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ORIGINAL ARTICLE

NADPH oxidase 1/4 inhibition attenuates the portal hypertensive syndrome via modulation of mesenteric angiogenesis and arterial hyporeactivity in rats

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Summary
Aim: NADPH oxidase (NOX)-derived reactive oxygen species (ROS) plays key roles in the development of portal hypertension (PHT) and represents a potential therapeutic method. The objective of this study was to investigate whether pharmacological inhibition of NADPH oxidase activity could ameliorate PHT in rats.
Method: PHT model was established by partial portal vein ligation (PPVL). Rats were treated with 30 mg/kg GKT137831 (the most specific Nox1/4 inhibitor) or vehicle daily by gavage for 14 days beginning at the day of PPVL or sham operation (SO). Hemodynamics, severity of portal-systemic shunting, vascular contractility, vascular endothelial growth factor (VEGF), VEGFR-2, CD31, AKT, phospho-AKT (p-AKT, at Ser473), endothelial nitric oxide synthase (eNOS), and phospho-eNOS (p-eNOS, at Ser1177) expressions were evaluated. Nitric oxide (NO) production and oxidative stress in mesenteric arteries, and hydrogen peroxide (H2O2) in both mesenteric tissues and arteries were measured.
Result: Inhibition of NOX1/4 with GKT137831 significantly decreased cardiac index, increased portal flow resistance, reduced portal pressure (PP), portal blood flow, mesenteric angiogenesis and portal-systemic shunting (PSS) in PPVL rats. GKT137831 reduced the production of H2O2, down regulated mesenteric angiogenesis markers (CD31, vascular endothelial growth factor (VEGF) and VEGFR-2 expression. Compared with controls), the mesenteric artery

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contraction to norepinephrine (NE) was impaired in PPVL rats, which was reversed by exposure to GKT137831. In addition, GKT137831 markedly decrease NADPH oxidase activity and ROS production in mesenteric arteries, and reduced NO production by decreasing the level of phosphor-AKT and eNOS.
Conclusion: Inhibition of NOX1/4 decreased PP, ameliorated hyperdynamic circulation, mesenteric angiogenesis and arterial hyporesonse in portal hypertensive rats. Pharmacological inhibition of NOX1/4 activity may be a potential treatment for PHT-related complications.

Introduction

Portal hypertension (PHT) is a serious complication of chronic liver diseases [1]. The PHT syndrome is characterized by pathological increase in portal pressure (PP), splanchnic blood flow, hyperdynamic circulation [2], and the formation of portal-systemic collaterals — shunting part of portal blood flow into the systemic circulation bypassing the liver [3]. The PHT syndrome results in severe clinical complications including hemorrhage from esophageal varices, hepatic encephalopathy, hepatorenal syndrome and ascites [4,5].

Essentially, hyperdynamic circulation is predominately elicited, among others of hyporesponse to vasoconstrictors, changes of vascular contractile signaling, and vascular remodeling etc., by increased vasodilatory substances release [6]. Nitric oxide (NO) released from endothelial cells is the major one among vasodilatory substances. Endothelial nitric oxide synthase (eNOS) of endothelium is activated and driven to synthesize and release NO which is currently the most recognized factor related to extrahepatic vasodilatation in PHT [7]. In addition, the development of splanchnic hemodynamic dysfunction is an active modulated angiogenic process induced by vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and placental growth factor (PlGF) [8,9,10]. As such, VEGF and PlGF-dependent splanchnic angiogenesis also plays a key role in the formation of portal-systemic collateral vessels [8,10]. However, the precise mechanisms by which the angiogenesis associated response in PHT is modulated still remain to be unknown.

NADPH oxidase (NOX) is a major source of reactive oxygen species (ROS) and an important determinant of the redox state of the vessels [11]. The NOX family including seven isoforms is NOX1 to 5, and DUOX1/2. NOX1, 2, 3, and 5 produce mainly superoxide anion (O^{2-}), but DUOX1/2 produce hydrogen peroxide (H_2O_2). Under pathological condition, endothelial cells mainly express NOX1, 2 and 4 [12]. During PHT, inhibition of NADPH oxidase not only attenuates redox-mediated endothelial dysfunction in the mesenteric artery [13], but also significantly decreases the formation of portal-systemic collaterals, and superior mesenteric arterial flow, as well [14]. These studies suggest that oxidative stress with increased generation of ROS acts as an important mechanism in main complications of PHT, including hyperdynamic circulatory syndrome and the formation of portal-systemic collaterals [15,16]. NADPH oxidase inhibitor apocynin effectively decreases ROS production, but this agent has significant off-target effects due to lack of specificity. Luckily, a specific NOX inhibitor GKT137831 has been developed recently [17]. GKT137831 does not affect NOX2-mediated phagocyte function, thus appears to be superior to other NOX/ROS inhibitors.

In summarize, we hypothesized that NOX1/4 derived ROS plays an important role in PHT syndrome, especial serious complications. In this study, we will explore that the underlying mechanism of NOX1/4 inhibition in the extent of portosystemic collateral vessels, the development of hyperdynamic circulation and the splanchnic neovascularisation in portal hypertensive rats.

Materials and methods

Animal model

Male Sprague—Dawley rats (280—320 g) were obtained from the Shanghai Slac Experimental Animal Centre (Shanghai, China). Rats were maintained at a constant room temperature of 24 °C with a 12-h light/dark cycle and allowed free access to water and standard rat chow. All experimental procedures in this study were conducted according to the local animal ethics committee and performed according to the guidelines of the Laboratory Animal Care and Use Committee at School of Medicine, Shanghai JiaoTong University (Shanghai, China).

Induction of portal hypertension and treatment

The rats underwent PPVL or sham operation (SO) as described previously [18]. Briefly, the portal vein was freed from surrounding tissue, and stenosis was induced by a single ligature (silk gut 3—0) placed around both the portal vein and a 20-gauge blunt-tipped needle. Then removal of the needle yielded a calibrated stenosis of the portal vein. Portal hypertension was considered present at 14 dads after surgery. In SO rats, the procedure was the same except that the ligature in the portal vein was not added. In this study, rats were divided into four groups ($n = 12$ in each group): SO-Vehicle, SO-GKT, PPVL-Vehicle, PPVL-GKT. From day 1 to day 14 after PPVL operation, rats were treated with 30 mg/kg of the NOX1/4 inhibitor GKT137831 (GenKyoTex, Geneva, Switzerland) or vehicle by intragastric injection daily [19].

Hemodynamic studies

According to our previous study [20], pressure (MAP) and portal pressure (PP) were measured by catheterizing PE50 to arteries. Microsphere analysis: Color microspheres (15 μm,

Triton Technologies, California, USA) with peak absorption at 448 nm (yellow), 530 nm (red) and 672 nm (blue), respectively, were used in our experiments [21,22,23]. The yellow spheres were injected into the left ventricle to determine the cardiac output and regional blood flow to the splanchnic tissues. The red spheres were injected into the portal vein to determine the portal-systemic shunts. The process of microsphere administration was started with collecting a reference blood sample from the left femoral artery catheter by a withdrawing syringe pump (ALC-IP900, Alcott Biotech, Shanghai, China) at a constant rate of 0.65 mL/min for 1 minute. 10 seconds after starting the syringe pump, 300,000 yellow microspheres and 30,000 red microspheres suspended in 0.3 mL saline containing 0.05% Tween 80 were injected by 30 seconds into the left ventricle and the portal vein respectively.

After injection of microspheres, rats were sacrificed by bilateral thoracotomy and then, the lungs, liver, kidneys, stomach, intestine, colon, mesentery and pancreas were collected. After removal of excessive blood, the tissues were carefully weighted and completely digested by boiling them in 5 M KOH solution containing 10% Tween 80. The microspheres were then collected by centrifugation and washed several times in PBS. The color on the collected microspheres was dissolved into 0.2 mL dimethylformamide and measured by spectrophotometry (BIO-RAD, California, USA) with respective absorptions. Blue microspheres were used as internal controls, which were added into each sample at the beginning of sample process.

Vascular function studies

Isolated mesenteric arterioles (140—200 μm in diameter) were cannulated in a vessel chamber. Vessels were perfused with oxygenated (95% O2 and 5% CO2) PSS containing, in mmol/L: NaCl, 117; KCl, 4.7; MgSO4, 1; KH2PO4, 1.2; glucose, 10; NaHCO3, 24; CaCl2, 2.5; and EDTA, 0.2 at 37 °C, pH 7.4. Intraluminal pressure was controlled at 80 mmHg throughout the experiment. After 1 h of stabilization, vessels developed spontaneous tone which reached to a basal tone of ∼25% maximal diameter. Test the viability of the vessel and integrity of the endothelium by stable and reproducible responses to the addition of phenylephrine (10^{-6} M) and acetylcholine (10^{-5} M). The arteries were considered unaccepted if they showed less than 60% relaxation of PE induced contractions. A cumulative concentration-contraction response curves were performed by sequential addition of increasing doses of norepinephrine (NE, $10^{-8.5}$ mol/L-$10^{-4.5}$ mol/L). The inner diameter was measured using a BA310 microscope camera system (Motic, Xiamen, China). The percentage of contraction was expressed by reduction in vessel diameter relative to baseline diameter before addition of NE. The vasoconstriction rate and the logarithm of the NE concentration were used as the vertical axis and the abscissa, respectively.

Vessel culture perfusion system

The vessel-culture procedure has been described in detail previously [24]. Briefly, first-order mesenteric arteries were isolated (8—10 mm in length) with all side-branches carefully ligated. The isolated arteries were then transferred into a vessel chamber and cannulated on two glass pipettes and secured with sutures. The vessel was perfused with 5 μL/min intraluminal flow at 40 mmHg transmural pressure and incubated with DMEM-F12 medium (Gibco, New York, USA) containing 1% penicillin-streptomycin at 37 °C under a closed environment containing 95% air—5% CO_2 for 24 hours. After the first 24-hour incubation, vessels were started to expose to different concentration of H_2O_2 (1, 10, 100 nM) or H_2O_2 (10 nM) plus GKT-137831 (10^{-4} M) for additional 48 hours. The medium with and without H_2O_2 or H_2O_2 plus GKT-137831 was refreshed in a 6-hour intermittent period. After incubation, vessels were collected and pulverized in liquid nitrogen for further analyses.

NADPH oxidase activity

This activity was performed as previous description in detail [25]. Briefly, isolated first-order mesenteric arteries were homogenized in a lysis buffer. The reaction was started via adding 0.1 mmol/L NADPH to the suspension containing sample, 5 mmol/L lucigenin (5 mmol/L) and assay phosphate buffer The luminescence was measured over next 10 min in a luminometer (AutoLumat Plus LB953, Berthold, Germany). Background signal was subtracted from each reading. At the end, the amount of total protein was measured by BCA Protein Determination Kit (Beyotime). Activity is expressed as relative light units/mg protein. The final NADPH activity is expressed as variations of the activity that were calculated as percentage of control condition.

ROS detection

The dihydroethidium oxidative fluorescence dye was used to evaluate ROS production in situ, as described previously [26]. Briefly, mesenteric arteries were embedded in OCT Tissue Tek and cut slices with 10 μm in thickness by means of a Frozen Slicer. Slices were incubated with DHE (10 μmol/L) in Krebs—HEPES buffer (in mM: 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, and 11.1 glucose, pH = 7.4) for 30 min at 37 °C in a light-protected humidified chamber. The sections were placed under a fluorescence microscope (Olympus, Tokyo, Japan), observed with 535 nm wavelength, and images were collected. All images were analyzed by image-pro plus 6.0.

H_2O_2 detection

According to the instructions of a hydrogen peroxide assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) [27], isolated mesentery and mesenteric vessels were homogenized in a lysis buffer and centrifuged at 12,000 g for 10 min to collect the supernatant. Protein in the supernatant was determined by BCA Protein Determination Kit (Beyotime). A 150 μl of reaction mixture containing 50 μl supernatant was incubated at room temperature for 20 min and the spectrophotometrical detection (560 nm) was carried out by using a microplate reader (Biotek-Synergy, Vermont, USA). The level of H_2O_2 in samples was determined using a H_2O_2 standard curve and expressed by nmol per mg sample proteins.

Table 1 Effects of NOX1/4 inhibition on systemic and splanchnic hemodynamics.

	SO-Veh ($n=6$)	SO-GKT ($n=6$)	PPVL-Veh ($n=8$)	PPVL-GKT ($n=7$)
BW (g)	486.33 ± 23.48	453.50 ± 11.44	353.63 ± 10.36	362.14 ± 16.43
HR (beats/min)	330.50 ± 8.73	327.50 ± 11.76	357.13 ± 15.72	347.00 ± 14.07
MAP (mmHg)	130.17 ± 2.04	130.00 ± 6.58	101.00 ± 3.88	106.14 ± 3.94
PP (mmHg)	6.04 ± 0.20	6.05 ± 0.35	14.37 ± 0.88	11.47 ± 1.21[a]
CO (mL/min)	121.93 ± 15.33	129.87 ± 10.71	143.40 ± 8.47	116.12 ± 7.46[a]
CI (mL/min/100 g)	25.17 ± 3.13	28.90 ± 2.92	40.85 ± 2.83	32.40 ± 2.33[a]
PBF (mL/min)	11.55 ± 1.15	16.60 ± 1.20	29.05 ± 2.03	17.61 ± 2.98[a]
PFR (mmHg/mL/min)	11.31 1.18	7.77 ± 0.90	3.09 ± 0.27	6.17 ± 0.91[a]

BW: body weight; HR: heart rate; MAP: mean blood pressure; PP: portal pressure; CO: cardiac output; CI: cardiac index; PBF: portal blood flow; PFR: portal flow resistance.
[a] $P<0.05$, GTK137831-treated PPVL group versus Vehicle-treated PPVL group.

Vascular NO measurement

According to the instructions of NO assay kit (Beyotime), we used Griess method to measure the $NO2^-$ and $NO3^-$ levels in mesenteric arteries, which serve as markers of NO production. The NO2- and NO3- levels were expressed as nmol/mg protein.

Western blot analysis

Tissues, including mesentery, intestine and isolated mesenteric arteries, were pulverized in liquid nitrogen and incubated for 30 min in ice-cold enhanced RIPA Lysis Buffer (Beyotime). The buffer contained 1% protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Missouri, USA). Then, the tissues were sonicated in ice water for three times (30-s duration and a 3-s interval). The samples were collected and total protein was quantified by BCA Protein Assay Kit (Beyotime). 25 μg proteins were loaded and separated by 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. After membranes were blocked in 5% (wt/vol) non-fat dry milk, membranes were incubated overnight with antibodies to VEGF, VEGFR-2, CD31 (1:200 dilution in TBS-T with 5% milk; Santa Cruz Biotechnology, California, USA), p-eNOS (Ser1177), eNOS, p-AKT(Ser473), AKT(1:1000 dilution in TBS-T with 5% milk; Cell Signaling Technology, Massachusetts, USA), and GAPDH, respectively. Immunoreactive bands were detected using the enhanced chemiluminescence western blotting system (Fusion Fx, Vilber Lourmat, France) and normalized to GAPDH.

Calculation and statistics

The calculation and statistics are as follows:

- regional blood flow to a specific tissue was calculated as: Q_s (mL/min) = Q_r (mL/min) × (A_s/A_r); where Q and A represent flow and absorbance of microspheres for the reference blood (r) and samples (s) respectively;
- cardiac output (CO) was determined by: CO (mL/min) = $Q_r \times (A_t/A_r)$. A_t refers to the amount of absorbance obtained from total yellow microsphere injected;
- cardiac index (CI) was determined by CO/body weight (g) × 100, and expressed as mL $min^{-1}\cdot 100\,g^{-1}$;
- portal blood flow (PBF) was presented by the sum of blood flow to the stomach, spleen, intestines, pancreas, colon and mesentery;
- portal flow resistance (PFR) was calculated as: PFR ($mmHg\cdot min\cdot mL^{-1}$) = (MAP-PP)/PBF;
- portal-systemic shunting (PSS%) was calculated as: PSS% = (absorbance of red microspheres in the lung)/(absorbance of red microsperes in lung and liver) × 100, and used as an index of portal-systemic collateral vessel formation.

Data are expressed as mean ± SEM and were analyzed by using one way ANOVA for multiple comparisons with SPSS 16.0. NE induced dose-response curve was fitted by nonlinear regression analysis with Graph Pad Software 6.0, and EC50 values were calculated from the fitted curve. $P<0.05$ was considered statistically significant.

Results

Systemic and splanchnic hemodynamics

Table 1 shows BW and hemodynamic parameters of vehicle- or GKT- treated rats, either sham- or PPVL-operated. In PPVL rats, GKT137831 significantly reduced portal pressure (PP) by 19% ($P=0.02$) as compared with those with vehicle treatment. Cardiac output (CO), Cardiac index (CI), and Portal blood flow (PBF) were markedly decreased by GKT137831. By contrast, Portal flow resistance (PFR) was significantly increased by GKT137831. Heart rate (HR) and mean arterial pressure (MAP) was not changed by GKT137831. Body weight (BW) was similar between the two PPVL groups. In sham groups, GKT137831 did not significantly influence BW and hemodynamic parameters. No mortality was found in all experimental groups.

Degree of PSS

Fig. 1 discloses the degree of PSS in four experimental groups. In vehicle-treated PPVL rats, PSS was significantly higher than that in vehicle- treated SO rats. GKT137831, as compared with vehicle, significantly attenuated sever-

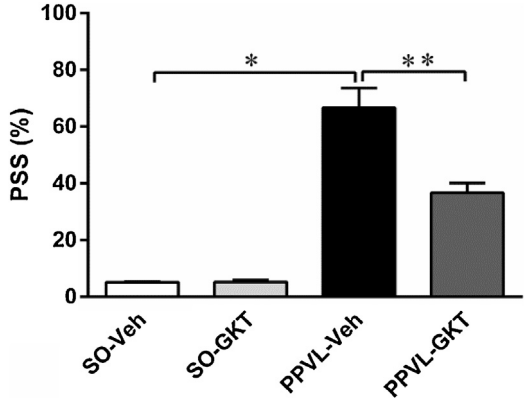

Figure 1 Portal-systemic shunting ratio in SO and PPVL rats treated with vehicle or GKT137831. Data are shown as mean ± SEM; $^{*}P < 0.001$ vs. SO-Veh; $^{**}P < 0.05$ vs. PPVL-Veh.

ity of shunting (PPVL-Veh vs. PPVL-GKT: 66.62 ± 6.97% vs. 36.67 ± 3.46%; $P = 0.003$).

Splanchnic angiogenesis

Western blot analysis of mesenteric and intestinal samples showed significantly higher splanchnic CD31, VEGF and VEGFR-2 expression in rats with PHT induced by PPVL compared to SO rats (Fig. 2). In PPVL animals, GKT137831 treatment resulted in a marked decrease of CD31, VEGF and VEGFR-2 expression in the mesentery and small intestines. In SO rats, no significant difference in angiogenesis related protein expression was observed between the treatment groups.

GKT137831 inhibits H_2O_2-induced VEGF expression of isolated mesenteric vessels

To further access the effect of GKT137831 on angiogenesis, rat mesenteric vessels were isolated and cannulated for 72 h culture. As results, VEGF and VEGFR-2 expressions of 10 nM H_2O_2-treated group were increased significantly compared with other concentrations (0, 1,100 nM) H_2O_2-treated groups (Fig. 3). After GKT137831 treatment, VEGF and VEGFR-2 levels were markedly decreased in 10 nM H_2O_2-treated vessels (Fig. 3). However, CD31 level of isolated vessels was not changed by H_2O_2 or GKT137831 treatment.

Inhibition of oxidative stress

Fig.4A shows that NADPH oxidase activity in mesentery vessels from PPVL rats was significantly increased than that from SO rats. The activity was inhibited by the NOX1/4 inhibitor, GKT137831. Accordingly, ROS production in the mesenteric arteries form PPVL rats in response to NADPH oxidase activation was significantly higher than that from SO rats, and this production was remarkably decreased after GKT137831 treatment (Fig. 4B). We also detected H_2O_2 levels in the mesentery and mesenteric arteries. As shown in Fig. 4C, the H_2O_2 levels of mesentery and mesenteric arteries of PPVL rats were significantly higher than that from SO rats. As described above, they were remarkably reduced in PPVL rats receiving GKT137831 than in those receiving vehicle. Therefore, GKT137831 as the antioxidant is in a position to decrease oxidative stress in mesenteric tissues of portal hypertensive rats.

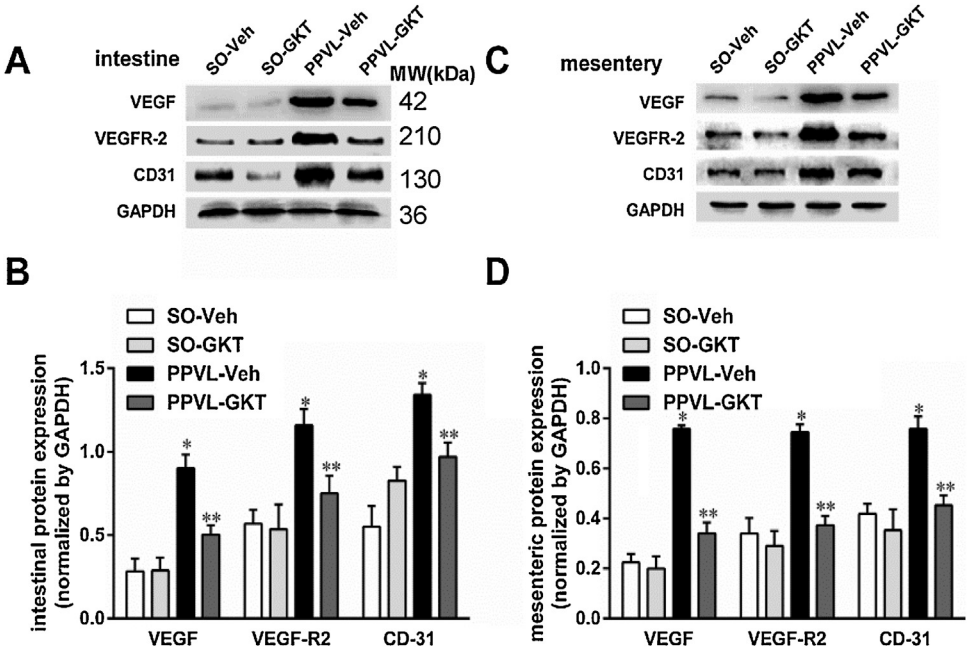

Figure 2 Intestinal and mesenteric protein expression in rats received vehicle or GKT137831 treatment. A, B. Intestinal protein expression and representative blots of angiogenesis markers in four groups. Results are expressed as mean ± SEM; $^{*}P < 0.05$ vs. SO-Veh; $^{**}P < 0.05$ vs. PPVL-Veh. C, D. Mesenteric protein expression and representative blots of angiogenesis markers in four groups. Results are shown as mean ± SEM. $^{*}P < 0.05$ vs. SO-Veh; $^{**}P < 0.05$ vs. PPVL-Veh.

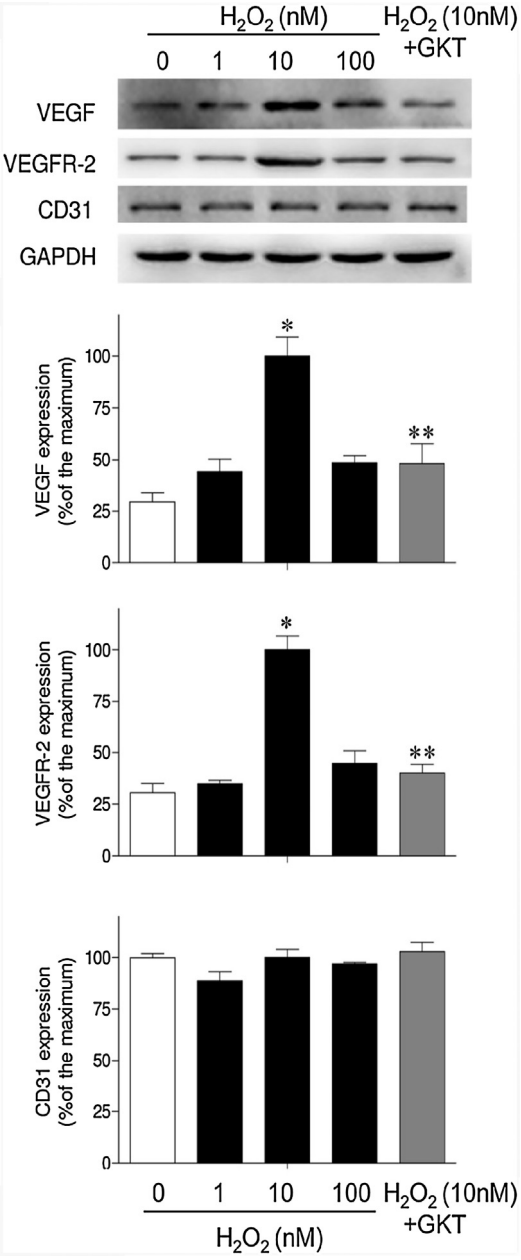

Figure 3 GKT137831 inhibits H_2O_2-induced angiogenesis of mesenteric arteries. Regulation of VEGF, VEGFR-2 and CD31 expressions of isolated vessels by H_2O_2 and GKT137831(10^{-4} M) treatment for 48 h. Data are expressed as means ± SEM of 3 independent experiments. $^{*}P < 0.05$ vs. 0 nM H_2O_2-pretreated vessels; $^{**}P < 0.05$ vs. 10 nM H_2O_2-pretreated vessels.

Vascular contractility in mesenteric arteriole

Fig. 5 shows that the dose-response curve of the mesenteric arteriole in response to NE moved to right with decreased Emax ($P < 0.01$) and EC50 significantly increased ($P < 0.01$) in PPVL group compared with SO group. After treatment with GKT137831, the dose-response curve markedly shifted to left with increased Emax ($P < 0.05$) and the EC50 significantly decreased ($P < 0.05$) in portal hypertensive rats. To evaluate whether NOX1/4 inhibition enhances the contraction of mesenteric arterioles via NO signaling, NO synthase inhibitor, L-NAME(10^{-4}M) were used to incubate vessels before the addition of NE. Pretreatment of L-NAME significantly increased the contractile response and caused a substantial attenuation of from PPVL-Vehicle-L-NAME group rats. Likewise, GKT137831 did not change the contractile response in the PPVL-Vehicle-L-NAME and PPVL-GKT-L-NAME group (Fig.5C, D). These data indicate that GKT137831ameliorates portal hypertension-induced hyporesponse to NE in mesenteric artery.

AKT and eNOS phosphorylation changes in mesenteric arteries

Western blot was used to analyze total and phosphorylated AKT and eNOS expression in mesenteric arteries (Fig. 6A, B). In PPVL rats, GKT137831 did not significantly change total AKT protein expression in mesenteric arteries. However, GKT137831 treatment induced a marked decrease in AKT phosphorylation at Ser473. Likewise, GKT137831 treatment reduced eNOS phosphorylation but did not affect total eNOS protein expression in mesenteric arteries. Consistent with western blot data, GKT137831 also reduced NO production in mesenteric arteries (Fig. 6C).

Discussion

The main findings of the present study are summarized in Fig7 that indicate 1) Inhibition of NOX1/4 with GKT137831 attenuates hyperdynamic circulation of PHT, as demonstrated by decreases in cardiac output, portal pressure, portal blood flow, and an increase in portal flow resistance. 2) GKT137831 reduces the degree of portal-systemic shunting in PHT, and inhibits splanchnic angiogenesis in vivo and vitro, as evidenced by the reduction of protein (CD31, VEGF and VEGFR-2) expressions in the mesenteric and small intestinal tissues, and the inhibition of H_2O_2-induced high VEGF expression of mesenteric arteries in vitro. 3) GKT137831 enhanced vascular contractility to vasoconstrictor of mesenteric arteries, which may be associated with the decreased H_2O_2 regeneration and the inhibition of AKT/eNOS pathway.

NOX is major resource of ROS, which plays a crucial role in PHT syndrome. ROS is involved in modulating angiogenesis-dependent processes (i.e., formation of portal-systemic collaterals, increased splanchnic vascularity and development of hyperdynamic splanchnic circulation) in the portal hypertensive rats [14]. Reducing ROS level with antioxidants could ameliorate the severity of portal-systemic shunting and splanchnic angiogenesis [28]. In this study, our data also show that decreased H_2O_2 level via inhibiting NOX1/4 activity significantly reduced the severity of portal-systemic collaterals, and if so, what is involved in.

We speculate that the reduction of collateralization and splanchnic blood flow caused by GKT137831 treatment is probably due to the inhibition of VEGF-induced neovascularization. This is based on the following reasons: first, previous studies have displayed significant overexpression of VEGF, VEGFR-2 and CD31 in splanchnic tissues from portal hypertensive rats [29,30], and inhibition of VEGFR-2 remarkably decreases the formation of portal-systemic collateral circu-

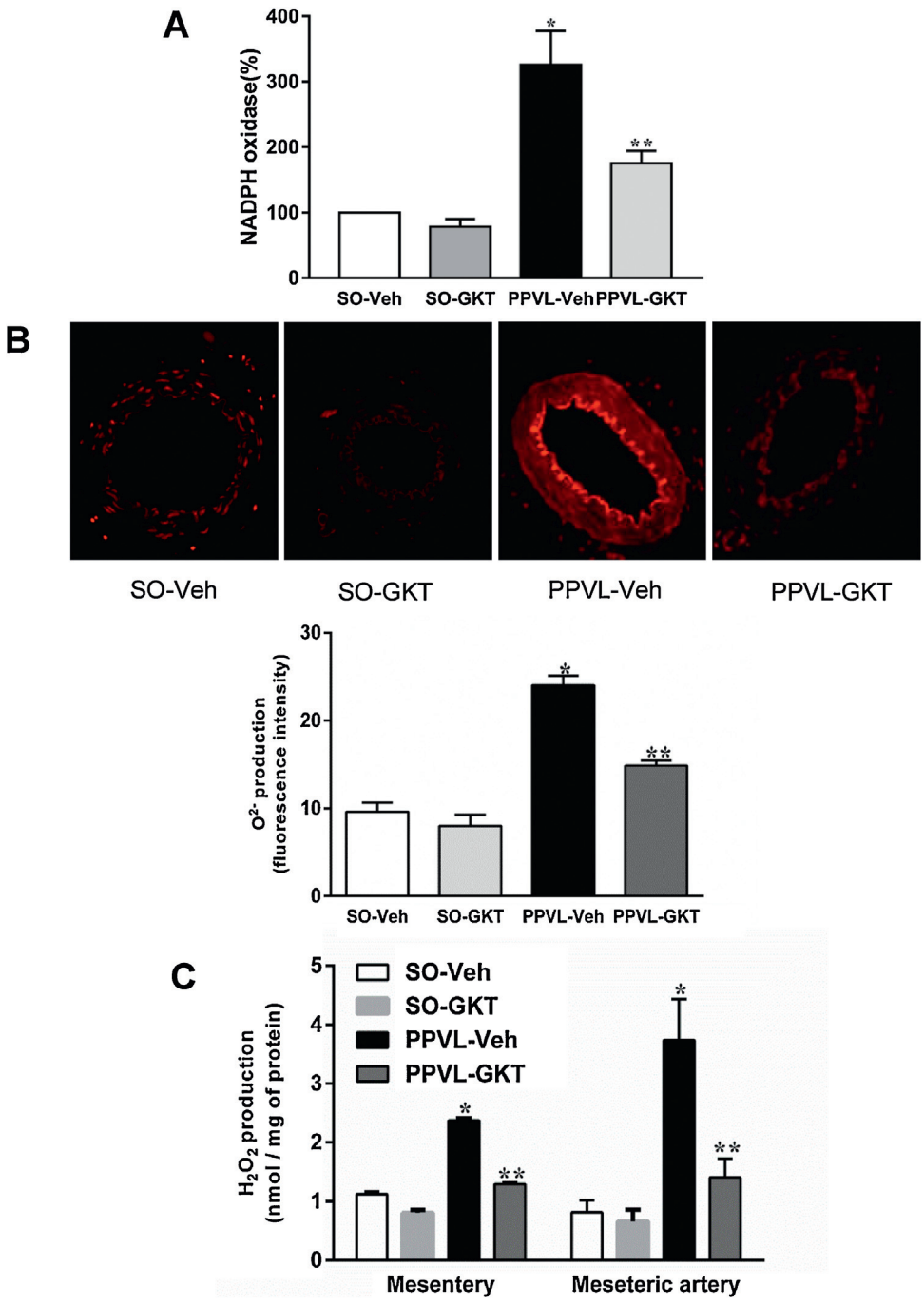

Figure 4 Oxidative stress changes in mesenteric tissues following GKT137831 treatment. A. NADPH oxidase activity in mesenteric artery from four groups. Data was expressed as a percent of SO-veh arteries. $^{*}P < 0.001$ vs. SO-Veh; $^{**}P < 0.05$ vs. PPVL-Veh. B. dye DHE (red fluorescence) was used to evaluate ROS level in mesenteric arteries in situ. Fluorescence intensity in PPVL-Veh group was the strongest, but reduced following GKT treatment. Quantitative analysis of fluorescence intensity was performed. Values are mean ± SEM; $n = 6$. $^{*}P < 0.01$ vs. SO-Veh; $^{**}P < 0.05$ vs. PPVL-Veh. C. H_2O_2 production in mesentery and mesenteric arteries after treatment with GKT. Values are mean ± SEM; $n = 6$. $^{*}P < 0.01$ vs. SO-Veh; $^{**}P < 0.01$ vs. PPVL-Veh.

lation, as well as attenuates the hyperdynamic splanchnic circulation [31]. These results suggest that the formation of collateral vessels in portal hypertension is not only a mechanical consequence of the increased portal pressure that will result in the opening and dilatation of pre-existing vascular channels [3], but it is also mediated by a VEGF-dependent angiogenic process. Second, our results show that NOX1/4 blockade in vivo significantly decreased the protein expression of VEGF and VEGFR-2 in the mesentery and intestine of portal hypertensive rats. The level of CD31 expression, as an index of vascular density [32], was also significantly decreased in GKT137831-treated portal hypertensive rats. Third,10 nM H_2O_2 rather than other concentrations(1 or 100 nM) of H_2O_2, in vitro, induced higher

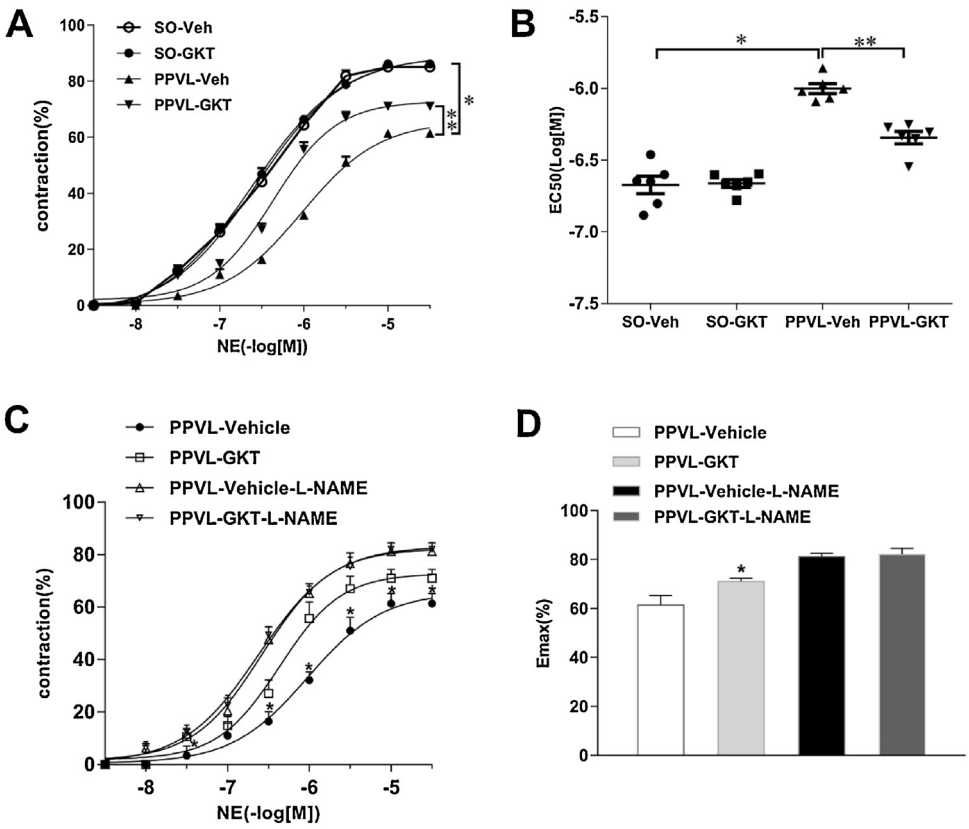

Figure 5 Response elicited by NE in rat mesenteric arterioles of SO-Veh, SO-GKT, PPVL-Veh, PPVL-GKT groups ($n = 6$). Values are mean ± SEM. A. $^{*}P < 0.01$ vs. SO-Veh group; $^{**}P < 0.05$ vs. PPVL-Veh group. B. $^{*}P < 0.01$ vs. SO-Veh group for EC50; $^{**}P < 0.05$ vs. PPVL-Veh group. C, D. Response elicited by NE from PPVL- Vehicle, PPVL-GKT, PPVL-Vehicle-L-NAME and PPVL-GKT-L-NAME groups ($n = 6$). Incubation of L-NAME in the PPVL- Vehicle-L-NAME group and PPVL-GKT-L-NAME group. Date are presented as mean ± SEM. $^{*}P < 0.05$ vs. PPVL+ Vehicle+ L-NAME group for % maximum response.

level of VEGF and VEGFR-2 expressions similar to M. Kubo [33], and, which was reversed by the NOX1/4 inhibitor. Interestingly, we did not detect any changes of CD31 expression, which may come from the distinction between vitro and vivo experiments. Taken together, these results suggest that blockage of NOX1/4 has a protective effect on VEGF-induced splanchnic angiogenesis in portal hypertensive rats.

Consistent with previous study, our data show that GKT137831 greatly ameliorates hyperdynamic circulatory syndrome, such as the reduced portal pressure, cardiac output and portal blood flow without significant alteration of blood pressure. This indicates that inhibition of NOX1/4 with GKT137831 is a reasonable and effective method for the treatment of PHT in further. However, the underlying mechanisms of the attenuation of hyperdynamic circulation remain unknown.

Previous studies indicated that ROS is involved in vascular dysfunction in various chronic diseases, such as obesity [34], hypertension [35], and liver cirrhosis [13]. In PHT, anti-oxidative treatment also ameliorates systemic and splanchnic hyperdynamic circulation [36]. In addition, NADPH oxidase inhibitor apocynin reduces vascular ROS level and attenuates hypocontractility of mesenteric artery to norepinephrine in cirrhotic rats with portal hypertension [37]. As such, our data in the present study indicate that treatment with GKT137831 has been capable of inhibition of arterial vasodilation to reduce blood flow to portal vein, leading to the improvement of circulation dysfunction in portal hypertensive rats. Actually, we also found that inhibition of NOX1/4 significantly decreased the levels of ROS and H_2O_2 in mesenteric vessels. Thus, we draw a conclusion that attenuation of splanchnic vasodilation, at least in part, results from the decreased ROS level induced by inhibition of NOX1/4.

In addition to ROS, we hypothesized that GKT137831 enhanced contractility of mesenteric arteries probably via modulation of NO production as well. In order to test this hypothesis, we performed a series of experiment and observed that NO concentration in PPVL-GKT group was markedly decreased compared with PPVL-vehicle group. It is well known that NO, as the most important vasodilator, contributes to excessive splanchnic vasodilation in PHT [38]. Therefore, GKT137831 regulating NO production in splanchnic vessels preferably explains that NOX1/4 inhibitor GKT137831 is able to decrease splanchnic vasodilation. In addition, the changes of eNOS activation levels are predominately responsible for the increased NO concentration in splanchnic arteries. Meanwhile, eNOS activation is charged by phosphorylation of AKT which elicited by various stimulator and mechanical forces such as shear stress [39,40]. As an important downstream target of PI3K, AKT directly phosphorylates eNOS at Ser^{1177} or Thr^{495} [41,42], activat-

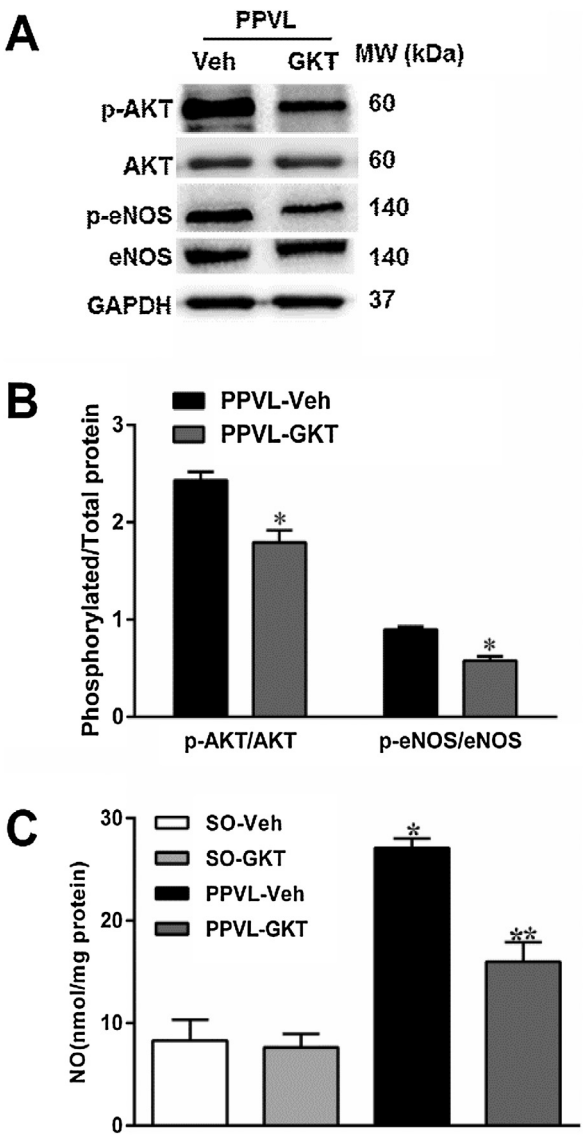

Figure 6 AkT/eNOS signaling and NO production in mesentery vessels. A, B. Analysis of AKT, p- AKT (Ser473), eNOS, and p-eNOS (Ser1177). Expression ratios were calculated for the optical density of p- AKT and total AKT relative to GAPDH. Values are mean ± SEM; $n = 3$. *Significant difference in quantitative analysis of western blot between two groups. C. The level of NO in mesenteric arteries from the SO, SO-GKT, PPVL-Veh, PPVL-GKT group ($n = 6$). Values are mean ± SEM. $^{*}P < 0.001$ vs. SO; $^{**}P < 0.01$ vs. PPVL-Veh.

ing these enzymes and eventually leading to NO production. In the pathogenesis of PHT, a process that AKT phosphorylates eNOS may be an initial step for an initial increase of NO production [43]. Indeed, we found in this study that GKT137831 treatment significantly decreased phosphorylation of AKT and eNOS at Ser1177 in mesenteric arteries from portal hypertensive rats, indicating that GKT137831 regulates NO level in vessels via AKT/eNOS signaling pathway.

In summary, NOX1/4 inhibition, as one therapeutic strategy, significantly ameliorates the development of portal-systemic collateral vessels and hyperdynamic splanchnic circulation in portal hypertensive rats. These

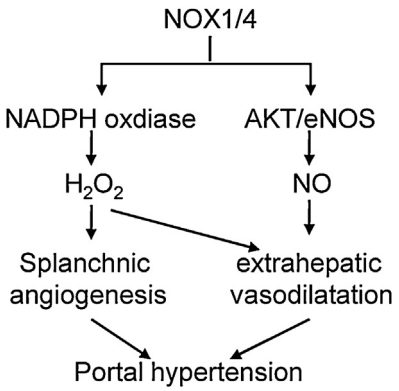

Figure 7 Schematic illustration of the mechanism of NOX1/4 contributes to portal hypertension. Enhanced NADPH oxidase induces H_2O_2 production in mesenteric tissues, which promotes splanchnic angiogenesis via upregulation of VEGF expression, and is directly involved in hyporeactivity of mesenteric arteries, coordinately aggravate the development of portal hypertension. On the other hand, NOX1/4 induced NO overproduction through activating the AKT/eNOS signaling pathway, substantially results in vasodilatation leading to portal hypertension.

novel findings support that GKT137831, a NOX1/4 inhibitor, has a great potential to prevent and treat vascular complications of PHT.

Author contributions

Meng Luo and Xiaoliang Qi conceived and designed the experiments; Wensheng Deng and Yiming Zhu performed the experiments; Ming Duan and Chihao Zhang analyzed the data; Lei Zheng and Jiayun Lin contributed reagents/materials/analysis tools; Wensheng Deng and Ming Duan wrote the paper. Meng Luo provided financial support for this work.

Disclosure of interest

The authors declare that they have no competing interest.

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