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Topical Rapamycin Suppresses the Angiogenesis Pathways Induced by Pulsed Dye Laser: Molecular Mechanisms of Inhibition of Regeneration and Revascularization of Photocoagulated Cutaneous Blood Vessels

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Abstract

Background and Objectives—Pulsed dye laser (PDL) is the most effective treatment for port wine stain (PWS) birthmarks. However, regeneration and revascularization of photocoagulated blood vessels may result in poor therapeutic outcome. We have recently shown that rapamycin (RPM), an angiogenesis inhibitor, can reduce the regeneration and revascularization of photocoagulated blood vessels. Herein, we attempt to further elucidate the molecular pathophysiology on the inhibition of the regeneration and revascularization of photocoagulated blood vessels by topical RPM in an animal model.

Materials and Methods—Two separate skin areas on each hamster were irradiated by PDL. After PDL exposure, topical RPM was applied daily to one of the randomly selected test sites. PDL, PDL + RPM and normal skin test sites were biopsied on day 3 after PDL exposure. The total ribonucleic acid (RNA) and protein were extracted from biopsied skin samples and quantified. Real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunoblot were subsequently performed to quantify the mRNA and protein levels of hypoxia-inducible factor-1alpha (HIF-1α), vascular endothelial growth factor (VEGF) and ribosomal protein S6 kinase (S6). The phosphorylation levels of S6 and AKT were also evaluated by immunoblot.

Results—The mRNA and protein levels of HIF-1α, VEGF, and S6 significantly increased after PDL exposure as compared to the normal hamster skin. Topical application of 1% RPM suppressed the PDL-induced increase in mRNA and protein levels of those genes on day 3 post-PDL exposure. The phosphorylation levels of S6 and AKT increased after PDL exposure but the increases were suppressed by the topical application of RPM.

Conclusion—The increase in expression of HIF-1α, VEGF, and S6 after PDL-exposure suggests that angiogenesis pathways play very active roles in the process of skin blood vessel

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regeneration and revascularization. Topical application of 1% RPM can suppress the angiogenesis pathways and, therefore, reduce the regeneration and revascularization of photocoagulated blood vessels.

Keywords

pulsed dye laser; port wine stain; rapamycin; angiogenesis; HIF-1 α ; VEGF

INTRODUCTION

Port wine stain (PWS) is a congenital, progressive vascular malformation of human skin involving post-capillary venules and occurs in an estimated 3–5 children per 1,000 live births [1–3]. Approximately 1,500,000 individuals in the United States and 32 million people worldwide have PWS birthmarks [4–6]. Since most malformations occur on the face, PWS is a clinically significant problem in the majority of patients. Personality development is adversely influenced in virtually all patients by the negative reaction of others to a “marked” person. Detailed studies have documented lower self-esteem in such patients and problems with interpersonal relationships [7–9]. Studies have indicated a high level of psychological morbidity in PWS patients resulting from feelings of stigmatization that are frequently concealed in casual social interactions.

In childhood, PWS are flat red macules, but lesions tend to darken progressively to purple and, by middle age, often become raised as a result of the development of vascular nodules [10,11]. The pulsed dye laser (PDL) is the current treatment of choice for PWS [12–18]. Yellow light (585–595 nm) emitted by the PDL is preferentially absorbed by hemoglobin, an endogenous chromophore in blood. PDL exposure induces blood vessel wall necrosis after incoming photon energy is converted to heat [19–21]. However, the degree of PWS blanching achieved following laser therapy can be variable and unpredictable with an average treatment success rate below 10%, if the ultimate standard required is complete blanching of the lesion, due to the recurrence of the blood vessels [22–24].

The regeneration and revascularization of blood vessels post-PDL treatment is a critical barrier to an adequate PWS therapeutic outcome. Our recent data suggest that activation of angiogenesis pathways induced by PDL in PWS contributes to this process [25] but the mechanism(s) remain incompletely understood. Although the complexity of angiogenesis pathways have been studied in normal and tumor vasculature, PDL-induced angiogenesis in PWS skin has not been fully investigated.

PDL treatment of PWS causes intense, acute damage to blood vessels [25]. The skin’s normal wound healing response detects hypoxia and initiates appropriate defense mechanisms, such as angiogenesis. The PDL-induced local hypoxia leads to upregulation of hypoxia-inducible factor-1 α (HIF-1 α), a master modulator for hypoxic response [26,27]. During hypoxia, HIF-1 α is stabilized and translocated which results in the transcription of numerous angiogenic genes, including vascular endothelial growth factor (VEGF) [26,27]. VEGF is the predominant growth factor that regulates angiogenesis pathways by signaling via VEGF receptor-2 (VEGFR-2) [28]. Activation of VEGFR-2 will lead to activation of many downstream pathways, including the phosphatidylinositol 3-kinases (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling [26,29–31]. mTOR is a serine-threonine protein kinase that can phosphorylate the 4E-binding protein 1 (4E-BP1) [32] and ribosomal protein S6 kinase (S6) [33,34] which then mediate efficient cap-dependent translation initiation and finally result in regeneration and revascularization of PWS blood vessels.

In this study we hypothesize that PDL combined with administration of angiogenesis pathways' inhibitors, such as RPM, can improve PWS lesion blanching and thus lead to a better therapeutic outcome as compared to PDL treatment alone. RPM is a FDA-approved anti-angiogenic agent with a relatively low side effect profile [35]. RPM can inhibit mTOR activity by forming a complex with FK-binding protein 12 (FKBP12) and then binding to mTOR directly [36–39]. RPM has been used: (1) for immunosuppression in renal transplantation subjects [40]; (2) as anticancer therapy due to inhibition of tumor cell survival and angiogenesis [36,41,42]; and (3) for the treatment of hypervascular anomalies including angiomyolipomas [43–46] and many skin diseases, including Kaposi's sarcoma [47–49], psoriasis [50], and angiofibromas [51]. In previous animal model studies, we demonstrated that regeneration and revascularization of blood vessels after PDL was dramatically reduced when the skin was subsequent treated with daily topical RPM application for 14 days as compared to PDL alone [25]. RPM administration abolished the upregulation of certain "stem cell" antigens, such as nestin, thereby interrupting the vascular repair process induced by PDL exposure [52]. Furthermore, we reported a case study from one PWS patient with an extensive lesion involving the left anterior chest and upper extremity. Our results showed that test sites treated with the combined PDL and oral RPM displayed an enhanced blanching response as compared to PDL alone [53]. These previous studies have demonstrated the feasibility and potential of a new therapeutic strategy for PWS. Herein, we attempt to further elucidate the molecular mechanism(s) of PDL combined with topical RPM to block the regeneration and revascularization of photocoagulated blood vessels after laser exposure.

MATERIALS AND METHODS

Animals

All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee, University of California, Irvine. Adult male Golden Syrian hamsters with an initial bodyweight of 90–120 g were used.

Laser Irradiation

Laser exposure was performed on the abdominal side of the hamsters. Skin was irradiated with a 585 nm PDL (Candela, Wayland, MA), pulse duration was 0.45 mseconds, energy density was 6 J/cm² delivered on a 10 mm spot diameter. Each animal had a pair of treatments in two designated areas (1.5 cm × 2 cm) side by side on the skin: PDL + vehicle and PDL + RPM. The vehicle contained the exact same ointment as the topical RPM formulation, but without RPM. The two areas were separated by at least 4 cm. Topical RPM was applied daily for 3 days post-PDL-exposure. The animals were euthanized on day 3 and four biopsy samples (4 mm diameter) were taken from each treated area. Biopsy samples collected from adjacent areas not exposed to PDL or combined PDL + RPM were designated as control.

Topical RPM

The topical RPM formulation used in this study contained 1% (w/w) RPM which was dissolved in benzyl alcohol and thoroughly mixed with a medical ointment and a skin penetration enhancer (Conrex Pharmaceutical, Newtown Square, PA). The mixture was stored at 4°C until use. Solvent, skin penetration enhancer, and ointment mixture were used as vehicle controls. Immediately after post-laser exposure on day 0, topical RPM was applied onto the treatment area and covered with Tegaderm (3M, St. Paul, MN). Every 24 hours after laser irradiation, the skin was cleaned gently with sterile swabs and water to remove any residual RPM and then the RPM ointment and Tegaderm were re-applied. The same procedure was followed for the application of vehicle controls.

RNA Extraction and Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from skin biopsy samples using the RNeasy Mini kit (Qiagen, Carlsbad, CA) according to the manufacturer's manual. To generate cDNA, 1.0 µg of total RNA was reverse-transcribed in a 20-µl reaction containing 1× RT buffer (Clontech, Mountain View, CA), 0.5 mM dNTPs, 0.5 µg of oligo (dT) 15-mer primer, 20 units of RNasin, and 5 units of SMART Moloney murine leukemia virus reverse transcriptase (Clontech). The RT reaction was carried out at 42°C for 2 hours. 0.5 µl of each sample (25 ng) was used directly for real-time PCR analysis which was performed using the LightCycler System (Roche, Pleasanton, CA). Table 1 listed the sequences and locations of the primers and the resulting amplicon sizes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA or the input amount of the total RNA into each reaction was used for normalization. The reaction for the multiplex real-time PCRs contained 1× SYBR Green qPCR Master Mix (Clontech), 25 ng of each template, and 50 nM of each specific primer in a 20-µl total volume. Each reaction was performed in triplicate under identical conditions. The PCR conditions were one cycle at 95°C for 2 min followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 40 seconds at 72°C. Relative quantification of the real-time PCR was based upon the amplification efficiency of the target and reference genes and the cycle number at which fluorescence crossed a prescribed background level, cycle threshold (Ct). A paired *t*-test was used to compare the expression significance of target genes between control and treatment groups. The results were presented as mean ± SD.

Protein Extraction and Immunoblot

After the previously described exposure protocols, the biopsy samples were completely homogenized in lysis buffer (5.1 M guanidinium thiocyanate, 50 mM sodium citrate, 50 mM EDTA, 0.5% β-mercaptoethanol). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose, and the membranes were probed with the indicated primary antibodies. Immunoreactive bands were visualized using a horseradish peroxidase-conjugated secondary antibody and the Amersham Biosciences ECL reagents as described by the manufacturer. Antibodies against VEGF (SC-507), HIF-1α (SC-10790), β-actin (SC-81178), phospho-S6 (SC-8416), S6 (SC-8418), AKT (SC-8312), and phospho-AKT (SC-7985-R) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Relative quantification of protein levels were compared with the level of β-actin in each sample based on densitometry of each band. A paired *t*-test was used to compare the expression significance of target proteins between control and treatment groups. The results were presented as mean ± SD.

RESULTS

Expression of HIF-1α was Suppressed by Topical Application of RPM

The mRNA level of HIF-1α was investigated using real-time RT-PCR. Typical real-time RT-PCR amplification curves of GAPDH and HIF-1α using SYBR green dye were shown in Figure 1A. The HIF-1α mRNA level in hamsters (*n* = 3) biopsied at day 3 post-PDL exposure significantly increased in the PDL (*P* = 0.0277) and PDL + RPM groups (*P* = 0.0370; Fig. 1B and Table 2) as compared to control. There was a statistically significant difference in the HIF-1α mRNA levels between the PDL and the PDL + RPM groups (*P* = 0.0487; Fig. 1B and Table 2).

The HIF-1α protein level of hamsters (*n* = 3) biopsied at day 3 post-PDL exposure significantly increased by a factor of 2.67 in the PDL group as compared to the control group (*P* = 0.0008; Fig. 2 and Table 2). Application of topical 1% RPM significantly suppressed PDL-induced HIF-1α protein levels (*P* = 0.0351; Fig. 2 and Table 2).

Topical RPM Suppressed PDL-Induced Expression of VEGF

The mRNA level of VEGF in hamsters ($n = 3$) biopsied at day 3 post-PDL exposure showed a significant increase in both the PDL and PDL + RPM groups as compared to control ($P = 0.0426$ and 0.0069 , respectively; Fig. 1B and Table 2). RPM decreased the PDL-induced VEGF mRNA level, but the reduction was not statistically significant ($P = 0.1903$; Fig. 1B and Table 2).

The VEGF protein levels in hamsters ($n = 4$) biopsied at day 3 PDL-laser exposure showed a significant increase in the PDL group as compared to control ($P = 0.0203$; Fig. 2 and Table 2). Topical RPM significantly decreased the PDL-induced VEGF protein level ($P = 0.0027$; Fig. 2 and Table 2).

PDL-Induced S6 Expression was Attenuated by Topical Application of RPM

The mRNA level of S6 in hamsters ($n = 3$) biopsied at day 3 post-PDL exposure showed a statistically significant increase in the PDL group ($P = 0.0255$) as compared to control (Fig. 1B and Table 2). There was a significant difference in the S6 mRNA levels between the PDL group and the PDL + RPM group ($P = 0.0193$; Fig. 1B and Table 2).

The protein levels of S6 in the hamsters ($n = 3$) biopsied at day 3 post-PDL exposure showed a significant increase in the PDL group ($P = 0.0275$) as compared to control (Fig. 2 and Table 2). There was a statistically significant decrease of the S6 protein level induced by PDL + RPM when compared to the PDL group alone ($P = 0.0369$; Fig. 2 and Table 2).

Topical RPM Suppressed Phosphorylation Levels of S6 and AKT Induced by PDL

The phosphorylation levels of both S6 (Ser411) and AKT (Ser473) showed a significant increase as compared to control ($P = 0.0153$ and 0.0204 , respectively; Fig. 2 and Table 2). Topical application of 1% RPM significantly suppressed the PDL-induced phosphorylation levels of both S6 (Ser411) and AKT (Ser473; $P = 0.0369$ and 0.0260 , respectively; Fig. 2 and Table 2).

DISCUSSION

In this study, we have determined that topical application of RPM can significantly suppress PDL-induced expression of HIF-1 α , VEGF, and S6. We hypothesize that these three molecules play critical roles in the pathophysiological process of regeneration and revascularization of PWS blood vessels after PDL exposure. Thus, inhibition of VEGF-activated mTOR signaling by its specific inhibitor, RPM [36,41], may potentially lead to better PWS therapeutic outcomes. Inhibition of mTOR by RPM results in suppression of the translation initiation process and, ultimately, inhibition of new blood vessel formation [54]. In this study, we demonstrate that topical RPM can suppress PDL-induced angiogenesis pathways via inhibition of the expression of HIF-1 α , VEGF, and S6 (Fig. 3).

Our study has shown that RPM can suppress the PDL-induced increase in HIF-1 α expression. HIF-1 α is known to control the expression of hundreds of genes involved in angiogenesis, inflammation, bioenergetics, proliferation, motility, and apoptosis [55,56]. As the key molecule acting as a function of oxygen concentration, HIF-1 α is regulated at multiple levels in response to hypoxia. First, HIF-1 α mRNA expression increases under hypoxia or ischemia. Many studies have shown that HIF-1 α mRNA increases in response to hypoxia in rodents [57–60] and humans [48,61]. These results are consistent with our findings. Furthermore, the stabilization of HIF-1 α mRNA may also contribute to the sustained increase of its mRNA [57]. Second, HIF-1 α protein is synthesized and accumulated as a result of hypoxia stimulation. The hypoxia activated mTOR signaling

pathway plays a very important role in stimulation of the synthesis of HIF-1 α protein and its transcriptional activities [56,62]. HIF-1 α has been shown as the downstream target of mTOR with a mTOR signaling motif located at its N terminus which can interact with the regulatory associated protein of mTOR (Raptor) [62]. This pathway affects the translational levels of HIF-1 α and serves as amplifiers for maximal expression of HIF-1 α rather than the essential triggers for its activation [62]. We also have found that protein levels of HIF-1 α , VEGF, and VEGFR-2 [63] increase in PDL-exposed hamster biopsy tissues as compared to control. In our hamster model, the blood vessels are destroyed by PDL. The hypoxia induced by laser exposure is very severe and persistent for days until the vessels are fully regenerated. The increase of HIF-1 α protein levels after PDL treatment may be the result from both the increase in its mRNA level and translation rate. We also suspect the increase in HIF-1 α protein and mRNA may be the consequences from not only activation of angiogenesis pathways, but also other intermingled biological processes, such as wound healing, defense, and inflammatory responses. Consistent with our findings, RPM has been demonstrated to downregulate hypoxia-induced HIF-1 α protein and mRNA levels by many other studies [62,64–67]. Inhibition of mTOR signaling by RPM can directly suppress the translation process of HIF-1 α and its transcriptional activities [62,68]. The mechanism underlying inhibition of HIF-1 α mRNA level by RPM remains incompletely understood but may be an indirect result of inactivation of the mTOR pathway.

VEGF is the growth factor that plays a predominant role in angiogenesis pathways. VEGF can activate VEGFR-2 which can render the full range of VEGF responses in endothelial cells, such as endothelial proliferation, migration and formation of vascular tubulin [28,69]. VEGF and HIF-1 α can be upregulated reciprocally through angiogenesis pathways. VEGF is one of the downstream targets of HIF-1 α . Hypoxia-activated-HIF-1 α can translocate into the nucleus and directly bind to the hypoxia response element of the VEGF promoter and activate its transcription, thus leading to an increase in VEGF mRNA levels [62,65,70,71]. Alternatively, VEGF can increase HIF-1 α mRNA translation into protein via PI3K/AKT signaling [70,72]. Many studies have shown that application of RPM can effectively suppress hypoxia-induced expression of VEGF in animal models and cancer patients [36,64,65,67,73]. One mechanism is that inhibition of mTOR by RPM downregulates HIF-1 α levels and thus suppresses its transcriptional activity to the VEGF promoter. However, the expression of VEGF can be also be regulated through HIF-1 α independent pathways. For example, in primary mesothelial cell culture, RPM has no effect on TGF β -induced VEGF but suppresses hypoxia-induced VEGF [65]. In colon cancer, hypoxia-induced VEGF does not require HIF-1 α but, rather, depends on activation of the PI3K/Rho/ROCK pathway and c-Myc [74]. In our study, topical RPM does not show a complete blockage of hypoxia-induced VEGF for two possible reasons: (1) Topical RPM at the site of action does not achieve a concentration high enough to completely block all mTOR signaling pathways. Thus, increasing the concentration of topical RPM may be one option to improve PWS outcomes; and (2) VEGF is also induced by HIF-1 α independent pathways which cannot be completely blocked by topical RPM. In this case, RPM in combination with other drugs to suppress HIF-1 α independent pathways should be considered for PWS treatment post-PDL exposure.

In this study, we chose RPM to inhibit regeneration and revascularization of blood vessels post-PDL. Indeed, the current study and our previous findings have showed that topical application of RPM can inhibit angiogenesis pathways in animals. RPM can inhibit the activities of kinases such as S6 and AKT [75,76]. In our animal model, RPM indeed suppresses PDL-induced both phosphorylation levels of S6 and AKT which further confirms the RPM-mediated inhibitory effects on angiogenesis. We did not observe any obvious acute and long-term side effects resulting from topical RPM application in the animals. Thus, topical RPM may be a safe anti-angiogenic agent with potential efficacy which can be used

as a novel therapeutic strategy for PWS patients. One potential critical barrier for topical RPM is whether or not the drug can penetrate the stratum corneum in concentrations sufficiently high enough to prevent blood vessel regeneration and revascularization. Based on the expression profiles of some key angiogenic molecules evaluated in our study, we can cautiously conclude that topical RPM does effectively penetrate into hamster skin and induce an inhibitory effect on angiogenesis pathways. However, to date the intradermal concentration of RPM after topical application has not been determined. Therefore, determination of the intradermal concentration of RPM after topical application at the drug action sites in animals and human PWS subjects will be the focus of a future study. Another future study will investigate the expression profiles of these genes during a time course post-PDL with continuous RPM application. We hope to be able to determine the critical window period of RPM application and eliminate possible bounce back expressions of these genes after topical RPM cessation. In one of our clinical reports, we showed that RPM taken by a patient orally and daily for 1 week before PDL and 4 weeks post-PDL resulted in a long-term enhanced blanching response in comparison to PDL treatment alone [53]. This result suggests that proper dosage and duration of RPM can maintain a long-term suppression of regrowth of PWS blood vessels. Collectively, the topical formulation of RPM developed in this study is effective in the hamster model and can be used as a prototype that can be enhanced and adapted for human use in PWS patients.

In conclusion, we have shown that the expression of three key angiogenic molecules, HIF-1 α , VEGF, and S6, increased significantly after PDL-exposure. Topical application of RPM can suppress PDL-induced expression of HIF-1 α , VEGF, and S6, and the phosphorylation levels of S6 and AKT in our animal model. Our results have provided essential information about the molecular basis of a potentially novel therapeutic strategy for the clinical management of PWS patients using combined PDL + RPM.

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Fig. 1. Topical application of RPM significantly suppressed PDL-induced mRNA levels of HIF-1 α , VEGF, and S6 in hamsters. **A:** Real-time PCR amplification curves of HIF-1 α and GAPDH with the RNA prepared from the hamster biopsy samples that received 1% topical RPM for 3 days post-laser exposure. **B:** mRNA levels of HIF-1 α , VEGF, and S6 in hamsters biopsied at day 3 post-laser exposure. The data were presented as “mean $\pm$ SD” and showed the fold changes of mRNA levels of target genes to the controls. *P* values were from a paired *t*-test.

Fig. 2. Topical application of RPM significantly suppressed PDL-induced protein levels of HIF-1 α , VEGF and S6, and phosphorylation levels of S6 (Ser411) and AKT (Ser473). Biopsy samples were collected from hamster skin after 3 days post-laser exposure. Tissues were completely homogenized and total lysates were prepared. The protein levels of HIF-1 α , VEGF and S6, and phosphorylation levels of S6 (Ser411) and AKT (Ser473) were determined by immunoblot analysis following SDS-PAGE.

Fig. 3. Schematic diagram of RPM mediated-inhibition of PDL-induced angiogenesis pathways. The PDL-induced local hypoxia leads to upregulation of HIF-1 α , which results in an increase in the transcription of numerous angiogenic genes, including VEGF. Secretion of VEGF will activate VEGFR-2 in the adjacent cells and lead to activation of many pathways, including the PI3K/AKT/mTOR signaling, which ultimately results in regeneration and revascularization of blood vessels. RPM can form a complex with FKBP12 and then bind to mTOR directly to block its activity, resulting in suppression of the phosphorylation of S6 kinase and leading to inhibition of angiogenesis pathways.

TABLE 1

Primers for HIF-1 α , VEGF-A, S6, and GAPDH

Gene name	5' primer	3' primer	Location	Genbank	Amplicon size (bp)
HIF 1 α	5'TGAGTTTCTGAACGTCGAAAAAGAAAAG 3'	5'TTCTCTCAATTTCTCTCATGGTCAC 3'	Exon 2-4	gij226061947	200
VEGFA	5'ATCATGCGGATCAAAACCTCACC 3'	5'TGTTCTGTCTTTCTTTGGTCACAT 3'	Exon 4-5	NM_001025257	95
S6 alpha	5'CTTGGCATGGAAACATTGTGAG 3'	5'TCCCTCTCTTTCTAACTGCAT 3'	Exon 2-6	NM_001114334	364
GAPDH	5'ATGGTGGAAGGTCGGTGTGAAC 3'	5'GCCTTCTCCATGGTGTGAAG 3'	Exon 2-3	NM_008084	316

TABLE 2

Topical Application of RPM Significantly Inhibited PDL-Induced mRNA and Protein Levels of HIF-1 α , VEGF, and S6 in Hamsters

Molecules	Type	Ctl	PDL	PDL + RPM	P*	P#
HIF-1 α	mRNA	1	4.02 $\pm$ 1.28	1.82 $\pm$ 0.41	0.0277	0.0487
	Protein	1	2.67 $\pm$ 0.31	1.22 $\pm$ 0.58	0.0008	0.0351
VEGF	mRNA	1	3.02 $\pm$ 1.98	1.84 $\pm$ 0.45	0.0426	0.1903
	Protein	1	3.16 $\pm$ 1.38	1.19 $\pm$ 0.52	0.0203	0.0027
S6	mRNA	1	2.67 $\pm$ 0.68	1.20 $\pm$ 0.25	0.0255	0.0193
	Protein	1	1.57 $\pm$ 0.29	1.05 $\pm$ 0.34	0.0275	0.0369
Phos-S6	Ser411	1	1.34 $\pm$ 0.25	0.98 $\pm$ 0.36	0.0153	0.0260
Phos-Akt	Ser473	1	1.95 $\pm$ 0.19	1.13 $\pm$ 0.01	0.0204	0.0272

The data were presented as "mean $\pm$ SD" and showed the fold changes of mRNA, protein, and phosphorylation levels of each molecule as compared to the controls (Ctl).

* P-value for the PDL group as compared to the control group (paired *t*-test).

P-value for the PDL group as comparison to the PDL + RPM group (paired *t*-test).