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Clofibrate improves myocardial ischemia-induced damage through regulation of renin-angiotensin system and favours a pro-vasodilator profile in left ventricle

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ABSTRACT

Myocardial ischemia initiates a chain of pathological conditions leading to cardiomyocyte death. Therefore, pharmacological treatment to stop ischemia-induced damage is necessary. Fibrates, have been reported to decrease inflammatory markers and to modulate the renin-angiotensin system (RAS). Our aim was to explore if clofibrate treatment, administered one week after myocardial event, decreases MI-induced cardiac damage. Wistar rats were assigned to: 1. Sham or 2. Coronary artery ligation (MI). Seven days after, rats were subdivided to receive vehicle (V) or clofibrate [100 mg/kg (C)] daily for 7 days. Blood samples and left ventricle were analyzed. RAS components [angiotensin II, angiotensin converting enzyme (ACE), and AT_1-receptor] decreased in MI-C compared to MI-V, while [Ang-(1–7), bradykinin, ACE-2, and AT_2-receptor] raised in response to clofibrate treatment. Oxidative stress markers increased in MI-V rats, a profile reverted in MI-C rats. Nitric oxide (NO) pathway (Akt, eNOS, and NO) exhibits a lower participation in MI-V, but clofibrate raised NO-pathway components and its production. MI-induced fibrosis and structural damage was also improved by clofibrate-treatment. In conclusion, clofibrate administration to 7 days MI-rats exerts an antioxidant, pro-vasodilator expression profile, and anti-fibrotic effect suggesting that PPARα activation can be considered a therapeutic target to improve cardiac condition posterior to ischemia.

Introduction

Acute myocardial infarction leads to a serious health condition, increasing the risk of morbi-mortality. Reperfusion of the ischemic area is a necessary condition to limit cell death. However, this maneuver is not suitable to be performed in every case; leading to extensive cell death.[1] Likewise, in order to maintain cardiac functioning at best possible condition, pharmacological treatment must be administered.[2]

Current treatment includes anti-platelet and antithrombotic treatment, adrenergic β-blockers, inhibitors of renin-angiotensin pathway such as angiotensin converting enzyme (ACE) inhibitors or angiotensin II type I (AT_1) receptor blockers,[3,4] as well as adenosine and nitric oxide (NO) donors.[5] Despite of these pharmacological treatments, the cardiac recovery is below optimal level; suggesting that highly relevant pathways or molecules have not been considered. Biopterins may be located into this category. Three types have been reported: BH_2, BH_3, and BH_4 differing from each other on the level of oxidation. Tetrahydrobiopterin (BH_4) plays an important cardiovascular role due to its participation as cofactor for endothelial nitric oxide synthase (eNOS) allowing this enzyme to couple and be able to transform L-arginine into L-citrulline and NO.[6] If dihydrobiopterin (BH_2) overcomes BH_4 concentration, eNOS would produce O_2^- instead; increasing oxidative stress and its detrimental consequences.[7] Likewise, the search for

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treatments to decrease oxidative stress, increase cardiac angiogenesis, to promote cardiac regeneration, and to improve ventricular dysfunction is highly active.[8]

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor family. They work as transcription factors modulating the expression of a wide range of genes. Currently, 3 isoforms have been described: α, β/δ, and γ. They possess a differential distribution, ligand- and PPAR response element (PPRE) specificity. Originally, the alpha isoform (PPARα) was related to the metabolism of fatty acids[9]; currently, many reports implicate this isoform with anti-inflammatory and antioxidant functions.[10–13]

Our group previously reported that clofibrate treatment, given as a pre-treatment or immediately after myocardial infarction decreases myocardial ischemia-induced inflammation[10,14] renin-angiotensin system (RAS)-mediated pro-hypertrophic/vasocontractile axis (ACE/angiotensin II/AT_1-receptor), favored the pro-vasodilator and anti-hypertrophic RAS axis (ACE-2/angiotensin 1-7/AT_2-receptor),[15–17] and exerted an antioxidant effect.[18,19] In the present study we aimed to explore if clofibrate treatment administered one week after the myocardial ischemic event is able of decreasing MI-induced cardiac damage.

Material and methods

Animals

Experimental animal procedures were conducted ethically in accordance with our Federal Regulations for Animal Experimentation and Care (Ministry of Agriculture, SAGARPA, NOM-062-ZOO-1999, Mexico) and they were approved by the Institutional Animal Care and Use Committee (Institutional Project Number 13–825).

Male Wistar rats (300–350 g) were assigned randomly to one of the following experimental groups: 1. Sham coronary artery ligation (Sham) or 2. Left anterior descending (LAD) coronary artery ligation (MI). Seven days after this procedure, MI rats were further divided to receive vehicle [vegetable oil (V), by intraperitoneal injection (I.P.)] or clofibrate [100 mg/kg (C), I.P.)] every day for 7 days. Sham rats received V for the same length of time. At the end of the 7 days of treatment, blood was withdrawn, the rats were sacrificed, and the heart was obtained to further analyze protein expression. A second set of experimental subjects was assigned for microscopy analysis. Rats had free access to pellet food and water *ad libitum*.

Myocardial ischemia

Anaesthetized rats [ketamine/xylazine (80/10 mg/kg)] were instrumented to be artificially ventilated (70 stroke/min, at 8–10 mL/kg) through a tracheal cannula. Chest was opened at the fourth intercostal space to reach LAD coronary artery and fully tied (6-0 polypropylene) permanently. Sham surgery was performed in the same way except for the LAD ligation.[20] At the end of the procedure, the heart was repositioned and the incision was sutured by layers. Corporal temperature was kept at physiological level along the surgery.

Electrophoretic determination of angiotensin II, angiotensin-(1–7), and bradykinin

AngII, Ang-(1–7), and bradykinin (Bk) production from plasma and left ventricle homogenate was evaluated by CZE,[21] following the same protocol previously reported.[22,23] Data are expressed as pmol per mL or per mg of protein, for plasma and tissue, respectively.

Protein analysis by Western blot

Frozen left ventricle from the different experimental groups was homogenized (25% w/v) in a lysis buffer containing 250 mM Tris–HCl, 2.5 mM EDTA, and protease inhibitors (10 μg/mL leupeptin and 20 μg/mL aprotinin; Complete ®, Roche Diagnostics, Indianapolis, IN, USA) by means of a polytron (PT-MR2100, Kinematica, Luzern, Switzerland) at 4 °C. Protein content was determinate by bicinchoninic acid assay (Pierce BCA protein assay kit, Thermo Scientific, Rockford, IL, USA). Fifty μg of protein was electrophoresed on a denaturing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were electrophoretically transferred onto PVDF membranes (Millipore, Billerica, MA, USA) and then blocked with 5% skim milk in PBS pH 7.4. The membranes were incubated overnight at 4 °C with primary antibodies against angiotensin II AT_1- and AT_2-receptors, Mas receptor, ACE, ACE2, eNOS, p-eNOS^{Ser1177}, AKT, p-AKT^{Ser473}, or VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were incubated 1 h at room temperature with secondary anti-rabbit- or anti-mouse-horseradish peroxidase-labeled antibody (Bio-Rad, CA, USA). Protein was detected by the Immobilon Chemiluminescent System (Millipore Merck, Darmstadt, Germany). Images from films were digitally acquired and analyzed using the calibrated densitometer (GS-800 USB; Bio-Rad). Blots were stripped and re-incubated with monoclonal β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as load control. Values of each band density are expressed as arbitrary units.[22,23]

Total antioxidant capacity

This parameter was determined on left ventricle and plasma samples as previously described.[24] Briefly, 35 μL of tissue homogenate or plasma were diluted with 145 μL of 0.1 M phosphate buffer at pH 7.5 and shook at 500 rpm for 200 sec. 100 μL of diluted sample were further treated with 50 μL of 0.01 M $CuCl_2$ and shook at 500 rpm for 200 s. Then 50 μL of 0.01 M batocuproin were added and vortexed again at 500 rpm for 200 s. The concentration of Cu^{2+} reduced to Cu^{+} was measured by means of a spectrometer to 490 nm (DW2000, SLM-Amico, Urbana, IL, USA).[25]

Quantification of malondialdehyde

Left ventricular homogenate or plasma samples (50 μL) were deproteinized with cold methanol (1:1 v/v) and centrifuged at 16,000×*g* for 15 min at 10 °C. The supernatants (80 μL) were filtered with a nitrocellulose membrane (0.22 μm, Millipore, Billerica MA, USA) and diluted (10:1) with 0.1 N NaOH. Finally, samples were analyzed by capillary electrophoresis (P/ACE MDQ Capillary Electrophoresis System, Beckman Coulter, CA, USA) following a previously reported protocol. The concentrations were obtained interpolating from standard curves of malondialdehyde (MDA) (Sigma Aldrich, St. Louis, MO, USA) treated in the same conditions as the samples.[26]

Quantification of biopterins

The reduced (BH_4) and oxidized (BH_2) forms of the biopterins were determined by CZE as previously reported.[27] Briefly, 50 μL of sample (tissue homogenate or plasma) was deproteinized with cold methanol (1:1 v/v) and centrifuged at 16,000×*g* for 15 min at 10 °C and filtered with a nitrocellulose membrane (0.22 μm, Millipore, Billerica MA, USA), before measurement using a P/ACETM MDQ electrophoresis system (Beckman Coulter, Mexico City, Mexico), using laser-induced fluorescence detection. A standard curve of BH_2

and BH_4 (Sigma Aldrich, St. Louis, MO, USA) was used to calculate concentrations.[28]

Quantification of NO

The NO was measured in 40 μL of either tissue homogenate or plasma. It was treated with 0.8% vanadium (III) chloride in 1 M phosphoric acid, 2% sulfanilamide in 5% phosphoric acid and 0.2% N-(1-naphthyl) ethylenediamine in H_2O. The reaction was incubated 50 min at room temperature. Samples were analyzed spectrophotometrically at 572 and 587 nm (Shimadzu 1800, Shimadzu Corporation, Koriyama, Japan). The difference between the measurements (Δ Abs = 572–587 nm) was used to calculate the NO concentration compared to a standard curve.[29]

Transmission electron microscopy

Approximately 0.5 cm of left ventricle was obtained from every group and rinsed with cacodylates buffer 0.16 M, pH 7.2. Then, these were cut in fragments (2 × 2 mm/approx) and fixed on glutaraldehyde 2.5% for 2 h. After, these were washed off thrice with cacodylates buffer 0.1 M, pH 7.2, 10 min. Later, these samples were post-fixed with osmium tetraoxide 1% in cacodylates buffer 0.16 M by 2 h and further rinsed with cacodylates buffer 0.16 M, three times for 10 min and subjected to gradual dehydration with alcohol (50, 70, 80, 96, and 100%). Later the sample was exposed to propylene oxide and washed twice. Preinclusion was carried on in Epon 812 resin (Electron microscopy sciences, Hatfield, PA, USA) and propylene oxide (1:1). Final inclusion was performed in pure resin for 2 h and later it was allowed to polymerize for 12–18 h at 60 °C. Ultrathin slices were obtained (50–60 nm) using a microtome (RMC PTXL, Boeckeler Instruments Inc., Tucson, AZ. USA), mounted on cooper grids and they were contrasted with uranyl acetate and lead citrate for random microscopy observation of 2–3 myocardial ischemic areas.[24]

Masson's trichrome staining

Left ventricle samples were formol buffered-fixed (10%), dehydrated using an alcohol gradient ending in xylol and embedded in Paraplast (Sigma-Aldrich, St. Louis, MO, USA). Three μm slices were obtained by means of a Leica RM2235 Microtome (Leica Biosystems, Buffalo Grove, IL, USA). Lately, samples were processed to determine the presence of fibrotic tissue dying with Masson's trichrome staining (MT) and observed by means of an Olympus BH2 microscope (Olympus, Lifesciences).[30]

Data analysis

Results are expressed as the mean ± standard error of the mean (SEM). For multiple comparisons, we applied one-way analysis of variance (ANOVA) followed by Tukey *post hoc*-test (Graph Pad Prism 8). Differences were considered significant when the *p* value was <0.05.

Results

The concentration of AngII, Ang-(1–7), and Bk were determined in left ventricle and plasma from rats of every experimental group. Our results show that Sham rats produce a basal amount of AngII in both, tissue and plasma. Fourteen days after MI, the production of AngII is elevated compared to Sham rats [left ventricle ($p < 0.001$) and plasma ($p < 0.0001$)]. This parameter was lower in the rats receiving 7 days clofibrate (100 mg/kg, i. p.) in tissue ($p < 0.0001$) and plasma ($p < 0.001$) (Fig. 1A and B). Because Ang-(1–7) is part of the RAS pathway, we also evaluated its production under the experimental conditions. As can be observed, the Ang-(1–7) amount in Sham- and MI-V-rats is comparable, but the treatment with clofibrate increased its cardiac ($p < 0.05$) and systemic production ($p < 0.001$) (Fig. 1C and D). We also evaluated Bk, a vasorelaxant component. The results show that in the left ventricle, there is no difference between Sham and MI-V rats. However, there is a significantly higher amount of Bk in MI-C ($p < 0.0001$). Regarding plasma Bk concentration, we observed that this parameter decreased in MI-V ($p < 0.001$), compared to Sham and it was raised in MI-C ($p < 0.001$) (Fig. 1E and F).

The expression of relevant participants of RAS pathway was explored. Thus, ACE and ACE-2 expression were determined. Myocardial infarction induced a rise in the tissue expression of ACE (MI-V, $p < 0.05$) (Fig. 2A and B). Treatment with clofibrate did not reduce the expression of the enzyme under our experimental conditions. ACE-2 expression significantly decreased in those rats subjected to MI-V ($p < 0.0001$), an effect that was reverted by clofibrate treatment ($p < 0.0001$) (Fig. 2C and D).

Regarding AngII receptors, we report that MI-V-rats show a higher AT_1-receptor expression compared to Sham rats ($p < 0.01$). Clofibrate treatment decreased AT_1-receptor expression ($p < 0.0001$) (Fig. 3A and B). The expression of AT_2-receptor was also evaluated. In the left ventricle from Sham rats there is a basal expression, MI-V also exhibit the presence of the receptor, but no statistical significance was reached comparing them. Clofibrate treatment raised AT_2-receptor expression, compared to MI-V, to a significant value ($p < 0.0001$) (Fig. 3C and D). Another important receptor in RAS pathway is the Mas receptor. As can be observed, MI increases Mas receptor expression, regardless of vehicle- ($p < 0.01$) or clofibrate-treatment ($p < 0.05$) (Fig. 3E and F).

Oxidative stress plays an important role mediating MI-induced tissue damage. Therefore, we evaluated if clofibrate treatment also exerts an effect on different players of this factor, as we previously show in other experimental models. First, we evaluated total antioxidant capacity. Our data show that MI decreases the tissue ($p < 0.01$) and systemic ($p < 0.0001$) antioxidant defense, an effect that was reverted by clofibrate treatment (tissue $p < 0.01$ and plasma $p < 0.001$) (Fig. 4A and B). In order to explore the effect of MI-induced oxidative stress on macromolecules, we measured tissue and plasma MDA content as lipoperoxidation marker. As shown in Fig. 4C and D, MI increased the level of MDA (tissue $p < 0.01$ and plasma $p < 0.0001$). Seven days of treatment with clofibrate exerted an antioxidant effect, observed as a decreased of MDA levels in both, left ventricle ($p < 0.001$) and plasma ($p < 0.0001$).

An important molecule highly susceptible to oxidation is BH_4. Our results show that Sham rats exhibit a basal content of BH_4 in the left ventricle. Myocardial infarction promoted an elevation of tissue content in both, vehicle- ($p < 0.05$) and clofibrate ($p < 0.0001$) treated rats. However, the increase observed with clofibrate is higher ($p < 0.01$). Regarding plasma content of BH_4 in the rats subjected to MI vehicle-treated, we observed that BH_4 concentration was lower compared to Sham ($p < 0.001$). Clofibrate-treatment could maintain BH_4 levels comparable to Sham ($p < 0.01$, Fig. 4E and F).

The concentration of BH_2 was also measured. It shows that in both, tissue and plasma, MI increased its production compared to Sham (tissue $p < 0.01$ and plasma $p < 0.001$) and clofibrate treatment decreased its concentration (tissue $p < 0.05$ and plasma $p < 0.01$) (Fig. 4G and H).

We also explored some components of NO pathway. The expression of eNOS and p-eNOS^{Ser1177} was evaluated in the left ventricle and it is expressed as the ratio of both proteins (Fig. 5A

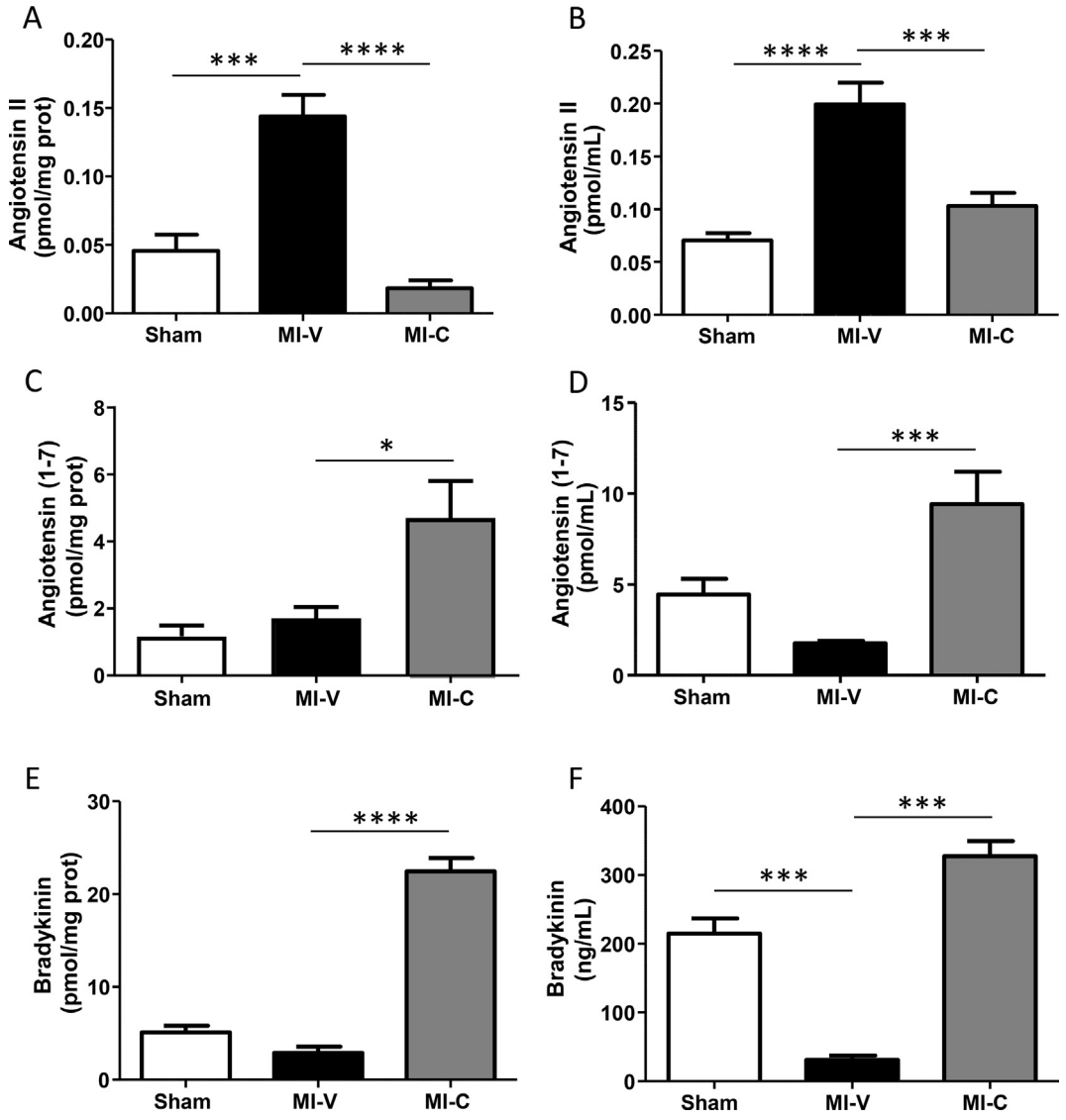

Fig. 1. Effect of clofibrate on myocardial infarction (MI)-induced changes in angiotensin II (A and B), angiotensin-(1–7) (C and D), and bradykinin (E and F). The left ventricle (A, C, E) and plasma (B, D, F) concentration of the metabolites was determined by capillary zone electrophoresis in samples from Sham-, MI vehicle-treated rats (MI-V), and MI rats treated with clofibrate (MI-C). Data are presented as the mean ± standard error of the mean of 6 independent experiments. One-way ANOVA followed by Tukey post hoc *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

and B). As can be observed, Sham rats express basal levels of these proteins. The expression observed in MI-V and MI-C remained comparable to Sham rats. The deleterious effect of MI may be localized upstream Akt signaling pathway since the ratio of p-Akt^{Ser473}/Akt is significantly lower in MI-V compared to Sham rats (Fig. 5C and D). The production of NO was lower in the plasma from MI-V compared to Sham values, MI-C reached a concentration comparable to Sham. Regarding NO in LV, the data show that Sham and MI-V exhibited comparable values, however MI-C produced a higher amount of NO (Fig. 5E and F).

It has been reported that NO could favour vascularization by inducing vascular endothelial growth factor (VEGF). Our data show that MI-V decreased the expression of VEGF ($p < 0.001$) and clofibrate treatment was able to increase its expression ($p < 0.0001$) (Fig. 5G and H).

Images from Sham rats showing Masson's staining exhibit well-organized sarcomere, the presence of nuclei, and intercalated discs (Fig. 6A). The hearts from MI-V did not develop a scar or fibrosis, however there is edema into sarcomere (Fig. 6B). The microscopic features observed on MI-V were not observed on MI-C; instead, sarcomere is well organized, intercalated discs look homogeneous, and intra-sarcolemma space is appropriate (Fig. 6C), suggesting a protector effect.

Through electron microscopy, we observed that MI-induced ultrastructural modifications are evident comparing Sham- and MI-V rats. Left ventricles from Sham rats exhibit a typical conformation of the fibers characterized by: longitudinal organized sarcomere connected in series at the Z-disc, a continuous membrane, as well as normal mitochondria size and distribution (Fig. 6D and G). Representative image for MI-V shows an evident damage, with loss uniformity and shortening of the fibers, edematous mitochondria and loss of continuity of the outer membrane (Fig. 6E and H). Clofibrate treatment improved the architecture of the left ventricle. Rats from MI-C group show slight myofibrillar damage and an architecture that resembles Sham conditions (Fig. 6F and I).

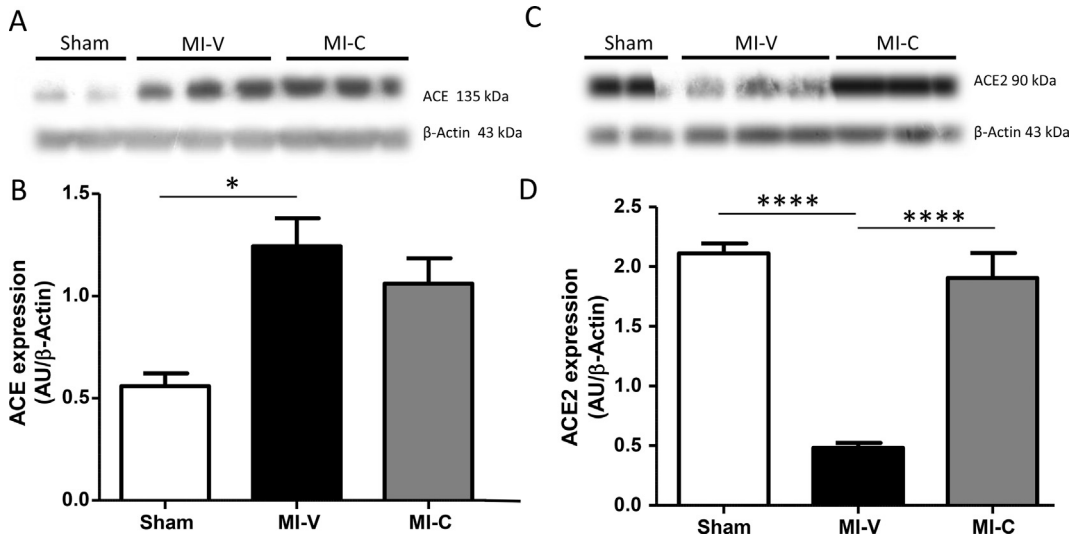

Fig. 2. Protein expression of angiotensin converting enzyme (ACE) and ACE-2 in the left ventricle of Sham-, myocardial infarcted vehicle-treated rats (MI-V) and MI clofibrate-treated rats (MI-C). A) and C) Exhibit representative Western blots and B) and D) show the densitometric analysis of ACE (B) and ACE-2 (D). β-Actin was used as load control and for densitometric normalizing, for both cases. Bars represent the mean ± standard error of the mean. n = 6 rats per group, ANOVA-Tukey *p < 0.05 and ****p < 0.0001.

Discussion

The current study shows that 7 days of clofibrate treatment to 1-week MI rats exerts an effect on key participants of RAS pathway, antioxidant system and NO pathway leading to an improved cardiac condition. This study complements an earlier report from our group showing that clofibrate was able to decrease late inflammation and partially reverse the left ventricular remodeling and functional damage.[10]

Myocardial infarction brings several early and late consequences including arrhythmias, cardiac insufficiency, heart failure, and eventually death.[31,32] Pharmacological treatment lowers the risk in post–MI conditions.[33–35] However, there is still a high prevalence of morbidity and mortality following the event[36–38]; showing that there are unattended factors in post–MI treatment. Previous reports have demonstrated that PPARα agonists (fibrates) induce changes in key participants involved in myocardial ischemia such as RAS, oxidative stress, NO-induced vasodilation, and inflammation.[10,11,23] However, most of them are based on experimental models given the pharmacological treatment before MI or immediately after the ischemic insult.[39] Our study was designed to explore the ability of clofibrate to revert or diminish MI-induced changes in those key pathophysiological factors, once damage is established.

The relevance of RAS has been widely documented in MI. Immediately after the ischemic process, the activation of RAS begins and the production of AngII rises significantly and stimulates mostly the AT_1-receptor leading to vasoconstriction, remodeling, and hypertrophy; together known as the ACE/AngII/AT_1-receptor vasoconstrictor axis. At lower rate, AngII may also interact with AT_2-receptor, as well as with Mas receptor; pathways reported to promote vasodilation.[40,41] Bradykinin also belongs to this pathway and its relevance as a vasodilator has been widely reported.[42] As reported, MI induced an increase in RAS activity favoring fibrotic/hypertrophic/vasocontractile axis. The administration for 7 days of clofibrate to MI rats reduced the participation of ACE/AngII/AT_1-receptor axis and increased that of ACE-2/Ang-(1–7)/AT_2-and Mas receptor pathway, as well as the level of Bk, consequently its physiological effects. These results resemble those previously reported by our group showing that fibrates decrease ACE/AngII/AT_1-receptor axis and increase ACE-2/Ang-(1–7)/AT_2-receptor and Mas receptor in rats given clofibrate previous to MI.[22] In a different experimental model, Jíchova S et al. reported that fenofibrate modulates RAS, being able to attenuate malignant hypertension by suppression of RAS in Cyp1a1-Ren-2 transgenic rats.[43] The experimental design used widens the therapeutic window previously set for fibrates and confirms the effect on RAS pathway at cardiac and systemic level.

Oxidative stress increases in hypoxic conditions.[44,45] Among the ROS producing systems are NOX subunits; NOX2 is present in cardiomyocytes and produces superoxide. Hypoxia also induces ROS formation through the activation of RAS. Nishida et al. reported that AngII stimulation in rat neonatal cardiomyocytes increases the production of ROS through the interaction with $AT_1R/G\alpha_{12/13}$/Rho/NADPH oxidase.[46] Here we report that AngII levels from tissue and plasma and, AT_1R expression decreased in those subjects treated with clofibrate; being a possible explanation for the lower oxidative stress observed in MI-C rats. On the other hand, clofibrate favors the ACE-2/Ang-(1–7)/AT_2-/Mas receptor axis and Bk concentration. This is also a relevant participant in both physiological and pathological conditions. The stimulation of this axis is related to anti-inflammatory effect, vasodilation, antiproliferation, antifibrosis, and promotion of cardiac contractility mediated by PI3K-Akt-eNOS pathway.[47] As we reported, clofibrate administration (7 days) to MI-rats with seven days of progression, exerted an anti-inflammatory effect evidenced by a significant decrease of MI-induced raise in TNF-α, IL-1β, IL-6, iNOS, as well as cellular addition molecules (ICAM and VCAM) and matrix metalloproteases in the left ventricle. These molecular changes improved MI-induced left ventricular dilatation determined by echocardiography in anaesthetized rats.[10]

Nitric oxide bioavailability, defined as the production and utilization of endothelial NO in organisms, is susceptible of being altered by oxidative stress leading to endothelial dysfunction. Endothelial NOS produces NO in physiological conditions, facilitating vasodilation and an antiaggregant vascular environment.[48–51] Under pathological conditions, like those reported in MI, the overexpression of iNOS raises the production of NO promoting inflammatory cell infiltrate, cardiac fibrosis, hypertrophy, and dilatation[52] Additionally, if anion superoxide ($O_2^{\cdot-}$) is present, it

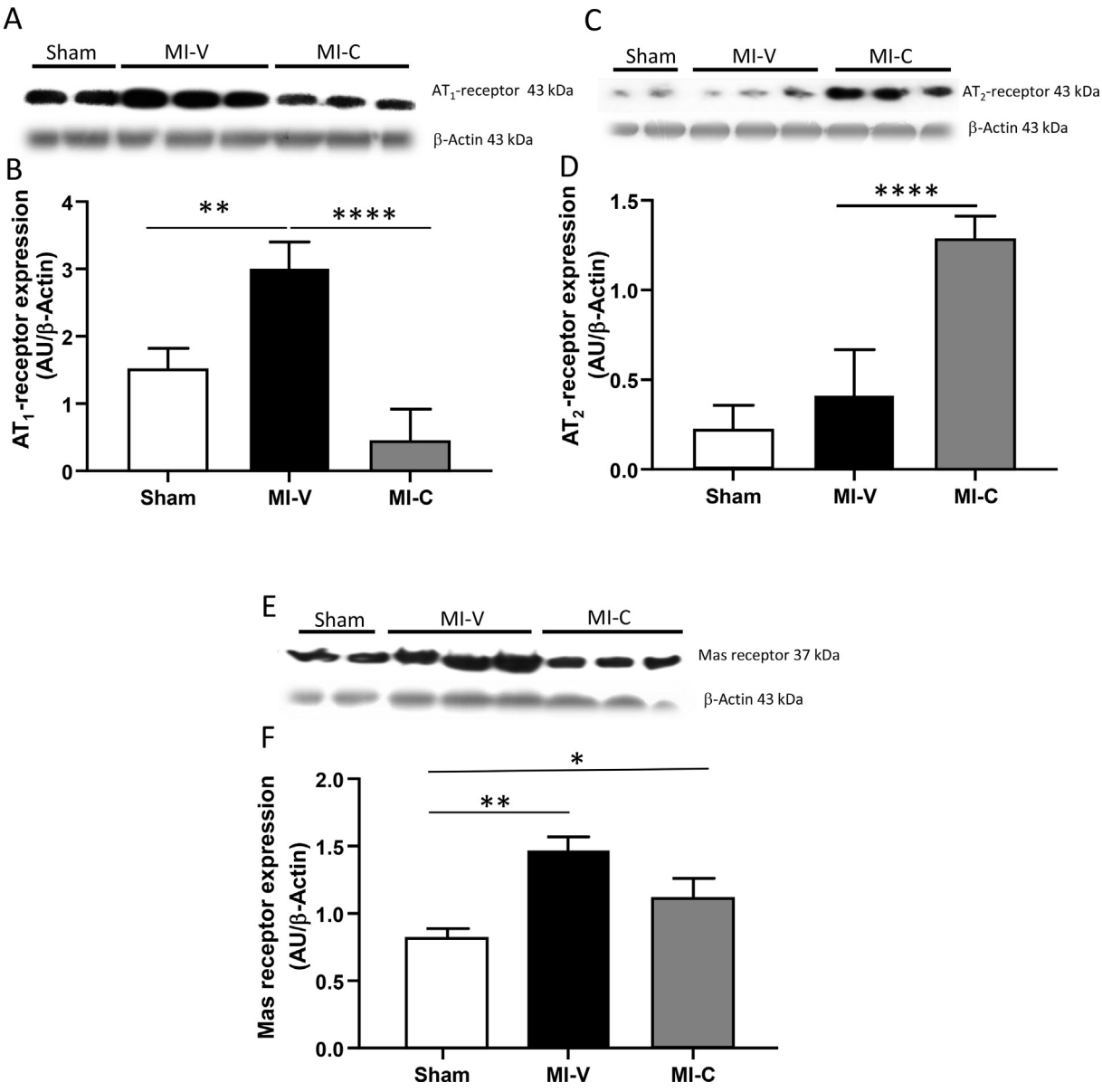

Fig. 3. Myocardial protein expression of angiotensin II type 1- (AT1), type 2- (AT2)- and Mas receptors in left ventricle from Sham, myocardial infarcted vehicle-treated rats (MI-V), and MI clofibrate-treated rats (MI-C). Representative Western blots are shown on panels A, C, and E while the densitometric analysis is presented on panel B) for AT1-receptor, D) for AT2-receptor, and F) for Mas receptor. β-Actin was used as load control and for densitometric normalizing in every case. Bars represent the mean ± standard error of the mean. n = 6 rats per group, ANOVA-Tukey *p < 0.05, **p < 0.01, and ****p < 0.0001.

reacts with NO to form peroxynitrite (ONOO-) and further reaction with lipids, proteins, and DNA enhances the damage.[53] Our data indicates that clofibrate decreased lipid peroxidation; most probably because of an improved antioxidant system and a reduction of ACE/AngII/AT_1-receptor axis lowering the stimulation of NOX enzymes. It is well-described that binding of clofibrate to PPAR alpha promotes a conformational change in the receptor facilitating its dimerization with RXR.[54,55] However, this is not the only interaction that PPARα establishes. A crosstalk between PPARα and other nuclear receptors exists and could explain the changes in expression of several proteins without PPRE in their promoter sequence.[56] PPARs also exert negative regulation of gene expression; a process known as transrepression. A relevant molecule affected by PPAR agonists is NF-kB. Reports from Ricote M &Glass CK document that the stimulation of PPARs decreases the activity of NF-kB and increases the expression of I-kB.[57] Being the AT_1R a protein transcriptionally controlled by NF-kB, it is clear that its expression is affected by PPAR agonists like fibrates and thiazolidinediones; a fact that has been demonstrated in vivo in cardiac tissue.[58]

Clofibrate also recovered NO bioavailability in MI-C; observed as a left ventricular NO production comparable to Sham rats.[59,60] This event could be explained by the responses promoted by clofibrate regarding: i) lower oxidant environment that maintain BH_4 levels, allowing the eNOS coupling, ii) an appropriate activation of Akt (p-Akt^{Ser473}) and eNOS (p-$eNOS^{Ser\ 1177}$), and iii) a lower expression of iNOS; as previously demonstrated.[10]

The VEGF promoter contains a PPRE and PPARα- and PPARγ-ligands increase VEGF expression both in vitro and in vivo experimental models.[61,62] Our data show that clofibrate increased its expression in the left ventricle of MI rats; supporting previous reports. Despite the damage induced by MI, an improvement in cardiac ultrastructure induced by clofibrate treatment was observed.

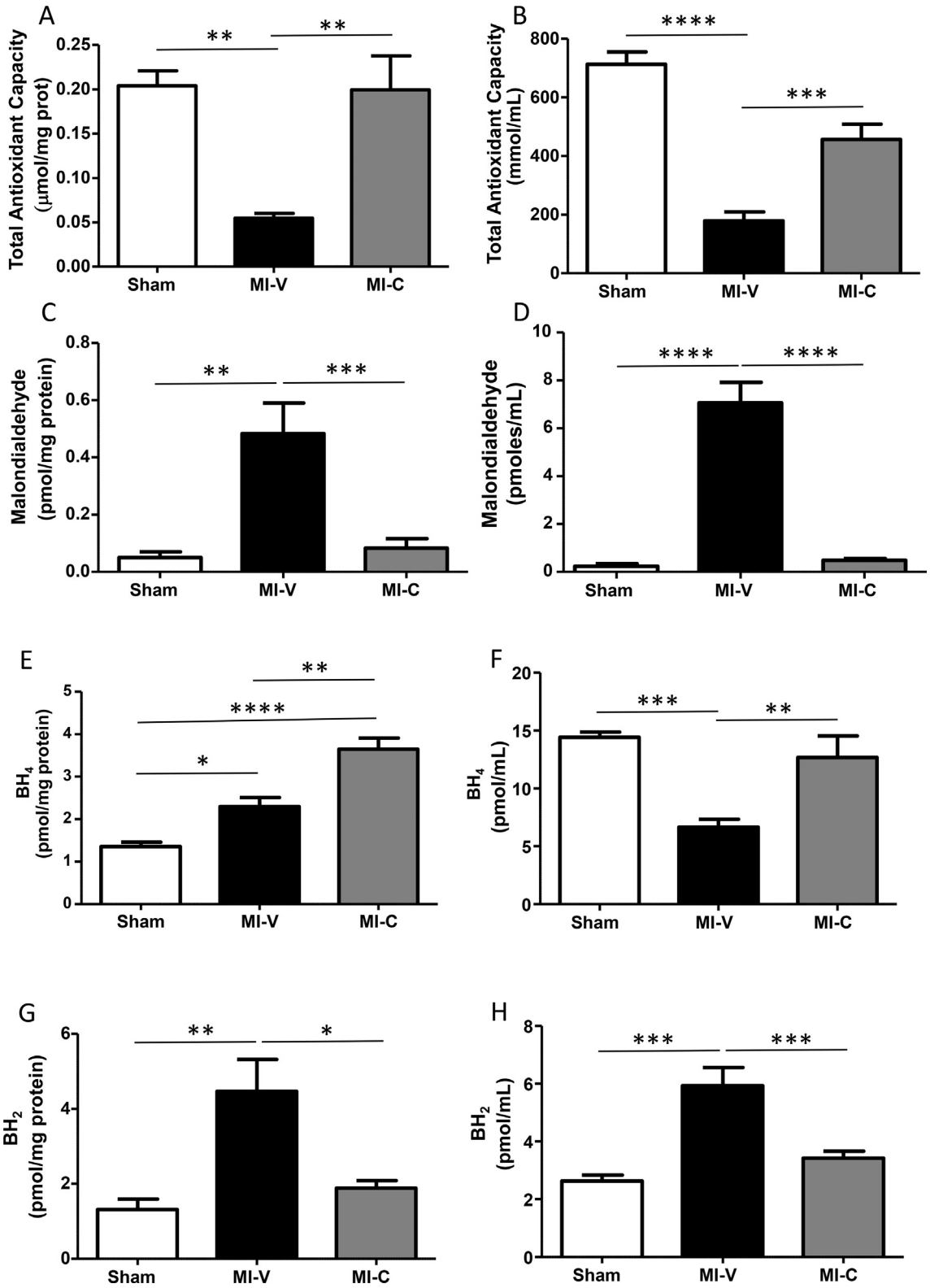

Fig. 4. Markers of oxidant environment in left ventricles. Left ventricle (A, C, E, and G) and plasma (B, D, F, and H) from Sham-, myocardial infarcted vehicle-treated rats (MI-V), and MI clofibrate-treated rats (MI-C) were analyzed by capillary zone electrophoresis to quantify A) and B) total antioxidant capacity, C) and D) malondialdehyde, E) and F) tetrahydrobiopterin (BH_4), and G) and H) Dihydrobiopterin (BH_2). Data are represented as the mean ± standard error of the mean. n = 6 rats per group, ANOVA-Tukey *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

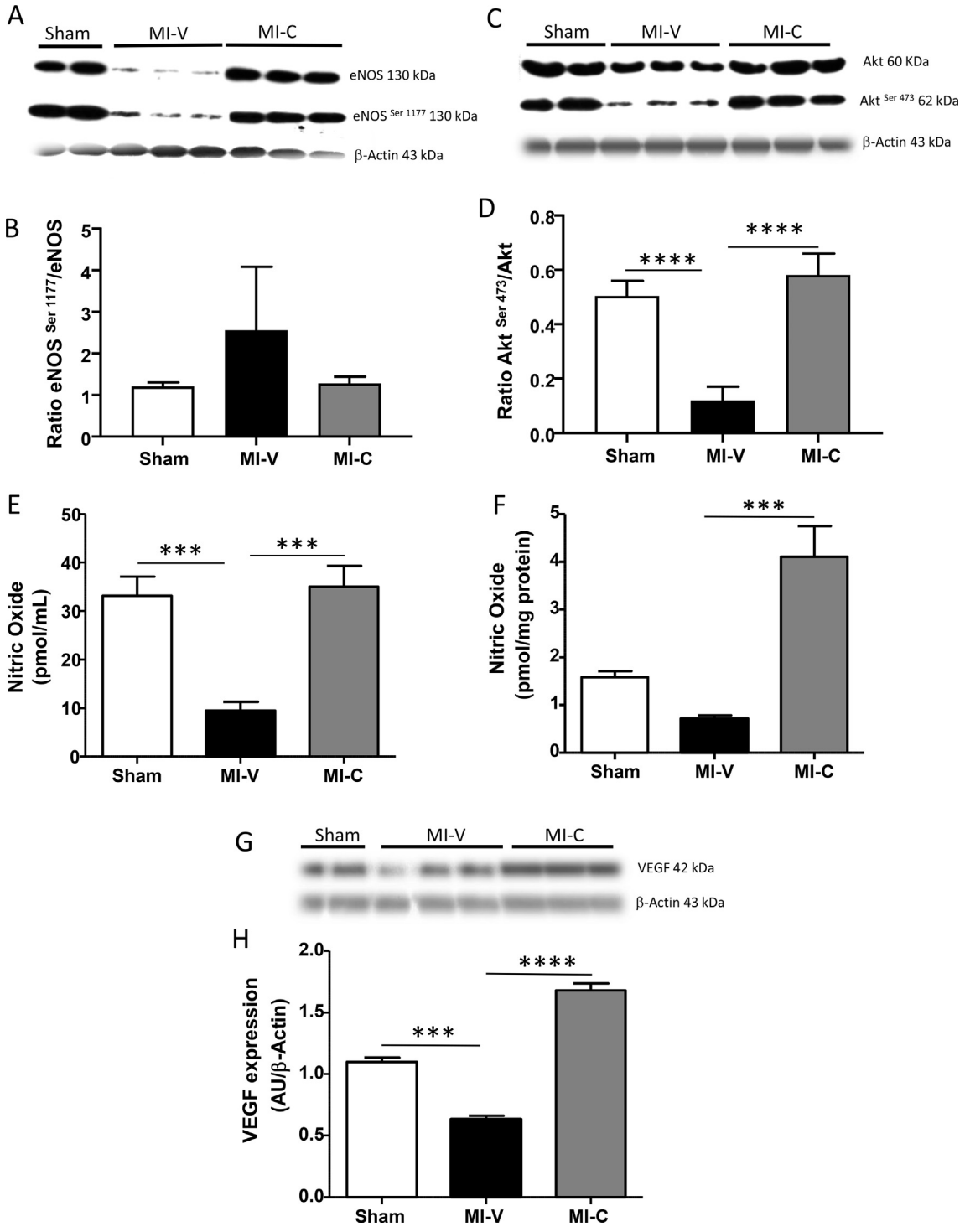

Fig. 5. Myocardial protein expression. The effect of clofibrate on tissue protein expression was evaluated by Western blot (A, C, and G) on the left ventricle from Sham-, myocardial infarcted vehicle-treated rats (MI-V), and MI clofibrate-treated rats (MI-C). Endothelial nitric oxide synthase (eNOS), p-eNOSSer 1177, and their ratio are shown on A) and B), Akt, p-AktSer 473, and their ratio are presented on C) and D), nitric oxide (NO, E and F), and vascular endothelial growth factor (VEGF, G and I). Nitric oxide production was evaluated spectrophotometrically in both plasma (E) and left ventricle (F). β-Actin was used, in every case, as load control and for densitometric normalizing. Data are represented as the mean ± standard error of the mean. n = 6 rats per group, ANOVA-Tukey **p < 0.01, ***p < 0.001, and ****p < 0.0001.

We hypothesize that PPARα stimulation promoted angiogenesis protecting myocardium from hypoxia; an effect previously reported by Zhao T et al.[63]

Hypoxia-induced cardiomyocyte damage and resolution is a complex process that depends on the time of evolution, cellular type affected and their condition at the time of the insult, as well as the availability of growth factors, cytokines, hormones, and enzymatic machinery. These factors would determinate the response of the heart in the inflammatory, proliferative, and maturation phase post–MI.[64] In our study, experimental subjects were treated with clofibrate, 7 days after myocardial hypoxia. According to this time frame, our experimental models most probably were in

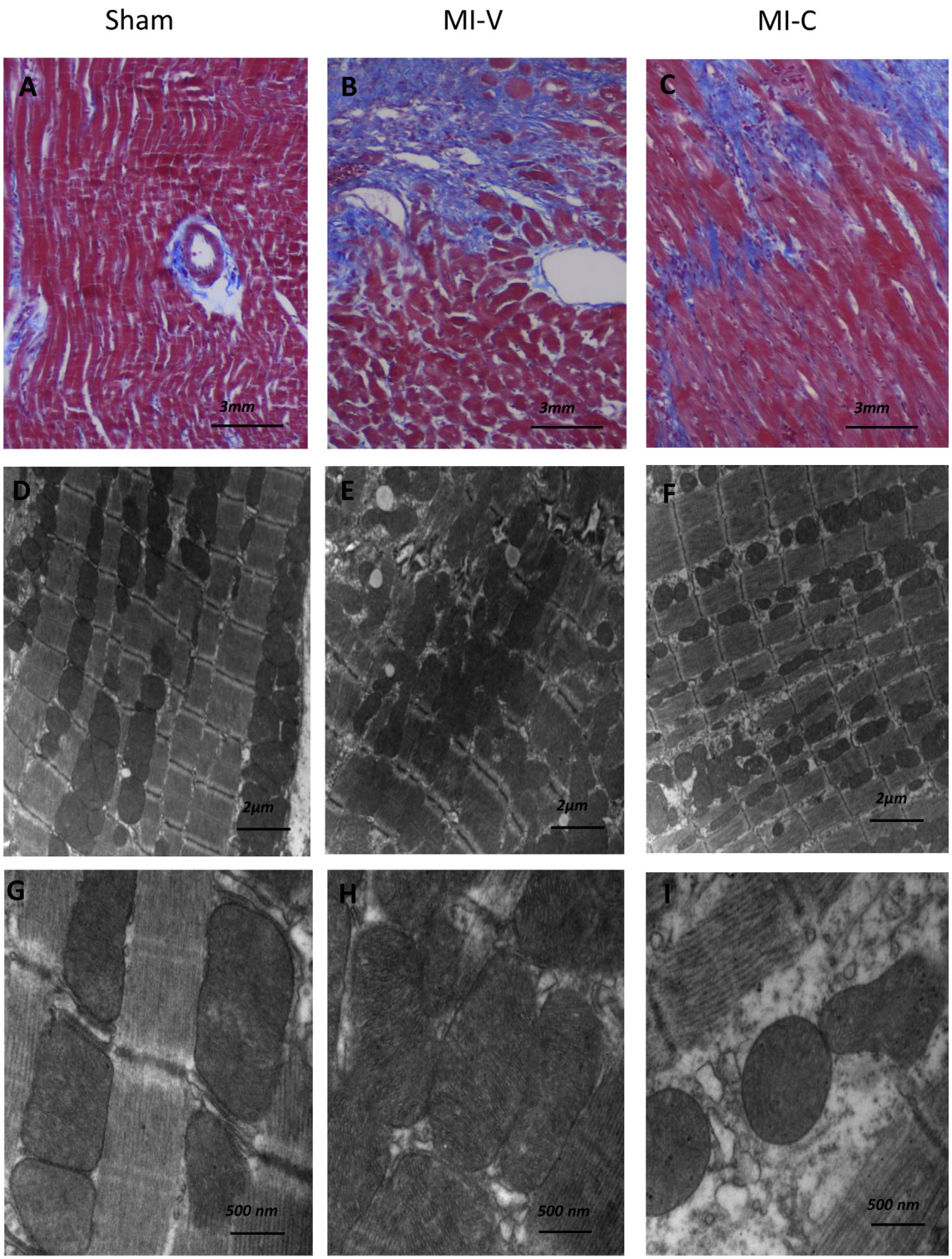

Fig. 6. Changes in left ventricle morphology after clofibrate treatment during MI-V. Masson 100x. from A) Sham, B) MI-V, or C) MI-C rats. Representative electronic micrography of the left ventricle from Sham (D and G), MI-V (E and H) or MI-C (F and I) -rats.

proliferative phase; which is characterized by the formation of a collagen-based matrix (scar), decreasing of inflammatory mediators, and presence of AngII, ET-1, TGF-β1, among others. In our study, collagen deposition decreased in clofibrate-treated rats. The effect could be explained by the reduction in the ACE/AngII/AT_1R axis. However, a lower inflammatory response could also be determinant; as was demonstrated.[10]

We acknowledge limitations of our study like the inclusion of a group of rats being treated with either an ACE inhibitor or an antioxidant. Even though the information obtained from those

groups would allow the comparison among the results, we believe that the data obtained is a strong evidence for the role of clofibrate decreasing ACE/AngII/AT_1R axis participation and oxidative stress, while improving NO bioavailability. We are aware that using a pharmacological antagonist of PPARα could demonstrate a direct association between PPARα activation by clofibrate and physiological consequences. Evidences of this interaction have been provided. Harada et al. studied the renoprotective effect elicited by a PPARα agonist in a murine model of kidney damage. Their data show that MK886, a PPARα antagonist, reversed the renal improvement observed in urine protein excretion, tubular injury, oxidative stress, and pro-inflammatory and apoptosis-stimulating response promoted by the agonist.[65] Another strategy to address this issue has been the study in PPARα knockout mice.[66] Deplanque D et al. showed that fenofibrate protects mice against cerebral injury through an anti-oxidant and anti-inflammatory mechanism. The neuroprotective effect produced by fenofibrate was completely absent in PPARα knockout mice suggesting that the receptor is the target for fibrates and they promote a response[67]

Taken together, the data obtained in this study showing the antioxidant, pro-vasodilator profile, and antihypertrophic effects produced by clofibrate and previously reported results demonstrating the anti-inflammatory effect promoted in MI rats allow us to suggest that PPARα activation can be considered a therapeutic target to further improve cardiac condition posterior to ischemia.

In conclusion, 7-day clofibrate treatment to rats with myocardial infarction and no reperfusion reduced the participation of RAS pathway and exerted an antioxidant effect that improved myocardial ultrastructure in ischemic cardiac conditions.

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Declaration of Competing Interest

Authors declare that they have no conflict of interest.

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