Mas receptor deficiency is associated with worsening of lipid profile and severe hepatic steatosis in ApoE-knockout mice

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1INCT-NanoBiofar, Department of Physiology and Biophysics, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; 2Department of Biochemistry, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; 3Department of Pathology, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; 4Max-Delbrück Center for Molecular Medicine, Berlin, Germany; 5Cardiology Division, Faculty of Medicine, University of Geneva, Geneva, Switzerland; 6First Medical Clinic, Laboratory of Phagocyte Physiopathology and Inflammation, Department of Internal Medicine, University of Genoa, Genoa, Italy; and 7Department of Pharmacology, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

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Silva AR, Aguilar EC, Alvarez-Leite JJ, da Silva RF, Arantes RM, Bader M, Alenina N, Pelli G, Lenglet S, Galan K, Montecucco F, Mach F, Santos SH, Santos RA. Mas receptor deficiency is associated with worsening of lipid profile and severe hepatic steatosis in ApoE-knockout mice. *Am J Physiol Regul Integr Comp Physiol* 305: R1323–R1330, 2013. First published October 2, 2013; doi:10.1152/ajpregu.00249.2013.—The classical renin-angiotensin system pathway has been recently updated with the identification of additional molecules [such as angiotensin converting enzyme 2, ANG-(1–7), and Mas receptor] that might improve some pathophysiological processes in chronic inflammatory diseases. In the present study, we focused on the potential protective role of Mas receptor activation on mouse lipid profile, liver steatosis, and atherogenesis. Mas/apopliprotein E (ApoE)-double-knockout (DKO) mice (based on C57BL/6 strain of 20 wk of age) were fed under normal diet and compared with aged-matched Mas and ApoE-single-knockout (KO), as well as wild-type mice. Mas/ApoE double deficiency was associated with increased serum levels of atherogenic fractions of cholesterol, triglycerides, and fasting glucose compared with wild-type or single KO. Serum levels of HDL or leptin in DKO were lower than in other groups. Hepatic lipid content as well as alanine aminotransferase serum levels were increased in DKO compared with wild-type or single-KO animals. Accordingly, the hepatic protein content of mediators related to atherosclerotic inflammation, such as peroxisome proliferator-activated receptor-α and liver X receptor, was altered in an adverse way in DKO compared with ApoE-KO. On the other hand, DKO mice did not display increased atherogenesis and intraplaque inflammation compared with ApoE-KO group. In conclusion, Mas deletion in ApoE-KO mice was associated with development of severe liver steatosis and dyslipidemia without affecting concomitant atherosclerosis. Mas receptor activation might represent promising strategies for future treatments targeting both hepatic and metabolic alterations in chronic conditions clustering these disorders. ANG-(1–7); lipodystrophy; liver steatosis; atherosclerosis; renin angiotensin system

THE METABOLIC SYNDROME refers to a clustering of metabolic abnormalities, including excess body fat, dyslipidemia, hypertension, and hyperglycemia (2). Although excess fat is considered as highly deleterious, adipose tissue deficiency, referred to as lipodystrophies, also leads to severe metabolic disorders resulting in cardiovascular complications (16).

In lipodystrophy, adipose tissue becomes unable to buffer excess circulating lipids and protect against its toxicity (15), leading to further ectopic fat deposition, such as muscle, liver, and arteries (16, 37).

Evidences in animal models and clinical studies indicate that the renin-angiotensin system (RAS) plays a major role in the development of cardiovascular and metabolic disorders, including vascular disease, diabetes, and nonalcoholic fatty liver disease (30, 33, 43).

In the “classic” pathway of the RAS, renin is secreted by juxtaglomerular cells at the renal afferent arterioles and cleaves the liver-derived precursor protein angiotensinogen into the decapeptide angiotensin I (ANG I). ANG I is further hydrolyzed into the octapeptide angiotensin II (ANG II) by the angiotensin converting enzyme (ACE), which represents the main effector peptide of the RAS.

The identification of both ACE homologue ACE2 (10, 57) and of the angiotensin-(1–7) [ANG-(1–7)] Mas receptor (47) added a new complexity to the RAS. The ACE2/ANG-(1–7)/Mas axis has been suggested as an important counterregulatory arm in the RAS with a number of important actions that oppose those of ANG II, including vasodilatation, antiproliferative, and antithrombotic effects (13, 46, 59).

Recently, the ACE2/ANG-(1–7)/Mas axis has been also implicated in features of the metabolic syndrome and its related disorders (25, 36, 48, 49, 55, 56). Previous studies from our group showed that Mas receptor deficiency induces to a metabolic syndrome-like state in FVB/N background mice (49). In addition, increased levels of ANG-(1–7) have been shown to improve lipid profile, insulin resistance, hepatic fibrosis, and atherosclerosis (25, 36, 48, 55, 56). These observations suggest that the RAS can play a pathophysiological role in metabolic syndrome-related disorders. However, further studies are necessary to clarify the role of specific component of the RAS, such as the ANG-(1–7) receptor, Mas, in these disorders.

Tesanovic and colleagues (55) reported that long-term ANG-(1–7) treatment in apolipoprotein E (ApoE)-knockout...
MAS RECEPTOR DEFICIENCY AND LIVER STEATOSIS

(KO) mice evoked improvement in endothelial function and atheroprotection, through an increase of nitric oxide bioavailability. However, the role of Mas in this effect was somehow inconclusive, since attenuation by both AT2 and Mas receptor antagonists was observed. No studies have been performed using the Mas receptor deletion in a model of atherosclerosis, such as the ApoE-KO mice. This would be particularly important for clarifying the role of Mas in atherosclerosis. In addition, the results obtained with PD-123319 should be reevaluated, considering our recent findings concerning the nonspecificity of this compound, which can block actions of the novel angiotensin, alamandine, by interference with MrgD receptor (23). This study aimed to start to elucidate these questions, specially the potential protective effects of Mas-triggered pathways in metabolic and inflammatory disorders (such as liver steatosis and atherosclerosis) using ApoE, Mas single KO, as well as Mas/ApoE-double-knockout (DKO) mice.

MATERIALS AND METHODS

Animals. To generate Mas/ApoE-DKO mice, Mas-KO and ApoE-KO mice (based on C57BL/6 strain, transgenic animal facilities of the Laboratory of Hypertension, Federal University of Minas Gerais, Brazil) were bred to yield heterozygous mice. These mice were crossed and intercrossed to yield DKO mice, with genetic identity confirmed by tail genotyping at each generation. Twenty-week-old male C57BL/6 wild-type (WT), Mas-KO, ApoE-KO, and DKO were used for the experiments.

Mice were kept under controlled light and temperature conditions, with free access to water and standard diet. The animals were maintained according to the ethical guidelines of our institution, and the experimental protocol was approved by the Ethical Committee in Animals Experimentation of the Federal University of Minas Gerais (protocol no. 139/11).

Tissue collection. At 20 wk of age, all mice were killed after anesthesia using an intraperitoneal injection of ketamine (130 mg/kg) and xylazine (0.5 mg/kg), followed by exsanguination via femoral artery. Samples of epididymal white adipose tissue and liver were collected, weighted, and immediately frozen in liquid nitrogen and stored at −80°C for posterior analysis. Aortae and aortic roots were dissected free of connective tissue, cut longitudinally, and stained with Sudan IV. Longitudinal aortic segments were then laid flat on a glass slide, and images were captured using a scanner and quantified using the appropriate software (Image Pro Plus 6.3, Bethesda, MD). Lesion area was expressed as a percentage of the total luminal surface area.

Immunohistochemistry in mouse aortic sinus plaques. Mouse aortic sinus was serially cut in 5-μm transversal sections through the heart and aortic roots, as previously described (29). Sections were fixed in aceton and immunostained with specific antibodies against mouse CD68 (macrophages, 1:1,000, Abcam), rabbit polyclonal anti-AT1 receptor (1:500, Millipore), rabbit polyclonal anti-AT2 receptor (1:500, Millipore); mouse monoclonal anti-ACE (mouse monoclonal anti-β-actin, 1:2,000, Sigma) was used as endogenous control. The blots were viewed using an infrared scanner from LICOR and analyzed using the Odyssey software.

Liver histology. Hepatic tissue was excised and fixed in formalin solution (4%) and embedded in Paraplast. Sections of the tissue were stained with hematoxylin and eosin. Sudan IV staining was performed on frozen sections 10 μm thick. The images were analyzed qualitatively by another operator blinded to the identities of the animals.

Atherosclerotic lesion analysis. Five sections per mouse aortic sinus were stained with Oil Red O, as previously described (29). Sections and aortas were counterstained with Mayer’s hemalum and rinsed in distilled water. Quantifications were performed with MetaMorph software. Data were calculated as ratios of stained area on total lesion area. Aortae were dissected free of connective tissue, cut longitudinally, and stained with Sudan IV. Longitudinal aortic segments were then laid flat on a glass slide, and images were captured using a scanner and quantified using the appropriate software (Image Pro Plus 6.3, Bethesda, MD). Lesion area was expressed as a percentage of the total luminal surface area.

Serum triglycerides, total cholesterol (TC), and high-density lipoprotein (HDL) cholesterol were routinely measured using commercial kits (Labtest), as previously described (12). Sections were rinsed twice with 0.01 N HCl for 1 min and then immersed in water. After dehydration with ethanol for 30 s and coverslipping, the sections were photographed with identical exposure parameters. Total collagen content was evaluated under polychromatic light. Quantifications were performed with MetaMorph software. Data were calculated as percentages of stained area on total lesion area or number of infiltrating cells on millimeters squared of lesion area.

Sirius red staining for collagen content. Five sections per mouse aortic sinus were rinsed with water and incubated with 0.1% Sirius red (Sigma Chemical, St. Louis, MO) in saturated picric acid for 90 min. Sections were rinsed twice with 0.1 N HCl for 1 min and then immersed in water. After dehydration with ethanol for 30 s and coverslipping, the sections were photographed with identical exposure settings under ordinary polychromatic or polarized light microscopy. Total collagen content was evaluated under polychromatic light. Quantifications were performed with MetaMorph software. Data were calculated as percentages of stained area on total lesion area.

Statistical analysis. Data are expressed as means ± SE. The statistical significance of the difference in mean values between groups was assessed by an unpaired Student t-test or one-way ANOVA followed by Newman-Keuls test. A value of P < 0.05 was considered statistically significant.

RESULTS

To characterize the DKO mouse line, we first investigated the potential relationship between body, fat, and liver weights. As observed in Table 1, DKO mice presented a decrease in body weight (vs. WT and ApoE-KO) and in epididymal adipose tissue (vs. ApoE-KO), indicating that DKO have a substantial decrease in fat mass compared with ApoE-KO mice. There were no differences in liver weight between groups.
We observed that the absence of Mas receptor in ApoE-KO mice worsened dyslipidemia usually observed in this model (Table 2). DKO mice presented a significant increase in TC compared with WT and single ApoE-KO (WT: 0.08 ± 0.006, n = 6; Mas-KO: 0.09 ± 0.01, n = 5; ApoE-KO: 0.09 ± 0.007, n = 6; DKO: 0.06 ± 0.008 arbitrary units, n = 6) (Fig. 4B). The hepatic protein expression of LXR was in ApoE-KO (WT: 9.7 ± 2.3 vs. ApoE-KO: 4.4 ± 0.6 arbitrary units; Fig. 4C). However, in Mas-KO mice, ApoE deficiency was associated to normal LXR expression (DKO: 11.3 ± 1.4, n = 6 vs. WT: 9.7 ± 2.3 arbitrary units; Fig. 4C).

To identify the molecular mechanisms underlying the development of hepatic steatosis in DKO mice, we focused on insulin receptor, PPAR-α, and LXR. As shown in Fig. 4A, Western blot analysis revealed a significant relative decreased hepatic expression of the insulin receptor in DKO mice compared with WT and single ApoE-KO (WT: 0.74 ± 0.08; Mas-KO: 0.57 ± 0.11; ApoE-KO: 0.70 ± 0.07; DKO: 0.32 ± 0.05 arbitrary units, n = 6), potentially indicating a hepatic insulin resistance. The liver expression PPAR-α protein was also reduced in DKO compared with ApoE-KO mice (WT: 0.08 ± 0.006, n = 6; Mas-KO: 0.09 ± 0.01, n = 5; ApoE-KO: 0.09 ± 0.007, n = 6; DKO: 0.06 ± 0.008 arbitrary units, n = 6) (Fig. 4B). The hepatic protein expression of LXR in ApoE-KO mice was lower than in WT mice (WT: 4.4 ± 0.6 arbitrary units; Fig. 4C). However, in Mas-KO mice, ApoE deficiency was associated to normal LXR expression (DKO: 11.3 ± 1.4, n = 6 vs. WT: 9.7 ± 2.3 arbitrary units; Fig. 4C).

**DISCUSSION**

The RAS and lipid profile alteration are two of the most relevant routes of metabolic regulation (24, 26, 36, 45). In the present study, we evaluated the potential participation of Mas receptor, a main component of the ACE2/ANG-(1–7)/Mas axis of the RAS, in the liver metabolism and atherosclerosis. We took advantage of the availability of Mas receptor and ApoE-deficient mice to generate a DKO mice. We observed that Mas deficiency in ApoE mice produced a lipodystrophy-like state (17, 24, 40, 48). In particular, ApoE mice showed inflammatory foci (Fig. 2, C and D). The histological analysis showed in vivo degeneration, and few mononuclear inflammatory foci (Fig. 2, C and D). In accordance with these histological observations, a marked increase in liver lipid accumulation was shown in the DKO mice compared with the other groups (Table 4). Furthermore, ApoE-KO and DKO presented a significant increase in serum alanine aminotransferase levels (WT: 11.3 ± 1.7; Mas-KO: 23.1 ± 6.8; ApoE-KO: 62.3 ± 5.8; DKO: 69.4 ± 10.4 U/ml, n = 6) (Fig. 3A). Since lipid peroxidation products play an important role in the pathogenesis of nonalcoholic fatty liver disease (5), we next examined serum and hepatic levels of TBARS. As shown in Fig. 3B, an increase in TBARS levels in liver of DKO mice was observed (WT: 0.58 ± 0.04; Mas-KO: 0.74 ± 0.09; ApoE-KO: 0.57 ± 0.02; DKO: 0.79 ± 0.09 μM malondialdehyde/g protein, n = 6).

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**ANG-(1–7)/Mas axis has been described as an important hormonal component involved in lipid and glucose metabolic regulation (17, 24, 40, 48). Mas-KO FVB/N mice were char-
characterized as a metabolic syndromelike state model with dyslipidemia, hypertension, and endothelial dysfunction (49, 59). On the other hand, apolipoproteins are described as fundamental in the lipid transport. ApoE is one of the most described apolipoprotein, and its major function is to remove triglycerides-rich lipoproteins from circulation (26). ApoE deletion commonly leads to atherosclerotic disease (38–39, 41). In this context, the new DKO mice Mas/ApoE is an innovative model to study metabolic regulation. Because ApoE genetic deletion produces a stronger phenotype in C57BL/6 mice (9), we used, in the present study, Mas-KO mice in this background, although, in C57BL/6 mice, Mas deletion produced only mild changes in lipid metabolism (49).

Indeed, we observed that Mas KO mice presented reduced adipose tissue mass, contrasting with our laboratory’s previous study showing an increased fat mass in FVB/N Mas-KO mice (49). A similar reduction of white adipose tissue mass was observed in the DKO mice. Accordingly, Mario and colleagues (27) demonstrated that the deletion of the Mas receptor decreases the PPAR-γ expression, transcription factor that is believed to be a master regulator of lipogenesis (44). To investigate if such changes in C57Bl6 background were due to a lipodystrophy-like state, we evaluated fat liver deposition. Strikingly, we observed a severe steatosis in DKO model, pointing out for a switched site of lipid deposition. This

Table 3. Immunohistochemical analysis of macrophage, neutrophil, collagen, MMP-9, MMP-8, lymphocyte, and smooth muscle cell expression in the aortic root atherosclerotic plaques from ApoE-KO and DKO mice

<table>
<thead>
<tr>
<th></th>
<th>ApoE-KO</th>
<th>DKO</th>
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<tr>
<td>n</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Macrophages, %plaque area</td>
<td>3.52 ± 0.39</td>
<td>2.27 ± 0.35†</td>
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<tr>
<td>Neutrophils, cells/mm²</td>
<td>0.95 ± 0.22</td>
<td>0.88 ± 0.13</td>
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<tr>
<td>Collagen, %plaque area</td>
<td>19.65 ± 2.7</td>
<td>16.07 ± 2.9</td>
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<tr>
<td>MMP-9, %plaque area</td>
<td>1.94 ± 0.34</td>
<td>1.73 ± 0.24</td>
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<tr>
<td>MMP-8, %plaque area</td>
<td>1.94 ± 0.54</td>
<td>1.64 ± 0.43</td>
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<tr>
<td>Lymphocytes, cells/mm²</td>
<td>10.18 ± 2.16</td>
<td>8.01 ± 0.89</td>
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<tr>
<td>SMC, %plaque area</td>
<td>23.54 ± 3.38</td>
<td>24.45 ± 3.57</td>
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Values are means ± SE; n, no. of mice. MMP-9, matrix metalloproteinase-9; MMP-8, matrix metalloproteinase-8; SMC, smooth muscle cell. †P ≤ 0.05 vs. ApoE-KO.
hypothesis was corroborated by the altered expression of insulin receptor, which is responsible for the liver lipid and glucose metabolism (19), and reduced PPAR-α, which controls lipid oxidation and transport (58). Furthermore, increased circulating triglyceride, TC, and diminished HDL-cholesterol support the theory of lipid metabolic unbalance.

It is well established that the intrahepatic expression of ACE/ANG II/AT1 axis is increased in experimental liver injury (3, 34). Moreover, deletion or blockade of components of this axis improves experimental hepatic steatosis and fibrosis (20, 22, 30, 53). In our study, increases in liver lipid content in DKO group were not accompanied by alterations in RAS components expression. In this regard, further studies are needed to identify the precise mechanisms involved in the possible association of the worsening of hepatic steatosis and the RAS members expression in the DKO mice. It should pointed out, however, that our present results suggest a primary role for the absence of Mas receptor in this increase of hepatic steatosis in the double ApoE/Mas mice.

Leptin is an important adipokine involved in the central satiety and also regulates local tissue lipid metabolism (1, 8). Several studies showed that circulating leptin reduction induced hyperphagia and a positive energy balance, contributing to ectopic fat deposition (50). Treatment with recombinant leptin in lipodystrophy patients reverses many of its metabolic disturbances (32, 37). Also, some animal models of lipodystrophy showed reduced leptin plasma levels (11, 18, 52), as observed in the present study in the DKO mice.

The ApoE-KO mice represent a well-recognized model of animals prone to develop atherosclerosis (38–39). As previously described (29), our laboratory evaluated aortic arch and aortic root lipid content as a validated method to estimate atherosclerotic entity. No difference between groups was observed on plaque size. Corroborating these data, we did not observe any increase in the intraplaque inflammatory profile disturbances.

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Table 4. Hepatic lipid content in WT, Mas-KO, ApoE-KO, and DKO mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Mas-KO</th>
<th>ApoE-KO</th>
<th>DKO</th>
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<tbody>
<tr>
<td>Total lipids, mg/g liver</td>
<td>51.9 ± 6.6</td>
<td>50.1 ± 6.7</td>
<td>79.7 ± 12.7***</td>
<td>123.7 ± 6.6†‡</td>
</tr>
<tr>
<td>Triglycerides, mg/g liver</td>
<td>18.6 ± 3.3</td>
<td>15.9 ± 7.2</td>
<td>40.5 ± 5.5†‡</td>
<td>70.3 ± 3.6†‡</td>
</tr>
<tr>
<td>Total cholesterol, mg/g liver</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>2.0 ± 0.1‡</td>
<td>2.1 ± 0.1†‡</td>
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Values are means ± SE; n, no. of mice. P ≤ 0.05 vs. *WT, †ApoE-KO, and ‡Mas-KO.

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**Fig. 2.** Representative microphotographs of the liver sections from wild-type (WT), Mas receptor (Mas)-KO, ApoE-KO, and DKO mice. A and E: WT group has normal characteristics. B and F: Mas-KO liver presents with intracytoplasmatic small droplets without clinical expression. In ApoE-KO group was observed inflammatory focus (C, *), with predominantly microvesicular steatosis (G). In DKO group was observed severe steatosis, macro- and microvesicular (H), but, with less inflammatory profile (D, arrow). Hematoxylin and eosin and Sudan IV staining; ×40 magnification.

**Fig. 3.** Serum ALT (alanine aminotransferase) and hepatic MDA (malondialdehyde) levels. A: serum levels of ALT in WT, Mas-KO, ApoE-KO, and DKO mice. B: liver lipid peroxidation in WT, Mas-KO, ApoE-KO, and DKO mice. Values are means ± SE; n = 6. P < 0.05 vs. *WT, #Mas-KO, and †ApoE-KO.
(assessed by neutrophil, MMP-9, MMP-8, macrophage, lymphocyte, smooth muscle cell, and collagen content). These results can be, at least in part, attributed to the increased liver expression of LXR. The LXR is considered a cholesterol sensor that regulates several genes involved in lipid metabolic regulation (60). Several studies showed the benefits of LXR in the atherosclerosis treatment (21, 35). Despite all of the metabolic improvement produced by LXR activation, some studies pointed out some side effects, including activation of hepatic lipogenesis and increased reverse cholesterol transport (31, 42, 51), which can explain partially the observed liver steatosis.

Furthermore, leptin has proinflammatory properties, and leptin-deficient mice exhibit an impaired immune function with diminished cellularity in spleen and thymus and reduced secretion of proinflammatory cytokines (6, 45). In addition, studies have shown that leptin deficiency reduces atherosclerotic lesions in both low-density lipoprotein receptor and ApoE-KO mice (7, 54). These mechanisms are probably contributing to the phenotype observed, especially considering that, in this study, we used mice under standard non-high-fat diet.

**Perspectives and Significance**

The present study showed for the first time that Mas deletion in ApoE-KO mice produced a devastating metabolic profile, leading to lipodystrophy-like state with severe liver steatosis and dyslipidemia. These results pointed out an important participation of Mas receptor in liver health, indicating that ANG-(1–7) could be considered as a putative new and innovative therapeutic drug for the treatment of liver diseases and metabolic-syndrome-associated disorders.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**Fig. 4. Hepatic expression of insulin receptor, peroxisome proliferator-activated receptor-α (PPAR-α), and liver X receptor (LXR).** A: Western blotting for insulin receptor showing a decrease in DKO compared with WT and ApoE-KO mice. B: Western blot for PPAR-α showing a decrease in DKO compared with ApoE-KO. C: Western blot for LXR showing an increase in DKO compared with ApoE-KO. Values are means ± SE; n = 6. P < 0.05 vs. *WT and #ApoE-KO.

**Fig. 5. Hepatic expression of AT2 and AT1 receptors and angiotensin converting enzyme (ACE).** A: there was no significant difference in AT2 receptor expression between groups. B: Western blot for AT1 showing a decrease in ApoE-KO compared with Mas-KO. C: Western blot for ACE showing a decrease in ApoE-KO compared with WT. Values are means ± SE; n = 6. P < 0.05 vs. *WT and #Mas-KO.
REFERENCES


