Effects of chronic L-NAME on nitrotyrosine expression and renal vascular reactivity in rats with chronic bile duct ligation

Antonia Alcaraz, David Hernández, David Iyú, Rubén Mota, Antonio José Ortiz, Noemí Marín Atucha, Joaquín García-Estañ, María Clara Ortiz

▶ **To cite this version:**

Antonia Alcaraz, David Hernández, David Iyú, Rubén Mota, Antonio José Ortiz, et al.. Effects of chronic L-NAME on nitrotyrosine expression and renal vascular reactivity in rats with chronic bile duct ligation. Clinical Science, 2008, 115 (2), pp.57-68. 10.1042/CS20070312 . hal-00479400

HAL Id: hal-00479400
https://hal.science/hal-00479400

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Clinical SCIENCE

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

EFFECTS OF CHRONIC L-NAME ON NITROTYROSINE EXPRESSION AND RENAL VASCULAR REACTIVITY IN RATS WITH CHRONIC BILE DUCT LIGATION

Alcaraz A[*], Hernández D[†], Iyú D*, Mota R[‡], Atucha NM[*], Ortiz AJ[§], García-Estañ J[*] and Ortiz MC[*]

[*]Department of Physiology, Faculty of Medicine, University of Murcia, 30100 Murcia, Spain.

[†]Department of Medicine, Regional Center for Blood Donation, University of Murcia, 30100 Murcia, Spain.

[‡]Department of Biochemistry, Molecular Biology and Immunology, University Hospital Virgen de la Arrixaca, 30120 Murcia.

[§]Department of Dermatology, Estomatology, Radiology and Medical Physics, University of Murcia, 30100 Murcia, Spain.

Short title: Renal nitrotyrosine during biliary cirrhosis

Author for correspondence:
María Clara Ortiz Ruiz
Departamento de Fisiología
Facultad de Medicina
Universidad de Murcia
30100 Murcia
SPAIN
Telephone: +34 968 364677
Fax: +34 968 364150
E-mail: clara@um.es

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE
Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

ABSTRACT

In liver cirrhosis, elevated levels of nitric oxide (NO) and reactive oxygen species (ROS) might greatly favour the generation of peroxynitrite. Peroxynitrite is a highly reactive oxidant and it can potentially alter the vascular reactivity and the function of different organs. In this research work, we evaluated whether peroxynitrite levels are related with the progression of renal vascular and excretory dysfunction during experimental cirrhosis induced by chronic bile duct ligation (BDL) in rats. Thus, we performed experiments at 7, 15 and 21 days after BDL, the appropriate controls and in 21 days-BDL rats chronically treated with L-NAME. We measured sodium balance, blood pressure (BP), basal renal perfusion pressure (RPP), and the renal vascular response to phenylephrine (PHE) and acetylcholine (ACH) in isolated-perfused kidneys. NO levels were calculated as 24-hours urinary excretion of nitrites, ROS as thiobarbituric reactive substances (TBARS), and peroxynitrite formation as the renal expression of nitrotyrosine. BDL rats exhibited progressive sodium retention, decreased BP, RPP and renal vascular responses to PHE and ACH as the BDL advanced. They also showed increasing levels of NO and ROS, and a renal nitrotyrosine accumulation, especially in the medulla. All of these changes were either prevented or significantly reduced by chronic L-NAME administration. In conclusion, these results suggest that the increasing levels of peroxynitrite might be contributing to the altered renal vascular response and sodium retention in the development of the experimental biliary cirrhosis. Moreover, the beneficial effects of decreasing NO synthesis are, at least in part, mediated by anti-peroxinitrite related effects.

Abstract word count: 250

Manuscript word count: 5299

Key words: peroxynitrite, nitrotyrosine, oxidative stress, nitric oxide, kidney, biliary cirrhosis.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

INTRODUCTION

Increased NO production contributes to the renal excretory and vascular alterations observed in experimental and human cirrhosis [1-6]. In fact, contrary to what happens under normal conditions, acute or chronic NO synthesis inhibition in cirrhosis increases sodium excretion without changing blood pressure [7-10]. In addition, the cirrhotic kidney has a reduced excretory response to maneuvers such as elevation of arterial pressure [11] or volume expansion [12], which normally induce a NO-dependent renal vasodilatation and increase sodium and water excretion. The mechanisms that account for this paradoxical renal effect of NO during cirrhosis remain still unclear.

Previous studies have also suggested that an elevated generation of radical oxygen species (ROS) participates in the systemic and renal functional alterations of experimental cirrhosis [13-18], and that antioxidants administration, that quenches superoxide anions (O_2^-), improves these alterations [19-23]. During cirrhosis, elevated levels of NO and O_2^- could easily react to form peroxynitrite ($ONOO^-$), a reactive nitroxy specie (RNOS). This reaction was initially viewed as a route for NO inactivation and it is at least three times faster than the dismutation of superoxide dismutase [24-25]. Peroxynitrite is a highly reactive intermediate and one of the most potent oxidants known in biological systems. The mechanism of injury caused by peroxynitrite involves multiple factors including initiation of lipid peroxidation, further increasing ROS and RNOS, and nitration of tyrosine-containing proteins (nitrotyrosine), a footprint left by peroxynitrite *in vivo* [24]. In addition, peroxynitrite is itself a vasodilator and can induce tachyphylaxis preventing further response to its own vasodilator actions. It also causes long-lasting impairment of the vasoactive response to other vasodilators [26], and even catecholamines [27].

Therefore, we hypothesized that during cirrhosis the progressive generation of peroxynitrite could perpetuate a high oxidative stress status and participate in the advance of renal vascular and excretory abnormalities of liver cirrhosis. Then, in this investigation we studied the renal excretory function and vascular reactivity in isolated kidneys, and measured the levels of NO and ROS, as well as peroxynitrite kidney levels at different phases of cirrhosis induced by chronic bile duct ligation (BDL) in rats. Furthermore, we examined the effect of blocking NO synthesis with L-NAME on the peroxynitrite formation and the renal alterations in this experimental model of cirrhosis.

Stage 2(a) POST-PRINT
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

METHODS

Animals and models

Male Sprague-Dawley rats (225-250 g) born and raised in the animal facilities of the Universidad de Murcia (Murcia, Spain) were used in all the experimental protocols of this study, according to the Spanish Ministerio de Agricultura, Pesca y Alimentación and the European Community guidelines for the use of experimental animals and approved by the Ethics Committee of the Universidad de Murcia.

Liver cirrhosis was induced by chronic bile duct ligation (BDL) as previously described [28-29]. Briefly, rats were subjected to either BDL or sham surgery. To that end, the rats were anesthetized with an intramuscular injection of a mixture of xylazine (50 mg/kg body wt i.m; Rompun®, Bayer, Spain) and ketamine (100 mg/kg body weight, i.m.; Imalgène; Merial, France). Then, the bile duct was exposed under aseptic conditions by a midline incision under the sternum, doubly ligated with a 2-0 silk suture, and excised between the ligatures. For sham or control surgery, the bile duct was exposed and dissected, but not ligated or cut. All rats were maintained under comparable conditions with ad libitum diet and free access to drinking water.

Study design

Rats were randomized into six groups: 1) Control, rats without any surgical intervention (n = 6); 2) Sham, rats studied 7 days after sham surgery (n = 6); 3) BDL7, rats studied 7 days after BDL (n = 7); 4) BDL15, rats studied at 15 days after BDL (n = 8); 5) BDL21, rats studied at 21 days after BDL (n = 6); and 6) BDL21+L-NAME, rats receiving chronic L-NAME ($N\text{-}^{w}$-nitro-L-arginine methyl ester, 10mg/Kg/day) in the drinking water, 7 days before the BDL surgery, and studied after 21 days of BDL (n = 6). The sham group (group 2) was considered the control for BDL7 (group 3) and BDL15 (group 4) groups, to evaluate any effect of the surgery itself; and group 1 served as the control (healthy rats) for the 21 days-BDL rats (groups 5 and 6). During the time course study, rats were progressively acclimated to individual metabolic cages, which provide a good separation of the urine and feces. After 2 days of adaptation, urine samples were collected during 2 consecutive days (days -2 and -1) before surgery (day 0) and 2 days right before the experimental day, days 6 - 7, 14 - 15, or 20 - 21 depending on the experimental group. Urine samples were centrifuged to remove solid matter before analysis and the values of urine volume obtained each experimental day were averaged for statistical purposes.

Experimental procedure

Preparation of isolated perfused kidneys and functional procedures.

The rats were anesthetized and placed on a heated table to maintain body temperature at 37°C.

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

A polyethylene cannula (PE-50) was placed in the right femoral artery for measuring blood pressure (BP) and to obtain plasma samples. Subsequently, we proceeded to isolate and perfuse the kidney as described previously [30-31]. Briefly, the left kidney was exposed by a midline laparotomy and the renal artery cannulated via suprarenal aorta to prevent or minimize interruption of blood flow. The kidney was perfused in situ with warm oxygenated Krebs' buffer (mM, NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; edetate calcium disodium, 0.026; and glucose, 5.6, pH 7.4) at 37° C and at a constant rate of 5 ml/min/g of kidney weight with a peristaltic pump (Master-flex 7518-00, Coler-Parmer Instrument Company, Niles, Illinois). The left renal vein was then cut and the ureter transected to allow exit of the perfusate. Finally, the kidney was excised from the surrounding tissues, decapsulated and placed in a chamber containing Krebs solution at 37º C. Renal vascular responses were recorded through a transducer connected to a Macintosh LCII computer and analyzed with MacLab software (AD Instruments, UK) as changes in renal perfusion pressure (RPP) downstream from the pump. Then, the right kidney was removed and weighed (as an index of the left kidney weight). Samples of kidney and liver tissue were either frozen or fixed with a 10%-formalin solution for later studies.

Renal responses to Phenylephrine (PHE) and Acetylcholine (ACH).

After a 30-min stabilization period, we determined a basal measure of RPP and then we performed a dose-response curve to increasing doses of PHE (10^{-7}, 5.10^{-7}, 10^{-6}, 5.10^{-6}, 10^{-6}, 5.10^{-5}, 10^{-5}, 5.10^{-4} and 10^{-4} M) administered as a 0.1 ml bolus. The approximate time between doses was 2 min, the estimated time to reach the plateau with each dose. After obtaining the PHE dose-response curve, we allowed a second 30-min stabilization period, and the renal vasculature was pre-constricted with krebs solution containing PHE (10^{-6} to 10^{-4} M) to reach a 75% of the maximal constriction observed in the previous PHE curve. Once a stable elevated perfusion pressure was reached, the vasodilator responses to ACH (10^{-8} to 10^{-4} M) and to sodium nitroprusside (SNP; 10^{-6} to 10^{-4} M), in this same order, were determined in all groups to assess the NO endothelium dependent or independent vasodilator responses, respectively. Changes in RPP in response to vasodilators are expressed as a percentage of the vasoconstriction obtained with PHE.

In a subgroup of 4 Control and 4 BDL21+L-NAME rats (acute), the experiments were performed in the presence of a high dose of L-NAME (10^{-4} M) to inhibit NO synthesis. L-NAME was added to the Krebs solution 30 min before the initiation of the PHE dose-response curve and maintained it during the rest of the experiment.

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Analytical measures

Thiobarbituric acid reactive substances (TBARS) were determined in plasma and kidney tissue as previously reported [23]. We used 25 µL of plasma sample or 50 µl of kidney lysate (between 1-3 mg of protein). Briefly, we added 0.5 mL of phosphate buffer to the samples or lysates. After mixing, we added 1 mL of a reagent solution containing 66 mg% deferoxamine mesylate, 7.5% TCA, 0.25M HCl and 0.37% of thiobarbituric acid. The mixture was vortexed, covered with aluminium foil and heated for 45 minutes in boiling water. TBARS from standards (prepared from 1,1,3,3-tetraethoxypropane), samples and lysates were extracted into 1 mL of 1-butanol. The color layer was read at 532 nm in a spectrophotometer (Varian Techtron, series 634, Australia) after a vigorous vortex and a brief centrifugation (2500 rpm, 5 minutes). The values were expressed as µg of TBARS per ml of plasma or per mg of kidney protein.

The urinary excretion of nitrites was determined by using the Griess reaction [29]. Briefly, sample volumes of 100 µL were mixed with 50 µL of 1% Sulfanilamide in 5% Potassium Phosphate. Then 50 µL of 0.1% N-(1-Naphthyl) Ethyl-Enediamine dihydrochloride was added and incubated for 15 minutes. The nitrite concentration was quantified in a spectrophotometer at 540 nm against the standards and subtracting a blank from each individual sample. The final concentration was expressed in µg/day.

Sodium balance was assessed as the difference between sodium intake and excretion, and factored by body weight. The sodium intake (mEq/day) was obtained by multiplying the consumption of food per day (g/day) by the sodium content of the diet (0.104 mEq/g). Sodium concentration was measured by a sodium electrode (Orion Research, Inc., U.S.A.) and urinary sodium excretion (mEq/day) determined as the product of sodium concentration and 24 hours urinary volume (ml/day).

Anti-nitrotyrosine inmunohistochemistry

For nitrotyrosine immunohistochemistry purposes, a peroxidase-antiperoxidase technique was carried out. Briefly, kidney sections were rinsed and endogenous peroxidase inhibited. Sections were blocked in blocking solution (0.1% Tween-TBS, 10% fetal calf serum, 5% BSA) for 30 minutes and incubated with a rabbit anti-nitrotyrosine polyclonal antibody (1:100; Cayman Chemical, Ann Arbor, MI, USA) overnight. Then, sections were washed three times in 0.1% Tween-TBS and incubated with anti-rabbit biotin-labelled secondary antibody (1:250; Vector Labs, Burlingame) and signal amplified with an ABC Kit (Vector Labs, USA). Sections were then washed twice in 0.1% Tween-TBS, once with TB, rinsed, developed with 3,3′-diaminobenzidine (0.5 mg/ml) in 50mM TB (pH 7.6), counterstained with hematoxylin, dehydrated and mounted.

Stage 2(a) POST-PRINT
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Average nitrotyrosine-labelled protein expression was evaluated by assigning subjective values to every single specimen after screening 6 fields (40x magnification) of three different kidney regions (glomerular, outer and inner medulla) for every experimental animal, as follows: 0, no expression; 1, Mild expression: only cytoplasmic accumuli of nitrotyrosine-labelled proteins in certain cells (25%); 3, Medium expression: medium expression of nitrotyrosine-labelled protein containing-cells in the field (25-75%); some cells present intracellular nytrotirosine-labelled protein accumuli. 5, Most cells contain clustered-like accumuli of nitrotyrosine-labelled proteins throughout the whole field (75%); most of them contain intracellular accumuli of nitrotyrosine-labelled proteins. All the immunohistological studies were carried out by an expert pathologist blinded to the different experimental groups.

Histologic Analysis

Liver tissue samples were fixed in 10% buffered formaldehyde, and then processed and embedded in paraffin and sectioned (~5µm), as previously reported [23]. These sections were then stained with hematoxylin and eosin, and viewed by light microscopy. A pathologist then performed morphologic evaluation in blinded randomized sections of the liver tissue.

Drugs

Except where indicated, all the drugs were purchased from Sigma Chemical (Madrid, Spain). Stock solutions of PHE, ACH and SNP were dissolved in distilled water and maintained frozen. Working solutions were freshly prepared in Krebs solution and concentrations expressed as final concentration.

Statistical Methods

Data are presented as means ± standard error of the mean (SEM). Differences between groups were compared by one-way repeated measures analysis of variance (ANOVA). For the dose-response curves, we applied a two-way repeated measures analysis of variance, and when significantly different, means were further compared by the Student-Neuman-Keuls' test. The ED_{50} (concentration of agonist producing half from its maximal response) is derived from logarithm transformation and regression analysis of each individual concentration−response curves. In the immunohistochemical study, an unpaired *t*-test was applied for the statistical purposes. A P-value < 0.05 was considered as statistically significant.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

RESULTS

General parameters

Cirrhotic rats had lower values of body weight and hematocrit than controls (table 1). The treatment with L-NAME normalized the body weight and elevated the hematocrit. In contrast, all BDL rats exhibited higher spleen weights and kidney-to-body weight rates than controls.

As represented in figure 2, BDL rats showed a progressive retention of sodium (panel A) with the advance of the disease (Control, -0.06±0.36; Sham, -0.33±0.25; BDL7, 0.13±0.48; BDL15, 0.90±0.07; BDL21, 2.52±0.36 mEq/Kg body weight) and decreases in BP (panel B; Control, 115±3; Sham, 125±3; BDL7, 114±6; BDL15, 104±5; BDL21, 86±3 mmHg) and RPP (panel C; Control, 57±8; Sham, 52±2; BDL7, 46±6; BDL15, 34±3; BDL21, 30±2 mmHg), with statistical significance at 15 and 21 days after the BDL. The BDL group receiving L-NAME showed similar values to controls in sodium balance (0.40±0.50 mEq/Kg body weight), BP (109±3 mmHg) and RPP (46±6 mmHg).

Renal vascular responses in the isolated and perfused kidneys

The administration of PHE (figure 3) induced dose-dependent increases in RPP that were significantly reduced with respect to the control animals, after 15 (panel A) and 21 (panel B) days following the BDL, as well as the maximum PHE response values (Control, 231±10; Sham, 240±11; BDL7, 226±15; BDL15, 196±10; BDL21, 191±12 mmHg). L-NAME administration (figure 3, panel B) normalized the dose-response curve and the maximum response to PHE in the 21-days BDL rats (251±18 mmHg).

The dose response-curve (figure 3, panel C and D) and the maximum response to ACH were reduced in the untreated BDL kidneys for all experimental days (Control, 63±9; Sham, 63±8; BDL7, 38±9; BDL15, 42±6; BDL21, 33±4 %) and chronic L-NAME administration normalized them in the 21-days BDL rats (61±4 mmHg).

The administration of acute L-NAME (10^{-4} M) to Control and BDL21+L-NAME kidneys (figure 3, panel B and D) did not modify the response to PHE respect to the observed after chronic L-NAME administration (maximum response: Control, 241±17; BDL21+L-NAME 243±10 mmHg) although the response to ACH was severely attenuated in both experimental groups (maximum response: 18±4 and 12±3 %, respectively).

SNP induced similar responses in all experimental groups.

Systemic and Renal Levels of OS and NO (TBARS and 24 hr Urinary Nitrites)

Figure 4 (panel A) illustrates plasma levels of TBARS which were significantly increased in all the BDL groups (Control, 11±1; Sham, 10±1; BDsL7, 36±3; BDL15, 35±5; BDL21, 36±3 nmol/ml)

Stage 2(a) POST-PRINT
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

and the treatment with L-NAME significantly decreased them (BDL21+L-NAME, 24±3 nmol/ml). On the other hand, TBARS in kidney tissue (panel B), increased progressively as the disease progressed (Controls, 5.5±0.8; Sham, 1.5±0.2; BDL7, 12.0±1.2; BDL15, 22.5±0.7; BDL21, 33.4±2.9 nmol/mg of protein), as well as the urinary excretion of nitrites (panel C; Control, 17±4; Sham, 14±3; BDL7, 62±17; BDL15, 99±3; BDL21, 141±17 µg/24 hours). Both, TBARS in kidney tissue and urinary excretion of nitrites, were significantly reduced after chronic administration of L-NAME to the 21-days BDL rats (17± nmol/ml and 90±5 µg/24 hours, respectively).

Nitrotyrosine Protein expression

Figures 5 and 6 represent the expression of nitrotyrosine. Glomerular nitrotyrosine had a tendency to increase in the cirrhotic kidneys but not significant differences were observed between groups (figure 5, panel A). Nevertheless, renal outer and inner medulla showed a significant accumulation of nitrotyrosine with the progression of the disease (figure 5, panels B and C, respectively), more pronounced in the inner medulla, where both vasa recta and tubules were affected since the earliest phase of the BDL (figure 6). BDL rats receiving chronic L-NAME exhibit similar expression of nitrotyrosine than controls in all the kidney regions, as shown in figures 5 and 6. All comparisons for nitrotyrosine expression were performed with respect to the sham group because the control group showed "not expression" and thus a value of zero in all kidney regions.

Liver histology

Chronic BDL-induced experimental cirrhosis produced a time-dependent increase in the inflammatory parameters in the livers. Thus, a mild inflammatory infiltrate (7 days) was widespread throughout the liver parenchyma (high numbers of PMNs [polymorphonuclear cells] and a lower number of MNs [mononuclear cells]). At this time, hepatocyte degeneration was minimal. This infiltrate became larger and clusters of PMNS and MNs (mainly monocytes) became evident at the periportal levels by 15 days post-BDL. Hepatocytes started to become eosinophilic and to accumulate biliary pigments. Although, signs of hepatocyte vacuolar degeneration were present, signs of cell apoptosis were not that frequent. Fibrosis relieved inflammation by 21 days post-BDL. Therefore, although, the diffuse inflammatory infiltrate was still evident and almost granulomatous formations were present at the portal levels, especially surrounding billiary conducts, greater areas of the hepatic parenchyma had been substituted for fibrotic tissue and the typical hepatic sinusoidal structure had been almost completely disrupted. Hepatocytes became very eosinophilic, as a sign of cytoplasmic degeneration, and contained greater amounts of biliary pigments within their cytoplasms. At this stage, signs of hepatocyte vacuolar degeneration and apoptosis appeared throughout the whole parenchyma.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE
Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

However, there was almost no evidence of fibrosis by 7 days post-BDL. Accumuli of fibrocytes and collagen fibers were almost restricted to near environment of the bile ducts. By 15 days, fibrosis had expanded wider to the surrounding areas of the hepatic lobule. By 21 days post-BDL, fibrosis was widespread, even disrupting the normal architecture of most hepatic lobules.

L-NAME administration attenuated partly liver cirrhotic changes by 21 days post-BDL (Figure 1). Inflammatory infiltrates, although still formed some cluster of cells, tended to be more diffuse. Hepatocytes, although damaged and displaying signs of cytoplasm degeneration and increased eosinophilia, had not suffered as much insult as those found in livers from non-treated BDL rats thereby not displaying that many features of cell degeneration and apoptosis. Finally, fibrosis, although still present was more restricted to periportal areas, rather than spread throughout the whole parenchyma.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

DISCUSSION

The main finding of this investigation is to report, we believe for the first time, that BDL induces a gradual increase of nitrotyrosine in the kidney, mainly in the renal medulla, and affecting both vascular and tubular components of this region. This accumulation might contribute to the onset and progression of renal vascular and excretory derangement in this experimental model of liver cirrhosis as we demonstrated that L-NAME administration that diminish NO synthesis and nitrotyrosine formation, also prevent the altered renal vascular responses and sodium retention of the BDL rats.

Experimental chronic cholestasis in the rat progressively alters liver structure and function leading to the development of portal hypertension and a hyperdynamic circulation, characterized by a systemic vasodilatation, high cardiac output and an altered response to vasoactive substances. As the disease advances, there is progressive sodium retention and eventual development of ascites, hepatorenal syndrome and death [5-6, 28]. The time course of these events is variable depending on several factors including the strain of rats, sex and even the laboratory [15, 28, 30, 32-34]. The animals used in the present study, in the fourth week of BDL, showed typical signs of cirrhosis, such as decreased growth, jaundice and coluria, and a decreased hematocrit. After death, the abdominal inspection revealed a little amount of ascites (1-5 mL) only in two rats of the untreated BDL group, but there was mesenteric edema and enlarged liver and spleen (indirect evidence of portal hypertension) in all of them. Morphologically the livers had marked ductal proliferation, fibrosis and signs of hepatocyte degeneration; and apoptosis appeared throughout the whole parenchyma. L-NAME treatment had a moderate effect on the liver morphology, improving the inflammatory process, but not on portal hypertension as suggested by a similar spleen weight. That is not entirely surprising since the L-NAME therapy is not addressing the primary insult, which is the surgical cholestasis. Comparable results have been described in other studies, using this and other treatments, where they found a recovery in the systemic hemodynamic and renal changes, although the improvement observed in liver function or portal pressures was small [20, 22-23, 35-36].

The incidence of renal dysfunction in patients and animals with obstructive jaundice is elevated in various clinical settings, such as bleeding or infection [33], as well as a special predisposition to renal failure [13, 35]. In our study, chronic BDL rats showed significant changes in the renal parameters measured after 15 days of BDL. Thus, we found a progressive sodium retention starting the 15th day post-BDL, an increased spleen weight and a decrease in blood pressure, basal renal perfusion pressure in the isolated kidneys and in the hematocrit. These results are similar to those obtained in our

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

previous studies in the same model of liver disease, showing that the systemic hemodynamic and renal excretory parameters start to change between the 12th and 14th days after the biliary occlusion [28]. At this stage, the antinatriuretic neurohormonal systems are activated as a consequence of the decreasing blood pressure, and sodium and water retention occurs through different vascular and tubular mechanisms along the different segments of the nephron [2, 6].

The mechanisms leading to the early decrease in blood pressure, are probably due to an excess of vasodilator substances, mainly NO, as we found increasing urinary excretion of nitrites, as soon as 7 days after BDL. NO has been widely implicated in the decreased vascular response to endogenous vasoconstrictors, a generalized phenomenon observed in clinical and experimental cirrhosis, in the setting of elevated circulating levels of vasoconstrictors [29, 31, 33, 37-38], although the mechanisms by which that happens are not completely elucidated. Our study also shows a reduced renal response to PHE at 15 and 21 days after BDL, coexisting with an increase in urinary nitrites excretion. In this regard, it has been reported that the cirrhotic serum progressively decreases the contractile responses to PHE, in aortic rings of healthy animals, starting at the first week after BDL [38]. This lower response was reversed by acute NO synthesis blockade, pointing to NO as the major factor for this vascular hyporesponse. In consonance with this, our data also demonstrate that chronic NOS inhibition with L-NAME completely avoids the renal vascular hyporesponsiveness to PHE in the BDL animals. It is possible that mechanisms derived from systemic NO synthesis inhibition can contribute to the beneficial effects on renal vascular reactivity of BDL rats, such as the increase in blood pressure and/or structural changes in the vessel wall. Indeed, it has been shown that a NO-dependent vascular remodeling occurs during liver cirrhosis [39] affecting conductive vessels and contributing to the profound circulatory dysfunction developed by experimental animals with advanced liver disease. Because L-NAME treatment results in a similar increase of blood pressure in control and cirrhotic rats, the reversal of the abnormal vascular remodeling in the cirrhotic animals was considered an effect of NO synthesis inhibition and not a consequence of the elevation of arterial pressure. In our study, 21-days BDL rats pre-treated with L-NAME had similar arterial pressure than controls. Nevertheless, the direct influence of blood pressure in the recovery of renal vascular reactivity to PHE in those rats must be partial because first, the kidneys from all groups were isolated from the systemic influences; and second, different factors, other than blocking the excess of NO synthesis, seem to be implicated since 24-hours urinary excretion of nitrites was not completely normalized by chronic L-NAME treatment in our animals with BDL-induced cirrhosis.

Stage 2(a) POST-PRINT
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE
Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

During the last years, ROS are the focus of many studies under diverse hepatic insults. Thus, ROS levels (malondialdehyde, isoprostanes, etc) are elevated in different models of chronic liver pathologies [14, 16, 18] and the levels correlated with the severity of the disease [17, 40]. We observed significant increased ROS levels, measured as TBARS (malondialdehyde), in plasma and kidney tissue, from the earliest stages of BDL. In the kidney, the increment was progressive with the advance of the disease and related to the hemodynamic and renal changes; whereas plasma TBARS remained elevated from the beginning of the disease. These results agree with previous studies by Panozzo et al [40] who found a correlation between the variation of hepatic and urinary TBARS, liver damage and functional glomerular and tubular renal alterations in rats following BDL, but not in plasma TBARS. It is possible that circulating TBARS represent a pool from several organs, affected differently by cirrhosis. However, the administration of chronic L-NAME reduced TBARS levels in plasma and kidney tissue, suggesting that in some way NO is involved rising ROS [24-25]. This is important since previous studies have shown that elevated generation of ROS contributes to the altered renal vascular reactivity and participates in the systemic and renal functional alterations induced by BDL [19, 22-23, 30].

With respect to NO, under physiological conditions, is a vasodilator. Thus, the progressive increase in NO production could explain the progressive decrease in the responses to PHE, since acute [30-31, 33] or chronic NO synthesis inhibition, as we show here, reverses the renal vascular hyporesponsiveness. However, this excess of NO should promote sodium and water excretion, opposite to what we found in the BDL rats, that is a decreased sodium excretion as BDL progress; and accordingly, the administration of NO inhibitors improved sodium and water excretion [7-10]. Additionally, the endothelium dependent relaxation was lower than normal from the initial periods of BDL and chronic L-NAME fully restored the altered vasodilatation to the BDL rats. This interesting finding suggests that this dose of L-NAME does not completely eliminate NO synthesis, which is supported by the fact that 24-hours urinary excretion of nitrites was not completely normalized and that a high dose of acutely administered L-NAME almost abolished the ACH-dependent vasorelaxation in the 21 days BDL rats chronically treated with L-NAME. And finally, it is possible that the excess of NO, or some NO-derived substances, is being harmful to the endothelial and renal function. Regarding this, we have recently showed that chronic administration of an antioxidant (vitamin E) to BDL rats paralleled the effects of L-NAME on renal vascular reactivity to PHE and ACH, kidney TBARS and most of the parameters studied in the present study; except for NO levels that were further increased (30). Taken together with our present study, these results suggest that during BDL the excess of NO in the presence of elevated oxidative stress is damaging the renal vascular and excretory function.

Stage 2(a) POST-PRINT
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

Although different substances may potentially alter vascular reactivity in the kidney [15, 41], in cirrhosis, elevated levels of NO and O_2^- can greatly favor the formation of peroxynitrite ($ONOO^-$). Peroxynitrite may alter the vasoactive responses to several substances by several ways. First, peroxynitrite formed endogenously, induces vascular relaxation and this effect is subjected to rapid tachyphylaxis thus altering subsequent vasorelaxant responses. In fact, after the development of tachyphylaxis to peroxynitrite, the hemodynamic effects produced by the systemic administration of acetylcholine (an endothelium-dependent vasorelaxant) but not to NO donors, were significantly attenuated [26]. In addition, it produces a substantial and selective attenuation of the hemodynamic effects *in vivo* produced by alpha- and beta-adrenoceptor agonists [27] and angiotensin II [42]; and these effects seem to be due to nitration of extracellular tyrosine residues by peroxynitrite [27, 42-44]. Despite of its non-radical nature, peroxynitrite is more reactive than its parent molecules [24-25] and can alter intracellular signaling pathways involve in the contractil vascular function [45-47]. Besides, it can promote the initiation of lipid peroxidation, yet again increasing oxidative stress derived products with the resultant consequences for the vascular and renal function [13, 48].

To date, only a few studies have reported the existence of increased nitrotyrosine in plasma, liver and heart during liver cirrhosis [49-51]. The present results now reveal, we believe for the first time, a progressive accumulation of nitrotyrosine in the BDL kidneys with the advance of disease, preferentially, in the endothelium of vasa recta and tubules of the inner medullary region, and more strikingly over 21 days after initiation of BDL. Thereby, these data suggest that excessive NO, by way of its transformation into peroxynitrite, can be noxious for the renal vascular and tubular function. Then, preventing the excess of the NO synthesis in the BDL rats with L-NAME decreases the formation of peroxynitrite (and therefore nitrotyrosine) and reverses the renal functional alterations evaluated in this study. According to this, it is tempting to speculate that the improvement observed in renal parameters after inhibiting NO synthesis in this or previous studies might be also a consequence of an anti-peroxynitrite effect. For example, previous *in vivo* studies demonstrated an inability of the cirrhotic kidney to adequately respond, increasing diuresis and natriuresis, to manoeuvres that vasodilate the kidney in a NO-dependent manner [11-12]. The same group showed later that administration of very low doses of L-NAME without having noticeable systemic or renal hemodynamic consequences, restored the lowered diuresis and natriuresis of cirrhotic animals, and these effects were reversed by L-arginine [7]. In addition, Mani et al [50] recently showed that decreasing the nitration of cardiac proteins with N-acetylcysteine and L-NAME led to normalization of *in vitro* cardiac chronotropic response of BDL animals. At the present, our study shows that

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

administration of a chronic dose of L-NAME, that avoids nitrotyrosine formation in the vasculature and tubules of BDL kidneys, prevents the changes in renal vascular reactivity (which can alter peritubular hemodynamic and sodium handling) and returns to normal sodium excretion in chronic BDL rats.

Limitations and unexplained findings

As mentioned in the introduction section of this work, we intended to study some of the paradoxical effect of elevated NO production during liver cirrhosis. We think that this work can help to explain part of these effects although some aspects would require further studies. One logical question emerged is about the existence of a decreased renal ACH response in the context of a decreased response to PHE, due to the increased NO production. In this respect, it is possible that basal and stimulated production of NO in the renal vasculature of BDL animals is affected differently [52]. The basal NO production might be increased considering that the lower response induced by PHE was reversed by L-NAME. Contrarily, the agonist-stimulated ACH release of NO is affected in an opposite way, suggesting a defective receptor or post-receptor activation of eNOS or a down regulation of this NO release mechanism. In this fashion, NO can be generated by iNOS from the endothelium and vascular smooth muscle cells resulting in a high NO output with potential damaging consequences, like the impairment in eNOS-derived NO production observed in vessels treated with inflammatory mediators [53]. Although both eNOS and iNOS seem to contribute to the high NO levels during BDL, the present study does not discriminate the individual contribution of each isoform.

The local damaging effects of overproduced NO during BDL seem to be mediated by its reaction with ROS to form peroxynitrite as we found that reducing NO levels, the altered renal response to ACH and PHE is reversed. If it is actually peroxynitrite that L-NAME is inhibiting, we think so as NO is an essential former of peroxynitrite [24, 25] and we found a striking reduction of the increased nitrotyrosine expression in the renal tissue of BDL+L-NAME rats. Other authors have obtained similar results in BDL rats [50] and in rats with endotoxemic shock (that causes comparable hemodynamic and vascular changes than BDL) by using a peroxynitrite decomposition catalyst [54]. In addition to the mechanisms cited above, peroxynitrite and other ROS can alter agonist eNOS activity *via* caveolae or uncoupling mediated mechanisms consequently driving to a lesser NO production [54-55]. Besides, they can affect iNOS expression that may increase NO output, later altering the eNOS-derived NO production in vessels [45, 53, 55]. Then, elevated NO levels in the setting of high oxidative status are infective and immersed in a vicious circle where both are continuously produced.

In conclusion, these results suggest that the effects of peroxynitrite might account for the altered renal vascular responses and abnormal renal sodium excretion during chronic BDL and thus contribute

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE

to the progression of renal dysfunction observed during this liver injury. The beneficial effects of decreasing NO synthesis with L-NAME are dual, first avoiding peroxynitrite formation and subsequently decreasing the re-initiation of lipid peroxidation and thus oxidative stress.

ACKNOWLEDGEMENTS
This work was supported by grants from the Spanish Ministerio de Ciencia y Tecnología (SAF2003-07467), from Fundación Séneca de Murcia (PB/45/FS/02) and from Instituto de Salud Carlos III (RNIHG, CO3/02). A. Alcaraz is supported by a predoctoral fellowship grant from SAF2003-07467.

Stage 2(a) POST-PRINT
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

REFERENCES

1 Angeli, P., Fernandez-Varo, G., Dalla Libera, V. et al. (2005) The role of nitric oxide in the pathogenesis of systemic and splanchnic vasodilation in cirrhotic rats before and after the onset of ascites. Liver Int. **25,** 429-437

2 Atucha, N.M. and García-Estañ, J. (1996) Intrarrenal alterations in experimental liver cirrhosis. News Physiol. Sci. **11,** 48-52

3 Atucha, N.M., Nadal, F.J., Iyu, D. et al. (2005) Role of vascular nitric oxide in experimental liver cirrhosis. Curr. Vasc. Pharmacol. **3,** 81-85

4 García-Estañ, J., Ortiz, M.C. and Lee, S.S. (2002) Nitric oxide and renal and cardiac dysfunction in cirrhosis. Clin. Sci. **102**, 213-222.

5 Hadoke, P.W. and Hayes, P.C. (1997) In vitro evidence for vascular hyporesponsiveness in clinical and experimental cirrhosis. Pharmacol. Ther. **75**, 51-68

6 Heneghan, M.A. and Harrison, P.M. (2000) Pathogenesis of ascites in cirrhosis and portal hypertension. Med. Sci. Monit. **6**, 807-816

7 Atucha, N.M., García-Estañ, J., Ramírez, A., Pérez, M.C., Quesada, T. and Romero, J.C. (1994) Renal effects of nitric oxide synthesis inhibition in cirrhotic rats. Am. J. Physiol. **267**, R1454-R1460

8 Martin, P.Y., Ohara, M., Gines, P. et al. (1998) Nitric oxide synthase inhibition for one week improves renal sodium and water excretion in cirrhotic rats with ascites. J. Clin. Invest. **101**, 235-242

9 Ortiz, M.C., Fortepiani, L.A., Martínez, C., Atucha, N.M. and García-Estañ, J. (1996) Renal and pressor effects of aminoguanidine in cirrhotic rats with ascites. J. Am. Soc. Nephrol. **7**, 2694-2699

10 Ortiz, M.C., Fortepiani, L.A., Martínez-Salgado, C. et al. (2001) Renal effects of the chronic inhibition of nitric oxide synthesis in cirrhotic rats with ascites. Nefrología **21**, 556-564

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

11 Atucha, N.M., Cegarra, M., Ramirez, A., Quesada, T. and Garcia-Estañ, J. (1993) Pressure diuresis and natriuresis in cirrhotic rats. Am. J. Physiol. **265,** G1045-G1049

12 Atucha, N.M., Quesada, T. and García-Estañ, J. (1993) Reduced renal papillary plasma flow in non-ascitic cirrhotic rats. Clin. Sci. **85,** 139-145

13 Bomzon, A., Holt, S. and Moore, K. (1997) Bile acids, oxidative stress, and renal function in biliary obstruction. Semin. Nephrol. **17**, 549-562

14 Ljubuncic, P., Tanne, Z. and Bomzon, A. (2000) Evidence of a systemic phenomenon for oxidative stress in cholestatic liver disease. Gut **47**, 710-716

15 Miyazono, M., Zhu, D., Nemenoff, R., Jacobs, E.R. and Carter, E.P. (2003) Increased epoxyeicosatrienoic acid formation in the rat kidney during liver cirrhosis. J. Am. Soc. Nephrol. **14**, 1766-1775

16 Moore, K. (2004) Isoprostanes and the liver. Chem. Phys. Lipids **128**, 125-133

17 Nanji, A.A., Khwaja, S., Tahan, S.R. and Sadrzadeh, S.M. (1994) Plasma levels of a novel noncyclooxygenase-derived prostanoid (8- isoprostane) correlate with severity of liver injury in experimental alcoholic liver disease. J. Pharmacol. Exp. Ther. **269**, 1280-1285

18 Orellana, M., Rodrigo, R., Thielemann, L. and Guajardo, V. (2000) Bile duct ligation and oxidative stress in the rat: effects in liver and kidney. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. **126**, 105-111

19 Ara, C., Karabulut, A.B., Kirimlioglu, H. et al. (2005) Protective effect of resveratrol against renal oxidative stress in cholestasis. Ren. Fail. **27**, 435-440

20 Fernando, B., Marley, R., Holt, S. et al. (1998) N-acetylcysteine prevents development of the hyperdynamic circulation in the portal hypertensive rat. Hepatology **28**, 689-694

21 Holt, S., Goodier, D., Marley, R. et al. (1999) Improvement in renal function in hepatorenal syndrome with N-acetylcysteine. Lancet **353**, 294-295

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

22 Marley, R., Holt, S., Fernando, B. et al. (1999) Lipoic acid prevents development of the hyperdynamic circulation in anesthetized rats with biliary cirrhosis. Hepatology **29**, 1358-1363

23 Ortiz, M.C., Manriquez, M.C., Nath, K.A., Lager, D.J., Romero, J.C. and Juncos, L.A. (2003) Vitamin E prevents renal dysfunction induced by experimental chronic bile duct ligation. Kidney Int. **64,** 950-961

24 Pryor, W.A. and Squadrito, G.L. (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am. J. Physiol. **268**, L699-L722

25 Beckman, J. and Koppenol, W. (1996) Nitric oxide, superoxide, and peroxynitrite; the good the bad and the ugly. Am. J. Physiol. **271**, C1424-C1437

26 Benkusky, N.A., Lewis, S.J. and Kooy, N.W. (1998) Attenuation of vascular relaxation after development of tachyphylaxis to peroxynitrite in vivo. Am. J. Physiol. **275**, H501-H507

27 Benkusky, N.A., Lewis, S.J. and Kooy, N.W. (1999) Peroxynitrite-mediated attenuation of alpha and beta-adrenoceptor agonist-induced vascular responses in vivo. Eur. J. Pharmacol. **364**, 151-158

28 Martínez-Prieto, C., Ortiz, M.C., Fortepiani, L.A., Ruiz-Maciá, J.A., Atucha, N.M. and García-Estañ, J. (2000) Haemodynamic and renal evolution of the bile duct-ligated rat. Clin. Sci. **98**, 611-617

29 Ortiz, M.C., Fortepiani, L.A., Martínez, C., Atucha, N.M. and García-Estañ, J. (1996) Vascular hyporesponsiveness in aortic rings from cirrhotic rats: role of nitric oxide and endothelium. Clin. Sci. **91**, 733-738

30 Alcaraz, A., Iyu, D., Atucha, N.M., Garcia-Estañ, J. and Ortiz M.C. (2007) Vitamin E supplementation reverses renal altered vascular reactivity in chronic bile duct-ligated rats. Am. J. Physiol. **292**, R1486-R1493

31 García-Estañ, J., Atucha, N.M. and Groszman, R.J. (1996) Renal response to methoxamine in portal hypertensive rats: role of prostaglandins and nitric oxide. J. Hepatol. **25**, 206-211

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

32 Graebe, M., Brond, L., Christensen, S., Nielsen, S., Olsen, N.V. and Jonassen, T.E. (2004) Chronic nitric oxide synthase inhibition exacerbates renal dysfunction in cirrhotic rats. Am. J. Physiol. **286**, F288-F297

33 Inan, M., Sayek, I., Tel, B.C. and Sahin-Erdemli, I. (1997) Role of endotoxin and nitric oxide in the pathogenesis of renal failure in obstructive jaundice. Br. J. Surg. **84,** 943-947

34 Katsuta, Y., Zhang, X.J., Ohsuga, M. et al. (2005) Hemodynamic features of advanced cirrhosis due to chronic bile duct ligation. J. Nippon Med. Sch. **72**, 217-225

35 Islas-Carbajal, M.C., Covarrubias, A., Grijalva, G., Alvarez-Rodriguez, A., Armendariz-Borunda, J. and Rincon-Sanchez, A.R. (2005) Nitric oxide synthases inhibition results in renal failure improvement in cirrhotic rats. Liver Int. **25,** 131-140

36 Wei, C.L., Hon, W.M., Lee, K.H. and Khoo, H.E. (2005) Chronic administration of aminoguanidine reduces vascular nitric oxide production and attenuates liver damage in bile duct-ligated rats. Liver Int. **25**, 647-656

37 Helmy, A., Newby, D.E., Jalan, R., Johnston, N.R., Hayes, P.C. and Webb, D.J. (2003) Nitric oxide mediates the reduced vasoconstrictor response to angiotensin II in patients with preascitic cirrhosis. J. Hepatol. **38**, 44-50

38 Schmandra, T.C., Folz, I.C., Kimpel, M., Fleming, I., Holzer, K. and Hanisch, E.W. (2001) Cirrhosis serum induces a nitric oxide-associated vascular hyporeactivity of aortic segments from healthy rats in vitro. Eur. J. Gastroenterol. Hepatol. **13**, 957-962

39 Fernandez-Varo, G., Ros, J., Morales-Ruiz, M. et al. (2003) Nitric oxide synthase 3-dependent vascular remodeling and circulatory dysfunction in cirrhosis. Am. J. Pathol. **162**, 1985-1993

40 Panozzo, M.P., Basso, D., Balint, L., Zaninotto, M., Bonvicini, P. and Plebani, M. (1995) Renal functional alterations in extrahepatic cholestasis: can oxidative stress be involved?. Eur. Surg. Res. **27**, 332-339

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

41 Tahseldar-Roumieh, R., Ghali-Ghoul, R., Lugnier, C. and Sabra, R. (2006) Effect of phosphodiesterase 5 inhibitor on alteration in vascular smooth muscle sensitivity and renal function in rats with liver cirrhosis. Am. J. Physiol. **290**, H481-H488

42 Kooy, N.W. and Lewis, S.J. (1996) The peroxynitrite product 3-nitro-L-tyrosine attenuates the hemodynamic responses to angiotensin II in vivo. Eur. J. Pharmacol. **315,** 165-170

43 Kooy, N.W. and Lewis, S.J. (1996) Nitrotyrosine attenuates the hemodynamic effects of adrenoceptor agonists in vivo: relevance to the pathophysiology of peroxynitrite. Eur. J. Pharmacol. **310,** 155-161

44 Lewis, S.J., Hoque, A., Walton, T.M. and Kooy, N.W. (2005) Potential role of nitration and oxidation reactions in the effects of peroxynitrite on the function of beta-adrenoceptor sub-types in the rat. Eur. J. Pharmacol. **518**, 187-194

45 Cooke, C.L. and Davidge, S.T. (2002) Peroxynitrite increases iNOS through NF-kappaB and decreases prostacyclin synthase in endothelial cells. Am. J. Physiol. **282**, C395-C402

46 Hattori, Y., Kasai, K. and Gross, S.S. (2004) NO suppresses while peroxynitrite sustains NF-kappaB: a paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO. Cardiovasc. Res. **63**, 31-40

47 Li, J., Li, W., Su, J., Liu, W., Altura, B.T. and Altura, B.M. (2004) Peroxynitrite induces apoptosis in rat aortic smooth muscle cells: possible relation to vascular diseases. Exp. Biol. Med. **229**, 264-269

48 Bomzon, A. and Ljubuncic, P. (2001) Oxidative stress and vascular smooth muscle cell function in liver disease. Pharmacol. Ther. **89**, 295-308

49 Cuzzocrea, S., Zingarelli, B., Villari, D., Caputi, A.P. and Longo, G. (1998) Evidence for in vivo peroxynitrite production in human chronic hepatitis. Life Sci. **63**, PL25-PL30

50 Mani, A.R., Ippolito, S., Ollosson, R. and Moore, K.P. (2006) Nitration of cardiac proteins is

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE
Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

associated with abnormal cardiac chronotropic responses in rats with biliary cirrhosis. Hepatology **43**, 847-856

51 Ottesen, L.H., Harry, D., Frost, M. et al. (2001) Increased formation of S-nitrothiols and nitrotyrosine in cirrhotic rats during endotoxemia. Free Radic. Biol. Med. **31**, 790-798

52 Busse, R., Mülsch, A., Fleming, I. et al. (1993) Mechanisms of nitric oxide release from the vascular endothelium. Circulation 87 (Supl V): V18-V25.

53 Andrew, P.J., Mayer, B. (1999) Enzymatic function of nitric oxide synthases. Cardiovascular Research 43: 521-531.

54 Cuzzocrea, S., Mazzon, E., Di Paola, R. et al. (2006) A role for nitric oxide-mediated peroxynitrite formation in a model of endotoxin-induced shock. J Pharmacol Exp Ther 319 (1): 73-81.

55 Münzel T, Li H, Mollnau H, Hink U et al. (2000) Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. Circ Res **86** (1): E7-E12.
Stage 2(a) POST-PRINT
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

FIGURES LEGENDS

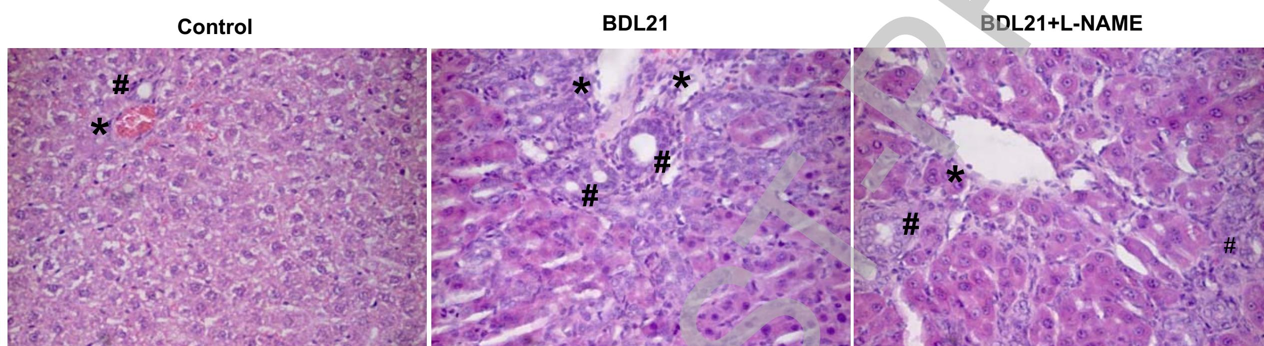
1. Photomicrographs (H&E stain; x40 magnification) of liver histology. As observed, the liver tissue is well preserved in control rats while non-treated 21 days BDL rats showed a diffuse inflammatory infiltrate (mainly monocytes) more evident at the periportal level (*), especially surrounding the proliferated billiary ducts (#). Hepatocytes are very eosinophilic, a sign of cytoplasmic degeneration, throughout the whole parenchyma which is substituted for fibrotic tissue, disrupting the normal architecture of most hepatic lobules. L-NAME administration partly attenuated liver cirrhotic changes by 21 days post-BDL. Damaged hepatocytes were more restricted to periportal areas and had not suffered as much insult (less eosinophilic) as those found in livers from non-treated BDL, and inflammatory infiltrates and fibrosis, although still present, tended to be more diffuse.
2. Sodium Balance (A), Blood Pressure (BP; B) and basal renal perfusion pressure in the isolated kidneys (RPP; C) in the different experimental groups. Values are means ± SE. (*) P <0.05 vs control group; (†) P<0.05 vs BDL7 group; (‡) P<0.05 vs BDL15 group; and (§) P<0.05 vs BDL21 group.
3. Renal vascular reactivity to phenylephrine (PHE; A and B panels) and acetylcholine (ACH; C and D panels) in the different experimental groups: Control (●), Sham (○), BDL7 (▲), BDL15 (Δ), BDL21 (■), BDL21+L-NAME (□, solid line), BDL21+L-NAME (acute) (□, dotted line). Values are means ± SE. (*) P <0.05 vs control group; (†) BDL21 group; and (‡) P<0.05 BDL21+L-NAME (acute) group.
4. Levels of TBARS in plasma (A) and kidney (B), and 24-hour urinary excretion of nitrites (C) in the different experimental groups. Values are means ± SE. (*) P <0.05 vs control group; (†) P<0.05 vs BDL7 group; (‡) P<0.05 vs BDL15 group; and (§) P<0.05 vs BDL21 group.
5. Semiquantitative score evaluation of renal nitrotyrosine in the three kidney regions (glomerular, A; outer medulla, B; and inner medulla, C) from the different experimental groups. Values are means ± SE. (*) P <0.05 vs sham group; (†) P<0.05 vs BDL7 group; (‡) P<0.05 vs BDL15 group; and (§) P<0.05 vs BDL21 group.
6. Representative images of nitrotyrosine expression in the inner medulla of kidneys from the different experimental groups (x100 magnification). Detail of the progressive nytrotirosine-labelled protein accumulation in the cytoplasm of endothelial (arrows) and tubular cells (crosses) at different stages of BDL (7, 15 and 21 days). As observed, chronic L-NAME treatment prevented this elevation in nitrotyrosine expression after 21 days of the BDL.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 1



Stage 2(a) POST-PRINT

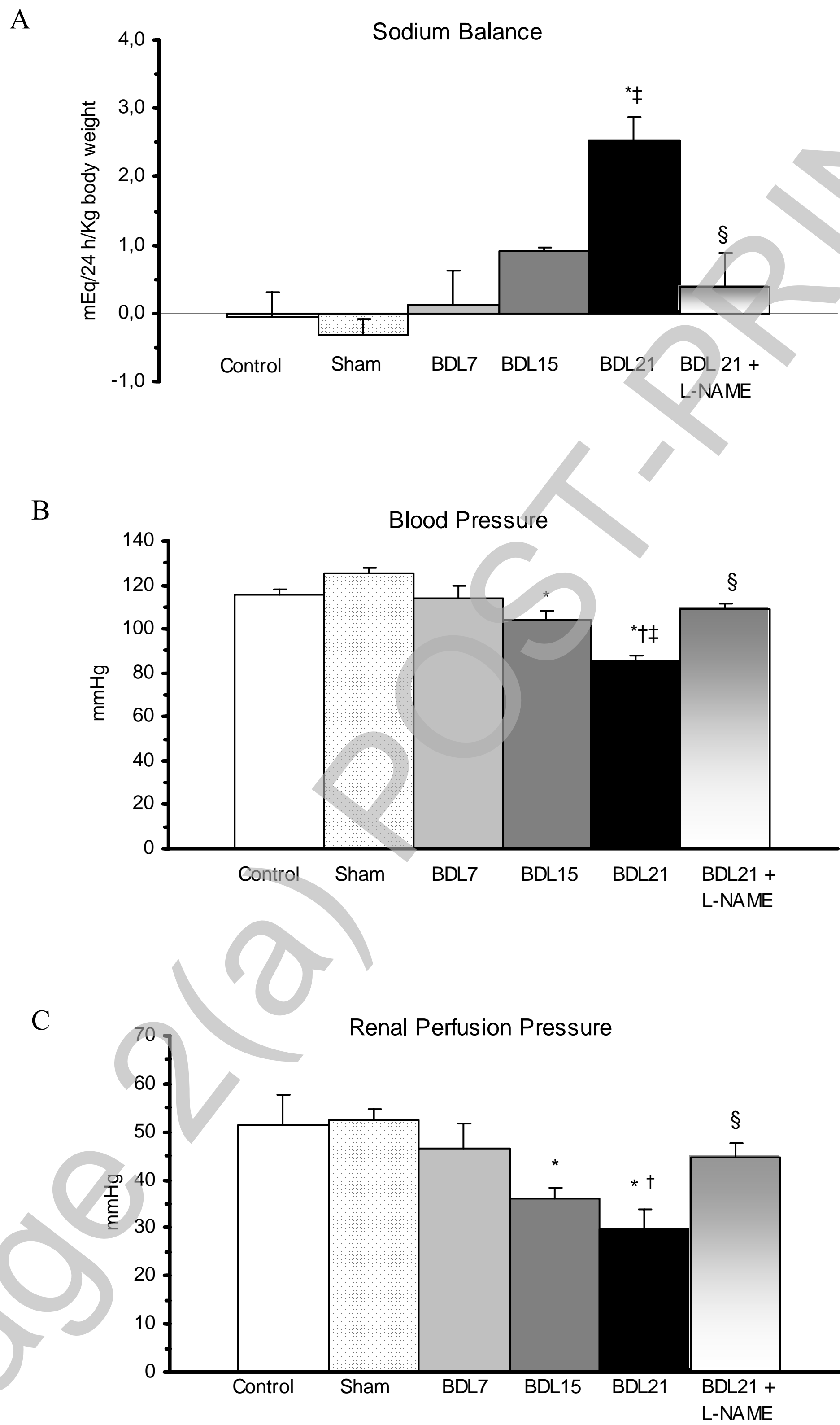
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

Figure 2

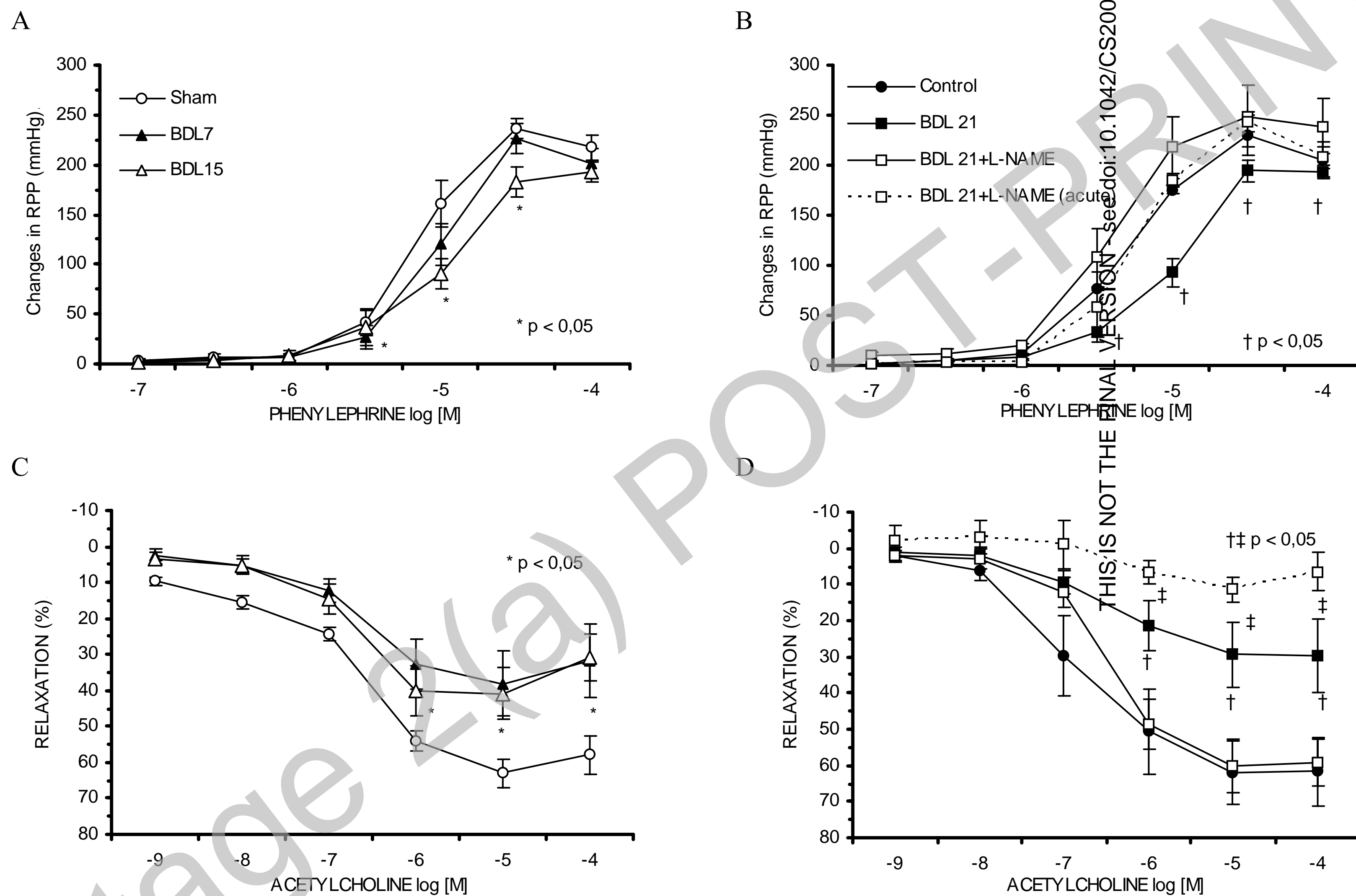


Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Figure 3

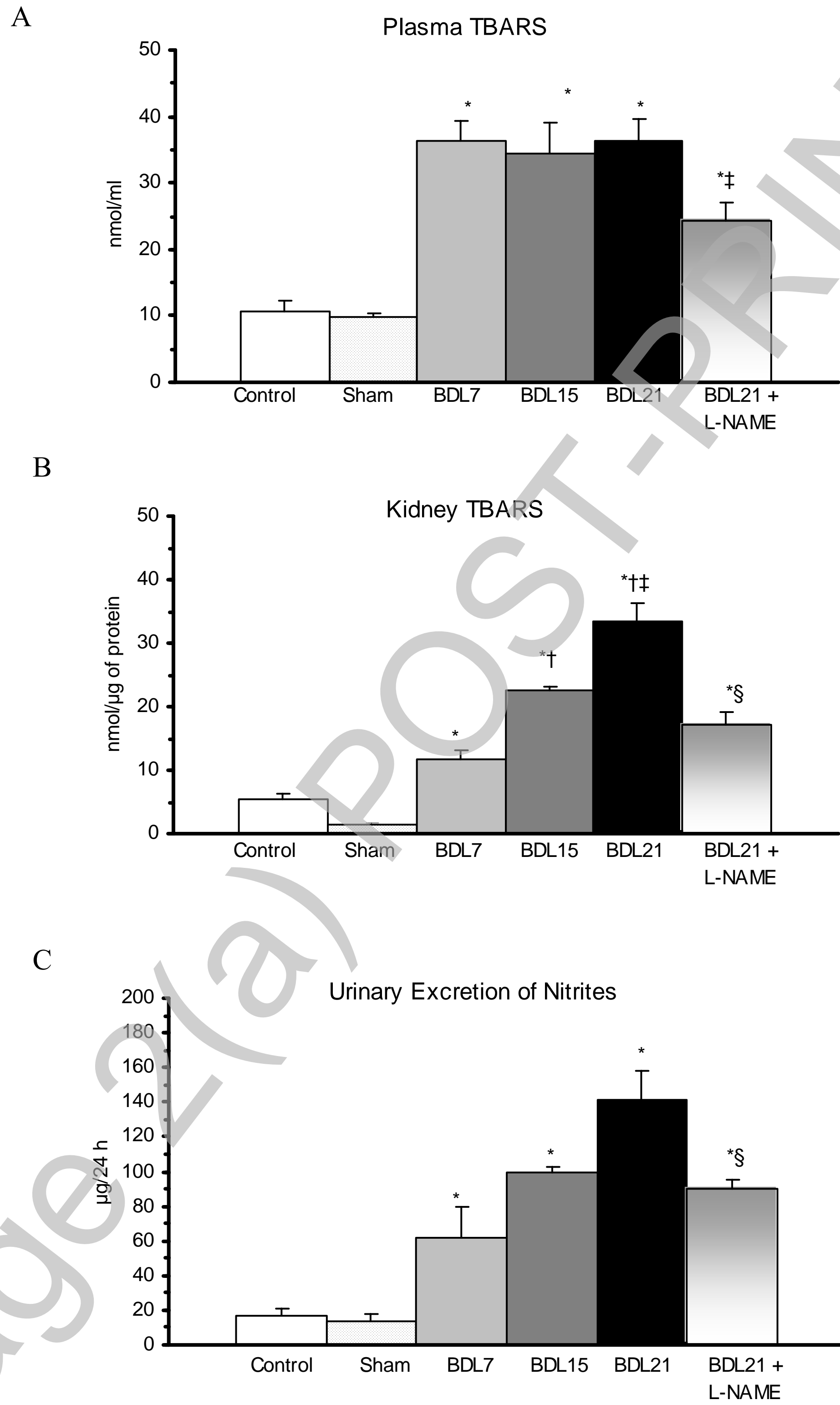


THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312
Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

Figure 4



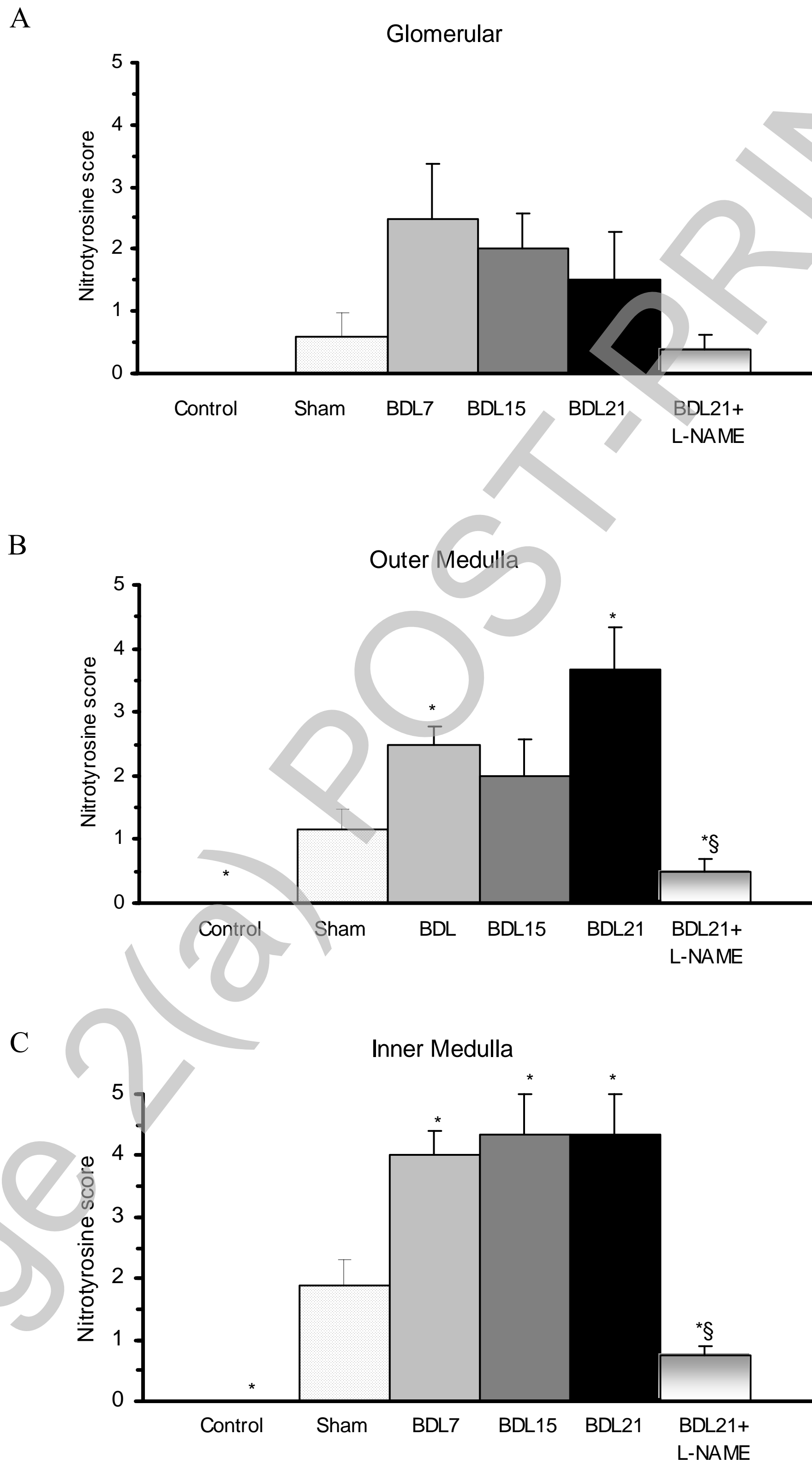
Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

Figure 5



Stage 2(a) POST-PRINT

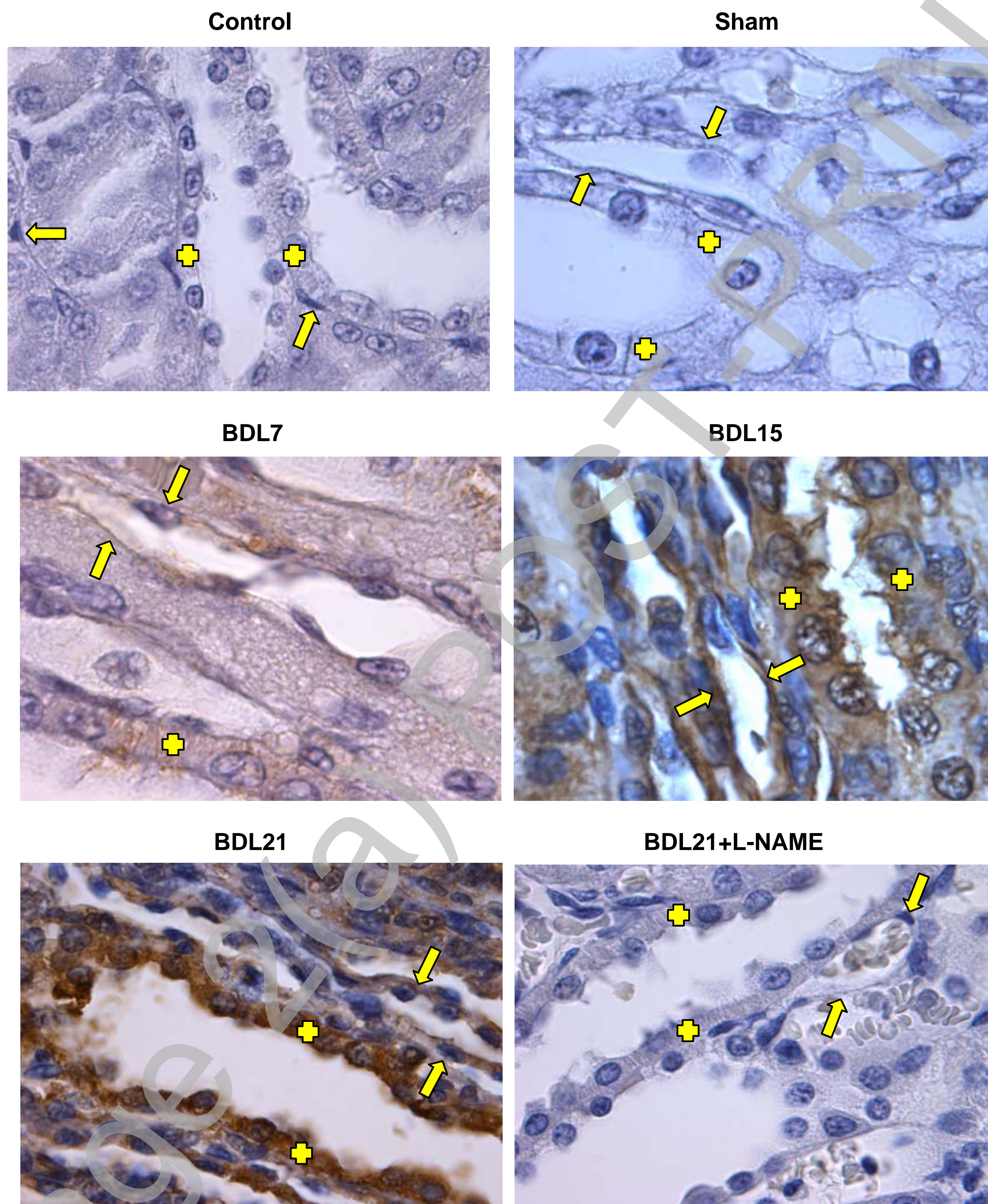
THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical Science

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

Figure 6



Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Clinical SCIENCE

Clinical Science Immediate Publication. Published on 9 Jan 2008 as manuscript CS20070312

Table 1. Values of the body weight, spleen weight, kidney to body weight and hematocrit in the different experimental groups.

	Body weight (g)	Spleen weight (g)	Kidney to Body weight (%)	Hematocrit (%)
Control	340 ± 18	0.86 ± 0.05	0.31 ± 0.02	46 ± 1
Sham	313 ± 8	0.92 ± 0.05	0.33 ± 0.01	44 ± 1
BDL 7	255 ± 8 *	0.88 ± 0.07	0.38 ± 0.01 *	45 ± 2
BDL 15	301 ± 14 †	1.61 ± 0.17 * †	0.39 ± 0.02 *	41 ± 1 * †
BDL 21	238 ± 6 * †	1.87 ± 0.14 * †	0.44 ± 0.02 * †	38 ± 1 * †
BDL 21+ L-NAME	295 ± 17 §	2.01 ± 0.19 *	0.44 ± 0.02 * §	52 ± 1 * §

Values are means ± SE. (*) $P < 0.05$ vs control group; (†) $P<0.05$ vs BDL7 group; (‡) $P<0.05$ vs BDL15 group; and (§) $P<0.05$ vs BDL21 group.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/CS20070312

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society