiments were replicated 4 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

Fig 1C. Amount of protein released in the efflux solution calculated as percent of total protein.

Values are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test. It was observed that the protein loss in the release solution did not depend on the strength of hypotonicity ($P>0.05$). Also evident that HTS application did not cause any significant damage to the cells during the course of the experiment as no significant difference was observed between basal and HTS (4:1/3:1/2:1) caused protein loss in the release solution for 2 or 5 min ($P>0.05$). The above experiments were replicated 4 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

Asc release values were recalculated after correcting for protein loss in the release solution (see details in methods) (Fig 2A). The results obtained after one-way ANOVA and Tukey-Kramer multiple comparisons statistical test were consistent with those obtained for Fig 1A where the release values were not corrected for protein loss.

Fig 2B shows the increase in release over basal (test - control) value for 2 and 5 min. A significant difference was observed when the HTS (4:1/3:1/2:1) induced release values for 2 and 5 min were compared to their respective basal values ($P<0.05$). Insignificant differences were observed when basal vs basal, 4:1 vs 4:1, 3:1 vs 3:1 and 2:1 vs 2:1 HTS induced release values for 2 and 5 min were compared ($P>0.05$).

The rate of release for 2 and 5 min in nmol/mg/min was calculated from all experiments (Fig 2C) to examine whether the Asc release tapers off with time and mean value was taken. As evident from the comparisons, there was a significant difference between 4:1 HTS induced release and their respective basal values for 2 min and 5 min. The same was true for 3:1 and 2:1 HTS for 2 and 5 min. Multiple group comparisons showed no significant difference in 4:1vs 4:1 and 3:1 vs 3:1 HTS induced release in 2 and 5 min, as the rate of release was higher for 2 min. ***Thus on the basis of mean rate of ^{14}C Asc release as a criteria, 2 min time limit could be used for these studies.***

Conclusively, based on parameters like relative ^{14}C-Asc and ^{3}H-DOG release, protein loss in the release solution and mean rate of release, it was decided to use 3:1 HTS and for 2 min for further characterization studies.

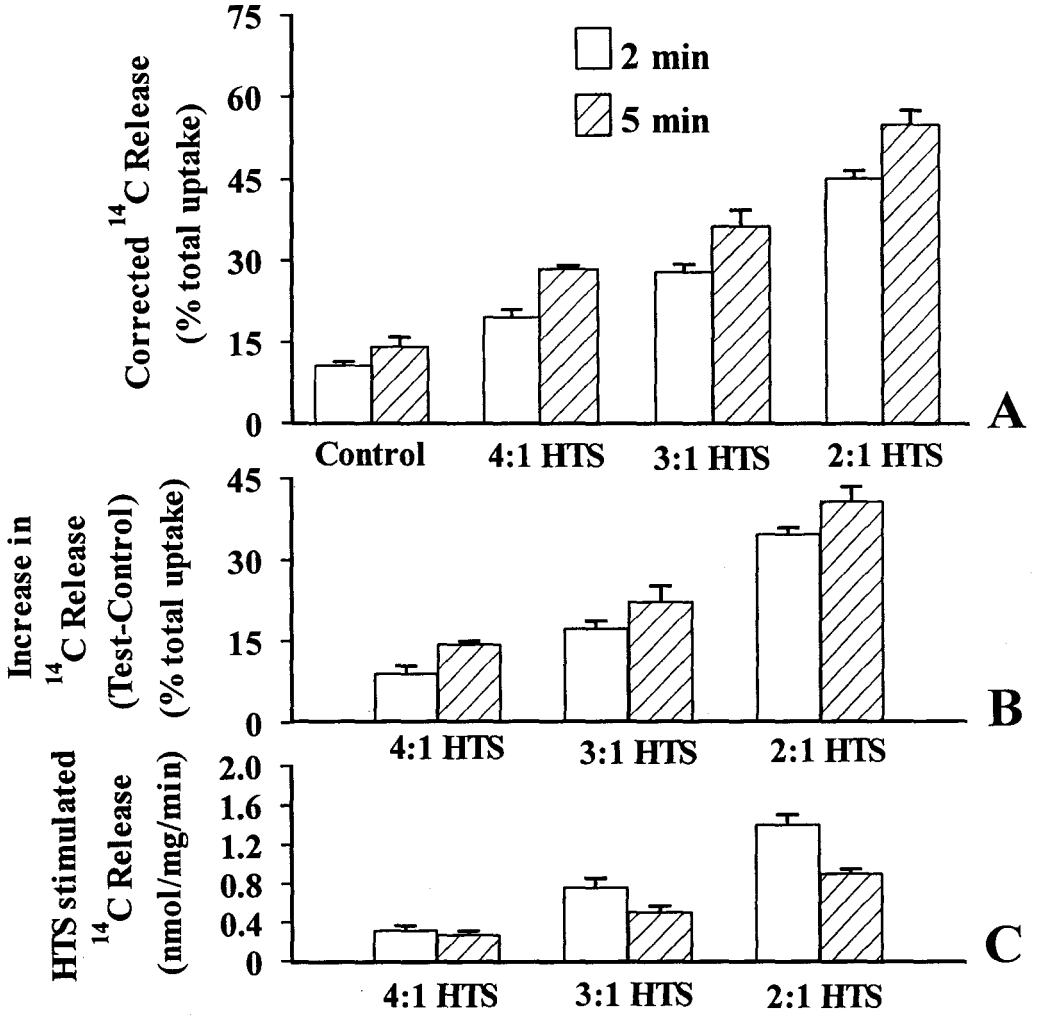

Corrected ^{14}C Release (% total uptake)
2 min
5 min
75
60
45
30
15
0
Control
4:1 HTS
3:1 HTS
2:1 HTS
A
Increase in ^{14}C Release (Test-Control) (% total uptake)
45
30
15
0
4:1 HTS
3:1 HTS
2:1 HTS
B
HTS stimulated ^{14}C Release (nmol/mg/min)
2.0
1.6
1.2
0.8
0.4
0
4:1 HTS
3:1 HTS
2:1 HTS
C

Figure 2. Analysis of comparison of the effects of time and strength of hypotonicity on ^{14}C-Asc release (Continued from Figure 1)

Details of determination of protein-corrected release and increase in release over control or basal calculations are given in the Methods. Values are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test. The following experiments were replicated 4 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

Fig 2A. ^{14}C release (corrected for the amount of protein loss in the release solution) calculated as percent of total uptake.

No significant differences were observed between the protein - loss corrected (data from Fig 1A) and uncorrected data.

Fig 2B. Increase in release over control computed from data in Fig 2A.

Detailed multiple group comparisons are given in Table III in the Appendix.

Fig 2C. Mean rate of Asc release in nmol/mg/min computed from all the experiments.

Detailed multiple group comparisons are given in Table IV in the Appendix.

To investigate whether the observed ^{14}C-Asc release in response to HTS is a concentration-gradient dependent phenomenon, values were calculated for Asc efflux as percent of total uptake corrected for protein loss and the amount of Asc loaded (nmol/mg) for control and 3:1 HTS groups (Fig 3A and B). The data was pooled from 18 experiments. The raw data was then subjected to regression analysis and r^2 values were calculated. The r^2 values for control and HTS groups were calculated as 0.069 and 0.099 respectively. Thus there is an insignificant correlation between basal (7 percent) or the 3:1 HTS (10 percent) induced ^{14}C-Asc release and the amount of Asc loaded into the cells. On correlating r and t values, a significant difference ($P<0.05$) between amount of loading and Asc release in control or HTS groups was observed. This confirmed that the Asc release observed from PCECs with or without HTS is not a concentration gradient dependent phenomenon.

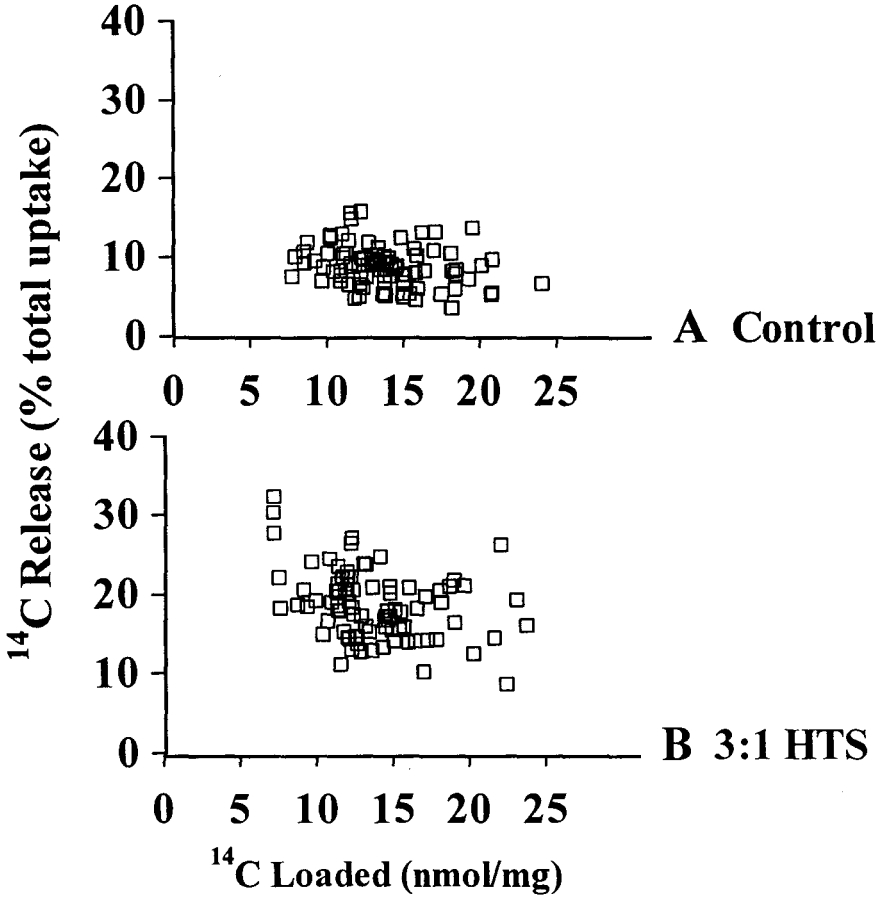
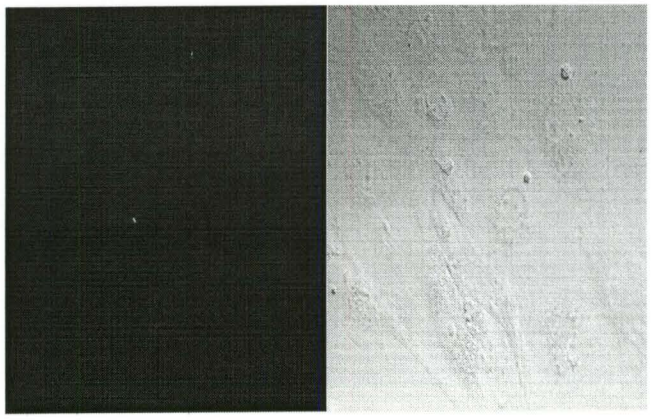


Figure 3. Correlation between ^{14}C-Asc loading (nmol/mg) and release

Values were computed for Asc release as percent of total uptake and the amount of Asc loaded (nmol/mg) for both control and 3:1 HTS groups from 18 experiments. Each data point represents an individual replica (90 replicates for each group). The data obtained were pooled and subjected to regression analysis. One-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test showed that the amount of ^{14}C Asc loaded into cells for control and 3:1 HTS groups was not significantly different ($P>0.05$) but the release calculated as percent of total uptake was significantly different ($P<0.05$).

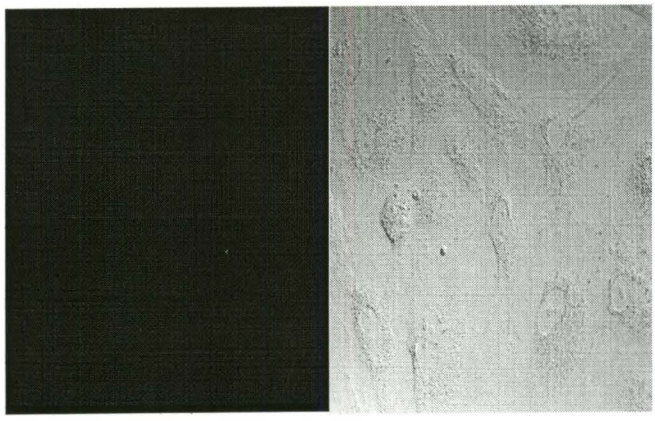
Cell membranes get damaged due to osmotic swelling under severe hypo-osmotic conditions. Therefore under milder hypo-osmotic conditions, there still exists a possibility that some cells might experience cell membrane damage due to an increase in cell membrane permeability leading to leaky membranes. As the ^{3}H-DOG release remained unaffected with increasing hypotonicity (as evident from Fig 1B), an increase in cell-permeability due to HTS is unlikely. Thus for further analysis of effect of HTS on cell-membrane permeability, ethidium bromide staining was undertaken. Ethidium bromide (MW 394.3) as a large compound doesn't readily cross the cell membrane but can go through pores created by saponin or HTS damage. In a total of 22 fields, 339 cells were examined for control. Of these 23 were stained with ethidium bromide. Similarly, 41 cells in 21 fields for 3:1 HTS were observed to be stained out of a total of 353. This corresponds to 7 ± 3 and 11 ± 4 percent cells being stained respectively. In contrast when cells were treated with saponin, 199 cells that accounted for 76±4 percent of the cells were stained out of 264 studied in 16 fields. One-way ANOVA followed by Tukey-Kramer multiple comparison statistical test showed no significant difference between control and 3:1 HTS groups ($P>0.05$) but the difference was extremely significant when these groups were compared to saponin group ($P<0.05$).



22 fields

28 fluorescent / 339 cells

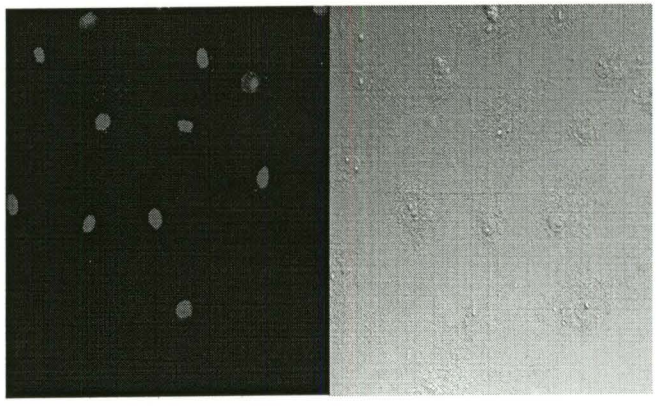
A. Control



21 fields

41 fluorescent / 353 cells

B. Hypotonic



16 fields

199 fluorescent / 264 cells

C. Saponin

Figure 4. Effect of HTS on cell-permeability studied by ethidium-bromide staining of PCECs

Fluorescence (left) and bright field images (right) of PCECs were taken after being exposed for 2 min to ethidium bromide with or without 3:1 HTS and in the presence of saponin (0.6mg/ml). Bright spots in the left panel indicate increased permeability to ethidium bromide. Data provided along the side are from all the fields examined.

Control cells showed on an average 7 ± 3 percent staining, 3:1 HTS with 11 ± 4 percent and saponin with 76 ± 4 percent. An insignificant difference was found when control and 3:1 HTS groups were compared for percentage of cells stained ($P>0.05$) but the difference was significant when these groups were compared to saponin group ($P<0.05$). The above experiment was replicated 4 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

To prove that the ^{14}C released during 3:1 HTS is Asc and not a decomposition product, HPLC analysis was performed. The loaded PCECs were observed for Asc release for 2 min with or without the presence of 3:1 HTS. There was a significant difference between basal release values of ^{14}C and HPLC ($p<0.05$) as the basal value for ^{14}C was higher than HPLC. The likely explanation for this phenomenon is the involvement of decomposed Asc in scintillation counting as compared to HPLC (Fig 5A). However, in computing the increase in release with HTS for ^{14}C and HPLC, there was no significant difference ($p>0.05$) (Fig 5B). This proves that the released material by PCECs when subjected to 3:1 HTS is authentic Asc and not a decomposition product.

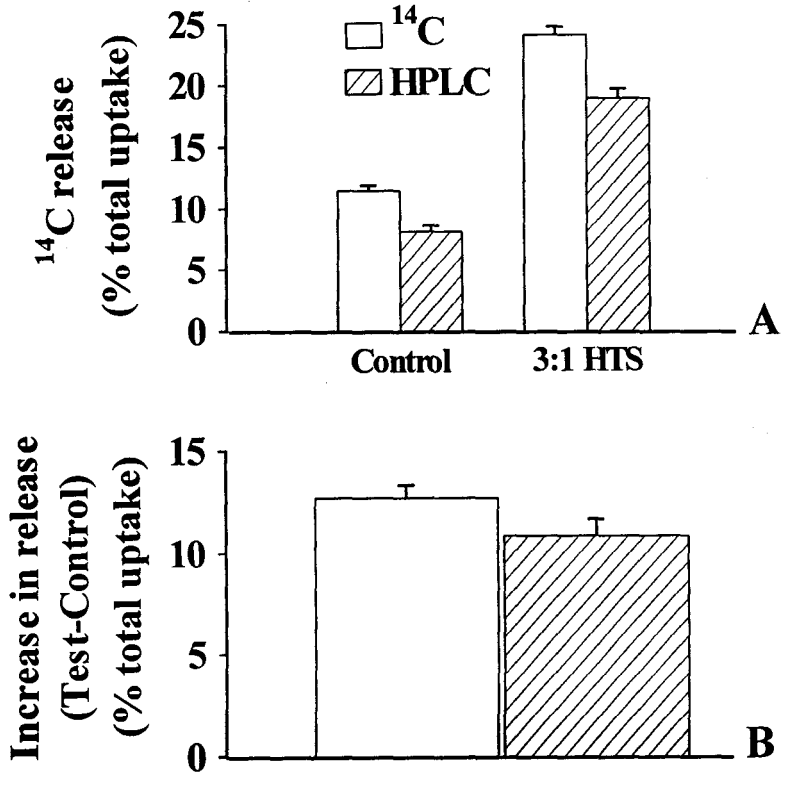


Figure 5. Confirmation of authenticity of released Asc during HTS by HPLC

Loaded PCECs were observed for Asc release with or without 3:1 HTS for 2 min using scintillation counting or HPLC. Details of experimental setup are given in the methods.

A. Asc release from the loaded cells as percent of total uptake.

Values obtained are mean ± SEM of 6 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test. On comparison basal values for scintillation counting and HPLC were observed as significantly different ($P<0.05$).

B. Percent increase in release by HTS computed from data in A.

No statistically significant difference was observed on comparing the values of scintillation counting and HPLC ($p>0.05$). The above experiment was replicated two times.

3.2 Role of ATP in hypotonic shock induced ascorbate release

In response to mechanical stress (shear or stretch), endothelial cells are known to release ATP(8; 71). Therefore to examine whether the Asc release caused by HTS was mediated by an ATP release, PCECs were subjected to 3:1 HTS with or without the presence of ATP scavenger apyrase (specific activity 2.5nmol/min) for 2 min (Fig 6). Based on the known total ATP content of PCECs(36), the above said amount of apyrase used was sufficient to hydrolyze all the cellular ATP in milliseconds if it were to be released simultaneously during the course of the experiment. Apyrase did not significantly alter the basal Asc release or that induced with HTS ($P>0.05$). Thus a potential role of release of intracellular ATP in the Asc release caused by 3:1 HTS in PCECs was ruled out.

ATP induced Asc release from PCECs is found to be mediated by the activation of P2Y2 receptors (unpublished results from our lab). This release was inhibited in the presence of suramin (300μM), a potent inhibitor of P2Y2 receptors. If any ATP was to be released, it is possible that it may have triggered an effect before being hydrolyzed with apyrase. To exclude this possibility, PCECs were subjected to 3:1 HTS with or without the presence of suramin (300μM) for 2 min (Fig 7). Suramin neither altered the basal nor the HTS induced ^{14}C-Asc release ($P>0.05$). This shows that ATP activated P2Y2 receptors has no role in the Asc release caused by 3:1 HTS in PCECs.

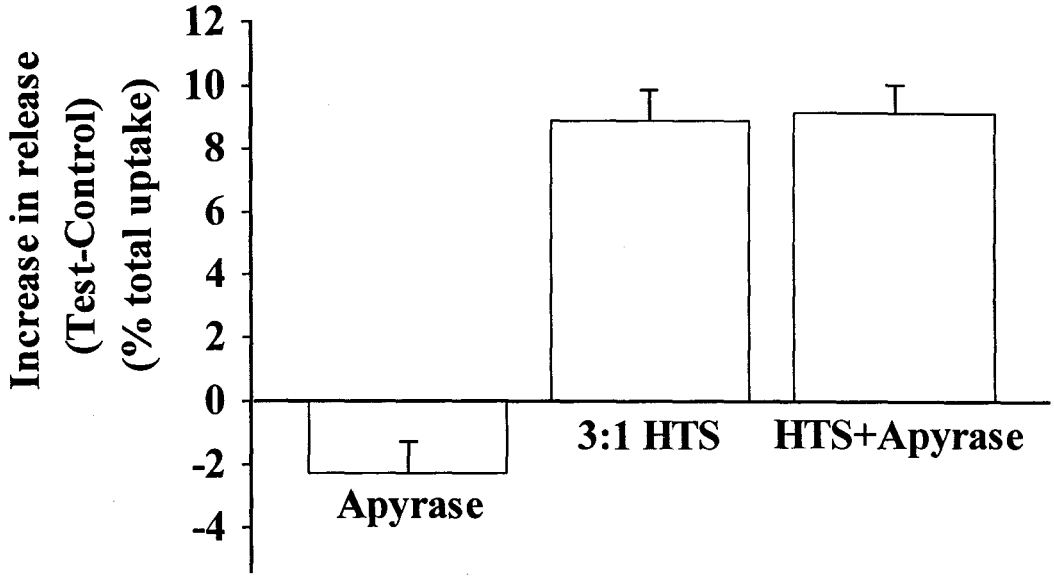


Figure 6. Effect of apyrase on ^{14}C-Asc release induced by HTS

The release was examined using 3:1 HTS with or without the presence of apyrase (specific activity 2.5 nmol/min) for 2 min. Values obtained are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer statistical test. It was observed that apyrase did not cause a significant change in basal release or that caused by 3:1 HTS ($P>0.05$). The above experiment was replicated 3 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

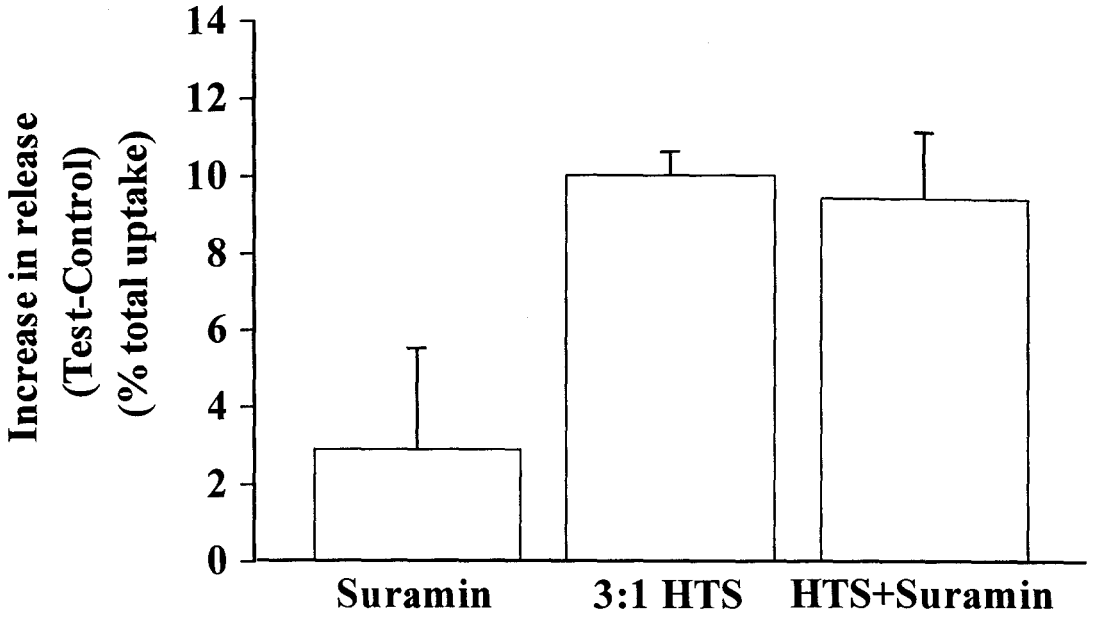


Figure 7. Effect of suramin on ^{14}C-Asc release induced by HTS

Loaded PCECs were observed for after being subjected to 3:1 HTS with or without the presence of suramin (300μM) for 2 min. Values obtained are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer statistical test. The release caused by suramin was not significantly different from basal ($P>0.05$). Also, the release caused by 3:1 HTS in the presence of suramin was found to be insignificantly different from what it caused by suramin independently ($P>0.05$). The above experiment was replicated 5 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

To examine whether there is an additive release caused by HTS and ATP, PCECs were subjected to 3:1 HTS with or without the presence of 1mM ATP for 2 min (Fig 8). 1mM ATP was used in this experiment because it causes the maximum ^{14}C-Asc release (unpublished results from the lab). Both ATP and HTS caused a significant increase in the Asc release greater than that produced by ATP or HTS independently ($P<0.05$). Thus this additive release caused by ATP and HTS together is consistent with the theory that there are two different pathways for Asc release: one mediated by ATP and another by HTS.

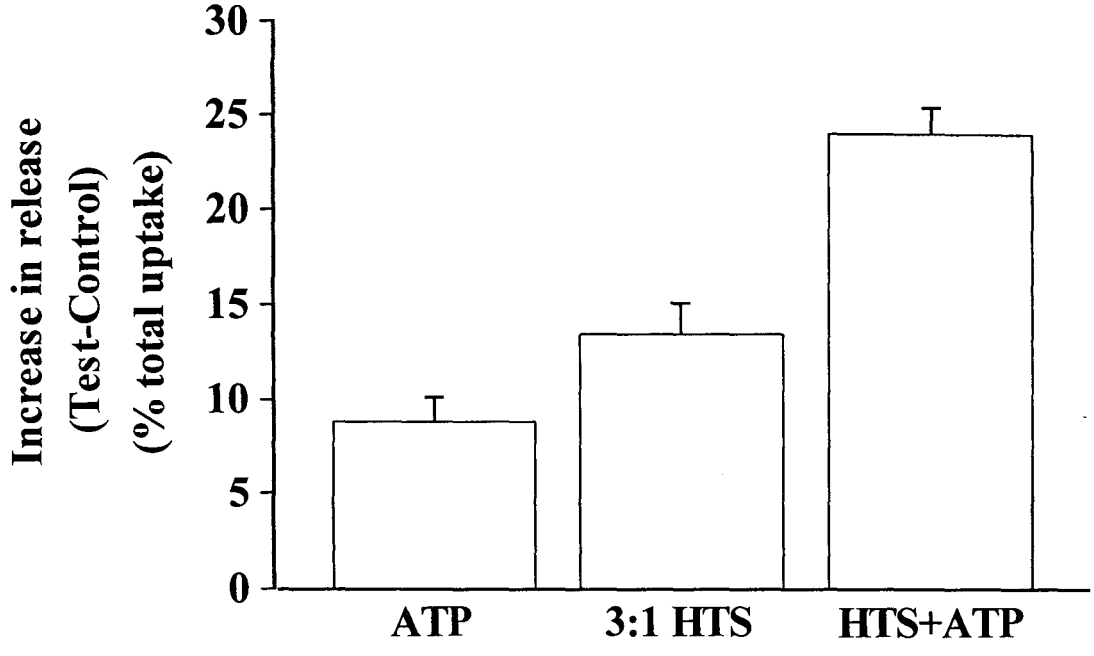


Figure 8. Comparison of ^{14}C Asc release induced by ATP, HTS and HTS+ATP

The release was examined with ATP (1mM), with or without the presence of 3:1 HTS for 2 minutes. Values obtained are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer statistical test. The release caused by 3:1 HTS and ATP together was observed to be significantly different from what caused by HTS or ATP independently ($P<0.05$). The above experiment was replicated 3 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

3.3 Role of Ca^{2+} in hypotonic shock induced ascorbate release

The ATP induced Asc release is Ca^{2+} dependent (unpublished results from the lab). An increase in $[Ca^{2+}]i$ was observed in PCECs in response to extracellular ATP. Furthermore, the Ca^{2+} ionophore A23187 also produced an increase in Asc release. To investigate a possible role of Ca^{2+} in HTS induced release 3:1 HTS was used in addition to Ca^{2+} ionophore A23187 at a concentration that has been observed previously to be sufficient to saturate Ca^{2+} mediated Asc release (unpublished data from the lab). The concentration used was 10 μM A23187 since it has been shown to produce the maximum Asc release. PCECs were subjected to 3:1 HTS with or without the presence of 10μM Ca^{2+} ionophore A23187 for 2 min (Fig 9). A23187 and HTS independently caused an increase in the Asc release. But the increase in release caused by HTS and A23187 together was greater than that by HTS or A23187 alone ($P<0.05$). Thus this additive release caused by A23187 and 3:1 HTS together is consistent with the concept that there are two different pathways causing Asc release: one mediated by Ca^{2+} and another by HTS.

To prove further the role of Ca^{2+} in HTS stimulated Asc release, PCECs were subjected to 3:1 HTS in solutions containing 1.5 mM or 0 mM $CaCl_2$ (nominally Ca^{2+} free) for 2 min (Fig 10). Nominally Ca^{2+} free did not significantly alter the basal ^{14}C-Asc release or that induced by HTS ($P>0.05$). Thus the HTS induced Asc release did not depend on the extracellular Ca^{2+}. This property is again different from the ATP induced ^{14}C-Asc release which requires extracellular Ca^{2+} (unpublished results from the lab).

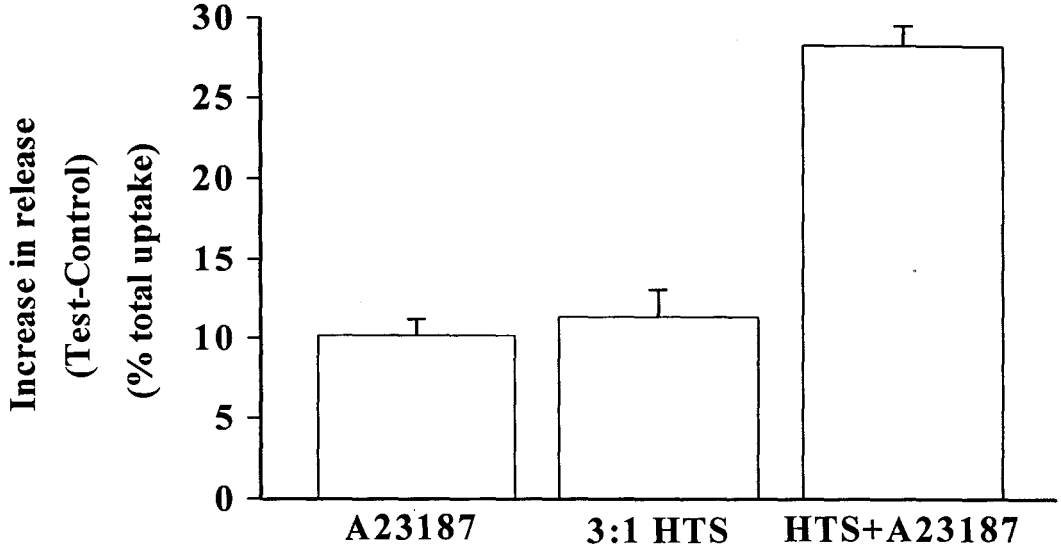


Figure 9. Comparison of ^{14}C Asc release induced by A23187, HTS and HTS+A23187.

Loaded PCECs were used for Asc release by 10 μM A23187 with or without the presence of 3:1 HTS for 2 minutes. Values obtained are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer statistical test. The release that was observed in the presence of A23187 or 3:1 HTS was found to be significantly different from basal ($P<0.05$). The release caused by A23187 + HTS was significantly different from that caused by A23187 or HTS alone ($P<0.05$). The above experiment was replicated 2 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

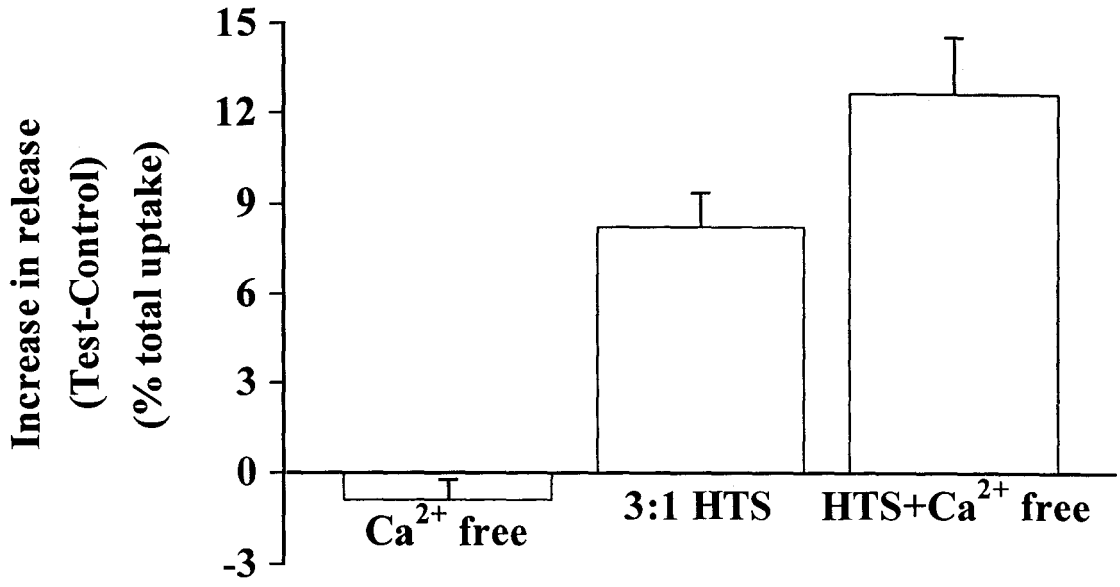


Figure 10. Effect of nominally Ca^{2+} free buffer on ^{14}C-Asc release induced by 3:1 HTS.

Loaded PCECs were used for Asc release with or without 3:1 HTS and with or without the presence of nominally Ca^{2+} free buffer for 2 min. Values obtained are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer statistical test. The release that was observed in the presence of Ca^{2+} free buffer was not significantly different from basal ($P>0.05$). Also, the release caused by 3:1 HTS with or without Ca^{2+} free buffer was not significantly different ($P>0.05$). The above experiment was replicated 4 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

3.4 Role of anion channel blockers in hypotonic shock induced ascorbate release

To prove that anion channels play a role in Asc release, PCECs were subjected to various anion channel blockers. It was observed that the ATP induced Asc release is inhibited nearly completely by the anion channel inhibitors: 300μM each of IAA-94, niflumic acid, NPPB and sulfinpyrazone (300μM or 1mM)(unpublished data from the lab). Therefore, the effects of these agents on the HTS induced Asc release was also examined. An experiment was conducted to compare the effects of niflumic acid on the release caused by ATP and HTS in 5 min (Fig 11). This release time was chosen because the ATP induced release was observed to be optimum at 5 min and also the inhibitors need some time to bind and show their effect. It was observed that niflumic acid completely inhibited the ATP induced Asc release but the inhibition of the HTS induced release was only partial.

In other experiment using HTS, various non-specific anion channels blocking agents (IAA-94, niflumic acid, NPPB and sulfinpyrazone) were also tested for the Asc release over 5 min (Fig 12A and B). Out of these agents only NPPB caused significant inhibition of the HTS induced Asc release ($P<0.05$). Thus the HTS induced Asc release differs from the ATP induced release, which is inhibited nearly completely by these agents.

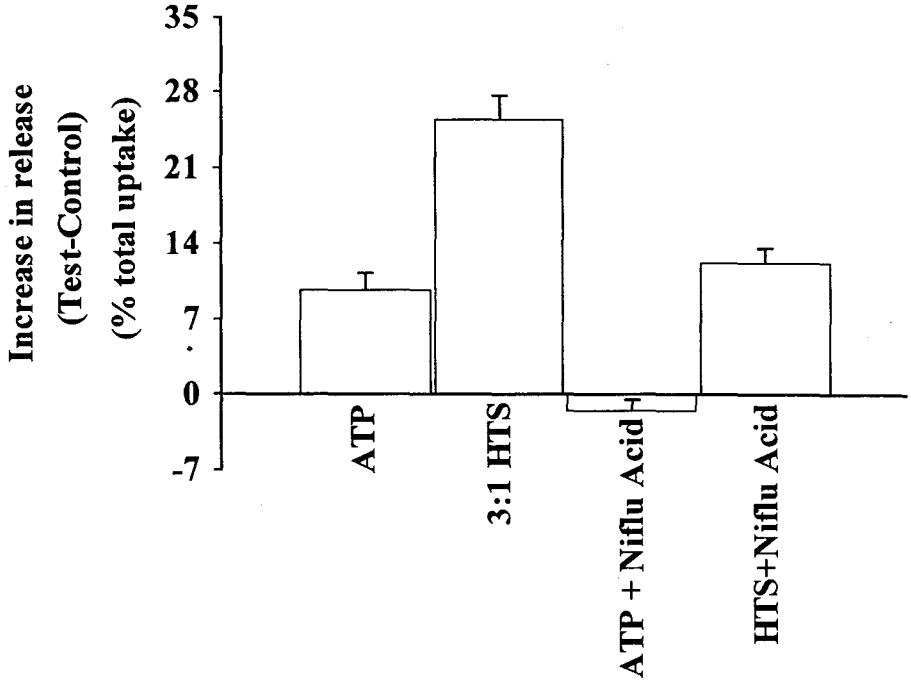


Figure 11. Effect of niflumic acid on ^{14}C-Asc release induced by ATP or HTS

Asc loaded PCECs were used for release after being subjected to ATP (1mM) and 3:1 HTS with or without the presence of niflumic acid (300μM) for 5 minutes. Values obtained are mean ± SEM of 8,6,6,6 replicates for ATP, ATP + niflumic acid, 3:1 HTS and HTS + niflumic acid groups respectively. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test. It was observed that niflumic acid completely blocked the ATP-mediated Asc release but partially blocked the HTS-mediated Asc release. The above experiment was replicated 3 times. The pooled data when subjected to statistical analyses gave similar results (not shown).

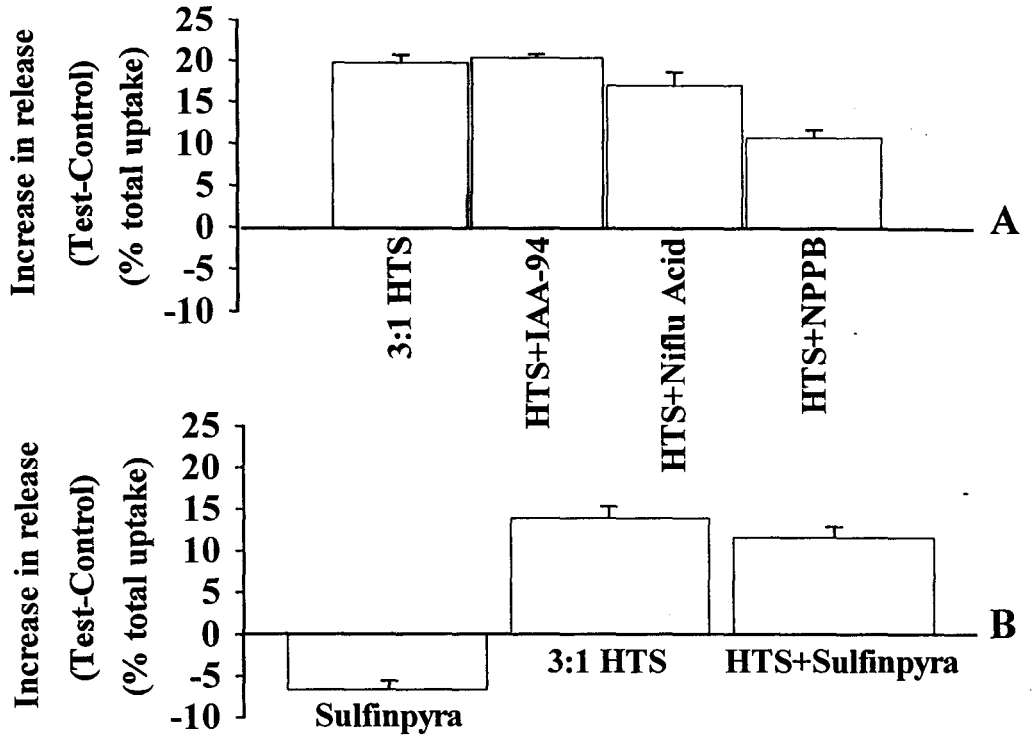


Figure 12A. Effect of anion channel blockers on ^{14}C-Asc release induced by HTS.

Loaded PCECs were observed for release after being subjected to HTS with or without the presence of IAA-94, niflumic acid and NPPB (300μM each) for 5 minutes. Values obtained are mean ± SEM of 7,5,5,5 replicates for HTS, HTS + IAA-94, HTS + niflumic acid, HTS + NPPB groups respectively. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test. Addition of IAA-94 and niflumic acid failed to cause any significant inhibition of HTS induced Asc release ($P>0.05$) as compared to NPPB ($P<0.05$). The above experiment was performed only once.

Fig 12B. Effect of Sulfinpyrazone on ^{14}C-Asc release caused by 3:1 HTS.

Loaded PCECs were observed for release after being subjected to HTS with or without the presence of sulfinpyrazone (300 μM) for 5 minutes. Values obtained are mean ± SEM of 5 replicates. Differences between various groups were evaluated by one-way ANOVA followed by Tukey-Kramer multiple comparisons statistical test. Addition of sulfinpyrazone did not significantly alter the HTS induced Asc release ($P>0.05$). The above experiment was performed only once.

4.0 DISCUSSION

According to this study, pig coronary artery endothelial cells (PCECs) release ascorbate in response to hypotonic shock (HTS). The observed release was authentic ascorbate and it did not result from increased membrane permeability as shown by ethidium bromide staining. No correlation was observed between the amount of ascorbate loaded and released in response to HTS. Further characterization of this release as a model for shear stress along with its relation to ATP induced release will be discussed here.

Most mammalian cells as a result of rapid alteration of intra and extracellular osmolality during HTS, exhibit passive water uptake thus resulting in osmotic-swelling. In most cell types, the coordinated action of K^+ and Cl^- channels is the principal mechanism to restore their volume to original state. Loss of these ions induces water egress and regulated volume decrease(40; 41). Cell membrane depolarization caused by the loss of Cl^- ions activates voltage sensitive calcium channels. Calcium influx through these channels results in consequent rise in intracellular calcium that in turn is responsible for the activation of calcium-activated K^+ channels. In order to maintain electroneutrality, the release of K^+ is paralleled by the release of Cl^- ions by volume-regulated anion channels(41). In aortic endothelial cells, regulated volume decrease occurs due to activation of both K^+ and Cl^- channels and the K^+-Cl^- cotransporter(90). Cortical astrocytes have been reported to respond to HTS via release of K^+ and Cl^- ions and organic osmolyte (taurine) through volume regulated anion channels(120).

4.1 Role of ATP in ascorbate release

Mechanical stress (shear stress and HTS) causes ATP release via vesicular exocytosis in endothelial cells(9). Extracellular ATP has been found to facilitate regulated volume decrease in rat neuroblastoma(11) as well as *Necturus* red blood cells(60). The released ATP binds to a specific class of G protein coupled receptors leading to phospholipase C activation and inositol 1,4,5 trisphosphate formation, thereby inducing Ca^{2+} mobilization. This in turn results in release of Cl^- ions via Ca^{2+} activated chloride channels. Osmotic swelling is also accompanied with dilution of intracellular medium resulting in decreased ionic strength. This decrease in ionic strength and depolarization (by extrusion of Cl^- ions) activates volume regulated anion channels. Thus there are two parallel anion release mechanisms getting activated in response to HTS one mediated by ATP involving Ca^{2+} activated chloride channels and other by cell swelling involving Ca^{2+} independent volume regulated anion channels. Human hepatocytes exposed to HTS have been reported to release ATP that serves as an autocrine trigger to restore the volume of swollen cells under hypotonic conditions resulting in release of anions via volume regulated anion channels(28).

Under isotonic conditions, PCECs subjected to extracellular ATP release ascorbate via activation of Ca^{2+} activated anion channels (unpublished results from the lab). To examine the role of endogenous ATP release in triggering HTS induced ascorbate release, PCECs were exposed to apyrase sufficient to degrade the whole cell content of ATP if it is to be released. A reduction in HTS induced release of ascorbate in the presence of apyrase would support a role for extracellular ATP. Surprisingly, apyrase

did not inhibit this release thus proving that ATP release is not a prerequisite for the activation of the volume regulated anion channels in PCECs through which ascorbate is released. The activation of volume regulated anion channels in neuroblastoma(11) and human fetal jejunum derived intestine 407 cells(37; 113) has been observed to be ATP independent.

In human hepatocytes, suramin was observed to inhibit regulated volume decrease and in turn inhibit activation of volume regulated anion channels(28). ATP induced ascorbate release from PCECs is observed to be inhibited by suramin. Occurrence of Asc release due to HTS even in the presence of apyrase suggested that ATP was not involved in this pathway. However, there remained a possibility that endogenously released ATP acted before it was degraded by apyrase. Therefore, ascorbate release in the presence of suramin was also examined. The presence of suramin did not alter the HTS induced release in PCECs suggesting that endogenously released ATP does not modulate the release.

The next experiment was conducted to examine whether HTS and extracellular ATP share the ascorbate release mechanism. An additive release was observed from PCECs subjected to HTS and ATP together that what did they cause independently. This proves that there are two different and independent release mechanisms at work elicited by extracellular ATP and HTS. Thus PCECs may have two parallel pathways to release ascorbate to combat different stressful conditions (shear stress and oxidative stress) efficiently in case of desensitization of one of the pathways.

4.2 Role of Ca^{2+} in ascorbate release

In most cells under HTS, Ca^{2+} plays a central role in regulatory volume decrease mechanisms and anions release via activation of volume regulated anion channels. However, this Ca^{2+} dependency is variable among different cell types. McCarty & O'Neil(68) summarized this variation by grouping cells into three general categories based on relative dependency of regulatory volume decrease on Ca^{2+}:

1. Cells that display little Ca^{2+} dependency. e.g. lymphocytes
2. Cells that require a certain basal $[Ca^{2+}]i$ level or transient release of Ca^{2+} from internal stores. e.g Ehlrich ascites tumor cells and medullary thick ascending limb cells.
3. Cells those are highly dependent on extracellular $Ca^{2+}$and the activation of Ca^{2+} influx. e.g. renal proximal straight tubule cells.

In case of *Necturus* red blood cells(60), regulated volume decrease was inhibited in low Ca^{2+} buffer. Rat caudal artery endothelial cells when faced with HTS require a basal Ca^{2+} level to achieve regulatory volume decrease via activation of volume regulated anion channels(106). HTS also increases cytosolic Ca^{2+} in bovine aortic endothelial cells by triggering ATP release in response to HTS(51). Thus to examine whether HTS stimulated ascorbate release in PCECs is sensitive to Ca^{2+} like the ATP induced release, PCECs were subjected to nominally Ca^{2+} free conditions and the release was examined. A reduction in the release would signify a role of Ca^{2+} in the HTS stimulated Asc release. Surprisingly, the release was not depressed suggesting that Ca^{2+} does not play a major role in HTS stimulated ascorbate release by volume regulated anion channels in PCECs.

Also note that volume regulated anion channels as already pre-activated under isotonic conditions require only a basal level of Ca^{2+} for activation in response to HTS. Thus there is still need to confirm the role of Ca^{2+} in HTS induced release by chelating extracellular and intracellular Ca^{2+} using agents like BAPTA and BAPTA-AM respectively.

In *Necturus* red blood cells(60), Ca^{2+} ionophore A23187 potentiates regulated volume decrease in response to HTS. To find out any link between HTS and ATP induced release pathways, a particular concentration of A23187 was used to saturate the Ca^{2+} mediated ascorbate release pathway. An additive release was observed by co-application of A23187 and HTS than what did they cause independently. This proves again that ascorbate release during HTS is Ca^{2+} independent and that extracellular ATP and HTS follow separate intracellular ascorbate release mechanisms.

4.3 Role of anion channels in ascorbate release

ATP stimulated ascorbate release from PCECs was completely blocked by anion channel inhibitors such as niflumic acid, IAA-94, sulfinpyrazone and NPPB. Niflumic acid and NPPB put a partial block (50 percent) on the HTS stimulated ascorbate release from PCECs. The usefulness of these blockers to determine the nature of the channels is limited by their low to moderate affinity and non-selectivity as niflumic acid has also been observed to inhibit calcium activated chloride channels(19). NPPB is not the most specific anion channels inhibitor either as its IC_{50} (half maximal inhibitory concentration) values for calcium activated and volume regulated anion channels are very similar. NPPB is also known as an effective inhibitor of Na^{+}-$2Cl^{-}$-K^{+} co-transporter and anion

exchangers(25). Furthermore these inhibitors also inhibit other ion-transporting systems, cation channels and intracellular processes.

Also note that volume regulated anion channels need a basal Ca^{2+} level to get activated and get inactivated in response to high extracellular ATP concentration. These channels allow large molecules like gluconate (MW 196) to pass through thus possibly allowing passage to ascorbate (MW 176). Thus volume regulated anion channels could be the possible candidates to mediate ascorbate release, as the observed release in response to HTS is not Ca^{2+} or ATP sensitive.

4.4 Model based on the results obtained

PCECs exhibit swelling due to water uptake in response to HTS. To regain their initial volume back, these cells release water along with anions (ascorbate) through volume regulated anion channels. The volume regulated anion channels could get activated due to membrane tension instigated by swelling and depolarization via release of anions. The observed release as is not sensitive to extracellular ATP and Ca^{2+} mobilization and thus could be mediated by volume regulated anion channels but not by calcium activated chloride channels.

4.5 Pathophysiological significance

ATP is released from the endothelial cells during shear stress and vascular injury(8). The beneficial effect of the released ATP is may be to produce vasodilation via activation of P2 receptors that leads to nitric oxide synthase activity. Ascorbate helps to

enhance the activity of nitric oxide synthase by keeping its cofactor tetrahydrobiopterin in reduced form(30; 43). The injurious effect of ATP is that it can lead to the development of atherosclerosis due to monocyte adherence to the vessel wall. Nitric oxide released by the endothelium into the blood stream inhibits monocyte adhesion to the walls before being inactivated by oxyhaemoglobin(115). Deficiency of the ascorbate leads to increased integrin expression on monocytes and thus elevated adhesion to endothelial cells(131). By releasing stored ascorbate in response to ATP and shear stress, endothelial cells can thus play a protective role by preventing such an adhesion that leads to atherosclerosis.

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6.0 APPENDIX

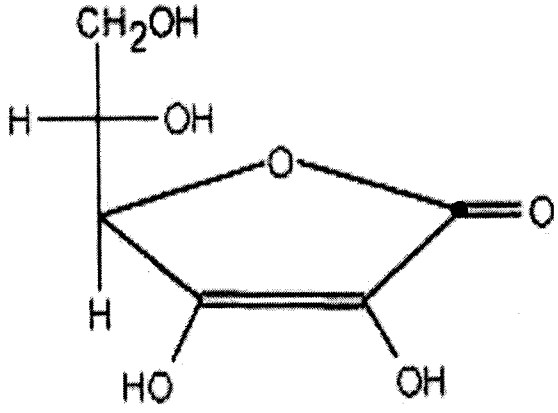

Fig 1. ^{14}C Ascorbate

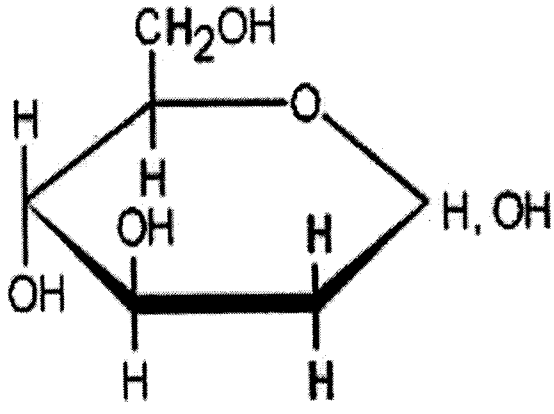

Fig 2. ^{3}H Deoxyglucose

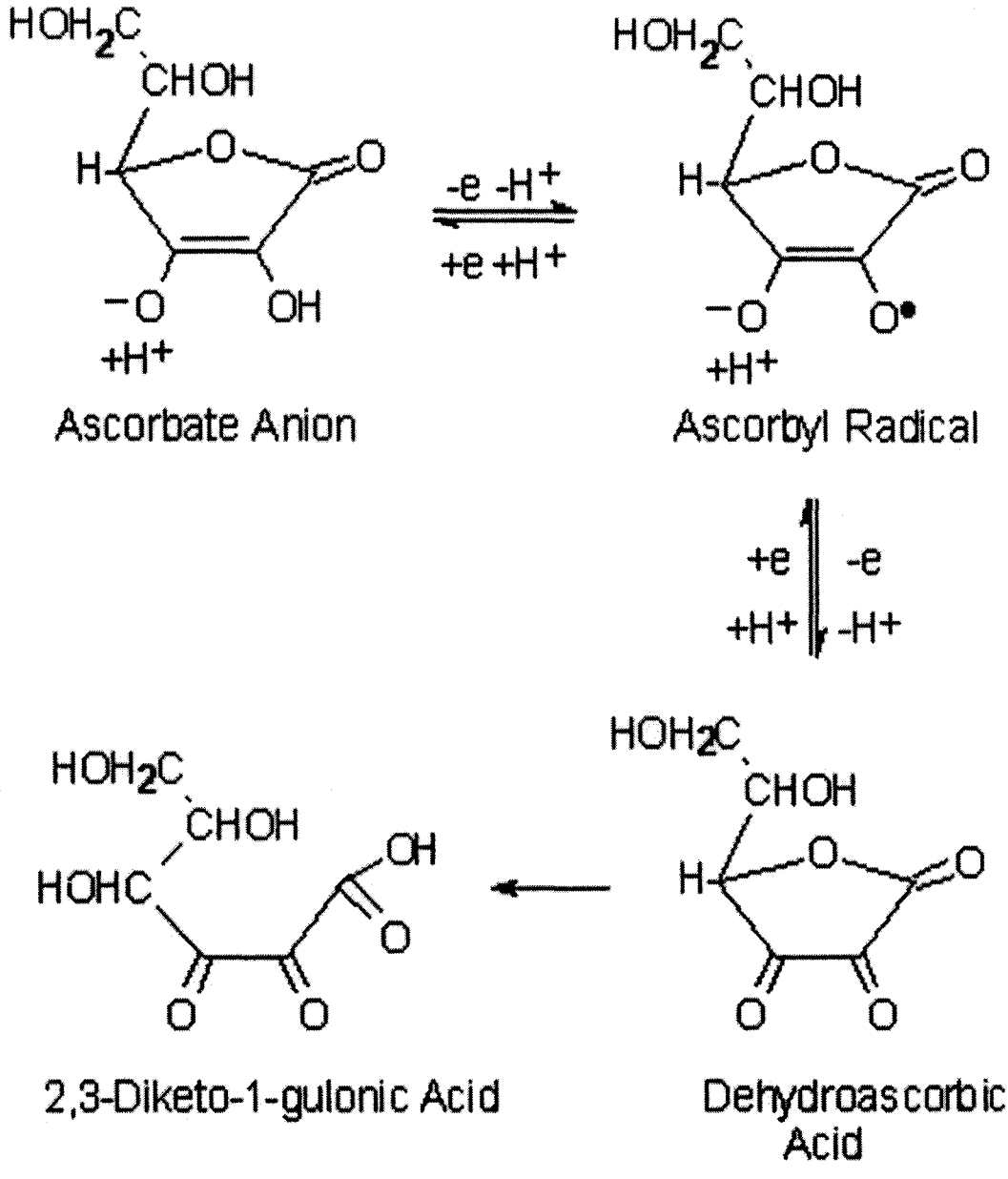

Fig 3. Metabolism of Ascorbic acid (66)

Table I Comparative ^{14}C-Asc release from PCECs in response to HTS

(Data from Fig 1A)

	Comparison	Resultant Significance	P value
1)	Control 2 min Vs 4:1 2 min	NS	P>0.05
2)	Control 2 min Vs 3:1 2 min	***	P<0.001
3)	Control 2 min Vs 2:1 2 min	***	P<0.001
4)	Control 2 min Vs Control 5 min	NS	P>0.05
5)	4:1 2 min Vs 3:1 2 min	NS	P>0.05
6)	4:1 2 min Vs 2:1 2 min	***	P<0.001
7)	4:1 2 min Vs 4:1 5 min	NS	P>0.05
8)	3:1 2 min Vs 2:1 2 min	***	P<0.001
9)	3:1 2 min Vs 3:1 5 min	NS	P>0.05
10)	2:1 2 min Vs 2:1 5 min	NS	P>0.05
11)	Control 5 min Vs 4:1 5 min	NS	P>0.05
12)	Control 5 min Vs 3:1 5 min	***	P<0.001
13)	Control 5 min Vs 2:1 5 min	***	P<0.001
14)	4:1 5 min Vs 3:1 5 min	**	P<0.01
15)	4:1 5 min Vs 2:1 5 min	***	P<0.001
16)	3:1 5 min Vs 2:1 5 min	***	P<0.001

"NS" indicates not significant difference between the two compared groups.

* indicates the degree of significant difference between the two compared groups

Table II. Comparative ^{3}H-DOG release from PCECs in response to HTS.

(Data from Fig 1B)

	Comparison	Resultant Significance	P value
1)	Control 2 min Vs 4:1 2 min	NS	P>0.05
2)	Control 2 min Vs 3:1 2 min	NS	P>0.05
3)	Control 2 min Vs 2:1 2 min	***	P<0.001
4)	Control 2 min Vs Control 5 min	***	P<0.001
5)	4:1 2 min Vs 3:1 2 min	NS	P>0.05
6)	4:1 2 min Vs 2:1 2 min	**	P<0.01
7)	4:1 2 min Vs 4:1 5 min	NS	P>0.05
8)	3:1 2 min Vs 2:1 2 min	**	P<0.01
9)	3:1 2 min Vs 3:1 5 min	NS	P>0.05
10)	2:1 2 min Vs 2:1 5 min	**	P<0.01
11)	Control 5 min Vs 4:1 5 min	*	P<0.05
12)	Control 5 min Vs 3:1 5 min	***	P<0.001
13)	Control 5 min Vs 2:1 5 min	***	P<0.001
14)	4:1 5 min Vs 3:1 5 min	NS	P>0.05
15)	4:1 5 min Vs 2:1 5 min	NS	P>0.05
16)	3:1 5 min Vs 2:1 5 min	NS	P>0.05

"NS" indicates not significant difference between the two compared groups.

* indicates the degree of significant difference between the two compared groups

Table III. Comparative increase in ^{14}C-Asc release over basal in response to HTS.

(Data from Fig 2B)

	Comparison	Resultant Significance	P value
1)	Control 2 min Vs 4:1 2 min	*	P<0.05
2)	Control 2 min Vs 3:1 2 min	***	P<0.001
3)	Control 2 min Vs 2:1 2 min	***	P<0.001
4)	Control 2 min Vs Control 5 min	NS	P>0.05
5)	4:1 2 min Vs 3:1 2 min	NS	P>0.05
6)	4:1 2 min Vs 2:1 2 min	***	P<0.001
7)	4:1 2 min Vs 4:1 5 min	NS	P>0.05
8)	3:1 2 min Vs 2:1 2 min	***	P<0.001
9)	3:1 2 min Vs 3:1 5 min	NS	P>0.05
10)	2:1 2 min Vs 2:1 5 min	NS	P>0.05
11)	Control 5 min Vs 4:1 5 min	***	P<0.001
12)	Control 5 min Vs 3:1 5 min	***	P<0.001
13)	Control 5 min Vs 2:1 5 min	***	P<0.001
14)	4:1 5 min Vs 3:1 5 min	NS	P>0.05
15)	4:1 5 min Vs 2:1 5 min	***	P<0.001
16)	3:1 5 min Vs 2:1 5 min	***	P<0.001

"NS" indicates not significant difference between the two compared groups.

* indicates the degree of significant difference between the two compared groups

Table IV. Comparative rate of ^{14}C-Asc release from PCECs in response to HTS.

(Data from Fig 2C)

	Comparison	Resultant Significance	P value
1)	Control 2 min Vs 4:1 2 min	*	P<0.05
2)	Control 2 min Vs 3:1 2 min	***	P<0.001
3)	Control 2 min Vs 2:1 2 min	***	P<0.001
4)	Control 2 min Vs Control 5 min	NS	P>0.05
5)	4:1 2 min Vs 3:1 2 min	***	P<0.001
6)	4:1 2 min Vs 2:1 2 min	***	P<0.001
7)	4:1 2 min Vs 4:1 5 min	NS	P>0.05
8)	3:1 2 min Vs 2:1 2 min	***	P<0.001
9)	3:1 2 min Vs 3:1 5 min	NS	P>0.05
10)	2:1 2 min Vs 2:1 5 min	***	P<0.001
11)	Control 5 min Vs 4:1 5 min	*	P<0.05
12)	Control 5 min Vs 3:1 5 min	***	P<0.001
13)	Control 5 min Vs 2:1 5 min	***	P<0.001
14)	4:1 5 min Vs 3:1 5 min	NS	P>0.05
15)	4:1 5 min Vs 2:1 5 min	***	P<0.001
16)	3:1 5 min Vs 2:1 5 min	**	P<0.01

"NS" indicates not significant difference between the two compared groups.

* indicates the degree of significant difference between the two compared groups.