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Resveratrol inhibits phorbol ester-induced membrane translocation of presynaptic Munc13-1

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Abstract

Background—Resveratrol (**1**) is a naturally occurring polyphenol that has been implicated in neuroprotection. One of resveratrol's several biological targets is Ca^{2+} -sensitive protein kinase C alpha (PKC α). Resveratrol inhibits PKC α by binding to its activator-binding C1 domain. Munc13-1 is a C1 domain-containing Ca^{2+} -sensitive SNARE complex protein essential for vesicle priming and neurotransmitter release.

Methods—To test if resveratrol could also bind and inhibit Munc13-1, we studied the interaction of resveratrol and its derivatives, (*E*)-1,3-dimethoxy-5-(4-methoxystyryl)benzene, (*E*)-5,5'-(ethene-1,2-diyl)bis(benzene-1,2,3-triol), (*E*)-1,2-bis(3,4,5-trimethoxyphenyl)ethane, and (*E*)-5-(4-(hexadecyloxy)-3,5-dihydroxystyryl)benzene-1,2,3-triol with Munc13-1 by studying its membrane translocation from cytosol to plasma membrane in HT22 cells and primary hippocampal neurons.

Results—Resveratrol, but not the derivatives inhibited phorbol ester-induced Munc13-1 translocation from cytosol to membrane in HT22 cells and primary hippocampal neurons, as evidenced by immunoblot analysis and confocal microscopy. Resveratrol did not show any effect on Munc13-1^{H567K}, a mutant which is not sensitive to phorbol ester. Binding studies with Munc13-1 C1 indicated that resveratrol competes with phorbol ester for the binding site. Molecular docking and dynamics studies suggested that hydroxyl groups of resveratrol interact with phorbol-ester binding residues in the binding pocket.

Conclusions and significance—This study characterizes Munc13-1 as a target of resveratrol and highlights the importance of dietary polyphenol in the management of neurodegenerative diseases.

Graphical abstract

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Author Contribution

Joydip Das designed the research. Satyabrata Pany, Anamitra Ghosh, Youngki You and Nga Nguyen performed experiments. Joydip Das, Satyabrata Pany, Anamitra Ghosh, and Youngki You analyzed the data, and wrote the paper.

Notes

The authors declare no competing financial interest.

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Keywords

resveratrol; presynaptic; munc13-1; neuroprotection; protein kinase C; activation; inhibition; membrane translocation; polyphenol

1. Introduction

Resveratrol (**1**, Figure 1A) is a naturally occurring polyphenol found in grapes, red wine, peanuts, olive oil, cranberries, and other food [1, 2]. Numerous studies highlighted the role of resveratrol in neuroprotection [3, 4]. Resveratrol exhibits neuro-protective benefits for many neuro-degenerative disorders [5]. Some of these benefits are due to its anti-oxidative and anti-inflammatory effects on neurons [6].

Resveratrol displays its biological response by acting on multiple targets [7-9]. The activity of resveratrol has been linked to cell-surface receptors, membrane signaling pathways, intracellular signal-transduction machinery, nuclear receptors, gene transcription, and metabolic pathways [10, 11]. Serine-threonine kinases, such as protein kinase C alpha (PKC α) is one of the many targets of resveratrol and recent studies showed that resveratrol binds to its activator-binding C1 domain [12-14].

Munc13-1 is a C1 domain-containing presynaptic active zone protein essential for synaptic vesicle priming [15, 16] and neurotransmitter release [17, 18]. It is a member of the Munc13 family of proteins consisting of four members. The other members are Munc13-2, Munc13-3 and Munc13-4[19]. Munc13-1 is expressed primarily in the hippocampus, cerebellum, cortex, striatum regions of the brain [20] and modulates short-term presynaptic plasticity [21, 22] and long-term potentiation through its interactions with an active zone protein RIM [23]. Studies have also shown that Munc13-1 mediates insulin release [24]. In addition, Munc13-1 and 13-2 double- knockout mice result in complete abolishment of neurotransmitter release [25-28]. Munc13-1 is critically involved in neuro-degenerative disorders and synaptic plasticity [29-31]. For example, Munc13-1 regulates A β -induced neurotoxicity in AD model [32, 33]. Clinical studies have reported that single-nucleotide polymorphism in Munc13-1 gene is associated with amyotrophic lateral sclerosis and fronto-temporal dementia [34-37]. Munc13-1 also regulates neuron cell survival at the time of amyotrophic lateral sclerosis disease progression [38]. Munc13-1 dependent neuro-secretion is essential for immune response and motor neuron degeneration [39].

Munc13-1 is a large peripheral membrane protein with a Ca²⁺ binding C2 domain (C2A) at the N-terminus, a diacylglycerol (DAG)/phorbol ester binding C1 domain, another C2 domain (C2B) next to it, two Munc13 homology domains (MHD) and another C2 domain

(C2C) at the C-terminus (Figure 1B) [27, 40]. Residues 859-1531 between the C2B and C2C domains form an autonomously folded domain is known as MUN domain [41]. The binding of DAG/phorbol ester to the Munc13-1 C1 domain stimulates synaptic vesicle priming and Munc13-1's translocation from the cytoplasm to the plasma membrane [42]. There are high similarities in the primary sequences of the C1 domains of Munc13, Dunc-13 and Unc13 and PKCs[43]. The Unc13 C1 domain also binds phorbol esters with high affinity, similar to that of PKC δ [44], although another study showed affinity of Munc13-1 is lower than that of PKC [17]. The binding of DAG/phorbol ester to Munc13-1 C1 domain lowers the energy barrier for vesicle fusion and facilitates neurotransmitter release [45]. However, the mutant, Munc13-1^{H567K} in which His-567 of the C1 domain is replaced by Lys, does not bind to phorbol esters, causing reduction in vesicle fusion [17, 45].

Structurally, the C1 domain of Munc13-1 is very similar to that of protein kinase C δ (PKC δ). The major structural features are that they both have two β -sheets, a short C-terminal α -helix and two Zn²⁺ binding sites and a DAG binding groove formed by two "rabbit-ear" loops [43, 46, 47]. Although the overall structure of these two C1 domains are similar, there are subtle differences in the orientation of the residues in the ligand binding pocket. Whereas the Trp-588 in Munc13-1 C1 occludes the ligand binding pocket [46], the homologous tryptophan in PKC δ C1B (Trp-252) is oriented outside the ligand binding pocket [43].

Resveratrol inhibits Ca²⁺-sensitive PKC α by binding to its C1 domain [12-14]. We hypothesized that resveratrol will also bind to the Ca²⁺-sensitive and C1-domain containing Munc13-1. To test this hypothesis we studied the effect of resveratrol and its derivatives (**1-5**, Figure 1A) on Munc13-1 activity in HT22 cells and primary hippocampal neurons. We observed that resveratrol inhibited phorbol-ester induced membrane translocation of Munc13-1 in both HT22 cells and in hippocampal neurons. Resveratrol binds to the C1 domain of Munc13-1 and competes with phorbol ester. The modulatory response of resveratrol on Munc13-1 signifies its role in the neuroprotection.

2. Materials and methods

2.1. General

Resveratrol, TPA, phorbol 13-acetate, and IPTG were from Sigma. Escherichia coli BL21 (DE3) competent cells were obtained from Stratagene (La Jolla, CA). LB (Luria-Bertani) broth medium, TB (terrific broth) medium, and SOC (super optimal culture) were obtained from Invitrogen (Carlsbad, CA). Protein estimations were conducted using the Bradford protein estimation kit from Bio-Rad (Hercules, CA). Resveratrol derivatives, (*E*)-1,3-dimethoxy-5-(4-methoxystyryl)benzene (**2**), (*E*)-5,5'-(ethene-1,2-diyl)bis(benzene-1,2,3-triol) (**3**), (*E*)-1,2-bis(3,4,5-trimethoxyphenyl)ethane (**4**), and (*E*)-5-(4-(hexadecyloxy)-3,5-dihydroxystyryl)benzene-1,2,3-triol (**5**) (Figure 1A) were synthesized according to the methods described earlier [12]. Munc13-1 and Munc13-1^{H567K} constructs were a gift from Dr. N. Brose of Max Planck Institute for Experimental Medicine, Göttingen, Germany. HT22 cell line (RRID: CVCL-0321) was a gift from Dr. S. Salim of University of Houston. All other reagents were purchased from Sigma and used without further purification.

2.2. DPPH free radical scavenging assay

Antioxidant activity of the resveratrol (**1**) and its derivatives (**2-5**) was measured by DPPH radical scavenging assay [48]. Briefly, DPPH (50 μ M) and varying concentrations of compounds (0.25-250 μ M) were incubated in a methanolic solution for 1 h at 25 °C. Absorbance spectra of DPPH were recorded from 400 to 600 nm. The percentage of inhibition of DPPH radical by each compound was calculated using formula: $(1-A/A_0) \times 100$, where A and A_0 represent the absorbance intensities at 517 nm in the presence and absence of the compound, respectively and plotted against concentration. The IC₅₀ values were obtained from the fitted curves using Hill equation in Igor Pro (WaveMetrics Inc, Lake Oswego, OR).

2.3. Cell Culture and Transfection

Mouse hippocampal cell line (HT22) was used for this study. Cells were cultured in DMEM media supplemented with FBS (10%), glutamine (1mM) and antibiotics. Cells were incubated at a 37 °C in a humidified atmosphere supplemented with 5% CO₂. HT22 cells were transfected with pEGFPN1-Munc13-1 or pEGFPN1-Munc13-1^{H567K} construct using Lipofectamine-3000 (Thermo-Fisher Scientific, San Jose, CA) and as per the manufacture's recommendations.

2.4. Cell Viability Assay

Cell viability was measured using the MTT assay [14]. Briefly, HT22 cells (10⁴ cells/well) were grown in 96-well plate for 24 h. Cells were treated with varying concentrations of different compounds (6.25-100 μ M) for 48 h. Cell viability was measured using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Scientific, Waltham, MA) as per the manufacture's recommendations.

2.5. Protein Purification

Bacterial expression and purification were carried out following the methods reported earlier [49]. Briefly, Munc13-1 C1 in pGEX-2TK (GE Healthcare Bio-Sciences, Pittsburgh, PA) vector was expressed as GST fusion protein. Munc13-1 C1 construct was transformed into BL21 Gold (DE3) cells (Agilent Technologies, Santa Clara, CA). Protein expression was induced with 5 mM IPTG at an optical density of 0.7 and cultures were allowed to grow for an additional 20 h at 20 °C. The cells were harvested and re-suspended in lysis buffer (PBS, 1 mg/ml lysozyme, 0.1 Triton-X-100). The cells were then sonicated (20 times, 3 s, 30% amplitude) using digital sonifier (Model 250, Branson, Danbury, CT) and soluble protein was recovered by centrifugation at 12,000g for 15 min at 4 °C. Soluble extract was further treated with PEI (0.5 %) to remove nucleic acids. Extracted lysates were loaded to GST affinity column (glutathione-sepharose) pre-equilibrated with PBS. Column was washed with PBS until maximum amounts of impurity was removed. Protein was eluted in PBS, after on-column digestion of the bound protein with thrombin. Eluted protein was further purified on a size exclusion column (Sephadex 75 10/300 GL, GE Life Science, Madison, CA) using gel filtration buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.4). The purity and molecular weight of the protein were examined by SDS-PAGE and mass spectrometry.

2.6. Fluorescence Studies

FRET between the tryptophan residue of Munc13-1 C1 and resveratrol (**1**) and effect of phorbol 13-acetate on FRET were measured using PTI fluorimeter (Photon Technology Instruments, Princeton, NJ) as described earlier [50]. Three samples, Munc13-1 C1 (1 μ M), resveratrol (10 μ M) and Munc13-1 C1 (1 μ M) plus resveratrol (10 μ M) incubated for 1 h at 25 °C, were prepared in buffer (50 mM Tris-HCl 150 mM NaCl and 5 mM ZnSO₄, pH 8). Samples were excited at 290 nm and emission spectra were recorded from 300 to 600 nm. The emission maxima for Munc13-1 C1 and resveratrol were 336 and 375 nm, respectively. The FRET values were calculated using equation: $[F_{i+protein} - F_{i-protein}] - [F_{0+protein} - F_{0-protein}]$, where $F_{i+protein}$ and $F_{i-protein}$ are the intensities of resveratrol in the presence and absence of Munc13-1 C1, respectively and $F_{0+protein}$ and $F_{0-protein}$ are fluorescence intensities of the buffer solution in the presence or absence of Munc13-1 C1, respectively. To determine the effect of phorbol 13-acetate on FRET, varying concentrations of phorbol 13-acetate were added to Munc13-1 C1 and resveratrol mixtures. The spectra were recorded after 1 h of incubation at 25 °C and FRET values were calculated. FRET value for each concentration of phorbol 13-acetate was normalized using the equation: $(F_i - F_0)/F_0$, where F_i and F_0 are FRET value in the presence or in the absence of phorbol 13-acetate. IC₅₀ was determined from Hill equation fitted curve in sigma plot (Systat Software Inc., San Jose, CA).

2.7. Cell Fractionations and Immunoblot Analysis

For cell fractionations, cells were suspended in fractionation buffer (20 mM HEPES, 10 mM KCl, 150 mM sucrose, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1mM DTT, protease/phosphatase inhibitor, pH 7.4) and lysed with brief sonication (2 times, 3 s, 10% amplitude). Lysate was centrifuged at 3500 rpm (Sorvall™ Legend™ Micro 17 microcentrifuge, Thermo Fisher Scientific, Waltham, MA) for 10 min at 4 °C to remove cell debris and nucleus. Supernatant (300 μ g protein/100 μ l) was centrifuged again at 40,000 rpm for 2 h at 4 °C using Optima XE ultracentrifuge (Beckman Coulter, Indianapolis, IN). This supernatant was cytosolic fraction. The pellet (membrane fraction) was incubated in fractionation buffer containing 1% Triton X-100 overnight at 4°C, and then homogenized by brief sonication. The cytosolic and membrane fractions were precipitated by trichloroacetic acid (20%). The pellets were washed twice with 70% acetone, and re-dissolved in 40 μ l of Laemmli sample buffer [51]. Cell fractions were subjected to SDS-PAGE and immunoblot analysis as described earlier [52]. The anti-rabbit GFP (1:1000); anti-rabbit β -actin (1:2000); and anti-rabbit HRP-conjugated (1:5000) (Cell Signaling Technology, Inc. Danvers, MA) were used for immunoblot analysis.

2.8. Confocal microscopic Analysis

Confocal microscopic analysis was carried out using Leica SP8 confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL) with 60x, NA 1.42 oil objective as reported earlier [52]. Munc13-1 transfected HT22 cells were either treated or co-treated with TPA and resveratrol on glass coverslips (VWR, Atlanta, GA) for 15 min. Cells were fixed in 4% PFA in PBS for 20 min. Coverslips were mounted in mounting medium (Vector Laboratories,

Inc., Burlingame, CA). ImageJ (<http://rsb.info.nih.gov/ij/>) was used to measure the sub-cellular distribution of Munc13-1 protein [52].

2.9. Primary neuronal culture

10 to 12-week-old timed pregnant female C57BL/6 mice weighing 24 to 28 g were received from Harlan labs, USA. Mice were housed in standard conditions: constant temperature ($22 \pm 1^\circ\text{C}$), relative humidity (30 %), and a 12-h light cycle. Animal experiments were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at University of Houston. Primary hippocampal neuronal cultures were prepared from the hippocampal tissues of gestational 18-day-old mouse embryos. Briefly, hippocampal tissues were dissected and maintained in ice-cold calcium-free Hank's balanced salt solution (HBSS) and then dissociated in HBSS containing trypsin-0.25 % EDTA for 20 min at 37°C . The dissociated cells were then plated at an equal density of 8×10^4 cells per well on 12-mm coverslips pre-coated with 50 $\mu\text{g/ml}$ poly-D-lysine. Cultures were maintained in neuronal feeding media fortified with B-27 supplement, 200 mM L-glutamine, 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and HEPES. The cells were maintained in a humidified CO_2 incubator (5% CO_2 and 37°C) for 24 h. One-half of the culture media was replaced every three days. Approximately 7 to 8-day-old cultures were used for experiments [53, 54]. Primary hippocampal neurons were co-treated with to 500 nM TPA and different doses of resveratrol (12.5-50 μM). Fifteen minute post-treatment cells were collected and processed for immunocytochemistry.

2.10. Immunocytochemistry

The primary hippocampal neurons were fixed with 4 % PFA in PBS for 15 min and processed for immunocytochemical staining. First, nonspecific sites were blocked with 0.4 % BSA, 0.2 % Triton X-100 and 0.02 % Tween-20 in PBS for 1 h at room temperature. Cells were then incubated overnight at 4°C with different primary antibodies such as Munc13-1 (1:500, Rabbit polyclonal, Synaptic Systems, Germany) and Tuj1 (1:1000, Mouse monoclonal, R&D, USA). Appropriate secondary antibodies (Alexa Fluor 488 or 594; Invitrogen, USA) were used followed by incubation with DAPI (Sigma, USA) for 5 min at room temperature to stain the nucleus. Coverslips containing immuno-stained cells were washed twice with PBS and mounted on slides. Images of cells were captured with a Leica SP8 confocal microscope (Leica Microsystem INC., USA).

2.11. Quantification of Munc13-1 in cytosol and plasma membrane

A total of three primary hippocampal neuronal culture wells per experimental group were immunostained with antibodies directed against Munc13-1 as mentioned above. Two independent experiments were performed for the quantification purpose. After immunofluorescence staining, three individual neurons were imaged from each well using 63x (3.5x zoom) magnification. The mean intensity of fluorescence of Munc13-1 in cytosol and plasma membrane in Tuj1-positive neurons were measured, as well as area of the cytosol and plasma membrane were quantified using LAS X software (Leica Microsystem INC., USA). Data were expressed as ratio of mean intensity of Munc13-1 in plasma membrane and cytosol per μm^2 area [48, 52].

2.12. Statistics

Data were analysed either with Prism 3.0 (GraphPad Software, Inc, La Jolla, CA) or Sigma Plot 11 (Systat Software Inc., San Jose, CA) software. Raw data were first analysed using one-way ANOVA and then either Tukey's or Bonferroni multiple comparison test was performed to compare all treatment groups. Differences with $P < 0.05$ were considered significant.

2.13. Molecular Docking

Resveratrol (**1**) and its derivatives (**2-5**) were docked into Munc13-1 C1 using SYBYL-X 2.1. NMR structure of Munc13-1 C1 (PDB: 1Y8F) was used as a template and the structures of **1-5** were created using ChemBioDraw (PerkinElmer, Akron, OH) [47, 55]. For docking space of ligand in protein, protomol was generated by selecting the homologous phorbol ester binding residues of the PKC δ C1B domain and residues within a radius of 3 Å. Threshold and bloat values were 0.5 and 2.0, respectively. Because Trp-588 occludes the phorbol ester binding site in Munc13-1 C1, protomol was generated with W588G mutant and the same protomol was used for Munc13-1 C1. After the protomol generation, the docking simulations on Munc13-1 C1 were performed using the SurFlexDock module in SYBYL.

2.14. Molecular Dynamics (MD) Simulation

MD simulation was performed on the Munc13-1 C1-resveratrol complex using GROMACS 4.6.5 package of programs with the Amber99sb force field [56, 57]. The topology and coordinate files of resveratrol were created by the Antechamber program in the AMBER tool and then were converted to GROMACS format using ACPYPE [58]. The models were solvated with 15,454 TIP3P water molecules with a cubic distance of 2.0 nm and neutralized by adding a Cl⁻ counter ions. Energy minimization was completed for 9594 steps using the steepest descent method to remove steric clashes generated while solvating the system. The minimized system was equilibrated for 160 ps by position-restrained MD simulation for maintaining the temperature and pressure of systems and relaxing the solvent. The equilibration was carried out in two phases. NVT optimization with 300 K was performed in the first phase, and the second phase was performed for NPT optimization at 1 bar. After equilibration, the MD production run was conducted using the Berendsen coupling method with 300 K and 1 bar for 2.5 ns [59]. The bond lengths were constrained by the LINCS algorithm allowing a time step of 2 fs [60]. Electrostatic interactions were computed by the particle mesh Ewald (PME) method [61]. The Coulombic interactions, electrostatic, and van der Waals were calculated with a 1 nm cut-off. For analysis, the atomic coordinates were saved every 2 ps during the MD simulation. The MD trajectories were analysed by GROMACS analysis tools, including g_rms and g_energy. The graphs were plotted with QtGrace. The structures and trajectories were visualized using Discovery Studio Visualizer 4.5 (Biovia Inc.) and PyMol version 1.7 (Schrodinger, LLC, Cambridge, MA).

3. Results

3.1. Antioxidant properties of resveratrol (1) and its derivatives (2-5)

The antioxidant activity of resveratrol (1) and derivatives (2–5) (Figure 1) was evaluated using DPPH radical scavenging assay and the results are shown in Figure 2. Resveratrol with three OH groups showed an IC_{50} value of $29.36 \pm 1.45 \mu M$, which is consistent with the value reported earlier [62]. Derivative 2 in which three OH groups of resveratrol were replaced with three OCH_3 groups showed no DPPH scavenging activity. Derivative 3 with six OH groups showed higher scavenging activity than resveratrol with an IC_{50} value $1.17 \pm 0.34 \mu M$. Derivative 4 with six OCH_3 groups was inert in scavenging the DPPH radical. Derivative 5 with five OH groups and a long aliphatic chain showed an IC_{50} value of $1.97 \pm 0.27 \mu M$. These results suggested that modification of phenolic hydroxyl groups alter the antioxidant potential of resveratrol.

3.2. Effect of resveratrol (1) and its derivatives (2-5) on the viability of HT22 cells

The cytotoxicity of resveratrol (1) and its derivatives (2–5) was measured with HT22 neuronal cells using MTT assay (Figure 3) at a concentration range of 0–100 μM . Resveratrol showed higher cytotoxicity as compared to its derivatives 2–5. Derivatives 3 and 5 are cytotoxic to cells at a high dose (50 and 100 μM) but not at low dose (6.25, 12.5 and 25 μM). Moreover, their cytotoxicity was 40–50% less as compared to resveratrol. Derivatives 2 and 4, on the other hand, did not show any significant cytotoxic effect on HT22 cells. These results suggest that chemical modification of resveratrol moiety can attenuate its cytotoxic property and the hydroxyl groups play important role in this process.

3.3. Resveratrol inhibits TPA-induced membrane translocation of Munc13-1 in HT22 cells

To determine the effect of resveratrol and its derivatives on Munc13-1 activity, we measured the effect of resveratrol on Munc13-1 translocation from cytosol to membrane in HT22 cells (Figure 4A). Munc13-1, like PKC, is a DAG/phorbol ester binding protein. DAG induces its translocation from cytosol to synaptic membrane for activation and subsequent vesicle docking and neurotransmitter release [63, 64]. When HT22 cells were transiently transfected with EGFP-tagged Munc13-1 and then treated with phorbol ester, TPA, Munc13-1 translocated from cytosol to plasma membrane. This is confirmed using immunoblot analysis of the cytosolic and membrane fractions (Figure 4A) and also by confocal microscopic analysis (Figure 4B). We observed complete translocation of Munc13-1 to plasma membrane at 500 nM of TPA. When the cells were treated with TPA (500 nM) and resveratrol (6.25–50 μM), there was significant reduction in the amounts of Munc13-1 in the membrane fraction suggesting that resveratrol inhibited translocation of Munc13-1 to the membrane. When the cells were treated with resveratrol (6.25–50 μM) alone, Munc13-1 level in cytosol and membrane were similar to the control cells, showing that resveratrol alone did not have any effect on Munc13-1 membrane translocation. The immunoblot results were further confirmed by confocal microscopic analysis (Figure 4B). In control cells Munc13-1 was primarily localized in cytosol but completely translocated to membrane on TPA (500 nM) treatment, an observation similar to the immunoblot analysis results. Again, we observed ~60% and ~80% inhibition of Munc13-1 translocation in the presence of 25 μM and 50 μM resveratrol, respectively together with TPA (500 nM). Resveratrol (25 and 50

μM) alone did not show any significant effects on the Munc13-1 translocations. Overall, the above results confirmed that resveratrol inhibited TPA-induced membrane translocation of Munc13-1.

To test if the inhibition of TPA-induced membrane translocation of Munc13-1 by resveratrol is indeed phorbol ester dependent and resveratrol's action is by binding in the phorbol binding site in Munc13-1, we examined the effect of resveratrol on Munc13-1^{H567K} in HT22 cells. This mutant of Munc13-1 does not bind to phorbol ester and inhibits activation of vesicle priming [17, 45]. Confocal microscopic analysis suggests that there was no significant effect of TPA on Munc13-1^{H567K} in HT22 at 500 nM and 2000 nM, the concentrations at which we observed complete translocation of wild type Munc13-1 to membrane. Similarly, co-treatment of TPA and resveratrol or treatment of resveratrol alone did not have any significant effect on Munc13-1^{H567K} translocation (Figure S1). These results suggest that resveratrol's effect is specific to the phorbol ester sensitive Munc13-1 and it could bind to the phorbol ester binding site of Munc13-1.

Next, we examined the effect of resveratrol derivatives (2-5) on TPA-induced Munc13-1 translocation and results are shown in Figure 5. Resveratrol derivatives (2-5) alone did have no effect on Munc13-1 translocation as levels of cytosolic and membrane Munc13-1 in treated cells were similar to the control cells. Similarly, we did not observe any significant effect of derivatives (2-5) on Munc13-1 when the cells were treated with TPA and resveratrol derivatives. These results suggest that chemical modification of resveratrol resulted in loss of its inhibition of TPA-induced Munc13-1 translocation.

3.4. Resveratrol inhibits translocation of Munc13-1 in primary hippocampal neurons

After demonstrating the inhibitory effect of resveratrol in TPA-induced translocation of Munc13-1 from cytosol to plasma membrane in transfected HT22 cells, we further investigated the effect of resveratrol in a mammalian *ex-vivo* system. Primary hippocampal neurons were cultured from 18-day-old prenatal pups and co-treated with 500 nM TPA and different doses of resveratrol (12.5 to 50 μM) for 15 minutes. As expected, TPA induced translocation of Munc13-1 from cytosol to plasma membrane (Figure 6A). When we checked the level of Munc13-1, we noticed reduced level of Munc13-1 in cytosol (as denoted by asterisks) in comparison to control cells (Figure 6A). However, resveratrol dose-dependently inhibited the TPA-induced translocation of Munc13-1 from cytosol to plasma membrane in Tuj-1-positive hippocampal neurons with nearly complete inhibition at 50 μM (Figure 6A and 6B). These results clearly suggest that resveratrol attenuated cytosol to plasma membrane translocation of Munc13-1.

3.5. Resveratrol inhibits phorbol ester binding to Munc13-1 C1

To examine if the observed inhibition of TPA-induced membrane translocation of Munc13-1 by resveratrol is by binding to the phorbol ester binding domain (i.e., C1 domain) of Munc13-1, we studied the binding of resveratrol to Munc13-1 C1 using *in vitro* FRET assay. Munc13-1 C1 was purified and characterized thoroughly before the study. The purified protein showed a single band at 7 kDa in SDS-PAGE (Figure S2) and MALDI-TOF analysis showed the molecular weight value of 7291 Da which is similar to the calculated value.

Munc13-1 C1 has a tryptophan residue at the 588 position located at the phorbol ester binding site (sequence shown in Figure 1B) [46]. When excited at 290 nm, Munc13-1 C1 and resveratrol showed emission maxima at 336 nm and 392 nm, respectively. Resveratrol quenched the intrinsic fluorescence of Munc13 C1 in a concentration-dependent manner with the decrease in protein fluorescence at 336 nm and with increase in the resveratrol fluorescence at 392 nm indicating FRET between Munc13-1 C1 and resveratrol (Figure S3). To test if phorbol ester could have any effect on the FRET between Munc13-1 C1 and resveratrol, we measured the fluorescence intensity of resveratrol in presence of increasing concentrations of phorbol 13-acetate added into the Munc13-1 and resveratrol mixture (Figure 7A). We observed a decrease in emission intensity of resveratrol with increasing concentrations of phorbol 13-acetate (Figure 7B), indicating that phorbol 13-acetate displaced resveratrol from the protein binding site to the solution thereby reducing its fluorescence intensity. The calculated IC_{50} value was found to be $3.954 \pm 1.2 \mu M$. Overall, these results suggest that resveratrol binds to the phorbol ester binding site of Munc13-1 C1.

3.6. Possible binding mode of resveratrol in Munc13-1 C1

To understand the site and the modes of resveratrol binding in Munc13-1 C1 from a structural point of view, we docked resveratrol molecule into the Munc13-1 C1 domain, and performed MD simulation for 2.5 ns. We found that resveratrol and its derivatives docked on site surrounded with residues, Trp-588, Gly-589, Ile-590 and Arg-592, which are within 3 Å of the resveratrol molecule.

The potential energy plot showed that the molecular system was quickly stabilized and remained stable during 2.5 ns MD simulation (Figure 8). The resveratrol-bound Munc13-1 C1 changed its conformation steadily from 700 ps to 1300 ps, and then maintained the conformation until 2.5 ns. These changes in conformation influenced the docking pose of resveratrol even though the two loops did not change dramatically (Figure 8). The resveratrol molecule rotated counter-clock wise during 1500 ps and two oxygen atoms of resveratrol molecule remained close to Thr-575. Finally, at the end of 2.5 ns the resveratrol molecule was found to maintain a stable orientation.

4. Discussion

To test the hypothesis that resveratrol will bind to the Ca^{2+} -sensitive and C1-domain containing Munc13-1, like PKC α , we studied the effect of resveratrol and its derivatives (1-5) on the membrane translocation of Munc13-1. Munc13-1 is a presynaptic active zone protein and primarily expressed in several brain regions including hippocampus. Therefore, we used hippocampal-derived HT22 cell line for our studies. In addition, we also used primary hippocampal neuron to see if we could replicate the HT22 results in the primary neuronal culture. Our results show that resveratrol inhibits TPA-induced membrane translocation of Munc13-1 and in both HT22 cells and in primary hippocampal neuron. In the HT22 cells, this inhibition was seen with as low as $6.25 \mu M$ of resveratrol treated for 15 min (Fig.3). Inhibition was also detected with a similar treatment of $50 \mu M$ resveratrol, but treatment for 48h caused cytotoxicity (Fig.3).

To understand the role of hydroxyl group of resveratrol in modulating the Munc13-1 activity, we used several resveratrol derivatives in which either we added extra hydroxyl groups (derivative **3**), or replaced the hydroxyl groups with OCH₃ groups (derivatives **2** and **4**), or modified hydroxyl group with a C₁₆ aliphatic chain (derivative **5**) to see its effect on membrane translocation. As expected, introduction of additional hydroxyl groups in the resveratrol moiety or modification of the hydroxyl groups with OCH₃ affected the DPPH radical scavenging in solution and cytotoxicity on HT22 cells. However, we do not see any significant effect of these derivatives on the membrane translocation of Munc13-1. These data also suggest that the inhibition of Munc13-1 by resveratrol and its derivatives is independent of their antioxidant properties and resveratrol is involved in fine-tuned interactions with the Munc13-1 residues that are different from that of the derivatives studied here. Earlier, similar observations were made for the TPA-induced membrane translocation of PKC α . Although HEK293 cells and much higher concentrations of resveratrol and its derivatives (100 μ M) were used in the PKC α experiments [12], the overall conclusion is that for both these proteins, resveratrol is more effective in inhibiting the membrane translocation than the derivatives.

What is the site of action of resveratrol in Munc13-1? Our preliminary result with the phorbol ester-insensitive mutant, Munc13-1^{H567K} that resveratrol does not inhibit it either by itself or co-treated with TPA, suggests that resveratrol could bind to the phorbol ester binding site, i.e. the C1 domain of Munc13-1. Furthermore, fluorescence binding studies with purified Munc13-1 C1 suggest that resveratrol binds to this domain. Phorbol 13-acetate decreased the FRET between Munc13-1 C1 and resveratrol in a concentration-dependent manner with an IC₅₀ of 3.9 μ M suggesting that resveratrol competes with phorbol ester in its binding site. When the resveratrol molecule was docked at the phorbol ester binding site of Munc13-1 C1, it formed two hydrogen bonds with the backbone oxygen atoms of Gly-589 and Ile-590 residues with a distance of 2.01 Å and 2.08 Å respectively (Figure 9). This docking resulted in a score of 2.66 and corresponding K_d was 2.22 mM. When similar docking methods were applied for derivative **3**, which contains six hydroxyl groups, a single hydrogen bond with the backbone oxygen of Gly-589 residue with a distance of 2.08 Å was detected with a docking of 0.87 and K_d value of 136.58 mM. These data suggest that the presence of higher number of hydrogen bond forming groups may not be the sole criterion for effective binding of the resveratrol derivative at the phorbol ester binding site. The MD simulation data further showed that these fine interactions could change at the nanosecond time scale. The recent crystal structure of the combined C1-C2B-MUN domains reveals that the C1 and C2B domains are packed at one end, interact with each other and cooperate in the plasma-membrane binding [65]. The surface residues, Arg-754, Lys-752 and Arg-750 of the C2B domain are in close proximity with the surface residues, Glu-584, Glu-582 and Tyr-581 of the C1 domain. Mutational studies with these surface residues suggested that the interaction between C1 and C2B are critical in the Ca²⁺ and DAG dependent presynaptic plasticity [65]. Trp-588 with which resveratrol is involved with FRET and Gly-589 with which resveratrol forms hydrogen bonds are not far from the interface residues of the C2B domain suggesting that resveratrol's binding with the C1 could influence its binding with the C2B and eventually the binding with the plasma-membrane.

In the present study we characterized a new target of resveratrol by presenting how resveratrol could modulate and bind to presynaptic Munc13-1. Is this just one more addition to the long list of resveratrol's target? Several studies have implicated the role of Munc13-1 in neurodegenerative diseases [29-33]. In addition, multiple studies also indicated the role of resveratrol in neuroprotection and neurodegenerative diseases [4, 66-68]. Munc13-1 is essential for fusion competence of predominantly glutamatergic synaptic vesicles [25]. Further, prolonged release of glutamate causes excitotoxicity and cell death and may play a role in the pathogenesis of chronic neurodegenerative disorders including Alzheimer's disease [69, 70]. Our results that resveratrol inhibits TPA-induced translocation of Munc13-1 suggests that it could mitigate glutamate release in the synaptic cleft and reduce neurotoxicity. While in the present study the effect of resveratrol was performed in the neuronal cells, it would be interesting to see if resveratrol exerts similar effects on Munc13-1 in other cell lines, such as, astrocytes which secrete glutamate and can regulate synaptogenesis through its interactions with neurons[71].

A critical issue that should be considered before the translational potential of this study is fully realized. Sometimes, a highly potent polyphenol in cell culture may show little or no potency in the in vitro experiments or in clinical trials because of its poor bioavailability. Bioavailability of resveratrol depends on several factors, such as its lipophilicity, first pass metabolism, phase II metabolism, stability in plasma etc. Resveratrol has a bioavailability of ~1% [72-74], which has been a major stumbling block in the drug development related to resveratrol and its derivatives. Furthermore, this poor bioavailability will also affect the concentration of resveratrol in the brain after crossing the blood brain barrier (BBB). However, the most encouraging aspect is that resveratrol reaches the brain both during short term and acute treatments [75, 76] and significant efforts are ongoing to increase the concentration of resveratrol in the brain using various delivery system [77, 78].

5. Conclusions

In summary, our results show that resveratrol inhibits Munc13-1 activity in both HT22 cells and primary hippocampal neurons by binding to the phorbol ester binding domain. Modulation of a presynaptic protein by a dietary polyphenol further reiterates the importance of natural compounds in the management of neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer's disease
BBB	blood brain barrier
DAG	diacylglycerol
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
GST	glutathione S-transferase
HRP	horseradish peroxidase
IPTG	isopropyl β -D-1-thiogalactopyranoside
MALDI-TOF	matrix assisted laser desorption ionization time of flight
MD	molecular dynamics
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PEI	polyethyleneimine
PFA	paraformaldehyde
PKC	protein kinase C
PS	phosphatidylserine
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SNARE	Soluble NSF (N-Ethylmaleimide-Sensitive Factor) Attachment Protein Receptor
TPA	12-Otetradecanoylphorbol 13-acetate

Highlights

- Resveratrol binds to the C1 domain of SNARE complex protein Munc13-1
- Resveratrol inhibits the activity of Munc13-1 in HT-22 cells and primary hippocampal neurons.
- Dietary polyphenols are important in the management of neurodegenerative diseases.

Figure 1.

A. Chemical structure of resveratrol (**1**) and its derivatives, (*E*)-1,3-dimethoxy-5-(4-methoxystyryl)benzene (**2**), (*E*)-5,5'-(ethene-1,2-diyl)bis(benzene-1,2,3-triol) (**3**), (*E*)-1,2-bis(3,4,5-trimethoxyphenyl)ethane (**4**), and (*E*)-5-(4-(hexadecyloxy)-3,5-dihydroxystyryl)benzene-1,2,3-triol (**5**). **B.** Schematic representation of the domains of Munc13-1: C1 regulatory domain binds to lipids, diacylglycerol, and phorbol esters and C2 regulatory domains (C2A, C2B and C2C) bind to Ca^{2+} . Residues 859-1531 between the C2B and C2C domains is known as MUN domain that binds to syntaxin. Amino acid sequence of Munc13-1 C1 domain used in the present study, is marked in bold letters and underlined extra residues were included for cloning and purification purposes.

Figure 2.

Anti-oxidant activity of **1-5** using DPPH radical quenching assay. The plots show percent of free DPPH (60 μM) in the presence of varying concentrations of **1-5** (1-250 μM) in methanol. Values are the average of triplicate experiments and represented as mean $\pm$ SEM. Solid lines indicate the Hill fit of dose response curves. The derived IC_{50} values for **1**, **3** and **5** were 29.36 ± 1.45 , 1.17 ± 0.34 and 1.97 ± 0.27 and μM, respectively. Absorbance was measured at 517 nm.

Figure 3.

Effect of **1-5** on the viability of HT22 cells measured using MTT assay. Bar graph shows the percent of viable HT22 cells treated with varying concentrations (6.25-100 μM). Data are expressed as mean ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA, followed by *Bonferroni* post hoc test. *P < 0.05 compared to **1**.

Figure 4.

A. Effect of resveratrol (1) on TPA-induced membrane translocation of Munc13-1 in HT22 cells. Upper panel, immunoblot analysis of cytosolic (C) and membrane (M) fractions after cells were either treated with varying concentrations of resveratrol (1) (12.5-50 μ M) or varying concentrations of TPA (125-2000 nM) or co-treated with TPA and resveratrol (1) for 15 min. Lower panel, bar graph of densitometry analysis of above immunoblots. Control (ctrl) refers to the sample where cells were treated with 0.1% DMSO. Data are expressed as mean $\pm$ SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA, followed by *Bonferroni* post hoc test. *P < 0.05 compared to TPA. Cell fractionation and immunoblot analysis was carried out as described in the method section. **B.**

Effect of resveratrol on TPA-induced membrane translocation of Munc13-1 in HT22 cells. Upper panel, confocal images of HT22 cells treated with either resveratrol (1) or TPA or co-treated with TPA and resveratrol (1) for 15 min. Lower panel, bar graph shows relative fluorescence intensity of Munc13-1 in cytosol and membrane, derived from above confocal images. Control (ctrl) refers to the sample where cells were treated with 0.1% DMSO. Data are expressed as mean $\pm$ SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA, followed by *Bonferroni* post hoc test. * $P < 0.05$ compared to TPA. Confocal analysis was performed as described in method section

Figure 5.

Effect of **1-5** on TPA-induced membrane translocation of Munc13-1 in HT22 cells. **A**, Immunoblot analysis of cytosolic (C) and membrane (M) fraction after cells were either treated with **1-5** (50 μ M) or TPA (500 nM) or co-treated with **1-5** (50 μ M) and TPA (500 nM) for 15 min. **B**, Bar graph showing the densitometry analysis of cytosolic and β -actin immunoblots. β -actin was used as loading control. Control (ctrl) refers to the sample where cells were treated with 0.1% DMSO. Data are expressed as mean $\pm$ SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA, followed by *Bonferroni* post hoc test. * $P < 0.05$ compared to TPA. Cell fractionation and immunoblot analysis was carried out as described in the method section.

Figure 6.

Effect of resveratrol on TPA-induced translocation of Munc13-1 in primary hippocampal neurons. Upper panel, confocal images of primary hippocampal neurons either treated with resveratrol (**1**) (50 μM) or TPA (500 nM) or co-treated with TPA (500 nM) and resveratrol (**1**) (12.5-50 μM) for 15 min. Images show double-label immunocytochemistry of Tuj1 (pan neuronal marker) and Munc13-1 in primary hippocampal neurons. Asterisks denote cytosol area. Lower panel, Quantification of mean intensity of Munc13-1 expression in hippocampal neurons. Scale bar, 40 μM . Data are means $\pm$ SEM. *** P < 0.001, ** P < 0.01. Data were analysed by one-way ANOVA with Tukey's multiple comparison tests. Details of immunocytochemistry are described in the method section.

Figure 7.

Effect of phorbol 13-acetate on resveratrol binding to Munc13-1 C1. **A**, Emission spectra of **a**) Munc13-1 C1 (1 μM), **b**) resveratrol (10 μM), **c**) Munc13-1 C1 (1 μM) + resveratrol (10 μM) and **d**) Munc13-1 C1 (1 μM) + resveratrol (10 μM) + phorbol 13-acetate (10 μM). Samples were incubated for 60 min at room temperature before spectra were recorded. Samples were excited at 290 nm and emission spectra were recorded from 300 nm to 600 nm. FRET between tryptophan residue of Munc13-1 C1 and resveratrol was calculated. **B**, Dose dependent effect of phorbol 13-acetate on FRET between Munc13-1 C1 and resveratrol. Data are expressed as mean ± SD of three independent experiments. The IC₅₀ was calculated from fitted curves (solid line) using Hill equation in Sigma Plot. The calculated IC₅₀ value was 3.97 ± 1.43 μM.

Figure 8.

A. MD simulation of Munc13-1 C1- resveratrol complex. Plot of potential energy (upper panel) and RMSD (lower panel) of Munc13-1 C1-resveratrol complex with respect to simulation time. Resveratrol was docked into phorbol 13-acetate site of Munc13-1 C1 domain and simulated for 2.5 ns as described in methods section. **B.** Snapshots of Munc13-1 C1 and resveratrol complex at a 0.5 ns time interval of MD simulation time. Resveratrol (res) (blue) and residues of Munc13-1 C1 within 4 Å distance from resveratrol (orange) The MD simulation was performed using the GROMACS 4.6.5 package of programs. The structures were visualized using Discovery Studio Visualizer 4.5 (Biovia Inc.).

Figure 9.

Location of resveratrol binding site in Munc13-1 C1. **A**, Surface diagram of resveratrol-Munc13-1 C1 complex. **B**, Enlarged view of the interaction site showing resveratrol (magenta) and residues within 3 Å of resveratrol (orange and yellow). The spheres indicate the zinc ions and hydrogen bond are shown in dotted lines. The structures were visualized using Discovery Studio Visualizer 4.5 (Biovia Inc.).