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# Heme oxygenase-1 inhibitor tin-protoporphyrin improves liver regeneration after partial hepatectomy

Monica Pibiri, Vera Piera Leoni, Luigi Atzori*

*Department of Biomedical Sciences, Oncology and Molecular Pathology Unit, University of Cagliari, Via Porcell 4, 09124 Cagliari, Italy*

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ABSTRACT

*Aims:* This study investigates the effects of the heme oxygenase-1 (HO-1) inhibitor tin protoporphyrin IX (SnPP), on rat liver regeneration following 2/3 partial hepatectomy (PH) in order to clarify the controversial role of HO-1 in the regulation of cellular growth.

*Main methods:* Male Wistar rats received a subcutaneous injection of either SnPP (10 μmoles/kg body weight) or saline 12 h before PH and 0, 12 and 24 h after surgery. Rats were killed from 0.5 to 36 h after PH. Bromodeoxyuridine (BrdU) incorporation was used to analyze cell proliferation. Immunohistochemistry, Western blot analysis and quantitative Real Time-PCR were used to assess molecular and cellular changes after PH.

*Key findings:* Data obtained have shown that administration of SnPP caused an increased entry of hepatocytes into S phase after PH, as demonstrated by labeling (L.I.) and mitotic (M.I.) indexes. Furthermore, enhanced cell cycle entry in PH-animals pre-treated with SnPP was associated with an earlier activation of IL-6 and transcription factors involved in liver regeneration, such as phospho-JNK and phospho-STAT3.

*Significance:* Summarizing, data here reported demonstrate that inhibition of HO-1 enhances rat liver regeneration after PH which is associated to a very rapid increase in the levels of inflammatory mediators such as IL-6, phopsho-JNK and phospho-STAT3, suggesting that HO-1 could act as a negative modulator of liver regeneration. Knowledge about the mechanisms of liver regeneration can be applied to clinical problems caused by delayed liver growth, and HO-1 repression may be a mechanism by which cells can faster proliferate in response to tissue damage.

## 1. Introduction

The ability of the liver to regenerate after 2/3 partial hepatectomy (PH) typifies the capacity of an organ to change instantly from an essentially quiescent state to a rapidly growing one [1,2] providing a robustexperimentalin vivo model to study cell cycle entry and cell proliferation. Although considerable advance has been made in the comprehension of the molecular mechanisms associated to the control of liver cell growth, their exact nature has not been fully elucidated yet. At present, the molecular signals triggering liver regeneration are thought to include cytokines, growth factors, reactive oxygen species, complement factors C3 and C5 and, possibly, bacterial endotoxin [3]. The priming phase of liver regeneration, which corresponds to the G0/G1 transition, is controlled by a cytokine network which acts through activation of pre-existing latent transcription factors, such as STAT3,NF-kB and AP-1, which, in turn, mediate the transcription of immediate early genes, such as c-jun, c-fos, c-myc, responsible for cell cycle progression [1,4,5]. A major role in signal transduction pathway induction has been attributed to a condition of oxidative stress [6–8], which may contribute to activation of oxidant-sensitive transcription factors (e.g. NF-kB and STAT3) and cytokines. It appears that the initial increase of cytokines associated with oxidative stress is beneficial for liver regeneration, as demonstrated by the finding that the response to PH is impaired in interleukin 6 (IL-6)-deficient mice [9,10].

Heme oxygenase (HO; EC 1.14.99.3), a member of the heat-shock protein family, plays a protective role in inflammation and oxidative stress [11,12]. Like other antioxidant enzymes, HO-1 contains the antioxidant response elements (ARE) which is a binding site for the transcription factor Nrf2 [13,14] whose activity is regulated by redox status [15]. HO-1 gene product, is a heme-catabolising enzyme that converts heme yielding carbon monoxide (CO), iron ($Fe^{2+}$) and biliverdin. HO-1, the inducible form of HO, is upregulated during oxidative stress and has been proposed to have a role in the regulation of inflammatory processes [16]. The enzymatic activity of HO-1 results in decreased oxidative stress and attenuation of the inflammatory response due to removal of heme, a potent prooxidant and

* Corresponding author.
*E-mail address:* latzori@unica.it (L. Atzori).

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proinflammatory agent, and to the generation of biologically anti-inflammatory active products, such as CO, bilirubin and ferritin. In addition, HO-1 exerts anti-inflammatory effects by inhibition of tumor necrosis factor-α and interleukin-1βand by upregulation of interleukin-10 [17]. Recent data suggest a key role for HO-1 in the regulation of cellular homeostasis and growth [18], although its effects are cell-type specific and can be opposite. Accordingly, while in vascular smooth muscle cells and in smooth muscle cells induction of HO-1inhibits proliferation [19,20], its over-expression in endothelial cells protects against apoptosis TNF-α-induced and promotes proliferation [21]. Moreover, HO-1 deficiency in humans is associated with susceptibility to oxidative stress and increased pro-inflammatory state in epithelium [22], fibroblasts [23], and T lymphocytes [24].

The synthetic heme analogue tin protoporphyrin IX (SnPP), is an extreme potent HO-1 inhibitor which exerts its action by a dual mechanism that involves competitive inhibition of the enzyme for the natural substrate heme and simultaneous enhancement of new enzyme synthesis in liver cells that, as a consequence, results completely inhibited [25,26]. Secondary increases of HO-1 mRNA and protein levels by SnPP can be due to enzyme "feedback inhibition" or to induction of a mild pro-oxidant state with counterbalancing HO-1 production [27]. However, this could potentially be offset by SnPP-induced HO-1 inhibition which lasts until SnPP elimination [27]. Inhibition of HO-1 activity by SnPP has been successfully used to avoid heme degradation to bilirubin *in vivo* and thus, to suppress various forms of jaundice [28,29]. Based on the potential role of HO modulation on cell proliferation, and on the strict association between HO-1 and inflammatory cytokines, key mediators of liver regeneration, the present study was designed to investigate whether inhibition of HO-1 by SnPP could affect liver regeneration after PH in rats.

## 2. Materials and methods

### 2.1. Animals and treatment

Male Wistar rats (180–220 g), purchased from Charles River (Milano, Italy), were maintained on a standard laboratory chow diet (Ditta Mucedola, Milano, Italy). The animals were provided food and water ad libitum, whit a 12 h light/dark daily cycle and were acclimated for 1 week before starting the experiment. Guidelines for the Care and Use of Laboratory Animals were used during the investigation. Two thirds hepatectomy was performed according to Higgins and Anderson [30]. SnPP (Frontier Scientific, Carnfort, UK) was dissolved in 0.2 N NaOH and then back titrate with HCl solution. Animals received subcutaneous injections of either SnPP (10 μmoles/kg of body weight) or vehicle 12 h before PH and 0, 12, and 24 h after PH. Rats were killed from 0.5 to 36 h after surgery. Control groups also include animal treated with SnPP or untreated and sacrificed at the same time points without been subjected to PH. To score S phase hepatocytes, rats received BrdU (SIGMA Chemical, St. Louis, MO) either in drinking water (1 mg/ml) all throughout the experimental period or in a single dose (50 mg/kg body weight i.p.) 2 h before sacrifice. Immediately after sacrifice, sections of the liver were fixed in 10% buffered formalin and processed for staining with hematoxylin-eosin or immunohistochemistry. The remaining liver was snap-frozen in liquid nitrogen and kept at −80 °C until use.

### 2.2. Western blot analysis

Preparation of total cell extracts and western blot analysis were performed as described previously [31]. Nuclear and cytosolic cell extracts were prepared according to the method of Timchenko et al. [32]. For immunoblotting experiments the following antibodies were used: mouse monoclonal antibodies directed against, PCNA (PC-10) (Santa Cruz Biotechnology, CA, USA), Cyp1/WAF-1/p21 (Upstate Biotech, New York) and actin (clone AC-40)(SIGMA); rabbit polyclonal antibodies directed against, E2F-1 (C20), p107 (C-18), cyclin A (C-19) and Lamin A/C(H-110) (Santa Cruz Biotechnology), SAPK/JNK, phospho SAPK/JNK, STAT3 and phospho STAT3 (Tyr705) (Cell Signalling Technology, Beverly, MA, USA) and goat monoclonal antibody directed against albumin (Bethyl Laboratories, Montgomery, TX).

### 2.3. Immunohistochemistry

BrdU-positive hepatocytes were stained with the peroxidase method as described previously [32]. Labelling index (L.I.) was expressed as number of BrdU positive hepatocyte nuclei/100 nuclei. Mitotic index (M.I.) was determined as number of mitoses/1000 hepatocytes. Results are expressed as means ± SE of four to five rats per group. At least 5000 hepatocyte nuclei per liver were scored.

### 2.4. Quantitative real time-PCR

Total RNA was extracted from frozen tissue with Trizol Reagent (Invitrogen, San Giuliano, Milan, Italy) and RNeasy MiniElute Cleanup Kit (Qiagen, Valencia, CA). Equal amounts of at last 3 samples for each group were used to create pools that were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expression analysis was performed with Real-time PCR analysis of 10 ng of cDNA mixed with 2× TaqMan Gene expression Master Mix and 20× specific TaqMan gene expression assays (Rn00561420_m1,Il6; Rn00477784_m1, HO-1; Rn00566528_m1, Nqo1) (Applied Biosystems) with an ABI PRISM 7300 Thermocycler (Applied Biosystems). Each sample was run in triplicate and gene expression analysis of Actin-beta or GAPDH was used as endogenous control. Relative quantification analysis for each gene was calculated by $2^{-\Delta\Delta Ct}$ method.

### 2.5. Statistical analysis

Results are expressed as the mean ± Standard Deviation (SD). Differences between groups were performed using either unpaired two-tail Student's *t* test or ANOVA for multiple groups comparison.

## 3. Results

To investigate whether inhibition of HO-1 by SnPP could affect liver regeneration response, male Wistar rats were subjected to 70% PH and injected s.c. with either 10 μmoles/kg body weight (bw) of SnPP or vehicle 12 h before surgery, at the time of PH and 12 h after. Animals were sacrificed 24 h after PH. Animals treated with 10 SnPP or untreated and sacrificed 12 h later, without been subjected to PH, served as control groups. To monitor the hepatocytes S-phase-entry, BrdU dissolved in drinking water (1 mg/ml) was given throughout the experimental period. As shown in Fig. 1A and B, the HO-1 inhibitor SnPP significantly increased the number of BrdU-positive hepatocytes during liver regeneration, without signs of liver toxicity. Indeed, 24 h after PH labelling index was 36%in SnPP pre-treated animals vs 23% of PH group (Fig. 1B).

In agreement with previous studies [25], we found that SnPP caused enhanced gene transcription of HO-1(Fig. 1C). Indeed, Real time PCR analysis revealed that SnPP treatment was associated to enhanced HO-1 mRNA levels, in both control and hepatectomized rats (Fig. 1C). This effect was specific, as the expression of Nqo1, anotherNrf2 target gene, was unaffected by the treatment with SnPP (Fig. 1D). One sample (SnPP + PH 30 min) resulted out of range in both HO-1 and Nqo1 RT-PCR analyses. To further characterize the effect of SnPP on liver regeneration, the kinetics of hepatocyte proliferation in the presence or absence of the inhibitor was examined by BrdU pulse labeling. Animals injected with SnPP or untreated prior to PH were given a single dose of BrdU (50 mg/kg bw, i.p.) 2 h before sacrifice at 18, 24, 30 and 36 h after surgery. As shown in Fig. 2C, while SnPP pre-treatment did not