Rapamycin is a neuroprotective treatment for traumatic brain injury

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The mammalian target of rapamycin (mTOR), commonly known as mTOR, is a serine/threonine kinase that regulates translation and cell division. mTOR integrates input from multiple upstream signals, including growth factors and nutrients to regulate protein synthesis. Inhibition of mTOR leads to cell cycle arrest, inhibition of cell proliferation, immunosuppression and induction of autophagy. Autophagy, a bulk degradation of sub-cellular constituents, is a process that keeps the balance between protein synthesis and protein degradation and is induced upon amino acids deprivation. Rapamycin, mTOR signaling inhibitor, mimics amino acid and, to some extent, growth factor deprivation. In the present study we examined the effect of rapamycin, on the outcome of mice after brain injury. Our results demonstrate that rapamycin injection 4 h following closed head injury significantly improved functional recovery as manifested by changes in the Neurological Severity Score, a neurobehavorial testing. To verify the activity of the injected rapamycin, we demonstrated that it inhibits p70S6K phosphorylation, reduces microglia/macrophages activation and increases the number of surviving neurons at the site of injury. We therefore suggest that rapamycin is neuroprotective following traumatic brain injury and as a drug used in the clinic for other indications, we propose that further studies on rapamycin should be conducted in order to consider it as a novel therapy for traumatic brain injury.

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Introduction

The mammalian target of rapamycin (mTOR) is a phosphatidylinositol kinase-related serine–threonine kinase (Schmelzle and Hall, 2000). Growth factors, mitogens and hormones activate the PI3K/Akt signaling pathway and consequently the mTOR signaling (Hay and Sonenberg, 2004). Nutrients (amino acids, glucose) also regulate mTOR activity (Proud, 2004). Thus, mTOR functions by integrating extracellular signals (growth factors and hormones), with amino acid accessibility and intracellular energy status to control translation rates and additional metabolic processes (Hay and Sonenberg, 2004). mTOR regulates a wide array of cellular functions, including translation, transcription, mRNA turnover, protein stability, actin cytoskeletal organization and autophagy (Harris and Lawrence, 2003). The best-characterized function of mTOR in mammalian cells is regulation of translation. Ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1), the most extensively studied substrates of mTOR, are key regulators of protein translation (Harris and Lawrence, 2003). mTOR regulates eIF4G, S6K, 4EBP1 and Atg1 proteins by phosphorylation. As a consequence, mTOR enhances translation initiation and affects cell growth and proliferation. Growth factors, such as insulin, and nutrients, such as amino acids or glucose, enhance mTOR function, as evidenced by an increased phosphorylation of S6K and 4EBP1 (Harris and Lawrence, 2003).

Rapamycin is a macrolide antibiotic first developed as an antifungal agent, however, it was discovered that rapamycin had potent immunosuppressive and antiproliferative properties. Rapamycin binds to the cytosolic protein FK-binding protein 12 (FKBP12). Thereby, the rapamycin–FKBP12 complex can inhibit mTOR preventing further phosphorylation of P70S6K, 4EBP1 and, indirectly, other proteins involved in transcription and translation and cell cycle control (Vignot et al., 2005). Inhibition of mTOR leads to, among others, cell cycle arrest in tumor cells resulting in growth retardation. Antiapoptotic signals mediated by mTOR are also antagonized by rapamycin (Guba et al., 2002). Rapamycin has been shown to inhibit growth of melanoma cells in mouse model, additionally, as an immunosuppressor it prevents transplant rejection in organ transplant recipients (Koehl et al., 2004). Apart from its immunosuppressive capacity, rapamycin was also recently shown to be capable of preventing coronary artery re-stenosis (Sousa et al., 2003).

The observation that rapamycin treatment also induces autophagy indicates that mTOR kinase activity is involved in this process (Kamada et al., 2004). Autophagy is a process of bulk degradation of cellular constituents through an autophagosomes–lysosomal pathway (Klionsky and Emr, 2000; Wang and Klionsky, 2003). Autophagy is important in normal growth control and may be defective in diseases (Larsen and Sulzer, 2002; Ogier-Denis and

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Codogno, 2003). This process permits the disposal of unwanted damaged organelles in the cell and allows recycling of free amino acids and nutrients at time of nutrient deprivation or other insults. Thus, autophagy is important for normal cell growth, differentiation and survival (Reggiori and Klionsky, 2002). Recent studies demonstrated that enhancement of autophagy may induce degradation of unwanted aggregates such as of mutant huntingtin, indicating that at least in some neurodegenerative diseases enhancement of autophagy may rescue neuronal cells (Ravikumar and Rubinsztein, 2004). It was previously shown that rapamycin can protect against mutant huntingtin-induced degeneration in cells, fly and mouse models of Huntington’s disease (Ravikumar et al., 2002, 2004). It was also demonstrated that induction of autophagy by rapamycin enhances the clearance of a wide range of aggregate-prone proteins and reduces their toxicity (Berger et al., 2006; Webb et al., 2003). The mammalian target of rapamycin (mTOR) negatively regulates autophagy (Schmelzle and Hall, 2000). Atg1–Atg13 complex is regulated by mTOR, which is also involved in the regulation of transcription, translation and cell cycle and plays a central role in cell metabolism. Thus, inhibition of mTOR may induce autophagy (Klionsky and Emr, 2000; Wang and Klionsky, 2003), may result in a reduction of protein synthesis and may inhibit cell proliferation (Dutcher, 2004).

Brain damage following closed head injury is divided to primary and secondary injuries. While primary brain injury results from the mechanical forces applied to skull and brain at the time of injury, the secondary neuronal injury is associated with a neuroinflammatory response characterized by microglial and astrocytic activation, resulting in the release of reactive oxygen species and inflammatory cytokines (Leker and Shohami, 2002). Several studies described the loss of neuronal cells that follows injury as both necrotic and apoptotic (Faden, 1993). Recently we have demonstrated that following closed head injury (CHI) or stab injury in mice the levels of Beclin 1, used as a marker for autophagy, are elevated near the injury site in neurons and astrocytes (Diskin et al., 2005). These findings suggest that Beclin 1 and autophagy may play a role in brain responses to head trauma.

In the present study we examined the effect of rapamycin treatment on recovery from traumatic brain injury (TBI). Our results do not exclude the existence of other mechanism except autophagy in response to rapamycin treatment. Beyond doubt, our results indicate that rapamycin treatment significantly improved the recovery from head injury and increased the number of surviving neurons at the injury site. Thus rapamycin treatment should be evaluated for patients suffering from traumatic brain injury.

Materials and methods

Materials

Antibodies specific to glial fibrillar acidic protein (GFAP) were purchased from Dako (Denmark), and antibodies specific to neurons (NeuN) were from Chemicon International, Inc (Temecula, CA). Antibodies specific to phospho-S6-kinase (p70S6K) were from Sigma (St. Louis, MO, USA). Antibodies specific to Beclin 1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to activated macrophages (Mac-2) are produced from hybridoma (ATCC TIB 166, M3/38.1.2.8.HL.2) and were kindly provided by Professor I. Witz from the Tel-Aviv University. Rapamycin was purchased from Calbiochem (La Jolla, CA). Unless otherwise indicated, all materials were purchased from Sigma (St. Louis, MO, USA).

Animals

The study was conducted according to the NIH Guidelines for the Care and Use of Laboratory Animals and was approved by the Animal Care Committee of the Hebrew University. Adult male Sabra mice (9–10 weeks old, about 35 g body weight) were used in all the experiments. Animals were bred in a specific pathogen-free environment; kept in cages, with four to six mice per cage, under standard conditions of temperature, light, food and water. Usually, 7–9 animals were included in each experimental group.

Rapamycin preparation

Rapamycin was dissolved in DMSO (25 mg/ml) and stored at −20 °C. For animal injection, the stock solution was diluted immediately before i.p. injections with 0.5 ml aqueous solution containing 5% polyethylene glycol 400 and 5% Tween 80. Drug-treated mice received 4 h post injury, one i.p. injection of 0.5 or 1 mg/kg rapamycin and control animals received the drug vehicle.

Closed head injury

The experimental closed head injury (CHI) paradigms developed in rats and mice (Chen et al., 1996; Yatsiv et al., 2005) closely resemble the clinical manifestations of focal head injury in humans. Briefly, under ether anesthesia, a midline longitudinal incision was performed, the skin retracted and the skull exposed. The left anterior frontal area was identified, and a tipped teflon cone was placed 1 mm lateral to the midline, in the mid-coronal plane. The head was fixed and a weight, calibrated according to the age and weight of the animal, was dropped on the cone from a height, resulting in a focal injury to the left hemisphere. After trauma, the animals received supporting oxygenation with 95% O₂ for no longer than 2 min and were returned to their cages.

Recovery of motor function

The neurobehavioral status of the mice was evaluated using a set of 10 tasks, collectively termed Neurologic Severity Score (NSS), which tests reflexes, alertness, coordination, and motor abilities. One point is awarded for failure to perform a particular task; thus, a score of 10 reflects maximal impairment, whereas a normal mouse scores 0 (Beni-Adani et al., 2001). Post-CHI, NSS was evaluated at 1 h (NSS 1 h) to define the severity of the injury and to ensure similar severity of injury in all groups and then every several days during 34 days. Each animal was assessed by an observer who was blinded to the animal treatment. The difference between the initial NSS and that at any later time was calculated for each mouse, and this value (ΔNSS) reflects the spontaneous or treatment-induced recovery of motor function. Rapamycin or vehicle was injected once, at 4 h following injury.

Western blot analysis

To assess levels of Beclin 1, sham, rapamycin or vehicle-treated mice were killed 5 h after CHI, their brains were removed and used to prepare total cell lysates as described below. To assess levels of p70S6K, rapamycin and vehicle-treated mice were killed 24 h after...
Membranes were blocked for 2 h in TBST buffer (0.02 M Tris gels and electrophoretically transferred to nitrocellulose membrane. By SDS Laboratories, GmbH, Munchen, Germany). Lysates were resolved by centrifugation and protein levels in the lysates were determined with the aid of the Bio-Rad protein assay (Bio-Rad Laboratories, GmbH, Munchen, Germany). Lysates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) through 7.5% gels and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked for 2 h in TBST buffer (0.02 M Tris–HCl pH 7.5, 0.15 M NaCl and 0.05% Tween 20) containing 5% milk, blotted with 1 μg/ml primary antibodies and incubated for 18 h, followed by 0.5 μg/ml secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham Corp).

Immunohistochemistry

Three days post injury and treatment mice were anesthetized with a mixture of ketamine (250 mg/kg; Ketaset, Fort Dodge, IA) and xylazine (2%) (in a ratio of 0.85:0.15, respectively) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and placed for prefixation for 5 days in 4% paraformaldehyde at 4 °C. The brains were blocked in the coronal plane and embedded in paraffin as described previously (Erlich et al., 2006; Erlich et al., 2000). Serial 7-μm-thick sections were cut and mounted on gelatin-coated slides. Deparaffinized coronal brain sections were used for immunohistochemical analyses of Beclin 1, Mac-2, GFAP and NeuN expression in CHI brains as described previously (Erlich et al., 2000; Pinkas-Kramarski et al., 1997). The frontal and dorsal borders of the trauma were determined by the presence of reactive astrocytes, and sections within this limited area were further evaluated. For each analysis between three and seven randomly selected sections were used. Specific stainings were visualized by diaminobenzidine (DAB) using the avidin–biotin peroxidase technique (Zymed Laboratories Kit). Sections were deparaffinized, rehydrated and incubated in 1% hydrogen peroxide in methanol to inhibit the activity of endogenous peroxidase. The sections were then washed in PBS for 10 min followed by washing with PBS containing 0.3% Tween for 10 min and blocked in blocking solution supplied by the Zymed kit for 30 min. Incubation with anti-NeuN or anti-Mac-2 antibodies diluted at 1:100 in PBS containing 10% normal goat serum was overnight at 4 °C. Primary antibody was detected using biotinylated goat anti-mouse or goat anti-rat secondary antibodies and the avidin–biotin complex method according to supplier directions (Zymed Laboratories Inc.). One series of sections was stained with hematoxylin for morphologic examination. Mac-2 stained sections were counted from 10 to 15 randomly chosen computer images at the level of dorsal hippocampus, representing a brain area of 1.54 mm². Data are the mean/mm²±SEM. In order to assess the cortical neuronal cell damage NeuN stained sections (from 7 animals in each experimental group, three sections per animal (between −2.18, and −2.54 mm from the bregma) were photographed in the cortical region of the injured hemisphere. Four randomly selected fields were photographed (0.4 mm² each field). The NeuN positive stained cells in the injured hemisphere were then counted manually by using a grid and mechanical counter. Data are the mean/mm²±SEM.

Groups were compared by Student’s t-test. To quantify the effect of rapamycin on astrocytes activation following CHI, coronal sections at bregma level −2.54 mm were immunostained using anti-GFAP antibodies. Immunostained hippocampi (from 7 animals in each experimental group, three sections per animal) were photographed randomly at 4 different hippocampus areas (0.23 mm² each field). The GFAP positive stained cells in the injured hemisphere were counted. Data are represented as mean/0.23mm²±SEM. Statistical significance was evaluated by the Student’s t-test.

Statistical analysis

Values of NSS are expressed as means±SEM and analyzed using the non-parametric Mann–Whitney test. p70S6K, Beclin 1 and tubulin were quantified by ImageJ computer program. The p70S6K level, normalized by tubulin level, is expressed as the mean fold reduction of the CHI rapamycin-treated brains over the control vehicle-treated brains±SEM. The Beclin 1 level, normalized by tubulin level, is expressed as the mean fold induction of the CHI rapamycin-treated or CHI vehicle-treated brains over the sham control brains±SEM. The results were analyzed using Student’s t-test. A p value of <0.05 is considered significant.

Results and discussion

Rapamycin, a lipophilic, macrolide antibiotic, inhibits mTOR and prevents further phosphorylation of proteins involved in transcription, translation and cell cycle control (Vignot et al., 2005). Anti-apoptotic signals mediated by mTOR are antagonized by rapamycin (Guba et al., 2002). Inactivation of mTOR induces also autophagy (Noda and Ohsumi, 1998) which plays a central role in cell metabolism. Activation of mTOR results in phosphorylation

Fig. 1. Rapamycin improves recovery of neurobehavioral function after CHI. One hour after CHI mice were evaluated for the severity of injury (NSS) and assigned to vehicle or rapamycin treatment such that NSS (1 h) was similar in both groups. Four hours after CHI control mice were treated with the vehicle (n=7) (filled bars), rapamycin (0.5 mg/kg body weight, n=7) (dotted bars) and rapamycin (1 mg/kg body weight, n=7) (hatched bars) was injected to the other group of mice. The vehicle solution was 5% Tween 80, 5% PEG 400 and 0.28% DMSO. NSS was assessed at various time intervals and functional recovery is presented as the difference between NSS at 1 h and at any other time (ΔNSS). From 48 h, and until 34 days post injury the ΔNSS of the rapamycin-treated mice was significantly higher as compared to controls, indicating greater recovery of neurobehavioral function. The mean ΔNSS values±SEM are depicted. The experiment was repeated twice with similar results. *p<0.05; **p<0.01 as compared by Mann–Whitney test to the vehicle-treated mice.
of several target proteins including eIF4G, S6K, 4EBP1 and Atg1. The most extensively studied substrates of mTOR are the key regulators of protein translation (Harris and Lawrence, 2003). Thus inhibition of mTOR results in cell cycle arrest and cell growth inhibition (Hay and Sonenberg, 2004). mTOR negatively regulates autophagy (Schmelzle and Hall, 2000). Atg1 protein phosphorylation by mTOR results in inhibition of autophagy (Klionsky and Emr, 2000; Wang and Klionsky, 2003). Moreover, rapamycin induces autophagy, even in a nutrient-rich medium (Blommaart et al., 1995; Noda and Ohsumi, 1998). In mammalian cells, autophagy is inhibited by amino acids and insulin. It was also demonstrated that activation of p70S6K is associated with inhibition of autophagy in rat hepatocytes, and the inhibition of autophagy by amino acids could be partially prevented by rapamycin (Blommaart et al., 1995; Shigemitsu et al., 1999). Thus, the mTOR protein appears to act as a master regulator of the balance between protein synthesis and degradation. We therefore examined the possibility that rapamycin treatment will enhance rescue mechanism and improve recovery following injury.

Three experimental groups (n=7/group) were examined, and the experiment was repeated twice: control vehicle-injected mice, rapamycin 0.5 mg/kg body weight and 1 mg/kg body weight-injected mice. The vehicle solution was 5% Tween 80, 5% PEG 400 and 0.28% DMSO. NSS was assessed at 1 h to ensure similar severity of injury in all treatment groups. Thus, 1 h after injury, before injection of the drug, mean NSS values were: 6.8±0.5 in the vehicle-treated, and 6.8±0.4 and 7.3±0.4 in the 0.5 and 1 mg/kg rapamycin-treated groups, respectively (difference is not significant). Fig. 1 depicts the temporal changes in functional recovery of the mice, expressed as ANSS. It is clear that rapamycin significantly improved the functional recovery of the injured mice between 2 and 34 days following CHI, and a single administration of the drug was sufficient to produce the beneficial effect. The effect was evident even at the lower dose (0.5 mg/kg) yet the effect of the higher dose (1 mg/kg) was more long lasting. Based on this long-term effect, and on the fairly wide therapeutic window (drug was given at 4 h post injury), we suggest that rapamycin treatment should be tested in the clinical setting to improve neurological recovery from head trauma.

In order to assess whether rapamycin indeed inhibited mTOR signaling pathway, we next examined its effect on p70S6K phosphorylation. CHI mice treated with rapamycin (1 mg/kg) or vehicle were examined for p70S6K phosphorylation 24 h following injury by Western blot analysis. As shown in Fig. 2, rapamycin treatment significantly reduced p70S6K phosphorylation (1.6-fold decrease, p=0.00024, in the left, contused, hemisphere and 1.9-fold decrease, p=0.0049 in the right, contralateral hemisphere). Thus, our results indicate that indeed rapamycin treatment inhibits brain p70S6K, the downstream effector of mTOR signaling.
In order to examine whether autophagy is enhanced following injury we examined the levels of Beclin 1 in the injured brains. Beclin 1 is a Bcl-2 interacting protein that was previously found to promote autophagy (Liang et al., 1999). Beclin 1 is a component of the phosphatidylinositol-3-kinase class III (PI3K) complex that is required for autophagy (Kihara et al., 2001). Using Beclin 1 protein as a marker for autophagy, we recently demonstrated its elevated expression following CHI in mice, at the cortical region bordering the site of injury (Diskin et al., 2005) and following Stab injury (Erlich et al., 2006). CHI mice treated with rapamycin (1 mg/kg) or vehicle and sham controls were examined for Beclin 1 levels 5 h following injury by Western blot analysis. As shown in Fig. 3, CHI significantly induced elevation in Beclin 1 levels compared to the sham controls indicating that injury induced elevation in Beclin 1 in the vehicle and rapamycin-treated mice. The elevation of Beclin 1 in the CHI rapamycin-treated animals was slightly higher, yet not significant, than that of the CHI vehicle-treated animals and significantly higher than the sham controls.

We next examined the effect of rapamycin treatment on various brain cell types at the cortical and hippocampal regions, near the site of injury, by immunostaining. CHI mice treated with rapamycin or vehicle 4 h following injury were sacrificed 3 days later. To investigate the effect of rapamycin on the inflammatory process in the CHI brains, the activation of microglia was examined using immunostaining with anti-Mac-2 antibodies (a microglia/macrophages marker). In the vehicle-treated animals, Mac-2-positive cells were detected at the cortical region near the site of injury (Fig. 4A), whereas in contrast, activated microglia/macrophages were barely detectable in the brains of the rapamycin-treated mice. Statistical analyses of the changes in number of Mac-2 positive cells at the site of injury indicated a mean of 83 ± 27 and 23 ± 11 cells per 1 mm² in the vehicle- and in the rapamycin-treated animals, respectively. These results indicate that rapamycin treatment inhibits the inflammatory responses to the injury.

In response to brain injury, astrocytes become activated (reactive). The process of astrocyte activation remains rather enigmatic in terms of their contribution to the neuronal damage or their protective effect following injury (Pekny and Nilsson, 2005). We next examined whether rapamycin treatment affected astrocytes activation by using anti-GFAP antibodies as a marker for
activated astrocytes. In contrast to the effect of rapamycin on microglia activation, no significant differences in astrocyte activation were found between the vehicle- and rapamycin-treated CHI mice at the hippocampal region of the injured hemisphere (Fig. 4B). The number of GFAP positive cells in the vehicle-treated CHI brains was similar to that of the rapamycin-treated CHI brains (153±14 and 148±18 respectively). Thus, rapamycin treatment does not affect astrocyte activation induced by the injury.

We next examined the effect of rapamycin treatment on the number of neurons at the injury site by immunostaining with anti-NeuN antibodies (neuronal marker). We also performed hematoxylin staining for morphology determination. As shown in Fig. 5, rapamycin treatment significantly increased the number of surviving neurons at the site of injury (677±120 in rapamycin-treated CHI brains compared to 218±48 in the vehicle-treated CHI brains). Serial sections stained with hematoxylin confirm the results of NeuN staining, showing that at the cortical site of injury, the number of cell nuclei is higher in the rapamycin-treated brains compared to the vehicle-treated brains. These results indicate that rapamycin treatment not only reduced microglia activation, but also increased the number of surviving neurons at the injury site.

There is a wealth of body of evidence to suggest that early (within hours) inflammatory response contributes to the late stages of brain injury and results in a worsening of the neurological outcome (Kirino, 2000; Shohami et al., 1999). Post trauma inflammation might contribute to neuronal damage probably through multiple mechanisms. In the active state, microglia produce cytotoxic molecules, such as nitric oxide, oxygen radicals, arachidonic acid derivatives and cytokines (Giulian, 1997). Thus, rapamycin treatment may act as an anti-inflammatory drug reducing microglia activation and enhancing the recovery from head trauma. Rapamycin was originally found to be an effective antifungal agent (Baker et al., 1978), and it was later found to have potent immunosuppressive qualities (Martel et al., 1977). Furthermore, it was demonstrated that rapamycin is not cytotoxic in vivo and can be used as an anti-angiogenic agent that inhibits tumor growth (Majumder et al., 2004) as well as immunosuppressant (Olsen et al., 1994). Thus, part of the neuroprotective effects of rapamycin shown in the present study may be due to its anti-inflammatory effects, yet, since it also enhances autophagy, these may be due to enhancing this specific cellular response. Indeed, head trauma induces Beclin 1 elevation at the site of injury (Diskin et al., 2005), however, the extent of its induction in rapamycin-treated mice was slightly higher than that of the non-treated animals (Fig. 3). The elevation in Beclin 1 levels reached the maximal level due to the injury. Thus our results do not rule out the possibility that the beneficial effect of rapamycin can be either from enhanced autophagy or from its anti-inflammatory effect.
action, nevertheless this treatment seems to be neuroprotective and to improve the recovery from CHI. While writing this paper, two Nature papers were published demonstrating that inhibition of autophagy leads to neurodegeneration. These studies are in good agreement with our present results (Hara et al., 2006; Komatsu et al., 2006).

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