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Portal Pressure Regulation following Kupffer Cell Activation: Control of Prostaglandin Production by Heme Oxygenases

Christian J. Steib[a] Leonore Gmelin[a] Manfred Bilzer[b] Burkhard Göke[a] Alexander L. Gerbes[a]

[a]Department of Medicine II, University of Munich, Klinikum Grosshadern, Munich, and [b]MSD, Haar, Germany

Key Words
Kupffer cell · Nitric oxide · Carbon monoxide · Portal hypertension · Microcirculation

Abstract
Background: Portal pressure (PP) results from the interplay of vasoconstrictors and vasodilators. Recently, we have shown that Kupffer cell (KC) activation increases PP. ***Aims:*** The role of the vasodilating compounds nitric oxide (NO) and carbon monoxide (CO) was studied. The hypothesis of the present study was that these vasodilators counteract the PP increase following KC activation. ***Methods:*** Livers of rats weighing 180–200 g were isolated and perfused. KCs were activated by zymosan A (cell wall particles from yeast; 150 μg/ml). The effects of NO and guanylate cyclase (GC) were evaluated by the NO synthase inhibitor N^G-nitro-L-arginine methylester (L-NAME; 0.3 mM, and the GC inhibitor 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one (NS-2028, 1.0 μM); the effects of the heme oxygenase (HO) derived compound CO were evaluated by direct administration of CO or inhibition of HO by zinc protoporphyrin IX (ZnPP IX, 1.0 μM). ***Results:*** In isolated perfused rat livers, administration of L-NAME or NS-2028 further raised PP increase following KC activation. This effect could be reduced by the cGMP analogue 8-Br-cGMP. Inhibition of HO caused marked amplification of PP increase in zymosan-treated organs. CO prevented this PP increase cGMP independently. Interestingly, KC activation and simultaneous inhibition of HO augmented the production of prostaglandins D_2 and $F_2\alpha$ and of thromboxane A_2. Accordingly, indomethacin blunted the increase of PP in zymosan/ZnPP-treated livers. ***Conclusions:*** NO restricts the initial PP increase after KC activation by GC-mediated cGMP. CO from heme degradation limits the increase of PP after KC activation eicosanoid dependently, but cGMP independently.

Introduction

Liver function is dependent on intrahepatic microcirculation. It has been shown that Kupffer cell (KC) activation affects the microcirculation of the liver [1–7]. The involved mechanisms are an increased production of vasoconstrictors [8–12], enhanced responsiveness to vasoconstrictors [13–15] and an altered production of vasodilators [16–20]. The best known vasoconstrictors are prostaglandins (PGs) such as thromboxane (TX) A_2 and cysteinyl leukotrienes like leukotriene C_4 and D_4 [6, 9]; to

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Christian J. Steib, MD
Department of Medicine II, Klinikum Grosshadern
Ludwig Maximilian University
Marchioninistrasse 15, DE–81377 Munich (Germany)
E-Mail christian.steib@med.uni-muenchen.de

the relevant vasodilators account nitric oxide (NO) and carbon monoxide (CO) [21–25].

KCs produce a significant amount of *vasoconstrictors* [11], which act on the contractile cells in the liver which are hepatic stellate cells and myofibroblasts [13, 26]. Contraction mechanisms are under the control of Ca^{2+}-dependent and Ca^{2+}-independent pathways [27].

The *vasodilator* NO is generated by different nitric oxide synthases (NOS). The constitutively expressed isoform endothelial NOS (eNOS) seems to be involved in the physiological regulation of hepatic perfusion [28]. For vasodilation, NO stimulates the soluble guanylate cyclase (GC) to produce cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells [29]. CO is produced by the degradation from protoheme IX to biliverdin. This step is catalyzed by heme oxygenases (HO) which are found in the liver in two different isoforms, the inducible isoform HO-1 and the constitutive isoform HO-2. In normal livers HO-2 influences the hepatic microcirculation. This has been shown by investigations with the HO inhibitor ZnPP IX [22].

KC activation has been found to influence hepatic microcirculation in normal livers [5]. To date, the mechanisms which counteract this portal pressure (PP) increase are not well defined. Here, we investigated the effects of NO and CO following KC activation in normal livers utilizing an in vitro model with the potential to better understand the clinical situation. The restriction of PP increase following KC activation is of clinical importance in healthy livers. These mechanisms and their therapeutic intervention possibilities are responsible to limit the deleterious effects of microcirculatory disturbances in liver injury, liver failure or following liver transplantation [30–33].

Materials and Methods

Animal Studies

Animals were ethically treated according to the criteria established by the National Academy of Sciences and published by the National Institutes of Health, in addition to the legal requirements of Germany. All animal experiments were approved by the local government (Regierung von Oberbayern, Munich, Germany) and were reported to the responsible authorities.

In situ Rat Liver Perfusion Study

Male Sprague-Dawley rats (180–200 g; Charles River, Sulzfeld, Germany) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg b.w.). After incision of the abdominal wall, the portal vein was cannulated with a 14-gauge Teflon intravenous catheter, and the liver was perfused at a constant flow rate. The inferior vena cava was cannulated through the right atrium and ligated above the right renal vein. For all experiments, the livers were perfused with Krebs-Henseleit solution (pH 7.4, 37°C) in a non-recirculating manner. The perfusion buffer was gassed with a mixture of 95% O_2 and 5% CO_2 using an oxygenator [34]. The portal perfusion pressure (PPP) was monitored continuously. The bile duct was cannulated with polyethylene tubing (PE10) in order to monitor bile flow. The liver was allowed to stabilize for 25 min prior to the addition of any substances. During this initial 25-min stabilization period, liver viability was determined using two criteria: (1) the maintenance of stable perfusion pressure and (2) lactate dehydrogenase efflux rates of <10 mU/min · g of liver. Lactate dehydrogenase activity was analyzed as reported elsewhere [5]. If one of the criteria for viability was not satisfied, the experiment was discarded.

KC Activation

Control experiments using Krebs-Henseleit buffer were performed over a 100-min time period (n = 6). To activate KCs, zymosan A (cell wall particles from yeast; 150 μg/ml, during min 40–46 after initiating perfusion, n = 11) was infused into the livers as described previously [11].

Infusion of NO, GC and cGMP

Rat livers were perfused with Krebs-Henseleit buffer alone for control experiments (n = 6) and zymosan (min 40–46, 150 μg/ml, n = 11) was infused for KC activation. To inhibit NOS, N^G-nitro-L-arginine methylester (L-NAME; min 30–100, 0.3 mM, n = 5) was infused additionally. Alternatively, for inhibition of GC, 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one (NS-2028; min 30–100, 1.0 μM, n = 5) was administered. The cGMP analogue 8-Br-cGMP (min 30–100, 50 μM) was infused in combination with L-NAME (min 30–100, 0.3 mM) and zymosan A (min 40–46, 150 μg/ml, n = 5), NS-2028 (min 30–100, 1.0 μM) and zymosan A (min 40–46, 150 μg/ml, n = 5) or in combination with zymosan A (min 40–46, 150 μg/ml, n = 5) alone.

Effects of HO on CO and cGMP

To investigate mechanisms following KC activation and inhibition of HO, livers were perfused with zymosan (150 μg/ml) from 40 to 46 min together with ZnPP IX (min 30–60, 1 μM, n = 5) and in comparison ZnPP IX alone (min 30–60, 1 μM, n = 5). Additionally, CO (2.5 μM, n = 5) was infused from min 30 to 100 in order to mimic HO-dependent CO formation. CO solutions were prepared as described by Wang [35]: 250 ml NaCl 0.9% was gassed for 15 min via frit. Saturated CO solutions were infused by microinfusion pumps reaching a final concentration of 2.5 μM in the effluent perfusate [21]. The hypothesis whether CO acts via cGMP was tested by additional infusion of 8-Br-cGMP instead of CO (min 30–100, 50 μM, n = 5).

PG Production and Its Functional Role

Production of the PGD_2, $PGF_2\alpha$ and TXA_2 was quantified by their release into perfusate. PGD_2 and TXB_2, the stable degradation product of TXA_2, was measured in duplicate using radioimmunoassays (Amersham, Freiburg, Germany). $PGF_2\alpha$ was measured by an enzyme immunoassay (Assay Designs, Inc., Ann Arbor, Mich., USA). To delineate the functional role of PGs, the cyclooxygenase (COX)-1/COX-2 inhibitor indomethacin (min 30–60, 50 μM, n = 5) was infused in combination with zymosan (min 40–46, 150 μg/ml) and ZnPP IX (min 30–60, 1 μM).

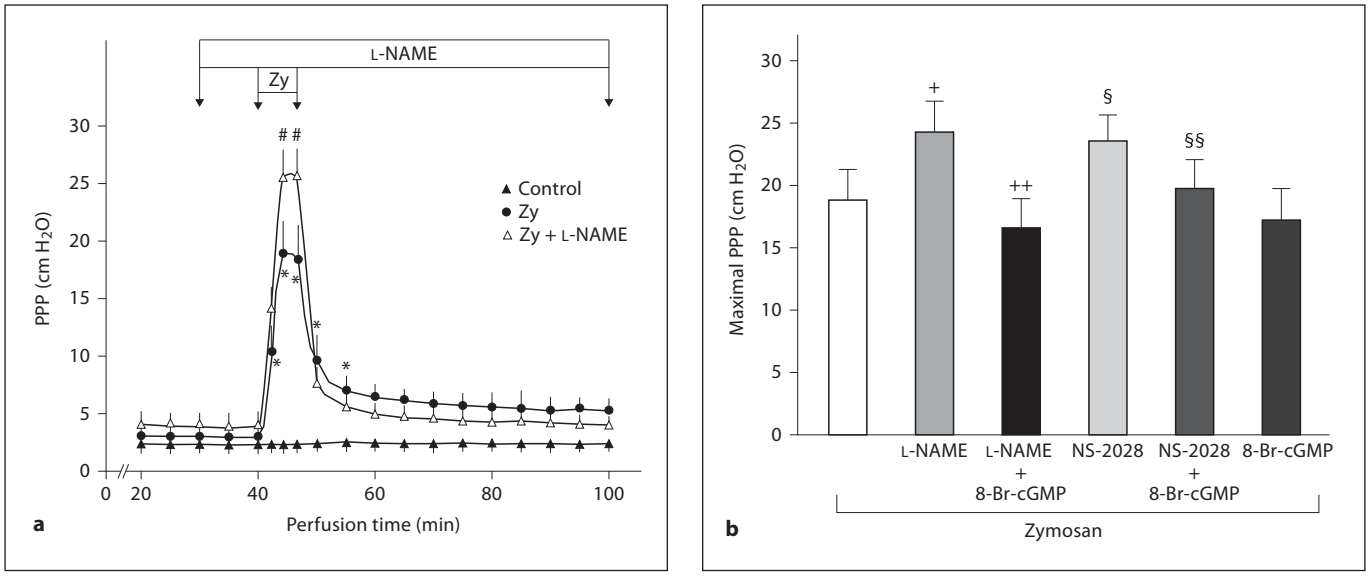

Fig. 1. Increase of PPP following KC activation and effects of NOS. **a** KCs were activated by administration of zymosan (Zy, 150 μg/ml) from min 40 to 46 (●, n = 11). Compared to control experiments (▲, n = 6) zymosan increased PPP transiently by about by 400% (* $p < 0.05$). The additional inhibition of NOS by L-NAME (0.3 mM, min 30–100) markedly enhanced this increase of PPP (△, n = 5, # $p < 0.05$). **b** The augmented increase of PPP by L-NAME (0.3 mM, min 30–100, + $p < 0.05$, n = 5) was reduced by infusion of the cGMP analogue 8-Br-cGMP (50 μM, min 30–100, ++ $p < 0.05$, n = 5). Inhibition of the GC by NS-2028 (1.0 μM, min 30–100, § $p < 0.05$, n = 5) markedly increased the PPP which was again reduced by addition of 8-Br-cGMP (50 μM, min 30–100, §§ $p < 0.05$, n = 5). Infusion of 8-Br-cGMP (50 μM, min 30–100, n = 5) did not lower the increase of PPP following zymosan infusion (150 μg/ml, min 40–46, n = 11).

Drugs and Reagents

Zymosan, indomethacin and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). NS-2028, 8-Br-cGMP and L-NAME were obtained from Calbiochem (Bad Soden, Germany). Zymosan was suspended directly in perfusion buffer. All other reagents were prepared as aqueous stock solutions (L-NAME) or DMSO stock solutions (NS-2028, 8-Br-cGMP, indomethacin). Stock solutions were infused into the portal inflow of the perfusion system by microinfusion pumps at a rate of 50 μl/min.

Statistics

All data are expressed as mean ± SD. Statistical analyses of data were performed using a Mann-Whitney U test. $p < 0.05$ was considered statistically significant.

Results

NO, GC and cGMP Pathway

The free radical NO is a well-known vasodilator. In the first part of the study the effects of NO on PP following KC activation were studied. The β-glycan-rich zymosan was infused to activate KCs. The PPP increased about 400% following zymosan infusion (fig. 1a). In control perfusion experiments the PPP did not change over 100 min (fig. 1a). Inhibition of the NOS by L-NAME increased the maximal PPP to a higher degree than the KC activation by zymosan alone (fig. 1a). The maximal PPP following zymosan and L-NAME infusion was diminished by the cGMP analogue 8-Br-cGMP (fig. 1b). The maximal PPP was also enhanced by inhibition of the soluble GC by NS-2028 (fig. 1b). Again, the infusion of 8-Br-cGMP reduced the maximal PPP following the combined administration of zymosan A and NS-2028 (fig. 1b). In contrast, infusion of 8-Br-cGMP had almost no effect on PPP following KC activation (fig. 1b).

Prolonged Increase of PPP by Inhibition of HO

The concomitant inhibition of HO by zinc protoporphyrin IX (ZnPP IX) resulted in a massive and prolonged increase of PPP compared to the PPP increase caused by the infusion of zymosan alone (fig. 2a). In contrast, the infusion of ZnPP IX alone increased the PPP only slightly (fig. 2a). The additional administration of CO following KC activation and inhibition of HO lowered the PPP during the time period from 60 to 100 min of perfusion time (fig. 2b). Interestingly, this

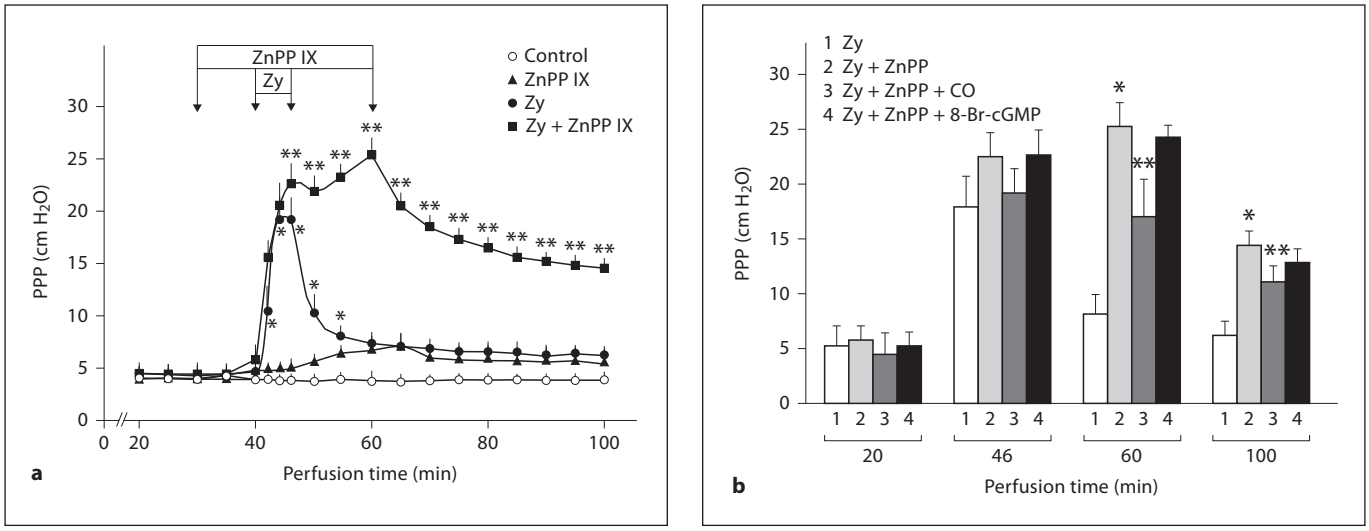

Fig. 2. Prolonged increase of PPP following KC activation and inhibition of the HO by ZnPP IX. **a** Activation of KCs by zymosan (Zy) (●, 150 μg/ml, min 40–46) increased the PPP (* $p < 0.05$, n = 11) compared to control experiments (○, n = 6). Inhibition of HO by ZnPP IX (1 μM, min 30–60) in addition to KC activation (■, zymosan 150 μg/ml, min 40–46, n = 5) increased the PPP to a higher maximum compared to the infusion of zymosan alone (** $p < 0.05$). Additionally, the PPP was elevated until end of experiments (** $p < 0.05$). The infusion of ZnPP IX (▲, 1 μM, min 30–60, n = 5) increased the PPP slightly over control values (○, n = 6). **b** The infusion of ZnPP IX (1 μM, min 30–100) in addition to KC activation by zymosan (150 μg/ml, min 40–46) ([2], n = 5) increased the PPP significantly (* $p < 0.05$) compared to the infusion of zymosan ([1], 150 μg/ml, min 40–46, n = 11). The infusion of CO ([3], 2.5 μM, min 30–100, n = 5) together with KC activation by zymosan (150 μg/ml, min 40–46, n = 5) and HO inhibition by ZnPP IX infusion (1 μM, min 30–100, n = 5) reduced significantly (** $p < 0.05$) the PPP increase compared to the simultaneous infusion of zymosan (150 μg/ml, min 40–46) and ZnPP IX (1 μM, min 30–100) ([2], n = 5). In contrast, the infusion of the cGMP analogue 8-Br-CGMP ([4], 50 μM, min 30–100, n = 5) did not lower the PPP compared to the combination of KC activation (zymosan 150 μg/ml, min 40–46) and inhibition of HO (ZnPP IX 1 μM, min 30–100) ([2], n = 5).

effect was not cGMP-dependent, because the infusion of 8-Br-cGMP instead of CO had almost no effect on the PPP (fig. 2b).

Enhanced PG Production by KC Activation and Inhibition of HO

The activation of KCs by zymosan resulted in a transient increase of the formation of PGD_2, $PGF_2\alpha$ and TXB_2, the stable degradation product of TXA_2 (fig. 3a–c). In parallel to the increase of PPP the formation of the PGD_2, $PGF_2\alpha$ and TXB_2 were enhanced by the inhibition of HO up from min 46 until the end of the experiments (fig. 3a–c).

Functional Role of the Increased PG Production

To address the functional role of the increased PG and TXB_2 efflux rates following KC activation and HO inhibition, the COX inhibitor indomethacin was additionally infused. Indomethacin, a simultaneous COX-1 and COX-2 inhibitor, reduced the profound hemodynamic effects by HO inhibition and KC activation (fig. 4).

Discussion

This study describes a novel mechanism of PP increase in normal livers by inhibition of HO. There were three principal findings of our study: (1) the inhibition of the NOS increased the PPP cGMP dependently; (2) the inhibition of HO extended the PPP increase from transient to long-lasting following KC activation, and (3) the effect of HO was cGMP-independent but based on the increased production of PGs and TXB_2.

NO and GCs

In the first part of this study we investigated the GC NO pathway and its potential role to counteract the increase of PPP following KC activation in normal livers. The inhibition of the intrahepatic NO formation by L-NAME as well as the inhibition of the GC by NS-2028 enhanced the increase of PPP following KC activation. These additional PPP increases were abolished by the cGMP analogue 8-Br-cGMP in both settings. Cells with contractile potential are mainly the hepatic stellate cells

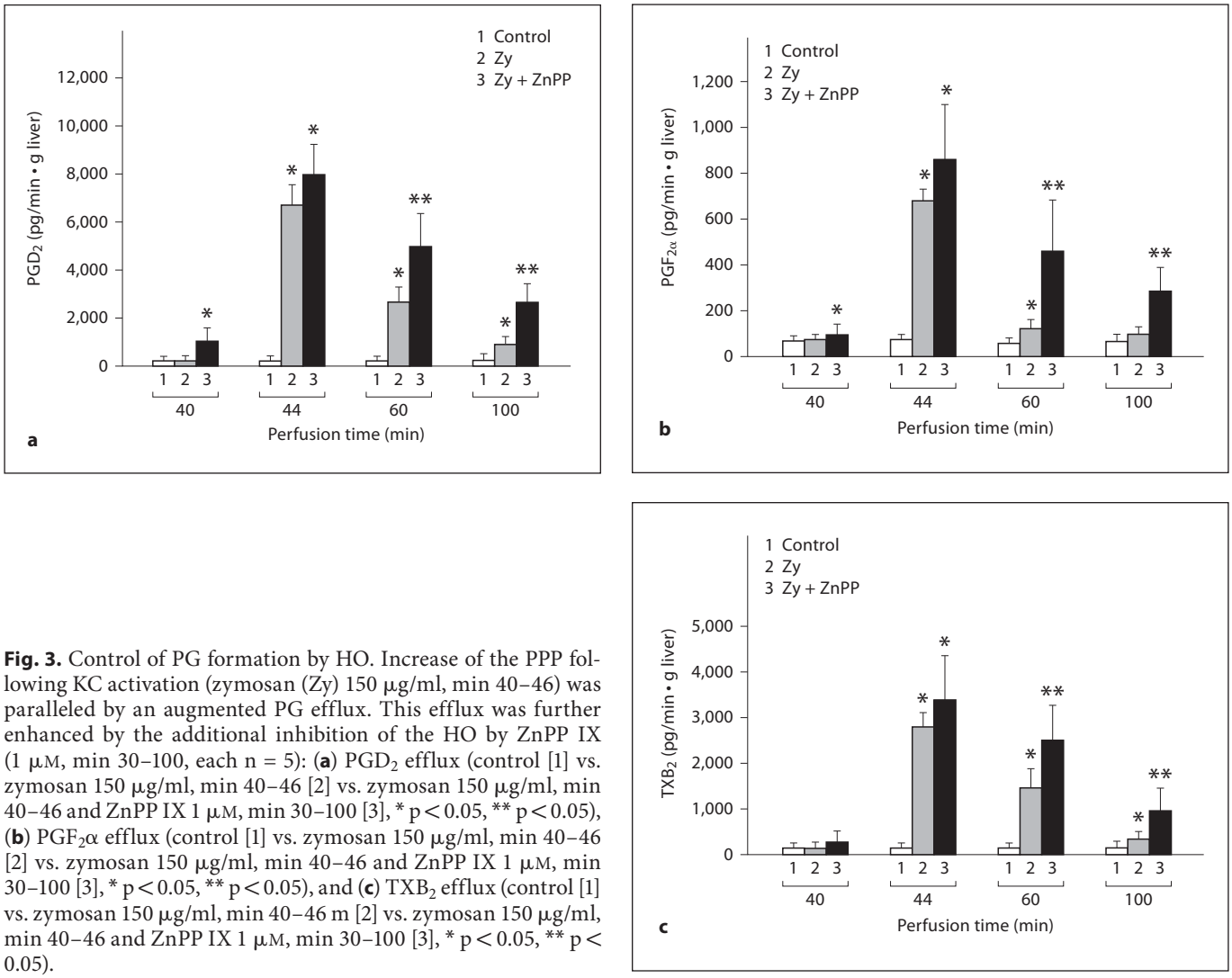

Fig. 3. Control of PG formation by HO. Increase of the PPP following KC activation (zymosan (Zy) 150 μg/ml, min 40–46) was paralleled by an augmented PG efflux. This efflux was further enhanced by the additional inhibition of the HO by ZnPP IX (1 μM, min 30–100, each n = 5): (**a**) PGD_2 efflux (control [1] vs. zymosan 150 μg/ml, min 40–46 [2] vs. zymosan 150 μg/ml, min 40–46 and ZnPP IX 1 μM, min 30–100 [3], * $p < 0.05$, ** $p < 0.05$), (**b**) $PGF_2\alpha$ efflux (control [1] vs. zymosan 150 μg/ml, min 40–46 [2] vs. zymosan 150 μg/ml, min 40–46 and ZnPP IX 1 μM, min 30–100 [3], * $p < 0.05$, ** $p < 0.05$), and (**c**) TXB_2 efflux (control [1] vs. zymosan 150 μg/ml, min 40–46 m [2] vs. zymosan 150 μg/ml, min 40–46 and ZnPP IX 1 μM, min 30–100 [3], * $p < 0.05$, ** $p < 0.05$).

and the myofibroblasts. Both cell types have been described previously to mediate contraction of the hepatic microvasculature NO dependently [18, 26, 27]. Furthermore, effects of NO on contractile cells are cGMP-mediated, which could explain the observed cGMP effects in the present study [36]. Additionally, liver sinusoidal endothelial cells have been described to play an important role for the NO pathway [28, 29, 37]; however, the interaction of all non-parenchymal cell types in sinusoidal blood flow regulation has not yet been understood in detail. Hypothetically the imbalance of vasoconstrictors and vasodilators in liver injury is not related to one of the cell types but to the interplay of all non-parenchymal cell types. It seems of major interest for future studies to compare the vasoconstrictive and vasodilative potential of each cell type.

HO and CO

In the second part of this study we investigated the HO-CO pathway. We demonstrated that the inhibition of HO in combination with KC activation increased the PPP. KCs and HO have been described to play a major role in many liver diseases like acute liver failure [38–40] and ischemia-reperfusion injury [41]. The hemodynamic effects of HO following KC activation have however not been investigated up to now. We have studied the mechanisms increasing PP in normal and fibrotic livers before following KC activation [5]. Interestingly, the transient PPP increase following KC activation was prolonged by the additional inhibition of HO. Inhibition of HO alone increased the PPP only slightly as it has been described in a previous study [21]. The PPP effects observed in the

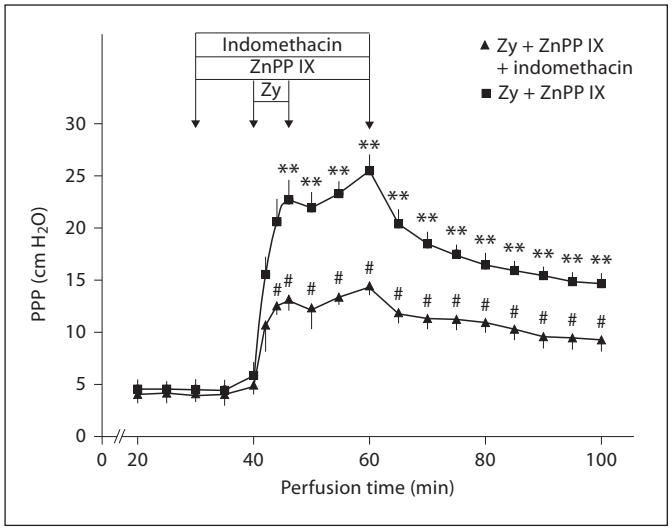

Fig. 4. Functional relevance of the enhanced amount of PGs in the effluent perfusate. The COX inhibitor indomethacin (▲, 50 μM, min 30–60, n = 5) reduced (# $p < 0.05$, ** $p < 0.05$) the increase of PPP following zymosan (Zy) infusion (150 μg/ml, min 40–46) and additional administration of ZnPP IX (■, 1 μM, min 30–100, n = 5).

present study can therefore not be the result of the simple addition of both PPP increases, resulting from zymosan infusion alone or ZnPP IX infusion alone.

In the next step we investigated the involved mechanisms. The vasodilator CO is produced by the degradation from heme to biliverdin. Indeed, infusion of CO attenuated the PPP increase by about 40%, but this effect was not dependent on cGMP. The lack of effect is not due to a low concentration, because the same concentration of 8-Br-cGMP was already used in the experiments with NO described above in the present study. Interestingly, the efflux of the PGD_2 and $PGF_2\alpha$ and the TXA_2 were enhanced by the inhibition of the HO in combination with KC activation. The functional role of the increased PG production was investigated by indomethacin, a combined COX-1 and COX-2 inhibitor. The additional infusion of indomethacin attenuated the PPP increase about 60%. Heme is an apoenzyme of COX [42]. Therefore, high concentrations of heme imply high activity of COX. Inhibition of HO therefore leads to high amounts of heme and consecutively high activity of COX. This could explain the increase of the PG efflux and the increase of the PPP following KC activation and HO inhibition.

Another relevant aspect concerning the protective effects of HO and CO could be the antioxidative property of CO [43]. Reactive oxygen species play an important role in ischemia/reperfusion injury [34] and in acute liver injury. This might on the one side activate hepatic stellate cells and on the other side impair endothelial dysfunction. Protective effects of CO in the present study might therefore additionally be related to its antioxidative property, but this was not investigated more in detail in the present study.

The HO pathway is also known to play an important role in fibrogenesis, liver fibrosis and liver cirrhosis. Recently it has been shown in Mdr2 knockout mice that induction of HO-1 was able to revert portal inflammation and fibrosis [44]. Furthermore, earlier studies demonstrated the role of HO and CO in the regulation of intrahepatic resistance in cirrhotic rat livers [24]. The role of KCs was not investigated in this earlier study but it could be hypothesized that the counteracting mechanism of HO and CO production is also of relevance in cirrhotic livers following KC activation.

Clinical Implications of This Study

This study describes novel mechanisms of counteraction of PP increase in normal livers following KC activation. Situations of KC activation in the clinical context are conceivable in each situation of an increased load in the portal vein of TLR agonists like bacterial or fungal products. Clinical examples are ischemia-reperfusion with increased amounts of pathogen-associated molecular patterns, biliary obstruction with cholangitis and sepsis. Microcirculatory disturbances are observed as shown by the present study immediately after the exposure to the TLR-2 and TLR-9 agonist zymosan. Interestingly, mechanisms of counteraction started almost simultaneously. To know the counteracting mechanisms seems of clinical relevance for potentially new therapeutic strategies. For example, induction of HO seems feasible in the near future as a therapeutic intervention.

In conclusion, our results show that in normal livers following KC activation there is a role for NO and for HO to influence PPP. We found that NO acts via GC and cGMP as a vasodilating mechanism following KC activation. In contrast, CO does not act via cGMP. The inhibition of the HO led to an increased PG and TX formation following KC activation, which led to a prolonged PPP increase. In the context of KC activation, HO are important regulators of liver hemodynamics. This knowledge can be considered for future therapeutical strategies like induction of HO.

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