Acta Pharmacologica Sinica (2010) 31: 1367–1375
 1671-4083/10 $32.00
www.nature.com/aps

Original Article

Pentoxifylline alleviates high-fat diet-induced non-alcoholic steatohepatitis and early atherosclerosis in rats by inhibiting AGE and RAGE expression

Jing WU[2, #], Miao-yun ZHAO[3, #], Hao ZHENG[2], Hua ZHANG[4], Ying JIANG[1, *]

[1]Department of Pathophysiology, Capital Medical University, Beijing 100069 , China; [2]Department of Diagnostic Ultrasound, Xuanwu Hospital, Capital Medical University, Beijing 100053 , China; [3]Municipal Laboratory of Liver Protection, Regulation and Regeneration, Capital Medical University , Beijing 100069 , China; [4]Department of Histology and Embryology, Capital Medical University, Beijing 100069, China

Aim: To investigate the expression of advanced glycation end products (AGEs) and their receptor RAGE in the livers and blood vessels of rats with non-alcoholic steatohepatitis (NASH) and the effect of pentoxifylline (PTX) on liver and artery function in rats with NASH.
Methods: Sprague-Dawley rats were fed a high-fat diet for 12 weeks and given PTX by gavage for 4 weeks. The effects of PTX on hepatic liver and vessel function as well as the expression of AGE and RAGE in rats with NASH were assessed. The intima-media thickness (IMT) of the aorta and carotid artery was evaluated using ultrasonography.
Results: Serum aspartic aminotransferase (AST) and blood levels of glucose (GLU) were reduced in the PTX group relative to the NASH group. The IMT of the aorta and carotid artery was increased in the NASH group compared with the control group. The IMT was reduced in NASH rats after treatment with PTX. Rats with NASH demonstrated higher AGE and RAGE protein levels in the liver and arteries compared with those of control rats. PTX treatment in NASH rats resulted in a decrease in AGE and RAGE protein levels in the liver and arteries compared with those in the NASH group.
Conclusion: Early atherosclerosis was observed in rats with NASH induced by a 16-week high-fat diet. High expression of AGE and RAGE in the livers and arteries of rats with NASH may contribute to the pathogenesis of NASH and early atherosclerosis. PTX showed protective effects on hepatic and arterial function, partially through inhibition of AGE and RAGE expression.

Keywords: nonalcoholic steatohepatitis; atherosclerosis; advanced glycation end products receptors; advanced glycation end products; pentoxifylline

Acta Pharmacologica Sinica (2010) 31: 1367–1375; doi: 10.1038/aps.2010.110; published online 13 Sep 2010

Introduction

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as a public health issue[1]. NAFLD represents a spectrum of liver disease from steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. NAFLD is also inextricably linked to metabolic syndrome and diabetes mellitus[2]. In particular, potential cardiovascular morbidity is considered to be associated with NAFLD[3]. Definitive medical therapies for individuals with NAFLD are currently lacking.

NASH, characterized by liver fatty infiltration, inflammation, hepatocellular injury and fibrosis, may easily develop into liver cirrhosis and hepatocellular carcinoma[4]. The mechanism of NASH is incompletely understood. Studies have found that serum levels of tumor necrosis factor-alpha (TNFα) are elevated in patients with NASH[5]. TNFα provokes inflammation, apoptosis, and fibrogenic responses that are involved in NASH[6].

Pentoxifylline (PTX) is a non-selective phosphodiesterase inhibitor. PTX is a methylxanthine derivative with known hemorheological activity. PTX has been used for many years in the treatment of peripheral vascular diseases. Interest in the other pharmacological actions of PTX is growing[7]. Apart from its effect on the rheology of blood cells, PTX also has anti-inflammatory and immune-modulating properties[8]. In particular, PTX has an inhibitory effect on TNFα and may be beneficial in a subset of patients with severe alcoholic hepatitis in which TNFα overproduction contributes to liver injury[9].

# The two authors contributed equally to the work.
* To whom correspondence should be addressed.
E-mail jiangy@ccmu.edu.cn
Received 2010-03-31 Accepted 2010-07-07

PTX has also been shown to improve aminotransferase levels and insulin resistance among patients with NASH[10]. Our previous study demonstrated that PTX inhibited TNFα expression in the livers of rats with NASH[11]. However, the exact mechanisms underlying the therapeutic effects of PTX on NASH are not fully recognized.

Advanced glycation end products of proteins (AGEs) are nonenzymatically glycosylated proteins that are associated with a variety of conditions including diabetes and other vascular disorders. These proteins regulate cellular functions via specific cell surface acceptor molecules, such as the receptor for AGEs (RAGE). RAGE is a multi-ligand member of the immunoglobulin superfamily of cell-surface molecules[12]. AGEs accumulate at an extremely high rate in the diabetic state and may trigger an intracellular signal transduction cascade that ultimately induces a series of inflammatory and other reactions, including activation of nuclear factor-kappa B (NF-κB), increased expression of cytokines, and induction of oxidative stress[13–15]. RAGE signaling plays a pivotal role in regulating the expression of TNFα, oxidative stress, and endothelial dysfunction in type-2 diabetes[16, 17]. TNFα appears to be involved in the enhancement of RAGE expression and in neointimal formation in obese Zucker rats[18]. RAGE also engages diverse ligands relevant to the pathogenesis of non-diabetic atherosclerosis[19]. In addition, the liver is the main site for metabolism of circulating AGEs[20]. AGE levels in serum were also increased in cirrhosis[21]. RAGE can be up-regulated if hepatic stellate cells are activated to transdifferentiate into myofibroblasts[22]. However, limited information is available on whether and how RAGEs are implicated in NAFLD. RAGE levels in the liver and its roles in the pathogenesis of NASH remain unclear.

We investigated whether vascular injury occurred in high-fat diet-induced NASH rats. We wanted to ascertain the levels of RAGE expression in the livers and blood vessels of NASH rats and determine whether PTX can affect RAGE expression. In other words, we wanted to determine whether PTX exerted protective effects on the livers and vessels of NASH rats. We concluded that the amelioration of hepatic and arterial functions by PTX in NASH rats fed a high-lipid diet was associated with a reduction in RAGE expression.

Materials and methods

Animals and experimental protocol

All protocols were approved by the Ethical Committee of Capital Medical University Beijing (Beijing, PR China). Animals were supplied from the Laboratory Animal Research Center of China at Capital Medical University.

Thirty male Sprague-Dawley rats (110–130 g) were used in the present study. After acclimatization for one week, rats were randomly divided into three groups of ten: control group, high-fat group (NASH group) and high-fat+PTX group (PTX group). Rats in the control group were fed a standard diet with *ad libitum* food intake. Rats in the high-fat group were fed a diet high in fat[23] with *ad libitum* food intake. Rats in the high-fat group were given PTX (PTX group; Ratiopharm GmbH, Germany) or physiological (0.9%) saline (NASH group) by gavage at 16 mg/kg daily[24] for 4 weeks following the 12-week dietary intervention period. All rats were fed between 8:00 am and 9:00 am each day. They were maintained on a 12-h light-dark cycle at 22–25 °C and fed tap water *ad libitum* for 16 weeks. Body weight was recorded each week.

After the 16-week intervention period, rats were killed by puncture of the abdominal aorta after overnight fasting. The livers, aortas and carotid arteries were rapidly removed and dissected. Partial liver specimens and artery specimens were snap-frozen in liquid nitrogen and stored at -80 °C for subsequent analyses. Serum activities of the liver-associated enzymes alanine aminotransferase (ALT) and aspartic aminotransferase (AST) as well as blood levels of glucose (GLU), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured using an autoanalyzer in the Clinical Chemistry Laboratory of Youan Hospital, Capital Medical University Beijing. Serum insulin was measured with a radioimmunoassay kit in the Radioimmunology Laboratory of Hospital 301 (Beijing, PR China). Insulin resistance was calculated by means of the homeostasis model assessment-insulin resistance ($HOMA_{IR}$) index[25].

Ultrasonography

We used Visual Sonics Vevo 770 ultra-high-frequency ultrasonic diagnostic equipment (frequency, 20–40 MHz) to perform ultrasonography. The brachydiagonal tangent plane of the common carotid artery was displayed by placing the probe beside the trachea. The sliver plane was then displayed by rotating the probe 90°. We measured the diameter and intima-media thickness (IMT) of the common carotid artery at the 5.0-mm proximal part and middle section of the aorta[26]. The sample volume was put in the center of vessels, and the angle between the sound beam and blood flow was <60°. We observed the spectrum and measured the systolic peak velocity and end-diastolic velocity of the aorta.

Histopathological evaluation

Bouin-fixed, paraffin-embedded sections of the liver, aorta and carotid artery were stained with hematoxylin-eosin (H&E). The slides of each liver tissue specimen were evaluated by the criteria proposed by Promrat and Brunt[27, 28]: grade 0, no foci of inflammation; grade 1, fewer than one foci per two 20×fields; grade 2, one foci per two 20×fields to one foci per one 20×field; grade 3, one to two foci per one 20×field; or grade 4, more than two foci per one 20×field.

Determination of AGE and RAGE protein and mRNA expression

Western blot analyses

Proteins from homogenized liver and aorta tissues were analyzed by Western blotting. Equal concentrations of protein from the liver and aorta were fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Incubation with primary antibodies to AGEs (clone 6D12, 1:1000 dilution; Trans Genic Inc, Kumamoto, Japan,) and

RAGE (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was followed by the addition of horseradish peroxidase-conjugated secondary antibodies. The positive reaction against special antibody was visualized using an electrochemiluminescent (ECL) reagent (Santa Cruz Biotechnology) and subsequent exposure to X-ray film (Kodak, Tokyo, Japan). The density of signals was quantitated with ImageJ software.

RNA extraction and the real-time reverse transcription-polymerase chain reaction (RT-PCR)

Liver and aorta tissues stored at -80 °C were homogenized. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was obtained using a random hexamer primer and a SuperScript III Reverse Transcriptase Kit according to the manufacturer's instructions (Invitrogen). RT-PCR was performed using a 7300 real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems). Sample cDNAs (equivalent of 2 μg of total RNA) were used as templates with gene-specific primers: RAGE, forward-ACA GAA ACC GGT GAT GAA GG, reverse-CTC TCC TCG AGT CTG GGT TG; and 18S, forward-GTA ACC CGT TGA ACC CCA TT, reverse-CCA TCC AAT CGG TAG TAG CG. Amplification was performed in duplicate for each sample in an ABI Prism 7300 Sequence Detector (PE Applied Biosystems), and the amount of mRNA was normalized using 18S as the endogenous control. Dissociation curves were analyzed to confirm that significant amounts of primer dimers were not formed.

Statistical analyses

Data are presented as means±SD or medians and interquartile ranges as appropriate. Some variables such as the ALT, AST and LDL-C had skewed distributions, thus the skewed variables were transformed with an inverse square root function to improve normality of the data before further analysis. Comparisons between groups were performed using ANOVA or Mann-Whitney U tests. Differences with a value of $P<0.05$ were considered statistically significant.

Results

PTX treatment ameliorated hepatic function in rats with NASH induced by a high-fat diet

In comparison with control rats, serum levels of ALT, AST, TC, LDL-C and GLU increased in rats in the NASH group. This result was similar to that observed in our previous study[23] but to a greater degree. Insulin levels and the $HOMA_{IR}$ index in the NASH group were markedly increased. This result suggested that there was insulin resistance in NASH rats. PTX treatment reduced the serum levels of AST and GLU compared with those in the NASH rats. Although the ALT levels, insulin levels, and the $HOMA_{IR}$ index decreased in the PTX group compared with those in the NASH group, the differences were not statistically significant. PTX had no effect on serum lipids levels in NASH rats (Table 1). These data suggested that PTX may alleviate hepatic dysfunction in NASH rats induced by a 16-week high-fat diet.

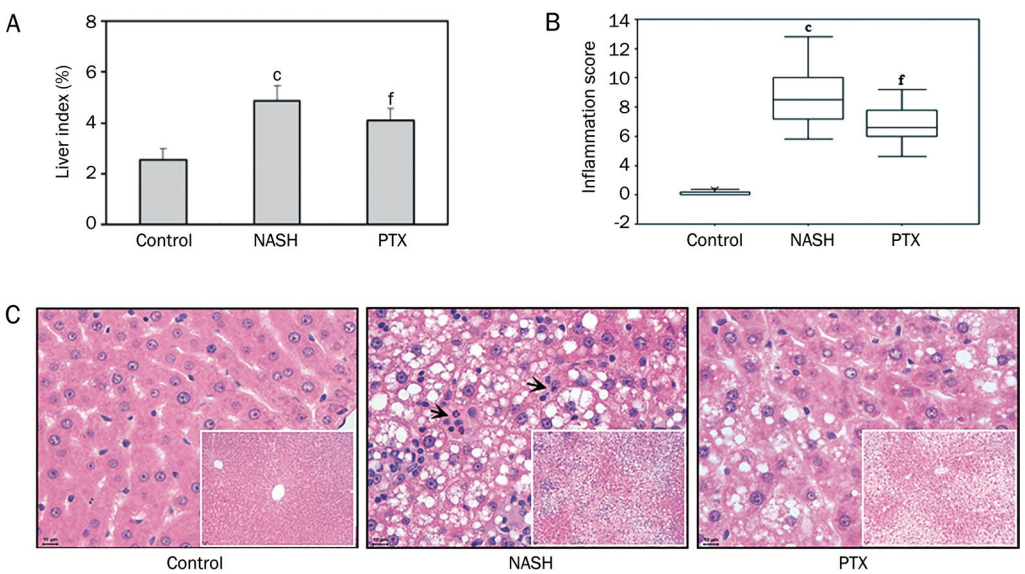

Figure 1. (A) The liver index in rats fed a normal diet (Control group), a high-fat diet (NASH group) or a high-fat diet+pentoxifylline (PTX group) for 16 weeks. The liver index indicates the relative liver weight, ie, the liver weight per 100 g of body weight (g). (B) The inflammation score of the liver in rats. The inflammation score is described in the "Materials and methods" section. (C) Hematoxylin and eosin (H&E) staining of hepatic sections (objective lens, ×40 and ×10). The livers of the NASH group rats show pronounced hepatic steatosis, infiltration of inflammatory cells, and liver cell necrosis (arrow). The inflammatory response was significantly reduced in the pentoxifylline-treated rats. Data are means±SD or medians (25/75th percentiles) for 10 rats per group. $^{c}P<0.05$ *vs* the control group; $^{f}P<0.05$ *vs* the NASH group.

Table 1. Biochemical changes in the blood. Data are mean±SD or median (25/75th percentiles), as appropriate, for 10 animals per group. [b]$P<0.05$ *vs* Control. [e]$P<0.05$ *vs* NASH.

Group	Control	NASH	PTX
ALT (U/L)	38 (30.75–41.75)	65 (39.75–117)[b]	49.5 (39.75–61.25)
AST (U/L)	98 (89.75–119.75)	187 (147.50–353.00)[b]	113.5 (96.75–149.75)[e]
GLU (mmol/L)	5.91±0.49	6.95±1.12[b]	5.84±0.63[e]
TG (mmol/L)	0.57±0.12	0.63±0.29	0.59±0.21
TC (mmol/L)	1.79±0.30	2.49±0.69[b]	2.21±0.62
HDL-C (mmol/L)	1.34±0.22	1.45±0.30	1.40±0.23
LDL-C (mmol/L)	0.22 (0.21–0.25)	1.21 (1.04–1.47)[b]	1.04 (0.81–1.28)
Insulin (μIU/mL)	22.60±5.02	41.06±9.02[b]	30.77±5.07
HOMAIR	5.93±1.52	10.53±3.18[b]	8.31±2.34

PTX treatment attenuated hepatic lesions in rats with NASH induced by a high-fat diet

The liver index of the PTX group was significantly less than that of the NASH group (Figure 1A). The mean body weight in the PTX group was less than that in the NASH group, but the difference was not statistically significant (data not shown). Histological analyses showed that rats in the PTX group and NASH group had significant accumulation of fat, ballooning degeneration, and inflammation in their livers compared with rats from the control group (Figure 1C). H&E slides were analyzed using a semi-quantitative score for inflammation. The PTX group had a significantly lower inflammation score than that of the NASH group (Figure 1B). However, the degree of steatosis observed in the PTX group was not significantly different from that in the NASH group (data not shown). These data suggested that PTX could lessen the degree of liver injury induced by a high-fat diet at least in part by attenuating the inflammatory response.

PTX treatment attenuated arterial lesions in rats with NASH induced by a high-fat diet

Ultrasonographic results showed that the IMT values of the abdominal aorta and carotid artery in the NASH group were significantly greater than those in the control and PTX groups ($P<0.05$) (Figure 2A & 2B). The diameters of the abdominal aorta and carotid artery in the NASH group were smaller than those in the control group, but the difference was not significant (data not shown). The end-diastolic velocity and the systolic peak velocity of the aorta in the NASH group were significantly smaller than those in the control group ($P<0.05$) but were not significantly different compared with the PTX group (Figure 3A & 3B). These results suggested that early atherosclerosis was present in NASH rats and that PTX treatment attenuated vascular lesions.

Plaques in the aortic intima were not observed by H&E staining or by macroscopic observation. Furthermore, H&E results showed few foam cells in the intima and slight hyperplasia of the media of the aorta and carotid artery in the NASH group. This finding demonstrated that vascular lesions occurred in the early stage of atherosclerosis. The degree of lesions in the PTX group was significantly lower than that in the NASH group (Figure 2C). The results of histopathological evaluation were consistent with ultrasonographic findings.

Effect of PTX on RAGE protein and mRNA expression and AGE protein expression in the livers and arteries of rats with NASH induced by a high-fat diet

To confirmation AGE-RAGE pathways associated with the pathogenesis of NASH, the expression of AGE and RAGE in the livers and aortas of rats with NASH was studied. Western blotting and RT-PCR were carried out to quantify the protein and gene expression of RAGE in these tissues. AGE and RAGE protein expression in the liver was significantly induced after administration of a high-fat diet. This induction was significantly inhibited by the administration of PTX. In the aorta, AGE and RAGE protein expression was also increased in the NASH group compared with that in the control group. However, there was also a significant decrease in AGE and RAGE protein levels in the PTX group relative to the NASH group (Figure 4), but little difference was observed in hepatic and vascular RAGE mRNA levels among the three groups (data not shown).

Discussion

We have reported previously that rats are susceptible to developing NASH when fed a high-fat diet for 12 weeks and SP1-mediated liver uncoupling protein 2 expression[23]. In the current study, we demonstrated that rats fed a high-fat diet *ad libitum* for 16 weeks showed severe histopathological NASH lesions (including steatosis, ballooning degeneration, inflammation and fibrosis). Additionally, levels of AST, ALT, TC, LDL-C, GLU, and insulin, as well as the $HOMA_{IR}$ index, increased remarkably in the NASH group. Thus, the histopathological and biochemical findings of the current study showed that a high-fat diet-induced model of NASH with insulin resistance in rats mimicked human NASH with respect to morphological and biological characteristics. We found that PTX treatment ameliorated hepatic function in rats administrated a high-fat diet by alleviating the inflammatory response and decreasing AST and GLU levels. This observation was

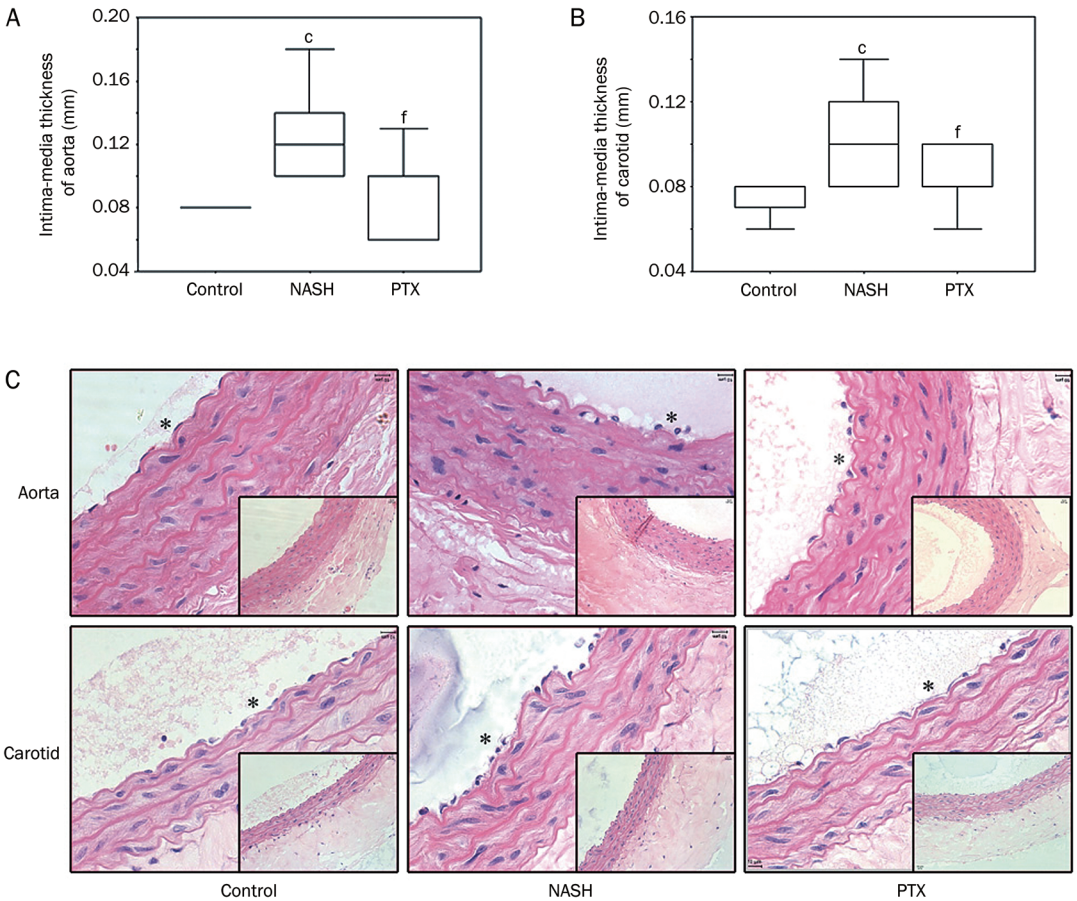

Figure 2. (A) The intima-media thickness (IMT) of the aortas in rats fed a normal diet (Control group), a high-fat diet (NASH group) or a high-fat diet+pentoxifylline (PTX group) for 16 weeks was measured by ultrasonography. (B) The intima-media thickness (IMT) of the carotid artery in rats was measured by ultrasonography. (C) H&E staining of thoracic and carotid artery sections (objective lens, ×40 and ×20). The thoracic and carotid arteries of the NASH group rats show mild intimal thickening and cellular hypertrophy in the intima and media of the aorta (NASH). Pentoxifylline treatment mitigates changes in vascular disease. Data are medians (25/75th percentiles) for 10 rats per group. $^{c}P<0.05$ *vs* the control group; $^{f}P<0.05$ *vs* the NASH group. Stars indicate the intima.

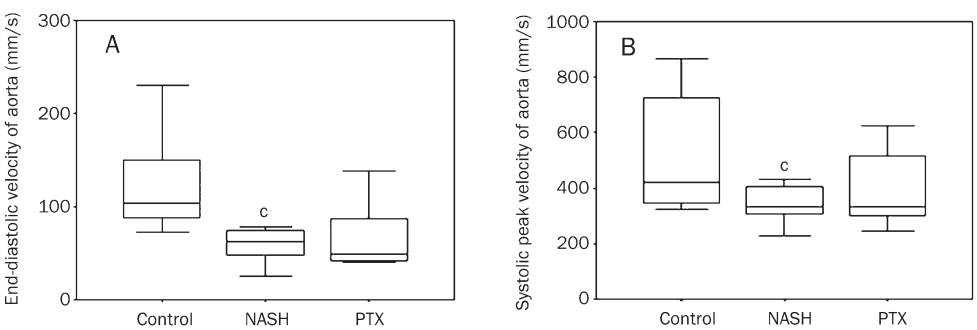

Figure 3. Hemodynamic parameters of aortic flow were measured by ultrasonography. (A) The end-diastolic velocity of the aortas in rats fed a normal diet (Control group), a high-fat diet (NASH group) or a high-fat diet+pentoxifylline (PTX group) for 16 weeks. (B) The systolic peak velocity of the aorta in rats. Data are medians (25/75th percentiles) for 10 rats per group. $^{c}P<0.05$ *vs* the control group; $^{f}P<0.05$ *vs* the NASH group.

in agreement with findings from studies of NASH patients[10] and an animal model of NASH induced by a diet deficient in methionine and choline[29], which suggests that PTX protected against high-fat diet-induced NASH in rats. PTX is a phosphodiesterase inhibitor with rheological and vasodilating properties. The primary site of action of PTX is the vasculature. PTX leads to increased tissue perfusion and improved regional microcirculation and tissue oxygenation. PTX improves liver

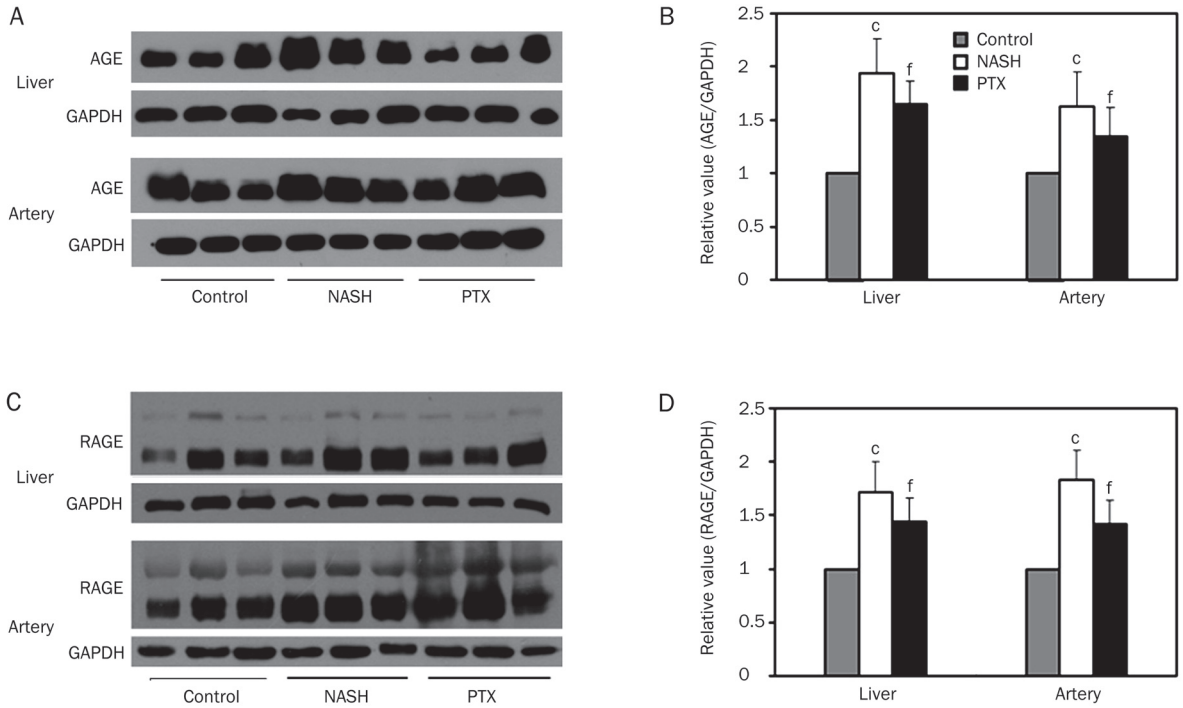

Figure 4. AGE and RAGE protein levels in the livers and arteries of rats. (A) AGE protein levels in the livers and aortas of rats were analyzed by Western blotting with the use of anti-AGE monoclonal antibody. Liver and artery protein from rats fed a normal diet (Control group), a high-fat diet (NASH group) or a high-fat diet+pentoxifylline (PTX group) for 16 weeks were isolated as described in the "Materials and methods" section. Protein samples were loaded onto 12% separating gels; the band associated with AGE displays molecular weight fragments between 25 and 30 kDa. (B) Quantitative analyses of AGE protein levels in the livers and arteries of rats. (C) RAGE protein levels in the livers and aortas of rats were analyzed by Western blotting analyses with the use of anti-RAGE monoclonal antibody. Protein samples were loaded onto 10% separating gels. Two variants of RAGE were detected: 55 kDa and 46 kDa. (D) Quantitative analyses of RAGE protein levels in the livers and arteries of rats. Data are means±SD for 10 rats per group. $^{c}P<0.05$ *vs* the control group; $^{f}P<0.05$ *vs* the NASH group.

perfusion in humans[30,31]. PTX may therefore lessen the acute hepatic injury induced by a high-fat diet and may be partially associated with increased hepatic arterial blood flow and alleviation of early hepatic circulatory disturbances in rats with NASH.

Although ALT levels decreased in the PTX group compared to those in the NASH group, the difference was not statistically significant. The reasons for the differential effects of PTX on AST and ALT are currently unknown, but they may be related to the antioxidant properties of PTX. AST is mainly located in mitochondria of hepatocytes. The mechanism of PTX protection of the liver may likely be to attenuate hepatic mitochondrial lesions via its antioxidant properties. Thereby, PTX decreased serum AST in NASH group rats more prominently than ALT. In addition, because AST and ALT values in the NASH group showed substantial variances, a larger sample size may lead to a statistically significant difference.

It has been reported that PTX improves insulin resistance in patients with NASH[32]. Our results showed that although PTX decreases the $HOMA_{IR}$ index, the difference was not statistically significant. The reason for this may be that the $HOMA_{IR}$ index is a product of fasting glucose and insulin levels, whereas serum insulin levels depend on not only the sensitivity of insulin but also its secretion, distribution and decomposition. Therefore, the measure of serum insulin may be too insensitive and nonspecific to reflect real changes in insulin. Hence, insulin and the $HOMA_{IR}$ index in NASH rats were found to not significantly decrease following PTX treatment.

Several experimental and clinical data suggest that NAFLD is the hepatic expression of the metabolic syndrome[33]. It has been confirmed that NAFLD is associated with an increased risk of type-2 diabetes and cardiovascular disease[34]. NAFLD patients are expected to also have a higher risk of vascular and coronary heart disease because of the underlying metabolic disorder. Follow-up mortality rates of NAFLD patients with coronary heart disease were found to be equal to those attributable to cirrhosis[3]. Clinical studies showed that the severity of histopathological features in NAFLD is strongly associated with early carotid atherosclerosis, insulin resistance, and the presence of metabolic syndrome[34]. IMT is marker of early generalized atherosclerosis[35]. A change in the IMT of the carotid artery was a risk factor for atherosclerosis in patients with NAFLD, and a diagnosis of NAFLD was an independent predictor of an increased IMT[36]. NAFLD patients showed increased carotid atherosclerosis with a higher mean IMT and higher prevalence of plaque formation. The present results of functional and histopathological features in the aorta and

carotid artery demonstrated that changes in IMT values appear before plaque formation. The IMT value in the NASH group was greater than that of the control group. This result illustrated that rats in the NASH group had a higher risk of atherosclerosis, and we demonstrated that NASH is a risk factor for atherosclerosis in a rat model of NASH. The decrease in end-diastolic velocity and systolic peak velocity in the aortas of rats in the NASH group illustrated that vessel function had been reduced, including an increase in vascular resistance and reductions in compliance and resilience. These changes may be associated with an increase in arterial IMT in rats with NASH. It was also demonstrated that functional disorder of vessel walls occurred in the early phase of atherosclerosis. These findings are in accordance with the results of Glagov *et al*[37]. The decrease in the IMT value after PTX treatment showed that PTX attenuated early atherosclerosis.

Though the function of RAGE is complicated by the existence of multiple ligands other than AGEs, including proinflammatory cytokines, S100-calgranulins, amphoterin, and fibrillar proteins such as beta-amyloid, it is unquestionable that AGE-RAGE is critical for protein homeostasis in the pathogenesis of diabetic complications and atherosclerosis. Several studies have suggested that the activation of different pathways is dependent upon the ligand and cell type[38]. RAGE, expressed by endothelial cells, smooth muscle cells, and mononuclear phagocytes, is hyperexpressed at sites of vascular injury[39]. There is enhanced expression of RAGE in diabetic vasculopathy and in arteriosclerosis. Engagement of AGEs by cellular RAGE affects critical properties of these cells in a manner that contributes to vascular dysfunction[13]. Chronic hyperglycemia has been considered to accelerate the formation of AGEs in various tissues. Binding of AGEs to RAGE results in the production of cellular oxidants. Hence, we evaluated AGE and RAGE expression in the livers and arteries of rats with NASH. The present study showed that AGE and RAGE expression was significantly increased in the liver arteries in rats with NASH. This finding suggested that the interaction between AGE and RAGE is involved in the pathogenesis of NASH. This mechanism may be related to activation of hepatic stellate cells in NASH. During liver fibrogenesis, RAGE can be up-regulated if hepatic stellate cells are activated to transdifferentiate into myofibroblasts[22], whereas activation of RAGE by AGE reportedly induces various pro-inflammatory responses resulting from the activation of NF-κB, including the expression of vascular cell adhesion molecule-1, TNFα and interleukin-6[40]. The interaction of AGE with RAGE induces the production of reactive oxygen species (ROS) that can stimulate the cascade leading to NF-κB-induced transcriptional events, such as the induction of TNFα and RAGE [41]. Further research on the mechanism of the increase in RAGE expression in NASH is therefore beneficial for the study of NASH pathogenesis.

Apart from the beneficial effects of PTX on microvascular perfusion and the preservation of vascular integrity, PTX possesses another important pharmacological action: PTX has been shown to suppress TNFα production in endotoxin-treated murine macrophages by inhibiting the transcription of the TNF gene. TNFα is an important cytokine in the development of steatohepatitis[6]. Increased expression of TNFα contributes to the development of steatosis, induces inflammatory and fibrogenic responses, and contributes to the progression of NAFLD. *In vitro* and *in vivo* studies have shown that PTX suppresses or reduces the production of TNFα, and beneficial effects of PTX have been reported in NASH[10, 29]. Our previous study demonstrated that PTX inhibited TNFα expression in the livers of rats with NASH fed a high-fat diet[11]. Few data regarding the effect of PTX on RAGE in NASH are available. The present study suggests that PTX treatment may decrease AGE and RAGE expression in the livers and arteries of rats with NASH. It also suggests that AGE-RAGE signaling plays an important role in the mechanism of the therapeutic effects of PTX on NASH. We believe that PTX decreases TNFα levels, probably in part by inhibiting AGE and RAGE expression in the liver and arteries. In addition, a report indicated that PTX has strong inhibitory effects on AGE formation and AGE crosslinking[42]. PTX also has been shown to block the activation of hepatic stellate cells in culture[43, 44]. Therefore, PTX decreases AGE and RAGE expression in the livers and arteries of rats with NASH, probably in part by inhibiting activation of hepatic stellate cells. The mechanism by which PTX decreased RAGE expression in the livers of rats with NASH remains to be determined.

In conclusion, the present study demonstrated that early atherosclerosis in NASH was induced by a high-fat diet in rats. This study is the first to show that AGE and RAGE expression is significantly increased in the livers and arteries of rats with NASH. The interaction between AGE and RAGE has a role in the pathogenesis of NASH in rats. PTX alleviated the hepatic inflammatory response, and hepatic function and vascular lesions were in part associated with a reduction in AGE and RAGE expression in the livers and arteries of rats with NASH.

List of abbreviations

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PTX, pentoxifylline; RAGE, receptor for advanced glycation end products; ALT, alanine aminotransferase; AST, aspartic aminotransferase; GLU, glucose; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; IMT, intima-media thickness.

Acknowledgements

This work was supported by the Beijing Natural Science Foundation of China (No KM201010025006) and the Beijing Municipal Education Commission Research Program (7102013). We are grateful to Xiao-bei ZENG, Qing XU, Hai-mei SUN and Hong-wei SHANG from the Department of Histology and Embryology and Lin-qiao LU from the Department of Pathophysiology at Capital Medical University for their expert technical assistance.

Author contribution

Ying JIANG and Jing WU designed the research; Ying JIANG, Jing WU, Miao-yun ZHAO, Hao ZHENG, and Hua ZHANG collected the primary data; Ying JIANG, Jing WU, and Hao ZHENG wrote the manuscript; and Ying JIANG, Miao-yun ZHAO, and Jing WU revised the manuscript.

References

1 Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002; 346: 1221–31.
2 Neuschwander-Tetri BA,Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. Hepatology 2003; 37: 1202–19.
3 Matteoni CA, Younossi ZM, Gramlich T, Bopari N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology 1999; 116: 1413–9.
4 Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, *et al*. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005; 129: 113–21.
5 Wigg AJ, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxeamia, and tumor necrosis factor alpha in the pathogenesis of nonalcoholic steatohepatitis. Gut 2001; 48: 206–11.
6 Tilg H, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. N Engl J Med 2000; 343: 1467–76.
7 Szczepanik AM, Wordliczek J, Serednicki W, Siedlar M, Czupryna A. Pentoxifylline does not affect nociception if administered postoperatively. Pol J Pharmacol 2004; 56: 611–6
8 Abdel-Salam OME, Baiuomy AR, El-Shenawy SM, Arbid MS. The anti-inflammatory effects of the phosphodiesterase inhibitor pentoxifylline in the rat. Pharmacol Res 2003; 47: 331–40.
9 Akriviadis E, Botla R, Briggs W, Han S, Reynolds T, Shakil O. Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: a double-blind, placebo-controlled trial. Gastroenterology 2000; 119: 1637–48.
10 Adams LA, Zein CO, Angulo P, Lindor KD. A pilot trial of pentoxifylline in nonalcoholic steatohepatitis. Am J Gastroenterol 2004; 99: 2365–8.
11 Zhang L, Jia JD, Zhang H, Wang D, An W. Therapeutic effects of pentoxifylline on rat nonalcoholic steatohepatitis is mediated by lowering tumor necrosis factor alpha. Liver 2007; 12: 261–4.
12 Schmidt AM, Stern DM. Receptor for age (RAGE) is a gene within the major histocompatibility class III region: implications for host response mechanisms in homeostasis and chronic disease. Front Biosci 2001; 6: 1151–60.
13 Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res 2004; 63: 582–92.
14 Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest 2001; 108: 949–55.
15 Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, *et al*. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med 2005; 83: 876–86.
16 Flyvbjerg A, Denner L, Schrijvers BF, Tilton RG, Mogensen TH, Paludan SR, *et al*. Long-term renal effects of a neutralizing RAGE antibody in obese type 2 diabetic mice. Diabetes 2004; 53: 166–72.
17 Gao X, Zhang H, Marie A, Zhang SC. AGE/RAGE produces endothelial dysfunction in coronary arterioles in type 2 diabetic mice. Am J Physiol Heart Circ Physiol 2008; 295: H491–H498.
18 Takeda R, Suzuki, E, Satonaka H, Oba S, Nishimatsu H, Omata M, *et al*. Blockade of endogenous cytokines mitigates neointimal formation in Obese Zucker rats. Circulation 2005; 111: 1398–406.
19 Falcone C, Emanuele E, D'Angelo AP, Buzzi M, Belvito C, Cuccia M, *et al*. Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men. Arterioscler Thromb Vasc Biol 2005; 25: 1032–7.
20 Horiuchi S, The liver is the main site for metabolism of circulating sdvanced glycstion end products. J Hepatology 2002; 36: 123–5.
21 Sebekova K, KupcovaV, Schinzel R, Heidland A. Markedly elevated levels of plasma advanced glycation end products in patients with liver cirrhosis-amelioration by liver transplantation. J Hepatology 2002; 36: 66–71.
22 Fehrenbach H, Weiskirchen R, Kasper M, Gressner AM. Up-regulated expression of the receptor for advanced glycation end products in cultured rat hepatic stellate cells during transdifferentiation to myofibroblasts. Hepatology 2001; 34: 943–52.
23 Jiang Y, Zhang H, Dong LY, Wang D, An W. Increased hepatic UCP2 expression in rats with nonalcoholic steatohepatitis is associated with upregulation of Sp1 binding to its motif within the proximal promoter region. J Cell Biochem 2008; 105: 277–89.
24 Raetsch C, Jia JD, Boigk G, Bauer M, Hahn EG, Riecken EO, *et al*. Pentoxifylline downregulates profibrogenic cytokines and procollagen I expression in rat secondary biliary fibrosis. Gut 2002; 50: 241–7.
25 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from plasma fasting glucose and insulin concentrations in man. Diabetologia 1985; 28: 412–9.
26 Heiss G, Sharrett AR, Barnes R, Chambless LE, Szklo M, Alzola C. Carotid atherosclerosis measured by B-mode ultrasound in populations: associations with cardiovascular risk factors in the ARIC study. Am J Epidemiol 1991; 134: 250–6.
27 Promrat K, Lutchman G, Uwaifo GI. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. Hepatology 2004; 39: 188–96.
28 Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999; 94: 2467–74.
29 Koppe S WP, Sahai A, Malladi P, Whitington PF, Green RM. Pentoxifylline attenuates steatohepatitis induced by the methionine choline deficient diet. J Hepatology 2004; 41: 592–8.
30 Farrell GC, Teoh NC, Mccuskey RS. Hepatic microcirculation in fatty liver disease. Anat Rec 2008; 291: 684–92.
31 Suren A, Bauer FE, Rosenkranz B. Effect of pentoxifylline on liver plasma flow in normal man. Eur J Clin Pharmacol 1991; 41: 233–7.
32 Satapathy SK, Sakhuja P, Malhotra V, Sharma BC, Sarin SK. Beneficial effects of pentoxifylline on hepatic steatosis, fibrosis and necroinflammation in patients with non-alcoholic steatohepatitis. J Gastroenterol Hepatol 2007; 22: 634–8.
33 Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 2003; 37: 917–23.
34 Targher G, Bertolini L, Padovani R, Rodella S, Zoppini G, Zenari L, *et al*. Relations between carotid artery wall thickness and liver histology in subjects with nonalcoholic fatty liver disease. Diabetes Care 2006; 29: 1325–30.
35 O'Leary DH, Polak JF. Intima-media thickness: a tool for atherosclerosis imaging and event prediction. Am J Cardiol 2002; 90: 18–21.
36 Aygun C, Kocaman O, Sahin T, Uraz S, Eminler AT, Celebi A, *et al*.

Evaluation of metabolic syndrome frequency and carotid artery intima-media thickness as risk factors for atherosclerosis in patients with nonalcoholic fatty liver disease. Dig Dis Sci 2008; 53: 1352–7.

37 Glagov S, Weisenberg E, Zarins CK. Compensatory enlargement of human atherosclerotic coronary arteries. N Eng J Med 1987; 316: 371–5.

38 Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H. Coregulation of neurite outgrowth and cell survival by amphoterin and s100 proteins through receptor for advanced glycation end products (RAGE) activation. J Biol Chem 2000; 275: 40096–100.

39 Wendt T, Bucciarelli L, Qu W, Lu Y, Yan SF, Stern DM, Schmidt AM. Receptor for advanced glycation endproducts (RAGE) and vascular inflammation: insights into the pathogenesis of macrovascular complications in diabetes. Curr Atheroscler Rep 2002; 4: 228–37.

40 Valencia JV, Mone M, Zhang J, Weetall M, Buxton FP, Hughes TE. Divergent pathways o gene expression are activated by the RAGE ligands S100b and AGE-BSA. Diabetes 2004; 53: 743–51.

41 De Martin R, Hoeth M, Hofer-Warbinek R, Schmid JA. The transcription factor NF-kappa B and the regulation of vascular cell function. Arterioscler Thromb Vasc Biol 2000; 20: E83–E88.

42 Rahbar S, Natarajan R, Yerneni KK, Scott S, Gonzales N, Nadler LJ. Evidence that pioglitazone, metformin and pentoxifylline are inhibitors of glycation. Clin Chim Acta 2000; 301: 65–77.

43 Lee KS, Cottam HB, Houglum K, Wasson DB, Carson D, Chojkier M. Pentoxifylline blocks hepatic stellate cell activation independently of phosphodiesterase inhibitory activity. Am J Physiol 1997; 273: G1094–100.

44 Hernández E, Bucio L, Souza V, Escobar MC, Gómez-Quiroz LE, Farfán B, *et al*. Pentoxifylline downregulates α (I) collagen expression by the inhibition of IκBα degradation in liver stellate cell. Cell Biol Toxicol 2008; 24: 303–14.