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Intrahepatic flow disturbance by clomipramine in the isolated perfused rat liver

Yasusuke Masuda*, Tomoko Edo, Sakiko Suzuki, Makoto Kawashima, Masaki Sato, Asami Hirota, Akira Kimura

Division of Toxicology, Niigata College of Pharmacy, 5-13-2, Kamishin'ei-cho, Niigata 950-2081, Japan

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Abstract

Intrahepatic flow disturbance may have important pharmacological and toxicological significance. However, apart from pathological conditions, flow disturbance by drugs has received little attention. In the isolated perfused rat liver (IPRL), we found that infusion of tricyclic antidepressants increased portal perfusion pressure (PP) with a coincidental decrease in oxygen uptake at concentrations of 3–30 μM; the order of potency was clomipramine > amitriptyline > imipramine ≈ nortriptyline ≫ desipramine. The characteristics of clomipramine action were as follows: (1) The extrahepatic portal vein was much less sensitive to clomipramine than the IPRL. (2) Changes in PP and oxygen uptake required Ca^{2+} in the perfusate and were inhibited by papaverine, staurosporine, sodium nitroprusside and indomethacin. (3) Compared with endothelin-1, clomipramine produced a greater decrease in the ratio of oxygen uptake/PP together with a greater overflow of loaded indocyanine green for an increase in PP by about 2 cm H_2O. (4) Vital staining with trypan blue and rhodamine 6G and histological examinations revealed that 3–10 μM clomipramine produced marked flow redistribution due to the constriction of portal vein branches. Portal flow was short-circuited to the hepatic vein at a deeper and hilar portion of the liver, with minimal perfusion of the periphery. These results demonstrate an example of drug-induced intrahepatic flow disturbance in vitro. Its contribution to the development of toxicity in vivo remains to be studied.

Keywords: Isolated perfused rat liver; Clomipramine; Tricyclic antidepressants; Intrahepatic flow redistribution; Portal vein branch constriction; Portal perfusion pressure

1. Introduction

The liver is supplied by blood from the portal vein and hepatic artery, especially the former. These vessels ramify and continue into sinusoids which perfuse the liver acinus, and the blood finally drains into the central vein and on to the large hepatic vein. This intrahepatic microcirculation is of primary importance for metabolic functions of the liver that are under nervous and hormonal control, and is altered by various pathological conditions [1–4].

Relatively, little is known about intrahepatic flow disturbance induced by drugs and its relation to toxicity [3,4]. For example, stimulation of the hepatic nerve increases the portal perfusion pressure (PP) due to contraction of the portal vein and produces intrahepatic flow disturbance [5–8]. Stachlewitz et al. [9] proposed that tacrine, a cholinesterase inhibitor used to treat dementia, causes ischemia-reperfusion liver injury through the activation of the hepatic sympathetic nerve via enhanced splanchnic ganglion transmission. Akerboom et al. [10], based on a study of perfused rat liver, suggested that the changes in hepatic microcirculation are responsible for cholestasis.

We previously found that in the IPRL, not only vasoconstrictors such as ET-1, angiotensin II and the thromboxan A_2 analogue U-46619, but also oxethazaine, a topical anesthetic, produce an increase in the PP accompanied by marked redistribution of the intrahepatic flow from the peripheral to the central portion of the liver [11]. Such flow redistribution, if it occurs in vivo, may have important toxicological and pharmacological significance: mal-perfused hepatocytes may suffer from oxygen

Abbreviations: ET-1, endothelin-1; ICG, indocyanine green; IPRL, isolated perfused rat liver; KHB, Krebs–Henseleit bicarbonate buffer; PP, portal perfusion pressure; R-6G, rhodamine 6G; TCAs, tricyclic antidepressans

* Corresponding author. Tel.: +81 25 269 3170x205; fax: +81 25 268 1230.
E-mail address: masuda@niigata-pharm.ac.jp (Y. Masuda).

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deficiency and a lack of nutritional supply. In addition, well-perfused portions may be exposed to greater amounts of drugs, while drugs absorbed through the gastrointestinal tract may escape the hepatic first-pass effect.

To search for drugs that can induce such hepatic flow redistribution, we tested some tricyclic antipsychotic drugs and related drugs in the IPRL. Chlorpromazine has long been known to be associated with liver injury [12,13]. TCAs have structural similarity to chlorpromazine and some of them are reported to be also potentially cholestatic [12,13]. However, the mechanism of hepatotoxicity by these tricyclic drugs remains uncertain. Apart from hepatotoxicity, TCAs have also serious cardiovascular toxicity [14,15], which may be exacerbated under disturbances of intrahepatic flow.

Among the drugs tested, some tricyclic drugs caused an increase in PP accompanied by a coincidental decrease in oxygen uptake. The most potent drug among them, clomipramine, was selected as a representative to characterize their action on hepatic microcirculation.

2. Materials and methods

2.1. Chemicals

The following chemicals were obtained commercially: clomipramine, imipramine, desipramine, chlorpromazine, clozapine, staurosporine, and R-6G (Sigma Chemical Co.); amitriptyrine, promethazine, papaverine, sodium nitroprusside and indomethacin (Wako Pure Chemical Ind., Ltd.); nortriptyline and ICG (ICN Biochemicals Inc.); paroxetine and sertraline (Tronto Research Chemicals Inc.); ET-1 (Peptide Institute Inc.). They were usually dissolved in 0.9% saline, but staurosporine was dissolved in dimethyl sulfoxide and infused at a rate that had no vehicle effect.

2.2. Animals

Male SPF-grade Sprague–Dawley rats were purchased from Japan SLC and housed in an air-conditioned animal room (temperature, 23 ± 1 °C, humidity, 50–60%) supplied with fresh air as well as food and water ad libitum. Well-nourished animals with a body weight of between 220 and 240 g were used throughout the liver perfusion experiments. For extrahepatic portal vein preparations, animals weighing 350 g were used. All animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

2.3. Liver perfusion

Livers were isolated under pentobarbital anesthesia and perfused as previously described [8,11]. To minimize possible uneven perfusion among liver lobes during disturbed circulation, the median and left lobes were perfused while the other smaller lobes were tied and cut-off. The portal vein and inferior vena cava were cannulated close to the liver, with the hepatic artery and branches of the portal vein being ligated and the bile duct being cut. The liver was suspended in a warmed vessel (37 °C) containing perfusion medium and perfused in a non-recirculating constant flow (20 mL/min) system using a rotary pump. KHB buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 5.6 mM D-glucose saturated with 95%O_2–5%CO_2 at 37 °C, pH 7.4) was used as the perfusion medium. The drug infusion device and the point of the outlet cannula were set at the level of the liver hilum. The PP was monitored at the drug infusion device insertion point using a pressure transducer (Safti-kit, DSK-101; Kawasumi Laboratories, Inc.). The oxygen concentration in the effluent perfusate was recorded using a pO_2 monitor (Type PO_2-100; Inter Medical Co. Ltd.) with and without attaching the liver. For the calibration of oxygen concentration, air-saturated saline at 37 °C (150 mmHg) was run through the system. Experiments were started 40 min after portal cannulation, and the liver weight was measured at the end of the experiment.

2.4. Portal vein perfusion

Animals were first subjected to the usual liver isolation procedure. The portal vein was cannulated 1.5 cm away from the final branch to the left and median lobes. Then, the bile duct was cut-off, the portal branches to the smaller lobes were ligated, and the portal vein was carefully peeled off to the final bifurcation. This vein segment was suspended in air in a warmed box and perfused with KHB at 20 mL/min. The preparations were equilibrated for 40 min after isolation and those that responded well to high K^+ (80 mM)-depolarization were used.

2.5. ICG leakage

ICG dissolved in distilled water was infused into the perfusion line for 20 s at 40 μM, a slightly higher concentration than the hepatic uptake threshold. The optical absorbance of the effluent at 780 nm was continuously recorded by leading the effluent to a 3 mL flow-cell. ICG overflow was calculated by fitting the sample AUC (area under the curve, concentration versus time) to the standard AUC curve prepared by infusing 0–40 μM ICG for 20 s into the line without the liver. Under these conditions, ICG overflow was slight during the basal state and unaffected by repeated infusion for at least 5 times. Drug infusion was stopped while determining ICG overflow.

2.6. Gross and microscopic observation

For gross observation of the perfusion state, trypan blue (10 μM) was infused into the perfusion line for 2 min and washed out for 1 min.

To visualize the intrahepatic flow state, R-6G, a lipophilic fluorescent probe, was infused into the perfusion line at 1.25 μM for 1 min (25 nmol/liver) [8]. Then, the liver was perfusion-fixed for 4 min with 2% glutaraldehyde solution containing 0.09 M phosphate buffer (pH 7.4) and 1% saccharose (total 430 mOsM) at 20 mL/min. In the case of clomipramine-treated liver, the perfusion-fixation was incomplete at the periphery, and therefore the liver was post-fixed in neutralized 10%-formalin until it was sectioned. The fixed livers were sectioned within the day. Vertical (100 μm thickness) and horizontal (200 μm) sections were prepared in cold 0.1 M phosphate buffer (pH 7.4) containing 10% glycerol using a microslicer (DYK-1000, Dosaka EM Co., Ltd.). The sections were mounted on glass slides and photographed under green (546 nm) excitation using a fluorescence microscope (BH2-RFCK; Olympus Optical Co., Ltd.).

Hematoxylin-eosin staining was performed according to the usual histological method. In this case, perfusion-fixed liver was post-fixed well in formalin-fixative.

2.7. Statistics

Data are presented as the means ± S.E.M. Statistical analyses were carried out using the calculating software StatView 5.0 (SAS Institute Inc.). For comparisons between two groups, Student's *t*-test was used. For comparisons among multiple groups, the Tukey–Kramer and Bonfferoni–Dunn methods were used for different and equal numbers of samples, respectively, after one- or two-way ANOVA. For comparisons of the correlations between two groups, ANCOVA was performed. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of TCAs and related drugs on PP and oxygen uptake in the IPRL

Fig. 1 shows changes in the PP and oxygen uptake following the infusion of various drugs whose concentrations were increased every 6 min. Since the responses were slow at lower concentrations, the values in the figure do not always indicate the maximum responses at those concentrations.

As shown in Fig. 1A (top), among the TCAs clomipramine increased the PP most strongly, exhibiting a marked effect at 10 μM (6 min infusion). The dechlorinated drug imipramine increased the PP at higher concentrations, whereas desipramine (*N*-demethylated imipramine) was less effective. In the other series, amitriptyline was as potent as imipramine, while nortriptyline (*N*-demethylated amitriptyline) was less effective. The increase in the PP induced by each drug was accompanied by a corresponding decrease in oxygen uptake (Fig. 1A, bottom). Thus, the order of potency for both effects was clomipramine > amitriptyline ≈ imipramine > nortriptyline ≫ desipramine. The clomipramine effect was still evident when the resting PP was elevated by 3 cm (H_2O) by raising the exit level (Fig. 1A, broken lines).

The effects of structurally and pharmacologically related drugs are shown in Fig. 1B; chlorpromazine induced a small but significant change in the PP and oxygen uptake, while promethazine, clozapine, and the selective serotonine re-uptake inhibitors paroxetine and sertraline had little or no effect.

The changes in the PP and oxygen uptake induced by each drug were correlated ($P < 0.001$) with a correlation coefficient of 0.81 ± 0.01 (for eight drugs that brought about a significant PP increase), the value for clomipramine being 0.80.

The liver did not swell despite the marked PP increase, indicating that portal venous and not hepatic venous resistance was increased.

3.2. Action of clomipramine on extrahepatic portal vein preparations

Extrahepatic portal vein preparations responded well to high K^+ (80 mM)-depolarization by increasing the perfusion pressure. However, in contrast to the IPRL, the response to clomipramine was quite small even though the concentration–response relationship was similar for both preparations (Fig. 2). This suggests that the large portal vein is not primarily involved in the PP increase in the IPRL.

In the following experiments, the action of clomipramine at concentrations of 10 μM or less was examined.

3.3. Effects of Ca^{2+} and some vasorelaxants on the action of clomipramine in the IPRL

Fig. 3A shows a time-course profile of the action of 10 μM clomipramine; the PP increased to a maximum at 10 min and was recovered to some extent by 40 min, while oxygen uptake rapidly decreased and remained suppressed. Both changes gradually recovered after terminating the clomipramine infusion. During and after infusion, the leakage of lactate dehydrogenase into the perfusate was minimal; $3.9\times 10^{-4} \pm 1.3\times 10^{-4}$% ($n = 6$) of the total liver activity leaked per min 30 min after the end of infusion, indicating that there was no apparent hepatocyte toxicity under these conditions.

Fig. 3B shows the results of Ca^{2+} (1.3 mM) infusion into the perfusion line. When Ca^{2+} was omitted during clomipramine infusion, the PP rapidly decreased and re-infusion

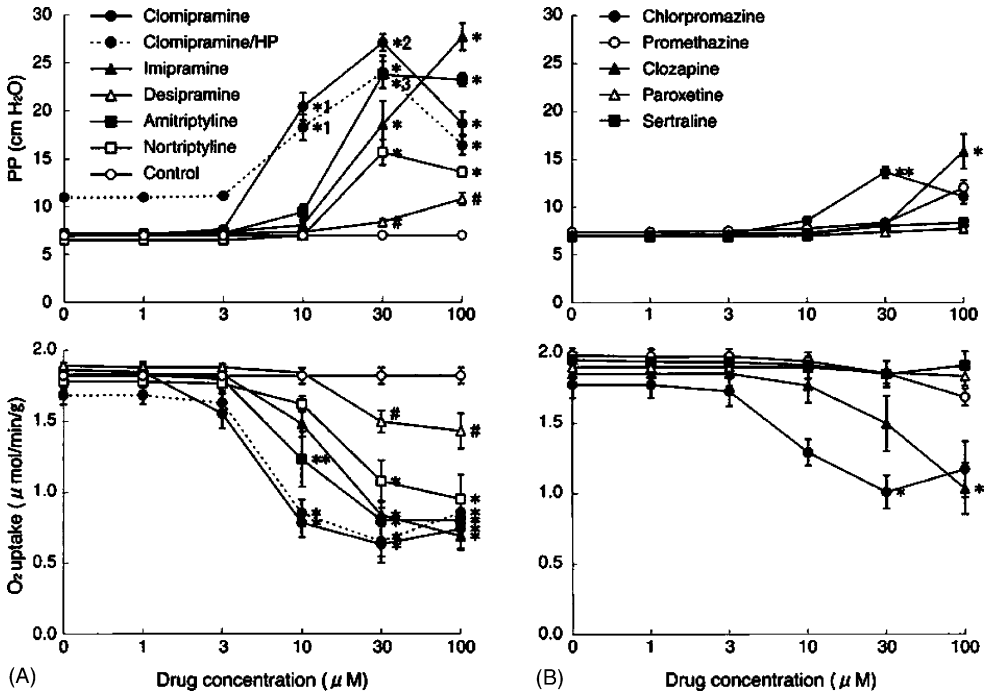

Fig. 1. The effects of tricyclic antidepressants (A) and related drugs (B) on the PP and oxygen uptake in the IPRL. Each drug (10 mM) dissolved in saline was cumulatively infused every 6 min at concentrations indicated in the figure using an infusion pump. The control received saline. Each point represents the mean ± S.E.M. ($N = 8$ for clomipramine and $N = 5$ for other drugs). *, **Significantly different from the control group at the same concentration at $^{*}P < 0.01$ and $^{**}P < 0.05$. *[1]Significantly different from all other groups. *[2]Significant difference between each other for the clomipramine, imipramine and desipramine groups at $P < 0.01$. *[3]Significantly different from the nortriptyline group at $P < 0.01$. #Significantly different from the 0 μM-value at $P < 0.01$.

of Ca^{2+} again increased it. Changes in oxygen uptake occurred coincidentally. Thus, the action of clomipramine was Ca^{2+}-dependent.

Fig. 3C–F shows the effects of some smooth muscle relaxants and inhibitors. Compared to the control (Fig. 3A), the effect of clomipramine was markedly inhibited in the presence of the phosphodiesterase inhibitor papaverine (50 μM), and the removal of papaverine reversed this effect (Fig. 3C). The protein kinase C inhibitor staurosporine (100 nM), and less potently the NO donor sodium nitroprusside (100 μM) were also suppressive (Fig. 3D and E). The cyclooxygenase inhibitor indomethacin (100 μM) suppressed the action of clomipramine (Fig. 3F); however the TXA_2 agonist SQ-29548 was ineffective at a concentration that inhibited any increase in PP by U-46619, a TXA_2 analogue. The Ca^{2+} cannel blocker nifedipine (~10 μM) and the selective α_1 adrenoceptor blocker prazosin (~1 μM) exerted no suppressive effect (data not shown).

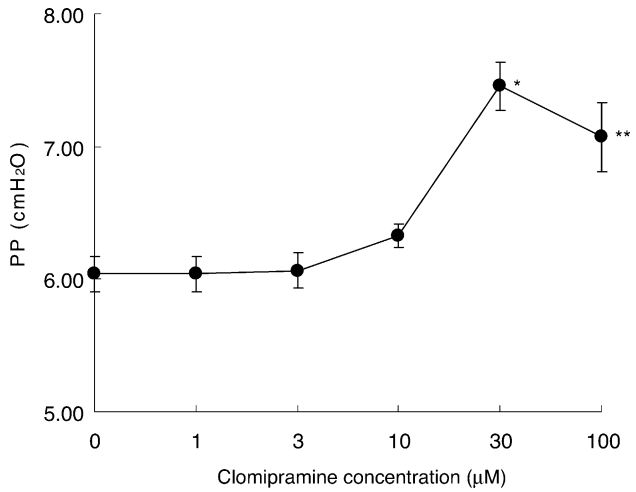

Fig. 2. The effects of clomipramine on perfusion pressure in an isolated perfused portal vein preparation. In the preparations used, high K^+ (80 mM)-depolarization increased the PP from 6.1 ± 0.1 to 21.6 ± 1.7 (cm H_2O). Each point represents the mean ± S.E.M. ($N = 6$). *, ** Significantly different from the 0 μM-value at $^{*}P < 0.001$ and $^{**}P < 0.01$.

As shown by the data in Fig. 3, the rate of oxygen uptake decrease was always greater than that of the PP increase, which is most evident in the transient changes observed in the presence of papaverine (Fig. 3C). This point was therefore further examined.

3.4. Comparison with ET-1

To compare the effects of ET-1 and clomipramine, increasing concentrations of clomipramine (0–20 μM) or ET-1 (0–4 nM) were cumulatively infused, and oxygen uptake was plotted against PP for each concentration (Fig. 4). Since ET-1-treated livers shrunk, oxygen uptake in this group was re-calculated by normalizing the liver weight to that of the clomipramine group (ET-1/norm). On

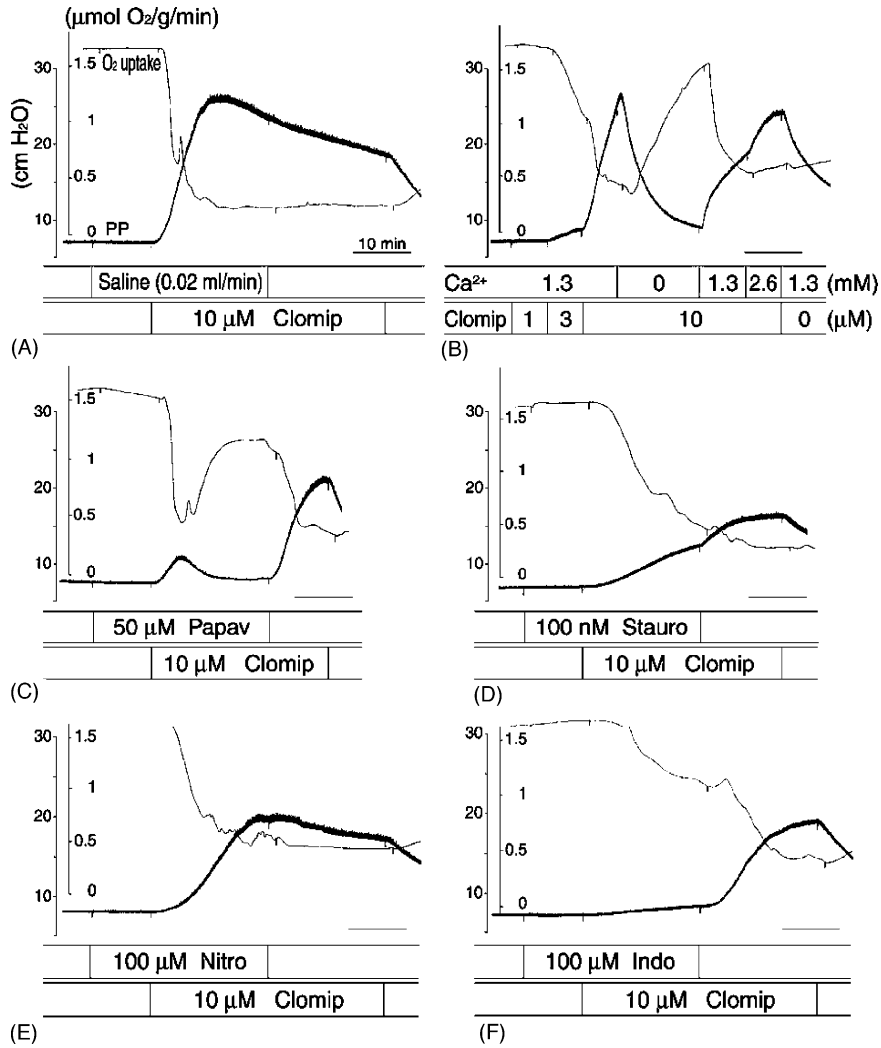

Fig. 3. The effects of Ca^{2+} and smooth muscle relaxants on clomipramine-induced changes in the PP and oxygen uptake in the IPRL. (A) Control experiment with clomipramine. (B) Ca^{2+} effect. Ca^{2+} was infused into a Ca^{2+}-free KHB perfusion line as indicated. (C–F): Pretreatment with papaverine (C), staurosporine (D), sodium nitroprusside (E) and indomethacin (F). Typical drawings for a series of at least six experiments for each drug are shown.

the basis of PP, clomipramine decreased the rate of the oxygen uptake to a greater extent than ET-1, even though the maximum PP increase was greater with ET-1. Analysis of both regression lines by ANCOVA confirmed a significant difference between the two ($P < 0.001$) irrespective of the liver weight normalization.

3.5. ICG overflow

As shown in Table 1, when ICG (40 μM for 20 s) was infused into the non-treated liver, its overflow into the effluent perfusate was small (about 3% at the basal level). When PP was elevated to the extent of 2 cm H_2O by 5 μM clomipramine or 0.25 nM ET-1, the ICG overflow induced by clomipramine (50%) was significantly greater than that induced by ET-1 (16%), corresponding to the degree of decrease in oxygen uptake. A similar tendency was observed at high PP levels under increased drug concentration. Thus, flow was impaired to a greater extent by clomipramine than ET-1.

3.6. Gross and microscopic observations

Fig. 5A shows the gross appearance of the liver infused with trypan blue. Compared with the control (a), clomipramine (10 μM) caused demarcated and uneven staining; darker staining occurred at the center of the concave side of the liver rich in large vessels (c), whereas little or no staining was observed at the periphery or surface of the convex side (b). Details of the flow disturbance were next examined by vital staining with R-6G (Fig. 5B). In the control liver, R-6G fluorescence was almost evenly distributed throughout the liver section (a), whereas clomipramine caused broader and stronger fluorescence around large vessels near the hilar portion with weak or no fluorescence at the periphery. These changes in the staining pattern already occurred at a PP increase of 2 cm H_2O induced by 3–5 μM clomipramine (b) and became demarcated at 10 μM (c). At higher magnification, the amount of R-6G used in the present study was seen to stain periportal areas preferentially in the control livers (Fig. 6A).

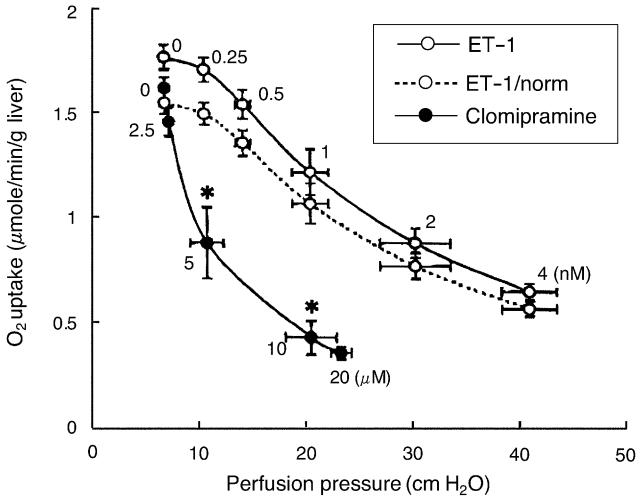

Fig. 4. Correlations between changes in the PP and oxygen uptake in the clomipramine- and ET-1-treated IPRL. Clomipramine (0–20 μM) and ET-1 (0–4 nM) were cumulatively infused every 8 min. Oxygen uptake vs. the PP were plotted for each concentration. Since the liver weight of the ET-1-treated group (5.67 ± 0.17 g) was less than that of the clomipramine-treated group (6.47 ± 0.15 g) at $P < 0.01$ because of shrinkage in the former group, oxygen uptake for the ET-1 group was normalized to that of the clomipramine-treated group on the basis of liver weight (ET-1/norm). Analysis of the oxygen uptake/PP regression lines of both groups by ANCOVA indicated a significant difference between the groups at $P < 0.001$ irrespective of the normalization. The points represent the means ± S.E.M. ($n = 6$). *Oxygen uptake was significantly lower for the clomipramine group (at 5 and 10 μM) than for the ET-1 group (at 0.25 and 1 nM) at $P < 0.001$, with no significant difference in the PP between the groups at the respective concentrations.

However, the staining area spread to pericentral areas upon clomipramine treatment (Fig. 6B, vertical section), and was remarkable around large portal veins near the hilar portion of the liver (Fig. 6C, horizontal section). These observations suggest that the flow was short-circuited around large portal veins. In accordance with this, the periphery of the clomipramine-treated liver, about one-third of the liver mass, was not fixed well even after a prolonged perfusion-fixation period.

Histology of the liver revealed that marked constriction of portal vein branches was induced by clomipramine treatment, as seen by a thickened portal vein wall, projecting endothelial cells, and hepatic cords drawn toward the constricted portal vein with narrowed sinusoids in this area (arrowheads, also note the increased nuclear density) (Fig. 6D–F). Portal vein branches of various sizes were constricted to variable degrees throughout the liver section. These changes were evident at a PP increase of 2 cm H_2O, and the number of constricted areas increased for high PPs.

4. Discussion

In the present study using the IPRL, the TCA clomipramine increased the PP at concentrations between 3–10 μM, which was accompanied by a decrease in oxygen uptake and marked flow redistribution.

The decrease in oxygen uptake induced by clomipramine may be largely due to intrahepatic flow disturbance, as seen by increased ICG overflow and a markedly uneven R-6G staining pattern, rather than to the inhibition of hepatocyte oxygen uptake. The good correlation between the PP increase and simultaneous decrease in oxygen uptake induced by various drugs (Fig. 1) and under different conditions (Fig. 3) also supports this notion.

The rate of decrease in oxygen uptake was much faster than that of increase in PP. This, in accordance with the R-6G distribution, indicates that the flow obstruction starts from the periphery (greater portion) of the liver mass, where constriction of portal vein branches was conspicuous, and then proceeds to deeper restricted portions where large portal veins run. Since our IPRL model is a constant flow system, the large portal vein may continue to respond, keeping balance with the increased PP with a smaller

Table 1
Effects of clomipramine and ET-1 on ICG overflow in relation to changes in portal pressure and oxygen uptake in the IPRL

	PP (cm H_2O)	Oxygen uptake (μmol/g liver/min)	ICG overflow (% recovery in effluent)
At basal PP			
Clomipramine	6.9 ± 0.2	1.66 ± 0.03*	2.5 ± 0.5
ET-1	6.7 ± 0.2	2.04 ± 0.06	2.6 ± 0.4
		(1.68 ± 0.06)	
At low PP increase			
Clomipramine	8.8 ± 0.2	0.96 ± 0.03*, (*)	49.9 ± 5.5*
ET-1	8.6 ± 0.2	1.76 ± 0.05	15.6 ± 3.3
		(1.45 ± 0.05)	
At high PP increase			
Clomipramine	24.0 ± 0.9*	0.60 ± 0.04*, (**)	113.0 ± 3.3*
ET-1	28.4 ± 1.1	1.07 ± 0.06	88.1 ± 3.8
		(0.88 ± 0.06)	

ICG overflow was examined at three points in one liver; first at a basal PP level before the infusion of drugs, at a low PP increase after the infusion of clomipramine (5 μM) or ET-1 (0.25 nM), and at high PP increase after the infusion of clomipramine (10 μM) or ET-1 (1 nM). ICG overflow was determined as described in Section 2. The values are the means ± S.E.M. ($n = 8$), and the values in parentheses show oxygen uptake for the ET-1 group normalized on the basis of liver weight (ET-1 group (5.2 ± 0.14) and for the clomipramine group (6.3 ± 0.11). *, **Significantly different from the ET-1 group at $^*P < 0.001$ or $^{**}P < 0.01$.

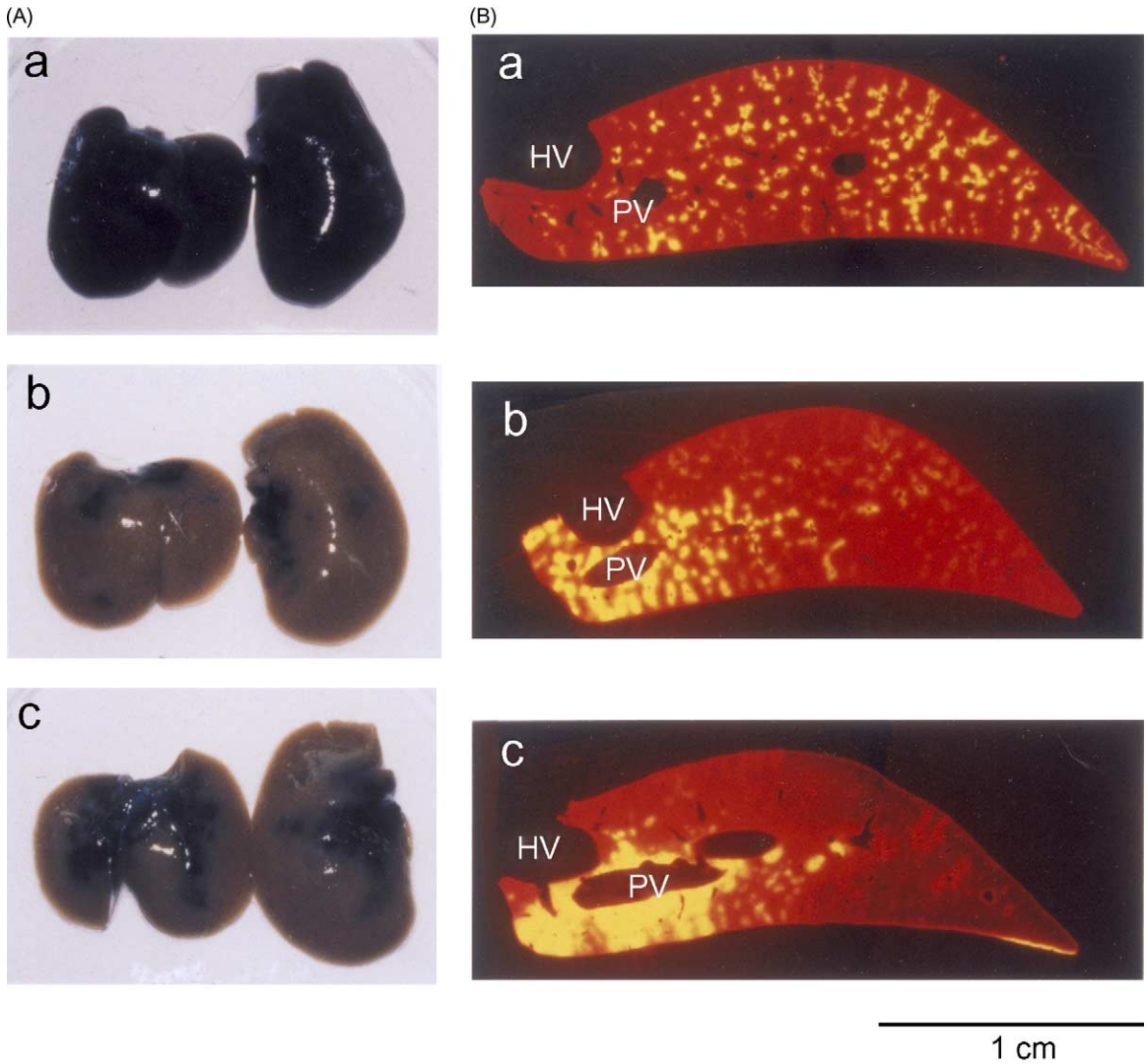

Fig. 5. Gross and microscopic observation of the perfusion state after clomipramine treatment. (A) Trypan blue infusion. (a) control liver, convex surface. (b) 10 μM clomipramine, convex side. (c) 10 μM clomipramine, concave side. The examples are from two livers per group. (B) Vitally staining with R-6G. Vertical sections of the liver near the entrance of the portal vein. (a) control. (b) and (c); 3 and 10 μM clomipramine, respectively. The examples are from at least three livers per group. PV: portal vein. HV: hepatic vein.

change in oxygen consumption. Inversely, under continued infusion of clomipramine (Fig. 3A), a gradual decrease in PP may occur in these larger portal veins, probably due to desensitization, while the peripheral branches continued to be constricted without much accompanying effect on the inhibition of oxygen uptake in the whole liver. Undetermined functional changes of the hepatocytes due to a longer exposure to clomipramine or prolonged oxygen deficiency cannot be ruled out, even though the leakage of lactate dehydrogenase was minimal.

Although not confirmed by vital staining, the TCAs amitriptyline, imipramine and nortripyline may exert similar effects on hepatic flow. Their ability to increase PP may be due to the tricyclic chemical structure and lipid solubility rather than their pharmacological properties, because (1) non-tricyclic selective serotonin re-uptake inhibitors, paroxetine and sertraline [15], unlike clomipramine had no effect, (2) the benzodizepine derivative clozapine containing a chlorine atom showed only a slight effect, (3) the PP increase by chlorinated (clomipramine and chlorpromazine) and *N*-dimethylated (imipramine and amitriptyline) drugs was greater than that by non-chlorinated (imipramine and promethazine) and monomethylated (desipramine and nortriptyline) drugs, respectively, (4) the selective and potent norepinephrine re-uptake inhibitor desipramine [15] showed only a weak effect, and (5) Ki values (nM order) for inhibition of norepinephrine or serotonin re-uptake are much lower than the concentrations used in the present study, and higher concentrations of TCAs usually block α_1 adrenoceptor [15]. Thus, the PP increase induced by TCAs may be independent of their usual pharmacological actions.

The PP increase induced by clomipramine was primarily due to contraction of intrahepatic portal vein branches (Fig. 6E and F), which required Ca^{2+} and was inhibited by smooth muscle relaxants (Fig. 3). The Ca^{2+} requirement

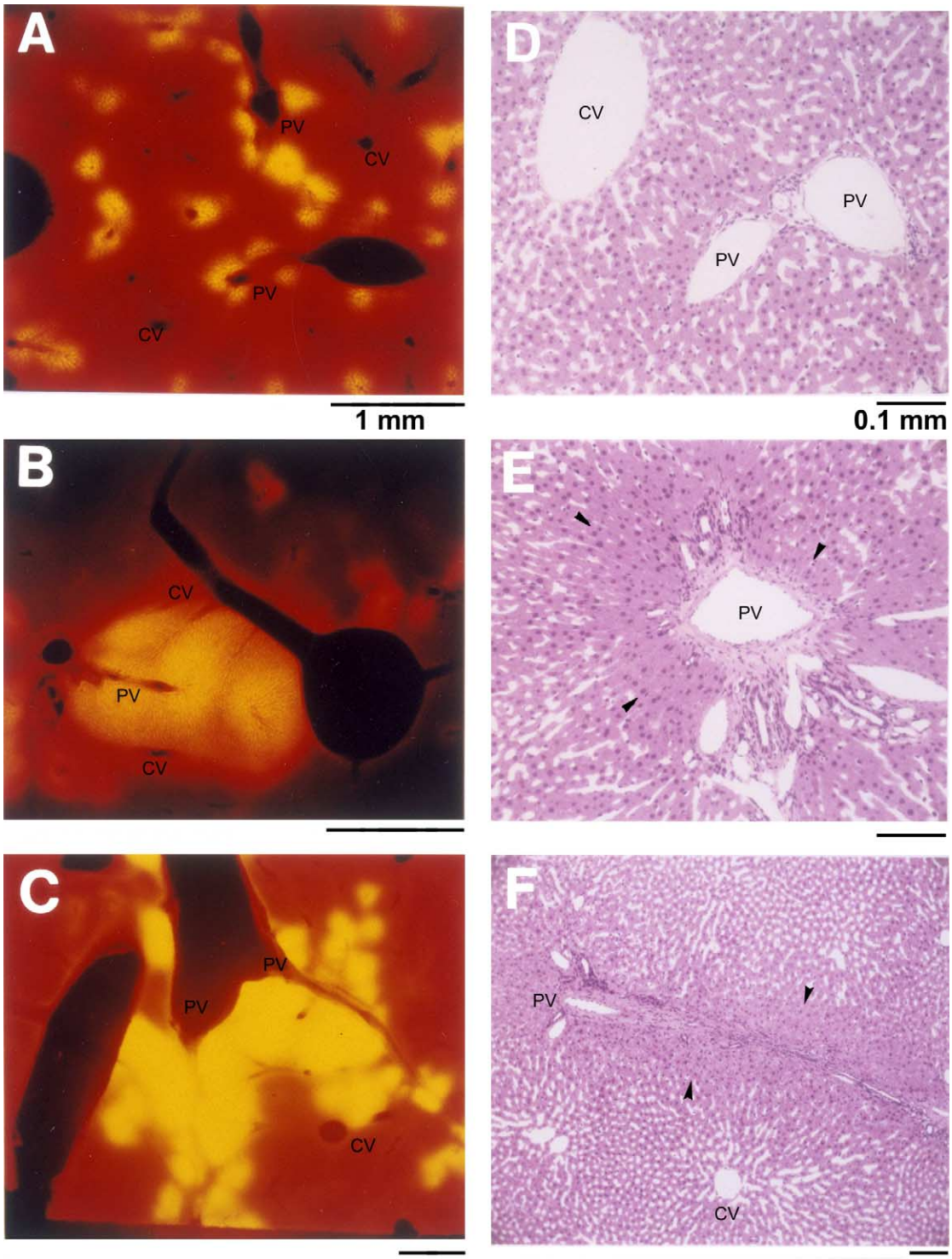

Fig. 6. Magnified observation of the flow state and histology of the liver after clomipramine treatment. (A–C) Vial staining with R-6G. Examples from at least three livers per group are shown. (D–F) Hematoxyline-eosin staining of a fixed liver. Examples from two livers per group are shown. (A and D): Control group, vertical sections. (B and E): Clomipramine (10 μM) group, vertical sections. (C and F): Clomipramine (10 μM) group, horizontal sections. PV: portal vein. CV: central vein. The arrow head indicates a contracted portion of the portal vein.

may be of primary importance for the PP increase, in agreement with the study of Akerboom et al. [16] on the calcium ionophore A23187 and our previous studies with OXZ [11,17]. Furthermore, in the rat mesenteric-portal vein, spontaneous or agonist-induced contractions are much more dependent on external calcium than those in the rat aorta and the sarcoplasmic reticulum plays little role as a source of calcium in normal contraction [18].

Papaverine, a non-specific phosphodiesterase inhibitor [19], produces a marked increase in intracellular cyclic AMP concentration in the mesenteric-portal vein [18]. Smooth muscle relaxation induced by cyclic AMP is

proposed to involve activation of protein kinase A and phosphorylation of effector proteins including myosin light chain kinase and ion channels that regulate cytosolic Ca^{2+} concentration [20]. Activation of protein kinase G by cyclic AMP has also been suggested as an important pathway in smooth muscle relaxation [20]. Sodium nitroprusside, an NO donor, increases the cyclic GMP concentration in smooth muscle cells (which activates guanylate cyclase) and produces similar functional changes in smooth muscle cells to those caused by cyclic AMP, and NO production by the endothelium is important in the regulation of vascular tension [21,22]. Smooth muscle relaxation by staurosporine, a protein kinase C inhibitor [23], is proposed to be brought about through the inhibition of phosphorylation of the regulatory protein caldesmon [24,25]. It also inhibits bradykinin-induced portal vein contraction [26]. Pharmacological interventions by these different types of smooth muscle relaxants confirm that the PP increase by clomipramine involves contraction of vascular smooth muscle cells and probably not a direct action on sinusoids. However, we cannot yet define the mechanism of clomipramine; considering the requirement for Ca^{2+}, an increased influx of extracellular Ca^{2+} could be a candidate mechanism.

To examine the possible involvement of prostanoids, the cyclooxygenase inhibitor indomethacin was used. This drug is reported to inhibit the contractile response of rat portal vein rings to kinins [26], and considerably inhibited the PP increase by clomipramine. However, TXA_2 may not be a mediator of the clomipramine action, since SQ-29548, a TXA_2 antagonist, was not inhibitory. Non-specific vasodilatory actions may also be involved at high concentrations.

From histological observations on perfusion-fixed livers, the primary sites of contraction are portal vein branches of various sizes, while the large portal vein trunk may have less contractility, similar to the extrahepatic portal vein, which showed less of a response to clomipramine. This may be a main cause for the mal-perfusion of the liver periphery; sinusoidal flow disturbance may also be involved, since the sinusoids around the constricted portal veins became narrowed. Kaneda et al. [27] showed that ET-1 causes localized constriction in the distal segments of preterminal portal venules, which continue to terminal venules and then on to sinusoids. Not only clomipramine but ET-1 and oxethazaine also exhibited similar histological profiles of constricted portal vein branches (data not shown). Therefore, intrahepatic portal vein branches appear to be sensitive to various contractile stimuli and to be responsible for obstruction of portal flow irrespective of different mechanisms being involved. It will be important to clarify the functional as well as anatomical differences along the portal vein tree.

On the basis of PP increases (e.g., at a small increase in the PP of less than 2 cm H_2O), clomipramine decreased oxygen uptake and increased ICG overflow to a greater extent than ET-1, one of the most potent vasoconstrictors [28,29], even though the maximum PP increase was much greater with ET-1. These findings indicate that the severity of intrahepatic flow disturbance is not always reflected by the degree of the PP increase. The mechanism of ET-1 induced vasoconstriction involves binding to ET_A receptor, G-protein-coupled activation of phospholipase C-β and release of inositol 1,4,5-triphosphate, release of Ca^{2+} from the endoplasmic reticulum and activation of Ca^{2+}/calmodulin-dependent myosin light chain kinase [30]. Regulation of intrahepatic flow is complex, involving the contribution of sinusoidal cells and various modifiers [30].

Flow redistribution caused by clomipramine was confirmed by vital staining and histology. As previously reported, the alteration of hepatic flow by oxethazaine, vasoconstrictors (ET-1, etc.) or hepatic nerve stimulation can be visualized by vital staining with fluorescent dyes such as acridine orange or R-6G [8,11,31]. Trypan blue and R-6G staining of clomipramine-treated liver indicated that the flow was biased toward the deeper and hilar portions of the liver with much less perfusion in the periphery and surface. This was evident at a low PP increase of 2 cm H_2O brought about by 3–5 μM clomipramine and became more demarcated at 10 μM. In the deeper portions, R-6G staining spread from periportal to pericentral areas, indicating a short-circuited flow. This is in agreement with the increased ICG overflow produced by clomipramine. By histological examinations, the constriction of portal vein branches as well as attraction of perivascular hepatic cords to the constricted portal vein appeared to obstruct the portal venous and sinusoidal flows. The resulting pressure increase in the proximal larger portal veins may have deviated the flow to smaller and shorter branches originating from them, and thus short-circuited the flow to hepatic veins, as observed in the R-6G staining pattern. Anatomical studies indicate that small and short portal vein branches sprout from large portal veins and are rich in perihilar portions of the liver [32–35].

Finally, the contribution of the flow redistribution seen in the IPRL to in vivo toxicities is still uncertain. In anesthetized rats, the infusion of 10 μM clomipramine through the mesenteric vein increased the PP accompanied by flow redistribution (preliminary experiment). However, it remains to be determined if such flow redistribution occurs in vivo. In humans, therapeutic plasma concentrations of clomipramine and its metabolite norclomipramine are less than 1.3 and 2.5 μM, respectively, and their plasma concentrations associated with acute serious toxicity are 3.2 and 6.3 μM, respectively [36]. Portal venous concentrations after ingestion of large doses would be higher; however less than 10% of a TCA circulates as free drug, the rest being bound to plasma proteins or lipids [14]. Taking these facts into consideration, the clomipramine concentration (3–5 μM) that caused flow redistribution in the IPRL may be not far from the concentrations that would be expected after ingestion of large doses of clomipramine.

However, at present, it is difficult to relate the clomipramine concentration used in the IPRL to human toxicity.

In summary, clomipramine at micromolar concentrations induced an increase in the PP and a coincidental decrease in oxygen uptake in the IPRL, both of which resulted from the constriction of portal vein branches. Under such conditions, marked flow redistribution occurs and the influent flow short-circuits in the deeper portions of the liver without perfusing the periphery. These actions may be common to TCAs. Whether there is a causal relationship between flow redistribution and the toxicities caused by these drugs in vivo remains to be studied.

References

[1] Campra JL, Reynolds TB. The hepatic circulation. In: Arias IM, Jakoby WB, Popper H, Schachter D, Shafritz DA, editors. The liver: Biology and Pathobiology. 2nd ed. New York: Raven Press; 1988. p. 911–30.

[2] Groszmann RJ, Loureiro-Silva MR, Tsai MH. The biology of portal hypertension. In: Arias IM, Boyrt JL, Chisari FV, Fauso N, Schachter D, Shafritz DA, editors. The Liver: Biology and Pathobiology. 4th ed. Phyladelphia: Lippincott Williams and Wilkins; 2001. p. 679–97.

[3] Greenway CV, Stark RD. Hepatic vascular bed. Physiol Rev 1971; 51:23–65.

[4] Lautt WW, Macedo MP. Hepatic circulation and toxicology. Drug Metab Rev 1997;29:369–95.

[5] Daniel PM, Prichard MML. Effects of stimulation of the hepatic nerves and of adrenaline upon the circulation of the portal venous blood within the liver. J Physiol London 1951;114:539–48.

[6] Ungváry G, Varga B. Intralobar redistribution of tissue circulation in the canine liver, II Effect of the stimulation of certain peripheral nerves. Physiol Acad Sci Hung 1971;40:347–57.

[7] Ji S, Beckh K, Jungermann K. Regularion of oxygen consumption and microcirculation by α-sympathetic nerves in isolated perfused rat liver. FEBS Lett 1984;167:117–22.

[8] Masuda Y, Oguma T. Examination of intrahepatic flow distribution by vital staining with Rhodamine 6G in isolated perfused rat liver. Jpn J Pharmacol 2000;83:215–24.

[9] Stachlewitz RF, Arteel GE, Raleigh JA, Connor HD, Mason RP, Thurman RG. Development and characterization of a new model of tacrine-induced hepatotoxicity: role of the sympathetic nervous system and hypoxia-reoxygenation. J Pharmacol Exp Ther 1997; 282:1591–9.

[10] Akerboom T, Schneider I, vom Dahl S, Sies H. Cholestasis and changes of portal pressure caused by chlorpromazine in the perfused rat liver. Hepatology 1991;13:216–21.

[11] Masuda Y, Ozaki M, Oguma T. Alteration of hepatic microcirculation by oxethazaine and some vasoconstrictors in the perfused rat liver. Biochem Pharmacol 1997;53:1779–87.

[12] Selim K, Kaplowitz N. Hepatotoxicity of psychotropic drugs. Hepatology 1999;29:1347–51.

[13] Saeian K, Reddy KR. Hepatotoxicity of psychotropic drugs and drugs of abuse. In: Kaplowitz, DeLeve LD, editors. Drug-Induced Liver Disease. New York: Marcel Dekker; 2003. p. 447–69.

[14] Dawson AH. Cyclic antidepressant drugs. In: Dart RC., editor. Medical Toxicology. 3rd ed. Philadelphia: Lippincott Williams and Wilkins; 2004. p. 834–51.

[15] Baldessarini RJ. Drugs and the treatment of psychiatric disorders. Depression and anxiety disorders. In: Hardman JG, Limbird LE, editors. Goodman and Gilman's The pharmacological basis of therapeutics. 10th ed. New York: McGraw-Hill; 2001. p. 447–83.

[16] Akerboom T, Lenzen R, Schneider I, Sies H. Cholestasis and changes in the microcirculation of perfused rat liver caused by the calcium ionophore A23187 and type 1 antiarrhythmic drugs. Biochem Pharmacol 1987;36:3037–42.

[17] Masuda Y, Oguma T, Kimura A. Biphasic effects of oxethazaine, a topical anesthetic, on the intracellular Ca^{2+} concentration of PC12 cells. Biochem Pharmacol 2002;64:677–87.

[18] Sutter MC. The mesenteric-portal vein in research. Pharmacol Rev 1990;42:287–325.

[19] Bergstrand H, Kristoffersson J, Lundquist B, Schurmann A. Effects of antiallergic agents, compound 48/80, and some reference inhibitors on the activity of partially purified human lung tissue adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate phosphodiesterases. Mol Pharmacol 1976;13:38–43.

[20] Dent G. Protein kinase A-independent responses to β-adrenoceptor agonists. Br J Pharmacol 2001;133:1199–200.

[21] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109–42.

[22] Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. Nature 1994;368:850–3.

[23] Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. Biochem Biophys Res Commun 1986;135:397–402.

[24] Lee MW, Severson DL. Signal transduction in vascular smooth muscle: diacylglycerol second messengers and PKC activation. Am J Physiol 1994;267:678.

[25] Gorenne I, Su X, Moreland RS. Caldesmon phosphorylation is catalyzed by two kinases in permeabilized and intact vascular smooth muscle. J Cell Physiol 2004;198:461–9.

[26] Campos AH, Calixto JB. Mechanisms involved in the contractile responses of kinins in rat portal vein rings: mediation by B_1 and B_2 receptors. J Pharmacol Exp Ther 1994;268:902–9.

[27] Kaneda K, Ekataksin W, Sogawa M, Matsumura A, Cho A, Kawada N. Endothelin-1-induced vasoconstriction causes a significant increase in portal pressure in rat liver: Localization of constrictive effect on the distal segment of preterminal portal venules as revealed by light and electron microscopy and serial reconstruction. Hepatology 1998;27: 735–47.

[28] Gandhi CR, Stephenson K, Olson MS. Endothelin, a potent peptide agonist in the liver. J Biol Chem 1990;265:17432–5.

[29] Tran-Thi TA, Kawada N, Decker K. Regulation of endothelin-1 action on the perfused rat liver. FEBS Lett 1993;318:353–7.

[30] Rockey DC. Vascular mediators in the injured liver. Hepatology 2003;37:4–12.

[31] Masuda Y, Oguma T, Kaneko H. Intrahepatic flow disturbance as detected by in vivo acridine orange staining in endothelin-1-treated and cirrhotic rats. Jpn J Pharmacol 1998;77:315–8.

[32] Elias H, Popper H. Venous distribution in livers. Comparison in man and experimental animals and application to morphogenesis of cirrhosis. AMA Arch Pathol 1955;59:332–40.

[33] Hase T, Brim J. Observation on the microcirculatory architecture of the rat liver. Anat Rec 1966;156:157–74.

[34] Matsumoto T, Kawakami M. The unit-concept of hepatic parenchyma-a re-examination based on angioarchitectural studies. Acta Pathol Jpn 1982;32:285–314.

[35] Saxena R, Theise ND, Crawford JM. Microanatomy of human liver-Exploring the hidden interfaces. Hepatology 1999;30:1339–46.

[36] Flanagan RF. Some drug/poison concentrations and mass/amount concentration conversion factors. In: Dart RC., editor. Medical Toxicology. 3rd ed. Philadelphia: Lippincott Williams and Wilkins; 2004. p. 1796–814.