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Rapamycin induces the expression of heme oxygenase-1 and peroxiredoxin-1 in normal hepatocytes but not in tumorigenic liver cells

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ABSTRACT

Rapamycin (sirolimus) is employed as an immunosuppressant following liver transplant, to inhibit the re-growth of cancer cells following liver resection for hepatocellular carcinoma (HCC), and for the treatment of advanced HCC. Rapamycin also induces the expression of antioxidant enzymes in the liver, suggesting that pretreatment with the drug could provide a potential strategy to reduce ischemia reperfusion injury following liver surgery. The aim of this study was to further investigate the actions of rapamycin in inducing expression of the antioxidant enzymes heme oxygenase-1 (HO-1) and peroxiredoxin-1 (Prx-1) in normal liver and in tumorigenic liver cells. A rat model of segmental hepatic ischemia and reperfusion, cultured freshly-isolated rat hepatocytes, and tumorigenic H4IIE rat liver cells in culture were employed. Expression of HO-1 and Prx-1 was measured using quantitative PCR and western blot. Rapamycin pre-treatment of normal liver in vivo or normal hepatocytes in vitro led to a substantial induction of mRNA encoding HO-1 and Prx-1. The dose-response curve for the action of rapamycin on mRNA expression was biphasic, showing an increase in expression at 0 - 0.1 μM rapamycin but a decrease from maximum at concentrations greater than 0.1 μM. By contrast, in H4IIE cells, rapamycin inhibited the expression of HO-1 and Prx-1 mRNA. Oltipraz, an established activator of transcription factor Nrf2, caused a large induction of HO-1 and Prx-1 mRNA. The dose response curve for the inhibition by rapamycin of HO-1 and Prx-4 mRNA expression, determined in the presence of oltipraz, was monophasic with half maximal inhibition at about 0.01 μM. It is concluded that, at concentrations comparable to those used clinically, pre-treatment of the liver with rapamycin induces the expression of HO-1 and Prx-1. However, the actions of rapamycin on the expression of these two antioxidant enzymes in normal hepatocytes are complex and, in tumorigenic liver cells, differ from those in normal hepatocytes. Further studies are warranted to evaluate preconditioning...
the livers of patients subject to liver resection or liver transplant with rapamycin as a viable strategy to reduce IR injury following liver surgery.

Keywords: Liver, Ischemia reperfusion, Rapamycin, Heme oxygenase-1, Peroxiredoxin-1, Quantitative PCR
Abbreviations

IR ischemia reperfusion

HCC hepatocellular carcinoma

ROS reactive oxygen species

HO-1 heme oxygenase-1

Prx-1 peroxiredoxin-1

CoPP cobalt protoporphyrin.
1. Introduction

Liver resection and liver transplant for end stage liver disease are often associated with ischemia reperfusion (IR) injury, which may lead to slow recovery, morbidity and, in some cases, death (Cotterell and Fisher, 2015; Nagai et al., 2015; Nakamura et al., 2017; Simillis et al., 2016; van Riel et al., 2016). The potential for IR injury presently limits the ability of liver surgeons to expand the number or amount of donor livers available for liver transplants (Dutkowski et al., 2015; Sapisochin and Bruix, 2017; Zhai et al., 2013). The inclusion of steatotic livers and livers from older subjects would greatly increase available donor livers (Dasari et al., 2017; Dutkowski et al., 2015; Sapisochin and Bruix, 2017; Zhai et al., 2013). However, these livers are particularly susceptible to IR injury (Dutkowski et al., 2015; Sapisochin and Bruix, 2017; Zhai et al., 2013). Moreover, for hepatocellular carcinoma (HCC) patients undergoing liver surgery, longer times of ischemia and associated potential IR injury can lead to increased recurrence of cancer (Cho et al., 2017; Cotterell and Fisher, 2015; Nagai et al., 2015). New strategies to reduce IR injury are therefore of great benefit in liver surgery (Dutkowski et al., 2015; Sapisochin and Bruix, 2017; Zhai et al., 2013).

Intracellular signaling pathways leading to IR injury are initiated by an increase in reactive oxygen species (ROS). This occurs principally at the time of initiation of reperfusion, which is accompanied by the re-admission of oxygen to the ischemic tissue. The resulting cascade of downstream events leads ultimately to hepatocyte injury, necrosis and apoptosis, and inflammation (Datta et al., 2013; Elias-Miro et al., 2013; Prieto and Monsalve, 2017; Quesnelle et al., 2015; Reiners et al., 2014). To reduce ROS-mediated liver damage following IR, surgical ischemic pre-conditioning and pharmacological
strategies have been developed and, in some cases, trialed and used clinically (Akhtar et al., 2013; Cheng and Rong, 2017; Liu and Qian, 2015). One such pharmacological strategy is to increase the capacity of hepatocytes to remove ROS by activating, or increasing the expression of, heme oxygenase-1 (HO-1) and other antioxidant enzymes (Amersi et al., 1999; Cheng and Rong, 2017; Kato et al., 2001; Lai et al., 2008; Li et al., 2018; Liu and Qian, 2015; Nakamura et al., 2018; Tullius et al., 2002; Wang et al., 2005; Yun et al., 2010). Moreover, in the absence of any pharmacological pre-treatment, increases in the activity of HO-1 initiated by IR itself may contribute to the beneficial effects of ischemic preconditioning (Liu et al., 2014; Su et al., 2006). While ROS are detrimental to normal hepatocytes, ROS promote the proliferation of hepatocellular carcinoma cells, and there is some evidence to indicate that reduction of ROS in hepatocellular carcinoma cells may inhibit the growth of these cells (Cabré N, 2016; Font-Burgada J, 2016; Karin M, 2016; Qi et al., 2014).

We have previously shown that rapamycin (sirolimus) can induce the expression of HO-1 and another antioxidant enzyme, peroxiredoxin-1 (Prx-1), in hepatocytes (Kist et al., 2012). Other recent studies have shown that pre-treatment with rapamycin or analogues of rapamycin attenuates IR injury (Lee et al., 2016; Liu et al., 2010; Zhu et al., 2015a; Zhu et al., 2015b). Taken together, these results suggest that pre-treatment with rapamycin may offer a strategy to reduce ROS and hence IR injury following liver resection or transplant (Kist et al., 2012). Rapamycin is presently employed as an immunosuppressant following liver transplant, and in HCC patients, to inhibit cancer cell regrowth (Cholongitas et al., 2014; Ghazal et al., 2017; Liu et al., 2017; Sanchez Antolin et al., 2011; Soll and Clavien, 2011; Toso et al., 2010; Zhang et al., 2018). Thus, the potential use of rapamycin pre-treatment in patients undergoing liver resection or liver transplant has the advantage that rapamycin is already approved for clinical use as an immunosuppressant (Cholongitas et al.,
Rapamycin inhibits mTORC1 kinase, leading to, among other consequences, inhibition of cell proliferation (Dibble and Cantley, 2015). The aim of this study was to further investigate the actions of rapamycin in inducing HO-1 and Prx-1 in hepatocytes. Since, in HCC patients who might be treated with rapamycin prior to liver surgery, tumorigenic liver tissue as well as normal liver tissue would be exposed to rapamycin, the effects of the drug on HO-1 and Prx-1 expression in tumorigenic liver cells have also been investigated.

2. Materials and methods

2.1 Animals

Male rats aged between 8 and 14 weeks and weighing 270-420 g were housed and bred in a controlled environment with a 12 h light-dark cycle, with access to food and water ad libitum. 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).

2.2 Surgical procedures for ischemia and reperfusion and the measurement of rates of bile flow and blood concentrations of rapamycin

An established rat model of segmental (60-70%) hepatic ischemia and reperfusion was employed (Hasselgren et al., 1982). The bilateral median and left lateral liver lobes of Sprague Dawley rats were made ischemic, whereas the right lateral and caudate lobes
remained non-ischemic (shown schematically in Fig. 1). Sham-operated rats were treated in a similar manner except that the portal triad was not clamped and there was no liver ischemia. Surgical procedures, measurement of bile flow, collection of liver samples from both ischemic and non-ischemic liver lobes, and the measurement of blood concentrations of rapamycin were all conducted as described previously (Kist et al., 2012; Peters et al., 2010).

2.3 Rapamycin solutions

Stock solutions of rapamycin (LC Laboratories, MA, U.S.A.) were prepared in filter-sterilized absolute ethanol and stored at -20°C. For administration to rats, the rapamycin stock solution was diluted in filter-sterilized phosphate buffered saline (137 mM NaCl, 10 mM sodium phosphate, 2.7 mM KCl, pH 7.4) to create a 2.5 mg/ml working solution. Before use, solutions of rapamycin and vehicle were pre-heated to 45°C and mixed well. For the pre-treatment of rats with rapamycin, the drug (4 mg/kg body weight) or vehicle was administered by i.p. injection 24 h prior to laparotomy, under a short period of isofluorane anesthesia. For the administration of rapamycin to hepatocytes in culture, rapamycin stock in absolute ethanol was diluted in ethanol. The maximum final concentration of ethanol in cell cultures was 0.2 % (v/v). Stock solutions of cobalt protoporphyrin IX chloride (CoPP) (Frontier Scientific Inc., Logan, UT) and oltipraz (LKT Laboratories Inc. USA) were prepared in 0.1 mM NaOH containing 0.9% (w/v) NaCl and DMSO, respectively, and diluted in DMEM (Kist et al., 2012) to give the final concentrations indicated in the figures.

2.4 Isolation and culture of hepatocytes

Hepatocytes were isolated from lean Zucker rats by collagenase digestion, as described previously (Aromataris et al., 2006; Castro et al., 2009), and cultured in T-25 flasks
in 5% (v/v) CO$_2$ in air at 37°C in the presence of 8 ml of Dulbecco’s Modified Eagle’s medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal bovine serum (FBS, Trace, Melbourne, Australia). After 4 h, when the cells had attached to the flask, the medium was replaced with fresh DMEM (8 ml) containing FBS, penicillin and streptomycin. The cells were then incubated for 24 h before the administration of rapamycin or vehicle. After a further incubation for 36 h, the medium was removed, and the cells harvested for the extraction of RNA.

2.5 Culture of H4IIE liver cells

Cultures of H4IIE rat liver cells (CRL-1548 cell line, ATCC, Rockville, MD, USA) (Evans and Kovacs, 1977; Pitot et al., 1964; Reuber, 1961) were maintained in 75 cm$^2$ sterile flasks in Dulbecco’s Modified Eagles Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (0.1 mg/mL) and 10 mM HEPES in 5% (v/v) CO$_2$ (pH 7.4) at 37°C (Wilson et al., 2015). The cells were used for a maximum of 25-30 passages.

To determine the effects of rapamycin and other agents on the expression of antioxidant enzymes, H4IIE cells were sub-cultured into T-25 flasks (seeded with 2.5 x 10$^6$ cells). After 24 h, the medium was replaced with fresh DMEM (8 ml) which contained FBS, penicillin and streptomycin, together with rapamycin, cobalt protoporphyrin, oltipraz or vehicle, as indicated in the figure legends. After a further incubation for 36 h, the medium was removed, and the cells harvested for the extraction of RNA.

Differentiation of H4IIE rat liver cells was achieved by culturing the cells in T-25 flasks in DMEM supplemented with FBS (10% v/v), penicillin (100 U/ml), streptomycin (100 μg/ml), insulin (100 nM final concentration) and dexamethasone (100 nM final
concentration) for 5 to 10 days at 37°C in 5% (v/v) CO$_2$ (pH 7.4), as described previously (Jones et al., 2011).

2.6 Extraction of RNA and quantitative PCR

The extraction of RNA from frozen liver sections from ischemic and non-ischemic liver lobes and from cultured hepatocytes, the synthesis of cDNA and semi-quantitative and quantitative PCR (qPCR) were conducted as described previously (Kist et al., 2012; Wilson et al., 2011). Quantitative PCR was conducted using probe-based strategies, β-actin as reference RNA, a Rotor-Gene 3000 (Corbett) and the ΔΔCT method (Wilson et al., 2011). Primers and fluorescent probes were synthesised by Geneworks, Adelaide, Australia, based on published sequences (Table 1). Triplicate incubations were conducted for each experimental condition. For qPCR, the cDNA from each RNA extract derived from liver tissue or cells in culture was amplified in triplicate, and the mean value determined.

Quantitative PCR was employed as the principle technique for measurement of the expression of HO-1 because it offers more precise quantitation than measurement of protein expression by western blot and can more readily be applied to the assay of multiple samples generated in dose-response experiments. Other studies of HO-1 expression in liver have shown a good correlation between results obtained using qPCR and those obtained using western blot (Atef et al., 2017; Ge et al., 2017).

2.7 Western blots
Western blots were performed as described previously (Wilson et al., 2015; Wilson et al., 2011). Proteins were separated by electrophoresis on a stain-free Bio-Rad AnyKD gel (mini gel, 10 wells per gel) (30 μg protein per lane) and transferred to PVDF membranes using a Trans-Blot® Turbo™ RTA Mini LF PVDF Transfer Kit. Stain-Free imaging of total protein and chemiluminescent imaging of the protein of interest on the PVDF membrane were conducted using a Bio-Rad Gel-Doc EZ Imager. Band intensities were quantified using Image Studio Lite software (LI-COR Biosciences). Local background was subtracted, and the value for a given band (Oatp2 or Mrp2) was normalised to the total protein on the membrane (Colella et al., 2012; Gilda and Gomes, 2013; Gurtler et al., 2013; Wilson et al., 2016). The origins and dilutions of the primary and secondary antibodies employed for the detection of HO-1 were: mouse monoclonal (heme oxygenase 1 (F-4) sc-390991, Santa Cruz Biotechnology, Dallas, Texas, USA) 1:100 with secondary antibody Donkey anti mouse-HRP 1:1000.

2.8 Statistical analysis

Results are expressed as means ± SEM. For the analysis of relative expression of mRNA, Student’s t-test or ANOVA followed by Post Hoc Tukey’s test was used. Means of two groups were compared using Student’s t-test. When the treatment groups were more than two, ANOVA was used for the comparison of the mean value. Statistical significance was confirmed when P values were less than 0.05. Equations were fitted to the experimental data in dose-response curves using Graphpad Prism 5 software.

3. Results
3.1 Rapamycin induces the expression of mRNA encoding heme oxygenase-1 and peroxiredoxin-1 in liver and in cultured hepatocytes

To investigate the effects of pre-treatment with rapamycin on the expression of HO-1 and Prx-1 in normal liver in the absence of IR, and in normal liver subsequently subjected to IR, rapamycin or vehicle was administered to rats by i.p. injection. After 24 h the bilateral median and left lateral liver lobes were subjected to segmental hepatic ischemia and reperfusion whereas the right lateral and caudate lobes remained non-ischemic (shown schematically in Fig. 1). At the end of the reperfusion period, samples were collected from both the bilateral median and left lateral (ischemic) lobes and the right lateral and caudate (non-ischemic) lobes, and the relative expression of mRNA encoding HO-1 and Prx-1 determined by qPCR. The concentration of rapamycin in the blood at the end of the 24 h period was 20 ± 4 nM (mean ± SEM, n = 4). In liver lobes pre-treated with rapamycin and subjected to normal blood flow, expression of mRNA encoding HO-1 and Prx-1 was increased 5-fold and 7-fold, respectively, compared to that in liver lobes pre-treated with vehicle and subject to normal blood flow (Fig. 2 (A,B), Non IR Rapamycin compared with Non IR Vehicle). In liver lobes pre-treated with rapamycin then subjected to IR, expression of both HO-1 and Prx-1 mRNA was not changed compared to that in liver lobes pre-treated with vehicle (Fig. 2 (A,B), IR Rapamycin compared with IR Vehicle). The effect of pre-treatment with rapamycin on expression of the HO-1 protein was also determined using western blot. Western blots with anti-HO-1 showed a principle band at about 35 kDa (Fig.3A). The reported size for HO-1 on western blot is 32 kDa (Ge et al., 2017; Kutty et al., 1995). The amount of HO-1 protein was increased about 8-fold in liver lobes pre-treated with
rapamycin and subject to normal blood flow (Fig. 3, Rapamycin Non IR compared with Vehicle Non IR).

The effects of IR alone (in the absence of pre-treatment with rapamycin) are also shown in Fig. 2 (A, B). In liver lobes pre-treated with vehicle 24h before being subjected to IR, the expression of each of HO-1 and Prx-1 mRNA was increased 4-fold compared to that in liver lobes pre-treated with vehicle 24h before being subjected to normal blood flow (Fig. 2 (A,B), IR Vehicle compared with Non IR Vehicle), as shown previously (Su et al., 2006; Wilson et al., 2011). (Previous studies on the effect of pre-treatment with rapamycin or with vehicle on bile flow recovery during reperfusion following ischemia have provided no evidence that pre-treatment with vehicle alone alters bile flow recovery following ischemia. Therefore, that it is unlikely that pre-treatment with vehicle alone alters the expression of mRNA encoding HO-1 and Prx-1.) The increase in HO-1 and Prx-1 mRNA expression caused by IR alone was less than that caused by rapamycin alone (Fig. 2 (A,B), IR Vehicle compared with Non IR Rapamycin). IR alone (in the absence of pre-treatment with rapamycin) also increased expression of the HO-1 protein, assessed by western blot (Fig. 3, Vehicle IR compared with Vehicle Non IR). Surprisingly, subjecting liver lobes to IR after pre-treatment with rapamycin ablated some of the increase in HO-1 and Prx-1 mRNA expression induced by pre-treatment with rapamycin (Fig. 2 (A,B), IR Rapamycin compared with Non IR Rapamycin).

The actions of rapamycin on the expression of HO-1 and Prx-1 were further investigated using rat hepatocytes in primary culture. Pre-treatment with 0.1 μM rapamycin caused a 3-fold increase in the expression of mRNA encoding each of HO-1 and Prx-1, compared to the vehicle control (Fig. 2 (C,E)). However, at a concentration of 0.5 μM, rapamycin, there was no significant increase in expression of HO-1 or Prx-1 mRNA compared to vehicle (Fig. 2 (C,E)). The dose-response curves for the action of rapamycin on
HO-1 and Prx-1 mRNA expression were each biphasic (Fig. 2 (D,F)). At concentrations from zero to 0.1 μM, a marked increase in expression of HO-1 and Prx-1 mRNA was observed, with a concentration of rapamycin of about 0.05 μM giving half-maximal increase (S₀.₅) (Fig 1(D,F)). At concentrations of rapamycin above 0.1 μM, induction of the expression of antioxidant enzyme mRNA decreased relative to the maximum achieved at 0.1 μM. At 5 μM rapamycin the expression of each of HO-1 and Prx-1 was about the same as that in untreated cells. However, overall it can be seen that, compared to the levels in untreated cells, rapamycin increased the expression of HO-1 and Prx-1 mRNA over the range zero to 1 μM rapamycin.

3.2 Rapamycin inhibits the expression of mRNA encoding heme oxygenase-1 and peroxiredoxin-1 in tumorigenic H4IIE rat liver cells

To investigate the ability of rapamycin to induce expression of HO-1 and Prx-1 in tumorigenic liver cells, we used the tumorigenic H4IIE rat liver cell line, which was originally derived from the rat H-35 Reuber hepatoma (Evans and Kovacs, 1977; Pitot et al., 1964; Reuber, 1961). Compared to cultured isolated hepatocytes, which are differentiated, cultured H4IIE cells are undifferentiated cells. In cultured H4IIE cells, the expression of mRNA encoding both HO-1 and Prx-1 was considerably lower than that in cultured rat hepatocytes (Fig. 4 A,B). In contrast to the results obtained with rat hepatocytes, the treatment of H4IIE cells with rapamycin at 0.1 and 0.5 μM inhibited the expression of HO-1 mRNA, although the degree of inhibition of HO-1 expression at 0.5 μM rapamycin was less than that at 0.1 μM rapamycin (Fig. 5A). Cobalt protoporphyrin (CoPP), an established inducer of HO-1 in liver (Tullius et al., 2002), caused a small, but not significant, increase in HO-1 mRNA expression in H4IIE cells (Fig 5 A). In these cells, rapamycin at 0.1 μM did not alter Prx-1
expression, and at 0.5 μM caused a small decrease in Prx-1 mRNA expression (Fig 5 C).

We have previously shown that incubation of tumorigenic H4IIE rat liver cells with insulin and dexamethasone for 5 to 10 days induces the cells to regain some characteristics of normal hepatocytes, including the expression of ATP-responsive P2Y receptors on the plasma membrane (Jones et al., 2011). Studies by others on the effects of insulin and dexamethasone on H4IIE cells have shown that the actions of these agents over several days include an increase the activity and amount of phosphofructokinase, expression of insulin-like growth factor receptor-1, and expression of the transcription factor c-fos (Messina et al., 1992; Vargas et al., 1994; Zahradka et al., 1998). Taken together, these observations indicate that the pre-treatment of H4IIE cells with insulin and dexamethasone induces a degree of differentiation in H4IIE cells.

Since the effects of rapamycin on expression of HO-1 and Prx-1 mRNA in H4IIE cells were the opposite of those in rat hepatocytes, we investigated whether the differentiation of H4IIE cells would alter the actions of rapamycin on HO-1 and Prx-1 expression. Compared to untreated cells, H4IIE cells pre-treated with insulin and dexamethasone, as described in Methods and Materials, were larger, more round in shape, exhibited a more clearly-defined nucleus, and glittered more noticeably, indicating the presence of lipid droplets. Differentiation of H4IIE cells caused a 3-fold increase in HO-1 mRNA expression and an 8-fold increase in Prx-1 expression, compared to that in undifferentiated H4IIE cells (Fig. 4). However, differentiation did not alter the actions of rapamycin on the expression of mRNA encoding HO-1 or Prx-1, compared to the effects of the drug on undifferentiated cells. Thus, in differentiated H4IIE cells, rapamycin inhibited HO-1 and Prx-1 expression, as it did in undifferentiated H4IIE cells (Fig 5 B compare with 5A, and 5 D compare with 5 C). Cobalt protoporphyrin caused a small but not significant increase in HO-1 mRNA expression
in differentiated H4IIE cells (Fig 5 A), similar to its actions on undifferentiated H4IIE cells.

It has previously been shown that the transcription factor Nrf2 is one of the main mediators of induction of the expression of HO-1 in liver (Ishii et al., 2000; Kim et al., 2007; Nguyen et al., 2009a; Paine et al., 2010). To evaluate whether Nrf2 mediates the actions of rapamycin in altering the expression of HO-1, we compared the actions of oltipraz, an established activator of Nrf2 (Ramos-Gomez et al., 2001), with those of rapamycin on H4IIE cells. At 25 and 50 μM, oltipraz caused a large induction of HO-1 mRNA expression and a modest induction of Prx1 mRNA expression (Fig 6 A,C). For each of HO-1 and Prx-1 the dose response curve for oltipraz was monophasic with an S_{0.5} value of about 50 μM (Fig 6 B,D). We used the ability of oltipraz to induce the expression of HO-1 and Prx-1 to further investigate the inhibitory action of rapamycin on the expression of these enzymes in H4IIE cells. In cells incubated in the presence of oltipraz (to maximally increase expression of HO-1 and Prx-1) rapamycin at 0.1 and 0.5 μM substantially inhibited HO-1 and Prx-1 mRNA expression (Fig 7 A,C). Dose response curves are shown in Figures 7B and 7D. Maximal inhibition was observed at the lowest rapamycin concentration tested, 0.01 μM. Thus, in cultured tumorigenic H4IIE rat liver cells incubated in the presence of oltipraz, rapamycin in the concentration range 0-0.1 μM, inhibited the expression of mRNA encoding HO-1 and Prx-1, whereas in rat hepatocytes in culture, rapamycin in this concentration range increased expression of mRNA encoding these enzymes (Fig. 7B,D compare with Fig 2D,F).

4. Discussion
The aim of this study was to investigate the actions of rapamycin on the expression of HO-1 and Prx-1 in hepatocytes and in tumorigenic liver cells. The main findings can be summarized as follows. In liver in vivo and in normal hepatocytes cultured in vitro, pre-treatment with rapamycin caused a large induction of HO-1 and Prx-1, with maximum induction at 0.1 μM rapamycin. The dose-response curve was biphasic, whereby at rapamycin concentrations higher than 0.1 μM, the induction was reversed, although some increase in mRNA expression, relative to that in untreated cells, was still observed at 1.0 μM rapamycin. In tumorigenic liver cells, the basal level of expression of HO-1 and Prx-1 mRNA was substantially lower than that in normal hepatocytes. Moreover, in contrast to its action on normal hepatocytes, pre-treatment of tumorigenic liver cells with rapamycin decreased expression of the antioxidant enzyme mRNA. Oltipraz, an activator of the transcription factor Nrf2 (Ramos-Gomez et al., 2001), induced the expression of HO-1 and Prx-1 mRNA in tumorigenic liver cells. In tumorigenic liver cells incubated in the presence of oltipraz, maximal inhibition by rapamycin of the expression of HO-1 and Prx-1 mRNA was observed at 0.01 μM rapamycin.

Our previous study provided some evidence that the pre-treatment of livers with rapamycin induces the expression of HO-1 and Prx-1 (Kist et al., 2012). The present results define the dose-response curve for the induction of HO-1 and Prx-1 expression by rapamycin in normal hepatocytes. While, in the majority of the experiments, expression of mRNA encoding HO-1 and Prx-1 was measured, western blot confirmed for HO-1 the key observation that the expression of this protein is increased in liver following pre-treatment with rapamycin. Moreover, previous studies on the expression of HO-1 and Prx-1 have shown a good correlation between results obtained with qPCR and western blot (Atef et al., 2017; Ge et al., 2017).
Several other interesting observations have arisen from the present study. These include the biphasic dose-response curve for the action of rapamycin on normal hepatocytes, attenuation of the rapamycin-induced increase in HO-1 and Prx-1 expression in livers by subsequent IR, the absence of any activation component in the action of rapamycin on tumorigenic liver cells, and the difference in the concentration of rapamycin which gave half maximal inhibition of mRNA expression in normal hepatocytes (about 0.5 μM) compared to that which gave half maximal inhibition of mRNA expression in tumorigenic liver cells (about 0.01 μM). It is also noteworthy that both HO-1 and Prx-1 responded to rapamycin in the same way in each of the conditions tested.

The intracellular signaling mechanisms involved in the induction by rapamycin of HO-1 and Prx-1 in normal hepatocytes most likely involve the transcription factor Nrf2. This is the major transcription factor which regulates the expression of HO-1, Prx-1, and several other antioxidant enzymes (Ishii et al., 2000; Kim et al., 2007; Nguyen et al., 2009b; Paine et al., 2010; Xu et al., 2017). Moreover, there is evidence that rapamycin can indirectly activate Nrf2. Thus, it has been shown that the inhibition of mTORC1 kinase by rapamycin leads to the inhibition of S6 kinase, which in turn, results in indirect activation of the phosphoinositide 3-kinase (PI3K)/ serine/threonine kinase protein kinase B (AKT) pathway and the activation of Nrf2 (Klempner et al., 2013; Yao et al., 2016; Zhang et al., 2016a; Zhang et al., 2016b). The observation that oltipraz, an established activator of Nrf2 (Weerachayaphorn et al., 2009), increases the expression of HO-1 and Prx-1 mRNA provides some evidence for the involvement of Nrf2 in induction of the expression of HO-1 and Prx-1 in hepatocytes and tumorigenic liver cells. The observation that rapamycin inhibits the ability of oltipraz to increase the expression of HO-1 and Prx-1 mRNA suggests that rapamycin activates another pathway which decrease or inhibits the activation of Nrf2 and/or alters the concentration of another transcription factor which inhibits the expression of HO-1 and Prx-1.
independent of Nrf2. Such a putative mechanism might also explain why rapamycin induction of HO-1 and Prx-1 mRNA is attenuated by subsequent IR. Further experiments are warranted to define the mechanisms involved, and the reasons underlying the difference in the dose response curves for the inhibition by rapamycin of HO-1 and Prx-1 mRNA expression in normal hepatocytes compared with tumorigenic liver cells.

It has previously been shown that pre-treatment of livers with rapamycin provides protection against IR injury (Lee et al., 2016; Liu et al., 2010; Zhu et al., 2015a; Zhu et al., 2015b). The mechanisms involved are thought to include reduction of endoplasmic reticulum stress, enhanced autophagy and activation of the mammalian target of rapamycin complex 2 (mTOR2) pathway (Liu et al., 2010; Zhu et al., 2015a; Zhu et al., 2015b). The present results suggest that an additional, or complimentary, mechanism may involve induction of the synthesis of the antioxidant enzymes HO-1 and Prx-1. Moreover, the observation that the rapamycin-induced increase in HO-1 and Prx-1 expression in isolated hepatocytes in culture is comparable to that in liver in vivo indicates that a substantial proportion of the enzyme induction observed in liver in vivo is due to induction of these enzymes in hepatocytes rather than other cell types present in liver. Elevated expression of HO-1 and Prx-1 would increase the capacity of hepatocytes to remove ROS formed at the beginning of liver reperfusion and reduce endoplasmic reticulum stress during reperfusion (Bozaykut P, 2016; Pagliassotti MJ, 2016). In this connection, it is interesting to note that one recent study has provided evidence that rapamycin can decrease the production of ROS in liver (Martinez-Cisuelo et al., 2016).

The concentration of rapamycin that gave half-maximal induction of the expression of HO-1 and Prx-1 mRNA in normal hepatocytes was 0.05 μM, with maximal induction at about 0.1 μM rapamycin. While, at rapamycin concentrations higher than 0.1 μM, the degree of enzyme induction decreased, elevated expression of mRNA encoding the anti-oxidant enzymes (relative to untreated cells) was observed at concentrations of rapamycin up to 1.0
µM. In liver transplant patients treated with rapamycin, the blood concentration of the drug is about 0.005-0.01 µM (5-10 nM). Thus, the pre-treatment of patients with rapamycin at doses currently employed for immunosuppression would be predicted to lead to elevated expression of HO-1 and Prx-1 in hepatocytes. The results of a recent study also suggest that maintenance of adequate levels of HO-1 in the liver following a liver transplant reduces post-transplant deterioration of liver function (Nakamura et al., 2017). Rapamycin pre-treatment of the livers of patients undergoing liver resection or liver transplant, or the pre-treatment of donor livers, in order to reduce IR injury might be considered for use in combination with one or more drugs targeting other sites in the pathways which mediate or enhance IR injury.

Increased expression of HO-1 and Prx-1 in livers subject to IR in the absence of any pre-treatment likely represents the activation of endogenous mechanisms to protect the liver from IR injury. Increases in expression and activity of HO-1, Prx-1 and other antioxidant enzymes are thought to mediate protection against IR injury in ischemic pre-conditioning (Liu et al., 2014; Liu et al., 2016). While subsequent IR in livers pre-treated with rapamycin attenuated the rapamycin-induced increase in HO-1 and Prx-1 mRNA, in livers pre-treated with rapamycin expression of these antioxidant enzymes was still increased about 4-5 fold compared to untreated liver at the end of the reperfusion period. Moreover, in the context of the potential ability of elevated levels of HO-1 and Prx-1 to protect against IR injury, the level of these enzymes at the beginning of the reperfusion is likely to be critical. Thus, in livers subject to IR, the generation of high levels of ROS, an increase in hepatocyte intracellular Ca\(^{2+}\) concentrations, and onset of apoptotic and necrotic pathways leading to injury occurs principally at the time of unclamping of the portal triad and re-admission of blood and oxygen (Elias-Miro et al., 2013; Han et al., 2018; Quesnelle et al., 2015). Pre-treatment with rapamycin leads to an increase in expression of antioxidant enzymes before or
at this time point, whereas IR alone leads to an increase in expression of antioxidant enzymes at a later time point.

While an increase in ROS is detrimental to normal hepatocytes, several studies indicate that ROS promote the survival and proliferation of tumorigenic liver cells (Cabré N, 2016; Font-Burgada J, 2016; Karin M, 2016). Moreover, interventions that decrease ROS reduce the proliferation of tumorigenic liver cells (Qi et al., 2014). We found that the expression of HO-1 and Prx-1 in tumorigenic H4IIE rat liver cells is substantially lower than that in normal rat hepatocytes. Moreover, pre-treatment with rapamycin led to a further decrease in HO-1 and Prx-1 expression in H4IIE cells. While the differentiation of H4IE cells increased the basal expression of HO-1 and Prx-1, it did not alter the effects of rapamycin, which inhibited expression of HO-1 and Prx-1 in differentiated as well as in undifferentiated H4IIE cells. These results suggest that the capacity of tumorigenic rat liver cells to remove ROS may be lower than that of normal rat hepatocytes. Moreover, treatment with rapamycin would further reduce the capacity of tumorigenic liver cells to remove ROS. If this situation applies in human HCC, these actions of rapamycin would not be relevant to the pharmacological treatment of livers before liver surgery for HCC since the tumorigenic liver tissue is surgically removed. However, they would be relevant to any HCC patient receiving rapamycin in the absence of surgical removal of the tumorigenic tissue. The level of expression of HO-1 and Prx-1 in tumorigenic liver may depend on several factors, including the mutations involved in the onset and progression of HCC and the stage of HCC. Thus, several studies have reported an increase, or no change in, the expression of Prx-1 in human HCC liver tissue compared with that in normal liver, and no change in expression of HO-1 (Aguilar-Melero et al., 2013; Cheng et al., 2015; Song et al., 2009; Sun et al., 2014).

Oltipraz has been shown to inhibit the growth of HCC cells and is being evaluated in clinical trials as a potential anti-cancer drug for HCC (Mann et al., 2009; Piton et al., 2005;
Yates and Kensler, 2007). The present observation that pre-treatment of tumorigenic liver cells with oltipraz causes a large induction of HO-1 and Prx-1 suggests that one mechanism by which oltipraz inhibits the growth of liver cancer may involve the reduction of ROS. This would be expected to reduce the downstream actions of ROS in promoting the progression of HCC.

5. Conclusion

It is concluded that, at concentrations comparable to those used clinically, pre-treatment of the liver with rapamycin induces the expression of HO-1 and Prx-1. However, the actions of rapamycin on the expression of these two antioxidant enzymes in normal hepatocytes are complex and, in tumorigenic liver cells, differ from those in normal hepatocytes. Further studies are warranted to determine the mechanisms involved in the action of rapamycin on the expression of antioxidant enzymes, and to evaluate the possibility that preconditioning the livers of patients subject to liver resection or liver transplant with rapamycin could provide a viable strategy to reduce IR injury following liver surgery.

Conflicts of interest

The authors declare no conflict of interest

Acknowledgements

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Fig. 1. Schematic representation of (A) the established rat model of segmental hepatic ischemia and reperfusion employed in the present study and (B) the protocol employed for the administration of rapamycin to rat liver. In the rat model of segmental hepatic ischemia, the bilateral median and left lateral lobes were made ischemic while the right lateral and caudate lobes remained non-ischemic (Hasselgren et al., 1982). This gives about 60-70% liver ischemia. Locations of the common bile duct (1), the bile duct cannula (4) and surrounding ligature (5), the hepatic artery (2), portal vein (3), and the position of the microvascular clamp (6) are indicated schematically. Ischemia time was 45 min and the time of reperfusion 90 min.

Fig. 2. Rapamycin induces the expression of heme oxygenase-1 (HO-1) and peroxiredoxin-1 (Prx-1) in liver in vivo and in rat hepatocytes in culture. (A,B) Effects of pre-treatment with rapamycin (Rapa) or vehicle (Veh) on the expression of mRNA encoding HO-1 (A) and Prx-1 (B) in rat liver lobes subject to normal blood flow (open bars, Non IR) or to ischemia and reperfusion (solid bars, IR). Rapamycin (4 mg/kg body weight) or vehicle was administered to rats by i.p. injection (time sequence shown schematically in Fig. 1B). After 24 h the rat was anesthetised and the bilateral median and left lateral (superior) lobes were made ischemic for 45 min followed by reperfusion for 90 min, whereas the right lateral and caudate lobes received normal blood flow. At the end of the reperfusion period, liver samples were collected from each lobe, frozen, and RNA subsequently extracted. (C,E). Effects of pre-treatment with rapamycin on the expression of mRNA encoding HO-1 (C) and Prx-1 (E) in cultured rat hepatocytes. Freshly-isolated rat hepatocytes were incubated in the presence of 0.1 or 0.5 μM rapamycin (Rapa) or with vehicle for 36 h. RNA was then extracted. (D,F).
Dose-response curves for the effects of rapamycin on the expression of mRNA encoding HO-1 (D) and Prx-1 (F) in cultured rat hepatocytes. The relative expression of mRNA encoding HO-1 and Prx-1 was measured using qPCR. Results show mRNA expression (normalized to β-actin expression) and referred to the value for the vehicle inferior lobe. The values in A and B are the means ± SEM (n = 4 separate livers). The values in C-F are the means ± SEM (n = 9, 3 individual hepatocyte preparations made on separate days). Degrees of significance, determined using ANOVA with post hoc contrast or Students t-test for unpaired samples, for comparison of rapamycin with vehicle for the non-ischemic and ischemic lobes, and for comparison of rapamycin with vehicle, are * P<0.05, ** P<0.01, and *** P<0.001.

**Fig. 3.** Effects of pre-treatment with rapamycin (i.p. injection 24 h) or of ischemia reperfusion in the absence of pre-treatment with rapamycin, on the expression of heme oxygenase-1 (HO-1) in the liver *in vivo*, assessed by western blot. Livers were pre-treated with rapamycin or vehicle, then subjected to sham operation (Rapamycin Non IR, lanes 5 to 8 and Vehicle Non IR, lanes 1 to 4), or were pre-treated with vehicle, and subject to ischemia for 30 min and reperfusion for 45 min (Vehicle IR, lanes 9 to 11). A) Chemiluminescence images of western blots showing HO-1 (35 kDa). A section of the Stain-Free protein image of the PVDF membrane after transfer of protein from the gel to the membrane (Protein loading control) is shown as a visual loading control only. B) Results expressed as band intensity (normalised to total protein on the membrane). Means ± SEM (n = 4 separate livers).

**Fig. 4.** Relative expression of heme oxygenase-1 (HO-1) (A) and peroxiredoxin-1 (Prx-1) (B) in cultured tumorigenic H4IIE rat liver cells compared to that in cultured rat hepatocytes. H4IIE cells, H4IIE cells differentiated by incubation with insulin and dexamethasone, or
freshly-isolated rat hepatocytes were incubated for 36 h following subculture. RNA was then isolated and the relative expression of mRNA encoding HO-1 and Prx-1 was measured using qPCR. Results show mRNA expression normalized to β-actin expression and referred to the undifferentiated H4IIE cells. The results are the means ± SEM (n = 9, 3 individual hepatocyte preparations conducted on separate days or 3 individual H4IIE cell cultures). Degrees of significance, determined using ANOVA with post hoc contrast or Students t-test for comparison of the values for hepatocytes and differentiated H4IIE cells with those of H4IIE cells, are ** P<0.01, and *** P<0.001.

Fig. 5. Rapamycin inhibits the expression of heme oxygenase-1 (HO-1) and peroxiredoxin-1 (Prx-1) in tumorigenic H4IIE rat liver cells. Effects of pre-treatment with rapamycin on the expression of mRNA encoding HO-1 (A,B) and Prx-1 (C,D) in normal H4IIE cells (A,C), and in H4IIE cells differentiated by pre-treatment with insulin and dexamethasone as described in Materials and Methods (B,D). H4IIE cells, or H4IIE cells differentiated by pre-treatment with insulin and dexamethasone, were incubated for 36 h in the presence of 0.1 or 0.5 μM rapamycin (Rapa), cobalt protoporphyrin (CoPP) or with vehicle. RNA was then isolated and the relative expression of mRNA encoding HO-1 and Prx-1 measured using qPCR. Results show mRNA expression normalized to β-actin expression and referred to the “no addition” control. The results are the means ± SEM (n = 9, 3 individual cell incubations). Degrees of significance, determined using ANOVA with post hoc contrast or Students t-test for comparison of rapamycin or CoPP with vehicle are * P<0.05, and *** P<0.001.

Fig. 6. Oltipraz induces the expression of heme oxygenase-1 (HO-1) and peroxiredoxin-1 (Prx-1) in tumorigenic H4IIE rat liver cells.
(A,C). Effects of pre-treatment with oltipraz (25 or 50 μM) on the expression of mRNA encoding HO-1 (A) and Prx-1 (C) in H4IIE cells. (B,D). Dose-response curves for the effects of oltipraz on the expression of mRNA encoding HO-1 (B) and Prx-1 (D) in cultured H4IIE cells. Cells were incubated in the presence of oltipraz (Olti) or vehicle for 36 h. RNA was then isolated and the relative expression of mRNA encoding HO-1 and Prx-1 measured using qPCR. Results show mRNA expression normalized to β-actin expression and referred to the “no addition” control. The results are the means ± SEM (n = 9, 3 individual cell incubations). Degrees of significance, determined using ANOVA with post hoc contrast or Students t-test for unpaired samples, for comparison of oltipraz with vehicle are * P<0.05 and *** P<0.001. The solid lines in B and D represent lines of best fit to the experimental data.

Fig. 7. Inhibition by rapamycin of the expression of heme oxygenase-1 (HO-1) and peroxiredoxin-1 (Prx-1) in tumorigenic H4IIE rat liver cells co-incubated in the presence of oltipraz. (A,C). Effects of pre-treatment with rapamycin (0.1 or 0.5 μM) in the presence of oltipraz (Olti) (50 μM) on the expression of mRNA encoding HO-1 (A) and Prx-1 (C). (B,D). Dose-response curves for the effects of rapamycin on the expression of mRNA encoding HO-1 (B) and Prx-1 (D) in H4IIE cells incubated in the presence of oltipraz (50 μM). H4IIE cells were incubated for 36 h in the presence of rapamycin, oltipraz, rapamycin plus oltipraz, or vehicle. RNA was then isolated and the relative expression of mRNA encoding HO-1 and Prx-1 measured using qPCR. Results show mRNA expression normalized to β-actin expression and referred to the “no addition” control. The results are the means ± SEM (n = 9, 3 separate cell incubations). Degrees of significance in, determined using ANOVA with post hoc contrast or Students t-test for comparison of rapamycin with vehicle are ** P<0.01 and *** P<0.001.
Table 1. Sequences of primers and probes used to measure expression of mRNA encoding HO-1, Prx-1 and bile acid transporters

<table>
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<tr>
<th>Name</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Probe sequence</th>
<th>Reference</th>
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<td>β-Actin</td>
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<td>CGCTCGGTCAG</td>
<td>FAM-</td>
<td>(Wilson et al., 2011)</td>
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<td></td>
<td>GAGGGTTACG</td>
<td>GATCTTCA</td>
<td>CTGGCCGGG</td>
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<td></td>
<td></td>
<td></td>
<td>ACCTGACAG</td>
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<td></td>
<td>ACTACCTC-BHQ</td>
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</tr>
<tr>
<td>HO-1</td>
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<td>CTGGCTTTGTG</td>
<td>FAM-</td>
<td>(Kist et al., 2012)</td>
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Highlights

- Rapamycin induces expression of heme oxygenase-1 and peroxiredoxin-1 in hepatocytes
- Biphasic dose-response curve for antioxidant enzyme induction in normal hepatocytes
- Rapamycin inhibits expression of the antioxidant enzymes in tumorigenic liver cells
- Rapamycin pre-treatment may provide strategy to reduce liver ischemia reperfusion injury
Administration of rapamycin (i.p.) 24h before commencing ischemia-reperfusion.
Figure 3

A

B
Figure 5

A

H4IIE cells
HO-1

B

Differentiated H4IIE cells
HO-1

C

H4IIE cells
Prx-1

D

Differentiated H4IIE cells
Prx-1

Relative expression (HO-1)

Relative expression (Prx-1)

No addition, Veh (CoPP), CoPP (10 μM), Veh (Rapa), Rapa (0.1 μM), Rapa (0.5 μM)

No addition, Veh (CoPP), CoPP (10 μM), Rapa (veh), Rapa (0.1 μM), Rapa (0.5 μM)

No Addition, Vehicle, Rapa 0.1 μM, Rapa 0.5 μM

No Addition, Vehicle, Rapa 0.1 μM, Rapa 0.5 μM
Figure 6

(A) H4IIE cells
HO-1

(B) H4IIE cells
HO-1

(C) H4IIE cells
Prx-1

(D) H4IIE cells
Prx-1

Relative expression (HO-1)

Relative expression (Prx-1)

Oltipraz concentration (µM)
Figure 7

**Panel A**: H4IIE cells

![Graph showing relative expression (HO-1) for different treatments](image)

- No Addition
- Veh (Rapa)
- Veh (OTZ)
- OTZ 50µM
- Rapa 0.1µM + OTZ 50µM
- Rapa 0.5µM + OTZ 50µM

**Panel B**: H4IIE cells

![Graph showing relative expression (HO-1) vs. varying Rapamycin concentrations](image)

**Panel C**: H4IIE cells

![Graph showing relative expression (Prx-1) for different treatments](image)

- No Addition
- Veh (Rapa)
- Veh (OTZ)
- OTZ 50µM
- Rapa 0.1µM + OTZ 50µM
- Rapa 0.5µM + OTZ 50µM

**Panel D**: H4IIE cells

![Graph showing relative expression (Prx-1) vs. varying Rapamycin concentrations](image)