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ORIGINAL ARTICLE

The effects of hepatic steatosis on thromboxane A_2 induced portal hypertension☆

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Abstract
Introduction and aim: Thromboxane (TX) A_2 was identified as an important vasoconstrictor during Zymosan induced portal perfusion pressure (PP) increase. We aimed at investigating whether hepatic steatosis influences the extent of TXA_2-induced portal hypertension.
Materials and methods: Sprague–Dawley rats were randomly divided into control and steatosis (induced by the special diet) groups. PP and TXB_2 (stable degradation product of TXA_2) in the perfusate were measured after *in situ* liver perfusion with Zymosan (150 μg/ml, 40–46 min) or U46619 (TXA_2 analog, 0.1 μM/ml, 40–46 min). The number of Kupffer cell (KC) was measured by immunohistochemistry with CD163.
Results: Zymosan induced more TXB_2 production and a higher PP increase in control group than in steatosis group despite more CD163 positive KCs in fatty livers. PP and TXB_2 efflux revealed a strong correlation in control group and a moderate correlation in steatosis group. Contrary to the effect of Zymosan, U46619 induced a much higher PP increase in steatosis group than in control group.
Conclusion: Severe steatosis increased number of KCs, however, PP increase and TXB_2 efflux caused by Zymosan infusion in fatty livers were lower than those in healthy livers. In contrast, TXA_2 analog caused higher PP increase in fatty livers. Targeting the more sensitive response to TXA_2 in fatty livers might be a potential therapy of severe steatosis.

Abbreviations: CLD, chronic liver diseases; H&E, haematoxylin & eosin; HPF, high power field; IHC, immunohistochemistry; KC, kupffer cell; NASH, nonalcoholic steatohepatitis; P_{min}, basal portal perfusion pressure; P_{max}, maximal portal perfusion pressure; PP, portal perfusion pressure.

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PALABRAS CLAVE
Esteatohepatitis no alcohólica;
Células de Kupffer;
Rata;
Zymosan

Efectos de la esteatosis hepática en la hipertensión portal inducida por tromboxano A_2

Resumen
Introducción y objetivos: Se ha identificado al tromboxano (TX) A_2 como importante vasoconstrictor durante el aumento de la presión de perfusión portal (PP) inducida por zymosan. El objetivo ha sido analizar si la esteatosis hepática influye en el grado de hipertensión portal inducida por TXA_2.
Materiales y métodos: Las ratas Sprague-Dawley® se han dividido aleatoriamente en grupos de control y esteatosis (inducida por una dieta especial). Se midieron la PP y el TXB_2 (producto de degradación estable de TXA_2) en la perfusión después de la perfusión hepática *in situ* de zymosan (150 μg/ml, minuto 40-46) o U46619 (análogo de TXA_2, 0,1 μM/ml, minuto 40-46). El número de células de Kupffer (CK) se midió mediante inmunohistoquímica con CD163.
Resultados: Zymosan provocó más producción de TXB_2 y mayor aumento de la PP en el grupo de control que en el grupo de esteatosis a pesar de hallar más CK positivas para CD163 en hígados grasos. El flujo de salida de la PP y el TXB_2 reveló una fuerte correlación en el grupo de control y una correlación moderada en el grupo de esteatosis. De manera diferente al efecto de zymosan, U46619 indujo un aumento de la PP mucho mayor en el grupo de esteatosis que en el grupo de control.
Conclusión: La esteatosis grave aumentó el número de CK; sin embargo, el aumento de la PP y el flujo de TXB_2 provocado por la perfusión de zymosan en hígados grasos fueron menores que en los hígados sanos. En cambio, el análogo de TXA_2 provocó un aumento de la PP en hígados grasos. Centrarse en la respuesta más sensible al TXA_2 en hígados grasos podría convertirse en un tratamiento potencial de la esteatosis grave.

Introduction

Chronic liver disease (CLD) is referred to as an immune dysfunction syndrome mostly caused by liver fibrosis and steatohepatitis.[1] Liver cirrhosis and various complications are the consequences of CLDs and a significantly higher survival rate can be achieved if CLDs are treated early and reasonably.[2] Yet causes and mechanism of progression from CLDs to liver cirrhosis remain obscure.

Steatosis is recognized more and more as an important source of liver-related morbidity and mortality.[3] Severe hepatic steatosis may induce inflammation and hepatocellular damage, leading to the definition of nonalcoholic steatohepatitis (NASH).[3,4] Previous publications reported that severe steatosis alone can significantly elevate the portal pressure.[5] Intrahepatic vascular hyperreactivity and increased thromboxane (TX) might significantly contribute to severe steatosis and be involved in the procedures of fibrosis and portal hypertension.[6]

Kupffer cells (KCs) and contractile cells are the key cells in the process of hypertension. KCs are one of the first liver cells to come into contact with microbial products and importantly increase portal pressure.[6,11] Zymosan, a mixture derived from yeast cell walls, could activate KCs and increase portal pressure in both *in vivo* and *in vitro* models of the isolated perfused rat liver.[7,8] TXA_2, prostaglandins (PG) D2 and F2a have been reported to be main vasoactive substances released by KCs during zymosan phagocytosis.[9,10] Previous results verified the vital role for TXA_2, but not for PGD2 and PGF2α, in mediating the Zymosan-induced portal pressure increase.[7] TXA_2 and cysteinyl leukotrienes produced by KCs induce contraction of contractile cells and subsequently lead to portal hypertension.[11,12] In one clinical study, contractile cells from the cases of fatty livers or NASH nearly all showed activation, supporting the association between steatosis and intrahepatic vasoconstriction.[13]

We have shown vasopressor response was augmented in fibrotic livers induced by bile duct ligation. However, the effect of severe steatosis on liver portal pressure has not been well investigated. In the present study, Zymosan and TXA_2 analog U46619 were administrated to rats with severe steatosis. Compared with healthy rats, we aimed to investigate whether hepatic steatosis influences the extent of TXA_2 induced portal hypertension.

Materials and methods

Animal studies

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

Induction of hepatic steatosis by high fat diet

To induce hepatic steatosis, the Sprague–Dawley rats received a special diet treatment: on first 10 days, the rats received control diet with 10% energy from fat (Altromin, Lage) to prepare the gastrointestinal tract. Then the rats received the high-fat diet with 70% energy from fat (Altromin, Lage) for 4 weeks *ad libitum* until start of experiments.[14]

In situ rat liver perfusion study

The procedures have been well described in earlier publications.[7,15,16] Briefly, rats were anesthetized by intraperitoneal injections of sodium pentobarbital (50 mg/kg body weight). Then, a 14-gauge Teflon intravenous catheter was used to cannulate the portal vein. The inferior vena cava was cannulated through the right atrium and ligated above the right renal vein. In all experiments, Krebs–Henseleit solutions (pH 7.4, 37 °C) were used for perfusing in a non-recirculating manner. The perfusion buffer was gassed with a mixture of 95% O_2 and 5% CO_2. The liver viability was determined by stable perfusion pressure during initial 20-min stabilization period.

Kupffer cell activation

KCs were activated by infusion of cell wall particles from *Saccharomyces cerevisiae* (Zymosan). Zymosan suspensions (150 μg/ml) preparation was described previously.[7,15,16] In short, Zymosan suspensions (37.5 mg/50 ml water) were boiled for 30 min at 95 °C, then, cold water and 10-fold concentrated Krebs–Henseleit stock buffers were added to a final volume of 250 ml. Final Zymosan suspensions in Krebs–Henseleit buffer were infused from 40 to 46 min after starting liver perfusion.

In further experiments, the special TXA_2 analog U46619 was used to activate contractile cells. Aliquots of an ethanol stock solution (10 mM) of U46619 (Axxora, San Diego, CA, USA) were stored at −80 °C before use.

TXA_2 measurement by ELISA

TXA_2 efflux was quantified by the release into liver perfusate. Perfusate samples were stored at −80 °C until measurement. The procedures were well described in our publications before.[7,12] TXB_2, the stable degradation product of TXA_2, was measured by TXB_2 ELISA kit (Cayman Chemical, USA).

Histological evaluation and immunohistochemistry

Sections of the livers were fixed in 4% buffered formalin, dehydrated in graded ethanol and embedded in paraffin. These sections were stained with H&E (hematoxylin & eosin). Simple grading and staging systems (0–4 scores represent no, low, mild, moderate and severe stages) were used to compare severities of inflammation, fat deposition, fibrosis and necrosis in control and steatosis groups.[17]

The number of KCs was compared by immunohistochemistry (IHC) with primary antibodies against CD163 (1:100; Acris, USA). The procedure was according to the instruction of the antibody.[18] In brief, paraffin sections were blocked with 0.3% H_2O_2 in PBS, followed by washing with three changes of PBS. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA). The sections were then incubated with CD163 antibody overnight. After washing in PBS, the slides were incubated with secondary antibody 1 h in room temperature the other day. The number of CD163 positive cells on every high power field (HPF) was compared between different groups.

Statistical analyses

Rats were randomly distributed to different groups, all data were presented as the mean ± standard deviation (SD). The Student's two-tailed *t*-test was used for paired or unpaired observations. A value of $P < 0.05$ was considered to be statistically significant; *n* denotes the number of animals used. SPSS and Graphpad were used for data analysis and figure drawing.

Results

Histological evaluation in different groups

Control (healthy rats without any treatment) and steatosis (induced by the special diet treatment) groups were included in the experiment. Histological evaluation with H&E staining (Fig. 1) and the relating scores (Table 1) confirmed hepatic steatosis: liver histology from control group was strictly normal (no steatosis, inflammation or fibrosis) while the special diet induced severe fat deposition and mild inflammation in steatosis group.

Zymosan infusion induced a higher portal perfusion pressure and TXB_2 efflux in control group

We determined the basal and maximal portal perfusion pressure (P_{min} and P_{max}) during the liver perfusion with Zymosan infusion (150 μg/ml, 40–46 min). Zymosan significantly increased portal perfusion pressure (PP) in both groups: from basal 5.58 ± 0.73 to maximum 18.46 ± 2.74 cmH_2O in control group and from 4.32 ± 0.66 to 11.60 ± 1.56 cmH_2O in steatosis group ($P < 0.05$ in each group). P_{max} in steatosis group was much lower than that in control group ($P < 0.05$) while P_{min} were almost the same in these groups (Fig. 2A). Meanwhile, Zymosan infusion also increased TXB_2 efflux from basal 41.77 ± 12.97 to maximal 1003.09 ± 384.69 and from 29.4 ± 4.43 to 532.72 ± 36.08 pg/ml × g liver in control and steatosis groups respectively ($P < 0.05$ in each group). Comparing between these groups, maximal TXB_2 caused by Zymosan was much higher ($P < 0.05$) in control group (Fig. 2B).

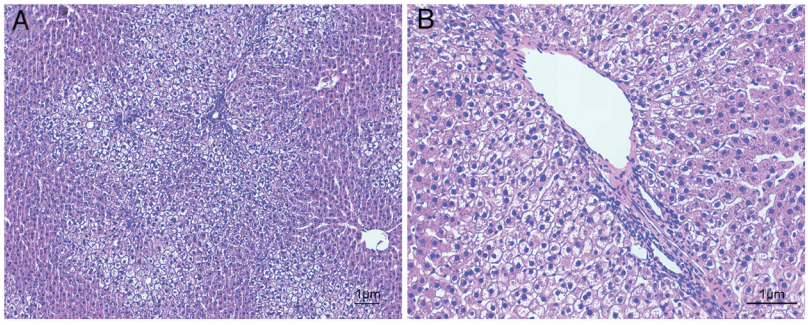

Figure 1 Hematoxylin and eosin staining results in steatosis group. The typical picture of hematoxylin and eosin (H&E) staining from five independent experiments in steatosis group (A, 100× magnification; B, 200× magnification). Special diet for 4 weeks induced severe fat deposition and mild inflammation in steatosis group.

Table 1 Histological evaluation in control and steatosis groups.

Group	Parameter	Description
Control	Inflammation: 0 Fat deposition: 0 Fibrosis: 0 Necrosis: 0	
Steatosis	Inflammation: 2 Fat deposition: 4 Fibrosis: 0 Necrosis: 0	Sprinkling of inflammatory cells in c.a.1/3 of portal areas >66% parenchymal involvement

Inflammation, fat deposition, fibrosis and necrosis were used to confirm the effects of treatment in steatosis group (4 weeks treatment with the special diet). Healthy rats were used as negative control, evaluation scores were based on the H&E results in every group ($n = 5$ in each group).

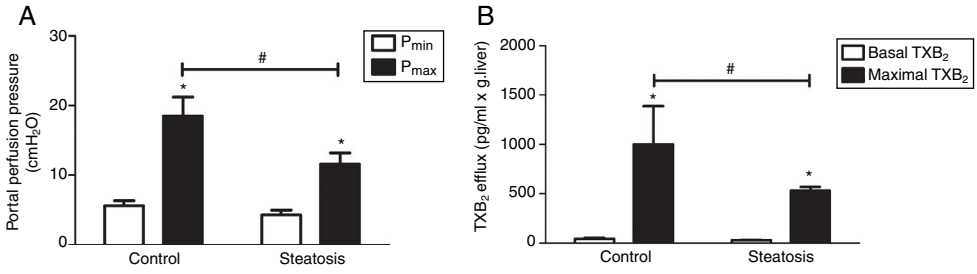

Figure 2 Influence of Zymosan on portal perfusion pressure and thromboxane B_2 efflux. (A) Infusion of Zymosan (150 μg/ml) into the portal vein of anesthetized rats from 40 to 46 min after starting liver perfusion significantly increased portal perfusion pressure in control and steatosis groups (*$P < 0.05$). Basal portal perfusion (P_{min}) had no difference between two groups while the maximal portal perfusion pressure (P_{max}) in control group is much higher (#$P < 0.05$). (B) Zymosan infusion significantly increased thromboxane (TX) B_2 efflux in both groups (*$P < 0.05$). Comparing between these two groups, Zymosan caused a much higher maximal TXB_2 efflux in control group (#$P < 0.05$). $n = 5$ in each group, data are expressed as mean ± SD.

Portal perfusion pressure was correlated with TXB_2 efflux

We compared the curves of PP and TXB_2 efflux over time after Zymosan infusion in order to confirm their correlations. The curve trends of PP and TXB_2 efflux in both groups were almost in parallel: Zymosan infusion caused a transient increase of PP or TXB_2 efflux from 40 to 46 min after starting perfusion, then, decreased from the summit until end (Fig. 3). Correlation coefficients (CC) and P values between PP and TXB_2 curves also revealed a strong correlation in control group (CC = 0.965, $P = 0.0004$) and a moderate correlation in steatosis group (CC = 0.725, $P = 0.065$).

Liver steatosis induced more KCs before and after Zymosan infusion

Number of KCs was induced by liver steatosis before and after Zymosan infusion (150 μg/ml, 40–46 min). IHC was performed to determine KC density. The steatosis group had more CD163 positive cells than the control group before

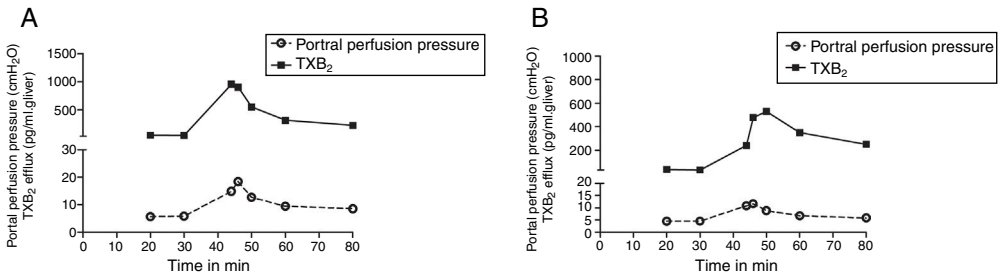

Figure 3 The curves of portal perfusion pressure and thromboxane B_2 efflux following Zymosan infusion. The curve trends of portal perfusion pressure and thromboxane (TX) B_2 efflux in both groups were almost in parallel: After a transient increase by Zymosan infusion (150 μg/ml, 40–46 min), portal perfusion pressure or TXB_2 efflux decreased until the end of the perfusion in both control (A) and steatosis (B) groups ($n = 5$ in each group).

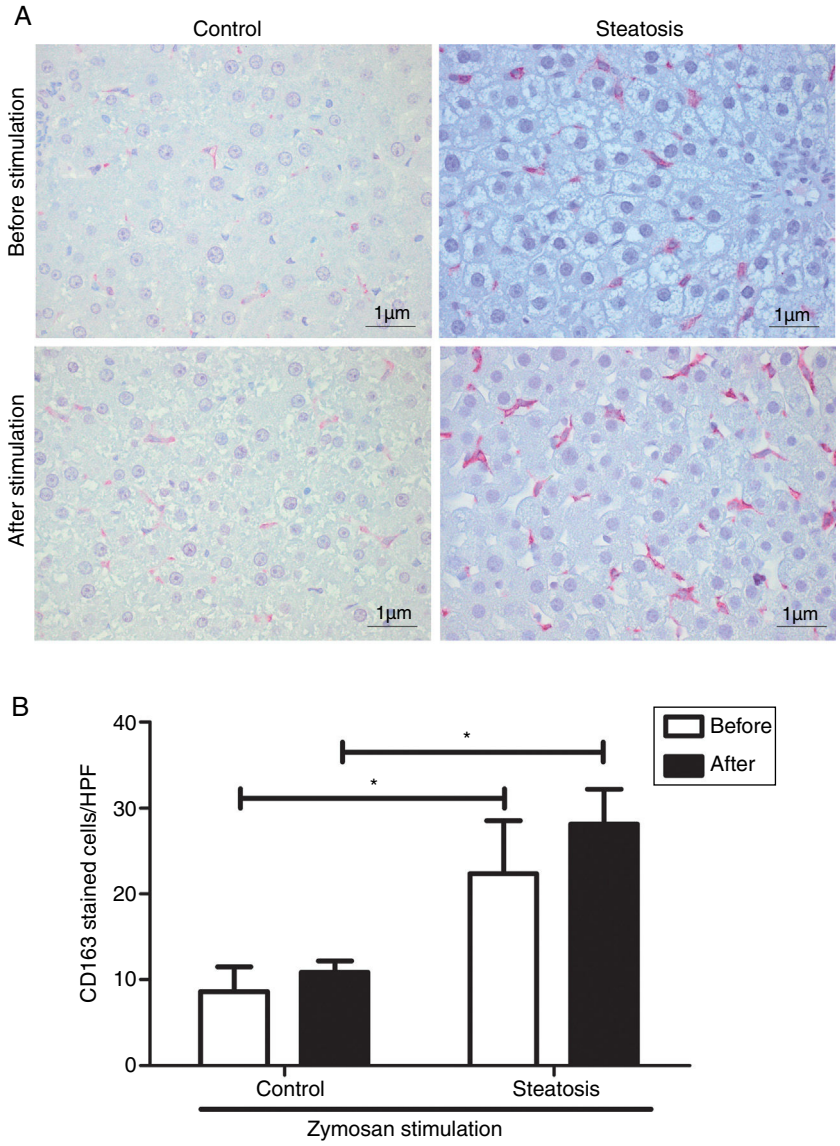

Figure 4 Kupffer cell density before and after Zymosan infusion. (A) The typical picture from five separate immunohistochemistry results in control and steatosis groups before and after infusion of Zymosan (150 μg/ml). Red color represents CD163 positive (400× magnification). (B) Steatosis group had significantly more (*$P < 0.05$) CD163 positive cells than control group every high power field (HPF) before and after stimulation. Data are expressed as mean ± SD.

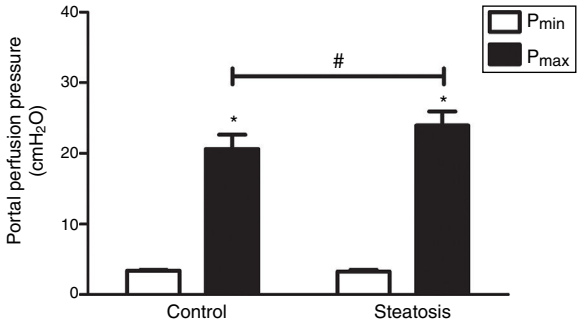

Figure 5 Influence of U46619 on portal perfusion pressure. Infusion of U46619 (0.1 μM/ml) from 40 to 46 min after starting liver perfusion caused a significant increase of portal perfusion pressure in both control and steatosis groups (*$P < 0.05$). Comparing between these two groups, basal portal perfusion pressure (P_{min}) were almost the same while the maximal portal perfusion pressure (P_{max}) was much higher in steatosis group (#$P < 0.05$). $n = 5$ in each group, data are expressed as mean ± SD.

and after the Zymosan stimulation ($P < 0.05$). There is a slight increasing trend of CD163 positive cells after Zymosan stimulation in steatosis group, however no statistical significance gained (Fig. 4).

Liver steatosis caused an enhanced reaction to TXA_2 analog

To compare the response ability to TXA_2 in these two groups, U46619 (the special analog of TXA_2, 0.1 μM/ml) was then infused into the portal vein of anesthetized rats from 40 to 46 min after starting liver perfusion. U46619 significantly increased PP in both groups however, P_{max} in control group (20.62 ± 2.02 cmH_2O) was much lower than that in steatosis group (23.98 ± 1.96 cmH_2O) (Fig. 5).

Discussion

This study investigated the influences of hepatic steatosis on portal perfusion pressure (PP). There were three main findings in our study: (1) Severe steatosis induced more CD163 positive KCs than healthy livers. (2) Zymosan infusion caused lower PP increase and TXB_2 efflux in steatosis group than in control group. (3) Fatty livers showed a more sensitive response to TXA_2 analog resulting in a higher PP increase following U46619 stimulation. These results were obtained using *in situ* perfused rat livers from control and steatosis groups. The diagnosis of hepatic steatosis was based on changes in liver histology (Fig. 1, Table 1).

The initial set of experiments showed that rat livers from steatosis group had much more severe steatosis than control group. Despite more severe inflammation in steatosis group, P_{min} and basal TXB_2 values in steatosis group were almost the same as control group, P_{max} and maximal TXB_2 values after Zymosan infusion were even much lower in steatosis group (Fig. 2). Since the correlation between PP and inflammation has been well proved,[19] we hypothesized that severe steatosis may influence the total number or function of KCs.

CD163 is exclusively expressed on monocytes and common used to identify macrophages in tissue sections.[20] Elevated CD163 expression was suggested to be associated with unfavorable clinicopathological features and prognosis in patients. There was a significant association between the numbers of CD163 positive cells and the severity of fibrosis, tumor stages, and survival patients.[21] In our experiment, nearly double CD163 positive cells were found in steatosis group before and after Zymosan infusion (Fig. 4), supporting the relation between severe steatosis and CD163 expression. Previous studies reported Zymosan to induce KCs in rat livers.[22] A slight increasing trend was observed in steatosis group after Zymosan stimulation, but no significant difference was found. That may due to the short stimulating and observing time in our experiments. A low dose of an anti-CD163-IgG-dexamethasone strongly reduced inflammation and hepatocyte ballooning in a rat model.[23] Thus, drugs targeting CD163 seems to be a promising therapy to severe steatosis.

The total duration of perfusion experiment in our research is nearly 1 h and Zymosan was added from 40 to 46 min after the start of perfusion. Several minutes of stimulation by Zymosan infusion caused a rapid and transient PP and TXB_2 increase (Fig. 3), which is in accordance with previous studies.[24] Additionally, we previously proved that TXA_2 receptor antagonist reduced PP increase both *in vivo* and in isolated rat liver perfusion, supporting TXA_2 as vital factor in zymosan induced PP increase.[7] As a result, the lower PP increase in steatosis group after Zymosan infusion in the present study might be related to decreased TXB_2 secretion. Besides TXA_2, PP increase may also be affected by other mechanisms like reduced NO production. However, NO was proved to be not related to the early mechanisms of alcohol induced portal pressure increase.[25] To rule out whether steatosis affects NO and other mechanisms after Zymosan infusion further models and experiments are needed.

However, numerous studies demonstrated the association between severe steatosis and portal hypertension, one clinical study with fifty consecutive patients found the degree of steatosis was the only independent predictor of the presence of portal hypertension.[26] We further investigated the influence of steatosis on contractile cells with TXA_2 analog. Higher P_{max} and increased value were found in steatosis group following U46619 infusion (Fig. 5), these results were in opposite with those by Zymosan (Fig. 2). Thus, the enhanced responses to TXA_2 caused by severe steatosis might play pivotal role on the procedure of portal hypertension. U46619 is supposed to act on primary HSCs from rats while primary KCs and sinusoidal endothelial cells failed to release vasoactive substance following U46619 stimulation.[27] High-affinity TXA_2 receptors and a specific binding site for TXA_2 analog were also found on HSCs.[28] The relationship between HSC activation and steatosis has been recognized and proved in recent years. HSC activation is the common pathogenic mechanism of both alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH). HSCs are quiescent in normal livers, however, with the stimulating effect of ASH or NASH, quiescent HSCs become transdifferentiated to myofibroblastic state.[29] A comparison of electronic microscopy between patients with fatty and healthy livers showed a more severe fat droplets

distribution in HSCs in fatty liver.[30] Thus, HSC activation by severe steatosis could explain the augmented response to TXA_2 analog in steatosis group in this experiment.

Conclusions

Zymosan and U46619 caused different effects of PP increase in healthy and fatty livers: Zymosan induced higher PP increase in healthy livers in contrast to U46619, while U46619 was more effective in fatty livers. The more sensitive response of fatty liver to TXA_2 may be a therapeutic target for steatosis.

Authorship statement

Jiang Zhang research design, performance of the research, data analysis, writing of the manuscript.

Julia Schewe research design, performance of the research, data analysis, writing of the manuscript

Hanwei Li performance of the research, data analysis.

Marie-Christine Makeschin performance of the research, data analysis.

Ujjwal M. Mahajan performance of the research, data analysis.

Alexander L. Gerbes research design, writing of the manuscript.

Christian J. Steib research design, performance of the research, data analysis, writing of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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