Journal of Surgical Research **129,** 306–312 (2005)
doi:10.1016/j.jss.2005.06.031

Pulmonary Expression of Inducible Heme-Oxygenase after Ischemia/Reperfusion of the Lower Extremities in Rats

Cherif N. Boutros, M.D.,*,[1] Rachid Zegdi, M.D.,† Nermine Lila, V.D.,† Michele Combillau, M.D.,‡ Paul Fornes, M.D.,§ Alain Carpentier, M.D.,† and Jean Noel Fabiani, M.D.†

*Laboratory of the Study of Cardiac Grafts and Prostheses, Broussais Hospital, Paris, France; †Department of Cardiovascular Surgery; ‡Department of Biochemistry; §Department of Pathology, European Hospital Georges Pompidou, Paris, France

Submitted for publication March 19, 2005

Background. **Expression of inducible heme-oxygenase (HO-1) has been shown to be increased in various inflammatory disorders, which may confer a protective role. The aim of our study was to assess pulmonary expression of HO-1 after ischemia/reperfusion (I/R) of the lower limbs in rats.**

Materials and methods. **We compared three groups of rats (n = 5/group): one Sham group, and two I/R groups (aorta cross-clamped for 2 h followed by 2 h of reperfusion), one of which pre-treated with Zn-protoporphyrin (Zn-PP), a competitive inhibitor of HO (50 μmol/kg, i.p.). At the end of experiment, lungs were harvested for determination of HO activity and HO-1 expression by Western blot and immunohistochemistry. Lung injury was assessed by bronchoalveolar lavage, histological study, and determination of the lung Evans Blue dye content, an index of microvascular permeability.**

Results. **I/R of the lower limbs was responsible for acute lung injury (ALI), characterized by neutrophilic infiltration (87 ± 20 × 10^3 neutrophils/mm^3, Sham group *versus* 191 ± 38 × 10^3 neutrophils/mm^3, I/R group; $P < 0.002$) and an increase in lung Evans blue dye content: (74 ± 6 μg/g, Sham group *versus* 122 ± 48 μg/g, I/R group; $P < 0.05$). Pre-treatment with Zn-PP further increases the Evans Blue content (122 ± 48 μg/g, I/R group *versus* 179 ± 23 μg/g Zn-PP group $P < 0.05$) and the neutrophilic infiltration. Pulmonary heme-oxygenase activity, and HO-1 content were increased after I/R. (10.5 ± 12 pmol bilirubin/mg protein/h, Sham group *versus* 101.2 ± 66 pmol bilirubin/mg protein/h, I/R group; $P < 0.02$). Immunohistochemistry revealed that the expression of HO-1 was mainly localized to inflammatory cells.**

Conclusions. **ALI following I/R of the lower limbs in rats is associated with an increase of pulmonary expression of HO-1, inhibition of this expression increase the severity of ALI.**

Key Words: **ischemia/reperfusion; heme-oxygenase; aorta; acute lung injury.**

INTRODUCTION

Heme oxygenase (HO) is the rate-limiting enzyme from heme catabolism. It converts heme into biliverdin while concomitantly producing carbon monoxide and releasing iron [1, 2]. Three isoforms of HO have been described, one inducible (HO-1) and two constitutive (HO-2 and HO-3). These isoforms differ by their cellular location and the regulation of their expression [3]. HO-1 expression has been shown to be primarily induced during oxidative injury [1].

ALI is a diffuse pulmonary inflammatory disorder, mainly characterized by interstitial or alveolar edema and leukocytes parenchymal infiltration [4, 5]. ALI is a frequent cause of respiratory failure occurring in various circumstances such as sepsis, trauma, or aspiration [6–8]. ischemia/reperfusion (I/R) of the lower limbs has also been shown to be a predisposing factor for ALI in humans and in animal species [9–11]. Several studies have demonstrated that oxidative stress occurred within lungs during ALI, contributing to the pathogenesis of the lung injury [12]. Recently, expression of HO-1 has been shown to be increased in some animal models of ALI [13, 14]. This up-regulated pulmonary expression of HO-1 is now considered as an important part of the host antioxidative response during ALI [15–17].

[1] To whom correspondence and reprint requests should be addressed at Department of Surgery, Monmouth Medical Center, 300 Second Avenue, Long Branch, NJ 07740. E-mail: cherifboutros@hotmail.com.

0022-4804/05 $30.00

The primary purpose of this rat study was to determine the influence of I/R of the lower limbs on the pulmonary expression of HO-1. We also investigated the potential effect of this expression on the severity of the lung injury by administrating a competitive HO inhibitor.

MATERIALS AND METHODS

The study was approved by the local institutional animal care and use committee and all animals were treated according to the European regulation for animal experimentation [18].

Animal Preparation

Male Wistar rats (250–350 g) were used for all experiments. They were anesthetized with an intramuscular administration of ketamine hydrochloride (80 mg/kg) and chlorpromazine hydrochloride (2 mg/kg). Anesthesia was maintained through the experiment with additional doses of intravenous Ketamine. Sustained muscle relaxation was achieved with an intravenous injection of 1 mg/kg of pancuronium bromide (Pavulon). Rats were continuously ventilated through a tracheostomy with air at 80 breaths/min, and 8 ml/kg tidal volume under 3 cm H_2O positive end-expiratory pressure (Harvard Rodent Respirator, model 680, SARL Ealing). Central temperature was monitored with an esophageal probe and maintained between 36 to 38°C by a heating lamp placed above the animal.

The right carotid artery was cannulated (catheter 22G, Jelco) to monitor systemic arterial pressure (Hewlett Packard, model 78342A). The left jugular vein was cannulated (catheter 22 G, Jelco) for drug administration and continuous perfusion of physiological saline (8 ml/kg/h).

After an equilibration period of 30 min, the infrarenal abdominal aorta was dissected and controlled through a median laparotomy and rats received heparin intravenously (500 IU/kg).

Protocol

Study 1

Three groups of animals (n = 5/group) were studied. In two groups, infrarenal aorta was cross-clamped with a microvascular clamp for 2 h followed by a 2-h period of reperfusion. Rats were intraperitonealy administered either Zn-Protoporphyrin IX (50 μmol/kg) 18 to 24 h before anesthesia (I/R-Zn-PP IX group) or the solvent (I/R-solvent group). In the third group, the abdominal aorta was exposed without being cross-clamped (Sham group).

Every experiment took place over a 4-h period, at the end of which the rats were sacrificed by exsanguination. A median sternotomy was performed and the left atrium was excised allowing free pulmonary drainage. The pulmonary circulation was flushed with 20 ml of physiological saline at 4°C, slowly injected into the pulmonary artery. Lungs and heart were harvested *en bloc*.

After cross-clamping the hilum of the right lung, a bronchoalveolar lavage of the left lung was performed by injecting physiological saline (20 ml/kg) through the tracheostomy catheter. Fluid was then collected by gentle suction and processed as described below. The right lung was then harvested and divided into three parts, one for lung histology (the inferior lobe), one for lung water content measurement and the remaining part (the superior lobe), used for determination of HO activity and HO-1 expression, was stored at −80°C.

Study 2

Three groups of rats (n = 5/group) were also studied. Rats received Evans blue dye (50 mg/kg, i.v.) either at reperfusion in the two I/R groups or at a matched-time in the Sham group. After sacrifice, the left lung was harvested and used for determination of pulmonary Evans blue dye content (see below).

Assessment of ALI after I/R of Lower Limbs

Lung Evans Blue Dye Content

Pulmonary microvascular permeability was assessed by measuring the lung concentration of Evans blue dye 2 h after intravenous administration. Evans blue dye binds rapidly to albumin and has been shown to increase in ALI [19, 20]. Evans blue dye (50 mg/kg) was intravenously administered after unclamping the aorta (or 120 min after baseline in Sham rats). After sacrifice, the left lung was harvested, weighed, and homogenized in 5 ml formamide for 1 min. The crude homogenate was incubated at 37°C for 16 h and then centrifuged at 10,000 × g for 20 min (25°C). The absorbency of the supernatant at 620 nm, measured against a formamide blank, was determined by spectrophotometry (DU-70, Beckman Coulter, Villepinte, France). The concentration of Evans blue dye in the left lung was determined by comparison against a standard curve and expressed as microgram dye per gram of wet lung weight.

Bronchoalveolar Lavage

After centrifugation (750 × g at 24°C for 5 min) the obtained deposit over a slide was fixed by Cytofix (Surgipath) and stained by rapid smear staining (RAL). Total cellularity and neutrophilic count were assessed.

Lung Histology

The right inferior lobe was immersed in 10% formalin for 48 h. After fixation, the lung tissue was embedded in paraffin. Two sections of 3-μm thickness were obtained from each block and stained with hematoxylin and eosin (H&E). A semi-quantitative assessment of the pulmonary injury was performed by a blinded observer. Lesions (interstitial and alveolar edema, cellular infiltration, hemorrhage) were graded (0-3+) according to their extent and their severity.

Lung Water Content

The wet weight of a small part of the right lung (around 200 mg) was first determined. The dry weight of the lung sample was measured after dessication for 20 h in a lyophilizator (RP-2V, CIRP, France). In our experience, the dry weight does not decrease any further after a 20-h dessication period. The lung water content (%) was calculated as follows: [(wet weight-dry weight)/wet weight]/100.

Assessment of Pulmonary HO-1 Expression

Heme Oxygenase Activity

The method was derived from Tenhunen *et al.* [21]. Briefly, as a source of biliverdin reductase, livers from fasted rats were harvested and immediately placed in cold 0.9% NaCL. The livers were then weighed and homogenized in four volumes of 2 mmol/L $MgCl_2$-100 mmol/L phosphate buffer (pH 7.4). The homogenate was centrifuged at 105,000 × g for 1 h at 4°C and the supernatant (liver cytosol) was used as a source of biliverdin reductase.

The frozen sample of the right lung was homogenized in 4 ml of a solution containing 50 mM Tris (pH 7.4), 250 mM sucrose and a mixture of proteases inhibitors (Complete, Boehringer Mannheim, 1 tablet/50 ml). The homogenate was centrifuged for 20 min at 10,000 × g and at 4°C. The supernatant was used for measuring HO activity. The reaction mixture consisted of 200 μl of lung supernatant, 50 μl of liver cytosol, 20 μl of 1 mmol/L heme b solution, 200 μl of 2.75 mmol/L β-NADPH solution, and 530 μl of 2 mmol/L $MgCl_2$-100 mmol/L phosphate buffer (pH 7.4). The samples were incubated