Resveratrol potentiates growth inhibitory effects of rapamycin in *PTEN*-deficient lipoma cells by suppressing p70S6 kinase activity

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ABSTRACT

Patients with phosphatase and tensin homolog (*PTEN*) hamartoma tumor syndrome and germline mutations in *PTEN* frequently develop lipomatosis, for which there is no standard treatment. Rapamycin was shown to reduce the growth of lipoma cells with heterozygous *PTEN* deficiency in vitro, but concomitantly induced an upregulation of AKT phosphorylation. Since it was shown that resveratrol stabilizes *PTEN*, we asked whether co-incubation with resveratrol could suppress the rapamycin-induced AKT phosphorylation in *PTEN*-deficient lipoma cells.

Resveratrol incubation resulted in decreased lipoma cell viability by inducing G1-phase cell cycle arrest and apoptosis. *PTEN* expression and AKT phosphorylation were not significantly changed, whereas p70S6 kinase (p70S6K) phosphorylation was reduced in *PTEN*-deficient lipoma cells after resveratrol incubation. Rapamycin/resveratrol co-incubation significantly decreased viability further at lower doses of resveratrol and resulted in decreased p70S6K phosphorylation compared to rapamycin incubation alone, suggesting that resveratrol potentiated the growth inhibitory effects of rapamycin by reducing p70S6K activation. Both viability and p70S6K phosphorylation of primary *PTEN* wild-type preadipocytes were less affected compared to *PTEN*-deficient lipoma cells by equimolar concentrations of resveratrol. These results support the concept of combining chemopreventive natural compounds with mammalian target of rapamycin (mTOR) inhibitors to increase the efficacy of chemotherapeutic drugs for patients suffering from overgrowth syndromes.

ABBREVIATIONS: AMPK 5⁰-, AMP-activated protein kinase; ERK, extracellular-signal-regulated kinases; IGF-I, insulin-like growth factor-I; IRS-1, insulin receptor substrate-1; mTORC1, mammalian target of rapamycin complex 1; PHTS, *PTEN* hamartoma tumor syndrome; PI3K, phosphatidylinositol 3-kinase; *PTEN*, phosphatase and tensin homolog; p70S6K, p70S6 kinase; TSC, tuberous sclerosis complex; SIRT1, sirtuin 1; FCS, fetal calf serum; PI, propidium Iodide; DMSO, dimethyl sulfoxide; WST, water-soluble tetrazolium salt; EDTA Ethylenediaminetetraacetic acid; RPIA, radioimmunoprecipitation.

Introduction

Phosphatase and tensin homolog (*PTEN*) counteracts phosphatidylinositol 3-kinase (PI3K) and therefore negatively regulates the AKT/mammalian target of rapamycin (mTOR) pathway (1). A deficiency in *PTEN* activity, which occurs in *PTEN* hamartoma tumor syndrome (PHTS) and various human cancers, affects cell cycle progression, apoptosis, cell metabolism, transcription, and translation through activation of signaling proteins belonging to the PI3K/AKT/mTOR pathway (1). Therefore, inhibitors of this pathway, especially inhibitors of mTOR, showed therapeutic efficacy in preclinical studies (1). Treatment attempts using rapamycin, a specific mTOR complex 1 (mTORC1) inhibitor, have been

reported for children with a germline *PTEN* deletion or mutation and suffering from PHTS (2–4). Growth of lipomatosis, a frequent PHTS-specific complication, was slowed down. However, no reduction in lipomatous tumor masses was observed (2,3). *In vitro*, rapamycin decreased cell viability in *PTEN*-deficient lipoma cells (LipPD1), but did not induce apoptosis (2). In particular, due to mTOR blockade, rapamycin upregulated AKT phosphorylation through a negative feedback loop

via insulin receptor substrate (IRS)-1, leading to enhanced cell survival and possibly to drug resistance (2,5,6). Resveratrol, a chemopreventive polyphenol (7–10), was described to increase *PTEN* levels and promote apoptosis through a decrease in AKT phosphorylation in breast cancer cells (11,12). It has been reported to inhibit cell proliferation and induce apoptosis in vitro in different types of human cancer (7,8,13–15) and 3T3L1 preadipocytes (16). Furthermore, it was shown that resveratrol inhibited not only AKT phosphorylation but also the phosphorylation of the mTORC1 target p70S6 kinase (p70S6K). P70S6K activation promotes synthesis of proteins involved, for example, in cell cycle progression via phosphorylation of the S6 ribosomal protein (17–19). A combined use of rapamycin and resveratrol showed a modest additive inhibitory effect on the growth of different breast cancer cell lines mainly by suppressing the rapamycin-induced phosphorylation of AKT (12). Resveratrol in combination with two other polyphenols (quercetin and catechin) was also described to be effective in reducing AKT activity, inhibiting mTOR signaling, and potentiating the inhibitory effect of the epidermal growth factor receptor inhibitor gefitinib in breast cancer cells (20). Another study highlighted the importance of combining rapamycin and polyphenols for cancer

therapy, based on their studies with isoflavones in combination with rapamycin (9). They described that the PI3K/AKT pathway was highly upregulated due to a deletion of *PTEN* and that treatment with isoflavones decreased AKT activation (9). We therefore investigated whether resveratrol could be used as a (co-)medication to improve the described effects of rapamycin on *PTEN*-deficient lipoma cells.

Methods

Cell culture

PTEN-deficient lipoma cells LipPD1 (2) were maintained in serum-containing medium [DMEM/F12 medium supplemented with 10% fetal calf serum (FCS), 1% of penicillin/streptomycin, biotin (33 mM), and pantothenic acid (17 mM)] (Biochrom AG) at 37°C and 5% CO₂. For experiments, cells were seeded in serum-containing medium overnight and then stimulated with resveratrol (1, 10, 25, 50, 100, 200 mM; dissolved in DMSO; Sigma Aldrich) or the combination of resveratrol and 100 nM rapamycin (Biomol GmbH). DMSO (0.1% in culture medium) was used as solvent control. Primary human subcutaneous (#16899 and #16847) and visceral (#14324) *PTEN* wild-type preadipocytes (LONZA) were treated like LipPD1 cells as described above.

Cell viability, apoptosis, and cell cycle arrest assay

To measure cell viability, cells were seeded at a density of 4000 cells per well. After stimulation with resveratrol alone or in combination with rapamycin, WST-1 assay (Roche, Mannheim, Germany) was used according to manufacturer's protocol. Apoptosis was detected using the FITC AnnexinV Apoptosis Detection Kit (BD Biosciences Pharmingen). Cells were seeded at a density of 50,000 per well in 6-well plates. After collection and resuspension in 100 ml AnnexinV-FITC-binding buffer, cells were incubated with 8 ml AnnexinV-FITC and 2 ml propidium iodide (PI) for 10 min at 4°C in the dark. AnnexinV-FITC-positive and AnnexinV-FITC and PI double-positive cells were considered to be apoptotic. For cell cycle analysis, cells were seeded in 6-well plates at a density of 35,000–40,000 cells per well, fixed in 70% ethanol overnight at 4°C and stained with 50 mg/ml PI for 30 min at 4°C in the dark.

Protein analyses

LipPD1 cells were seeded at a density of 200,000 cells per well, incubated for 48 h and stimulated with 10 nM recombinant human insulin-like growth factor-I (hIGF-I) (Pharmacia Biotech) for 15 min. Cells were lysed with modified RIPA buffer (50 mM Tris HCl, pH 7.4; 1% NP40; 0.25% sodium deoxycholate; 1x Roche complete proteases inhibitor cocktail (Roche); 1 mM EDTA; 1 mM sodium

orthovanadate; and 1 mM sodium fluoride). Immunoblots were performed to detect the amount of PTEN, phospho-AKT (Thr308), AKT, phospho-mTOR (Ser2448), mTOR, phospho-p70S6K (Thr389), and p70S6K (New England Biolabs). GAPDH (EMD Millipore) served as loading control. Appropriate secondary antibodies (Dako) were used. Protein bands were detected by Classico LuminataTM (Millipore) or AmershamTM ECL (GE Healthcare Life Sciences). Western blots were quantified using ImageJ densitometry software.

Statistical analysis

Data represent mean $\pm$ SEM of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 5 software. All statistics were calculated by unpaired student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test as posthoc test. Two-way ANOVA with Bonferroni post-test was used to analyze the effects of resveratrol when comparing LipPD1 cells with primary *PTEN* wild-type preadipocytes. Two-way ANOVA with Bonferroni post-test was also used for analyzing cell cycle experiments. Significant differences were indicated as

**p* < 0.05 compared to control cells or rapamycin incubated cells and ^x*p* < 0.05 compared to resveratrol incubated cells.

Results

Resveratrol enhances the antiproliferative effect of rapamycin on PTEN-deficient lipoma cells (LipPD1) by inducing G1-phase cell cycle arrest and apoptosis

Previously we showed that rapamycin incubation of LipPD1 cells resulted in a dose-dependent decrease in cell viability (2). We asked whether this effect could be enhanced by resveratrol. Therefore, viability of LipPD1 cells in response to various concentrations of resveratrol alone or a resveratrol/rapamycin combination was determined following 72-h incubation. Both, stimulation with resveratrol alone and in combination with 100 nM rapamycin resulted in a dose-dependent inhibition of cell viability. At low concentrations of resveratrol (10 mM), the combination with 100 nM rapamycin resulted in a significant further decrease of viability of $\downarrow 28.0 \pm 12.2\%$ compared to 10 mM resveratrol alone. Cell viability was also further decreased at 25 mM resveratrol/rapamycin combination ($\downarrow 26.1 \pm 11.0\%$) compared to 25 mM resveratrol alone. At high resveratrol concentrations, the addition of rapamycin had no significant effect ($\downarrow 72.0 \pm 13.4\%$ decrease in cell viability at 200 mM resveratrol/ rapamycin combination vs. $\downarrow 70.2 \pm 3.7\%$ at 200 mM resveratrol alone) (Fig. 1A).

To further characterize the effect of resveratrol on LipPD1 cells and the mechanism underlying

the decrease in cell viability, we asked whether or not resveratrol co-incubation had an effect on induction of apoptosis or on cell cycle progression. After stimulation for 24 h with either 100 nM rapamycin (Supplementary Fig. 2A) or 25 and 50 mM resveratrol (Supplementary Fig. 2B) the number of cells in the G1-phase was significantly increased. Rapamycin/resveratrol co-incubation resulted in a significantly

increased number of cells in the G1-phase of cell cycle compared to untreated cells (10 mM resveratrol C 100 nM rapamycin: 16.24 § 6.09%; 50 mM resveratrol C 100 nM rapamycin: 32.02 § 4.2%) and rapamycin incubated cells (10 mM resveratrol C 100 nM rapamycin: 13.08 § 2.74%; 50 mM resveratrol C 100 nM rapamycin: 13.78 § 1.34%; %) (Fig. 1C, D).

Figure. 1. Resveratrol enhances the antiproliferative effect of rapamycin on *PTEN*-deficient lipoma cells (LipPD1) by inducing G1-phase cell cycle arrest and apoptosis. LipPD1 cells were incubated with increasing doses of resveratrol or resveratrol in combination with 100 nM rapamycin for 72 h. A: Cell viability was measured by WST-1 assay. B: Cells were stained with AnnexinV/PI for apoptosis detection. C, D: Cells were stained with PI for cell cycle analysis. For cell viability analysis, data were normalized to untreated cell values (*not shown*). 0.1% DMSO was used as solvent control. All data represent the mean § SEM of at least three independent experiments performed in triplicates (A) or duplicates (B-D). Statistical analysis was done by one-way ANOVA for cell viability and apoptosis analysis (* $p < 0.05$ vs. control cells; $xp < 0.05$, compared to cells with resveratrol incubation alone). Two-way ANOVA was used for cell cycle analysis. Significant differences were determined in comparison to values of rapamycin incubated cells, * $p < 0.05$. The interaction between cell cycle phase and stimulation was significant. resv: resveratrol; rapa: rapamycin.

Figure 2. Resveratrol incubation of *PTEN*-deficient lipoma cells (LipPD1) reduces p70S6K phosphorylation. A-E: Western blot analysis was carried out using lysates of LipPD1 cells that were incubated with different resveratrol concentrations for 48 h and 10 nM IGF-I for 15 min. GAPDH served as loading control. DMSO (0.1%) + IGF-I (solvent control) incubated cells did not differ from IGF-I stimulated control cells in densitometric analyses. One typical blot out of four independent experiments is shown. Data were normalized to serum free + IGF-I stimulated values and are presented as mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA and Student's *t*-test (* $p < 0.05$ vs. control cells). resv: resveratrol; con: control.

No additive effect compared to 10 or 50 mM resveratrol incubated cells was detected (Fig. 1C, D).

Resveratrol incubation (10, 25, 50, 100, 200 mM) for 72 h significantly increased the number of apoptotic cells starting from 50 mM (by 27.2 $\pm$ 5.3%) (Fig. 1B). The combination with rapamycin did not have an additive effect, although a significant induction of apoptosis compared to control cells could be detected (Fig. 1B).

Reduction of p70S6K phosphorylation after resveratrol and combined resveratrol/rapamycin incubation

Previously it was shown that resveratrol increased PTEN protein levels, thereby negatively regulating AKT and p70S6K activation (11,12). However, in our study, PTEN protein expression was not elevated (Fig. 2A, B) and AKT phosphorylation did not decline significantly (Fig. 2A, C) in LipPD1 cells treated with resveratrol. P70S6K phosphorylation was decreased by 75.8 $\pm$ 17.6% after incubation with 50 mM resveratrol for 48 h (Fig. 2A, D). In addition, mTOR activation was significantly decreased after resveratrol incubation (49.5 $\pm$ 9.9%, Fig. 2E). Total AKT and p70S6K expression were not affected. We then asked whether resveratrol could potentiate the rapamycin-induced decrease in p70S6K activation. When examining the combination of rapamycin and resveratrol stimulation, an additive effect was

observed and p70S6K phosphorylation was reduced by 97.7 $\pm$ 1.7% (rapamycin alone: 75.6 $\pm$ 20%) (Fig. 3A, B). Phosphorylation of the mTOR target 4E-binding protein (4E-BP)-1 was not influenced (Fig. 3C). We asked whether combined effects of resveratrol and rapamycin could be mediated via activation of AMP-activated kinase (AMPK) and subsequent Sirtuin 1 (SIRT1) activation (21). No significant effect was observed on AMPK phosphorylation, SIRT1 protein levels, or global lysine acetylation as a measure for activity of NAD-dependent deacetylases after incubation of LipPD1 cells with resveratrol and rapamycin compared to controls (Fig. 3D).

PTEN wild-type preadipocytes are less affected than lipPD1 preadipocytes at equimolar concentrations of resveratrol

To estimate the effects of resveratrol on normal preadipocytes, we determined cell viability and p70S6K phosphorylation of three lots of primary human *PTEN* wildtype preadipocytes compared to LipPD1 cells after 72-h incubation with resveratrol and the combined incubation as described above. *PTEN* wild-type preadipocytes showed a dose-dependent reduction of cell viability. However, we observed that *PTEN* wild-type preadipocytes were significantly less viable at a concentration of 200 mM resveratrol, whereas LipPD1 cell viability was already significantly reduced at 50 mM of resveratrol.

Figure 3. Reduction of p70S6K phosphorylation after resveratrol/rapamycin incubation of *PTEN*-deficient lipoma cells (LipPD1) is not influenced by AMPK/SIRT1 signaling. Western blot analysis was carried out using lysates of A-D: LipPD1 cells that were incubated with a 50 mM resveratrol/100 nM rapamycin combination for 48 h and 10 nM IGF-I for 15 min. GAPDH served as loading control. DMSO (0.1%) + IGF-I (solvent control) incubated cells did not differ from IGF-I stimulated control cells in densitometric analyses. One representative blot is shown. B: Data were normalized to serum free + IGF-I stimulated values and are presented as mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA and Student's *t*-test (**p* < 0.05 vs. control cells). resv: resveratrol; rapa: rapamycin.

(Fig. 4A). The co-stimulation also led to a dose-dependent inhibition in LipPD1 cells and *PTEN* wild-type human preadipocytes. In contrast to the results obtained with resveratrol alone, cell viability of both cell cultures was significantly decreased by 100 nM rapamycin/ 200 mM resveratrol combination (Fig. 4B). Since we observed a significantly different response in cell viability at 50 mM resveratrol, we determined p70S6K activation of LipPD1 cells and *PTEN* wild-type preadipocytes after stimulation with 50 mM resveratrol. LipPD1 cells showed a stronger decrease of p70S6K phosphorylation than *PTEN* wild-type preadipocytes. Total p70S6K protein was not affected by resveratrol incubation (Fig. 4C).

Discussion

Rapamycin as inhibitor of mTORC1 (22) has been clinically used to treat cancers associated with increased activity of the PI3K/AKT/mTOR pathway (23,24). Individual treatment attempts applying rapamycin have been described for patients with germline deletions or mutations of *PTEN* and PHTS (2-4). However, resistance to rapamycin was observed in treated cell lines, mouse models, and human tumor samples (2,9,12,25-27). This effect was found to be frequently due to p70S6K-mediated downregulation of IRS-1 serine phosphorylation

and concomitant AKT activation (2,6,28). P70S6K is a target of mTORC1 and regulates protein synthesis (29). It also mediates mTOR signaling in the negative feedback regulation of the PI3K/AKT/mTOR pathway by phosphorylation of IRS-1 that is upstream of PI3K (28,30-32). Recent studies focused on a combined use of rapamycin with other agents to overcome the hyperactivation of AKT. The combined treatment of breast cancer or glioblastoma cell lines with rapamycin and chemopreventive agents such as isoflavones or resveratrol was shown to decrease cell viability and activation of AKT (9,12). Based on these studies and the reported capability of resveratrol to elevate *PTEN* levels (11,12), we sought to evaluate the combined therapy of rapamycin and resveratrol on activation of AKT and the mTORC1 target p70S6K and cell survival of *PTEN*-deficient lipoma cells (LipPD1) *in vitro*. In contrast to the studies mentioned above, we could not detect elevated *PTEN* protein levels

Figure 4. Viability and p70S6K phosphorylation of *PTEN* wild-type preadipocytes (PP) are less affected by resveratrol incubation than *PTEN*-deficient lipoma cells (LipPD1). LipPD1 cells and primary human *PTEN* wild-type preadipocytes were incubated with A: increasing doses of resveratrol alone or B: in combination with 100 nM rapamycin for 72 h. WST-1 assay was performed to assess cell viability. Data are presented as mean $\pm$ SEM of three independent experiments. Cells treated with 0.1% DMSO were set to 1 and served as control cells. Statistical analysis was performed by two-way ANOVA and Bonferroni post-test ($p < 0.05$ vs. control cells). C: LipPD1 cells and primary human *PTEN* wild-type PP (two different lots) were incubated with 50 mM resveratrol for 48 h to analyze p70S6K phosphorylation levels. GAPDH served as loading control. Densitometric analysis data are shown above the bands. One typical blot out of two independent experiments is shown. PP: primary human *PTEN* wild-type preadipocytes; resv: resveratrol; rapa: rapamycin.

(11,12) and significant decrease of AKT phosphorylation (12,15,33,34) after treatment of LipPD1 cells with resveratrol, although similar resveratrol concentrations were used. However, other studies also described an activation of AKT after resveratrol treatment (34,35). Emphasizing that resveratrol did not significantly decrease the activation of AKT in LipPD1 cells, we focused on p70S6K activity as being most affected after incubation with resveratrol alone. Compared to incubation with rapamycin alone, a combined therapy potentiated the reduction of p70S6K activity and subsequently reduced phosphorylation of the S6K target ribosomal protein S6. Interestingly, 4E-BP-1 as another target of mTORC1 besides p70S6K was not significantly affected by resveratrol. This would point to a direct inhibition of p70S6K (18,19). Resveratrol was more effective on LipPD1 cells, since primary human *PTEN* wild-type preadipocytes were less affected at equimolar concentrations.

The described decrease in cell viability caused by resveratrol incubation alone could be explained by either induction of cell cycle arrest or apoptosis depending on the resveratrol concentration. Lower concentrations, especially 10 and 50 mM, resulted in a significant increase of G1-phase cell cycle arrest after 24 h, whereas a longer incubation period (72 h) with higher resveratrol concentrations (50, 100, and 200 mM) led to apoptosis induction. We found that 50 mM resveratrol affected cell cycle arrest after 24 h, but significantly induced apoptosis after 72 h. Interestingly, the significant difference in the reduction of LipPD1 cell viability after incubation with the rapamycin/resveratrol co-incubation could be explained by an additive effect of resveratrol on

cell cycle arrest, but not on apoptosis. The diminishing difference in cell viability between resveratrol incubation alone and rapamycin/resveratrol co-incubation might be due to the cytotoxic effects of resveratrol at higher concentrations (36,37). mTOR activity is regulated by the upstream complex tuberous sclerosis complex (TSC)1/TSC-2, which inhibits the mTOR activator RHEB and is inhibited through phosphorylation by AKT (38,39). Other signaling molecules that act upon TSC1/TSC2 to regulate mTOR activity are SIRT1 (7,40,41) and WNT (42). AMPK (43–45) and PRAK can suppress mTORC1 by acting upon mTORC1 itself and TSC2 or its upstream activator RHEB, respectively. Resveratrol as an activator of the AMPK/ SIRT1 axis had no influence on AMPK phosphorylation or SIRT1 protein levels in LipPD1 cells. In line with these results, global lysine acetylation as a measure for the activity of NAD-dependent deacetylases, like sirtuins, was not affected by resveratrol treatment. Overall, our results emphasize the importance of chemopreventive natural compounds, such as resveratrol, as potentiating agents in combination with mTOR inhibitors (47) to increase the efficacy of drugs applied as treatment option for patients with PHTS-associated lipomatosis.

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