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Rapamycin Induces Heme Oxygenase-1 in Liver but Inhibits Bile Flow Recovery after Ischemia

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Background/Aims. **Rapamycin, which is employed in the management of patients undergoing liver surgery, induces the synthesis of heme oxygenase-1 (HO-1) in some non-liver cell types. The aim was to investigate whether rapamycin can induce HO-1 expression in the liver, and to test the effects of rapamycin on liver function in the early phase of ischemia reperfusion (IR) injury.**

Methods. **Isolated rat hepatocytes and a rat model of segmental hepatic ischemia and reperfusion were employed. Bile flow was measured gravimetrically or by using indocyanine green. mRNA and protein (by quantitative PCR and Western blot, respectively) and blood concentrations of rapamycin, bilirubin, and liver marker enzymes were measured.**

Results. **In isolated hepatocytes, rapamycin induced a 6-fold increase in HO-1, comparable to that induced by cobalt proporphyrin (CoPP), and a 2-fold increase in peroxiredoxin-1. Pretreatment of rats with rapamycin resulted in a small increase in liver HO-1 expression, a 20% inhibition of the basal rate of bile flow, and a 50% inhibition in the rate of bile flow recovery after ischemia. CoPP increased basal bile flow by 20% and inhibited bile flow recovery by 50%. These effects were associated with small increases in the blood concentrations of bilirubin and liver marker enzymes.**

Conclusions. **Rapamycin, through HO-1 induction, has the potential to protect the liver against damage in the late phase of IR. The inhibition by rapamycin of bile flow indicates that its actions on liver function in the acute phase of IR injury are complex.**

Key Words: **liver; isolated hepatocytes; ischemia reperfusion injury; bile flow; rapamycin; heme oxygenase-1; quantitative PCR; rat.**

INTRODUCTION

Liver resection or transplantation is presently the only effective treatment for advanced hepatocellular carcinoma and metastatic cancer of the liver [1–3]. However, these surgical procedures are commonly associated with ischemia and reperfusion (IR) injury, especially in patients with advanced age, obesity, and fatty liver disease, and downstaged cancers [3–6]. Moreover, tumor recurrence following surgery for hepatocellular carcinoma is a major concern [7]. Limiting the extent of IR injury would improve the outcome for patients undergoing liver resection, especially for the recipients of non-heart-beating and fatty donor livers in transplantation [3].

The onset of hepatocyte damage induced by IR is chiefly due to the deleterious actions of an elevated intracellular Ca^{2+} concentration and reactive oxygen species (ROS) [2, 6]. IR is associated with the induction and/or post-translation modification of several antioxidant enzymes [2, 8, 9]. Two of these enzymes, which seem particularly important, are heme oxygenase-1 (HO-1) and peroxyredoxin-1 (Prx-1) [2, 8–10]. These exhibit powerful antioxidant effects and also other actions that can protect against ROS-induced damage in IR. Pharmacologic induction of HO-1 and Prx-1 is a potentially useful strategy to reduce ROS-induced IR injury

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[2, 8, 11]. However, many effective inducers of HO-1, such as cobalt protoporphyrin (CoPP), are toxic, and there are no known effective and potentially useful pharmacologic induces of Prx-1. Rapamycin (sirolomus), which is currently in use in liver transplant surgery as an immunosuppressive agent and in liver resection to inhibit the regrowth of hepatocellular carcinoma cells [12–16], has been shown to induce HO-1 expression in heart, kidney and pulmonary vascular cells [9, 17–19]. However, whether rapamycin can induce the expression of HO-1 and/or Prx-1 in hepatocytes is not known. The aims of the present study were to determine whether rapamycin can induce the increase expression of these antioxidant enzymes in hepatocytes, and to investigate the effects of pretreatment with rapamycin on liver function (assessed by measurement of bile flow) in the early phase of IR injury.

MATERIALS AND METHODS

Animals and Surgical Procedure

Rats (10–12 weeks weighing 250–400 g) were bred and housed in the Flinders Medical Centre Animal House at 22°C, 60% humidity, with a 12-h light/dark cycle, and free access to food and water. Animals received humane care, and the experimental protocols were conducted according to the criteria outlined in the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (National Health and Medical Research Council of Australia). A rat model (male Sprague Dawley rats) of segmental (60%–70%) hepatic ischemia in which the bilateral median and left lateral liver lobes are made ischemic [20], including the measurement of bile flow from the bilateral median and left lateral liver lobes by cannulation of the common bile duct, was employed as described previously [21]. Blood samples were taken from the inferior caval vein, and at the end of the protocol the rat was euthanized and tissue samples from the ischemic and non-ischemic lobes collected for RNA extraction. During sham operations, clamping was not performed, and there was no induction of IR. For rapid removal of the liver, the liver was flushed with saline and removed directly after midline laparatomy.

Rapamycin, CoPP, or vehicle (approx 1 mL) was administered by intraperitoneal injection (i.p.) 20–24 h prior to the surgical procedure. Rapamycin (LC Laboratories, Woburn, MA) stock solution (25 mg/mL in 100 % (vol/vol) sterilized ethanol was diluted 1:1 with sterile phosphate buffered saline (PBS) just before injection. Cobalt protoporphyrin IX chloride (Frontier Scientific Inc., Logan, UT) (40 mg) was dissolved in 5 mL of 0.1 M NaOH. Subsequently it was diluted in 5 mL of 0.9% (wt/vol) saline, and the pH was adjusted to 7.4 with 1 M HCl. The concentration of the solution injected was 4 mg/mL CoPP.

The dose of rapamycin employed and the time of pretreatment with this agent (4 mg/kg body weight, 24 h) were chosen on the basis of doses previously used in experiments *in vivo* with rats and mice [22–24] in order to achieve a blood concentration of rapamycin within range employed clinically [25]. The dose of CoPP employed and time of pretreatment (20 mg/kg body weight, 24 h) were chosen on the basis of those employed previously in experiments with rats *in vivo* for the induction of HO-1 in the liver [26].

Measurement of Bile Flow

Bile flow was measured gravimetrically as described previously [21, 27]. Total bile secretion during the reperfusion period was determined by measuring the area under the curve. Initial rates of increase in bile flow after commencement of the 60-min reperfusion were determined by linear regression (PRISM) using the first five time points [28]. Bile flow was also measured using indocyanine green (ICG) [27, 29]. ICG (Cardiogreen; Sigma, St. Louis, MO, USA) dissolved in sterile water (0.2–0.3 mL) was injected as a bolus dose (0.5 mg/kg body weight) into the vena cava. The concentration of ICG in the liver was measured by near infrared spectroscopy using a USB4000 Fiberoptic Spectrophotometer (Ocean Optics, Dunedin, FL) fitted with a 700–950 nm diffraction grating. The light source was a tungsten-halogen 360–2000 nm 20W globe (Ocean Optics) and light was delivered to the liver by a 1000 μm fiberoptic light guide connected to a VIS/NIR reflection probe. Reflected light was collected using a second VIS/NIR reflection probe coupled to the spectrophotometer by a 400 μm fiberoptic light guide. The two probes were positioned over the surface of the left lateral liver lobe at an angle of about 70° to the liver surface, gently touching the surface and held in place by two clamps. The distance between the centers of the probes was 10mm. Data were collected for 60 min.

Preparation of Isolated Hepatocytes

Hepatocytes were isolated from Hooded Wistar rats by colagenase digestion, and were cultured in multiwell plates or on glass coverslips, as described previously [30].

Plasma Concentrations of Liver Marker Enzymes and Rapamycin

Liver marker enzymes (AST, ALT, LDH) and bilirubin in rat plasma were measured spectrophotometrically using an Hitachi 917 auto analyzer and standard Roche-Hitachi methodology in the SA Pathology laboratories (Flinders Medical Centre, Bedford Park, S.A). Plasma rapamycin concentrations were measured using LC-MS by Dr. Ray Morris (Queen Elizabeth Hospital, Woodville, S.A.).

RNA Extraction and PCR

The isolation of RNA, synthesis of cDNA and semiquantitative PCR were conducted as described previously [10, 31]. Quantitative PCR (qPCR) was conducted using probe-based strategies, β-actin as reference RNA, a Rotor-Gene 3000 (Corbett), and the $\Delta\Delta$CT method. Sequences of primers and probes used for quantitative PCR were: β-actin sense 5′ - TGC CCA TCT ATG AGG GTT ACG - 3′ , anti-sense 5′ - CGC TCG GTC AGG ATC TTC A - 3′, and probe 5′ FAM - CTG GCC GGG ACC TGA CAG ACT ACC TC - BHQ 3′ (product size 100 base pairs); and HO-1 sense 5′ - CAC AGG GTG ACA GAA GAG GCT AA - 3′ , antisense 5′ - CTG GTC TTT GTG TTC CTC TGT CAG - 3′ and probe 5′ FAM - CAG CTC CTC AAA CAG CTC AAT GTT GAG C - BHQ 3′ (product size base 160 pairs). The probes were 5′ labeled by a 6-carboxy-fluorescein reporter (FAM) and 3′ labeled by a black hole quencher (BHQ).

Protein Extraction and Western Blot

The extraction of proteins, 1D gel electrophoresis, western blots and quantitation of HO-1 expression using β-actin were conducted as described previously [30]. Cells were washed three times in ice cold phosphate-buffered saline (PBS), then lysed by addition of 1mL sodium phosphate extraction buffer (154mM NaCl, 1% w/v Triton X-100, 0.5% wt/vol sodium deoxycholate, 0.1% wt/vol sodium dodecylsulphate, 0.2% wt/vol sodium azide, 0.004% wt/vol NaF), centrifuged at 4°C and the supernatant stored at –80°C. Protein concentration was determined using EZQ protein quantitation using ovalbumin as a standard. For Western blots, 25 μg of total protein run on a 10 well SDS-PAGE gel with a dual stained molecular marker for reference. Proteins were transferred to a PVDF membrane, blocked with TBST with 5% skim milk powder and probed with a 1:1000 dilution