THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON GROWTH AND CYCLOOXYGENASE EXPRESSION IN MURINE MELANOMA (BL6) CELLS

THESIS

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ABSTRACT

This thesis examines the effect of combined vitamin E succinate and Asc supplementation on the *in vitro* growth of a non-malignant monkey kidney (LLCMK) and a malignant melanoma (BL6) cell line, with nutritional concentration ranges of 5-20μg/ml and 25-50μg/ml respectively. Vitamin E and C are thought to interact synergistically to inhibit tumour cell growth by virtue of their antioxidant properties, whereby they quench free radicals and terminate lipid peroxidation. Furthermore vitamin E and C are thought to modulate the biosynthetic pathways in arachidonic acid metabolism at a number of different points. This may also offer a means of regulating tumour cell growth. It is well documented that vitamin E and C are distributed in the lipid and aqueous phases in the cell respectively. However, the cells need to obtain the vitamins from the environment in which they are found in order to exert a growth inhibitory effect. Supplementation of combined vitamin E succinate and Asc on BL6 and LLCMK cells resulted in a significant increase in LLCMK cell growth, and a significant decrease in cell growth was observed in BL6 cells.

Vitamin E succinate in its esterified form cannot function as an antioxidant and requires the cleavage of the succinate to become an active antioxidant. The metabolism of vitamin E succinate to form free vitamin E in LLCMK and BL6 cells resulted in the cleavage of the succinate group from the vitamin E molecule in BL6 cells only, thus suggesting that an esterase may be present in BL6 cells. This would allow for a synergistic interaction between the two vitamins.

The arachidonic acid cascade generates a family of bioactive lipids that modulate diverse physiological and pathological responses including tumour growth and promotion. The enzyme prostaglandin endoperoxide synthase (PGHS) or cyclooxygenase (Cox) is the key enzyme in the biosynthetic pathway leading to the formation of prostaglandins. Two enzyme isoforms of Cox have been identified, Cox 1 and Cox 2.

Supplementation with vitamin E succinate and Asc at a combination 20:25μg/ml respectively resulted in a trend of increasing Cox activity over 12 hours suggesting that vitamin E and Asc have a stimulatory effect on Cox activity in BL6 cells. The inhibitors of Cox 2, dexamethasone, showed a decreasing trend in Cox activity at the 20:25μg/ml combination, while cycloheximide showed an initial stimulatory effect and then a gradual decrease in Cox activity. The elimination of the Cox activity by dexamethasone suggests that transcriptional regulation may be occurring in BL6 cells.

We examined by Northern blot analysis whether combined supplementation of vitamin E succinate and Asc caused an elevation of Cox 2 RNA expression in BL6 cells. An inducible effect of Cox 2 was observed after 2 hours of supplementation with a combination of vitamin E succinate and Asc in BL6 cells, however the results are inconclusive and further studies are required to substantiate this finding.

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ABBREVIATIONS

α-TOH	Vitamin E = α-Tocopherol
aa	Amino acid
AA	Arachidonic acid
AMP	5' Adenosine monophosphate
Asc	Ascorbic acid
BL6	Murine melanoma cells
Ca^{2+}	Calcium
Chx	Cycloheximide
Cox	Cyclooxygenase
CPM	Counts per minute
Dex	Dexamethasone
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
FCS	Foetal calf serum
GAPDH	Glyderaldehyde phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
HNO_2	Nitrous acid
HPLC	High performance liqid chromatography
kDa	Kilodallton
LDL	Low-density lipoprotein
LOOH	Lipid hydroperoxide
LLCMK	Monkey kidney cells
MEM	Minimum essential medium
Mg^{2+}	Magnesium
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phospate

NO	Nitrous oxide
NO_2^-	Nitrite
N_2O_3	Nitrogen(III) oxide
NSAIDs	Non steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PG	Prostaglandin
PGHS	Prostaglandin endoperoxide synthase
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PLA_2	Phospholipase A_2
PKC	Protein kinase C
PNK	Polynuclear kinase
PUFA	Polyunsaturated fatty acid
RDA	Recommended daily dietary allowances
RNA	Ribonucleic acid
Rnase	Ribonuclease
SEM	Standard error of the mean

CHAPTER 1

LITERATURE REVIEW

1.1 CANCER

Cancer is the uncontrolled growth of abnormal cells in which two types of tumours have been identified. Benign tumours are formed by cells that cannot invade the surrounding tissues and remain as local growths. Those that spread from their site of origin and disseminate into the bloodstream and lymphatic system are called malignant tumours. The classification of cancers is based on which organs they originate in and by the kind of cell involved.[1]

The transformation of a normal cell to a cancerous cell can be induced by a variety of different agents that are generally chemical or physical in nature, this transformation of a normal cell into a neoplastic cell is considered to proceed through three phases: induction, promotion, and progression (Fig 1). Malignant tumours are rarely formed if initiation is not followed by promotion and progression. Initiation is a phenomenon of gene alteration, which may result from the interaction of carcinogens with DNA. However, mutations do not necessarily lead to cancer but are indicative of increased risk of neoplasia. The modifications that occur in the cell during initiation are permanent and irreversible. The stages of initiation can be altered by exogenous and endogenous factors. The principle characteristic of promotion that distinguishes it from the stages of initiation and progression is its operational reversibility.[2-4] Promotion has been suggested to involve epigenetic processes that stimulate the expression of the altered genotype within the initiated cells in the absence of initiators. Promoters can generate reversible changes in cell proliferation, however the molecular mechanisms of tumour promoters are not understood. Promotion can be divided into two steps: conversion (I) and propagation (II). Thus promotion results in a high yield of benign tumours. Promotion is a very slow process, and is reversible, thus it is an attractive target for the intervention with cancer chemopreventive agents.[2-4] Free radicals have been said to play a role in the initiation and promotion stages of carcinogenesis and antioxidants have been suggested as inhibitors of the neoplastic process.[5] Progression may be characterised primarily by its karyotypic instability and evolution, and the development of irreversible, aneuploid malignant neoplasms. The alterations that occur in the structure of the

genome during this stage of the neoplastic cell is directly related to the increased growth rate, invasiveness, metastatic capability, and the biochemical changes in the malignant cell.[4]

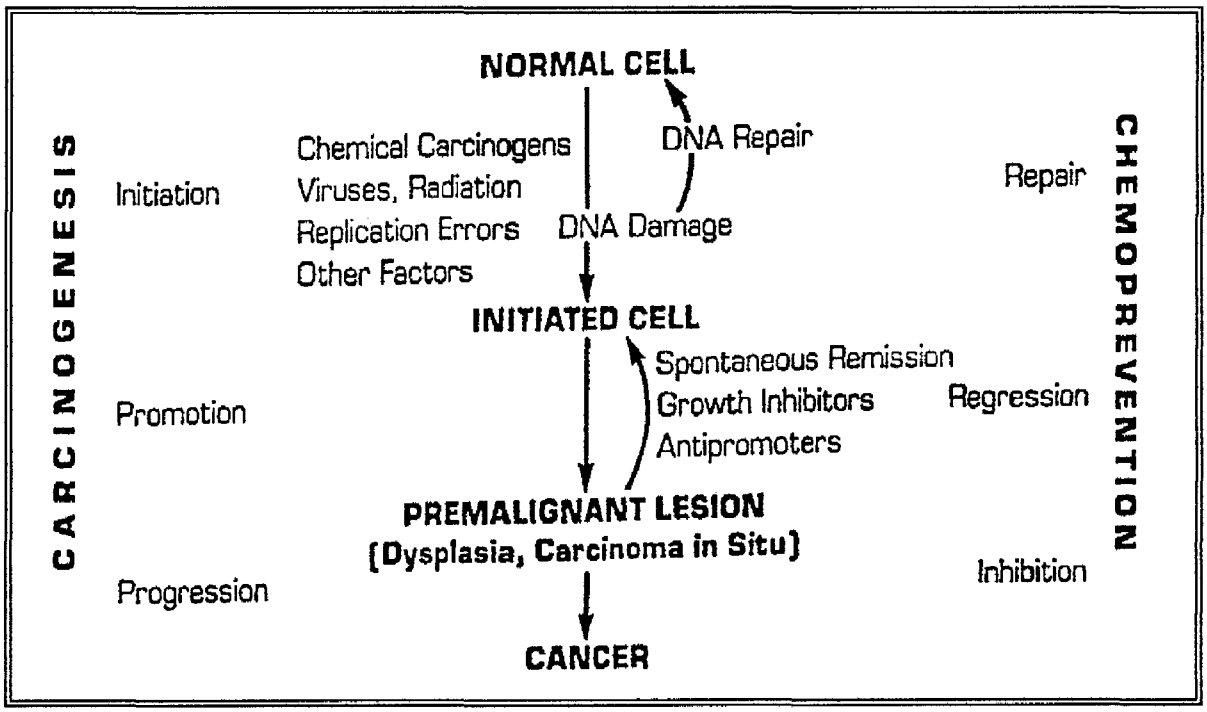


Figure 1: A multistep carcinogenesis model.[3]

The bulk of cancer deaths are not caused by the primary tumour but rather by the secondary tumour growth elsewhere in the body. The ability of malignant tumour cells to spread can be considered to be the most lethal aspect of cancer.[6] Joseph Claude Recamier in 1829 coined the term "metastasis."[7] Metastasis is a multi-stage process. Initially a primary tumour is formed, from here the cells exit by penetrating the walls of channels in the vascular and lymphatic circulatory systems. The tumour cells are carried in the circulatory system until becoming lodged in a capillary bed. Penetration and invasion of the blood vessel walls and surrounding tissues occurs followed by the proliferation of the tumour cells. Local growth factors and hormones provided by the host, as well as autostimulatory growth factors produced by the tumour cells provides the stimuli for growth and proliferation of the tumour cells, thereby forming a secondary tumour.[6, 7]

Epidemiological studies have estimated that 80% of the cancers occurring in humans are as a result of environmental factors and that the majority of these cancers could be prevented if one could identify the main risk and anti-risk factors. Cancer and the significance of diet in its etiology have led to a number of studies that have suggested that the absence of certain dietary components, the antioxidants, contribute to a substantial proportion of human malignancies.[8]

Nontoxic agents which block, arrest, or reverse the development of neoplasia are termed chemopreventive agents, which has lead to a major new field in cancer research.[9] Chemoprevention is defined as, "the systemic use of specific natural or synthetic chemical agents to reverse or suppress the progression of a premalignancy to invasive cancer."[10] A large number of studies have been undertaken to improve the understanding of the role of antioxidants in protecting the body against cancer.[11] Animal carcinogenic models show that specific agents can prevent premalignant lesions from progressing to invasive cancers. Epidemiological studies suggest that dietary inhibitors for carcinogenesis in humans exist.[10]

Cell culture offers a powerful tool to assess the carcinogenic potential of physical and/or chemical agents at a cellular level and to identify factors that inhibit transformation and act as cancer preventative agents.[12]

Over the last century, information about the importance of vitamins and other micronutrients in the maintenance of health has arisen. The ability of some vitamins in preventing cancer has been extensively researched. The micronutrient requirements of cancer patients may be influenced by several factors such as the metabolic state of the malignancy and its effects on host metabolism, the effects of antineoplastic therapy catabolism and physiological stresses with regard to the treatment of cancer.[12] Evidence suggests that vitamin C and E may alter cancer incidences and growth by functioning as anticarcinogens, quenching free radicals or reacting with their products.[13, 14] Nutritional factors potentially can inhibit critical phases of tumour promotion. The correct combination of these factors is more effective in inhibition than isolated substances because of their ability to complement each other in their mechanisms of action.[15]

Since ascorbic acid (Asc) and vitamin E and their role in the neoplastic process is the subject of this thesis, it is important to consider the general role of both Asc and vitamin E in cell metabolism.

1.2 ASCORBIC ACID

1.2.1 HISTORY

Micronutrients, which include vitamins, minerals and trace elements are dietary components essential to normal metabolic functions. The term "essential" implies that Asc has to be acquired from the diet as it cannot be synthesised *de nova*.[16] Sir Richard Hawkins in the 16th century discovered that oranges and lemons were effective in curing scurvy in British sailors, however this was only verified in the 18th century by James Lind. In 1909 Holst and Frolich first formally proposed that the disease was a dietary deficiency. Albert Szent-Gyorygyi in the late 1920's isolated the active agent, the enolic form of 3-keto-L-gulofuranlactone or hexuronic acid, which was later named vitamin C or Asc, from oranges, cabbages, and adrenal glands.[17-20] Synthesis of the first vitamin was undertaken by Reichstein in 1933.[17]

Asc unlike the other vitamins, is required in the diet by only a few species of animals:- man, other primates, the guinea pig, the red-nested bullbill and some related species of Passeriform birds.[21]

1.2.2 STRUCTURE AND METABOLISM

Vitamin C is shown to be a γ-lactone (Fig 2), although other tautomeric forms may exist in small quantities. It reacts as monobasic acid and is a strong reducing agent.[19, 22] Asc is a weak acid and is stable in weak acids. Exposure to air causes oxidation and it is sensitive to heat and light.[19]

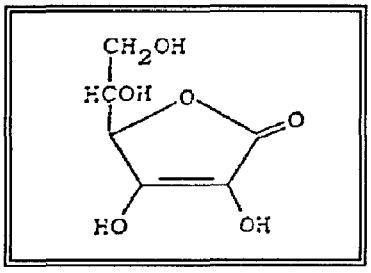


Figure 2: Ascorbic acid.[23]

In the late 1950's L-Asc was shown to be synthesised from D-glucose (Fig 3). It involves two enzymatic reactions, viz, lactonization and oxidation, with the latter step being catalysed by L-gulono-γ-lactone oxidase, which is lacking in humans, other primates, and guinea pigs, and thus vitamin C needs to be provided in the diet.[24]

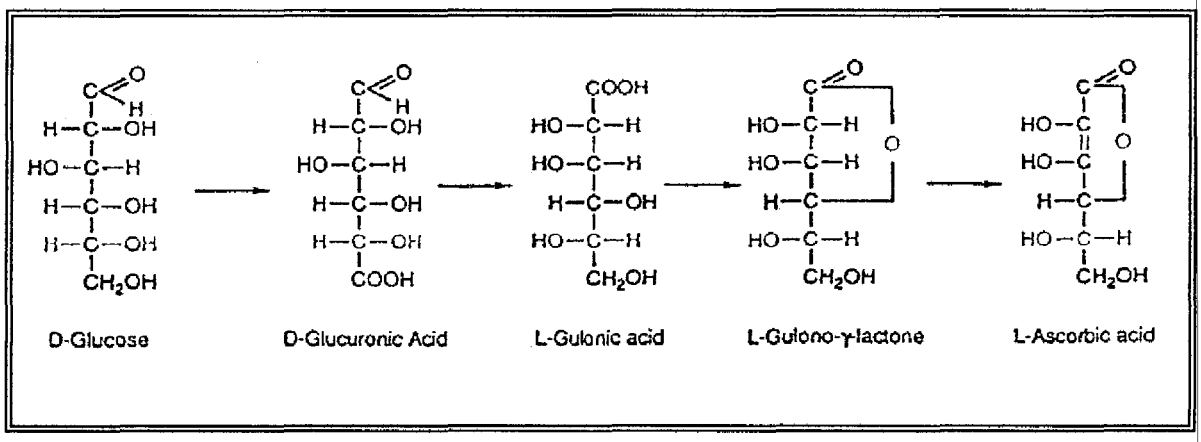


Figure 3: The metabolic pathway of L-ascorbic acid biosynthesis in animals.[24]

1.2.2.1 Metabolism

The requirements for Asc vary and may be higher according to state of health, age, weight, activity, energy levels, and general metabolism. The requirements of vitamin C are further increased under stress, illness and injuries. The recommended daily dietary allowances (RDA) for adults is considered to be 60mg, while infants require 35mg. The best know sources of Asc are found in citrus fruits (lemons, limes, tangerines, and grapefruit), and leafy vegetables.[19]

Transport is required for any material that cannot be synthesised within cells, transport is required across membranes and through biological fluids. Three mechanisms exist for the transport through biological fluids, namely simple diffusion, hydrodynamic flow and protein binding.[25]

The possibility of protein binding of vitamin C is limiting, as it is filtered by the kidney, ultrafiltered from plasma, and is rapidly lost from energy-depleted cells. The polarity of the membrane, which hinders its passage across hydrophobic cell membranes and its ability to be maintained at high concentrations within the cell, suggests that a carrier mechanism probably facilitated transport is required for vitamin C to enter cells.[25, 26]

Asc is excreted through the urine, which occurs within 4-6 hours following ingestion. Some Asc may be excreted in the feces.[27]

1.2.3 FUNCTIONS OF ASCORBIC ACID

Asc is increasingly being recognised as an agent with broad biological functions and importance.[27] Established functions include synthesis of hormones and neurotransmitters, immune system functions, antioxidant functions, free radical scavenging, protection against lipid peroxidation, and a role in reconstituting the active form of vitamin E.[28, 29]

1.2.3.1 Ascorbic acid and Nitrosamines

N-Nitroso compounds are a major class of chemical carcinogens which induce tumours in a wide variety of animals and organs. This implies that N-Nitroso compounds are important in human carcinogenesis. Exogenous and endogenous sources of nitrosamines exist.[30, 31] The formation of nitrosamines occurs when nitrite (NO_2^-) reacts with amines under conditions of low pH (pH2.4), as in the stomach. Nitrite can react with primary, secondary, or tertiary amines, but stable nitrosamines are usually the product of the nitrosation of secondary amines. The reaction of a given amine depends on its basicity at the pH of the environment, which influences the concentration of nitrous acid (HNO_2). Nitrogen(III) oxide (N_2O_3) is formed from nitrous acid which is the reactive species for nitrosation.[17] Nitrosamine products are inhibited by Asc, by reacting with nitrite to form a non-nitrosating species before nitrite can react with amines in the diet to form the toxic and carcinogenic nitrosamines.[32] This has been supported by studies performed by Ivankovic *et al* and Mirvish.[11, 32, 33] Dietary supplementations have been carried out to determine the effectiveness of vitamin C in inhibiting cancer by these chemicals. Studies showed protective effects of vitamin C against skin, respiratory and kidney cancer.[11]

1.2.3.2 Hydrogen peroxide (H_2O_2) formation by and the antioxidant role of ascorbic acid

It is suggested in literature that Asc can generate hydrogen peroxide (H_2O_2) upon oxidation by molecular oxygen (O_2) in biological systems.[34] The cytocidal property of Asc is believed to be due to the intracellular generation of toxic H_2O_2 produced upon oxidation of the ascorbate by the cells.[34] H_2O_2 can be formed at the cell surface or within the cell. The transport and control of uptake of Asc in normal cells is stringent, however, a few studies have shown that tumour cells

lose their homeostatic control for the transport of vitamin C, thus cells treated with high doses of vitamin C would die by the mechanism involving intracellular H_2O_2.[34, 35]

Catalase and peroxidation activity is found in normal cells, and therefore the inhibition of either of these enzymes may affect intracellular levels of H_2O_2. Asc may potentiate H_2O_2-mediated toxicity *in vitro* since the vitamin is an inhibitor of the catalase activity, thus in tumour cells inhibition would cause an accumulation of H_2O_2 which would cause cell death.[35, 36] This was shown to occur in Ehrlich Ascites carcinoma cells *in vitro*.[34]

In mammalian systems, Asc is probably the most effective and least toxic antioxidant identified.[37] It is a water-soluble, chain-breaking antioxidant that reacts directly with superoxide, hydroxyl radicals, and singlet oxygen.[37-39] Asc is known to act as an antioxidant both *in vitro* and *in vivo*. Free radicals attack various biological molecules, membranes, and tissues to induce free radical-mediated chain oxidations. Evidence implies that free radicals cause pathological events, such as cancer.[40] Damage to DNA, enzymes, proteins, and other macromolecules may be caused by oxidative processes.[37-39] Studies have shown that Asc completely protects lipids in plasma and low-density lipoproteins (LDL) against peroxidative damage.[37] In addition Asc interacts with the tocopheroxyl radical to regenerate tocopherol, thus allowing for a synergistic action to exist between the two vitamins, where vitamin C spares vitamin E.[39]

1.2.4 Deficiency and toxicity of ascorbic acid

A deficiency of vitamin C causes scurvy which is characterised by bleeding gums, impaired wound healing, perifollicular haemorrhage, anaemia, fatigue, depression and sudden death.[19, 37] A number of medical problems have been found to be associated with low blood levels of vitamin C, which include various infections, colds, high blood pressure, arthritic vascular fragility, allergies, ulcers, and cholesterol gallstones.[19]

Vitamin C in its many forms has been found to be nontoxic. Appreciable amounts are not stored by the body, as most excess amounts are rapidly eliminated through the urine. Side effects include nausea and skin sensitivity. Very high doses may cause hemolysis of red blood cells and may cause kidney stones.[17,19]

1.2.5 Ascorbic acid and Cancer

Of all cancer incidence 37% have been related to diet, and epidemiological and animal data suggests that vitamins and/or minerals act as anticarcinogens, altering cancer incidence, differentiation and growth thus, proving to be useful adjuncts to conventional therapies or in cancer prevention.[33]

In recent years there has been considerable interest regarding the therapeutic significance of Asc in various conditions, especially cancer.[41] Asc has numerous and varying effects on cancer induction and growth, and its antitumour activity has been investigated both *in vitro* and *in vivo* against human and animal tumours.[42]

Epidemiological and biochemical studies indicate that vitamin C may play an important role in the prevention of cancer as evidence links increased intake of fruits and vegetables with decreased risk of most cancer types. Of 170 epidemiological studies of cancer at all sites, 132 showed a statistically significant protective effect associated with the highest intakes of fruit and vegetables. Individuals with low fruit and vegetable intake have cancer values twice that of individuals with high intakes.[43] Cameron *et al.* (cited in[41]) showed that patients with terminal cancer, showed complete tumour regression and prolonged life expectancy with administration of 10g of vitamin C on a daily basis, while Creagan *et al.* (cited in[41]) reported that high doses of Asc had no therapeutic value. More specifically, studies have indicated the therapeutic value of vitamin C in the treatment of estrogen-induced renal cancer, mammary tumours, and UV light-induced skin tumours.[44]

This suggests that vitamin C has a unique advantage when compared to other preventives for cancer, in that it is almost completely safe and harmless even when high doses are administered for prolonged periods of time.[18] Reports state that vitamin C's antitumour activity is due to its chemical properties and not to its metabolism.[45]

1.3 VITAMIN E (α-TOCOPHEROL)

1.3.1 HISTORY

Evans and his co worker Bishop (cited in[46-48]) in 1922 undertook studies in which they reported that reproduction failed in pregnant females fed diets containing all of the then known nutrients. With these results Bishop and Evans demonstrated the existence of an unknown dietary factor, initially called factor X. Inadequate amounts of factor X resulted in fetal death and resorption in the laboratory rats. Wheat germ, lettuce, and dried alfalfa leaves were recognised as sources of the fat-soluble factor X, and prevented gestation reabsorption. In 1925 Evans adopted the letter E as the next serial alphabetical designation.[49-51] Further research carried out by Evan and co workers led to the isolation of a homogeneous substance which was named α-tocopherol.[51] Erhard Fernholz in 1938 undertook thermal degradation of the vitamin, and showed it contained a phytyl chain and a hydroquinone moiety, and suggested a structure of the vitamin later proven to be true.[48] The structure of vitamin E was subsequently determined and given the name tocopherol. Tokos and phere are the Greek words for "offspring" and "to bear," so tocopherol (TOH) literally means "to bear children."[46-48]

1.3.2 Structure, chemistry and metabolism of vitamin E

"Vitamin E" is a generic term which represents a family of compounds that can be divided into two groups, the tocopherols and the tocotrienols on the basis of the degree of saturation of their side chains.[48,52] The tocopherols are phenolic compounds, and there are 8 natural analogs. The series is made up of four compounds with tocol-structure bearing a saturated phytyl C_{16} side chain (α-, β-, γ-, δ-tocopherol) and 4 compounds with tocotrienol structures bearing 3 double bonds in the phytyl side chain (α-, β-, γ-, δ-tocotrienols).[46] The three tocopherols, α, β, and δ have known biopotency. Of the known forms α-TOH is the most active *in vivo*. The 6-hydroxyl group is the biological active site of the molecule.[53] α-TOH is a derivative of 2-methyl-6-chromonol on to which a saturated C-16 isoprenoid tail or chain is attached at C-2 and which is methylated at C-5, C-7 and C-8.[54] The side chain in the 2-position facilitates the incorporation and retention of vitamin E in biological membranes, so that the 6-position is optimal for scavenging free radicals and terminating lipid peroxidation.[46] Of the various forms of vitamin E for antiproliferative effects on tumour cells, vitamin E succinate has shown to be the most potent *in vitro* (Fig 4). Although the precise reason for this is unknown, the following possibilities have

been suggested: 1) vitamin E succinate may be more stable and soluble in the growth media or in solution; 2) it may easily cross cell membranes; 3) α-TOH succinate acts as a carrier for vitamin E thus enabling a slow release and accumulation of vitamin E at a unique and critical cellular site, so that intracellular levels of vitamin E remain high for a longer period of time; 4) it could have its antiproliferative effect at the nuclear level.[35, 55] Thus is appears that the antiproliferative activity of vitamin E is unique among the tocopherols.[55]

Vitamin E is found in both plant and animal foods. Oils of vegetable seeds, particularly wheat, soya-bean, cotton, sunflower and corn are rich sources of vitamin E. Milk, butter, cheese and other dairy products also supply a variable, but relatively low amount of vitamin E.[55-56] The amount of vitamin E required depends on two factors; body size and the amount of polyunsaturated fats present in the diet. The different forms of vitamin E have various potencies.[47] The RDA for vitamin E is 6-10mg of α-TOH or the equivalent.[57] α-TOH occurs as free phenols in most food sources, however α-TOH esters are widely used in pharmaceutical formulations and dietary supplements because the esters are much more resistant to oxidation than α-TOH. The phenol moiety is masked because of the esterification, thus the esters themselves display no antioxidant activity and must undergo enzymatic hydrolysis to release α-TOH. α-TOH esters undergo metabolism *in vivo* to α-TOH.[58-60]

The mechanism by which vitamin E is absorbed and transported in plasma are well known, however its intracellular transport is not well known. Vitamin E requires a transport system in aqueous environments of the plasma, extracellular space, and cell cytoplasm because of its hydrophobicity. Absorption occurs mainly in the upper and middle third of the small intestine of animals.[61] Vitamin E is absorbed via the lymphatic pathway and transported in association with chylomicrons. Proteins with binding capacity for α-TOH have been described in the cytosol, and nuclear fraction, which may function as intermembrane carriers for α-TOH. The hydroxyl group in the chromanol ring of α-TOH may be important for the transfer process between cell organelles.[46,61] Once absorbed, vitamin E is distributed throughout the body. α-TOH is taken up by all tissues of the body and it is concentrated in the membrane containing structures of the cells such as the mitochondria, microsomes, nucleus, and plasma membrane. The major route of excretion is by fecal elimination.[51]

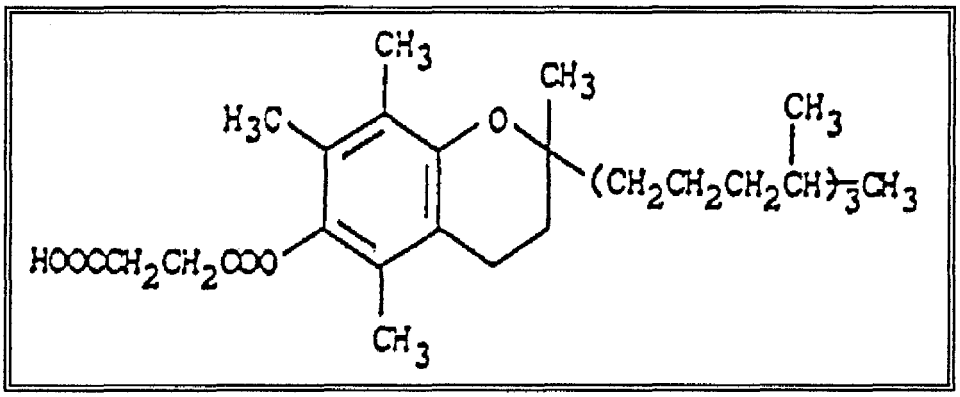


Figure 4: Vitamin E acid succinate.[23]

1.3.3 FUNCTIONS OF VITAMIN E

Vitamin E may have important roles in biological processes, such as in DNA synthesis, the stimulation of immune responses and the suppression of inflammation, however, the functions to be considered are those relevant to cancer.[61] Vitamin E is a potent anticancer agent in terms of both cancer prevention and cancer regression.[62] The antioxidant function of vitamin E seems to play a major role in retarding the process of carcinogenesis, thereby reducing tumour incidence.[56]

1.3.3.1 Vitamin E and Nitrosamines

α-TOH's effect in preventing nitrosamine formation is similar to that as described for vitamin C, and may also inhibit N-nitroso compound formation in the stomach. However unlike vitamin C, vitamin E inhibits nitrosation in the nonaqueous lipid compartments.[11,14,63] Vitamin E has been shown to block the formation of nitrosamines *in vivo*.[64]

1.3.3.2 Stabilizing membranes by functioning as an antioxidant

Numerous observations have suggested that vitamin E plays a structural role in membranes. Studies have proposed that uncontrolled changes in membrane permeability and stability may occur in the absence of vitamin E, which would lead to the failure of compartmentalization by biological membranes.[65]

Vitamin E is a potent chain breaking antioxidant which inhibits lipid peroxidation in membranes.[54] Lucy suggested that vitamin E stabilises the membrane by virtue of specific physicochemical interactions between its phytyl chain and the fatty acyl chains of polyunsaturated fatty acids (PUFA) particularly those derived from arachidonic acid (AA).[65] The hydrophobic tail is the means by which TOH inserts into lipoproteins or anchors into membranes next to unsaturated fatty acids, whereby stability occurs by London-van der Waal's dispersion-attraction forces. Proper orientation of the molecule occurs via the isoprenoid chain, while the chromanol nucleus lies at the surface of a lipoprotein or at the surface of membranes, where the phenolic hydroxyl group is at an optimal position to quench free radicals, thus terminating lipid peroxidation.[54]

1.3.3.3 Antioxidant role of vitamin E

Excess free radicals are believed to be involved in the initiation and growth of many cancers. Vitamin E may decrease the incidence of cancer and tumour growth by functioning as anticarcinogens, quenching free radicals or reacting with their products.[54] Vitamin E is the major lipid soluble chain-breaking antioxidant in biological systems, and because of its location and structure it plays a pivotal role in the antioxidant defence system in the membrane.[54,66] The prime targets of the free radicals are the unsaturated bonds in membrane lipids. Consequently, peroxidation results in a loss of membrane fluidity and receptor alignment and potentially causes cellular lysis. Potential biological targets of radical attack include nucleic acids, proteins and enzymes.[2, 38] Oxidative damage to carbohydrates can alter any of the cellular receptor functions including those associated with hormonal and neurotransmitter responses.[38] α-TOH's antioxidant properties have been ascribed to hydrogen abstraction from the hydroxyl (OH) group in the tocopherol molecule.[67] The initial chain is terminated via reaction 1, which yields a molecule of lipid hydroperoxide, (LOOH), and a tocopheroxyl radical, (α-TOH$^{\cdot}$). The second chain is terminated by the fast coupling of a peroxyl radical (LOO$^{\cdot}$) with the α-TOH$^{\cdot}$ radical, reaction 2. Each tocopherol molecule can react with two peroxyl radicals, as shown in Fig 5.[66, 67]

$$LOO^{\cdot} + \alpha\text{-TOH} \rightarrow LOOH + \alpha\text{-TOH}^{\cdot} \quad (1)$$

$$LOO^{\cdot} + \alpha\text{-TOH}^{\cdot} \rightarrow LOO\text{-}\alpha\text{-TOH} \quad (2)$$

Figure 5: α-TOH inhibition of phospholipids.[66, 67]

Evidence continues to accumulate indicating that the processes of tumour initiation, promotion and progression can be effected by oxygen-radical-mediated events, and numerous studies have shown that vitamin E's activity is predominantly in membranes where it functions as a free-radical scavenger and serves to remove reactive oxygen species as well as free radicals produced by lipid peroxidation, and thus may alter cancer incidence.[68-70]

1.3.4 Deficiency and toxicity of vitamin E

Vitamin E deficiencies are fairly rare with vague symptoms that are difficult to diagnose.[47] Deficiencies of vitamin E are often due to some abnormality or disease that slows vitamin E absorption or transport.[71] A deficiency may cause muscular lesions, characteristic of dystrophy and paralysis, testicular degeneration, disturbed uterine physiology, abortion of foetuses, encephalomalacia, functional failure of the heart due to degeneration of heart muscle, kidney injury, increased fragility of red blood cells and haemolysis.[46, 56] Alterations in vitamin E status have also been associated with development of certain forms of cancer.[46]

The possibility of vitamin E toxicity is unlikely as it is not stored as readily as the other fat soluble vitamins. Excess is usually eliminated in the urine and feces. Animal studies have shown that very high amounts of vitamin E retard growth and decrease muscle tone, and cause poor bone calcification.[55]

1.3.5 Vitamin E and Cancer

The role of vitamin E in cancer is being more frequently researched since increased observations have indicated that an increased risk of certain human neoplastic diseases may be connectd with a vitamin E deficiency.[56] Vitamin E may protect against cancer by functioning as an intracellular antioxidant and as a free radical scavenger.[72] Several epidemiological studies have shown that an inverse relationship between serum levels of vitamin E and cancer rates exist. Animal studies have shown that vitamin E in the presence of an induced tumour can inhibit growth.[8] Contradictory reports do exist, while some studies indicate that vitamin E can inhibit and restrict tumour growth and incidence, others fail to support such observations.[56]

One important aspect of the anticancer effect of vitamin E is its ability to inhibit tumour cell proliferation *in vitro* as vitamin E has been shown to inhibit growth in several types of cells. Inhibition in cultured cell lines include murine neuroblastoma, murine melanoma, rat glioma, and myeloid leukemia cells. However the mechanism(s) whereby vitamin E inhibits tumour cell proliferation remains unclear.[73] Vitamin E has been shown to inhibit skin, cheek pouch, and forestomach cancers, to enhance or inhibit colon carcinogenesis, and to have no effect or to inhibit mammary gland carcinogenesis. The possible effect may depend on several factors, such as the amount of vitamin E, the method of vitamin E administered, the level of dietary selenium or dietary fat, the level of modifying factors, the type, dose and route of administration of the carcinogen used, and the animal species studied.[11, 33, 64] Studies have shown that α-TOH succinate inhibits growth and induces morphological differentiation in melanoma cells and is the most potent form of vitamin E to do this.[74, 75]

Vitamin E's direct effect on cancer cells may also be that it enters cancer cells and destroys them by altering cell metabolism and perhaps by inactivating the oncogenes or oncoproteins that maintain the viability of the abnormal cells.[62]

1.4 SYNERGISTIC INTERACTION OF VITAMIN C AND VITAMIN E

Golumbic and Mathill over 40 years ago reported that even though vitamin C was a poor antioxidant for hydrogenated cottonseed oil, it enhanced the antioxidant activity of vitamin E. They showed that the length of the induction period produced by a mixture of vitamin C and E was much greater than the sum of their individual induction periods. Thus they showed that a synergistic antioxidant effect was obtained in the presence of these two vitamins.[69, 76, 77] Further work by Tappel in 1969 suggested that vitamin C might reduce the tocopheroxyl radical formed during the scavenging of free radicals found *in vivo* during metabolism, and this would link vitamin C to the protection of membranes against free-radical damage, as vitamin C represents a large reservoir of soluble antioxidant potential which could be delivered to peroxidising membranes through regeneration of the lipid soluble vitamin E.[69, 78] Both vitamins C and E function as chain-breaking antioxidants, in their specific aqueous and lipid compartments *in vivo*. Vitamin C being water soluble cannot scavenge radicals in the membranes, and since vitamin E

forms the only protective agent within the membranes, vitamin E may be the last line of defence against membrane peroxidation.[77] Free-radical generation is generally accepted as a steady state phenomenon in respiring cells, and it is now recognised that uncontrolled radical production may contribute to the etiology of cancer.[38, 79] Thus it has been suggested that dietary antioxidants may become an effective strategy for cancer control.[14]

1.4.1 Mechanism

Polyunsaturated fatty acids (PUFA) found in membranes are especially susceptible to peroxidation because of the easily oxidizable bis-allylic hydrogens. Thus many oxidising agents can initiate lipid peroxidation (Fig 6).[80]

$$PUFA\text{-}H + X^{\cdot} \rightarrow PUFA^{\cdot} + X\text{-}H \qquad \text{(Fig 6a)}$$

Propagation of the chain reaction is favoured because PUFA$^{\cdot}$, the pentadienyl radical reacts with oxygen:

$$PUFA^{\cdot} + O_2 \rightarrow PUFA\text{-}OO^{\cdot} \qquad \text{(Fig 6b)}$$

The propagation of the chain reaction is further favoured with the production of the lipid peroxyl radical. Once this lipid peroxyl radical is formed a significant dipole is present which allows the peroxly radical moiety and the lipid chain to partition to the membrane-water interface where it is positioned for repair by α- TOH, which ultimately brings about the oxidation of other unsaturated lipids.[80]

$$PUFA\text{-}OOH \rightarrow PUFA^{\cdot} \qquad \text{(Fig 6c)}$$

Vitamin E can out compete the propagation reaction and repairs the PUFA-OO$^{\cdot}$ to form PUFA-OOH.[80]

$$TOH + PUFA\text{-}OO^{\cdot} \rightarrow PUFA\text{-}OOH + TO^{\cdot} \qquad \text{(Fig 6d)}$$

As a chain breaking antioxidant each tocopherol molecule has the capacity to donate two electrons, and in order for it to be an effective antioxidant each oxidised tocopherol must be recycled to tocopherol. Vitamin E quenches the free radical by providing hydrogen atoms which

can pair up with the unpaired electron on the free radical. However during this process vitamin E becomes oxidised. The ascorbate then recycles tocopherol via TO˙, producing the ascorbate radical:

$$AscH^{\cdot} + TO^{\cdot} \rightarrow Asc^{\cdot} + TOH \qquad \text{(Fig 6e)}$$

By the process of dismutation the Asc can be removed to yield AscH˙ and dehydroascorbate. These active reduced forms of the vitamin can be regenerated by reduction with NADH/NADPH, thus recycling ascorbate. In membranes the phenolic OH group of TOH is found at the membrane-water interface which makes it more accessible for the ascorbate molecule to react with the tocopheroxyl radical.[80]

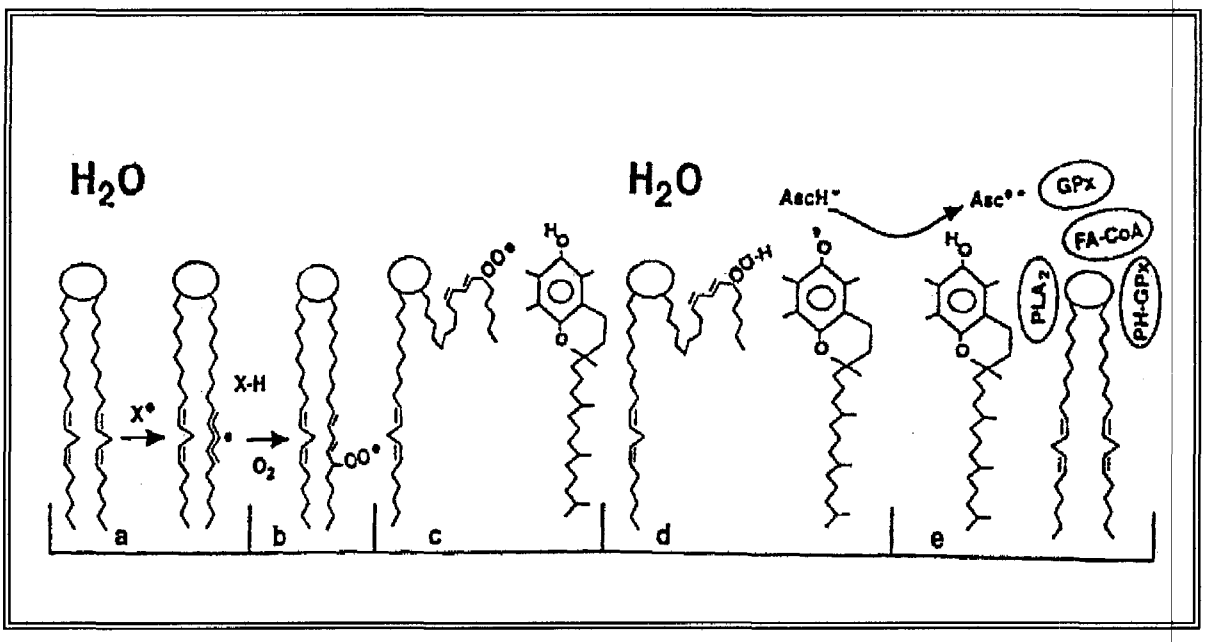


Figure 6: Lipid peroxidation in membranes.[80]

Thus vitamin C serves as a reservoir of antioxidant potential while vitamin E may function as the specific, highly effective delivery system.[78]

1.5 PROSTAGLANDINS

A significant link between cancer development, cancer progression and invasiveness, and prostaglandins (PGs) has been reported over the last 2 decades. An important relationship between PGs and vitamins has also been suggested by recent reports.[81] The biosynthetic pathways in arachidonic acid (AA) metabolism may be modulated at a number of different points by vitamin E and other antioxidants.[82]

Prostaglandins are lipids produced enzymatically and nonenzymatically from 20 carbon fatty acids, particularly AA. High levels of PGs and the synthetic enzymes, the PG synthases/cyclooxygenases have been associated with many types of cancer. Several mechanisms exist by which PG may contribute to the cancer processes, these include increased proliferation, apoptosis, enhanced carcinogen metabolism or modulation of the immune system.[83] PGs represent a diverse group of autocrine and paracrine hormones that are important mediators of many cellular functions.[84] In spite of extensive research on the effect of specific eicosanoids, little is known about the mechanism(s) of action at the cellular and molecular levels. For most tissues, it is not clear whether the effect on a given tissue of a particular PG is the same for normal and malignant cells.[83]

The biosynthetic pathway for eicosanoids consists of multiple steps which may be possible sites for regulation (Fig 7).[85] Prostanoid synthesis occurs when a hormone or proteinase interacts with an appropriate receptor or proteinase target on the cell surface. Each stimulus appears to act in a cell-specific manner. The formation of a prostanoid occurs either by a phospholipase A_2 or by phospholipase C. Both pathways allow for the mobilization of AA in stimulated cells. A prostaglandin H synthase (PGHS) acts upon the AA once it is released. This enzyme exhibits both bis-oxygenase (cyclooxygenase) activity catalysing PGG_2 formation and a peroxidase action catalysing a two-electron reduction of PGG_2 to PGH_2. PGH synthase is a integral membrane protein found mainly in microsomal membranes. Conversion of PGH_2 by a set of synthases leads to the formation of biologically active eicosanoids, namely PGD_2, PGE_2, and $PGF_{2\alpha}$. The PGs formed then exit the cells, probably via carrier-mediated transport, and interact with receptors on neighbouring cells to modulate second messenger levels.[86]

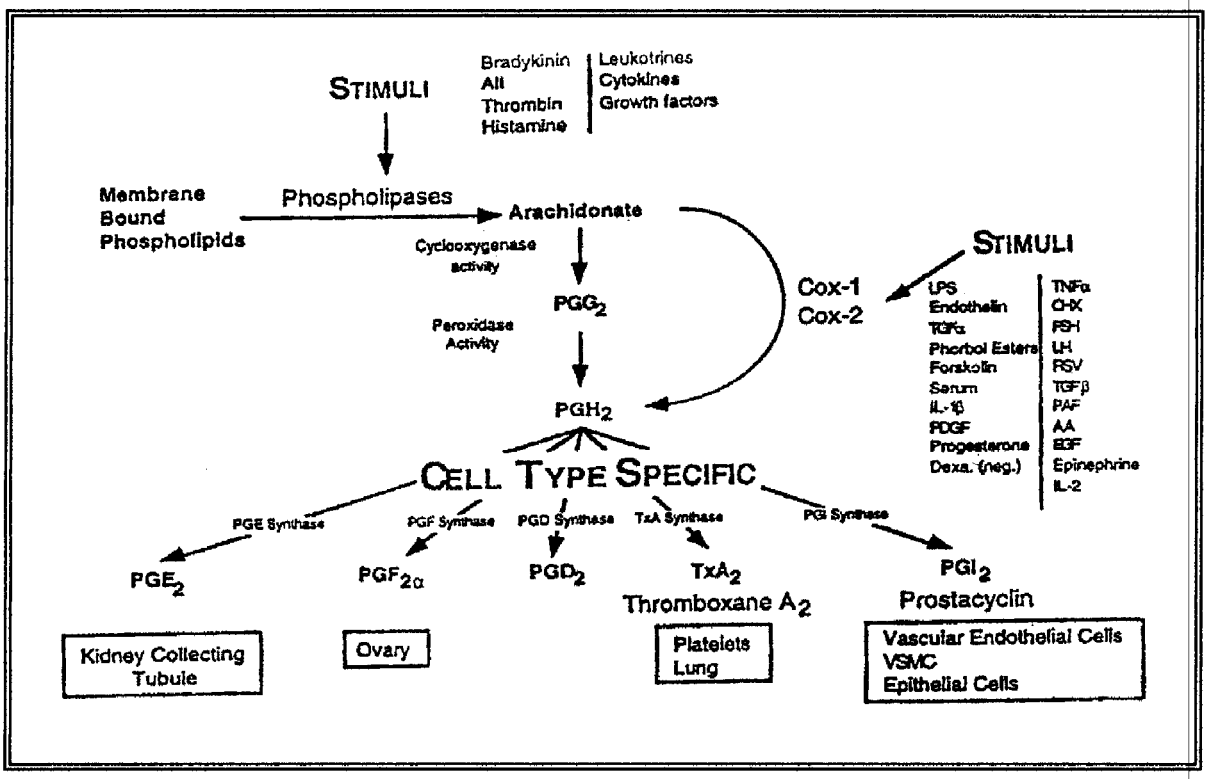


Figure 7: Biosynthetic pathway for prostanoid formation.[85]

Given the multiple role of eicosanoids in human health and disease, it will clearly be important to further elucidate the molecular mechanism regulating the mobilization and subsequent metabolism of AA.

1.5.1 Prostaglandin endoperoxide synthase/cyclooxygenase

A major mechanism for the control of PG synthesis occurs at the level of cyclooxygenase (Cox). The induction of the Cox enzyme by growth factors, cytokines, and tumour promoters appear to modulate PG synthesis.[87] Two isoforms of Cox have been identified in eukaryotic cells: Cox 1 and Cox 2. Cox 2 has only just recently been identified as a distinct isoform from Cox 1. Cox 2 existence was first suspected by Needleman and his group, who showed that bacterial lipopolysaccharides increased the synthesis of PG's in human monocytes *in vitro*, and in mouse peritoneal macrophages *in vivo* this increase was inhibited by dexamethasone and was associated with de nova synthesis of a new Cox protein.[88] The two isoforms are about 60% homologous within a species. [89-90] It has been proposed that Cox 1 and Cox 2 have different physiological

functions largely because of the striking differences in their tissue expression and regulation. Cox 1 displays the characteristic of a "housekeeping" gene and is constitutively expressed, that is messenger RNA (mRNA) is being constantly transcribed from the gene and the protein is synthesised at a steady rate independent of extracellular signals, in almost all tissues. Cox 1 appears to be responsible for the product of PG's responsible for homeostatic functions. Cox 2 forms a sharp contrast with Cox 1, as it is the product of an 'immediate early gene" that is rapidly inducible and tightly regulated, and the gene is thought to be sensitive to extracellular signals and its expression may be increased by activation of cell surface receptors.[91-92]

1.5.1.1 Cyclooxygenase protein structure

At the protein level, the isozymes Cox 1 and Cox 2 share over 60% (homology) in human and mouse. The functionally conserved relevant sites include; the heme coordinating histidines, the catalytic site tyrosine and the serine residue which is acetylated by aspirin.[93] Cox 1 and Cox 2 are both membrane bound, but have different patterns of subcellular localization. Cox 1 is an integral membrane glycoprotein which is localised in the endoplasmic reticulum (ER), while Cox 2 is localised to the ER and the nuclear envelope. Cox 1 exists as a homodimer of 72-kDa subunits. Four consensus sequences for *N*-glycosylation (Asn-X-Ser/Thr) are conserved in human and murine Cox 1. Three out of the four sites are located with the 19-amino acid (aa) polypeptide which is unique in Cox 2.[93-94] A model predicting the Cox topography in the ER with various putative functional domains has been proposed (Fig 8). Cox 1 and Cox 2 have been predicted to have similar orientation in the membrane because of their high degree of homology. According to the model, two transmembrane sparing regions separates the amino-terminal and carboxyl-terminal end of the proteins. The amino termini of the enzymes are thought to reside in the lumen, due to the presence of signal peptides in the deduced aa sequences which are found to be cleaved in the native enzyme. *N*-Glycosalation sites at Asn^{68}, Asn^{144} and Asn^{410} in Cox 1 are predicted to be located on the lumenal side of the ER. Asn^{550} of Cox 2 is also expected to be in the lumen because it can be *N*-glycosylated.[94]

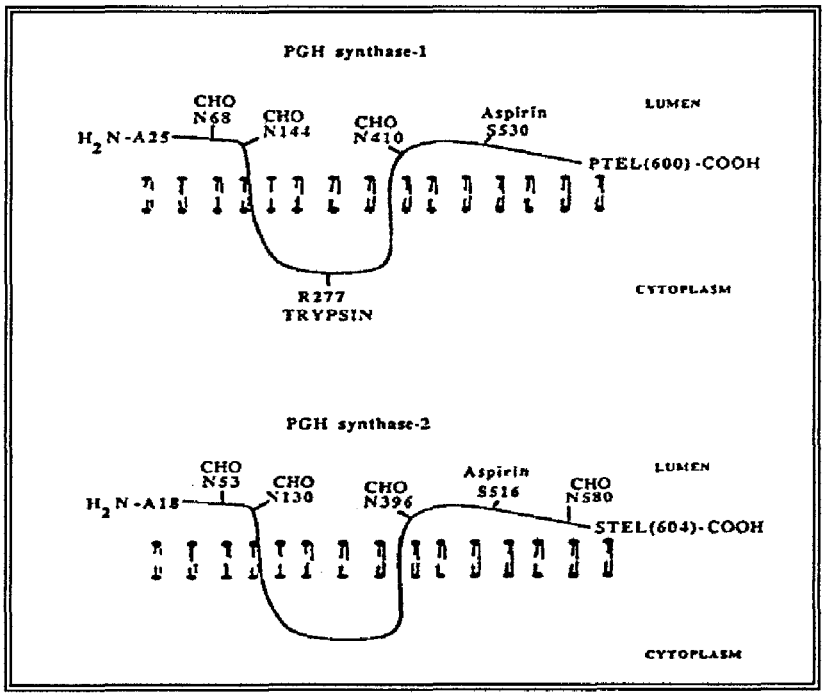


Figure 8: Models for the orientation of Cox 1 and Cox 2 in the endoplasmic reticulum.[94]

Two notable differences between the aa sequence of Cox 1 and Cox 2 are apparent. Firstly, Cox 2 lacks the large hydrophobic signal peptide of Cox 1, and instead contains a short, cleaved signal sequence at its N-terminus. Secondly, Cox 2 contains an 18-aa insert located near the carboxyl terminus which is missing in Cox 1. The overall length of the two isozymes is almost identical; Cox 1 has 600-602 aa, while Cox 2 has between 603-604 aa.[93-94] No TATA box is present in Cox 1 which is a common characteristic of "housekeeping" genes, and the promoter has not shown any significant inducible transcription. The Cox 2 promoter on the other hand contains a TATA box and a number of transcriptional elements that are common in highly regulated genes (Fig 9).[91]

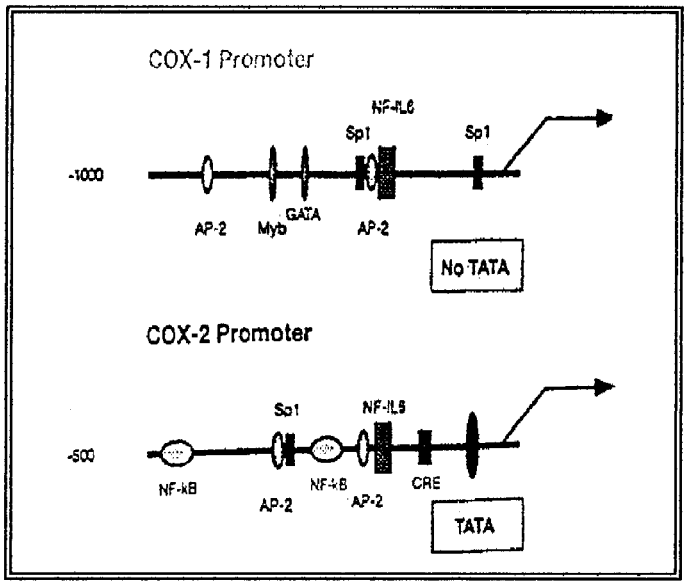


Figure 9: Cox 1 and Cox 2 promoters with transcription factor binding sites.[91]

1.5.1.2 Cyclooxygenase gene structure

Significant differences between Cox 1 and Cox 2 occur at the gene and promoter structure, the stability of the mRNA's and the intracellular localization of the gene products.[90] Both PGHS genes are single copy genes.[93] Cox 1 gene has been mapped to chromosome 2 and 9 in the murine and human systems respectively, while Cox 2 is located in chromosome 1 in both systems.[91, 93] The intron-exon structure is almost identical, Cox 1 is composed of 11 exons and 10 introns, the only difference between the two is that exon 1 and 2 of Cox 1, containing the translational start site and signal peptide, are condensed into a single exon in Cox 2 (Fig 10). The length of the two genes vary, as the introns of Cox 2 are smaller, thus Cox 2 is 8-9kb in length, while Cox 1 is 22.5kb. These features are consistent with rapid transcription and mRNA processing.[85, 91, 93, 95]

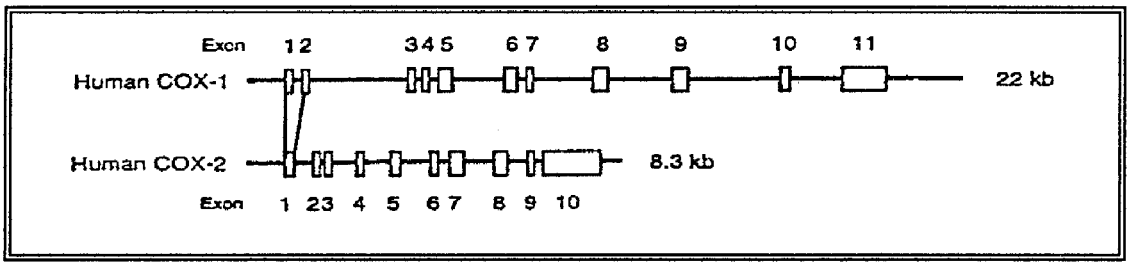


Figure 10: Human cyclooxygenase 1(Cox 1) and cyclooxygenase 2 (Cox 2) genomic structure.[85]

Cox 1, the first isolated form is encoded by 2.8kb mRNA, while Cox 2 is encoded by 4kb mRNA.[88] The Cox 2 mRNA is unstable and this feature has been attributed to the presence of a long 3' untranslated region containing 17 copies of a Shaw-Kamen sequence (AUUUA) that mediates rapid degeneration of the transcript.[90-91]

Cox 2 is a "primary-response" gene, which means that ligand stimulation can induce mRNA accumulation of the primary response genes in the presence of an inhibitor of protein synthesis. Ligand-induction of primary response genes does not require induced synthesis of intervening proteins.[95] Little is know about the molecular mechanisms controlling PGHS gene transcription, and it appears to be quite complex (Fig 11). Factors that increase PG synthesis by increasing PGHS synthesis appears to enhance transcription of the gene. The activation of protein kinase C (PKC), leads to changes in PGHS expression, and it appears that the second most common intracellular signalling mechanism controlling PGHS levels involves adenylate cyclase. Indirect evidence obtained with inhibitors of protein synthesis and transcription indicates that most of the factors that increase de nova synthesis of PGHS also increase transcription of the PGHS gene. The most commonly described mechanism for elevating mRNA levels is by increasing transcription of a gene, however it is also possible that increased stability of mRNA contributes to increased PGHS mRNA levels. Thus in some instances increased PGHS expression may result from increased translation of extant PGHS mRNA. A decrease in mRNA levels may result from the degradation of PGHS mRNA during translation and thus limit synthesis. PGHS protein could also attenuate transcription of the gene or translation of the mRNA. PGHS expression also appears to decrease in the presence of glucocorticoids, although whether it is at the level of transcription or translation, is not known.[96]

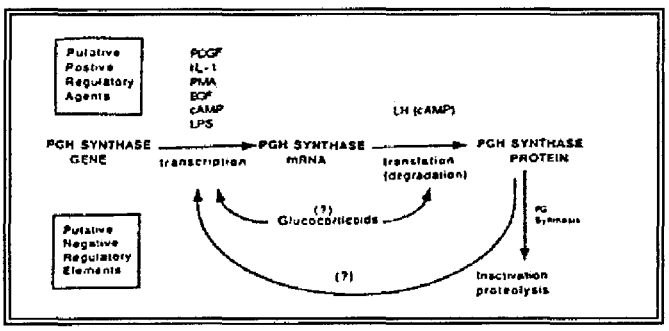


Figure 11: A scheme for the regulation of PGHS expression.[96]

1.5.1.3 Reaction mechanism of cyclooxygenase

PGHS catalyses the first committed step in the formation of PG's and thromboxane from AA. (Fig 12) PGHS has two different enzymatic activities, a cyclooxygenase which mediates the formation of PGG_2 from two molecules of O_2 and a molecule of AA, and a hydroperoxidase which catalyses a net two electron reduction of the 15-hydroperoxyl group of PGG_2 yielding PGH_2. Both activities require heme, and it appears that there is one heme per enzyme subunit.[94,97]

The Cox reaction requires the continued presence of hydroperoxide, and it has been suggested that the peroxidase plays an important role in the hydroperoxide-dependent initiation of Cox.[98] The substrate molecule is held by PGHS in a conformation in which a kink is presented by virtue of rotation about the C-9/C-10 single bond. Initially the removal of the *pro*-S hydrogen atom produces a carbon radical that is trapped by O_2 at C-11. Serial cyclization of the 11-peroxyl radical produces a bicyclic peroxide with *trans* aliphatic side chains. A second molecule of O_2 attaches at C-15 of the carbon radical. Reduction of the resultant peroxyl radical produces PGG_2. As Cox has an obligatory requirement for a hydroperoxide "activator," a reduction of the hydroperoxide concentration below 10nM causes an inhibition of Cox.[86, 98]

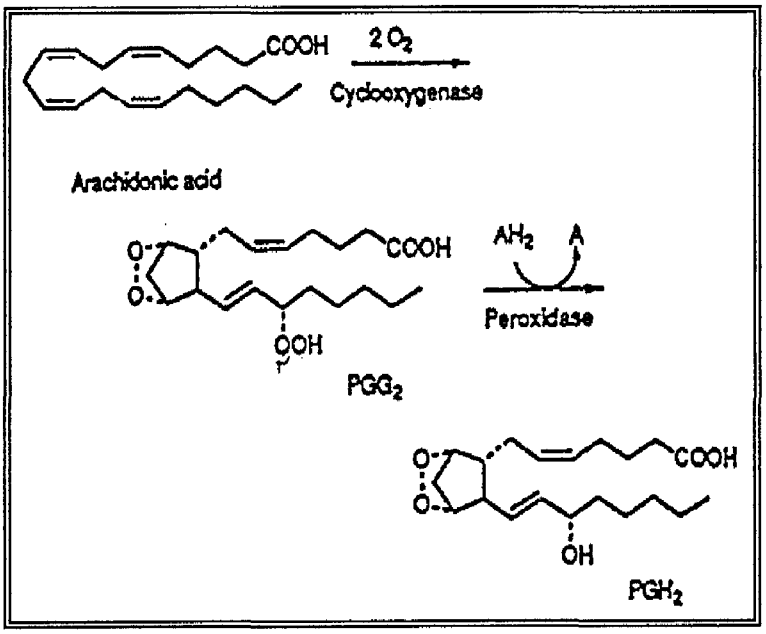


Figure 12: Reaction catalysed by prostaglandin endoperoxide synthase.[99]

There have been numerous publications (cited [81, 91, 100]) describing the roles of AA metabolites in various types of tumours and mechanism for inhibiting PGHS may provide a means for cancer prevention. A variety of drugs known collectively as non-steroidal anti-inflammatory drugs (NSAID) have shown to inhibit Cox activity, and so effect tumour growth and promotion.[100] However, it has been shown that both Cox -dependent and -independent pathways are involved in the cancer chemoprevention properties of NSAID.[101]

1.6 PROSTAGLANDINS AND CANCER

1.6.1 Prostaglandins and tumour growth

Prostaglandins and their analogs endoperoxides are a large series of compounds which mainly enhance cancer development and progression, by acting as tumour promoters, thereby having profound effects on carcinogenesis. A number of mechanisms of action of PG on cancer cells have been postulated: 1) they act mainly by specific receptors, 2) they stimulate DNA, RNA and protein synthesis, 3) PG exert their tumour promoting effect in a manner similar to that of hormones, 4) they increase cyclic AMP levels, 5) they enhance the transformation of precancer cells into cancer cells, and 6) they inhibit apoptosis of cancer cells.[81] It is implied by numerous studies that eicosanoids play a role in metastasis, however, there is no concensus on which eicosanoid is responsible or the mechanism(s) by which it acts.[100]

Considerable variation has been observed in the results of experiments conducted in different model systems with regards to the effect of PG on tumour cells.[100] It has been demonstrated that the level of several eicosanoids are increased in breast cancer in comparison to benign breast tumours.[100] Cancers of the head, neck, lung and colon have been found to form more PG than the normal tissues from which they arise.[90] Many soluble factors are secreted by tumour cells, such as PGE_2, which may alter tumour migration *in vitro* or metastasis in vivo.[100] $PGF_{2\alpha}$ and PGE_2 have been found to promote the growth and development of basal cell carcinoma (BCC). Substantial amounts of PGE_2 and $PGF_{2\alpha}$ have been found in bronchial carcinoid, pheochromocytoma, neuroblastoma and pancreatic α-cell tumours.[81] The increased amounts of PG in tumours reflect enhanced synthesis, which occurs by Cox-catalysed metabolism of AA. The increased production of PG in transformed cells and tumours has been associated with the upregulation of Cox 2. Studies showed that the overexpression of Cox 2 in epithelial cells inhibits

apoptosis and increases the invasiveness of tumour cells.[90] Epidemiological studies have shown that individuals using NSAIDs showed a decreased relative risk of colon cancer. It has been shown that Cox 2 expression is upregulated in colorectal adenomas and carcinomas, with a marked increase of Cox 2 mRNA in 86% of carcinomas compared with paired normal mucosa.[91]

1.6.2 Antitumour activity of prostaglandins

While many reports have described the effect of PG in tumour promotion and metastasis, literature regarding the antitumour activity of synthetic PG analogs also exists. It has been well know since the 1970's that PG of the E and A series inhibit the growth of many tumour cell lines *in vitro* and *in vivo*.[100] Studies by ElAttar *et al.* showed that supplementation with PGE_2 and vitamin E succinate caused a dose-dependent inhibition in DNA in tongue squamous carcinoma cells (SCC-25) *in vitro*. A combination of the two resulted in a greater significant inhibition.[102] The antitumour effect of PGD_2 on the L1210 murine leukemia cell lines was reported by Fukushima *et al.* (cited in [100]). Ellis *et al.* (cited in[100]) examined the role of PGE_1 in Lewis lung carcinoma in male B6D2F1 mice and found a decrease in the tumour weight of the mice receiving 6μg/kg/min of PGE_1.

Despite the considerable amount of evidence, and the expanded role of PG in tumour biology, the mechanism(s) of action of PG at the molecular and cellular levels remains unclear.[81]

1.7 OBJECTIVES

The roles of vitamin E and C as chemopreventive agents are uncertain. However, their effect may in part be due to their synergistic interaction as antioxidants or due to their ability to effect various metabolic pathways, particularly the AA cascade, as numerous reports as indicated above state that PGs are linked to cancer development.

The effects of vitamin E succinate and Asc on the growth of malignant BL6 and non malignant LLCMK cells have been shown in an earlier study to be mediated through the AA cascade which in turn influences cell growth. AA is the substrate for Cox, and Cox is the first enzyme involved in the cascade. The possibility that vitamin E may regulate gene expression via a ligand receptor

mode of action, and the fact that Cox 2 is inducible, is sensitive to extracellular signals and is found in the nuclear membrane, may offer a means by which vitamin E may regulate this pathway at the level of transcription of the Cox 2 gene.

Thus the objectives of this thesis are:

1) to determine the growth effect of combined vitamin E succinate and Asc supplementation at varying concentrations on BL6 and LLCMK cells.
2) to determine whether vitamin E succinate is cleaved to form free vitamin E in BL6 and LLCMK cells.
3) to determine the effect of combined vitamin E succinate and Asc supplementation on cyclooxygenase expression in BL6 cells over a period of time.
4) to determine the effect of combined vitamin E succinate and Asc supplementation on cyclooxygenase activity in the absence and presence of cyclooxygenase inhibitors.

Hence, the effect of combined vitamin E succinate and Asc supplementation in BL6 cells was investigated to determine whether this gene (Cox 2) and the enzyme encoded by this gene are possible target for chemopreventive strategies in conjunction with vitamin E and Asc.

CHAPTER 2

EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID ON CELL GROWTH

2.1 INTRODUCTION

Considerable interest in the therapeutic significance of vitamin E and C in cancer has arisen over the years, and it has been suggested that vitamin C and E may be some of the endogenous substances which could exert selective pressure against transformed cells. The transformation of a normal cell to a cancer cell does not always result in clinical cancer. This suggests that the host exerts considerable selection pressure against the first or first few transformed cells. Mutations may be a reason for certain transformed cells escaping the selection pressure exerted by certain endogenous substances at physiological conditions. However, these substances at pharmacological concentrations could also inhibit tumour activity by either inducing a normal phenotype or by causing cell death.[35]

Vitamin C has been suggested and at the same time disputed as an anti-cancer agent, and both epidemiological and biochemical studies indicate that vitamin C may play an important role in the prevention of cancer.[43, 103] The culturing of animal cell lines is becoming a widely used technique in studying the effect of substances such as vitamins in cancer metabolism.[104]

In vitro studies show that vitamin C can exert multiple mechanisms of action depending upon the cell type and experimental conditions.[37, 42, 105] Bram *et al.* showed that melanoma cells *in vitro* preferentially incorporate vitamin C when compared to other tumour types.[103] Benade *et al.* (cited in [106]) showed that Asc is highly toxic or lethal to Ehrich ascites carcinoma cells *in vitro,* while Park *et al.* reported that Asc suppressed the *in vitro* growth of leukemia cells. It has been shown that Asc produced a cytotoxic effect on neuroblastoma (NB) cells in culture, similarly in Ehrlich ascitic carcinoma, chicken embryo fibroblasts and other mammalian cells in culture. Mulligan (cited in[107]) observed that sodium L-Asc at 1μg/ml caused a cytotoxic effect on human melanoma

cells in culture. However, the concentration for a cytotoxic effect differs from one cell line to another.[107]

Studies have indicated the therapeutic value of vitamin C in the treatment of estrogen-induced renal cancer, mammary tumours, and in the inhibition of neoplastic transformations.[44] Asc was found to inhibit the growth of solid sarcoma -180 in Swiss mice, Ehrlich ascite carcinoma in Swiss or CF_1 mice, and mammary carcinoma in Balb/ C_f/Had/ Se mice, while vitamin C was also shown to increase the survival of C3H/ HEJ mice with C3HBA mouse mammary adenocarcinoma and in Swiss mice with carcinoma -180.[45] Substantial epidemiological data indicates that a diet high in vitamin C may reduce the risk of cancer at several sites in the body.[43] Approximately 90 epidemiological studies have examined the role of vitamin C or vitamin-C-rich-food in cancer prevention, and the vast majority have found statistically protective effects. [106] In a report where a dietary vitamin C index was calculated, 33 of the 46 studies found statistically significant protection of vitamin C, with high intake conferring approximately a twofold protection effect compared to low intake.[28] Block found evidence of a protective effect against cancers of the oesophagus, larynx, oral cavity, and pancreas. Eighteen of 20 studies of cancer at these sites showed a statistically significant association between low intake of dietary vitamin C and increased risk of cancer.[43]

In vitro studies have shown that tocopherol also inhibits growth and/or induces morphological differentiation in several types of tumour cells.[73] Of the various forms of vitamin E, vitamin E succinate has been shown to be the most potent form of vitamin E as an antitumour proliferative agent, which has been established in numerous animal experiments and in a variety of cell culture studies using numerous melanotic cell lines.[35, 55, 109-110] Studies by ElAttar *et al.* demonstrated that supplementation of vitamin E succinate on human tongue squamous carcinoma (SCC-25) cells caused significant dose-dependent inhibition of cell growth.[62] Prasad *et al.* showed that the most inhibitory effective concentrations of vitamin E succinate on BL6 cells is narrow and that concentrations of 6μg/ml produced marked morphological differentiation and growth inhibition when compared to 5μg/ml, while 10μg/ml was found to be lethal.[35] Vitamin E succinate was shown to inhibit proliferation of ovian retrovirus-transformed lymphoid tumour cell line (C4#1) in a dose-dependent manner, with a maximal growth inhibition occurring at 10μg/ml. The effect

of vitamin E succinate in cell proliferation appears to be cell type dependent.[55]

Numerous *in vitro* studies on the effect of vitamin C and vitamin E individually on cell growth in numerous cell lines and their possible synergistic interaction as antioxidants have been well documented.[42, 69, 73] However, the assumption that the supplementation of a combination of the two vitamins to cell lines and the subsequent growth effects has only been reported by Midgley.[111]

2.2 MATERIALS AND METHODS

MATERIALS

Basal Minimum Essential (MEM), McCoys 5A Modified Media, L-ascorbic acid, (+) -α-tocopherol acid succinate and L-serine were purchased from Sigma Chemicals Co. USA. Nova streptomycin and nova penicillin were purchased from Nova Industries Pharmaceuticals, Ltd, South Africa. Foetal Calf Serum (FCS) and sterile tissue culture flasks ($25cm^2$ and $75cm^2$) were purchased from Corning, USA. Glycine, D-glucose, phenol red, KH_2PO_4, $Na_2HPO_4.H_2O$, dimethylsulphoxide (DMSO) and ethylenediamenetetracetic acid (EDTA) were purchased from BDH Chemicals Ltd, England. Trypsin was purchased from Boehringer Mannheim, Germany. A haemocytometer was purchased from Neubauer, Germany (double ruling).

Malignant murine melanoma (BL6F10) and non malignant monkey kidney (LLCMK) cell lines were obtained from Stellenbosch University, South Africa.

METHODS

2.2.1 PREPARATION OF CULTURE REAGENTS

2.2.1.1 Preparation of the medium

The following compounds were added to McCoys 5A Media (12.1g/l) for the growth and maintenance of BL6 cells.

0.01g/l Serine	2.2g/l $NaHCO_3$
0.006g/l Glycine	5ml/l Nova-strep and nova-pen mixture

(One vial of sodium benzylpenicillin and one vial of streptomycin sulphate were combined and made up to 100ml with milli Q water.)

2.2.1.2 Filtration of the medium

The medium was filtered through a Millipore filtration unit (Millipore Corporation, USA), using 3 filters of different pore size: a prefilter, type AW 03 "Membr filter" 50k (size 130); 0.45μm type HA filter (HAWP 14250); and a 0.22μm type GS filter (GSWP 14250). Under sterile conditions 500ml of milli Q water was pumped through, followed by 100ml of medium, both of which were discarded. The remainder of the medium was filtered into autoclaved Schott bottles. The bottles of medium were incubated at 37°C for 72 hours to test for contamination.

2.2.1.3 Preparation of growth and freezing media

FCS was filtered through a 0.45μm Millipore filter using a Swinnex-25 holder (Millipore Corporation, USA) into the media, so growth media containing 10% (v/v) FCS was obtained. The media was incubated at 37°C for 72 hours to test for contamination. The pH of the medium was adjusted by the addition of a few drops of sterile 1M NaOH if too acidic. Freezing media was prepared by adding 10% DMSO and 20% FCS to 100ml media, and frozen until required.

2.2.1.4 Preparation of trypsin solution

The trypsin required was a 0.33% solution, containing the following compounds:

0.8g/l NaCl	0.4g/l KCl
1.0g D-glucose	0.58g/l $NaHCO_3$
0.2g/l EDTA	33.33mg/l Trypsin
0.02g/l Phenol red	

10ml/l Nova-strep and Novapen Mixture (prepared as in 2.2.1.1)

The trypsin was filtered directly into the culture flasks, through a 0.45μm Millipore filter using a Swinnex-25 holder, and kept at -20°C until required.

2.2.1.5 Preparation of (+) vitamin E succinate and L-ascorbic acid stock solutions

Stock solutions of vitamin E succinate and Asc were freshly prepared. Stock solutions of vitamin E succinate (5-20mg/ml) were prepared in ethanol and diluted 1:1000 in media containing 10% (v/v) FCS, to give a final concentration of 5, 10 and 20μg/ml vitamin E succinate respectively, in a final 0.1% final volume of ethanol. Stock concentrations of L-Asc 25 and 50 mg/ml were prepared in 1ml of milli Q water and diluted 1:1000 in media containing 10% (v/v) FCS to give a final concentration of 25 and 50μg/ml, respectively. Thus, four sets of media containing the following combined vitamin E succinate:Asc concentrations were obtained: 5:25, 10:50, 10:25 and 20:25μg/ml. The concentrations were calculated as described by Midgley in order to obtain the most effective inhibitory concentrations of combined vitamin E succinate and Asc.[111]

2.2.1.6 Preparation of phosphate buffered saline solution

Phosphate buffered saline (PBS), pH 6.6, in milli Q water was prepared as follows:

8g/l NaCl	0.2g/l KCl
0.2g/l KH_2PO_4	0.15g/l $Na_2HPO_4.H_2O$

The PBS solution was kept at 4°C until required.

2.2.2 CELL CULTURE

2.2.2.1 Routine cell culture procedure

Sterile techniques were employed at all times. All experiments were performed under a laminar flow bench which had previously been exposed to UV light, and all equipment used was either sterilized by autoclaving or purchased sterile. Regular swabbing of the hands and bench top with 70% ethanol were employed throughout experimentation. Solutions were filtered through millipores using Swinnex-25 holders.

Non-malignant LLCMK and malignant BL6 cells were maintained in 75cm^2 sterile tissue culture flasks containing 30ml of 10% (v/v) FCS of their respective growth media. The flasks were incubated at 37°C and media was changed frequently when the cells were not required. To passage the cells, the growth media was discarded and 10ml of sterile trypsin added. The flasks were incubated at 37°C for 10 minutes to allow for cell detachment from flask surfaces. The cells were then passaged into three new sterile flasks and 30ml 10% (v/v) FCS medium was added to

each flask.

2.2.2.2 Freezing of cells

Storage of all cell lines involved the harvesting of cells under sterile conditions with 10ml of sterile trypsin, and incubation at 37°C. The detached cells were then transferred to sterile tubes with caps under sterile conditions under the laminar flow bench and centrifuged. The trypsin was discarded, 2ml of freezing media was added, and the contents transferred to a cryogenic vial. The cells were stored in liquid nitrogen until required.

2.2.2.3 Experimental cell culture procedure

LLCMK or BL6 cells that were nearing confluency in 75cm^2 flasks were trypsinised with 10ml of sterile trypsin. The cells were incubated at 37°C to allow for the detachment of the cells from the flask surface. The cells were then poured into sterile tubes with caps and centrifuged at 3500g (Eppendorf Centrifuge 5403, Germany) for 5 minutes. The trypsin was poured off and the pellet was resuspended in 2ml of 10% (v/v) FCS medium. A haemocytometer was used to count the total number of cells in suspension, so that the volume required to seed 5 x 10^5 cells per 75cm^2 flask, or 3 x10^5 cells per 25cm^2 flask could be calculated.

LLCMK or BL6 cells, 5 x10^5 were seeded in 6 sets of 3 flasks (75cm^2). The first set of flasks contained 30ml of 10% (v/v) FCS medium and was referred to as the O control culture (O). To the second set a final 0.1% volume of ethanol in 30ml of 10% (v/v) FCS medium was added, and was referred to as the ethanol control culture (OE*). The remaining 4 sets of flasks contained the varying levels of combined vitamin E succinate (5-20µg/ml) and Asc (25-50µg/ml) which were added to 30ml of 10% (v/v) FCS medium. These 6 sets of flasks were incubated at 37°C for approximately 5 days with one media change. The analytical studies for these experiments can be found in sections 3.1, 4.2 and 4.3.

2.2.2.4 Harvesting of the experimental cell cultures

When the first flask reached confluency, the cells in all the flasks were harvested under non-sterile conditions. Using 10ml of trypsin and incubated at 37°C. The cell suspensions were transferred to tubes with caps and centrifuged at 3500g for 5 minutes. The pellet was resuspended in 2ml of

PBS buffer and the cells numerated. The cell counts were used as an indication of cell growth and hence determining the effect of combined vitamin E succinate Asc on cell growth to be determined. The data was expressed as a percentage growth inhibition in relation to the untreated vehicle control. The cells were then used for further experimentation and analysis.

2.2.3 STATISTICAL ANALYSIS

The results obtained from the experiments were analysed using a one way analysis of variance (ANOVA) followed by the Student-Newman Keuls Multiple Range Test.

2.3 RESULTS

The effect of vehicle treatment on LLCMK and BL6 cell growth is shown in Table 1a and Table 1b. A control experiment using the solvent of vitamin E (absolute ethanol) was done to determine whether the ethanol had any effect on the parameters of this study. The results show that no significant inhibitory effect on cell growth in both LLCMK and BL6 cells occurred with ethanol (OE*) supplementation when compared to the control culture (O)

Table 1a: The effect of vehicle treatment on LLCMK cell growth. Results are the mean of three cultures ±SEM.

Treatments	Growth inhibition [% of untreated controls]
	LLCMK cells
	Exp 3.1
O	100.00 ±6.15
OE*	93.45 ±12.05

O - control culture

OE* - control culture containing 0.1% ethanol

Table 1b: The effect of vehicle treatment on BL6 cell growth. Results are the mean of three cultures ±SEM.

Treatments	Growth inhibition [% of untreated control]			
	BL6 cells			
	Exp 3.1	Exp 4.2.1.3.1	Exp 4.2.1.3.2	Mean
O	100.00 ±3.63	100.00 ±2.92	100.00 ±4.96	100.00 ±3.84
OE*	94.76 ±6.58	103.26 ±1.65	130.74 ±5.80	110.91 ±12.14

O - control culture

OE* - control culture containing 0.1% ethanol

The effect of the combination of vitamin E succinate and Asc supplementation on cell growth of LLCMK cells is shown in Table 2a. Supplementation of combined vitamin E succinate and Asc at the varying concentrations resulted in significant inhibitory growth effect on LLCMK cells at concentrations of 10:50; 10:25; and 20:25µg/ml, when compared to the LLCMK control culture (OE*), while at 5:25µg/ml a decrease in cell growth was observed, although this was not significant.

Combined Asc and vitamin E succinate supplementation on BL6 cells at the varying concentrations showed an overall marked decrease in cell proliferation when compared to the control culture (OE*) (Table 2b). Significant decreases in cell growth were observed at 5:25µg/ml ($p \leq 0.01$), 10:50µg/ml ($p \leq 0.001$), 10:25µg/ml ($p \leq 0.001$), and 20:25µg/ml ($p \leq 0.001$) when compared to the BL6 control culture (OE*). The greatest growth inhibitory effect on BL6 cells was observed with the supplementation of 20:25µg/ml vitamin E succinate:Asc relative to the control culture (OE*) and the other groups.

Table 2a: The effect of combined vitamin E succinate and ascorbic acid supplementation on cell growth in LLCMK cells. The values are the mean of three cultures ±SEM.

[vitamin E succinate: ascorbate] µg/ml	Growth inhibition [% of untreated controls] LLCMK cells Exp 3.1
OE*	100.00 ±3.62
5:25	86.60 ±7.70
10:50	50.34[a] ±5.09
10:25	60.55[a] ±5.97
20:25	47.49[a] ±2.80

a - p≤ 0.001 relative to control cultures (OE*)

Table 2b: The effect of combined vitamin E succinate and ascorbic acid supplementation on cell growth in BL6 cells. The values are the mean of three cultures ±SEM.

[vitamin E succinate: ascorbate] µg/ml	Growth inhibition [% of untreated controls]			
	BL6 cells			
	Exp 3.1	Exp 4.2.1.3.1	Exp4.2.1.3.2	Mean
OE*	100.00 ±3.14	100.00 ±1.59	100.00 ±2.65	100.00 ±2.46
5:25	87.22[b] ±2.13	82.06[b] ±2.26	75.40[a] ±1.21	81.56[b] ±3.42
10:50	60.78[a] ±3.85	58.16[a] ±1.69	61.83[a] ±2.13	60.26[a] ±1.09
10:25	68.04[a] ±2.83	74.96[a] ±5.89	67.78[a] ±1.15	70.26[a] ±2.35
20:25	30.24[a] ±0.76	46.24[a] ±2.83	52.64[a] ±2.81	43.04[a] ±6.67

a - $p \leq 0.001$ relative to the control cultures (OE*)

b - $p \leq 0.01$ relative to the control cultures (OE*)

2.4 DISCUSSION

Vitamin E has been found to inhibit carcinogenesis *in vitro* and to suppress tumour formation in different experimental systems, while Asc has been shown to both inhibit and stimulate tumour growth in cell lines in culture.[35, 42, 44, 45, 64, 73, 75, 109]

Results from this study showed that supplementation of combined vitamin E succinate and Asc produced a significant growth inhibitory effect in BL6 cells with a lesser inhibitory effect occurring in the LLCMK cells. Supplementation of combined vitamin E succinate and Asc in BL6 cells resulted in a decreasing trend in growth showing a significance ($p \leq 0.001$) at all combined

concentrations (5:25; 10:25; 10:50 and 20:25μg/ml). Inhibition in BL6 cells increased as the concentration of vitamin E succinate relative to Asc increased, suggesting that the ratio of vitamin E succinate to Asc is an important factor in determining the growth inhibitory effectiveness of these vitamins. This may be due in part to the synergistic interaction of the two vitamins, where vitamin C regenerates the vitamin E molecule, and so produces a greater inhibitory effect on cell growth by virtue of their combined antioxidant properties. In order for this synergistic interaction to occur, the succinate group needs to be cleaved off from the vitamin E molecule. Thus the effect of combined vitamin E succinate and Asc supplementation on the metabolism of vitamin E succinate in BL6 and LLCMK cells was investigated.

Asc ability to inhibit tumour cell growth has been attributed to certain inherent properties of the molecule. The toxicity of Asc has been suggested to be due to its ability to inhibit the catalase enzyme which leads to the toxic accumulation of H_2O_2 in the cell, as well as the inhibition of the hyaluronidase enzyme, thereby causing cell death.[105, 107] Asc inhibition may depend on the extent to which a given cell incorporates Asc, and the sensitivity of the cell to its cytotoxic effects.[103] The concentration required for a cytotoxic effect has been shown to differ from one cell line to another.[107] Work by Gardiner *et al.* showed that Asc supplementation of between 25-200μg/ml did not have a significant inhibitory or stimulatory effect on LLCMK cells but reduced the growth of transformed BL6 cells.[112] Furthermore, Bram *et al.* showed that vitamin C directly inhibited the growth of proliferating BL6 cells, while the non-malignant cells tested were less sensitive to this vitamin.[103] It was observed by Prasad *et al* that no morphological changes occurred in mouse melanoma (B-16), mouse neuroblastoma (NBP_2), rat glioma (C-6) and mouse fibroblasts (L cells) in culture when Asc, up to 200μg/ml, was supplemented to the cells, while concentrations from 500-1000μg/ml were shown to be lethal.[35]

α-TOH succinate has been found to have several unique biochemical properties *in vitro* and *in vivo*. A number of *in vitro* studies have shown that α-TOH succinate induces differentiation and growth inhibition in certain human and animal tumour cells in culture.[109] Prasad *et al.* reported that the effective concentration range of α-TOH succinate is very narrow, while a concentration of 10μg/ml being found to be lethal, and morphological changes were observed in mouse melanoma cells (B-16) in culture including enlargement of soma, elongation of bipolar

cytoplasmic processes, and the tendency of cells to arrange themselves parallel to each other. They also noted that the growth inhibitory effect of α-TOH succinate on B-16 cells in culture is primarily irreversible.[35] Studies have also demonstrated that α-TOH succinate inhibits HL-60 human promyelocytic leukemia cell proliferation, while estrogen receptor-positive and estrogen receptor-negative human breast cancer cell lines were inhibited in a dose-dependent manner *in vitro*.[109]

CHAPTER 3

THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON THE METABOLISM OF VITAMIN E SUCCINATE

3.1 INTRODUCTION

It is generally accepted that DL-α-TOH is an antioxidant and a radical scavenger in various metabolic processes, and stabilises cell surface membranes and the membranes of subcellular organelles by its physicochemical interactions.[113] For vitamin E administration, ester derivatives of α-TOH are commonly used for both experimental and therapeutic purposes. A stable dosage of vitamin E can be provided by these esters, which are broken down by cellular esterases to supplement endogenous α-TOH. The α-TOH esters are hydrolysed during the absorptive process which occurs in the gut lumen.[60]

α-TOH succinate, but not α-TOH and its analogs, have been found to have several unique biochemical properties *in vitro* and *in vivo*. *In vitro* studies have shown that α-TOH succinate is the most potent form of vitamin E for inducing morphological differentiation and growth inhibition in certain animal and human tumour cells in culture, however, the exact reasons for this are unknown.[35, 109] Pascoe and Reed showed that in tissue culture α-TOH succinate was more effective than free TOH in protecting against the toxic effect of the free radical generating drug adriamycin. They proposed that vitamin E may be ineffective in protecting against toxicity because of its high lipophilicity which might result in its immediate distribution to the outer membrane lipid environment. The tocopheryl ester has a lower lipophilicity than the nonesterified vitamin E compound, and so is not expected to be trapped in the outer lipid bilayer to the same extent as the free vitamin E molecule. They also suggested that with vitamin E succinate supplementation, the tocopheryl esters are first absorbed into the cell (by unknown transfer mechanisms), after which they are hydrolysed by esterases to release the free vitamin E compound. These vitamin E molecules derived from the cleavage of vitamin E succinate are then subsequently transported by unknown transfer mechanisms and distributed throughout the internal

cell environment. The result of the more extensive intracellular distribution of the deesterified TOH would therefore be its localization in internal membrane sites and subsequent greater availability for protection against oxidative stress in comparison to the free vitamin E molecule.[114]

Vitamin E succinate cannot exhibit any antioxidant properties since the phenolic hydroxyl of the tocol nucleus is chemically protected by the succinate esterified to it. If it is to act as an antioxidant and to interact synergistically with vitamin C, the succinate group must be hydrolysed to remove the protective succinate group from the hydroxyl group.

To determine whether inhibition of BL6 cell growth is the result of a synergistic interaction between vitamin C and E or vitamin E succinate, experiments were carried out to examine the effect of combined supplementation of vitamin E succinate and Asc on the metabolism of vitamin E succinate.

3.2 CLEAVAGE OF VITAMIN E SUCCINATE TO FREE VITAMIN E AND SUCCINATE AND ANALYSIS BY REVERSE PHASE HPLC

3.2.1 MATERIALS AND METHODS

MATERIALS

Nitric acid was purchased from Holpro Chemical Co, South Africa. Iminodiacetic acid and neostigmine were purchased from Sigma Chemical Co, USA. High Performance Liquid Chromatography (HPLC) grade methanol, acetonitrile, hexane and ethanol were supplied by BDH Chemicals Ltd, England. Reverse Phase μBondaclone ID C^{18} HPLC column (300 x 390mm) was purchased from Phenomenex, USA, while the Gaurd-Pak precolumns [5m, ODS] were obtained from Metachemicals, USA.

METHODS

3.2.1.1 Glassware preparation

Prior to use, all glassware was treated by a modified method of Buettner (cited in[115]). This

involved the glassware being soaked for 48 hours in a 25% solution of HNO_3. The glassware was then rinsed in milli Q water and soaked for a further 48 hours in a solution of milli Q water containing the chelating resin, iminodiacetic acid (5g/l).

3.2.1.2 Preparation of Ca^{2+} and Mg^{2+} free phosphate buffered saline, pH 7.4

To minimise oxidation of vitamin E succinate, trace amounts of catalytic transition metals such as Ca^{2+} and Mg^{2+} were omitted from the assay buffer, PBS. The buffer was prepared as described by Kelly *et al.*[115] The buffer contained:

2.68mM KCL	1.47mM K_2HPO_4
136.8mM NaCl	8.06mM NaH_2PO_2
0.5mg/ml EDTA	10nm Neo-stigmine bromide

3.2.1.3 Cell culture procedure

Refer to sections 2.2.2.3 and 2.2.2.4.

The cell pellets were resuspended in 1ml ice cold Ca^{2+} and Mg^{2+} free PBS buffer. 20µl of a 200mM Asc solution was added to the cell suspensions.

3.2.1.4 Extraction of vitamin E and vitamin E succinate

The extraction of vitamin E and vitamin E succinate was carried out using a modified version of the method described by Kelly *et al.*[115] Following cell enumeration, the cell suspensions were centrifuged at 750g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed in 1ml of PBS containing 200mM Asc and centrifuged. A 1ml volume of PBS was added to the final washed pellet and the cell suspension was finger vortexed and placed on ice for 2 minutes. The cell suspension was then transferred to a cold Dounce homogeniser and 500µl of 95% ethanol was added. The samples were homogenised 30 times with a tight plunger followed by the addition of 500µl ice cold PBS. 500µl of ice cold hexane (non-polar solvent) was added. The cell suspensions were transferred to pre-soaked glass tubes and centrifuged at 300g for 3 minutes to separate the phases. Aliquots of the top phase (hexane) were transferred to glass storage vials using Pasteur pipettes. The head space air was replaced with nitrogen gas (N_2) and stored at -70°C until required.

The extracted samples were analysed by reverse phase HPLC, using a modified method of Scalia *et al.*[116] The hexane extracts were dried under a stream of N_2 and reconstituted with 100µl of mobile phase containing methanol:acetonitrile (60:40, v/v). Separation of vitamin E and vitamin E succinate was performed on a µBondaclone C^{18} column fitted with a GaurdPak precolumn and eluted isocratically with methanol:acetonitrile at a flow rate of 1.5ml.min^{-1}. The reconstituted samples were injected (50µl injected volume) onto the column. Detection was performed using a UV-visible spectrophotometer (Beckman, System Gold, Programmable detector module, Mode 166, USA). The identity of the separated compounds was determined by co-chromatography with authentic standards of (+) vitamin E and vitamin E succinate. The quantity of each sample was determined by using peak areas and the standard curve recorded in Appendix 1.

3.2.2 RESULTS

The effect of combined vitamin E succinate and Asc supplementation in LLCMK and BL6 cells and the subsequent effect on the levels of vitamin E succinate and vitamin E found is shown in Table 3.

Supplementation of combined vitamin E succinate and Asc supplementation in LLCMK cells showed that vitamin E succinate levels increased with increasing concentrations of vitamin E succinate and Asc, and significance was shown at 20:25µg/ml ($p \leq 0.001$) when compared to the 5:25µg/ml combined vitamin supplemented cultures. No vitamin E was detected which suggests that the esterase needed for cleavage of the vitamin E succinate molecule is absent in LLCMK cells.

In BL6 cells, vitamin E succinate supplementation at various concentrations in each case resulted in the production of some vitamin E, suggesting the presence of an esterase. However, there was no significant, parallel increase in vitamin E production with increased vitamin E succinate supplementation possibly because the esterase was already saturated with vitamin E succinate substrate at the lowest concentration and, therefore already operating at V_{max}.

Table 3: The effect of combined vitamin E succinate and ascorbic acid supplementation in LLCMK and BL6 cells and the subsequent effect on the levels of vitamin E succinate and vitamin E found. The values are the mean of three cultures ±SEM.

[vitamin E succinate: ascorbate] µg/ml	vitamin E succinate (nmoles/10^4 cells)		vitamin E (nmoles/10^4 cells)	
	LLCMK	BL6	LLCMK	BL6
OE	ND	ND	ND	ND
5:25	0.026 ±0.003	0.012 ±0.007	ND	0.002 ±0.02
10:50	0.009 ±0.004	0.012 ±0.006	ND	0.003 ±0.001
10:25	0.0501 ±0.017	0.007 ±0.001	ND	0.004 ±0.008
20:25	0.249[a] ±0.048	0.012 ±0.001	ND	0.006 ±0.008

ND - not detected
a - $p \leq 0.001$ relative to 5:25µg/ml LLCMK cells (vitamin E succinate)

3.3. DISCUSSION

Combined supplementation of vitamin E succinate and Asc in LLCMK cells showed (Table 3) that with an increase in the concentration of vitamin E succinate, there was an increased accumulation of vitamin E succinate in the cells, however, no free vitamin E was detected. This suggests that there are no esterases present in LLCMK cells to cleave the succinate group from the vitamin E molecule. In BL6 cells, an increase in the concentration of vitamin E succinate resulted in an increased accumulation of vitamin E, while no increasing or decreasing trend was observed in relation to the level of vitamin E succinate present in BL6 cells. Thus, an esterase is probably present in BL6 cells which brings about the removal of the succinate to form the free vitamin E molecule. It is thus proposed that a synergistic interaction may occur between vitamin E and Asc

in BL6 cells. Further studies were consequently done using only BL6 cells and not the nontransformed LLCMK cells.

α-TOH esters are believed to undergo hydrolysis via any number of nonspecific esterases in the liver, vascular tissue, and other organs.[117] Although vitamin E does not exhibit any antioxidant properties unless the succinate is cleaved off, evidence suggests that cells hydrolyse it to the free vitamin E form, which is an active antioxidant.[55] Pascoe and Reed showed that a marked elevation in cellular TOH content, after supplementation of the cell incubation medium with TOH succinate was due to the enhanced intracellular hydrolysis of the TOH ester in calcium depleted cells. However, the reason for the enhanced intracellular hydrolysis of the TOH esters is at present unknown.[117] Farris proposed that the TOH succinate may act as a carrier for TOH, enabling the release and accumulation of TOH at a unique and critical cellular site.[60]

Vitamin E has been suggested to modulate the regulatory proteins in signal transduction pathways and those of the eicosanoid pathway.[82] The synthesis of eicosanoids is initiated by signal transduction cascades which result in the hydrolysis of free arachidonic acid from membrane phospholipids.[118] The key step in the conversion of AA to PGs is catalysed by Cox, and it is not inhibited by vitamin E *in vitro*.[82] Prasad and co-workers (cited in[55]) reported that a significant amount of vitamin E was found bound to the chromatin fraction in glioma and neuroblastoma cells, thus suggesting that vitamin E may be regulating gene expression in mammalian cells via a ligand receptor mode of action. Since Cox 2 is sensitive to extracellular signals and its expression may be increased by activation of cell surface receptors,[91] the effect of combined vitamin E succinate and Asc supplementation on the expression of Cox 2 in BL6 cells was subsequently investigated.

CHAPTER 4

THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON CYCLOOXYGENASE EXPRESSION IN BL6 CELLS

4.1 INTRODUCTION

For many years it has been realised that cancer has a genetic component. Boveri, in 1914, suggested that an aberration in the genome might be responsible for the origin of cancers.[119] In molecular biology and tissue culture, technical advances have permitted the detailed study of specific genes in the pathogenesis of cancer. Cell culture techniques and the use of genetically defined experimental animals allow for the identification of factors necessary for transformation. Gene expression begins with the synthesis of ribonucleic acid (RNA). Eukaryotic gene expression studies entail the elucidation of which RNAs are produced in which cells at which times. The study of RNA in general is facilitated by its smaller size relative to DNA, however, RNA is sensitive to degradation by a ribonuclease (RNase) that resists common sterilisation procedures, thereby necessitating careful handling.[120] The purification of high quality undegraded RNA from biological sources allows for the investigation of the regulation of gene expression.[121]

Transcription is the process whereby single-stranded RNA molecules are synthesised from a defined sequence of double-stranded DNA. Transcription at any phase is subjective to variation, which offers potential points in the regulation of gene expression. The four major potential regulatory points in gene regulation include, 1) regulation at the transcriptional level; 2) regulation at the postranscriptional level; 3) regulation at the translational level; 4) regulation at the postranscriptional level; however, the chemical stability of cytoplasmic RNA may also be a point of regulation.[121]

PGs are ubiquitous tissue hormones which exert pleiotropic effects on cancer cells. However, their mechanism(s) of action at the molecular and cellular level have not yet been clarified. Evidence suggests that PGs exert their tumour promoting effects by stimulating DNA, RNA and

protein synthesis in cancer cells and consequently accelerate cell division and proliferation.[81] Cox is a key enzyme in the biosynthetic pathway leading to the formation of PG, which are potent biological mediators with diverse normal physiological effects.[122] A question has been raised as to why cells need an inducible Cox. Nearly all cells produce Cox 1 constitutively. If phospholipase is activated by cells being stimulated by external stimuli and AA is released, and the Cox 1 enzyme is present in excess and is available to this AA, a paradox is seen. Why should cells produce a second form of Cox in response to ligand stimulation if the AA present can be converted to PGH_2 by Cox 1? Studies reveal that AA released from membranes cannot be converted to PGE_2 by constitutive Cox 1. However, this pool of AA can only be converted to PGE_2 by Cox 2 synthesized in response to ligand stimulation.[95]

Recent reports have suggested that a relationship between PGs and vitamins exists.[81] It has been suggested that vitamin E and other lipid antioxidants modulate the biosynthetic pathways in AA metabolism at a number of points, including the Cox enzyme.[82] Midgley reported that the supplementation of combined vitamin E succinate and Asc on BL6 cells significantly increased PGE_2, which suggests that the observed increases in PGE_2 levels in BL6 cells may be a result of the increased Cox activity.[111]

Numerous studies have shown that there is a significant relationship between levels of Cox 2 and cancer, and that Cox 2 is a target molecule by which the incidence of cancer might be modulated.[90] Prasad and co-workers reported that vitamin E is capable of binding to cytosolic proteins and that a significant amount of vitamin E is bound by the chromatin fraction in glioma and neuroblastoma cells treated with vitamin E succinate.[123] These observations suggest the possibility that vitamin E could have its antiproliferative effects at the nuclear level by a ligand receptor mode of action.[55, 123] Cox 2 is localised in the ER and nuclear membrane and the gene is thought to be sensitive to extracellular signals and its expression may be increased by activation of cell surface receptors.[90, 92] Cox 2 is also known to be regulated at the level of transcription.[89] Hence, the effect of combined vitamin E succinate and Asc supplementation on the expression of Cox 2 in BL6 cells was investigated. Initial studies were conducted over a 7 day period since this was the length of time the vitamins were supplemented in all previous studies. Problems were, however, experienced in detecting Cox 2 expression over this period as will be discussed.

Consequently, subsequent experiments were conducted over a 12 hour period.

4.2 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON CYCLOOXYGENASE EXPRESSION IN BL6 CELLS OVER A TIME COURSE OF SEVEN DAYS.

4.2.1 MATERIALS AND METHODS

MATERIALS

Oligonucleotide AVM1 was purchased from The Great American Gene Company, USA. Oligotex mRNA Mini Kit and RNeasy Mini Kit were purchased from Qiagen, South Africa. RNA molecular weight marker (II), formamide and DNA Herring Sperm, Bovine serum albumin (BSA) fraction V, ethidium bromide (EtBr) were purchased from Boehringer Mannheim, Germany. Kodak Scientific Imaging Film (Biomax MS) and a Kodak BioMax Transcreen-HE (high energy) Intensifying Screen were purchased from Eastern Kodak company, NY. Photographic fixitive, developer, and starter were purchased from Protea Medical Services, South Africa. Formaldehyde, diethyl pyrocarbonate (DEPC), ficoll-type 400-DL and polyvinylpyrrolidone were purchased from Sigma Chemicals Co, USA. [alpha-32P]dCTP (3000CI/MMol), a Hypercassette and MOPS [3-(N-Morpholine) propanesufonic acid], Hbond-N+Nylon membranes were purchased from Amersham International, England. Agarose (genetic technology grade) was purchased from FMC Bio-products,USA. RNase Away was obtained from Molecular Bio-products, (MβP), CA. Sodium lauryl sulphate (SDS), bromophenol blue, glycerol and sodium citrate were purchased from Saarchem, South Africa. T4 Polynuclear kinase (PNK) was purchased from USB (United States Biochemical),USA. Tris-free base (ultra pure) was purchased from B&M Scientific, South Africa. Acetic acid glacial was purchased from BDH, England.

METHODS

4.2.1.1 PREPARATION OF GLASSWARE, PLASTICWARE AND REAGENTS

Test tubes, pipette tips, and eppendorphs were autoclaved before being used. Gloves were worn at all times. The work surface was cleaned with RNase Away before each experiment to remove any RNase present.

4.2.1.1.1 Preparation of glassware

Glassware was treated prior to experiments to ensure that it was RNase-free, and treated with DEPC as described by Farrell.[121] The glassware was rinsed with DEPC (0.1% in water) overnight (12 hrs) at room temperature and then autoclaved for 15 minutes to remove residual DEPC.

4.2.1.1.2 Preparation of solutions and DEPC water

Solutions and water were treated with 0.1% DEPC. DEPC (0.1%) was added and shaken vigorously to bring the DEPC into solution. The solutions and water were left overnight at room temperature and then autoclaved for 15 minutes to remove any trace amounts of DEPC.

4.2.1.1.3 Preparation of nondisposable plasticware, the electrophoresis tank and the gel support

Nondisposable plasticware, the electrophoresis tank and the gel support were thoroughly rinsed with 0.1N NaOH/1mM EDTA solution, followed by RNase-free water.

4.2.1.1.4 Preparation of TE buffer pH 8.0

TE buffer, pH 8.0 in RNase-free water, was prepared a follows:

10mM Tris-HCl (pH 8.0)

1mM EDTA (pH 8.0)

4.2.1.1.5 Preparation of 1.2% Formaldehyde (FA) gel (100 ml)

1.2g agarose

10ml 10x FA gel buffer

DEPC-water to 100ml

Agarose was melted in a microwave and allowed to cool to 65°C in a water bath. 1.8ml of 37% (12.3M) formaldehyde and 1µl EtBr (10mg/ml) was added, mixed thoroughly and poured onto the gel support. Prior to running the gel, the gel was equilibrated in 1x FA gel running buffer for 30 minutes.

4.2.1.1.6 Preparation of 10x FA Gel buffer:

50mM Sodium acetate

10mM EDTA

200mM MOPS

pH was adjusted to 7.0 with NaOH

4.2.1.1.7 Preparation of 1x FA gel running buffer

100ml 10x FA gel buffer

20ml of 37% (12.3M) formaldehyde

880ml DEPC-water

4.2.1.1.8 Preparation of 5x RNA Loading buffer

16µl saturated bromophenol blue

80µl 500mM EDTA (pH 8.0)

720µl 37% (12.3M) formaldehyde

2ml 100% glycerol

3084µl formamide

4ml 10x FA gel buffer

Add DEPC to make up to 10ml

4.2.1.1.9 Preparation of 20x SSC buffer

175.3g NaCl (3M)

88.2g NaCitrate (0.3M)

Adjust pH to 7.0 using 10N NaOH

Adjust volume to 1L

Sterilize by autoclaving

4.2.1.1.10 Preparation of DNA Herring Sperm (100µg/ml)

DNA herring sperm (10mg/ml stock) was sonicated on ice for 30 seconds and then left to stand for 30 seconds on ice. This was repeated 6 times and thereafter the solution was autoclaved for 15 minutes.

4.2.1.1.11 Preparation of 100x Denhardt's solution

2% Ficoll type 400

2% BSA (fraction V)

2% polyvinylpyrrolidone

4.2.1.1.12 Preparation of hybridization buffers

Prewarm the formamide

5x SSC, 5x Denhardt's solution and 0.1% SDS was mixed together and prewarmed to 45°C.

100µg/ml of Herring sperm DNA was added and boil for 8-10 minutes.

The Herring sperm DNA and the SSC, Denhardt's solution and SDS mixture were added to the prewarmed 50% deionized formamide. The mixture was swirled to mix it and stored at 42°C until ready to use.

4.2.1.1.13 Preparation of hybridization buffers (Zeta probe)

1mM EDTA (pH 8.0)

0.5M NaH_2PO_4 (pH 7.2)

7% SDS

4.2.1.1.14 Preparation of wash I and wash II (Zeta probe)

Wash #I

1mM EDTA

40mM NaH_2PO_4 (pH 7.2)

5% SDS

Wash #II

1mM EDTA

40mM NaH_2PO_4 (pH 7.2)

5% SDS

4.2.1.2 CELL CULTURE PROCEDURE

Refer to section 2.2.2.3 and 2.2.2.4.

BL6 cells were only used for this study.

4.2.1.3 MOLECULAR PROCEDURE

Two different protocols were used for the extraction of total RNA (tRNA) and for the Northern blots analysis.

4.2.1.3.1 Protocol for total RNA extraction I

The extraction method was taken from the RNeasy Mini Kit handbook (Qiagen 1997).[124] After cell enumeration, the cells were centrifuged at 480g in centrifuge tubes for 5 minutes. The supernatant was discarded. 600µl of Buffer RLT (supplied by the kit) was added to the pellet and gently flicked (to release the pellet). The lysate was passed at least 5 times through a 20-G needle fitted to a sterile syringe. 600µl of 70% ethanol was added to the homogenised lysate and mixed by pipetting. 700µl of sample was transferred to a RNeasy mini spin column sitting in a 2 ml collection tube (supplied) and microfuged for 15 minutes at 10 000 rpm (Biofuge, Heraes instraments GmbH). The flow-through was discarded but the collection tube was reused. 700µl Buffer RW1 (supplied) was pipetted onto the RNeasy column and centrifuge for 15 seconds at 10 000 rpm to wash. Again the flow-through was discarded and the collection tube. The RNeasy column was transferred into a new 2 ml collection tube to which 500µl of Buffer RPE (supplied) was added, and microfuged for 15 seconds at 10 000 rpm to wash. The flow-through was discarded, but the collection tube was reused. 500µl of Buffer RPE was pipetted onto the RNeasy column and microfuged for 2 minutes at a maximum speed to dry the RNeasy membrane. The RNeasy column was then transferred into a new 1.5ml collection tube (supplied) and 30-50µl of RNase-free water was pipetted onto the RNeasy membrane, and microfuged for 1 minute at 10 000 rpm to elute.

An example for the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100µl

Dilution = 10µl of RNA sample + 490µl of dH_2O

Absorbance of diluted sample was measured in a 1ml RNase-free cuvette

A_{260} = 0.23

When measured in water, an A_{260} value of 1 is equal to 40µg/ml of RNA;

therefore, concentration of original RNA sample = 40 x A_{260} x dilution factor

= 40 x 0.23 x 50

= 460µg/ml

Total yield = concentration x volume of sample (ml)

= 460µg/ml x 0.1 ml

= 46µg

4.2.1.3.2 Protocol for total RNA extraction II

The RNA extraction method used was a modified version taken from Farrell.[121] All work was carried out on ice. The cells were washed twice in 2 ml of ice-cold PBS (pH 6.5) and centrifuged at 480g for 5 minutes at 4°C. The pellets were resuspended in 1ml 10mM EDTA (pH 8.0), 0.5% SDS. The cells were then homogenised using a 20-G sterile needle attached to a sterile syringe by plunging 10 times. 1 ml of 0.1M Na acetate (pH 5.2), 10mM EDTA (pH 8.0) and 2ml of phenol (equilibrated in water) was added. The samples were rinsed by shaking for 2 minutes at room temperature and then phase separation was performed by centrifuging at 480g for 10 minutes at 4°C. The phenol extraction step was then repeated. The upper aqueous phase was transferred to a sterilized tube containing 220µl ice-cold 1M Tris-Cl (pH 8.0) and 90µl 5M NaCl, 2 volumes of ice-cold ethanol was added and the mixture was allowed to stand for 30 minutes on ice after mixing. RNA was collected by centrifuging at 480g for 10 minutes at 4°C. The ethanol was removed and the tubes were stored in an inverted position until all the ethanol had been removed. The RNA was redissolved in 200µl ice-cold TE buffer (pH 8.0) and left to stand on ice for 30 minutes. The samples were transferred to sterile eppendorphs and 4µl 5M NaCl and 500µl ice-cold ethanol was added. The samples were microfuged at 12 000 rpm for 5 minutes. The ethanol was removed as described above, and the RNA was redissolved in 50µl TE buffer (pH

8.0). The samples were then concentrated by adding 125µl ice-cold ethnol and 3M NaAc to a final concentration of 0.3M. The samples were mixed and microfuged at 12 000 rpm for 5 minutes. The supernate was discarded and the pellets were allowed to dry. The pellets were redissolved in 20µl of TE buffer (pH 8.0).

4.2.1.3.3 Protocol for mRNA extraction

The extraction method was taken from the Oligonucleotide mRNA Kit handbook (Qiagen 1997).[125] After cell enumeration the amount of total RNA in the RNA sample was determined using the following formula:

Example: Volume of RNA = 470µl

Dilution 1 = 10µl of RNA + 90µl DEPC-water (1:10 dilution)

Dilution 2 = 20µl of dilution 1 + 780µl DEPC-water (1:40 dilution)

Measure dilution 2 in a 1ml cuvette (RNase free)

- $A_{260} = 0.23$
- Concentration of RNA stock = 40 x A_{260} x dilution factor 1 x dilution factor 2
  = 40 x 0.23 x 10 x 40
  = 3680µg/ml
- Total yield = concentration x volume of stock in ml
  = 3680µg x 0.47 ml
  = 1730µg

The oligotex suspension (supplied by the kit) was heated to 37°C and mixed immediately before use. The elution buffer (supplied) was heated to 70°C in a water path. It was calculated that 250µl of total RNA was to be pipetted into an RNase-free 1.5 ml eppendorph, 250µl of 2x binding buffer (supplied) and 15µl of oligotex suspension was added, and was brought to a total volume of 515µl with RNase-free DEPC-water. The contents was mixed by gently flicking the bottom of the tube, and incubated for 3 minutes at 65°C in a water bath. The sample was removed and allowed to cool at room temperature for 10 minutes. The sample was microfuged for 2 minutes at 12000 rpm to pellet the oligotex resin containing the mRNA. The supernatant was removed by aspirating or pipetting. 50µl of the oligotex resin was left in the tube so as not to

remove any mRNA. 400µl of wash buffer OW2 was added and pellet was resuspended by vortexing. The contents was transferred to a spin column (supplied), and spun in a microfuge at 12 000 rpm for 30 seconds. The spin column was transferred to a new RNase-free 1.5 ml eppendorph (supplied) and 400µl of wash buffer OW2 was added to the spin column and microfuged at 12000 rpm for 30 seconds. The flow-through was discarded. The spin column was transferred to a new RNase-free 1.5 ml eppendorph (supplied). 20µl to 100µl of preheated elution buffer was pipetted onto the spin column, the resin was resuspended by pipetting up and down three or four times, and centrifuged as above. The elution step was repeated to ensure maximal yield. The second elution step was performed in the same eppendorph.

4.2.1.3.4 Preparation of tRNA, mRNA and molecular marker for RNA visualisation

RNA Molecular Weight Marker II:

Size distribution: 5 Bands: 6948; 4742; 2661; 1821; 1517 bases

Concentration: 1mg/ml total RNA in water

5µl of sample from the tRNA and mRNA extraction were transferred to autoclaved 1.5ml eppendorphs, respectively and made up to a final volume of 10µl with loading buffer. 1µl of marker (II) was placed in a autoclaved eppendorph and made up to 5µl with DEPC-water and 5µl of loading buffer was added. The samples and marker were then incubated at 65°C for 3-5 minutes in a water bath. The samples and marker were chilled immediately on ice and loaded onto the equilibrated 1.2% agarose gel. The gel was run in 1 x FA gel running buffer at 75 -100 volts for 3 hours. The gel was visualised by UV-light (2011 Macrovue transilluminator), and a polaroid photograph was taken of the gel using a Mitsubishi video copy processor and camera.

4.2.1.3.5 Preparation of RNA transfer by Passive Capillary Diffusion

4.2.1.3.5.1 Destaining of the formaldehyde gel

The method was taken from Farrell.[121]

The gel was soaked in 1x MOPS buffer, 20x SSC or DEPC-water 3 times for 5 minutes.

4.2.1.3.5.2 Gel transfer for Northern Blot

The left and right edges of the gel were trimmed, and the filter membrane was cut to the size of the gel. The nitrocellulose filter was allowed to float on the surface of the DEPC water and then submerged for a minimum of 5 minutes. The filter was then equilibrated in transfer buffer (10-20x SSC) until used. A wick was cut from Whatman 3MM paper approximately 2.54 cm wider and at least 20.32 cm longer than the gel, and saturated in transfer buffer for 10 seconds and then draped over a glass sheet (RNase free) supported by a baking dish (Fig 13). The dish was filled with transfer buffer so that a minimum of 2-3cm of each end of the wick was submerged. Air bubbles were removed using a sterile pipette.

The gel was placed in the middle of the wick, with the wells facing down, and again, any air bubbles were removed. The area surrounding the gel was marked with strips of parafilm to prevent short circuiting of the system. The membrane filter was placed on top of the gel and air bubbles were removed. 2-3 sheets of Whatman 3MM paper were cut to the same size as the gel, with one of the sheets being pre-wet in transfer buffer. The wet sheets of Whatman 3MM paper were placed on top of the filter membrane, and air bubbles were removed as before, and then the remaining sheets were placed on top of this (Fig 13). A stack of paper towelling was cut and positioned on top of the Whatman 3MM paper, and then a 500g weight was placed on this. The transfer was left overnight. At the end of the transfer, the filter membrane was peeled away from the gel with forceps, and the filter membrane was marked asymmetrically for orientation. The filter membrane was then washed for 30 seconds in 5x SSC buffer and air dried on a piece of Whatman paper. The RNA was then cross-linked to the filter membrane by exposing it to UV-light for 3 minutes. A polaroid photograph of the blot was taken. The filter membrane was then stored until used.

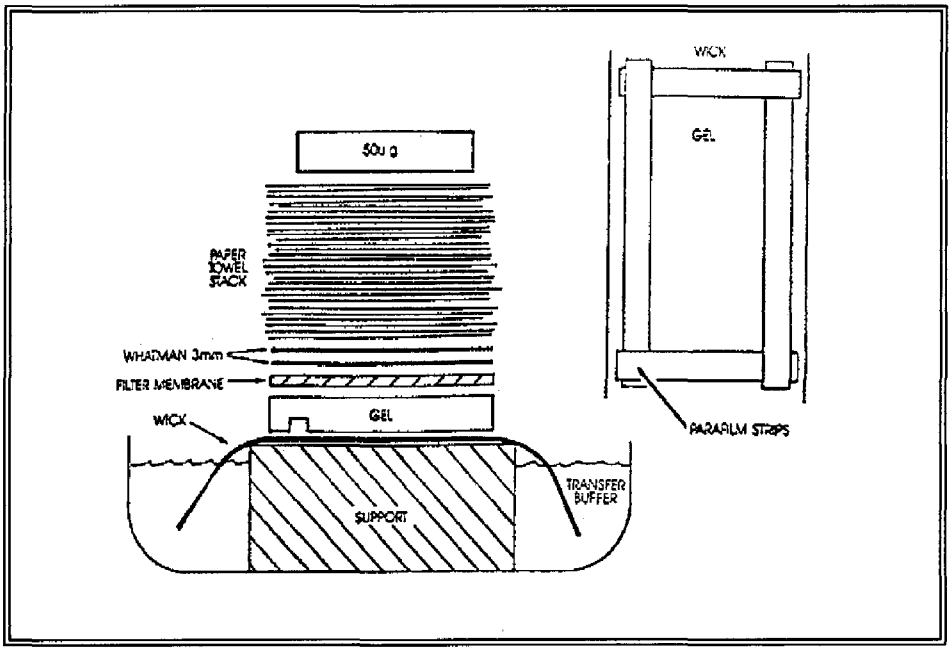


Figure 13: Set up for the capillary transfer of RNA.[121]

4.2.1.3.6 Oligonucleotide probe preparation

Oligonucleotide sequences

With reference to the gene bank, numerous mouse nucleotide sequences for Cox 1 and Cox 2 were compared and the following Cox sequence was found in both the Cox 1 and Cox 2 gene, in order to construct a antisence oligonucleotide complementary to the sequence chosen.[126]

Oligonucleotide AVM 1 (Cyclooxygenase)

Oligo number: e957682

Oligo sequence: ggt ccc ggg aag gag ggc atg ctc tac tga cgg g

Sequence length = 34

Base count: A - 6 G - 15

C - 8 T - 5

MW = 10590

OD (260) /µMol = 327.4

The following was added into an eppendorph:

2μg DNA (respective oligonucleotide)	2μl
[alpha- ^{32}P]dCTP (30μCi)	3μl
1U PNK	3μl

And made up to a total volume of 30μl using DEPC-water

The probe was incubated at 37°C for 1 hour

A 2.5 vol of 70% ice-cold ethanol and 3M NaAcetate to a final concentration of 0.3M, was added to the probe, to remove any unreacted ^{32}P, and incubated at -40°C for 1 hour. The oligonucleotide was then microfuged at 13 000 rpm for 10 minutes to pellet the DNA. The pellet was washed twice in 70 % ethanol (150μl) and spun at 13 000 rpm for 5 minutes. The supernatant was discarded and the pellet was dried in a desiccator for 15 minutes. The pellet was redissolved in 100μl of TE buffer (pH 8.0) and stored at 20°C until used. Approximately 10^6 cpm of probe was used per blot.

4.2.1.3.7 Prehybridization I

This method was taken from Farrell.[121] The filter was pre-wet in 5x SSC buffer for 20-30 minutes. The filters were prehybridized for 3-4 hours at 42°C in prehybridization buffer. At the conclusion of the prehybridization period, the buffer was poured off and a fresh aliquot of hybridization buffer was added.

4.2.1.3.8 Hybridization I

The probe was denatured by adding 0.1 vol 1N NaOH and incubated at 37°C for 10 minutes. The probe was added to the buffer and allowed to hybridize for 12-16 hours (overnight) at 42°C.

4.2.1.3.9 Posthybridization stringency washes I

Depending on the nature of the probe and the stability of the hybrid, the adjustment to the exact posthybridization washing conditions for each probe was made. At the conclusion of the hybridization period, the hybridization buffer was poured off and the filter was washed to remove all the unreactive probe molecules.

a) the filter was washed for 30 seconds in a solution of 2x SSC, 0.1% SDS to remove

most of the probe on the filter.

b) the filter was washed twice for 15 minutes each time, in 200-300 ml of a solution of 2x SSC, 0.1% SDS at room temperature.

c) the filter was washed twice for 15 minutes each time, in 200-300 ml of a solution of 0.1x SSC, 0.1% SDS at 37°C.

The filter was rinsed in 2x SSC briefly and placed on a piece of Whatman paper to blot excessive buffer from the filter. The filter was then placed in a plastic sleeve and placed in a cassette.

4.2.1.3.10 Prehybridization II (Zeta-probe)

This method was taken from the Zeta-probe Blotting Membranes, Instruction manual supplied by Bio-Rad.[127] The filter membrane was presoaked in hybridization buffer and then transferred to a dish containing Herring sperm (100μg/ml) which had been preboiled for 8-10 minutes. Hybridization buffer (200μl - 1000μl/cm^2) was added and allowed to prehybridize for 2-3 hours at 42°C in a shaking water bath.

4.2.1.3.11 Hybridization II (Zeta probe)

The probe was denatured by adding 0.1 vol 1N NaOH and incubated at 37°C for 10 minutes. The prehybridization buffer was discarded. The filter membrane was placed into a container containing the denatured probe and the hybridization buffer, and incubated at 42°C overnight in a shaking water bath.

4.2.1.3.12 Posthybridization stringency washes II (Zeta probe)

The hybridization buffer was poured off and the filter membrane was washed to remove all the unreacted probe molecules. The filter membrane was washed in the various washes (wash I and wash II) and the length of time was dependent on the counts detected with a Geiger-counter.

a) the filter was washed in 50ml of wash I for 30 minutes (refer to section 4.2.1.14).

b) the filter was washed in 50ml of wash I for 30 minutes at 42°C

c) the filter was washed in 50ml of wash II for 15 minutes at 42°C (refer to section 4.2.1.14).

The filter membrane was then placed in a plastic sleeve and sealed. The radioactive filter membrane was attached to the outer sleeve of the intensifying screen in a dark room and Kodak X-ray film was placed inside the intensifying screen of the cassette and sealed. The cassette was stored at -40°C over night.

4.2.1.3.13 Development of X-ray film

To expose the X-ray film, all work was conducted in the dark. The cassette was opened and the X-ray film was removed and placed in developer for 2 minutes. It was then rinsed in 2% acetic acid for 1 minute and then placed in fixer for 2 minutes. The X-ray film was then washed in water and left to dry.

4.2.2 RESULTS

Cox 2 tRNA or mRNA was not detected on the autoradiograms after the Northern blot analysis, using either of the methods described. A number of factors were considered to account for this:

1) Initially it was thought that the RNA was being degraded by RNase and stricter sterility techniques were used.
2) The possibility that insufficient RNA was being loaded onto the gels would explain its absence on the autoradiogram.
3) The possibility that the oligonucleotide probes were not specific enough and were not binding to the RNA was considered and more specific commercially obtainable Cox 1 and Cox 2 probes were purchased.
4) No RNA was detected on the autoradiogram with the new probes, therefore in order to test the new probes for binding capabilities a Southern Blot procedure was done and the probes were shown to bind.
5) The X-ray film was also taken into consideration and film with a higher sensitivity was purchased. Only the probes were being detected on the autoradiogram at a concentration of 20ng.
6) A new [alpha -^{32}P] dCTP of higher specific activity (6000CI/MMol) was purchased in an attempt to detect the extracted RNA samples.

The literature suggests that because Cox 2 is an immediate early gene, and its mRNA is unstable. This instability is presumably caused by the presence of the Shaw-Kamen motif in the 3' untranslated region of the cDNA, which would result in the rapid degradation of its mRNA. The instability of Cox 2 mRNA has been shown in several systems.[88, 92] Thus, it is possible that no RNA would have been detected with the supplementation of vitamin E succinate and Asc after a weeks growth. Taking these possibilities and those above into account, new methods were considered.

A time course study for Cox activity was also done to determine when the Cox gene was being switched on (described in chapter 5), so that Cox 2 RNA studies at these times could be performed to verify whether the combined vitamin E succinate and Asc supplementation stimulates Cox 2 expression in BL6 cells.

4.3 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON CYCLOOXYGENASE EXPRESSION IN BL6 CELLS OVER A TIME COURSE OF 12 HOURS

4.3.1 MATERIALS AND METHODS

MATERIALS

Prostaglandin H synthase 1 (murine) cDNA Probe and Prostaglandin H synthase 2 (murine) cDNA Probe were purchased from Cayman Chemicals Company, USA. [alpha -^{32}P] dCTP (6000CI/MMol) was purchased from Amersham International, England. Molecular Grade Phenol was purchased from Sigma Chemicals Co, USA. Prime-a-gene kit was purchased from Promega Corporation, UK

METHODS

4.3.1.1 PREPARATION OF GLASSWARE, PLASTICWARE AND REAGENTS

As described in section 4.2.1.1.

4.3.1.1.1 Preparation of 1% Agarose gel in 1x MOPS buffer

0.5g Agarose (1%) and 36.5 ml DEPC-water were mixed together. The agarose was melted in a microwave and allowed to cool to 65°C in a water bath. 1.8ml formaldehyde and 5ml 10x MOPS was added. The mixture was mixed thoroughly and poured onto a gel support. Prior to running the gel, the gel was equilibrated in 1x MOPS buffer for 30 minutes.

4.3.1.1.2 Preparation of Formaldehyde Gel Loading Buffer:

50µl Formamide

20µl Formaldehyde

10µl 10x MOPS

10µl EtBr (1mg/ml)

4.3.1.1.3 Preparation of 10 x MOPS buffer

200mM MOPS	10.46g/l
50mM NaAcetate	1.03g/l
10mM Na_2EDTA	0.93g/l

The pH was adjusted to 7.0 with acetic acid glacial

4.3.1.1.4 Preparation of lysis buffer

0.5% SDS

10mM EDTA (pH 8.0)

4.3.1.1.5 Preparation of equilibrated phenol

A suitable amount of phenol was melted at 65°C in a water bath. The equilibration was carried out in a separation funnel. An equal volume of DEPC-water was added to the phenol and mixed

gently by inverting the funnel. The phases were allowed to separate and the phenol, the bottom phase, was removed and again equilibrated with DEPC-water. The equilibrated phenol was tested for acidity and the bottle was wrapped in foil.

4.3.1.2 CELL CULTURE PROCEDURE

It was decided that the combination 20:25μg/ml vitamin E succinate and Asc would only be used because this combination showed the greatest growth inhibitory effect on BL6 cells, and Midgely found that Cox activity was significantly increased at the combination 20:25μg/ml in BL6 cells.[111]

BL6 cells were seeded into 9 sets of 3 ($75cm^2$) flasks, at 5×10^5 cells/flask. McCoys medium, 30ml containing 10% (v/v) FCS, was added to all the flasks. The cells were incubated at 37°C and grown to near confluency (≈5 days). The medium was then discarded under sterile conditions. The first 4 sets of 3 flasks received fresh medium containing 30ml of 10% (v/v) FCS and the vitamin E succinate and Asc combination 20:25μg/ml. The second, 4 set of 3 flasks received fresh medium containing a final 0.1% volume of ethanol in 30ml of 10% (v/v) FCS, and was referred to as the ethanol control culture (OE*). The flasks were then incubated at 37°C. This was referred to as time interval 0.

4.3.1.2.1 Harvesting of the experimental cell cultures

The cells were treated as follows over a period of 12 hours. At time 0, a set of 3 flasks, the control culture (O) was harvested as described in section 2.2.2.4. Subsequently the remaining 8 sets, one set of 3 flasks from the 20:25μg/ml and one set of 3 flasks from the OE* set of flasks, were harvested at 2, 4, 8, and 12 hours, respectively as described in section 2.2.2.4. The cells were enumerated and the 3 flasks of cells from each respective treatment and time interval were combined just prior to RNA extraction.

4.3.1.3 MOLECULAR PROCEDURES

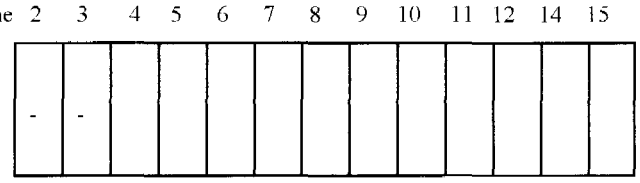
4.3.1.3.1 Protocol for total RNA extraction II

Refer to section 4.2.1.3.2.

4.3.1.3.2 Preparation of the RNA sample for visualization

The total amount of concentrated total RNA from each sample was used. The total RNA samples were separated by electrophoresis. 20µl of each sample and 10µl loading buffer were added to prepare the samples to be run on an agarose (RNase-free) gel. PGH synthase 1 and 2 probes (cold) were run as standards (std), 1µl of each was made up to a volume of 5µl in DEPC-water in its respective eppendorph and 10µl of loading buffer was added. The samples and std were heated to 65°C for 15 minutes and chilled on ice. The samples were loaded onto the equilibrated gel and run at 75- 100V for ±3 hours.

Illustration of the gel:



Lane 2: PGH1 synthase 1 probe (1.1 kb)
3: PGH synthase 2 probe (1.2 kb)
4: Blank
5: 2 hrs 20:25 vitamin E succinate:Asc
6: 4 hrs 20:25 vitamin E succinate:Asc
7: 8 hrs 20:25 vitamin E succinate:Asc
8: 12 hrs 20:25 vitamin E succinate:Asc
9: Blank
10: 2 hrs OE* (0.1% ethanol control culture)
11: 4 hrs OE* (0.1% ethanol control culture)
12: 8 hrs OE* (0.1% ethanol control culture)
13: 12 hrs OE* (0.1% ethanol control culture)
14: Blank
15: O# (control culture)

4.3.1.3.3 Preparation of RNA transfer by passive capillary diffusion

Refer to section 4.2.1.3.5.

4.3.1.3.4 Prehybridization II (Zeta probe)

Refer to section 4.2.1.3.10.

4.3.1.3.5 Preparation of the prostaglandin H synthase 1 and 2 probes

Prostaglandin H synthase 1 (murine) cDNA probe:

Concentration: 2µg DNA/100µl buffer

Ends: 5' overhang

Purity: ≥98%

Length: ≈ 1.1 kbp

Prostaglandin H synthase 2 (murine) cDNA probe:

Concentration: 2µg DNA/100µl buffer

Ends: 5' overhang

Purity: ≥98%

Length: ≈ 1.2 kb

4.3.1.3.6 Probe preparation

The method was taken from Prime-a-Gene® Labelling System for product U1100[128] All work was done on ice. The cDNA of PGHS 1 and 2 probes were dissolved individually in TE buffer (25µg/ml) and denatured by heating at 95-100°C for 2 minutes. Once heated, the cDNAs were immediately chilled on ice. The Prime-a-gene Labelling System kit was thawed on ice, except for the Klenow solution.

The probes were made as follows in a sterile 1.5ml eppendorph using buffers provided by the Prime-a-gene kit:

(dNTPs mix = 1µl of the following dNTPs were added to a sterile 1.5ml eppendorph; dATP, dTTP, and dGTP)

	Volume
1) DEPC-water (supplied)	25µl
2) 5x buffer (supplied)	10µl
3) dNTPs mix (supplied)	2µl
4) cDNA PGHS 1or 2	5µl
5) BSA (supplied)	2µl
6) α-^{23}P dNTP	5µl

7) Klenow (5U/µl) (supplied)	1µl
	50µl

The contents of each probe was mixed and incubated at room temperature for 60 minutes. The reactions were terminated by heating at 95-100°C for 2 minutes and then chilled on ice. 2µl of a 20mM EDTA solution was added and the probes were allowed to hybridise overnight at -20°C.

4.3.1.3.7 Removal of the unreactive α-^{32}P

A 2.5 volume of 70% ice-cold ethanol and 3M Na acetate to a final concentration of 0.3 M, was added to the probes and incubated at -40°C for 1 hour. The probes were then microfuged at 13 000 rpm for 10 minutes to pellet the DNA. The supernatant was discarded and 150µl of ice-cold ethanol was added to wash the pellets, and they were then microfuged at 13 000 rpm for 5 minutes. The wash step was repeated, the ethanol discarded and the pellets were vacuum dried in a desiccator. The pellets were redissolved in 100µl TE buffer (pH 8.0) and left to stand on ice for 30 minutes. The probes were stored at -20°C until used.

4.3.1.3.8 Hybridization II (Zeta probe)

Refer to section 4.2.1.3.11.

Both the PGHS 1 and PGHS 2 probes were added to the hybridization buffer.

4.3.1.3.9 Posthybridization stringency washes (Zeta probe)

Refer to section 4.2.1.3.12.

4.3.1.3.10 Development of X-ray film

Refer to section 4.2.1.3.13.

4.3.2 RESULTS

The procedures used for the 12 hour time course study took into consideration the RNA stability, the sterility techniques, the RNA availability and the previous protocols used. Firstly, with reference to the stability of the RNA, time course studies (chapter 5) were performed to

determine at what stage the RNA was being expressed. Secondly, to ensure that there would be enough RNA present to bind to the probes the total amount of RNA extracted was loaded on to the gels. Initially [alpha -^{32}P] dCTP (3000CI/MMol) was used to make the Cox 1 and Cox 2 probes, however only the probes were being detected on the autoradiogram. The possibility that not enough Cox 1 or Cox 2 DNA was being added to the labelling procedure for binding to occur was considered and instead of adding 1.25µl of Cox 1 or Cox 2 DNA, 5µl of Cox 1 or Cox 2 DNA was added, however, still no Cox 1 or Cox 2 RNA was detected on the autoradiograph. Lastly the increase in the [alpha -32P] dCTP(6000CI/MMol) specific activity resulted in bands being detected on the autoradiograph.

The effect of combined vitamin E succinate and Asc on the expression of Cox 2 over a time course of 12 hours in BL6 cells is shown in Figure 14.

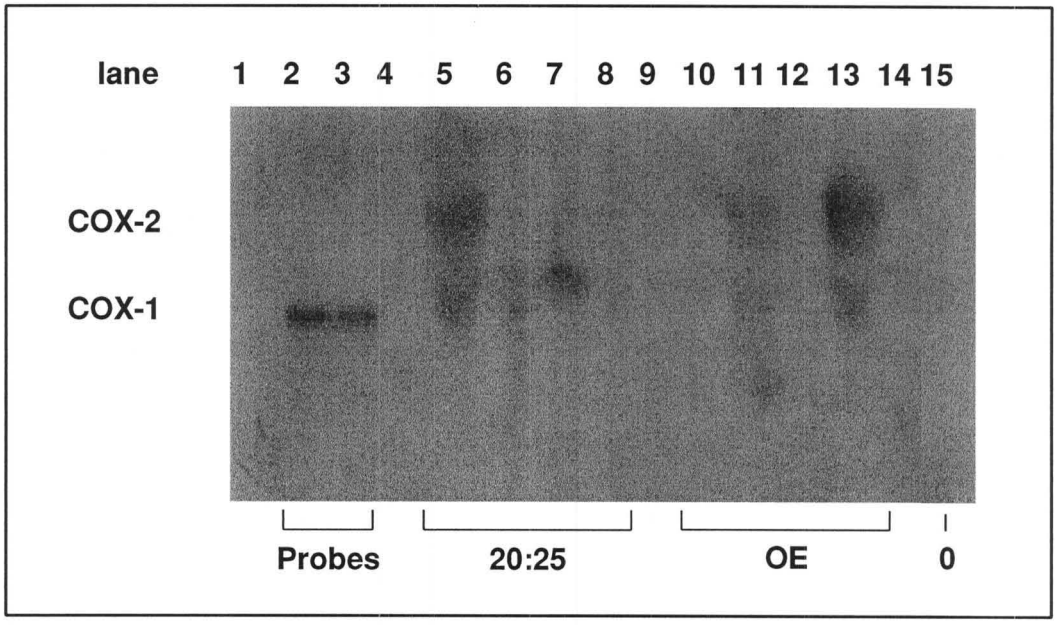


Figure 14: Autoradiogram of Cox 1 and Cox 2 expression in BL6 cells supplemented with a combination of vitamin E succinate and Asc, 20:25µg/ml respectively.

Lanes 2 and 3 contain the standards of Cox 1 and Cox 2 and their product sizes are 1. 1 and 1.2kb, respectively. The lengths of mouse Cox 1 and Cox 2 are 3kb and 4.1kb, respectively.

The data in Fig 5 may be interpreted in two ways:

A) The upper band seen may be Cox 2, because if it is compared to the probe sizes, it appears to be about 4.1kb. The larger the RNA the smaller the distance it moves through the gel. The middle band may be Cox 1, which appears to be 3kb and below that a third band appears to be present, which may be the 18S rRNA. Lanes 5-8 contains the combination 20:25μg/ml vitamin E succinate and Asc treatment at 2, 4, 8 and 12 hours, respectively. In lanes 5-7 Cox 1 is present, however, the inducible Cox 2 RNA is only observed at 2 hours. Lanes 10-13 contain the OE* control cultures and it appears that Cox 2 and Cox 1 are present at the time intervals 4 and 12 hours. The O control culture, lane 15, had no Cox 1 or Cox 2 present. From the band intensity of the Cox 1 and Cox 2 probes which contain 20ng it appears that for the 20:25μg/ml at 2 hours Cox 2 may contain just less than 20ng of RNA, similarly for the OE* at 12 hours.

B) The upper band may be 28S rRNA and below it may be the 18S RNA.

4.3.3 DISCUSSION

It has always been assumed that free AA controls PG synthesis, however, recent evidence suggests that significant regulation also occurs at the level of Cox gene expression.[83] Cox exists in two isoforms, Cox 1 is the constitutive enzyme that produces the PGs for maintaining the physiological functions, and are maintained at constant levels for the most part. Cox 2 is an inducible enzyme that produces PGs involved in cell growth.[129] The regulation of 4.1kb mRNA for Cox 2 has the potential for occurring at several levels including transcription, stability, and alternative polyadenylation. The rapid turnover of Cox 2 mRNA allows tightly controlled responses to multiple stimuli.[130] The inducible PGHS, Cox 2, appears to be the major PGHS regulated by many agents with influence in PG production.[131]

Due to the problems encountered by the initial molecular work done, in terms of the sterile techniques used, the RNA stability and availability, the protocols used, the analysis of that data and the considerations make a new study was conducted. The shorter time course study did produce some positive results which may be interpreted in two ways for the following reasons:

A) There is possibility that the upper band is Cox 2, which for mouse has a molecular size of 4.1 kb, if the position of the band is compared to the probe sizes, while the middle band, assuming

it is Cox 1, has a size of 3 kb and the bottom band the 18S rRNA for mouse is 1.9 kb. However, the sizes of our bands cannot be determined accurately because no molecular size marker was run, as it would not have been detected on the autoradiogram. However, from the sizes of the probes one may assume that the bands visualised are approximately those sizes. If this assumption is made for the results of this time course study for the levels of RNA for Cox 2, it appears that maximum induction of Cox 2 RNA in BL6 cells treated with vitamin E succinate and Asc occurs within 2 hours of treatment, with its subsequent disappearance over time. Feng *et al.* showed that the maximum induction of Cox 2 mRNA in cells treated with interleukin-1 (IL-1) occurred within 2 hours and then decreased gradually.[89] Cox 1 appears to be present in the vitamin E succinate and Asc supplementation, (20:25μg/ml) samples from 2-8 hours, as well as the OE* 4 and 12 hour samples. This may be explained by the fact that Cox 1 is the constitutive isoform. The amount of Cox 1 and 2 visualised on the autoradiogram varies because of the uneven amount of total RNA loaded on the gel at the different time intervals, and the absence of bands for Cox 2 during the time intervals for the vitamin E succinate and Asc supplemented samples at 4, 8, and 12 hours, as well as the absence of Cox 1 and Cox 2 for the OE*samples at 2 and 8 hours may be accounted for by this. There is a possibility that non-specific binding of the probe to the 18 S rRNA could occur as Kutchera *et al.*[132] found that non-specific binding occurred with the 18S RNA which might account for the bands below Cox 1. The broad bands of Cox 2 may be due to the exposure time of the autoradiogram as studies by Hla *et al.*[87] showed that the longer the autoradiogram was left to expose, the less sharp the bands became, and a type of band broadening was observed. In the OE* control cultures both Cox 1 and Cox 2 are visualised at 4 and 12 hours. The O control culture, however, contains neither Cox 1 or Cox 2. The time courses for Cox 1 and Cox 2 RNA expression suggest that Cox 2 is much less stable than Cox 1 RNA, as the autoradiograph shows that Cox 1 is present at 2-8 hours for the vitamin treated samples but Cox 2 is only detected at 2 hours. The rapid disappearance suggests a "tight message regulation", and the rapidity of the disappearance of Cox 2 RNA is similar to those found in several other cell types.[89, 133] This rapid disappearance of the RNA may be due to the presence of the Shaw-Kamen motif, which causes instability of the RNA.[89]

B) The bands visualised for the time course for the expression of RNA for Cox 2 may possibly be the non-specific binding of the 18S rRNA and 28S rRNA, and not Cox 1 or Cox 2.

If the first interpretation is considered, the possibility of the rapid induction of Cox 2 *in vitro* within 2 hours after the administration of vitamin E succinate and Asc suggests that Cox 2 may play a role in producing the increased cyclooxygenase-derived products of AA as found by Midgely.[111] However, whether Cox 2 in BL6 is transcriptionally regulated by vitamin E succinate and Asc still remains to be elucidated.

The results are clearly not conclusive and the following experiments would need to be done to make them more conclusive:

1) The same amount of RNA needs to be loaded on the gel for the different time intervals and treatments.
2) Instead of running tRNA samples, mRNA could be extracted, which would eliminate the possibility of the 18S and 28S rRNA binding.
3) A molecular size marker is needed to determine the band sizes.
4) As an internal control for mRNA loading, one could probe with the addition of a murine cDNA for glyceraldehyde phosphate dehydrogenase (GAPDH).
5) The autoradiogram could be exposed for different time periods to see if band broadening occurs.

CHAPTER 5

THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON CYCLOOXYGENASE ACTIVITY IN BL6 CELLS

5.1 INTRODUCTION

PGs are synthesised from the precursor AA, stored esterified within cellular phospholipid stores. For synthesis of PGs, AA must be liberated by the action of phospholipases.[92] Over the last decade, there has been increasing emphasis on the enzymes that initiate the oxidation of AA. The enzyme PGHS (Cox) is a key enzyme in the biosynthetic pathway leading to the formation of PGs.[122] Two isoforms of Cox have been identified, Cox 1 is a continuously transcribed stable message, providing relatively constant levels of enzyme in most cell types. Cox 1 appears to be responsible for the production of PGs that are important for homeostatic functions.[91] Cox 2 is the product of an "immediate-early" gene that is rapidly inducible by a variety of stimuli.[91, 122] Cox is a rate limiting enzyme in PG synthesis because of its rapid autoinactivation, and this activity seems to be determined by the PGHS protein concentration.[89, 93, 129] PGHS has two activities, a cyclooxygenase and a peroxidase activity. Its cyclooxygenase activity oxygenates AA to the hydroperoxyl endoperoxide PGG_2 and its peroxidase activity reduces PGG_2 to the alcohol PGH_2 in the presence of a reducing substrate.[99] The peroxidation function of Cox 1 and Cox 2 appears to be similar in both enzymes.[91] Both forms of Cox are heme proteins composed of two 70-kDa subunits containing one heme per subunit and each monomer appears to catalyse both activities.[99] The Cox enzymes have a half life of approximately four minutes and undergo a suicide reaction during catalysis. This short half life suggests that there must also be an increase in the synthesis of this enzyme.[92] Evidence indicates, however, that despite the conservation of the enzyme structure and function, Cox 1 and Cox 2 may function as separate enzyme systems.

Cox 1 and Cox 2 have been reported to use different pools of AA that are mobilised in response to different stimuli for PG synthesis.[91] Cox 2 requires the release of a pool of membrane bound AA which is converted to PGE_2 by Cox 2, synthesised in response to ligand stimulation. In

contrast Cox 1 is accessible to exogenous AA only.[95] Antioxidants may inhibit, have no effect on, or stimulate Cox. The role of vitamin E in Cox reactions has been disputed for some time and depending on the system, vitamin E either has no effect on or enhances PG biosynthesis from AA.[82] PGs and the Cox 2 enzyme may be involved in the initiation and/or promotion of carcinogenesis.[90, 129] Cox 2 has been found to be upregulated in human cancers of the colon, stomach and breast. Increased levels of Cox 2 have also been detected in premalignant intestinal tumours in humans and in experimental animals.[90] Recently considerable literature regarding the role of PGs in cancer cell growth and cell proliferation and their role in carcinogenesis has been published. However, some of these reports are conflicting and the exact role of PGs in cancer development and progression has not yet been clarified.[81]

Since the molecular studies described in section 4.2 were unsuccessful, probably because of the duration the cells were supplemented with vitamin E succinate and Asc a shorter time course study was undertaken in order to determine when the Cox 2 gene was being "switched on." Thus, this present study was undertaken to determine the effect of combined vitamin E succinate and Asc supplementation on Cox activity in BL6 cells over a time course of 12 hours, so as to determine when Cox was being expressed so that the RNA could be extracted from the cells at the appropriate time intervals and be analysed by Northern blots as was done and described in section 4.3.

5.2 MATERIALS AND METHODS

MATERIALS

Arachidonic acid, N,N,N',N'-tetramethyl-p-phenylenediamine was purchased from Sigma Chemicals Co, USA.

METHODS

5.2.1 Experimental cell culture procedure

BL6 cells were seeded into 9 sets of 5($25cm^2$) flasks, at 3 x10^5 cells/flask. McCoys medium, 30ml containing 10% (v/v) FCS, was added to all the flasks. The cells were grown to near confluency and incubated at 37°C for ≈4 days. The medium was then discarded under sterile conditions from 8 sets of the flasks and this was referred to as time 0. To 4 sets of the 5 flasks, a final volume

of 0.1% ethanol in 30ml of 10% (v/v) FCS medium was added and was referred to as the ethanol control culture (OE*). The second set was made up of 4 sets of 5 flasks and contained the combination of vitamin E succinate:ascorbate 20:25µg/ml and 30ml of 10% (v/v) FCS medium. The cells were then incubated at 37°C for a further 2-12 hours.

The cells were harvested as described in section 2.2.3.2, however, 5 ml of trypsin was used. Initially a set of 5 flasks containing 30ml of 10% (v/v) FCS medium and referred to as O control culture (O) was harvested at time 0. The OE* and 20:25 flasks were harvested as before at the following time intervals 2, 4, 8, and 12 hours after treatment. The pellets were resuspended in 1 ml of PBS (pH 6.5) buffer, and cell counts were done. The cells were once again centrifuged at 3500 rpm for 5 minutes and the pellets resuspended in 1M Tris-HCl (pH 8.5) buffer.

5.2.2 Homogenization of cells and separation of cellular components

The membrane fraction of the cells were obtained as follows. Following cell enumeration, the cell suspensions were centrifuged at 3500 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellets were resuspended in 1 ml of Tris-HCl pH 8.5. The cell suspension were then transferred to a Dounce homogeniser and the tubes were rinsed with a further 500µl ice-cold Tris-HCl which was added to the cell suspension in the Dounce homogeniser. The samples were homogenised 30 times with a tight plunger. The Dounce homogeniser was rinsed with 500µl ice-cold Tris-HCl. The membrane fractions of the various homogenates were obtained by differential centrifugation. The cell homogenates were centrifuged (Beckman Model J2-21 Centrifuge) in thick walled tubes at 480g for 20 minutes to remove cell nuclei and non disrupted cells. The supernatant was retained and centrifuged at 20 000g for 30 minutes. In order to remove the stroma fraction the supernatant was discarded and the pellet containing the final membrane fraction was resuspended in 1000µl of ice-cold Tris-HCl (pH 8.5) and transferred to 1.5ml eppendorph, and stored at -20°C until assayed.

5.2.3 Cyclooxygenase assay using a spectrophotometer

The extracted samples were analysed using a modified method of Benedetto C *et al.*[134] 1ml of the sample was placed into a cuvette and 20µl of 4mg/ml TMPD was added. The absorbance (611nm) was recorded as a function of time for about 1 minute to establish the background rate

of TMPD oxidation. To this 5µl of 20mM arachidonic acid was added and the change in absorbance was recorded for a further 3 minutes. The results are represented as a percentage of the O control.

5.3 STATISTICAL ANALYSIS

The results obtained were analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman Keuls Multiple range Test. However, the statistical analysis in not shown on the graphs due to the large amount of variation observed between the groups, which resulted in high SEM's.

5.4 RESULTS

The time course of Cox activity in the presence or absence of the combination of vitamin E succinate and Asc and ethanol is shown in Figure 15.

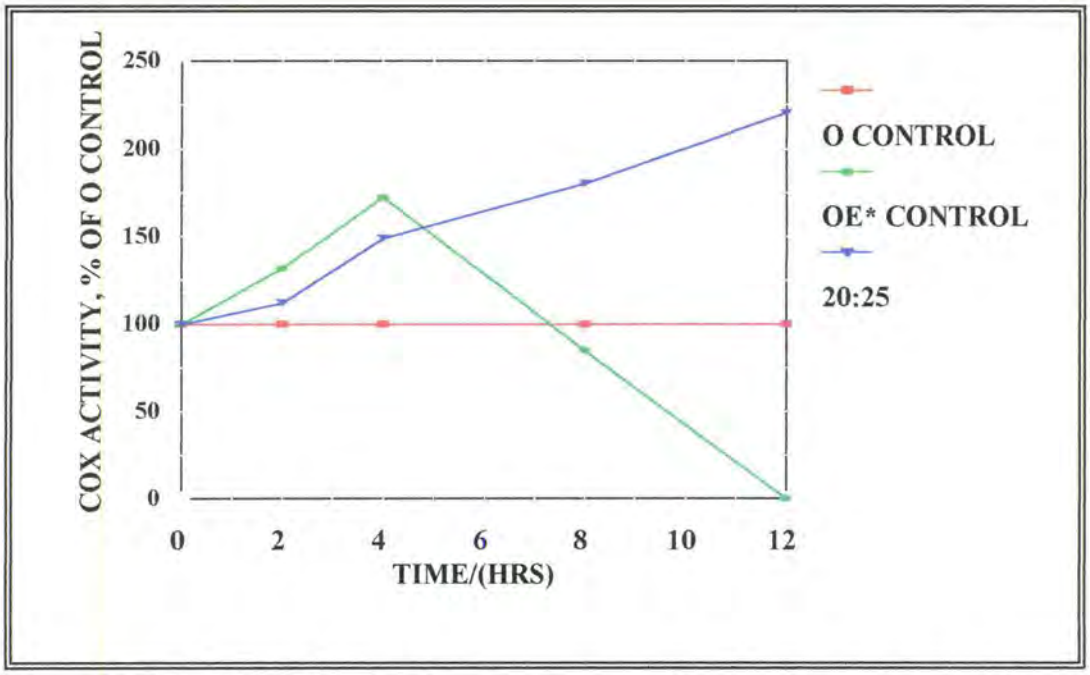


Figure 15: The effect of combined supplementation of vitamin E succinate and Asc on Cox activity in BL6 cells. The values are the mean of three cultures.

In BL6 cells a basal level of Cox activity was observed in the O control culture. The supplementation of the combination 20:25μg/ml vitamin E succinate and Asc respectively in BL6 cells shows an increasing trend in Cox activity. A significant ($p \leq 0.05$) increase in Cox activity was observed in the 20:25μg/ml combination at 12 hours relative to the O control. The comparison of the 20:25μg/ml at 2 and 4 hours with the 20:25μg/ml at 12 hours shows a significant ($p \leq 0.01$ and $p \leq 0.05$ respectively) increase. The OE* control culture however shows an initial increase and then a decrease in Cox activity over time when compared to the O control.

5.5 DISCUSSION

The time course for the recovered Cox activity in cells treated with vitamin E succinate and Asc showed that Cox activity continued to increased over 12 hours, whereas the OE* control culture reached a maximum activity at 2 hours and was decreased by 12 hours. The decline in the OE* control culture suggests that ethanol does not have a stimulatory effect on Cox. Significant ($p \leq 0.05$) stimulation by vitamin E succinate and Asc supplementation was not detected until 12 hours after incubation when compared to the O control, which is suggestive of an induction process. The literature suggests that serum (FCS) in the medium may have a slight initial stimulatory effect on Cox activity within 2 hours, which may account for the stimulation observed in the OE* control culture at 2 hours.[89, 135] Therefore, increased Cox activity in BL6 cells during the subsequent incubation in medium containing the combination 20:25μg/ml vitamin E succinate and Asc, respectively, suggests that vitamin E and Asc may induce Cox at the level of translation. To determine whether vitamin E succinate and Asc stimulatory action required the synthesis of new protein, the effects of protein synthesis inhibitors on vitamin E succinate and Asc action was tested.

CHAPTER 6

THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION TOGETHER WITH CYCLOOXYGENASE INHIBITORS ON CYCLOOXYGENASE ACTIVITY IN BL6 CELLS

6.1 INTRODUCTION

One of the primary approaches to the understanding of the function of PGs in any tissue has been through the use of inhibitors of Cox activity. Several drugs called PG-synthesis inhibitors or PG-antagonists are found to markedly inhibit Cox activity.[81] The NSAIDs that have most commonly been used in *in vitro* and *in vivo* studies include drugs such as aspirin, indomethacin, ibuprofen all of which inhibit PG synthesis although by different mechanisms.[83] PG synthesis is also inhibited by glucocorticoids steroids. Dexamethasone (dex) is the most widely used glucocorticoid steroid, and earlier evidence suggested that glucocorticoids induced a protein that modulated PLA_2 activity, thus interfering with the release of AA substrate. More recently evidence indicates that glucocorticoids directly inhibit the synthesis of Cox, although the mechanism has not yet been fully elucidated. *In vitro*, the inhibition of Cox activity by glucocorticoids occurs rapidly (within hours).[130] Selective inhibition of Cox 2 expression by dex has been demonstrated in various cell types.[89] Studies by Masferrer *et al.* showed that Cox 2 was inhibited by dex in a dose-dependent manner.[135] Another inhibitor of Cox is cycloheximide. Cycloheximide inhibits only eukaryotic cytoplasmic ribosomes, as it inhibits peptidyl transferase of the 60S ribosome, and thus blocks the elongation of the peptide chain.[133, 136-137]

In an attempt to determine whether Cox activity required the synthesis of new Cox protein, the effects of protein synthesis inhibitors, dexamethasone and cycloheximide together with vitamin E succinate and Asc in BL6 cells were tested.

6.2 MATERIALS AND METHODS

MATERIALS

Dexamethasone and cycloheximide were purchased from Sigma Chemicals Co, USA.

METHODS

6.2.1 PREPARATION OF CULTURE REAGENTS

6.2.1.1 Preparation of cycloheximide stock solution (25mM)

The cycloheximide stock solution was prepared as follows:

7mg Cycloheximide

10µl of Tris-HCl buffer (pH 8.5)

6.2.1.2 Preparation of dexamethasone stock solution (1mM)

The dexamethasone stock solution was prepared as follows:

3.9mg Dexamethasone

10µl of Tris-HCl buffer (pH 8.5)

6.2.2 Experimental cell culture procedure

BL6 cells were seeded into 29 sets of 5 ($25cm^2$) flasks, at 3 $x10^5$ cells/flask. McCoys medium, 30ml containing 10% (v/v) FCS, was added to all the flasks. The cells were grown to near confluency and incubated at 37°C for ±4 days. The medium was then discarded under sterile conditions from 28 of the sets of flasks and this was referred to as time interval 0. To 4 sets made up of 5 flasks, a final volume of 0.1% ethanol in 30ml of 10% (v/v) FCS medium was added with the addition of 100µl Chx (25mM) and was referred to as the ethanol/Chx control culture (OE*/Chx). The second set was made up of 4 sets of 5 flasks containing the combination of vitamin E succinate:ascorbate 20:25µg/ml and 30ml of 10% (v/v) FCS medium with the addition of 100µl Chx (25mM) and referred to as the 20:25/Chx culture. Similarly 100µl Dex (1mM) was added to 4 sets of 5 flasks of a OE* and 20:25, respectively, and were referred to as the OE*/Dex control culture (OE*/Dex) and 20:25/Dex culture, respectively. The fifth set was made up of 4 sets of 5 flasks containing a combination of 100µl Chx (25mM) and 100µl Dex (1mM) and a final volume of 0.1% ethanol in 30ml of 10% (v/v) FCS medium and referred to as

the OE*/Chx/Dex control culture (OE*/Chx/Dex). Similarly the sixth set made up of 4 sets of 5 flasks containing a combination of 100µl Chx (25mM) and 100µl Dex (1mM) and the combination 20:25 in 30ml of 10% (v/v) FCS medium and was referred to as the 20:25/Chx/Dex culture. The seventh set was made up of 4 sets of 5 flasks containing 100µl Dex (1mM) in 30ml of 10% (v/v) FCS medium was referred to as the control culture (Dex). The eighth set was made up of 4 sets of 5 flasks containing 100µl Chx (25mM) in 30ml of 10% (v/v) FCS medium and was referred to as the control culture (Chx). To the final set made up of 4 sets of 5 flasks a combination 100µl Chx (25mM) and 100µl Dex (1mM) was added to flasks containing 30ml of 10% (v/v) FCS medium, and was referred to as the control culture (Chx/Dex). The cells were then incubated at 37°C for a further 2-12 hours.

The cells were harvested as described in section 2.2.3.2, however, 5 ml of trypsin was used. Initially a set of 5 flasks containing 30ml of 10% (v/v) FCS medium and referred to as O control culture (O) were harvested at time 0. One set of 5 flasks from each treatment; OE*/Chx, 20:25/Chx, OE*/Dex, 20:25/Dex, OE*/Chx/Dex, 20:25/Chx/Dex, Chx, Dex and Chx/Dex were harvested as before at the following time intervals 2, 4, 8, and 12 hours after treatment. The pellets were resuspended in 1 ml of PBS (pH 6.5) buffer, and cell counts were done. The cells were once again centrifuged at 3500 rpm for 5 minutes and the pellets resuspended in 1M Tris-HCl (pH 8.5) buffer.

6.2.2.1 Homogenization of cells and separation of cellular components

As describes as in section 5.2.2

6.2.3 Cyclooxygenase assay using a spectrophotometer

As described in section 5.2.3

6.3 STATISTICAL ANALYSIS

As described in section 5.3

6.4 RESULTS

Treatment of BL6 cells (Figure 16) with 1μM dexamethasone and the combination of vitamin E succinate and Asc (20:25μg/ml) resulted in a decreasing trend over time when compared to the O control culture. The OE* control culture and the Dex control culture shows a decreasing trend over time when compared to the O control culture.

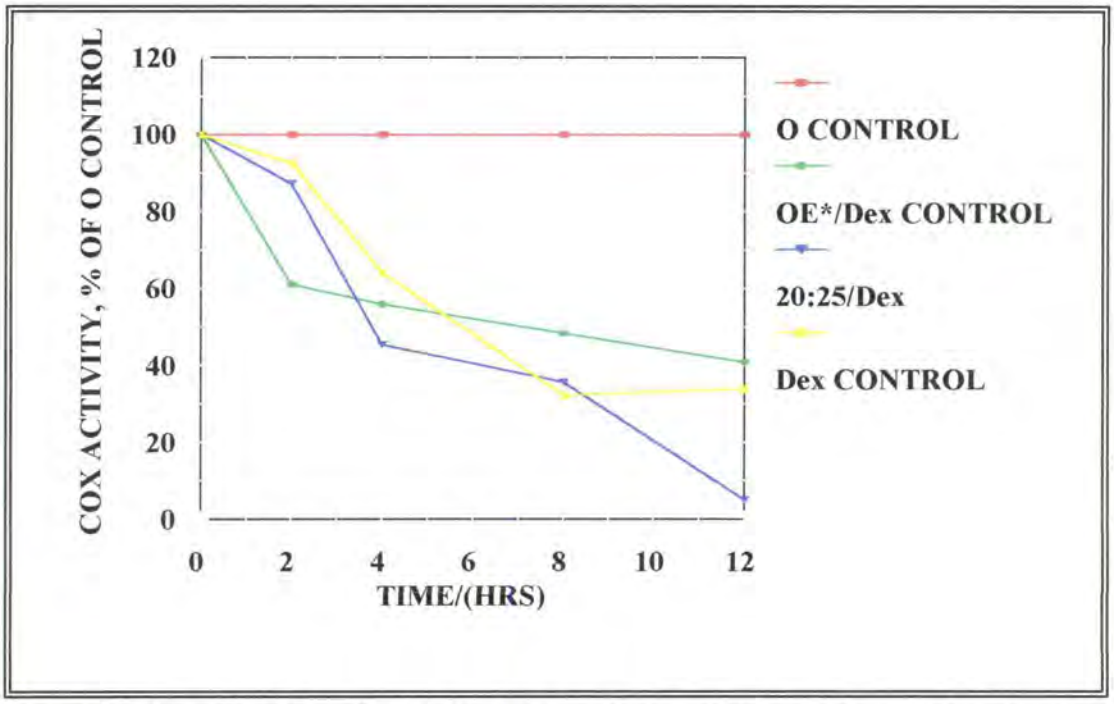


Figure 16: The effect of combined vitamin E succinate and Asc supplementation, together with dexamethasone on Cox activity in BL6 cells. The values are the mean of three cultures.

Treatment of BL6 cells (Figure 17) with 25μM cycloheximide and the combination of vitamin E succinate and Asc (20:25μg/ml) resulted in an initial increase and then an overall decrease of Cox activity over time, however not significant when compared to the O control culture. However, a significant ($p \leq 0.05$) decrease was observed when comparing the 20:25μg/ml at 8 and 12 hours with the Chx control culture at 2 hours. The OE* control culture shows a decreasing trend and a significance ($p \leq 0.01$) at 12 hours when compared to the Chx, 2 hour control culture.

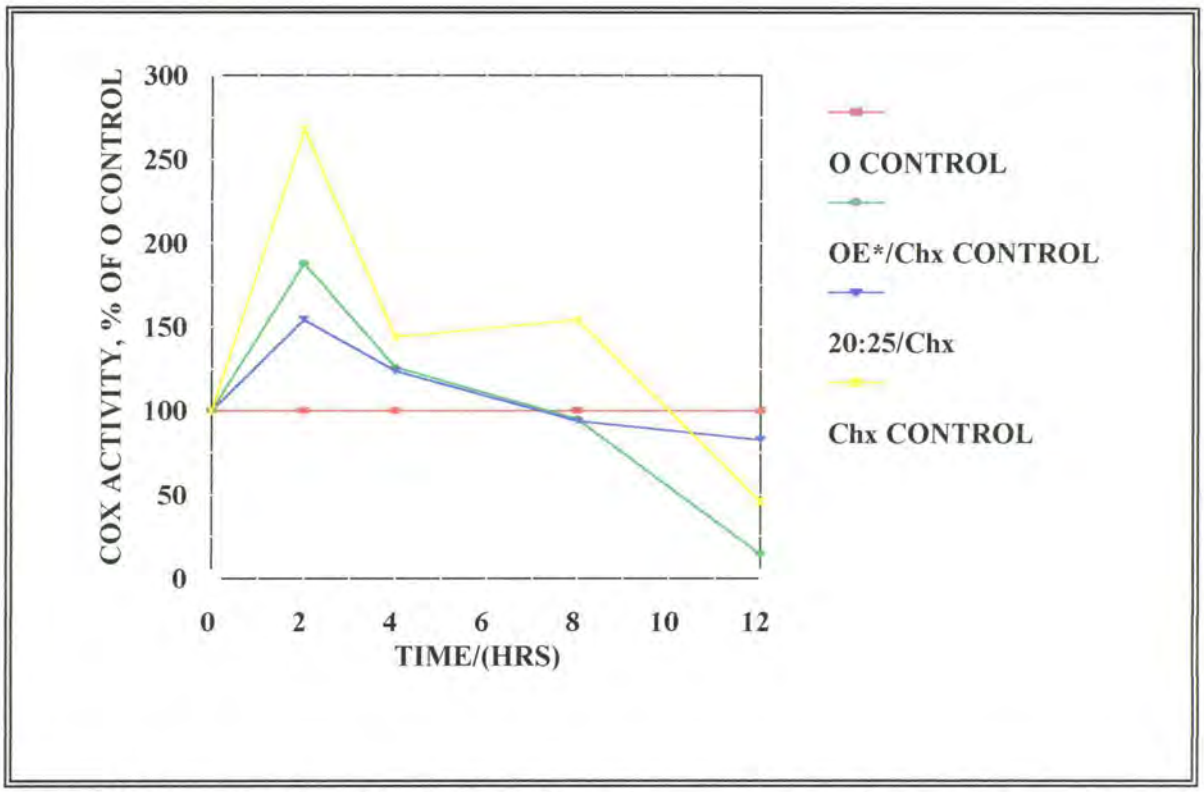


Figure 17: The effect of combined vitamin E succinate and Asc supplementation, together with cycloheximide on Cox activity in BL6 cells.

Treatment of BL6 cells (Figure 18) with 25μM cycloheximide, 1μM dexamethasone and the combination of vitamin E succinate and Asc (20:25μg/ml) resulted in no increasing or decreasing trend over time when compared to the O control culture, however a decrease is observed from 8-12 hours. A decreasing trend is observed in the OE* control culture and the Chx/Dex control culture from 2-12 hours when compared to the O control culture.

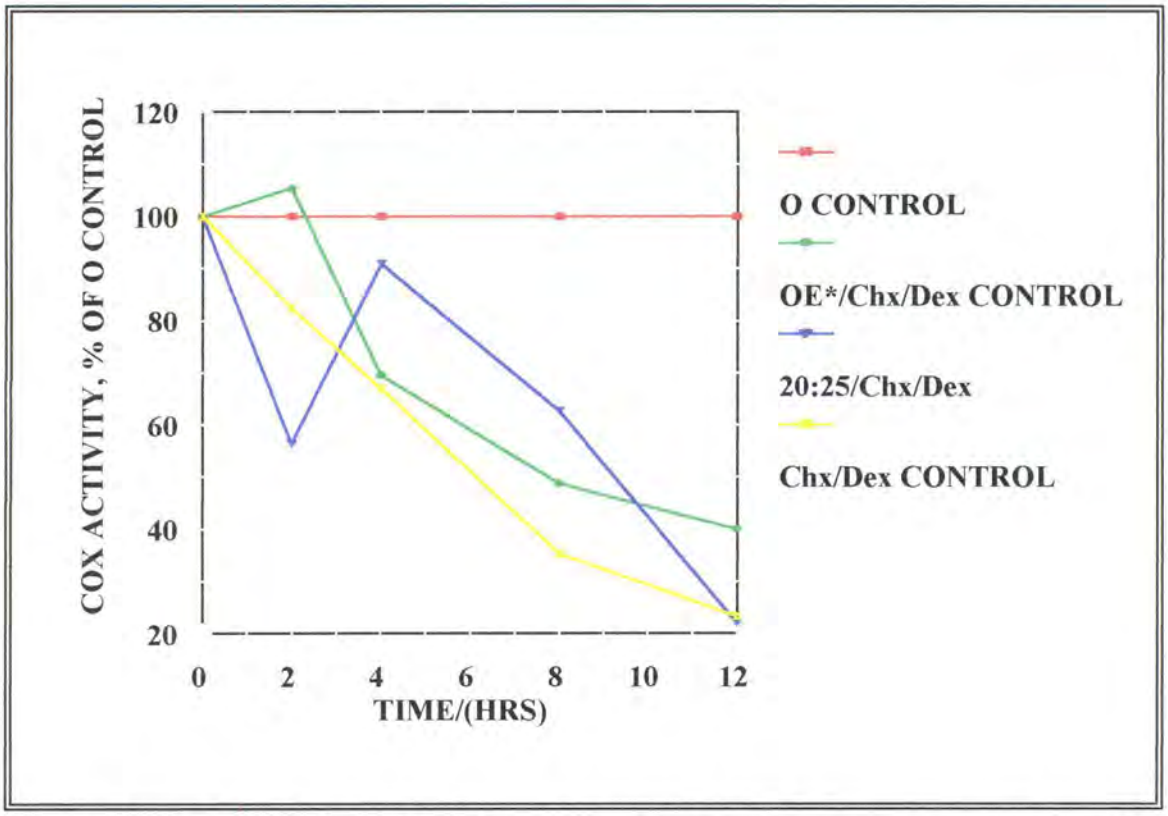


Figure 18: The effect of combined vitamin E succinate and Asc supplementation, together with dexamethasone and cycloheximide on Cox activity in BL6 cells. The values are the mean of three cultures.

6.5 DISCUSSION

Cox 1 the constitutive isoform is present in most tissues and mediates the synthesis of PGs required for normal physiological functions. Cox 2 is not detectable in most normal tissues, but is inducible. In transformed cells and tumours the increased production of PGs is associated with upregulation of Cox 2.[90]

Stimulation of Cox induced by vitamin E succinate and Asc at the combination 20:25µg/ml was almost eliminated by Dex after 12 hours. Dex has been reported to strongly inhibit Cox 2 expression (protein and mRNA) without affecting the constitutive Cox 1 mRNA.[135, 138] Dex appears to decrease Cox activity in the vitamin supplemented cells, however, whether it is at the level of transcription or translation in BL6 cells it is not known. Selective inhibition of Cox 2

expression by Dex has been demonstrated in various cell types, thus, suggesting that Dex inhibits Cox at the level of transcription.[89, 130, 135] The increase that may be caused by serum has been shown to be abolished by Dex.[89] O'Banion *et al.* reported that Cox 2 protein synthesis was repressed by Dex and suggests that translational regulation may occur.[130] This stimulation is dependent on new RNA and protein synthesis since the action of vitamin E succinate and Asc is almost abolished with the addition of Dex, suggesting that glucocorticoids induce a protein that modulates the translation of Cox.

The treatment with Chx in both the OE* and vitamin treated (20:25μg/ml) samples shows an initial stimulation at 2 hours and then a gradual decrease over time to below the established basal level. Mitchell *et al.* reported that doses of 1μg/ml or greater of Chx eliminated PGE_2 production treated with IL-1β.[133] This initial increase observed in the Cox activity may be due to the effect the Chx has on the mRNA of Cox 2, as O'Banion found that in the presence of Chx there was an accumulation of the Cox mRNA, thereby stabilizing the mRNA, which suggests transcriptional regulation.[130] The presence of the AUUUA repeats in the 3' untranslated region of Cox 2, allows for longer half-lives of mRNA in cells treated with Chx, while on the other hand the AUUUA repeats are found to be less stable in cells exposed to glucocorticoids.[130] This may account for the initial increase in Cox activity detected in the Chx treated samples and the decrease in Cox activity observed in the Dex treated samples.

Cox 1 is a constitutively expressed enzyme that is involved in the physiological synthesis of PGs. The increased Cox activity levels seen in BL6 cells, may thus as a result of the stimulation of Cox 2 and the coordinated down regulation by Dex, suggest that Cox may play a role in the increased synthesis of PGs in BL6 cells.

CHAPTER 7

GENERAL DISCUSSION

Tumour growth, vascularization, invasion and metastasis require activation and/or proliferation of specific host cells, and perhaps, deviation and/or suppression of others. The network of communication through intracellular signalling continues to be implicated in many of the checks and balances regulating cell growth in various normal and malignant tissues. Nutritional factors potentially can inhibit critical phases of tumour promotion. The proper combination of these factors is more effective in this inhibition than isolated substances because of their ability to complement each other in their mechanisms of action.[15]

The supplementation of combined vitamin E succinate and Asc at nutritional concentrations in BL6 cells and LLCMK with reference to the overall mean growth, showed a decreasing cell growth trend in both cell lines. Individually vitamin E[35, 55, 109] and Asc [45, 103, 106-107] have been shown to have a cytotoxic effect on a number of cell lines.

Vitamin E and Asc being antioxidants were presumed to be causing an inhibitory effect in BL6 cells by virtue of their antioxidant properties. Vitamin E and Asc are known to interact synergistically, whereby vitamin C regenerates vitamin E.[69, 76] However, in order for vitamin E succinate to function as an antioxidant in BL6 cells, cleavage of the succinate group is required. Analysis by HPLC showed that in BL6 cells the supplementation of combined vitamin E succinate and Asc showed no increasing or decreasing trend with reference to the levels of vitamin E succinate detected, however, an increase in the level of vitamin E was detected although not significant. This suggests that an esterase may be present in BL6 cells to allow for the cleavage of the vitamin E succinate molecule to form free vitamin E. Thus, vitamin E and Asc may interact synergistically as antioxidants.

The AA cascade generates a family of bioactive lipids that modulate diverse physiological and pathological responses including tumour growth and promotion. Recently there have been numerous publications describing the roles of AA metabolites in various types of tumours.[100]

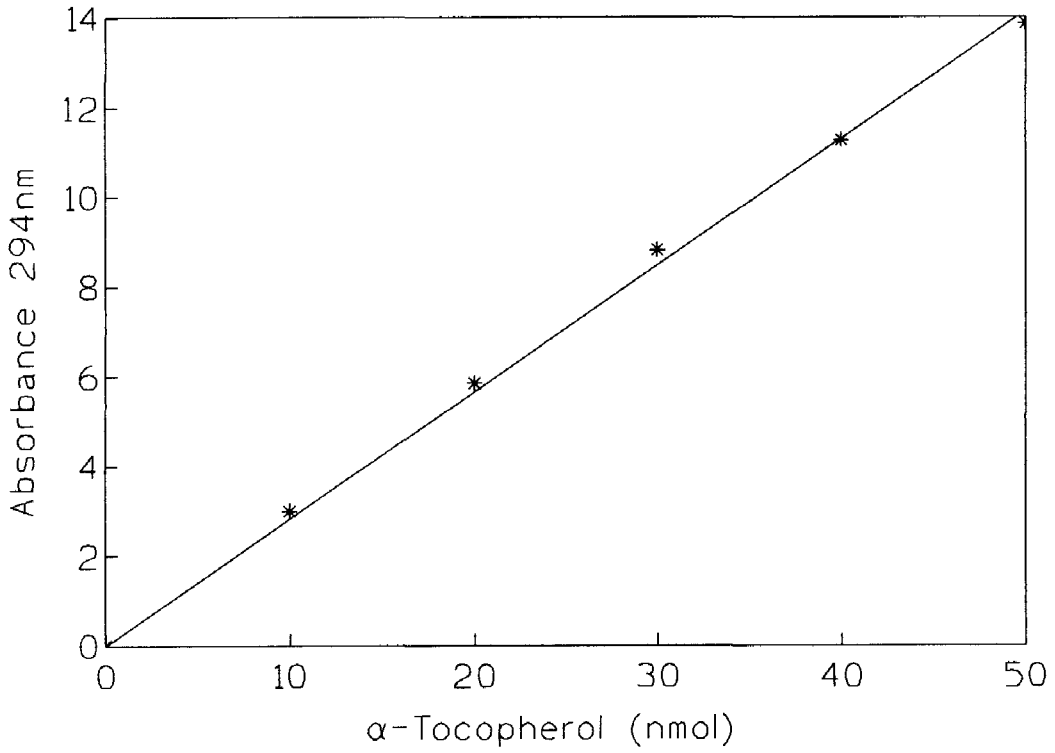
Traditionally, Cox has been regarded as an enzyme that is passively present in cells reacting to the varying supply of AA. This feature seems to hold true in most cells for the Cox 1 isozyme, which is regulated in the course of developmental processes but appears to be constantly expressed in most (patho)physiological situations. In contrast Cox 2 isozyme has evolved as a dynamically regulated enzyme. The structural characteristics, the relatively small gene, multiple binding sites for transcription factors in the 5'-untranslated region and the multiple AUUUA sites in the 3'-untranslated region of the mRNA, provide the basis for the observed rapid up- and downregulation of Cox 2 mRNA. Transcriptional, as well as post-transcriptional, mechanisms are involved in the upregulation by numerous stimuli.[93] Detailed studies of the interrelationships of the enzymes involved in the AA cascade is a challenge since these enzymes are regulated not only at the transcriptional level but also post-transcriptionally and post-translationally.[93]

Studies have proposed that the inhibitory effect of vitamin E succinate and Asc on BL6 cells may be mediated through the AA cascade. In an attempt to provide further evidence of an increase in Cox enzyme activity which may mediate the growth inhibitory effect on BL6 cells, the BL6 cells were supplemented with combined vitamin E succinate and Asc over a time course of 12 hours. A significant increase in Cox activity was observed after 12 hours following supplementation of vitamin E succinate and Asc with the combination 20:25μg/ml, respectively. In an attempt to determine if this increase in Cox activity required the synthesis of new protein, dexamethasone and cycloheximide, inhibitors of Cox were supplemented in combination with the vitamin treatment. Analysis of Cox activity following dexamethasone, cycloheximide and combined vitamin E succinate and Asc supplementation resulted in a decreasing trend in Cox activity with dexamethasone supplementation, while cycloheximide initially stimulated Cox activity at 2 hours and then a slow decrease was observed over time. Therefore it is reasonable to conclude that the inhibitory effect on Cox activity by dexamethasone suggests that new Cox protein was synthesized with the supplementation of combined vitamin E succinate and Asc, as dexamethasone is know to be a inhibitor of transcription. Initial attempts to determine the effects of vitamin supplementation on Cox using molecular studies were unsuccessful for a number of reasons but probably primarily as these were done on cells subjected to 7 days of vitamin supplementation whereas the above study showed that the effects on Cox expression were probably only found in the first 12 hours after supplementation. Northern blot analysis studies

over the initial 12 hour period of Cox 1 and Cox 2, while inconclusive, did show that there is a possibility that Cox 2 may be present in BL6 cells after 2 hours of supplementation with combined vitamin E succinate and Asc. Therefore vitamin E and Asc may be assumed to possibly have a stimulatory effect on Cox expression through the activation of cell surface receptors, which ultimately would result in the increase in PGs observed by Midgley,[111] especially those of the E series.

It appears from these studies that vitamin E and Asc play a role in stimulating Cox activity which would result in the increased PG synthesised in BL6 cells, however, these results are not conclusive and further investigation is required to clarify vitamin E and Asc role in the AA cascade and as chemopreventive agents.

APPENDICES



APPENDIX 1: Vitamin E standard curve.

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