Progesterone with Vitamin D Affords Better Neuroprotection against Excitotoxicity in Cultured Cortical Neurons than Progesterone Alone

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Because the complex heterogeneity of traumatic brain injury (TBI) is believed by many to be a major reason for the failed clinical trials of monotherapies, combining two (or more) drugs with some potentially different mechanisms of action may produce better effects than administering those agents individually. In this study, we investigated whether combinatorial treatment with progesterone (PROG) and 1,25-dihydroxyvitamin D_3 hormone (VDH) would produce better neuroprotection than PROG alone following excitotoxic neuronal injury *in vitro*. E18 rat primary cortical neurons were pretreated with various concentrations of PROG and VDH separately or in combination for 24 h and then exposed to glutamate (0.5 μmol/L) for the next 24 h. Lactate dehydrogenase (LDH) release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays were used to measure cell death. Both PROG and VDH significantly ($P < 0.001$) reduced neuronal loss when tested independently. Primary cortical cultures treated with VDH exhibited a U-shaped concentration-response curve. PROG at 20 μmol/L and VDH at 100 nmol/L concentrations were the most neuroprotective. When the drugs were combined, the "best" doses of PROG (20 μmol/L) and VDH (100 nmol/L), used individually, did not show substantial efficacy; rather, the lower dose of VDH (20 nmol/L) was most effective when used in combination with PROG ($P < 0.01$). We also examined the effect of combinatorial treatment on mitogen-activated protein kinase (MAPK) activation as a potential neuroprotective mechanism and observed that PROG and VDH activated MAPK alone and in combination. Interestingly, the best combination dose of PROG and VDH (20 μmol/L and 20 nmol/L, respectively), as observed in cell death assays (LDH and MTT), resulted in increased MAPK activation compared with either the most neuroprotective concentration of individual PROG (20 μmol/L) and VDH (100 nmol/L) or the combination of these individual best doses. Such interactions must be considered in planning individualized combinatorial therapies. In conclusion, the findings of the present study can be taken to suggest that VDH warrants study as a potential partner for combination therapy with PROG.

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INTRODUCTION

For more than 30 years, researchers in the field of neuroprotection have been trying to find a safe and effective agent to treat acute-stage traumatic brain injury (TBI). In the past two decades, almost all phase II and phase III clinical trials for moderate and severe TBI failed (1) because no single drug proved clinically effective. These failures may be attributable in part to the heterogeneity of TBI and the limitations of monotherapies, which target a single mechanism of the injury cascade. Because TBI is a very complex systemic illness that results in long-term neurodegenerative processes that last for months or even years after initial injury (2,3), a logical strategy would be to combine pleiotropic agents that can simultaneously target different injury mechanisms to stem the cascade of destructive events. Addressing this problem, a National Institutes of Health (NIH) panel has recently called for studies to investigate combining therapeutic agents that have individually shown promising effects against TBI. These investigations would determine whether such combinations could lead to better, more clinically relevant, functional outcomes than agents given alone (4).

Fortunately a number of pleiotropic drugs that work via multiple mechanisms to enhance central nervous system (CNS) repair (for example, citicoline, erythropoietin, statins, and fibrates) are now under clinical investigation, but only one, progesterone (PROG), has demonstrated proof of safety and efficacy in clinical trials. In one phase II trial for the use of PROG in moderate to severe TBI, ProTECT II,

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Wright *et al*. (5) reported a 50% reduction in mortality in patients with a severe TBI and a statistically significant improvement in functional outcomes in patients with moderate TBI. The second human trial (6), studying only patients with severe brain injuries, reported results replicating those of the ProTECT trial, but extending over a longer period of time—6 months postinjury.

We explored combining vitamin D hormone (1,25-dihydroxyvitamin D3; VDH), the active form of vitamin D, with PROG. This combination was chosen for several reasons. First, VDH is a neurosteroid (like PROG), with a cholesterol backbone and steroid-like effects in various tissues, and shares some mechanisms of neuroprotection with PROG (7). The VDH receptor affects the expression of more than 1000 genes and leads to the activation of many pathways in the CNS that are similarly modified by PROG (8–10). Thus VDH might potentiate and improve functional outcomes in combination with PROG administration. PROG itself has been shown to limit TBI and stroke injury by protecting the blood-brain barrier and reducing cerebral edema, inflammatory response, necrosis, and apoptosis and by stimulating remyelination (11).

VDH in its own right has a number of physiological properties that render it neuroprotective in some types of experimental brain injury (12–14). Further, VDH deficiency can impair various physiologic processes associated with healthy CNS functions such as mitosis, mitogenesis, neurite outgrowth, and possibly adult neurogenesis in hippocampal cells (15), so correcting the deficiency might improve clinical outcomes following TBI. Perhaps more importantly, our laboratory's recent *in vivo* research has shown that vitamin D deficiency can reduce the beneficial effects of PROG treatment after TBI, especially in older subjects (16).

In this *in vitro* study, we investigated combining PROG and VDH as a challenge against glutamate-induced excitotoxic cell death in rat primary cortical neurons. The rationale for using an *in vitro* injury model is that such models can provide a rapid screening of the effects of various dosages, durations, and treatment delays on specific types of cell survival and metabolic outcomes, compared with *in vivo* injury models, in which many different extracellular variables can play a role in the injury cascade and eventual recovery from it. We hypothesized that VDH would enhance the neuroprotective efficacy of PROG by either synergistic or additive effects. We examined glutamate-mediated excitotoxicity because this highly effective mechanism of initiating cell death is widely accepted as a crucial substrate in several types of CNS injury, such as TBI (17), spinal cord injury (18), and ischemia (19). Mitogen-activated protein kinase (MAPK) is known to play a critical role in a number of intracellular activities like metabolism, mitosis, differentiation, inflammation, cell death, and survival (20). PROG has been reported to protect against glutamate toxicity in primary hippocampal neurons via activation of extracellular signal-regulated kinase 1/2 (ERK1/2) (21,22). Accordingly, we examined the combination treatment effects of VDH on PROG-mediated MAPK activation and associated neuroprotection.

MATERIALS AND METHODS

Neuronal Culture

NeuroPure™ E18 primary rat cortical cells were commercially procured (Catalogue # N200200; Genlantis, San Diego, CA, USA) as microsurgically dissected regions from 18-d-old embryonic Sprague-Dawley rat brain. The tissues were processed for culturing according to manufacturer specifications. Prior to mechanical dissociation, enzymatic pretreatment of the tissue was done by incubating the tissues in sterile NeuroPapain enzyme solution at 30°C for 30 min. Following incubation, the cells were centrifuged and transferred to fresh plating medium, where they were then dissociated into isolated neurons by use of a P-1000 pipettor with a sterile 1-mL plastic tip (opening diameter 0.8–1.0 mm). The cells were again centrifuged and seeded in multiwell plates precoated with poly-D-lysine (0.15 mL/cm^2, 50 μg/mL) and maintained at 37°C in a humidified 5% CO_2 atmosphere. All experiments were performed after 9–10 d in culture.

Induction of Glutamate Excitotoxicity and Drug Treatment

Twenty-four h before glutamate exposure, cultures were pretreated with both PROG (Cat. #P3972; Sigma-Aldrich, St. Louis, MO, USA) and VDH (Cat. #D1530; Sigma) separately or in combination, with VDH at various concentrations. Stock solutions of PROG and VDH were prepared in dimethylsulfoxide (DMSO; Cat. #D2650; Sigma) and ethanol, respectively, both of which were further diluted in culture medium so that the final concentrations of DMSO and VDH were <5 μL/mL and 0.01%, respectively. Glutamate was diluted in phosphate-buffered saline (PBS) (pH 7.4). All reagents were filter sterilized before being added to cultures. At d 11, cortical neurons in fresh media were separated into five treatment groups: (i) control; (ii) 24-h treatment with 0.5 μmol/L glutamate (23); (iii) 24-h pretreatment with different concentrations of PROG (0.1, 1, 5, 10, 20, 40, 80 μmol/L) with subsequent exposure to glutamate for 24 h; (iv) 24-h pretreatment with VDH (1, 5, 10, 20, 40, 80, 100 nmol/L) with subsequent exposure to glutamate for 24 h; and (v) 24-h pretreatment with different combinations of PROG and VDH (PROG: 20 μmol/L + VDH: 1, 5, 10, 20, 40, 80, 100 nmol/L) with subsequent exposure to glutamate for 24 h.

Evaluation of Neuronal Death

We used two widely accepted assays, lactate dehydrogenase (LDH) release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, for the measurement of cell viability. These assays are considered very reliable and reproducible with high predictive validity and are widely used in various pharmacological studies (21).

LDH release assay. Cytotoxicity was assessed 24 h after the start of the exposure by quantitative measurement of LDH in the bathing medium, an index that is proportional to the total number of neurons damaged by excitotoxic exposure (24). LDH activity was measured using a Cytotoxicity Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and quantitated by measuring absorbance at 490 nm. Data were normalized against the amount of LDH activity released from vehicle-treated control cultures receiving no glutamate.

MTT reduction assay. Neuronal death was assessed by MTT assay, which is based on the cleavage of the tetrazolium ring of the pale yellow MTT into dark-blue formazan crystals by mitochondrial dehydrogenase enzyme in viable cells. These blue formazan crystals accumulate within the cells owing to their impermeability to cell membrane, and are then solubilized by adding DMSO. The intensity of blue-colored formazan solution is directly proportional to the number of surviving cells. Concentrations were determined by photometric analysis. Briefly, 10 μL of MTT was added per well and incubated at 37°C for 4 h until purple precipitate was visible. DMSO (50 μL) was added to solubilize the crystals, and the absorbance was read at 570 nm.

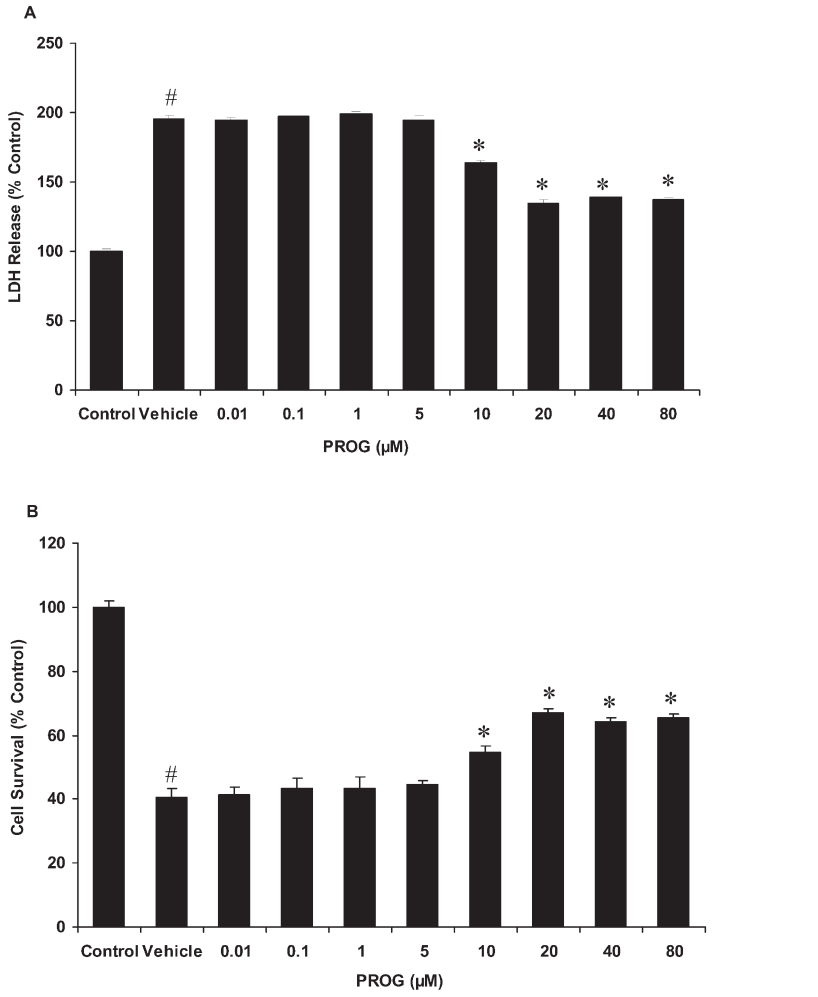

Figure 1. Effect of PROG on glutamate-induced LDH release (A) and MTT reduction (B) in rat primary cortical neurons. Primary cells were pretreated with different concentrations of PROG for 24 h and subsequently exposed to glutamate (0.5 μmol/L) for 24 h. PROG was present in the culture medium during the glutamate exposure. The values are expressed as mean ± SEM of four experiments. Significant difference: $^{\#}P < 0.001$ compared with control; $^{*}P < 0.001$ compared with vehicle.

Morphological Analysis of Cortical Cultures

Changes in the morphology of neurons treated with different drugs in various groups were observed by use of a phase-contrast microscope (Nikon Instruments Inc., Melville, NY, USA). Primary cultures were mainly observed for neurite outgrowth, a hallmark feature of healthy cells, and the density of healthy cells in different groups.

MAPK Phosphorylation

PROG and VDH were added to the primary cultures, as indicated, for 30 min (21), and the cells were lysed using an RIPA lysis buffer kit (sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein was determined in cell lysates by bicinchoninic acid protein assay (Cat. # 23225; Pierce, Rockford, IL, USA). Cell lysates (40 μg protein each sample) were separated under reducing and denaturing conditions by 12.5% acrylamide Criterion gel (Bio-Rad, Hercules, CA, USA) at 200 V for 1 h and transferred to a polyvinylidene difluoride membrane at 100 V for 30 min. The nonspecific binding sites of the membrane were blocked with 5% nonfat dry milk in PBS-T (PBS containing 0.05% Tween-20). For MAPK phosphorylation, membrane was probed with p-ERK1/2 antibody (sc-101761; Santa Cruz Biotechnology) recognizing the dual threonine (Thr 202) and tyrosine (Tyr 204) phosphorylation sequence from MAPK. Total ERK1/2 protein was detected using ERK2 (C-14) antibody (sc-154; Santa Cruz Biotechnology). Membranes were then incubated in horseradish peroxidase–conjugated secondary antibody (Goat antirabbit IgG, 074-1506; KPL, Gaithersburg, MD, USA). β-Actin was probed as a loading control. Blots were developed using a chemiluminescent substrate (Pierce) for 5 min. Chemilumi-

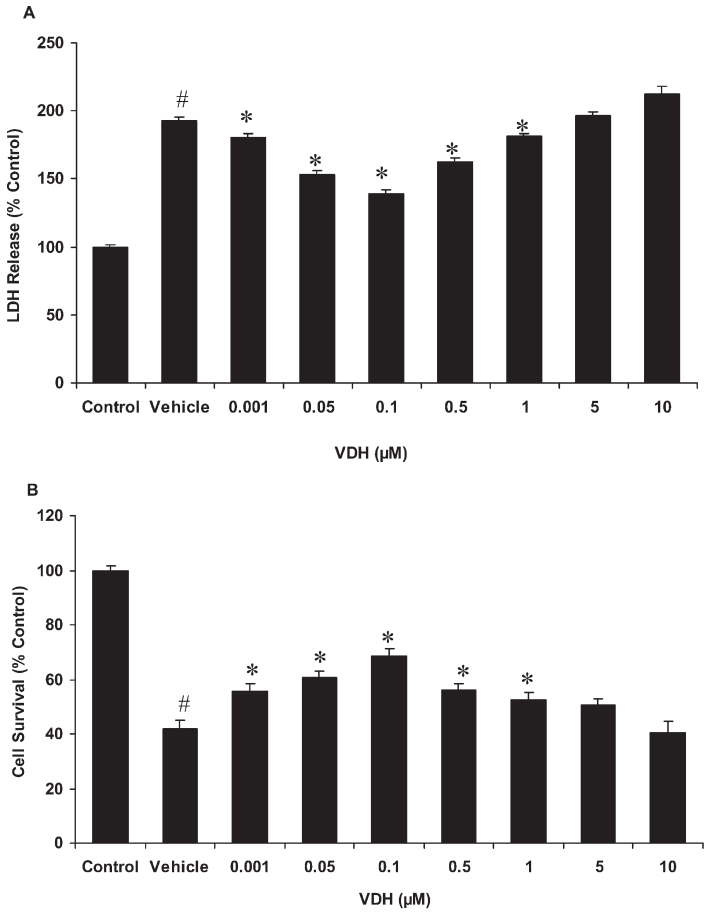

Figure 2. Effect of VDH on glutamate-induced LDH release (A) and MTT reduction (B) in rat primary cortical neurons. Primary cells were pretreated with different concentrations of VDH for 24 h and subsequently exposed to glutamate (0.5 μmol/L) for 24 h. VDH was present in the culture medium during the glutamate exposure. The values are expressed as mean ± SEM of four experiments. Significant difference: $^{\#}P < 0.001$ compared with control; $^{*}P < 0.001$ compared with vehicle.

nescent bands were detected on Kodak autoradiography film in a dark room, and their densities were measured using Bio-Rad Gel-Doc software (Quantity-One 4.6.1). MAPK activation was calculated by normalizing p-ERK1/2 with total ERK1/2 protein values.

Statistical Analysis of Data

We employed analysis of variance (ANOVA) and *post hoc* tests. The Neuman-Keuls test was used for independent comparisons among groups. The significance of results was set at $P < 0.05$ two-tailed. All data are presented as mean ± standard error of the mean (SEM).

RESULTS

Neuroprotective Effect of PROG against Glutamate-Induced Excitotoxic Cell Death

Glutamate exposure (0.5 μmol/L for 24 h) resulted in a significant ($P < 0.001$) increase in cell death in primary cortical neurons compared with control cells exposed to solvent. The concentration-response curve for PROG against glutamate-induced cell death revealed that PROG at lower concentrations (0.01, 0.1, 1, 5 μmol/L) did not show any decrease in cell death compared with the vehicle-only group. At higher concentrations (10, 20, 40, and 80 μmol/L), a significant reduction ($P < 0.001$) in cell death was observed as measured by both LDH and MTT assays (Figure 1A, B). The best concentration of PROG against glutamate-induced neuronal death was found to be 20 μmol/L.

Neuroprotective Effect of VDH against Glutamate-Induced Excitotoxic Cell Death

Different concentrations of VDH were tested against glutamate insult in primary cortical neurons. It was observed that VDH exhibited a U-shaped concentration-response curve for neuroprotection against glutamate toxicity. Lower concentrations (0.001–0.5 μmol/L) were significantly protective ($P < 0.001$), whereas higher concentrations (1–10 μmol/L) did not prevent neuronal loss compared with the vehicle-only control group. Both cell death assays suggested that VDH is most effective at a 0.1 μmol/L concentration (Figure 2A, B).

Combinatorial Effect of PROG and VDH against Glutamate-Induced Excitotoxic Cell Death

On the basis of the concentration-response curve, we combined and tested the most effective concentrations of PROG (20 μmol/L) and VDH (0.1 μmol/L) against glutamate toxicity in primary cortical neurons. The rationale behind combining only the most effective concentrations was that both the drugs were most neuroprotective individually at these concentrations ($P < 0.001$) and therefore likely to show an additive or synergistic effect in combination at the same concentrations. To our surprise, we found that this combination treatment with PROG and VDH did not prevent cell death compared with vehicle (Figure 3A, B). In light of this finding and because VDH showed a U-shaped response curve against glutamate toxicity, it is unlikely that higher concentrations of VDH would have a better outcome in combination with the most effective concentration of PROG.

Next, the most protective concentration of PROG (20 μmol/L) was combined

with various lower concentrations of VDH (1, 5, 10, 20, 40, 80, and 100 nmol/L) to evaluate the best combination of VDH with PROG. Interestingly, both cell death assays showed that PROG and VDH given together produced a U-shaped concentration-response curve for neuroprotection against glutamate-induced neuronal death (Figure 4A, B). The most effective combination was PROG (20 μmol/L) + VDH (20 nmol/L), which significantly reduced neuronal loss ($P < 0.001$) compared with vehicle. Also, this combinatorial effect was significantly better ($P < 0.01$) than the individual effect of either PROG (20 μmol/L) or VDH (0.1 μmol/L) at their most effective concentrations.

Morphological Changes in Primary Cortical Cultures after Drug Treatments

Changes in the morphology of neurons treated with the different agents and doses are shown in Figure 5. The control group shows neuritic processes characteristic of neurons *in vitro*. The glutamate (0.5 μmol/L for 24 h) group shows the loss of neuronal processes and number of neurons. PROG and VDH alone rescued neurons independently, but this neuroprotective effect was more pronounced when neurons were exposed to both hormones in combination.

Effect of PROG and VDH on MAPK Activation

To examine the involvement of MAPK in the synergy of PROG- and VDH-mediated neuroprotection, MAPK phosphorylation was assessed in primary cortical neurons after 30-min hormone treatment alone or in various combinations. Treatment with the most neuroprotective concentrations of PROG (20 μmol/L) and VDH (100 nmol/L) resulted in a 2× and 1.7× increase, respectively, in p-ERK1/2 level compared with base-line phosphorylation values in the control group (Figure 6). The combination of these doses showed no additive effect, and ERK1/2 phosphorylation was less (1.6×) than for PROG alone. Interestingly, the best combination dose of PROG and VDH (20 μmol/L and 20 nmol/L, respectively) seen in cell death assays (LDH and MTT) resulted in a 2.7× increase in MAPK activation.

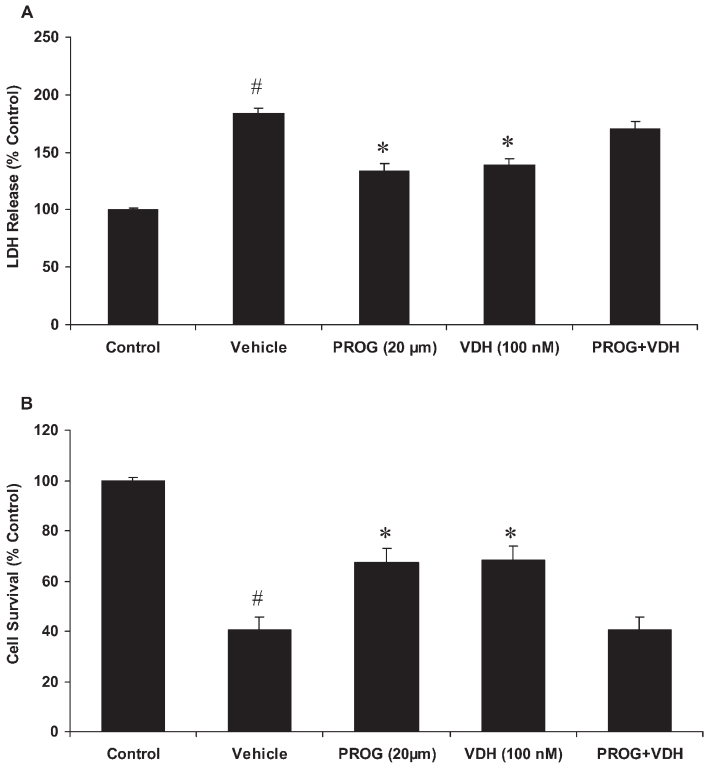

Figure 3. Effect of combinatorial treatment of PROG and VDH on glutamate-induced LDH release (A) and MTT reduction (B) in rat primary cortical neurons. Primary cells were pretreated with either best concentrations of PROG and VDH or their combination for 24 h and subsequently exposed to glutamate (0.5 μmol/L) for 24 h. Drugs were present in the culture medium during the glutamate exposure. The values are expressed as mean ± SEM of three experiments. Significant difference: $^{\#}P < 0.001$ compared with control; $^{*}P < 0.001$ compared with vehicle.

DISCUSSION

This study evaluated the *in vitro* neuroprotective effects of the combinatorial treatment of PROG and VDH against glutamate-induced excitotoxic cell death in primary cortical neurons. Our data can be taken to indicate that both PROG and VDH each independently reduce glutamate-induced neuronal death, as evidenced by decreases in LDH release and MTT reduction assays. These data are in agreement with previously reported literature on PROG and VDH neuroprotection. For example, PROG protects primary hippocampal neurons against glutamate insult by activating MAPK signaling as a mechanism of neuroprotection (21). Similarly, VDH also shows neuroprotection against various excitotoxic insults and also downregulates L-type calcium channel expression in hippocampal neurons (25). The neuroprotective efficacy of VDH has been evidenced in various *in vivo* and *in vitro* models of neurodegeneration, for example, stroke, zinc-induced neurotoxicity, experimental autoimmune encephalomyelitis, lipopolysaccharide-induced oxidative stress, Parkinson's disease, and in multiple sclerosis in human patients (12–14,26–29).

We observed that rat primary cortical cultures treated with VDH exhibited a U-shaped concentration-response curve

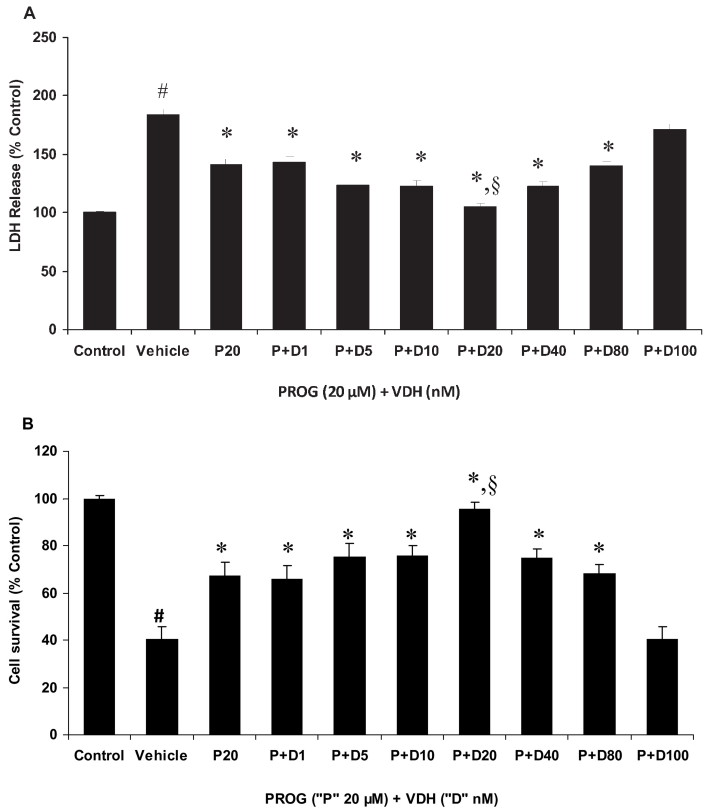

Figure 4. Effect of combinatorial treatment of PROG and VDH on glutamate-induced LDH release (A) and MTT reduction (B) in rat primary cortical neurons. Primary cells were pretreated with different combinations of PROG and VDH for 24 h and subsequently exposed to glutamate (0.5 μmol/L) for 24 h. Drugs were present in the culture medium during the glutamate exposure. The values are expressed as mean ± SEM of four experiments. Significant difference: $^{\#}P < 0.001$ compared with control, $^{*}P < 0.001$ compared with vehicle, and $^{\S}P < 0.01$ compared with P20 group.

for neuroprotection after excitotoxic insult. Lower concentrations of VDH were highly neuroprotective in our injury model, but higher concentrations were either less protective or not protective at all. In fact, VDH at 100 nmol/L concentration (physiological concentration) showed the best protective effect against glutamate insult. This response against glutamate- and NMDA (N-methyl-D-aspartate)-induced excitotoxicity *in vitro* has been previously reported by Brewer *et al*. (25). The authors proposed two novel actions of low concentrations of VDH on neurons: a direct neuroprotective action against excitotoxic insults and a decrease in both L-type voltage-sensitive Ca^{2+} channel activity and mRNA levels of the corresponding pore-forming subunits (α_{1C} and α_{1D}) of the L-type calcium channel. We observed a decrease in neuroprotection with increasing concentrations of VDH. Although the exact mechanism behind this phenomenon is not clear, there are previous reports suggesting that VDH has a direct toxic effect on motor (400 ng/d) (30) and hippocampal neurons (500-1000 nmol/L) (25) at high concentrations in different *in vitro* injury models.

Following the dose-response efficacy studies of PROG and VDH, we examined the combinatorial effects of these two hormones on neuronal survival. We first tested a combination of the most effective concentrations of PROG (20 μmol/L) and VDH (100 nmol/L) against glutamate toxicity, but the combination treatment was not effective, and surprisingly, it suppressed the individual protective effects of PROG and VDH. We then tested lower concentrations of VDH (1, 5, 10, 20, 40, 80, 100 nmol/L) in combination with the

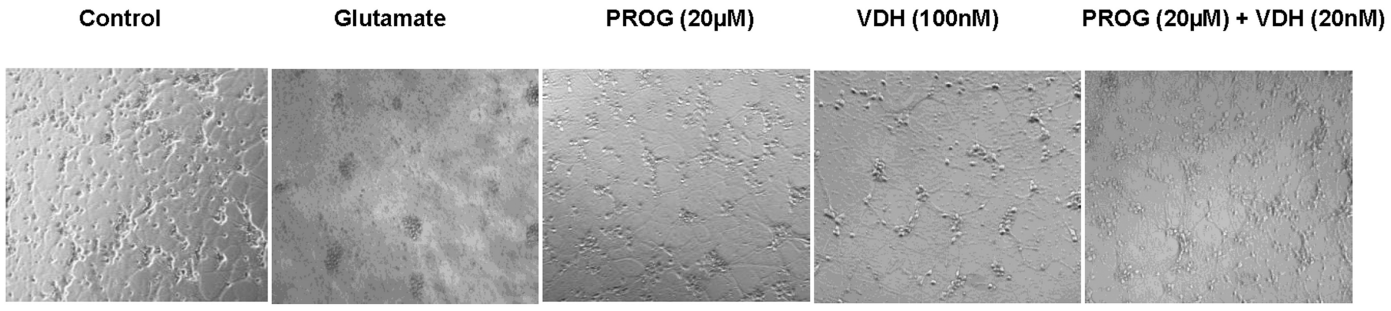

Figure 5. Changes in the morphology of neurons treated with VDH or PROG. Control group shows neuritic processes characteristic of neurons *in vitro*. The glutamate (0.5 μmol/L for 24 h) group shows the loss of neuronal processes and number of neurons. PROG (20 μmol/L) and VDH (100 nmol/L) alone rescued neurons independently, but this neuroprotective effect was more pronounced when neurons were exposed to their combination, that is, PROG (20 μmol/L) and VDH (20 nmol/L).

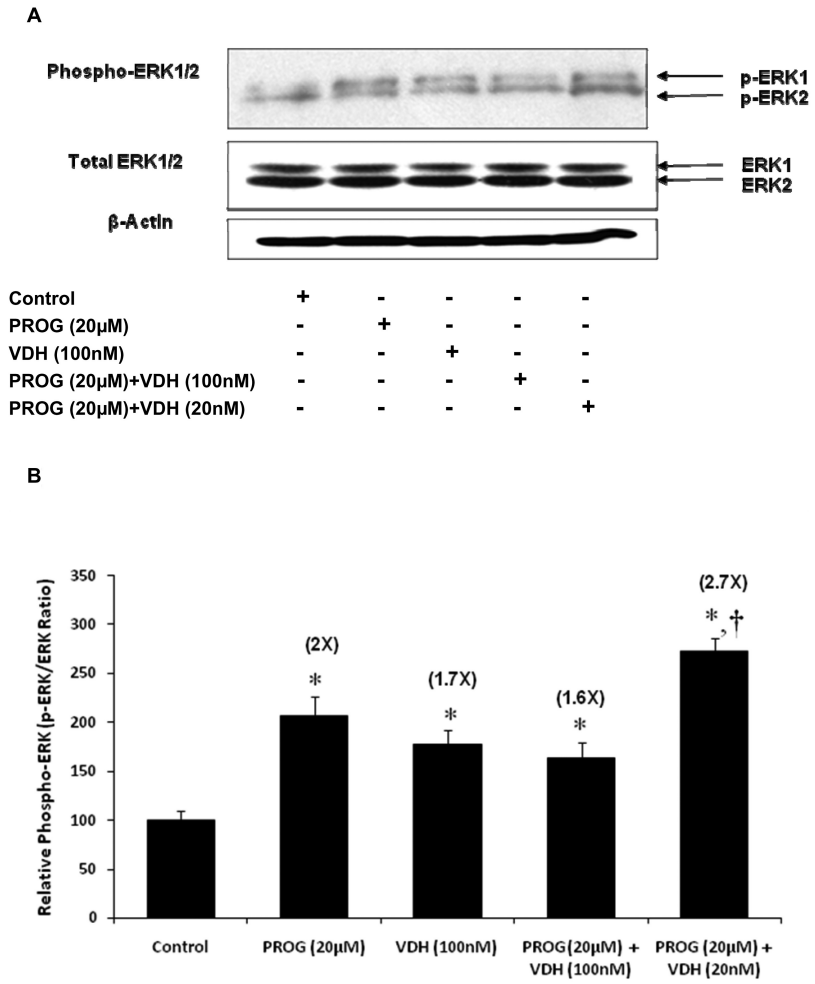

Figure 6. Effect of PROG and VDH exposure on the activation of MAPK in primary cortical neurons. Cells were exposed to hormones either separately or in various combinations for 30 min. Cells were lysed after incubation, and lysates were separated on 12.5% SDS gel and transferred onto polyvinylidene difluoride membrane. Membrane was probed with either phospho-ERK1/2 or total ERK1/2 protein. Phospho-ERK1/2 data were normalized with total ERK1/2 protein. Data were analyzed using ANOVA and the Neuman–Keuls test. Values are expressed as SEM of three independent experiments. Significant difference: $^{*}P < 0.05$ compared with control; $^{\dagger}P < 0.05$ compared with PROG (20 μmol/L) and VDH (20 nmol/L) groups. Values in parentheses represent fold increase in MAPK over control values.

best concentration of PROG (20 μmol/L) to determine whether a lower concentration of VDH could enhance the protective effect of PROG on cultured neurons. Here, we observed that PROG, when combined with a lower concentration of VDH (20 nmol/L), produced a significantly better neuroprotective effect than when given alone. Not only do these findings suggest that the combination of VDH with PROG merits investigation as a therapy for TBI, but they also demonstrate the variability in individual and combinatorial effects of two neuroprotective drugs and the importance of carefully testing combination dose–response efficacy rather than selecting the best effective doses worked out for single drugs independently.

The exact mechanism of the synergistic effect of VDH with PROG clearly needs to be explored. There is evidence that VDH interacts with PROG and estradiol to stimulate their secretion in human placenta (31) and also acts in maintaining bone health in postmenopausal women. It is important to note that many of the physiological properties of VDH are also attributed to PROG (11,32,33)—for example, both are natural hormones present in males and females. In the present study, we examined the role of MAPK in the synergistic effect of PROG and VDH and found that neuroprotective concentrations of PROG and VDH resulted in the specific activation of MAPK in primary cortical neurons. PROG has previously been reported to activate MAPK in unchallenged primary hippocampal neurons after 30 minutes of exposure (21). Thus upregulation induces expression of antiapoptotic genes like *BCl-2*, which then protects cells from toxic injury (21,22). We observed that VDH activates MAPK in primary cortical neurons. There are reports suggesting that VDH activates MAPK in different experimental models (34–37). In combination with PROG, the lower concentration of VDH (20 nmol/L) showed the maximum increase in ERK1/2 phosphorylation (2.7×), whereas a higher concentration of VDH (100 nmol/L) showed less ERK1/2 phosphorylation. Our cell death data demonstrate that a lower concentration of VDH (20 nmol/L) significantly enhanced the neuroprotective efficacy of PROG, as demonstrated by a marked MAPK activation in the combination therapy. We also noted that a higher VDH concentration (100 nmol/L) reduced PROG-mediated neuroprotection but still activated MAPK. In general, our findings can be interpreted to indicate that MAPK activation may be one of the mechanisms of synergistic neuroprotection offered by PROG and VDH in a cell culture model of injury. There is supporting evidence that MAPK is a necessary but not sufficient condition for neuroprotection by combinatorial treatment. For example, medroxyprogesterone acetate (MPA), despite activating MAPK, does not afford neuroprotection against glutamate insult in hippocampal neurons. Interestingly, MPA in combination with estrogen activates MAPK but blocks the neuroprotec-

tive effect of estrogen (21). Nilsen and Brinton (22) suggested that a possible reason behind this paradox is that nuclear translocation of phosphorylated ERK is necessary to obtain steroid-induced neuroprotection.

In addition to MAPK, on which combined PROG and VDH treatment showed a synergistic effect, other mechanisms of VDH could compensate for PROG's lesser or missing effects and would be very interesting to investigate. For example, compared with PROG, VDH more directly protects mitochondria by inducing the expression of γ-glutamyl transpeptidase and significantly increases intracellular glutathione in response to lipopolysaccharide-induced oxidative stress in astrocytes (27). Unlike PROG, VDH protects against the toxic effects of heme breakdown products. It efficiently maintains intracellular calcium (38,39) and has been reported to protect cells from free heme-induced oxidative injury by upregulating glial heme oxygenase-1 immunoreactivity associated with the reduction in GFAP (glial fibrillary acidic protein) immunoreactivity in cortical regions affected by focal cortical ischemia (40). Furthermore, VDH is also known to exert a regulatory effect on the renin-angiotensin system by suppressing renin biosynthesis and regulating blood pressure homeostasis (41–43). Another important feature of VDH is its role in growth and regeneration mediated by nerve growth factor and glial-derived neurotrophic factor (44,45). Clearly, VDH, like PROG, affects and enhances CNS repair after injury, but VDH at the right dose can potentiate the neuroprotective effects of PROG in a cell culture model of CNS injury and thus provide additional evidence of enhancing neuronal repair.

Our finding that VDH significantly enhances the neuroprotective efficacy of PROG *in combination* also suggests that PROG could be given to prevent the initial inflammatory cascade and edema, and then be coupled with VDH to stimulate the neurotrophic and regenerative events that take place later in the reparative process. Because only a few studies have been done in this area, it is obvious that more preclinical research will be needed to determine the benefits of this combinatorial therapy in clinical trial for TBI or other CNS injuries such as stroke.

ACKNOWLEDGMENTS

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DISCLOSURES

DG Stein is entitled to royalties from products of BHR Pharmaceuticals Ltd related to the research described in this presentation and may receive research funding from BHR Pharmaceuticals, which is developing products related to this research. In addition, he serves as a consultant to BHR Pharmaceuticals and receives compensation for these services. The terms of this arrangement have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

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