Cicletanine stimulates eNOS phosphorylation and NO production via Akt and MAP kinase/Erk signaling in sinusoidal endothelial cells

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Liu S, Rockey DC. Cicletanine stimulates eNOS phosphorylation and NO production via Akt and MAP kinase/Erk signaling in sinusoidal endothelial cells. Am J Physiol Gastrointest Liver Physiol 305: G163–G171, 2013. First published May 2, 2013; doi:10.1152/ajpgi.00003.2013.—The function of the endothelial isoform of nitric oxide synthase (eNOS) and production of nitric oxide (NO) is altered in a number of disease states. Pharmacological approaches to enhancing NO synthesis and thus perhaps endothelial function could have substantial benefits in patients. We analyzed the effect of cicletanine, a synthetic pyridine with potent vasodilatory characteristics, on eNOS function and NO production in normal (liver) and injured rat sinusoidal endothelial cells, and we studied the effect of cicletanine-induced NO on stellate cell contraction and portal pressure in an in vivo model of liver injury. Sinusoidal endothelial cells were isolated from normal and injured rat livers. After exposure to cicletanine, eNOS phosphorylation, NO synthesis, and the signaling pathway regulating eNOS activation were measured. Cicletanine led to an increase in eNOS (Ser1177) phosphorylation, cytochrome c reductase activity, l-arginine conversion to l-citrulline, as well as NO production. The mechanism of the effect of cicletanine appeared to be via the protein kinase B (Akt) and MAP kinase/Erk signaling pathways. Additionally, cicletanine improved NO synthesis in injured sinusoidal endothelial cells. NO production induced by cicletanine in sinusoidal endothelial cells increased protein kinase G (PKG) activity as well as relaxation of stellate cells. Finally, administration of cicletanine to mice with portal hypertension induced by bile duct ligation led to reduction of portal pressure. The data indicate that cicletanine might improve eNOS activity in injured sinusoidal endothelial cells and likely activates hepatic stellate cell NO/PKG signaling. It raises the possibility that cicletanine could improve intrahepatic vascular function in portal hypertensive patients.

protein kinase B; stellate cell; portal hypertension; signaling; endothelial isoform of nitric oxide synthase; mitogen-activated protein kinase; extracellular/signal-regulated kinase

THE ENDOTHELIUM PLAYS A CENTRAL role in the maintenance of vascular homeostasis largely by virtue of its synthesis of nitric oxide (NO). It is now well appreciated that the endothelial isoform of nitric oxide synthase (eNOS) produces endothelium-derived NO. Furthermore, endothelial dysfunction contributes to the development and clinical course of vascular diseases such as pulmonary and portal hypertension, both characterized by reduced NO bioactivity (19, 23–24, 27).

MATERIALS AND METHODS

Cell isolation and culture. Sinusoidal endothelial cells were isolated from male Sprague-Dawley rats (450–500 g) (Harlan, Indianapolis, IN). In brief, after in situ perfusion of the liver with 20 mg/100 mg promace (Roche Molecular Biochemicals, Indianapolis, IN), followed by collagenase (Worthington Biochemical, Lakewood, NJ), dispersed cell suspensions were removed from a layered discontinuous density gradient of 8.2 and 15.6% Accudenz (Accurate Chemical and Scientific, Westbury, NY), further purified by centrifugal elutriation (18 ml/min flow), and were grown in medium containing 20% serum (10% horse/calf). The purity of endothelial cells was documented by their uptake of fluorescently labeled di-I-acetoacetylated low-density lipoprotein as described (21). Only primary sinusoidal endothelial isolates of 95% purity were used for study. Experiments were performed with cells from a minimum of three different isolations (for all experiments).

For coculture experiments, freshly isolated stellate cells and freshly isolated sinusoidal endothelial cells were isolated separately and mixed together on collagen-coated plates and cultured for 5 days. Sinusoidal endothelial cells were plated at a density of 200,000 cells/cm², and stellate cells were at a density of 65,000 cells/cm².

Adenoviruses. Adenovirus (Ad) control empty vector (Ad-EV), constitutively active Akt (Ad-myrAkt), and dominant-negative Akt (Ad-dnAkt) have been described previously (17). Sinusoidal endothelial cells were exposed to Ad in 2% serum for 16 h, and medium was exchanged; cells were then harvested at the specified time points.

Immunoblotting. Immunoblotting was performed by using specified primary antibodies, including anti-eNOS antibody (1:1,000; BD Transduction Laboratories, San Jose, CA), anti-phospho-eNOS-Ser1177 (1:1,000; BD Transduction Laboratories or Cell Signaling Technology), anti-phospho-Akt, anti-Akt (Cell Signaling Technology, Beverly, MA), and horseradish peroxidase-conjugated secondary antibody. Specific signals were visualized using enhanced chemiluminescence as per the...
incubated with a reaction buffer containing 3 pmol of L-[3H]arginine (Cayman Chemical). In brief, triplicate samples of cell lysates were measured and used to determine L-NAME-inhibitable NOS activity (thus, Radiolabeled counts per minute of generated L-citrulline were measured stop buffer and passed over a Dowex AG 50WX-8 resin column. (L-NAME). The reaction mixture was terminated by the addition of 45 Ci/mmol, 0–50 M arginine, 1 mM NADPH, 100 nM calmodulin (CaM), 2 mM CaCl2, and 30 μM BH4 in a volume of 50–100 μl at room temperature for 30 min and at 37°C for an additional 30 min in the presence and absence of 1 mM N^6-nitro-L-arginine methyl ester (L-NAME). The reaction mixture was terminated by the addition of stop buffer and passed over a Dowex AG 50WX-8 resin column. Radiolabeled counts per minute of generated L-citrulline were measured and used to determine L-NAME-inhibitable NOS activity (thus, each experiment included total counts and background counts).

NOS activity assay. NOS activity from sinusoidal endothelial cell lysates was assessed by measuring the conversion of L-[3H]arginine to L-[3H]citrulline as described (6) per the manufacturer’s instructions (Cayman Chemical). In brief, triplicate samples of cell lysates were incubated with a reaction buffer containing 3 pmol of L-[3H]arginine (45 Ci/mmoll, 0–50 μM arginine, 1 mM NADPH, 100 nM calmodulin (CaM), 2 mM CaCl2, and 30 μM BH4 in a volume of 50–100 μl at room temperature for 30 min and at 37°C for an additional 30 min in the presence and absence of 1 mM N^6-nitro-L-arginine methyl ester (L-NAME). The reaction mixture was terminated by the addition of stop buffer and passed over a Dowex AG 50WX-8 resin column. Radiolabeled counts per minute of generated L-citrulline were measured and used to determine L-NAME-inhibitable NOS activity (thus, each experiment included total counts and background counts).

Cytochrome c reductase assay. The reductase activities of sinusoidal endothelial cells with or without cicletanine exposure were determined using the NADPH-dependent cytochrome c assay. The reaction was carried out in a total volume of 1.1 ml containing cell lysates (30 μg) in 50 mM Tris·HCl, pH 7.2, 1 mM CaM, 0.2 mM CaCl2, manganese superoxide dismutase (Mn-SOD, 400 U/ml), and 100 μM cytochrome c. The reaction was initiated by the addition of NADPH to a final concentration of 0.5 mM. The reduction of cytochrome c was monitored at 550 nm at 25°C (Beckman Spectrophotometer-Model DU650). Mn-SOD (400 U/ml) was included to eliminate the cytochrome c reduction contributed by O2. The linear portion of the kinetic traces was used to calculate the rate of cytochrome c reduction and reductase activity of eNOS. Turnover number was calculated using the absorbance change during this 30-s interval and an extinction coefficient of 0.021/μM.

Collagen lattice assay. Contraction of hepatic stellate cells was performed as previously described with minor modifications (25). Briefly, individual wells of a 24-well culture dish were incubated with PBS containing 1% BSA (500 μl/well) for 1 h at 37°C and then washed two times with PBS and allowed to air dry. Collagen gels

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**Fig. 1.** Effect of cicletanine on the function of endothelial isoform of nitric oxide synthase (eNOS) in sinusoidal endothelial cells. A: sinusoidal endothelial cells were isolated from rat livers and exposed to cicletanine (100 nM) for 2 h, cell lysates were harvested, and phosphor (P)-eNOS (Ser^1177), total eNOS, and β-actin were detected by immunoblotting. In the graph shown on the bottom, bands corresponding to phospho-eNOS were quantified (n = 4, *P < 0.01 vs. control). B: the activity of nitric oxide synthase (NOS) from normal sinusoidal endothelial cells was examined in the same cells. NOS activity without cicletanine, **P < 0.01 vs. cicletanine. L-NAME, G-nitro-L-arginine methyl ester. C: cells were treated with cicletanine, S-cicletanine, and R-cicletanine as indicated for 2 h, conditioned medium was harvested, and nitrite levels were measured as in MATERIALS AND METHODS (n = 3, *P < 0.001 and **P < 0.05 vs. control).

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- **Portal pressure measurement.** Portal hypertension was induced in male BALB/c mice by bile duct ligation as above. Cicletanine (5 mg·kg^-1·day^-1) or vehicle was given by gavage as a single daily dose for 10 days before portal pressure measurement as described (17). In brief, mice were anesthetized, and an intravenous catheter (Becton Dickinson Vascular Access, Sandy, UT) was introduced in the portal vein and secured with a silk tie. A calibrated low-pressure transducer (Digi-Med, Louisville, KY) was connected to the portal vein catheter, and portal pressure was recorded continuously using the Digi-Med Integrator 200 System (Digi-Med).

- **NO measurement.** To assess NO production, we analyzed the release of nitrite, the stable breakdown product of NO, using a nitrite/nitrate assay kit (Cayman Chemicals, Ann Arbor, MI), following the manufacturer’s instructions. Briefly, conditioned medium from sinusoidal endothelial cells with or without cicletanine treatment were loaded on a 96-well plate and mixed with both enzyme cofactor and nitrate reductase. After incubation at room temperature for 3 h, 100 μl Griess reagent mix were added to each well. After 10 more minutes at room temperature, the absorbance of each well was determined on a microplate reader at 540 nm, and the nitrite concentrations were deduced from a standard curve.

- **NOS activity assay.** NOS activity from sinusoidal endothelial cell lysates was assessed by measuring the conversion of L-[^3]H]arginine to L-[^3]H]citrulline as described (6) per the manufacturer’s instructions (Cayman Chemical). In brief, triplicate samples of cell lysates were incubated with a reaction buffer containing 3 pmol of L-[^3]H]arginine (45 Ci/mmoll, 0–50 μM arginine, 1 mM NADPH, 100 nM calmodulin (CaM), 2 mM CaCl2, and 30 μM BH4 in a volume of 50–100 μl at room temperature for 30 min and at 37°C for an additional 30 min in the presence and absence of 1 mM N^6-nitro-L-arginine methyl ester (L-NAME). The reaction mixture was terminated by the addition of stop buffer and passed over a Dowex AG 50WX-8 resin column. Radiolabeled counts per minute of generated L-citrulline were measured and used to determine L-NAME-inhibitable NOS activity (thus, each experiment included total counts and background counts).

- **Cytochrome c reductase assay.** The reductase activities of sinusoidal endothelial cells with or without cicletanine exposure were determined using the NADPH-dependent cytochrome c assay. The reaction was carried out in a total volume of 1.1 ml containing cell lysates (30 μg) in 50 mM Tris·HCl, pH 7.2, 1 mM CaM, 0.2 mM CaCl2, manganese superoxide dismutase (Mn-SOD, 400 U/ml), and 100 μM cytochrome c. The reaction was initiated by the addition of NADPH to a final concentration of 0.5 mM. The reduction of cytochrome c was monitored at 550 nm at 25°C (Beckman Spectrophotometer-Model DU650). Mn-SOD (400 U/ml) was included to eliminate the cytochrome c reduction contributed by O2. The linear portion of the kinetic traces was used to calculate the rate of cytochrome c reduction and reductase activity of eNOS. Turnover number was calculated using the absorbance change during this 30-s interval and an extinction coefficient of 0.021/μM.
were prepared by mixing 60% type I tail collagen (Upstate Laboratories), 10% 10× MEM (GIBCO), 10% 0.2 HEPES, and 20% DMEM (GIBCO) to make a final concentration of collagen of 2.4 mg/ml. The solution was added to the culture wells and incubated for 1 h at 37°C, and hepatic stellate cells and sinusoidal endothelial cells were isolated separately from normal rat livers and cocultured (each at a density of 100,000 cells/lattice) on collagen lattices for 5 days. Cells were changed to serum-free medium overnight, then exposed to cicletanine, and then stimulated with endothelin-1 (ET-1, 10 nM). Collagen lattices were released from their substrata, and gel contraction was measured from 0 to 30 min.

Statistical analyses. All experiments were performed in replicate using cells isolated from different rats. All results were expressed as means ± SE. We performed statistical analysis using the two-tailed Student’s t-test, and P < 0.05 was considered statistically significant.

RESULTS

Cicletanine stimulates eNOS in sinusoidal endothelial cells.

We examined whether cicletanine is capable of activating eNOS in sinusoidal endothelial cells; since eNOS is typically phosphorylation dependent, we initially examined eNOS phosphorylation (Ser1177). After exposure to cicletanine (100 nM), total eNOS expression was unchanged, whereas phosphorylation at Ser1177 was stimulated (Fig. 1A). Consistent with eNOS phosphorylation and activation, exposure of sinusoidal endothelial cells to cicletanine (100 nM) led to robust eNOS activity (i.e., conversion of arginine to citrulline), inhibitable by L-NAME (Fig. 1B). In addition, nitrite levels in conditioned medium were significantly increased after exposure to cicletani-
CICLETANINE STIMULATES eNOS

Fig. 3. Cicletanine potentiates eNOS cytochrome c reductase activity. Sinusoidal endothelial cells were isolated from rat livers and exposed to cicletanine (100 nM) for 2 h. Lysates were then harvested and subjected to erythrocyte cytochrome c reductase activity assay. Results are representative of 3 experiments. (P < 0.05 and **P < 0.01 vs. control.)

Fig. 4. Cicletanine-induced eNOS activity is protein kinase B (Akt) dependent. A: sinusoidal endothelial cells were isolated from rat livers and were exposed to 50 or 100 nM cicletanine for 2 h, and cell lysates were harvested and subjected to immunoblotting to detect phospho-eNOS (Ser1177) and total Akt. B: sinusoidal endothelial cells were exposed to 50 or 100 nM cicletanine for 1 or 2 h, and cell lysates were harvested and subjected to immunoblotting to detect phospho-eNOS, total eNOS, phospho-Akt (Ser473), total Akt, and β-actin. Blots representative of 3 others are shown.

The results of phosphorylation and citrulline activity assays. Consistent with previous studies, we found that exposure to cicletanine for 1 to 2 h increased eNOS activity and nitrite production, as measured by citrulline accumulation over time (Fig. 2). Cicletanine’s effects on nitrite production were also dose dependent (Fig. 2B). Cicletanine induced eNOS phosphorylation at Ser1177 as well as Akt phosphorylation at Ser473 without a change in total Akt expression (Fig. 4, A and B). To determine whether eNOS phosphorylation by cicletanine was Akt dependent, we transduced sinusoidal endothelial cells with Ad-myrAkt, Ad-dnAkt, or Ad-EV for 24 h before exposure to cicletanine (100 nM) for 0–4 h. These data suggest that eNOS activation induced by cicletanine involves the MAP kinase signaling partners, including Erk1 and Erk2. First, we examined the

Increased eNOS activity caused by cicletanine can be attributed to enhanced reductase activity. We found a significant increase in cytochrome c reductase activity after exposure of sinusoidal endothelial cells to cicletanine (100 nM) (Fig. 3).

Cicletanine-induced eNOS activity is Akt dependent. To understand the mechanism by which cicletanine stimulates eNOS, we performed studies examining the known eNOS-signaling partner Akt. Again, cicletanine increased eNOS phosphorylation at Ser1177 as well as Akt phosphorylation at Ser473 without a change in total Akt expression (Fig. 4, A and B). To determine whether eNOS phosphorylation by cicletanine was Akt dependent, we transduced sinusoidal endothelial cells with Ad-myrAkt, Ad-dnAkt, or Ad-EV for 24 h and exposed cells to cicletanine (100 nM) for an additional 2 h. Cicletanine stimulated nitrite production while the dominant active Akt further increased it and the dominant negative Akt blocked cicletanine’s effects (Fig. 4C). These data suggest that cicletanine’s mechanism of action requires Akt signaling.

Erk activation is involved in cicletanine-induced eNOS activity. We also explored the possibility that eNOS activation induced by cicletanine involved the MAP kinase signaling partners, including Erk1 and Erk2. First, we examined the
We and others have previously shown that endothelial cells. B phosphorylation (Fig. 5) Erk inhibitor PD-98059 blocked cicletanine-induced eNOS (24, 27); Fig. 6A significantly reduced compared with that from normal endothelial cells, concomitant with an increase in eNOS activity cells; Fig. 6A). Additionally, the sinusoidal endothelial cells injured by bile duct ligation (note tanine restored eNOS phosphorylation to above basal levels in endothelial cells to cicletanine for 2 h (Fig. 5A). Findings of Erk phosphorylation and eNOS activity is reduced after liver injury and in portal hypertensive rats and mice (17, 24, 27). We next tested whether cicletanine could rescue the abnormal relationship between Erk and eNOS phosphorylation. We found that phosphorylation of Erk and eNOS was increased in a dose-dependent manner after exposure of sinusoidal endothelial cells to cicletanine for 2 h (Fig. 5A). Additionally, the Erk inhibitor PD-98059 blocked cicletanine-induced eNOS phosphorylation (Fig. 5B) and NO production (Fig. 5C).

Cicletanine stimulates eNOS activity in injured sinusoidal endothelial cells. We and others have previously shown that Akt phosphorylation and eNOS activity is reduced after liver injury and in portal hypertensive rats and mice (17, 24, 27). We next tested whether cicletanine could rescue the abnormal endothelial cell/eNOS phenotype typical of liver injury. Cicletanine restored eNOS phosphorylation to above basal levels in sinusoidal endothelial cells injured by bile duct ligation (note that there is little to no phosphorylated eNOS in unstimulated cells; Fig. 6A), concomitant with an increase in eNOS activity [eNOS activity in injured sinusoidal endothelial cells is significantly reduced compared with that from normal endothelial cells (24, 27); Fig. 6B] and nitrite production (Fig. 6C). Cicletanine also restored Akt phosphorylation to above normal levels (Fig. 6D) and increased Erk1/2 phosphorylation (Fig. 6E).

Cicletanine-induced NO inhibits stellate cell contractility via activation of NO/protein kinase G. To determine whether cicletanine-induced NO has a physiological function, we developed a coculture model in which we cultured sinusoidal endothelial cells with hepatic stellate cells, the latter of which generate considerable contractile force (25). We used a model

Fig. 5. Cicletanine-induced eNOS activity is Erk dependent. A: sinusoidal endothelial cells were isolated from rat livers and were exposed to 50 or 100 nM cicletanine for 2 h, and cell lysates were subjected to immunoblotting to detect phospho-eNOS, total eNOS, phospho-Erk, total Erk1/2, and β-actin. In the graphs shown on bottom, bands corresponding to phospho-Erk were quantified (n = 3, *P < 0.05 vs. control). B: sinusoidal endothelial cells in A were pretreated with the MAP kinase inhibitor PD-98059 (10 µM) for 30 min and then stimulated with cicletanine (100 nM) for an additional 2 h; eNOS and Erk phosphorylation were detected by immunoblotting. In the graph shown on bottom, bands corresponding to phospho-Erk were quantified (n = 3, *P < 0.01 vs. no cicletanine control, ***P < 0.005 vs. cicletanine control). C: conditioned supernatants from cells in B were collected, and nitrite levels were measured (n = 3, *P < 0.05 vs. no cicletanine control, **P < 0.01 vs. cicletanine control).

Fig. 6. Effects of cicletanine in injured sinusoidal endothelial cells. A: sinusoidal endothelial cells were isolated from rat livers 10 days after bile duct ligation (BDL), allowed to adhere overnight, and then exposed to the indicated concentrations of cicletanine for 2 h; cells were harvested, and cell lysates were subjected to immunoblotting to detect phospho- and total eNOS. In the graph shown on bottom, bands corresponding to phospho-eNOS were quantified (n = 3, *P < 0.01 vs. control). B: sinusoidal endothelial cells in A were harvested, and NOS activity was measured in cell lysates; NOS activity was normalized to that of control cells and is presented graphically (n = 3, *P < 0.005 vs. control). C: conditioned medium as in A was harvested, and nitrite levels were measured as in MATERIALS AND METHODS (n = 3, *P < 0.05 vs. control). D and E: sinusoidal endothelial cells as in A were harvested, and cell lysates were immunoblotted to detect phospho- and total Akt and phospho- and total Erk1/2, respectively. Bands corresponding to phospho-Akt and phospho-Erk were quantitated, normalized, and shown in the lower graphs (D: n = 3, *P < 0.01 vs. control; E: n = 3, *P < 0.005 vs. control).
in which stellate cells were cultured on thick collagen lattices; after their activation by ET-1, they contract. Cicletanine inhibited stellate cell contraction in a dose-dependent manner (Fig. 7A), consistent with a relevant physiological effect. Next, we studied protein kinase G (PKG) activation by assaying phospho-vasodilator-stimulated phosphoprotein (VASP) in stellate cells cocultured with or without sinusoidal endothelial cells. VASP, a key downstream target of the NO/cGMP signaling pathway, is a vasodilator-stimulated phosphoprotein. We found that cicletanine did not alter VASP phosphorylation in stellate cells, but it stimulated VASP phosphorylation in stellate cells cocultured with sinusoidal endothelial cells (Fig. 7B) in a dose-

Fig. 7. Cicletanine-induced nitric oxide (NO) production in sinusoidal endothelial cells causes protein kinase G (PKG) activation and stellate cell relaxation. A: hepatic stellate cells and sinusoidal endothelial cells were isolated separately from rat livers and cocultured (each at a density of 100,000 cells/lattice) on collagen lattices for 5 days as in MATERIALS AND METHODS. Cells were then serum starved overnight, exposed to 50 or 100 nM cicletanine for 2 h, and then stimulated with endothelin-1 (ET-1, 10 nM). Collagen lattices were released from their substrata, and gel contraction was measured over time. A representative experiment with images of changes in gel area at the indicated time points is shown on left. Gel areas were measured, quantified, and depicted quantitatively in the graph on the right (n = 4, *P < 0.05 and **P < 0.001 vs. control). B: hepatic stellate cells (HSC) and sinusoidal endothelial cells (EC) were isolated, and HSC were cultured alone or cocultured (HSC+EC) as in MATERIALS AND METHODS. Cells were harvested, and cell lysates were immunoblotted to detect phospho-vasodilator-stimulated phosphoprotein (VASP) for PKG activity. HSC or HSC+EC without cicletanine served as controls. Bands corresponding to phospho-VASP were quantitated, normalized, and shown in the lower graph (n = 3, *P < 0.01 and **P < 0.001 vs. HSC+EC control). C: cells (HSC+EC) as in B were preexposed to L-NAME (1 μM) for 4 h and then exposed to cicletanine (100 nM) for an additional 1–2 h. Phospho-VASP, PKG, and β-actin were detected in cell lysates by immunoblotting, and representative images are shown.
Cicletanine increases portal pressure in vivo. To investigate whether cicletanine may have an effect on intrahepatic relaxation in vivo, we measured portal pressure following cicletanine gavage in bile duct-ligated (BDL) mice. As expected, portal pressure was significantly elevated in BDL mice compared with sham-operated mice (Fig. 8). However, cicletanine treatment significantly reduced portal pressure after BDL. Slight and nonsignificant decreases in portal pressure were observed in sham-operated mice in response to cicletanine.

DISCUSSION

We have shown that cicletanine directly stimulates eNOS in sinusoidal endothelial cells in a concentration- and dose-dependent manner; the mechanism of eNOS activation appears to be linked to an Akt/Erk pathway and regulation of stellate cell contractility via the NO/PKG signaling pathway (Fig. 9). Additionally, cicletanine stimulated eNOS in injured sinusoidal endothelial cells, suggesting that it may rescue abnormal signaling pathways.

Previous literature suggests that cicletanine leads to vasodilation by increasing intracellular Ca2+ concentrations and stimulating the formation of prostaglandins (5, 10) and cGMP phosphodiesterases in cultured vascular smooth muscle cells (28–29). Another study revealed that cicletamine enhanced eNOS coupling to BH4, leading to NO production. Cicletamine also stimulated Ca2+/K+ channel opening in cultured cells consistent with cell vasorelaxation (3, 20). Finally, cicletamine caused NO/O2 release in human umbilical vein endothelial cells (16). Here, our data extend these previous studies by indicating that the mechanism by which cicletamine acts is by posttranslational eNOS phosphorylation and activation.

Currently, posttranslational mechanisms of eNOS activation are an area of active investigation. eNOS catalytic function is influenced by phosphorylation, acylation, and protein interactions (4, 6, 11). Because phosphorylation of eNOS at Ser1177 by Akt is critical for activation of eNOS (7, 11), we investigated the effects of cicletanine in this regard; our results clearly indicate that cicletanine activates the protein kinase Akt, leading to posttranslational activation of eNOS via phosphorylation of Ser1177 (Figs. 4 and 6).

The interrelationships of eNOS and MAP kinase pathways are not entirely understood. Of note, eNOS has many putative MAP kinase phosphorylation consensus binding site sequences (2). Additionally, recent studies suggest that eNOS and Erk physically associate in the cell cytoplasm (2) and that Erk appears to promote eNOS activity (18). Therefore, we inquired as to whether cicletanine stimulates Erk and thus eNOS. We found that cicletanine robustly induced Erk activation in sinusoidal endothelial cells (Figs. 5 and 6). PD-098059, an MEK inhibitor and downstream inactivator of Erk, abolished cicletanine stimulation of Ser1177 phosphorylation and NO generation, suggesting a role for Erk. Thus, it is possible that cicletanine regulates eNOS in a dual pathway involving Akt and Erk.

The biochemical basis by which NOS functions is via the regulation of electron flux form NADPH through flavins in the reductase portion of NOS protein to the heme domain in the oxygenase domain and control the rate of NO synthesis (8, 22). We therefore examined eNOS cytochrome c reductase activity (12) and found that cicletanine is able to enhance the rate of cytochrome c reduction twofold more compared with no cicletanine treatment in both the basal level and CaM stimulation (Fig. 3), suggesting that cicletanine plays a critical role on the electron flux from the reductase domain to the oxygenase domain and contributes to increase NO generation in sinusoidal endothelial cells.

Portal hypertension is associated with deficient endothelial cell NO production, which results in increased intrahepatic resistance...
and portal pressure (24, 27). Thus, providing the liver with NO is a potentially novel therapeutic strategy. Here, we found that cicletanine, which has been shown to have effects on the pulmonary endothelium (15, 26, 30, 32), not only enhances eNOS activity in normal sinusoidal endothelial cells but also improves the reduced eNOS activity typical of injured sinusoidal endothelial cells (Fig. 6). The mechanism appears to involve enhancement of Akt-Erk-eNOS signaling in injured sinusoidal endothelial cells. Overall, our data substantially extend previous work and emphasize a mechanism involving ERK responsible for the impairment of sinusoidal endothelium-dependent NO production in injured sinusoidal endothelial cells. The data also raise the possibility that cicletanine may augment eNOS bioactivity and may be useful for treatment of portal hypertension (Fig. 9).

In summary, our data support a physiological role for the use of pharmacological agents such as cicletanine to stimulate eNOS function in sinusoidal endothelial cells, which had paracrine effects on stellate cells (Fig. 7), and additionally reduced portal pressure (Fig. 8). The data highlight the possibility that pharmacological intervention in patients with increased intrahepatic resistance and portal hypertension may be clinically feasible.

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DISCLOSURES

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Author contributions: S.L. and D.C.R. conception and design of research; S.L. performed experiments; S.L. and D.C.R. analyzed data; S.L. and D.C.R. interpreted results of experiments; S.L. and D.C.R. prepared figures; S.L. and D.C.R. drafted manuscript; S.L. and D.C.R. edited and revised manuscript; S.L. and D.C.R. approved final version of manuscript.

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