

IN VITRO AND *EX VIVO* EFFECTS OF VITAMIN D
ON REGULATION OF VASCULAR TONE

LAILA ALHARBI

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Division of Nutritional Sciences
School of Biosciences
University of Nottingham
Sutton Bonington Campus
Leicestershire
England

ABSTRACT

Hypertension is a key factor in the progression of cardiovascular diseases, and vitamin D is now recognised as a significant factor affecting blood pressure. Low serum concentrations of calcidiol may be related to the pathogenesis and progression of hypertension.

The effect of both calcidiol and calcitriol on the proliferation and gene expression of HUVECs and on the subsequent proteins produced, secreted, or regulated by endothelial cells is investigated. This was followed by studies on the thoracic and abdominal segments of aortas isolated from adult female Wistar rats to determine direct vasomodulatory effects of calcitriol using a wire myograph system. The result showed that calcitriol (10^{-13} M) significantly enhanced the proliferation of HUVECs whereas a relatively high concentration of calcidiol (10^{-9} , 10^{-7} M) significantly inhibited cell proliferation after the same incubation period: 24 and 48 hours. Calcidiol had the greatest effect on the expression of genes, particularly those involved in regulating blood pressure, with decreased ET-1 mRNA expression, and there was a significant increase in eNOS mRNA relative to the control, particularly with 10^{-11} M of calcidiol. However, calcitriol had little effect on the expression of these genes so its effects on blood vessels may relate to non-genomic mechanisms. *Ex vivo* studies have shown that calcitriol acutely reduced endothelium-independent contraction in rat thoracic and abdominal aortas and does not involve NO. The acute effect of calcitriol reduced or inhibited the dose-dependent (10^{-9} to 10^{-5} M) contractions induced by PE, and there was a significant inhibition of the subsequent dose-dependent (10^{-9} to 10^{-5} M) relaxation induced by acetylcholine. The acute vasorelaxant effects of calcitriol do not appear to be mediated via the AC-cAMP-PKA signalling pathway but were similar to the effects of the Ca^{2+} channel blocker, verapamil.

In summary, this thesis reports novel information regarding the effect of calcitriol on regulation of vascular tone through enhancement of a potential relaxation of arteries in an endothelium-independent manner, which is mediated by a non-genomic mechanism. Future studies need to confirm these results with *in vivo* studies. Hence, inducing relaxation in smooth muscle cells *ex vivo* may facilitate to use calcitriol as an antihypertensive drug.

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LIST OF ABBREVIATION

[Ca]	Serum calcium
[Ca²⁺]_i	Intracellular Ca ²⁺
[Pi]	Serum phosphate
μl	microliter
1,25(OH)₂D₃	Calcitriol
25(OH) D₃	Calcidiol
KCL	Potassium chloride solution
AC	Adenylate Cyclase
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
Ang II	Angiotensin II
BMPs	Bone morphogenic proteins
cAMP	Cyclic adenosine monophosphate
Cdk2	Cyclin-dependent kinase 2
CO₂	Carbon dioxide
CVD	Cardiovascular disease
CYP27b1	1-alpha-hydroxylase enzyme
cGMP	Cyclic guanosine monophosphate
CYP24	24-hydroxylase enzyme
CYP24a1	24-hydroxylase enzyme
CYP2R1	25-hydroxylase enzyme
D₂	Ergocalciferol
D₃	Cholecalciferol
DAG	Diacylglycerol
dbcAMP	Dibutryl-Cyclic adenosine monophosphate
DBPs	Vitamin D Binding Proteins
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EBM-2	Endothelial basal media-2
ECes	Endothelium Converting Enzymes
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
FBS	Fetal bovine serum
FGF23	Fibroblast-like growth factors
GPCRs	G-protein-coupled receptors
FPL 64176	Ca ²⁺ channel (L-type) activator
H₂O₂	Hydrogen peroxide
H-89	Dihydrochloride hydrate (PKA inhibitor)
HCASMCs	Human coronary artery smooth muscle cells
HUVECs	Human Umbilical Vein Cord Endothelial cells
iNOS	Inducible Nitric Oxide synthase
IP3	Inositol 1,4,5-trisphosphate
kDa	Kilo Daltons

L-NAME	Endothelial NO synthase inhibitor, N ω -Nitro-L-arginine methyl ester hydrochloride.
M	Molar
mg/ml	Milligram per millilitre
mRNA	Messenger ribonucleic acid
mVDRs	membrane Vitamin D Receptors
nNOS	Neuronal Nitric Oxide synthase
NO	Nitric Oxide
NO²⁻	Nitrite concentration
NOA	NO analyser
NSCC	Ca ²⁺ -permeable nonselective cation channel
O₂	Oxygen
O²⁻	superoxide
ONOO⁻	peroxynitrate
PBS	Phosphate buffered saline
PE	Phenylephrine
PI3K	Phosphatidylinositol-3 kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PLA2	Phospholipase A2
PLC	Phospholipase C
PTH	Parathyroid hormone
qRT-PCR	Quantitative reverse transcriptase polymerase chain reactions
ROC	Receptor-Operated Calcium channel
ROS	Reactive oxygen species
RXR	Retinoid x Receptors
RyR	Ryanodine Receptor
SD	Standard Deviation
SDS-PAGE	Sodium doecyl sulphate polyacrylamide gel electrophoresis
SOC	Store-Operated Calcium Channel
SR	Sarcoplasmic Reticulum
UV-B	Ultraviolet B radiation
VDR	Vitamin D Receptors
VDRE	Vitamin D Responsive Elements
VOC	Voltage Operated Calcium Channel
VSMCs	Vascular Smooth Muscle Cells

1

INTRODUCTION

1 Introduction

Calcitriol, which is the active form of vitamin D, provides a pivotal benefit for bone development and maintenance (Esper et al., 2006). However, recently, it has been recognized that vitamin D has a role beyond maintaining skeletal health and calcium homeostasis (Goel and Lal, 2011, Resnick, 1994). With the publication of new reports on the role of vitamin D in the pathophysiology of several diseases, such as osteoporosis, vascular calcification, and hypertension, greater attention has been given to vitamin D deficiency. There is also greater recognition of vitamin D's contribution to fundamental biological functions such as the inhibition of cell growth, and cell proliferation.

In the circulatory system, vitamin D is found in two main forms: i) calcidiol or 25(OH)D₃ (the inactive precursor form), with concentrations in the blood ranging between 30 and 50 nmol/L, and ii) calcitriol or 1,25(OH)₂D₃ (the active form), with concentrations in the blood ranging between 50 and 180 pmol/L (Kragt et al., 2009).

It has been suggested that a low serum concentration of calcidiol may be a significant risk factor for cardiovascular disease, cancers, autoimmune disease, and metabolic syndrome (Feneis and Arora, 2010). Furthermore, there is now a great deal of observational data linking blood pressure with vitamin D concentrations (Beveridge et al., 2015, Mandarino et al., 2015).

Hence, the objective of this chapter is to thoroughly examine the published literature on the link between vitamin D status and the control of blood pressure. Specifically, the topic of this review covers the physiology of vitamin D, vascular structure and function of the blood vessels and the implications of vitamin D deficiency on the progression of hypertension.

1.1 Vitamin D

1.1.1 Overview

Vitamin D comprises a group of fat-soluble seco-sterols present in two isoforms ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) (Gouni-Berthold et al., 2009, Kulie et al., 2009). Vitamin D₂ is manufactured by the action of ultraviolet B radiation (UV-B) on ergosterol, which occurs in plants. Vitamin D₃ occurs in a few dietary sources, such as mushrooms, but is primarily synthesized in the skin from 7-dehydrocholesterol after exposure to sunlight (Del Valle et al., 2011). Both vitamin D₂ and vitamin D₃ can be made commercially and found in dietary supplements or used to fortify foods (Del Valle et al., 2011). The chemical difference between D₂ and D₃ is in their side chain structure. Vitamin D₂ has a double bond between its carbon atoms 22 and 23, as well as an extra methyl group on carbon 24 (Figure 1-1) (Norman, 2008).

1.1.2 Sources and synthesis of vitamin D

Limited sources of vitamin D₃ are found in foods such as fatty fish; fish liver oil and egg yolk. In addition to these sources, most milk and dairy products, margarine and spreads and some breakfast cereals are fortified with either vitamin D₃ or D₂ (Jäpelt and Jakobsen, 2013, Spiro and Buttriss, 2014). Hence, vitamin D can be obtained from dietary sources to a minor degree (10 to 20%), compared to formation by the skin (Holick, 2007).

Humans primarily (80 to 90%) gain vitamin D₃ from exposure to sunlight under natural conditions, since human skin is able to synthesise cholecalciferol, the precursor to vitamin D₃ (Calvo et al., 2004, Glerup et al., 2000). More specifically, UV-B is responsible for the cutaneous synthesis of vitamin D₃ (Wolpowitz and Gilchrest, 2006, Jones et al., 1998). The optimum wavelength of UV-B to produce vitamin D₃ is between 280–315 nm (MacLaughlin et al., 1982, Engelsen et al., 2005). The process of converting 7-dehydrocholesterol, which is the last precursor of cholesterol synthesis,

by UV-B to vitamin D₃ (and subsequently to calcitriol) occurs in the epidermis and dermis.

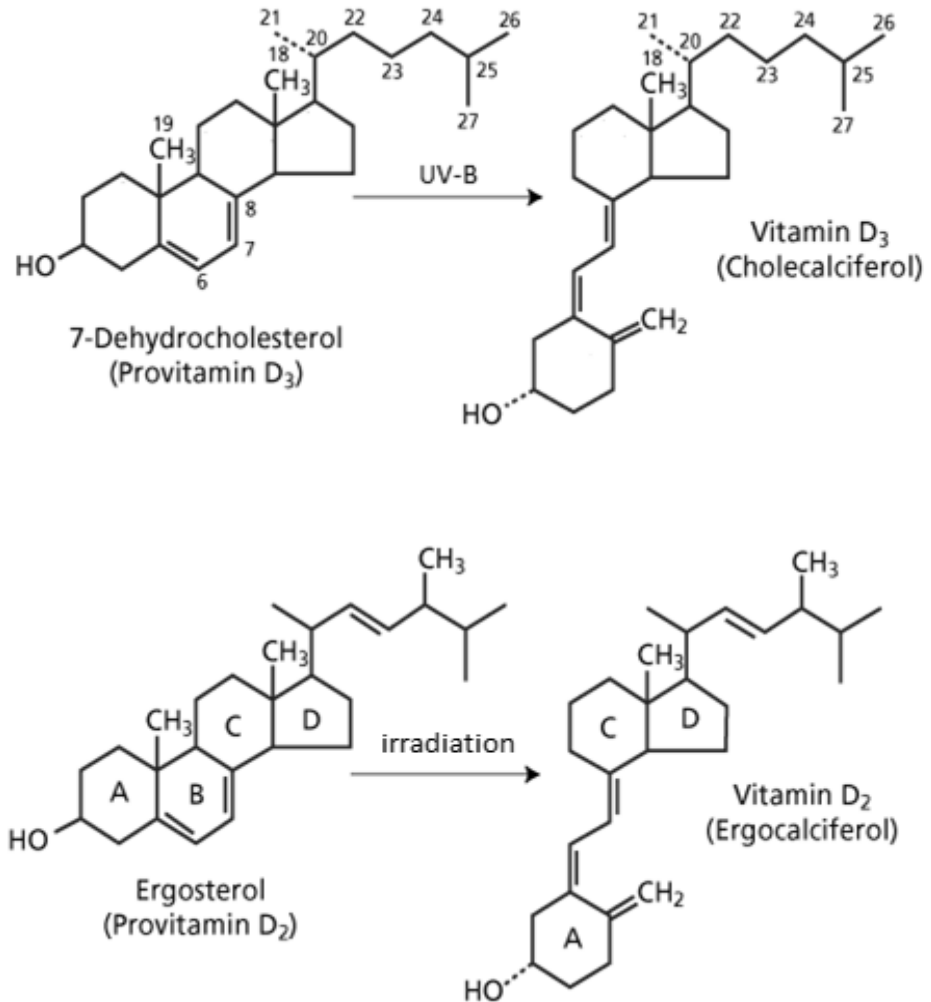


Figure 1-1 The chemical structure of vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol) and their respective precursors. Cholecalciferol is produced in the skin through the action of ultraviolet UV-B, which converts 7-dehydrocholesterol to vitamin D₃. Ergocalciferol is obtained artificially by the irradiation of ergosterol (Holick, 2006).

1.1.2.1 Factors that Influence Cutaneous Vitamin D Synthesis

There are many external factors that might interfere with the production of vitamin D₃ by the skin, and therefore affect vitamin D status. The intensity of UV-B radiation is one such critical factor that is itself affected by latitude (Nair and Maseeh, 2012), the winter season (Rostand, 1997), aging, cover-up clothing (Matsuoka et al., 1992) and/or sunscreen use (Matsuoka et al., 1987) and skin pigmentation (Holick et al., 1981, Tsiaras and Weinstock, 2011, Holick, 2011). The cutaneous synthesis of vitamin D₃ requires UV-B radiation, with epidemiological studies showing a declined capacity for vitamin D₃ synthesis during winter months when UV-B radiation is low (Rostand, 1997). It is also been reported that the risk for vitamin D deficiency is greater for people living at higher latitudes than for those living at lower latitudes because of the lower intensity of sunlight (Nadir et al., 2010, Woodhouse et al., 1993). In addition, melanin (the dark pigment in skin) reduces the effectiveness of vitamin D₃ synthesis in the skin, acting as a natural sunscreen by competing with 7-dehydrocholesterol the absorption of UV-B (Holick et al., 1981, Tsiaras and Weinstock, 2011, Holick, 2011). As such, a sunscreen with a sun protection factor (SPF) of 8 will reduce the capacity of skin to synthesise vitamin D₃ by approximately 95% (Holick, 2004a). Ageing also affects vitamin D synthesis, reducing it by about 50% every 25 years; thus, a 70-year-old person has only about a quarter of the capacity of a 20-year-old to produce vitamin D₃ (Gallagher, 2013). In addition, exposure to sunlight through glass does not result in vitamin D synthesis as the glass absorbs all UV-B radiation (Holick, 2011).

1.2 Metabolism of Vitamin D

1.2.1 Absorption

Vitamin D, either D₂ or D₃, is absorbed from the intestinal tract and is dependent on an intact fat absorption mechanism (Del Valle et al., 2011, Pappa et al., 2008). Subsequently, clinical and experimental studies have confirmed that the efficient absorption of vitamin D is highly likely when it is consumed with a large meal (Mulligan and Licata, 2010, Johnson et al., 2005) and, conversely, that disorders associated with fat malabsorption, such as bile salt deficiency, celiac disease, Crohn's disease and pancreatic insufficiency, impair the absorption of vitamin D (Pappa et al., 2008).

Due to it being a fat-soluble compound, vitamin D requires the presence of bile salts and pancreatic lipase for absorption. The absorption of vitamin D is relatively low and variable with only 30% to 50% being absorbed. It requires the presence of fat in the lumen; this induces the release of bile salts and pancreatic lipase. The bile salts emulsify lipids and assist the formation of lipid-containing micelles, which diffuse into enterocytes.

Within the intestinal wall, vitamin D is packaged into chylomicrons and absorbed into the lymphatic system (Holick, 2006). From there, chylomicrons, which are associated with vitamin D binding proteins (DBPs) and lipoproteins, enter the circulation to reach the liver (Holick, 2004b, DeLuca, 2004, Haddad et al., 1993). Thereafter, vitamin D dissociates from protein binding and precedes to the liver, where it undergoes the main pathway of vitamin D uptake, the first hydroxylation step in order to obtain the major circulating metabolite of vitamin D, (25(OH) D₃). Chylomicron lipids are metabolized through the action of lipoprotein lipase where they are expressed in peripheral tissues, such as adipose tissue and muscle (Jones, 2008). The liver takes up any vitamin D left in the chylomicron remnant and removes it from the bloodstream for storage (Mawer et al., 1971, Jones, 2008). Thus, whether taken up by adipose tissues or metabolised in the

liver, there is a logical explanation for the fact that vitamin D in plasma has a short lifespan of approximately 4-6 hours (Jones, 2008).

1.2.2 Transport in Circulation

As mentioned above, vitamin D and its metabolites are initially transported in chylomicrons via the lymph and blood plasma (Holick et al., 2011). Once entering into circulation, they are bound to the proteins and transported to the liver. The most important carriers in this process are the vitamin D binding proteins (DBPs), which are predominantly produced in the liver (Haddad, 1995).

1.2.3 Conversion to Active Metabolite, Calcitriol

The biological activation process of either vitamin D₂ or D₃ involves 25-hydroxylation, followed by 1-alpha-hydroxylation, to produce the active metabolite form, calcitriol (Anderson et al., 2003). The activation steps are illustrated in Figure 1-2. The first hydroxylation step occurs in the liver, where vitamin D₃ is converted to Calcidiol, which is a major circulating form in blood. Then, in the kidney, Calcidiol undergoes a second hydroxylation in order to produce the active form, calcitriol by 1-alpha-hydroxylase (CYP27b1) (Hamilton, 2010).

1.2.3.1 Hydroxylation of Vitamin D₃ to Calcidiol

With exposure to UV-B radiation, 7-dehydrocholesterol found naturally in the skin is converted into previtamin D₃. Over the course of a few hours, this is slowly converted into vitamin D₃ by body heat. Vitamins D₃ and D₂ bind with DBP, a 458-amino acid polymorphic human serum protein. This is a specific plasma carrier of Calcidiol and its metabolites (Verboven et al., 2002, Bhan, 2014). The vitamins are transported to the liver.

In the liver, both vitamins D₃ and D₂ convert to Calcidiol by the actions of a cytochrome P450 (CYP) enzyme, namely 25-hydroxylase (CYP2R1) (Henry, 2011); Calcidiol is still inactive and it is a major circulating form (Bhan, 2014).

1.2.3.2 Formation of Calcitriol

The active metabolite of vitamin D₃, calcitriol mainly occurs in the kidney via endocrine actions; however, many cell types can produce calcitriol, primarily as an autocrine/paracrine action (Henry, 2011). In the kidney, calcidiol undergoes a further hydroxylation in order to produce either the active form calcitriol or to inactivate calcidiol to (24,25)-hydroxycalcidiol (24,25(OH) D₃) to prevent vitamin D intoxication (Norman, 2008). The production of calcitriol is catalyzed by CYP27b1, a mitochondrial P450 enzyme (Hamilton, 2010), whereas (24,25)-hydroxycalcidiol is catalyzed by the 24-hydroxylase enzyme CYP24 (Norman, 2008). The 1- α -hydroxylase (CYP27b1) gene is expressed predominantly in the kidney, but also in other tissues (Del Valle et al., 2011, Tangpricha et al., 2001, Schwartz et al., 1998).

1.2.4 Physiological Regulation

1.2.4.1 Regulation of Calcitriol Production

The activity of CYP27b1 in the kidney is highly regulated by calcitriol itself, where the parathyroid hormone (PTH) signals serum calcium (Ca) status and fibroblast-like growth factors (FGF23) signal phosphate status (Pi) (Figure 1-2) (Bikle, 2007, Barthel et al., 2007, Henry, 2011).

First, PTH essentially plays a central role in the regulation of calcium homeostasis in blood serum affecting the control of vitamin D metabolism (Jones et al., 2012, Brown, 2000). In response to low levels of serum calcium, PTH is secreted and causes the kidney to increase expression of CYP27b1, thus facilitating the production of calcitriol, which then increases intestinal absorption of Ca²⁺.

As with PTH, FGF23 control the production of calcitriol as well as regulation of serum phosphate. They have also been seen to impede the production of renal calcitriol through the inhibition of CYP27b1 and stimulation of 24-hydroxylase (CYP24a1) *in vivo* and *in vitro* (Shimada et al., 2004, Perwad et al., 2007). Low levels of phosphorus

in serum stimulate calcitriol synthesis, whereas high levels of phosphorus inhibit it (Del Valle et al., 2011).

Extra-renal CYP27b1 is also regulated at external sites, although this differs slightly from the regulation of the renal enzyme (Dusso et al., 2005). The calcium and phosphate regulating hormones do not regulate this enzyme's activity, although it may be regulated by changes that are specific to the function or environment of the cell, such as in cytokines and growth factors; these cell-specific changes can optimize the levels of calcitriol via mechanisms that are not yet completely understood (Dusso et al., 2005, Henry, 2011).

1.3 Catabolism and Excretion

It has been shown that the 24-hydroxylation reaction is a major step in the degradation and inactivation of both calcidiol and calcitriol and, and is mediated by CYP24a1, which places a hydroxyl group on C-24 (DeLuca, 2008). This hydroxylation reaction allows for its metabolism into calcitroic acid and its excretion in the bile into the feces and a very little is eliminated through the urine (Henry, 2011).

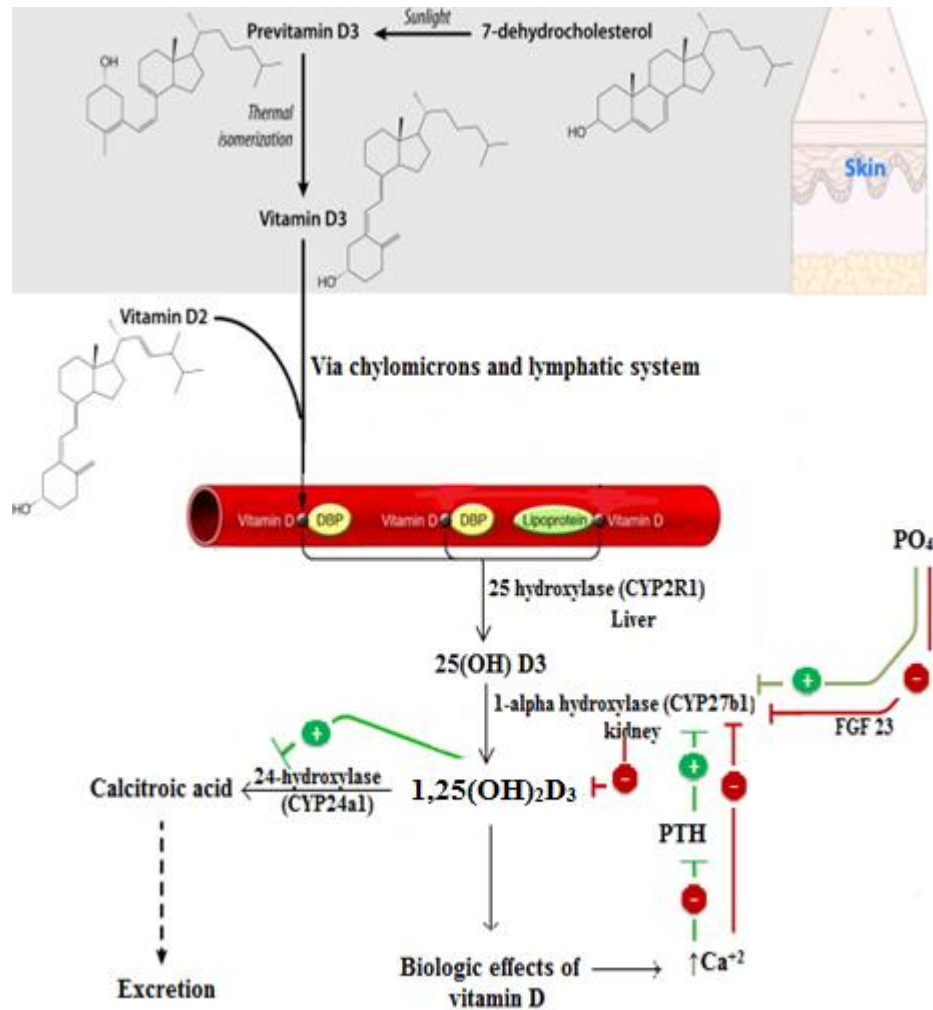


Figure 1-2 Sources, synthesis and metabolism of vitamin D. Vitamin D is either produced in the skin by exposure to sunlight, where 7-dehydrocholesterol converted to previtamin D₃, or is ingested through diet as either Vitamin D₃ or vitamin D₂. In the liver, vitamin D (vitamin D₂ or vitamin D₃) is enzymatically hydroxylated by the 25-hydroxylase (CYP2R1) to calcidiol (25(OH) D₃). Thereafter, in the kidney, calcidiol is converted by CYP27b1 to the active metabolite of vitamin D, calcitriol (1,25(OH)₂D₃). The biologically active forms of vitamin D calcitriol enhance intestinal calcium and phosphorus absorption. The activity of CYP27b1 is highly regulated by calcitriol itself, as calcitriol inhibits the CYP27b1 and stimulates the expression of the renal 24-hydroxylase (CYP24a1). Stimulation of the CYP24a1 results in the destruction of calcitriol into a water-soluble inactive metabolite known as calcitroic acid. In response to low levels of calcium serum, PTH is secreted and signals the kidney to increase expression of CYP27b1, thus facilitating the production of calcitriol, which then increases intestinal absorption of Ca²⁺. Adapted from (Querfeld and Mak, 2010, Jones, 2012).

1.4 Toxicity

Excessive intake or supplementation of vitamin D₃ can cause a state of vitamin D intoxication, or hypervitaminosis D (Alshahrani and Aljohani, 2013, Sidhra, 2014). In fact, from the 1930s to 1950s, the widespread consumption of fortified food and drinks with vitamin D₃, such as milk, bread and hot dogs, in the United States and Europe led to numerous reported cases of toxicity (Holick, 2003a, Holick, 2003b). Hypercalcemia can result from vitamin D toxicity; this leads to calcium being deposited in soft tissues, resulting in renal and cardiovascular damage (Brown et al., 2002, Marcus et al., 2012). Vitamin D toxicity is not caused by prolonged exposure to the sun as thermal activation degrades previtamin D₃ in the skin to non-vitamin D forms such as lumisterol and tachysterol (Holick et al., 1981, Webb et al., 1989).

Three major hypotheses have been posed concerning the mechanism of vitamin D toxicity (Jones, 2008). All of these involve the increased levels of vitamin D metabolite that enter the target cells and interact with the VDR in the nucleus, leading to exaggerated gene expression (Jones, 2008). The three proposed explanations are as follows:

1. Vitamin D excess raises the plasma calcitriol, leading to increased intracellular calcitriol levels. Although this theory might be the most obvious explanation of elevating vitamin D in plasma, as a result of overproduction of calcitriol from excessive precursor levels of calcidiol, studies conducted on vitamin D production/overproduction in humans do not strongly support this hypothesis (Shepard and Deluca, 1980, Jones, 2008).
2. Increased concentrations of calcidiol in plasma leading to an increased DBPs capacity and free calcidiol, which enters into cells and has a direct effect on gene expression.

3. Increased intake of Vitamin D intensifies the concentrations of calcitriol and Calcidiol. This leads to exceeding the DBPs binding capacity and causing the release of 'free' calcitriol, which enters target cells.

1.5 Functions of Vitamin D

In its hormonal form, calcitriol acts to maintain and regulate plasma calcium and phosphorus levels; these are needed for the mineralization of bone (DeLuca, 2008). The major function of calcitriol is to promote calcium absorption from intestine by stimulating the formation of epithelial calcium channels such as transient receptor potential vanilloid type 6 (TRPV6) and calcium-binding protein calbindin (Christakos et al., 2011). A slight decrease of plasma calcium concentrations to below the normal physiological range levels stimulates the parathyroid gland to secrete PTH in circulation within seconds, which stimulates synthesis of calcitriol (Silver et al., 1996). Following synthesis of calcitriol, calcitriol increases the plasma calcium concentration by increasing absorption of intestinal calcium and bone resorption (DeLuca and Zierold, 1998). However, if plasma calcium concentrations above the normal levels, then the thyroid gland secretes calcitonin that block mobilisation of calcium from bone to restore homeostasis (Chambers and Magnus, 1982).

Calcitriol increases the absorption of phosphorus in the intestine and induces the secretion of FGF23 by osteocytes which assists in the prevention of phosphate accumulation in the body (Liu et al., 2008, Kolek et al., 2005). Deficiency of vitamin D is associated with skeletal diseases such as rickets and osteoporosis (Holick, 2008). Recently, calcitriol has physiologic effects beyond its role in mineral homeostasis and bone function (Lips, 2006, Díaz et al., 2015). An example of the action of vitamin D is in calcitriol /VDR signaling is its suggested contribution to the modulation of growth, the prevention of cancer, cardiovascular processes, and the regulation of immune responses and insulin secretion (Holick, 2010).

1.6 Determination of Vitamin D status

Vitamin D status is most commonly assessed by measuring serum calcidiol levels (Makariou et al., 2011). The optimal plasma levels of calcidiol are between the ranges of 30-100 ng/ml. The term ‘insufficient vitamin D’ is commonly used when serum calcidiol is within the 21-29 ng/ml range, while levels below 20ng/ml are considered to be indicative of a deficiency (Makariou et al., 2011).

1.6.1 Risks Associated with Vitamin D Deficiency

As well as synthesis by the body, vitamin D can be obtained from both food and supplementation. It has been shown that, worldwide, 1 billion people have vitamin D deficiency (Holick, 2006, Nair and Maseeh, 2012). According to the United States’ third National Health and Nutrition Examination Survey (NHANES III), the prevalence of vitamin D deficiency among American adults is 57% (Holick, 2006). Deficiency or insufficiency is even more common in children, young adults, and women who are pregnant or lactating (Pearce and Cheetham, 2010). A recent study suggested that more than half of the elderly population of the United States and Europe is vitamin D deficient (Nadir et al., 2010). Ageing and obesity are the most common factors underlying vitamin D deficiency. As described earlier, ageing results in decreased levels of 7-dehydrocholesterol in the skin. Obesity also increases the sequestering of calcidiol by fat cells (Scragg and Camargo, 2008, Wortsman et al., 2000). Hypertension has also been linked with vitamin D deficiency with studies reporting an increased incidence of hypertension associated with vitamin D status and supplementation, and one study concluding that most patients with high blood pressure have insufficient levels of vitamin D (Vacek et al., 2012). It is also been reported that low vitamin D levels due to diet and limited exposure to sunlight resulted in increased incidence of hypertension (Feneis and Arora, 2010).

1.7 Mechanism of Action

In its hormonal form, calcitriol acts to maintain and regulate plasma calcium and phosphorus levels; these are needed for the mineralization of bone (DeLuca, 2008). As well as its roles in maintaining bone and muscle health and in mineral homeostasis and bone function, it has been shown that calcitriol has broader physiological effects (Díaz et al., 2015), with biological responses to calcitriol involving the regulation of gene expression at the transcriptional level (the classic genomic response) as well as non-genomic responses such as the activity of signal transduction pathways (Jones et al., 1998, Jurutka et al., 2001). Two types of receptor mediate these actions: nuclear vitamin D receptors (VDRs) and putative plasma membrane vitamin D receptors (mVDRs) (Zúñiga et al., 2011). Frequently, genomic responses have taken several hours to days to be fully apparent. This contrasts with non-genomic effects, which have rapid responses that usually occur within 1-2 minutes to 45 minutes (Haussler et al., 2011).

1.7.1 Genomic Action of Vitamin D

The genomic effect of calcitriol results from its interaction with its VDR (Barsony et al., 1997). The VDR is a protein of 53 kDa, which is a member of the nuclear receptor family acting as a high affinity ligand-activated transcription factor (Fleet, 2004). The expression of VDR in target tissues determines the magnitude of the response evoked by calcitriol. The levels of VDR expression in target tissues might be dynamically auto-regulated by calcitriol, in addition to several other factors (Pike et al., 2011, Pike et al., 2014). Calcitriol induces up-regulation of the amount of VDR, whereas growth factors may lead to up- or down-regulation of receptor abundance.

In the classic genomic mechanism, following synthesis, calcitriol in the kidney binds DBP to be transported to the target cell. Upon entering the cell, it then binds to and activates the VDR, leading it to interact with retinoid x receptors (RXR) and to the recruitment of cofactors to form a VDR/RXR/cofactor complex. Next, this complex

binds to vitamin D responsive elements (VDRE) in the promoter region of target genes to regulate gene transcription (Lips, 2006, Tagami et al., 1998).

Research indicates that VDRs are present and frequently expressed in the nuclei of many tissues involved in calcium and phosphorus homeostasis such as the intestine, kidney and thyroid gland (Jones et al., 1998). Furthermore, they are semi-frequently expressed in the tissues of nearly all organs, including the heart, as well as in vascular smooth muscle, endothelium and various cells of the immune system (Holick, 2006, Norman, 2008, Bookout et al., 2006).

Calcitriol has physiologic effects beyond its role in mineral homeostasis and bone function (Lips, 2006, Díaz et al., 2015). An example of the action of vitamin D is in calcitriol /VDR signaling is its suggested contribution to the modulation of growth, the prevention of cancer, cardiovascular processes, and the regulation of immune responses and insulin secretion (Holick, 2010).

1.7.2 Non-genomic Action of Vitamin D

Besides regulating gene expression via its intracellular VDR, calcitriol can also exert responses via non-genomic mechanisms, which induces a rapid and activated signal transduction pathway. However, non-genomic signaling may indirectly affect transcription via cross-talk with other signaling pathways (Zanatta et al., 2011).

Hence, The localization of mVDRs in the plasma membrane activates signal transduction pathways that can generate rapid responses (Haussler et al., 2011). The response of non-genomic actions occurs within 1-45 minutes, and includes a variety of signal transduction activities, such as phospholipase C (PLC) and phospholipase A2 (PLA2), as well as phosphatidylinositol-3 kinase (PI3K) When it interacts with mVDR, calcitriol rapidly initiates the synthesis of second messengers, including calcium influx and release of Ca^{2+} from intra-cellular stores, cyclic AMP (cAMP), cGMP, fatty acids and 3-phosphoinositides such as phosphatidylinositol 3,4,5 trisphosphates (Sylvia et al.,

1996, Santillán and Boland, 1998, Vazquez et al., 1995, Barrera et al., 2008, Wali et al., 1990, Bellido et al., 1993, Morelli et al., 1993). Afterwards, these second messengers are accompanied by the consequent activation of a cascade of protein kinases, such as protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and Ca^{2+} -calmodulin kinase (CaMK) (Morelli et al., 2001, Fleet, 2004, Bellido et al., 1993).

1.8 Control of Blood Pressure

1.8.1 Vascular Structure and Function

The walls of blood vessels are composed of three layers, with three distinct functions (Wang et al., 2015). These layers are tunica intima, tunica media and tunica externa (Figure 1-3). Tunica intima, which is the inner layer, and tunica externa, which is the outer layer, are composed mainly of endothelial cells and connective tissue, respectively, whereas the tunica media, which is the muscular middle layer, contains smooth muscle cells (Cherng et al., 2010).

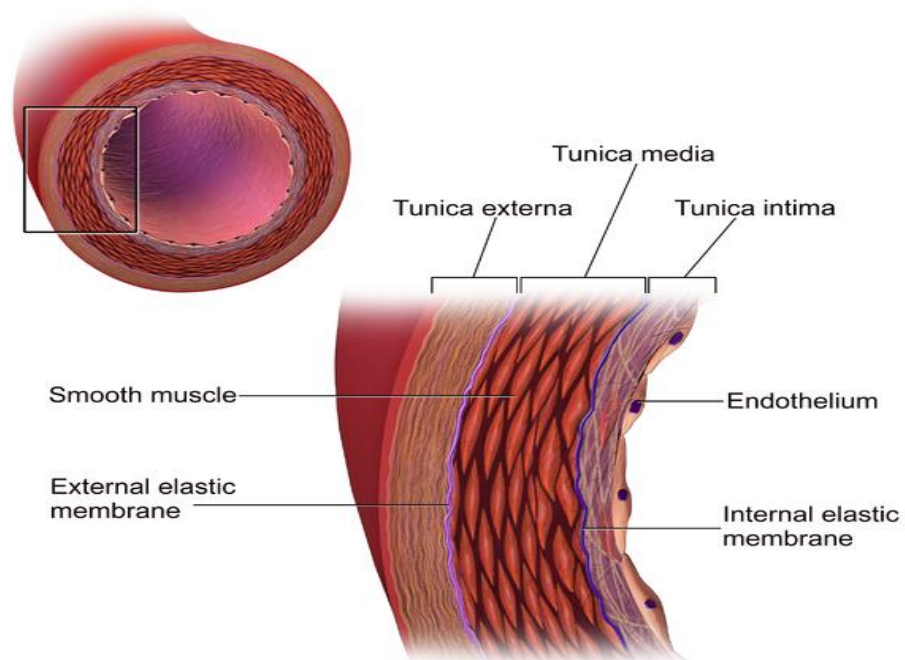


Figure 1-3 Schematic diagram of an artery wall. Blood vessel walls have three layers: the inner tunica intima composed mainly of endothelial cells; the tunica media, the muscular middle layer composed of smooth muscle cells; and the tunica extrema, the outer layer composed of connective tissue.

<http://blogs.furman.edu/wellness/2014/11/07/your-guide-to-heart-health/>

1.8.2 Vascular Endothelium

Endothelial cells line the interior surface of blood vessels and lymphatics and are single nucleated cells which compose the endothelium (Sumpio et al., 2002). The endothelium is a thin mononuclear layer lining all the interior of blood vessels within the cardiovascular system (CV), separating the circulating blood from the tissue (Esper et al., 2006).

The vascular endothelium has numerous physiological and pathological functions, including modulation of vascular tone, blood flow as well as the regulation, along with effects on cellular proliferation, immune and inflammatory mechanisms and blood coagulation (Böhm and Pernow, 2007). Hence, vasomotor tone is characterized by the level of contraction or relaxation of vascular smooth muscle cells, which administers the level of local blood pressure and distributes to the flow according to metabolic need. Therefore, the vascular endothelium is a dynamic endocrine organ. It modulates vasomotor function and affects homeostasis by releasing various substances, such as endothelin-1 (ET-1) and nitric oxide (NO) in response to physiologic stimuli (Egashira, 2002).

The endothelium behaves as a receptor effector organ that responds to different chemical or physical stimuli by releasing a variety of both agonistic and antagonistic substances, which may maintain the vascular homeostasis and its function (Esper et al., 2006). The endothelium plays an essential role in the proper function and formation of new blood vessels. It is important to emphasize that endothelial cells are crucial in determining the health of blood vessels, as well as in the maintenance and development of new blood vessels through the process of angiogenesis, which is required for tissue growth.

1.8.2.1 The Role of Endothelial Cells in Vasoregulation

As mentioned, the endothelium plays a critical function in regulating vascular flow and vascular tone (hence, blood pressure) by producing a large number of biologically active substances. These are vasodilators and vasoconstrictors, and both participate in the regulation of blood pressure.

Endothelium derived Vasodilatory Factors

Nitric oxide (NO)

Nitric oxide is a highly reactive soluble gas that is continuously generated through the oxidation of the amino acid L-arginine, NADPH and molecular oxygen by a family of three enzymes known as nitric oxide synthases (NOS). These isoforms are referred to as endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) (Förstermann and Sessa, 2012). Endothelial nitric oxide synthase (eNOS) is constitutively expressed in endothelial cells (Figure 1-4) and responsible for the production of NO from L-arginine (Behrendt and Ganz, 2002, Cannon, 1998).

Nitric oxide has a wide range of biological function that maintain vascular homeostasis (Cannon, 1998). It is a critical factor in maintaining vascular homeostasis by controlling blood pressure, regulating local cell growth and inhibiting platelet adhesion. In addition, it is an extremely important signal for protection of the vessels from injuries as a consequence of platelets and cells circulating in the blood, which could lead to heart attacks and strokes. Indeed, it is an essential biological regulator and a fundamental substance in the field of vascular homeostasis. Nitric oxide is a potent vasodilator that has a biological half-life of ~30seconds in plasma (Palmer et al., 1988). The principal physiologic stimulus for nitric oxide production and release from endothelium is likely to be either the shear stress (Britten et al., 1999) or the variety of agonist like as Acetylcholine (ACh) or activated by receptor-dependent endothelial nitric oxide synthase (eNOS) (Figure 1-4) (Cannon, 1998). Therefore, ACh has been

used as a practical stimulus for assessment of the endothelial dysfunction in human (Britten et al., 1999). The Activation of G protein receptors in endothelium by ACh increases the intracellular calcium $[Ca^{2+}]_i$, which, in turn induces eNOS activation and NO production (Figure 1-4). Then, NO diffuse from the endothelium to smooth muscle cells. In smooth muscle cells, the vasodilation effect of NO is mediated by stimulating the activation of guanylate cyclase and increased production of cyclic guanosine 3',5' monophosphate (cGMP) (Figure1-4) (Förstermann and Sessa, 2012), leading to decreased $[Ca^{2+}]_i$, which in turn allows vasodilation and increased blood flow (Förstermann and Sessa, 2012).

In addition to its potent vasodilating effect, NO also acts against leukocyte adhesion to the endothelium (Egashira, 2002, Cannon, 1998). For instance, the release of NO into the lumen of blood vessels increases cyclic cGMP in platelets and inhibits platelet adhesion to the surface of the endothelium (Egashira, 2002).

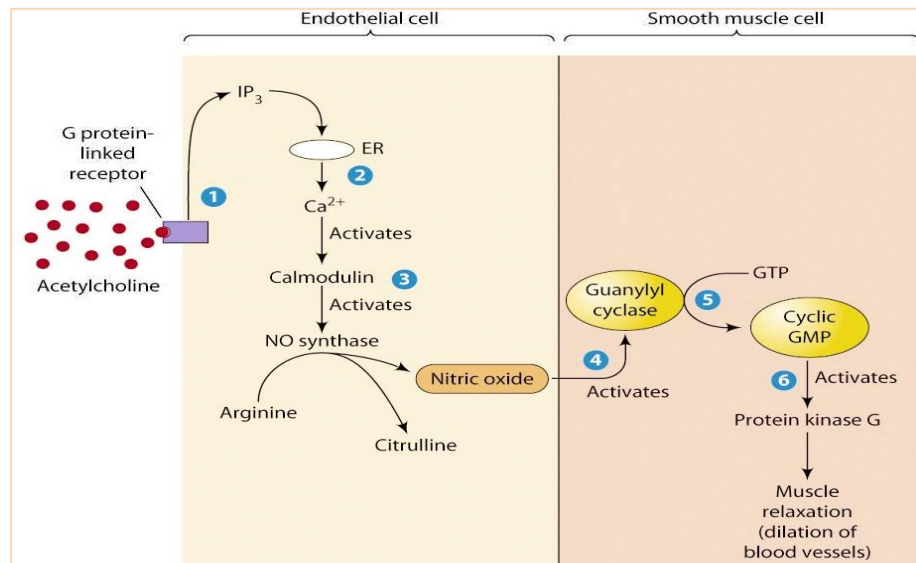


Figure 1-4 The NO signalling pathway. The activity of eNOS is determined by the intracellular calcium $[Ca^{2+}]_i$. Binding of acetylcholine (ACh) to G protein receptors causes production of inositol 1,4,5-trisphosphate (IP₃). IP₃ release calcium from endoplasmic reticulum. $[Ca^{2+}]_i$ and calmodulin form complex activates NO synthase which in turn , produce NO. NO diffuses from endothelial cell into smooth muscle cells. In smooth muscle cell, NO stimulate the activation of dissoluble guanylate cyclase, triggering an increased production of cyclic GMP, which leads to decreased intracellular calcium by activates protein kinase G which phosphorylates several muscle proteins, enabling vasodilation and increased blood flow (Hardin et al., 2012).

1.8.2.2 Endothelium-derived Contractile Factors

Endothelin-1

The endothelin family consists of three isoforms, termed endothelin-1, endothelin-2 and endothelin-3 (ET-1, ET-2, and ET-3). They have been described as peptides that are produced by the vascular endothelium (Haug et al., 2001, Hickey et al., 1985, Schiffrin, 2005, Dhaun et al., 2008). They regulate a variety of cells (Schiffrin, 2001) through two types of receptors: endothelin receptor type A (ET_A) and endothelin receptor type B (ET_B). These isoforms are vasoconstrictor peptides that are produced from a 212-amino acid prepro-endothelin peptide. This peptide is cleaved into ET-1 through the action of endothelium converting enzymes (ECEs) to produce the biologically active form, ET-1 and a C- terminal fragment (Schiffrin, 2005, Dhaun et al., 2008). The main enzyme responsible for the genesis of ET-1 is ECE (D'Orleans-Juste et al., 2003).

Endothelin-1 is a powerful vasoconstrictor peptide in the vascular system that has pressor properties, regulates blood flow and is produced by vascular endothelial cells of blood vessels. It acts as an autocrine or paracrine factor by binding to specific G-protein-coupled receptors, ET_A and ET_B. Both ET receptors are located on the vascular smooth muscle (Dhaun et al., 2008, Haug et al., 2001). Under the influence of numerous signals of cardiovascular stress, such as thrombin, cytokines and angiotensin II (Ang II), ET-1 induces vasoconstriction. In addition, ET_B is also present on endothelial cells and induce relaxation by mediating release relaxing factor such as nitric oxide (Mazzuca and Khalil, 2012). Moreover, ET_B mediates clearance of endothelin-1 from the circulation (Kedzierski and Yanagisawa, 2001).

The intracellular action of ET-1 includes activation of phospholipase C (PLC), which leads to an increase in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG); IP₃ causes the release

of calcium by the sarcoplasmic reticulum (SR), as well as increased smooth muscle contraction and vasoconstriction (Mazzuca and Khalil, 2012).

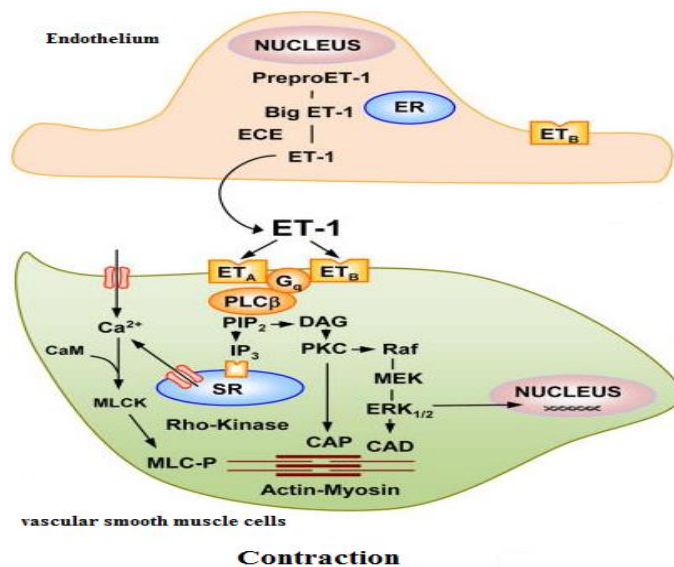


Figure 1-5 Endothelin receptor type B (ET_B) mediated signalling pathways in the vascular system. In the endothelium, a large precursor prepro-endothelins peptide is cleaved through the action of ECEs to produce the biologically active form ET-1. Thereafter, ET-1 engages in paracrine actions on smooth muscle cells by binding to ET_{RB} and activating PLC. This leads to an increase in the hydrolysis of PIP₂ into IP₃ and DAG. In turn, IP₃ causes the release of calcium by the sarcoplasmic reticulum (SR). [Ca²⁺]_i binds to calmodulin (CaM) to form a complex (Ca²⁺/CaM), which in turn, activates the MLCK enzyme to phosphorylate myosin light chain (MLC), enabling the molecular interaction of myosin with actin. Following this, MLC phosphorylation induces cross-bridge formation between the myosin heads and the actin filaments, thus producing a contraction of the smooth muscle. In Meantime, DAG activates either PKC, which phosphorylates the actin-binding protein calponin (CAP) or initiates a protein kinase cascade involving Raf, MAPK kinase (MEK) and MAPK (ERK1/2), leading to phosphorylation of caldesmon (CAD) and thereby increases the myofilament force sensitivity to Ca²⁺ (Mazzuca and Khalil, 2012).

Angiotensin II

Angiotensin II (Ang II) is the principal effector molecule of the renin angiotensin system and is implicated in the aetiology and progression of hypertension (Wackenfors et al., 2004, Crowley et al., 2006). Angiotensin II is a potent vasoconstrictor and plays a significant role in vascular tone (Hilgers et al., 2001). It is released from the endothelium, as well as being present in blood circulation (Montón et al., 1997, Ide et al., 2008, Hilgers et al., 2001, Rajendran et al., 2013). There is considerable evidence indicating that endothelial and smooth muscle cells are both able to produce angiotensin peptides (Kifor and Dzau, 1987, Re et al., 1982, Hilgers et al., 2001). Several studies have reported that the generation of angiotensin I (Ang I) and Ang II occurs in the walls of blood vessels and in the heart (Admiraal et al., 1990, Danser et al., 1992, Dzau and Re, 1994, Campbell, 1985). In addition, the angiotensin converting enzyme (ACE) is localised on the surface of the endothelial cells and plays an obligatory role in the conversion of Ang I to Ang II (Gohlke et al., 1992, Igic and Behnia, 2003). Previous studies have reported that ACE is predominantly localized on endothelial cells, and that Ang II receptors are also localized on endothelial cells, indicating that endothelial cells are the principal sites of the Ang II production (Hayashi et al., 2000). However, not much is known about Ang II signalling in endothelial cells (ECs).

The biological actions of Ang II in increasing blood pressure are mediated, in both paracrine and autocrine fashions, by a plasma member receptor, namely angiotensin receptor (AT1), which is predominantly, expressed by vascular smooth muscle cells (Shanmugam and Sandberg, 1996, Higuchi et al., 2007, Allen et al., 2000). The AT1 is localized in the outermost layers of the vascular smooth muscle cells (Shanmugam and Sandberg, 1996, Allen et al., 2000). The intracellular action of Ang II in vascular smooth muscle cells is mediated by the AT1 receptor, which interacts with multiple heterotrimeric G-proteins, including Gq/11. This activates an IP3-dependent

mechanism, leading to an increase $[Ca^{2+}]_i$ and ultimately causing contraction (Griendling et al., 1997, Suzuki et al., 2005, Touyz and Berry, 2002).

1.8.3 Vascular Smooth Muscle

Vascular smooth muscle cells (VSMCs) are the main cellular components of the vascular wall (Metz et al., 2012, Lacolley et al., 2012), contributing to the regulation of blood pressure and blood flow (Rzucidlo et al., 2007). Hence, The main functions of vascular smooth muscle cells are contraction and relaxation (Yoshida and Owens, 2005, Goulopoulou and Webb, 2014).

The balance between the cellular signalling pathways that mediate the generation and release of contractions is controlled by the tone of VSMCs, which in turn regulate blood pressure and flow (Woodrum and Brophy, 2001). Regardless of the stimulus, the process of smooth muscle cells' contraction is mediated through the cross-bridge cycling between actin and myosin filaments, which is initiated and regulated by $[Ca^{2+}]$ in a number of ways (Webb, 2003, Hilgers and Webb, 2005).

1.8.3.1 Contraction

Contraction of vascular smooth muscle cells is principally regulated by receptor or mechanical (i.e., stretch) activation of actin and myosin, the contractile proteins (Webb, 2003, Somlyo and Somlyo, 1993, Wynne et al., 2009). The contractile activity is then determined by the phosphorylation state of myosin light chains (MLC) (Webb, 2003). These are regulated by the activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (Kamm and Stull, 1985, Marston, 1983, Sobieszek, 1985, Trybus and Lowey, 1985, Wynne et al., 2009). The cell-surface receptors are activated by circulating vasoconstriction peptides, such as Ang II and ET-1; this results in the activation of various types of guanosine 5'-triphosphate-binding proteins (G proteins) coupled with various ion channels and enzymes, thus regulating their activity (see Figure 1-6).

In smooth muscle cells, specific agonist stimuli such as Ang II, ET-1 or norepinephrine/noradrenaline bind to receptors, coupled with heterotrimeric G proteins, which in turn, stimulate PLC activation (Higuchi et al., 2007, Ohtsu et al., 2006, Touyz and Berry, 2002, Alexander et al., 1985). PLC activation hydrolyses phosphatidylinositol 4,5-bisphosphate into two potent second messengers, IP₃ and diacylglycerol (DAG) (Berridge, 1987, Touyz and Berry, 2002, Ushio-Fukai et al., 1998, Nishizuka, 1992, Horowitz et al., 1996). Next, IP₃ regulates the release of Ca²⁺ from intracellular stores in the sarcoplasmic reticulum into the cytosol, which is mediated by the ryanodine receptor (RyR) (Rogers and Lokuta, 1994, Alexander et al., 1985, Touyz and Schiffrin, 1997, Kanashiro and Khalil, 1998, Horowitz et al., 1996, Somlyo and Somlyo). Meanwhile, DAG, together with Ca²⁺, activates PKC that phosphorylates specific target proteins to regulate cross-bridge cycling and L-type Ca²⁺ channels (Rasmussen et al., 1987, Alexander et al., 1985, Ushio-Fukai et al., 1998, Salamanca and Khalil, 2005, Horowitz et al., 1996, Webb, 2003, Jin and Burnett, 2006). Additionally, cytosolic Ca²⁺ is increased through Ca²⁺ release from the extracellular space through different Ca²⁺ entry channels, such as voltage-operated (VOC), receptor-operated (ROC) and store-operated (SOC) Ca²⁺ channels, as well as Ca²⁺-permeable nonselective cation channels (NSCC), leading to the elevation of [Ca²⁺]_i (Wynne et al., 2009).

Following the elevation of [Ca²⁺]_i, Ca²⁺ adheres to a special calcium-binding protein called calmodulin (CaM) (Salamanca and Khalil, 2005, Kanashiro and Khalil, 1998, Horowitz et al., 1996). This complex (Ca²⁺/CaM) activates the MLCK enzyme to phosphorylate the 20-kDa light chain of myosin, enabling the molecular interaction of myosin with actin (Kanashiro and Khalil, 1998, Horowitz et al., 1996). Following this, MLC phosphorylation induces cross-bridge formation between the myosin heads and the actin filaments, thus producing a contraction or the shortening of the smooth muscle (Horowitz et al., 1996, Salamanca and Khalil, 2005).

Conversely, relaxation is usually initiated when the Ca^{2+} concentration begins to decline. Intracellular free Ca^{2+} can be taken up by the sarcoplasmic reticulum and Ca^{2+} extrusion by the Ca^{2+} -ATPase pump on the SR membrane, and also by $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Salamanca and Khalil, 2005, Kanashiro and Khalil, 1998, Horowitz et al., 1996). Despite the view that the activation of smooth muscle cell contraction proceeds predominantly in a Ca^{2+} -dependent manner, some evidence suggests that additional Ca^{2+} -sensitization mechanisms play a substantial role in the contraction of smooth muscle cells (Webb, 2003, Karaki et al., 1997). In fact, Ca^{2+} -sensitization mechanisms involve the inhibition of MLCP (MLC phosphatase) by the RhoA/Rho-kinase pathway, thereby maintaining force generation (Hirata et al., 1992).

Hence, any alterations in these cellular signalling components might lead to pathological conditions such as hypertension by stimulating a state that causes maintained vasoconstriction and blood pressure elevation, as discussed in the section below.

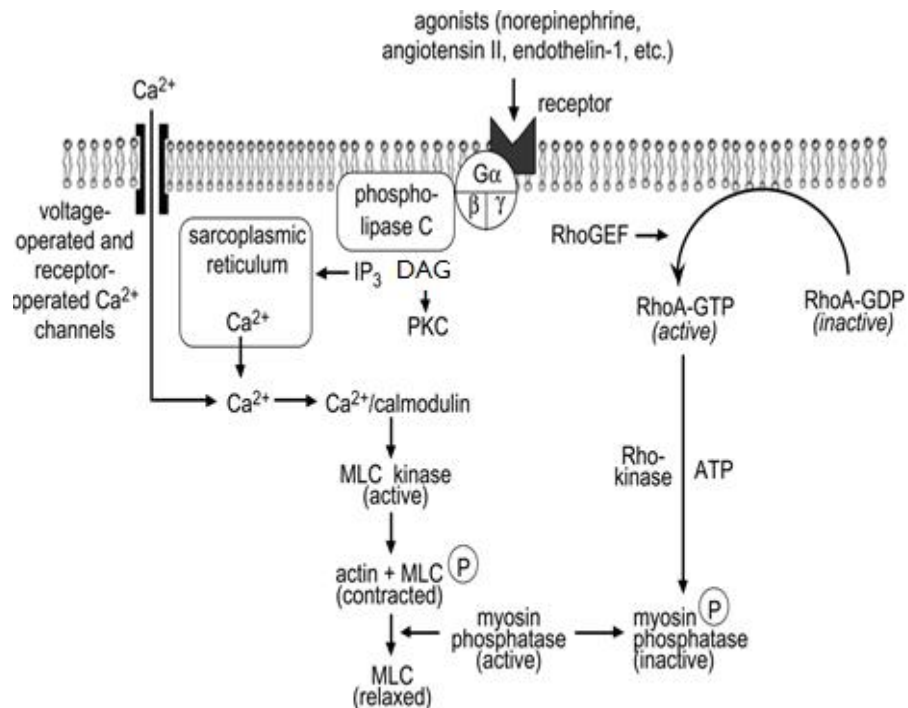


Figure 1-6 Ca²⁺-dependent and Ca²⁺-sensitive regulation of smooth muscle contraction. In smooth muscle contraction, agonists such as Ang II and ET-1 bind to specific receptors leading to the activation of phospholipase C (PLC), which, in the classic Ca²⁺-dependent pathway, hydrolyzes phosphatidylinositol 4,5-bisphosphate into two potent second messengers: inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ stimulates the release of Ca²⁺ from the sarcoplasmic reticulum, and extracellular calcium enters through Ca²⁺ channels. DAG then activates PKC, which phosphorylates specific target proteins. [Ca²⁺]_i binds to calmodulin, resulting in the activation of MLCK and initiating contraction of the MLC by phosphorylates. During the interaction with actin, cross-bridge cycling occurs, initiating the contraction of the smooth muscle cell. Relaxation occurs when MLCP dephosphorylates the MLC. It is possible in some circumstances for the generation of force to be mediated via a Ca²⁺-independent mechanism. The activation of G protein-coupled receptors stimulates guanine nucleotide exchange factor Rho activity, which facilitates the exchange of GTP for GDP on RhoA and the dissociation of RhoA-GTP from RhoA-GDP. The active RhoA-GTP then migrates to the plasma membrane and activates Rho-kinase, inhibiting myosin phosphatase (Webb, 2003).

1.9 Hypertension

1.9.1 Overview

Hypertension is a major risk factor for cardiovascular disease (CVD) (Rapsomaniki et al., 2014). It is a chronic condition that indicates an elevated blood pressure in the arteries (Reule and Drawz, 2012). It has been estimated that over one billion people suffer from hypertension worldwide (Pavlović and Nikola, 2011). Excessive blood pressure in the arteries can lead to damaged organs as well as it might be leading to increase the risk of variety of cardiovascular diseases such as stroke, coronary artery disease, heart failure (Rapsomaniki et al., 2014, Angeli et al., 2014). Normal blood pressure is within the range of 120 mmHg (systolic) and less than 80 mmHg (diastolic), and high blood pressure as clinically defined is above 140/90 (systolic/ diastolic) mmHg (Mary 2009).

Hypertension results from a multifactorial and complex interaction of genetics and lifestyle (e.g. diet and physical activity) (Oparil et al., 2003). The genesis of hypertension implicates multiple pathophysiologic factors including overproduction of vasoconstrictors; long-term high sodium intake; inadequate dietary intake of potassium and calcium; increased or inappropriate renin secretion with resultant increased production of angiotensin II and aldosterone; deficiencies of vasodilators (e.g. nitric oxide [NO]) (Oparil et al., 2003, Beevers et al., 2001).

Hypertension has also been linked with vitamin D deficiency with studies reporting an increased incidence of hypertension associated with vitamin D status and supplementation, and one study concluding that most patients with high blood pressure have insufficient levels of vitamin D (Vacek et al., 2012).

1.9.2 Pathogenic Hypothesis Related to Hypertension

1.9.2.1 Dysfunction Endothelium

Once considered to be a selectively permeable barrier between circulating blood and the tissues, the endothelium is now regarded as a dynamic organ in the regulation of vascular tone and the maintenance of vascular homeostasis (Rubanyi, 1993, Sandoo et al., 2010, Galley and Webster, 2004). The endothelium is able to produce a large number of biologically active substances. These are vasodilators and vasoconstrictors, and they participate in the regulation of blood pressure (described in detail above).

Studies have shown hypertension to be associated with changes in endothelial function that contribute to increased arterial resistance, which is characteristic of hypertensive states (Watts et al., 1996, Zeiher et al., 1993). Other studies have reported in detail on the role of the endothelium in mediating arterial relaxation, which is impaired in patients with essential hypertension (Egashira, 2002, Panza et al., 1995). However, not all studies have supported this view (Cockcroft et al., 1994, Hutri-Kähönen et al., 1999).

Hypertension causes oxidative stress, which leads to altered endothelial cell capacity and in turn to endothelial dysfunction (Buntgen et al., 2006). A growing body of evidence suggests that activation and dysfunction of endothelial cells comprise one of the earlier markers in patients with hypertension (Egashira et al., 1995). One study (Panza et al., 1990) described endothelial dysfunction in the forearm vasculature of humans with hypertension.

Injury or activation of the endothelium leads to an imbalance between relaxing and contracting factors (Singh et al., 2011). This may cause a failure in blood pressure control, angiogenesis and hemostasis. Much of the evidence to date indicates that the uncontrolled growth of endothelial cells is responsible for common pathological processes, including hypertension, atherosclerosis and heart failure (Cai and Harrison, 2000).

Endothelial dysfunction has been implicated in the various pathological processes of different forms of cardiovascular diseases (Egashira, 2002). It is characterized by the enhanced expression of adhesion molecules as a result of increased leukocyte-endothelial adhesions, increased production and activity of the potent vasoconstrictor ET-1 and decreased activity or bioavailability of NO in the vessel wall (Böhm and Pernow, 2007). Previous studies have shown that a deficiency of NO might trigger vasoconstriction, which can cause decreased elasticity among blood vessels via platelet aggregation and thrombus formation, increased proliferation of smooth muscle and enhanced expression of leukocyte adhesion to the endothelium (Cooke and Dzau, 1997).

The mechanisms behind the progression of impaired endothelial function are of great importance. One of the possible mechanisms is decreased bioavailability of NO. The effect of declining NO levels in the vessel wall might be due to the down-regulation of endothelial NO synthase expression (Wilcox et al., 1997), lack of cofactors (Pou et al., 1992) or degradation of NO via reactive oxygen species (ROS) production (Harrison, 1997).

Reactive oxygen species are highly sensitive and are produced as a consequence of normal aerobic metabolism. Under physiological conditions, endogenous antioxidant defenses minimize the interaction between O_2 and $NO^\cdot$. The excess of ROS, especially superoxide anion, may oxidize NO (Esper et al., 2006). This condition has commonly been referred to as oxidant stress (Cai and Harrison, 2000).

Another change in endothelial dysfunction of similar importance is the increased bioavailability and expression of ET-1 in a wide range of cell types, including vascular smooth muscle and endothelial cells (Ito et al., 1993). This may lead to a decreased expression of eNOS, thus reducing production of NO. Furthermore, in endothelial dysfunction, the ET_{RA} and ET_{RB} in smooth muscle work to produce superoxide $O_2^{\cdot-}$,

which contributes to an increased formation of peroxynitrate (ONOO^-), followed by more oxidation (Böhm and Pernow, 2007). Hence, ONOO^- decreases the biological activity of NO (Böhm and Pernow, 2007).

Endothelin-1 has been implicated as a main factor in the development and progression of endothelial cell dysfunction, which causes vascular diseases. Furthermore, it may be involved in the pathophysiology of hypertension and cardiovascular diseases, due to its contribution to the remodelling of large and small arteries (Schiffrin, 2005). Current studies in models of experimental hypertension, such as deoxy-corticosterone acetate (DOCA) - salt hypertensive rats, have emphasized the important role of ET-1 in vascular disease (Amiri et al., 2004), specifically in elevating blood pressure. In addition, numerous studies involving moderate to severe cases of hypertension have proved that there is an overexpression of preproET-1 mRNA in the endothelium of small arteries that are promoted to produce ET-1, which plays a role in the hypertrophic remodelling of small arteries, thus, causing high blood pressure.

1.9.2.2 Vitamin D Deficiency

In recent years, vitamin D deficiency has been thought to be the result of vascular diseases, and it has been used as a marker to detect the function of vascular disease before its clinical manifestation (Maggio et al., 2014). This hypothesis has been investigated in a systematic review, documenting the discrepancy between the existing observational and interventional studies on the role of calcidiol concentrations in a number of non-skeletal disorders, including hypertension (Autier et al., 2014).

Numerous studies have reported that vitamin D deficiency plays a significant role in the regulation of blood pressure and cardiac health (Judd and Tangpricha, 2009, Ullah et al., 2009). There has been an abundance of evidence to support that calcitriol has an essential function in regulating tissues, including vascular smooth muscle, myocardium, endothelium and skeletal muscle (Zittermann and Koerfer, 2008). With regard to

endothelial effects, (Wong et al., 2008) have demonstrated that calcitriol regulates vascular tone via reducing calcium influx into endothelial cells, which leads to a decrease in the production of contracting factors. Furthermore, accumulating evidence has shown that there is a strong link between vitamin D supplementation and decreased blood pressure (Pilz et al., 2009, Beveridge et al., 2015, Mandarino et al., 2015). The majority of these studies indicate that vitamin D supplements have an important role to play in the control of blood pressure. Moreover, observational data support the idea that low levels of calcidiol in serum are implicated in the pathogenesis of arterial hypertension (Pilz et al., 2009). Essentially, a wealth of observational data has reported that there is a strong link between levels of calcidiol in serum and blood pressure (Beveridge et al., 2015, Mandarino et al., 2015). Beyond all of this evidence, the final explanation of vitamin D as a therapeutic option in the improvement of vascular function (Liu et al., 2013) and its effect on vascular system is yet not fully understood.

1.9.2.3 Effects of Vitamin D on Endothelial Cells

A large body of evidence suggests that vitamin D has direct effects on endothelial cells, due both to the expression of VDR and the presence the enzyme 1-alpha-hydroxylase, which is required for the conversion of calcidiol to calcitriol in the endothelium (Merke et al., 1989, Zehnder et al., 2002). This conversion has a paracrine or intracrine effect that results in the protection of vascular walls (Min, 2013, Zehnder et al., 2002). There is now evidence that has demonstrated the beneficial effect of vitamin D on improvement of vascular endothelial function in some populations (Chitalia et al., 2014, Harris et al., 2011, Tarcin et al., 2009, Sugden et al., 2008, Hashemi et al., 2015). Indeed, although the relationship between the vitamin D and cardiovascular disease is well established (Ke et al., 2015), little is known about the effect of vitamin D on endothelial function (Molinari et al., 2011).

Endothelial cell signalling has a strong effect on vascular stiffening; also, deficiency of vitamin D is associated with arterial stiffening (London et al., 2007). It is suspected that

stiffening of the arteries may be the main cause of numerous disease processes, including hypertension, atherosclerosis and others, as a result of a complex interaction between the cellular and structural mechanisms (Zieman et al., 2005).

Endothelial cells and VSMCs produce reactive oxygen species (ROS) (Sorescu et al., 2002, Channon and Guzik, 2002, Griendling et al., 2000, Suzuki and Ford, 1999) primarily from NADPH oxidase (Griendling and Ushio-Fukai, 1997, Griendling et al., 2000). These ROS, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), as well as reactive nitrogen species such as NO and ONOO $^-$, play an important role in vascular biology through their reduction-oxidation (redox) potential (Griendling et al., 2000). Vitamin D also has anti-oxidant potential to protect endothelial cells from oxidative stress (H_2O_2) by inhibiting superoxide anion generation, hence counteracting apoptosis (Uberti et al., 2013) through the activation of MAPKs signalling pathway cascades (Polidoro et al., 2013). A study by Tarcin et al. (2009) demonstrated that vitamin D replacement improved the function of endothelial cells by reducing lipid peroxidation and oxidative stress. In addition, a recent study (Hirata et al., 2013) showed that 22-oxacalcitriol (OCT), which is a vitamin D analog of calcitriol, improved endothelial dysfunction in rats with type 2 diabetes, in part via reduction in generation of ROS through suppression of p22phox, NADPH oxidase subunit, expression which might contribute to improving endothelial nitric oxide synthase (eNOS) uncoupling. Confirming the *in vitro* data, (Wong et al., 2010) demonstrated that treating spontaneously hypertensive rats (SHRs) for long periods of time with calcitriol led to a significant reduction in ROS concentrations in endothelial cells.

Besides vitamin D's antioxidant effect in protecting cells from oxidative stress (Danilenko et al., 2003, Goodwin et al., 2006), it is also suspected to play a significant role in the regulation of vascular tone. There is evidence to indicate that vitamin D participates in the regulation of the vascular tone via inhibiting the release of vasoconstrictor metabolites of arachidonic acid by reducing calcium influx into the ECs

(Wong et al., 2008). The vasodilatory effect of vitamin D on endothelial cells is mediated through both genomic and non-genomic mechanisms. Hence, low levels of vitamin D are viewed as a key biological predictor of reduced bioavailability of NO (Andrukhova et al., 2013). Furthermore, (Andrukhova et al., 2013) demonstrated that calcitriol acts as a direct transcriptional regulator of endothelial NO synthase in endothelial cells by a VDR-dependent mechanism. In addition to its genomic effects, (Molinari et al., 2011) reported that calcitriol stimulates the production of NO in HUVECs via the rapid non-genomic activation of eNOS through the phosphorylation of intracellular kinases such as p38, protein kinase B (AKT) and extracellular signal-regulated kinases (ERK), which results in eNOS activation. Furthermore, (Ben-Shoshan et al., 2007) showed that under hypoxic conditions, calcitriol inhibited the expression of ET-1.

Apart from its vasodilatory effect, there is considerable data to suggest that the physiological effect that vitamin D exerts by its binding with VDR in turn regulates the expression of the genes responsible for cellular proliferation (Thompson et al., 2012, Haussler et al., 1998). (Zehnder et al., 2002) reported that treatment with calcitriol [25(OH) D₃] for 72 hours inhibited HUVECs' proliferation. Therefore, this anti-proliferative effect might be mediated through interference with the cell cycle. Presumably this is done by inhibiting the cell's mitotic cycle in the G0/G1 phase through the 1,25(OH)₂D₃ receptor VDR complex as shown in tumour cells (Muindi et al., 2002).

1.9.3 Effects of Vitamin D on VSMCs

In addition to the effect of active vitamin D on endothelial cells, it exerts multiple biological functions in vascular smooth muscle cells (VSMCs) involving regulation of vascular tone, growth and migration, as well as the evaluation of vascular calcification (Kawashima, 1988, Inoue and Kawashima, 1988, Wakasugi et al., 1991, Somjen et al., 2005, Aoshima et al., 2012). Similar to those performed on endothelial cells, several

studies have reported that vascular smooth muscle cells express both vitamin D receptors (Wu-Wong et al., 2007, Rajasree et al., 2002) and 1-alpha-hydroxylase (Somjen et al., 2005). As such, vitamin D exhibits both systemic and local effects on VSMCs, which are complex and also regulated by other hormones (Somjen et al., 2005).

The anti-proliferative effects of calcidiol and calcitriol on VSMCs have been shown in several studies; however, the mechanism by which these operate is still unclear (Carthy et al., 1989a, Wu-Wong et al., 2006). In cultured neonatal rat aortic VSMCs (Chen et al., 2010), it has been demonstrated that calcitriol inhibits the proliferation of VSMCs via a reduction in the activity of cyclin-dependent kinase 2 (Cdk2), which is actually a key cell cycle kinase that regulates the cell cycle machinery. In addition, (Carthy et al., 1989b) revealed that calcitriol, the active metabolite of vitamin D, inhibits proliferation of VSMCs and endothelial cells. Additionally, (Wu-Wong et al., 2006) have shown that calcitriol regulates the expression of genes that modulate cell proliferation, including IGF 1, Wilms tumors 1 and TGF β in human coronary artery smooth muscle cells. Moreover, calcitriol is crucial to the inhibition of antigen-induced, cytokine-mediated endothelial activation and TNF- α adhesion molecule. Lastly, (Zittermann and Koerfer, 2008), have indicated that the active metabolite of vitamin D, calcitriol down-regulates the proliferation of vascular smooth muscle cells and their migration into the intima.

Other aspects besides the vasodilatory effect of vitamin D on VSMC biology have been studied; for instance, vitamin D plays a role in the regulation and expression of anti-fibrotic factors. Bone morphogenic proteins (BMPs) in particular BMP2, which is produced from endothelial cells have been implicated in the dysfunction of the endothelium via promoting the alteration of VSMS to an osteoblast-like phenotype, which in turn induces vascular calcification (Boström et al., 1993, Demer, 1995). In this process, BMP2 produced in endothelium and exposed to high blood pressure, which

contributes to osteogenic differentiation of VSMCs in the vessel wall (Johnson et al., 2006).

In another area, (Absi and Ward, 2013) studied the effects of chronic calcitriol treatment on intracellular signalling pathways in human coronary artery smooth muscle cells (HCASMCs) and they found that HCASMCs treated with 100nM of calcitriol for 24 hours significantly enhanced endothelin responsiveness, and that the effect was mediated through endothelin receptor type A (ET_{RA}).

1.10 Vitamin D and Hypertension

Low serum levels of calcidiol have been shown to play a central role in regulating blood pressure (Li, 2003). There is now strong evidence supporting an inverse relationship between serum calcidiol concentrations and high blood pressure, which has been interpreted as indicating that vitamin D is regulating the expression of the renin gene. Randomized controlled trials have confirmed an inverse association between hypertension and plasma calcidiol (Feneis and Arora, 2010). Other studies have suggested that vitamin D may have a direct impact on cardiovascular functions (Li, 2003).

Animal and human studies (Weng et al., 2013, Kota et al., 2011) have suggested that the link between vitamin D and hypertension may be through vitamin D's involvement in increased activation of the renin-angiotensin-aldosterone system, which is the main regulator of blood pressure and electrolyte and volume homeostasis, thus contributing to the development of arterial hypertension (Atlas, 2007).

The relationship between plasma calcidiol concentrations and the risk of all-cause death has been evaluated in cross-sectional studies. For example, out of 13,331 adults in the US from the National Health and Nutrition Examination Survey (NHANES III), those whose serum vitamin D level was in the lowest quartile had a 26% higher risk of all-cause death over eight years than those whose level was in the highest quartile

(Melamed et al., 2008). Intervention studies and meta-analyses have shown that vitamin D supplementation is associated with an improvement in survival, with a large study indicating an inverse association between low serum levels of calcidiol and survival (Stechschulte et al., 2009, Vanga et al., 2010). It has also been suggested that vitamin D supplementation may reduce overall mortality (Stechschulte et al., 2009, Vanga et al., 2010). A cross-sectional study investigated the relationship between vitamin D and endothelial function in older people, assessing 852 subjects aged 70 years who were taking part in the prospective study of vasculature in Uppsala seniors (PIVUS); this revealed a positive association between serum levels of calcidiol in women and endothelium-independent vasodilation measured by an invasive forearm technique (Maggio et al., 2014). The strong association between vitamin D and regulation of blood pressure has been recognized for decades (Resnick, 1994).

There are currently several intervention studies investigating the role of vitamin D supplementation in the regulation of blood pressure (Pfeifer et al., 2001, Margolis et al., 2008, Vaidya et al., 2011). Epidemiological and clinical studies have suggested that the risk of hypertension can be reduced considerably by vitamin D₃ supplementation. A few studies have reported the effect of vitamin D₃ supplementation on reducing blood pressure (Pfeifer et al., 2001). One such study compared the reduction in blood pressure in hypertensive patients between 33,000 IU of daily vitamin D₃ supplementation and antihypertensive drugs; both groups showed a significant decrease in blood pressure after three months, leading the authors to suggest that antihypertensive medication could be supplemented with vitamin D₃ (Goel and Lal, 2011).

As mentioned previously, vitamin D deficiency is caused primarily by a lack of exposure to UV-B, which is dependent on many factors that may consequently limit vitamin D₃ production in the skin. Cross-sectional studies have reported that decreased calcidiol levels are associated with higher blood pressure (BP) levels (Scragg et al., 2007, Fraser et al., 2010), and with an increased incidence of hypertension (Kunutsor

et al., 2013). Such observations are based on a number of biologically logical mechanisms, along with the fact that 1-alpha-hydroxylase enzyme (CYP27b1) and vitamin D receptors (VDR) are expressed on endothelial cells and smooth muscle cells, which have special relevance in the genesis of hypertension (Norman, 2008).

Related to this, epidemiological studies have shown that low UV-B radiation during winter can result in a reduced capacity for vitamin D synthesis (Rostand, 1997). One study concluded that reduced serum calcidiol levels due to diet and decreased exposure to sunlight were associated with an increased incidence of hypertension (Feneis and Arora, 2010). According to epidemiological observational studies, people who live at higher latitudes have an increased risk of hypertension compared with those who live at lower latitudes (Nadir et al., 2010, Woodhouse et al., 1993). Conversely, several studies of children and young adults have shown that the incidence of hypertension is lower in those with high concentrations of serum calcidiol resulting from greater exposure to sunlight (Chang et al., 2005).

However, there have been mixed results from clinical studies that controlled UV-B exposure. One of these reported that, compared with untreated hypertension, there was a slight reduction in blood pressure with increased UV-B exposure (Krause et al., 1998), whereas another study in vitamin D deficient population failed to find any such change in blood pressure with increased exposure to UV-B (Scragg et al., 2011). Both of these studies were based on increasing the blood concentration of calcidiol through UV-B-induction therapy; the different results may be due to the short duration of the follow-up period (6 and 12 weeks, respectively) for detecting changes in arterial functions.

Conversely, several studies have failed to find a relationship between vitamin D supplementation and the risk of hypertension. One aim of the Women's Health Initiative was to investigate in postmenopausal women whether 400 IU of vitamin D₃ plus 1000 mg calcium daily reduced blood pressure or the risk of developing hypertension

compared with placebo; no change in systolic or diastolic blood pressure was found during a mean follow-up period of seven years (Margolis et al., 2008). A further study (Forman et al., 2005) also failed to find an association between vitamin D supplementation and the regulation of blood pressure. Together, the results of these studies show a mixed picture. On the one hand, one of these studies (Goel and Lal, 2011) showed a correlation between increasing vitamin D₃ supplementation and decreasing high blood pressure over a short follow-up period. In contrast, the Women's Health Initiative study found no decrease in blood pressure in postmenopausal women who received vitamin D₃ supplementation over seven years. It may be that increasing the dose of vitamin D₃ supplementation over a short follow-up period could play an important role in reducing high blood pressure.

Despite the varied results reported above, it is clear that a low concentration of 25(OH) D₃ in serum may lead to adverse effects on cardiovascular systems, an observation which may have significant therapeutic implication for the immediate treatment of hypertension.

1.11 Aims and Objectives

Calcitriol plays a pivotal role in a number of physiological and clinical processes (Pérez-López et al., 2011). In addition to its traditional role in calcium and bone metabolism, it has also been shown to regulate blood pressure and maintain cardiovascular health (Judd and Tangpricha, 2009). Many studies indicate that low serum concentrations of calcidiol may be related to pathogenesis and/or the progression of hypertension (Wang, 2009, Jafari and Paknahad, 2014). Hypertension is considered a key factor in the progression of cardiovascular diseases (WHO, 2015), with vitamin D now recognised as a significant factor affecting blood pressure (Kienreich et al., 2013). Over the last few years, the focus on vitamin D deficiency and health-related problems has increased. This increased attention has resulted from the identification of VDRs in vascular endothelial cells (ECs) and smooth muscle cells (VSMCs)

(O'CONNELL and Simpson, 1996, Sandgren et al., 1991, Norman, 2008, Simpson et al., 2007), with VDRs now thought to activate signal transduction pathways that generate rapid non-genomic responses, as well as the more recognised genomic responses (Haussler et al., 2011).

Hence, based on these findings, the main hypothesis of my PhD was both calcidiol and calcitriol regulate vascular tone by inducing vasodilation, and therefore reduces blood pressure. Therefore, the overall aim of this project was to study the direct effect of vitamin D on the proliferation and gene expression of endothelial cells and the subsequent proteins produced, secreted or regulated by endothelial cells, using HUVECs, as well as utilising thoracic/abdominal segments of aortas isolated from adult female Wistar rats to determine direct vasomodulatory effects using a Wire Myography System.

1.11.1 Chapter 3 Aims

An accumulation of evidence from experimental, clinical and epidemiological studies suggests that a deficiency of vitamin D is associated with the development and progression of cardiovascular diseases. Moreover, it has been recognized that vitamin D contributes to a wide range of fundamental biological functions including cell proliferation and the inhibition of cell growth. Thus, the aim of the studies presented in this chapter was to investigate whether vitamin D (either the inactive precursor form calcidiol or the active form calcitriol) had any effects on the proliferation of HUVECs.

1.11.2 Chapter 4 Aims

Experimental studies reported that vitamin D seems to affect the vascular wall and act locally in ECs and VSMCs through its binding with VDRs (Norman, 2008, Simpson et al., 2007). In these tissues, vitamin D influences a wide range of biological responses through the classic genomic effect (Norman et al., 2004). The main aim of the study presented in this chapter was to investigate the effect of both form of vitamin D,

calcidiol and calcitriol on the expression of two key genes involved in the endothelial regulation of blood pressure: endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS).

1.11.3 Chapter 5 Aims

Vitamin D has been shown to regulate vascular tone using a calcium-dependent process in which calcium influx into ECs is reduced, leading to a decrease in the production of contracting factors (Wong et al., 2010, Vanhoutte et al., 2005, Wong et al., 2008). In contrast, some studies suggest that the effects of vitamin D extend beyond the endothelium to VSMCs (Kawashima, 1987, Somjen et al., 2005). What is certain is that calcium signalling plays a significant role in cellular function (Firth et al., 2013, Van Hove et al., 2009). Therefore, intracellular calcium concentration ($[Ca^{2+}]_i$) is an important factor in the regulation of peripheral vascular resistance, as it modulates the ability for VSMCs to contract (Spence and Weaver, 2013). Recently, researchers have been investigating the rapid non-genomic effects of vitamin D, which are likely to be mediated by specific membrane receptors beyond the classical genomic effects that typically take place over a longer period of time (Falkenstein et al., 2000). Non-genomic actions, which occur within seconds or minutes, involve a variety of signal transduction activities, such as calcium (Ca^{2+}) influx and the release of Ca^{2+} from intracellular stores. These activities also include the modulation of adenylate cyclase (AC), phospholipase C (PLC), and protein kinase activities (e.g. MAP kinase) and changes in the phosphorylation status of cellular proteins and other traditional messengers (de Boland and Nemere, 1992, Falkenstein et al., 2000). Therefore, the aim of the studies presented in this chapter was to investigate the direct dose-dependent effects of calcitriol on contraction or relaxation of aortic rings. Thoracic/abdominal segments of aortas were isolated from adult female Wistar rats and their contractility was studied using a Wire Myography System, as first described by Mulvany and Halpern (Mulvany and Halpern, 1976).

2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Cell Culture

2.1.1 Overview

Human umbilical vein endothelial cells (HUVECs) are isolated from the umbilical cord of babies, and are the most common source of vascular endothelial cells. In the field of vascular biology, HUVECs have been used as a model system to examine the effects of different compounds, such as vitamin D, on their proliferation and the behaviour of the endothelium in response to the stretching of blood vessel walls, shear forces and the development of atherosclerotic plaques and angiogenesis.

2.1.2 Culturing HUVECs

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics through Cambrex Inc. Primary cultures of HUVECs were initially grown to 80-90% confluence in Endothelial basal media -2(EBM-2, Lonza) with 2% (v/v) fetal bovine serum (FBS), as shown in Table 2-1. Subsequently, HUVEC cultures were incubated at 37 °C (5% CO₂) for 48 hours by transferring 1 ml of cells into T75 cm² culture flasks containing 13 ml of EBM-2 medium with the same supplements mentioned in Table 2-1. Cells were grown to 80% confluence and used between passages 3 and 8.

Table 2-1 Endothelial basal media (EBM-2)

	EBM-2 growth media	Final concentration*
Endothelial Basal Media (EBM-2)	500ml	
Foetal bovine serum (FBS)	10ml	2%
Ascorbic acid	0.5ml	0.1%
GA 1000	0.5ml	0.1%
Heparin	0.5ml	0.1%
Hydrocortisone	0.2ml	0.04%
rhEGF	0.5ml	0.1%
rhFGF	0.5ml	0.1%
R3-IGF-I	0.2ml	0.04%
VEGF	0.5ml	0.1%

*Absolute concentration is unknown as ingredients supplied by manufacture

2.1.3 Passaging of Cells

Once HUVECs reached 80-90% confluence, the media was removed from the flask and the cells were washed gently with 10 ml PBS. Then the PBS was taken off and replaced with 2 ml of Trypsin-EDTA solution 0.25% (Invitrogen) to detach the cells from the surface of the flask. After 3-4 minutes of incubation, 8 ml of EMB-2 growth media was added and the contents were transferred to a universal tube (30 ml), which was centrifuged at 2500 rpm (Centrifuge-Eppendorf 5702 R) for 10 minutes (4°C). The supernatant was removed and cell pellet was re-suspended into 10 ml of EMB-2 media. Cells were then diluted with an appropriate volume of EBM-2 Endothelial basal media to provide 0.75×10^6 cells/ml.

2.1.4 Counting Cells

Once the cells had gone through passage, they were counted under a microscope using a haemocytometer. The following equation was used to determine the appropriate volume of cell suspension needed for seeding into culture plates:

Volume required (μl) = (cell number needed/total cell number) $\times$ Total volume of cells suspension (μl)

2.1.5 Freezing Cells

In order to be used in later experiments, HUVECs were frozen at a high concentration and at low passage numbers in liquid nitrogen. Briefly, once cells were detached from culture flasks and pelleted (see 2.1.3), cell pellets were re-suspended in freezing medium (foetal bovine serum [FBS] with 10% (v/v) Dimethyl sulfoxide [DMSO]; (Sigma Aldrich, UK)). Thereafter, 1 ml of cell suspension was dispensed into cryovials and slowly frozen (using a Mr. Frosty container) at -80°C overnight before being transferred to liquid nitrogen.

2.1.6 Preparation of Vitamin D

Stock (10^{-3} M) calcitriol and calcidiol (Sigma Aldrich, UK) were prepared in Dimethyl Sulfoxide (DMSO); (Sigma Aldrich, UK). The stock solution was used to prepare different dilutions of vitamin D in DMSO, ranging from 10^{-13} M – 10^{-7} M , to examine the effect of different doses of both active and inactive vitamin D on the proliferation of vascular endothelial cells and gene expression in the presence or absence of growth factors, compared with vehicle (0.01% DMSO) as a control.

2.1.7 Preparation of Treatment Media

Treatments were prepared in EBM-2 control media with 2% (v/v) FBS, supplemented with 0.1% (v/v) ascorbic acid, 0.1% (v/v) GA1000, 0.1% (v/v) heparin, and 0.04% (v/v) hydrocortisone. Following this, HUVECs were cultured in 96 well plates and were treated with different concentrations of vitamin D, as shown in Table 2-2.

Table 2-2 Treatments in EBM-2 control media (per well)

	EBM-2 Control media	Treatment
DMSO, 0.01%	999 μ l	1 μ l of DMSO
Dose of 10^{-7} M	999 μ l	1 μ l of 10^{-4} M
Dose of 10^{-9} M	999 μ l	1 μ l of 10^{-6} M
Dose of 10^{-11} M	999 μ l	1 μ l of 10^{-8} M
Dose of 10^{-13} M	999 μ l	1 μ l of 10^{-10} M

2.1.8 Determination of the Effects of Vitamin D on the Proliferation of HUVECs

To study the effect of vitamin D at different concentrations on HUVEC proliferation, DNA content assays were performed after 0 (T0), 8, 24 and 48 hours of incubation. The HUVECs were cultured in EBM-2 Endothelial basal media (2% FBS plus growth factors), following the method detailed in Section 2.1.2. They were then directly treated with different doses of calcidiol and/or calcitriol, ranging from 10^{-13} M – 10^{-7} M, and compared with DMSO (0.01%) as a control. The cells were maintained in two different culture media: a medium with growth factors and 2% FBS and a medium with 2% FBS but without growth factors, both of which were supplemented with different doses of calcidiol and/or calcitriol. The DNA content assay relates to the number of viable cells and depends upon the balance between cell proliferation and cell death (apoptosis). These DNA concentration assays were used to determine the effect of different doses of both calcidiol and calcitriol on the proliferation of HUVECs by measuring the DNA content. In order to do this, HUVECs were harvested and plated out onto 96 well plates in a volume of 200 μ l of EBM-2 media/well in wells A4-H10. Then, the plates were incubated at 37°C (5% CO₂) for 20 hours. At that point, the time zero plate was stopped and cells were washed with 100 μ l of PBS, and then 100 μ l of sterile water was added and incubated for one hour at 37°C, 5% CO₂. Next, the plate was frozen at -20°C for 30 minutes and thawed again, which helped to lyse the cells and release the DNA. This step was repeated for the other treatment plates after 8, 24, and 48 hours. As for the

other plates, the media was changed and treatment was added (as detailed in Table 2-2), and they were incubated at 37°C, 5% CO₂ for 8, 24 or 48 hours. The cells were thawed a final time and standard calf thymus DNA (40 µg/ml) was added to the wells A1-H3 on 96 well plates to produce a DNA standard curve. A volume of 30 µl of bis-benzimide dye solution (1 mg/ml stock) was added to 15 ml of 2x TNE buffer (see Table 2-3) and covered with aluminum foil to protect the dye from the light. Then, 100 µl of dye solution was added to each well, including the standard curve, on the time 0, 8, 24 and 48 hour plates. The plates were then scanned using a fluorescent plate reader (Cytofluor™ 2350, Millipore Corp) using 355-nm excitation and 465-nm emission wavelength, to produce a standard curve, which was used to calculate the DNA concentration of control and treated cells in wells A4- H10.

Table 2-3 10x TNE buffer (Tris; NACL; EDTA), pH 7.4

10 x TNE buffer	
Tris	100mM
NACL	1 M
EDTA	10mM

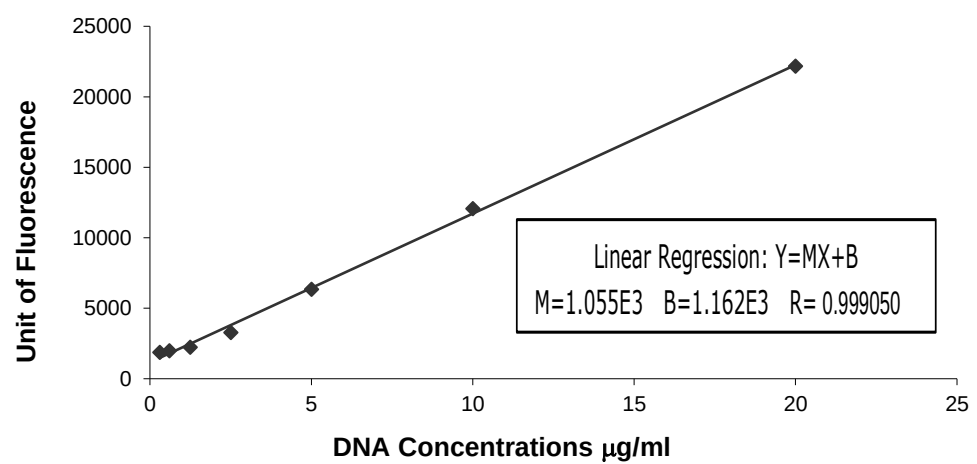


Figure 2-1 Standard curve for the DNA concentration determination assay.

2.2 Gene Expression Analysis

2.2.1 Overview

Total RNA was extracted from HUVECs, which was then reverse transcribed into cDNA. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify relative gene expression.

2.2.2 Effects of Vitamin D on Gene Expression as Measured by RT-PCR

To determine the effects of calcidiol and calcitriol on gene expression, HUVECs were harvested as described in Section 2.1.2 and incubated in 12 ml of EBM-2 growth media (see Table 2-1) containing growth factors in 75cm² culture flasks. Cells were then plated out onto 6 well plates and grown to 90% confluence. At this stage, the culture media was replaced with 2 ml of endothelial basal media (EBM-2) with growth factors and 2% FBS, supplemented with different doses of either calcidiol or calcitriol and incubated for 24 hours (37°C, 5% CO₂). The media was removed from the wells and 200 µl of RNase-free PBS was added to each well. A cell scraper was used to scrape the cells off the bottom of the wells and into the RNase-free PBS. Then, the 200 µl RNase-free PBS containing the cells was transferred into an autoclaved 1.5ml micro centrifuge tube and immediately put on ice. The samples were stored at -80°C until required for RNA extraction.

2.2.3 RNA Extraction of HUVECs

Total RNA was extracted from HUVECs and eluted in 50 µl of elution buffer using the High Pure RNA isolation kit, according to the manufacturer's instructions (High Pure; RAS, Mannheim, Germany) (Appendix a) . Cells had been frozen at -80°C in 200 µl RNase-free PBS, so samples were defrosted on ice and 400µl of the Lysis/-Binding Buffer was added to each sample immediately. Each sample was vortexed for 15 seconds to mix the samples, and then 600 µl was transferred to an upper reservoir of the high pure filter, which was inserted in one collection tube. Eppendorfs were spun for 15 seconds at 8000 xg. After centrifugation, the filter tube was removed from the

collection tube; the flow through liquid was discarded and again combined the filter tube and the used collection tube. Each sample was incubated at room temperature for 15 minutes at 15 to 25°C after adding 100 µl containing 10 µl DNase I with 90 µl DNase incubation buffer. Next, 500 µl of wash buffer II was added to the upper reservoir of the filter tube assembly and centrifuged for 15 seconds at 8000 xg. Moreover, in this step, flow through liquid was discarded and the filter tube was combined with the used collection tube. Next, 200 µl of Wash Buffer II was added to the upper reservoir of the filter tube and spun for 2 minutes at 13000 xg. This step was performed to remove any residual wash buffer. Following this, RNA present in the upper reservoir of the filter tube was transferred to sterile 1.5ml micro centrifuge tubes. To elute the RNA, a volume 50 µl of elution buffer was added and spun again for 1 minute at 8000 xg. Thereby, RNA was present in sterile 1.5 ml micro centrifuge tubes, then stored at -80°C

2.2.4 Determination of RNA Concentration and Integrity

The concentration and purity of extracted RNA in each sample was quantified using a Nanodrop spectrophotometer ND-1000 (The Thermo Scientific, Wilmington, USA) blanked with RNase-free water. A good quality of RNA has a ratio of absorbance readings at 260 nm and 280 nm between 1.8 and 2.0. All samples used had RNA integrity ratio between 1.8 and 2.0. The integrity and purity of the RNA (250ng/lane) were confirmed by denaturing agarose gel stained with ethidium bromide (EtBr). 1% agarose gel dissolved in 100 ml 1xTAE buffer and left it for 40 minutes. The gel was placed in an electrophoresis tank and run at 100V for 30 minutes. UV light was used to visualize the bands in the gel. The presence of two bands which are ribosomal RNA (18S and 28S) was required to ascertain RNA integrity (Figure 2-2). Sample concentrations were adjusted to 50 ng/µl with RNase-free water.

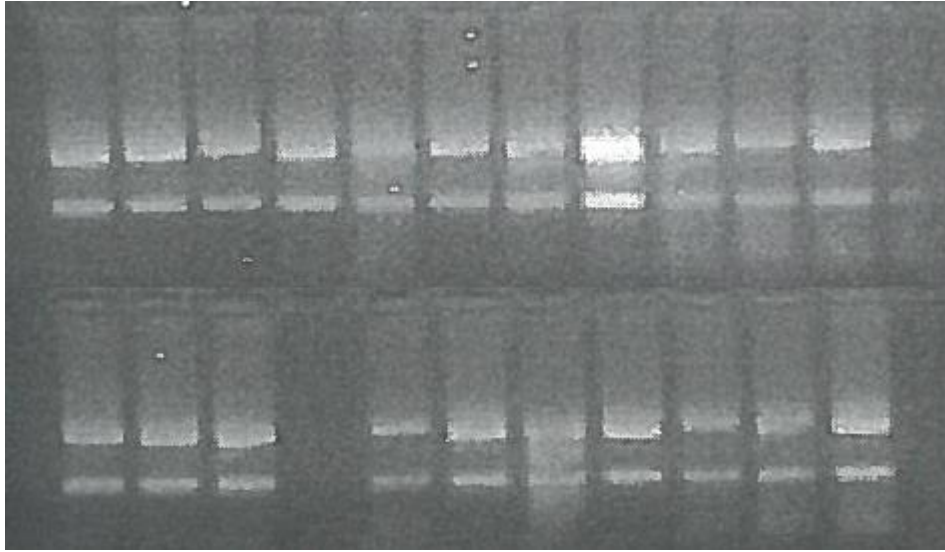


Figure 2-2 RNA analysis by agarose gel electrophoresis

2.2.5 Synthesis of cDNA by Reverse Transcriptase for RT-PCR

Total RNA (50 ng/μl) was defrosted on ice and used to synthesise cDNA using random hexamer primers to give a final volume of 100 μl for real-time polymerase chain reaction (RT-PCR). First-strand cDNA synthesis was carried out using the cDNA synthesis kit (Transcriptor First Strand cDNA Synthesis Kit, Roche, Burgess Hill, UK). Specifically, 10 μl of total RNA (50 ng/μl), 2 μl of hexamer primer (60 μM) and 1 μl of sigma water were incubated for 10 minutes at 65 °C in a thermal block cycler with a heated lid then immediately kept on ice. After adding 4 μl of 5x reaction buffer, 0.5 μl of Protector RNase inhibitor (40 U/ μl), 2 μl of Deoxynucleotide mix (10 mM), and 0.5 μl of Transcriptor reverse transcriptase (20 U/ μl), the reaction was incubated at 50 °C for 60 minutes then immediately put on ice. A volume of 80 μl of sigma water was added to the 20 μl of cDNA for a total of 100 μl, and the samples were stored at -20 °C until required for RT-PCR.

Table 2-4 cDNA synthesis by reverse transcriptase

Component	Template Primer Mix for one reaction	Final concentration
Total RNA	10 μl	
Random Hexamer Primer 600 pmol/ μl	2 μl	60 μM
Water	1 μl	
Transcriptor Reverse Transcriptase	4 μl	1x (8 mM
Reaction Buffer 5x concentrations		MgCl ₂)
Proctoer RNase Inhibitor, 40 U/ μl	0.5 μl	20 U
Deoxynucleotide triphosphate Mix, 10mM each	2 μl	1mM each
Transcriptor Reverse Transcriptase, 20U/ μl	0.5 μl	10 U
Total	20 μl	

2.2.6 Primer Design

Primers were designed using the primer analysis software Primer Express v3.0 (Applied Biosystems). Designs were based on available sequences of the NCBI Reference Sequence database and the Ensembl database. Once the primers had been chosen, a general BLAST of each primer sequence was run to ensure their unique specificity to the desired target sequence. The expression level of target genes, including Endothelin 1 (ET-1), endothelial nitric oxide synthase (eNOS), Vitamin D receptor (VDR) and 1-alpha-hydroxylase (CYP27b1), were determined using the standard curve method (Larionov et al., 2005). Quantifying all target genes relative to a housekeeper gene was not possible, since using cyclophilin and GAPDH, as a housekeeping gene showed that these were altering in response to calcitriol and calcidiol treatment (data not shown). Thus, all target genes were normalized to total first-strand cDNA content measured using Oligreen (Fisher).

Table 2-5 Primer sequences for candidate normalization genes

Symbol	Gene name	Foreword primer (5' to 3')	Reverse primer (5' to 3')
ET-1	Endothelin 1	CGTCCCTGATGGAAA GAGTGT	AACGTGCTCGGGAGTGTG
eNOS	Endothelial nitric oxide synthase	GGTCTGCACAGGAAA TGTTTCA	GATGGCCGAGCGAAGGTT
VDR	Vitamin D receptor	GCGCTCCAATGAGTC CTTCA	GCTTGTCCGCTTTGGT
CYP27b1	1-alpha- hydroxylase activity	GGCCCAGATCCTAAC ACATTTT	TGTAGGTTGATGCTCCTTTCA GGTA
CYP24a1	24 hydroxylase	GACCCACCTACTCC CACTTCT	GGCTCCCTCCACCATCATT
GAPDH	Glyceraldehyd e 3-phosphate dehydrogenase	TGGAAGGACTCATGA CCACAGT	GCCATCGCCACAGTTTC
Cyc	Cyclophilin	GGAGATGGCCAGGA AA	CGTAGTGCTTCAGTTTGAAG TTCTCA

2.2.7 Measurement of Gene Expression by RT- PCR (Lightcycler LC 480, Roche)

In order to measure gene expression, RT-PCR was conducted using a LightCycler 480 (Roche, Burgess Hill, UK). Furthermore, quantitative RT-PCR was carried out in triplicate using SYBR1 master mix (Roche, as shown in Table 2-6), and 384-well micro-plates and the LightCycler LC480 (Roche) configured for SYBR green detection. A standard curve was produced using serial dilutions of a pool of cDNA made from all samples, in order to check the linearity and efficiency of the PCR for every gene (Figure 2-3).

Table 2-6 SYBR Master Mix

	Volume	Final concentration
SYBR Master solution* (2x)	7.5ul	
Forward primer (10 uM)	0.45ul	0.3 mM
Reverse primer (10 uM)	0.45ul	0.3 mM
Water (molecular grade)	1.6ul	
	10ul total volume	

*For PCR each reaction in a total of 15µl the following constituents are required

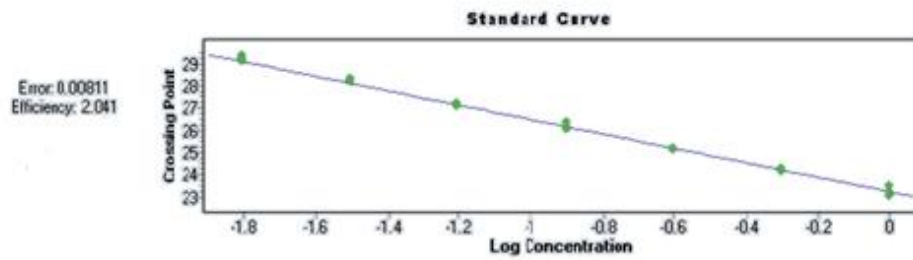
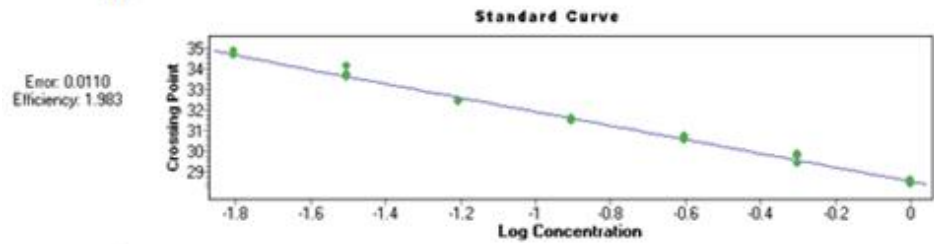
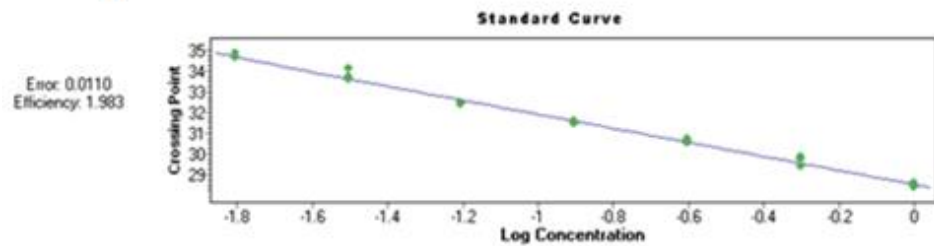
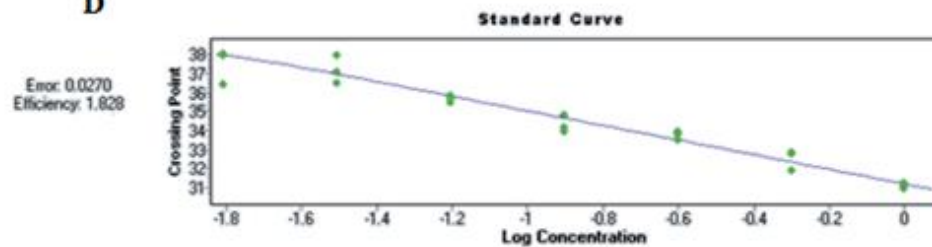
A**B****C****D**

Figure 2-3 Standard curve generated by using serial dilutions (typically 1:8) of pooled cDNA samples in Q RT-PCR reaction for relative **(A)** ET-1 **(B)** eNOS **(C)** VDR **(D)** CYP27b1. PCR efficiency was between 1.8 and 2.04 for all primers.

2.3 Western Blot Analysis

2.3.1 Overview

Western blotting, also known as protein immunoblotting is an important technique used to identify protein expression in samples. Producing a western blot involves several steps. These steps include: protein extraction and quantification, polyacrylamide gel electrophoresis, membrane transfer, incubation of antibodies and protein band detection. Western blot was performed as described by (Mahmood and Yang, 2012).

2.3.2 Preparation of Cell Lysate

In this part of the procedure, HUVECs were seeded as described in Section 2.1.2 and incubated in 12 ml of EBM-2 growth media (see Table 2-1) containing growth factors in T75 culture flasks. Cells were plated out onto 6 well plates and grown to 90% confluence. At this stage, the culture media were replaced with 2 ml of endothelial basal media (EBM-2) with 2% (v/v) FBS and growth factors supplemented with different doses of calcitriol, and incubated for 24 hours (37°C, 5% CO₂). Before starting the extraction of protein, 50 µl protease inhibitor (Complete Protease Inhibitor Cocktail tablets, Roche Diagnostics) was added to 200 µl 1x SDS, (which was prepared as a 2x stock solution in distilled water, as shown in Table 2-7), to avoid any protease enzyme action on protein samples. At the end of the incubation period, culture media was removed from the wells and washed with cold PBS, which was then discarded. Thereafter, 200 µl 1x SDS+50 µl protease inhibitor was added to each well. Cells were dislodged by a cell scraper and transferred to an autoclaved 1.5ml micro centrifuge tube. The 1.5ml micro centrifuge tube was immediately placed on ice and stored at -80°C until required for protein concentration and western blot.

Table 2-7 Preparation of 2x Sodium Dodecyl Sulfate (2x SDS) mix solution

Chemical	Amount
Glycerol	2 ml
1M Tris/HCl, (pH6.8)	1.25ml
10% SDS	4ml
DTT	0.154g
Bromophenol Blue	Few grains (approximately 1-5 mg)

2.3.3 Protein Determination

Protein was extracted from HUVECs and measured by kit2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions. A standard curve was produced using serial dilutions of a 2 mg/ml bovine serum albumin (BSA) stock to determine the protein concentrations of the samples. Prior to performing the assay, working colour reagent was prepared by mixing 100 parts of colour reagent A with one part colour reagent B (100:1); 1-50 µl of samples was added to the tubes. Next, 500 µl of precipitant was added to each tube, then briefly vortexed and incubated for 2-3 minutes at room temperature. Following this, 500 µl of co-precipitant was added to each tube and vortexed. The samples were then centrifuged at 10,000×g for five minutes. The supernatants were discarded and 100 µl of copper solution and 400 µl of distilled water were added before the samples were vortexed to dissolve the precipitated protein. One ml of working colour reagent was added, followed by incubation for 15-20 minutes. The absorbance was read at 480 nm. The protein concentration was determined from the standard curve.

2.3.4 Electrophoresis

The mix of samples and SDS were boiled for 5 minutes at 90°C to denature the protein. Protein samples (20 µl) and protein marker ladder (10 µl, Biorad Precision Plus Protein Standard, Biorad) were then loaded onto a 4-10% mini Protean® TGX precast polyacrylamide gradient gel (Biorad). Afterwards, a tank was filled with 1x running buffer (5mM Tris, 50 mM glycine (Fisher Scientific)) and the gel was run for one hour at 200V. The next step was to transfer proteins from the gel to nitrocellulose membrane (Hybond ECL, GE Healthcare) or polyvinylidene fluoride (PDVF) membrane. The nitrocellulose membrane was activated by soaking in water for 10 minutes, whereas the PDVF membrane was activated using methanol for 10 minutes, followed by transfer buffer for at least 15 minutes. The gel was placed in a transfer sandwich as follows: sponges, filter papers, the membrane, gel, filter papers and sponge. These were all pre-soaked in the transfer buffer. The sandwich was placed in a tank filled with transfer buffer (24 mM Tris, 192 mM glycine, 20% (v/v) methanol in distilled water) then run at 200 mA for two hours with cooling. Following the transfer, the nitrocellulose membranes were washed with TBS-T. The membranes were then blocked with 2% (w/v) ECL prime blocking reagent (GE Healthcare) in 1% Tween in 1x Tris Buffer Saline TBS (TBS-T) for one hour. The primary antibody was diluted to 1:5000 (with TBS-T) and incubated overnight at 4 °C in a shaker. The membrane was washed with the washing buffer for at least 30 minutes. It was then incubated for one hour with secondary antibody Conjugated to HRP at a dilution of 1:30,000 (with TBS-T). After washing the membrane, the targeted protein was detected by incubation with enhanced chemiluminescence detection kit (ECL advanced western blot detection kit, Fisher Scientific) for 1-2 minutes, while being protected from light.

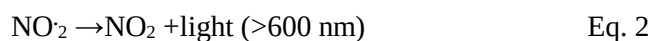
2.3.5 Detection

This process was carried out in a dark room. Equal volumes of the ECL western blotting detection reagents (advanced western blot detection kit, Fisher Scientific) were mixed together in ratio (1:1) poured over the membrane surface and incubated for 1-2 minutes. Then, the membrane was blotted dry to remove the excess liquid, then it placed in cling film and positioned in film cassette. In the dark room, an x-ray film (Hyperfilm-ECL, GE Healthcare) was applied on top of cling film and was exposed for 1 to 3 minutes, depending on the strength of the signal. The film was then developed under dark condition by placing it in the developer tank (Kodak LX24) for 90 seconds, rinsing it in the water tank for 90 seconds and fixing it in the fixer tank (Ilford Hypam fixer liquid) for 90 seconds. The film was then dried.

2.4 Nitric Oxide (NO) Production Measurement

2.4.1 Overview

Sievers' nitric oxide analyser (NOA) (Model 280i) was used for NO detection in the culture media. This technique is based on a gas-phase chemiluminescence reaction between nitric oxide and ozone, which leads to the rapid formation of Nitrogen dioxide (NO_2) in the excited state, as seen in Eq. 1:



Thereafter, NO_2 returns to the ground state, as seen in Eq. 2, emitting light in the form of chemiluminescence.

This model is considered the most commonly used assay for NO detection in culture media. The major advantages of using the NOA include its specificity, sensitivity, versatility and dependable operating performance.

2.4.2 Effect of Calcitriol on NO Production

Cells were grown as described in Section 2.1.2 and incubated in 12 ml of EBM-2 growth media (see Table 2-1) containing growth factors in 75cm² culture flasks. Thereafter, cells were plated in 6 well plates and allowed to adhere; then, all culture media were replaced with 2 ml of Dulbecco Modified Eagle's Medium (DMEM), which was supplemented with 2 mM glutamine, 1% (v/v) penicillin and streptomycin, 1% (v/v) serum (FBS) and without red phenol for cell starvation for 4-6 hours. Then, cells were directly treated with different doses of calcitriol, ranging from 10^{-13}M – 10^{-7}M , and compared with DMSO (0.01%) containing media as control for 24 hours. As positive control, some samples were stimulated with 10^{-5}M acetylcholine (Sigma Aldrich, UK). After 24 hour incubation, the media was transferred to autoclaved 1.5ml micro centrifuge tubes, and then spun for 30 seconds at 8000 xg to discard any cells. After centrifugation, the media was transferred to clean 1.5ml centrifuge tube and kept at -20°C for further analysis. Finally, NO production was measured in cell culture

supernatants using Sievers' nitric oxide analyser (NOA) (Model 280i) and normalized to the total protein in the media.

2.4.3 Nitrite Measurement

To determine the total synthesis of NO, nitrite was measured in the culture media of HUVECs after 24 hours. The purge vessel was filled with 5 ml of a reducing agent (1% w/v of sodium iodine (NaI)] solution) with the following composition: 50 mg sodium iodide, 0.5 ml of pure water, 4.5 ml glacial acetic acid). The reducing agent converted nitrite to nitric oxide at room temperature, as seen in Eq. 3. Figure 2-4 shows the setup of the purge vessel for nitrite reduction.

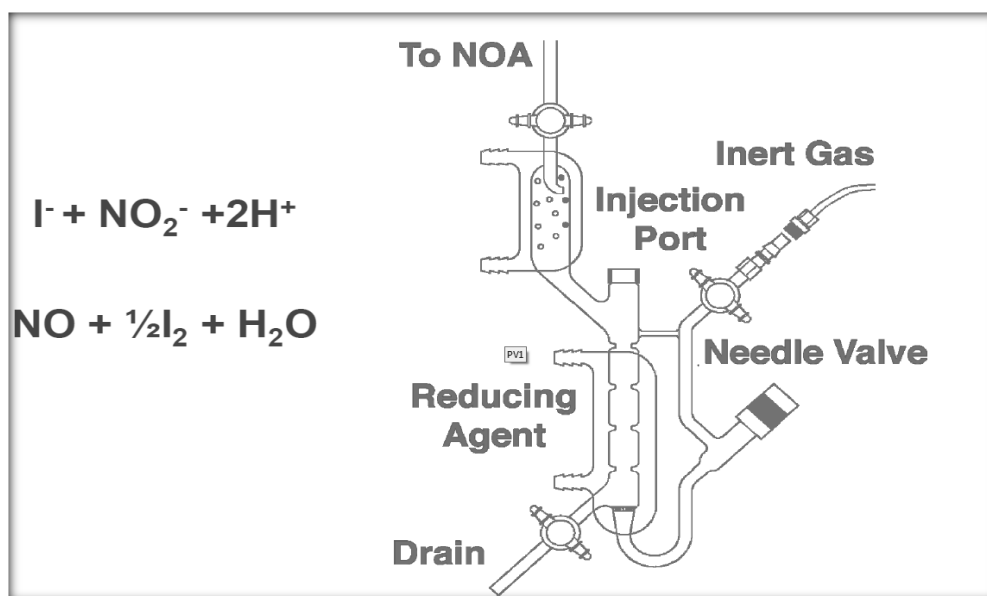
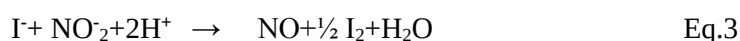


Figure 2-4 Setup for nitrite measurement. Standard and samples are injected into a glass purge vessel containing a reducing agent, which is bubbled with nitrogen and oxygen cylinders. The NO in the headspace is swept into an NOA chamber, where it reacts with ozone to form electronically-excited nitrogen dioxide. This reaction produces emission chemiluminescence, which is detected by the red-sensitive photomultiplier tube, and the signal is noted by data recording software.

2.4.3.1 Preparation of Nitrite Standard Dilutions

The 10^{-1} M of nitrite stock solution (Sigma Aldrich, UK) was stored at 4 °C. It was used to prepare fresh serial dilutions in order to construct the standard curve for NOA. This standard curve contained nitrite at concentrations greater than those of the samples, concentrations lower than those of the samples and concentrations approximately the same as those of the samples. Subsequently, analysis of standard solutions containing 10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M and 10^{-6} M, allowed construction of a calibration curve suitable for samples. To prepare 1 ml of the dilute standards, 900 μ l of nitrite-free water was added to five micro centrifuge tubes (1.5 mL). The first tube (10^{-2} M) was prepared by adding 100 μ l of the stock solution, then vortexed for 15 seconds. Importantly, new pipette tips were used for each dilution to minimize nitrite contamination, which might remain in the tips. Thereafter, 100 μ l of the 10^{-2} M standard was transferred to the next tube and vortexed to obtain 10^{-3} M. These steps were repeated to prepare 10^{-4} M, 10^{-5} M and 10^{-6} M concentrations of the standard curve.

2.4.3.2 Preparation of Calibration Curve and Analysis Samples

Before analysing the standards, 50 μ l of nitrite-free water was injected into the purge vessel to check the nitrite levels in the water used. After obtaining clean water blank, the calibration curve was constructed by injecting 50 μ l of the standard into the Purge Vessel, and measuring the peak area for each standard. Each time a dose of standard was added, it was allowed to reach its peak and to return to the baseline before adding the next one (Figure 2-5A). Once the injection of all standards was complete, NOA software was used to generate a standard curve, based on information about the peak areas (Figure 2-5B). After generating a standard curve, 50 μ l of samples were injected into the glass purge vessel, the peak was allowed to return to baseline after each sample. Samples were injected in duplicate into the purge vessel using a 50 μ l syringe. Nitric oxide levels in samples were determined directly from the standard curve.

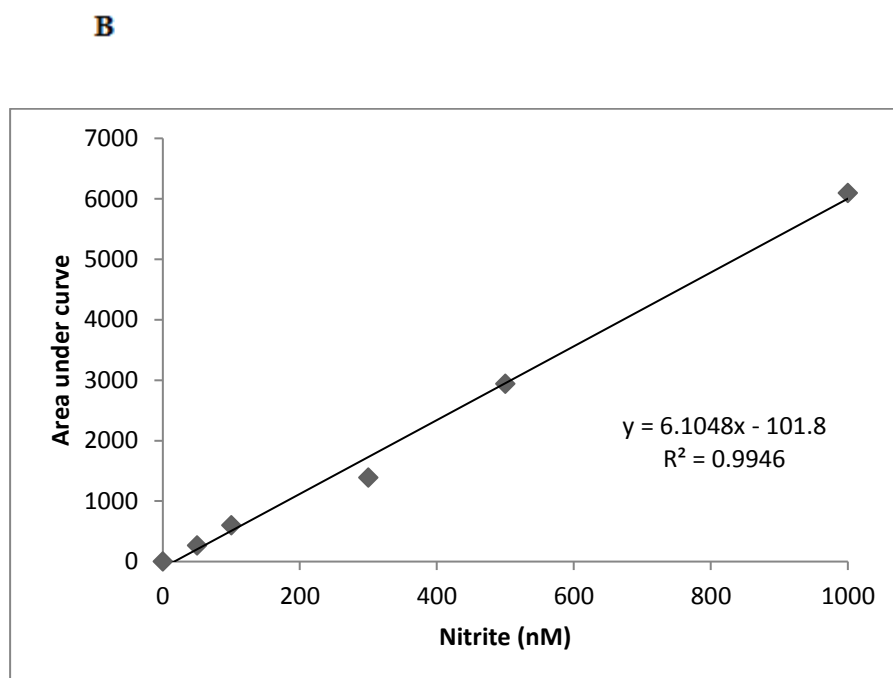
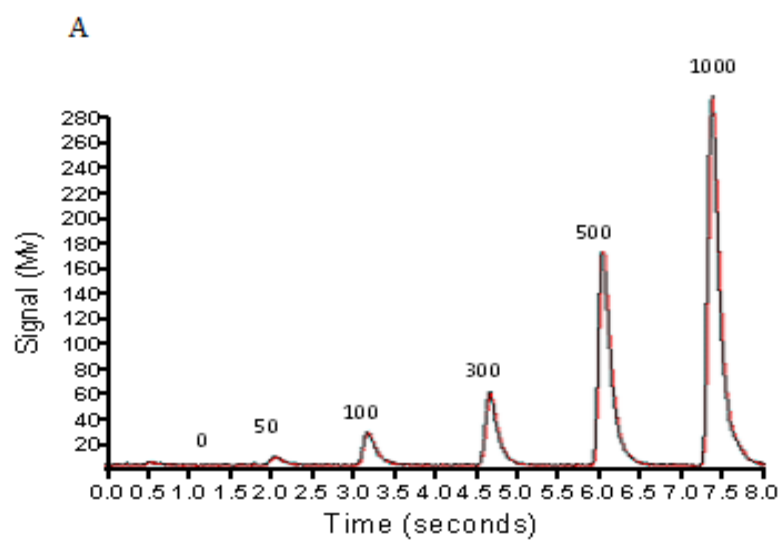


Figure 2-5 Standard curve achieved for NO₂. **(A)** is the raw data plotted on the analysis program, NOA software. The figures above each peak represent the concentrations of NO₂ in nM. **(B)** A typical standard curve of this data is shown underneath (n=2, r²= 0.9946).

2.5 Ex Vivo Myography Experiments

2.5.1 Overview of Myography

Myography is an *ex vivo* technique that allows researchers to investigate the functional responses and mechanical properties of small, isolated arterial segments ((Spiers and Padmanabhan, 2005). It was introduced by Mulvany and Halpern to investigate highly isometric responses from vessels with internal diameters as small as 100 μm . (Mulvany and Halpern, 1976). Blood vessels of different arteries from different species can be examined using this technique. In all experiments carried out in these studies, thoracic and/or abdominal aorta were isolated from adult female Wistar rats and their contractility was studied with the multi-wire Myograph system model 620M (Danish Myo Technology, A/S). This system has four separate channels, which allow for four segments of vessels to be examined simultaneously.

2.5.1.1 Calibration of the Myograph

Calibration of the Myograph was done regularly, especially prior to the start of any experiment. The force calibration kit consists of a 2 g weight, bridge and T-balance. In brief, chambers were filled with 5 ml of double-distilled water. In the heat menu of the myo-interface, the heating was turned on to reach the target temperature of 37°C after 20 minutes. As illustrated in Figure 2-6, the calibration bridge/T-balance was placed on the Myograph unit, and they were warmed up together. Then, the balance was placed over the bath, resting upon the bridge. More importantly, the tip of the transducer arm on the balance was placed as close as possible to the pin, without touching it. In the calibration menu on the myo-interface, the force transducer 1 was selected to initiate calibration. Once the relative force reading in the display was stable, the 2 g weight was carefully placed on the pan. Then, once the relative force reading was stable, the calibration was selected to finish at an output around 9.81 mN. All the force transducers were calibrated to an output 9.81 mN, which displayed automatically as the last step of calibration. Lastly, the weight was carefully removed, and with the balance and

calibration bridge, it was moved from Myograph unit 1 to Myograph unit 2. This process was then repeated for force transducers 2, 3 and 4.

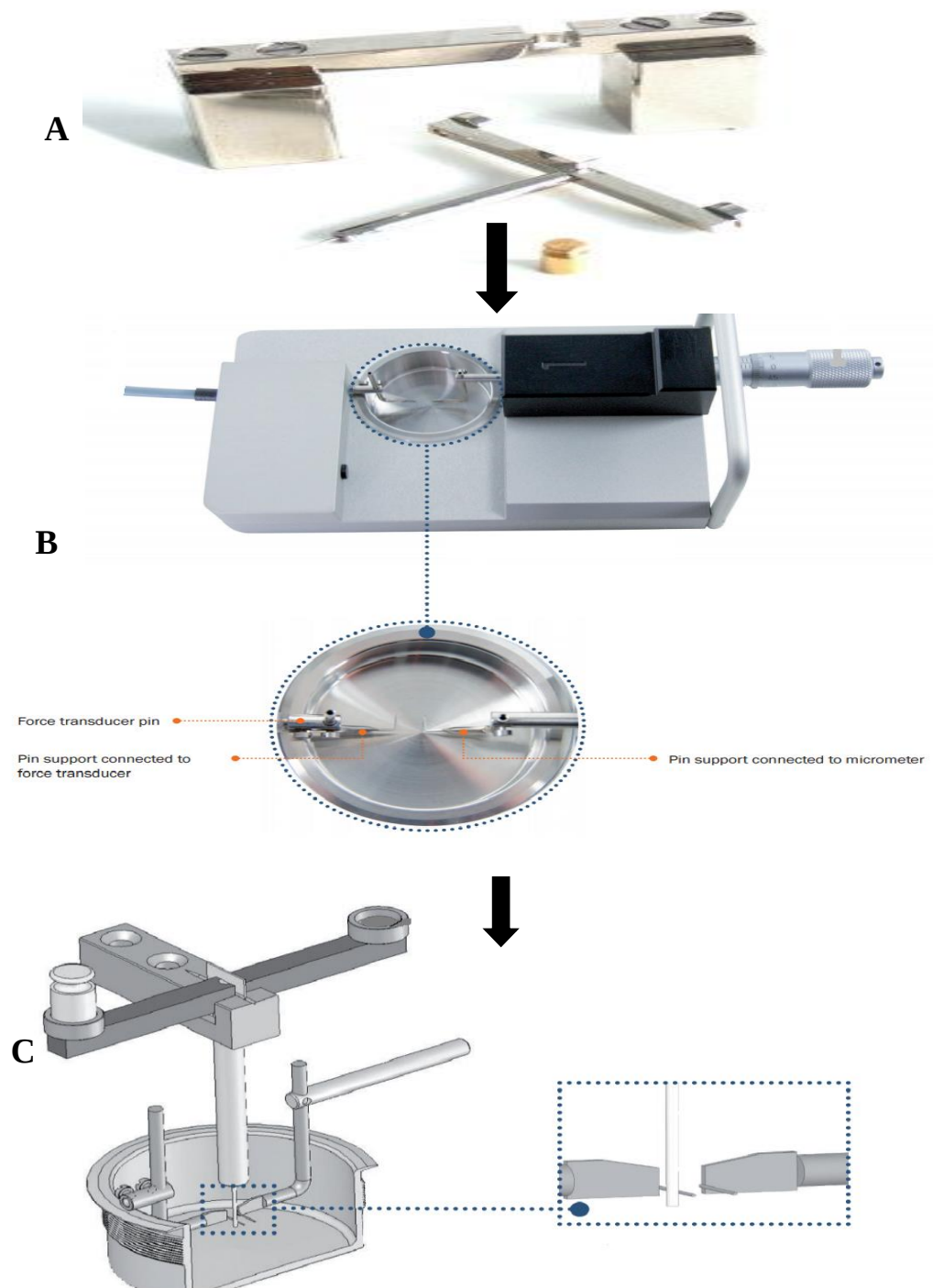


Figure 2-6 Calibration of the Myograph. (A) The force calibration kit which consists of a bridge, T-balance and 2 g weight. (B) The chamber unit. (C) Weight calibration setup. Pictures and information Taken from <http://www.dmt.dk>

2.5.1.2 Maintenance and Cleaning of the Myograph

The most delicate component of the Myograph system is the force transducer which is located in the 4 chambers. Therefore, the force transducer should always be handled with the utmost care. At the end of the experiment, the vacuum pump and oxygen supply were turned on to remove any buffer remaining in the chamber. To dissolve calcium deposits and other salt build up on the chambers or surrounding surface, chambers were filled with an 8% acetic acid solution, which was incubated for a few minutes. Following this, the acetic acid was removed and the chamber was washed several times with double distilled water.

2.5.2 Animal Experiment

Female Wistar rats weighing about 150-250 g were used in the experiments. All animals were purchased from Charles River UK Limited, were housed in a room with standardized temperature (21°C) and exposed to a 12-h:12-h dark-light cycle. Animals had *ad libitum* free access to a standardized chow diet and tap water. The welfare of animals and procedure carried out in this study was conducted in accordance with the UK Home Office regulations and the UK Animals (Scientific Procedures) Act, 1986. Ethical approval was obtained from the University of Nottingham Ethical Review Committee.

2.5.3 Chemicals

A brief description of the concentrations of inhibitors employed for the *ex vivo* experiments are listed in Table 2-8. All concentrations used are expressed as the final molar concentration in the organ bath

Table 2-8 Summary of drugs used in *ex vivo* experiment

Drug	Concentration used	Stock	Manufacturer
Phenylephrine (PE)	10^{-9} to 10^{-5} M in Krebs solution	10^{-1} M in H_2O	Sigma Aldrich, UK
Angiotensin II (Ang II)	10^{-11} to 10^{-8} M in Krebs solution	10^{-3} M in H_2O	Sigma Aldrich, UK
Acetylcholine chloride (ACh)	10^{-9} M to 10^{-5} M in Krebs solution	10^{-1} M in H_2O	Sigma Aldrich, UK
N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME)	10^{-5} M in Krebs solution	10^{-1} M in H_2O	Sigma Aldrich, UK
Verapamil(L-type calcium channel blocker)	10^{-10} M to 10^{-6} M in Krebs solution	10^{-3} M in DMSO	Sigma Aldrich, UK
FPL 64176 (Potent Ca^{2+} channel (L-type) activator)	10^{-10} to 10^{-6} M in Krebs solution	10^{-3} M in DMSO	Sigma Aldrich, UK
H-89 dihydrochloride hydrate (PKA inhibitor)	10^{-6} M in Krebs solution	10^{-6} M in H_2O	Sigma Aldrich, UK
SQ 22,536 (adenylate cyclase inhibitors (AC)	10^{-5} M in Krebs solution	10^{-1} M in DMSO	Sigma Aldrich, UK

2.5.4 Preparation of Aortic Rings and Isometric Tension Measurement

The thoracic and abdominal aorta of the rats was carefully removed after they were euthanized by raised CO₂. These aorta were immediately placed into cold Krebs solution (119mM NaCl, 4.7mM KCl, 1.17mM MgSO₄, 25mM NaHCO₃, 41.18mM KH₂PO₄, 0.02mM EDTA, 5mM glucose and 2mM CaCl₂) pH7.4. The fat and adherent tissue was dissected away in a petri dish containing Krebs solution; this was done under a microscope with an external light source. The aorta was cut into rings about 3 mm in length, keeping the endothelium intact. In some experiments, the endothelium was gently damaged mechanically, as described elsewhere (Wong et al., 2010), in order to assess endothelium contribution to vitamin D responses. Each ring was mounted to a pair of pins in a DMT myograph chamber (the multi wire myograph system model 620M [Danish Myo Technology, A/S]) in order to measure isometric force (Figure 2-7). Each chamber was filled with 5 ml of Krebs solution (pH 7.4), constantly gassed with 95% O₂ and 5% CO₂ and maintained at 37°C. The aortic rings were then equilibrated in Krebs solution and maintained under an optimal tension of 1 g for 60 minutes, with three changes of Krebs solution, before the experimental procedure began. Isometric force was recorded by a force transducer connected to a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO).



Figure 2-7 Mounting arterial rings on pin.

Pictures and information Taken from <http://www.dmt.dk>

2.5.4.1 Observation and Analysis of Calcitriol-induced Relaxation

To investigate the effect of calcitriol, aortic rings of vessels were mounted by pins in a Myograph chamber containing Krebs solution (pH7.4) that had previously been gassed with 95% O₂ and 5% CO₂ at 37 °C, and were maintained at 1 g of initial tension, as illustrated in 2.5.4. To obtain submaximal contraction, aortic rings were initially exposed to 60 mM KCl. After washing with Krebs solution at 37°C to return to the rested state, the arteries were exposed to vehicle (DMSO) or increasing concentrations of calcitriol (10⁻¹³M to 10⁻⁷M). All vessels were then treated immediately (with no washing) with increasing concentrations of phenylephrine (PE, 10⁻⁹ to 10⁻⁷M) to induce vasoconstriction, followed by increasing concentrations of acetylcholine (ACh, 10⁻⁹ to 10⁻⁵M) to induce vasodilation. For certain experiments, after cumulative concentrations of calcitriol, all vessels were washed out for 15 minutes before they were treated with increasing concentrations of phenylephrine (PE, 10⁻⁹ to 10⁻⁷M) to induce vasoconstriction, followed by increasing concentrations of acetylcholine (ACh, 10⁻⁹ to 10⁻⁵M) to induce vasodilation. This was to establish whether the observed effects of calcitriol altered the response to these known regulators.

Moreover, the relaxant effect of calcitriol was investigated by stimulating rings with increasing concentrations of PE (10⁻⁹-10⁻⁵ M) until a stable contraction was obtained. All vessels were then treated immediately (with no washing) with increasing concentrations of calcitriol (10⁻¹³ to 10⁻⁷M), followed by increasing concentrations of acetylcholine (ACh, 10⁻⁹ to 10⁻⁵M) to induce vasodilation.

2.5.4.2 The Relaxant Effect of Vitamin D: Role of Nitric Oxide,

Involvement of the L-type Calcium Channel

In separate experiments, aortic rings were pre-incubated with different blockers prior to treatment with calcitriol or other agents. In all experiments vessels were pre-contracted with Phenylephrine (PE, 10⁻⁹ to 10⁻⁷M); in certain experiments, vessels were pre-contracted with Angiotensin II (Ang II, 10⁻¹¹-10⁻⁸ M). These ranges of concentrations

had previously been shown to cause sub-maximum constriction in the arteries of rats (Paul et al., 2006, Brito et al., 2013). To obtain the cumulative concentration response curve to each drug, the response to given concentrations of the drugs was allowed a two minute interval between each drug addition.

A description of the blockers/ activators and concentrations employed is described below.

➤ First pre-incubation for 30 minutes:

❖ **L-NAME** (Sigma Aldrich, UK): The role of nitric oxide (NO) in calcitriol-induced relaxation was investigated by adding a potent endothelial NO synthase inhibitor, namely L-NAME (10^{-4} M), for 30 minutes prior to the addition of vehicle (DMSO) or increasing concentrations of calcitriol (10^{-13} M to 10^{-7} M). After this, the vasodilatory effects of calcitriol were analysed in mechanically endothelium-denuded arteries compared with intact vessels. Intact and denuded rings of thoracic or/and abdominal arteries were pre-contracted using 10^{-9} to 10^{-5} M phenylephrine (PE) to obtain endothelium-independent contractions. Thereafter, the rings were relaxed with increasing concentrations of ACh (10^{-9} - 10^{-5} M) to test the endothelial integrity. Intact endothelium and denuded arterial rings were then pre-contracted again with PE (10^{-9} - 10^{-5} M) and treated immediately (with no washing) with increasing concentrations of calcitriol (10^{-13} to 10^{-7} M).

❖ **Verapamil** (Sigma Aldrich, UK): In order to investigate the role of intracellular Ca^{2+} in relaxant responses induced by calcitriol, arteries were incubated with 10^{-4} M L-NAME for 30 minutes, and exposed to vehicle (DMSO) or increasing concentrations of the Ca^{2+} channel blocker, verapamil (10^{-10} - 10^{-6} M). All vessels were then treated immediately (with no washing) with increasing concentrations of phenylephrine (PE, 10^{-9} to 10^{-7} M) to induce

vasoconstriction, followed by increasing concentrations of acetylcholine (ACh, 10^{-9} to 10^{-5} M) to induce vasodilation.

- ❖ **FPL 64176** (Sigma Aldrich, UK): The role of Ca^{2+} in the relaxant responses of calcitriol, were tested using a potent Ca^{2+} channel (L-type) activator, FPL 64176. This experiment was designed to clarify the mechanism of action of calcitriol on rat arteries by comparing its action with verapamil, which is a calcium channel blocker.

In all experiments, three arteries were exposed to increasing concentrations of FLP (10^{-10} - 10^{-6} M), while one artery was used as control, with vehicle (DMSO) added instead of FPL. Next, all vessels were incubated (with FPL or DMSO) for 20 minutes, and then exposed to vehicle (DMSO) or increasing concentrations of verapamil (10^{-10} M to 10^{-6} M). Afterwards, they were treated immediately (with no washing) with increasing concentrations of phenylephrine (PE, 10^{-9} to 10^{-7} M) to induce vasoconstriction, followed by increasing concentrations of acetylcholine (ACh, 10^{-9} to 10^{-5} M) to induce vasodilation. In similar experiments, vessels were exposed to increasing concentrations of calcitriol (10^{-13} M to 10^{-7} M) instead of using verapamil, to clarify the mechanism of action of vitamin D on rat arteries by comparing its action with verapamil a calcium channel blocker.

2.5.4.3 Investigation of the Mechanisms Underlying the Relaxation

Induced by Calcitriol

These experiments were carried out to identify the possible signalling pathways underlying the vasodilator action of calcitriol. It was hypothesised that vitamin D-induced relaxation may be mediated via the Adenylate Cyclase (AC) /cAMP/ Protein Kinase A (PKA) pathway. Therefore, we set out to block at different points in that pathway using AC and PKA inhibitors.

A description of the blockers and concentrations employed for the experiments is described below.

- ❖ **SQ 22,536** (Adenylate Cyclase inhibitor (AC) (Sigma Aldrich, UK): After washing vessels from 60 mM KCl with Krebs solution at 37 °C, to return to the rested state, three aortic rings were incubated with SQ22,536 (10^{-5} M), whereas one arterial ring was incubated with DMSO instead of SQ22,536 for 30 minutes. Following incubation, all vessels were pre-contracted with increasing concentrations of Ang II (10^{-11} - 10^{-8} M), before being exposed to increasing concentrations of calcitriol (10^{-13} M to 10^{-7} M) or vehicle (DMSO). All vessels were then incubated immediately (with no washing) with 10^{-5} M dbcAMP for 10 mins to test responsiveness.
- ❖ **H-89 dihydrochloride hydrate (PKA inhibitor)** (Sigma Aldrich, UK): To study the relaxation effect of calcitriol, and to investigate the role of vascular smooth muscle cells on vasodilation, a potent inhibitor of cAMP-dependent protein kinase (H-89) was used. Three arterial rings were incubated with H-89 (10^{-6} M) for 30 minutes, whereas one channel was incubated with PBS as control. All vessels were pre-contracted with increasing concentrations of angiotensin II (Ang II, 10^{-11} - 10^{-8} M), before they were then immediately treated with either increasing concentrations of calcitriol (10^{-13} - 10^{-7} M) or vehicle (DMSO). The subsequent effect of 10^{-5} M dibutyryl cAMP (dbcAMP) on the relaxation of arteries following calcitriol administration was then studied. Vessels were incubated with 10^{-5} M dbcAMP for 10 minutes.

3

EFFECTS OF VITAMIN D ON VASCULAR ENDOTHELIAL CELL PROLIFERATION

3 Effects of vitamin D on vascular endothelial cell proliferation

3.1 Introduction

It is well known that calcitriol, which is the most biologically active form of vitamin D, plays a significant role in the cellular function of a wide range of extra-skeletal organs, including the heart and vasculature. In addition, it has a beneficial effects seen in the cardiovascular system as a whole (Zittermann and Koerfer, 2008, Franczyk et al., 2014). Recently, it has demonstrated that calcitriol has a wide range of fundamental biological functions, including the inhibition of cell growth (Chakraborti, 2011).

In fact, there is considerable data to suggest that the physiological effect that calcitriol exerts by its binding with vitamin D receptors (VDR) in turn regulates the expression of the genes responsible for cellular proliferation (Thompson et al., 2012, Haussler et al., 1998). The expression of the biosynthetic enzyme 1-alpha-hydroxylase (CYB27b1) and VDR in human umbilical vein cord endothelial cells (HUVECs) (Zehnder et al., 2002) suggests a possible autocrine/intracrine mechanism (Zehnder et al., 2002), which supports the idea that vitamin D is essential in the modulation of endothelial functions (Holick, 2004b).

Hence, the identification of vitamin D receptors and the biosynthetic enzyme 1-alpha-hydroxylase (CYB27b1) in endothelial cells (Wang et al., 2008), as well as the wall of blood vessels (Gonzalez-Parra et al., 2012), suggest a potential role of vitamin D in the cardiovascular system. As such, it has been proposed that vitamin D₃ supplementation is a useful approach for vascular regeneration (Wong et al., 2014). Thus, this chapter aimed to examine the effect of a range of doses of calcidiol and calcitriol, on proliferation of human umbilical vein cord endothelial cells (HUVECs).

3.2 Hypothesis

The hypothesis of this study was that calcidiol and calcitriol have an inhibition effect on growth and proliferation of HUVECs.

3.3 Aim

The overall aim of the work described in this chapter was to investigate whether calcidiol and/or calcitriol had any effect on the proliferation of HUVECs.

3.4 Objectives

- To determine whether different concentrations of calcidiol and/or calcitriol have a direct effect on the growth of HUVECs cells.
- To verify whether growth factors in the culture medium might alter the HUVECs' behaviour or affect the various treatments.

3.5 Materials and methods

A brief description of all experimental procedures undertaken is detailed below. A more detailed explanation of procedures is described in the Materials and Methods (Chapter 2).

3.5.1 Cell culture

To begin, HUVECs were cultured in EBM-2 endothelial basal media (2% FBS plus growth factors) following the method detailed in Section 2.1.2. Once the cells reached 80% confluence, they were plated onto 96 well plates in a volume of 200µl of EBM-2 control media/well and allowed to adhere, in order to evaluate the effect of calcidiol and calcitriol on cell proliferation.

3.5.2 Cell Proliferation

After the cells were cultured, DNA content assays were performed at 0 (T₀), 8, 24 and 48 hours of incubation to determine cell viability and proliferation. The cells were maintained in two different culture media: one containing growth factors and 2% FBS and another without growth factors. Both media were supplemented with different doses of calcidiol and/or calcitriol. Cells for DNA content assays were washed with 100 µl of PBS, and then 100 µl of sterile water was added and the cells were incubated for one hour at 37°C, 5% CO₂. Next, the plates were frozen at -20°C for 30 minutes and thawed again, which helped to lyse the cells and release the DNA. Plates were stored at -20°C until use.

3.5.3 DNA assay

Prior to determination of DNA contents, cells were thawed a final time and standard calf thymus DNA (40 µg/ml) was added to the wells: A1-H3 on each 96 well plate (0, 8, 24 and 48 hour plates) to produce a DNA standard curve. A volume of 30 µl of bis-benzimide dye solution (1 mg/ml stock) was added to 15 ml of 2x TNE buffer. Next, 100 µl of dye solution was added to each well, including the standard curve. The plates

were then scanned using a fluorescent plate reader (Cytofluor™ 2350, Millipore Corp.) to produce a standard curve, which was used to calculate the DNA concentration of control and treated cells in wells A4- H10.

3.5.4 Statistical analyses

All data from cell proliferation studies (DNA content) were evaluated by one- or two-way analysis of variance (ANOVA) using Genstat statistical analysis software (Version 17th). Statistical significance was identified at $p < 0.05$.

3.6 Results

3.6.1 Effect of Vitamin D on Endothelial Cell Proliferation

The cells were maintained in two different cultured media: one containing growth factors and 2% FBS (referred to as A), and another without growth factors (referred to as B). Both of these were supplemented with different doses of vitamin D calcidiol, and calcitriol ranging from 10^{-13} M to 10^{-7} M and compared with DMSO (0.01%) containing media as control.

3.6.1.1 Determination of the Effects of Calcidiol on Proliferation of HUVECs

As expected in the DMSO control cells treated with growth factors, the DNA concentration increased with time (Figure 3-1), indicating cell proliferation. In contrast, in the absence of growth factors, there was little change with time, indicating either the cells were not proliferating or they were proliferating and dying at the same rate.

As shown in Figure 3-1, the duration of treatment revealed that calcidiol had a significant effect on endothelial cell proliferation in a dose-dependent and time-dependent manner in both media ($p < 0.001$ for time x treatment interaction). The highest concentration of calcidiol, 10^{-9} M and 10^{-7} M, significantly decreased DNA content in both media, particularly at 24 and 48 hours relative to DMSO ($p < 0.001$) (see Figure 3-1), suggesting a toxic or anti-proliferative effect of this dose. Therefore, despite its status as an inactive form of vitamin D, it is shown to significantly inhibit HUVECs' proliferation.

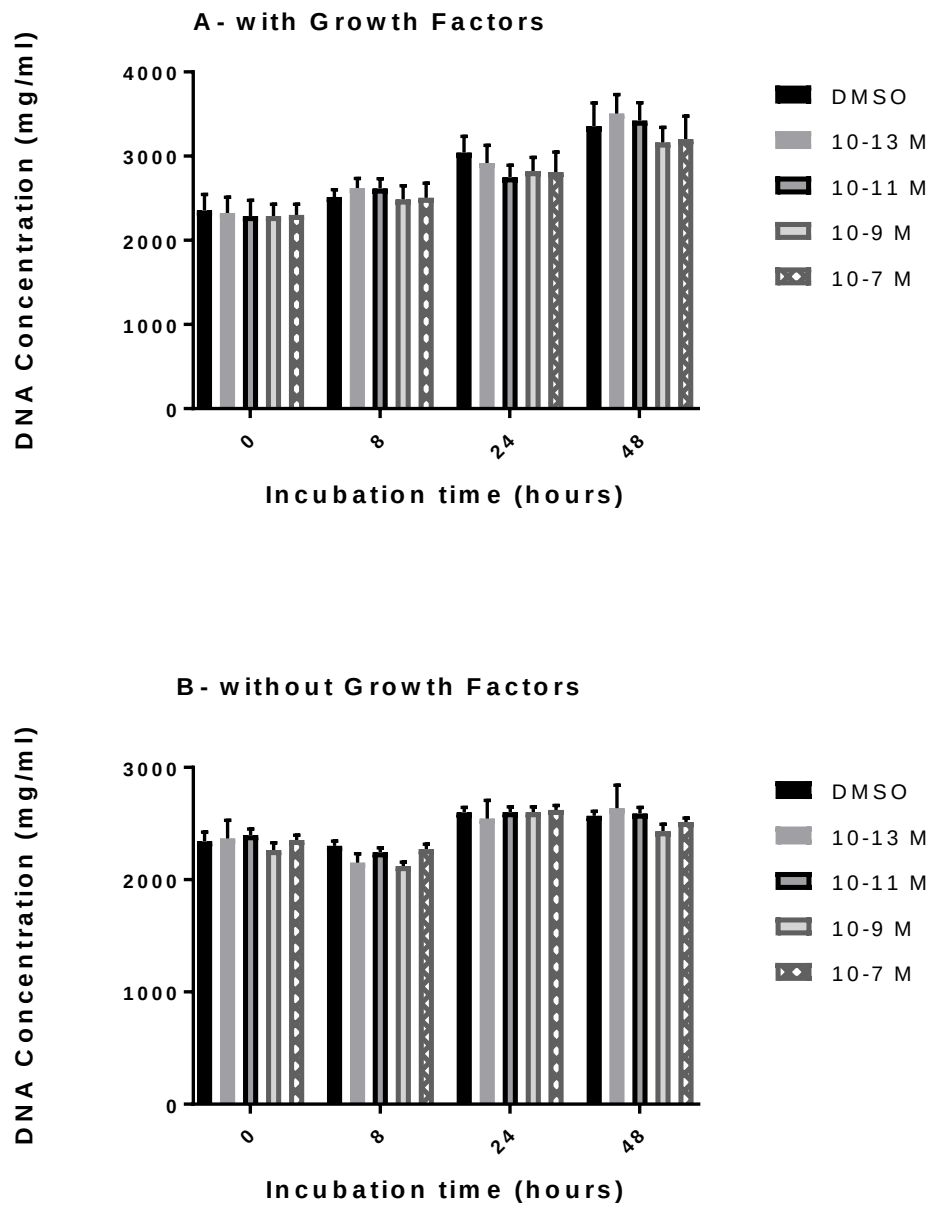


Figure 3-1 Dose-dependent and time-dependent effects of calcidiol on DNA content. **(A)** HUVECs cultured in a medium with growth factors supplemented with increasing doses (10^{-13} to 10^{-7} M) of calcidiol for up to 48 hours. **(B)** HUVECs cultured in a medium without growth factors supplemented with increasing doses (10^{-13} to 10^{-7} M) of calcidiol for up to 48 hours. (Mean $\pm$ SD, $n=4$; $p<0.001$ and $p=0.002$ for interaction 2 way ANOVA for A and B, respectively).

3.6.1.2 Determination of the Effects of Calcitriol on Proliferation of HUVECs

In addition to the effect of inactive vitamin D, calcidiol on cell proliferation, the effect of calcitriol on the proliferation of HUVECs was also studied. Once again, HUVECs were treated with various concentrations ranging from 10^{-13} M to 10^{-7} M for up to 48 hours, and DNA content was determined as described previously.

As shown in Figure 3-2A, at low concentrations of calcitriol (10^{-13} M), DNA content, and therefore the proliferation of HUVECs cells, significantly increased at 48hrs, whereas high concentrations (10^{-7} M) had no effect compared with DMSO, control ($p < 0.001$). However, this effect was not observed for calcitriol in medium without growth factors (see Figure 3-2B). Data from this study showed that calcitriol had a significant effect on endothelial cell proliferation in a dose-dependent and time-dependent manner in media containing growth factors ($p = 0.01$ for interaction). However, it had no effect in the absence of growth factors ($p = 0.937$ for interaction). These results indicate that the lowest levels (10^{-13} M) of calcitriol enhance the proliferation of HUVECs.

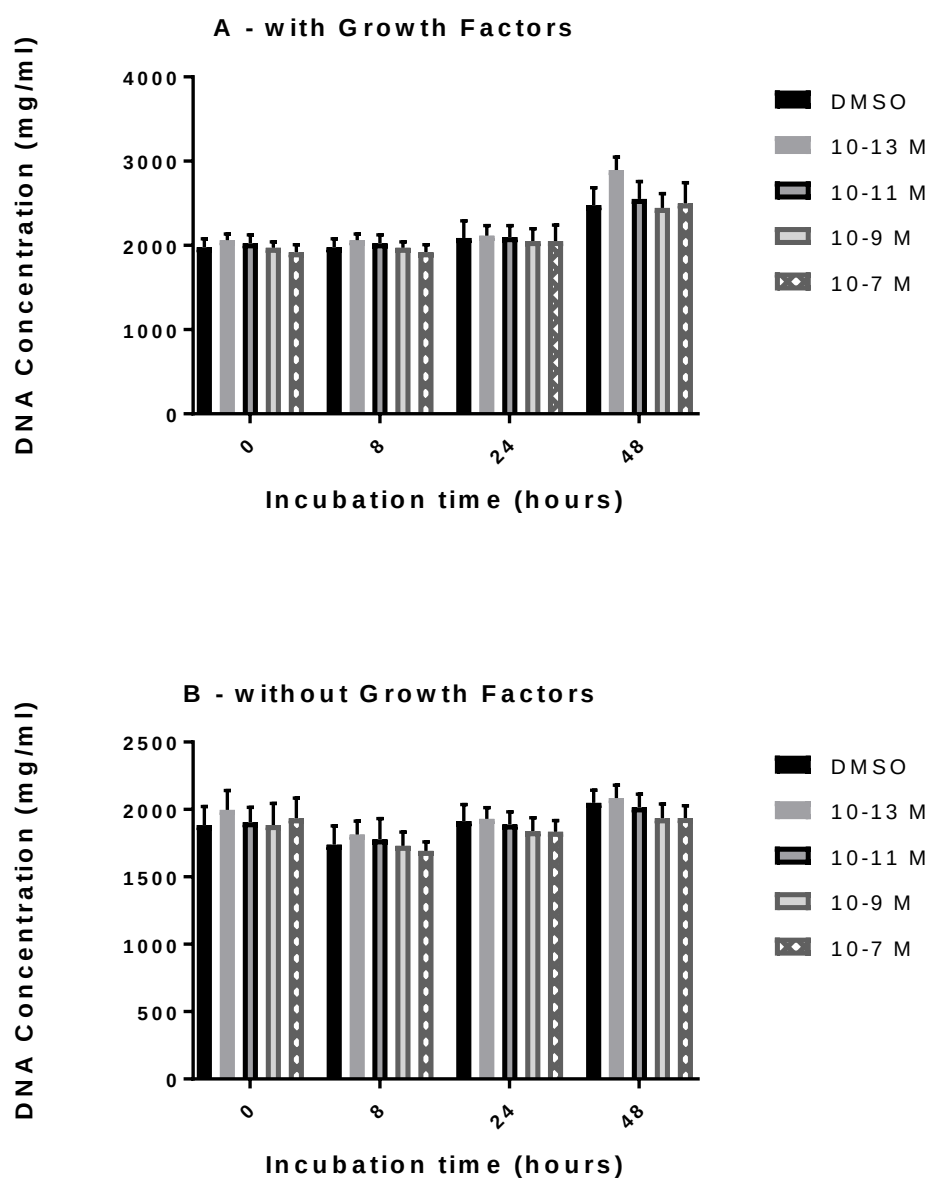


Figure 3-2 Dose-dependent and time-dependent effects of calcitriol on DNA content. **(A)** HUVECs cultured in a medium with growth factors supplemented with increasing doses (10^{-13} to 10^{-7} M) of calcitriol for up to 48 hours. **(B)** HUVECs cultured in a medium without growth factors supplemented with increasing doses (10^{-13} to 10^{-7} M) of calcitriol for up to 48 hours. (Mean $\pm$ SD, $n=4$; $p=0.01$ and $p=0.937$ 2-way ANOVA for interaction for A and B respectively).

3.7 Discussion

The main aim of this chapter is to investigate whether the vitamin D (both calcidiol and/or calcitriol) had any effect on the proliferation of HUVECs. Hence, Cells were treated with different doses for different periods of time in order to evaluate the effect of the vitamin D on cell proliferation. Experiments were carried out both in the presence and absence of growth factors in the culture medium, in order to verify whether these factors might alter the HUVECs' behaviour or the effects of the various treatments.

Data in this chapter clearly demonstrated that the lowest concentration of calcitriol (10^{-13} M) induced cell proliferation after 24 and 48 hours, as indicated via increasing DNA content. In addition, despite its status as an inactive metabolite of vitamin D, calcidiol at that high concentrations (10^{-9} , 10^{-7} M) significantly inhibit HUVEC proliferation, suggesting the presence of a vitamin D micro-endocrine system in endothelial cells. These results support previous *in vitro* studies using HUVECs, showing that human endothelial cells are capable of producing the calcitriol, active form of vitamin D, due to the presence of the 1-alpha-hydroxylase (CYB27b1) enzyme (Merke et al., 1989, Wang et al., 2008). Furthermore, it has been reported by Zehnder et al., (2002) that 1-alpha-hydroxylase (CYB27b1) is expressed in endothelial cells of large and small arteries and veins. In another study, (Zehnder et al., 2001) revealed that endothelial cells express the VDR as well as 1-alpha-hydroxylase (CYB27b1) enzyme.

In addition to the direct growth-inhibitory effects of vitamin D in tumor cells, it also hinders the proliferation of endothelial cells and angiogenesis (Bernardi et al., 2002, Merke et al., 1989, Oikawa et al., 1990). In fact, the anti-proliferative and anti-angiogenic effects appear to require vitamin D receptors (Whitfield et al., 1995, Zehnder et al., 2001). Moreover, vitamin D and its active form are involved in the process of proliferation, as well as apoptosis, through interference with the cell cycle. Presumably this is done by inhibiting the tumour cell's mitotic cycle in the G0/G1 phase through the calcitriol receptor VDR complex (Muindi et al., 2002). Interestingly, our

study showed that the highest doses of calcidiol (10^{-9} - 10^{-7} M) inhibited cell proliferation. This result is consistent with previous studies, by (Zehnder et al., 2002) who showed that treatment with 10^{-7} M calcidiol for 72 hours inhibited the proliferation of HUVECs. Hereby, this result demonstrated that the anti-proliferative action of calcidiol on HUVECs might be due to autocrine activation by 1-alpha-hydroxylase (CYB27b1) enzyme.

In contrast to other studies, our results showed that low concentration of calcitriol (10^{-13} M) significantly induced proliferation of HUVECS after 24, and 48 hours. In contrast, recent study (Pittarella et al., 2015) showed that administration of different doses of calcitriol (ranging from 10^{-8} - 10^{-7} M) to HUVECs induced a significant increase in cell proliferation after 48 hours. Comparatively, data from the current study showed that there was no inhibitory effect of calcitriol on proliferation of HUVECs. In support of our finding, one study (Masood et al., 2000) who reported that despite an increase in VDR protein levels, as a result of increasing doses of calcitriol ranging from 10^{-10} - 10^{-6} M, there was no effect of calcitriol on proliferation of endothelial cells (HUVECs) after 24 hours. In contrast, (Mantell et al., 2000) reported that calcitriol (10^{-9} - 10^{-7} M) had a significant inhibitory effect on vascular endothelial growth factor, VEGF-induced cell proliferation, after 24 hours in a dose-dependent manner. Moreover, previous observations in tumour system concluded that calcitriol exhibits anti-proliferative effects among *in vitro* and *in vivo* tumour systems (Hannah and Norman, 1994, Holick, 1995, Chung et al., 2007). Moreover, (Merke et al., 1989) reported that administration of calcitriol (10^{-8} M) to cultured bovine aortic endothelial cells (BAEC) inhibited cell proliferation after 24 hours. In addition, (Chung et al., 2007) in tumour-derived endothelial cells (TDCE), the high dose of calcitriol (10^{-8} M) inhibited cell proliferation and promoted apoptosis after 48 hours. In turn, these effects were mediated by G₀/G₁ cell cycle arrest and by promoting apoptosis (Chung et al., 2007).

In conclusion, inactive form of vitamin D calcidiol has been observed to possess potent anti-proliferative effects in this experiment. In addition, these biologic effects are associated with the calcitriol concentration. The results further showed that calcidiol and calcitriol affects cell proliferation in a dose- and time-dependent manner. Additionally, a significant difference was observed in cell proliferation between the two media used: medium with growth factors and medium without growth factors. It has been observed that calcitriol (10^{-13} M) significantly enhanced the proliferation of HUVECs whereas relatively high concentration of calcidiol (10^{-9} , 10^{-7} M) significantly inhibited cell proliferation after the same incubation period: 24 and 48 hours. Lastly, this study suggests that HUVECs may be capable of synthesising the active form of vitamin D, calcitriol, implying that it might have localised (autocrine or paracrine) effects possibly related to the regulation of blood pressure, angiogenesis or both.

4

EFFECTS OF VITAMIN D ON THE EXPRESSION OF KEY GENES INVOLVED IN CARDIOVASCULAR HEALTH

4 Effects of vitamin D on the Expression of Key Genes Involved in Cardiovascular Health

4.1 Introduction

Hypertension is a major risk factor for cardiovascular disease (CVD). Observational data has reported that low serum calcidiol concentrations are viewed as a key biological predictor of an increased risk for cardiovascular diseases, including hypertension (Roger et al., 2011; Martins et al., 2007). In addition to its well-known effects on calcium and bone metabolism, vitamin D is thought to play a role in regulating the cardiovascular system, with the vitamin D receptor (VDR) expressed in several different tissues including vascular smooth muscle cells and endothelium (Schwartz et al., 1998, Tangpricha et al., 2001), suggesting a myriad of potential biological functions (Kienreich et al., 2013). Hence, the research to date has tended to investigate a wide range of non-calcitropic effects of vitamin D, via both genomic and non-genomic signalling in the cardiovascular system.

Based on previous *in vitro* and *in vivo* studies, it is reasonable to hypothesize that vitamin D may affect vascular tone within vessels via direct transcriptional (genomic pathway) influences on the expression of cellular proteins. Thus, in order to provide direct evidence of the biological action of vitamin D, human umbilical vein endothelial cells (HUVECs) were cultured in EBM-2 media with 2% fetal bovine serum containing increasing concentrations (0, 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} and 10^{-7} M) of both calcidiol and calcitriol for 24 hours. Following this, the expression of two key genes involved in the endothelial regulation of blood pressure, endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS), were measured.

4.2 Hypothesis

Both calcidiol (inactive form of vitamin D) and calcitriol (the active form), have an effect on altering the expression of two key genes involved in the endothelial regulation of blood pressure, (ET-1) and (eNOS) as well as eNOS secretion and NO production in HUVECs.

4.3 Aim

The primary aim of this study is to investigate the effect of both forms of vitamin D, calcidiol and calcitriol, on expression of 2 key genes involved in the endothelial regulation of blood pressure, (ET-1) and (eNOS).

4.4 Objective

- Investigation of the dose response effects of both forms of vitamin D, calcidiol and calcitriol on the mRNA expression of ET-1 and eNOS.
- Investigation of the dose response effects of both forms of vitamin D, calcidiol and calcitriol on the mRNA gene expression of VDR and the vitamin D hydroxylating enzyme 1-alpha-hydroxylase mRNA (CYP27b1 mRNA), which is involved in the metabolism of vitamin D.
- Investigation of the dose response effects of the calcitriol on the protein expression of eNOS.

4.5 Material and methods

A brief description of all experimental procedures undertaken is detailed below. A more detailed explanation of procedures is described in the Materials and Methods (Chapter 2).

4.5.1 Cell culture

Prior to the experimental procedures described below, HUVECs were cultured in 6 well plates until they reached 80-90% confluence, and then exposed to increasing concentrations (0- 10^{-7} M) of both calcidiol and calcitriol for 24 hours.

4.5.2 RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted from HUVECs using a High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions (section 2.2.3). Next, RNA integrity was assessed using NanoDrop and first-strand cDNA synthesis was carried out using the Transcriptor First-strand cDNA Synthesis Kit according to manufacturer's instructions (section 2.2.5). All target genes were normalized to total first-strand cDNA content measured using Oligreen.

4.5.3 Western blotting

Protein samples, cell lysate, were heated to 95-100° C for -5 minutes, loaded on 10% SDS-PAGE gel and subjected to electrophoresis, then transferred to nitrocellulose membranes. After blocking, blots were probed with specific primary antibodies anti-vitamin D receptor (Millipore) or anti-eNOS (Santa Cruz biotechnology, INC) followed by the appropriate secondary antibodies. The bound antibody was visualized using an enhanced chemiluminescent (ECL) detection kit (advanced western blot detection kit, Fisher Scientific) and exposed onto x-ray film.

4.5.4 Measurement of Nitric oxide

Next, HUVECS were plated out in six well plates and allowed to adhere, and then the complete cell culture medium was replaced with DMEM medium (without phenol red).

Cells were then treated in the absence and presence of calcitriol ($0-10^{-7}$ M) for 24 hours. It has been reported that endothelial cells produce NO following stimulation with acetylcholine (ACh) (Figueroa et al., 2002). Therefore, in this experiment, some cells were treated with 10^{-5} M ACh as a positive control. For the measurement of eNOS production, nitrite concentration (NO^2) was measured in cell culture supernatants using Sievers NO analyser instrument (NOA, model 280i, GE Analytical Instruments, Boulder, CO, USA) as described in Chapter 2, section 2.4.3.

4.5.5 Statistical analysis

All data from gene expression, protein and nitrite levels were evaluated by one-way analysis of variance (ANOVA) using Genstat statistical analysis software (Version 17th) To identify treatments significantly different from controls, post-hoc Dunnett's tests were performed when there was a significant treatment effect determined by one-way ANOVA. Statistical significance was identified at $p < 0.05$. Data in figures is displayed as mean $\pm$ the standard error of the mean (s.e.m).

4.6 Results

4.6.1 Effects of Calcidiol on the Expression of ET-1 and eNOS mRNA

To assess the effects of calcidiol on endothelium-dependent vasodilation and contraction, HUVECs were cultured in the absence or presence of a broad range of concentrations of calcidiol (0 - 10^{-7} M) for 24 hours. Expression of all genes was normalized to total first-strand cDNA content, which was measured using Oligreen. We found that all concentrations (10^{-13} - 10^{-7} M) of calcidiol significantly decreased ET-1 mRNA relative to control ($p=0.005$, Figure 4-1A), suggesting that calcidiol might be activated to calcitriol locally in HUVECs. This result is in agreement with (Merke et al., 1989), who showed that both 1-alpha-hydroxylase (CYP27b1) and VDR are expressed in bovine aortic endothelial cells (BAEC). Alternatively, the effect of calcidiol on ET-1 mRNA expression might be indicating that calcidiol binds to VDR directly, without being activated to calcitriol by CYP27b1. In support of this, two studies (Lou et al., 2004, Lou et al., 2010) revealed that the activation of calcidiol by CYP27b1 is not essential for its biological activity. To verify these results, the incubation of HUVECs for 24 hours with calcidiol was repeated but additional concentrations. There was a similar decrease in ET-1 mRNA for 10^{-12} , 10^{-10} , and 10^{-8} M relative to control ($p=0.06$, Figure 4-1B).

In contrast to its effects on ET-1, a high physiological concentrations (10^{-11} M) of calcidiol tended to increase eNOS mRNA relative to control ($p=0.06$, Figure 4-2A). In the repeat experiment, when HUVECs were stimulated with extra doses of calcidiol, there was a significant increase ($P=0.005$) in eNOS mRNA relative to control, particularly with 10^{-11} M calcidiol (Figure 4-2B). However, supra-physiological concentrations (10^{-8} M) of calcidiol, which is higher than the concentrations found in blood (50-180 pmol/l) (Kragt et al., 2009), reduced eNOS mRNA back to control (DMSO) levels (Figure 4-2), possibly suggesting toxic effects at these higher

concentrations as resulting in decrease DNA content and thereby potential cell death (Figure 3-1A).

Therefore, despite supposedly being an inactive form of vitamin D, calcidiol appears to have biologically relevant effects on cultured HUVECs. In fact, the results of this study demonstrate that physiological doses (10^{-11} M) of calcidiol alter gene expression, decreasing ET -1 mRNA and increasing eNOS mRNA. This would be expected to result in a potent vasodilation, and therefore reduced blood pressure.

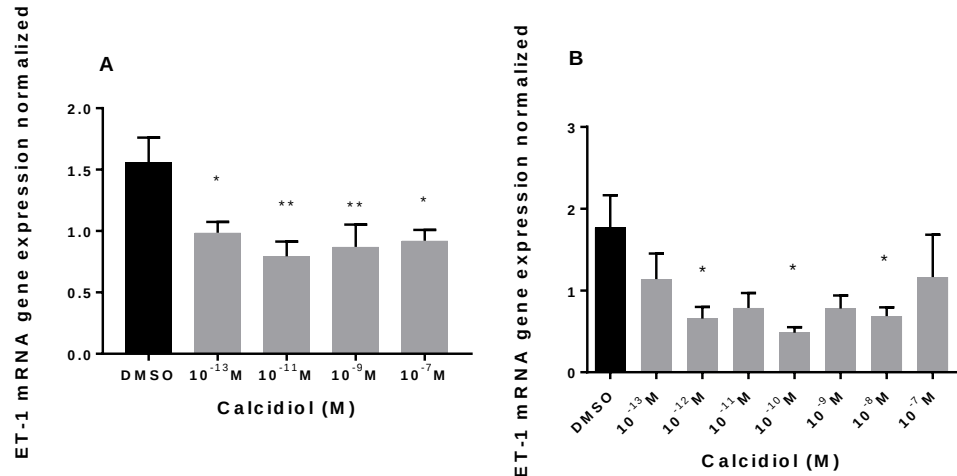


Figure 4-1 Dose-dependent effects of calcidiol on expression of ET-1 mRNA determined by quantitative RT-PCR analysis. Levels of ET-1 mRNA were quantified in HUVECs cultured in the absence or presence of increasing doses (10^{-13} to 10^{-7} M) of calcidiol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions. Mean $\pm$ s.e.m, n=8; P= 0.005 and P=0.06 for A and B respectively. *p<0.05, **p<0.01 post-hoc Dunnett's test.

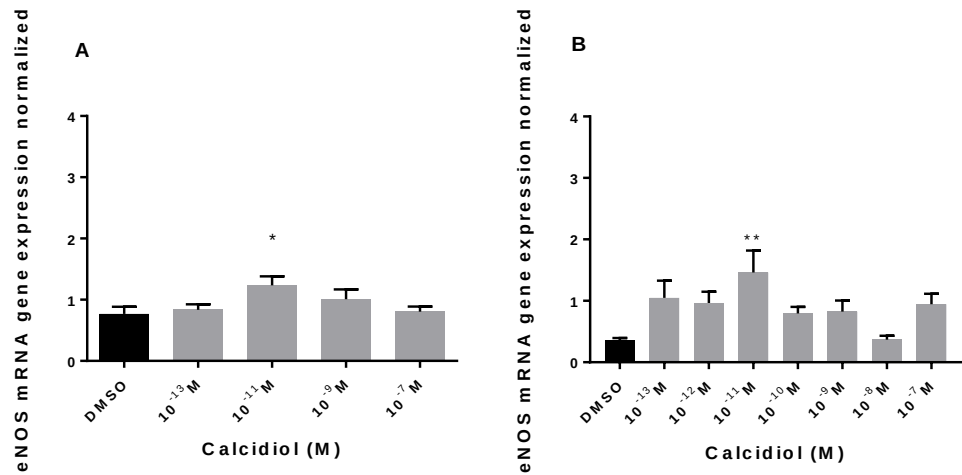


Figure 4-2 Dose-dependent effects of calcidiol on expression of eNOS mRNA determined by quantitative RT-PCR analysis. Levels of eNOS mRNA were quantified in HUVECs cultured in the absence or presence of increasing doses (10^{-13} to 10^{-7} M) of calcidiol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions. Mean $\pm$ s.e.m, n=8, P= 0.06 and p=0.005 for A and B respectively. *p<0.05, **p<0.01 post-hoc Dunnett's test.

4.6.2 Effects of Calcitriol on the Expression of ET-1 and eNOS mRNA

The results for ET-1 and eNOS mRNA expression in the presence or absence of increasing concentrations (0 - 10^{-7} M) of the calcitriol for 24 hours are shown in Figures 4-3 and 4-4. As can be seen, ET-1 mRNA was not significantly decreased by increasing concentrations (10^{-13} to 10^{-7} M) of calcitriol relative to control ($p=0.108$, Figure 4-3A). However, in the repeat experiment with 10^{-12} M calcitriol, a similar trend was observed wherein ET-1 mRNA expression tended to be decreased relative to control ($p=0.06$, Figure 4-3B).

Endothelium-derived NO is considered a major mediator of endothelium-dependent vasodilation (Landmesser et al., 2004), and consequently it has been hypothesised that the expression of this gene would be increased by calcitriol in HUVECs. However, this hypothesis was refuted by the present experiments, since eNOS mRNA expression was not significantly affected when HUVECs were treated with increasing doses (10^{-13} - 10^{-7} M) of calcitriol ($p=0.238$, Figure 4-4A). However, in the repeat experiment there is a trend for a decrease in eNOS mRNA was observed with high concentration compared to control ($p=0.075$, Figure 4-4B). There are several possible explanations for this result. For one, it might be related to rapid inactivation of calcitriol by 24-hydroxylase (CYP24a1). In support of this is the fact that the calcitriol half-life is only a few hours, whereas calcidiol has a half-life of months (Peters et al., 2004), suggesting that calcidiol is more stable and may potentially have more effect on gene expression. From the findings reported here it might be suggested that cells are generally more sensitive to calcidiol than calcitriol. Another explanation could be that different results might be observed if performed over shorter (4-6 hours) or longer (48 hours) periods of time.

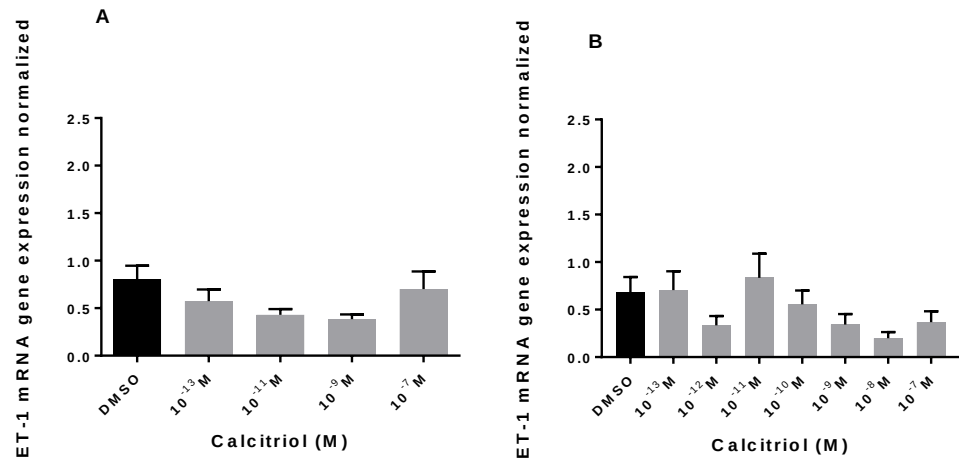


Figure 4-3 Dose-dependent effects calcitriol on expression of ET-1 mRNA determined by quantitative RT-PCR analysis. Levels of ET-1 mRNA were quantified in HUVECs cultured in the absence or presence of increasing concentrations (10^{-13} to 10^{-7} M) of calcitriol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions. Mean $\pm$ s.e.m, n=8; P=0.108 and p=0.063 for A and B respectively.

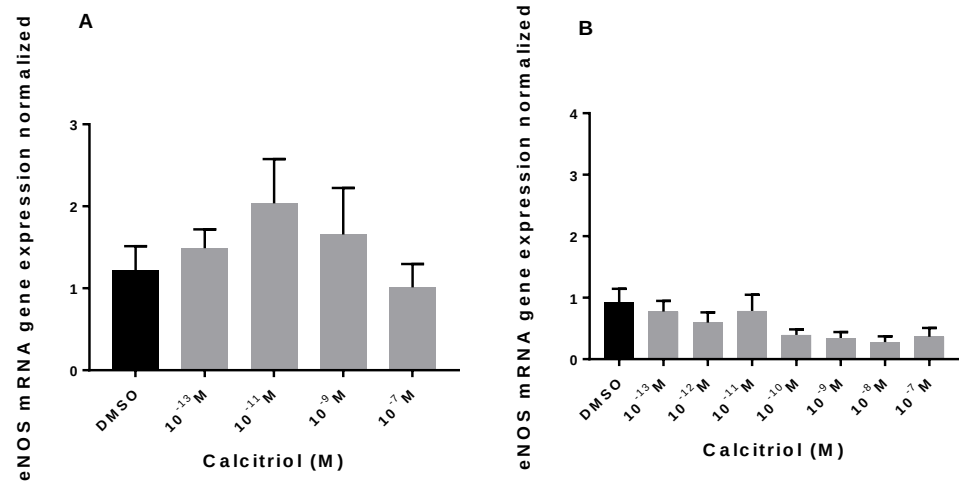


Figure 4-4 Dose-dependent effects of calcitriol on expression of eNOS mRNA determined by quantitative RT-PCR analysis. Levels of eNOS mRNA were quantified in HUVECs cultured in the absence or presence of increasing concentrations (10^{-13} to 10^{-7} M) of calcitriol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions. Mean $\pm$ s.e.m, n=8; P=0.238 and p=0.075 for A and B respectively.

4.6.3 Effects of Calcidiol and Calcitriol on Expression of vitamin D receptor (VDR) mRNA and the vitamin D hydroxylating enzyme, 1-alpha-hydroxylase mRNA (CYP27b1 mRNA)

The available evidence indicates the presence of a micro endocrine system in endothelial cells, including VDR and 1-alpha-hydroxylase and 24-hydroxylase (Merke et al., 1989). Numerous factors appear to determine the responsiveness to vitamin D, including VDR, 1-alpha-hydroxylase and 24-hydroxylase. In the current study, the presence of both VDR mRNA and 1 alpha- hydroxylase mRNA in the HUVECs was confirmed by qRT-PCR. The high physiological concentrations (10^{-11} M) of calcidiol significantly increased the expression of VDR mRNA ($p=0.001$ and $p=0.007$; Figures 4-5A and B, respectively). In contrast, VDR mRNA expression tended to decrease in response to the highest concentrations (10^{-10} M and above). These results were mimicked by calcitriol, the active form of vitamin D, ($p=0.038$ and $p=0.002$; Fig 4-7A and B).

In terms of the vitamin D hydroxylating enzymes, emerging evidence indicates CYP24a1, the gene responsible for inactivation of calcitriol is expressed in various human tumours (Bortman et al., 2002, Anderson et al., 2006). The findings (data not shown) reported here show it was not expressed in endothelial cells. This means either the primers did not work or HUVECs could not express it.

With regard to the vitamin D hydroxylating enzyme 1alpha-hydroxylase (CYP27b1) mRNA, as Figure 4-6A shows, treating HUVECs with the highest concentration (10^{-9} M) of calcidiol and the supra-physiological concentration (10^{-7} M) of calcidiol significantly decreased the expression of CYP27b1 mRNA relative to control ($p<0.001$ Fig 4-6A). To identify the dose response of CYP27b1 mRNA expression, HUVECs were exposed for 24 hours with extra doses of calcidiol, and there was no difference in the expression of CYP27b1 mRNA between treated and control cells ($p=0.124$, Fig 4-6B).

It can be seen from the data in Figures 4-8A and B that a low physiological concentration (10^{-13}M) of calcitriol increased the expression of CYP27b1 mRNA relative to control ($p=0.005$ and $p<0.001$, respectively). Interestingly, the high physiological concentration (10^{-9}M) and supra-physiological concentrations (10^{-7} and 10^{-8}M) of calcitriol decreased the expression of this gene. The most striking result to emerge from the data is shown in Figure 4-8B, where the physiological concentration 10^{-12}M of calcitriol significantly decreased the expression of CYP27B1 mRNA relative to control ($p<0.001$).

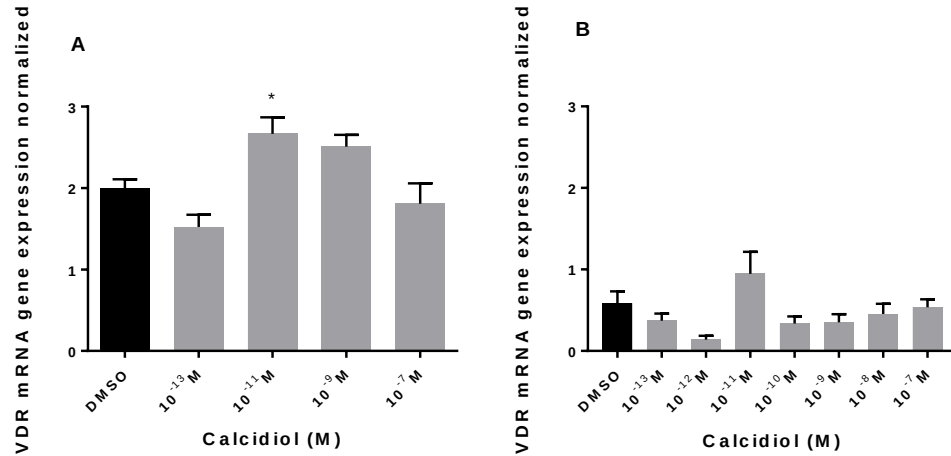


Figure 4-5 Dose-dependent effects of calcidiol on expression of VDR mRNA determined by quantitative RT-PCR analysis. Levels of VDR mRNA were quantified in HUVECs cultured in the absence or presence of (10^{-13} to 10^{-7} M) calcidiol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions Mean $\pm$ s.e.m, n=8; P= 0.001 and p=0.007 for A and B respectively) *p<0.05, post-hoc Dunnett's test

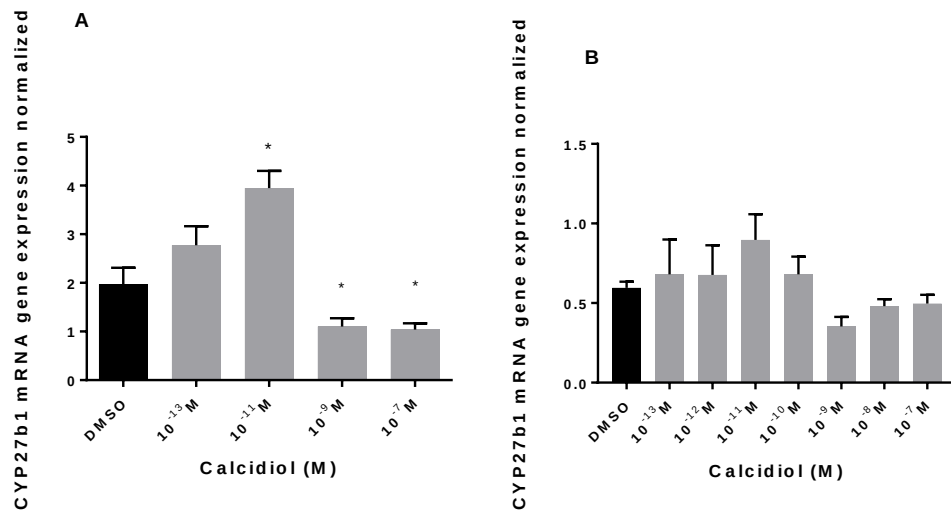


Figure 4-6 Dose-dependent effects of calcidiol on expression of CYP27b1 determined by quantitative RT-PCR analysis. Levels of CYP27b1 mRNA were quantified in HUVECs cultured in the absence or presence of (10^{-13} to 10^{-7} M) calcidiol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions. Mean $\pm$ s.e.m, n=8; P<0.001 and p=0.124 for A and B respectively. *p<0.05, post-hoc Dunnett's test

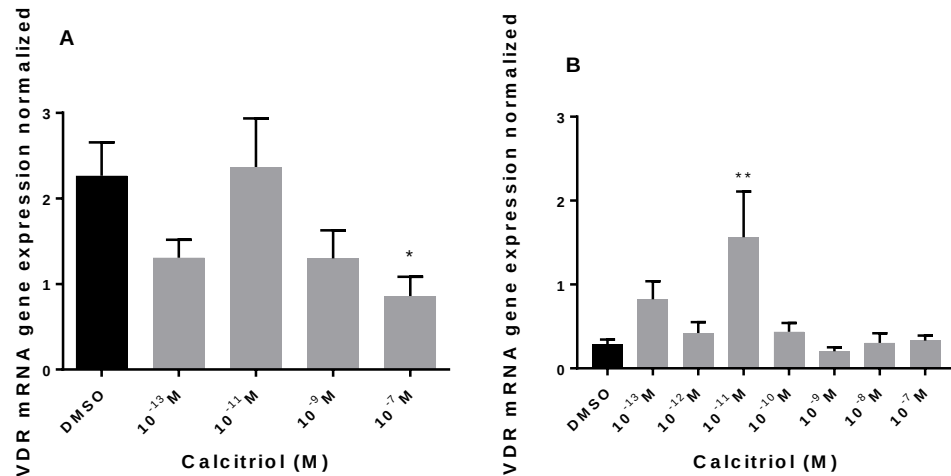


Figure 4-7 Dose-dependent effects calcitriol on expression of VDR mRNA determined by quantitative RT-PCR analysis. Levels of VDR mRNA were quantified in HUVECs cultured in the absence or presence of (10^{-13} to 10^{-7} M) calcitriol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions. Mean $\pm$ s.e.m, n=8; P= 0.038 and p=0.002 for A and B respectively. *p<0.05, **p,0.01 post-hoc Dunnentt's test

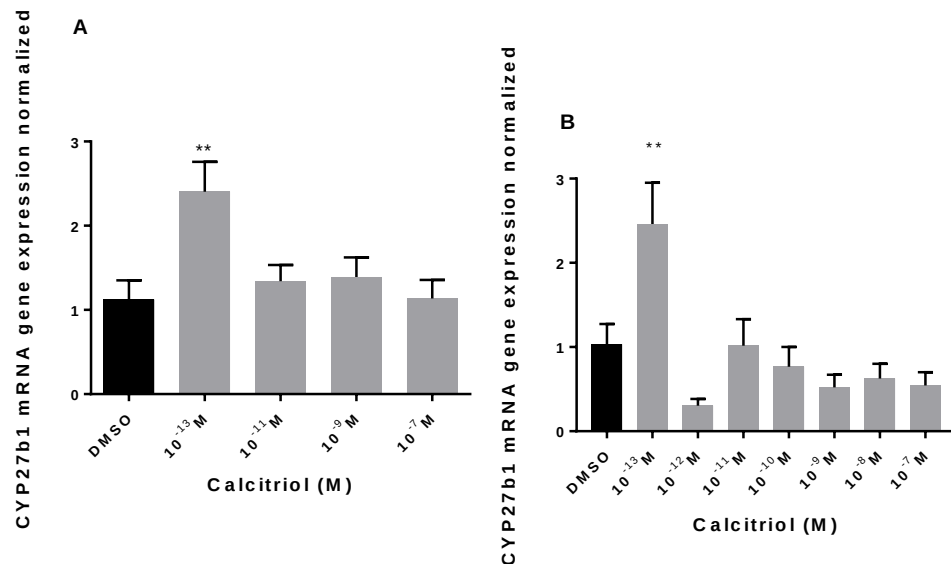


Figure 4-8 Dose-dependent effects of calcitriol on expression of CYP27b1 mRNA determined by quantitative RT-PCR analysis. Levels of CYP27b1 mRNA were quantified in HUVECs cultured in the absence or presence of (10^{-13} to 10^{-7} M) calcitriol for 24 hours. **A** shows 1000-fold dilutions, whereas **B** shows 100-fold dilutions Mean $\pm$ s.e.m, n=8; P= 0.005 and p<0.001 for A and B respectively). **p,0.01 post-hoc Dunnentt's test

4.6.4 The Presence of Vitamin D Receptor (VDR) Protein in Endothelial Cells

The expression of VDR mRNA has been demonstrated by qRT-PCR in a previous experiment. Thus, to confirm that the VDR protein is present in the HUVECs, western blot analysis was used in the present experiment. As shown in Figure 4-9, VDR was expressed at 53 kDa in untreated cells but the effects of treatment were not investigated.

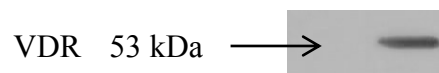


Figure 4-9 Western blot analysis of VDR in HUVECs. Representative blot showing the expression of VDR protein in HUVECs.

4.6.5 Effects of Calcitriol on eNOS Secretion and NO Production in HUVECs

To analyse the effect of calcitriol on eNOS protein and NO production, HUVECs were treated with different doses of calcitriol (0 - 10^{-7} M) for 24 hours. Western blot data showed a clear single band for eNOS at ~ 140 kDa, and there was no difference between increasing concentrations of calcitriol compared to DMSO, control (Figure 4-10A).

To further evaluate our finding that calcitriol did not have any effect on eNOS secretion in HUVECS, the nitrite content of the HUVEC culture's supernatants was measured by a chemiluminescence technique using Silvers Instruments Model 280/280i NO Analyser. In this technique, NO was detected and quantified by measuring the nitrite (NO^{2-}) anion concentration, which is a major oxidation product of NO in a cell culture system (Tiso and Schechter, 2015). As shown in Figure 4-10B, exposing cells to increasing concentrations of calcitriol did not significantly change the supernatant nitrite levels compared with control ($p=0.644$). Whereas exposing cells with 10^{-5} M of ACh, as was expected, significantly increased supernatant nitrite levels compared with control ($p<0.001$).

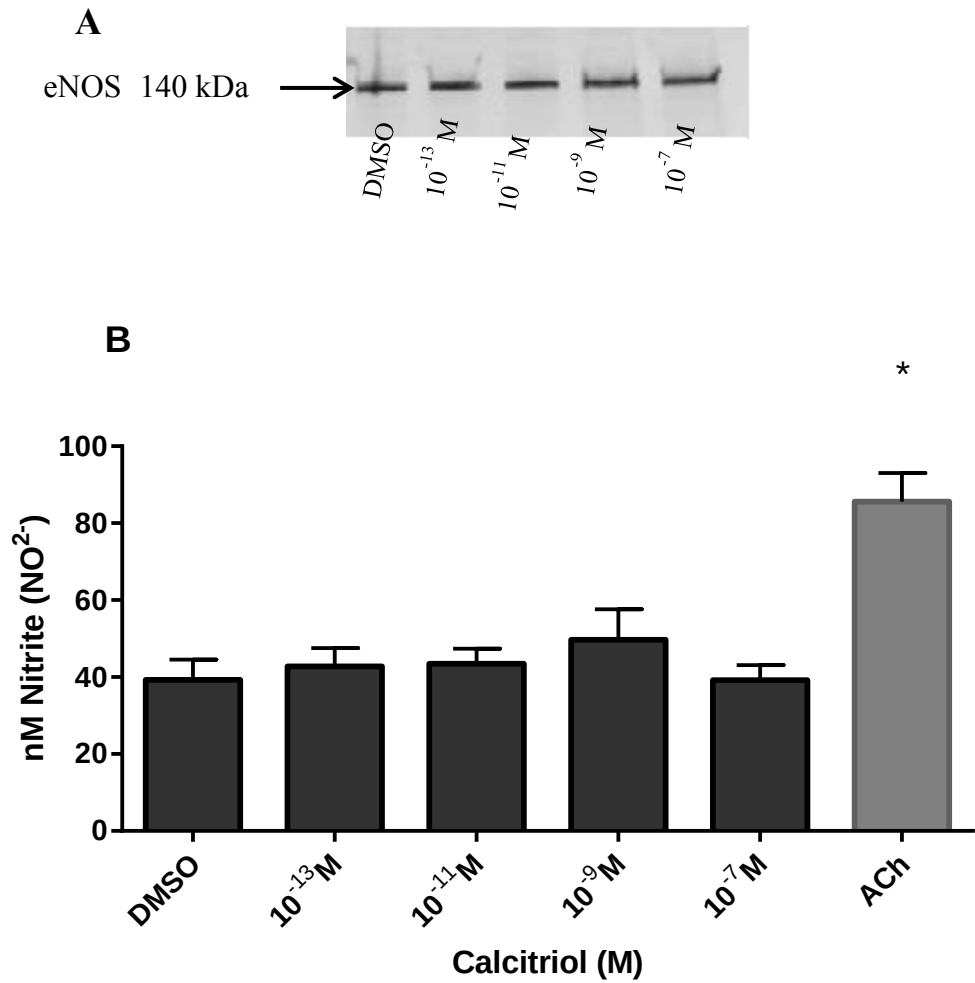


Figure 4-10 Effects of calcitriol on eNOS secretion and Nitric Oxide production in HUVECs. **(A)** western blot analysis of expression of eNOS protein in HUVECs cultured in the absence or presence of (10^{-13} to 10^{-7} M) calcitriol for 24 hours **(B)** Effect of calcitriol on production of Nitric Oxide in culture supernatants which measured through Silvers Instruments Model 280/280i NOA in the absence or presence of increasing concentrations (10^{-13} to 10^{-7} M) of calcitriol for 24 hours. Mean $\pm$ s.e.m, n=3; p=0.644 (ANOVA) or 10^{-5} M of ACh for 24 hours. (Mean $\pm$ s.e.m, n=3; p=0.005 (t.test)).

4.7 Discussion

The cellular effects of calcitriol, an active metabolite of vitamin D, are mediated by a high-affinity nuclear receptor, VDR (Chawla et al., 2001), which acts primarily by regulating the expression of genes whose promoters contain specific DNA sequences known as vitamin D response elements (VDRE) (Haussler, 1986, Rosen et al., 2012). Importantly, the fact that VDR is found in virtually all tissues, including vascular smooth muscle cells and cardiomyocytes, as well as skin, bone and liver (Lee et al., 1994, Wang et al., 2012), has led to the realization that vitamin D has multiple biological functions in regulating gene expression beyond bone tissue mineralization (Moukayed and Grant, 2013, Rosen et al., 2012). Our data confirmed the presence of VDR protein in HUVECs using western blot analysis, supporting previous studies (Lee et al., 1994, Wang et al., 2012). For instance, Andrukhova et al. (2014) used a VDR^{-/-} mouse to demonstrate that the effect of vitamin D was dependent on its interaction with VDR signalling. Despite the low affinity of calcidiol for VDR, many studies suggest that it has the ability to interact with VDR directly (Lou et al., 2010, Lou et al., 2004). In accordance with this view, Rowling et al. (2007) fed CYP27b1 KO mice a diet high in vitamin D and observed that elevated serum calcidiol sufficiently activated VDR, suggesting that high serum calcidiol concentrations can induce VDR-mediated molecular events *in vivo*. Moreover, another studies (Lou et al., 2010, Lou et al., 2004) reported that both calcidiol and calcitriol had the potential to bind the VDR but with differing affinity. Data from the current study suggested that supra-physiologic levels of calcidiol, such as 10^{-9} , 10^{-8} and 10^{-7} M, trend to decrease the expression of VDR mRNA. Furthermore, pre-treatment of HUVECs with supra-physiological levels of calcidiol at 10^{-9} , 10^{-8} and 10^{-7} M reduced CYP27b1 mRNA. This is consistent with what others have reported: that wild type (WT) mice fed a high dose of cholecalciferol D₃ 20,000 IU VD₃/kg diet for 12 weeks demonstrated reduced serum renal CYP27b1 mRNA (Rowling et al., 2007). However, (Turunen et al., 2007) found that

administering HEK 293 with 10^{-8} M of calcitriol suppressed 1 α -hydroxylase (CYP27b1) promoter activity.

Our experiments showed that HUVECs are able to express VDR and CYP27b1 mRNA. In addition, previous studies such as a study by (Verstuyf et al., 1999).who reported that extra renal 1- α -hydroxylase (CYP27b1), which is hydroxylated calcidiol to the active metabolite of vitamin D calcitriol, induced non-classical vitamin D effects. Thus, this experiment sought to investigate the effects of calcidiol and calcitriol on the expression of key genes involved in cardiovascular health via direct transcriptional (genomic pathway) influences on the expression of cellular proteins.

As hypothesized, ET-1 appeared to show a modest down regulation with calcidiol in HUVECs. This reduction occurred in all treatments of calcidiol and with 10^{-12} M calcitriol. This result is consistent with previous studies (Ben-Shoshan et al., 2007) that found increasing concentrations of calcitriol (0 - 10^{-6} M) significantly inhibited the expression of ET-1 in various human cancer cells under hypoxic conditions. Whereas (Martínez-Miguel et al., 2014) reported that 10^{-7} M of calcitriol was able to increase the synthesis of ET-1 in a cultured human endothelial cell line after 16 hours, and recent study (Widener, 2013) demonstrated that there was a non-statistically significant reduction of ET-1 mRNA expression when human embryonic kidney cells (HEK293) were exposed to 10^{-8} M of calcitriol for 24 hours; however, over 48 hours they found that 10^{-8} M of calcitriol had an effect on ET-1 mRNA expression, suggesting that the responsive effect of the active metabolite of vitamin D was a rapid one that decays over 24-48 hours. Similarly, recent study (Absi and Ward, 2013) studied the effects of chronic calcitriol treatment on intracellular signalling pathways in human coronary artery smooth muscle cells (HCASMCs), and they found that HCASMCs treated with 10^{-7} M of calcitriol for 24 hours significantly caused an increase in ET-1-induced intracellular calcium mobilization, and that the effect was mediated through endothelin receptor type A (ET_{RA}).

For its part, NO is a potent vasodilator, and it is a critical factor in maintaining vascular homeostasis by way of controlling blood pressure. Furthermore, eNOS is constitutively expressed and is responsible for the production of NO from L-arginine (Behrendt and Ganz, 2002, Cannon, 1998). The results of this study demonstrated that treating HUVECs with different concentrations of calcitriol ranging from 10^{-13} - 10^{-7} M, failed to alter eNOS mRNA expression, as well as subsequent eNOS protein and levels of nitrite. This is consistent with results reported by (Willems et al., 2012), who incubated osteoblasts cells (MC3T3-E) for 24 hours with 10^{-11} - 10^{-9} M of calcitriol. They showed that calcitriol increased expression of inducible nitric oxide synthase (iNOS) and stimulated NO production, likely via nuclear actions of the VDR, but did not alter neuronal nitric oxide synthase (nNOS) nor eNOS gene expression. In contrast, recent studies (Widener, 2013) revealed that exposing HEK293 cells to 10^{-8} M of calcitriol for 24 hours decreased the expression of iNOS. In accordance with this finding, the exposure of HUVECs to 10^{-9} , 10^{-8} and 10^{-7} M of calcidiol, which are all higher than the freely available concentrations of calcidiol in human blood (30 and 50nM) (Kragt et al., 2009), significantly decreased the expression of eNOS mRNA. Comparing results obtained by the treatment of HUVECs with calcidiol to those obtained by treating them with calcitriol revealed that calcidiol appears to have biologically relevant effects on cultured HUVECs beyond those of calcitriol. This result suggests that calcidiol might be activated locally in HUVECs, in agreement with (Merke et al., 1989), who showed that both 1- α -hydroxylase (CYP27b1) and VDR are expressed in bovine aortic endothelial cells (BAEC).

Another potential explanation is that the effects of calcidiol on ET-1 and eNOS mRNA are indicating that calcidiol binds to VDR directly, without activation by CYP27b1. In support of this, some studies (Lou et al., 2004, Lou et al., 2010) revealed that the activation of calcidiol through 1 α -hydroxylase (CYP27b1) is not essential for its biological activity. Moreover, Lou et al. (2004, 2010) reported that both calcidiol and

calcitriol had the potential to bind the VDR but with differing affinity. In contrast, in organ culture, (Andrukhova et al., 2014) concluded that pre-treatment of aortic rings from Wild-type (WT) mice with 10^{-8} M of calcitriol and 10^{-7} M of calcidiol induced up regulation of eNOS mRNA expression *in vitro*, whereas this effect was abrogated in aortic rings from VDR mutant mice, implying that this effect is VDR dependent.

To conclude, returning to the hypothesis posed at the beginning of this study, it appears that ET-1 is a prime candidate for genes targeted by calcidiol in the prevention of hypertension.

In conclusion, the current experiments demonstrated that, at the molecular level, calcidiol affected the expression of genes involved in cardiovascular health. Importantly, calcidiol affects gene expression at physiological concentrations and also induces the expression of the activating enzyme 1-alpha-hydroxylase (CYP27b1) and vitamin D receptor (VDR). The finding that calcidiol, at physiological concentrations, possesses an inherent hormonal activity provides a novel view of the vitamin D₃ endocrine system in HUVECs, suggesting that calcidiol may offer a cardio-protective role through endothelial-mediated vasoregulatory effects of vitamin D, and therefore potentially reduce blood pressure. Moreover, calcitriol did not appear to have much effect on the expression of these genes and therefore its effects on blood vessels may relate to non-genomic mechanisms.

5

CALCITRIOL INDUCES VASODILATION IN ISOLATED RAT AORTAS

5 Calcitriol Induces Vasodilation in Isolated Rat Aortas

5.1 Introduction

In the previous chapter, it was shown that different concentrations of calcitriol ranging from 10^{-13} to 10^{-7} M, failed to alter eNOS mRNA expression, as well as subsequent eNOS protein and nitrite levels. This indicates that the effect of calcitriol appear not to be via the chronic/genomic mechanism, therefore this chapter investigates the possible effect of calcitriol via acute/non-genomic mechanism.

Recently, researchers have been investigating the rapidly occurring non-genomic effects of vitamin D, which are likely to be mediated by specific membrane receptors, beyond the classical genomic effects that typically occur over a longer period of time via nuclear hormone mediated effects of VDR on gene expression (Falkenstein et al., 2000, Hii and Ferrante, 2016). Non-genomic actions, which occur within seconds or minutes, involve a variety of signal transduction activities, such as calcium (Ca^{2+}) influx and the release of Ca^{2+} from intracellular stores (Sylvia et al., 1996, Santillán and Boland, 1998, Vazquez et al., 1995, Barrera et al., 2008, Wali et al., 1990, Bellido et al., 1993, Morelli et al., 1993). These activities also include the modulation of adenylate cyclase (AC), phospholipase C (PLC) (Rebsamen et al., 2002), and protein kinase activities (e.g. MAP kinase) and facilitate changes in the phosphorylation status of cellular proteins and other traditional messengers (de Boland and Nemere, 1992, Falkenstein et al., 2000, Hii and Ferrante, 2016, Rebsamen et al., 2002).

This study, therefore, aimed to investigate the direct dose-dependent effects of calcitriol on the contraction or relaxation of aortic rings, as well as the mechanism(s) involved. To succeed in this, thoracic/abdominal segments of aortas were isolated from adult female Wistar rats and their contractility was studied using a Wire Myograph System, as first described by Mulvany and Halpern (Mulvany and Halpern, 1976).

5.2 Summary of the Signalling Pathway of Vascular Smooth Muscle Cells for the Modulation of Contraction and Relaxation

The potential signalling pathways of vascular smooth muscle cells that modulate the contraction and relaxation in response to certain stimuli are summarised in the diagram below (Figure 5-1). In this Figure, red circles indicate the vasoconstrictors used in this chapter. Briefly, angiotensin II (Ang II) and phenylephrine (PE), bind to receptors, leading to the activation of phospholipase C (PLC) signalling pathway, which is the classic Ca^{2+} dependent pathway. IP₃ stimulates the release of Ca^{2+} from the sarcoplasmic reticulum (SR), and extracellular calcium enters through L type Ca^{2+} channels known as voltage-operated calcium channel (VOC). Intracellular calcium levels $[\text{Ca}^{2+}]_i$ binds to calmodulin (CaM) to form Ca^{2+} /CaM complex, resulting in the activation of MLCK and initiating contraction of the MLC by phosphorylation. During the interaction with actin, cross-bridge cycling occurs, initiating the contraction of the smooth muscle cell (illustrated in more detail in chapter 1).

The green circles in the diagram, indicate the targets for the relaxation drugs used in this chapter. In brief, relaxation of vascular smooth muscle cells occurs when MLCP dephosphorylates the MLC. The activation of G protein coupled receptors by ACh stimulates activation of endothelial nitric oxide synthase (eNOS) which leads to NO production (Cannon, 1998). In smooth muscle cells, the vasodilation effect of NO is mediated by stimulating the activation of guanylate cyclase and increasing production of cGMP, leading to decreased intracellular calcium, which, in turn allows vasodilation and increased blood flow, as explained in detail in chapter 1.

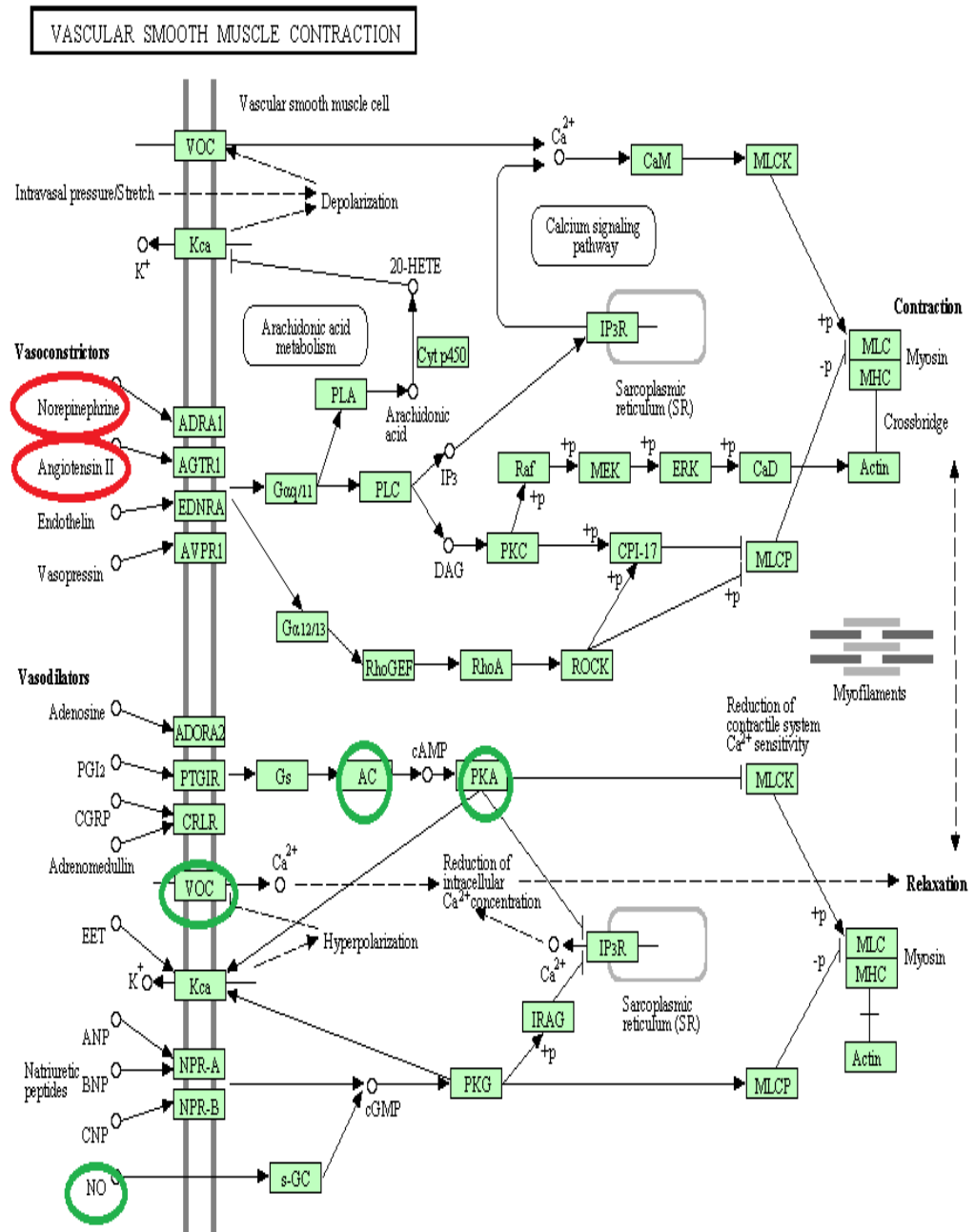


Figure 5-1 Summary of the signalling pathway of vascular smooth muscle cells for the modulation of contraction and relaxation. Red circle indicates the vasoconstrictor, while green circle indicates to inhibitors and activators were used in all experiments

http://www.genome.jp/kegg-bin/show_pathway?hsa04270

5.3 Hypothesis

The original hypothesis for this study predicted: i) calcitriol has a rapid relaxant effect on isolated rat aortas and ii) the relaxant effects of calcitriol on vascular smooth muscle cells are mediated via the adenylate cyclase (AC)/cAMP/protein kinase A (PKA) signalling pathway.

5.4 Aim

The overall aim of these studies was to investigate the acute direct effects of the active form of vitamin D, calcitriol, on the contractility of isolated rat aortas, as well as the mechanism(s) involved.

5.5 Objectives

- Investigation of the effect of calcitriol on the relaxation of arteries that are stimulated with either phenylephrine (PE) or angiotensin II (Ang II).
- Investigation of the effect of cumulative doses of calcitriol on the response to known regulators, including phenylephrine (PE) and acetylcholine (ACh).
- Determination of the reversibility of the vasodilatory effect of calcitriol.
- Comparison of the relaxing effects of calcitriol in intact and endothelium-denuded arteries.
- Investigation of the involvement of the L-type calcium channel in the relaxant effect of calcitriol.
- Investigation of the mechanism underlying the relaxation induced by calcitriol.

5.6 Material and methods

The Experimental procedures described here are a brief overview only. A full comprehensive explanation of procedures undertaken herein can be found in the Materials and Methods (Chapter 2).

5.6.1 Animal Experiment

All procedures were approved by the University of Nottingham Ethical Review Committee and this study was conducted in accordance with the UK Home Office regulations and the UK Animals (Scientific Procedures) Act, 1986.

5.6.2 Isometric Force Measurement

After the rats were killed (as described in chapter 2, section 2.5.4) the thoracic and abdominal aorta was isolated and dissected under a light microscope. Four segments were mounted in pair of pins in a DMT myograph chamber (the multi wire myograph system model 620M [Danish Myo Technology, A/S]) (section 2.5.4). The aortic rings were then equilibrated in Krebs solution and maintained under an optimal tension of 1 g for 60 minutes, and subsequently contracted with 60mM KCl to check the viability of vessels and to obtained maximal contraction before the experimental procedure began. Isometric force was recorded by a force transducer connected to a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO).

For the relaxation studies, aortic rings were pre-contracted with cumulative doses of phenylephrine (PE, 10^{-9} - 10^{-5} M) or Angiotensin II (Ang II, 10^{-11} - 10^{-8} M) until a stable contraction over two minutes was obtained, then arteries were treated with calcitriol (10^{-13} - 10^{-7} M) or acetylcholine (ACh, 10^{-9} - 10^{-5} M), which were added cumulatively to the bath until a maximal relaxation response was achieved.

For certain experiments, to establish whether the observed effects of calcitriol might alter the response to known regulators, all vessels were washed out for 15 minutes before they were treated with increasing concentrations of phenylephrine (PE, 10^{-9} to

10⁻⁷M) to induce vasoconstriction, followed by increasing concentrations of acetylcholine (ACh, 10⁻⁹ to 10⁻⁵M) to induce vasodilation.

For experiments to investigate the mechanism incorporating inhibitors/ activators, aortic rings were pre-incubated with different drugs prior to treatment with calcitriol or other agents. A brief description of the inhibitors/ activators and concentrations employed for each experiment is described below:

- ❖ **L-NAME** (Sigma Aldrich, UK): endothelial NO synthase inhibitor, namely Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 10⁻⁴ M).
- ❖ **Verapamil** (Sigma Aldrich, UK): In order to investigate the role of intracellular Ca²⁺ in relaxant responses induced by calcitriol, arteries were incubated with 10⁻⁴M L-NAME for 30 minutes, and exposed to vehicle (DMSO) or increasing concentrations of the Ca²⁺ channel blocker, verapamil (10⁻¹⁰-10⁻⁶M).
- ❖ **FPL 64176** (Sigma Aldrich, UK): The role of Ca²⁺ in the relaxant responses of calcitriol were tested using a potent Ca²⁺ channel (L-type) activator, FPL 64176. This experiment was designed to clarify the mechanism of action of calcitriol on rat arteries by comparing its action with verapamil, which is a Ca²⁺ channel blocker.
- ❖ **SQ 22,536** (Sigma Aldrich, UK): (Adenylate Cyclase inhibitor (AC) namely SQ22, 536 (10⁻⁵ M)).
- ❖ **H-89** (Sigma Aldrich, UK): dihydrochloride hydrate (PKA inhibitor) (H-89, 10⁻⁶ M).

5.6.3 Statistical analyses

Results are presented as mean ± s.e.m with n referring to the number of aortic rings, and the numbers of rats used also indicated. Other data are represented as a percentage of the maximum contraction induced by Ang II. Data were analysed using Students T-test for comparison of two groups, repeated measures ANOVA or 2-way repeated

measures ANOVA using Genstat statistical analysis software (Version 17th). Differences were considered to be statistically significant when p was at <0.05 .

5.7 Results

5.1.1 The Active Form of Vitamin D, Calcitriol, Induces Relaxation in Rat Thoracic/Abdominal Arteries

At the start of each experiment and following an equilibration period, arterial rings were exposed to 60 mM KCl (Kpss) to determine the maximum contractile response, before then washing and allowing the vessels to re-equilibrate again. In all experiments, 60 mM KCl caused a strong contraction (Figure 5-2A; $p < 0.001$). As shown in Figure 5-2B, from a rested (untreated) state, calcitriol elicited a dose-dependent (10^{-13} to 10^{-7} M) relaxation compared to vehicle (DMSO) control ($p = 0.007$ for interaction). The highest concentration of calcitriol (10^{-7} M) induced a marked relaxation, compared to DMSO-treated control vessels in which no change in force was observed.

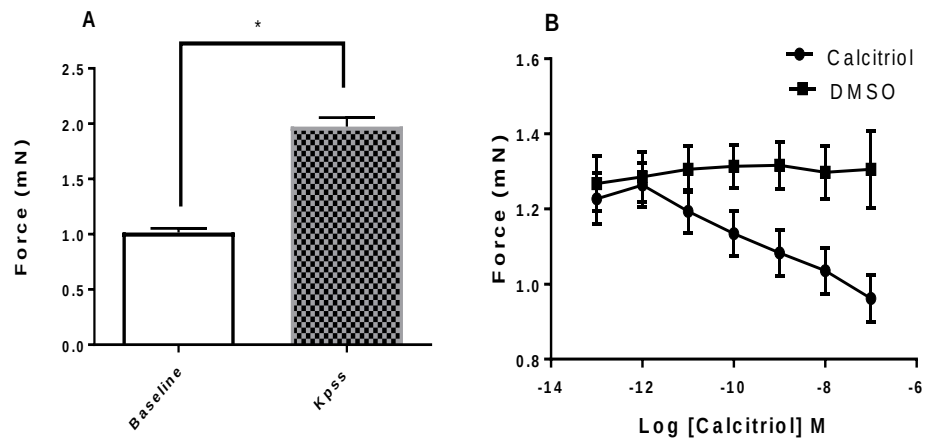


Figure 5-2 The active form of vitamin D, calcitriol, induces relaxation in rat thoracic/abdominal arteries. **(A)** 60 mM KCl (Kpss) induced contractions in thoracic/abdominal rings of rat aorta ($p < 0.001$ [t test]). **(B)** Dose-dependent vasorelaxation induced by calcitriol ($p = 0.007$ for interaction (2-way repeated measures ANOVA)). The values on the x-axis represent the increasing concentration of calcitriol used, however for the DMSO (control) group there was no increase in concentration but the vehicle was added at the same time as the concentration of calcitriol, therefore these values represent the force developed by the DMSO controls at the point when the indicated dose of calcitriol was administered to the test thoracic/abdominal arteries All data are shown as means $\pm$ s.e.m of aortic rings ($n = 5-15$ per treatment group) from five different rats.

Pre-treatment with calcitriol tended to reduce/inhibit the dose-dependent (10^{-9} to 10^{-5} M) contraction induced by phenylephrine (PE) compared to DMSO pre-treatment ($p=0.072$ for interaction, Figure 5-3A) and there was a significant inhibition of the subsequent dose-dependent (10^{-9} to 10^{-5} M) relaxation induced by Acetylcholine (ACh, $p<0.001$ for interaction, Figure 5-3B).

To determine the reversibility of the vasodilatory effect of calcitriol, further experiments ($n=3$ rats) were carried out, whereby the arteries were washed for 15 minutes after adding the highest concentration (10^{-7} M) of calcitriol. In this case, increasing concentrations of PE (10^{-9} to 10^{-5} M) induced dose-dependent contractions (Figure 5-3C; $p<0.001$), with subsequent addition of acetylcholine (10^{-9} to 10^{-5} M) also causing significant relaxation (Figure 5-3D; $p=0.002$) in rings treated previously with either calcitriol or DMSO followed by a washout (PE, $p=0.251$ and ACh, $p=0.271$ for interactions). This indicated that the acute vasodilatory effect of calcitriol was reversible and the vessels remained responsive to other regulators.

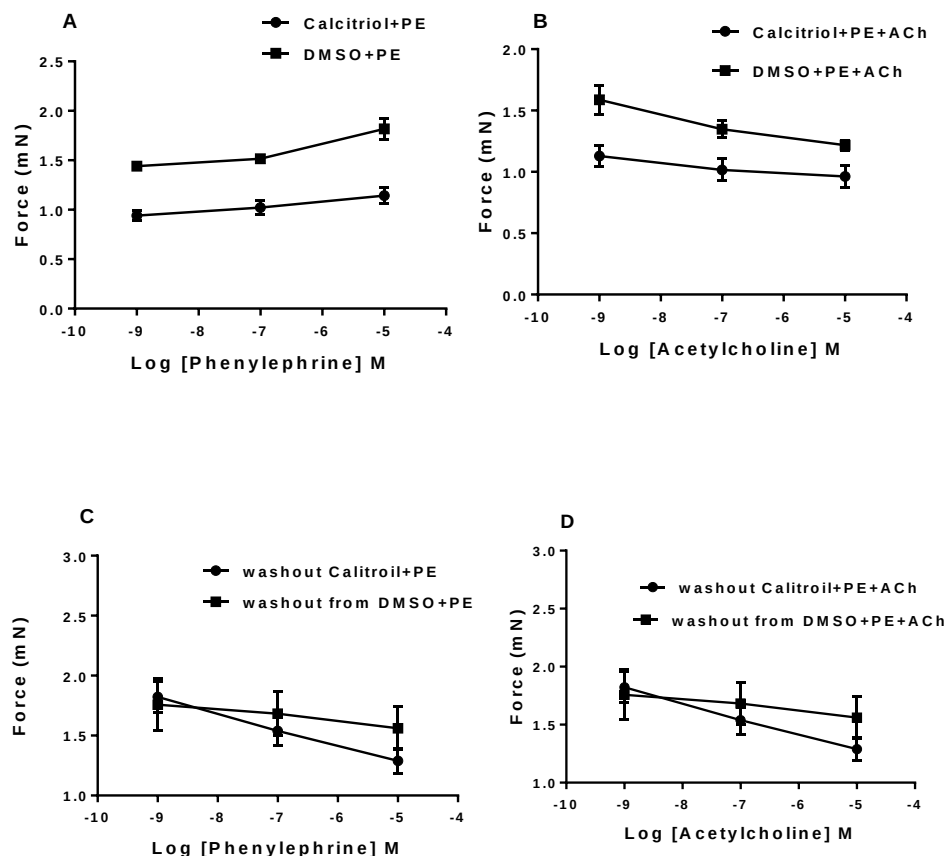


Figure 5-3 Effects of calcitriol on the response of rat thoracic/abdominal arteries to phenylephrine (PE) and Acetylcholine (ACh). Pre-treatment with calcitriol **(A)** tended to inhibit the vasoconstriction induced by phenylephrine (PE) and subsequently **(B)** significantly inhibited the subsequent dose-dependent (10^{-9} to 10^{-5} M) relaxation induced by Acetylcholine ($p=0.072$ and $p<0.001$ for interactions respectively (2-way repeated measures ANOVA)). The values on the x-axis represent the increasing concentration of calcitriol used, however for the DMSO (control) group there was no increase in concentration but the vehicle was added at the same time as the concentration of calcitriol, therefore these values represent the force developed by the DMSO controls at the point when the indicated dose of calcitriol was administered to the test thoracic/abdominal arteries. All data are shown as means $\pm$ s.e.m of aortic rings ($n=5-15$ per treatment group) from five different rats. In further experiments ($n = 3$ rats, $n=3-9$ aortic rings per treatment). Following the incubation in **(B)** the calcitriol was washed out for 15 min, before vessels were treated with **(C)** increasing concentrations of PE, followed by **(D)** increasing concentrations of acetylcholine (ACh). In both cases there was no effect of the preincubation with calcitriol on the response to PE or ACh ($p=0.251$, $p=0.271$ for interactions respectively (2-way repeated measures ANOVA), with PE inducing a consistent dose-dependent vasoconstriction ($p<0.001$) and ACh inducing a dose-dependent vasodilation ($p=0.002$).

5.7.1 Effects of Calcitriol on the Contraction of Rat Thoracic/Abdominal Arteries Induced by Phenylephrine

The calcitriol-induced vasorelaxation was investigated further by firstly stimulating rings with increasing concentrations of PE (10^{-9} - 10^{-5} M) until a stable contraction over two minutes was obtained (Figure 5-4A; $p < 0.001$). Cumulative doses of calcitriol caused a concentration dependent relaxation of arteries pre-treated with an accumulating dose of PE (10^{-9} - 10^{-5} M), with no effect of DMSO control (Figure 5-4B; $p < 0.001$ for interaction). Subsequent addition of ACh (10^{-9} to 10^{-5} M) caused significant relaxation in rings treated previously with PE and the DMSO control, whereas there was very little response to ACh in rings treated previously with PE and calcitriol (Figure 5-4C; $p < 0.001$ for interaction), suggesting that the calcitriol had already induced maximal relaxation.

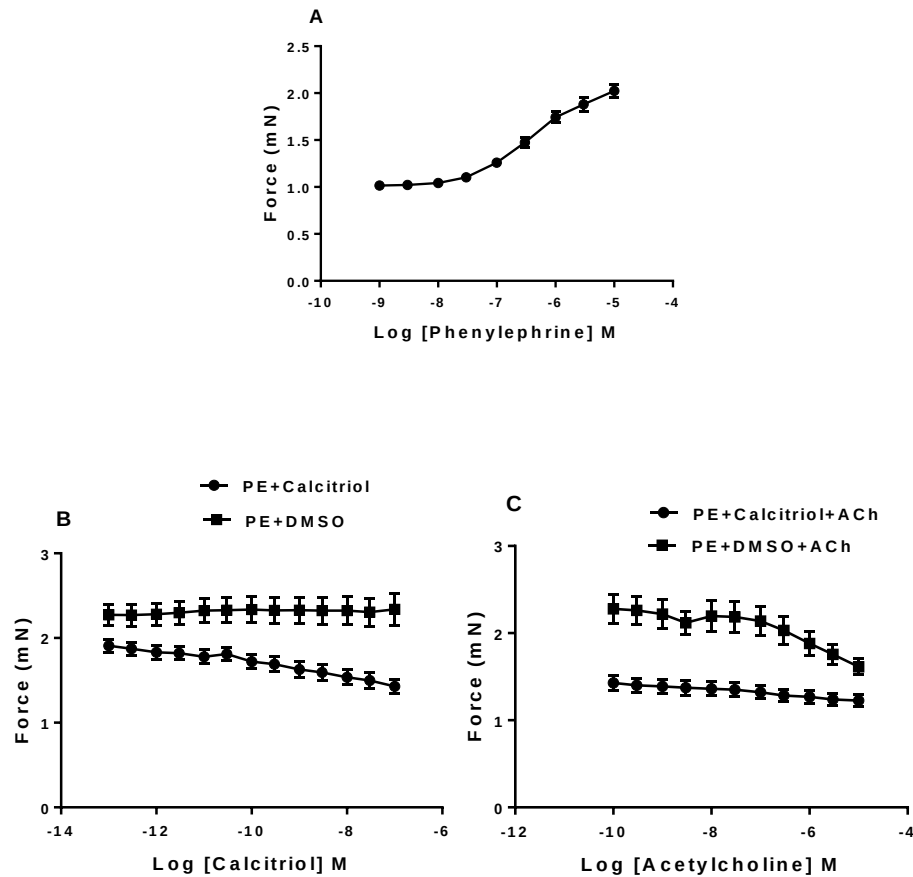


Figure 5-4 Effects of calcitriol on the contraction of rat thoracic/abdominal arteries induced by phenylephrine. **(A)** Increasing concentrations of PE (10^{-9} to 10^{-5} M) induced significant vasoconstriction of isolated rat arteries ($p < 0.001$). **(B)** Increasing concentrations (10^{-13} – 10^{-7} M) of calcitriol induced relaxation of arterial rings pre-treated with PE (10^{-9} - 10^{-5} M), but there was no effect of vehicle (DMSO; $p < 0.001$ for interaction (2-way repeated measures ANOVA). The values on the x-axis represent the increasing concentration of calcitriol used, however for the DMSO (control) group there was no increase in concentration but the vehicle was added at the same time as the concentration of calcitriol, therefore these values represent the force developed by the DMSO controls at the point when the indicated dose of calcitriol was administered to the test thoracic/abdominal arteries. **(C)** Acetylcholine (10^{-10} to 10^{-5} M) induced relaxation in vessels treated previously with PE and DMSO control, but this magnitude of induced relaxation was reduced in vessels previously treated with PE and calcitriol ($p < 0.001$ for interaction (2-way repeated measures ANOVA)). All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n=6-18$ per treatment group) derived from three different rats.

5.7.2 Role of Endothelial Nitric Oxide in the Relaxant Effects of Calcitriol on Rat Thoracic/Abdominal Arteries

The role of nitric oxide (NO) in calcitriol-induced relaxation was investigated by adding the potent endothelial NO synthesis inhibitor, N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME, 10^{-4} M) for 30 min prior to the addition of vehicle (DMSO) or increasing concentrations of calcitriol (10^{-13} M to 10^{-7} M). Incubating the arteries with L-NAME (10^{-4} M) resulted in a significant induction of contraction ($p < 0.001$, Figure 5-5A). However, pre-incubation with L-NAME did not affect the relaxation response to the increasing concentrations of calcitriol (Figure 5-5B, $p < 0.001$ for interaction).

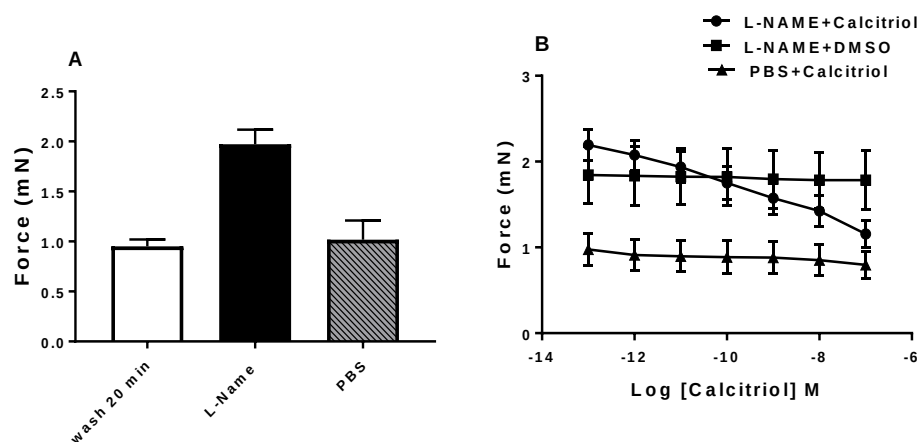


Figure 5-5 Role of endothelial nitric oxide in the vasodilatory effects of calcitriol of rat thoracic/abdominal arteries. **(A)** Pre-incubation with L-NAME (10^{-4} M) for 30 min induced significant contraction in isolated rat arteries compared to PBS Control ($p < 0.001$ (ANOVA)). **(B)** Pre-incubation with L-NAME had no effect on subsequent calcitriol-induced relaxation ($p < 0.001$ for interaction (2-way repeated measures ANOVA). The values on the x-axis represent the increasing concentration of calcitriol used, however for the DMSO (control) group there was no increase in concentration but the vehicle was added at the same time as the concentration of calcitriol, therefore these values represent the force developed by the DMSO controls at the point when the indicated dose of calcitriol was administered to the test thoracic/abdominal arteries. All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n=5-15$ per treatment group) derived from five different rats.

5.7.3 Comparison of Relaxant Effects of Calcitriol in Intact and Endothelium-Denuded Arteries on Rat Thoracic/Abdominal Arteries.

To further investigate the role of the endothelium in the calcitriol-induced relaxation, the effects were analysed in mechanically endothelium-denuded arteries compared with intact vessels. Before loading onto the myography probes, the endothelium was gently damaged mechanically (i.e. denuded). Following an equilibration period of approximately 60 min, arterial rings were exposed to 60 mM KCl to obtain submaximal contraction. KCl (60 mM) significantly induced contractions in both intact and denuded arteries (Figure 5-6A; $p < 0.001$).

Intact and denuded rings were pre-contracted using 10^{-9} to 10^{-5} M PE, which induced a concentration-dependent contraction ($p < 0.001$) in both intact and denuded vessels (Figure 5-6B; $p = 0.155$ for interaction). However, only the contracted intact arteries were significantly relaxed by subsequent addition of increasing concentrations (10^{-9} to 10^{-5} M) of ACh (Figure 5-6C; $p = 0.004$ for interaction), presumably due to ACh stimulating eNOS activity and NO formation in the endothelium, which could not occur in the denuded arteries.

The arterial rings were then washed and allowed to re-equilibrate again. Once the arterial rings were restored to the rested state, they were treated with increasing concentrations of PE (10^{-9} to 10^{-5} M) again, which induced contractions in a dose-dependent manner similar to Figure 5-6A. Subsequent addition of increasing concentrations of calcitriol (10^{-13} to 10^{-7} M) induced a concentration-dependent relaxation ($p < 0.001$) in both denuded and intact endothelium arteries (Figure 5-6D; $p = 0.307$ for interaction), suggesting that the endothelium was not involved in the relaxant effects of calcitriol.

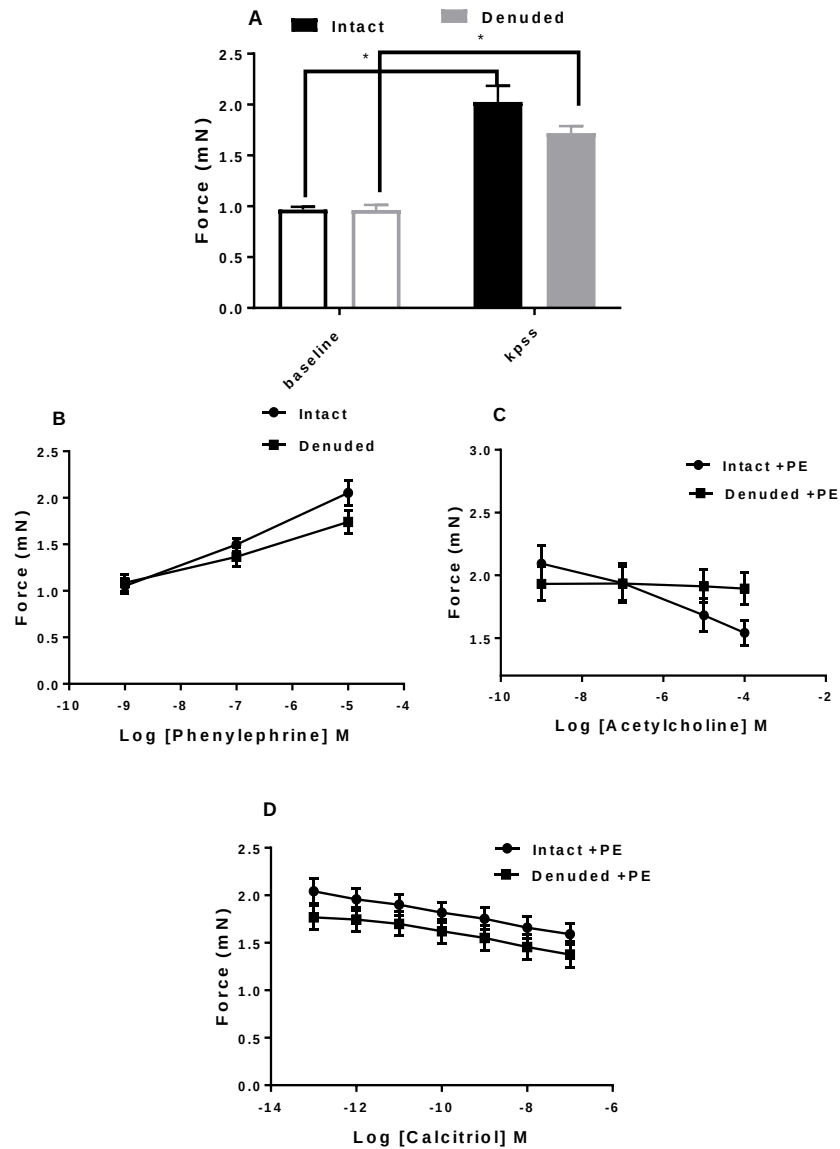


Figure 5-6 Comparison of relaxant effects of calcitriol in intact and endothelium-denuded rat thoracic/abdominal arteries. **(A)** Influence of 60 mM KCl (Kpss) on the contraction responses of both intact and denuded rat arteries ($p < 0.001$). **(B)** Contraction responses of intact and denuded arteries to increasing concentrations (10^{-9} to 10^{-5} M) of PE ($P < 0.001$ for PE dose and $p = 0.155$ for interaction (2-way repeated measures ANOVA)). **(C)** Subsequent relaxation responses of intact and denuded arteries to increasing concentrations (10^{-9} to 10^{-5} M) of Ach following pre-treatment with PE (10^{-9} to 10^{-5} M) ($P = 0.004$ for interaction (2-way repeated measures ANOVA)). **(D)** Relaxation responses of intact and denuded arteries following washout and pre-treatment with PE (10^{-9} to 10^{-5} M) to increasing concentrations (10^{-13} to 10^{-7} M) of calcitriol ($p < 0.001$ for calcitriol dose and $p = 0.307$ for interaction (2-way repeated measures ANOVA)). All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n = 10$ per treatment group) derived from five different rats.

5.7.4 Involvement of the L-type Calcium Channel in the Relaxant Effect of Calcitriol

5.7.4.1 Effects of the Ca²⁺ Channel Blocker, Verapamil, on L-NAME-Induced Vasoconstriction of Rat Thoracic/Abdominal Arteries.

These experiments were carried out to investigate the role of calcium channels in the vasorelaxant effect of calcitriol. First of all, to investigate the role of L-type calcium channels in vasoconstriction/relaxation, arterial rings were exposed to increasing concentrations of the Ca²⁺ channel blocker, verapamil (10⁻¹⁰ to 10⁻⁶ M), following pre-incubation with either L-NAME (10⁻⁴ M) or PBS as a control (Figure 5-7A). The L-NAME-induced contraction was significantly attenuated by addition of the highest dose of verapamil compared to DMSO, and maximum relaxation was reached after incubation for a further 20 min, but there was no effect of verapamil following pre-treatment with PBS control (Figure 5-7B, $p=0.004$ for interaction), presumably because the vessels were already relaxed.

As shown in Figure 5-7C, the relaxant effect of verapamil was still apparent and did not alter ($p=0.420$ for interaction) the dose-dependent (10⁻⁹ to 10⁻⁵ M) PE-induced contraction (Figure 5-7C; $p<0.001$). In contrast, verapamil did appear to inhibit/block the subsequent dose-dependent (10⁻⁹ to 10⁻⁵ M) relaxation induced by ACh (Figure 5-7D; $p=0.010$ for interaction).

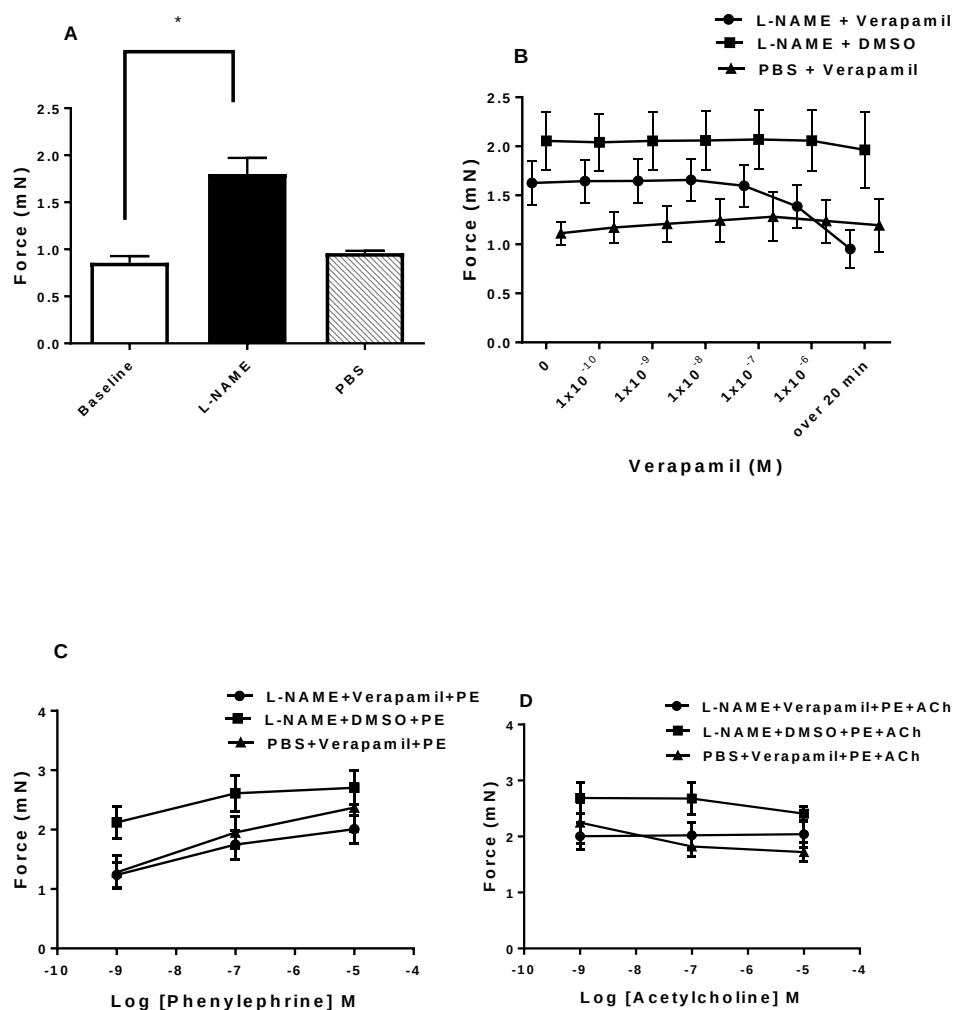


Figure 5-7 Effects of the Ca^{2+} channel blocker, verapamil, on L-NAME-induced vasoconstriction of rat thoracic/abdominal arteries. **(A)** Incubation of vessels with L-NAME (10^{-4} M) induced contraction ($p < 0.001$ (ANOVA)), with no effect of PBS. **(B)** Verapamil induced a potent vasorelaxation in vessels pre-treated with L-NAME, but not in vessels exposed to PBS control ($p = 0.004$ for interaction (2-way repeated measures ANOVA)). **(C)** Pre-treatment with verapamil (after L-NAME or PBS) had no effect on the vasoconstriction induced by PE ($p < 0.001$ for PE dose, $p = 0.420$ for interaction (2-way repeated measures ANOVA)); whereas **(D)** pre-treatment with verapamil (after L-NAME or PBS) did inhibit the vasorelaxation induced by subsequent treatment with ACh ($p = 0.010$ for interaction (2-way repeated measures ANOVA)). All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n = 4$ -12 per treatment group) derived from four different rats.

5.7.4.2 Effects of the Ca²⁺ Channel activator FPL-64176 (FPL) on Calcitriol-Induced Vasorelaxation in Rat Thoracic/Abdominal Arteries.

Based on previous results, the relaxant effect of calcitriol might be similar to that of verapamil (i.e. calcitriol might inhibit calcium channels in some way). This experiment was designed to investigate this by comparing the effects of calcitriol with that of verapamil, following activation using the calcium channel activator, FPL-64176. Addition of the highest concentration (10⁻⁶ M) of FPL-64176 induced a significant contraction on arterial rings, particularly after a short (20 min) delay (Figure 5-8A, $p < 0.001$). Subsequent treatment with both verapamil and calcitriol evoked significant reductions in contraction (Figure 5-8B, $p < 0.001$ for both verapamil and calcitriol), back to the level of the control (DMSO). However, the relaxant effect of verapamil was delayed or required much higher concentrations compared to calcitriol (Figure 5-8B, C), suggesting that the effects of calcitriol are either more potent or may not be via the blocking of Ca channels.

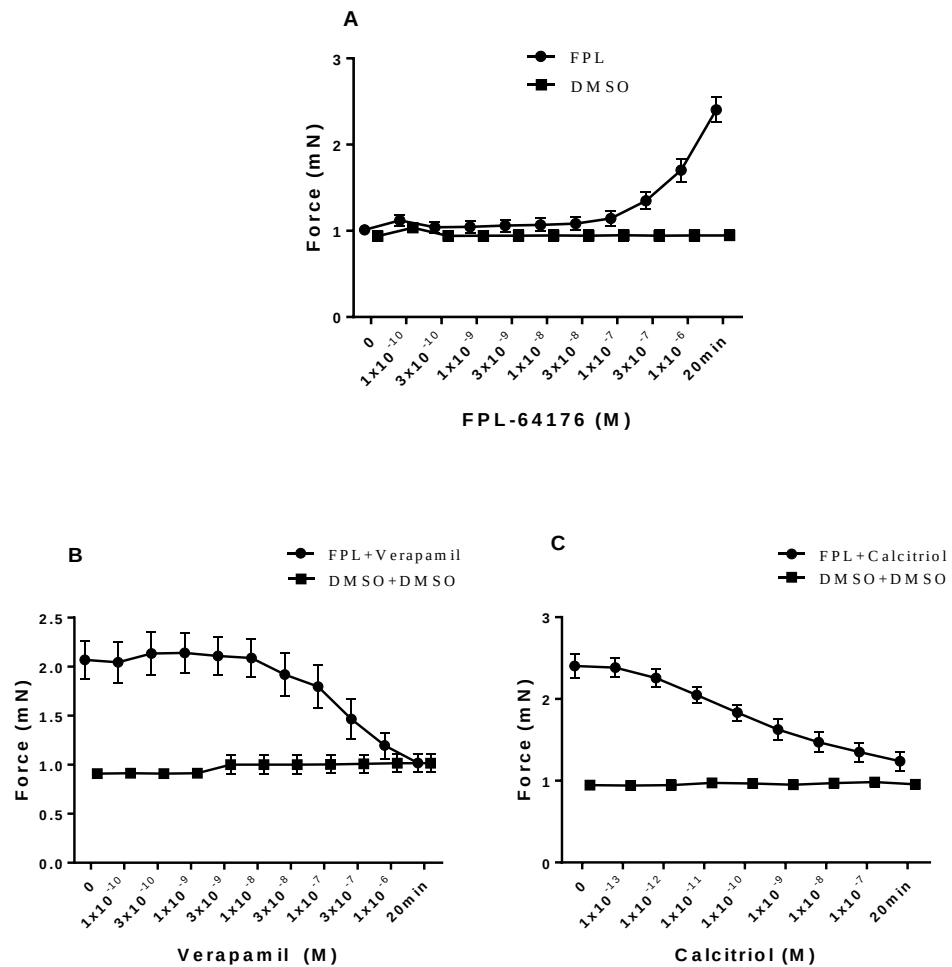


Figure 5-8 Effects of the Ca^{2+} channel agonist FPL-64176 (FPL) on calcitriol-induced vasorelaxation in rat thoracic/abdominal arteries. **(A)** Isometric contraction induced by cumulative concentrations of FPL-64176 compared to DMSO control ($p < 0.001$ for interaction (2-way repeated measures ANOVA)). **(B)** Verapamil dose-dependently reduced the contraction induced by pre-treatment with FPL-64176 compared to pre-treatment with DMSO control ($p < 0.001$ for interaction (2-way repeated measures ANOVA)). **(C)** Similarly with pre-treatment with FPL-64176, calcitriol induced a dose-dependent vasorelaxation compared to pre-treatment with DMSO control ($p < 0.001$ for interaction (2-way repeated measures ANOVA)), but much earlier, at lower doses. The values on the x-axis represent the increasing concentration of calcitriol used, however for the DMSO (control) group there was no increase in concentration but the vehicle was added at the same time as the concentration of calcitriol, therefore these values represent the force developed by the DMSO controls at the point when the indicated dose of calcitriol was administered to the test thoracic/abdominal arteries. All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n = 4-18$ per treatment group) derived from four different rats.

5.7.5 Investigate the Mechanism Underlying the Relaxation of Rat Thoracic/Abdominal Arteries Induced by Calcitriol

In order to study the vasodilatory effects of calcitriol on vascular smooth muscle cells, we first investigated the effects of calcitriol on Angiotensin II (Ang II)-induced contraction and the role of the cAMP-signalling pathway, which is known to be involved in adenosine-induced relaxation (Figure 5-1).

As expected, a clear dose-dependent (10^{-11} - 10^{-8} M) vasoconstriction was induced by Ang II (Figure 5-9A, $P < 0.001$). Interestingly, the response to Ang II was observed within 2 min for each dose (data not shown), similar to the previously described responses to ACh, but unlike the effects of the Ca^{2+} channel agonist and blocker, which were much more delayed. Even though the vessels had been pre-contracted with high concentrations of Ang II, calcitriol was still able to induce a concentration dependent relaxation (Figure 5-9B, $p = 0.012$ for interaction). In contrast, the vehicle (DMSO) control, had no effect (Figure 5-9B, $p = 0.348$).

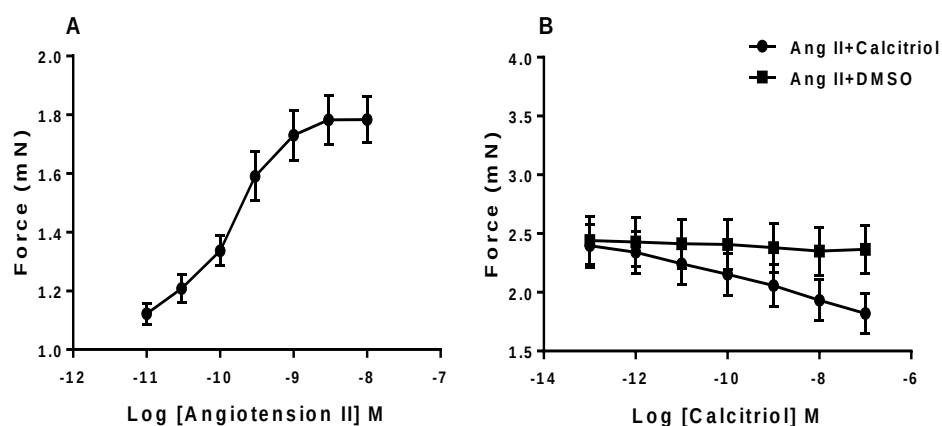


Figure 5-9 Effects of calcitriol on the contraction induced by Angiotensin II in rat thoracic/abdominal arteries. **(A)** Ang II induces a concentration-dependent (10^{-11} to 10^{-8} M) vasoconstriction ($p < 0.001$ [repeated measures ANOVA]). **(B)** Calcitriol was able to overcome the Ang II-induced vasoconstriction and induce a dose-dependent relaxation, whereas DMSO had no effect ($p < 0.012$ for interaction (2-way repeated measures ANOVA)). The values on the x-axis represent the increasing concentration of calcitriol used, however for the DMSO (control) group there was no increase in concentration but the vehicle was added at the same time as the concentration of calcitriol, therefore these values represent the force developed by the DMSO controls at the point when the indicated dose of calcitriol was administered to the test thoracic/abdominal arteries. All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n=5-12$ per treatment group) derived from three different rats.

5.7.5.1 Effect of the Adenylyl Cyclase Inhibitor SQ22536 on Calcitriol-induced Vasodilation of Rat Thoracic/Abdominal Arteries

Based on previous data, we hypothesised that calcitriol -induced relaxation might be mediated via the Adenylate Cyclase (AC)/ cAMP/Protein Kinase A (PKA) signalling pathway (Figure 5-1). We therefore set out to block at different points in that pathway using AC and PKA inhibitors. In this experiment, the effect of inhibiting adenylyl cyclase on the calcitriol-induced relaxation was investigated. Pre-incubation of arterial rings with 10^{-5} M of SQ22536 for 30 min induced a small but significant constriction (Figure 5-10A; $p < 0.001$). As expected, the maximal contraction induced by increasing concentrations of Ang II (10^{-11} to 10^{-8} M) occurred in the presence of SQ22536 (Figure 5-10B, $p < 0.001$). However, the magnitude of contraction induced by subsequent increasing concentrations of Ang II differed between the groups (Figure 5-10B). This difference might be related to the channels used on the myograph, hence, in order to investigate effects of SQ22536 on subsequent response to calcitriol, we present both raw data (Figure 5-10C) and data normalised to the % maximal Ang II-response (Figure 5-10D). Increasing concentrations of calcitriol induced a concentration-dependent relaxation (Figure 5-10C, D, $p < 0.001$), with no effect of the AC inhibitor, 10^{-5} M SQ22536 (Figure 5-9C, D; $p < 0.001$).

As such, Addition of 10^{-5} M Dibutyryl-Cyclic adenosine monophosphate (dbcAMP) did not alter the tone in the presence of AC inhibitors (Figure 5-11, $p > 0.7$).

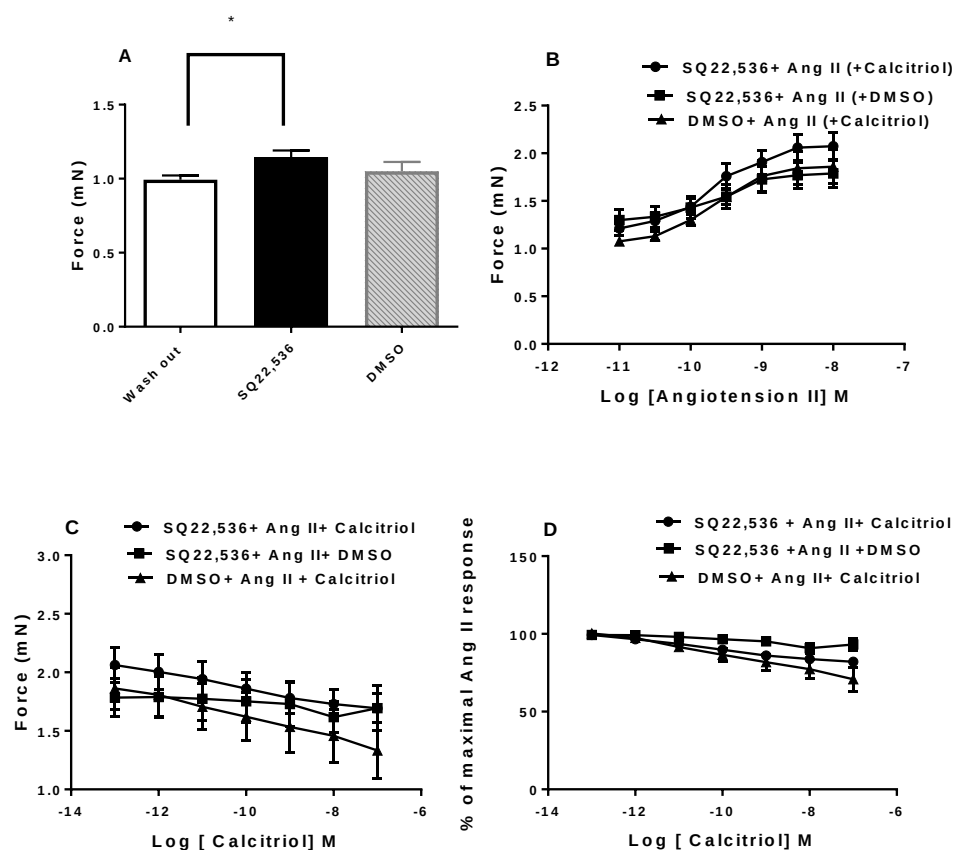


Figure 5-10 Effect of the adenylyl cyclase inhibitor SQ22536 on Calcitriol-induced vasodilation of rat thoracic/abdominal arteries. **(A)** The effect of incubation with SQ22536 (10^{-5} M) for 30 min contraction in rat arteries, $p < 0.001$ (t Test)). **(B)** The effect of incubation with increasing doses of Ang II (10^{-11} - 10^{-8} M) on artery rings of arteries pre-incubated in the presence and absence of SQ22536, ($p < 0.001$, [repeated measures ANOVA]). Two incubations of SQ22536 followed by increasing doses of Ang II (10^{-11} - 10^{-8} M) are shown as they were subsequently used either to be exposed to calcitriol or control (DMSO), as indicated in the figure as the appropriate agent indicated in the brackets. **(C)** Subsequent incubation of arteries shown in (B) with calcitriol (10^{-13} to 10^{-7} M) or control (DMSO). The values on the x-axis represent the increasing concentration of calcitriol used, however for the DMSO (control) group there was no increase in concentration but the vehicle was added at the same time as the concentration of calcitriol, therefore these values represent the force developed by the DMSO controls at the point when the indicated dose of calcitriol was administered to the test thoracic/abdominal arteries. **(D)** Data shown in (C) normalized to % maximum Ang II response for each treatment group ($p < 0.001$, calcitriol; $p = 0.161$, DMSO, [repeated measures ANOVA]). All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n=10-20$) per treatment group) derived from six different rats.

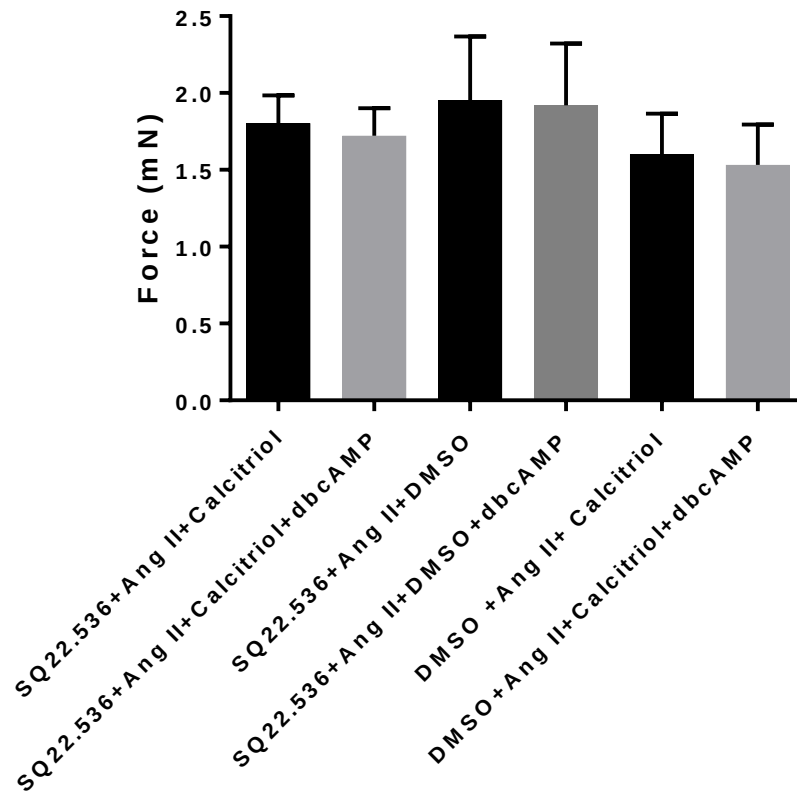


Figure 5-11 Effect of 10^{-5} M dbcAMP on relaxation of rat thoracic/abdominal arteries (means $\pm$ SEM, $n = 6$; $P > 0.787$, (ANOVA) for all groups. All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n=10-20$) per treatment group) derived from six different rats.

5.7.5.2 Effects of PKA Inhibitor (H-89) on Calcitriol-Induced

Vasodilation of Rat Thoracic/Abdominal Arteries

In the final experiment, pre-incubation of vessels with the protein kinase A inhibitor (H-89, 10^{-6} M) for 30 min. The expectation was that if the vasodilation was via cAMP mediated mechanism this inhibitor would have prevented this change in vascular tone. Unexpectedly it had no effect (Figure 5-12A, $p = 0.841$). However, the magnitude of contraction induced by subsequent increasing concentrations of Ang II differed between the groups (Figure 5-12B). We suspect that this difference might be related to the channels used on the myograph, rather than an effect of H-89, but in order to investigate effects of H-89 on subsequent response to calcitriol, we present both raw data (Figure 5-12C) and data normalised to the % maximal Ang II-response (Figure 5-12D).

As seen in Figure 5-12, the PKA inhibitor did not block the effect of calcitriol ($P < 0.001$), with similar relaxant effects seen in vessels pre-treated with either H-89 or PBS (prior to Ang II and then calcitriol), with no effect of DMSO, as expected (Figure 5-12D). Interestingly, addition of 10^{-5} M dibutyryl cAMP (dbcAMP) after calcitriol treatment had no effect, irrespective of whether the vessels had been exposed to the PKA inhibitor, H-89, or not (Figure 5-13, $P > 0.15$). Whereas 10^{-5} M dbcAMP induced significant relaxation (Figure 5-13; $p = 0.02$) following treatment with vehicle (DMSO), even in vessels pre-incubated with H-89. This suggests an inhibitory effect of calcitriol on dbcAMP-induced relaxation and that the PKA inhibitor, H-89, did not work in these studies.

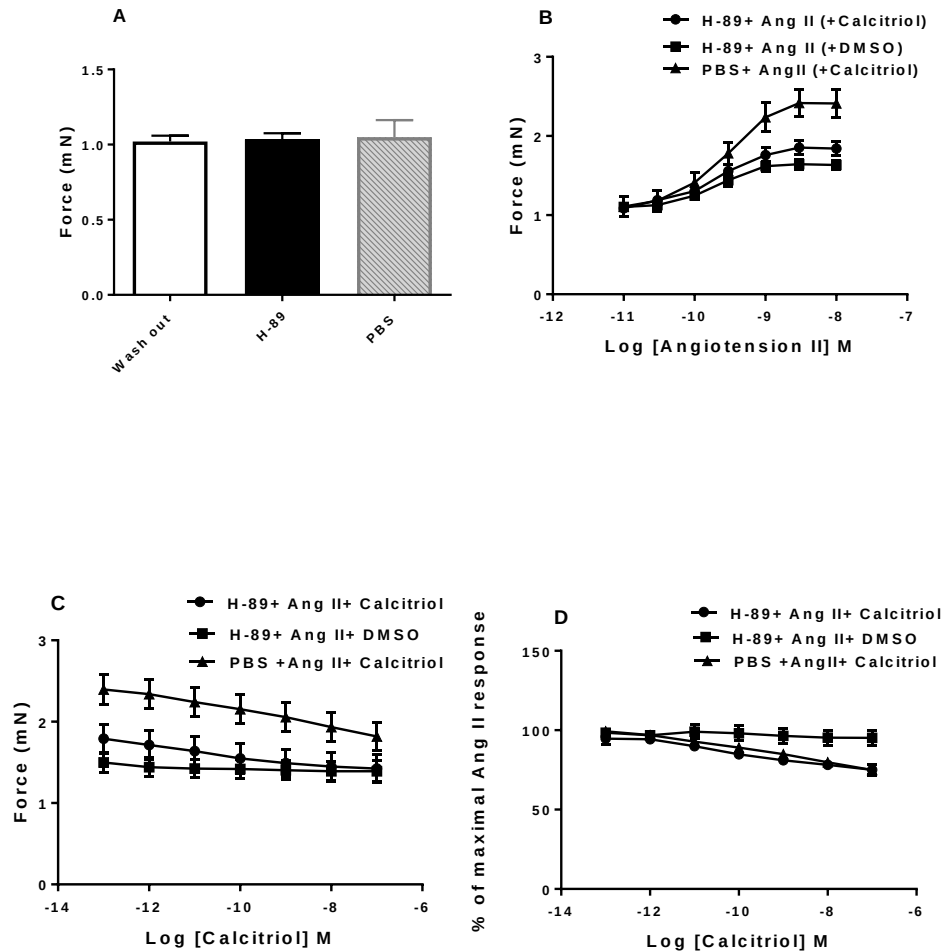


Figure 5-12 Effects of PKA inhibitor (H-89) on calcitriol-induced vasorelaxation of rat thoracic/abdominal arteries. **(A)** Pre-incubation with H-89 (10^{-6} M) for 30 min did not alter isometric tension in rat arteries ($p = 0.841$ [t test]). **(B)** The effect of incubation with increasing doses of Ang II (10^{-11} - 10^{-8} M) on artery rings of arteries pre-incubated in the presence and absence of H-89, ($p < 0.001$, [repeated measures ANOVA]). Two incubations of SQ22536 followed by increasing doses of Ang II (10^{-11} - 10^{-8} M) are shown as they were subsequently used either to be exposed to calcitriol or control (DMSO), as indicated in the figure as the appropriate agent indicated in the brackets. **(C)** Subsequent incubation of arteries shown in **(B)** with calcitriol (10^{-13} to 10^{-7} M) or control (DMSO). **(D)** Data shown in **(C)** normalized to maximum Ang II response for each treatment group ($p < 0.001$, calcitriol; $p=0.089$, DMSO, [repeated measures ANOVA]). All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta ($n=9-28$ per treatment group) derived from six different rats.

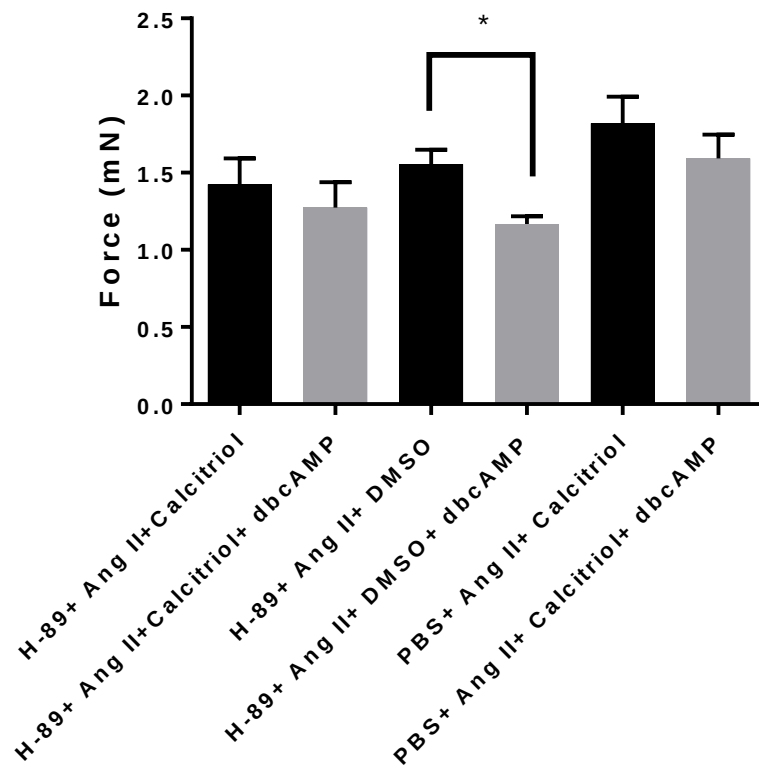


Figure 5-13 Effect of 10^{-5} M dibutyl cAMP on the relaxation of rat thoracic/abdominal arteries following calcitriol administration (means $\pm$ SEM, $n = 6$; $P > 0.179$ for vitamin D, $P = 0.02$ for DMSO [ANOVA]). All data are shown as means $\pm$ s.e.m of experiments on thoracic/abdominal rings of aorta($n=9-28$ per treatment group) derived from six different rats.

5.8 Discussion

5.8.1 Calcitriol Acutely Reduce Endothelium Independent Contraction in Rat Thoracic/Abdominal Aorta

These studies demonstrate a novel direct vascular effect of calcitriol in isolated rat aorta. The primary goal was to determine the direct effect of calcitriol on the contractility function of an arterial aorta isolated from a female Wister rat. The main findings were that acute administrations of calcitriol modulate vascular tone by inducing a potent relaxation on the arterial rings of aorta, both in a rested state and when aortic rings were pre-contracted with either phenylephrine (PE) or Angiotensin II (Ang II), which is consistent with previous studies, although previous studies suggested an endothelium-dependent mechanism (ÜN et al., 2013, Wong et al., 2010). Recent study (ÜN et al., 2013) showed that exposing the aortal rings of mice to increasing concentrations of calcitriol (10^{-5} - 10^{-6} M) significantly relaxed rings of the arterial aorta that had been pre-contracted by phenylephrine (PE, 10^{-5} M), suggesting that this effect might be mediated via a nitric oxide (NO) mechanism. On another note, (Wong et al., 2010) showed that chronic treatment of SHR rats with physiological dose of calcitriol (10 ng/ 100 g body) for six week had an effect on the thoracic aorta contraction *ex vivo* by reduction in ROS levels and downregulation of cyclooxygenase-1(COX-1) expression.

Our results showed that the acute effect of calcitriol tended to reduce or inhibit the dose-dependent (10^{-9} to 10^{-5} M) contractions induced by PE. These data are in accordance with studies reported that pre-incubation of arterial rings of mice with synthetic vitamin D₃ analog, (alfacalcidol, 10^{-5} M) for one hour, but not calcitriol (10^{-5} M), significantly inhibited PE –induced contraction (10^{-8} - 10^{-5} M) via an endothelium dependent nitric oxide mediated mechanism (ÜN et al., 2013). In contrast, (dos Santos et al., 2014) showed that vitamin D₃ supplementation induced an increased contractile response to PE in aortas with denuded endothelium. In addition, our studies showed that there was a significant inhibition of the subsequent dose-dependent (10^{-9} to 10^{-5} M) relaxation

induced by acetylcholine. These findings are in line with (Wong et al., 2008) reported that calcitriol had an inhibitory effect on acetylcholine (ACh, 10^{-7} - 10^{-5} M) when pre-incubated rings of the thoracic aorta of spontaneously hypertensive rat (SHR) with 10^{-7} M calcitriol for 30 minutes, suggesting that this effect is mediated via an endothelium-dependent mechanism. In addition, other studies that have examined normotensive rats whose chow was supplemented with 3,000 IU or 10,000 IU of vitamin D₃/kg for two months; the result was impaired relaxation to acetylcholine (ACh) (10^{-11} to 10^{-5} M) in aorta rings that were pre-contracted with PE (10^{-5} M) (dos Santos et al., 2014).

Consistent with a previous report (Wong et al., 2008), our data showed that the acute relaxant effect of calcitriol was reversible. Observational studies have reported an inverse association between the serum levels of calcidiol and blood pressure (Tamez and Thadhani, 2012). Recent studies (Chou et al., 2015) showed that the systolic blood pressure in male Wistar-Kyoto rats was significantly reduced by 14 ± 4 and 9 ± 4 mmHg after calcitriol treatment with a high dose (25 ng / 100 g per day) and a low dose (10 ng / 100 g per day), respectively. Furthermore, the treatment also partially improved the impairment of endothelium-dependent aortic relaxation in the fructose-fed rats (Chou et al., 2015). However, this contrasts with previous findings (Hatton et al., 1994), which have shown that subjecting an SHR to daily subcutaneous injections of calcitriol (48 ng/kg) enhanced vascular reactivity to norepinephrine and potassium chloride without altering baseline blood pressure on the rings of the aorta and mesenteric arteries. Furthermore, vitamin D supplementation of both normotensive and hypertensive rats showed an increased contractile response to PE in the aorta with denuded endothelium (dos Santos et al., 2014). In either male spontaneously hypertensive (SHR) or Wistar-Kyoto (WKY) rats (Bukoski et al., 1990) showed that intraperitoneal administration of calcitriol at 50 ng / day over a 3-day period did not alter blood pressure as measured using the tail-cuff method

Vascular endothelium plays a critical function in regulating vascular tone (hence, blood pressure) by producing a large number of biologically active substances (Rubanyi, 1993, Böhm and Pernow, 2007). Vitamin D₃ replacement in deficient people significantly improved the flow-mediated relaxation of the brachial artery, suggesting that vitamin D plays a role in endothelial function (Tarcin et al., 2009). Nitric oxide (NO) is a potent vasodilator that is synthesized by NO synthase (NOS). The endothelium releases the nitric oxide as a gas that stimulates the activation of soluble guanylate cyclase in smooth muscle cells, leading to the increased production of cyclic GMP, which leads to decreased intracellular Ca²⁺, which, in turn, allows vasodilation and increased blood flow (Behrendt and Ganz, 2002). Incubation of vessels with the NO synthase inhibitor L-NAME leads to the blocking of NO synthase, thus reducing the production of NO. However, our data showed that the relaxant effect of calcitriol was unaffected by the nitric oxide synthase (NOS) inhibitor (L-NAME) and was thus presumed independent of NO, which is in accordance with previous studies (Borges et al., 1999). Borges et al. (Borges et al., 1999) concluded that cholecalciferol treatment of spontaneously hypertensive rats (SHR) with impaired endothelial function significantly improved the endothelium-dependent vascular relaxation, which may modulate the pathway involving the endothelium-derived hyperpolarising factor but not NO. In contrast, *in vitro* studies showed that calcitriol, an active metabolite, is capable of effecting endothelial-dependent relaxation in cultured HUVECs by increasing the production of NO (Dalan et al., 2014, Molinari et al., 2011, Andrukhova et al., 2013). To explain the discrepancy between our findings and the previous studies, we suggest that other factors rather than eNOS might be more relevant in the modulation of relaxation in arteries. Interestingly, endothelium removal did not alter the relaxant effect of calcitriol, indicating that calcitriol might modulate relaxation by an endothelium independent mechanism, which is in accordance with previous studies (Li et al., 2002, Maggio et al., 2014, Norman and Powell, 2005, Ishibashi et al., 1995). Recent studies (Maggio et al., 2014) have addressed a link between calcitriol serum levels and

endothelial function in a large community-based population of older subjects. They found that there is a strong relationship between calcidiol serum concentrations and endothelium-independent vasodilation in women, as evaluated using an invasive forearm technique. Additionally, *in vivo* studies, several lines of evidence suggest that Calcitriol plays a significant role in the regulation of blood pressure, which might be indirectly modulated via an endothelium-independent function by suppressing the renin angiotensin system (RAS) (Li et al., 2002). On another note, one studies (Norman and Powell, 2005) have shown how calcitriol affects vascular tone by decreasing vascular resistance. Additionally, (Ishibashi et al., 1995) demonstrated that a Wistar rat that received intraperitoneal injections of calcitriol (20 ng/100 g body) for three days and had its mesenteric arteries isolated on the fourth day, showed calcitriol significantly enhanced the force generation of resistance in the mesenteric arteries to 10 μ mol/l norepinephrine, with no change in either the content of total myosin light chain or myosin heavy chain in these arteries.

Although previous studies reported that vitamin D suppresses contractions via an endothelium-dependent mechanism (Tarcin et al., 2009, Ün et al., 2013, Wong et al., 2008, Wong et al., 2010), here, we showed that calcitriol reduces contraction via an endothelium independent mechanism. The effect of calcitriol on pre-constricted thoracic and/or artery rings of the aorta appears to be via effects on vascular smooth muscle cells rather than on the underlying of endothelium. This conclusion is based on the observations that calcitriol induced a similar relaxation effects on both endothelium-intact and endothelium-denuded rings, and no significant difference was observed between endothelium-intact rings in the absence or presence of L-NAME. This observation is in accordance with previous studies (Borges et al., 1999). In spontaneously hypertensive rats (SHR) with impaired endothelial function, oral cholecalciferol (vitamin D₃) treatment significantly improved the endothelium-dependent vascular relaxation induced by acetylcholine. Furthermore, they concluded

that cholecalciferol treatment may modulate the pathway involving the endothelium-derived hyperpolarizing factor, but not nitric oxide. Moreover, in older women (but not in men), serum levels of calcidiol was positively and independently associated with endothelium-independent vasodilation (Maggio et al., 2014). Several lines of evidence suggested that vitamin D prevents hypertension, which may indirectly modulate endothelial-independent function by suppressing the renin-angiotensin system (Li et al., 2002), and/or decreasing vascular resistance (Norman and Powell, 2005). *In ex vivo* studies, (Li et al., 2002) isolated the left carotid artery from both wild type and VDR^{-/-} mice and administered intraperitoneal injections of calcitriol at 30 pmol/daily over a three day period. They found that both systolic and diastolic pressure readings, as measured using a pressure transducer model 60-3002, of VDR^{-/-} mice were higher than those of wild type mice due to elevated expressions of renin and angiotensin II (Ang II).

5.8.2 Involvement of the L-Type Calcium Channel in the Relaxant Effect of Calcitriol on Rat Thoracic/Abdominal Arteries

Calcium signalling plays a significant role in cellular function. In addition, an increase in intracellular calcium levels ($[Ca^{2+}]_i$) in vascular smooth muscle cells has been implicated in the pathogenesis and progression of diseases such as hypertension (Firth et al., 2013, Van Hove et al., 2009). Evidence from epidemiological surveys and human clinical trials suggests that disturbances in whole animal Ca^{2+} metabolism play a role in the hypertensive process (McCarron, 1995, Resnick et al., 1986, Young et al., 1988). In addition, vitamin D has been identified to regulate Ca^{2+} homeostasis (Del Valle et al., 2011, Holick and Chen, 2008). Especially, non-genomic effects of vitamin D have been described as involving Ca^{2+} as a second messenger (Revelli et al., 1998). However, recent study (dos Santos et al., 2014) showed that two-month supplementation with cholecalciferol (3,000 IU VD/kg of chow) in normotensive rats was associated with higher blood pressure (systolic pressure), with higher aortic contractility, and with the

impairment of aorta relaxation. This elevation on blood pressure was not associated with serum calcium, suggesting an alteration in aortic function and structure.

Phenylephrine has potent vasoconstrictor properties with a sympathomimetic effect, which causes vascular smooth muscle contraction by activating the selective α -1 adrenergic receptor (Yamada et al., 1989). When vascular smooth muscles contract because of PE, the cytosolic Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) are increased mainly via extracellular Ca^{2+} influx and Ca^{2+} release from intracellular sarcoplasmic reticulum (SR) Ca^{2+} stores (Van Hove et al., 2009). PE mediates the contraction of smooth muscle cells via the G protein-coupled receptor subtype (Gq-coupled receptors) (Keys et al., 2002). Binding PE to these receptors (Gq-coupled receptors) activates multiple heterotrimeric G proteins that link the receptor to the activation of the phospholipase C (PLC) signalling pathway (Chen and Bache, 2003). This, in turn, hydrolyses polyphosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Figure 5-1) (Berridge and Taylor, 1988). IP3 regulates the release of Ca^{2+} from the internal stores, which are primarily located in the endoplasmic/sarcoplasmic reticulum (ER/SR), whereas DAG stimulates protein kinase C (PKC) (Nishizuka, 1992), which may be involved in the maintenance of sustained contractions (Hamada et al., 1997). Calcitriol significantly reduced PE-induced vasocontraction in the present study, which is in line with previous observations using the same preparation (ÜN et al., 2013, Wong et al., 2008). This suggests that calcitriol may mediate relaxation through a calcium-dependent manner, which could relate to it acting as an antagonist of L-type calcium channels in vascular smooth muscle cells. This is in accordance with previous studies. Recent studies (ÜN et al., 2013) reported that a supra-physiological dose of calcitriol (both 10^{-6} and 10^{-5} M) significantly relaxed PE (10^{-5} M) induced vasocontraction in mice aortas, but with an endothelium-dependent nitric oxide-mediated mechanism. Another study (Wong et al., 2008) reported that 10^{-7} M of calcitriol modulates endothelium-dependent contractions in the thoracic aortae of adult SHR through a calcium-dependent

mechanism via partial inhibition of Ca^{2+} influx into the endothelium, which explained the reduced production of endothelial-dependent contraction factors (EDCFs).

Until now, the effect of vitamin D on the electrophysiological properties of the vascular smooth muscle Ca^{2+} channel and its importance in relaxation has not been investigated. Hence, based on our results, it is reasonable to hypothesise that the relaxant effect of calcitriol is similar to that of verapamil, which is a Ca^{2+} channel blocker. In this study, we found that both verapamil and calcitriol evoked significant relaxation in the arterial rings pre-contracted with the calcium channel agonist, FPL-64176, suggesting that a decrease in intracellular calcium levels ($[\text{Ca}^{2+}]_i$) in vascular smooth muscle cells was the crucial step in the relaxant effect induced by calcitriol. Although some studies reported that vitamin D may indirectly antagonise the effect of calcium-channel blockers via increasing the absorption of calcium in gastrointestinal tract (Vitamin and Mason, 2006), we found the relaxant effect of vitamin D may be due to the antagonised calcium channels in smooth muscle cells. The relaxant effect through chronic treatment with dietary vitamin D (12.5 μg (500 IU) vitamin D3 per 100 g body weight) in spontaneously hypertensive rats (SHR) may be due to the calmodulin-dependent activation of calcium-dependent potassium channels (Feres et al., 1992).

However, the vasodilatory effect of verapamil was delayed or required much higher concentrations compared to calcitriol, suggesting that the effects of this form of vitamin D are more potent and may not be due to the blocking of Ca^{2+} channels. However, the underlying calcitriol mechanism of action on arteries still requires further investigation.

Angiotensin II is a principal vasoactive factor in mammalian species that induces a potent and direct vasocontraction in smooth muscle cells (Mogi et al., 2007). It exerts its contractile effects through interaction with seven specific transmembrane heterotrimeric G-protein-coupled receptors (GPCRs) (Douglas et al., 2000, Mogi et al., 2007). Animal and human studies have shown that calcitriol controls vascular tone

(hence, blood pressure) through its role as a negative regulator of the renin-angiotensin system (RAS,) and renin expression supports that this effect is mediated by the calcitriol-VDR complex (Li et al., 2002, Vaidya and Williams, 2012, Vaidya et al., 2012). Our findings showed that calcitriol induced relaxation in aortic rings that were pre-contracted with angiotensin II. However, this effect occurred within minutes, suggesting that it was mediated via non-genomic effects.

5.8.3 Investigation of the Mechanisms Underlying the Relaxation of rat thoracic/abdominal arteries induced by Calcitriol

Hence the last experiment was designed to investigate the mechanism mediated by calcitriol. In vascular smooth muscle cells, adenylate cyclase is an important signalling system mediating relaxation (Nelson et al., 2011). Activation of adenylate cyclase leads to the increased stimulation of cAMP-dependent protein kinase A (PKA), which prompts the phosphorylation of a variety of cellular targets, including the activation of a number of K⁺ channels present in VSMCs (Figure 5-13). This triggers an increase in K⁺ efflux, which in turn causes hyperpolarization and relaxation (Nelson and Quayle, 1995, Nelson et al., 2011). Hence, most of the non-genomic effects of calcitriol are mediated by its interaction with plasma membrane-associated receptors, namely mVDR (Haussler et al., 2011), leading to activation of the adenylate cyclase-cAMP-PKA pathway in non-vascular tissue, such as the cerebral cortex of young rats and cardiac muscle cells (Zanatta et al., 2012, Revelli et al., 1998). Moreover, one study (Kawashima, 1987) suggested that vascular smooth muscle cells might be target tissues of calcitriol. Consequently, it is reasonable to hypothesise that the relaxant effect of calcitriol might be mediated via AC/cAMP/ PKA in the rat aorta. In fact, several studies have reported that the cellular effect of calcitriol is mediated by the mVDR receptor, which is coupled with activation of the adenylate cyclase-cAMP-PKA pathway in non-vascular tissues (Revelli et al., 1998, Vazquez et al., 1995, Selles and Boland, 1991). A very rapid increase (1-10 min) in the levels of cAMP occurs in cardiac muscle after

calcitriol treatment, and this effect is abolished by a specific inhibitor of adenylate cyclase or PKA, suggesting that the cellular effect of calcitriol is mediated by the cAMP pathway (Selles and Boland, 1991). However, this hypothesis was refuted by the present experiments, since the relaxant effect of calcitriol was not significantly impacted when arteries were incubated with AC or PKA inhibitors. However, we expected that adding of 10^{-5} M of dbcAMP to ring of arteries incubated with H-89 did not alter the relaxation, however, administration of 10^{-5} M of dbcAMP significantly induce relaxation, indicating that (H-89) did not block PKA. Hence, although the protein kinase inhibitor (H-89) did not work in our studies, since adding 10^{-5} dbcAMP showed significant relaxation of arteries in presence of inhibitor. Hence, further experiment done with different protein kinase inhibitor might be useful in order to confirm our results.

To conclude, the results of these studies clearly demonstrate that the calcitriol-induced vasodilation in rat thoracic/abdominal aorta is endothelium independent and does not involve NO. This relaxant effect was observed with both denuded and intact endothelium. Moreover, calcitriol modulated vascular tone by inducing a potent relaxation on the arterial rings of an aortas, both in a rested state and when aortic rings were pre-contracted with either phenylephrine (PE) or Angiotensin II (Ang II). Importantly, the acute vasorelaxant effects of calcitriol do not appear to be mediated via the AC/ cAMP/ PKA signalling pathway, but were similar to the effects of the Ca^{2+} channel blocker, verapamil. Hence, inducing relaxation in smooth muscle cells may be one of the modes of the action of calcitriol in reducing hypertension.

6

CONCLUSIONS AND FUTURE WORK

6 General Conclusions and Future work

The main aim of this chapter is to summarise and draw the last conclusion for all finding that obtained from all the experiments that have been detailed in this thesis. Then, Possibilities for future work as a result of these findings will be suggested.

6.1 Summery and Future Work Direction

Calcitriol, the most biologically active form of vitamin D, has been shown to have physiologic effects much broader than its role in mineral homeostasis and bone function (Díaz et al., 2015). In addition, it has been recognized that calcitriol has a wide range of fundamental biological functions, including the inhibition of cell growth (Chakraborti, 2011) and in the regulation of blood pressure (Judd and Tangpricha, 2009, Ullah et al., 2009). Although recent evidence supports a role for vitamin D in maintaining cardiovascular health (Aggarwal et al., 2010, McGreevy and Williams, 2011), the mechanism behind this effect of vitamin D is unclear. Hence, the body of work presented in this thesis aimed to expand knowledge of the fundamental biological effect of both calcidiol and calcitriol on the regulation of blood pressure. First, *in vitro* studies used human umbilical vein endothelial cells (HUVECs) as a model system to examine the effects of vitamin D on the proliferation and expression of key genes involved in hypertension. One advantage of using HUVECs is that we are primarily interested in the effect of vitamin D on human responses. In addition, the isolation of thoracic and abdominal aortas from Wistar rats, as well as the measurement of vascular tension in an *ex vivo* preparation, have been accomplished using myography, which is an important method for analysing arterial tone, one of the physiological processes that regulate blood pressure. This technique is useful to study the physiological properties of isolated vessels in response to calcitriol.

6.1.1 Effect of Vitamin D on HUVECs Proliferation

The work detailed in chapter 3 has shown the lowest dose of calcitriol (10^{-13} M) had clear proliferative effects in HUVECs (Figure 3-2). On the other hand, despite its status as a less potent form of vitamin D, supra-physiologic levels of calcidiol (10^{-9} , 10^{-7} M) had potent anti-proliferative effects on cell proliferation (Figure 3-1) during the same incubation period (24 and 48 hours). Moreover, the experiments presented in chapter 3 aimed also to investigate the effects of presence and absence of growth factors in the culture medium. This is particularly important because it demonstrates that both calcidiol and calcitriol alone, in the absence of any other stimuli, are able to affect HUVECs proliferation. Thus, the effect of the lowest dose of calcitriol (10^{-13} M) on HUVEC proliferation changed over time and was dependent on serum-related growth factors. On the other hand, the highest concentrations of calcidiol 10^{-9} M and 10^{-7} M significantly decreased DNA content in both media independently of serum-related growth factors. In addition, to support the fact that the proliferative and anti-proliferative effects appear to require vitamin D receptors (Whitfield et al., 1995, Zehnder et al., 2001), our data in chapter 4 confirmed the presence of VDR protein receptors in HUVECs using Western blot analysis (chapter 4; Figure 4-9). The data also demonstrated the expression of 1-alpha-hydroxylase enzyme (CYP27b1 mRNA) in HUVECs. This suggests the presence of a micro-endocrine system in HUVECs, which may be capable of synthesising the active form of vitamin D calcitriol, implying that this form of vitamin D might have localised (autocrine or paracrine) effects possibly related to the regulation of blood pressure, angiogenesis or both.

6.1.2 Effect of Vitamin D on HUVECs Gene Expression and Protein Secretion

Following the identification of factors that determine the responsiveness to vitamin D, including VDR and 1-alpha-hydroxylase, in HUVECs, it was speculated that the effect of vitamin D in HUVECs might be mediated by a genomic effect. Hence, the research

detailed in chapter 4 found that calcidiol had the greatest effect on the expression of genes, particularly those involved in regulating blood pressure, with decreased ET-1 mRNA expression (Figure 4-1), and there was a significant increase in eNOS mRNA relative to control, particularly with 10^{-11} M of calcidiol (Figure 4-2 B). However, treating HUVECs with different concentrations of calcitriol (ranging from 10^{-13} - 10^{-7} M for 24 hours) failed to alter ET-1 and eNOS mRNA expression (Figure 4-3 and Figure 4-4), as well as subsequent eNOS protein and levels of nitrite in media (Figure 4-10). Therefore, despite supposedly being the inactive form of vitamin D, calcidiol appears to have biologically relevant effects on cultured HUVECs, unlike calcitriol, which would be expected to result in potent vasodilation and therefore reduced blood pressure.

The data presented in chapter 4 suggest that calcitriol did not appear to work via genomic effects, so the work detailed in chapter 5 aimed to study the biological effect of calcitriol through non-genomic effects, because previous studies have shown that calcitriol affects the activity of a variety of signal transduction pathways (non-genomic responses) (Jones et al., 1998, Jurutka et al., 2001). By activating such pathways calcitriol is potentially having a more acute and transient effect compared to the stimulatory effect of influencing gene expression and subsequent change in protein concentration. This biological action is mediated by putative plasma membrane vitamin D receptors (mVDRs) (Zúñiga et al., 2011).

6.1.3 Effect of Calcitriol on Regulation of Vascular Tone in *ex vivo* Studies

No study to date has investigated the non-genomic effect of calcitriol on the physiology and pharmacology of isolated vessels. This is the first study to provide novel findings regarding the direct vascular effect of calcitriol treatments on arterial rings of aortas mediated through a non-genomic mechanism. The isometric force of arterial rings was measured in a rested state and after pre-contraction with PE and Angiotensin II (Ang II). It is advantageous to use Ang II to induce contractions of the arterial rings because we are primarily interested in creating a condition that mimics human arteries. We then

want to investigate if it might be useful to use calcitriol as an antihypertensive in humans. Therefore, in chapter 5, we show that calcitriol acutely reduced endothelium-independent contraction in rat thoracic/abdominal aortas when aortic rings were pre-contracted with either PE or Angiotensin II (Ang II) (Figure 5-4 and Figure 5-9). In addition, the acute effect of calcitriol tended to reduce or inhibit the dose-dependent (10^{-9} to 10^{-5} M) contractions induced by PE. In addition, there was a significant inhibition of the subsequent dose-dependent (10^{-9} to 10^{-5} M) relaxation induced by acetylcholine. Interestingly, the acute relaxant effect of calcitriol was reversible.

Until now, the effect of calcitriol on the electrophysiological properties of the vascular smooth muscle Ca^{2+} channel and its importance in relaxation has not been investigated. Therefore, particular emphasis was placed on investigating the effect of calcitriol on the vascular smooth muscle L-type Ca^{2+} channel. Verapamil was used as a positive control. For the first time, we found that both verapamil and calcitriol evoked significant relaxation in the arterial rings pre-contracted with the calcium channel activator FPL-64176, suggesting that a decrease in intracellular calcium levels ($[\text{Ca}^{2+}]_i$) in vascular smooth muscle cells might be the crucial step in the relaxant effect induced by calcitriol. Importantly, the acute vasorelaxant effects of calcitriol do not appear to be mediated via the AC/cAMP/ PKA signalling pathway, but they were similar to the effects of the Ca^{2+} channel blocker, verapamil. Hence, inducing relaxation in smooth muscle cells *ex vivo* may facilitate the use of calcitriol as an antihypertensive.

6.2 Future work

The findings obtained from all experiments examined in this thesis raise some interesting questions; however, in several areas, further experiments would be advantageous. The initial experiment described at the beginning of this thesis, which studied the effect of both forms of vitamin D on cell proliferation and gene expression, demonstrated vitamin D's involvement in the regulation of blood pressure at the molecular level. Calcidiol affects gene expression in physiological concentrations and also induces the expression of the activating enzyme 1-alpha-hydroxylase and vitamin D receptor (VDR). The finding that calcidiol at physiological concentration possesses an inherent hormonal activity provides a novel view of the vitamin D endocrine system in HUVECs, suggesting that calcidiol may offer a cardio protective role through vasodilation and therefore reduce blood pressure. Hence, previous studies (Lou et al., 2010, Lou et al., 2004) have revealed that the activation of calcidiol through 1-alpha-hydroxylase (CYP27b1) is not essential to its biological activity. Further investigation to provide direct evidence of the biological action of calcidiol by using a 1-alpha-hydroxylase-specific inhibitor should be carried out. In addition, further investigation using calcidiol in *ex vivo* studies is recommended. In the future, further experiments would be advantageous to evaluate the existence of potential non-genomic effects of calcitriol *in vitro* and how these signalling pathways may alter vascular tone, leading to relaxation. Future work should focus on measuring intracellular calcium concentrations in smooth muscle cells, as well as measuring the presence of membrane vitamin D receptor (mVDR) in smooth muscle cells. Moreover, additional studies are needed to further investigate the underlying mechanism of vasodilation induced by calcitriol in vascular smooth muscle cells. Finally, it will be advantageous to study the direct effect of calcidiol and calcitriol with *in vivo* studies.

6.2.1 Final summary of novel finding from this thesis

To conclude, the findings of this thesis showed, for the first time, the following:

1. Calcidiol had the greatest effect on anti-proliferation and gene expression, particularly expression of genes involved in the regulation of blood pressure (e.g., decreased ET-1 mRNA expression). In addition, calcidiol caused a significant increase in eNOS mRNA relative to the control.
2. Calcitriol induced a potent acute vasodilation in rat thoracic/abdominal aortas, which is endothelium-independent and does not involve nitric oxide (NO). This relaxant effect was observed with both denuded and intact endothelium.
3. The effects of calcitriol do not appear to be mediated via the AC/cAMP/ PKA signalling pathway, but they were similar to the effects of the Ca²⁺ channel blocker verapamil.

7

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8

APPENDIX

8 APPENDIX

8.1 High Pure RNA isolation kit, according to the manufacturer's instructions.

Efficiently purify total RNA

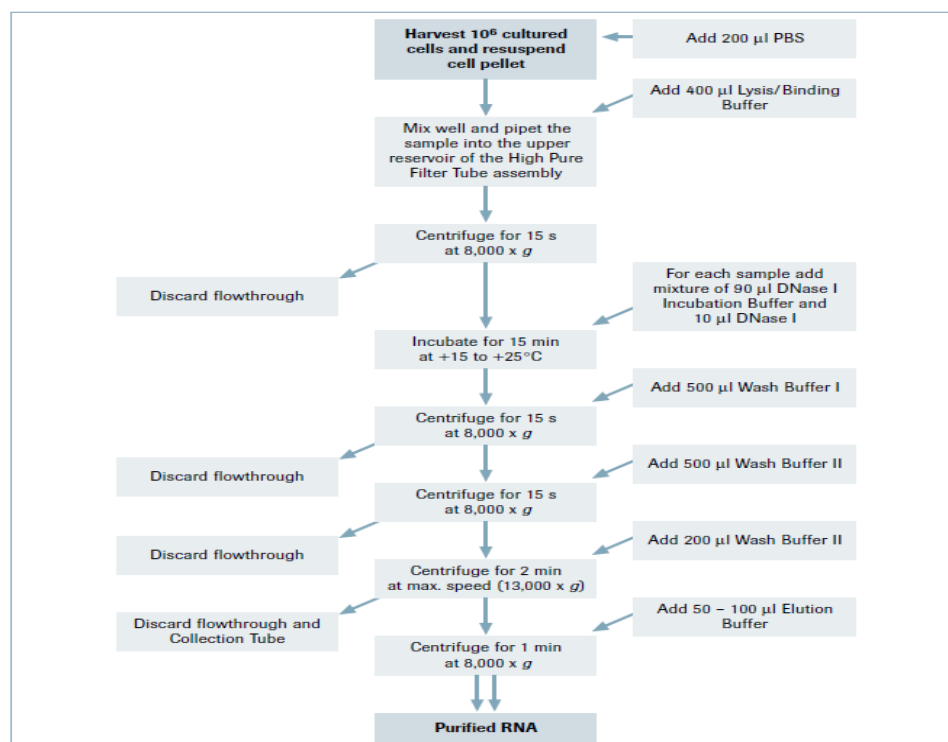
Procedure

Protocol for isolating total RNA from up to 10^6 cultured cells.

View detailed procedures for other sample materials in the pack insert at www.roche-applied-science.com

1	Resuspend cells in 200 μ l PBS.
2	Add 400 μ l Lysis/Binding Buffer and vortex for 15 s.
3	To transfer the sample to a High Pure Filter Tube: • Insert one High Pure Filter Tube into one Collection Tube. • Pipet the entire sample into the upper reservoir of the Filter Tube (max. 700 μl).
4	• Insert the entire High Pure Filter Tube assembly into a standard table-top centrifuge. • Centrifuge the tube assembly 15 s at $8,000 \times g$.
5	After centrifugation: • Remove the Filter Tube from the Collection Tube; discard the flowthrough, and again combine the Filter Tube and the used Collection Tube.
6	After re-inserting the Filter Tube: • For each sample, pipet 90 μl DNase I Incubation Buffer into a sterile reaction tube, add 10 μl DNase I, mix, and pipet the solution onto the glass fiber fleece in the upper reservoir of the Filter Tube. • Incubate for 15 min at +15 to +25°C.
7	• Add 500 μl Wash Buffer I to the upper reservoir of the Filter Tube assembly and centrifuge 15 s at $8,000 \times g$. • Discard the flowthrough and combine the Filter Tube with the used Collection Tube.
8	• Add 500 μl Wash Buffer II to the upper reservoir of the Filter Tube assembly and centrifuge 15 s at $8,000 \times g$. • Discard the flowthrough and combine the Filter Tube with the used Collection Tube.
9	Add 200 μ l Wash Buffer II to the upper reservoir of the Filter Tube assembly and centrifuge for 2 min at maximum speed (approx. $13,000 \times g$) to remove any residual Wash Buffer.  The extra centrifugation time ensures removal of residual Wash Buffer.
10	Discard the Collection Tube and insert the Filter Tube into a clean, sterile 1.5 ml microcentrifuge tube.
11	To elute the RNA: • Add 50 – 100 μl Elution Buffer to the upper reservoir of the Filter Tube. • Centrifuge the tube assembly for 1 min at $8,000 \times g$.
12	The microcentrifuge tube contains the eluted, purified RNA, which can be used directly in RT-PCR or stored at -80°C for later analysis.

High Pure RNA Isolation Kit workflow



Typical RNA yields from different sample materials

Starting Material	Sample Size	Average RNA Yield
Cultured cells (K-562)	10 ⁶ cells	5 µg
Whole blood, human	200 µl	Enough for 10 RT-PCR reactions
Yeast (<i>S. cerevisiae</i>)	10 ⁸ cells	20 µg
Bacteria (<i>E. coli</i>)	10 ⁹ cells	50 µg
Bacteria (<i>B. subtilis</i>)	10 ⁹ cells	35 µg

Figure 2: Typical yields obtained with the High Pure RNA Isolation Kit.

Ordering information

Product	Cat. No.	Pack Size
High Pure RNA Isolation Kit	11 828 665 001	Up to 50 isolations

For more information about the **High Pure RNA Isolation Kit** and other products for nucleic acid isolation and purification, visit www.roche-applied-science.com/napure

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