The Metabolic and Hemodynamic Effects of Oxethazaine in the Perfused Rat Liver

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ABSTRACT—Alteration of hepatic microcirculation and its effects on hepatic metabolism were examined using oxethazaine (OXZ). The infusion of OXZ into isolated perfused livers rapidly increased the portal perfusion pressure (PP) and inhibited oxygen (O2) uptake, which was followed by a decrease in tissue ATP content and an increase in lactate, pyruvate and glucose release into the perfusate. P-450-dependent reductive drug metabolism was enhanced by OXZ, whereas oxidative drug metabolism was suppressed, and this was accompanied by a decrease in substrate uptake. During OXZ infusion, a time delay between the inhibition of O2 uptake and the release of cellular and xenobiotic metabolites was observed. The actions of OXZ required Ca2+. It is unlikely that the inhibition of O2 uptake is due to the inhibition of cellular respiration. The PP increase induced by OXZ was inhibited by papaverine, but not by prazosin, sodium nitroprusside and verapamil, whereas all of these vasodilators were effective against norepinephrine. Under retrograde perfusion, the PP increase by OXZ was abolished, but norepinephrine, uridine 5'-triphosphate, angiotensin II and endothelin 1 were still effective. The extrahepatic portal vein preparation contracted at high concentrations of OXZ. The results suggest that OXZ acts differently from other known vasoconstrictors and possibly narrows hepatic sinusoids to reduce the rate of substance exchange between the sinusoids and hepatocytes, including a reduction in O2 extraction.

Keywords: Oxethazaine, Perfused rat liver, Metabolic effect, Hemodynamic effect, Hepatic microcirculation

The hepatic microcirculatory system is composed of branches of the portal vein and hepatic artery that supply blood to the liver acinus, the sinusoids where substances are exchanged between the blood and hepatocytes, and the terminal hepatic venules. Constriction of the inlet blood vessel branches and sinusoids may cause a decrease in sinusoidal flow and result in a decrease in the rate of substance exchange between hepatocytes and sinusoids, thus affecting hepatocellular function. In a perfused rat liver preparation, in which the liver is usually perfused through the portal vein alone, various substances reportedly increase portal perfusion pressure (PP), including phenylephrine (1, 2), ATP (3, 4), uridine 5'-triphosphate (UTP) (4), arachidonic acid (2), the thromboxane A2 (TXA2) analogue U-46619 (9,11-dideoxy-9a,11a-methanoepoxy prostaglandin F2α) (1, 5), leukotrienes C4 and D4 (5), prostaglandin F2α (6, 7), 12-O-tetradecanoyl phorbol 13-acetate (TPA) (8) and endothelins (9, 10). Stimulation by most of these compounds is accompanied by the inhibition of hepatic oxygen (O2) uptake and ensuing glycogenolysis. However, the mechanisms by which these substances cause constriction of the microcirculatory system are not clear.

During a search for agents that prevent peroxidative liver damage using the liver perfusion system, we found that the topical anesthetic oxethazaine (OXZ), at a concentration of 10 μM, prevented liver injury induced by t-butyl hydroperoxide and also suppressed hepatic O2 uptake. The other membrane-acting drugs, nifedipine, diltiazem, promethazine, chlorpromazine, quinidine, lidocaine, propranolol and prenylamine, had no such effects at that concentration. In addition, OXZ markedly increased PP and decreased the uptake of hydroperoxide itself.

OXZ, HOCH2CH2-N(CH2CON(CH3)C(CH3)2CH2C6H5)2, has long been prescribed clinically for esophagitis, chronic gastritis and peptic ulcers (11). Its potent local anesthetic action, even at low gastric pH, is partly
explained by its unique chemical characteristics. As a weak base, it is relatively non-ionized in acidic solutions, whereas its hydrochloride salt is fairly soluble in organic solvents and thus it can penetrate cell membranes (11, 12). OXZ also inhibits gastric acid secretion (11, 13). A toxicological study on animals showed that oral administration of the drug has a considerable margin of safety; however, toxicity following intravenous injection is high, as it depresses myocardial contractility and impairs conduction (14). Some studies on the other pharmacological actions of OXZ have been done, but its direct effects on the liver have not yet been reported.

Thus, we studied the effects of OXZ on perfused livers, especially on 

MATERIALS AND METHODS

Chemicals

OXZ was provided by Eisai Co., Ltd., Tokyo, and dissolved in an equimolar HCl solution at a concentration of 10 mM, pH 3.5. Infusion of this solution did not affect the pH of the perfusion medium at the final concentrations used in this study. Prazosin hydrochloride was provided by Taito-Pfizer Co., Ltd., Tokyo. Other reagents were of analytical grade and commercially available.

Animals

Male, SPF-grade Sprague-Dawley rats (Japan SLC, Hamamatsu) weighing between 170–190 g were used for the liver perfusion studies. For some experiments, the animals were fasted overnight (16–20 hr). Animals were sacrificed between 9:00–12:00 for metabolic studies and between 9:00–15:00 for the other studies.

Liver perfusion

The livers were isolated according to basic surgical procedures (15), except that the smaller lobes (proc. papillaris, proc. caudatus and lobus dexter) were tied and cut off (16). This procedure may be appropriate not only to conserve perfusion medium but also to minimize uneven perfusion among lobes during disturbed circulation. Thus, the portal vein was cannulated close to where it branches to the median and left lobes. The moment the portal vein was cannulated was defined as 0 min. The hepatic artery was ligated, and the bile duct was cut. Another cannula was placed through the right atrium into the inferior vena cava, with the tip of the cannula advanced to the entry of the hepatic vein. The liver was kept in a small warmed vessel containing perfusion medium and perfused in a non-recirculating, constant flow system, using a rotary pump. The flow rate was 25 ml/min/liver; this was necessary to supply sufficient O₂ to the liver. Krebs-Henseleit bicarbonate buffer (KHB: 118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃ and 5.6 mM glucose, saturated with 95% O₂–5% CO₂ at 37°C, pH 7.4) was the standard perfusion medium (95% O₂–KHB). The hypoxic medium was saturated with 95% N₂–5% CO₂ (95% N₂–KHB). The actual oxygen concentrations of the 95% O₂–KHB and 95% N₂–KHB solutions were about 0.62 and 0.08 mM, respectively. The test compounds were given using infusion pumps through an injection device placed between the rotary pump and the liver. The liver weight was measured at the end of each experiment.

PP was measured using an air-trap tube (inner diameter, 3 mm; height, 50 cm) placed between the roller pump and the drug injection device.

Retrograde perfusion was conducted by displacing the inflow and outflow connecting tubes to the venous and portal sides, respectively, while the perfusate flow was stopped, and the reflow was started within 15 sec.

Effluent monitoring and assays

The effluent temperature was continuously checked by a thermister, and it was maintained at about 35°C. The oxygen concentration in the effluent perfusate was monitored by a Clark type-oxygen electrode (K-IC oxygraph; Gilson Medical Electronics, Inc., Middleton, WI, USA) connected to the venous outlet of the perfusion system. Air-saturated water at 37°C (0.217 mM O₂) was run through the perfusion system as the routine standard. OXZ infusion through the system without the liver did not disturb the oxygen measurement.

The effluent perfusate was collected for 10-sec periods at appropriate intervals, unless otherwise noted. Lactate and pyruvate were measured by clinical assay kits (Determiner LA and PA; Kyowa Medics, Tokyo). Glucose was determined by a clinical assay kit (Glucose B-Test; Wako Pure Chemicals, Osaka). Hexobarbital, p-nitrophenol, p-aminobenzonic acid and the reduction products of the nitro group of chloramphenicol in the perfusate were determined as described by Mazel (17). p-Nitroanisole was determined by HPLC using a Shim-pack CLC-ODS column (Shimadzu, Kyoto) with 50% methanol as the eluate.

Hepatic ATP assays

The liver was instantaneously freeze-clamped using an aluminum tongue chilled in liquid nitrogen. ATP in the perchloric acid extract of the liver, after neutralization with K₂CO₃, was enzymatically assayed with hexokinase and glucose-6-phosphate dehydrogenase (18).
Measurements of respiration in isolated mitochondria and hepatocytes

In both cases, a Clark type electrode assembly unit (Gilson Medical Electronics, Inc.) was used. Liver mitochondria were isolated in 0.25 M sucrose - 0.1 mM EDTA - 20 mM Tris-HCl (pH 7.4), and O2 uptake was measured at 30°C (19); mitochondria (1.5 mg protein/ml) and 7.5 mM substrates (malate-pyruvate, glutamate or succinate) were added to the suspension medium (0.3 mM sucrose, 10 mM potassium phosphate, 10 mM Tris-HCl, 10 mM KCl, 5 mM MgCl2 and 0.2 mM EDTA, pH 7.4). Hepatocytes were isolated using the collagenase method (20), with a viability of about 90%, and O2 uptake was measured at 37°C in Hanks balanced salt solution.

Preparation of the extrahepatic portal vein

Rats weighing between 230 - 250 g were subjected to the usual liver isolation procedure. The portal vein was cannulated at a point about 1.5 cm away from where it enters the left and median lobes, while the other branches were ligated. The portal vein was dissected from the liver, suspended in a warm box and perfused at a rate of 25 ml/min with the effluent allowed to drop freely.

Statistical analyses

Data are presented as means ± S.E. For paired samples, the paired t-test was used, and for unpaired samples, the t-test or the Welch’s test was performed after analysis of variance by the F-test. P < 0.05 was considered statistically significant. In some figures, typical data are shown.

RESULTS

Some aspects of the effects of OXZ on hepatic O2 uptake and PP

As shown in the cumulative dose response profile (Fig. 1A), the infusion of 0.3 μM OXZ into perfused rat livers caused a slight and transient increase in O2 uptake. By increasing the OXZ concentration to 10 μM, O2 uptake
was rapidly inhibited to near maximum, which was slightly preceded by a rapid increase in PP. Calculated from the pooled data throughout the present study, the O₂ uptake before and after OXZ (10 μM) infusion amounted to 2.19±0.06 and 0.44±0.03 μmol O₂/min/g liver (P<0.001, n=33), respectively; thus the O₂ uptake inhibition by OXZ was about 80%. After stopping the OXZ infusion, these changes recovered gradually. As the concentration of OXZ and the exposure time increased, the recovery time was prolonged, and the sensitivity to a repeated challenge of OXZ decreased. The increase in PP was not due to swelling of the liver; the liver size may be rather decreased since the water level of the container, in which the liver was suspended, was decreased.

In the present study, a rather high flow rate was used to supply sufficient O₂ to the liver. Therefore, the effects of the flow rate was examined (Fig. 1B); OXZ was still effective under a low flow rate of 6.25 ml/min, although the response was slightly delayed.

In the absence of Ca²⁺ in the perfusate, neither the PP increase nor the inhibition of O₂ uptake occurred, and O₂ uptake was rather enhanced (Fig. 1C).

**Effects on energy metabolism**

The hepatic ATP content decreased to about one fifth of the control value within 10 min of OXZ infusion and remained at this level for at least 30 min, while in the absence of Ca²⁺, the ATP content was not significantly affected (Fig. 2).

Following OXZ infusion, the effluent lactate and pyruvate levels transiently decreased in response to the rapid change in PP and then gradually increased (Fig. 3, cen-
ter). Both of their releases rapidly increased soon by changing the perfusate to 95% N₂–KHB, i.e., under cellular hypoxia (Fig. 3, left). By cyanide infusion (cellular hyperoxia), lactate release was enhanced, but pyruvate release was lowered (Fig. 3, right) with no alteration in PP. Thus, the release profiles of OXZ resembled those of hypoxia rather than those of cyanide intoxication.

Glucose output was increased by OXZ and also delayed compared with the control experiments with norepinephrine and the TXA₂ analogue U-46619 (Fig. 4); with norepinephrine and U-46619, glucose output significantly increased 5 min after their infusion ($P < 0.05$), whereas with OXZ, glucose levels did not significantly change at 10 min and increased maximally and significantly ($P < 0.05$) at 5 min after cessation of the OXZ infusion.

With isolated mitochondria, OXZ at concentrations above 100 μM inhibited malate plus pyruvate-sustained

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**Fig. 4.** Comparison of the effects of oxethazaine (OXZ), norepinephrine (NE) and U-46619 on glucose output and perfusion pressure in perfused livers. OXZ, NE and U-46619 were infused as indicated, and effluent glucose concentrations (●) and perfusion pressure (○) were monitored. Each point represents a mean ± S.E. (n = 3–4).

**Fig. 5.** Effects of oxethazaine (OXZ) on the respiration of isolated mitochondria (Mit.) and hepatocytes. In the mitochondrial respiration experiments, malate plus pyruvate (M-P, each 7.5 mM; upper trace) or succinate (Succ, 7.5 mM; middle trace) was added as the substrate. Experimental details are described in the Materials and Methods. Typical data from 2 to 3 experiments are shown.
respiration, whereas it enhanced succinate-sustained respiration (Fig. 5, top and middle traces). O$_2$ uptake by isolated hepatocytes was unaffected by 200 µM OXZ, although it was inhibited at much higher concentrations.

**Effects on P-450-dependent drug metabolism**

This experiment was done to examine whether OXZ produces hepatocellular hypoxia or hyperoxia. First it was noticed that the oxidative metabolism and accompanying O$_2$ uptake during infusion of hexobarbital or p-nitroanisole was suppressed by OXZ. However, this effect cannot simply be explained by a cellular O$_2$ deficiency, since it was found that the uptake of these substrates by the liver was considerably lowered during OXZ infusion, and this was also Ca$^{2+}$-dependent (Table 1).

In contrast to oxidative metabolism, the P-450-dependent nitro reduction of p-nitrobenzoic acid and chloramphenicol to their respective amino compounds was markedly enhanced by OXZ infusion, like under 95% N$_2$–KHB perfusion (Fig. 6). With OXZ, we also observed a delay between the inhibition of O$_2$ uptake and the release of metabolites, whereas under hypoxia, both occurred almost simultaneously.

**Effects of vasodilators on the increased PP induced by OXZ**

In the following experiments, characteristics of the PP increase by OXZ were examined in comparison with the effects of some vasoconstrictors. The PP increase induced by norepinephrine was antagonized by prazosin, papaverine, sodium nitroprusside and verapamil, whereas the action of OXZ was antagonized only by papaverine (Fig. 7).

**Table 1. Effects of oxethazaine (OXZ) on hepatic substrate uptake during oxidative drug metabolism in isolated perfused livers**

| Substrates         | Ca$^{2+}$ | Substrate uptake (%) |
|--------------------|-----------|-----------------------|
|                    | −Oxz      | +Oxz                  |
| Hexobarbital       | +         | 38.0 ± 1.1            | 7.3 ± 2.3*             |
| Hexobarbital       | −         | 35.8 ± 2.4            | 33.0 ± 3.2             |
| p-Nitroanisole     | +         | 18.1 ± 0.9            | 3.5 ± 0.9*             |

Initially, hexobarbital or p-nitroanisole (each 0.2 mM) was infused for 3 min in the presence or absence of Ca$^{2+}$ (1.3 mM) and in the absence of OXZ, and then, with the same liver preparation, the substrates were infused again in the presence of 10 µM OXZ. The OXZ infusion was started 5 min before the substrate infusion and continued for 10 min. The substrate uptake by the liver was calculated from the recovery in the effluent perfusate which was pooled for 6 min after the start of substrate infusion. Values represent means ± S.E. (n = 4–6). *Significantly lower than the controls (P < 0.01).

**Retrograde perfusion**

As Fig. 8 shows, the PP increase induced by OXZ completely disappeared under retrograde perfusion, which was accompanied by a slight enhancement of O$_2$ uptake (data not shown). U-46619 was also ineffective under these conditions. However, the other vasoactive agents such as norepinephrine, UTP, angiotensin II and endothelin 1 were effective under both antero- and retro-

**Fig. 6. Effects of oxethazaine (OXZ) and hypoxia on the reductive metabolism of p-nitrobenzoic acid (PNBA) and chloramphenicol (CP) in perfused livers.** The livers from fasted rats were used, and glucose and MgSO$_4$ in the regular KHB were replaced with 2 mM xylitol and MgCl$_2$, respectively, to enhance P-450 dependent metabolism and to suppress the conjugation reaction of the products. Hypoxia was produced by exchanging to the above medium saturated with 95% N$_2$. PNBA or CP was infused for 10 min in the absence and presence of OXZ or under hypoxic conditions (95% N$_2$), and the effluent O$_2$ concentrations (traces) and metabolite levels (●) were monitored. Upper panels: Nitro-reduction of PNBA. The release of p-amino benzoic acid (PABA) was measured. Lower panels: Nitro-reduction of CP. The release of the CP reduction product (CP-NH$_2$) was measured. Changes in the order of the treatment did not essentially alter the oxygen and metabolite profiles of the perfusate. In both cases, typical data from 3 experiments are shown.
grade perfusion systems. In the retro-direction, the increase in PP was always accompanied by marked swelling of the liver and leakage of the perfusate.

Fig. 7. Effects of some vasodilators on the increased perfusion pressure induced by oxethazaine (OXZ) and norepinephrine (NE). Vasodilators were co-infused at the concentrations indicated (shaded bars) during the elevated perfusion pressure in the presence of NE or OXZ (open bars). Typical data from at least 2 experiments are shown.

Fig. 8. Effects of oxethazaine (OXZ) and other vasoactive compounds on the perfusion pressure in the retrograde perfusion system. The livers were initially perfused in an anterograde manner, and then the direction of the perfusion was changed to the retrograde. For retrograde infusion of ET-1, new livers were used since the action of ET-1 was long-lasting. The compounds were infused cumulatively and removed at the indicated times by the upward and downward arrows. NE, norepinephrine; UTP, uridine 5'-triphosphate; ANG, angiotensin II; ET-1, endothelin 1. Typical data from at least 2 experiments are shown.
**Effects of OXZ on the extrahepatic portal vein**

As Fig. 9 shows, OXZ contracted extrahepatic portal vein preparations at concentrations about 10 times higher than those required for the PP increase in perfused livers, whereas the dose-dependency on norepinephrine was nearly parallel in both preparations, suggesting that norepinephrine mainly acts on the portal vein to increase hepatic perfusion pressure. It is also clear that the cumulative dose-response curves of OXZ and norepinephrine differed in terms of their ED₅₀ concentrations, maximum responses and slopes in both preparations.

**DISCUSSION**

Infusion of 10 μM OXZ into isolated livers rapidly increased PP and suppressed O₂ uptake. The action of OXZ appears to be unique, since the local anesthetics procaine, lidocaine, tetracaine and dibucaine (data not shown), and some other membrane-acting drugs described in the Introduction had no such effects. The action of OXZ is probably not due to altered hepatic microcirculatory sensitivity resulting from the unphysiologically high flow rate, 25 ml/min/about 5 g liver, which we used in this study, since a decrease in the flow rate to 6.25 ml/min did not essentially alter the effect of OXZ.

OXZ is a fairly lipid-soluble compound (11, 12), and in our preliminary experiments, roughly 70% of the OXZ was taken up by the liver during 5-min infusion of 10 μM OXZ at a flow rate of 25 ml/min. Therefore, the actual dosage to which the liver is exposed would be higher than that expected from the infusing OXZ concentration, although hepatic metabolism of OXZ cannot be ignored. Pharmacodynamics of OXZ in experimental animals and humans has not been reported. It is unlikely, however, that the actions of OXZ observed in the isolated liver are operative at clinical therapeutic dosages, e.g. 15–40 mg/day, divided into 3 to 4 administrations, p.o.

Inhibition of hepatic O₂ uptake by OXZ was accompanied by impaired cellular metabolism, such as a marked decrease in the tissue ATP content and an enhanced release of glucose and glycolytic metabolites. This may be due to a decreased supply of O₂ to hepatocytes rather than the inhibition of hepatocyte respiration that causes cellular hyperoxia for the following reasons: 1) the release profiles of lactate and pyruvate by OXZ were comparable to those observed under hypoxic conditions, rather than those observed in the presence of cyanide; 2) cytochrome P-450-dependent reductive metabolism of p-nitrobenzene and chloramphenicol to their respective amines was markedly enhanced by OXZ, as it is under hypoxic conditions; and 3) inhibition of mitochondrial and hepatocellular respiration occurred only at OXZ concentrations greater than those that inhibited hepatic O₂ uptake. The actions of OXZ on mitochondrial respiration may not be simple, since OXZ rather enhanced succinate-induced respiration despite inhibiting NADH-linked respiration, which may partly explain the increase in hepatic O₂ uptake observed transiently after infusion of low concentrations of OXZ or under OXZ infusion in the absence of Cat⁺⁺.

Consistent with the above notion, OXZ may reduce the rate of substance exchange between the sinusoids and hepatocytes as suggested by: 1) a delay in the time between O₂ uptake inhibition and the enhanced release of various substances including lactate, pyruvate, glucose
and xenobiotic metabolites, in contrast to their rapid and almost simultaneous changes under hypoxic conditions, and; 2) a considerable decrease in the uptake of hexobarbital and p-nitroanisole during their oxidative metabolism. In addition, OXZ accelerated the elution rate of trypan blue, nigrosin and azoalbumin (30-sec infusion), but did not affect the elution profile of erythrocytes that are not present in the Disse space (data not shown).

A causal relationship of the increase in PP to these metabolic and exchange disturbances may be obvious from the Ca\(^{2+}\) dependency of all of these OXZ actions. Thus, in the constant flow system used in this study, OXZ may narrow hepatic sinusoids by still unknown mechanisms, e.g., contraction or swelling of sinusoidal cells, to cause a decreased sinusoidal exchange area. This may reduce the exchange of substances between the sinusoids and the Disse space, including a decrease in O\(_2\) extraction by the hepatocytes. Contraction of portal venules and sphincters cannot be ruled out, because higher concentrations of OXZ also contracted extrahepatic portal vein preparations. However, contraction of portal venules alone cannot explain the metabolic effects of OXZ in our forced flow system. Conversely, the fact that perfused livers are about one order more sensitive to OXZ than portal vein preparations favors the involvement of other mechanisms as well. In this context, the sinusoidal endothelial cell fenestrae reportedly change in size and number under the influence of chemical stimuli or pathological conditions, affecting the transport of substances between the sinusoids and hepatocytes (21, 22). It is supposed that the fenestrae would become narrower if sinusoidal cells contracted in the presence of OXZ, but no morphological evidence by means of scanning electron microscopy has been obtained. Another possibility that OXZ may alter microcirculatory flow pattern to cause partial perfusion of hepatic lobules remains to be clarified.

The PP increase caused by OXZ may have different mechanisms from known vasoconstrictors. First, it is obvious that OXZ has quite different mechanisms from norepinephrine as evidenced by the differences in their dose-response curves and responses to vaso dilators. Insensitivity of the OXZ-induced PP increase to the Ca\(^{2+}\) antagonist verapamil and diltiazem implies that the Ca\(^{2+}\) channel may not be a principal site of OXZ action; unfortunately, nifedipine could not be used because of its too low solubility and separating out in the KHB. Antagonism by papaverine suggests that a smooth muscle type of cells are involved in the OXZ action, but the portal vein may not be a principal site of the OXZ action since nitroprusside did not antagonize OXZ. Second, in retrograde perfusion, OXZ did not increase PP, whereas norepinephrine, UTP, angiotensin II and endothelin 1 did with swelling of the liver. This suggests that the latter vasoconstrictors may increase PP primarily by acting on portal vein branches. Like OXZ, U-46619 was ineffective during retrograde perfusion, but OXZ was not antagonized by the TXA\(_2\) antagonist SQ-29548. From the directional nature of the OXZ action, we speculate that some mediators could be released at portal vein branches and cause sinusoidal alteration.

The Ca\(^{2+}\) requirement is a distinctive feature of the action of OXZ in both its metabolic and hemodynamic effects. Ca\(^{2+}\) may be required not only for contraction of the portal vein but also for contractile mechanisms of sinusoidal cells. The sinusoidal endothelial cells contain contractile actin microfilaments (23). It is also reported that the sinusoidal lipocytes (Ito cells) develop contractility after a few days of culture, and the contraction is stimulated by various vasoactive substances such as endothelin 1 and antagonized by nitrooxide producing compounds and Ca\(^{2+}\) antagonists (24–27). However, normal lipocytes are not contractile, and therefore a role for lipocytes in organ contraction or vasoconstriction is thought to be confined to injured, not normal liver (28). Thus, sinusoidal contractile mechanisms are not yet fully elucidated.

Although the mechanisms of the OXZ action largely remain unknown, OXZ may be a new type of hepatic microcirculatory constrictor that can be useful for clarifying the regulatory mechanisms of the hepatic microcirculation.

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