Elucidation of the Molecular Actions of 1,25 Dihydroxyvitamin D_3 and Docosahexaenoic Acid that may Mediate Cardiovascular Health

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Tim Widener
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Mentors: Peter Jurutka, PhD and Mark Haussler, PhD

Abstract

Omega 3 polyunsaturated fatty acids (PUFAs), composed of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been demonstrated to be beneficial in primary and secondary cardiovascular disease (CVD) prevention. The mechanism of action of PUFAs is not yet fully understood. Vitamin D, via its active form, 1,25-dihydroxyvitamin D_3 (1,25 D_3), functions through the vitamin D receptor (VDR), regulating serum calcium and phosphorus, and ultimately bone health. There is now evidence that 1,25 D_3 may be cardioprotective as well, but the mechanism is also not fully understood. Evidence supports DHA as a weak VDR agonist, therefore there may be crosstalk between the two ligands and their known and yet to be discovered receptors. In the present research, we probed six genes as potential VDR targets, identified both through literature searches as well as their logical association with proposed 1,25 D_3 and DHA cardioprotective mechanisms. Treating human embryonic kidney cells (HEK293) with 1,25 D_3 and DHA independently, and in combination, we demonstrate changes of expression of three genes through quantitative real time polymerase chain reaction analysis (qRT-PCR). Nitric oxide synthase (NOS2), involved in the immune system nitric oxide burst, was significantly repressed by 1,25 D_3 (fold effect 0.84, p value 0.04), DHA (fold effect 0.85, p value <0.01), with the greatest repression in the 1,25 D_3 and DHA combination (fold effect 0.74, p value 0.01). Serpin peptidase inhibitor (SERPINE1), for which expression results in increased thrombus formation through tissue plasminogen activator inhibition, was repressed in the 1,25 D_3 treatment group (fold effect 0.78, p value <0.01). Thrombomodulin (THBD), which indirectly activates protein C and increases thrombolysis, was repressed in the DHA (fold effect 0.69, p value <0.01) and combination 1,25 D_3 and DHA groups (fold effect 0.75, p value 0.04). SERPINE1 and NOS2 repression is consistent with cardioprotective decreases in thrombus formation and immunomodulation, but THBD repression is not consistent with this hypothesis.

Table of Contents

List of Figures and Tables:

Introduction

Polyunsaturated ω3 fatty acids (PUFAs) have been shown to have a profound clinical benefit in cardiovascular disease (CVD) with little associated side effects. While the mechanisms through which the PUFA produce the clinical benefit in CVD is not entirely understood, their potency is such that the American Heart Association (AHA) has issued guidelines outlining recommendations on dietary intake. Another potential player in CVD is vitamin D, most accurately characterized as an essential nutrient for bone health, now with inconclusive but intriguing evidence of a potential CVD therapeutic benefit. Many prospective and retrospective studies and clinical trials have been performed seeking to quantify the benefit, if any, of these dietary components. However, little is known about the synergistic effect that the combination of vitamin D and PUFAs may have together. This area is one of significant interest, such that currently a large, randomized, double blinded, placebo controlled clinical trial, titled The Vitamin D and Omega 3 Trial (VITAL), is underway seeking to demonstrate the effects of supplementation of both vitamin D and ω3 PUFAs [1]. Vitamin D and ω3 fatty acids each have hypothesized mechanisms in which they might influence cardiovascular health, one of which is by modulating the immune system and subsequently decreasing inflammation, as well as possibly reducing atherosclerosis and thrombogenesis. While the results of the VITAL trial will not be known for some time, we seek to identify indirectly a synergistic effect, defined as an observed effect greater than that demonstrated by each ligand alone, between 1,25-dihydroxyvitamin D_3 (1,25 D_3), the active metabolite of vitamin D, and docosahexaenoic acid (DHA), a primary component of the much studied PUFAs. We approached this question in a human embryonic kidney cell model (HEK293) by measuring induction and repression of possible key mediator genes in cardiovascular health and disease.

Cardiovascular disease remains the number one cause of mortality worldwide, accounting for approximately 17.3 million deaths in 2008, equivalent to 30% of all global deaths [2]. In addition to the high cost of lives, CVD carries a staggering financial burden. Estimates of cost to the US economy in 2010 are approximately $171 billion, with projections skyrocketing

to approximately $300 billion by 2030 [3]. The increased scrutiny of how health care dollars are spent is progressively becoming an ever more important medical and political issue. Many of the risk factors linked to CVD are readily modifiable, including tobacco use, diet, obesity, and physical inactivity. Other modifiable risk factors, albeit less amenable to lifestyle adjustments, include comorbidities such as diabetes, dyslipidemia, and hypertension. The combination of high prevalence rate, modifiable risk factors, and increased financial and political demands make CVD prevention and efficacious treatment an area of intense research, both for clinicians and basic scientists. The National Health and Nutrition Examination Survey (NHANES) showed more than 50% of the middle to elder aged women, as well as approximately 30% of similarly aged men, to be insufficient in vitamin D [4] [5] [6]. Additionally, the US diet is generally poor in fish and other foods rich in ω3 PUFAs. Thus, the plausibility of substantially impacting the CVD burden throughout the world with inexpensive dietary changes, possibly including increased vitamin D and ω3 fatty acid intake through dietary changes or supplementation, remains very tantalizing.

The current research focuses on demonstrating changes in expression at the molecular level of genes thought to encode mediators of cardiovascular health, in the presence and absence of 1,25 D_3 and DHA, independently and in combination with one another. To achieve this, we utilize a robust cell culture model consisting of human embryonic kidney cells (HEK293), commonly used in biomedical research laboratories, exposing them to treatments with active 1,25 D_3 and DHA. Using quantitative real time polymerase chain reaction technology, we quantify relative changes in expression of these potential cardiovascular modifying genes to better characterize their association with vitamin D and DHA. Insights garnered through this research may be extrapolated into future experiments to help further the understanding of the molecular pathways and the possible synergy between vitamin D and DHA.

Vitamin D and Cardiovascular Disease

Current dietary recommendations for vitamin D intake are 600 IU/day for those less than 70 years old and 800 IU/day if older than 70 years, so long as there is at least a minimal amount of sun exposure [7]. In the United States, it is thought that at least one third of middle aged and older individuals have some level of insufficiency or deficiency, with the elderly being the most affected due to their decrease in outdoor activity [8] [6] [9]. Outdoor physical activity presumably increases serum vitamin D levels through sunlight exposure and also strengthens bones through biofeedback. African-Americans appear to be particularly at risk for vitamin D deficiency or insufficiency, presumably due to the increased pigmentation which leads to decreased pro-hormone synthesis in the skin when exposed to sunlight relative to lighter skin, as well as lower dietary intake and rates of supplementation within the population [10]. The obese population also appears to be at risk of vitamin D deficiency or insufficiency, and although the mechanism is not fully understood, it is presumed to occur due to increased deposition of the fat soluble vitamin D hormone in adipose tissue, not decreased sun exposure [11].

1,25 D_3 is a fat soluble hormone acquired in its pro-hormone form through diet and supplementation, being absorbed in the gastrointestinal tract, as well as photosynthesized in the skin with exposure to ultraviolet radiation in sunlight. This pro-hormone is then metabolized to 25-hydroxyvitamin D (25 D_3) in the liver, and is further metabolized in the kidney by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) to produce the active form of the hormone, 1,25-dihydroxyvitamin D (1,25 D_3) [12]. The potent hormone, 1,25 D_3 exerts its effects through activation of the nuclear vitamin D receptor (VDR) and transcription control of downstream genes. An individual's 1,25 D_3 status is clinically measured indirectly through serum 25-hydroxyvitamin D concentration, which is also the generally used method in clinical studies. Renal production of 1,25 D_3 is stimulated by plasma parathyroid hormone (PTH) under conditions of low serum calcium. Parathyroid hormone stimulates a predominately renal conversion of 25-hydroxyvitamin D to its active form 1,25-dihydroxyvitamin D, increasing gastrointestinal absorption of calcium and renal tubular reabsorption of calcium and

phosphorus [13] [14]. Homeostatic levels of 1,25 D_3 are maintained by the enzymatic breakdown of the hormone via 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), for which expression is responsive to altering levels of 1,25 D_3 [14]. In summary, diet and sunlight provide the pro-hormone vitamin D substrate for the body to activate, which is then regulated by PTH, calcium, and phosphorus serum status, and degradation occurs via CYP24A1.

Bone health is strongly tied to adequate levels of 1,25 D_3. The disease rickets represents one end of the spectrum, occurring because of extreme vitamin D-deficiency, or with inactivating mutation of the vitamin D receptor (VDR), through which the 1,25 D_3 performs its main functions of calcium and phosphate regulation [15]. More salient forms of bone disease can occur because of 25 D_3 deficiency (less than 20 ng per milliliter, or 50 nmol per liter) or insufficiency (21-29 ng per milliliter, or 57 to 72 nmol per liter), however serum levels are only loosely tied to disease once outside the spectrum of extreme deficiency. The Women's health initiative showed that those with serum levels less than 26 ng/mL did not have an increased risk of fracture, but those women in the study undergoing consistent vitamin D and calcium supplementation had a 29% reduced risk of fracture [16]. This suggests that serum levels of the surrogate marker 25 D_3 by itself is not a reliable predictor of fracture risk. Quality clinical trials of dietary supplementation with vitamin D and calcium in elderly women, those thought to be most at risk of deficiency, have resulted in dramatically decreased rates of osteoporotic hip and other nonvertebral fractures, implicating relative deficiency as a major source of disease in this subpopulation [17] [18]. Meta-analysis of seven randomized clinical trials giving 400 IU vitamin D daily alone showed little benefit in terms of hip and other nonvertebral fracture reduction, however studies using 700 to 800 IU vitamin D_3 daily alone showed a decrease in the relative risk of hip fracture by 26% and nonvertebral fractures by 23%, suggesting dose responsiveness [19]. These data, when combined, shows that 25 D_3 status is an unreliable indicator of osteoporotic fracture risk, and that the decision to supplement should not be based solely on this measure. Instead, supplementation in specific populations, mainly the elderly with other risk factors not mentioned here, should occur.

Strong evidence for a causal relationship between low 25 D_3 status and CVD is found in studies of patients with end stage renal disease (ESRD) on hemodialysis or peritoneal dialysis, a population with chronically low 25 D_3, where adjusted mortality from cardiovascular events is 10-20 times the normal population rates [20]. Similar populations that are supplemented with 1α hydroxyvitamin D and the vitamin D analogue paracalcitriol show markedly reduced rates of cardiovascular events [21] [22]. Observational evidence supporting a relationship between low 25 D_3 and CVD has shown mixed results [23]. Several large epidemiological studies have shown a relationship between low levels of vitamin D and CVD, although the relationship plateaued between 20 and 30 ng/mL, and even suggested a slight increase of CVD risk at higher levels [24] [25]. Epidemiological studies have shown a protective effect of vitamin D against CVD [24] [25] [26] [27]. Thus the totality of the evidence briefly reviewed here shows a trend toward supporting increased CVD risk in the presence of low levels of 25 D_3.

The mechanism by which vitamin D influences the cardiovascular system is not well understood. There are a number of theories, with a common theme of reducing known CVD risk factors, including reductions in hypertension, insulin insensitivity and diabetes, generalized inflammation, atherosclerosis, and thrombosis. In the present research, we have identified six potential target genes of 1,25 D_3 and DHA that have proven physiological role in the cardiovascular system and thus may have altered expression in the presence of either 1,25 D_3 or DHA, or the combination of the two (Table 1). Given that DHA, and to a lesser extent Vitamin D3, have been demonstrated to affect cardiovascular health, we hypothesize that genes integral to cardiovascular health, including the six genes studied, will show altered expression in the presence of each ligand and may additionally demonstrate a synergistic effect in combination. The hypothesized change in expression of each gene in response to the different treatment groups was developed under the presumption that the ligands decrease inflammation and thrombogenicity and generally improved the integrity of the cardiovascular system. Further characterization of the role vitamin D and DHA, including the combination of the two, may have as potential CVD modifying genes could be important in future research to fully understand the mechanism by which they perform their modest benefit, which in turn may be exploited in the future.

Gene	Gene product and physiological role	Hypothesis when exposed to:		
		+1,25 D_3	**+DHA**	**+1,25 D_3 +DHA**
VEGFA **(Vascular Endothelial Growth Factor A)**	Growth factor signaling molecule Induces angiogenesis in embryos and hypoxic tissues Increases vascular permeability Promotes cellular migration Inhibits apoptosis	**++**	**+**	**+++**
NOS2 **(Nitric Oxide Synthase)**	Synthesizes nitric oxide Vasodilation Produces nitrous oxide burst as part of the immune defense system	**-**	**-**	**--**
EDN1 **(Endothelin 1)**	Protein signaling molecule Potent inducer of vasoconstriction	**--**	**--**	**---**
PDGFA **(Platelet Derived Growth Factor A)**	Growth factor signaling molecule Induces cellular differentiation, particularly in the vascular system	**+**	**+**	**++**
SERPINE1 **(Serpin Peptidase Inhibitor, or endothelial plasminogen inhibitor)**	Plasminogen activator inhibitor Promotes thrombus formation by inhibiting thrombolysis	**--**	**--**	**---**
THBD **(Thrombomodulin)**	Thrombin cofactor Promotes thrombolysis through indirectly activating Protein C	**+**	**++**	**+++**

Table 1. Genes studied, predominant gene product and physiological role, and the hypothesized result when cells are treated with 1,25-dihydroxyvitamin D_3 and docosahexaenoic acid (DHA); (+) increased and (-) decreased expression, magnitude proportional to number of plus or minus signs

DHA and Cardiovascular Disease

Evidence for the heart health benefits of ω3 PUFAs has been building for approximately six decades and has reached a level sufficient for the AHA to issue dietary guidelines recommending 2 oily fish meals per week, equivalent to 500 mg/day of ω3 PUFA, and 1000 mg/day of ω3 PUFA supplementation for individuals with coronary heart disease (CHD) [28]. In addition, many other organizations including the World Health Organization, American Diabetes Association, and others have issued their recommendations for dietary fish and supplementation for those with heart disease.

There is a long history of epidemiological evidence supporting the association between high levels of dietary ω3 PUFAs and decreased rates of CVD. As early as 1944, the PUFA rich diet of Greenland Eskimos, which included whale, seal, and fish, was associated with rates of CVD near zero [29]. Later observational studies in populations with similar diets supported these earlier findings, culminating today in a strong body of epidemiological evidence supporting ω3 PUFAs as mediators of a modest cardiovascular benefit.

Clinical trials of ω3 PUFA have shown similar results. Eicosapentaenoic acid (EPA) and docosahexaenoic acid, the two constituents of ω3 PUFAs, have been tested in clinical trials together and independently. Serum levels of both DHA and EPA have been shown to correlate inversely with the incidence of major cardiovascular events, with DHA exhibiting a slightly stronger effect [30]. Evidence supporting ω3 PUFAs in CVD has been demonstrated through clinical trials for primary and secondary prevention, with secondary prevention exhibiting the strongest and most consistent results. A large randomized controlled trial of post-MI patients receiving 850 mg of ω3 PUFA versus normal treatment for a period of one year showed a 21% reduction in total mortality, and 30% reduction in cardiovascular mortality [31]. Another large trial in Japan comparing a statin to statin plus 1800 mg/day of EPA showed a similar 19% reduction in major cardiovascular events [32]. A third large trial, conducted more than 20 years ago, demonstrated similar results when supplementing post-MI patients with either oily fish or

fish oil capsules, in which the reduction in mortality was due almost entirely to decreased CHD [33]. When the data from these three trials are pooled and analyzed, there is also a prevention component evident in the treatment groups [34]. Thus EPA and DHA, separately or in combination, have demonstrated through quality clinical studies as markedly reducing the likelihood of cardiovascular events, both through primary and secondary prevention.

The mechanism by which ω3 PUFA, and DHA in particular, mediates their beneficial effect appears to be multifactorial. There is strong evidence that high serum levels of ω3 PUFA have antiatherosclerotic effects by a non-specific mechanism [35]. Evidence supports ω3 PUFA in decreasing the occurrence of sudden cardiac death (SCD), possibly due to their ability to inhibit fast voltage-dependent sodium channels and L-type calcium channels [36] [37]. This is thought to result in stabilization of autonomic system function, which is supported by a large study of 5,096 subjects in which it was demonstrated those with high dietary intakes of fish had lower heart rates, slower atrial ventricular conduction, and a decreased QT intervals [38]. Similarly, another study demonstrated a decrease in average heart rate in patients with complex ventricular arrhythmias when supplemented with 1260 mg of ω3 PUFA [39].

More recently, ω3 PUFA has been associated with primary prevention of heart failure (HF) in groups with high dietary intake of baked or broiled fish [40] [41]. A large, randomized placebo controlled trial of 1000 mg ω3 PUFA versus and in combination with a statin showed a 9% reduction in total mortality in all of the ω3 PUFA groups [42]. It is also well established that high doses of ω3 PUFA lower serum triglyceride levels significantly [43]. It is thought that the ω3 PUFA acts as a ligand for the peroxisome proliferator-activator receptor (PPAR) alpha, a regulator of myocardial fatty acid uptake regulating genes, as well as genes involved in the formation of very-low-density lipoprotein carrying triglycerides from the liver [44]. This serves to decrease the hepatic synthesis of triglycerides and increase hepatic fatty acid oxidation.

Additional mechanisms by which ω3 PUFA may improve CV health are being discovered and expanded upon at staggering rates. Omega3 PUFAs have been shown improve arterial and endothelial function and reduce platelet aggregation at relatively high doses [45] [46]. This ability of ω3 PUFA to decrease the ability of platelets to aggregate, a process integral to the

ruptured plaque and thrombosis theory accounting for the majority of MIs, could partially explain the decreased rates of initial and repeat MIs with supplementation. In addition, ω3 PUFAs have been shown to stabilize the already formed carotid artery plaques [47]. This new evidence is intriguing and may eventually shed more light on the mechanisms behind the cardioprotective effects of DHA.

This brief review implicating ω3 PUFA as a mediator of improved cardiovascular health is by no means meant to be complete. However, it is clear that the clinical evidence is strongly supportive. Research in these areas is ongoing and potentially lucrative in terms of impacting the understanding of the mechanisms and the disease processes. There is much uncertainty regarding the mechanism by which ω3 PUFAs act, although there appears to be a number of reasonable hypotheses. The mechanism is most likely multifactorial, not the result of a single process or pathway but rather the effect of the accumulation of a number of mechanisms, each with a small contribution by itself, but a cumulative effect that is rather profound when working in concert.

Potential Genes Influenced by Vitamin D and DHA

In the current work, we seek to elucidate changes in expression of potential CVD altering mediators that were identified through literature searches, as well as their logical association given their physiological role and relationship to the cardiovascular system. Several of the proposed mechanisms by which DHA and vitamin D may mediate their effects are overlapping, including immune system mediation, rendering genes possessing known associations with both systems particularly interesting. One such gene is nitric oxide synthase, or NOS2. Induction of NOS2 has been recently demonstrated in human osteoblast cell lines with the addition of 1,25 D_3 [48], and there is also evidence of its suppression in the presence of DHA in human umbilical vein cell lines [49]. Literature searches for investigations of combination 1,25 D_3 and DHA yields no evidence of their having been studied at the molecular level. Given both 1,25 D_3 and DHA appear to have the potential to influence NOS2 in specific cell lines,

albeit antagonistically, it is probable that in some tissues they both may act as agonistic or antagonistic VDR ligands.

Plasminogen activator inhibitor (PAI-1), also known as endothelial plasminogen activator inhibitor or serpin peptidase inhibitor (SERPINE1), is actively involved in both the inflammation and clotting process. SERPINE1 plays an important role in inactivating tissue plasminogen activator, and thus its levels have an inverse relationship with thrombus formation. Modulation of its activity by both 1,25 D_3 and the clinically important analogue paracalcitriol has been demonstrated in human coronary artery smooth muscle cells, showing a dose responsive decline in activity with the hormone [50], and paradoxically has shown an up-regulation in human endothelial vein cells [51]. Interestingly, vitamin D receptor knockout mice show increased thrombogenicity and no change in blood coagulation, implicating the 1,25 D_3 / VDR pathway as critical to normal control of the thrombus formation [52]. However, there has not been a documented study of the effects that DHA may have on the activity of the SERPINE1 gene. Thus there is the possibility that either 1,25 D_3 or DHA alone may influence the activity of SERPINE1, and there is the possibility of a combinatorial effect.

Thrombomodulin (THBD) is another potentially interesting gene in terms of possible modulation by 1,25 D_3 and DHA. The normal physiological role of THBD is to act as a cofactor for thrombin, in turn aiding the activation of protein C, which when activated performs proteolytic deactivation of clotting factors and reduces thrombus formation. Recent evidence shows impressive up-regulation by 1,25 D_3 in microarray analysis and confirmatory rt-PCR in osteoblasts [53]. Data for the effects of DHA on THBD expression appear to be lacking, although there are clinical trials with DHA supplementation wherein DHA appears not to alter the levels of plasma soluble thrombomodulin [54]. Combination 1,25 D_3 and DHA appears not to have been studied. Given the impressive apparent induction by 1,25 D_3 in osteoblasts, the question of whether the same effect can be reproduced in other tissues remains to be answered, as well as the effect that a combination of 1,25 D_3 and DHA may produce.

Vascular endothelial growth factor alpha (VEGFA) is a potent signaling molecule integral to angiogenesis and formation of the vasculature, and as such could potentially be a target

through which 1,25 D_3 and DHA could exert some portion of their effects. Similarly, platelet derived growth factor alpha (PDGFA) is a signaling molecule that influences the differentiation of cells in the vasculature. Lastly, endothelin 1 is a potent vasoconstrictor of the vasculature, and may be differentially expressed in presence or absence of 1,25 D_3 and/or DHA. Each of these genes plays a key role in the genesis and maintenance of the vascular network, and as such could possibly play a role in the mechanism by which 1,25 D_3 and DHA exert their effects.

Research Materials and Methods

Cell Culture and Treatment

Human HEK293 cells were obtained from the laboratory of James Hsieh and were subcultured according to readily available practices. HEK293 cells were selected as a cell model due to their relative ease to subculture and robust VDR expression. Growth media consisting of minimal essential media (MEM) obtained from American Type Culture Collection (ATCC) was used with the addition of 10% fetal bovine serum, 100 U/mL penicillin G, and 100 U/mL of streptomycin. Cells were subcultured approximately every 3-5 days and maintained at 37° C and with 5% CO_2. Treatment of cells occurred in Becton, Dickinson and Company 60 mm tissue culture plates with cells of identical passages 40-45 with a confluency between 90-95%. Treatments of 1,25 D_3 and DHA were both suspended/dissolved in equal quantities of cell culture grade ethanol and the control group was ethanol alone.

Quantitative Real Time Polymerase Chain Reaction Experiments

Total RNA extraction was performed from the HEK293 cells after treatment using the Aurum Total RNA Mini-Kit in accordance with the manufacturer's instructions, including the addition of RNAse inhibitor. Total RNA was re-suspended from the supplied column using the manufacturer's Elution Buffer. Yield was determined using an optical density of 260 nm and total RNA was aliquoted and stored at -80° C.

Reverse transcription of 2 μg total RNA was performed using the iSript cDNA Synthesis Kit according to the manufacturer's instructions in a total volume of 40 μL. Quantitative real time polymerase chain reaction (qrt-PCR) was performed using the cDNA product via the SYBR Green Quantitative PCR kit according to the manufacturer's instructions in a total reaction volume of 10 μL. Forward and reverse primers for the genes of interest were either obtained from publications or engineered using IDT Technologies online primer design tools (Table 2).

PCR amplification was achieved using a melt temperature of 95° C, 1.0 minute of annealing at 62° C, and 1.5 minutes of extension at 70° C, for a total of 2 hours and 30 minutes. Reactions were performed and analyzed using an Applied Biosystems 7500 Fast Real Time System. Melt curves were analyzed and compared between experiments and were consistent with single products. Positive controls consisting of primers for the human CYP24A1 gene were used and showed excellent induction with exposure to 1,25 D_3, generally 50-60 fold over ethanol only. Negative controls were GAPDH primers, which showed consistent expression across all treatment groups.

Gene	Forward	Reverse
VEGFA	5'-CCGAAACCATGAACTTTCTGC-3'	5'-CTCCTTCTGCCATGGGTG-3'
NOS2 [55]	5'-TACTCCACCAACAATGGCAA-3'	5'-GATGAGCTGAGCATTCCACA-3'
EDN1 [56]	5'-CTCTCTGCTGTTTGTGGCTTGC-3'	5'-GTGGACTGGGAGTGGGTTTCTC-3'
PDGFA	5'-CCGCCAACTTCCTGATCTG-3'	5'-TTCCTGACGTATTCCACCTTG-3'
SERPINE1 [57]	5'-GCGCTGCAGAAAGTGAA-3'	5'-TGTGCCGGACCACAAA-3'
THBD	5'-GCGCTGCAGAAAGTGAA-3'	5'-TGTGCCGGACCACAAA-3'

Table 2: Primer information for each of the genes studied. Unless citation is given, genes were engineered via IDT Technologies online primer design tool.

Results

Treatment with either ethanol vehicle, 1,25 D_3, DHA, or the combination of both ligands did not appear to alter the morphology or growth rate of the HEK293 cells at any stage of the experiments. Consistent RNA preparations were obtained from all treatment groups with similar concentrations. Quantitative real time polymerase chain reactions showed melt curves that were consistent between experiments, and additionally consistent with single PCR products. Expression of all genes was normalized to GAPDH mRNA levels and are reported as fold effect (or decimal fraction) of control mRNA level.

Figure 1 illustrates the results for the six genes under study in the presence or absence of 1,25 D_3, DHA, or the combination of both ligands . All results discussed refer to HEK293 treatment conditions of 24 hours with $1X10^{-8}$ M 1,25 dihydroxyvitamin D_3 (1,25 D_3) and $5X10^{-5}$ M docosahexaenoic acid (DHA). Endothelin 1 (EDN1) exhibited a non-statistically significant reduction in expression when HEK293 cells were exposed to 1,25 D_3 (fold effect of 0.75, p value 0.23) and DHA (fold effect 0.79, p value 0.28) independently, as well as in combination (fold effect 0.81, p value 0.36). Thus, the trend is in part consistent with the hypothesis (Table 1), and suggests that 1,25 D_3 may indeed repress endothelin 1 as a mechanism of retarding the formation of atherosclerotic plaque and consequent myocardial infarction. However, more replicate experiments are required to establish statistical significance. Notably, analysis of 4 hour treatment of HEK293 cells revealed a similar trend, with combined 1,25 D_3 and DHA eliciting statistically significant repression of EDN1 expression (data not shown). Because neither 1,25 D_3 nor DHA affected EDN1 after 48 hours treatment (data not shown), we conclude that the repressive effect of 1,25 D_3 and DHA on EDN1 is a relatively rapid one which decays over 24-48 hours.

In contrast, nitric oxide synthase 2 (NOS2) revealed a statistically significant decrease in expression with exposure of HEK293 cells to 1,25 D_3 (fold effect 0.84, p value 0.04) and DHA (fold effect 0.85, p value <0.01), and statistically significant and greater degree of decrease in expression with the combination of 1,25 D_3 and DHA (fold effect 0.74, p value 0.01) (Fig. 1). The

results were confirmed in HEK293 cells treated 48 hours with 1,25 D_3 and DHA (data not shown). These data support the hypothesis (Table 1), and indicate that NOS2 is a prime candidate gene targeted by 1,25 D_3 in the prevention of immune-related inflammation leading to coronary artery disease. Intriguingly, DHA functions equally well in suppressing NOS2, and the combination of DHA and 1,25 D_3 elicits a synergistic suppression of this inflammatory gene. It will be of interest to see if these effects in cultured cells translate to patients in the VITAL trial, where DHA and vitamin D are being supplemented individually and in combination to determine their effects on the incidence of a number of chronic conditions with an inflammatory origin, including myocardial infarction.

Platelet derived growth factor alpha (PDGFA) is generally observed to be a vitamin D-induced gene [58] and, consequently, we hypothesized in Table 1 that the expression of this gene would be increased by 1,25 D_3 in HEK293 cells. However, this hypothesis was disproven in the present experiments because PDGFA displayed a non-significant *decrease* in expression with 1,25 D_3 (fold effect 0.89, p value 0.06) and DHA (fold effect 0.92, p value 0.09) independently, and a non-significant *decrease* in expression when treatments were combined (fold effect 0.89, p value 0.13) (Fig. 1). Therefore, PDGFA expression exhibits non-statistically significant pattern of repression similar to that of endothelin 1, although the magnitude of the repressive trend is more modest. Interestingly, 48 hours treatment of HEK293 cells with DHA, but not 1,25 D_3, generated a statistically significant repression of PDGFA (data not shown). PDGFA is believed to act analogously to EDN1 in promoting smooth muscle cell growth in the raised atherosclerotic plaque and consequent myocardial infarction, indicating that further experiments exploring the influence of 1,25 D_3 and/or DHA on EDN1 and PDGFA are warranted, perhaps in arterial cell culture models.

Serpin peptidase inhibitor (SERPINE1) exhibited a significant decrease in expression with 1,25 D_3 (fold effect 0.78, p value <0.01), and non-significant decrease with DHA (fold effect 0.75) and combined treatment (fold effect 0.80, p value 0.08) which trended such that the data appeared to mimic those of EDN1 and PDGFA (Fig. 1). Therefore, we can conclude that the SERPINE1 suppression by 1,25 D_3 observed in Fig. 1 conditionally confirms the hypothesis that

vitamin D represses the expression of this thrombus-promoting gene product, perhaps reducing the risk of MI and ischemic stroke. The same may be true for DHA, but more replicate experiments are required at the 24 hour time period to establish statistical significance, although 4 and 48 hours treatment of HEK293 cells with DHA or 1,25 D_3 plus DHA does produce statistically significant repression of SERPINE1 (data not shown).

Thrombomodulin (THBD) displayed a non-statistically significant increase in expression with 1,25 D_3 alone (fold effect 1.43, p value 0.28), but a statistically significant decrease in expression with DHA (fold effect 0.69, p value <0.01) and combined treatment (fold effect 0.75, p value 0.04) (Fig. 1). These data are independently confirmed by treating HEK293 cells for 48 hours with 1,25 D_3 and DHA (data not shown). Thus, thrombomodulin is the only gene probed wherein 1,25 D_3 and DHA function as agonistic and antagonistic ligands, respectively. Therefore, the THBD results in Fig. 1 support the hypothesis in Table 1 that vitamin D potentiates thrombolysis by inducing thrombomodulin, signifying that vitamin D supplementation may be protective of the cardio- and cerebrovascular systems by eliciting both anti-thrombosis and thrombolysis. Data from the VITAL trial should test this conclusion in patients. On the other hand DHA has the opposite effect on THBD (Fig. 1), suggesting that DHA is anti-thrombolytic, and perhaps should not be supplemented in combination with vitamin D.

The final gene studied, vascular endothelial growth factor alpha (VEGFA) showed a non-significant decrease with 1,25 D_3 alone (fold effect 0.72, p value 0.14), a non-significant increase in expression with DHA alone (fold effect 1.06, p value 0.59), and a non-significant decrease with combined treatment (fold effect 0.90, p value 0.24). Thus, VEGFA manifests statistically significant responses to neither vitamin D nor DHA, at least in HEK293 cells. This is in contrast to published data on the modulation of VEGFA by 1,25 D_3 [59].

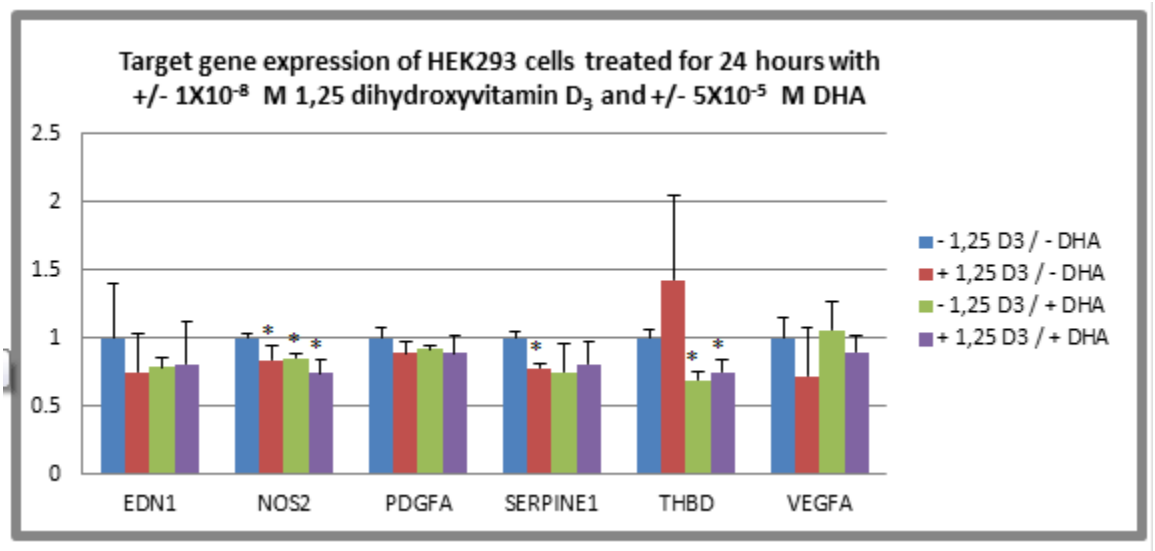

Figure 1: Treatment of human HEK293 cells with either ethanol vehicle control, $1X10^{-8}$ M 1,25 D_3, or $5X10^{-5}$ M DHA independently or in combination for a time period of 24 hours. Both reagents were suspended/dissolved in ethanol vehicle, for which the volume was equal between treatment groups. Three replicate samples for each treatment were obtained and compared to controls within each experiment using a two-tailed t-test. Experiments were repeated a minimum of three times with consistent results. Fold effect averages for + 1,25 D_3 / - DHA, - 1,25 D_3 / +DHA, and + 1,25 D_3 / + DHA treatments across all experiments for each gene are respectively as follows: EDN1 0.75, 0.817, and 0.69; NOS2 0.94, 0.933, and 0.78; PDGFA 0.93, 0.96, 0.88; SERPINE1 0.89, 0.79, and 0.81; THBD 1.43, 0.74, and 0.81; VEGFA 0.86, 1.03, and 0.90.

* denotes p value < 0.05

Discussion

It is generally agreed that the majority of the physiological effects mediated by 1,25 D_3 occur in the nucleus through VDR and its heterodimerization with the retinoid X receptor (RXR) to control gene transcription. Although DHA has been shown to have a weak affinity for VDR [60], and stronger affinity for RXR [61], it is likely the majority of its physiological functions occur outside the VDR mediated pathway.

As hypothesized, NOS2 appears to have a modest down regulation with exposure to 1,25 D_3 and DHA independently in HEK293 cells. This repression occurs across all treatment groups, but is most pronounced in the combination 1,25 D_3 and DHA treatment group, implicating a cooperation in the antagonism of the expression of NOS2. If the changes seen with addition of DHA were VDR mediated it would be expected that the repression would be reduced with the weak VDR agonist DHA competing for binding sites, thus the mechanisms for this observed increased repression of 1,25 D_3 in the presence of DHA is most likely not predominately VDR mediated. The repression of NOS2 is in itself interesting and is supported by experiments in macrophages, the hypothesis being that 1,25 D_3 acts as a paracrine antagonist of local inflammatory changes and helps to control the nitric oxide burst of macrophages [62]. Thus there is the possibility that 1,25 D_3 may offer a cardioprotective role through attenuation of the immune system by reducing the oxidative nitric oxide burst at the local level. The exact mechanism by which the combination of 1,25 D_3 and DHA exert their repression of NOS2 is beyond the scope of these experiments, but may be a result of crosstalk between common receptors other than VDR.

SERPINE1 showed a rather robust decrease of expression with the addition of 1,25 D_3, but the results with addition of DHA were not statistically significant. The statistically significant decrease in expression of SERPINE1 by exposure of HEK293 cells to 1,25 D_3 is of great interest, and supports study results observed in human primary coronary artery cells [50], yet refutes results in studies of human primary umbilical vein cell [51]. The natural activity of SERPINE1 is to inhibit the activator of plasminogen, thus at above normal levels it has a role of augmenting

thrombus formation. It logically would be expected to decrease in activity in the presence of 1,25 D_3 if it were truly to be antithrombotic. It is very interesting that the activity of SERPINE1 varies so dramatically in similar tissue types. It is beyond the scope of this project to elucidate the mechanism behind the apparent repression 1,25 D_3 has on SERPINE1, but it most likely is a result of VDR activation. This is supported by the observations of increased thrombogenicity in rat VDR knockout models. We have not as yet been able to develop the statistical significance to draw conclusions regarding the SERPINE1 DHA treatment groups with this study. Future studies will address this with increased numbers of experiments for pooling of data and attempts to make the system more sensitive. However, it appears that in HEK293 cells 1,25 D_3 downregulates SERPINE1 expression, another potential pathway for mediating a cardioprotective effect through possibly attenuating the thrombus formation pathway.

Thrombomodulin showed a moderate but non-statistically significant increase in expression with the 1,25 D_3 treatment alone. This increase, were it proven to be statistically significant, would support prior evidence in other studies. Interestingly, the addition of DHA caused a repression both by itself and in the presence of 1,25 D_3, results that did reach statistical significance. This is contrary to the hypothesized (Table 1) result of increased expression. Clinical studies measuring soluble thrombomodulin with PUFA supplementation have demonstrated no change in plasma levels. Thus the current result with DHA and THBD may be the product of a nonspecific function of DHA in the cell model, or may represent a result specific to the HEK293 cells.

The remainder of our results with PDGFA, EDN1, and VEGFA did not reach statistical significance and thus drawing conclusions from them is subject to reinterpretation. Nevertheless, it is tempting to speculate that in the cases of EDN1 and PDGFA, the observed repressive trend of 1,25 D_3 illuminates two mechanisms whereby the vitamin D hormone is beneficial to the cardio- and cerebrovascular systems to lower the risk of ischemia. Repeat experiments and pooling of data will be necessary to solidify this conclusion, and continued optimization of the cell model and rt-PCR system may be beneficial, something we may address in the future. Also, there is the possibility that we have missed an actual change in expression of

PDGFA, EDN1, and VEGFA by limiting the treatments to a single time point. Therefore, a time course experiment with times of 4, 12, 24 and 48 hour treatment protocols is under consideration. Future plans may also include repeats of the experiments in a more physiologically correct, but more expensive and difficult to culture, human cell model. While our HEK293 cells provided a robust and species specific model for testing the genes of interest in the cardiovascular system, a cell line derived from endothelium or smooth muscle may better fit the purpose. Unfortunately, these cell lines are difficult and expensive to harvest and grow and were beyond the scope of this research. Finally, we have not demonstrated a linear dose-response to 1,25 D_3 or DHA, which would provide further evidence that the present results are pathophysiologically relevant.

Future Directions

The current insight gained through this research is encouraging. We demonstrated at the molecular level the effects that 1,25 D_3 and DHA has on the expression of genes intimately involved with cardiovascular health. For the future, these experiments should be further qualified and quantified to better understand the mechanisms behind the findings. More investigation of these genes needs to occur in the HEK293 cell line in the form of repeat experiments, including experiments to identify if a dose response can be demonstrated. Also a time course experiment should be performed, as some of our genes did not show statistical significance, but may with shorter or longer periods of treatment. Repeat experiments should be performed in cell lines with a closer physiologic association to the cardiovascular system. Examples include cardiac myocytes, endothelial cells, and vascular smooth muscle cells, all of which differentially express VDR as well as the genes being investigated. In terms of PUFAs, the current study examined exclusively DHA, which only represents one half of the PUFA equation; thus, it may prove beneficial to attempt experiments with EPA as well. While EPA has not been shown to be an agonist for VDR, it also may operate in a yet characterized synergy with vitamin D or independently to control the expression of the genes we studied.

Conclusion

The cardioprotective benefit of vitamin D and DHA independently and in combination is an area of very active research. Here we demonstrated at the molecular level changes in expression of NOS2, for which decreased expression in the presence of all treatments may prove to be important in the understanding of decreased inflammation in the cardiovascular system. This insight might prove useful as a stepping stone to a more complete understanding of the complex mechanisms behind the actions of 1,25 D_3 and DHA. Also, we demonstrated decreases in activity of SERPINE1 with both treatments. Combined with the observations from VDR knockout rats and clinical evidence of decreased thrombogenicity with DHA, this evidence may also be important in future studies to understand the cardioprotective benefits of combination 1,25 D_3 and DHA, through decreased thrombus formation. It is our hope that this evidence will be expanded upon both in research at the University of Arizona College of Medicine-Phoenix, as well as through research in other laboratories around the world. Eventually this may lead to greater understanding of the mechanism (s) defining the cardioprotective benefits of 1,25 D_3 and DHA, separately and in combination.

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