THE ROLE OF LYSINE DEACETYLASES ON THE GLUCOCORTICOID-RECEPTOR MEDIATED REPRESSION

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ABSTRACT

The Role of Lysine Deacetylases on the Glucocorticoid-Receptor Mediated Repression – Nuclear receptors regulate transcription through histone acetylation by using lysine acetyltransferases and lysine deacetylases (KDACs). Upon binding to an antagonist, KDACs have been shown to interact with steroid receptors. We have previously shown that KDACi exposure has a significant impact on GR signaling and that Class I lysine deacetylases facilitate glucocorticoid-induced transcription. In the current study, we extended this analysis to the target genes repressed by the GR. We found that VPA impairs the GR induced repression through KDAC inhibition, which was also observed using a structurally distinct KDACi. This inhibition is either due to impaired transcription at certain genes or through post-transcriptional effects, but not because of defective GR processing. Depletion of KDAC1 expression mimicked the effects of KDACi in four of six genes found to be impaired in GR mediated repression. Co-depletion of KDAC1 and 2 caused similar impairment of the same four genes. Altogether we found that Class I KDAC activity plays both a transcriptional or post-transcriptional role in GR-mediated repression and that KDAC1 alone or in coordination with KDAC2 is required for GR repression at many of these target genes.

INTRODUCTION

According to recent proteomic studies, lysine acetylation is a post-translational modification that occurs on over 1500 proteins with various essential cellular functions[1,2]. Lysine acetylation is directly regulated by lysine acetyltransferases (KATs) and deactylases (KDACs). Prior to these findings, histones were the only known proteins to be acetylated. The current models of histone acetylation and its role in transcription depict KATs as transcriptional coactivators and KDACs as transcriptional corepressors[4]. However, the variety of proteins found to be acetylated suggests that KATs and KDACs participate in cellular processes beyond solely regulating histone acetylation. In particular, the functions of acetylation in signaling-regulated transcription are poorly understood.

KDACs are a family of proteins divided into four classes: I, II (subdivided into IIA & IIB), III, and IV[3]. This study focuses solely on inhibition of Class I KDACs (KDACs 1, 2, 3, and 8). Class I KDACs are located in the nucleus and are involved in numerous nuclear processes. There are numerous lysine deacetylase inhibitors (KDACi) that target the various classes of KDACs with different potencies and specificities[5,6]. Some Class I –selective KDACi, such as valproic acid (VPA), are clinically used to treat epilepsy, bipolar disorder, migraines, and cancer[7]. However, the use of KDACi in humans may expand as they are also under investigation for potential use in treating additional diseases such as HIV, inflammatory and neurological disorders[3,8]. It has been found that in about 50% of its users, VPA (Class I KDACi) causes significant metabolic and reproductive side effects[9]. This calls for a greater understanding of the physiological impact of KDACi drugs to improve their benefits and reduce unwanted side effects.

The glucocorticoid-receptor (GR) is a transcription factor that belongs to the nuclear receptor family. The GR can interact with specific sequences in DNA that are found in the vicinity of target genes to activate or repress transcription through various mechanisms. It has been shown from studying steroid receptor signaling that antagonist-bound steroid receptors associate with corepressor complexes containing KDACs[10-12] to prevent transcriptional activation. It has also been shown that agonist-bound steroid receptors recruit KATs to target genes and that transcriptional activation results from histone acetyltransferase activity[13,14]. The model based on these findings for the role of KDACs in steroid receptor signaling depicts KDACs as countering steroid receptor-mediated transactivation of target genes. However, in a previous study, we have shown that about half of GR target genes depend on KDAC1 expression and activity for transcriptional activation by GR[15], suggesting that KDACs in addition to KATs are required for glucocorticoid-induced transcriptional activation. Although the results of the previous study call for an expansion in the model to include an activating function in the role of KDACs in GR signaling pathway, it does not address KDACs role in GR-regulated transcriptional repression.

Once in the nucleus, the GR can inhibit expression by binding to specific DNA glucocorticoid response elements (GRE) at the promoter or enhancer level of responsive genes. It has also been shown that the GR can repress transcription by mechanisms independent of direct DNA binding, mediated through protein-protein interactions with other transcription factors. The GR also represses its own synthesis in a hormone-dependent manner (restricted to repressing GR ligands) and that this auto regulation occurs at both a transcription and post-transcriptional level[15]. Members of the nuclear receptor family involved in transrepression have been shown to exist in a repressor complex with histone deacetylase activities[17]. Transrepression mediated by

the GR has been shown to occur in both KDAC-dependent and –independent fashion. Since we have previously shown that KDACs cooperate with GR to activate transcription, the current study investigated the involvement of KDACs in the repression of GR target genes in the same cell line. We show that the repression of GR target genes is impaired with exposure to VPA, a KDACi, when co-administered with dexamethasone (Dex), a synthetic glucocorticoid.

METHODS

RNA Analysis – Cells were plated in 6-well dishes at $2x10^5$ cells/well and treated the following day with VPA (5 mM) or apicidin (0.5 μg/ml) for 5 hours and Dex (100 nM) for 4 hours.. After treatment, cells were collected by lysis in TRIzol (Invitrogen). Total RNA was isolated with the NucleospinRNAII kit (Clontech). cDNA was produced using the iScript cDNA synthesis kit (Bio-Rad). Real time quantitative PCR (qPCR) was performed using SYBR Green Master Mix (Bioline) with the Applied Biosystems StepOne instrument. Exon-exon and exon-intron primer pairs for each gene tested are listed in Table 1. For each experiment, the probed gene Ct values were normalized against a geometric means of GAPDH and HPRT1 Ct values to obtain ΔCt values for each sample. The primer efficiency for each experiment was calculated using standard curves and used to change the Ct values into the graphed fold change values. The ΔΔCt values found from two different treatments were analyzed using a paired *t* test (two-tailed and one-tailed) to determine statistical significance.

Cell Culture – Murine hepatoma cells (Hepa-1c17) were kept in minimum essential medium α (Invitrogen) containing 10% fetal bovine serum (FBS) (Gemini Bio-Products) and 0.1% gentamicin (Invitrogen).

Antibodies and Reagents – Lamin A/C (sc-6215), GAPDH (sc-25778), and HDAC3 (sc-11417) antibodies were purchased from Santa Cruz Biotechnology. The antibodies against HDAC2 (2540S) were purchased from Cell Signaling Technology. The HDAC1 antibody (05-100) was purchased from Millipore. The secondary anti-rabbit (11-035-144) and anti-goat (sc-2056) antibodie were purchased from Jackson ImmunoResearch Laboratories and Santa Cruz Biotechnology, respectively. VPA and apicidin were purchased from Sigma-Aldrich.

Western Blotting – Cell lysates for analysis of KDAC expression were prepared by 2X SDS-PAGE buffer added to treated cells. Proteins were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad) at 400mA for 2 h. The membrane was blocked with 2% nonfat dry milk for 1 h and then exposed to the primary antibodies overnight at 4 °C. The following day, the membrane was washed three times with 1 X TBS with 0.1% Tween 20 (TBS-Tween) followed by exposure to secondary antibodies for 1 h at RT. After exposure to the secondary antibodies, the membrane was washed again for three times with the TBS-Tween solution. The proteins were visualized with the ChemiDoc XRS + molecular imager (Bio-Rad) using a 1:1 ratio of hydrogen peroxide and luminol (Pierce).

siRNA-mediated KDAC Knockdown – Cells were plated in 24-well dishes at 2 X 10^4 cells/well in antibiotic-free minimum essential medium α. The cells were transiently transfected with siRNA using the DharmaFECT Reagent 1 (Dharmacon) according to the manufacturer's specifications. Expression of KDACs 1, 2, and 3 was depleted using their corresponding ON-TARGETplus

SMARTpool ORF siRNAs (Dharmacon). Knockdowns were confirmed by Western blotting. Lamin siRNA and non-targeting siRNA were used as controls.

TABLE 1
PCR primers used in the study

Gene	Forward primer	Reverse primer
Exon primers		
Gapdh	CATGTTCCAGTATGACTCCACTC	GGCCTCACCCCATTTGATGT
Hprt1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Mex3a	CAAGGGAGCGACCATCAAAC	CGCCGGTTATCTCAAACACG
Cdon	CAAAGCTGAGGTGCGCTATAA	CCATTCGAGGAAGGACGACTC
Fam20c	GATGTGACGCGGGATAAGAAG	GCTCGGTGGAACAGTAGTAGG
Hes1	ATAGCTCCCGGCATTCCAAG	GCGCGGTATTTCCCCAACA
Rgs16	CGAGTGGGCCAGTAAGCATAA	CGAAAGACTCTCTCCATCCCAG
Igsf9	TGACCGTCCTCAGCCTGAT	GGACTCGTCCCACGTAATCG
Hbegf	ATGCCTCCCTGGTTACCAC	AGTCAGCCCATGACACCTGT
Ikbke	GCGGAGGCTGAATCACCAG	GAAAGCCCGAACGTGTTCTCA
Mllt11	CCTACCTACGAGAGCAAGGAT	AAGGTGCTGTACTCAAGGAGG
Exon-intron primers		
Mex3a	TCTCCTCTTCTCCCTTCCACCACC	CCCCCGAAACATCCTAGTTCTCCA
Fam20c	ACAGCGACATCAGGTTTAACCCCA	TGCCTGGCGTGTGAAATAGCTTTC
Igsf9	CAGAGCCCCTTCCTTCATTCAACA	GGCACTCGTACCAACCCTGGTCTT

RESULTS

Effect of VPA Treatment on Expression of GR-regulated Genes - In our previous study, we carried out expression profiling of the Hepa-1c1c7 cells treated with VPA in the presence and absence of Dex[15]. We found that the majority of the genes that were significantly regulated by glucocorticoids were activated with Dex exposure. However, the microarray data also shows a group of genes repressed in the presence of Dex[15]. This group is indicated to the right of the heat map (Supplementary Fig. 1), in which the green signal in *Column 4* denotes repression of these genes by Dex. The *red* signal in *Column 1* indicates that for that particular GR-repressed target gene, the Dex-induced repression is impaired in the presence of VPA,. This suggests that, for those GR target genes, KDACs may cooperate with GR to induce repression.

VPA and Apicidin, Two Structurally Different KDACis, Impact GR-Regulated Repression Similarly – The microarray data shows that VPA can impair Dex-mediated repression at GR target genes[15]. VPA, however, is known to have off-target effects. To determine whether the negative effect of VPA on Dex-mediated repression occurs through KDAC inhibition and not through VPA's off target effects, we investigated whether a structurally distinct KDACi, apicidn, has similar outcomes to VPA on a set of 9 GR-repressed genes. Apicidin is a cyclic tetrapeptide, whereas VPA is a simple aliphatic acid (Fig. 1A). However, both small molecules are Class I-selective KDACi. Because they have major structural differences, they are highly likely to cause different off-target effects. Therefore, if VPA and apicidin have different effects on Dex-mediated repression of GR target genes, the impaired repression observed in the presence of

VPA might not be mediated through KDAC inhibition. However, if the effects are similar, we can conclude that the small molecules are mediating their actions through KDAC inhibition.

We treated Hepa-1c1c7 cells with either VPA or apicidin in the presence or absence of Dex and measured their effects on expression of nine Dex-repressed genes through RT-qPCR. All genes showed significant decreases expression with Dex treatment (Fig. 1b-d). For six of these genes exposure to VPA significantly impaired Dex-mediated repression of gene expression (compare VPA/Dex to Dex alone condition Fig. 1b,c). For one gene, Isgf9, the effect of VPA co-treatment on Dex-mediated repression just missed statistical significance, with a p value of 0.07 (Fig. 1b). For the same group of seven genes, treatment with apicidin had very similar effects on Dex-mediated repression. Five of the seven genes, including Igsf9, showed significant impairment of Dex-induced repression (Fig. 1b,c). However, for two of the genes, Hbegf1 and Hes1, apicidin cotreatment did not significantly impair Dex-mediated repression (Fig. 1c), suggesting that VPA mediates its effects on these genes through KDAC-independent mechanisms.

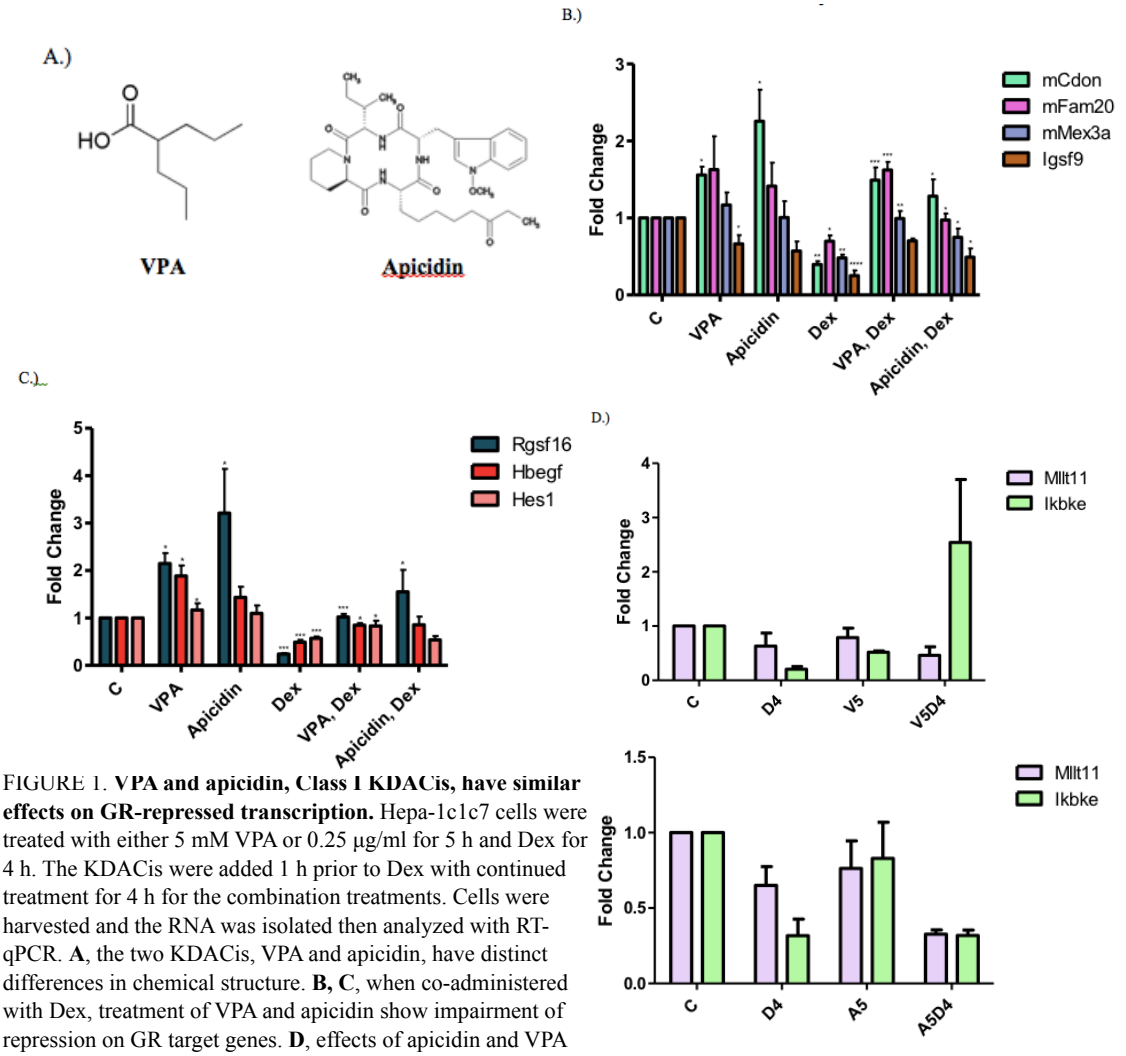

FIGURE 1. **VPA and apicidin, Class I KDACis, have similar effects on GR-repressed transcription.** Hepa-1c1c7 cells were treated with either 5 mM VPA or 0.25 μg/ml for 5 h and Dex for 4 h. The KDACis were added 1 h prior to Dex with continued treatment for 4 h for the combination treatments. Cells were harvested and the RNA was isolated then analyzed with RT-qPCR. **A**, the two KDACis, VPA and apicidin, have distinct differences in chemical structure. **B, C**, when co-administered with Dex, treatment of VPA and apicidin show impairment of repression on GR target genes. **D**, effects of apicidin and VPA on GR target genes at which transcriptional repression was unaffected by VPA. *Asterisks* denote significant changes between Dex alone and control, VPA alone and control, and Dex/VPA co-treatment relative to Dex alone.

Mllt11 and Ikbke consist of the second set of genes we investigated. In the microarray data, these Dex-repressed genes were unaffected by VPA co-treatment [15]. To confirm these results, we carried out further analysis with RT-qPCR. In the case of Mllt1, neither VPA nor apicidin treatment had any significant effect on the Dex-induced repression. In the case of Ikbke, apicidin co-treatment had no effect on Dex-induced repression but VPA co-treatment had variable effects between four biological replicates. Two replicates showed that VPA had no effect on Dex-mediated repression, but the other two replicates showed the opposite. Perhaps with more replicates, the effect of VPA in the presence of Dex can be clarified at that particular gene. Altogether, VPA and apicidin showed similar effects on the Dex-mediated repression of 6 out of the 9 GR target genes tested. This strongly suggests that the observed impairment of GR-induced repression is largely mediated through KDAC inhibiting activity of VPA and apicidin.

KDAC1 Knock Down Impairs GR-Regulated Repression at Select KDACi-impaired Genes – Because VPA and Apicidin are Class I-selective KDAC inhibitors, the results above imply a significant role for Class I KDACs in GR-regulated repression. To test this directly, we performed siRNA knockdowns of selected Class I KDACs and measured mRNA expression levels of 6 genes impaired by VPA treatment. Lamin A/C-specific siRNAs were used as a control for off target effects. The responses of these genes can be classified into two groups.

In the first group, KDAC1 depletion fully or partially mimicked VPA-mediated impairment of GR-induced repression as shown in Fig. 1B. Only two of the four genes, Mex3a and Cdon, showed a significant difference in expression between Lamin and the knockdown after statistical analysis. However, after the co-depletion of KDACs 1 and 2, all four genes showed a significant difference in expression compared to Lamin (Fig. 2a). The magnitude of the impairment (Lamin versus KDAC1+2) was similar to that seen when comparing fold induction by Dex in the presence and absence of VPA (compare VPA/Dex with Dex in Fig. 1b).

In contrast, depletion of KDACs 2 or 3 alone showed no effect on the expression level of these four genes (Fig. 2a,c). This indicates that KDAC1 facilitates the repression of these four GR target genes, either alone or together with KDAC2. Interestingly, the co-depletion of KDAC1 and 3 showed no effect on the expression level of this group, although the data represents only one biological replicate.

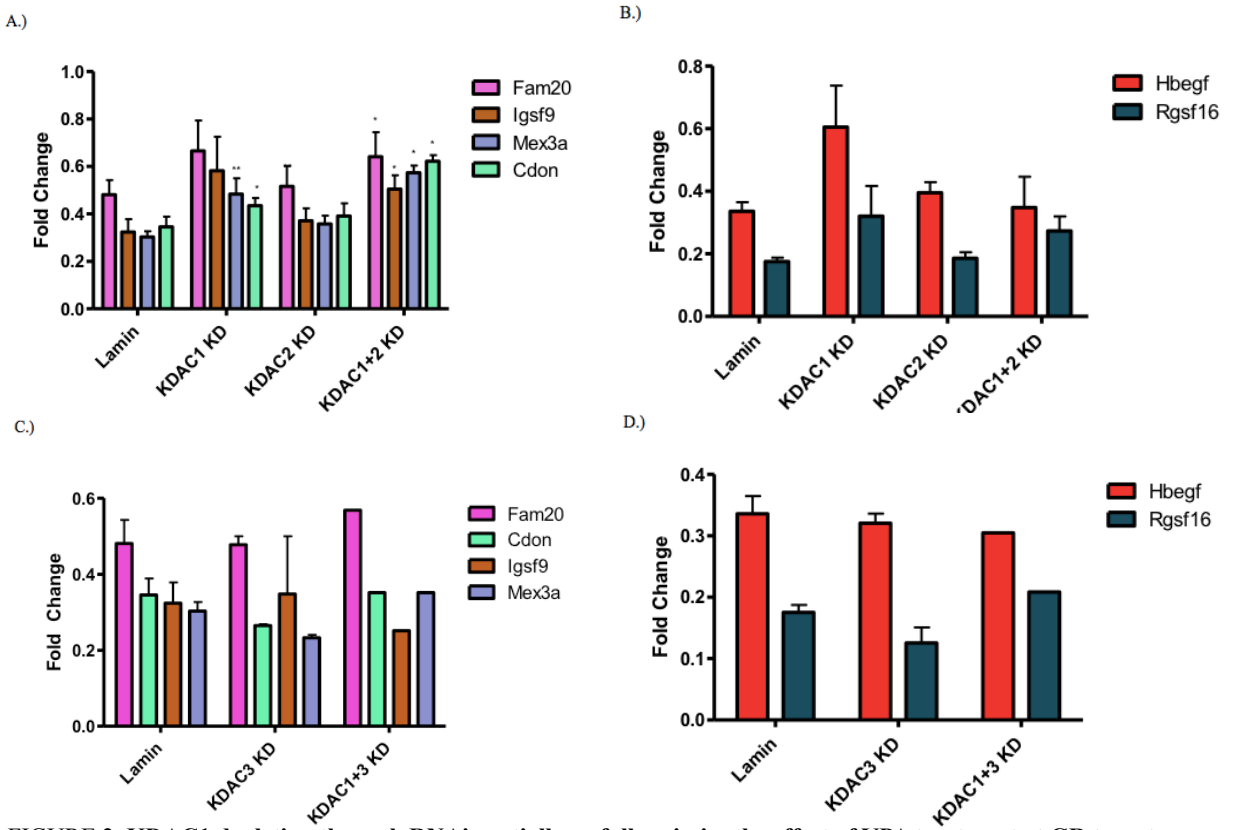

FIGURE 2. **KDAC1 depletion through RNAi partially or fully mimics the effect of VPA treatment at GR target genes.** Hepa-1c17 cells were transfected with Lamin or KDAC siRNA. Following 48 h after transfection, cells were treated with or without 100nM Dex for 4 h. RNA was isolated from the cells and subjected to RT-qPCR. **A, B**, cells were transfected with Lamin or KDAC 1, KDAC 2, or a combination of KDAC1 and KDAC2. **C, D**, cells were transfected with siRNA against lamin, KDAC3 or a combination of KDAC 1 and KDAC 3. *Asterisks* denote significant changes between Dex-treated cells transfected with respective KDAC siRNA and Lamin siRNA. *, $p \leq 0.05$; **, $p \leq 0.01$. *Error bars* represent S.E.

The second group of genes consists of those resistant to all the KDAC depletions performed. Individual depletions of KDAC1, 2, or 3 as well as co-depletions of KDACs 1 and 2, or KDACs 1 and 3, had no significant effect on the Dex-mediated repression these genes (Fig. 2b,d). Ttreatment with VPA or apicidin showed an effect on GR-repressed gene expression of Rgs16, both apicidin and VPA significantly impaired Dex-mediated repression (Fig. 1c), strongly suggesting a role for Class I KDACs. . It is possible that KDAC 8 or depletion of other combinations of Class I KDACs would show an effect that mimics VPA or apicidin treatment on Rgs16 In the case of Hbegf, apicidin and VPA had different effects on Dex-mediated repression. The lack of effect of any of the KDAC depletions tested confirms that VPA impairs the repression of Hbegf through KDAC-indpendent mechanisms.

Effects of VPA on Dex-repressed Transcription – The experiments described above show that the activity of KDACs 1 and 2 are required for repression of at least four genes by GR. Next we investigated the mechanics behind the observed effects. We have previously demonstrated When no ligand is present, the GR interacts with hsp90, a molecular chaperone, in order to maintain its ligand binding-competent conformation. It is known that acetylation of hsp90 stunts its ability to interact with the GR, which impairs subsequent ligands from binding and receptor degradation[14]. Hsp90 is deacetylated by KDAC6, a Class IIB KDAC. If KDAC6 is inhibited by VPA, then the effects of VPA on GR-regulated genes could be due to the loss of integrity in the

GR-hsp90 interaction. In our previous study, however, we showed that VPA treatment of Hepa-1c1c7 cells did not change GR levels nor did it affect the GR-hsp90 complex, which is consistent with studies that show that VPA does not inhibit KDAC6[15].

Since the inhibition of GR processing as a potential mechanism behind the effects of KDACi on GR action, it is likely that the effect of the drugs is downstream of processing at the level of transcription. To test this hypothesis we measured levels of nascent, unspliced transcripts of three GR-repressed genes using exon-intron primer sets (Table 1) in RT-qPCR.Hepa-1c1c7 cells were treated with VPA or Dex or a combination of both for up to 4 h. The results for Igsf9, a gene whose Dex-mediated repression was significantly impaired in VPA/Dex co-treatment, showed that Dex induces a drop in transcription which was completely compromised by VPA co-treatment (Fig. 3a). The results for Mex3a were very similar (Fig. 3c). In both cases, VPA appeared to increase transcription initially. However, statistical analysis shows that the increases are not statistically significant. At the Fam20c gene, there was no significant difference between the combination treatment and Dex alone in levels of nascent transcripts (Fig. 3b), even though the Dex-induced decline in Fam20c mRNA levels was significantly impaired by KDACi (see Fig. 1b). Further investigation in to the mechanism of VPA in other significantly repressed GR target genes such as Cdon or Rgsf9 could lend more insight on VPA's role on GR induced repression.

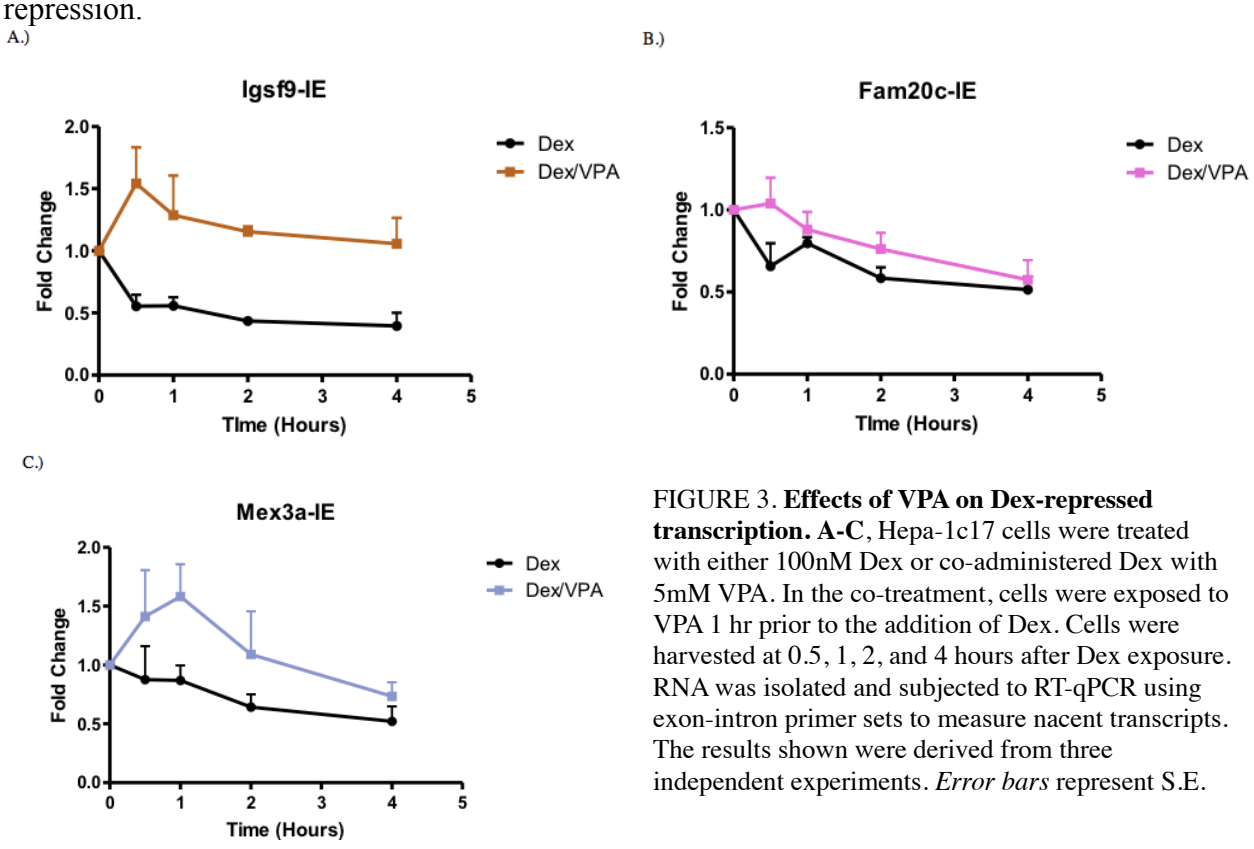

FIGURE 3. **Effects of VPA on Dex-repressed transcription. A-C**, Hepa-1c17 cells were treated with either 100nM Dex or co-administered Dex with 5mM VPA. In the co-treatment, cells were exposed to VPA 1 hr prior to the addition of Dex. Cells were harvested at 0.5, 1, 2, and 4 hours after Dex exposure. RNA was isolated and subjected to RT-qPCR using exon-intron primer sets to measure nacent transcripts. The results shown were derived from three independent experiments. *Error bars* represent S.E.

DISCUSSION

Longstanding models of GR action posit that KDACs serve only a repressive role in GR-regulated transcription. Our previous study established that KDACi have a major impact on the GR-regulated transcriptome. We showed that Class I KDAC activity cooperates with GR to activate a sizable fraction of GR target genes. Previous studies of GR-mediated transcriptional repression show that it is dependent on KDACs in a gene-selective manner. Because KDACs play a major role in GR transactivation, we wanted to determine whether they can also facilitate GR transrepression in the same model cell line. The current study demonstrates that KDACs 1 and 2 can also cooperate with GR to mediate gene repression.

This study clearly shows that VPA impairs Dex-induced repression through its ability to inhibit KDACs. First, seven of nine GR-repressed genes showed extremely similar responses to VPA and the structurally distinct KDACi apicidin, which suggests that the effects of VPA at most GR-repressed genes are not due to non-KDAC target effects. Second, siRNA-mediated depletion of KDACs mimicked the effects of these KDACi on six GR-repressed genes.

The siRNA experiments establish that KDAC1 plays a role in facilitating GR transrepression. At two of six genes at which VPA impaired Dex-mediated repression, KDAC1 depletion alone was sufficient to impair GR-induced repression to approximately the same extent as VPA treatment. At two additional genes, co-depletion of KDACs1 and 2 fully-mimicked the effect of VPA or apicidin on Dex-induced repression.. Thus, at these four genes KDACs 1 and 2 facilitate repression by GR. Two of the six genes were completely resistant to any of the knockdowns. This was expected in the case of the Hbegf gene because VPA and apicidin had contrasting effects on its repression by Dex. However, at the other gene, Rgs16, both VPA and apicidin impaired Dex-mediated repression, strongly suggesting a role of Class I KDACs in GR action. It is possible that there is redundancy in the system where one KDAC can rescue functionality in the loss of one or two other KDACs. It is also possible that another Class I KDAC is the major player in facilitating GR transrepression, like KDAC8, or perhaps a combination of different KDACs. We did not deplete KDACs1, 2, and 3 because of the potential deleterious effects on cell survival. Altogether, this study shows that KDAC1, either acting alone or with KDAC2, contributes to GR-induced repression in certain gene contexts.

VPA treatment does not disrupt the GR-hsp90 interaction nor induce GR degradation, which implies that VPA does not cause defects in GR processing. Analysis of nascent transcripts showed that VPA impaired Dex-repressed transcription at the Igsf9 and Mex3a genes. Coupled with the unaffected GR processing, this result suggests that KDACs cooperate with GR to mediate repression at the transcriptional level. Nothing is known about the mechanism by which GR transcriptionally represses these genes. There are GR binding sites within 25 kb of the transcription start sites of these genes (G. Hager, NIH, personal communication), but it is not known whether GR binds directly to DNA in these regions or whether it tethers to another transcription factor.

At the Fam20c gene, VPA had no effect on Dex-repressed transcription. However, it did have a significant impact on mRNA accumulation, suggesting that KDACs are involved in regulation of this gene at a post-transcriptional level. Interestingly, our previous study identified two GR-activated genes that were sensitive to VPA downstream of transcription. In recent proteomic studies numerous proteins involved in RNA processing were found to be acetylated.

However, the functional effect of the acetylation is unknown. The results of our studies predict that the acetylation of these proteins may serve a role in gene expression.

The current study establishes that KDACs 1 and 2 cooperate with GR to repress gene transcription in a gene-selective fashion. Combined with the results of our previous study on GR transactivation, we conclude that KDACs 1 and 2 are versatile transcriptional coregulators in GR signaling. Depending on the gene context, they can either cooperate with GR to activate or repress transcription. Future studies will focus on the mechanisms by which KDACs 1 and 2 work with GR.

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