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Effects of Nitroprusside as a Nitric Oxide Donor on Anoxia/Reoxygenation and D-galactosamine Hepatic Injuries: a Study in Perfused Hepatocytes

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Summary

At present, the physiological role of $NO^{\bullet}$ synthesis in the liver is ambiguous. Studies directed to reveal the role of $NO^{\bullet}$ in relation to liver function were primarily initiated by an interest in the hepatic response to infections and the consequent modulation of liver function. The purpose of the present investigation was to use perfused rat hepatocytes to test the ability of the latter to produce $NO^{\bullet}$ and to delineate the relationship between exogenously delivered $NO^{\bullet}$ and any alteration in the degree of injury as produced by anoxia/reoxygenation (AR) or D-galactosamine (GalN, 5 mM) intoxication. $NO^{\bullet}$ production in rats was stimulated by a single dose of lipopolysaccharide (LPS, 20 mg/kg i.p.) from which hepatocytes were isolated and perfused. Exogenous $NO^{\bullet}$ was delivered to the perfusate of hepatocytes that were isolated from untreated rats, by the addition of sodium nitroprusside (SNP, 2 mM and 0.2 mM). AR and GalN hepatocyte injury was followed after the addition of SNP. Rat hepatocytes were immobilized in low-gelling agarose and perfused with Williams E medium. Endogenous synthesis of $NO^{\bullet}$ and exogenous $NO^{\bullet}$ as produced by SNP was evaluated by estimating the end products of $NO^{\bullet}$ ($NO_2^- + NO_3^-$) in the perfusion medium. The functional and structural integrity of hepatocytes was evaluated from lactate dehydrogenase (LD) leakage and urea synthesis in the perfusion medium. Normal, AR- and GalN-injured hepatocytes did not exhibit measurable $NO^{\bullet}$ while LPS-treated hepatocytes produced $NO^{\bullet}$ (80 μM $NO_2^- + NO_3^-$). SNP-produced $NO^{\bullet}$ significantly increased or decreased LD leakage in AR at 2 mM or 0.2 mM, respectively, and also reduced or increased the rate of urea synthesis, respectively. 0.2 mM SNP increased trypan blue exclusion by hepatocytes. On the other hand, GalN toxicity was not significantly altered by SNP as demonstrated by LD leakage and the rate of urea synthesis was increased by SNP addition. The present data suggest both deleterious and beneficial role of $NO^{\bullet}$ in AR liver injury model depending on the level of $NO^{\bullet}$ generated.

Key words
Perfused hepatocytes – Anoxia/Reoxygenation – Nitric oxide

Introduction

Nitric oxide ($NO^{\bullet}$) has been shown to be an important messenger in many vertebrate signal transduction processes (Stryer 1995). This short-lived molecule (half-life is measured in seconds) can be produced endogenously from its precursor L-arginine by nitric oxide synthase (NOS) as part of the integrated urea cycle with the nitric oxide pathway. During the last several years, it has been established that expression of the inducible isoform of NOS (iNOS) occurs in several cell types (Nathan 1992, Kuo *et al.* 1996). Recently, studies directed to unravel the role of $NO^{\bullet}$ in relation to liver function, which were initiated by an interest in hepatic responses to infections, revealed that liver cells can also be stimulated to produce $NO^{\bullet}$. It was assumed that an immune stimulus would activate Kupffer cells to secrete inflammatory mediators, including cytokines, which in turn might modulate hepatocellular function in sepsis where the stimulation of iNOS expression and $NO^{\bullet}$ production are a part of the consequences (Freeswick *et al.* 1994). Functionally, $NO^{\bullet}$ was described to possess potentially opposing effects, depending on the organ system or

pathophysiological situation, which might be deleterious (Beckman *et al.* 1990, Yu *et al.* 1994, Walker *et al.* 1995) or beneficial (Harbrecht *et al.* 1992a,b, Kuo *et al.* 1996).

However, the role of NO• remains unidentified in various types of tissue or organ injuries and the net effects are not known under many pathophysiological conditions. The most studied item in relation to NO• is oxidative cell injury. Some authors suggest that NO• interacts with the superoxide radical to form the toxic peroxynitrite (OONO•) which may lead to the formation of highly toxic hydroxyl radicals (Beckman *et al.* 1990, Radi *et al.* 1991). Wink *et al.* (1993) have postulated that NO• forms reactive nitrogen oxide species NOx in the presence of oxygen, which possess strong nitrosating and oxidative properties and that endogenously produced or exogenously delivered NO• may contribute to cytotoxic reactions in certain pathophysiological states of oxidative stress. On the other hand, some authors reported that NO• acts as a cytoprotective agent in oxidative hepatocyte injury (Kuo *et al.* 1996).

In rat hepatocytes, we assume that endogenous or exogenous NO• could modulate hepatic insults, specifically those related to oxygen species, and that the degree of modulation is related to the level of NO•. Therefore, the overall aim of the present investigation using isolated and immobilized perfused rat hepatocytes was to test the ability of the latter to produce NO• and to delineate the relationship between exogenously delivered NO• and the degree of hepatocyte injury produced by anoxia/reoxygenation (AR) or D-galactosamine (GalN, 5 mM) intoxication. The latter hepatotoxic agent was used for comparative study since its mode of toxicity does not include oxygen species.

Materials and Methods

Reagents

Sea Plaque agarose (low-temperature gelling agarose) was a product of FMC (Rockland, ME), trypan blue, ethylene glycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), bovine serum albumin, D-galactosamine (GalN), Williams E cell culture medium, L-arginine, sodium nitroprusside (SNP), sulfanilamide, naphthylethylenediamine, lipopolysaccharide from *Escherichia coli* K-235 (LPS), diagnostic kits for lactate dehydrogenase (LD) measurement and kits for urea biosynthesis measurement were obtained from Sigma (St. Louis, MO), thin-wall Chem fluor TFE tubing was used for threading (I.D. 0.5 mm, Berghof/America, Concord, CA). The nitric oxide test-combination kit was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). All other chemicals were analytical grade and were obtained from standard sources.

Isolation of rat hepatocytes and purification

Adult male Wistar rats of 220–300 g body weight were used throughout the present study. All rats received standard care and the study protocol complies with the general guidelines of the First Faculty of Medicine, Charles University. Hepatocytes were isolated from either LPS- treated rats (as mentioned below) or from untreated animals. Hepatocytes were isolated by the standard two phase perfusion method as reported earlier (Moldeus *et al.* 1978) with minor modifications (Farghali *et al.* 1984). After hepatocyte harvesting, and before immobilization, the cells were counted and their viability was evaluated by the trypan blue exclusion method.

Immobilization and perfusion of hepatocytes in agarose gel threads

Hepatocytes were immobilized in agarose gel threads as previously described (Farghali *et al.* 1992, 1994, 1996). Briefly, a 1.8 % low-gelling agarose solution was prepared in a warm Krebs-Henseleit medium at 70 °C. The agarose solution was brought to 37 °C and mixed with hepatocytes. The cells were immobilized in agarose threads by extruding the agarose-cell mixture through teflon tubing into a bioreactor tube containing Williams E medium. The immobilized hepatocytes were allowed to stabilize for one hour after thorough washing to remove unattached cells and cell debris (non-recirculating system). Then the cells were perfused for another hour in a recirculating system under efficient carbogen bubbling of the perfusion medium to allow for stabilization of the cells.

Perfusion of the immobilized hepatocytes with Williams E medium with or without added L-arginine

L-arginine (5 mM) was added to Williams E medium during the perfusion period of hepatocytes obtained from LPS-treated rats and from the untreated control rats to test the inducibility of NOS and the production of endogenous NO•. In all other experiments with exogenously delivered NO•, hepatocytes were perfused with Williams E medium without additional arginine.

Generation of NO• in isolated perfused rat hepatocytes and in vitro hepatocyte injury

Endogenous NO• production was induced by administration of 20 mg/kg of LPS (i.p.) and cells were isolated 5 h later. Exogenous NO• delivery to perfused hepatocytes (obtained from untreated rats) was done by the addition of SNP to the perfusion medium and during the perfusion time. After perfusion of the hepatocytes for one hour in a recirculating system under normoxia, cells were subjected to anoxia which was initiated by replacing carbogen with nitrogen and anoxia was continued for 2 or 2.5 h. Then the reoxygenation was resumed by carbogen for another

hour, so that the whole perfusion lasted 4.5 h. In experiments with GalN, intoxication was initiated by perfusion of GalN at a final concentration of 5 mM in the perfusion medium. GalN perfusion continued for 4.5 h. SNP was added to the perfusion medium at concentrations 2 mM or 0.2 mM in the course of perfusion in AR or GalN experiments. Control experiments were simultaneously carried out without the addition of SNP (AR and GalN controls). In saline control experiments, the perfusion time was the same but without anoxia or the addition of GalN to the perfusion medium. In addition, SNP control experiments were also performed where 2 mM SNP was added to the perfusion medium.

Urea biosynthesis was followed up in the perfusate of hepatocytes by taking the appropriate samples after cell stabilization and during the perfusion time as indicated in the results. Simultaneously, the same sample was used to evaluate the integrity of the immobilized hepatocyte plasma membrane during various treatments by measuring LD leakage in the perfusion medium.

Assays

Measurement of NO• released endogenously or delivered exogenously into the perfusion medium was quantified by the assessment of the stable NO• end product $NO_2^- + NO_3^-$. Total $NO_2^- + NO_3^-$ was determined after conversion of NO_3^- to NO_2^- using nitrate reductase. This was achieved by customizing the nitrate UV kit (Boehringer, Mannheim, Germany), where NO_3^- was reduced by nicotinamide-adenine dinucleotide phosphate (NADPH) to nitrite in the presence of the enzyme nitrate reductase. The amount of NADPH oxidized during the reaction is stoichiometric with the amount of nitrate. The decrease in NADPH is measured by UV absorbance at 340 nm. NO_2^- was assayed spectrophotometrically, using the procedure based on the Griess reaction (Pastor *et al.* 1995). The functional integrity and metabolic competence of the hepatocytes were evaluated by measuring LD release and urea synthesis respectively, using the relevant Sigma kit. In addition, trypan blue exclusion was also measured after termination of the perfusion experiments of the immobilized cells. Values are presented as the mean ± S.E.M. The significance of differences was determined using Student's t-test. P-values less than 0.05 were considered to be significant.

Results

Normal, AR- and GalN-injured hepatocytes did not exhibit measurable NOS activity or only expressed very low activity (data not shown). Hepatocytes that were isolated 5 h after *in vivo* LPS treatment of rats, expressed NOS activity and $NO_2^- + NO_3^-$ production which was already measurable in the perfusate several hours after perfusion. The level of $NO_2^- + NO_3^-$ reached 80 μM within 3 h after initiation of perfusion. When the blood was collected 5 h after LPS-treatment, the plasma concentration of $NO_2^- + NO_3^-$ was 270 μM demonstrating NO• production *in vivo* by various cellular types.

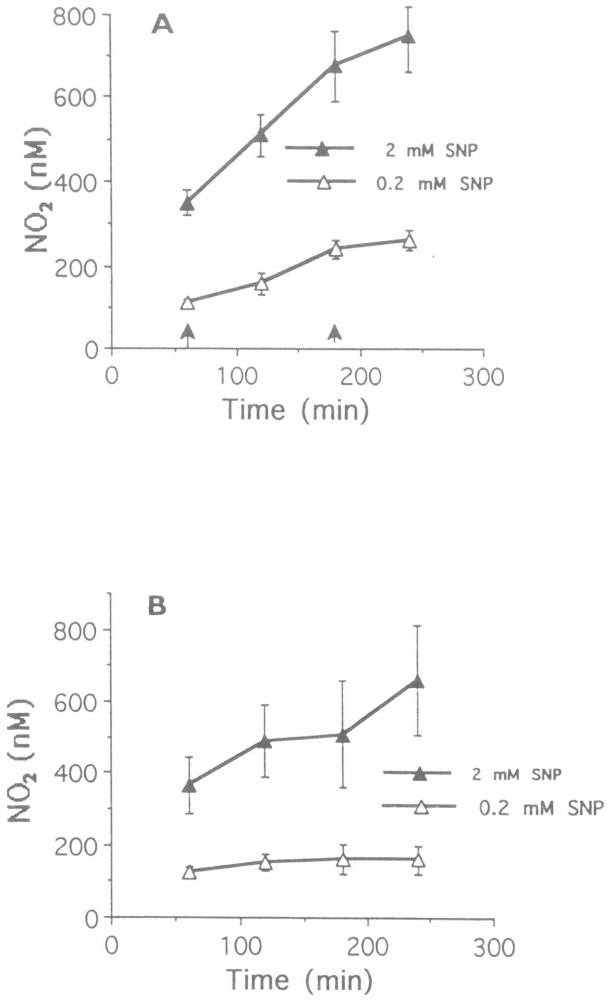

Fig. 1. *The average time course of $NO_2^- + NO_3^-$ levels under 2 mM and 0.2 mM of SNP in the perfusion medium after A) anoxia/reoxygenation (arrows) and B) D-galactosamine intoxication (means ± S.E.M., n = 4).*

Two types of hepatocyte injury models were produced. They were investigated under high and low exogenous NO• concentrations that were achieved by the addition of the NO• donor SNP to the perfusion medium. Figure 1 demonstrates the time course of $NO_2^- + NO_3^-$ level under 2 mM and 0.2 mM of SNP in the perfusion medium under AR and GalN intoxication. It is clear that the levels of $NO_2^- + NO_3^-$

produced were independent of the type of injury applied but were dependent on the initial level of SNP added to the perfusion medium.

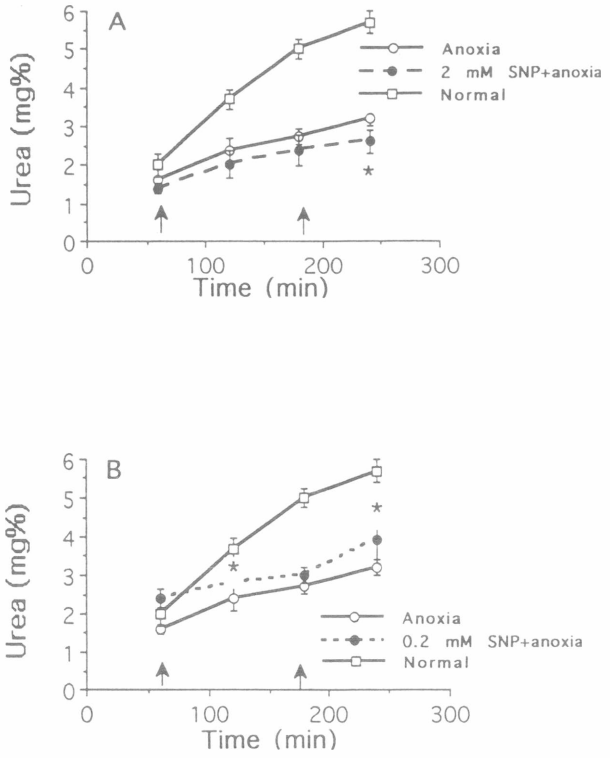

Fig. 2. *The effect of A) 2 mM SNP and B) 0.2 mM SNP on the average cumulative urea synthesis in the perfusion medium by hepatocytes during anoxia/reoxygenation, arrows (means ± S.E.M., n = 4–6). The asterisk indicates significant difference (P<0.05) vs anoxia.*

Figure 2 shows that the rate of urea synthesis, during 2 h anoxia, was affected in two opposite ways depending on the initial SNP level added. At high initial SNP concentration, urea synthesis was significantly reduced by NO• production after 3 h perfusion. On the other hand, at the lower SNP initial concentration, urea synthesis was significantly increased during perfusion. It should be noted, however, that high and low SNP concentrations did not significantly affect the rate of urea production which was comparable to the value obtained from untreated cells (data not shown). These opposite NO• effects were also evident on LD leakage under various treatments (Fig. 3). A 2 mM concentration of SNP potentiated LD leakage produced by AR to more than twofold values. In contrast, 0.2 mM SNP significantly reduced LD leakage produced by AR and the LD time course was similar to that obtained from normoxic cells. After termination of the perfusion experiments under AR, hepatocyte viability was 75 ±6 % and 57±7 % as at 0.2 mM and 2 mM SNP concentrations, respectively.

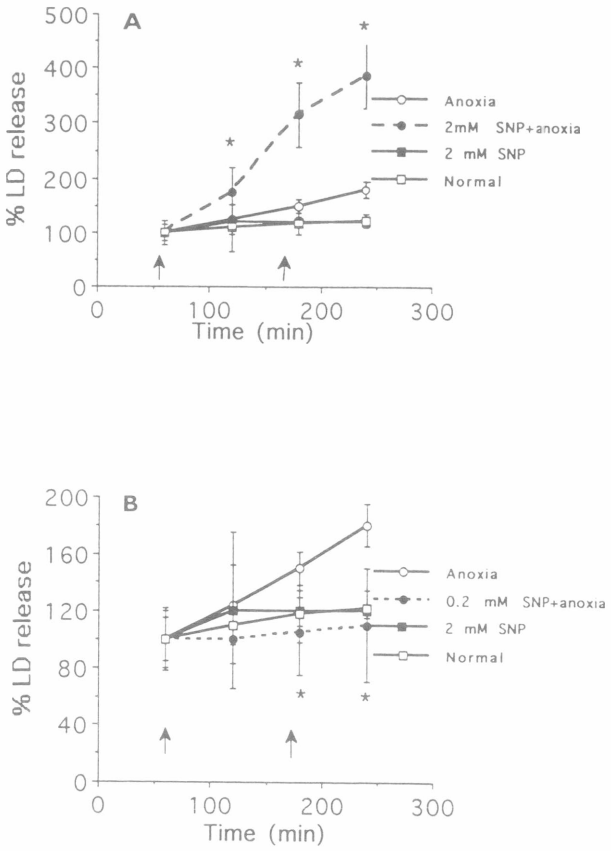

Fig. 3. *The effect of A) 2 mM SNP and B) 0.2 mM SNP on the average lactate dehydrogenase release during anoxia/reoxygenation, arrows, (means ± S.E.M, n = 4–6). The asterisk indicates significant difference (P<0.05) vs anoxia.*

For unknown reasons, both the initial concentrations of SNP increased urea synthesis after 4 h of GalN perfusion (Fig. 4). However, this was not reflected by any significant effect on GalN toxicity as evidenced from LD leakage data (Fig. 5). It is clear that SNP at both its initial concentrations neither increased nor reduced GalN toxicity as revealed by the LD leakage.

Discussion

With the available techniques of measuring NO• production by liver cells, it is difficult to establish a measurable level of NO• produced endogenously by hepatocytes. However, it is now well established that NO• production by liver cells can be induced during infections and sepsis. This was demonstrated by using isolated perfused liver (Pastor *et al.* 1995) or cultured rat hepatocytes (Kuo *et al.* 1996).

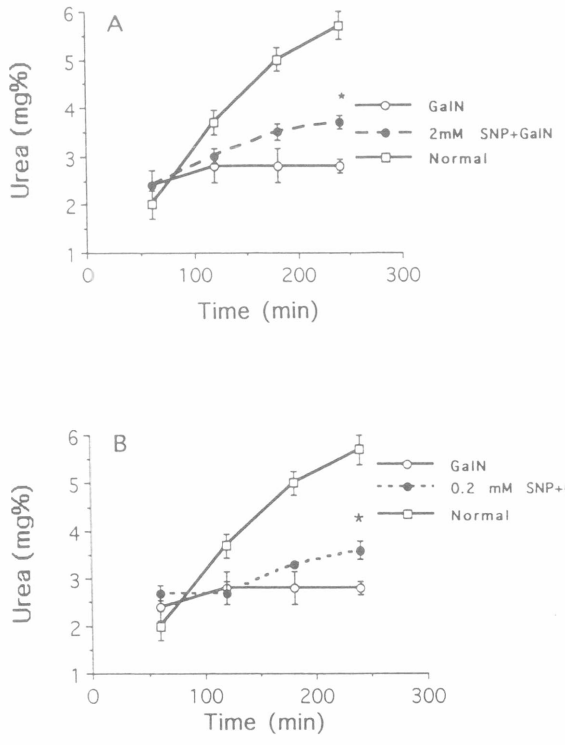

Fig. 4. *The effect of A) 2 mM SNP and B) 0.2 mM SNP on the average cumulative urea synthesis in the perfusion medium by hepatocytes during D-galactosamine intoxication (means ± S.E.M., n = 4–6). The asterisk indicates significant difference (P<0.05) vs GalN treatment.*

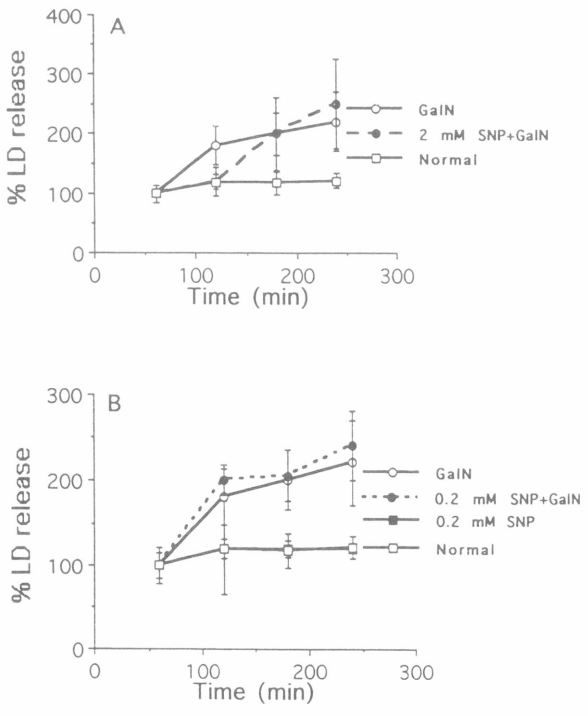

Fig. 5. *The effect of A) 2 mM SNP and B) 0.2 mM SNP on the average lactate dehydrogenase release during D-galactosamine intoxication (means ± S.E.M., n = 4–6).*

In the present study, we have used a cellular system of immobilized and perfused hepatocytes. Hepatocytes that were prepared from LPS pretreated rats exhibited low but measurable NO• production already 3 hours after recirculating perfusion. In spite of the finding that NOS is expressed by liver cells, the exact role of NO• production during septic liver injury and other types of injuries is not yet known. Therefore, two types of hepatocyte injury models were investigated under high and low exogenously delivered NO• concentrations that were achieved by the addition of the NO• donor SNP. Consequently, the NO•-dependent influence on hepatocellular injury evoked by AR or by GalN was followed up. The data reported herein indicate that NO• delivered exogenously modulates AR injury but not GalN injury. The presence of higher concentrations of exogenous NO• (2 mM SNP) exacerbated AR injury whereas lower exogenous NO• (0.2 mM SNP) ameliorated the injury. On the other hand, exogenously delivered NO• (within the studied concentration range) had no effect on GalN toxicity.

Under some pathophysiological conditions of oxidative injury, as for instance during AR, the potential harmful or protective role of NO• is controversial. In some studies, NO• has been implicated as a toxic metabolite and product which can react with superoxide to form peroxynitrite. The latter can be decomposed to the highly oxidant hydroxyl radical, concomitantly promoting protein nitration (Beckman *et al.* 1990, Radi *et al.* 1991). NO• can also form reactive nitrogen oxide species NOx in the presence of oxygen which possess strong nitrosating (forming nitroso derivatives) and oxidative properties (Wink *et al.* 1993). The endogenously produced or exogenously delivered NO• may contribute to the cytotoxic reactions in certain pathophysiological states of oxidative stress. In other studies, it was shown that NO• formation has a protective function in oxidative injury such as inactivation of superoxide radicals produced by activated human leukocytes, and that inhibition of NO• synthesis promotes oxygen free radical-induced damage manifested as intrahepatic thrombosis in an *in vivo* model of endotoxin-mediated

liver injury (Harbrecht *et al.* 1992a,b). Several other authors reported a beneficial effect of NO• as a cytoprotective agent in oxidative cell injury (Kuo *et al.* 1996, Konorev *et al.* 1995, Wink *et al.* 1995). The hepatoprotective effects of NO• against various types of oxidative stress have also been described (Kuo and Slinka 1994, Kuo and Abe 1995).

In summary, we have presented some data which are suggestive of both a deleterious and a beneficial role of NO• in the AR liver injury model depending on the level of NO• generated. We propose that this may be applicable to other types of liver oxidative stress.

Acknowledgments

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