

# Inhibition of aldose reductase ameliorates diet-induced nonalcoholic steatohepatitis in mice via modulating the phosphorylation of hepatic peroxisome proliferator-activated receptor $\alpha$

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**Abstract.** Aldose reductase (AR) is involved in the pathogenesis of nonalcoholic steatohepatitis. This study aimed to determine the mechanism by which AR affects the development of murine diet-induced nonalcoholic steatohepatitis. Steatohepatitis was induced in C57BL/6 mice by administration of a methionine-choline-deficient (MCD) diet, a commonly used nutrition-induced model of steatohepatitis. Hematoxylin and eosin staining was used for histological analyses. Western blot analyses were used to determine protein expression levels and quantitative polymerase chain reaction was used to analyze mRNA expression levels. The results showed that the AR protein expression level was significantly higher in C57BL/6 mice fed the MCD diet than in mice fed the control diet. Diet-induced hepatic steatosis and necroinflammation were attenuated in the MCD diet-fed mice treated with the AR inhibitor, zopolrestat. The ameliorating effect of AR inhibition on steatohepatitis was associated with decreased levels of serum alanine aminotransferase and hepatic lipoperoxides, reduced expression of phosphorylated peroxisome proliferator-activated receptor (PPAR) $\alpha$  and increased mRNA expression of acyl coenzyme A oxidase. These data indicated that induction of hepatic AR expression in mice with steatohepatitis resulted in the phosphorylation of PPAR $\alpha$  and suppression of PPAR $\alpha$  activity. Inhibition of AR decreased lipid accumulation and inflammation in the liver, at least in

part through the modulation of PPAR $\alpha$  phosphorylation and PPAR $\alpha$  transcriptional activity.

## Introduction

Nonalcoholic steatohepatitis (NASH) is a form of metabolic liver disease. The features of NASH on liver biopsy include steatosis, inflammation, liver cell injury and varying degrees of fibrosis (1). Although NASH has become a worldwide public health issue, the underlying causes remain poorly understood. However, it is generally hypothesized that lipid accumulation precedes hepatocellular injury and liver inflammation (2). Peroxisome proliferator-activated receptor (PPAR)  $\alpha$ , a metabolic nuclear receptor, regulates hepatic lipid disposal through direct transcriptional control of genes involved in peroxisomal and mitochondrial  $\beta$ -oxidation pathways, fatty acid uptake and triglyceride catabolism (3). It is hypothesized that PPAR $\alpha$  is involved in the development of NASH, and it has been shown that treatment with PPAR $\alpha$  agonists improves liver lipid accumulation and inflammation (4-6).

Aldose reductase (also termed AR, AKR1B1 and EC1.1.1.21) catalyzes the rate-limiting reduction of glucose to sorbitol with the aid of co-factor nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (7). The role of AR in the development of diabetic complications is well-established. However, AR expression has been found to be induced in certain tissues in conditions other than diabetes. Notably, hepatic AR is induced in several types of liver disease, including alcoholic liver disease, hepatitis, cirrhosis and hepatocellular carcinoma (8-10). This indicates that AR is involved in the development of hepatitis and fibrosis. We previously reported that AR regulated PPAR $\alpha$  phosphorylation and activity, thus influencing lipid homeostasis, and that inhibition of AR improved hepatic steatosis in db/db diabetic mice (11,12). This suggested that inhibition of AR may curtail the development of NASH. The present study aimed to investigate the effect of AR inhibition on the development of nutrition-induced murine NASH in C57BL/6 mice fed a

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methionine-choline-deficient (MCD) diet and to investigate the mechanism underlying the effects of AR on the accumulation of lipid in the liver.

## Materials and methods

**Animal experiments.** All experiments were conducted according to protocols and guidelines approved by Longyan University Institutional Animal Care and Use Committee (Longyan, China). C57BL/6 mice were obtained from The Shanghai Laboratory Animal Center (Shanghai, China). All animals were maintained under a 12/12-h light/dark cycle. Male mice, 7–8 weeks of age, were randomly divided into four experimental groups (each containing six animals), namely, control diet-fed mice, control diet-fed mice + zopolrestat (zopol), MCD diet-fed mice, MCD diet-fed mice + zopol. The control diet was identical to the MCD diet (MP Biomedicals, Aurora, OH) but supplemented with DL-methionine (3 g/kg) and choline chloride (2 g/kg). Mice were administered with 50 mg/kg body weight/day zopol as a single daily intraperitoneal injection for 4 weeks. The same volumes of saline were also administered to the other groups of mice as a control.

**Quantitative analyses of mRNA expression by quantitative polymerase chain reaction (qPCR).** Total RNA was isolated from tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from hepatic mRNA using RevertAid First Strand cDNA synthesis kits (Fermentas, Vilnius, Lithuania). Hepatic PPAR $\alpha$ , acyl coenzyme A oxidase (ACO), liver fatty acid binding protein (L-FABP) and carnitine palmitoyl transferase-1 (CPT-1) were analyzed with the specific primers listed in Table I. Quantitative PCRs were assayed using the FastStart Universal SYBR Green Master mix (Rox; Roche Applied Science, Mannheim, Germany). The reaction was run at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 55–58°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 10 min. Each Ct value was normalized to  $\beta$ -actin.

**Western blot analyses.** Tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer (Beyotime, Haimen, China). Each protein sample (40  $\mu$ g) was loaded and separated on a 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Blotted membranes were then incubated with goat polyclonal anti-AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500), rabbit polyclonal anti-PPAR $\alpha$  (Santa Cruz Biotechnology; 1:500) or rabbit polyclonal anti-phospho-PPAR $\alpha$  (Abcam, Cambridge, UK; 1:1000) in Tris-buffered saline with 0.1% Tween-20 (TBST) and 5% non-fat milk at 4°C overnight. After several washes, the membranes were incubated with horseradish peroxidase-conjugated monoclonal anti-goat IgG or anti-rabbit IgG (Sigma, St. Louis, MO, USA; 1:2000) in TBST and 5% non-fat milk. Detection was achieved using the Supersignal chemiluminescent substrate kit (Pierce, Rockford, IL, USA).

**Histological examination.** Formalin-fixed liver tissue was processed and 5- $\mu$ m-thick paraffin sections were stained

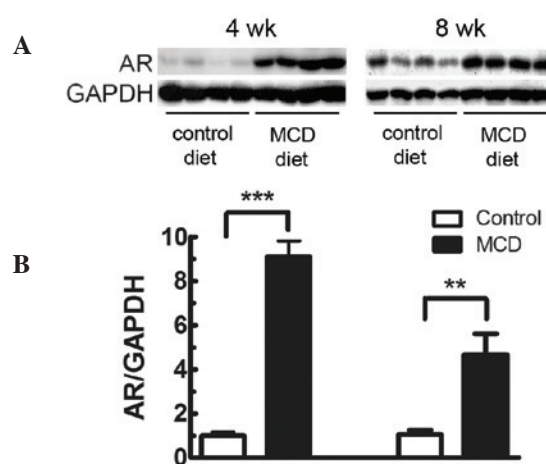


Figure 1. AR is induced in the MCD diet-fed C57BL/6 mice. (A) Representative western blots show the induction of hepatic AR protein expression in MCD diet-fed mice. (B) Average densitometric analyses of AR were calculated as fold increases over the control diet (n=4); values are expressed as the mean  $\pm$  standard error of the mean \*\*\*P<0.001 and \*\*P<0.01. MCD, methionine-choline-deficient.

with hematoxylin and eosin (H&E) for histological analyses. A hepatopathologist who was blinded to the experimental conditions examined all sections for steatosis and inflammation as previously described (13). Hepatic steatosis was graded according to the percentage of lipid-laden hepatocytes as 0, 0%; 1, 1–33%; 2, 34–67%; or 3, 68–100%. Hepatic necroinflammation were scored from 0 to 3, as follows: 0, no inflammatory foci; 1, mild; 2, moderate; and 3, severe.

**Tissue and serum biochemical measurements.** Serum alanine aminotransferase (ALT) was measured using an IDEXX analyzer (IDEXX Laboratories, Inc., Westbrook, ME, USA). Total lipoperoxides were measured as thiobarbituric acid reactive substances (TBARS) in 100  $\mu$ l of liver homogenate using Lipid Peroxidation Assay kits (Beyotime). TBARS were quantified using malondialdehyde as a standard. Liver lipid was extracted using chloroform/methanol. Briefly, pulverized liver was homogenized in phosphate-buffered saline (PBS), then extracted with chloroform/methanol (2:1), dried overnight and resuspended in a solution of 60% butanol and 40% Triton X-114/methanol (2:1). The liver triglyceride level was measured using colorimetric assays (Sigma).

**Statistical analysis.** All data were processed and analyzed using the GraphPad software Prism 5.0 (GraphPad Software, Inc., Chicago, IL, USA) and are expressed as the mean  $\pm$  standard error of the mean. Student's t-test was used for pair-wise comparisons and one-way analysis of variance with Bonferroni's Multiple Comparison test was used for multi-group analyses. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Inhibition of AR attenuates diet-induced hepatic steatosis and inflammation.** Feeding mice with a lipogenic MCD diet was previously shown to be capable of inducing a liver injury similar

Table I. Primer sequences used for the amplification of mRNA by quantitative polymerase chain reaction (qPCR).

Gene	Forward primer	Reverse primer
PPAR $\alpha$	5'-AAGAGGGCTGAGCGTAGGT-3'	5'-GGCCGGTTAAGACCAGACT-3'
ACO	5'-CCACATATGACCCCAAGACC-3'	5'-AGGCATGTAACCCGTAGCAC-3'
CPT-1	5'-GTCAAGCCAGACGAAGAACA-3'	5'-CGAGAAGACCTTGACCATAG-3'
L-FABP	5'-GTGGTCCGCAATGAGTTTAC-3'	5'-GTATTGGTGATTGTGTCTCC-3'
$\beta$ -actin	5'-CTATTGGCAACGAGCGGTTCC-3'	5'-GCACTGTGTTGGCATAGAGGTC-3'

PPAR, peroxisome proliferator-activated receptor; ACO, acetyl CoA oxidase; CPT-1, carnitine palmitoyl transferase-1; L-FABP, liver fatty acid binding protein.

Table II. Effect of ARI treatment on scores for hepatic steatosis and necroinflammation.

Measurement	Controls	Controls + ARI	MCD	MCD + ARI
Steatosis	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	2.20 $\pm$ 0.29	1.15 $\pm$ 0.10 <sup>a</sup>
Necroinflammation	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.95 $\pm$ 0.19	0.19 $\pm$ 0.10 <sup>a</sup>

Severity of hepatic steatosis and necroinflammation were scored as described in the Materials and methods section. Values are presented as the mean  $\pm$  standard error of the mean (n=4 per group). <sup>a</sup>P<0.01, compared with the MCD diet-fed mice. ARI, aldose reductase inhibitor; MCD, methionine-choline-deficient.

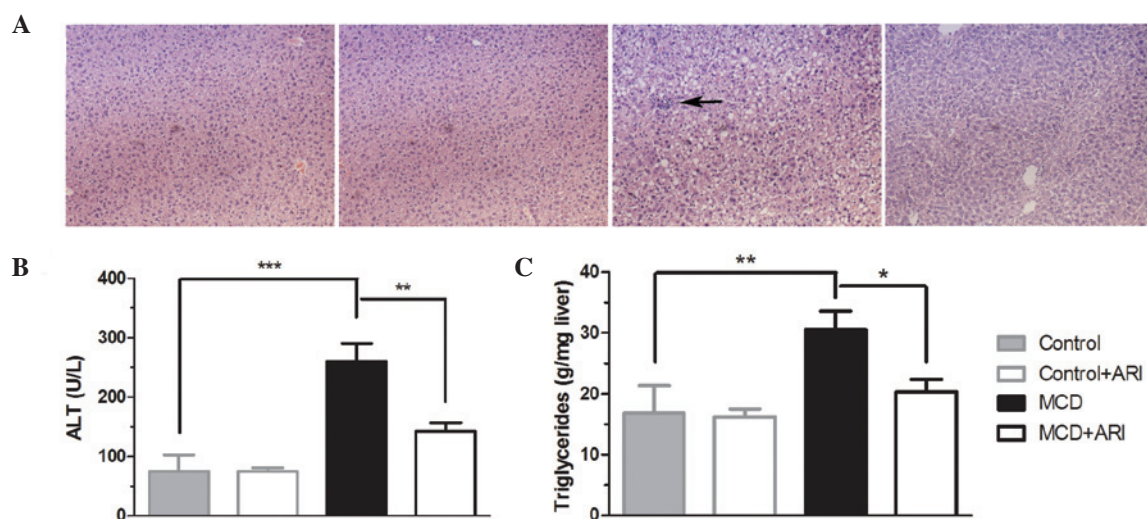


Figure 2. AR inhibitor treatment improved the MCD diet-induced steatohepatitis. (A) Hematoxylin and eosin-stained liver sections from: (Aa) Control diet-fed mice, (Ab) control diet-fed mice + zopol, (Ac) MCD diet-fed mice and (Ad) MCD diet-fed mice + zopol. Arrows point to foci of necroinflammation. Slides are representative of four separate experiments (original magnification,  $\times 100$ ). (B and C) Effect of AR inhibitor treatment on serum ALT levels and hepatic triglyceride levels in C57BL/6 mice fed MCD diet for 4 weeks (n=6). Values are expressed as the mean  $\pm$  standard error of the mean. \*\*\*P<0.001, \*\*P<0.01 and \*P<0.05. MCD, methionine-choline-deficient; AR, aldose reductase; ALT, alanine transaminase.

to human NASH. MCD-fed mice are therefore a useful small animal model of this disease (6,14). As an initial step in investigating whether AR was involved in the development of MCD diet-induced steatohepatitis, protein expression levels of hepatic AR in C57BL/6 mice fed the MCD diet were measured. As shown in Fig. 1, hepatic AR protein expression in mice fed the MCD diet for 4 weeks was 9.11-fold higher (P<0.001) than that in mice fed the control diet. The elevation of hepatic AR was also observed after feeding the MCD diet for 8 weeks (4.68-fold; P<0.01). To further investigate the role of AR in the development

of diet-induced steatohepatitis, MCD diet-fed mice were treated with zopol, an inhibitor of AR, for 4 weeks. As shown in Fig. 2A and Table II, examination of H&E-stained sections demonstrated marked steatosis and lobular inflammation in mice fed the MCD diet for 4 weeks, while mice fed the control diet did not exhibit significant histological steatosis or inflammation. Treating MCD diet-fed mice with zopol significantly improved the levels of hepatic steatosis and inflammation. Consistent with these histological findings, AR inhibition reduced serum ALT by 44.8% (P<0.01; Fig. 2B) and reduced liver triglycerides by

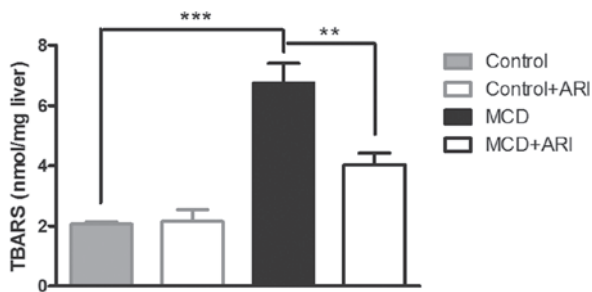


Figure 3. AR inhibition attenuated the increase of hepatic lipoperoxide contents in C57BL/6 mice fed the MCD diet. Hepatic lipoperoxide contents were determined in mice fed the control diet or the MCD diet for 4 weeks (n=4). Values are expressed as the mean  $\pm$  standard error of the mean. \*\*\*P<0.001 and \*\*P<0.01. MCD, methionine-choline-deficient; AR, aldose reductase; TBARS, thiobarbituric acid reactive substances.

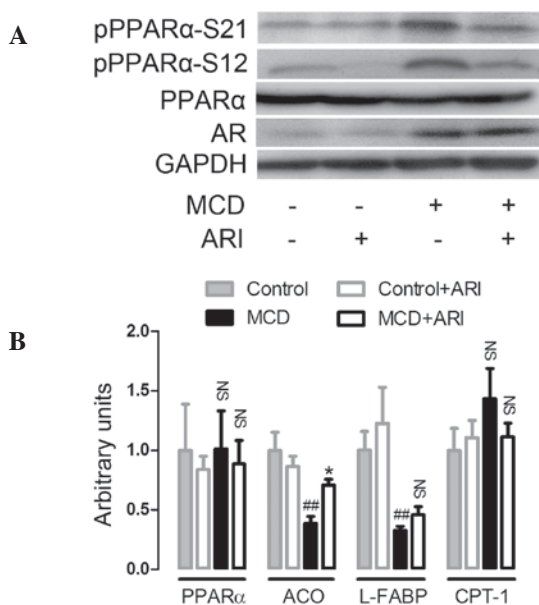


Figure 4. AR inhibition increased hepatic PPARα transcriptional activity in MCD diet-fed C57BL/6 mice. (A) The effects of AR inhibition on PPARα expression and phosphorylation in mice fed the MCD diet for 4 weeks. Western blotting was performed as described in the Materials and methods section (n=3). (B) Hepatic mRNA expression of PPARα, ACO, L-FABP and CPT-1 analyzed by quantitative polymerase chain reaction, standardized against an internal control (β-actin) and are expressed as fold differences compared with values obtained in mice fed the control diet (n=4). Values are expressed as the mean  $\pm$  standard error of the mean. \*\*P<0.01, \*P<0.05, compared with mice fed the control diet. NS, no significant difference; PPAR-α, peroxisome proliferator-activated receptor-α; pPPARα S12, phosphoserine-12 PPARα; pPPARα S21, phosphoserine-21 PPARα; MCD, methionine-choline-deficient; AR, aldose reductase; ACO, acetyl coenzyme A oxidase; L-FABP, liver fatty acid binding protein; CPT-1, carnitine palmitoyl transferase-1.

33.4% (P<0.05; Fig. 2C) compared with levels in mice on the MCD diet. These data indicate that AR inhibitor treatment ameliorates the development of MCD diet-induced steatohepatitis in C57BL/6 mice.

This study also investigated the effect of AR inhibition on hepatic lipoperoxide production. As shown in Fig. 3, intake of the MCD diet for 4 weeks resulted in a prominent increase in the hepatic TBARS level, compared with those in mice administered the control diet. This increase was attenuated when the

MCD-fed mice were also administered zopol (P<0.01). These data suggest that hepatic AR elevation may exacerbate the MCD diet-induced oxidative stress in the livers of mice with steatohepatitis and that inhibition of AR may reverse this process.

*AR inhibition suppresses the MCD diet-induced phosphorylation of hepatic PPARα and increases its activity.* PPARα is an important metabolic nuclear receptor that regulates lipid metabolism (3). Activation of PPARα decreases hepatic steatosis in MCD diet-fed C57BL/6 mice (5,6). To clarify the mechanism whereby AR exacerbates the hepatic steatosis in MCD diet-fed mice, the effect of AR inhibition on the activity of PPARα was investigated. PPARα is a phosphoprotein and phosphorylation is one of the most rapid and efficient mechanisms through which its activity can be modulated. Firstly, the protein expression level and the phosphorylation of PPARα was analyzed. As shown in Fig. 4A, no significant differences were identified in hepatic PPARα protein expression amongst all four groups of mice. Western blot analysis using antibodies that recognize phospho-PPARα at either serine 12 (S12) or serine 21 (S21), two major phosphorylation sites located at the A/B domain (AF-1) of PPARα, showed that the phosphorylation of PPARα at these sites was markedly increased in the MCD diet-fed mice compared with the levels in mice on the control diet. Furthermore, this induction of PPARα phosphorylation was suppressed in ARI-treated mice. In addition, with the phosphorylation of PPARα, hepatic mRNA expression of ACO and L-FABP, two target genes regulated by PPARα, were downregulated in mice fed the MCD diet. Whilst the downregulation of ACO mRNA expression was reversed in ARI-treated mice, the downregulation of L-FABP was not significantly altered (Fig. 4B). mRNA expression of CPT-1, another target gene regulated by PPARα, was not significantly altered in all four groups of mice. These data indicate that administration of an MCD diet resulted in the phosphorylation of hepatic PPARα, which suppressed PPARα activity. The results also suggest that AR inhibition attenuated the MCD diet-induced phosphorylation of PPARα and thus suppression of its activity.

## Discussion

AR induction has been observed in a number of liver diseases, including alcoholic liver disease, chronic hepatitis B and C, and hepatocellular carcinoma in humans, and in hereditary hepatitis in rats (8-10). We previously demonstrated that hepatic AR induction in db/db mice with diet-induced steatohepatitis contributed to the development of liver inflammation and fibrosis (15). However, the importance of AR in the development of hepatic steatosis in db/db mice with steatohepatitis was not established. The present study demonstrated that hepatic AR protein levels were also increased in MCD diet-induced steatohepatitis in C57BL/6 mice. It also showed that the induction of AR protein resulted in an increase in PPARα phosphorylation and thus led to the accumulation of lipid in the liver. Furthermore, AR inhibition ameliorated MCD diet-induced hepatic steatosis and inflammation, suppressed the phosphorylation of PPARα and increased its transcriptional activity. The results therefore indicate that AR may affect the development of hepatic steatosis by modulating the phosphorylation of PPARα and suppressing the activity of this protein.



Notably, the current study demonstrated the beneficial effect of pharmacological inhibition of AR on hepatic steatosis whereas a previous study did not report a significantly beneficial effect of genetic ablation of AR, which results in complete loss of activity, on hepatic steatosis in the same rodent model (15). One possible explanation for the discrepancy between complete AR deficiency and ARI treatment is the possibility that any of a number of AR-related enzymes, such as mouse vas deferens protein, mouse fibroblast growth factor regulated protein and AR-like-1, may be upregulated to compensate for the AR deficiency. These AR-like enzymes have a number of the same functions as AR. However, little data is available regarding the significance of these enzymes in lipid disorders. Further studies are required using genetic ablation of AR-like enzymes in order to investigate their involvement in hepatic steatosis.

It is well established that PPAR $\alpha$  is a central regulator of hepatic lipid catabolism. The ablation or inhibition of PPAR $\alpha$  causes the development of a number of lipid disorders including hepatic steatosis and non-alcoholic fatty liver disease (16,17). Post-translational modification by phosphorylation is one of the most important mechanisms whereby PPAR $\alpha$  transcriptional activity is modulated (18,19). Multiple phosphorylation sites have been identified on different domains of mouse PPAR $\alpha$ , which include the A/B domain, the DNA-binding domain and the ligand-binding domain. Further, PPAR $\alpha$  phosphorylation was shown to be catalyzed by a diverse group of kinases, including protein kinase A, protein kinase C, extracellular signal-regulated kinases and glycogen synthase kinase. Depending on the types of cells and stimuli involved, phosphorylation can either lead to activation or inactivation of PPAR $\alpha$  (19-21). In the current study, it was demonstrated that MCD diet-induced PPAR $\alpha$  phosphorylation at S12 and S21 contributed to suppression of PPAR $\alpha$  transcriptional activity in the mouse liver and that AR inhibition attenuated this PPAR $\alpha$  phosphorylation, which may contribute to the amelioration of diet-induced steatosis. However, further studies are required to determine the signaling pathways involved in the MCD diet-induced phosphorylation of PPAR $\alpha$ .

The molecular mechanisms involved in progression from liver steatosis to NASH remain unclear. However, oxidative stress is a possible candidate (22,23). 4-Hydroxynonenal (4HNE) is a cytotoxic byproduct of lipid peroxidation that is hypothesized to participate in the pathogenesis of a number of diseases (24). In addition to glucose, AR can catalyze the reduction of a number of aldehydes and carbonyls, including 4HNE (25). Thus, AR has been postulated to serve a cytoprotective function by rapidly detoxifying aldehydes. *In vitro* studies have shown that AR expression is induced by 4HNE in rat vascular smooth muscle cells and that inhibition of AR sensitizes cells to 4HNE cytotoxicity (26). Furthermore, an *in vivo* study showed that inhibition of AR was associated with increased numbers of apoptotic cells and increased 4HNE content in the arterial wall of a murine model of giant cell arteritis (27). However, AR inhibitors have also been reported to exert beneficial effects on injuries in a number of rodent models, including allergic airway inflammation, ischemic myocardial injury, arterial balloon injury and uveitis (28-31). The current study demonstrated that zopol, an

AR inhibitor, attenuated MCD diet-induced oxidative stress and improved liver inflammation. This provides further evidence of the beneficial effect of AR inhibitors on inflammation.

In conclusion, the present study demonstrated the protective effect of an AR inhibitor against MCD diet-induced hepatic steatosis and liver damage. This effect was mediated, at least in part, through modulation of the phosphorylation of PPAR $\alpha$  and its transcriptional activity.

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