Assessment of the Mechanism of Astrocyte Swelling Induced by the Macrolide Immunosuppressant Sirolimus Using Multinuclear Nuclear Magnetic Resonance Spectroscopy

Natalie Serkova,†‡ Uwe Christians,*,† Ulrich Flögel,† Josef Pfeuffer,† and Dieter Leibfritz†

Fachbereich Chemie/Biologie, Universität Bremen, Bremen, Germany, and Department of Biopharmaceutical Sciences, School of Pharmacy, University of California, San Francisco, California

Received May 6, 1997

The toxic effect of the macrolide immunosuppressant sirolimus on cell metabolism of primary astrocytes was studied by multinuclear NMR spectroscopy of viable cells and perchloric acid (PCA) extracts and compared to the effects of the immunosuppressant cyclosporine. The addition of 5 mg/L sirolimus (5.5 μmol/L) induced swelling of primary astrocytes to 110% of the original volume. Alteration in astrocyte volume in the presence of sirolimus was accompanied by reduction of the following important cell osmolytes and amino acid metabolites: myo-inositol, −58 ± 12% (mean ± standard deviation, n = 5); taurine, −44 ± 5%; glutamine, −13 ± 2%; compared with control. Sirolimus altered glucose metabolism and partially inhibited the tricarboxylic acid (TCA) cycle: ΣTCA/Σglycylate = 1.36 ± 0.09 (control, n = 3), 0.96 ± 0.08 (with sirolimus). The increased concentration of phosphodiesters by sirolimus addition (glycerophosphoethanolamine, 52 ± 18% glycerophosphocholine, 61 ± 14% compared with control, n = 5) indicated disorders in phospholipid metabolism of cellular membranes. Addition of sirolimus led to a decline of the energy state in astrocytes: the concentration of phosphocreatine (PCr) decreased to 75% of control value within 60 min of perfusion with sirolimus and the nucleotide triphosphate (NTP) concentration to 85% within 90 min (n = 3). The effect of sirolimus on the cell metabolism of astrocytes equals that of the immunosuppressants cyclosporine and tacrolimus, the neurotoxicity of which is well-established in clinical studies. The results of this in vitro study indicate that sirolimus possesses neurotoxic potential as well.

Introduction

Maintenance of viability and function of a transplanted organ requires lifelong immunosuppressive therapy. Today, most immunosuppressive protocols are based upon the undecapeptide cyclosporine. Cyclosporine therapy is complicated by its small therapeutic index in combination with severe toxicity (1). In clinical studies, between 25% and 55% of cyclosporine-treated organ transplant patients showed neurological symptoms such as tremor, seizures, paraesthesia, cortical blindness, cerebellar dysfunction, coma, and encephalopathy (2–5). Clinical computer tomography and magnetic resonance imaging studies revealed diffuse hypoxic neuronal damage and brain edema in cyclosporine-treated patients with neurological side effects (6–8). Postmortem, axonal degeneration, denyelination, multiple infarctions, and diffuse cerebral edema were found (9). In an earlier study analyzing extracts of glial and neuronal cells using NMR spectroscopy, we showed that cyclosporine partially inhibited the tricarboxylic acid (TCA) cycle, reduced the energy status, and interfered with membrane synthesis (10).

Recently, interest has focused on the macrolide immunosuppressant sirolimus. Sirolimus, which was isolated from Streptomyces hygroscopicus, has antifungal and antitumor activity (11) and is currently in the clinical phase of development as an immunosuppressive drug in combination with cyclosporine after kidney transplantation (12). Its mechanism of action is distinct from that of cyclosporine or tacrolimus (13). Coadministration of sirolimus and cyclosporine seems promising since combination of both drugs results in synergistic immunosuppressive effects (12). Additionally, in clinical studies, sirolimus showed a pattern of side effects different from those of cyclosporine. Among the most important were a reversible decrease of the platelet and white blood cell count and an alteration of the blood lipid profile (14). Neurotoxicity associated with sirolimus immunosuppression has not yet been reported.

* Address for correspondence: Uwe Christians, M.D., Ph.D., Department of Biopharmaceutical Sciences, School of Pharmacy, University of California at San Francisco, 513 Parnassus Ave., RmS-834, San Francisco, CA 94143-0446. Phone: (+1) 415 502 4968. Fax: (+1) 415 476 8887. E-mail: uwech@itsa.ucsf.edu.
† Universität Bremen.
‡ University of California.

1 Abbreviations: CT, computer tomography; DMEM, Dulbecco’s modified Eagle’s medium; DW, diffusion-weighted; FCS, fetal calf serum; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; MRI, magnetic resonance imaging; NDP, nucleotide diphosphate; NMR, nuclear magnetic resonance; NTP, nucleotide triphosphate; PBS, phosphate-buffered saline (pH 7.4); PC, phosphorylcholine; PCA, pericholic acid; PCK, phosphocreatine; PE, phosphoryl ethanolamine; Pi, inorganic phosphate; TCA, tricarboxylic acid; TMS, trimethylsilane; TSP, (trimethylsilyl)proponionic-2,3,3-d$_3$ acid.
It was our aim to study the effect of sirolimus on volume regulation, energy status, tricarboxylic acid cycle, and membrane synthesis in primary astrocytes using nuclear magnetic resonance spectroscopy. Since the effect of cyclosporine on volume regulation had not been evaluated in the earlier study (10), here the changes in the volume of cyclosporine-treated cells were determined and compared with the effects of sirolimus. The effect of cyclosporine and sirolimus on cell volume of perfused astrocytes was examined by diffusion weighted (DW) \(^1\)H NMR spectroscopy (15). \(^{31}\)P NMR spectra of perfused cells were used to verify the energy status of the astrocytes.

**Experimental Procedures**

**Materials.** Sirolimus was purchased from Sigma Chemicals (Deisenhofen, Germany), and cyclosporine was a kind gift from Sandoz (Nuremberg, Germany). Primary cultures of astroglial cells were prepared from cortex regions of 3-day-old newborn Wistar rats (10). Cell cultures were used 32 days after preparation of the rats.

Culture Petri dishes, tissue culture flasks, and centrifugation tubes were obtained from Nunc (Wiesbaden, Germany). DMEM, FCS, PBS, penicillin/streptomycin, and trypsin/EDTA were purchased from Gibco (Eggenstein, Germany).

Detergents as well as EDTA were obtained from E. Merck (Darmstadt, Germany). \([1-13\text{C}]\) Glucose was purchased from Cambridge Isotope Laboratories (Promococh, Wesel, Germany), and TSP was from Aldrich (Steinheim, Germany).

**Drug Treatment.** Astrocytes were incubated with either cyclosporine or sirolimus for 3 h. The concentration of cyclosporine was 10 µg/mL (8.3 µmol/L) and that of sirolimus 5 µg/mL (5.5 µmol/L). Stock solutions at 1 g/L were prepared for cyclosporine in 50/50 acetonitrile/water (v/v) and for sirolimus in 80/20 acetonitrile/sulfuric acid, pH 3 (v/v), and kept at 4 °C. The drug solutions were directly added to the cells in culture medium. The final concentrations of organic solvents in the medium did not exceed 0.5%. It was shown that the maximum concentration of organic solvent itself had no significant effect on cell metabolism.

For experiments with viable cells, astrocytes were embedded in basement membrane gel (BMG) threads as previously described (16). Approximately 10⁷ cells were resuspended in 500 µL of DMEM and mixed with 500 µL basement membrane extract (matrigel solution) at 4 °C. The cold cell suspension was drawn into a 1-mL syringe and immersed in a water bath at 37 °C for 2 min. The threads were extruded using a sterile Teflon tubing into Petri dishes filled with DMEM. The BMG threads were kept in an incubator at 37 °C and 90%/10% atmospheric air/CO₂ for 3 days.

**Determination of Cell Volume.** For NMR measurement of perfused astrocytes, the BMG threads were transferred into an 8-mm (for DW \(^1\)H NMR experiments) or 10-mm (for \(^{31}\)P NMR experiments) NMR tube. Cell cultures in the NMR tubes were perfused with culture medium oxygenated with 95% air/CO₂ for 3 days.

\[ \frac{\text{V}}{\text{t}} = \frac{\text{dV}}{\text{dt}} \]

For quantification of \(^1\)H NMR spectra, phosphocreatine (PCr) was determined in the \(^1\)H NMR spectra as described above. Phosphometabolite concentration was obtained from peak area ratios using phosphocreatine as reference peak. The absolute concentration of phosphocreatine was determined in the \(^1\)H NMR spectra as described above.

**Statistical Analysis.** All results are given as means ± standard deviation (SD) for each series of experiments. The results of the controls and drug-treated astrocytes were compared using unpaired Student's t-test (procedure TTest, SAS, version 6.05, SAS Institute, Cary, NC) or analysis of variance in combination with Duncan grouping (procedure GLM, SAS).

**Results**

**Volume Alterations during Immunosuppressive Treatment.** As assessed using DW \(^1\)H NMR spectroscopy, addition of cyclosporine at a concentration of 10 mg/L (8.3 µmol/L) to perfused astrocytes led to significant cell swelling to 130% of the volume of untreated control cells after an induction period of 50 min (n = 3) (Figure 1a). The cells remained in this swollen state during the...
perfusion period with cyclosporine of 2.5 h. During reperfusion with cyclosporine-free medium, the cells returned to their original volume. Recovery from the swollen state to the initial volume took 7–10 min (Figure 1a). Perfusion with 5 mg/L (5.5 µmol/L) sirolimus increased the cellular volume of astrocytes to 110% of the control volume (n = 3) (Figure 1b). The cells returned to their original volume when perfused without sirolimus.

**31P NMR Studies of Phospho Metabolites.** 31P NMR spectra of perfused astrocytes indicated a significant deterioration in the energy state during incubation with either sirolimus or cyclosporine. 31P NMR spectra of living cells showed characteristic signals of phosphocreatine (PCr), α-β-γ-nucleotide triphosphate (NTP), phosphomonoesters (PME) in high concentrations, and small signals of inorganic phosphate (Pi) and glycerophosphocholine (GPC) (Figure 2). The time-dependent concentration changes of PCr, NTP, and GPC in perfused astrocytes in the presence of sirolimus are depicted in Figure 3. Addition of 5 mg/L (5.5 µmol/L) sirolimus to perfused cells caused reduction of the concentrations of high-energy phosphates such as PCr and NTP (Figures 2 and 3). A significant PCr decrease to 75% of controls could be observed within 60 min of perfusion with sirolimus (n = 3) (Figure 3). A small reduction of NTP (−15%) was observed 90 min after addition of sirolimus. An intensive increase of intracellular Pi (25%) was distinctly detectable 50 min after addition of sirolimus. Additionally, 31P NMR spectra of perfused astrocytes showed an intensive increase of the phosphodiester concentration (GPC) after 60, 120, and 180 min of sirolimus treatment (Figure 3). The maximal GPC increase to 160% was detected 2 h after perfusion with 5 mg/L (5.5 µmol/L) sirolimus. The corresponding 31P NMR spectra of viable astrocytes with 10 mg/L (8.3 µmol/L) cyclosporine detected a reduction of NTP by 10% and of PCr by 15% and an increase of the cytosolic concentration of Pi by 10% and of GPC by 35% compared with untreated control (n = 3).

31P NMR spectra of PCA extracts confirmed a significant decline of the energy state after addition of sirolimus as indicated by the reduced concentration of PCr and the increased concentration of Pi in sirolimus-treated cells. Addition of 5 mg/L (5.5 µmol/L) sirolimus reduced the PCr concentration in astrocytes by 70% in comparison with untreated controls (n = 5) (Table 1). This reduction of PCr was accompanied by a significant increase of the Pi signal (Table 1). In consequence, an increased NDP/NTP ratio was observed after 3 h of incubation with 5 mg/L sirolimus or 10 mg/L cyclosporine: controls, 0.31 ± 0.01 (n = 5), with cyclosporine: 0.34 ± 0.01 (n = 5; P < 0.05), with sirolimus: 0.42 ± 0.03 (n = 5, P < 0.01). Addition of sirolimus to the cell cultures increased the cytosolic levels of the sum of phosphomonoesters, PME [phosphoethanolamine (PE) and phosphocholine (PC)], and the sum of phosphodiester, PDE [glycerophos- phocholine (GPE) and glycerophosphoethanolamine (GPE)] and the sum of phosphomonoesters, PME [phosphoethanolamine (PE) and phosphocholine (PC)] (Table 1). The increased concentrations of all intracel-

**Figure 2.** 31P NMR spectra of perfused astrocytes during immunosuppressive treatment with 5.5 µmol/L (5 mg/L) sirolimus. A representative experiment is shown (total n = 3). Abbreviations: glycerophosphocholine (GPC), nucleotide triphosphate (NTP), inorganic phosphate (Pi), phosphocreatine (PCr), and phosphomonoesters (PME).

**Figure 3.** Time-dependent changes of the phospho metabolite concentrations in primary astrocytes in the presence of 5.5 µmol/L (5 mg/L) sirolimus. Primary astrocytes were perfused for 30 min with cell culture medium (control). Then 5.5 µmol/L (5 mg/L) sirolimus was added to the perfusion medium. The time of addition of sirolimus after 30 min is marked by a vertical line. The relative cell metabolite concentrations were determined in living cells by 31P NMR spectroscopy. Each data point is the mean of three experiments. Symbols: ◆, glycerophosphocholine; □, inorganic phosphate; ◆, nucleotide triphosphate; ○, phosphocreatine.

**Table 1.** Changes in the Metabolite Concentrations of Primary Astrocytes after Incubation with 5.5 µmol/L (5 mg/L) Sirolimus for 3 h.

<table>
<thead>
<tr>
<th>metabolite</th>
<th>control (nmol/mg of protein)</th>
<th>+5 mg/L sirolimus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-inositol</td>
<td>45.9 ± 4.1</td>
<td>−58 ± 12***</td>
</tr>
<tr>
<td>taurine</td>
<td>63.3 ± 5.2</td>
<td>−44 ± 5***</td>
</tr>
<tr>
<td>hypotaurine</td>
<td>65.1 ± 3.3</td>
<td>−20 ± 2**</td>
</tr>
<tr>
<td>glutamine</td>
<td>51.9 ± 4.9</td>
<td>−13 ± 2*</td>
</tr>
<tr>
<td>PE</td>
<td>20.8 ± 6.2</td>
<td>+102 ± 28***</td>
</tr>
<tr>
<td>PC</td>
<td>6.5 ± 1.6</td>
<td>+152 ± 32***</td>
</tr>
<tr>
<td>GPE</td>
<td>5.8 ± 1.5</td>
<td>+52 ± 18***</td>
</tr>
<tr>
<td>GPC</td>
<td>20.2 ± 2.9</td>
<td>+61 ± 14***</td>
</tr>
<tr>
<td>PCr</td>
<td>23.1 ± 5.5</td>
<td>−71 ± 6***</td>
</tr>
<tr>
<td>P1</td>
<td>15.7 ± 4.8</td>
<td>+142 ± 11***</td>
</tr>
</tbody>
</table>

*The values are means ± standard deviations (n = 5); % = percent of control. Values significantly different from control: *P < 0.05, **P < 0.02, ***P < 0.01. Abbreviations: glycerophosphocholine (GPC), glycero phosphoethanolamine (GPE), inorganic phosphate (P1), phosphocreatine (PCr), phosphocholine (PC), and phosphoethanolamine (PE).
ular phospholipids indicated enhanced degradation of cell membranes.

**Changes in Concentrations of Cell Metabolites.** Alterations in the cytosolic concentrations of several osmotolytes and amino acid metabolites after incubation with 5 mg/L (5.5 µmol/L) sirolimus were determined from 1H NMR spectra of PCA extracts. The most distinct effect was a decreased concentration of PME, while PDE decreased. The most likely explanation for the observed reduction of PME as well as that of PDE is increased dephosphorylation of PME in the presence of sirolimus (21). Sirolimus also caused reduction of the cytosolic glutamine (Gln) concentration, an important amino acid in neural tissue (∼13 ± 2% compared with untreated controls, n = 5, P < 0.01). This was confirmed by 13C NMR measurements of PCA extracts after 3-h incubation with 5 mg/L (5.5 µmol/L) sirolimus and 5 mmol/L [1-13C]glucose. This assay allowed to follow the metabolic pathways of glucose, in particular the synthesis of intermediates of glycolysis (such as lactate) and derivatives of the TCA cycle (such as glutamine) and PDH) was calculated from the isotopomer pattern of glutamine (C-2+C-3/C-4). This was possible since Gln labeled in position C-2 and C-3 is considered to be synthesized only via the anaplerotic pathway (PC activity), while the label of Gln-C-4 resulted only from PDH activity (23). After incubation with sirolimus, the ratio of PC/PDH was increased in primary astrocytes. Incubation of astrocytes with the immunosuppressants cyclosporine and tacrolimus caused reduction of all tricarboxylic acid cycle derivatives and an increase of glycolysis intermediates (10). Addition of sirolimus showed a similar effect on glucose metabolism. This suggests that sirolimus, like cyclosporine and tacrolimus, reduces the contribution of the TCA cycle to anaerobic pathways in astrocytes.

While the potential of cyclosporine and tacrolimus to cause neurotoxicity in transplant patients is well-established and the underlying mechanisms were characterized by our previous study (10), neurotoxicity in patients has not yet been described for sirolimus. The same restrictions in respect to transferring the results of this in vitro study to the in vivo situation in patients as discussed for cyclosporine and tacrolimus in our previous study (10) apply for the results of the present study with sirolimus. The tissue concentrations of sirolimus in the central nervous system and peripheral nerve tissue are as yet unknown. However, the effects of sirolimus on cell metabolism and volume regulation of astrocytes as identified in the present study parallel those of cyclosporine and tacrolimus (10). Thus, it can be assumed that sirolimus has the potential to cause cytotoxic brain edema and diffuse disorders of the neuronal tissue as observed in clinical MRI studies (6-8) in transplant patients under immunosuppressive therapy with cyclosporine or tacrolimus. On the basis of the results of this study, special attention should be paid to neurological side effects in individual patients during the further clinical development of sirolimus.

**Acknowledgment.** The study was supported by DFG Grant Ch 956/1 and Grant SFB 265, Project A7, and the Alexander von Humboldt-Foundation, Grant V-3-FLF-1052812.

**References**


TX970071K