Research Article

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Inactivation of extrahepatic vascular Akt improves systemic hemodynamics and sodium excretion in cirrhotic rats

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Background & Aims: Increased activity of the vascular Akt/eNOS signaling pathway is involved in the hemodynamic and renal complications developed by patients and rats with cirrhosis and ascites. This occurs in the setting of impaired Akt/eNOS activity within the cirrhotic liver. Here we assessed the feasibility of selectively inhibiting vascular eNOS without further impairing the intrahepatic activity of this enzyme. Ultimately, we sought to determine whether endothelial transduction of a constitutively inactive mutant of Akt (AA-Akt) improves circulatory function and sodium excretion in cirrhotic rats with ascites.

Methods: First, we administered recombinant adenoviruses that encode the β-galactosidase gene (β-gal) to 5 control rats and 5 cirrhotic rats with ascites and analyzed their tissue distribution by chemiluminescence. Next, urine samples were obtained from 18 cirrhotic rats with ascites and then the animal randomly received saline or adenoviruses containing the β-gal or the AA-Akt genes. Following a 24-h urine collection period, hemodynamic studies were performed and tissue samples were obtained to analyze Akt and eNOS expressions.

Results: No β-gal activity was detected in the liver of cirrhotic rats compared to that of controls. This was paralleled by increased β-gal activity in other territories such as the thoracic aorta. AA-Akt transduction improved systemic hemodynamics, splanchnic perfusion pressure and renal excretory function in comparison with cirrhotic rats transduced with β-gal adenoviruses or receiving saline. Moreover, the AA-Akt transgene did not modify portal pressure.

Conclusions: Inactivation of extrahepatic vascular Akt and the concomitant decrease in nitric oxide expression ameliorate systemic hemodynamics and renal excretory function in experimental cirrhosis.

Keywords: Cirrhosis; Ascites; Arterial vasodilation; Systemic hemodynamics; Renal function; Nitric oxide (NO); Endothelial nitric oxide synthase (eNOS); Serine-threonine kinase Akt; AA-Akt adenoviruses.

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Abbreviations: NO, nitric oxide; AA-Akt, constitutively inactive mutant of serine-threonine kinase Akt; eNOS, endothelial nitric oxide synthase; BLMVECs, bovine lung microvascular endothelial cells; β-gal, β-galactosidase; HA, hemagglutinin; m.o.i., multiplicity of infection; P-Akt, phosphorylated Akt; CCl4, carbon tetrachloride; pfu, plaque-forming units; UV, urine volume; UNaV, urinary excretion of sodium; UmOsmV, urinary excretion of mOsmol; UCreatV, urinary excretion of creatinine; MAP, mean arterial pressure; PP, portal pressure; HR, heart rate; CO, cardiac output; CI, cardiac index; TPR, total peripheral resistance; SPP, splanchnic perfusion pressure; P-eNOS, phosphorylated eNOS; L-NAME, Nϖ-nitro-L-arginine-methyl-ester.

Introduction

Derangements in systemic hemodynamics and renal function are common findings in advanced liver disease. These complications frequently jeopardize the life of cirrhotic patients. Therefore, amelioration of these clinical dysfunctions has become a major challenge in this condition [1]. It is currently widely accepted that arterial vasodilatation with the concomitant hyperdynamic circulation and the activation of the endogenous vasoconstrictor/antinatriuretic systems trigger these complications [2]. Several molecular mediators have been implicated in the pathogenesis of arterial vasodilation. Among them major attention has been attracted by nitric oxide (NO) [3]. This vasodilator substance has, however, a dual behavior. There are numerous studies demonstrating the increased NO production in peripheral vessels of human and rats with cirrhosis, but an impaired activity of this substance in the cirrhotic liver is also well established [3]. Therefore, any maneuver addressed to improve peripheral vasodilation by systemically inhibiting vascular NO production could also theoretically result in a further deterioration of the intrahepatic vascular resistance.

The current investigation sought to assess whether extrahepatic inhibition of vascular NO without affecting the intrahepatic production of this substance is a feasible objective. This hypothesis was raised taking advantage of the marked porto-systemic shunting occurring in rats with cirrhosis and ascites [4]. This

would facilitate the selective delivery of adenoviral constructs containing a constitutively inactive mutant of serine-threonine kinase Akt (AA-Akt) to the peripheral circulation with almost negligible infection of the hepatic vascular tree. Akt phosphorylates endothelial NO synthase (eNOS), thereby enhancing its catalytic activity which results in eNOS activation and NO production. Thus selective delivery of AA-Akt would inactivate NO production in the extrahepatic circulation of cirrhotic rats. Whether this maneuver results in an improvement in systemic hemodynamics and excretion of sodium and water was also assessed.

Materials and methods

Cell purification and culture

Bovine lung microvascular endothelial cells (BLMVECs, Vec Technologies) were cultured as previously described [5]. Human Embryonic Kidney (HEK) 293 cells (American Type Culture Collection) were maintained in DMEM with 10% heat-inactivated FCS, penicillin/streptomycin (50 U/ml and μg/ml), and L-glutamine (2 mmol/L).

Adenoviral constructs

Two replication-defective recombinant adenoviral constructs, under the control of the cytomegalovirus promoter, expressing β-galactosidase (β-gal) or hemagglutinin (HA)-tagged inactive phosphorylation mutant Akt (AA-Akt) were used [6,7]. The amino acids threonine (Thr) 308 and serine (Ser) 473 have been substituted by alanine in the wild-type sequence of Akt to synthesize the AA-Akt mutant. All vectors were propagated in the HEK 293 cell line, and titres were determined by standard plaque assay [8].

In vitro viral transduction

BLMVECs were infected with 100 multiplicity of infection (m.o.i.) of adenovirus containing β-gal or AA-Akt for 3 h. The adenoviruses were removed, and the cells were left to recover for 24 h in a complete medium. Next, the cells were serum starved during 12 h. Finally, the cells were harvested and the cell lysates were subjected to Western blot analysis with antibodies specific for phospho-Akt (P-Akt) and total Akt. Eighty micrograms and 30 μg of the denaturated proteins per lane were loaded for P-Akt and total Akt, respectively.

Induction of cirrhosis in rats

The study was performed in 23 male Wistar rats with ascites and in 5 control Wistar rats (Charles-River, Saint Aubin les Elseuf, France). Cirrhosis was induced by repetitive CCl_4 inhalation [9]. The rats were fed 'ad libitum' with standard chow and water containing phenobarbital (0.3 g l^{-1}) as drinking fluid. The animals were exposed to CCl_4 vapor atmosphere twice a week starting with 0.5 min per exposure. Afterwards, the duration of exposure was increased to 1 min after three sessions, to 2 min after three more sessions, to 3 min after three more sessions, to 4 min after three more sessions, and then to 5 min until the animals developed ascites. The ascites volume ranged between 5 and 100 ml.

Adenoviral tissue distribution

The β-galactosidase reporter gene chemiluminescent assay (Roche Diagnostics, Mannheim, Germany) was used to measure adenoviral tissue distribution in 5 cirrhotic rats with ascites and 5 control rats. The animals were lightly anesthetized with isoflurane and injected in the tail vein with 5×10^{10} plaque-forming units (pfu) of recombinant adenoviruses under the control of the cytomegalovirus promoter encoding for the β-galactosidase gene. The viruses were diluted in 500 μl of saline solution (B. Braun Melsungen AG, Melsungen, Germany). Following 72 h, the animals were killed by isoflurane overdose and the liver, mesentery, kidney, heart, lung, spleen and thoracic aorta were obtained to assess the adenoviral organ distribution. A tissue sample from each organ was placed in dry ice and kept at −80 °C until further analysis. Thereafter, the samples were homogenized in lysis buffer and aliquots were obtained to measure β-galactosidase activity.

Effect of AA-Akt transduction on renal excretory function in cirrhotic rats

Eighteen cirrhotic rats with ascites were administered with recombinant adenoviruses or saline by the tail vein. The animals included in the protocol were randomly assigned to one of the following groups: (A) six animals were administered with adenoviruses encoding β-gal (10^{11} pfu in 500 μl of saline), (B) seven animals were administered with adenoviruses encoding AA-Akt (10^{11} pfu in 500 μl of saline) and (C) six animals received saline (500 μl). Two weeks after the cirrhotic rats had developed ascites and 72 h before the administration of adenoviruses or vehicle, all the animals were placed in metabolic cages. Measurements of the body weight, urine volume (UV) and urinary excretion of sodium ($U_{Na}V$), mOsmol ($U_{mOsm}V$) and creatinine ($U_{Creat}V$) were made 24 h prior and 24 h after the animals being administered with adenoviruses or saline.

Effect of AA-Akt transduction on systemic hemodynamics in cirrhotic rats

At the end of the renal function study and 24 h after the iv administration of adenoviruses or vehicle, cirrhotic rats were anesthetized with Inactin® (100 mg/kg bw, Sigma–Aldrich Chemie Gmbh, Steinherim, Germany) and prepared with a PE-50 polyvinyl catheter in the left femoral artery. A blood sample (1 ml) from each animal was obtained to analyze the standard liver and renal function tests. Hemodynamic parameters were allowed to equilibrate for 30 min and the values of mean arterial pressure (MAP), portal pressure (PP), heart rate (HR) and cardiac output (CO) were recorded for two time periods of 30 min. Each value represents the average of two measurements. The cardiac index (CI) was calculated as CO/body weight (bw); total peripheral resistance (TPR) was obtained using the formula TPR = MAP/CO; and splanchnic perfusion pressure (SPP) was defined as MAP-PP. The ascites volume was measured by aspiration. Animals were sacrificed by isoflurane overdose and tissue specimens were collected (liver and thoracic aorta) to analyze the protein abundance of Hemagglutinin (HA), Akt, eNOS and iNOS.

Western blot analysis of HA, total Akt, P-Akt, total eNOS, P-eNOS and iNOS

Liver samples and thoracic aortas were homogenized as described previously [10]. For P-Akt, Akt, HA, eNOS and iNOS analysis, 40 μg of the denaturated proteins per lane was loaded and separated on a 7.5% SDS–polyacrylamide gel (Mini Protean III; Bio-Rad, Richmond, CA). Membranes were transferred to nitrocellulose membranes (Transblot Transfer Medium, Bio-Rad), which were stained with Ponceau-S red as a control for protein loading. Subsequently, membranes were blocked with 5% powdered defatted milk in TTBS buffer (50 mmol/L Tris–HCl, pH 8, containing 0.05% Tween 20 and 150 mmol/L NaCl) for 2 h. For P-Akt, Akt and HA, the membranes were probed at room temperature with rabbit polyclonal anti-P-Akt-Ser-473, anti-Akt (Cell Signaling Technologies, Beverly, MA) and mouse monoclonal anti-HA-12CA5 (Roche Diagnostics) for 2 h in a 1:1000 dilution in TTBS buffer containing 5% powdered defatted milk. For eNOS, membranes were incubated at room temperature with mouse monoclonal anti-eNOS (BD Transduction Laboratories, Lexington, KY) for 2 h in a 1:2500 dilution in TTBS buffer containing 5% powdered defatted milk. For iNOS, membranes were incubated at room temperature with rabbit polyclonal anti-iNOS (BD Transduction Laboratories, Lexington, KY) for 2 h in a 1:500 dilution in TTBS buffer. A cell lysate of murine macrophage cell line (RAW 264.7, ATCC TIB71) stimulated (12 h) with γ-interferon and LPS, obtained from Transduction Laboratories (Lexington, KY), was used as the positive control. For phospho-eNOS (P-eNOS), 80 μg of the denaturated proteins per lane was loaded and then separated, transferred, and subsequently blocked as described. Membranes were incubated overnight at 4 °C with rabbit polyclonal anti-P-eNOS-Ser-1177 (Cell Signaling Technologies, Beverly, MA) in a 1:1000 TTBS buffer containing 5% bovine serum albumin. After incubation with primary antibody, the membranes were washed (three times, 10 min each) with TTBS buffer. For eNOS and HA, membranes were incubated for 60 min at room temperature with a 1:2000 dilution with goat anti-mouse horseradish peroxidase-conjugated antibody (Amersham Biosciences). For P-Akt, Akt, P-eNOS and iNOS, the membranes were incubated for 60 min at room temperature with a 1:2000 dilution with donkey anti-rabbit horseradish peroxidase–conjugated antibody (Amersham Biosciences). The bands were visualized by chemiluminescence (ECL Western blotting analysis system; Amersham Biosciences).