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ORIGINAL ARTICLE

Beneficial effects of mineralocorticoid receptor blockade in experimental non-alcoholic steatohepatitis

Margarita Pizarro[1], Nancy Solís[1], Pablo Quintero[1], Francisco Barrera[1], Daniel Cabrera[1,2], Pamela Rojas-de Santiago[1], Juan P. Arab[1], Oslando Padilla[3], Juan C. Roa[4], Han Moshage[5], Alexander Wree[6], Eugenia Inzaugarat[6], Ariel E. Feldstein[6], Carlos E. Fardella[7], Rene Baudrand[7], Arnoldo Riquelme[1] and Marco Arrese[1]

1 Departamento de Gastroenterología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile
2 Departamento de Ciencias Químico-Biológicas, Universidad Bernardo O Higgins, Santiago, Chile
3 Departamento de Salud Pública, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile
4 Departamento de Patología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile
5 Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
6 Department of Pediatrics, University of California, San Diego, CA, USA
7 Departamento de Endocrinología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

Keywords
fatty liver – fibrosis – inflammation – NASH – steatohepatitis

Abbreviations
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CDAA, choline-deficient and amino acid-defined; Col1a1, collagen type I, alpha 1; CSAA, choline-supplemented L-amino acid-defined; FDA, Food and Drugs Administration; GADPH, Glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; Hep, Hepatocytes; Hox-1, haem-oxigenase 1; HSC, hepatic stellate cells; HTC, hepatic triglyceride content; iNOS, inducible nitric oxide synthase; IR, insulin resistance; KC, Kupffer cells; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NRF2, Nuclear factor (erythroid-derived 2)-like 2; PCR, Polymerase chain reaction; SREBP1c, sterol response element binding protein 1c; TGF-β, transforming growth factor beta; Timp-1, tissue inhibitor of metalloproteinase 1; TNF-α, tumour necrosis factor alpha; VAT, visceral adipose tissue; α-SMA, alpha smooth muscle actin.

Correspondence
Marco Arrese, MD, Departamento de Gastroenterología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Marcoleta #367, 833-0024 Santiago, Chile
Tel: 56 2 6397780
Fax: 56 2 6397780
e-mail: marrese@med.puc.cl

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Abstract

Background: Therapeutic options to treat Non-alcoholic steatohepatitis (NASH) are limited. Mineralocorticoid receptor (MR) activation could play a role in hepatic fibrogenesis and its modulation could be beneficial for NASH. *Aim:* To investigate whether eplerenone, a specific MR antagonist, ameliorates liver damage in experimental NASH. *Methods:* C57bl6 mice were fed a choline-deficient and amino acid-defined (CDAA) diet for 22 weeks with or without eplerenone supplementation. Serum levels of aminotransferases and aldosterone were measured and hepatic steatosis, inflammation and fibrosis scored histologically. Hepatic triglyceride content (HTC) and hepatic mRNA levels of pro-inflammatory pro-fibrotic, oxidative stress-associated genes and of MR were also assessed. *Results:* CDAA diet effectively induced fibrotic NASH, and increased the hepatic expression of pro-inflammatory, pro-fibrotic and oxidative stress-associated genes. Hepatic MR mRNA levels significantly correlated with the expression of pro-inflammatory and pro-fibrotic genes and were significantly increased in hepatic stellate cells obtained from CDAA-fed animals. Eplerenone administration was associated to a reduction in histological steatosis and attenuation of liver fibrosis development, which was associated to a significant decrease in the expression of collagen-α1, collagen type III, alpha 1 and Matrix metalloproteinase-2. *Conclusion:* The expression of MR correlates with inflammation and fibrosis development in experimental NASH. Specific MR blockade with eplerenone has hepatic anti-steatotic and anti-fibrotic effects. These data identify eplerenone as a potential novel therapy for NASH. Considering its safety and FDA-approved status, human studies are warranted.

Key Points

- Mineralocorticoid receptor (MR) activation play a role in hepatic fibrogenesis and its modulation could be beneficial for non-alcoholic steatohepatitis (NASH).
- Hepatic MR mRNA levels correlate with the expression of pro-inflammatory and pro-fibrotic genes in a dietary model of NASH.
- The present work shows that specific MR blockade with eplerenone has hepatic anti-steatotic and anti-fibrotic effects in experimental NASH.
- These data identify eplerenone as a potential novel therapy for NASH.

The acronym non-alcoholic fatty liver disease (NAFLD) refers to a spectrum of liver abnormalities ranging from isolated steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by steatosis plus necro-inflammatory changes and various degrees of hepatic fibrosis (1, 2). Nowadays, NAFLD is considered the most common form of liver disease worldwide affecting 25–30% of the general population (3, 4). NAFLD has a high prevalence among patients with diabetes and obesity and is almost universally present among morbidly obese diabetic patients (5, 6) and is also considered the hepatic manifestation of the metabolic syndrome (7). The clinical relevance of this condition lays in its association with an increased liver-related mortality because of the progression to cirrhosis and hepatocellular carcinoma of a variable proportion of patients mainly those with NASH (8). In addition to lifestyle modifications, many pharmacological therapeutic options aiming to halt disease progression by decreasing hepatic inflammation and/or fibrosis have been studied. However, the therapeutic armamentarium to treat NASH is currently rather limited and only vitamin E and pioglitazone are recommended in selected patients although its long-term benefit has not been demonstrated (9).

As in other liver diseases, the presence and severity of fibrosis is closely related to both overall and liver-related mortality in patients with NAFLD (10). Thus, effective anti-fibrotic compounds would be likely beneficial in this condition. The important advances in the knowledge of the mechanisms underlying hepatic fibrogenesis (11) allows to explore different pathways as potential targets for NASH in pre-clinical models. Among the pathways with potential to be targeted in the liver, the activation of the mineralocorticoid receptor (MR), which has been explored as a relevant target for modulating fibrosis development in other organs such as heart and kidney, remains insufficiently explored (12). Experimentally, it has been shown that aldosterone may be produced locally during hepatic fibrogenesis and contribute significantly to organ fibrosis since MR receptor blockade with spironolactone significantly reduces collagen deposition (13). Interestingly, local activation of the MR in the liver could not only be related to aldosterone but also to the activation by other steroids such as glucocorticoids. In fact, cortisol (corticosterone in rodents) is another important physiological ligand of MR having the same affinity for the MR. This could be relevant in the setting of NAFLD where increased local cortisol production and portal hypercortisolism has been described (14, 15) and a dysregulation of MR expression in the adipose tissue has been found (16). Thus, MR blockade could be a potential therapeutic strategy to treat NAFLD. MR blockade is commonly achieved clinically with spironolactone but is long-term use is frequently associated to several unwanted effects. Thus, newly agents, such as eplerenone, has been recently developed and designed to enhance selective binding to the MR avoiding adverse effects related to the long half-life of spironolactone and the action of these compounds on androgen, glucocorticoid and progesterone receptors in various tissues (17). Eplerenone is approved by the FDA to treat hypertension and cardiac failure after an acute myocardial infarction and has a good safety profile. In this study, we aimed to examine the effects of eplerenone administration on the development of liver injury in a diet-induced murine model of NASH. The choline-deficient and amino acid-defined (CDAA) diet was used since it has been shown to mimic the features of human liver injury including hepatic steatosis and inflammation as well as liver fibrosis and hepatic stellate cells (HSC) activation as observed in human NASH (18).

Methods

Animals and diets

The use and care of the animals were reviewed and approved by the local institutional animal care and use committee. Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were aged 10 weeks at the beginning of this study and divided into four experimental groups (n = 7–10) receiving either the CDAA diet (Catalog # 518753; Dyets Inc., Bethlehem, PA, USA) to induce NASH or the choline-supplemented L-amino acid-defined (CSAA, Catalog # 518754; Dyets Inc.) diet as control with or without eplerenone (Pfizer Pharmaceuticals, Caguas, Puerto Rico) supplementation at a dose of 1 mg/g of diet as described (19). Each group of animals were housed in transparent polycarbonate cages subjected to 12 h light/darkness cycles under a temperature of 21°C and a relative humidity of 50%. Feeding and eplerenone administration lasted for 22 weeks without any interruption. After ending the feeding course, mice were euthanized by exsanguination and serum, liver

and visceral adipose tissue (VAT) samples were collected and processed or stored at −80°C until analysed. In separate experiments specified pathogen-free male Wistar rats [purchased from Charles River Laboratories Inc. (Wilmington, MA, USA)] were used for Hepatocyte and Kupffer cell isolation as described in the supporting information files.

Biochemical determinations

Serum aspartate-aminotransferase (AST) and alanine aminotransferase (ALT) were quantized with a commercial kit from Kovalent Ltd. (Río de Janeiro, Brazil). Serum aldosterone was assessed with Alpha Diagnostic International ELISA kit (San Antonio, TX, USA). Hepatic triglyceride content (HTC) was assessed according to Carr *et al.* (20).

Histological studies

Liver sections from the right lobe of all mouse livers were routinely fixed in 10% formalin and embedded in paraffin. Then 4 μm tissue sections were stained with haematoxylin/eosin, 0.1% picrosirius red solution and Oil Red-O as described. Immunohistochemical staining for α-smooth muscle actin (α-SMA; Dako, Glostrup, Denmark) and MR1 (Abcam, Cambridge, MA, USA) were also performed in formalin-fixed, paraffin-embedded liver sections according to the Histostain®-Plus 3rd Gen IHC Detection Kit (Invitrogen, Carlsbad, CA, USA), the reaction was developed using a high-sensitivity substrate-chromogen system for use in peroxidase-based immunohistochemical. To improve the immunohistochemistry performance, endogenous peroxidase and biotin were blocked before the immunohistochemistry staining. To avoid background by using the mouse anti MR1 antibody, endogenous mouse immunoglobulins were blocked using the Vector® M.O.M.™ Kit (Vector, Burlingame, CA, USA). Nuclei were counterstained with Haematoxylin. A blind investigator assigned a score for steatosis and inflammation as described (21). Scores were given as it follows: Steatosis: grade 0, none present; grade 1, steatosis of ≤25% of parenchyma; grade 2, steatosis of 26–50% of parenchyma; grade 3, steatosis of 51–75% of parenchyma; grade 4, steatosis of ≥76% of parenchyma and inflammation: grade 0, no inflammatory foci; grade 1, 1–5 inflammatory foci per high power field; grade 2, >5 inflammatory foci/high power field. Liver fibrosis was quantified using digital image analysis of the red-stained area in Sirius red-stained samples (IMAGEJ; NIH, Bethesda, MD, USA).

Quantitative real-time PCR analysis

RNA was isolated from liver samples using SV Total RNA Isolation System (Promega, Madison, WI, USA) and quantified spectrophotometrically in a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed with one microgram of total RNA, then it was reverse transcribed in 25 μl total volume (Improm II system; Promega) and 150 pmol random hexamers according to the manufacturer's guidelines. The reaction was terminated by heating the cDNA to 70°C for 5 min. We measured the hepatic expression of key genes of hepatic inflammation (tumour necrosis factor alpha [TNF-α] and monocyte chemoattractant protein-1 [MCP-1]) as well as hepatic mRNA levels of fibrogenic genes such as collagen-α1 (Col1a1), α-SMA, tissue inhibitor of metalloproteinase-1 (Timp-1), transforming growth factor beta (TGF-β), Collagen alpha-1(III), (Col3A1) and Matrix metalloproteinase-2 (MMP-2). We also assessed genes related to ongoing oxidative stress such as the nuclear factor (erythroid-derived 2)-like 2 (NRF2), haem-oxigenase 1 (Hox-1), glutathione reductase-1 (GSH Reductase 1), glutathione synthase (GSH synthase). In addition, we assessed the hepatic expression of MR (official full name: nuclear receptor subfamily 3, group C, member 2, Nr3c2). All probes were obtained from Applied Biosystems (Foster City, CA, USA). The relative amounts of all mRNAs were calculated using the comparative threshold cycles (ΔC_T) method and normalized to 18S RNA as an internal control.

Isolation of hepatocytes, Kupffer cells and hepatic stellate cells

In additional experiments we sought to assess the expression of MR in different liver cell populations. To that end, hepatocytes, Kupffer cells (KC) and HSC were simultaneously obtained from control 20 week-old C57BL/6 mice and both CSAA and CDAA-fed mice by in situ perfusion followed by density gradient cell separation as previously described (22). Cells were harvest after a 24 h cultivation period, total RNA was isolated, and reverse transcript was synthesized as described above. Expression levels were calculated using the 2-ΔΔCt method using following primer pairs: MR F-5′-GAAGAGCCCCTCTGTTTGCAG-3′ R-5′- TCCTTGAG TGATGGGACTGTG-3′ and GADPH F-5′-TGGAAAG CTTGTGCGTGAT-3′ R-5′-TGCTTCACCACCTTGTT GAT-3′. We also assessed the effects of eplerenone on hepatocytes and KC isolated from livers harvested from Wistar rats as described in the supportive information files.

Statistical analysis

Shapiro-Wilk test was applied to determine if the variables were parametric (P value >0.05) or non-parametric (P value <0.05). Accordingly, non-parametric variables were expressed as median (Q1–Q3) unless specified. Data between groups were analysed by using Mann–Whitney U-test or Kruskal–Wallis to compare

more than two groups. *Post hoc* Bonferroni analysis was carried out for correction of multiple comparisons. Percentages were used for histological variables and chi-square was used to compare groups. Spearman test was used to determine correlations between hepatic MR expression and expression of pro-inflammatory and pro-fibrotic genes. A P value of less than 0.05 was considered statistically significant in primary analysis and $P < 0.017$ was considered statistically significant in *post hoc* Bonferroni analysis.

Results

CDAA diet feeding induces fibrotic NASH and up-regulation of the mineralocorticoid receptor in HSC

Table 1 shows the anthropometric and serum parameters after 22-week period of experimental diet feeding. CDAA feeding was associated to an increased liver weight, elevated serum levels of aminotransferases and greater HTC compared to CSAA fed animals. As previously described (18, 23) and shown in Figure 1A, CDAA diet feeding for 22 weeks resulted in a florid histological picture resembling human NASH and characterized by severe steatosis, inflammatory cell infiltration, hepatocytes ballooning and development of hepatic fibrosis. Also, α-SMA-positive cells appeared in the perisinusoidal space was associated to the presence of some fibrous septa in CDAA diet-fed mice indicating activation of HSC. Although mild steatosis was caused by the control CSAA diet, this diet induced neither liver inflammation nor fibrosis. In agreement with the histological picture of NASH, the expression of both pro-inflammatory (MCP-1 and TNF-α) and pro-fibrotic (Col1a1, Timp-1 as well as Col III and MMP-2) markers as well as the expression of oxidative stress-associated genes (GSH reductase 1, GSH synthase and NRF2) were significantly increased in CDAA-fed mice (Fig. 2). Although we only observed a trend to a higher expression of MR in CDAA-fed mice, a positive correlation was found between the hepatic mRNA levels of MR and the expression of both pro-inflammatory and pro-fibrotic genes (Fig. 3).

Eplerenone decreases steatosis and fibrosis in CDAA diet-induced NASH

As shown in Table 1, eplerenone administration was associated to a slightly decreased weight gain during the experimental period which was reflected in a lower final weight in animals receiving the compound. This reached significance only in the CDAA + eplerenone group and was not associated to differences in food intake among groups (mean food intake approximately 2.8 g/day). In addition, eplerenone administration prevented the increase in liver weight observed in CDAA diet-fed mice and was associated to a lower HTC although the latter did not reach statistical significance. As expected when blocking MR, eplerenone administration increased serum levels of aldosterone in both CSAA and CDAA diet-fed mice. Histological scoring of steatosis and inflammation in the different experimental groups are shown in Figure 1B. Consistent with the decrease in liver size, livers from eplerenone-treated mice exhibited a decrease in liver steatosis although no effect of eplerenone administration was seen on liver inflammation. In addition, levels of liver fibrosis assessed by Sirius red staining were reduced markedly in CDAA diet-fed mice supplemented with eplerenone as illustrated in Figure 1C. Analysis of the hepatic gene expression in the different groups showed that eplerenone did not influence mRNA levels of pro-inflammatory genes (Fig. 2A) but efficiently attenuated the CDAA diet-induced up-regulation of fibrosis markers Col1a1, Col3A1 and MMP-2 (Fig. 2B). The observed increase in oxidative stress-associated genes in CDAA diet-fed mice was mostly not influenced by eplerenone administration (Fig. 2C).

Studies in liver cell sub-populations

To explore if mRNA levels of the MR are present in different hepatic cells we isolated hepatocytes, KC and HSC and quantified expression of MR via qPCR determinations. MR was detected in the three cell types being slightly lower in HSC in comparison with both hepatocytes and KC (data not shown). In addition, we assessed the MR expression in hepatocytes and HSC

Table 1. Anthropometric and serum parameters in experimental groups

	CSAA	CSAA + EPLERENONE	CDAA	CDAA + EPLERENONE
Final body weight (g)	39.3 (37–41.6)	35.4 (34.2–36)[a]	35.8 (33.7–36.3)	32.5 (26.8–33.8)
Weight gain (%)	69.6 (50.7–77.8)	47.2 (40–55.6)	43.3 (35.4–55.1)	38.8 (13.6–52.9)
Liver weight (g)	1.8 (1.5–2.4)	1.8 (1.6–1.9)	2.7 (2.7–3)[b]	2 (1.5–2.2)
Body weight/liver weight	0.048 (0.036–0.058)	0.054 (0.05–0.054)	0.079 (0.075–0.082)[b]	0.06 (0.053–0.067)[c]
ALT (IU/L)	48.2 (36.1–57.8)	108.3 (72.2–180.6)	144.4 (144.4–252.8)[b]	132.4 (72.2–144.4)
AST (IU/L)	57.8 (57.8–57.8)	115.6 (57.6–115.6)	144.4 (108.3–180.6)[b]	114.4 (72.2–144.4)
Serum aldosterone (pg/ml)	478.6 (416.9–575.4)	831.8 (758.6–1047)[a]	371.5 (331.1–389)	562.3 (512.9–676.1)[c]
Hepatic TG content (mg/g liver)	78.9 (66–79.6)	66.7 (34.5–82.2)	99.8 (93.5–114.8)[b]	87.8 (80–93.5)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; CSAA, choline-supplemented L-amino acid-defined; CDAA, choline-deficient and amino acid-defined. Data represent Median and Interquartile Range (Q1–Q3), n = 7–10/group.

[a]$P < 0.017$ compared to untreated groups. [b]$P < 0.017$ vs. CSAA. [c]$P < 0.017$ compared to CSAA + Eplerenone group.

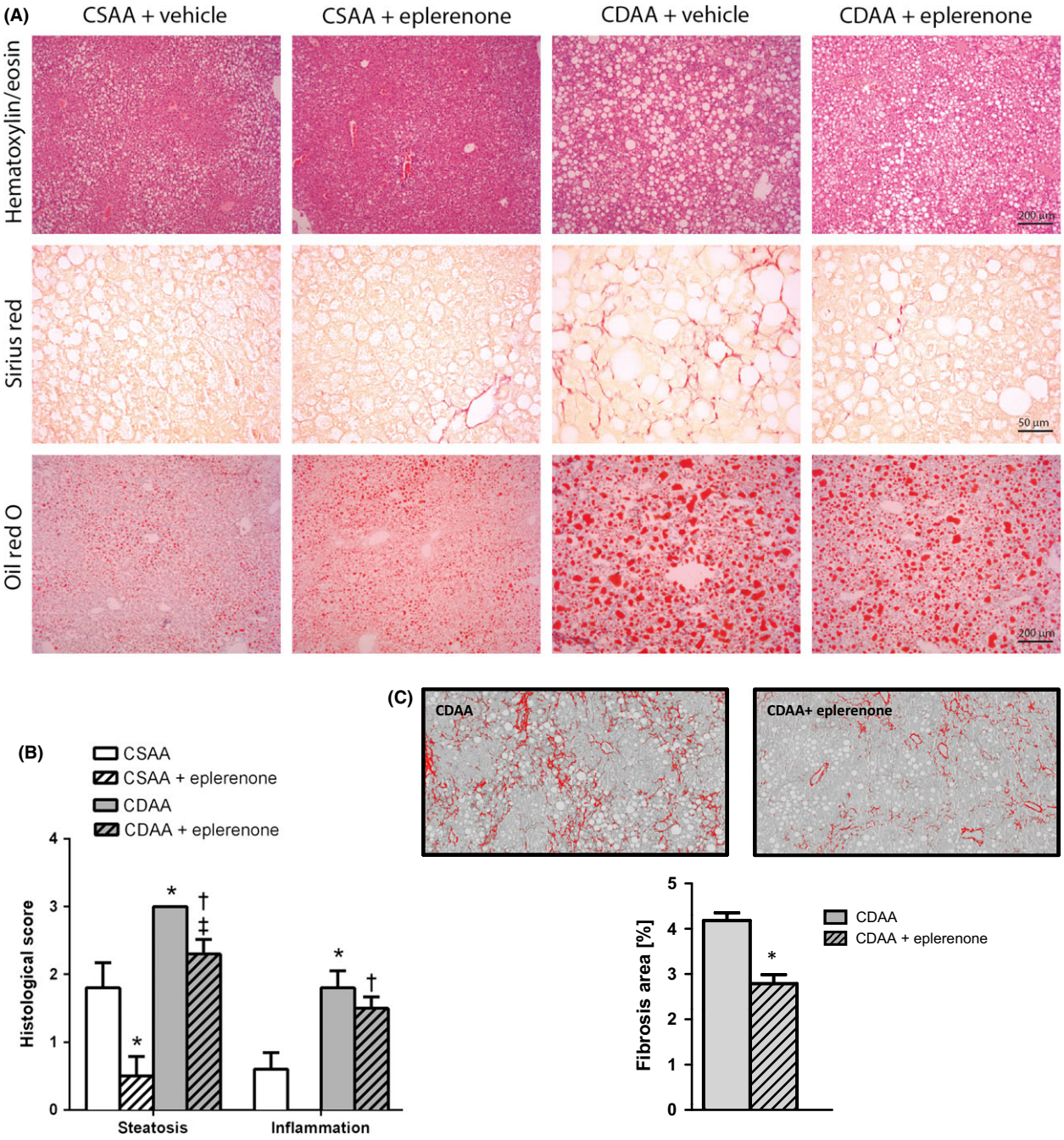

Fig. 1. Effect of eplerenone on histological features of choline-deficient and amino acid-defined (CDAA) diet-induced non-alcoholic steatohepatitis (NASH). (A) Representative histological images of livers from experimental groups after 22 weeks of feeding with either control [choline-supplemented L-amino acid-defined (CSAA)] or CDAA diets. Paraffin or frozen sections of the liver were stained with H-E (Hematoxylin-Eosin), Sirius Red, Oil Red O staining and α-SMA (α-smooth muscle actin) antibody. While CSAA diet induced a mild predominantly bland microvesicular steatosis, CDAA diet induced a florid NASH histological picture. Eplerenone supplementation attenuated both steatosis and fibrosis. (B) Histological scores for liver steatosis and inflammation in experimental groups. Data represent mean ± SEM. n = 7–10/group. *P < 0.05 compared to CSAA untreated group; †P < 0.05 compared to CSAA + eplerenone group; ‡P < 0.05 compared to CDAA untreated group. The score for hepatic inflammation of the CSAA + Eplerenone group was 0.05. (C) Histological images of Sirius Red-stained liver slices from mice fed with CDAA diet with or without Eplerenone supplementation. Fibrosis area was quantified using digital image analysis of the red-stained area in Sirius red-stained samples (IMAGEJ, NIH, Bethesda, MD, USA).

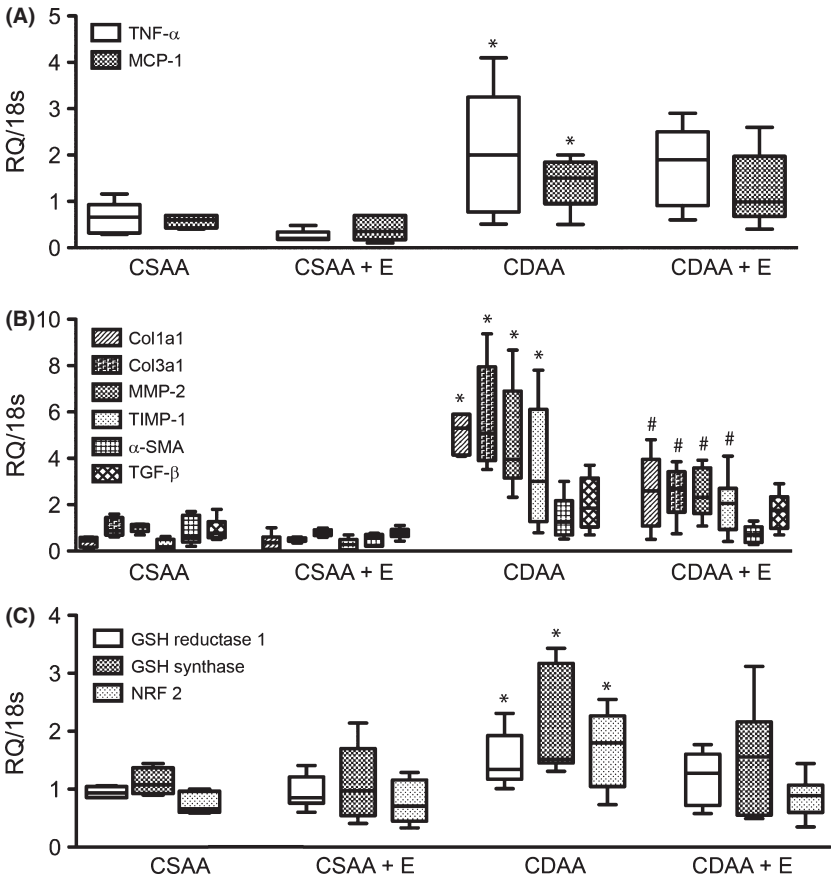

Fig. 2. Effect of eplerenone on Hepatic mRNA expression of pro-inflammatory (A), pro-fibrotic (B) and oxidative stress (C) markers in experimental NASH. Hepatic mRNA expression of pro-inflammatory markers in livers from mice fed with either choline-supplemented L-amino acid-defined; (CSAA) diet or choline-deficient and amino acid-defined (CDAA) diet with or without Eplerenone (E) supplementation. TNFα: tumour necrosis factor alpha; MCP1: monocyte chemoattractant protein 1; Co1a1: Collagen, type I, alpha 1; Col3A1: Collagen alpha-1(III); MMP-2: Matrix metalloproteinase-2; TIMP-1:, tissue inhibitor of metalloproteinase 1; TGFβ: transforming growth factor beta; α-SMA: smooth muscle actin. GSH: glutathione, NRF2: Nuclear factor (erythroid-derived 2)-like 2. Data represent median (Q1–Q3). n = 7–10/group. *P < 0.017 compared to CSAA; #P < 0.017 compared to CDAA without E.

obtained from livers harvested from mice fed either CSAA or CDAA diets and found that while MR mRNA decreases in hepatocytes, its levels are increased in HSC (Fig. 4A). Immunostaining for mineralocorticoid receptor in liver sections obtained from either CSAA or CDAA diets with or without Eplerenone supplementation showed a more intense signal in livers from CDAA-fed mice which was slightly reduced by Eplerenone supplementation (Fig. 4B). Finally, in trying to reproduce the findings of Wada *et al.* (16) we conducted experiments in KC isolated from Wistar rats to evaluate potential anti-inflammatory effects of eplerenone. To that end, we treated both hepatocytes and KC with Lipopolysaccharide (LPS) and assessed inducible nitric oxide synthase (iNOS) and TNF-α expression. While iNOS was strongly induced in hepatocytes, both iNOS and TNF-α were markedly induced in KC. Neither 1 μmol/L nor 10 μmol/L of eplerenone affected the expression of inflammatory markers in either hepatocytes or Kupffer cells (Fig. S1). In contrast, the NF-κB inhibitor MG-132 strongly reduced expression of inflammation markers in both hepatocytes and Kupffer cells. Taken together, these results demonstrate that eplerenone has no direct anti-inflammatory effect on hepatocytes or Kupffer cells.

Discussion

Aspects related to transition to or development of NASH, the progressive form of the disease, are key issues in the NAFLD field. However, factors involved in the development of more aggressive forms of the disease remains only partially unveiled (24). Well-known factors involved in NASH pathogenesis and progression are the degree of obesity, the magnitude of insulin resistance (IR), the coexistence of Type 2 Diabetes Mellitus, adipokine imbalance and excessive dietary fructose intake. Thus, correction and management of these conditions are reasonable goals of NAFLD/NASH treatment. Also, the excessive oxidative stress, mainly driven

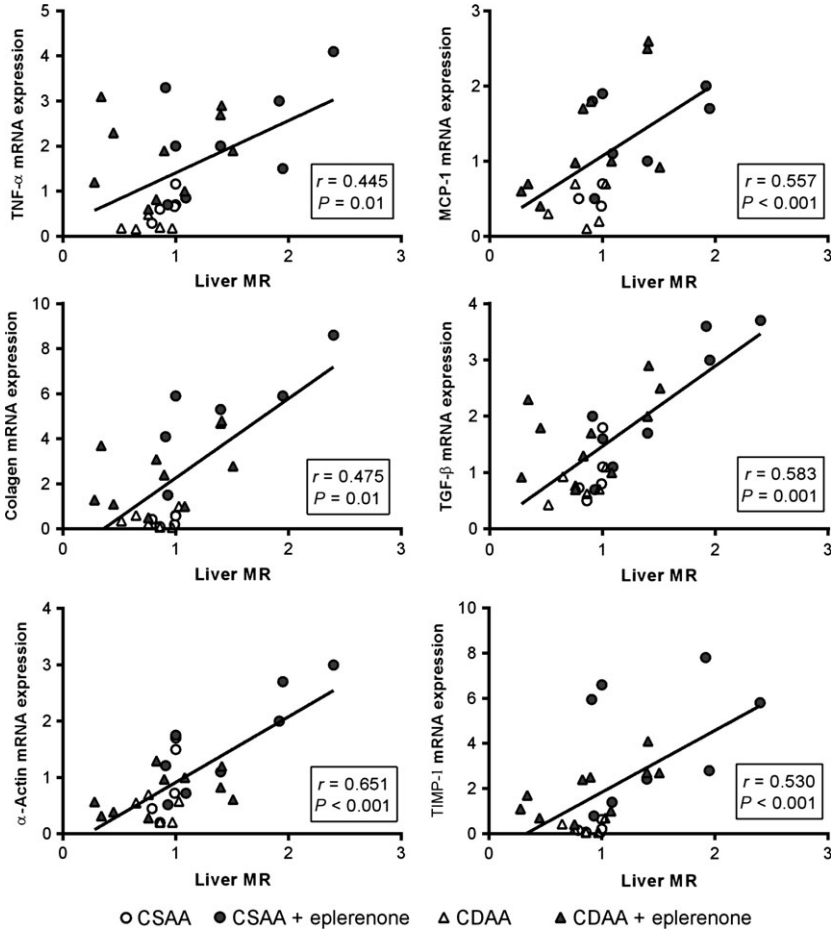

Fig. 3. Hepatic expression of the mineralocorticoid receptor (MR) correlates with the expression of pro-inflammatory and pro-fibrotic markers in experimental NASH. Correlations between gene expression of the MR and the expression of pro-inflammatory and pro-fibrotic markers in mice fed a choline-deficient and amino acid-defined (CDAA) diet with or without Eplerenone supplementation and their respective controls (CSAA and CSAA + Eplerenone fed mice) are shown. TNF-α: tumor necrosis factor alpha; MCP-1: monocyte chemoattractant protein-1; TGF-β: transforming growth factor beta; TIMP-1: tissue inhibitor of metalloproteinase 1.

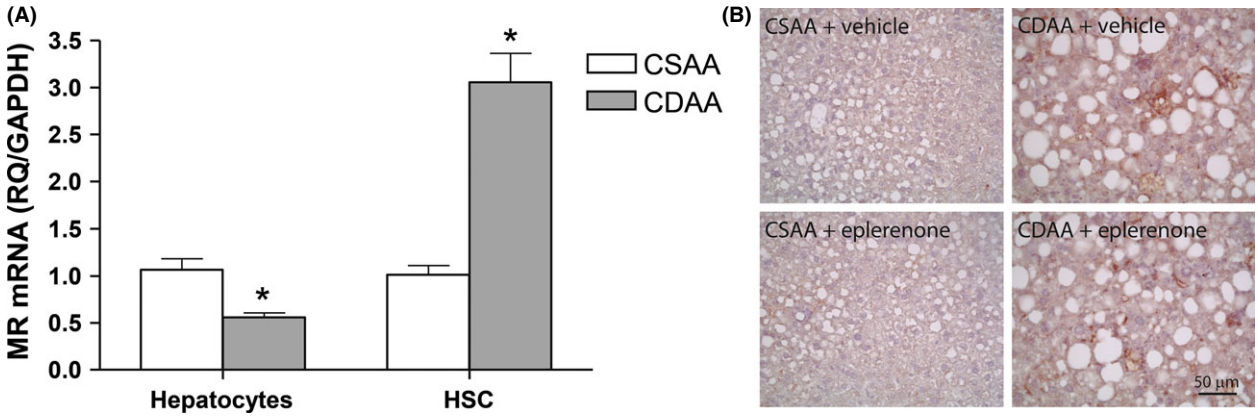

Fig. 4. Hepatic Mineralocorticoid receptor (MR) in experimental NASH. (A) Expression of the MR in hepatocytes and hepatic stellate cells (HSC) obtained from choline-deficient and amino acid-defined (CDAA) diet-fed mice. *$P < 0.05$ compared to MR expression in cells obtained from animals fed a choline-supplemented L-amino acid-defined (CSAA) diet. (B) Immunostaining for MR in liver sections obtained from animals fed either CSAA or CDAA diets with or without Eplerenone supplementation. Staining was more evident in CDAA-fed mice and slightly reduced by Eplerenone supplementation.

by lipotoxic metabolites of saturated fatty acids, plays a critical role and represents another amenable therapeutic target (25). Recently, the MR have been also suggested to be involved in hepatic fibrogenesis in the setting of NASH, based on observations made in several experimental models such as the liver-specific transgenic mice overexpressing the active form of sterol response element binding protein 1c (SREBP1c) fed with a high-fat and fructose diet (16) and the ob/ob and db/db mice, (19, 26, 27). In these models, inappropriate activation of MR has been also associated with diabetes and IR (28, 29) and MR antagonism significantly inhibited adipose tissue inflammation and improved systemic glucose metabolism also influencing liver steatosis and inflammation. These data allow considering MR as a novel potential therapeutic target for IR and NAFLD. Moreover, MR blockade has been shown to ameliorate experimental organ fibrosis in the heart (30), lung (31) and kidney (32). If that beneficial effects also occurs in the liver then MR blockade would provide action on both metabolic and fibrogenic phenomena rendering this class of drugs potentially useful agents for NASH. Thus, this study aimed to evaluate the effects of eplerenone, a selective MR antagonist, on liver fibrosis in the CDAA diet model of NASH, which is a model with a more robust hepatic phenotype than that obtained by feeding mice with high-fat diets which induce mild inflammation and no fibrosis. In fact, feeding of a CDAA diet during 22 weeks induced a florid histological picture of NASH including liver steatosis, inflammation and fibrosis, which was associated to an up-regulation of the expression of both pro-inflammatory and pro-fibrotic markers. Interestingly, CDAA diet was also associated to an increased expression of MR in HSC, which was not seen in VAT (data not shown), highlighting the primary hepatic effect of the CDAA diet NASH model in contrast to other models with metabolic dysfunction in which the primary effect is seen in the adipose tissue (19). Also, hepatic mRNA levels of MR were positively correlated with the hepatic expression of both pro-inflammatory and pro-fibrotic genes.

In our model, eplerenone significantly decreased histological steatosis and fibrosis, but did not influence hepatic inflammation assessed either histologically or through mRNA expression of pro-inflammatory markers in contrast with recent findings of Wada *et al.* (16). Also, in the latter study eplerenone suppressed lipopolysaccharide (LPS)-induced TNFα expression in both primary-cultured KC and bone marrow-derived macrophages which we did not observe when performing similar experiments. These differences are likely related to technical issues and differences in the animal model used. Moreover, although the general concept is that tissue inflammation and fibrosis are closely linked, it is also likely that both phenomena could be influenced separately. As mentioned, the CDAA diet feeding model is rather intense model of NASH with a liver-predominant features compared to more 'metabolic' models such as high-fat diet feeding. In this setting, more potent agents are needed to counteract inflammation as we have shown preliminarily with andrographolide (33).

Despite a positive effect on fibrosis with eplerenone, our study is not designed to elucidate if the MR ligand is Aldosterone or corticosterone. Corticosterone can activate MR, especially in non-epithelial cells including macrophages, which do not express 11β-HSD2, that inactivate glucocorticoids to cortisone (34, 35). A role of glucocorticoid local activation in NAFLD related to 11β-HSD1 expression that could increase MR activation has been previously described by our group in human studies (14, 15, 36). Interestingly, Hirata and coworkers have also shown that MR blockade decrease 11β-HSD1 expression, suggesting that eplerenone could stop a vicious circle related to glucocorticoids and aldosterone on MR activation (19). On the other hand, aldosterone synthase deficient mice exposed to high-fat diet presented significantly lower liver steatosis, fat mass, and higher adiponectin serum levels suggesting a pathogenic role of aldosterone in NAFLD (37), suggesting a potential role for aldosterone in NAFLD.

Among the limitations of the present, the following issues deserve consideration. First, although our animal model developed a full-blown NASH histological picture, currently no animal model is completely representative of physiopathological changes observed in human NAFLD. Second, the specific pathogenic pathways involved in eplerenone protective effect were not explored in our study. Prior data suggest a significant effect of MR antagonist in reducing insulin resistance, increasing adiponectin and reducing KC activation, constituting potential pathways involved in eplerenone action (16, 26, 38, 39). However, a detailed analysis of the effects of eplerenone on these pathways and on derangements of both carbohydrate and lipid metabolism in the CDAA model (18, 40) will be focus of future studies. Finally, since we did not include a study group exposed to increased MR activation, the role of MR in NAFLD pathogenesis remains not fully demonstrated. Unpublished work from our laboratory using MR knockout mice suggest that these mice develop a less intense phenotype when fed methionine choline-deficient diets which also support a role of MR in NAFLD development and progression (Cabrera D, personal communication). However, further data on effectiveness of MR antagonist therapy in NAFLD is needed.

In summary, since eplerenone effectively ameliorated histological steatosis and hepatic fibrosis in a mouse model of NASH, our results provide basis for therapeutic exploitation of MR blockade in NASH therapy. Thus, we think that this therapeutic option could be formally explored in clinical trials for the treatment of NASH in humans, considering its favourable safety profile (FDA-approved) and its potential positive impact in other components of the metabolic syndrome such as insulin resistance, hypertension and adipose tissue inflammation (41).

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Author contributions

Rene Baudrand, Arnoldo Riquelme and Marco Arrese conceived and designed the research project and drafting the final manuscript. Margarita Pizarro, Nancy Solís, Pablo Quintero, Pamela Rojas-de Santiago and Daniel Cabrera carried out animal experiments, summarized data and constructed final figures. Juan Carlos Roa blindly analyzed histological pictures and assigned scores for steatosis, inflammation and fibrosis in liver slices. Alexander Wree and Eugenia Inzaugarat isolated liver cells and performed PCR experiments in these cells. Oslando Padilla provided expert statistical advice. Francisco Barrera, Juan Pablo Arab, Han Moshage, Ariel E. Feldstein and Carlos E. Fardella critically revised and substantially contributed to the final draft, which was approved by all authors.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Methods S1. Studies assessing the effects of eplerenone on hepatocytes and KC isolated from livers harvested from Wistar rats.

Table S1. Sequences of primers and probes used for quantitative real-time PCR.

Fig. S1. Effect of eplerenone on cytokine-induced inducible nitric oxide synthase (iNOS) and Tumour Necrosis Factor alpha (TNF-α) expression in isolated Kupffer cells and hepatocytes.