

## Research Article

# Nonclassical Axis of the Renin-Angiotensin System and Neprilysin: Key Mediators That Underlie the Cardioprotective Effect of PPAR-Alpha Activation during Myocardial Ischemia in a Metabolic Syndrome Model

**María Sánchez-Aguilar** <sup>1</sup>, **Luz Ibarra-Lara** <sup>1</sup>, **Leonardo del Valle-Mondragón** <sup>1</sup>,  
**Elizabeth Soria-Castro** <sup>2</sup>, **Juan Carlos Torres-Narváez** <sup>1</sup>, **Elizabeth Carreón-Torres** <sup>3</sup>,  
**Alicia Sánchez-Mendoza** <sup>1</sup> and **María Esther Rubio-Ruíz** <sup>4</sup>

<sup>1</sup>Department of Pharmacology, Instituto Nacional de Cardiología Ignacio Chávez, Juan Badiano 1, Sección XVI, Tlalpan, Mexico City 14080, Mexico

<sup>2</sup>Department of Cardiovascular Biomedicine, Instituto Nacional de Cardiología Ignacio Chávez, Juan Badiano 1, Sección XVI, Tlalpan, Mexico City 14080, Mexico

<sup>3</sup>Department of Molecular Biology, Instituto Nacional de Cardiología Ignacio Chávez, Juan Badiano 1, Sección XVI, Tlalpan, Mexico City 14080, Mexico

<sup>4</sup>Department of Physiology, Instituto Nacional de Cardiología Ignacio Chávez, Juan Badiano 1, Sección XVI, Tlalpan, Mexico City 14080, Mexico

Correspondence should be addressed to María Esther Rubio-Ruíz; [esther\\_rubio\\_ruiz@yahoo.com](mailto:esther_rubio_ruiz@yahoo.com)

María Sánchez-Aguilar and Luz Ibarra-Lara contributed equally to this work.

Received 14 September 2020; Revised 10 November 2020; Accepted 11 November 2020; Published 28 November 2020

Academic Editor: Sainan Li

Copyright © 2020 María Sánchez-Aguilar et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The activation of the renin-angiotensin system (RAS) participates in the development of metabolic syndrome (MetS) and in heart failure. PPAR-alpha activation by fenofibrate reverts some of the effects caused by these pathologies. Recently, nonclassical RAS components have been implicated in the pathogenesis of hypertension and myocardial dysfunction; however, their cardiac functions are still controversial. We evaluated if the nonclassical RAS signaling pathways, directed by angiotensin III and angiotensin-(1-7), are involved in the cardioprotective effect of fenofibrate during ischemia in MetS rats. Control (CT) and MetS rats were divided into the following groups: (a) sham, (b) vehicle-treated myocardial infarction (MI-V), and (c) fenofibrate-treated myocardial infarction (MI-F). Angiotensin III and angiotensin IV levels and insulin increased the aminopeptidase (IRAP) expression and decreased the angiotensin-converting enzyme 2 (ACE2) expression in the hearts from MetS rats. Ischemia activated the angiotensin-converting enzyme (ACE)/angiotensin II/angiotensin receptor 1 (AT1R) and angiotensin III/angiotensin IV/angiotensin receptor 4 (AT4R)-IRAP axes. Fenofibrate treatment prevented the damage due to ischemia in MetS rats by favoring the angiotensin-(1-7)/angiotensin receptor 2 (AT2R) axis and inhibiting the angiotensin III/angiotensin IV/AT4R-IRAP signaling pathway. Additionally, fenofibrate downregulated neprilysin expression and increased bradykinin production. These effects of PPAR-alpha activation were accompanied by a reduction in the size of the myocardial infarct and in the activity of serum creatine kinase. Thus, the regulation of the nonclassical axis of RAS forms part of a novel protective effect of fenofibrate in myocardial ischemia.

## 1. Introduction

The renin-angiotensin system (RAS) is a complex hormone system that plays a critical role in cardiovascular physiology. Indeed, RAS has a central role in the development of metabolic syndrome (MetS), insulin resistance, and heart failure [1].

The RAS is a system composed of different angiotensin peptides with different biological actions mediated by distinct receptor subtypes. The classical RAS comprises the renin angiotensin-converting enzyme (ACE)/angiotensin II (Ang II)/angiotensin receptor 1 (AT1R) axis and promotes vasoconstriction and increases oxidative stress, fibrosis, cellular growth, and inflammation [1]. In contrast, the nonclassical RAS, composed mainly by the angiotensin-converting enzyme 2 (ACE2)/angiotensin-(1-7) (Ang-(1-7))/Mas receptor (MasR)/angiotensin receptor 2 (AT2R) pathway, improves the cardiac function of hearts subjected to myocardial infarction (MI) and has a beneficial role in insulin resistance, hypertriglyceridemia, fatty liver disease, and obesity [1–4].

Currently, additional metabolites of RAS and some of their biological functions have been described. Ang III, formed from Ang II by aminopeptidase A (APA), is cleaved by aminopeptidase N (APN) forming Ang IV. Ang IV binds to the angiotensin type 4 receptor (AT4), identified as an insulin-regulated aminopeptidase (IRAP), and it plays a potential role in the regulation of glucose homeostasis and inflammatory processes and in the metabolism of various hormones including vasopressin, oxytocin, and somatostatin [5, 6]. Ang III and APA are potential therapeutic targets for the treatment of hypertension; however, the roles of Ang III and Ang IV in cardiac function remain controversial [7–10].

On the other hand, neprilysin (NEP) is a ubiquitous endopeptidase of RAS, essential for the metabolism of the biologically active natriuretic peptides and several other vasoactive compounds, including adrenomedullin, endothelin 1, and bradykinin [11]. Moreover, plasmatic NEP levels have been positively associated with heart failure, obesity, and MetS [12, 13]. This enzyme is a target of multiple clinical trials given its importance in cardiovascular diseases, and a new class of drugs called “ARNI” (angiotensin receptor blocker-neprilysin inhibitor) is currently being used as therapy for hypertension and heart failure [14, 15].

Fenofibrate acts as an agonist of the peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) that regulates the expression of target genes, including some components of RAS. Fenofibrate has pleiotropic effects besides lowering lipids, such as improving vascular endothelial function and reducing oxidative stress, inflammation, and fibrosis during cardiac ischemia [1, 16, 17]. However, the exact mechanism underlying the beneficial effect of fenofibrate on cardiovascular diseases remains uncertain.

Our previous work demonstrated that fenofibrate treatment decreased ischemic damages by favoring an antioxidant environment as a consequence of reducing the Ang II/AT1R signaling pathway and reestablishing the cardiac insulin signaling pathway. Although numerous experimental studies have focused on an alternative RAS cascade, to our knowl-

edge, there are no reports on the effect of fenofibrate on these novel important components of RAS in myocardial ischemia. Therefore, the aim of the current study was to evaluate if the nonclassical RAS signaling pathways, directed by Ang III, Ang-(1-7), and NEP expression, are involved in the cardioprotective effect of fenofibrate during ischemia in MetS rats.

## 2. Methods

**2.1. Animals and Surgical Procedures.** All the experiments were conducted in accordance with our Institutional Ethical Guidelines (Ministry of Agriculture, SAGARPA, NOM-062-ZOO-1999, Mexico). Male 25-day-old Wistar rats weighing  $45 \pm 9$  g were randomly separated into two groups of 10–12 animals: group 1, control rats that were given tap water for drinking; group 2, MetS rats that received 30% sugar in drinking water during 24 weeks. The animals were maintained under standard conditions of light and temperature with water and food *ad libitum* (LabDiet 5001; Richmond, IN, USA). Systolic arterial blood pressure was determined in conscious animals by a plethysmographic method described previously [1].

Animals from each experimental group were divided to receive one of the subchronic (two weeks) oral gavage treatments: (a) vehicle (NaCl 0.9%) or (b) fenofibrate (100 mg/kg/day). This dose was selected based on previous publications and from a dose-response curve to fenofibrate [1, 16, 17]. At the end of the treatment, the animals were anesthetized (ketamine hydrochloride 80 mg/kg and xylazine hydrochloride 10 mg/kg, I.M.) and they were assigned to either sham-operated (Sh) or myocardial infarction (MI) for 60 min. As previously reported, MI was achieved occluding the left anterior descending coronary artery with 7-0 PROLENE® polypropylene suture (Ethicon, São José dos Campos, Brazil) [1]. Then, the rats were sacrificed, the heart was cut out, and the ischemic area was separated to perform the analysis. Additionally, the abdominal white adipose tissue was removed and weighed.

**2.2. Measurement of Serum Biochemical Parameters.** The fasting measurements of glucose, HDL-C, non-HDL-C, and triglycerides were performed with commercial enzymatic kits. Serum insulin levels were measured using a rat-specific insulin radioimmunoassay (Linco Research, Inc., Missouri, USA). Insulin resistance was estimated from the homeostasis model (HOMA-IR) [18].

Creatine kinase (CK) activity was spectrophotometrically determined at 340 nm (UV-test, Roche Cobas C-501, Roche Diagnostics, Indianapolis, IN, USA) after the MI procedure. The determination of CK was carried out using the reverse reaction and activation by N-acetylcysteine (NAC) at 37°C. Equimolar quantities of NADPH and ATP are formed at the same rate. The photometrically measured rate of formation of NADPH is directly proportional to the CK activity [19].

**2.3. Electrophoretic Determinations.** Ang II, Ang III, Ang IV, and Ang-(1-7) concentrations were evaluated in myocardial

ischemic areas from the different experimental groups by capillary zone electrophoresis (CZE, P/ACE MDQ Capillary Electrophoresis System, Beckman Coulter, Inc., Fullerton, CA, USA) according to the methods previously described [1, 18]. Bradykinin was evaluated by capillary electrophoresis with a laser-induced fluorescence detector, as previously reported [20].

**2.4. Western Blotting Analysis.** The frozen myocardial ischemic area was homogenized with a polytron (model PT-MR2100; Kinematica AG, Lucerne, Switzerland) (25% *w/v*) in a lysis buffer pH = 7.4 (Tris-HCl 250 mM, NaF 0.2 M, Na<sub>3</sub>VO<sub>4</sub> 10 mM, and NP40 1%) and a protease inhibitor cocktail (cOmplete® tablets, Roche Applied Science, Mannheim, Germany) at 4°C. A total of 100 µg protein was separated on a SDS-PAGE (12% bis-acrylamide-Laemmli gel) and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were blocked for 3 h at room temperature using Tris-buffered saline with 0.05% Tween (TBS-T) and 5% non-fat dehydrated milk. Afterwards, membranes were incubated overnight with primary antibodies at 4°C. ACE, ACE2, AT1R, AT2R, MasR, APA, APN, and NEP primary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA); IRAP antibody was from Cell Signaling Technology (Danvers, MA, USA). Secondary horseradish peroxidase-labeled antibodies were from Jackson ImmunoResearch (Suffolk, UK). All blots were incubated with β-actin antibody as a load control. After incubation, the blots were visualized using the Immobilon chemiluminescent system (Immobilon Western, Millipore, MA, USA) [1]. Images from films were digitally obtained using a GS-800 densitometer with the Quantity One software (Bio-Rad Laboratories, Inc.) and are reported as arbitrary units (AU).

**2.5. Determination of the Infarct Size.** After 60 min of ischemia, the rats were euthanized, and the hearts were rapidly excised. Later, the hearts were perfused with 1.5 mL of 0.05% Evans blue dye on the Langendorff system; this procedure was performed to outline the ischemic myocardium (and area at risk). The hearts were frozen at -20°C for 1 hour, and then 2 mm thick cross-sections were obtained. The slices were covered completely with 2,3,5-triphenyl tetrazolium hydrochloride (TTC) at 1% in phosphate-buffered saline (PBS) (1 M, pH 7.2) and incubated for 20 min at 37°C to distinguish the viable myocardium from the necrotic tissue. Later, the slices were incubated in 10% formalin for one hour and maintained in PBS until the image was obtained. The slices were photographed to show the ischemic area with a Multiphot Canon camera EOS 6D (Tokyo, Japan) [17].

**2.6. Statistical Analysis.** Results are expressed as mean ± standard error of the mean (SEM). Experimental data were examined using the one-way ANOVA followed by the Newman-Keuls *post hoc* test. Differences were considered statistically significant when  $p < 0.05$ . All analyses were performed using the statistical package GraphPad Prism version 5.03 (GraphPad Software, La Jolla, CA).

### 3. Results

The characterization of the MetS model was done by analyzing the animal's body weight, blood pressure, and intra-abdominal fat and by the serum biochemical analysis. As shown in Table 1, MetS animals developed central obesity, hypertension, dyslipidemia (high levels of triglycerides and non-HDL-C and low levels of HDL-C), hyperinsulinemia, and insulin resistance (HOMA-IR).

As expected, the treatment with fenofibrate significantly reduced the concentration of triglycerides and non-HDL-C levels and restored the insulin resistance index (HOMA-IR) in the MetS group. In the CT group, fenofibrate administration significantly reduced the concentration of non-HDL-C and did not affect the other parameters.

Serum CK activity was determined in all groups. No significant difference was found between the CT and MetS sham-operated groups ( $533.30 \pm 48.14$  vs.  $626.50 \pm 101.40$ ). After MI, CK activity was significantly higher in MetS than in CT rats treated with vehicle (Table 1). Fenofibrate treatment significantly reduced CK activity in serum from the CT and MetS animals (23% and 14%, respectively).

Figure 1 shows the expression of ACE and ACE2 in the homogenate from the left ventricles from each experimental group. ACE expression was slightly higher in the MetS group than in CT. Ischemia promoted an increase in ACE expression in the CT group, while the expression remained essentially unchanged in hearts from MetS rats. Fenofibrate treatment significantly diminished ACE expression in the same proportion in both experimental groups (Figures 1(a) and 1(b)).

Due to the relevance of ACE2 for the production of Ang-(1-7), we studied whether fenofibrate exerts an effect on this enzyme. ACE2 expression was significantly decreased in the MetS group compared to the CT group (Figure 1(c)). When the hearts were subjected to ischemia, ACE2 increased in the MetS group and no change was observed in CT rats. The administration of fenofibrate significantly increased ACE2 expression in the CT group and had no effect on MetS animals.

According to the pathophysiology, the left ventricular Ang II levels increased in MetS rats in comparison to CT rats (Figure 2(a)). When the hearts were under ischemic conditions, Ang II levels increased in CT and there was a further increase in MetS rats; however, fenofibrate administration significantly diminished Ang II concentrations. Importantly, in the MetS group, the Ang-(1-7) levels were significantly higher in basal conditions, but under ischemic conditions, Ang-(1-7) levels decreased (Figure 2(b)). Fenofibrate treatment significantly increased the concentration of this peptide in both groups (Figure 2(b)).

Western blot analyses revealed differences in the expression of AT1R, AT2R, and MasR in hearts from the CT and MetS groups (Figures 3(a)–3(d)). As expected, AT1R expression was higher in MetS rats compared to CT rats. Ischemia promoted an increase in AT1 expression in both the CT and MetS groups. Fenofibrate treatment significantly diminished the AT1R expression in both groups, although this effect was more evident in the MetS rats (Figures 3(a) and

TABLE 1: The effects of fenofibrate administration on body characteristics and biochemical parameters from the control (CT) and MetS rats.

	CT-V	CT-F	MetS-V	MetS-F
Body weight (g)	503.0 ± 18.3	490.5 ± 19.8	518.7 ± 14.6	502.5 ± 13.5
Blood pressure (mmHg)	98.6 ± 5.8	101.5 ± 1.3	148.1 ± 6.5 <sup>a</sup>	141.5 ± 11.3 <sup>a</sup>
Intra-abdominal fat (g)	6.4 ± 0.7	5.2 ± 0.5	13.4 ± 0.8 <sup>a</sup>	12.9 ± 0.9
Triglycerides (mg/dL)	60.8 ± 11.3	48.8 ± 5.3	135.6 ± 10.9 <sup>a</sup>	53.7 ± 10.2 <sup>b</sup>
HDL-C (mg/dL)	42.5 ± 4.8	41.7 ± 1.3	21.8 ± 3.9 <sup>a</sup>	23.5 ± 5.8
Non-HDL-C (mg/dL)	19.9 ± 2.1	11.7 ± 1.8 <sup>b</sup>	34.8 ± 2.8 <sup>a</sup>	12.8 ± 2.6 <sup>b</sup>
Total cholesterol (mg/dL)	58.6 ± 4.3	52.5 ± 3.8	61.7 ± 1.2	48.8 ± 6.4
Glucose (mg/dL)	101.3 ± 6.7	98.5 ± 3.2	110.6 ± 10.3	100.4 ± 5.8
Insulin (ng/mL)	0.16 ± 0.05	0.11 ± 0.03	0.39 ± 0.06 <sup>a</sup>	0.14 ± 0.07 <sup>b</sup>
HOMA-IR	1.1 ± 0.2	0.9 ± 0.1	4.1 ± 0.9 <sup>a</sup>	1.6 ± 0.7 <sup>b</sup>
CK activity (U/L)	1556.0 ± 77.77	1190.33 ± 73.12 <sup>c</sup>	1964.0 ± 52.03 <sup>a</sup>	1688.6 ± 54.18 <sup>a,c</sup>

Values are mean ± SEM. The serum biochemical determinations were performed after the myocardial insult. CT-V: control vehicle-treated; CT-F: control fenofibrate-treated; MetS-V: metabolic syndrome vehicle-treated; MetS-F: metabolic syndrome fenofibrate-treated; HOMA-IR: homeostatic model assessment of insulin resistance; HDL-C: high-density lipoprotein cholesterol; CK: creatine kinase.  $n = 6$ . <sup>a</sup> $p < 0.01$  MetS vs. CT same treatment; <sup>b</sup> $p < 0.05$  against vehicle corresponding group; <sup>c</sup> $p < 0.01$  against vehicle corresponding group.

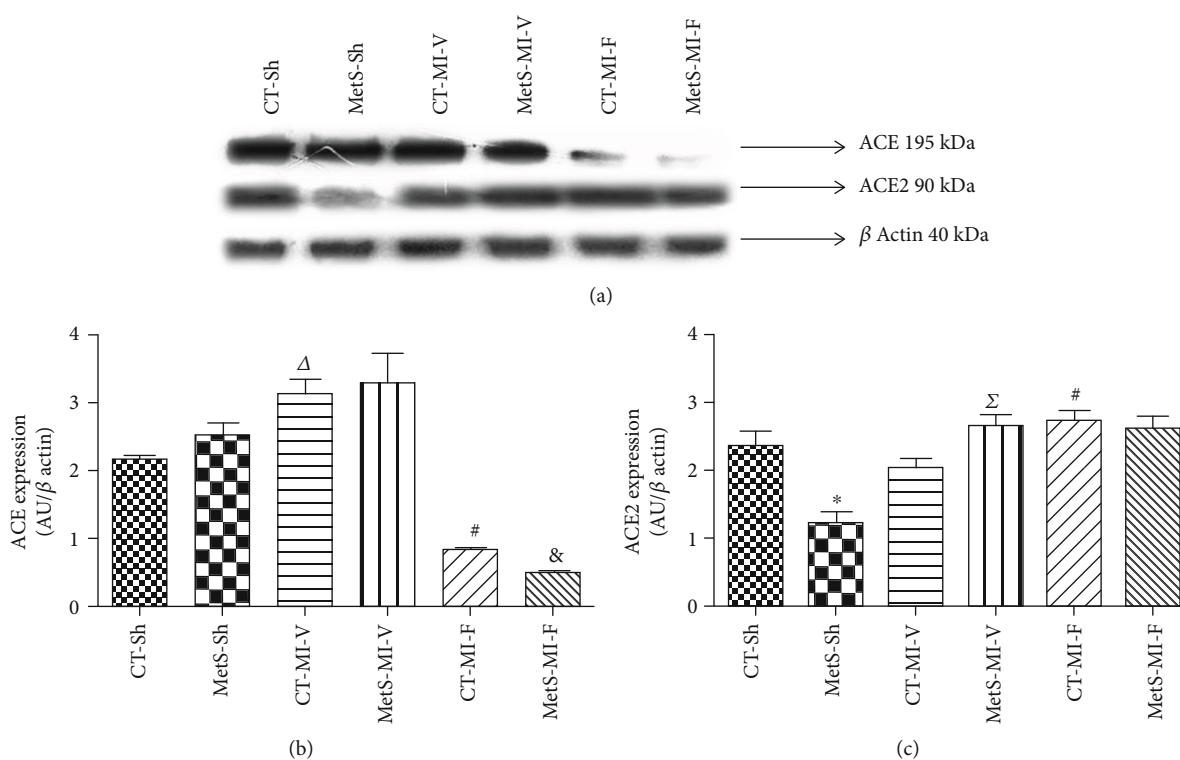


FIGURE 1: Effect of fenofibrate on the expression of renin-angiotensin-converting enzyme (ACE) and ACE2. The expression was evaluated in the left ventricles from the control (CT) and metabolic syndrome (MetS) rats subjected to sham (Sh) or myocardial infarction (MI) and treated for two weeks with either vehicle (V) or fenofibrate (F). (a) Representative western blot analysis. (b) ACE protein expression. (c) ACE2 protein expression. Data represent mean ± SEM ( $n = 5$  per group). \* $p < 0.05$  vs. CT-Sh;  $\Delta$   $p < 0.05$  vs. CT-MI-V;  $\Sigma$   $p < 0.05$  vs. MetS-Sh; # $p < 0.05$  vs. CT-MI-V;  $\&$   $p < 0.05$  vs. MetS-MI-V. Analysis of variance-Newman-Keuls.

3(b)). Results show that AT2R expression did not differ between the two sham-operated groups. When the hearts were subjected to ischemia, a significant decrease of AT2R expression in the CT and MetS groups was observed. The administration of fenofibrate significantly increased the

expression of this receptor in both groups (Figures 3(a) and 3(c)). MasR expression was significantly higher in MetS-Sh compared to CT-Sh animals; the expression of this receptor under ischemic conditions was comparable to values from sham experimental groups. The treatment with fenofibrate

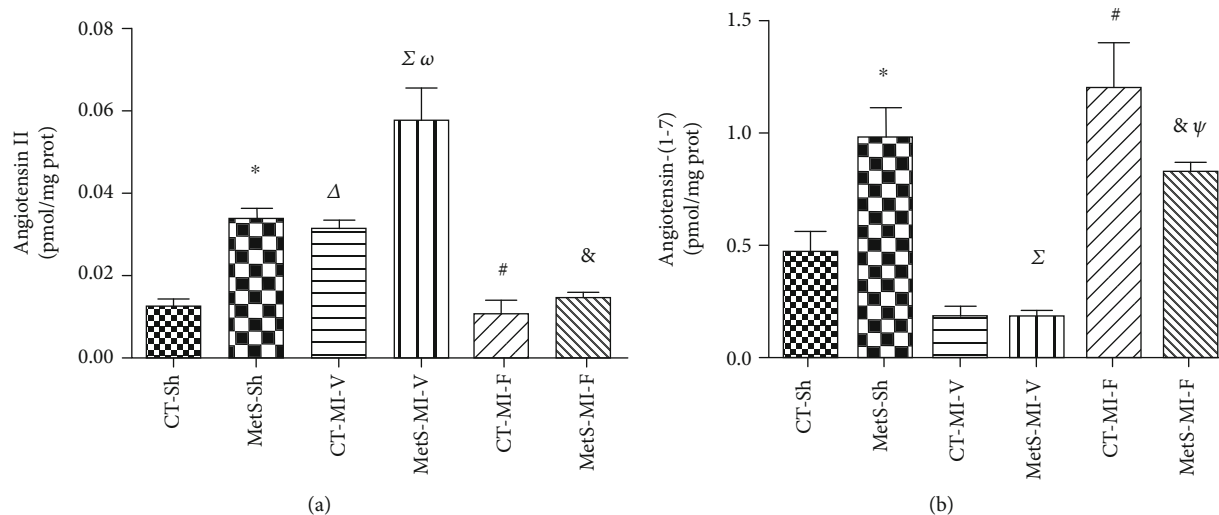


FIGURE 2: Effect of fenofibrate on angiotensin II and angiotensin-(1-7) levels. (a) Angiotensin II and (b) angiotensin-(1-7) concentrations were determined in the homogenate from myocardial ischemic areas from the sham (Sh), myocardial infarction (MI) vehicle-treated, and MI fenofibrate-treated experimental groups. The values show the mean  $\pm$  SEM ( $n = 5$  per group). \*  $p < 0.05$  vs. CT-Sh;  $\Delta$   $p < 0.05$  vs. CT-Sh;  $\Sigma$   $p < 0.05$  vs. MetS-Sh; #  $p < 0.05$  vs. CT-MI-V;  $\omega$   $p < 0.05$  vs. CT-MI-V; &  $p < 0.05$  vs. MetS-MI-V;  $\psi$   $p < 0.05$  vs. CT-MI-F. Analysis of variance-Newman-Keuls.

promoted an increase in MasR expression in CT rats, while its expression remained unchanged in hearts from MetS (Figures 3(a) and 3(d)).

Since Ang III and Ang IV have been identified as new biologically active peptides of RAS, we studied the effect of fenofibrate on the production of these metabolites. Figures 4(a)–4(c) show that the expression of APA (the major enzyme metabolizing Ang II to Ang III) and Ang III concentration were significantly increased in the left ventricle of MetS-Sh animals compared to CT-Sh. This effect was also observed under ischemic conditions. Fenofibrate was able to prevent the increase in APA and Ang III levels in MetS animals.

We also investigated if fenofibrate induced variations in Ang III concentrations which might be associated with the Ang IV-IRAP pathway. Our results showed that hearts from MetS-Sh had significantly higher levels of APN, Ang IV, and IRAP when compared to the corresponding CT group (Figures 5(a)–5(d)). Ischemia was accompanied by a decrease in the components analyzed in hearts from MetS rats; however, the levels of APN and Ang IV did not change significantly in the CT group. Fenofibrate treatment was able to prevent the activation of the Ang IV-IRAP axis in the left ventricles from MetS rats (Figures 5(a)–5(d)).

Due to the beneficial effects of the inhibition of NEP and the cardioprotective effects of bradykinin, we evaluated the effect of fenofibrate on NEP expression and bradykinin production. Figures 6(a) and 6(b) show that under basal conditions, MetS hearts expressed more NEP when compared to CT hearts.

In CT-MI vehicle-treated animals, the expression of this enzyme increased under ischemic conditions; nevertheless, in the MetS-MI vehicle-treated group, NEP levels were not modified. The concentrations of bradykinin showed the opposite effect (Figure 6(c)). The administration of fenofibrate was associated with a significant decrease in NEP

expression and a consequent increase in bradykinin production in both groups.

The infarct size was evaluated using Evans blue dye plus TTC at 1%. Blue-dyed myocardial tissue represents viable tissue. Left ventricles obtained from the CT-Sh group exhibit an extensive blue area. Tissue from MetS-Sh shows a blue periphery that signals the border of the central zone colored in red. The red area represents the area at risk (Figure 7). In MI groups treated with vehicle, the TCC-stained heart slice confirms infarcted areas (white spots within the viable area); nevertheless, the area at risk (red-colored region) was greater in hearts from MetS rats. The treatment with fenofibrate attenuated the tissue injury in the ischemic area of the MetS and CT rats. The improvement was larger in the CT group where the area at risk and ischemic regions decreased (limited at the central area). However, these areas were widely distributed in the tissue in hearts from MetS rats (Figure 7).

#### 4. Discussion

This work shows that fenofibrate treatment generates cardioprotection in an experimental model of MetS subjected to ischemia, by regulating the nonclassical pathways of RAS. Our study demonstrated that the pharmacological treatment was associated with the activation of Ang-(1-7)/AT2R and inhibition of Ang III/Ang IV/IRAP pathways. Moreover, another novel finding of this study was that the treatment with fenofibrate may decrease the expression of NEP with the consequent increase of bradykinin production.

Extensive research has revealed that the activation of specific molecules of RAS participates in the development of MetS and heart failure. Fenofibrate therapy reverts some of the effects caused by these pathologies by regulating several processes mediated by Ang II/AT1, such as energy metabolism, oxidative stress, inflammation, and cell differentiation

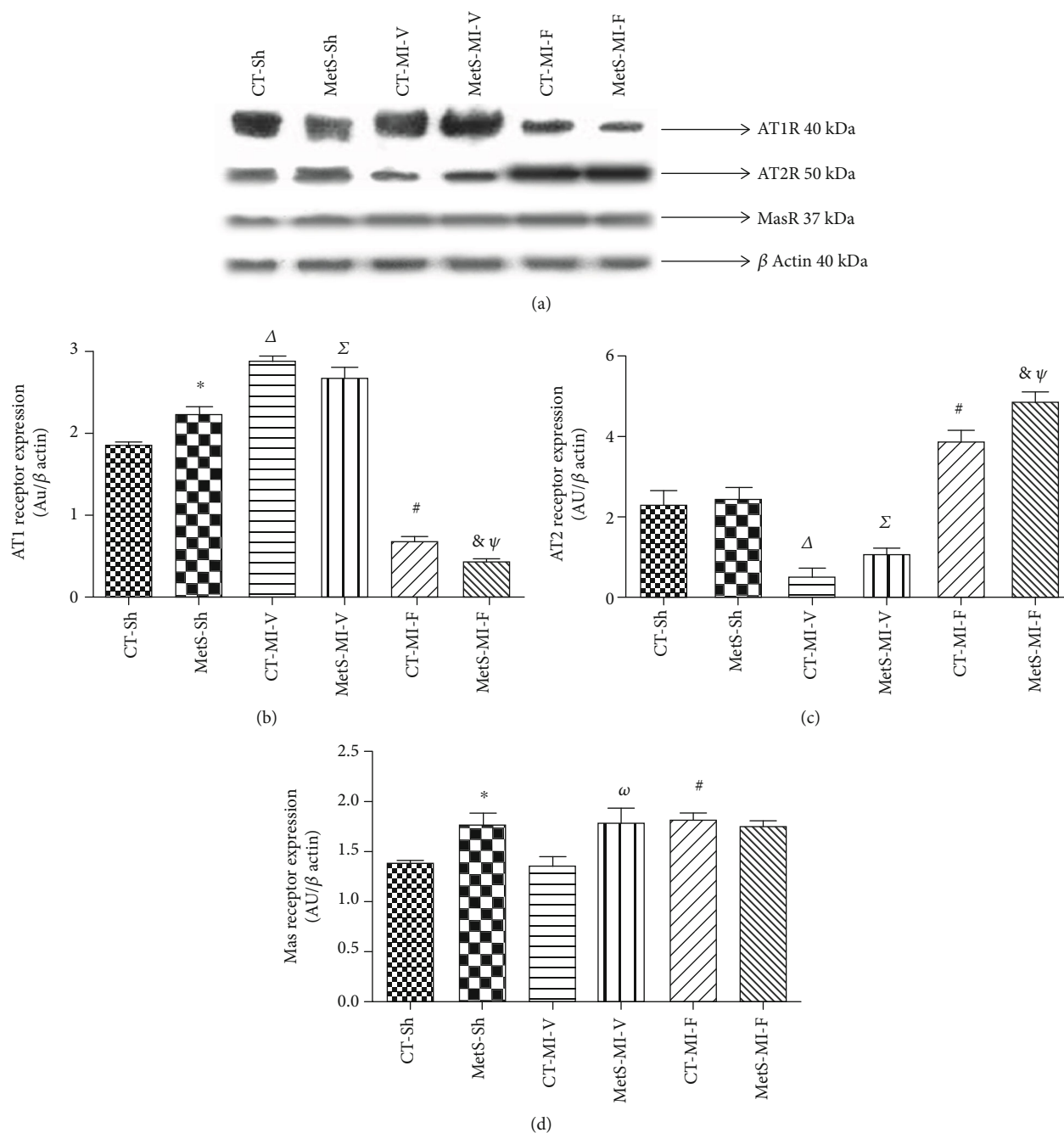


FIGURE 3: Expression of cardiac angiotensin receptors. The proteins were evaluated in the left ventricles from the control (CT) and metabolic syndrome (MetS) rats subjected to sham (Sh) or myocardial infarction (MI) and treated for two weeks with either vehicle (V) or fenofibrate (F). (a) Representative immunoblot. (b) Angiotensin II-type 1 receptor (AT1R) protein expression. (c) Angiotensin II-type 2 receptor (AT2R) protein expression. (d) MasR protein expression. The values show the mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$  vs. CT-Sh;  $^{\Delta} p < 0.05$  vs. CT-Sh;  $^{\Sigma} p < 0.05$  vs. MetS-Sh;  $^{\#} p < 0.05$  vs. CT-MI-V;  $^{\omega} p < 0.05$  vs. CT-MI-V;  $^{\&} p < 0.05$  vs. MetS-MI-V;  $^{\psi} p < 0.05$  vs. CT-MI-F. Analysis of variance-Newman-Keuls.

[1]. On the other hand, an accumulating amount of data signals the importance of an alternative pathway of RAS, such as Ang-(1-7) and its cardioprotective properties. Our data are in line with previous findings; however, there are no reports on the effect of fenofibrate on the important counterparts of the RAS pathways analyzed in this paper as far as we know.

Data in Table 1 show that the fenofibrate treatment reversed the signs of MetS such as dyslipidemia and insulin

resistance, and it did not affect the other parameters. These data are in accordance with those previously published [1].

Serum CK activity is well known as a cardiac risk biomarker in human and animal models; however, this parameter is unspecific [21–23]. The results in Table 1 show that CK activity was higher in serum from MetS-MI rats than the CT-MI group treated with vehicle. In addition, we demonstrated that fenofibrate treatment significantly reduced the increase

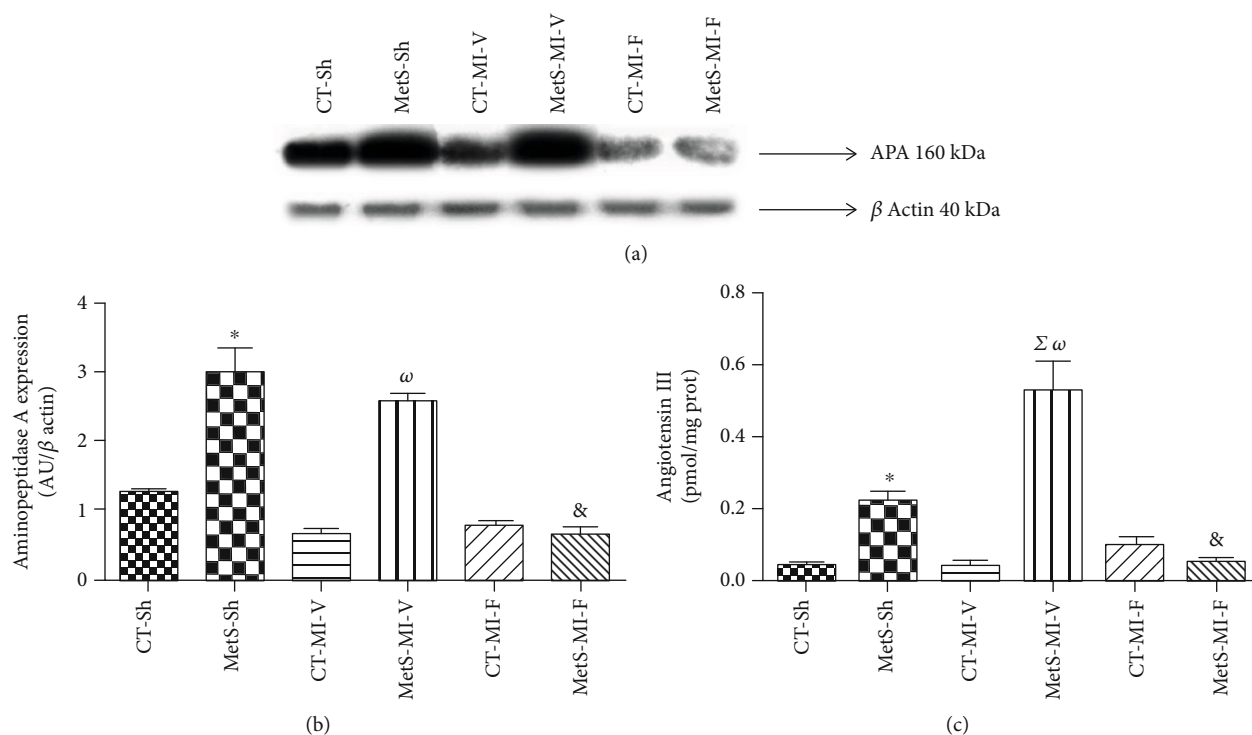


FIGURE 4: Effect of fenofibrate on the expression of aminopeptidase A (APA) and angiotensin III (Ang III) concentrations in the control (CT) and metabolic syndrome (MetS) rats under ischemic conditions (MI). (a) Representative western blot analysis. (b) APA protein expression. (c) Ang III concentration. Arbitrary units (AU). The values show the mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$  vs. CT-Sh;  $\Sigma p < 0.05$  vs. MetS-Sh;  $\omega p < 0.05$  vs. CT-MI-V; & $p < 0.05$  vs. MetS-MI-V. Analysis of variance-Newman-Keuls.

of CK in the serum from both the CT and MetS rats. Nevertheless, this effect was more evident in the CT group. Therefore, our results show that CK activity is a good biomarker of myocardial damage in our experimental model.

In this study, the classical and nonclassical RAS axes were studied. The ACE/Ang II/AT1R axis has been the main RAS pathway studied, and its roles in cardiac damage and therapeutic implications have been extensively reviewed [1]. As expected, the ACE expression and the Ang II concentration were higher (slightly and significantly, respectively) in hearts from MetS animals compared to the CT-Sh group. Ischemic conditions were associated with an increase in the expression of the enzyme and its product (Figures 1(a) and 2(a)). Fenofibrate treatment prevented the increase of ACE expression in both the CT and MetS groups. Consistent with these findings, the Ang II concentration decreased in both experimental groups (Figures 1(b) and 2(a)). As far as we know, there are no reports on the determination of Ang II levels in patients undergoing fenofibrate therapy. There are conflicting data regarding the effect of PPAR agonists on blood pressure in humans, due to heterogeneity of populations, the mechanism underlying the high blood pressure, and differences in concurrent treatments or dietary salt intakes [24]. However, Walker et al. [25] demonstrated a beneficial effect of fenofibrate on vascular endothelial function in humans. Fenofibrate reduces oxidative stress and increases eNOS expression. These effects might be related to the reduction of Ang II concentrations and agree with our previously reported results [1].

On the other hand, ACE2 efficiently hydrolyses Ang II to form Ang-(1-7), a peptide that exerts opposite actions to those of Ang II. Our results show that under basal conditions, the expression of ACE2 was not accompanied by similar changes in Ang-(1-7) concentration. Surprisingly, the ischemic insult promoted an increase in ACE2 expression while the levels of Ang-(1-7) decreased in the MetS group (Figures 1(c) and 2(b)). The increase in ACE2 expression under ischemia conditions suggests a protective role of this enzyme and is in agreement with the data published by other authors in human and animal models [26, 27]. However, the activity of ACE2 should be evaluated to explain the discrepancy between ACE2 expression and Ang-(1-7) concentration. This constitutes a limitation of the present study. The levels of Ang-(1-7) were significantly higher in the ischemic hearts in the presence of fenofibrate in both the CT and MetS animals (Figure 2(b)). Ang-(1-7) decreases hypertension, cardiac hypertrophy, oxidative stress, and insulin resistance [28, 29]. Furthermore, in a previous report, we demonstrated that clofibrate treatment increased Ang-(1-7) concentration in the ischemic myocardium [20].

Figure 3 shows that there is an overexpression of AT1R and MasR in hearts from MetS rats under basal conditions. Under ischemic conditions, AT1R levels increased and AT2R levels decreased in both experimental groups; fenofibrate therapy reversed this effect. The expression of Mas increased only in the CT-MI-F group (Figures 3(a)–3(d)). It has been reported that Ang-(1-7) interacts with both MasR and AT2R [30–32]. Therefore, it is reasonable to suggest that

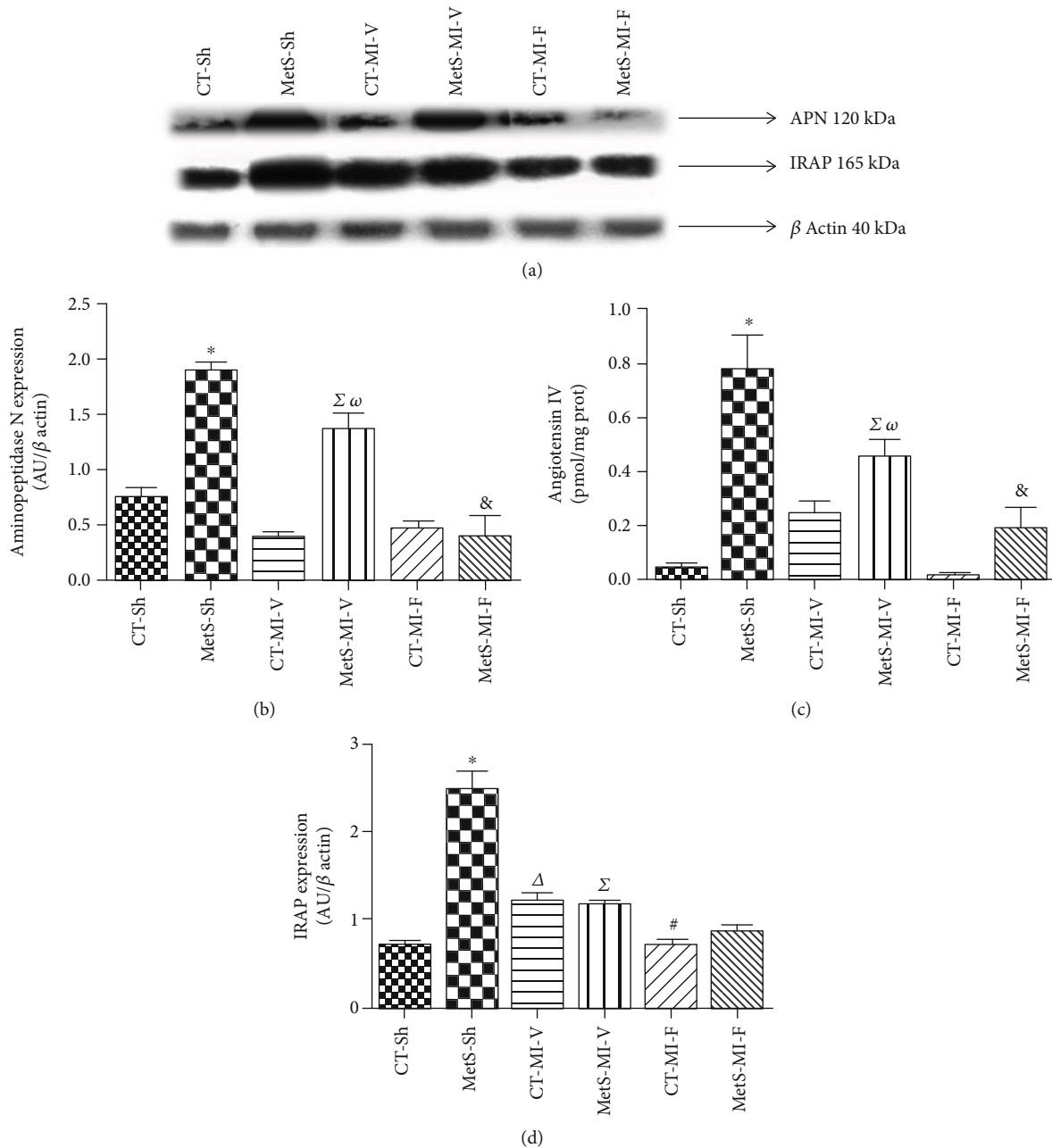


FIGURE 5: Effect of fenofibrate administration on myocardial aminopeptidase N (APN) expression in myocardial tissue from the control (CT) and metabolic syndrome (MetS) animals. (a) Representative image. (b) Angiotensin IV concentration. (c) Insulin-regulated aminopeptidase (IRAP) protein expression. The values show the mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$  vs. CT-Sh;  $^{\Delta}p < 0.05$  vs. CT-Sh;  $^{\Sigma}p < 0.05$  vs. MetS-Sh;  $^{\#}p < 0.05$  vs. CT-MI-V;  $^{\omega}p < 0.05$  vs. CT-MI-V;  $^{\&}p < 0.05$  vs. MetS-MI-V. Analysis of variance-Newman-Keuls.

the cardioprotective actions could be mediated through these receptors.

Altogether, our results show that ischemia promotes the activation of the ACE/Ang II/AT1R pathway and that the activation of the Ang-(1-7)/MasR-AT2 signaling pathway is involved in the cardioprotective role of fenofibrate.

Subsequently, we studied the effect of fenofibrate therapy on the nonclassical components of the RAS pathway. Two smaller angiotensin peptides, Ang III and Ang IV, have been reported to have harmful effects via AT1R activation [33, 34]. Ang III and Ang IV levels were increased in the left ventricles

in MetS-Sh rats (Figures 4(c) and 5(c)). After the ischemic insult, Ang III concentrations were significantly increased, while Ang IV levels diminished in MetS vehicle-treated hearts. We suggest that this may be a compensatory effect to the ischemic damage. These results were consistent with the expression of APA and APN, respectively (Figures 4(b) and 5(b)). The administration of fenofibrate prevents the rise in the small peptides in MetS rats, while the values remained unchanged in CT animals. Clearly, the decrease in the concentration of the small peptides was directly related to the decrease of its precursor, Ang II; however, our results show



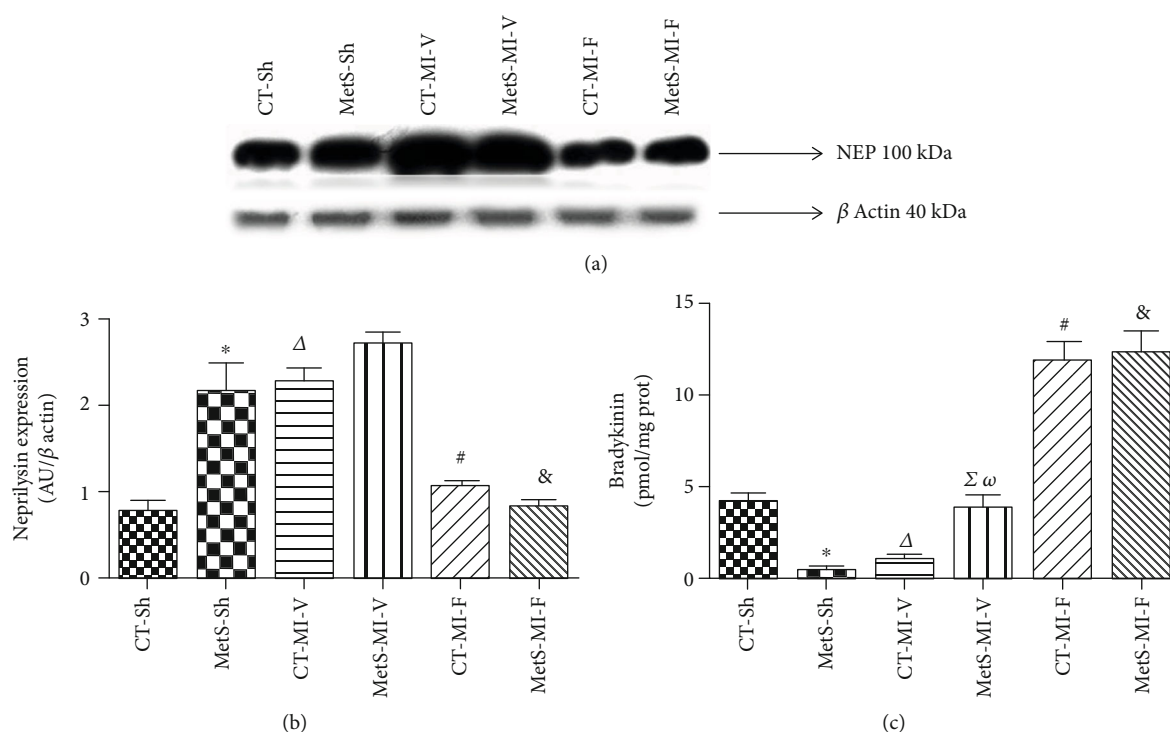


FIGURE 6: Effect of fenofibrate on the expression of neprilysin (NEP) and bradykinin concentration in control and under ischemic conditions. (a) Representative western blot analysis. (b) NEP protein expression. (c) Bradykinin concentration. The values show the mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$  vs. CT-Sh;  $\Delta p < 0.05$  vs. CT-Sh;  $\Sigma p < 0.05$  vs. MetS-Sh; # $p < 0.05$  vs. CT-MI-V;  $\omega p < 0.05$  vs. CT-MI-V; & $p < 0.05$  vs. MetS-MI-V. Analysis of variance-Newman-Keuls.

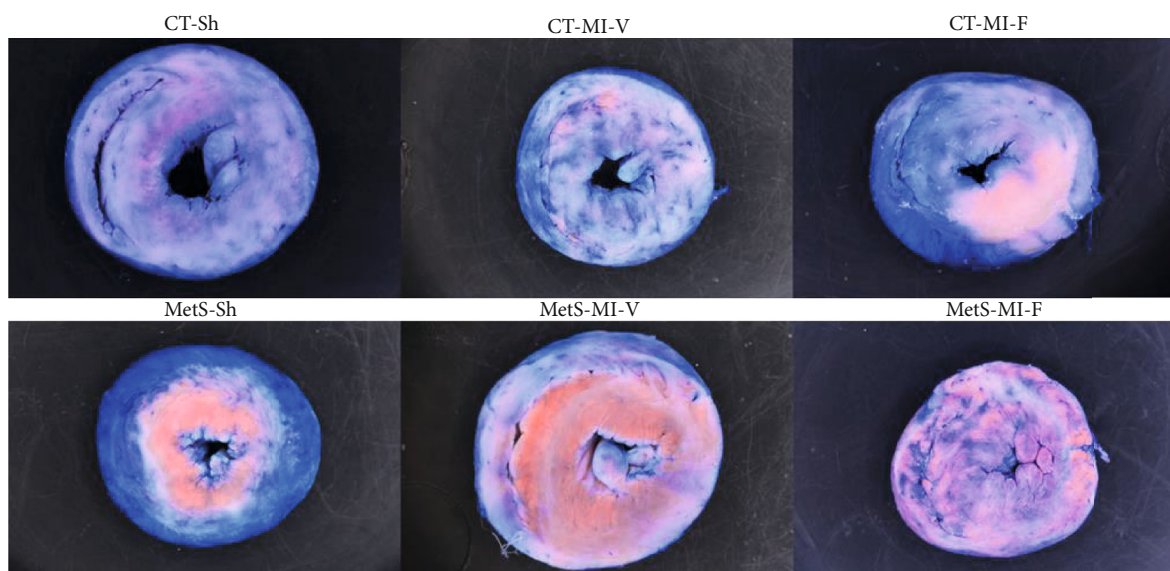


FIGURE 7: Fenofibrate treatment decreased the area at risk in myocardial infarcted rats. Representative images of TTC staining. Blue areas indicate viable tissue; the red-colored region represents tissue at risk, and the white regions indicate the infarcted myocardium. CT-Sh and MetS-Sh: control and metabolic syndrome sham-operated groups; CT-MI-V: control myocardial infarction vehicle-treated; CT-MI-F: control myocardial infarction fenofibrate-treated; MetS-MI-V: metabolic syndrome myocardial infarction vehicle-treated; MetS-MI-F: metabolic syndrome myocardial infarction fenofibrate-treated.

that the administration of fenofibrate had a direct association with the expression of APA and APN. Moreover, the receptor to Ang IV has been identified in several tissues as an IRAP.

The presence of IRAP is important for the translocation of the insulin-stimulated GLUT4 and in the metabolism of oxytocin [33, 35, 36]. MetS and ischemic conditions were

associated with IRAP overexpression, and the therapy with fenofibrate decreased the levels of this protein (Figure 5(d)).

Ang IV is the endogenous inhibitor of the catalytic binding site of IRAP preventing the metabolism of various IRAP substrates, including oxytocin. Oxytocin exerts cardioprotection, either directly or via stimulation of mediators such as the natriuretic peptides and nitric oxide [37]. Furthermore, this hormone enhances glucose uptake via the translocation of GLUT4 to the cell surface [36, 38]. Therefore, we speculate that the Ang IV/AT4R/IRAP axis is also involved in cardiac insulin resistance under ischemic conditions, regulating oxytocin concentrations. Fenofibrate treatment might improve glucose uptake by increasing oxytocin levels as a consequence of the downregulation of the IRAP expression. These suggestions are in agreement with our previous study, in which we showed that ischemia impairs myocardial insulin action and that fibrates reestablish the cardiac insulin signaling pathway [1]. There are few studies reporting that the IRAP deficiency/inhibition protects against cardiac damage; therefore, further studies demonstrating the pathophysiological role of the Ang IV/AT4R/IRAP axis are needed.

We studied whether the administration of fenofibrate had an effect on the expression levels of NEP and bradykinin, due to the interaction between the RAS and the NEP system and the role of peptides, such as bradykinin, in heart homeostasis. We observed that MetS and ischemic conditions were accompanied by a NEP overexpression with the consequent reduction in the concentration of bradykinin (Figures 6(b) and 6(c)). Fenofibrate treatment favored the production of bradykinin by suppressing NEP expression in hearts from both the CT and MetS rats. Our findings suggest the involvement of NEP in the cardioprotective effect of fenofibrate, and this effect could be mediated by genomic actions through the previously reported PPAR activation [39]. Thus, the increase in bradykinin levels might mediate several cardiac functions, as has been previously reported by other authors [40, 41]. Further studies are needed to prove the effect of the pretreatment with fenofibrate on the production of other hormones metabolized by NEP during heart failure, such as natriuretic peptides.

Finally, in order to evaluate the efficacy of our treatment, we analyzed the ischemic size by the TTC staining analysis. Evans blue dye has been used extensively to stain hearts, and it accurately reflects the extent of viable areas and irreversible myocardial ischemic damage. Figure 7 shows that hearts from MetS-Sh rats present a larger area at risk when compared to the CT group. Our results are in line with experimental and clinical trial evidence which suggests that MetS is associated with myocardial infarction [42]. After MI insult, the MetS heart had a larger area at risk and infarcted area when compared to the heart from CT rats. Fenofibrate treatment was able to attenuate MI-induced damage evidenced by the reduction of the area at risk and ischemic area (red- and white-colored regions). This effect is more evident in the CT group. The results of our work are in agreement with those of Mo et al. [22], who showed that fenofibrate reduced the myocardial infarct size.

Cardiac myocytes in the ischemic zone die by apoptosis and necrosis during a myocardial infarction; nevertheless,

the magnitudes of each form of cell death remain unclear. Krijnen et al. [43] described that upon permanent occlusion of a coronary vessel in rats, apoptosis occurred in the ischemic region, in the area immediately bordering the ischemic region, and in remote regions from ischemia. Therefore, apoptosis is the major determinant of infarct size. Necrosis occurred less often and was seen only in the ischemic region.

Nevertheless, we did not perform an analysis of cell necrosis and apoptosis in the present study. In a previous report of our group, we found that PPAR- $\alpha$  stimulation with clofibrate augmented the expression of antiapoptotic proteins Bcl-2 and 14-3-3 $\epsilon$  and decreased the expression of proapoptotic proteins Bax and the phosphorylation of Bad [44]. Moreover, oxidative stress coexists with apoptosis after myocardial infarction [43]. Our model also presents oxidative stress [1], and therefore, we cannot exclude an effect similar to the one previously reported by our group.

## 5. Conclusion

Overall, our results show that PPAR- $\alpha$  activation by fenofibrate prevents damage due to ischemia in MetS rats by changing the angiotensin metabolites and their receptor profiles. It favors the Ang-(1-7)/AT2 axis and inhibits the Ang III/Ang IV/IRAP signaling pathway. In addition, PPAR- $\alpha$  signaling downregulates the expression of NEP and increases the production of bradykinin. Thus, the regulation of the nonclassical axis of RAS is a novel protective effect to myocardial ischemia of fenofibrate.

## Data Availability

The data in our study are available from the corresponding author upon reasonable request.

## Conflicts of Interest

The authors declare no conflict of interest.

## Authors' Contributions

M. S-A. and L. I-L. were responsible for planning and performing the experiments and data analysis; L. DV-M. was responsible for performing the electrophoretic analysis; E. S-C. and M. S-A. were responsible for the histochemistry analysis; J.C. T-N. was responsible for performing some physiological experiments; E. C-T. was responsible for serum biochemical analysis; A. S-M. obtained financial support to partly cover expenses and reviewed the manuscript; M.E. R-R. was responsible for planning the experiments, performing the physiological experiments and data analysis, and writing the paper. M. S-A. and L. I-L. contributed equally to this work.

## Acknowledgments

The authors would like to thank Biol. Oscar Infante (Departamento de Instrumentación Electromecánica) for his helpful advice and expertise on blood pressure determination. The authors also thank Biol. Armando Zepeda Rodríguez and

Francisco Pasos Nájera for their support in acquiring photos and José Saúl Carreón Cervantes, Mario Pérez, and Jhony Pérez for excellent technical assistance. This work was supported by Consejo Nacional de Ciencia y Tecnología (CONACyT) (Grant numbers 222720 and 280458 to Alicia Sánchez-Mendoza).

## References

- [1] L. Ibarra-Lara, M. Sánchez-Aguilar, A. Sánchez-Mendoza et al., “Fenofibrate therapy restores antioxidant protection and improves myocardial insulin resistance in a rat model of metabolic syndrome and myocardial ischemia: the role of angiotensin II,” *Molecules*, vol. 22, no. 1, p. 31, 2017.
- [2] Y. Marcus, G. Shefer, K. Sasson et al., “Angiotensin 1-7 as means to prevent the metabolic syndrome: lessons from the fructose-fed rat model,” *Diabetes*, vol. 62, no. 4, pp. 1121–1130, 2013.
- [3] R. A. S. Santos, A. J. Ferreira, T. Verano-Braga, and M. Bader, “Angiotensin-converting enzyme 2, angiotensin-(1-7) and Mas: new players of the renin-angiotensin system,” *Journal of Endocrinology*, vol. 216, no. 2, pp. R1–R17, 2013.
- [4] M. Slamkova, S. Zorad, and K. Krskova, “Alternative renin-angiotensin system pathways in adipose tissue and their role in the pathogenesis of obesity,” *Endocrine Regulations*, vol. 50, no. 4, pp. 229–240, 2016.
- [5] S. R. Keller, “Role of the insulin-regulated aminopeptidase IRAP in insulin action and diabetes,” *Biological and Pharmaceutical Bulletin*, vol. 27, no. 6, pp. 761–764, 2004.
- [6] C. A. McCarthy, L. J. Facey, and R. E. Widdop, “The protective arms of the renin-angiotensin system in stroke,” *Current Hypertension Reports*, vol. 16, no. 7, p. 440, 2014.
- [7] J. Gao, Y. Marc, X. Iturrioz, V. Leroux, F. Balavoine, and C. Llorens-Cortes, “A new strategy for treating hypertension by blocking the activity of the brain renin-angiotensin system with aminopeptidase A inhibitors,” *Clinical Science (London)*, vol. 127, no. 3, pp. 135–148, 2014.
- [8] V. Esteban, M. Ruperez, E. Sanchez-Lopez et al., “Angiotensin IV activates the nuclear transcription factor- $\kappa$ B and related proinflammatory genes in vascular smooth muscle cells,” *Circulation Research*, vol. 96, no. 9, pp. 965–973, 2005.
- [9] P. L. Moraes, L. M. Kangussu, L. G. da Silva, C. H. Castro, R. A. S. Santos, and A. J. Ferreira, “Cardiovascular effects of small peptides of the renin angiotensin system,” *Physiological Reports*, vol. 5, no. 22, p. e13505, 2017.
- [10] B. M. Park, S. A. Cha, S. H. Lee, and S. H. Kim, “Angiotensin IV protects cardiac reperfusion injury by inhibiting apoptosis and inflammation via AT4R in rats,” *Peptides*, vol. 79, pp. 66–74, 2016.
- [11] S. A. Hubers and N. J. Brown, “Combined angiotensin receptor antagonism and neprilysin inhibition,” *Circulation*, vol. 133, no. 11, pp. 1115–1124, 2016.
- [12] J. Kim, D. Han, S. H. Byun et al., “Neprilysin facilitates adipogenesis through potentiation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway,” *Molecular and Cellular Biochemistry*, vol. 430, no. 1-2, pp. 1–9, 2017.
- [13] M. Kassi, B. Hannawi, and B. Trachtenberg, “Recent advances in heart failure,” *Current Opinion in Cardiology*, vol. 33, no. 2, pp. 249–256, 2018.
- [14] T. Prasad, L. C. W. Roksnoer, P. Zhu et al., “Beneficial effects of combined AT1 receptor/neprilysin inhibition (ARNI) versus AT1 receptor blockade alone in the diabetic eye,” *Investigative Ophthalmology & Visual Science*, vol. 57, no. 15, pp. 6722–6730, 2016.
- [15] A. T. Owens, S. Brozena, and M. Jessup, “Neprilysin inhibitors: emerging therapy for heart failure,” *Annual Review of Medicine*, vol. 68, no. 1, pp. 41–49, 2017.
- [16] E. A. Abd El-Haleim, A. K. Bahgat, and S. Saleh, “Resveratrol and fenofibrate ameliorate fructose-induced nonalcoholic steatohepatitis by modulation of genes expression,” *World Journal of Gastroenterology*, vol. 22, no. 10, pp. 2931–2948, 2016.
- [17] V. H. Oidor-Chan, E. Hong, F. Pérez-Severiano et al., “Fenofibrate plus metformin produces cardioprotection in a type 2 diabetes and acute myocardial infarction model,” *PPAR Research*, vol. 2016, Article ID 8237264, 14 pages, 2016.
- [18] M. E. Rubio-Ruiz, L. Del Valle-Mondragón, V. Castrejón-Tellez, E. Carreón-Torres, E. Díaz-Díaz, and V. Guarner-Lans, “Angiotensin II and 1-7 during aging in metabolic syndrome rats. Expression of AT1, AT2 and Mas receptors in abdominal white adipose tissue,” *Peptides*, vol. 57, pp. 101–108, 2014.
- [19] G. Schumann, R. Bonora, F. Ceriotti et al., “IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 degrees C. Part 2. Reference procedure for the measurement of catalytic concentration of creatine kinase,” *Clinical Chemistry and Laboratory Medicine*, vol. 40, no. 6, pp. 635–642, 2002.
- [20] L. Ibarra-Lara, M. Sánchez-Aguilar, E. Hong et al., “PPAR $\alpha$  stimulation modulates myocardial ischemia-induced activation of renin-angiotensin system,” *Journal of Cardiovascular Pharmacology*, vol. 65, no. 5, pp. 430–437, 2015.
- [21] M. Kurata, T. Iidaka, Y. Sasayama, T. Fukushima, M. Sakimura, and N. Shirai, “Correlation among clinicopathological parameters of myocardial damage in rats treated with isoproterenol,” *Experimental Animals*, vol. 56, no. 1, pp. 57–62, 2007.
- [22] H. Mo, S. Zhao, J. Luo, and J. Yuan, “PPAR $\alpha$  activation by fenofibrate protects against acute myocardial ischemia/reperfusion injury by inhibiting mitochondrial apoptosis,” *International Journal of Clinical Experimental Pathology*, vol. 9, no. 11, pp. 10955–10964, 2016.
- [23] W. Chen, J. Liang, Y. Fu et al., “Cardioprotection of cortistatin against isoproterenol-induced myocardial injury in rats,” *Annals of Translational Medicine*, vol. 8, no. 6, p. 309, 2020.
- [24] K. Gilbert, H. Nian, C. Yu, J. M. Luther, and N. J. Brown, “Fenofibrate lowers blood pressure in salt-sensitive but not salt-resistant hypertension,” *Journal of Hypertension*, vol. 31, no. 4, pp. 820–829, 2013.
- [25] A. E. Walker, R. E. Kaplon, S. M. S. Lucking, M. J. Russell-Nowlan, R. H. Eckel, and D. R. Seals, “Fenofibrate improves vascular endothelial function by reducing oxidative stress while increasing endothelial nitric oxide synthase in healthy normolipidemic older adults,” *Hypertension*, vol. 60, no. 6, pp. 1517–1523, 2012.
- [26] A. B. Goulter, M. J. Goddard, J. C. Allen, and K. L. Clark, “ACE2 gene expression is up-regulated in the human failing heart,” *BMC Medicine*, vol. 2, no. 1, 2004.
- [27] S. Keidar, M. Kaplan, and A. Gamliellazarovich, “ACE2 of the heart: from angiotensin I to angiotensin (1-7),” *Cardiovascular Research*, vol. 73, no. 3, pp. 463–469, 2007.
- [28] D. G. Passos-Silva, T. Verano-Braga, and R. A. S. Santos, “Angiotensin-(1-7): beyond the cardio-renal actions,” *Clinical Science (London)*, vol. 124, no. 7, pp. 443–456, 2013.

- [29] B. Liang, Y. Li, Z. Han et al., "ACE2-Ang (1-7) axis is induced in pressure overloaded rat model," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 2, pp. 1443–1450, 2015.
- [30] S. Bosnyak, E. S. Jones, A. Christopoulos, M. I. Aguilar, W. G. Thomas, and R. E. Widdop, "Relative affinity of angiotensin peptides and novel ligands at AT1 and AT2 receptors," *Clinical Science (London)*, vol. 121, no. 7, pp. 297–303, 2011.
- [31] M. P. Ocaranza and J. E. Jalil, "Protective role of the ACE2/Ang-(1-9) axis in cardiovascular remodeling," *International Journal of Hypertension*, vol. 2012, Article ID 594361, 12 pages, 2012.
- [32] E. J. Tassone, A. Sciacqua, F. Andreozzi et al., "Angiotensin (1-7) counteracts the negative effect of angiotensin II on insulin signalling in HUVECs," *Cardiovascular Research*, vol. 99, no. 1, pp. 129–136, 2013.
- [33] J. L. Zhuo, F. M. Ferrao, Y. Zheng, and X. C. Li, "New frontiers in the intrarenal renin-angiotensin system: a critical review of classical and new paradigms," *Frontiers in Endocrinology*, vol. 4, 2013.
- [34] K. Kramkowski, A. Mogielnicki, and W. Buczko, "The physiological significance of the alternative pathways of angiotensin II production," *Journal of Physiology and Pharmacology*, vol. 57, no. 4, pp. 529–539, 2006.
- [35] S. Y. Chai, R. Fernando, G. Peck et al., "What's new in the renin-angiotensin system?," *Cellular and Molecular Life Sciences*, vol. 61, no. 21, pp. 2728–2737, 2004.
- [36] S. Diwakarla, E. Nylander, A. Grönbladh et al., "Binding to and inhibition of insulin-regulated aminopeptidase by macrocyclic disulfides enhances spine density," *Molecular Pharmacology*, vol. 89, no. 4, pp. 413–424, 2016.
- [37] M. Jankowski, T. L. Broderick, and J. Gutkowska, "Oxytocin and cardioprotection in diabetes and obesity," *BMC Endocrine Disorders*, vol. 16, no. 1, p. 34, 2016.
- [38] M. Florian, M. Jankowski, and J. Gutkowska, "Oxytocin increases glucose uptake in neonatal rat cardiomyocytes," *Endocrinology*, vol. 151, no. 2, pp. 482–491, 2010.
- [39] L. Katsouri, C. Parr, N. Bogdanovic, M. Willem, and M. Sastre, "PPAR $\gamma$  co-activator-1 $\alpha$  (PGC-1 $\alpha$ ) reduces amyloid- $\beta$  generation through a PPAR $\gamma$ -dependent mechanism," *Journal of Alzheimer's Disease*, vol. 25, no. 1, pp. 151–162, 2011.
- [40] N.-E. Rhaleb, X.-P. Yang, and O. A. Carretero, "The kallikrein-kinin system as a regulator of cardiovascular and renal function," *Comprehensive Physiology*, vol. 1, no. 2, pp. 971–993, 2011.
- [41] R. Sharma, P. K. Randhawa, N. Singh, and A. S. Jaggi, "Bradykinin in ischemic conditioning-induced tissue protection: evidences and possible mechanisms," *European Journal of Pharmacology*, vol. 768, pp. 58–70, 2015.
- [42] J. D. Tune, A. G. Goodwill, D. J. Sassoon, and K. J. Mather, "Cardiovascular consequences of metabolic syndrome," *Translational Research*, vol. 183, pp. 57–70, 2017.
- [43] P. A. J. Krijnen, "Apoptosis in myocardial ischaemia and infarction," *Journal of Clinical Pathology*, vol. 55, no. 11, pp. 801–811, 2002.
- [44] L. Ibarra-Lara, M. Sánchez-Aguilar, E. Soria-Castro et al., "Clofibrate treatment decreases inflammation and reverses myocardial infarction-induced remodeling in a rodent experimental model," *Molecules*, vol. 24, no. 2, p. 270, 2019.