Eur J Med Res (2008) 13: 327-329 

# Tissue Specific Activation of the Endothelin System in Severe Acute Liver Failure

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**Abstract**
The endothelin system has been implicated in the pathogenesis of acute liver failure. However, it has not yet been assessed in a tissue specific manner.

Acute liver failure was induced in rats by two intraperitoneal injections of galactosamine (1.3 g/kg, interval of 12 hours, n = 20). The animals were sacrificed after 48 hours.

Plasma measurements demonstrated that animals receiving galactosamine had a laboratory constellation of severe liver injury and they histologically presented with hepatic necrosis and inflammation. Plasma concentrations of endothelin-1 were elevated 60-fold in the animals receiving galactosamine (p = 0.005). In contrast endothelin-1 tissue contents were decreased in the kidneys and unchanged in the liver. Western blot analysis showed that animals receiving galactosamine had a significantly lower endothelin B receptor concentration in liver and kidney tissue, whereas no differences were detected for endothelin A receptors.

This study demonstrates that the local endothelin system of liver and kidneys is not responsible for the increase of plasma endothelin-1 concentrations in acute liver failure. Since it is well established that the endothelin B receptor acts as a clearance receptor, its decreased density might contribute to the strongly elevated plasma endothelin-1 concentrations seen in this model of acute liver injury.

*Key words:* Endothelin, endothelin receptors, liver failure, galactosamine

## Introduction

Although acute liver failure may have a varied etiology, its clinical forms do have many features in common. The liver cell damage seen in acute liver failure is not only due to direct effects of the precipitating cause, but also due to a secondary release of cytokines and cytotoxic mediators from activated Kupffer, stellate and sinusoidal endothelial cells, thus creating a vicious circle.

Many previous studies have shown that plasma levels of endothelin-1 (ET-1) are increased in patients with severe liver disease [1-3]. The mechanisms responsible for the rise of plasma ET-1 and its role in the development of acute liver failure remain unclear.

Liver injury induced by galactosamine (GalN) is an established model for the investigation of hepatotoxic pathomechanisms [4, 5]. After injections of GalN rats develop acute liver failure with elevated liver enzymes and increased plasma ET-1 concentrations. Using this model, our study is set out to investigate the tissue specific ET system of liver and kidneys in acute liver injury.

## Materials and Methods

### Study Design

Animal studies were carried out in accordance with German law governing the use and care of laboratory animals. Animals were housed under standardized conditions with access to water and food ad libitum. Male Sprague Dawley rats (7-8 weeks of age) were randomly allocated to two study groups. They received two intraperitoneal injection of either GalN at a dose of 1.3 g/kg with a 12 hour interval (GalN group, n = 20), or the same volume of saline (control group, n = 9).

The animals were sacrificed 48 hours after the first injection, blood samples were obtained and liver and kidneys were harvested and immediately frozen in liquid nitrogen.

### Clinical Chemistry

Final plasma concentrations of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma glutamyltransferase (GGT), bilirubin, creatinine, urea, glucose, lipase, amylase, creatine kinase, total protein and sodium were determined using the appropriate kits in an automatic analyzer (Cobas Integra 800, Roche, Grenzach, Germany).

### Histological Studies

Liver tissue samples were embedded in paraffin, cut into 3 µm sections and subjected to hematoxylin-eosin staining. Histological evaluation was performed similar to previous publications [6]. A semi-quantitative score was used to grade the extent of hepatic necrosis and neutrophil infiltration. A score from 0 to 3 was assigned to each specimen according to the following system: Necrosis: 0, normal histology; 1, minor necrosis; 2, widely distributed patchy necrosis of hepato-

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cytes; and 3, complete lobular disruption and diffuse hepatocyte necrosis. Neutrophil infiltration: 0, normal histology; 1, perilobular inflammatory reaction; 3, panlobular inflammatory reaction.

### Tissue and Plasma ET-1 and Big-ET-1

Tissue samples were prepared as described recently [7]. ET-1, big-ET-1 and protein were determined using a commercially available immunoassay according to the manufacturer's instruction (Immundiagnostik AG, Bensheim, Germany). Plasma samples were analyzed with the same assays.

### Western Blot Analysis

Snap frozen tissue was pulverized with mortar and pestle under liquid nitrogen. 100 mg of the powder was dissolved in 1 ml of RIPA Buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium desoxycholic acid, 0.1% SDS and 1% Triton X100) supplemented with a proteinase inhibitor cocktail (Roche, Grenzach, Germany). The samples were sonicated five times for 5 s. The samples were incubated for 10 min at room temperature and then centrifuged for 12 min (13,000 rpm, 4°C). Protein concentration in the supernatant was measured using the BCA assay from Novagen (Madison, WI, USA) and the samples were diluted accordingly to assure equal loading. Proteins (25 µg per lane) were separated by SDS-PAGE (10%). The proteins were transferred onto a nitrocellulose membrane for 70 min (2 mA/cm²) using semidry transferring equipment. The unspecific sites were blocked with TBS-Tween/5% milk. Antibodies against the ETA and ETB receptor (1:1000 and 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and beta-actin (1:4000, Sigma-Aldrich, Steinheim, Germany) were incubated at room temperature for 1 hour. After extensive washing blots were incubated with a peroxidase-linked anti-rabbit IgG (1 hour, 1:4000, Sigma-Aldrich, Steinheim, Germany). Immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany) and were subsequently quantified with the AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). Results of ET receptors were normalized to beta-actin.

### Statistical Analysis

Data was analyzed with SPSS 11.5 (Chicago, IL, USA). Results are expressed as mean ± standard error. Differences between groups were compared by the nonparametric Mann-Whitney-U test. All tests were two-sided and p-values $<0.05$ were considered significant.

## Results and Conclusion

Plasma measurements two days after the first administration of GalN demonstrated that the animals receiving GalN had a laboratory constellation of severe liver injury. ASAT, ALAT, GGT and bilirubin were markedly elevated, whereas glucose and total protein were significantly reduced in this group (Table 1). As expected from previous reports GalN leads to enhanced necrosis and inflammation in liver tissue (Table 2).

As shown in Table 3, ET-1 was significantly elevated in plasma of animals that received GalN. This is in line with previous reports [1-3]. In contrast, ET-1 tissue contents were decreased in the kidneys of the animals that received GalN. No significant differences were detected in liver tissue. The analysis of the precursor big-ET-1 showed no differences between the groups.

The western blot analyses demonstrated that animals receiving GalN had a significantly lower ETB re-

*Table 1.* Plasma measurements two days after the administration of galactosamine.

| | Control | Galactosamine | p |
|---|---|---|---|
| N | 9 | 20 | |
| **ASAT** (U/l) | 266 ± 27 | 5802 ± 1299 | 0.007 |
| **ALAT** (U/l) | 46.2 ± 6.9 | 2937 ± 700 | 0.001 |
| **GGT** (U/l) | 2.0 ± 0.01 | 8.4 ± 1.6 | 0.002 |
| **Bilirubin** (mg/dl) | 0.05 ± 0.001 | 3.17 ± 0.57 | 0.001 |
| **Creatinine** (mg/dl) | 0.27 ± 0.02 | 0.33 ± 0.02 | not significant |
| **Urea** (mg/dl) | 37.4 ± 3.14 | 42.9 ± 2.24 | not significant |
| **Glucose** (mg/dl) | 159 ± 7.0 | 89 ± 12.5 | 0.001 |
| **Lipase** (U/l) | 8.33 ± 0.47 | 18.2 ± 7.6 | not significant |
| **Amylase** (U/l) | 1823 ± 148 | 1249 ± 364 | 0.038 |
| **Creatine kinase** (U/l) | 1891 ± 169 | 2687 ± 1483 | 0.024 |
| **Total protein** (g/dl) | 4.96 ± 0.13 | 4.23 ± 0.12 | 0.001 |
| **Sodium** (mmol/l) | 143 ± 0.91 | 141 ± 0.38 | not significant |

ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; GGT, gamma glutamyltransferase.

*Table 2.* Necrosis and inflammation scores in histological liver sections.

| | Control | Galactosamine | p |
|---|---|---|---|
| N | 9 | 20 | |
| **Necrosis score** | 0.61 ± 0.26 | 1.95 ± 0.23 | 0.003 |
| **Inflammation score** | 0 ± 0 | 0.97 ± 0.16 | 0.001 |

*Table 3.* Plasma and tissue measurements of endothelin-1 and big-endothelin-1 two days after the administration of galactosamine.

| | Control | Galactosamine | p |
|---|---|---|---|
| N | 9 | 20 | |
| **Plasma** (pg/ml) | | | |
| Endothelin-1 | 0.02 ± 0.01 | 1.23 ± 0.38 | 0.001 |
| Big-Endothelin-1 | 0.13 ± 0.05 | 0.66 ± 0.22 | not significant |
| **Liver** (pg/100mg protein) | | | |
| Endothelin-1 | 40.1 ± 16.1 | 18.6 ± 3.0 | not significant |
| Big-Endothelin-1 | 29.1 ± 8,73 | 37.7 ± 4.09 | not significant |
| **Kidney** (pg/100mg protein) | | | |
| Endothelin-1 | 121.6 ± 22,5 | 59.0 ± 11.4 | 0.027 |
| Big-Endothelin-1 | 17.8 ± 3.0 | 17.9 ± 3.9 | not significant |

*Table 4.* Western Blot analyses of endothelin receptors (ETAR and ETBR) in liver and kidney tissue two days after the administration of galactosamine. Arbitrary units, normalized to control group.

| | Control | Galactosamine | p |
|---|---|---|---|
| N | 6 | 6 | |
| **Liver** | | | |
| ETAR | 1.00 ± 0.13 | 1.09 ± 0.15 | not significant |
| ETBR | 1.00 ± 0.06 | 0.24 ± 0.07 | < 0.001 |
| **Kidney** | | | |
| ETAR | 1.00 ± 0.16 | 0.76 ± 0.26 | not significant |
| ETBR | 1.00 ± 0.16 | 0.46 ± 0.02 | 0.018 |

ceptor concentration in liver and kidney tissue. There were no significant changes for ETA receptors (Table 4). There are no reports on ETB receptors in acute liver injury in the literature so far. In contrast, a recent study reported increased hepatic ETA receptors after 2 hours in a very aggressive model of acute liver failure, measured by immunohistochemistry [3].

In conclusion, we have demonstrated that the local ET system of liver and kidney is not responsible for the strong increase of plasma ET-1 concentrations. We also showed that GalN treatment reduces the number of overall ETB receptors in liver and kidney but has no influence on ETA receptors. Since it is well known that the ETB receptor acts as a clearance receptor [8, 9], the decreased ETB receptor density seen in our model might contribute to the elevated plasma ET-1 concentrations in acute liver failure.

Through our findings, it is indicated that an activated vascular ET system results in an increased plasma ET-1 level, since the endothelium is the most probable structure able to liberate large amounts of ET-1 [10]. Downregulation of endothelial ETB receptors has also been described in diseases associated with endothelial dysfunction [11]. However, additional experiments are needed to reappraise this hypothesis.

*Acknowledgments:* The work of Philipp Kalk and Katharina Relle was supported by a grant from the Deutsche Forschungsgemeinschaft (PE 388/20-1).

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*Received: January 9, 2008 / Accepted: February 15, 2008*

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