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• BASIC RESEARCH •

Effects of tumor necrosis factor, endothelin and nitric oxide on hyperdynamic circulation of rats with acute and chronic portal hypertension

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Abstract

AIM: To evaluate the effect of tumor necrosis factor (TNF), endothelin (ET) and nitric oxide (NO) on hyperdynamic circulation (HC) of rats with acute and chronic portal hypertension (PHT).

METHODS: Chronic portal hypertension was induced in Wistar rats by injection of carbon tetrachloride. After two weeks of cirrhosis formation, L-NMMA (25 mg/kg) was injected into one group of cirrhotic rats via femoral vein and the experiment was begun immediately. Another group of cirrhotic rats was injected with anti-rat TNFα (300 mg/kg) via abdominal cavity twice within 48 h and the experiment was performed 24 h after the second injection. The blood concentrations of TNFα, ET-1 and NO in portal vein and the nitric oxide synthase (NOS) activity in hepatic tissue were determined pre-and post-injection of anti-rat TNFα or L-NMMA. Stroke volume (SV), cardiac output (CO), portal pressure (PP), superior mesenteric artery blood flow (SMA flow) and iliac artery blood flow (IAflow) were measured simultaneously. Acute portal hypertension was established in Wistar rats by partial portal-vein ligation (PVL). The parameters mentioned above were determined at 0.5 h, 24 h, 48 h, 72 h and 120 h after PVL. After the formation of stable PHT, the PVL rats were injected with anti-rat TNFα or L-NMMA according to different groups, the parameters mentioned above were also determined.

RESULTS: In cirrhotic rats, the blood levels of TNFα, NO in portal vein and the liver NOS activity were significantly increased ($P<0.05$) while the blood level of ET-1 was not statistically different ($P>0.05$) from the control animals (477.67±83.81 pg/mL *vs* 48.87±32.79 pg/mL, 278.41±20.11 μmol/L *vs* 113.28±14.51 μmol/L, 1.81±0.06 u/mg·prot *vs* 0.87±0.03 u/mg·prot and 14.33±4.42 pg/mL *vs* 8.72±0.79 pg/mL, respectively). After injection of anti-rat TNFα, the blood level of TNFα was lower than that in controls (15.17±18.79 pg/mL *vs* 48.87±32.79 pg/mL). The blood level of NO and the liver NOS activity were significantly decreased, but still higher than those of the controls. The blood level of ET-1 was not significantly changed. PP, SV, CO, SMAflow and IAflow were ameliorated. After injection of L-NMMA, the blood level of NO and the liver NOS activity were recovered to those of the controls. PP and CO were also recovered to those of the controls. SV, SMAflow and IAflow were ameliorated. In PVL rats, the blood levels of TNFα, NO in portal vein and the liver NOS activity were gradually increased and reached the highest levels at 48 h after PVL. The blood level of ET-1 among different staged animals was not significantly different from the control animals. PP among different staged animals (2.4±0.18 kPa at 0.5 h, 1.56±0.08 kPa at 24 h, 1.74±0.1 kPa at 48 h, 2.38±0.05 kPa at 72 h, 2.39±0.16 kPa at 120 h) was significantly higher than that in controls (0.9±0.16 kPa). After injection of anti-rat TNFα in 72 h PVL rats, the blood level of TNFα was lower than that in controls (14±14 pg/mL *vs* 48.87±32.79 pg/mL). The blood level of NO and the liver NOS activity were significantly decreased, but still higher than those of the controls. The blood level of ET-1 was not significantly changed. PP was decreased from 2.38±0.05 kPa to 1.68±0.12 kPa, but significantly higher than that in controls. SV, CO, SMAflow and IAflow were ameliorated. After injection of L-NMMA in 72 h PVL rats, the blood level of NO and the liver NOS activity were recovered to those of the controls. PP, SV, CO, SMAflow and IAflow were also recovered to those of the controls.

CONCLUSION: NO plays a critical role in the development and maintenance of HC in acute PHT and is a key factor for maintenance of HC in chronic PHT. TNFα may not participate in the hemodynamic changes of HC directly, while play an indirect role by inducing the production of NO through activating NOS. No evidence that circulating ET-1 plays a role in both models of portal hypertension has been found.

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INTRODUCTION

Associated with hyperdynamic circulatory syndrome (HCS), the portal hypertension (PHT) is characterized by systemic vasodilatation, increase of plasma volume, cardiac output and regional blood flow[1-8]. Although it is most likely initiated by vasodilatation resulted from an increase of vasodilator activity[9], the etiology of HCS is still controversial. Two potent vasodilators, endogenous nitric oxide (NO) and tumor necrosis factor (TNF) may play important roles in the pathogenesis of hemodynamic changes of PHT[1,10]. As a powerful vasoconstrictor, endothelin (ET) could influence the

pathogenesis of hemodynamic changes of PHT as well[5,11-15]. Since ET has contradictory effect on blood vessels in comparison with the former two, it is hard to imagine that they synergistically take part in the hemodynamic changes. It is thus necessary to find out what kind of role the three factors play in the pathogenesis of HCS, respectively.

MATERIALS AND METHODS

Reagents

Carbon tetrachloride was purchased from Chongqing Chemical Reagents Factory (Chongqing, China). A rabbit anti-rat TNFα antibody was purchased from PharMingen Company (USA). N^G-methyl-L-arginine (L-NMMA) and endothelin EIA kit were purchased from Cayman Company (USA). Rat TNFα ELISA kit was purchased from Endogen Company (USA). NO and NOS determining kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animal model of acute PHT (aPHT)

Partial portal vein ligation (PVL) was performed to establish the aPHT model as described previously[10]. In brief, male Wistar rats (220-280 g, offered by the Animal Center of Chongqing University of Medical Sciences) had free access to water and standard rat chow. After fasted overnight, the rats were anesthetized with pentobarbital intra- abdominally at a dose of 60 mg/kg. The portal vein was isolated and two ligatures were placed around both the portal vain and a 16-gauge blunt-end needle. One ligature was placed 1 mm distal to the bifurcation of portal vein and the other ligature was placed 1-2 mm to the input point of splenic vein. The needle was ligated together with the portal vein and immediately removed to allow the portal vein to expand to the limit imposed by the ligature. The abdomen was closed. In sham-operated rats, surgery consisted of dissection and visual inspection of the portal vein without ligature.

Animal model of chronic PHT (cPHT)

Carbon tetrachloride induced cirrhosis was made as the cPHT model. Male Wistar rats (150-200 g, provided by the Animal Center of Chongqing University of Medical Sciences) had free access to standard rat chow and 100mL/L alcohol. Cirrhosis model was established by injection with 600mL/L carbon tetrachloride mixed with paraffin liquid subcutaneously at a dose of 0.3 mL/100g at the lateral abdomen of both sides, twice a week for 17 times. The rats were allowed to stabilize for 2 weeks.

Determination of hemodynamic indexes

Stroke volume (SV) and cardiac output (CO) Ultrasonic probe of HEWLETT PACKARD 5500 type ultrasonic instrument (USA) was placed on the parasternum of rats at the left ventricle long axis and mitral valve level and then exchanged by M type ultrasonic image. The inner computer system of this instrument would calculate and display the data we needed.

Superior mesenteric artery (SMA) and iliac artery (IA) blood flow SMA and IA were isolated and embraced by a cuff of electromagnetic flowmeter (NIHON KHDEN, Japan) respectively. Its blood flow was determined while blood passed a photoelectric sensor.

Portal pressure (PP) and right atrial pressure (RAP) A 7 gauge needle was penetrated into portal vein in the direction of liver and a catheter was inserted through the internal jugular vein into the right atrium and connected to a pressure transducer respectively. PP and RAP were recorded with a four-channel physiometer (NIHON KOHDEN, Japan).

Mean arterial pressure (MAP) Rat' s tail was placed in a photoelectric channel and MAP was determined with an RBP-1 type blood pressometer.

Calculation of superior mesenteric artery vascular resistance (VR_{SMA}) and iliac artery vascular resistance (VR_{IA}) VR_{SMA} and VR_{IA} were calculated according to the following formula reported by Colombato *et al*[16].

$$VR_{SMA}(kPa/L\cdot min)=\frac{MAP-PP}{SMA\text{-}flow}$$

$$VR_{IA}(kPa\cdot/L\cdot min)=\frac{MAP-RAP}{IA\text{-}flow}$$

Serum levels of TNFα, ET-1 and NO and hepatic activity of NOS

Blood was obtained from the portal vein at the time of sacrifice. Hepatic tissue was obtained from the left lobe of rat' s liver. Serum level of TNFα was measured by ELISA according to the manufacturer' s instructions (Phar Mingen Co., USA). Serum samples were analyzed for ET-1 content by EIA according to the manufacturer' s instructions (Cayman Co., USA). Serum samples and hepatic tissues were analyzed for NO content and NOS activity according to the manufacturer' s instructions by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Experimental protocol

The rats involved in this experiment were divided into experimental group, treated group and control group. Fifty aPHT(PVL) rats were divided into five staged subgroups (0.5 h, 24 h, 48 h, 72 h and 120 h after PVL, 10 rats each group) and 10 cPHT rats were used as the experimental group. Twenty cPHT rats and 20 aPHT rats were divided into two groups (10 rats each group) respectively as the treatment group, and the method of treatment was as following. Ten cPHT rats were injected with anti-rat TNFα twice within 48 h and the experiment was performed 24 h after the second injection and 10 aPHT rats were injected with anti-rat TNFα at 0.5 h and 48 h after PVL respectively and the experiment was performed at 72 h after PVL (one dose of 300 μg/kg, via intra-abdominal cavity). The other 10 aPHT rats and 10 cPHT rats were injected with L-NMMA (25 mg/kg) through femoral vein 72 h after PVL and the experiment was performed immediately. Ten normal rats and 10 sham operated rats were used as the cPHT and the aPHT control groups respectively. The following parameters were determined, namely the hemodynamic indexes, serum levels of TNFα, ET-1 and NO, hepatic NOS activity.

Statistical analysis

Data were expressed as mean±SD and analysis of variance was performed using SPSS 8.0 software. Differences between groups were analyzed using *t*-test. One-way analysis of variance was used for multiple comparisons, and Newman-Keuls test was used for intra-group comparisons. $P<0.05$ was considered statistically significant.

RESULTS

Hemodynamic change

In PVL rats, SV, CO, PP, SMAflow and IAflow were significantly increased from 0.16±0.04 mL/s, 0.058±0.008 L/min, 0.9±0.16 kPa, 8.24±1.16 ml· min^{-1} and 10±0.89 ml· min^{-1} to 0.27±0.02 mL/s, 0.113±0.004 L/min, 1.74±0.1 kPa, 17.58±0.7 mL/min and 20.42±1.07 mL/min respectively at 48 h after PVL($P<0.05$). VR_{SMA} and VR_{IA} were significantly decreased from 15.57±2.75 kPaL/min and 13.58±2.19 kPaL/min to 5.96±0.35 kPaL/min and 5.62±0.33 kPaL/min respectively at 48 h after PVL($P<0.05$).

These hemodynamic variables no matter increased or decreased, all reached a maximal level at 72 h after PVL(P<0.05) and did not change thereafter (P>0.05). In cirrhotic rats, SV, CO, PP, SMAflow and IAflow were significantly increased from 0.162±0.04 mL/s, 0.058±0.017 L/min, 0.91±0.16 kPa, 8.42±1.16 mL/min and 10±0.89 mL/min to 0.59±0.06 mL/s, 0.159±0.031 L/min, 2.26±0.39 kPa, 27±3.19 mL/min and 27.33±1.21 mL/min respectively at the time of cirrhosis formation (P<0.05). VR_{SMA} and VR_{IA} were significantly decreased from 15.57±2.74 kPaL/min and 13.58±2.19 kPaL/min to 4.07±0.43 kPaL/min and 4.44±0.13 kPaL/min respectively at the time of cirrhosis formation (P<0.05).

Alteration of serum TNFα, ET-1 and NO and hepatic NOS activity

Serum TNFα and NO levels and NOS activity were significantly increased at 24 h compared with those of control (P<0.05) and reached a maximal level at 48 h after PVL and did not change thereafter. An increase of serum ET-1 levels was also observed at different time points post PVL, however, all of them did not reach the significant level as compared with control (P>0.05). The serum TNFα and NO levels were significantly increased compared with control in cirrhotic rats (P<0.05) and their increment was markedly greater than those rats 48 h after PVL (P<0.05). The NOS activity was also markedly increased (P<0.05) and the serum ET-1 level was slightly increased, but did not reach the significant level (P>0.05) compared with control in cirrhotic rats (Table 1).

Effects of anti-rat TNFα and L-NMMA on serum TNFα, ET-1 and NO and hepatic NOS

The serum level of TNFα was markedly decreased and lower than that of controls in both rat models, and the serum level of NO and hepatic NOS activity were significantly decreased, but still markedly greater than those of controls. Injection of anti-rat TNFα had no effect on the level of ET-1. The serum level of NO and hepatic NOS activity were significantly decreased to the levels of the controls in both rat models after injection of L-NMMA (Table 2).

Table 1 Serum levels of TNFα, ET-1 and NO and hepatic NOS activities in PVL and cPHT rats

Groups	TNFα (pg/min)	ET-1 (pg/min)	NO (μmol/L)	NOS (u/mg·prot)
Control	48.67±32.79	8.72±0.79	113±15	0.9±0.03
aPHT				
PVL0.5 h	50±15	13.4±2.6	128.64±18.29	0.86±0.14
PVL24 h	328±100[ac]	12.8±5.7	169.40±21.07[ac]	1.45±0.17[ac]
PVL48 h	428±69[a]	13.5±2.6	223.71±35.44[a]	1.7±0.12[a]
PVL72 h	416±48[a]	13.1±3.2	215.49±12.75[a]	1.67±0.16[a]
PVL120 h	425±49[a]	13.4±3.5	225.36±18.66[a]	1.7±0.12[a]
cPHT	477.67±83.81[ae]	14.33±4.42	278.41±20.11[ae]	1.81±0.06[a]

[a]P<0.05 *vs* control, [c]P<0.05 PVL 24 h group compared with PVL 48 h, 72 h, 120 h and cPHT groups respectively, [e]P<0.05 cPHT group compared with PVL 48 h, 72 h, 120 h groups respectively.

Effects of anti-rat TNFα and L-NMMA on hemodynamic variables

In both rat models, SV, CO, PP, SMAflow and IAflow were significantly decreased, but were still markedly higher than those of the controls. However, VR_{SMA} and VR_{IA} were significantly increased but still markedly lower than those of the controls after injection of anti-rat TNFα. In PVL rats, SV, CO, PP. SMAflow, IAflow, VR_{SMA} and VR_{IA} were all recovered to the levels of the controls after injection of L-NMMA. In cPHT rats, CO and PP were exclusively recovered to the levels of controls. SV, SMAflow, IAflow, VR_{SMA} and VR_{IA} were markedly increased or decreased, but still significantly different from those of the controls after injection of L-NMMA (Table 3).

Table 2 Effects of anti-rat TNFα and L-NMMA on levels of TNFα, ET-1 and hepatic NO and NOS activity in PVL and cPHT rats

Groups	TNFα (pg/mL)	ET-1 (pg/mL)	NO (μmol/L)	NOS (u/mg·prot)
Control	48.67±32.29	8.72±0.79	113±15	0.9±0.03
cPHT	477.67±83.8[ac]	14.33±4.42	278.41±20.11[ac]	1.81±0.06[ac]
cPHT+anti-rat TNFα	15.17±18.79[a]	14.33±4.42	190.61±10.9[a]	1.39±0.04[a]
cPHT+L-NMMA	–	–	119.18±11.51[e]	4.92±0.03[e]
PVL72 h	416±48[ag]	13.1±3.2	215.49±12.75[ag]	1.67±0.16[ag]
PVL72 h+anti-rat TNFα	14±14[a]	13.5±2.6	178.59±14.61[a]	1.34±0.09[a]
PVL72 h+L-NMMA	–	–	104.61±18[i]	0.95±0.08[i]

[a]P<0.05 *vs* control, [c]P<0.05 cPHT group compared with cPHT+anti-rat TNFα and cPHT+L-NMMA groups respectively, [e]P<0.05 cPHT+anti-rat TNFα compared with cPHT+L-NMMA groups, [g]P<0.05 PVL72 h group compared with PVL72 h+anti-rat TNFα and PVL72 h+L-NMMA groups respectively, [i]P<0.05 PVL72 h+anti-rat TNFα compared with PVL72 h+L-NMMA.

Table 3 Effects of anti-rat TNFα and L- NMMA on homodynamic variables

Groups	SV (mL/s)	CO (L/min)	PP (kPa)	SMAflow (mL/min)	IAflow (mL/min)	VR_{SMA} (kPa/.L·min)	VR_{IA} (kPa/.L·min)
Control	0.162±0.04	0.05±0.017	0.91±0.16	8.42±1.16	10±0.89	15.57±2.74	13.58±2.19
cPHT	0.59±0.06[ac]	0.159±0.031[ac]	2.26±0.34[ac]	27±3.19[ac]	27.33±1.21[ac]	4.07±0.43[ac]	4.44±0.51[ac]
CPHT+anti-rat TNFα	0.39±0.08[a]	0.138±0.029[a]	1.53±0.13[a]	20.75±1.92[a]	24.15±1.67[a]	5.56±0.59[a]	5.21±0.51[a]
cPHT+L-NMMA	0.32±0.02[a]	0.076±0.005[e]	1.12±0.08[e]	12.75±0.82[ae]	16.08±0.74[ae]	10.13±0.26[ae]	8.84±0.66[ae]
PVL72 h	0.42±0.03[ag]	0.143±0.029[ag]	2.38±0.05[ag]	23.27±1.52[ag]	25.43±1.44[ag]	4.44±0.28[ag]	4.54±0.32[ag]
PVL72 h+anti-rat TNFα	0.33±0.02[a]	0.127±0.008[a]	1.68±0.12[a]	19.3±1.1[a]	20.7±2[a]	5.9±0.1[a]	6.0±0.5[a]
PVL72 h+L-NMMA	0.24±0.04[i]	0.072±0.013[i]	0.85±0.15[i]	9.82±0.96[i]	10.77±1.11[i]	12.58±0.93[i]	12.42±0.99[i]

[a]P<0.05 *vs* control, [c]P<0.05 cPHT group compared with cPHT+anti-rat TNFα and cPHT+L-NMMA groups respectively, [e]P<0.05 cPHT+anti-rat TNFα compared with cPHT+L-NMMA groups, [g]P<0.05 PVL 72 h group compared with PVL 72 h+anti-rat TNFα and PVL 72 h+L-NMMA groups respectively, [i]P<0.05 PVL 72 h+anti-rat TNFα compared with PVL 72 h+L-NMMA.

DISCUSSION

In our study, HCS was observed in rats with acute and chronic PHT, and characterized by the increase of SV, CO, regional blood flow and PP, as well as the decrease of peripheral and splanchnic vascular resistance. This agreed with a lot of literature[1-8]. Lopez-Talavera *et al*[9,10] studied the correlation between hemodynamic changes and TNFα on days 5, 13 and 14 after PVL, and found that TNFα might play a role in HCS of portal hypertension. In this study, we found that the serum level of TNFα in portal vein was markedly increased at 24 h, reached a peak at 48 h and maintained stable thereafter in PVL rats. Whereas, the obvious hemodynamic changes occurred at 48 h and HCS was induced at 72 h, about 24 h later than the obvious increase in TNFα level. The serum level of TNFα was much more higher in cPHT rats than that in rats 48 h after PVL. There was no obvious difference between the hemodynamic indexes of both groups. Therefore, we speculated that TNFα might play a role in the early stage of HCS, and that overproduction of TNFα might have a mild effect on hemodynamic changes. In the anti-rat TNFα experiment, we found that the serum level of TNFα was lower than that of the controls and the effect of TNFα was completely inhibited by the injected anti-rat TNFα. Although the hemodynamics was significantly changed, it still had a remarkable difference in comparison with the controls. In other words, HCS was improved and a new HCS balanced on a lower basis formed. At the same time, the NO levels and hepatic NOS activity in rats with hepatic cirrhosis and PVL were decreased by 20-25% and 15-30%, respectively. Kaviani *et al*[17] revealed that after gastric strips from PVL rats were incubated with TNFα neutralizing antibody, inducible NOS mRNA expression was significantly decreased by 40%, 70%, and 80% after 1, 2, and 6 h. This suggested that the vasoactive effect of TNFα itself on the development and formation of HCS in portal hypertension was little, and that corresponding hemodynamic changes after injection of TNFα antibody were due to the elimination of TNFα activation on NOS and the decreased production of NO. This conclusion disagreed with the report of Lopez-Talavera *et al*[9] that anti-rat TNFα treatment of rats after PVL significantly inhibited hyperdynamic circulation and reduced portal pressure. It was also inconsistent with the report of Munoz *et al*[18]. In their experiment, anti-TNFα polyclonal antibodies were injected into rats before and 4 days after portal vein stenosis (PVS) (short-term inhibition) and at 24 h and 4, 7, 10 d after PVS (long-term inhibition). After a short-term inhibition or a long-term inhibition, portal pressure kept unchanged. Tabrizchi[19] found that cardiac output, blood pressure and mean circulatory filling pressure were significantly reduced, but the arterial resistance increased following treatment with TNFα in anaesthetized rats. This, obviously, did not accord with the features of HCS at PHT, and also suggested that TNFα did not directly take part in the hemodynamic changes at PHT.

In our study, we found the serum NO level and hepatic NOS activity in the two animal models with portal hypertension were decreased by 20-25% and 15-30% respectively after injection of anti-rat TNFα antibody. This suggested that the increase of serum NO level was stimulated by the combination of TNFα and other media such as IL-6 and INFα,*etc.*, which agreed with what was reported[10,20,21]. Wiest *et al*[21] reported that upregulation of eNOS release and increase of NO by SMA endothelium occurred before the development of hyperdynamic splanchnic circulation, suggesting a primary role of NO in the pathogenesis of arterial vasodilatation. But the results reported by Albornoz *et al*[22] disagreed with those of ours. Their results showed that dexamethasone (an inhibitor of the expression of the iNOS) administration did not modify systemic and splanchnic hemodynamic parameters in endotoxemic cirrhotic rats and suggested that stimulation of iNOS might not play a role in increasing NO production in portal hypertension. We found that the NO level in portal vein and the liver NOS activity were significantly decreased to the level of controls by injecting L-NMMA in cirrhotic rats and in rats 72 h after PVL. In PVL rats, the hemodynamics was recovered to the controls. In cirrhotic rats, the PP was also recovered to the control. But the SV was still significantly greater than that of control and the systemic vasodilatation was not recovered to the state of control. These results suggested that NO played a critical role in the development and maintenance of HCS in acute PHT and was a key factor in maintenance of HCS in chronic PHT. This conclusion was consistent with those of most authors[1,5-7,23-26]. In patients with chronic portal vein hypertension, since the tissue structure of vascular wall was changed due to the long term dilatation of systemic blood vessels, the dilated blood vessels would be hard to recover, even if the effect of vasodilators had been completely eliminated.

Elevated ET-1 level in blood and its active role in portal hypertension in cirrhotic patients and a variety of animal models have been reported by many authors[13-16,27-29]. Nevertheless, Poo *et al*[30] reported that the liver paracrine ET system did not play a major role in the pathogenesis of portal hypertension, but took part in the development of liver fibrogenesis. Varagic *et al*[31] reported that circulating endothelin-1 did not play a role in spontaneously hypertensive rats. In this study, the blood level of ET-1 in portal vein was mildly increased but not significantly higher in comparison with the controls in cirrhotic and PVL rats. This finding suggested that ET-1 might not play a role in the development of hemodynamic abnormalities in PHT. It might keep the tension of blood vessels and antagonize the effect of vasodilators. Therefore, ET-1 may have a regulating effect on the vasodilatation and vascular refilling. Our finding was consistent with Poo *et al*[30], but inconsistent with the other authors[12-15,27-29].

Based on the result of a combination study of TNFα, NO and ET, we draw a conclusion that TNFα may not directly participate in the hemodynamic changes of HCS, while exert an indirect effect by inducing the production of NO. NO is the primary factor for forming and maintaining HCS at PHT. ET does not directly take part in the hemodynamic changes of PHT either, while keeps the tension of blood vessel and prevents it from overdilating under the effect of vasodilatation factors.

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