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Valproate recovers the inhibitory effect of dexamethasone on the proliferation of the adult dentate gyrus-derived neural precursor cells via GSK-3 β and β -catenin pathway

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Abstract

Neurogenesis in the adult dentate gyrus (DG) is decreased in rodent models for mood disorders. Mood stabilizers including lithium (Li) and valproate (VPA) increase it. These increasing effects of Li and VPA on neurogenesis in adult DG are considered to be one of therapeutic action of Li and VPA, but its molecular mechanism remains unclear.

We have already reported that Li recovers the inhibitory effects of dexamethasone (DEX), an agonist of glucocorticoid receptor, on the proliferation of adult rat DG-derived neural precursor cells (ADP) via GSK-3 β and β -catenin pathway. Following it, here we investigated the mechanism underlying the recovery effects of VPA on DEX-induced decrease of ADP proliferation.

VPA is an inhibitor of histone deacetylase (HDAC). However, Trichostatin A, a HDAC inhibitor, had no effect on ADP proliferation. In contrast, SB415286, a specific GSK-3 β inhibitor, recovered DEX-induced decrease of ADP proliferation. In addition, quercetin (Que), a β -catenin pathway inhibitor, abolished such recovery effect of VPA. Moreover, nuclear β -catenin and the expression of cyclin D1 were altered by DEX, VPA and Que like the proliferation. Moreover, VPA increased the phosphorylation of Ser⁹, which is known as the inhibitory phosphorylation site of GSK-3 β .

These suggest that HDAC is not involved in the recovery effect of VPA on ADP proliferation and that VPA recovers the inhibitory effects of DEX via increasing the phosphorylation of Ser⁹ on GSK-3 β and following up-regulation of β -catenin pathway. Therefore, GSK-3 β and β -catenin pathway might play a role in the increasing effects of VPA on neurogenesis on adult DG.

Keywords: neurogenesis; glucocorticoid; mood disorder; mood stabilizer; hippocampus

1. Introduction

Neurogenesis in the adult dentate gyrus (DG) is affected by a lot of factors, including environment, stress, hormones, and drugs. For example, neurogenesis in adult DG is decreased in rodent models for stress-related disorders (Gould et al. 1997; Malberg and Duman 2003; Pham et al. 2003; Jayatissa et al, 2006). Although it remains unclear how neurogenesis in DG is decreased in these models, some studies have suggested that glucocorticoids might be involved in the decrease of adult hippocampal neurogenesis (Gould et al, 1997; Cameron and McKay, 1999; Kim JB et al, 2004; Boku et al, 2009). In human, elevated levels of glucocorticoids is one of causal events in stress-related disorders (de Kloet et al, 1995; Swaab et al, 2005). In contrast, recent reports have described that lithium (Li) and valproate (VPA), common mood stabilizers and often used for the treatment of stress-related disorders, affect neurogenesis through increasing cell proliferation and/or promotion of neuronal differentiation of neural precursor cells (Chen et al., 2000; Son et al., 2003; Hao et al., 2004; Hsieh et al., 2004; Kim JS et al., 2004; Laeng et al., 2004; Wexler et al., 2008; Boku et al, 2009; Boku et al, 2011) and that Li blocks the effects of stress on depression-like behaviors through increasing hippocampal neurogenesis in adult rodent models (Silva et al., 2008). Taken together, these studies suggest that

neurogenesis in adult DG may play an important role in the therapeutic action of mood stabilizers for stress-related disorders. Therefore, to elucidate how glucocorticoids and lithium regulate neurogenesis might lead to further understanding of the pathophysiology of stress-related disorders and the development of new therapeutic targets.

We have already established the culture system of adult DG-derived neural precursor cells (ADP) and that Li recovers dexamethasone (DEX), a specific agonist of glucocorticoid receptor, -induced decrease of ADP proliferation via glycogen synthase kinase-3 β (GSK-3 β) and β -catenin pathway (Boku et al., 2009). In addition, we have shown that VPA also recovers the inhibitory effect of DEX on ADP proliferation like Li (Boku et al, 2011). However, the detailed mechanism underlying this recovery effect of VPA on DEX-induced decrease of ADP proliferation is poorly understood.

Some previous studies have reported that VPA promotes β -catenin pathway through inhibition of GSK-3 β (Chen et al., 1999; Kim et al., 2005). These suggest that VPA might regulate ADP proliferation through GSK-3 β and β -catenin pathway. In contrast, other past studies have reported that the activity of GSK-3 β is not directly inhibited by VPA (Williams et al., 2002; Kozlovsky et al., 2003; Ryves et al., 2005).

Additionally, it has been shown that VPA actions are partly mediated by histone deacetylase (HDAC) inhibition (Phiel et al., 2001).

Here we investigated the mechanism underlying the recovery effects of VPA on DEX-induced decrease of ADP proliferation, focusing on HDAC, GSK-3 β and β -catenin pathway.

2. Materials and Methods

2.1 Isolation and culture of ADP ADP were isolated from the DG of adult male Sprague–Dawley rats (8 weeks old), as described in our previous report (Boku et al., 2009). ADP were maintained with Neurobasal (Invitrogen, Carlsbad, CA)/ B27 supplement minus vitamin A (Invitrogen)/ 1 mM L-glutamine (Invitrogen)/ 20 ng/ml bFGF (Invitrogen) at 37°C on laminin (Invitrogen)-ornithine (Sigma, St. Louis, MO)-coated dishes and fed with new medium every two or three days by replacing 50% of the medium. When cell confluency reached 80–90%, cells were passaged by trypsinization, and the cell density for plating was approximately 1×10^4 cells/cm².

2.2 Drugs DEX was purchased from Sigma. VPA was kindly donated by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). Trichostatin A was purchased from Sigma. Quercetin was purchased from Calbiochem (San Diego, CA).

2.3 Cell counting with Alamar Blue assay Alamar Blue assay is a rapid and simple non-radioactive assay used to estimate the number of living cells (Ahmed et al., 1994). Alamar Blue dye is a fluorogenic redox indicator is converted from the oxidized form to the reduced form in cells. The reduced form of Alamar Blue dye is highly fluorescent; fluorescence in Alamar Blue assay reflects the number of cells. Although BrdU-based assays are often used for cell counting, BrdU-positiveness reflects the duplication of DNA, but not the number of cells. Additionally, we confirmed that fluorescence in Alamar Blue assay is proportional to the simply counted number of ADP (data not shown). Therefore, we used Alamar Blue assay to estimate the effects of drugs on the number of cells. First, 1×10^4 cells/ well were seeded on laminin–ornithine coated 96-well plates in 100 μ l/ well of medium. After 24h, cells were treated with each drug at each concentration. After 3 days, 10 μ l/ well of Alamar Blue solution (Invitrogen) was added to medium, and cells were incubated at 37°C for 3 hr. Subsequently, 50 μ l of medium was dispensed into plates and the fluorescence of samples was measured and calculated as described in the manufacturer’s manual. Data were expressed as the means \pm S.E.M. of four independent cultures. Statistical analysis was performed using one-way ANOVA and Bonferroni’s post hoc test. Significance was inferred for $P < 0.05$.

2.4 Quantitative RT-PCR 1×10^5 cells/ well were seeded on laminin-ornithin coated 6-well plates in 2 ml /well of medium. After 24 h, cells were treated with each drug at each concentration. After 3 days, total RNA was extracted from cells with RNeasy extraction kit (Qiagen, Hilden, Germany). Total RNA was converted to cDNA with Quantitect Reverse Transcription kit (Qiagen). PCR was performed with SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) in ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA). The conditions of PCR were: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The sequences of forward and reverse primers used were: *AGC TGG TCA TCA ATG GGA AA* and *ATT TGA TGT TAG CGG GA TCG* for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), *CAA CGC ACT TTC TTT CCA GAG* and *AGG GCT TCA ATC TGT TCC TG* for cyclin D1, respectively. GAPDH was used as a control. The results were analyzed by using SDS 2.0 software (Applied Biosystems). Data were expressed as the means \pm S.E.M. of three independent cultures. Statistical analyses were performed by one-way ANOVA and Bonferroni's post hoc test. Significance was defined as $P < 0.05$.

2.5 Western Blotting For preparation of total protein, 1×10^5 cells/ well were seeded on laminin-ornithin coated 6-well plates in 2 ml/ well of medium. After 24 h, cells were

treated with each drug at each concentration. After 3 days, lysis of cells and preparation of total protein were performed with Mammalian Cell Lysis Kit (Sigma).

For preparation of nuclear protein, 4×10^5 cells/ well were seeded on laminin-ornithin coated 100 mm dishes in 10 ml medium. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, lysis of cells and preparation of nuclear proteins were performed with Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein concentration was measured with Protein Assay Kit (Pierce, Rockford, IL), and equal amount of proteins (20 μ l/ well) were loaded onto a 10 % SDS gel. The gel was transferred onto a nitrocellulose membrane (GE Healthcare, Milwaukee, WI) and incubated with primary antibodies, which were used at following concentrations: mouse monoclonal anti- β -catenin antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-GAPDH antibody (1:200; Santa Cruz Biotechnology), rabbit polyclonal anti-GSK-3 β antibody (1:1000; Cell Signaling, Danvers, MA), rabbit polyclonal anti-pGSK-3 β (Ser⁹) antibody (1:1000, Cell Signaling), rabbit polyclonal anti-pGSK-3 β (Tyr²¹⁶) antibody (1:1000; Biosource, Camarillo, CA). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary anti-mouse IgG antibody (1:10000, GE Healthcare) or horseradish peroxidase-conjugated secondary anti-rabbit IgG

antibody (1:10000, GE Healthcare). Protein expression was detected with Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). The pictures were converted to digital files and the intensity of each band was analyzed with Image J (National Institute of Health, Bethesda, MD). Data are expressed as the means \pm S.E.M. of three independent cultures. Statistical analyses were performed by one-way ANOVA and Bonfferoni's post hoc test (nuclear β -catenin) and unpaired t-test (phosphorylation sites on GSK-3 α). Significance was defined as $P < 0.05$.

3. Results

3.1 Effects of VPA and TSA on DEX-induced decrease of ADP proliferation

We have already shown that VPA has no effect on ADP proliferation (data not shown) but recovers ADP proliferation decreased by dexamethasone (DEX), a specific agonist of glucocorticoid receptor (Figure 1A and Boku et al., 2011). To investigate whether the inhibitory effects of VPA on HDAC mediates the recovery effects of VPA on ADP proliferation decreased by DEX, we examined the effects of Trichostatin A (TSA), a common inhibitor of HDAC, on ADP proliferation with Alamar Blue assay in the presence of DEX. Cells were treated with 0, 1, 3, 10, 30, 100 nM

TSA in the presence of 5 μ M DEX for 3 days, and Alamar Blue assay was performed.

DEX significantly decreased ADP proliferation, but TSA had no effect on ADP proliferation at any concentrations in the presence of 5 μ M DEX (One-way ANOVA and Bonferroni's post hoc test, $F(6, 21)=13.37$, $p<0.01$, Fig. 1B). Therefore, HDAC may not mediate the recovery effect of VPA on ADP proliferation decreased by DEX.

3.2 Que abolishes the recovery effect of VPA on ADP proliferation decreased by DEX

We have already shown that the inhibitory effects of DEX on ADP proliferation is recovered by Li and SB415286, a specific inhibitor of GSK-3 β and that quercetin (Que), an inhibitor of β -catenin pathway, abolishes such this recovery effects of Li (Boku et al, 2009). Therefore, by using Alamar Blue assay, we examined whether Que abolishes the recovery effects of VPA on DEX-induced decrease of ADP proliferation. Cells were treated with 5 μ M DEX, 300 μ M VPA and/ or 10 and 30 μ M Que for 3 days, and Alamar Blue assay was performed. The concentration of VPA was determined as 300 μ M based on our previous study (Boku et al, 2011). Que abolished the recovery effects of 300 μ M VPA on DEX-induced decrease of ADP proliferation as in the case of Li (One-way ANOVA and Bonferroni's post hoc test, $F(4,15)=75.49$, $p<0.01$, Fig. 2). Therefore, GSK-3 β / β -catenin pathway may mediate

the recovery effects of VPA on DEX-induced decrease of ADP proliferation.

3.3 Effects of DEX, VPA, and Que on nuclear β -catenin and mRNA expression of cyclin D1

β -catenin translocates from cytosol to nucleus and works as a transcription factor (Salic et al, 2000). Cyclin D1 is a common cell cycle regulator that controls G1-S transition via controlling cyclin-dependent kinases, and its expression is regulated by β -catenin pathway (Tetsu and McCormick, 1999; Shtutman et al, 1999). To confirm the involvement of β -catenin pathway in the recovery effect of VPA on DEX-induced decrease of ADP proliferation, we examined the effects of DEX, VPA and Que on nuclear β -catenin and mRNA expression of cyclin D1, like as performed in our previous study (Boku et al, 2009). Cells were treated with 5 μ M DEX, 300 μ M VPA and/ or 30 μ M Que for 3 days. Then, nuclear protein and total RNA were extracted. The change of nuclear β -catenin was estimated with Western blotting for nuclear protein. The change of mRNA expression of cyclin D1 was estimated with quantitative RT-PCR for total RNA. Nuclear β -catenin is considered to regulate the transcription of mRNA of cyclin D1 (Tetsu and McCormick, 1999; Shtutman et al, 1999). It means that mRNA of cyclin D1 is more useful as an indicator of the involvement of β -catenin than protein. Therefore, we choose quantitative RT-PCR to

estimate the effects of DEX, VPA and Que on the expression of cyclin D1. As in the case of Li, VPA recovered both nuclear β -catenin and mRNA expression of cyclin D1 decreased by DEX and Que abolished these recovery effects of VPA (One-way ANOVA and Bonferroni's post hoc test, $F(3,8)=32.19$, $p<0.01$, Fig. 3A and $F(3,8)=32.19$, $p<0.01$, Fig. 3B).

3.4 Effects of VPA on the phosphorylation states of GSK-3 β

Nuclear translocation of β -catenin is inhibited by GSK-3 β -induced phosphorylation of β -catenin (Aberle et al, 1997; Orford et al, 1997). The activity of GSK-3 β is regulated by two phosphorylated residues; Ser⁹ to render it inactive (Cross et al, 1995) and Tyr²¹⁶ to render it active (Hughes et al, 1993). Our previous results suggest that VPA may recover the inhibitory effects of DEX on β -catenin pathway as in the cases of Li and SB415286, both are well-known direct inhibitors of GSK-3 β (Boku et al, 2009). However, it remains unclear whether VPA inhibits GSK-3 β through direct inhibition of its catalyzed reaction or indirect inhibition by affecting its phosphorylation states. To elucidate it, we examined the effects of VPA on the phosphorylation states of Ser⁹ and Tyr²¹⁶ on GSK-3 β . Cells were treated with 5 μ M DEX and 300 μ M VPA for 3 days. Then, Western Blotting was performed. As our past study showed, Tyr²¹⁶ is well phosphorylated but Ser⁹ is slightly phosphorylated by 5

μM DEX (Boku et al, 2009). 300 μM VPA remarkably increased the phosphorylation of Ser⁹ (unpaired t-test, $t=6.127$, $df=4$, $p<0.05$, Fig.4B) but had no effect on the phosphorylation of Tyr²¹⁶ (unpaired t-test, $t=0.07624$, $df=5$, $p=0.9429$, Fig.4C), in the presence of 5 μM DEX. On the other hand, the treatment of VPA alone had no effect on all of them (data not shown). Taken together, these suggest that VPA may indirectly inhibit GSK-3 β through increasing the phosphorylation of Ser⁹ on GSK-3 β .

4. Discussion

Here we showed that VPA recovers the inhibitory effects of DEX on ADP proliferation, nuclear β -catenin and mRNA expression of cyclin D1 in parallel and that Que, an inhibitor of β -catenin, abolished such recovery effects of VPA. In addition, VPA may inactivate GSK-3 β via increasing the phosphorylation of inhibitory Ser⁹ on GSK-3 β . Our previous study has already shown that SB415236, a specific inhibitor of GSK-3 β , also recovers the inhibitory effects of DEX on ADP proliferation (Boku et al, 2009). Taken together, these suggest that VPA recovers the inhibitory effect of DEX on ADP proliferation via inhibiting GSK-3 β and following activation of β -catenin pathway. It is the same as in the case of Li except for the difference of the action on GSK-3 β □ □ VPA increases Ser9 phosphorylation, but not Li (Boku et al, 2009).

VPA is well known as an inhibitor of HDAC (Phiel et al, 2001). DEX inhibits transcription factors Lef/Tcf, activated by binding of β -catenin (Novak and Dedhar, 1999) via GSK-3 β -dependent pathway and HDAC-dependent pathway in osteoblasts (Smith and Frenkel, 2005). In addition, HDAC binds to β -catenin in its enzymatic activity-dependent manner and inhibits the binding of β -catenin to Lef/Tcf (Billin et al, 2000). Therefore, we first hypothesized that VPA recovers DEX-induced decrease of ADP proliferation via inhibiting HDAC. To test this hypothesis, the effects of TSA, a specific inhibitor of HDAC, was examined. However, TSA had no effect on DEX-induced decrease of ADP proliferation. It suggests that the recovery effect of VPA on DEX-induced decrease of ADP proliferation may be HDAC-independent.

In our past study, we concluded that Li directly inhibits the activity of GSK-3 β because Li had no effect on the phosphorylation of both Ser⁹ and Tyr²¹⁶ (Boku et al, 2009). On the other hand, we have shown that VPA increases the phosphorylation of Ser⁹, which inactivates GSK-3 β in our present study. In addition, some past studies have reported that VPA does not directly inhibit the activity of GSK-3 β (Williams et al., 2002; Kozlovsky et al., 2003; Ryves et al., 2005). These suggest that VPA may indirectly inhibit GSK-3 β via increasing the phosphorylation of Ser⁹. However, others have reported that VPA can directly inhibit the activity of GSK-3 β (Chen et al., 1999;

Kim et al., 2005). Therefore, our results do not rule out the possibility that VPA has the direct inhibitory effect on GSK-3 β in addition to the indirect inhibitory effect.

It has been shown that VPA increases the phosphorylation of Ser⁴⁷³ on Akt kinase (De Sarno et al, 2002; Aubry et al, 2009). Akt kinase is activated by the phosphorylation of Ser 473 on Akt kinase and increases the phosphorylation of Ser⁹ on GSK-3 β (Fresno Vara et al, 2004). Therefore, there is a possibility that VPA inactivates GSK-3 β via activating Akt kinase. In the near future, we are going to examine the involvement of Akt kinase in the recovery effects of VPA on DEX-induced decrease of ADP proliferation. To elucidate how VPA increases the phosphorylation of Ser⁹ on GSK-3 β in ADP may lead to the further understanding the action mechanism of VPA.

5. Conclusions

Here we have shown that VPA recovers DEX-induced decrease of proliferation of adult rat dentate-gyrus derived neural precursor cells via increasing the phosphorylation of Ser⁹ on GSK-3 β and following its inactivation. However, the detailed mechanism underlying it remains unclear. To elucidate how VPA increases the phosphorylation of Ser⁹ on GSK-3 β may lead to the further understanding the

action mechanism of VPA.

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Figure Legends

Fig1. Effects of VPA and TSA on DEX-induced increase of ADP proliferation

VPA recovers 5 μ M DEX-induced decrease of ADP proliferation in dose-dependent manner (A). On the other hand, TSA had no effect on 5 μ M DEX-induced decrease of ADP proliferation. Drugs were added to medium 24 h after cell seeding. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm S.E.M. of four independent cultures. # $P < 0.01$, compared with control.

Fig 2. Que abolishes the recovery effect of VPA on DEX-induced decrease of ADP proliferation in dose-dependent manner

Drugs were added to medium 24 h after cell seeding. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm S.E.M. of four independent cultures. # $P < 0.01$, compared with control, DEX or 0 μ M.

Fig 3. Effects of DEX, VPA, and Que on nuclear β -catenin and mRNA expression of Cyclin D1

A. DEX decreased nuclear β -catenin, VPA recovered it, and Que abolished the recovery effect of VPA. Cells were treated with 5 μ M DEX, 300 μ M VPA and/ or 30 μ M Que 24 h after cell seeding. Nuclear proteins were prepared, 20 μ g of them were loaded into each lane and western blotting was performed 3 days after the treatment. Pictures are shown from a typical experiment. Data are shown as the means \pm S.E.M.

of three independent cultures. # $P < 0.01$, compared with control, DEX or VPA.

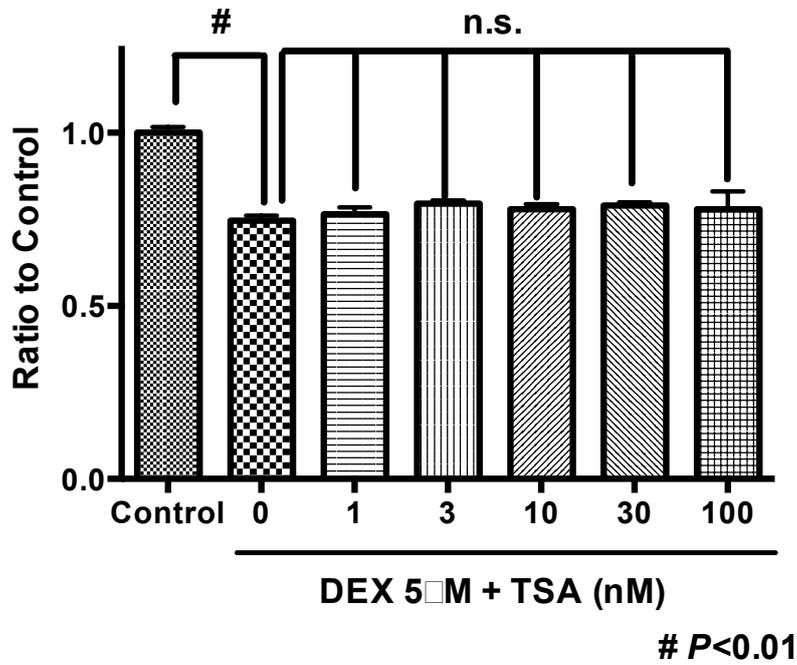
B. DEX decreased mRNA expression of cyclin D1, VPA recovered it, and Que abolished the recovery effect of VPA. Cells were treated with 5 μM DEX, 300 μM VPA and/ or 30 μM Que 24 h after cell seeding. RNA isolation and quantitative RT-PCR were performed 3 days after the treatment. Values are shown as the ratio of cyclin D1 mRNA to GAPDH mRNA. Data are shown as the means \pm S.E.M. of three independent cultures. # $P < 0.01$, compared with control, DEX, or VPA.

Fig 4. Effects of VPA on the phosphorylation state of GSK-3 β

VPA increased the phosphorylation of Ser⁹ (A and B), but had no effect on that of Tyr²¹⁶ (A and C) on GSK-3 β . Cells were treated with 5 μM DEX or 5 μM DEX + 300 μM VPA. After 3 days, 20 μg of total cell lysates were loaded into each lane and western blotting were performed. In A, pictures are shown from typical experiment that was repeated five times. In B and C, Values are shown as the ratio total GSK-3 β . Data are shown as the means \pm S.E.M. of five independent cultures. # $P < 0.05$, compared with control.

Figure 1

A



B

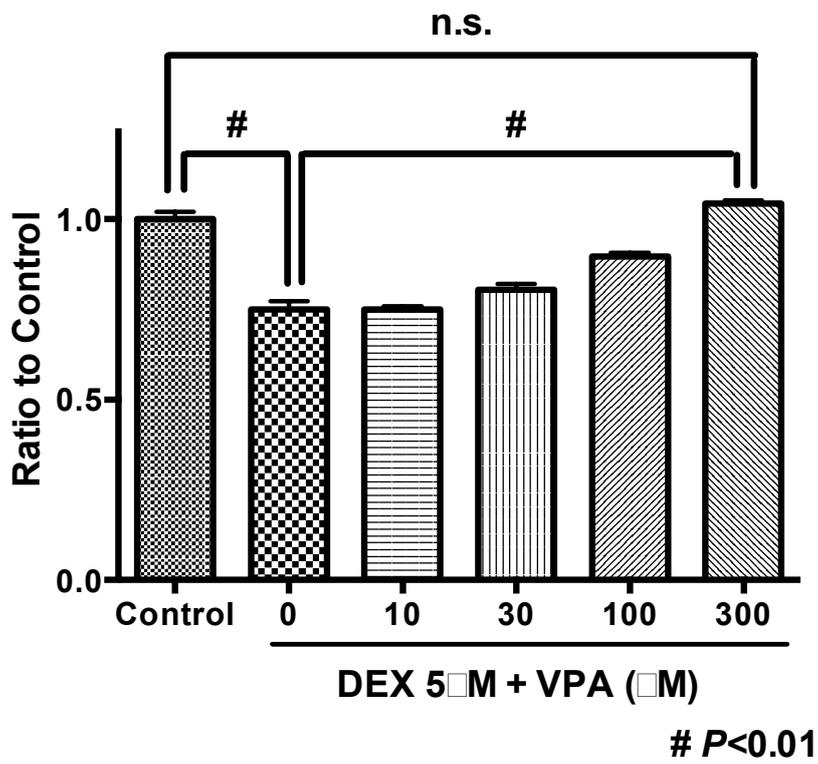


Figure 2

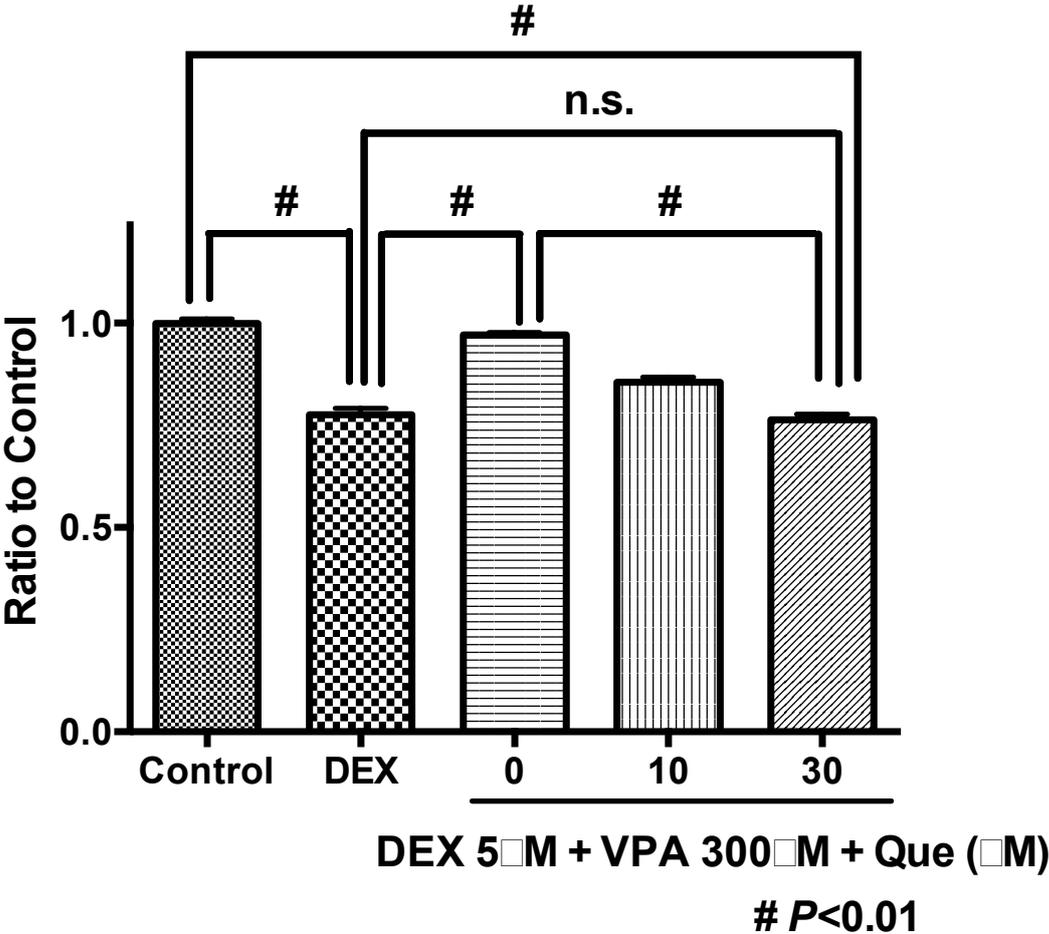
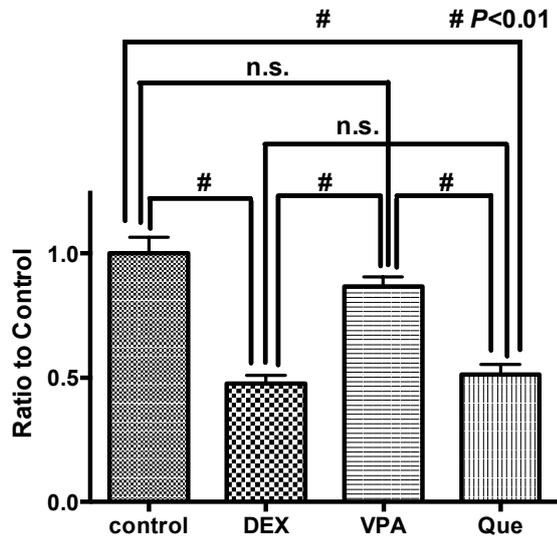
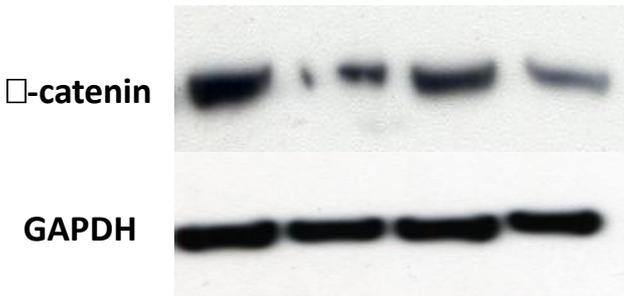
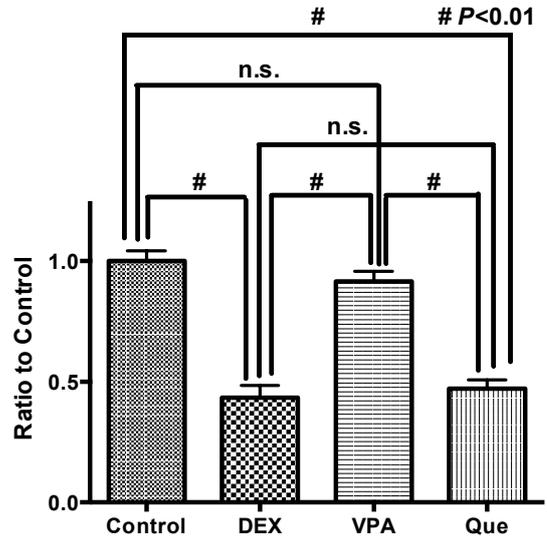


Figure 3

A



B

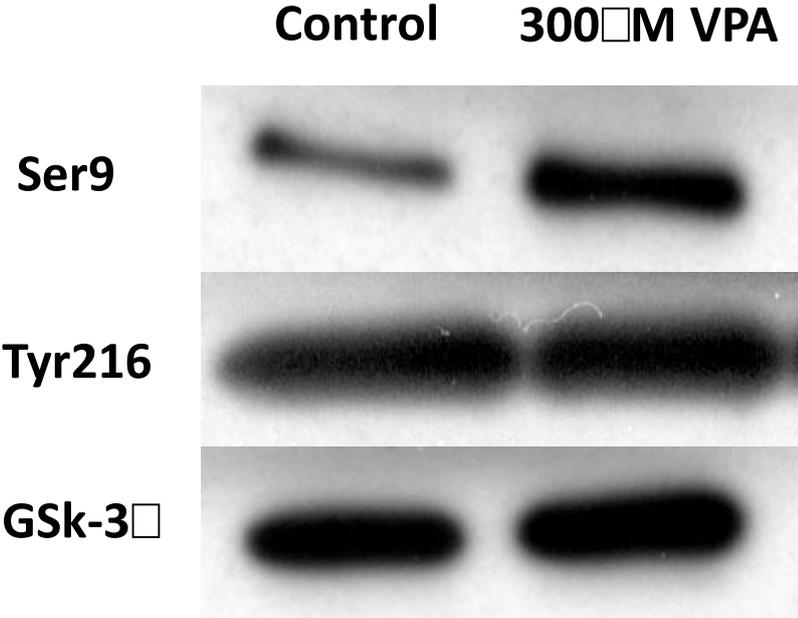


DEX 5 μ M	-	+	+	+
VPA 300 μ M	-	-	+	+
Que 30 μ M	-	-	-	+

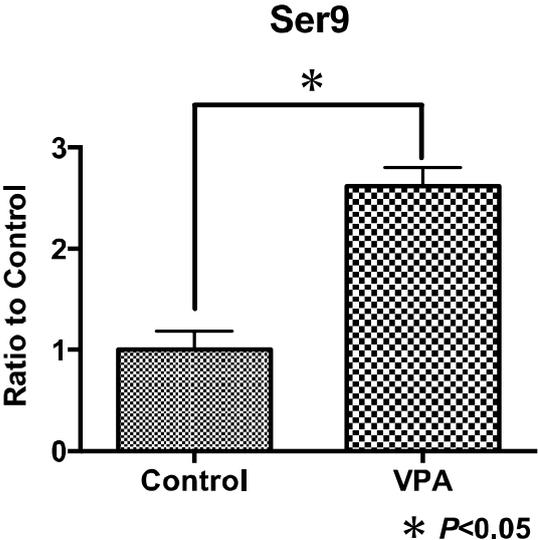
DEX 5 μ M	-	+	+	+
VPA 300 μ M	-	-	+	+
Que 30 μ M	-	-	-	+

Figure 4

A



B



C

