Rapamycin inhibits poly(ADP-ribosyl)ation in intact cells

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A B S T R A C T

Rapamycin is an immunosuppressive drug, which inhibits the mammalian target of rapamycin (mTOR) kinase activity inducing changes in cell proliferation. Synthesis of poly(ADP-ribose) (PAR) is an immediate cellular response to genotoxic stress catalyzed mostly by poly(ADP-ribose) polymerase 1 (PARP-1), which is also controlled by signaling pathways. Therefore, we investigated whether rapamycin affects PAR production. Strikingly, rapamycin inhibited PAR synthesis in living fibroblasts in a dose-dependent manner as monitored by immunofluorescence. PARP-1 activity was then assayed in vitro, revealing that down-regulation of cellular PAR production by rapamycin was apparently not due to competitive PARP-1 inhibition. Further studies showed that rapamycin did not influence the cellular NAD pool and the activation of PARP-1 in extracts of pretreated fibroblasts. Collectively, our data suggest that inhibition of cellular PAR synthesis by rapamycin is mediated by formation of a detergent-sensitive complex in living cells, and that rapamycin may have a potential as therapeutic PARP inhibitor.

Introduction

The antibiotic rapamycin is currently used as an immunosuppressive drug to prevent graft rejection after organ transplantation. Since immunosuppressive therapy increases the posttransplantation malignancy risk, rapamycin, as a new-generation immunosuppressant compound, attracts attention due to its apparently lower incidence of de novo cancer [1,2]. Furthermore, there is an increasing interest in rapamycin as an anti-cancer drug because it exerts inhibitory effects on tumor growth, proliferation, and angiogenesis of various types of cancer [3,4]. Rapamycin forms a complex with the 12-kDa FK506 binding protein (FKBP12), which binds to mammalian target of rapamycin (mTOR) kinase, a member of the phosphoinositide-3-OH-kinase (PI3-kinase) family, and modulates its activity [5–7]. mTOR is a central kinase, which controls many cellular processes such as cell growth, cell proliferation, protein synthesis, and gene expression [8,9]. However, the precise mechanism whereby rapamycin exerts its activity poorly understood.

Protein-conjugated poly(ADP-ribose) (PAR) is a nuclear biopolymer synthesized by poly(ADP-ribose) polymerase 1 (PARP-1) using NAD+ as substrate [10,11]. PAR produced by PARP-1 facilitates base excision repair, regulates genomic stability following oxidative damage, controls transcription, and acts as a survival factor for proliferating cells under genotoxic stress [11–14]. PAR polymer also interacts non-covalently with proteins involved in DNA damage and repair with high affinity [15]. Beside its cytoprotective physiological functions, PARP-1 can mediate pathophysiological effects through PAR overproduction, which may result in depletion of cellular NAD+ and ATP pools or trigger the release of AIF leading to cell death [16–18]. Therefore, PARP-1 inhibitory compounds may have a therapeutic potential in diseases where PARP-1 overactivation mediates pathological effects, such as ischemia–reperfusion damage in brain, heart, kidney or bowel; hemorrhagic and septic shock; type I diabetes; Parkinson disease; and multiple organ failure. Furthermore PARP-1 inhibition may be useful as a co-treatment in the context of cytotoxic tumor therapy [16,19,20] and as a selective, single-agent anti-tumor strategy in BRCA-2-deficient tumors [19]. In addition to the regulation of PARP activity by inhibitors, there are other mechanisms through which activation of the enzyme is controlled such as signal transduction [21–23]. Therefore, rapamycin as an mTOR inhibitor may affect PARP-1 activation.

In this paper we demonstrate a new effect of rapamycin in intact human fibroblasts, i.e. inhibition of poly(ADP-ribose)ylation. We show that rapamycin does not act as a competitive PARP-1 inhibitor, either by preventing PARP-1 from binding to DNA ends or by interfering with PARP-1 automodification. In addition, rapamycin did not affect cellular NAD levels or influence the activation of PARP-1 in cell lysates obtained from rapamycin-pretreated fibroblasts. Our results suggest that besides the potential involvement of cellular signal transduction, rapamycin-dependent inhibition of poly(ADP-ribose)ylation in living cells may be mediated by a hitherto unknown formation of a detergent-sensitive complex.

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Materials and methods

Materials. 3-Aminobenzamide, Trizma base, dithiothreitol (DTT), NAD+, histone type IIa, and rapamycin were from Sigma. Sodium pyrophosphate and MgCl₂ were from Merck. Trichloroacetic acid (TCA) was from Roth. The octameric palindromic oligonucleotide GGAATTCC was purchased from Invitrogen. Mouse monoclonal antibody 10H directed against poly(ADP-ribose) was immuno-purified on a protein A column (Sigma). Recombinant PARP-1 was overexpressed and purified as described [15].

Cell culture and treatments. Neonatal human dermal fibroblasts (NHDF, Bioproducts Boehringer Ingelheim, Germany) in passage 6–8 were cultured to confluence in Dulbecco’s modified Eagle’s medium (DMEM, 1 g/L glucose; GibcoBRL) supplemented with 580 mg/ml L-glutamine, 10 mM HEPES, penicillin G (100 U/ml, GibcoBRL), streptomycin (100 μg/ml, GibcoBRL), and 10% fetal calf serum (Sigma), at 37 °C.

For immunohistochemical analyses of PAR, cells were plated on sterile coverslips at a density of 2–3 × 10⁴/cm² and incubated. A stock solution of rapamycin (1 mg/ml) was prepared with 99.9% ethanol (EtOH), stored at –80 °C and diluted to the appropriate concentrations in culture medium prior to use. Cells were treated with 1–10 ng/ml rapamycin and control cells were exposed to solvent (0.001% EtOH) in serum-free DMEM for 10 min at 37 °C.

Immunohistochemical analysis of PAR formation. The content of PAR in intact cells was assessed by immunofluorescence analysis. Following the experimental treatments, cells were washed with PBS and treated with 1 mM of hydrogen peroxide in PBS for 10 min at 37 °C. Subsequently, cells were fixed and PAR was detected by staining with monoclonal antibody 10H in conjunction with a FITC-coupled secondary antibody (Goat-anti-mouse, Sigma, Germany). The fluorescence of the PAR-antibody conjugate was detected using 10H primary antibody and a secondary peroxidase-conjugated anti-mouse IgG (DakoCytomation). Bands were visualized in the FujiLAS1000 device using enhanced chemiluminescence and signal intensities were quantified using AIDA software (Raytest).

NAD⁺ cycling assay. Cellular NAD⁺ levels were quantified using an enzymatic cycling assay as described [24].

In vitro PARP activity assay. Poly(ADP-ribose) formation was measured in vitro using a non-radioactive immuno-dot blot technique. The reaction buffer contained 100 mM Tris–HCl pH 7.8, 10 mM MgCl₂, 1 mM DTT, 200 μM NAD⁺, 400 μg/ml histone Type IIa, and 50 μg/ml of the “activator” oligonucleotide. The reaction was started by adding 100 ng PARP-1 (8.9 nM), incubated at 37 °C for 10 min and terminated by adding 3-aminobenzamide (5 mM) in PBS on ice. For NAD⁺ competition experiments a serial dilution of rapamycin (1 mg/ml in EtOH) was prepared in PBS and the reaction mixture was supplemented with the indicated amounts of rapamycin prior to PARP-1 addition. To investigate the possibility of competition against activator oligonucleotide, PARP-1 activity was monitored with increasing amounts of the oligonucleotide ranging from 0 to 5 μg/100 μl reaction mixture at different rapamycin concentrations. To evaluate the possible influence of rapamycin on PARP-1 automodification histones were omitted and the enzyme concentration was raised to 89 nM for improved detection. Control experiments were performed with serial dilutions of the solvent (EtOH).

Subsequently, samples were vacuum aspirated on a positively charged nylon membrane (Hybond N+, Amersham) followed by a crosslinking step at 90 °C for 1 h. The membrane was then blocked with 3% (w/v) skim milk powder in TBS-T and bound PAR was detected using 10H primary antibody and a secondary peroxidase-conjugated anti-mouse IgG (DakoCytomation). Bands were visualized in the FujiLAS1000 device using enhanced chemiluminescence and signal intensities were quantified using AIDA software (Raytest).

PARP activity blot. To analyze PARP activation in cell extracts of NHDF pretreated with rapamycin or not, an activity blot technique was used. Briefly, cells were preincubated with 10 ng/ml rapamycin for 10 min or left untreated. In some experiments, cells were additionally exposed to 1 mM H₂O₂ for 10 min at 37 °C. To address possible effects of rapamycin on PARP phosphorylation, cells were treated for 30 min with 1 μM of the selective protein kinase C inhibitor Gö 6976 (Calbiochem, UK) prior to rapamycin incubation. Cells were then harvested, counted and resuspended in two volumes of extraction buffer containing 50 mM glucose, 25 mM Tris–HCl, 10 mM EDTA, and 1 mM PMSF. After addition of one volume SDS
loading buffer, the samples were heated at 65°C for 15 min and mechanically sheared. Proteins were separated by 10% SDS–PAGE and subsequently incubated in transfer buffer supplemented with 0.7 M 2-mercaptoethanol before being blotted. To restore protein folding and activity, the transferred proteins were incubated for 1 h in equilibration buffer (50 μg/ml "activator" oligonucleotide, 20 μM zinc acetate and 2 mM MgCl₂) followed by another incubation step in reaction buffer (equilibration buffer + 200 μM NAD⁺). After a final renaturation step in buffer containing 50 mM Tris–HCl pH 8, 100 mM NaCl, 0.3% Tween 20, and 1 mM DTT, PAR was detected with the specific antibody LP96–10 (BD Bioscience) as described above. Blots were stripped and reprobed for PARP-1 to normalize for cellular PARP protein levels.

Statistical analysis. Statistical analysis was performed with One Way Analysis of Variance using the statistical program SigmaStat, (Version 2.03, SPSS Science, Chicago). All values are expressed as means and standard deviations (SD).

Results

Incubation of normal human fibroblasts with rapamycin at 10 ng/ml for 10 min before challenging the cells with hydrogen peroxide, a well-known inducer of PARP-1 activity, inhibited nuclear poly(ADP-ribosyl)ation significantly compared to the untreated culture, as demonstrated by immunofluorescence analysis. No immunostaining was observed in the nuclei of cells without hydrogen peroxide treatment regardless of rapamycin co-treatment (Fig. 1A). Inhibition of PAR synthesis by rapamycin occurred in a dose-dependent manner, with maximal inhibition reaching 70% at 10 ng/ml rapamycin applied 10 min before hydrogen peroxide (Fig. 1B).

The very short time period of rapamycin exposure that was sufficient to inhibit cellular PAR formation suggested that rapamycin might act as a direct PARP-1 inhibitor. Therefore, we analyzed the enzyme activity of purified human PARP-1 in vitro in the presence or absence of rapamycin. The assay was performed in the presence of histones as known target proteins for poly(ADP-ribosyl)ation. The results showed that recombinant PARP-1 was not inhibited by rapamycin at concentrations of 10 ng/ml or higher (Fig. 2A and B). By contrast, 3-aminobenzamide (3-AB; a commonly used NAD⁺ analog and competitive inhibitor of PARP formation) repressed PAR synthesis significantly at a concentration of >30 μM (Fig. 2C). The vehicle ethanol exerted no influence on PARP-1 activity.
ence with auto-poly(ADP-ribosyl)ation of PARP-1 or inhibition of PARP-1 binding to DNA. To study if rapamycin interferes with PARP-1 automodification, the PARP-1 activity assay was carried out in the absence of histones and revealed no influence on PARP-1 automodification, even at a concentration of 10 μg/ml (Fig. 3A). An additional possibility is that inhibition of the DNA-binding activity of PARP-1 by rapamycin may prevent PARP-1 activation. Varying the concentration of the octamer activator oligonucleotide [25] allowed testing the hypothesis that rapamycin might compete with the octamer for the DNA-binding domain of PARP-1, thereby blocking PARP activation. However, rapamycin displayed no inhibitory effect on PAR synthesis at lower octamer concentrations (Fig. 3B).

To gain more information on the effect mediated by rapamycin, we investigated if rapamycin down-regulates PAR production by modulating cellular NAD⁺ content, as NAD⁺ is the PARP-1 substrate. Therefore we measured the NAD⁺ content in fibroblasts after 10 min rapamycin treatment with concentrations up to 20 ng/ml. As shown in Fig. 4A, the cellular NAD⁺ content was not significantly affected by rapamycin and revealed similar NAD⁺ concentrations in the range of 322–410 pmol/10⁶ cells for untreated and rapamycin-treated cultures. Finally, we addressed the question if rapamycin modulates PARP-1 activity by acting on signal transduction pathways, i.e. by stimulating PKC-mediated phosphorylation of PARP-1, which was described to inhibit poly(ADP-ribosyl)ation [23]. To this end, fibroblasts pretreated with rapamycin or not were harvested and PARP-1 activity was measured in whole cell lysates using an activity blot technique, but displayed no significant difference compared to untreated control cells (Fig. 4B and C). Likewise, co-incubation of cells with rapamycin and Gö 6976, a selective inhibitor of PKC, revealed no effects on poly(ADP-ribosylation) capacity.

Discussion

Our results demonstrate for the first time a fast and dose-dependent down-regulation of PAR synthesis in intact human fibroblasts exposed to rapamycin at low concentrations. This effect could not be explained by a putative acute depletion of the cellular NAD⁺ pools, as attested by our measurements of cellular NAD⁺ levels. Furthermore, although the chemical structure of rapamycin does not resemble that of conventional PARP-1 inhibitors [26], we hypothesized that rapamycin might act as a PARP-1 inhibitor, in view of the low concentration of rapamycin and the short time period of exposure (10 min) needed for inhibition of PAR formation in intact cells. In principle, rapamycin could modulate activation of PARP-1 by acting as a competitive substrate inhibitor; by preventing binding of PARP-1 to DNA breaks; or by interfering PARP-1 automodification [27]. To investigate how rapamycin causes PARP-1 inhibition, we used an in vitro PARP-1 activity assay. Our data revealed that rapamycin does not act as a competitive PARP-1 inhibitor by blocking the catalytic domain like 3-AB or other benzamide compounds [26]. Furthermore, we used a double-stranded octameric oligonucleotide for stimulating PARP-1 activity [25]. Inhibition of PARP-1 binding to DNA by rapamycin may be an alternative mechanism to prevent PARP-1 activation. However, PARP-1 activity was not affected by rapamycin at any concentration of oligonucleotide tested. From these results we conclude that rapamycin does not inhibit the binding of DNA to PARP-1. In addition, this in vitro assay permits the examination of PARP-1 automodification reaction by omitting other acceptor proteins like histones. Our data reveals that rapamycin likewise has no influence on the automodification of PARP-1 in vitro. So far, our results revealed that rapamycin down-regulates PAR synthesis by some novel mechanism that is distinct from the common pathways that inhibit PARP-1 interactions.

In addition to the above described regulation of PARP-1 activity, activation of the enzyme can also be controlled by phosphorylation-mediated signal transduction [27]. The action of rapamycin is mediated by two cellular proteins. First, rapamycin exerts its effect by binding with high affinity to FKBP12 [5] and second, the rapamycin-FKBP12 complex binds to mTOR and modulates its kinase activity [6,28]. FKBP12, which is inhibited by immunosuppressant ligands, is an immunophilin exhibiting prolyl isomerase activity implicated in associated protein folding [5,29]. FKBP12 stabilizes the membrane associated inositol 1,4,5-triphosphate receptor (IP₃R), which is primarily responsible for the IP₃ mediated release of endogenous Ca²⁺. Therefore, dissociation of FKBP12 from IP₃R by rapamycin results in an increase of cytosolic calcium [29]. Several authors had demonstrated that phosphorylation of PARP-1 by protein kinase C (PKC) inhibits its activity, and this reaction is
entirely Ca\textsuperscript{2+} dependent [21–23,30]. Based on these studies, it is attractive to speculate that PKC signaling is involved in the rapamycin-induced down-regulation of PAR synthesis in vivo. However, activation of PARP-1 in whole cell lysates of fibroblasts pretreated with rapamycin was not affected as monitored by an activity blot technique. Furthermore, supplementation with G6 6976, a potent PKC inhibitor, during rapamycin treatment did not promote the activation of PARP-1, arguing against an involvement of PKC. In fact, the results obtained by the activity blot assay suggests that the fast inhibitory effect of rapamycin on poly(ADP-ribosyl)ation may be mediated by a complex formation in vivo, which is disrupted upon cell lysis and denaturing SDS–PAGE and may implicate other signaling proteins.

In summary, our study demonstrates that low-dose rapamycin decreases PAR synthesis in intact human fibroblasts and that this effect is not due to a direct interaction of rapamycin and PARP-1. Thus we have added a novel pharmacological effect of rapamycin, a compound that is increasingly used in clinical settings. Several immediate effects of rapamycin are known that influence cellular signal transduction, such as increasing cytosolic Ca\textsuperscript{2+} by FKBP12 binding and inhibition of mTOR kinase activity. It will be interesting to identify in future work the precise molecular mechanisms, which are most likely related with signal transduction cascades, by which rapamycin induces the swift and strong inhibition of cellular poly(ADP-ribosyl)ation formation we are reporting in the present paper. Our data suggest that rapamycin may have a potential for use (i) in the therapy of pathophysiological conditions caused by overactivation of PARP, such as ischemia–reperfusion damage, or (ii) as a sensitizing agent in cytotoxic cancer therapy, by blocking DNA repair and tumor cell resistance.

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