**Vitamin D, Resveratrol, and Control of LCE3 Genes with Implications for Psoriasis**

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Abstract

Psoriasis (Psx) is a chronic inflammatory skin disease with abnormal keratinocyte proliferation and differentiation. One genetic risk factor for psoriasis (denoted PSORS4) is a deletion of LCE3B and LCE3C genes encoding structural proteins in terminally differentiated keratinocytes. Analogs of the hormonal form of vitamin D, 1,25-dihydroxyvitamin $D_3$ (1,25D) are routinely used to treat Psx, a skin disease that affects over 7 million patients in the US. However, this therapy, even when combined with an anti-inflammatory (e.g., betamethasone), is ineffective in some patients, particularly those with a severe disease phenotype, underscoring the need for better agents. Further, the mechanism of action of vitamin D analogs is not understood, although their ability to reduce proliferation and promote differentiation of psoriatic keratinocytes is both valued in therapy and is complementary to anti-inflammatory agents. Given that 1,25D acts via the vitamin D receptor (VDR) to regulate gene expression, this project is focused on elucidating expression alterations in psoriasis-relevant genes mediated by the 1,25D-liganded VDR in human keratinocytes. Whereas VDR activity is increased when bound to 1,25D, less is known about the ability of other nutritionally-derived lipids to act on VDR. The current study is designed to: 1) evaluate resveratrol, an antioxidant found in the skin of red grapes, as an effector of VDR signaling and potent activator of LCE gene transcription in human keratinocytes, and 2) determine whether resveratrol acts synergistically with 1,25D to regulate the expression of LCE3 genes, with the potential to boost skin repair and ameliorate the symptoms of psoriasis.

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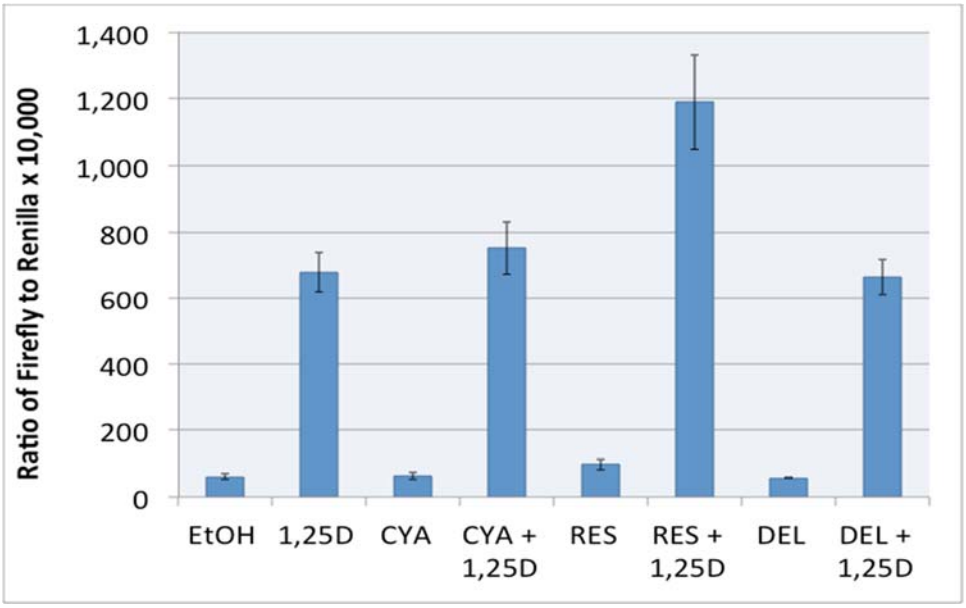


Figure 1: Luciferase assay measuring transcriptional activation from the LCE3 VDRE in transfected HEK293 cells. Standard error of the mean for each treatment group is represented by the error bars. Results are the compilation of 3 independent experiments.

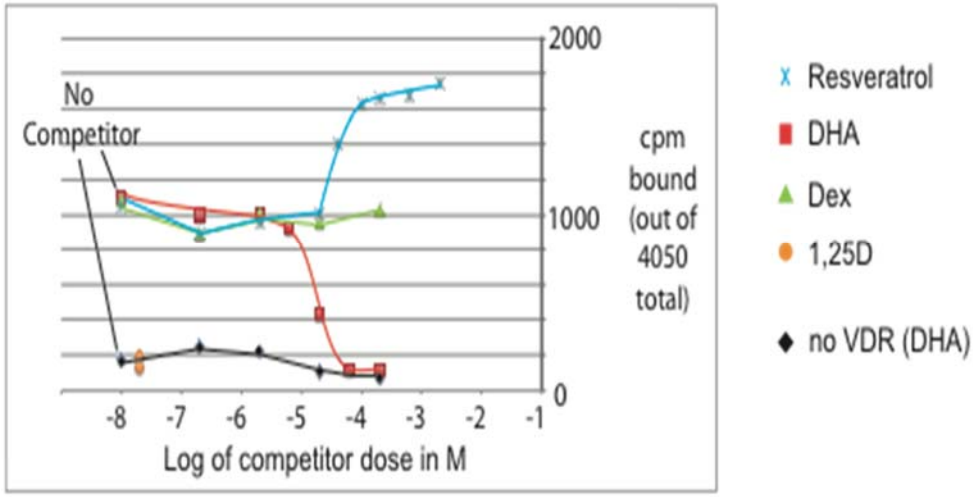


Figure 2: Binding curve demonstrating the Ability of candidate ligands to compete for binding to VDR with approximately 0.4 nM [$^3$H]1,25D in VDR-containing cell lysates.

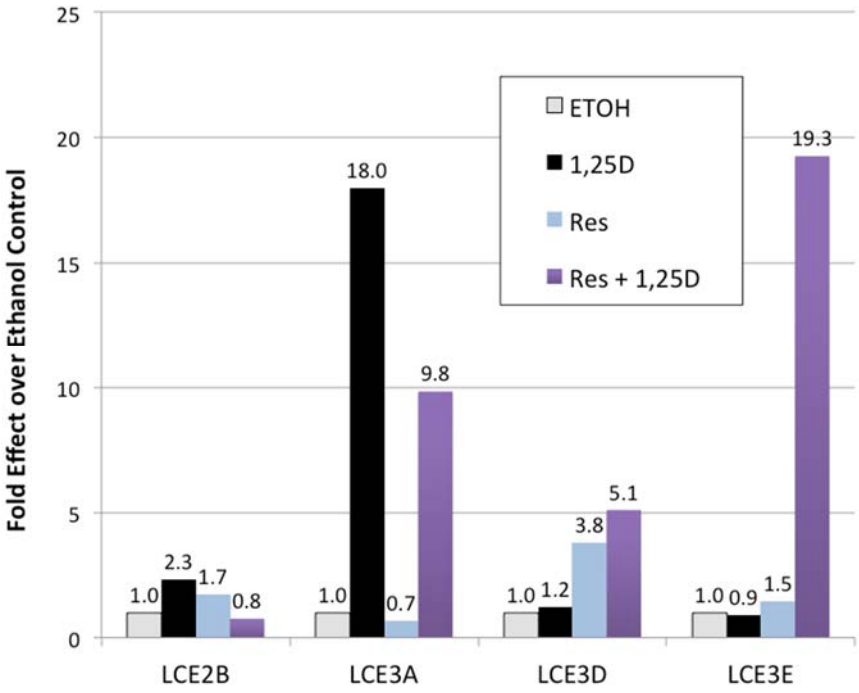


Figure 3: Activation of genes LCE2B, LCE3A, LCE3D, and LCE3E by 1,25 vitamin D, resveratrol, and the combination of resveratrol and vitamin D is shown here as fold effects over the negative control, Ethanol. CYP24A1 gene has been omitted from this figure so the fold effects on the LCE family of genes can be more readily interpreted.

## Introduction and Significance

Psoriasis (Psx) is a relatively common skin disorder characterized by hyperproliferation and inflammation of the skin. The etiology of this disorder is not known, but it was discovered in the 1980's that treatment with 1,25-dihydroxyvitamin $D_3$ (1,25D) has beneficial effects on psoriatic lesions[9]. Subsequent studies have determined that many (but not all) patients with mild/moderate disease show some degree of response after topical treatment with 1,25D or one of its analogs such as calcipotriol [24], such that forms of vitamin D have become front-line therapies for mild/moderate Psx. Although novel biologic agents can control severe psoriasis [25] these powerful anti-inflammatory agents are not available to patients with mild/moderate disease. Given this limitation, plus the fact that a large subset of patients do not respond to 1,25D or its analogs, even when combined with a topical steroid, the development of novel therapies for mild/moderate psoriasis remains an important priority.

The current understanding of the nuclear vitamin D receptor (VDR), largely derived from studies in its primary target tissues of kidney, intestine, and bone, is as follows: upon binding its 1,25D ligand, VDR heterodimerizes with its partner RXR (retinoid X receptor), then binds to specific vitamin D responsive elements (VDREs) within DNA and activates (or represses) nearby target genes. Although VDR is known to be present in skin, and 1,25D, as described above, is used to treat Psx, the gene targets that underlie this effect still await identification.

One genetic risk factor for psoriasis (denoted PSORS4) is a deletion of LCE3B and LCE3C genes encoding structural proteins in terminally differentiated keratinocytes[4]. The PSORS4 deletion removes the LCE3B and LCE3C genes from the LCE3 cluster, leaving the LCE3A, -3D and 3E genes intact. Prior research has shown that indeed, 1,25D is able to upregulate the intact LCE3A, -3D, and 3E genes in a PSORS4 deleted cell population. Given the current understanding that 1,25D acts via the VDR to regulate gene expression, this project will elucidate expression changes in psoriasis-relevant genes mediated by the liganded VDR in human keratinocytes. A potential lipid ligand of great interest is resveratrol, a molecule similar to vitamin D in that it is nutritionally derived, but is found in the skin of red grapes and a component in red wine. While 1,25D binds to and activates VDR with high affinity, resveratrol only mildly activates the VDR when tested alone. When cells are treated with both 1,25D and

resveratrol, however, there is an apparent synergistic effect and transcription rates of an artificial, VDRE-linked reporter construct significantly increase[15, 23]. However, whether resveratrol has a similar effect on LCE3 gene transcription in human keratinocytes remains to be tested. It should be noted that resveratrol has been reported to activate SIRT-1, a known activator of skin differentiation[16], giving support to the idea that resveratrol is active and beneficial in skin repair mechanisms. In this study, we tested the ability of resveratrol to synergize with 1,25D in the upregulation of LCE3A, -3D and 3E genes as a way of compensating for the loss of LCE3B and -3C in many psoriasis patients [68%] that bear the PSORS4 deletion. We also present direct evidence that resveratrol can activate VDR via a mechanism that is independent of traditional binding in the VDR ligand binding pocket.

## Materials and Methods

*Source of ligands*: Crystalline 1,25D was a kind gift from Milan Uskokovic of Hoffmann-LaRoche. Crystalline Resveratrol was a kind gift from Peter Jurutka of Arizona State University. The human keratinocyte cell lines HaCaT and CCD-1106 KERTr (KERTr), and the human embryonic kidney cell line HEK-293, were obtained from ATCC (Manassas, VA). HaCaT and HEK-293 were cultured in DMEM high glucose with 10 % fetal bovine serum, and KERTr cells were maintained in SFM Keratinocyte medium containing the recommended supplements (Invitrogen Corp, Carlsbad, CA) along with 5 µg/mL gentamicin. Culture media, fetal bovine serum, penicillin–streptomycin and gentamicin stocks were obtained from Gibco (Invitrogen Corp.). Human primary neonatal keratinocytes (HEKn) were purchased from Invitrogen Corp. (Carlsbad, CA) and cultured in serum-free EpiLife medium containing the recommended HKGS supplement kit reagents. Genotyping of these cell lines was as follows: genomic DNA was isolated from 4 to 5 million cells using a DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A triple set of primers was utilized to detect the presence or absence of the LCE3B_LCE3C deletion as described by de Cid et al. [4]: LCE3CF 50 - TCACCCTGGAACTAGACCTCA; LCE3CR 50 -CTCC AACCACTTGTTCTTCTCA; and LCE3CR2D 50 - CATC CCAGGGATGCTGCATG. PCR method included 50–80 ng of DNA, 0.5 µL of an 18 µM primer stock and 5 µL of Fast Start Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) in a 10-µL total volume. An ABI 2400 machine was programmed for 35 cycles of 94 C for 30 s, 60 C for 30 s and 72 C for 1 min, followed by a 72 C step for 10 min. PCR products were resolved on 3 % agarose gels. A single band at 199 bp indicates a homozygous deletion, a single band at 240 bp indicates a homozygous intact locus and the presence of both bands indicates a heterozygote. Real-time PCR analysis of LCE gene expression RNA was isolated using an Aurum Total RNA Mini Kit (BioRad, Hercules, CA, USA) from cells grown in 60 mm dishes to approximately 70 % confluence. Total RNA was assessed for purity and quantity by OD260/280 readings. First strand cDNA was synthesized using an iScript kit (Bio-Rad) and 2 µg of total RNA in a 40-µL reaction. Quantitative real-time PCR (qPCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) in a System 7500 Fast thermal cycler using 2 µL of first strand DNA and 1 µL of 18 µM primer mixture in 20

µL total volume. For detection of human LCE transcripts, the primers were as follows: LCE2B, forward primer 50 -GCCAGCCCC CTCCCAAGTGT and reverse primer 50 -GGGCACTGGGG CAGGCATTT; LCE3A, forward primer 50 -GAGTC ACCACAGATGCC and reverse primer 50 -CTTGCTGA CCACTTCCCA; LCE3B, forward primer 50 -CCCAAAGAGCTCAGCACAGT and reverse primer 50 -TGCCTCTGTCACAGGAGTTG; LCE3C, forward primer 50 -AGTTGTCCCTCACCCAAGTG and reverse primer 50 -ATTGATGGGACCTGAAGTGC; LCE3D, forward primer 50 -CTCTGCACCTGGACAACTCA and reverse primer 50 -CACTTGGGTGAGGGACACTT; LCE3E, forward primer 50 -CTGATGCTGAGACAAGCGATCTT and reverse primer 50 -GATCCCCCACAGGAAAACCT. Human CYP24A1 was detected using forward primer 50 -CAGCGAACTGAACAA ATGGTCG and reverse primer 50 -TCTCTTCTCATACAAC ACGAGGCAG, and human GAPDH was amplified using forward primer 50 -TGACAACTTTGGTATCGTGGAAGG and reverse primer 50 -AGGGATGATGTTCTGGAGAGCC. Given the high similarity among LCE3 genes, the LCE2B, 3A, 3B, 3C, 3D and 3E PCR products were sequenced to confirm their specificity. Data were analyzed using the comparative Ct method, normalized to GAPDH. Fold effects were calculated relative to vehicle-treated control samples and expressed as 2-DDCt according to instructions in the Applied Biosystems software.

Total RNA from culture was prepared employing primary human HEKn neonatal keratinocytes using an Aurum kit (BioRad) yielding a 50 µL volume containing 2-10 µg RNA from $10^7$ cells. $1^{st}$ strand cDNA synthesis was performed using an iScript kit (BioRad) and 2 µg RNA in a 20 µL reaction.

Real time PCR was performed in a ABI 7500 Fast machine using the Roche Fast Start Universal SYBR Green Master Mix, custom primers for each mRNA to be tested, and 2 µL of $1^{st}$ strand cDNA in a 10 µL reaction. A VDRE sequence was identified ~29 kb upstream of the LCE3A gene. Two copies of this sequence with four bases on either side were synthesized with an additional four base overhang for cloning into the HindIII/BglII sites of the pLUC-MCS reporter plasmid to create pLUC-LCE3.

HEK-293 cells were transfected in 24-well plates at 60,000 cells/well. Each well received 2.0 µl Express-In Reagent, 250 ng of either empty pLUC-MCS plasmid or pLUC-LCE3, 25 ng of pSG5-hVDR, 20 ng of pRL-null and 1 µl of 100X sodium pyruvate. After transfection, wells were

treated with ligands or ethanol control for 20 hours. Whole cell lysates were harvested and analyzed for Firefly luciferase and Renilla luciferase activity using a Dual Luciferase assay kit and a Sirius Luminometer according to the manufacturers' protocols.

## Results

Luciferase assay measuring transcriptional activation from the LCE3 VDRE in transfected HEK293 cells was performed (Fig. 1). Cyanidin (CYA), resveratrol (RES), and delphinidin (DEL) showed modest, if any activation over the ethanol control vehicle. When 1,25D was combined with CYA or DEL, there was no apparent increase in activation over 1,25D alone. However, when cells were treated with RES and 1,25D in combination, an apparent synergistic relationship was observed in transcriptional activation of the artificial LCE3 VDRE luciferase gene construct.

The ability of candidate ligands to compete for binding to VDR with approximately 0.4 nM [$^{3}$H]1,25D in VDR-containing cell lysates was explored (Fig. 2). Dexamethasone (Dex, green) is a negative control with no appreciable binding to VDR. Radioinert 1,25D (orange circles) is the high affinity VDR ligand (nanomolar range) that shows the full range of competition. Docosahexaenoic acid (DHA, red squares) is a known low affinity ligand (micromolar range). Resveratrol (blue) does not compete with vitamin D, but rather shows an increase in binding affinity of radioactive 1,25D. The black line with diamonds shows a DHA competition curve using lysates from COS-7 cells that were not transfected with the VDR expression plasmid. These results are a compilation of two similar assays performed independently.

Real time PCR of total RNA from cells treated with ethanol, vitamin D, resveratrol, and the combination of resveratrol and vitamin D were performed. As anticipated, the positive control gene CYP24A1 demonstrated the highest fold effect of 1,25D over the ethanol control (1154-fold, not shown), in keeping with the exquisite sensitivity of this gene to regulation by 1,25D. Omitting the CYP24A1 results in this plot allows us to examine the genes of interest on a scale which allows for comparison of their regulation by the tested ligands. Resveratrol alone showed a modest activation of LCE2B, LCE3D, and LCE3E genes. When cells were treated with the combination of resveratrol and 1,25D, however, a synergistic fold effect over the ethanol and 1,25D controls was observed with the LCE3D and LCE3E genes. The LCE3A gene was regulated in a different pattern, although inconsistencies in the results with the 1,25D (alone) treatment leave open the possibility that the pattern of regulation of LCE3A may in fact be similar to that of LCE3D and LCE3E. Consistent with this conclusion is the fold-induction of

LCE3A (9.8-fold) by the combination of resveratrol and 1,25D, which falls between the 5.1- and 19.3-fold induction of LCE3D and LCE3E, respectively, with the dual treatment. In contrast, the LCE2B gene behaved in a very different manner, with no activation by the combination of resveratrol and 1,25D, but modest induction by either resveratrol or 1,25D alone, suggesting that the LCE2 gene cluster (separated from the LCE3 cluster by insulator elements) may be regulated quite differently by these two compounds.

## Discussion

The data collected in this study confirm the original hypothesis that resveratrol can synergize with 1,25D to activate the LCE3 gene cluster beyond the traditional 1,25D therapy. Although other compounds tested in our laboratory, such as cyanidin, delphinidin, and curcumin, seem to act by binding in the VDR ligand binding pocket (in competition with 1,25D), there is recent evidence that resveratrol acts via completely different mechanisms[23]. Also, the present results in a competitive binding assay reveal that resveratrol does not compete with 1,25D for VDR binding, but in fact actually enhances 1,25D binding. Resveratrol is now thought to function by activating SIRT1 which then may, in turn, potentiate VDR action on transcription by a mechanism such as deacetylation. Indeed, resveratrol enhances rather than competes for 1,25D binding to VDR (Fig. 2). This scenario would explain why 1,25D and resveratrol can synergize in the activation of VDR since they do so by separate and independent means.

Although overall positive, our study does have limitations. The keratinocytes tested in our system were shown to be heterozygous for the PSORS4 deletion. Ideally, these experiments would be performed in a homozygous deletion lineage. This strategy would yield a clearer picture of the potential for resveratrol and 1,25D to upregulate the surrounding LCE3 genes. Obtaining a homozygous deletion lineage has proven difficult, as the company we purchase the cells from does not test for the genotype, but future work could address this limitation.

## Future Directions

Given that we have shown that the combination of resveratrol with 1,25D is a more potent activator of the LCE3 genes that 1,25D alone, future studies are needed to show a clinical benefit of treating psoriasis with not only the classical vitamin D treatment, but also the potential novel addition of resveratrol as a therapeutic option. It would also be prudent to perform these experiments in a test system that included a cell lineage homozygous for the PSORS4 deletion. This will likely be difficult as we have been unable to locate a commercially-available cell line homozygous for the PSORS4 deletion.

## Conclusions

Vitamin D has long been used as a treatment for psoriasis, but the molecular mechanisms by which this treatment is successful have been in question. In a specific subset of patients with psoriasis, namely those with the PSORS4 deletion, vitamin D therapy may potentially be augmented with compounds shown to improve 1,25D-induced gene expression, such as the polyphenol red wine constituent, resveratrol.

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