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Molecular hydrogen attenuates hypoxia/reoxygenation injury of intrahepatic cholangiocytes by activating Nrf2 expression

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HIGHLIGHTS

- H_2 ameliorates cholangiocytes H/R injury *in vitro*.
- Nrf2 signaling is activated in cholangiocytes after exposed to H_2.
- H_2 could not protect Nrf2-silenced cholangiocytes from H/R injury.
- H_2 protects cholangiocytes from I/R injury through *in vivo*.

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ABSTRACT

Hypoxia/reoxygenation (H/R) injury of cholangiocytes causes serious biliary complications during hepatobiliary surgeries. Molecular hydrogen (H_2) has been shown to be effective in protecting various cells and organs against oxidative stress injury. Human liver cholangiocytes were used to determine the potential protective effects of hydrogen against cholangiocyte H/R injury and explore the underlying mechanisms. We found that H_2 ameliorated H/R-induced cholangiocytes apoptosis. Our study revealed that H_2 activated NF-E2-related factor 2 (Nrf2) and downstream cytoprotective protein expression. However, the protective function of H_2 was abolished when Nrf2 was silenced. Apoptosis in cholangiocytes isolated from a rat model of liver ischemia/reperfusion injury indicated that H_2 significantly attenuates ischemia/reperfusion cholangiocyte injury *in vivo*. In conclusion, our study shows that H_2 protects intrahepatic cholangiocytes from hypoxia/reoxygenation-induced apoptosis *in vitro* or *in vivo*, and this phenomenon may depend on activating Nrf2 expression.

1. Introduction

Ischemia/reperfusion (I/R) injury is a severe deleterious postsurgical complication in liver transplantation, partial hepatic resection and trauma settings (Liu et al., 1996). Hypoxia/reoxygenation (H/R) is the root of I/R injury and has been regarded as an important model to study the pathophysiology of I/R injury *in vitro* (Monno et al., 2010; Pardo and Tirosh, 2009). During hepatobiliary surgeries, hepatic I/R injury or H/R injury is caused by interruption of hepatic blood inflow, which result in massive liver parenchyma injury (Jaeschke, 2003). Several studies have demonstrated that generation of reactive oxygen species (ROS), proinflammatory cytokines and oxidants are associated with hepatic I/R or H/R injury (Abu-Amara et al., 2010; Ren et al., 2011; Jaeschke, 1991; Jaeschke, 1998).

Abbreviations: H_2, molecular hydrogen; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; ROS, reactive oxygen species; Nrf2, NF-E2-related factor 2; HRM, hydrogen-rich medium; HRS, hydrogen-rich saline; NS, normal saline; BCL2, B-cell CLL/lymphoma 2; BAX, BCL2-associated X protein; HO-1, heme oxygenase 1; CK7, keratin 7; CK19, keratin 19; AQP1, aquaporin 1; EPCAM, epithelial cell adhesion molecule; Icam1, intercellular adhesion molecule 1; ALB, albumin; G6PC, glucose-6-phosphatase; Nqo1, nicotinamide adenine dinucleotide phosphate (NADPH) quinine oxidoreductase 1; GSTA2, glutathione S-transferase alpha 2; Prdx1, peroxiredoxin 1; MnSOD, manganous superoxide dismutase; SOD, superoxide dismutase; MDA, malondialdehyde.

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Regulation of ROS and proinflammatory cytokines is suggested to be an important therapeutic strategy for hepatic H/R injury.

Cholangiocytes, also known as biliary epithelial cells, are an important cell type in the liver parenchyma, in addition to hepatocytes. As a kind of important but delicate cells, cholangiocytes are also sensitive to oxidative stress and involved in the process of cholangiopathies (Mizuguchi et al., 2014; Brain et al., 2013). Although an increasing number of studies have investigated intracellular mechanisms and therapeutic targets to prevent H/R-induced hepatocyte injury (Pardo and Tirosh, 2009; Chen et al., 2012; Spencer et al., 2013), only a few have focus on H/R-induced cholangiocyte injury. It's worth noting that H/R injury also induces cholangiocytes necrosis or apoptosis and results in serious complications, such as biliary obstruction or stricture (Sanchez-Urdazpal et al., 1992; Xu et al., 2004).

Several studies have demonstrated that molecular hydrogen (H_2) is an important gaseous signaling molecule with antioxidant and anti-inflammatory protective effects on cells and organs (Huang et al., 2010). H_2 can act as a scavenger to selectively alleviate ROS and activate potent cellular protective effects. Recent research has revealed that H_2 protected tissue from oxidative stress injury via activating NF-E2-related factor 2 (Nrf2) expression (Kawamura et al., 2013). H_2 has been shown to be effective in protecting various cells, including hepatocytes, and organs against I/R injury (Ohsawa et al., 2007; Fukuda et al., 2007; Nakao et al., 2010), but its exact effect on cholangiocytes with H/R injury is still unclear.

To avoid the influence from other cells and to accurately investigate underlying mechanisms, our study explored the potential function of H_2 on intrahepatic cholangiocytes *in vitro* and *in vivo*.

2. Materials and methods

2.1. Isolation of cholangiocytes

After informed consent was obtained from patients and the tissue acquisition protocol was approved by the Zhejiang University Institutional Review Board, liver explant tissue from a female patient with hepatic hemangioma without other hepatic diseases was obtained. Cholangiocytes were isolated from the normal liver tissue samples with minor modifications to previously described protocols (Tabibian et al., 2014; Coots et al., 2012). Briefly, about 5 g of liver tissue was cut into small pieces using sterile blades and incubated in Dulbecco's modified eagles medium (DMEM) solution containing 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.2% collagenase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 0.07% DNase for 45 min in a shaking water bath at 37 °C. The suspension was filtered through a 100 μM and 40 μM cell strainer in sequence. After washed with DMEM, cells contained in the 40 uM cell strainer were resuspended and subjected to further digestion with a DMEM solution containing hyaluronidase for 45 min at 37 °C. The resulting digestant was filtered as described above. The isolated cells were plated on collagen-coated flasks (BD Biosciences, San Jose, CA) and allowed to grow to confluence. Cholangiocyte cells were positively selected by magnetic separation using the Epithelial Enrich magnetic bead (Miltenyi Biotec, Germany).

2.2. Cell culture and establishment of the H/R cell model

Cholangiocytes cells were plated on collagen-coated culture dish (Corning Life Science, Corning, NY, USA), and cultured with mediums, which contained DMEM/F12 medium (Invitrogen), 10% FBS, 100 μg/ml penicillin–streptomycin, 2 mM glutamine, 10 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), 20 ng/ml HGF (Peprotech), 2 μg/ml hydrocortisone, 10 ng/ml choleratoxin, 2 nM tri-iodothyronine (all from Sigma, St. Louis, MO, USA), and 1× ITS (Insulin, Transferrin, Selenium Solution, Invitrogen). In all subsequent experiments, cholangiocytes cells were used between passage 2 and 8 depending on the initial yield of the primary isolate.

When the cholangiocytes cells were at 70–80% confluence, the culture inserts were incubated under hypoxic conditions (94% N_2, 5% CO_2 and 1% O_2) using a hypoxia chamber (MIC-101, Billups–Rothenberg, Del Mar, CA, USA). After hypoxia for 2, 4 or 8 h, the cells were then reoxygenated by incubation in a standard 5% CO_2 incubator for 6 h. In the process of reoxygenation, the hydrogen-rich medium (HRM) or normal medium were exchanged in the respective group every three hours.

The normal human hepatic cell line HL-7702 and the normal rat hepatic cell line BRL were obtained from the Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China). HL-7702 cells and BRL cells were cultured in RPMI 1640 medium and DMEM respectively, with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO_2.

Table 1
Primer sequences for PCR assay.

	Name	Symbol	Forward (5′-3′)	Reverse (5′-3′)
Human				
	β-actin	ACTB	AGAAGGAGATCACTGCCCTGGCACC	CCTGCTTGCTGATCCACATCTGCTG
	CK7	KRT7	TGTGGATGCTGCCTACATGAGC	AGCACCACAGATGTGTCGGAGA
	CK19	KRT19	AGCTAGAGGTGAAGATCCGCGA	GCAGGACAATCCTGGAGTTCTC
	GGT	GGT	TGACGTACCACCGCATCGTAGA	CAGCGAAGAACTCGGAGGTCAT
	AQP1	AQP1	TATGCGTGCTGGCTACTACCGA	GGTTAATCCCACAGCCAGTGTAG
	EPCAM	EPCAM	GCCAGTGTACTTCAGTTGGTGC	CCCTTCAGGTTTTGCTCTTCTCC
	Icam1	ICAM1	GCCAACCAATGTGCTATTCAAAC	AGCACCGTGGTCGTGACCTCAG
	ALB	ALB	GATGAGATGCCTGCTGACTTGC	CACGACAGAGTAATCAGGATGCC
	G6PC	G6PC	GCTGTGATTGGAGACTGGCTCA	GTCCAGTCTCACAGGTTACAGG
	HO-1	HMOX1	CCAGGCAGAGAATGCTGAGTTC	AAGACTGGGCTCTCCTTGTTGC
	Nrf2	NFE2L2	CACATCCAGTCAGAAACCAGTGG	GGAATGTCTGCGCCAAAAGCTG
	Nqo1	NQO1	CCTGCCATTCTGAAAGGCTGGT	GTGGTGATGGAAAGCACTGCCT
	GSTA2	GSTA2	CTGCCCTTTAGTCAACCTGAGG	ACAAGGTAGTCTTGTCCGTGGC
	MnSOD	SOD2	CTGGACAAACCTCAGCCCTAAC	AACCTGAGCCTTGGACACCAAC
	Prdx1	PRDX1	CTGCCAAGTGATTGGTGCTTCTG	AATGGTGCGCTTCGGGTCTGAT
Rat				
	β-actin	ACTB	CAACTGGGACGATATGGAG	TGGCTACGTACATGGCTG
	CK19	KRT19	GTCACGGAACTCCGACGTAA	TCCGTAACGGGCCTCTATCT
	ALB	ALB	GTGAGCGAGAAGGTCACCAA	TTTCACCAGCTCAGCGAGAG