Resveratrol inhibits type II phosphatidylinositol 4-kinase: A key component in pathways of phosphoinositide turn over

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Abstract

Resveratrol has anti-inflammatory, cardio protective and cancer chemopreventive properties. The molecular targets for resveratrol in early signaling cascades are not well understood. Resveratrol inhibits type II PtdIns 4-kinase but not PtdIns 3-kinase activity in vitro. Resveratrol directly binds to the enzyme with a $K_d$ of 7.2 μM. Kinetic studies show that resveratrol competes with PtdIns binding. Inhibition of PtdIns 4-kinase activity by resveratrol/phenylarsine oxide reduces Jurkat cell adhesion to matrigel/fibronectin coated surfaces, suggesting a role for type II PtdIns 4-kinase in lymphocyte infiltration to the sites of inflammation.

Keywords: Signal transduction; T lymphocytes; Cell adhesion

1. Introduction

Resveratrol is a naturally occurring stilbene (3,4',5'-trihydroxystilbene) synthesized by plants in response to injury or fungal attack [1]. It exists in cis and trans configuration. Trans resveratrol has been shown to possess many biological functions in vitro and in animal model systems. These include antioxidant activity, modulation of hepatic apolipoprotein and lipid synthesis, inhibition of platelet aggregation as well as the production of pro-atherogenic eicosanoids by human platelets and neutrophils resulting in protection against atherosclerosis [2–5]. In addition to its cardio-protective activity, resveratrol has been implicated as chemopreventive agent in carcinogenesis, through its actions on ribonucleotide reductase activity, protein kinase C and Cdk kinases [6–12]. Apart from these activities, resveratrol is known to induce apoptosis through CD95 receptors in tumor cell lines [13]. While most of these effects are the results of long-term incubation of cells with resveratrol, the early effects of resveratrol on cell signaling mechanisms are not well understood.

Resveratrol is shown to inhibit cell proliferation in human peripheral blood lymphocytes and this inhibition is due to an arrest of $G_0$-$G_1$ transition [14]. Phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)P$_2$) hydrolysis is an early and critical step in T cell proliferation and differentiation, and very little is known about the effects of resveratrol on PtdIns (4,5)P$_2$ biosynthetic enzymes. PtdIns (4,5)P$_2$ is synthesized by sequential phosphorylation of PtdIns by PtdIns 4-kinases and PtdIns 4P-5-kinases in the canonical pathway of PtdIns (4,5)P$_2$ biosynthesis. PtdIns 4-kinases have been classified into types II and III PtdIns 4-kinases. While type II PtdIns 4-kinases represent a new class of enzymes, type III PtdIns 4-kinases share homology in their catalytic domain with PtdIns 3-kinases [15,16]. A type II PtdIns 4-kinase activity has been suggested to be an integral part of early signal transduction machinery in T cells stimulated with polyclonal mitogen Con A or through TCR-CD3 receptors [17–21]. The present manuscript describes resveratrol as an inhibitor of type II PtdIns 4-kinase without significant effects on PtdIns 3-kinase.

Abbreviations: PtdIns (4,5)P$_2$, d-myo-phosphatidylinositol 4,5-bisphosphate; PtdIns 4P, d-myo-phosphatidylinositol 4-phosphate; PtdIns, d-myo-phosphatidylinositol
2. Materials and methods

2.1. Materials

Human type II PtdIns 4-kinase β clone (Gen Bank accession #AY065990) was a generous gift from Tamas Balla, Endocrinology and Reproduction Research Branch, NICHD, Bethesda, MD, USA. Glutathione, thrombin and glutathione sepharose beads were from Amersham International plc. Phosphatidylinositol, Triton X-100, N-lauroylsarcosine, phenylmethyl-sulfonylflouride (PMSF), resveratrol, fibronectin, matrigel and all restriction enzymes were from Sigma, MO, USA or from Roche, Germany. [γ-32P] ATP was from Board for Radiation and Isotope Technology (BRIT), Mumbai, India. All other chemicals were of analytical grade. Primers used for PCR was obtained from Microsynth, Switzerland.

2.2. Cloning of human type II PtdIns 4-kinase β

Human type II PtdIns 4-kinase was amplified using PI4KF (forward) 5’-TTATTGTGAGATGGGATCCC-TCCG-3’ and PI4KB (backward) 5’-AAATAGCGGCC-GCCACAGGAGAAAAATGGCT-GTCGACATGGAGBCCC-3’ primers. The amplified product was sub-cloned at SalI/NorI sites in pGEX-4T3 vector. The construct was expressed in BL21(DE3) strain of E. coli using IPTG induction (0.5 mM) at 37 °C for 3 h.

2.3. Purification of GST tagged protein

Bacterial cells expressing GST tagged proteins were centrifuged at 14,700 × g for 1 min. Bacterial cell pellet was lysed in ice in STE buffer [10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl] and lysozyme at 100 μg/ml was added. Cells were sonicated for 1 min in presence of 10 mM DTT and 1.5% N-lauroysarcosine. The lysate was centrifuged at 14,700 × g for 20 min. The supernatant was diluted 1:1 with STE buffer and Triton X-100 to give a final concentration of 0.7% N-lauroylsarcosine and 0.9% Triton X-100. It was incubated for 30 min in ice. The lysate was added to glutathione beads, that were prewashed with PBS (pH 7.6), and incubated for 45 min at room temperature. The beads were washed thrice with PBS. The washed beads were incubated with thrombin protease for 4 h at 4 °C for removal of GST tag. The purified protein was dialyzed against PBS and analyzed on SDS–PAGE.

2.4. Resveratrol treatment of Jurkat cells

Jurkat cells (1 × 10^6) were washed twice with serum free RPMI buffer. The cells were suspended in 200 μl of RPMI. Cells were incubated with different concentrations of resveratrol for 45 min at 37 °C. Cells were washed with PBS and lysed in 100 μl lysis buffer [25 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 0.25 mM sodium orthovanadate, 1 mM PMSF]. The lysates were assayed for PtdIns 4-kinase activity.

2.5. PtdIns 4-kinase assay

PtdIns 4-kinase assay was carried out in a final volume of 50 μl containing 50 mM Tris (pH 7.6), 10 mM MgCl2, 0.25 mM EGTA, 0.1 mM sodium orthovanadate, 20 μg/ml PtdIns, 100 μM (γ-32P) ATP (200–300 cpm/pmol) and 0.3% Triton X-100. The reaction was initiated with the addition of labeled ATP and incubated at room temperature (~25 °C) for 6 min. The reaction was terminated with 50 μl of 12N HCl. The labeled phospholipids were extracted with 500 μl of chloroform:methanol:water (15:5:5, v/v/v). The organic phase was washed with 100 μl of methanol:1N HCl (1:1, v/v) and applied on Merck Silica gel TLC plates pretreated with 60 mM EDTA, 2% sodium potassium tartarate in 50% ethanol (pH 8.0). The chromatograms were developed with chloroform:methanol:ammonium hydroxide:water (90:90:7:20, v/v/v/v). The phospholipids were visualized by autoradiography and quantified by scintillation counting [17].

2.6. Immunoprecipitation and PtdIns 3-kinase assay

Jurkat cells (10 × 10^6) were pelleted and lysed with 200 μl lysis buffer [25 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 0.25 mM sodium orthovanadate, 1 mM PMSF]. Lysate was centrifuged at 13,000 × g for 30 min. Supernatants were incubated with 1 μg of the antibody (p85, or p110) for 2 h at 4 °C. At the end of this incubation, bovine serum albumin pre-coated protein A agarose beads were added and incubated for 1 h at 4 °C with gentle tumbeling. The beads were washed 3× with PBS and 2× with lysis buffer without Triton X-100. The beads were assayed for PtdIns 3-kinase activity in a 100 μl reaction volume containing 50 mM Tris (pH 7.6), 10 mM MgCl2, 0.25 mM EGTA, 0.1 mM sodium orthovanadate, 20 μg/ml PtdIns, 100 μM (γ-32P) ATP (200–300 cpm/pmol). The reaction was initiated with the addition of labeled ATP and incubated at room temperature (~25 °C) for 6 min. The reaction was terminated with 50 μl of 12N HCl. The labeled phospholipids were extracted and separated as described previously for PtdIns 4-kinase assay. The phospholipids were visualized by autoradiography and quantified by scintillation counting.

2.7. Resveratrol binding studies

Fluorescence studies were performed using a JASCO fluorescence spectrophotometer. Spectra were taken by multiple scans and buffer blanks were subtracted from all measurements. A quartz cuvette of 3 mm path length was used. The band pass for excitation and emission monochromators was 10 nm. Emission spectra were...
recorded over a range of 340–500 nm using 320 nm as excitation wavelength and fluorescence intensity at 389 nm was used to calculate the dissociation constant.

The dissociation constant of resveratrol with type II PtdIns 4-kinase β was determined by using ligand (resveratrol) titration procedure. Free resveratrol has a fluorescence maximum around 389 nm, upon binding to type II PtdIns 4-kinase β the fluorescence intensity increases several fold. Increased fluorescence of resveratrol at 389 nm, upon binding with type II PtdIns 4-kinase β was used to determine the affinity of resveratrol and type II PtdIns 4-kinase β interaction. Type II PtdIns 4-kinase β (5 μM) was allowed to interact with different concentrations of resveratrol (2–30 μM) in PBS at 25°C. The fluorescence intensity was measured at 389 nm upon binding. Buffer blank containing different concentrations of resveratrol were subtracted from experimental data. ΔF_m (maximum fluorescence change when protein in completely liganded with resveratrol) was determined by plotting 1/C (resveratrol concentration) and I/ΔF (increase in fluorescence). The concentration of bound resveratrol was calculated using the relationship, [bound ligand] = - protein concentration × ΔF/ΔF_m. The free resveratrol concentration was calculated as [free resveratrol] = [total resveratrol] – [bound resveratrol]. The dissociation constant was calculated by plotting 1/[bound resveratrol] with 1/[free resveratrol] and calculating the slope [22].

2.8. Cell adhesion assay

Fibronectin (5 μg/ml) and matrigel (10 μg/ml) were coated onto 96 well flat bottomed tissue culture plates overnight at 37°C and then the wells were blocked with 1% BSA in PBS for 1 h at 37°C. Jurkat cells, suspended in serum free RPMI, were pre-incubated with resveratrol (100 μM) for 45 min at 37°C. The treated cells were washed with 0.1% BSA containing RPMI and resuspended at a density of 3 × 10^5 cells/ml in 0.1% BSA containing RPMI before addition to the plate. The Jurkat cells were added to the well in 100 μl aliquot and incubated for 90 min at 37°C. The non-adherent cells were washed off with PBS by two rounds of gentle pipetting. To the well 100 μl of serum containing RPMI was added with final addition of 20 μl of MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) solution (5 mg/ml). The plates were incubated at 37°C for 12 h. The plates were centrifuged at 7000 × g for 30 min and supernatant was removed. The formazan crystals were dissolved by addition of 100 μl of dimethylsulfoxide. Optical densities were determined using an enzyme linked ImmunoSorbent adsorption plate reader at 540 nm with 690 nm reference. Background values were determined in wells coated with 1% BSA alone, were subtracted from each point. Adhesion was expressed as a percentage of the respective total number of cells (wells containing adherent and non-adherent cells) [23].

3. Results

3.1. Resveratrol inhibits human type II PtdIns 4-kinase β in vitro

The effect of resveratrol on phosphoinositide kinases was studied in vitro. Human type II PtdIns 4-kinase β was expressed in E. coli as GST-fusion protein. The GST-tag was removed and analyzed on SDS–PAGE (Fig. 1A). The purified type II PtdIns 4-kinase β was assayed for activity in presence of increasing concentrations of resveratrol. PtdIns 4-kinase activity was inhibited by resveratrol in a dose dependent fashion (Fig. 1B). To address whether this inhibition is specific to type II PtdIns 4-kinase or not, PtdIns 3-kinase activity was immunoprecipitated from Jurkat cells with antibodies against p85 and p110 subunits.

![Fig. 1. Resveratrol inhibits type II PtdIns 4-kinase activity in vitro. (A) Type II PtdIns 4-kinase β was sub cloned and expressed in E. coli. The expressed protein was purified and analysed on SDS–PAGE. (B) The effect of resveratrol on type II PtdIns 4-kinase activity. Values are representative of four experiments. Error bars represent standard deviation (S.D.). (C) Resveratrol affects type II PtdIns 4-kinase activity but not PtdIns 3-kinase activity. Recombinant type II PtdIns 4-kinase β was purified from E. coli (a). Human PtdIns 3 kinases were immunoprecipitated with anti p85 (b) and anti p110 (c) subunits. All these enzymes were assayed for phosphatidylinositol kinase activities as described in Section 2. 32P labeled products were analyzed on tlc and the upper panel shows the autoradiogram. Lane 1: enzyme activity in the absence of resveratrol. Lane 2: enzyme activity in presence of 50 μM resveratrol. Lane 3: enzyme activity in presence of 100 μM resveratrol. The bar diagram depicts the quantification of the data. Open bars: 0 μM resveratrol, hatch bars: 50 μM resveratrol, and cross-bars: 100 μM resveratrol. The values are mean of three experiments ± S.E. The P value is ≤ 0.002 (one-way ANOVA).]
of PtdIns 3-kinase. Resveratrol did not inhibit PtdIns 3-kinase activity in these immunoprecipitates while the same concentrations of resveratrol showed a strong inhibition of type II PtdIns 4-kinase activity (Fig. 1C). These results suggest that type II PtdIns 4-kinases are selectively/preferentially inhibited by resveratrol.

3.2. Resveratrol competes with phosphatidylinositols binding site on type II PtdIns 4-kinase

To rule out any artifacts in resveratrol mediated inhibition of type II PtdIns 4-kinase activity, the direct interaction of resveratrol with type II PtdIns 4-kinase was addressed by fluorescence spectroscopic techniques. Incubation of resveratrol with increasing concentrations of type II PtdIns 4-kinase β showed an increase in resveratrol fluorescence (Fig. 2A). The $K_d$ value of the two molecules was 7.2 μM (Fig. 2B). These results provide evidence for a direct binding of resveratrol to type II PtdIns 4-kinase. The binding of resveratrol to type II PtdIns 4-kinase β was not affected by ATP (results not shown) but presence of increasing concentrations of phosphatidylinositol abolished resveratrol binding to type II PtdIns 4-kinase (Fig. 3). These results suggest that resveratrol and phosphatidylinositol may compete for the same or overlapping binding sites on the enzyme.

Kinetic analysis of the enzyme activity suggests that resveratrol did not alter the $V_{max}$ of the enzyme but affected its $K_m$ for phosphatidylinositol, supporting the hypothesis that phosphatidylinositol and resveratrol compete for the same site or an overlapping site (results not shown).

3.3. Resveratrol inhibits PtdIns 4-kinase activation in Jurkat cells

The studies on in vitro inhibition of type II PtdIns 4-kinase activity by resveratrol was extended to cell culture assays. Pre-incubation of Jurkat cells with increasing concentrations of resveratrol showed a dose dependent reduction in PtdIns 4-kinase activity (Fig. 4). Under these experimental conditions resveratrol did not induce any cell death as assayed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by trypan blue dye staining methods, suggesting that the reduction in PtdIns 4-kinase activity is not due to possible cytotoxic effects of resveratrol. Earlier studies from our lab and others have shown activation of a type II PtdIns 4-kinase in Jurkat cells in response to anti-CD3 cross-linking. Pre-incubation of Jurkat cells with resveratrol showed inhibition of type II PtdIns 4-kinase activation in anti-CD3 stimulated cells, suggesting that resveratrol interferes with type II PtdIns 4-kinase functions (Fig. 5).
3.4. Resveratrol inhibits Jurkat cell adhesion

T lymphocytes need to adhere to the extra cellular matrix molecules like fibronectin, laminin and collagen at the site of inflammation. Type II PtdIns 4-kinases are shown to be associated with integrin receptors and are suggested to play a role in cell adhesion and motility [24]. The effect of resveratrol on cell adhesion was analyzed with Jurkat cells on two extra cellular matrix substrates, viz. fibronectin and matrigel. Resveratrol inhibited the adhesion of Jurkat cells on both these substrates. A ~50% decrease in adhesion of Jurkat cells was observed in fibronectin coated plates (Fig. 6A), while a ~70% decrease in cell adhesion was observed in matrigel coated plates (Fig. 6B). These results support the hypothesis that type II PtdIns 4-kinase activity is necessary for cell adhesion and resveratrol negatively modulates cell adhesion by inhibiting the enzyme activity.

The role of type II PtdIns 4-kinase in cell adhesion was addressed in parallel experiments in which PtdIns 4-kinase

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Fig. 4. Resveratrol inhibits type II PtdIns 4-kinase activity in Jurkat cells. Jurkat cells were incubated with increasing concentration of resveratrol for 45 min at 37 °C. At the end of incubation, cells were washed and were lysed in lysis buffer. Cell lysates were assayed for PtdIns 4-kinase activity. The upper panel shows autoradiograph of PtdIns 4P formed and the line diagram shows the quantification of the data. Values are representative of three experiments. Error bars represent S.D.

Fig. 5. Resveratrol inhibits type II PtdIns 4-kinase activation in anti-CD3 stimulated Jurkat cells. Jurkat cells were pre treated with resveratrol (100 μM) and stimulated with anti-CD3 crosslinking. The cells were lysed and assayed for PtdIns 4-kinase activity. Lane 1: lysates from control cells (absence of resveratrol treatment and CD3 cross linking). Lane 2: lysates from CD3 cross linked cells in the absence of resveratrol. Lane 3: lysates from cells pre-incubated with resveratrol (100 μM) in absence of CD3 cross linking. Lane 4: cell lysates from cells pre-treated with resveratrol (100 μM) followed by CD3 cross linking. Graph shows mean ± S.E. of four experiments. The P value is ≤0.0015 (one-way ANOVA).

Fig. 6. Resveratrol affects Jurkat cell adhesion. Jurkat cells were pre-treated with resveratrol (100 μM) and PAO (20 μM) and checked for adhesion on matrigel coated plates (A) and on fibronectin coated plates (B). Cell adhesion was assayed using MTT method. The graph represents percentage of adherent cells in comparison to control (untreated cells) taken as 100%. Values are representative of six experiments ±S.E. The P values are ≤0.003 (one-way ANOVA).
activity was inhibited with phenylarsine oxide (PAO). Pretreatment of Jurkat cells with PAO showed ~60% decrease in cell adhesion in matrigel coated plates. These studies suggest that type II PtdIns 4-kinases can be potential targets for pharmacological intervention in treatment of inflammation and cancer metastasis.

4. Discussion

Type II PtdIns 4-kinase(s), in addition to their role in biosynthesis of PtdIns(4,5)P₂, are also implicated in other physiological functions like cytoskeletal rearrangement, intracellular vesicular transport, cell secretion and mitogenesis [15,25–27]. Nonavailability of inhibitors for type II PtdIns 4-kinases makes it difficult to assess their individual contributions in these signaling cascades. The present manuscript describes resveratrol as an inhibitor for type II PtdIns 4-kinases and suggests its use as a pharmacological tool to distinguish type II PtdIns 4-kinase signaling cascades from PtdIns 3-kinase pathways. This suggestion is supported by the following observations: (a) it showed dose dependent inhibition of type II PtdIns 4-kinase β isoform activity in in vitro assays, while the same concentrations have no significant effects on PtdIns 3-kinase activity, (b) it binds to type II PtdIns 4-kinase β as shown by fluorescence spectroscopic studies, (c) resveratrol is a competitive inhibitor of PtdIns, and (d) resveratrol showed a dose dependent inhibition of type II PtdIns 4-kinase activity in Jurkat cells. Resveratrol did not inhibit yeast PtdIns 4-kinase PIK (a homologue of type III PtdIns 4-kinases, which share homology with PtdIns 3-kinase) under these experimental conditions (results not shown). These results strongly support that resveratrol inhibits type II PtdIns 4-kinase activity and can discriminate over PI 3-kinase signaling pathways.

In addition to type II PtdIns 4-kinases, resveratrol has been shown to bind and inhibit cyclooxygenases, protein kinase C and transcriptional factor NF-κB [6,12,28–31]. Even though PKC is activated by pharmacological agent PMA in vivo, PtdIns (4,5)P₂ is implicated as the physiological ligand for PKC [32–34]. PtdIns (4,5)P₂ is suggested to be a competitor for PMA binding on PKC [32–34]. The binding sites of resveratrol on protein kinase C are in the region where PMA binds [12]. It would be interesting to know whether resveratrol mediated inhibition of PKC may be due to its competition with PtdIns (4,5)P₂ binding in vivo.

Resveratrol has been implicated in PtdIns 3-kinase mediated signaling cascades [35,36]. The concentrations required for inhibition of PtdIns 3-kinase is significantly on the higher side compared to our studies. It could be possible that inhibition of type II PtdIns 4-kinases at these concentrations may limit the synthesis of PtdIns 4P and subsequent productions of PtdIns (3,4)P₂ or PtdIns (3,4,5)P₃, and activation of Akt/PKB pathways.

Recently Haider et al. have reported that resveratrol may not inhibit PtdIns 3-kinase activity per se but affects upstream of PtdIns 3-kinase signaling cascades in vascular smooth muscle cells. Resveratrol is shown to activate Shp2, a phosphotyrosine phosphatase in vascular smooth muscle cells. Activation of Shp2 leads to dephosphorylation of adaptor molecule Gab 1 and prevents Gab 1/PtdIns 3-kinase interaction thus attenuating PtdIns 3-kinase signaling cascades [37]. Further, serum-induced PtdIns 3-kinase signaling pathways are not inhibited by resveratrol, supporting that resveratrol affects PtdIns 3-kinase signaling pathways by indirect means. These results are in support of present observations, in which we demonstrated that resveratrol does not inhibit PtdIns 3-kinase activity. In addition to its direct effects on type II PtdIns 4-kinase, resveratrol may also negatively modulate PtdIns 4-kinase signaling events through Shp2. Type II PtdIns 4-kinase activity in rat splenic cells has been shown to increase upon tyrosyl phosphorylation [18,19]. While p56lck has been implicated in phosphorylation of type II PtdIns 4-kinases, phosphatase(s) responsible for dephosphorylation were not identified. It would be interesting to know whether Shp2 can be a potential regulator of type II PtdIns 4-kinases.

Resveratrol is known to inhibit cell proliferation and induce apoptosis in cancer cells. Cellular proliferation involves the stimulation of mitogen activated protein kinase (MAPK) pathway, and the PtdIns 3-kinase pathway consisting of PI3K/phosphoinositide-dependent kinase 1 (PDK1)/Akt cascade. Both MAPK and PDK1 affect cell division by regulating multiple cyclin dependent kinases [38–43]. Inhibition of type II PtdIns 4-kinase by resveratrol may down regulate PtdIns 4P levels which are substrates for PtdIns 3-kinases thus affecting PI3K/PDK1/Akt cascade. Recently, a type III PtdIns 4-kinase (PI4K92) has been shown to have potential cdc 2 phosphorylation sites, which suggest a role of these kinases in rearrangement of the Golgi apparatus during cell division [44]. These studies suggest that phosphatidylinositol 4-kinases may play a role in cell division at different levels.

Inhibition of type II PtdIns 4-kinase activity in Jurkat cells by resveratrol suggests that resveratrol can be used as pharmacological tool to address the role of PtdIns 4-kinase signaling cascades. Type II PtdIns 4-kinases are shown to associate with integrin molecules and are implicated in cell adhesion [24]. Cell adhesion plays a major role in lymphocyte migration to sites of inflammation and also in metastasis of tumor cells. Inhibition of Jurkat cell adhesion to fibronectin coated surfaces by resveratrol with a concomitant reduction in PtdIns 4-kinase activity strongly correlates with a regulatory role for type II PtdIns 4-kinases in lymphocyte infiltration and a possible role in tumor metastasis.

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