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Bimodal Effects of $P2Y_{12}$ Antagonism on Matrix Metalloproteinase–Associated Contractile Dysfunction in İnsulin-Resistant Mammalian Heart

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Abstract
The matrix metalloproteinases (MMPs) contribute to matrix remodeling in diabetes via tissue degradation; however, their contributions can be different depending on the pathology. For instance, MMPs are elevated in acute stress hyperglycemia, whereas they can be degraded in chronic hyperglycemia. Since studies emphasize the possible cardioprotective effect of ticagrelor (Tica) beyond its antiplatelet action, we aimed to examine whether Tica treatment can reverse the depressed heart function of metabolic syndrome (MetS) rats via affecting the expression levels of MMPs. Tica treatment of high-carbohydrate-induced MetS rats could not affect significantly the depressed contractile activity of Langendorff-perfused heart preparations. On the other hand, the Tica treatment provided a significant recovery in the reduced relaxation activity of the aortic preparations from the same animals. Histological examination of the hearts demonstrated marked damages in Mets rats, such as increases in the number of foamy cells and accumulation of collagen fiber and increases in the elastic lamellar irregularity of tunica media, while Tica treatment provided a slight improvement in the structure of left ventricle tissue. We also could not obtain a significant reverse in the high cytosolic labile Zn^{2+} ($[Zn^{2+}]_i$) with the treatment of cardiomyocytes with Tica. Furthermore, Tica treatment of MetS rats could not significantly reverse the degraded protein levels of MMP-2 and MMP-9 in the heart, as well. Overall, we demonstrated that Tica treatment of MetS rats has no significant benefits on the depressed heart function, although provide a significant beneficial impact on vascular relaxation. This action of Tica may be through its lack of action on both MMP degradation and high $[Zn^{2+}]_i$, which can further precipitate in cleavage of extracellular matrix in the heart.

Keywords Metabolic syndrome · Heart dysfunction · Ticagrelor · Matrix metalloproteinase · Zinc

Introduction

Metabolic syndrome (MetS) is a clustering of hyperglycemia/insulin resistance, obesity, and dyslipidemia, and its prevalence is increasing rapidly in the current century, at most, depending on an unbalanced energy intake and expense [1, 2]. Metabolic syndrome represents a series of cardiovascular risk factors, in particular, high lipidemia and blood pressure levels, and insulin resistance [3, 4]. Particularly, insulin resistance is getting more important through its coordinated effects on peripheral glucose use, vascular tone, blood flow, and cardiac function. The mechanism of cardiac dysfunction associated with MetS is very complex. The factors contributing to MetS-associated cardiac dysfunction include increased lipid accumulation, fibrosis, and stiffness as well as the reduced ratio of antioxidant defense to oxidant production in the heart in parallel with insulin resistance [5, 6]. In these contents, we previously have shown that there are marked functional and structural alterations in high-carbohydrate intake–induced Mets rat heart [3].

The experimental studies demonstrated that there is a marked prolongation in the single-cell action potential, intracellular Ca^{2+}-dyshomeostasis, mitochondrial dysfunction,

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and increased production of reactive oxygen species (ROS) in the left ventricular cardiomyocytes isolated from the MetS rats besides the marked prolonged QT-interval in their ECGs together with significantly depressed contractile activity [4, 7, 8]. Furthermore, we have also previously demonstrated that there was a significant increase in the level of labile Zn^{2+} ($[Zn^{2+}]_i$) in the left ventricular cardiomyocytes from hypertrophic rat heart, which was closely associated with the depression in the mechanical activity of the cardiomyocytes [9, 10]. More importantly, the relationship between increased $[Zn^{2+}]_i$, oxidative stress, and degradation in the cardiac matrix metalloproteinases (MMPs) in MetS rats are very correlated with each other [11]. In line with the previous statement, some experimental studies examined the relationship between an MMP induction system, including their decreased synthesis/activity contribution to increased collagen deposition, and pathological remodeling in diabetes [12–15]. Some further studies also examined the modulatory role of matrix stiffness in the activity of MMP-9-associated fibrosis in hepatic stellate cells [16], MMP-mediated collagen degradation in acute myocardial infarction [17], and the role of hyperoxia on the decreases of MMP-9 [18]. On the other hand, MMPs comprise a large family of multidomain Zn^{2+}-endopeptidases enzymes, which play role in the degradation of extracellular matrix proteins and other important biological procedures [19]. In other words, Zn^{2+} is the catalytic component of proteins that regulate MMPs, and accordingly, an increase in $[Zn^{2+}]_i$ seems to alter the activity of MMP-2 and MMP-9, contributing to the occurrence of malignancy [20].

Diabetes is associated with a high risk of recurrent cardiovascular events such as an increased tendency to activate and aggregate platelets despite antiplatelet therapy besides abnormalities in the heart itself. Those patients have also impaired response to some oral antiplatelet therapy, particularly characterized by recurrent ischemic events, including stent thrombosis [21]. These observations emphasize the need for more potent platelet-inhibiting therapies in diabetic patients. Ticagrelor is an oral, direct-acting cyclopentyltriazolopyrimidine antiplatelet agent and has a more rapid onset and robust antiplatelet effect [22]. Beyond its antiplatelet action, ticagrelor (Tica) has clinically relevant "off-target" pleiotropic effects on heart function under disease states [23, 24]. We have previously shown a detectable amount of mRNA expression of the $P2Y_{12}$ receptor in palmitate-induced insulin-resistant H9c2 cell lines, while Tica treatment elicited substantial improvement on mitochondrial function through prevention of increases in oxidant production and modulated the autophagosomes pathway(s) [25]. An antiplatelet agent aspirin provided a positive effect on the modulation of MMPs in the diabetic heart [14]. Moreover, it also could reduce vascular inflammation in aortic aneurysms via modulation of MMPs [26]. In addition, the relationship between increased $[Zn^{2+}]_i$, oxidative stress, and degradation in the cardiac matrix metalloproteinases (MMPs) in MetS rats is very correlated with each other [11]. However, the exact mechanisms of those relations are not known yet. Since MMPs have important roles in cardiac fibrosis under hyperglycemia, it would be an important contribution if one can show the interplay of their roles and new therapeutic. Therefore, taken into consideration our previously published data associated with degraded MMP-2 and MMP-9 in the MetS rat heart and the increased $[Zn^{2+}]_i$ in parallel with increased oxidative stress, we aimed to examine whether Tica treatment has a positive effect on the mechanical activity of the MetS rat heart, through affecting the degradation of those MMPs.

Materials and Methods

All experimental protocols were performed following the standards of the European Community guidelines on the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the Ankara University with a reference number of 2016–18-165.

Induction of Metabolic Syndrome

Male Wistar rats were housed in a temperature-controlled condition with a 12-h light/dark cycle, and induction of MetS was performed as described, elsewhere [4]. Briefly, the experimental group (MetS group) rats (2-month-old) were receiving tap water including 32% sucrose (935 mM) for 16 weeks, whereas the control group rats were receiving only tap water. All rats had free access to standard rat chow ad libitum, and there was no significant difference between these two groups in terms of the amount consumed either drinking water or food. The content of the rat diet was similar to those used in our previous studies [27–29]. The rat diet is a standard local commercial diet, and its composition includes (as a percentage) torula yeast 30.0, corn oil 2.0, sucrose 59.0, and DL-methionine 0.3 together with AIN-76 mineral and vitamin mixture as 5.0 and 1.0, respectively.

The development of MetS in rats was validated by measuring their body weights, fasting blood glucose levels, and oral glucose tolerance test (OGTT). We have shown marked insulin resistance with increased body weight and serum insulin levels (HOMA index), similar to our previous studies [8].

Following validation of MetS, the rats were randomly divided into two groups: the ticagrelor (Tica; dissolved in tap water and orally administered as 150 mg/kg/day for 15 days, $n = 5$ rats) and the MetS group (received with the vehicle, $n = 4$ rats) [30]. Age-matched rats were kept as

controls, gavaged with the vehicle for 15 days to exclude stress-dependent alterations ($n = 5$ rats).

Experiments with Langendorff-perfused hearts

The rats were anesthetized with pentobarbital sodium (30 mg/kg by intraperitoneal injection), and hearts were removed and cannulated to the Langendorff perfusion apparatus, as described previously [31]. The hearts were electrically stimulated (DCS, Harward) at 300 beats/min with 1.5 ms^2 waves (at twice the threshold voltage). Changes in the left ventricular developed pressure (LVDP) were measured with a water-filled latex balloon inserted into the left ventricle, and all data were recorded online then stored and processed (Model 1050BP; BIOPAC Systems, Goleta, CA, USA).

Tension Measurements in the Left Ventricular Papillary Muscle Strips

Following deep anesthesia, the hearts were quickly removed and placed in cold modified Krebs–Henseleit solution (mmol/l: 119 NaCl; 4,8 KCl; 1,8 $CaCl_2$; 1,2 $MgSO_4$; 1,2 KH_2PO_4; 20 $NaHCO_3$; and 10 glucose; pH 7.4). Left ventricular papillary muscles were quickly separated and mounted in an organ bath with an attached force generator. Under initial tone (1 g), the muscle was electrically stimulated for 45 min to reach the equilibrated muscle tone. All muscles were monitored before and after Tica (1 µM or 10 µM) treatment during the experiments. Twitch traces were analyzed, and time to peak tension (TP) and decay time of 50% of the peak tension (DT50) were calculated. All experiments were performed at 37 °C.

Contractile Activity of Aortic Rings

Experimental rats were anesthetized and the thoracic aorta was removed and placed in iced Krebs–Henseleit solution (mM): NaCl, 119; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 1.8; $NaHCO_3$, 25; $KH2PO_4$, glucose 10 (bubbled with 95% O_2–5% CO_2). The segments were carefully cleaned from fatty tissues sectioned into 3-mm long rings. Aortic rings were stretched to a 1-g initial tension and were equilibrated for 60 min. Tension was developed by using an isometric force transducer (GRASS FT03) connected to an amplifier. All data were processed and analyzed with BIOPAC (Model 1050BP; BIOPAC Systems, Goleta, CA, USA).

Preparation of Cell line

The H9c2 cell line was derived from the left ventricle of the embryonic rat heart (purchased from The American Type Culture Collection, CRL1446). The cells were grown at 37 °C, as described previously. Briefly, a modified Dulbecco's modified Eagle's medium (DMEM) was used which included 5.5 mM glucose instead of 25 mM glucose and supplemented with 10% fetal calf serum (F2442, Sigma-Aldrich, USA), 50 U/mL penicillin-G, and 50 µg/mL streptomycin. To obtain insulin-resistant cells, we used the previously published protocol [32].

Experimental groups designed as follows: the cells were incubated with 50-µM palmitic acid (PA-group) and palmitic acid plus Tica (1-µM) (PA + Tica group) for 24 h. The cells incubated with only the normal medium were accepted controls (Con group).

Determination of Intracellular Labile Zn^{2+} in Palmitate-Induced Insulin-Resistant H9c2 Cell line

To monitor the intracellular level of labile Zn^{2+} ($[Zn^{2+}]_i$) in H9c2 cells, a Zn^{2+}-sensitive fluorescence dye, FluoZin-3 (3 µM), was used and monitored in a confocal microscope (LEICA SP5), as described previously [33]. Fluorescence intensities were acquired at 1 Hz, at 490-nm excitation wavelength, and collected at 525 nm. The steady-state fluorescence intensity (F) was measured; then maximum and minimum ratios of the fluorescence intensity changes were determined to calculate labile Zn^{2+} level using the following formula: $[Zn^{2+}] = K_d\,(F - F_{min})/(F_{max} - F)$, where the K_d for FluoZin-3 is 15 nM. The maximum fluorescence (F_{max}) was obtained upon Zn^{2+} saturation with a zinc ionophore, Zn^{2+}-salt of 1-hydroxypyridine-2-thione, and Zn^{2+}-pyrithione (ZnPT; 10 µM), and the minimum ratio (F_{min}) was obtained upon an intracellular Zn^{2+} chelation with *N*, *N*, *N*′, *N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; 50 µM).

Histological Analysis

For structural investigation, we used light microscopic evaluation as described previously [34]. Tissue samples (left ventricle and aortic rings) fixed in 10% neutral buffered formalin and routine histological procedures were applied. Samples were embedded in paraffin for histologic sectioning (5-µm slices) and stained with either hematoxylin–eosin (HE) or Masson's trichrome (MT) stain. The preparations were investigated under a Carl Zeiss Axioscope photomicroscope.

Western Blot Analysis

The left ventricular tissue was prepared for western blot analysis, as described elsewhere. Briefly, equal amounts of protein preparations were run on SDS polyacrylamide gels and blotted with a primary antibody against MMP-2 (Santa Cruz, sc-6838, 1:500), MMP-9 (Santa Cruz, sc-6841, 1/500), and β-actin (Santa Cruz, sc-47778, 1:5000), and to

detect their protein levels. Band densities were presented as a ratio to β-actin.

Reagents and Statistical Analysis of Data

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Data were presented as mean ± SEM with GraphPad Prism 8.1 (GraphPad Software, Inc., La Jolla, CA). Statistical significance ($p < 0.05$) was considered either with the Student's t-test or with the Kruskal–Wallis test.

Results

No Beneficial Effects of Ticagrelor Treatment on the Depressed Contractile Activity of MetS Rat Heart

As presented previously, the development of Mets in rats was validated by measuring the body weight, fasting blood glucose level, and oral glucose tolerance test of high-glucose-intake rats. We have shown marked insulin resistance with increased body weight and serum insulin levels (HOMA index), previously [8]. Notably, Tica treatment could not have any benefits on systemic metabolic indexes of the rats (data not shown).

We examined the global effects of chronic Tica treatment on the depressed contractile activity of working-heart preparations of MetS rats (similar to those presented with our previous studies) [3, 8]. Figure 1A represents the original LVDP traces of the rats. Interestingly, Tica treatment was not sufficient to improve the depressed contractile activity in MetS hearts in comparison to the non-treated group (Fig. 1B). It has also no effects on kinetics including time to peak (TP; Fig. 1C) and decay time of 50% of the maximum (DT50; Fig. 1D) as well as the calculated changes of LVDPs with time (Fig. 1E).

To reconcile the contractile dysfunction in the MetS group with the vascular activity, we investigated the possible role(s) of the Tica treatment in aortic ring experiments. The maximum contractile responses were achieved by submaximal phenylephrine (Phe; 10 μM), whereas aortic rings were relaxed with acetylcholine (Ach; 10 μM) stimulation. The original traces of contraction-relaxation traces of the aortic rings are given in Fig. 2A. Although contractile activity is not altered among the groups (Fig. 2B), the vascular relaxation tone reduced in the MetS group in comparison to that in the Con group (Fig. 2C), while Tica treatment provided a substantial recovery in that activity (Fig. 2C).

We also examined the papillary muscle contractile activity by measuring twitches elicited by electrical stimulation to elucidate an acute application of Tica in the MetS group compared to that in the age-matched controls. As can be seen in Fig. 3B, Tica with 2 concentrations reduced the contractile activity of the Con group, significantly, whereas these Tica-applications could not induce any significant effect in the MetS group.

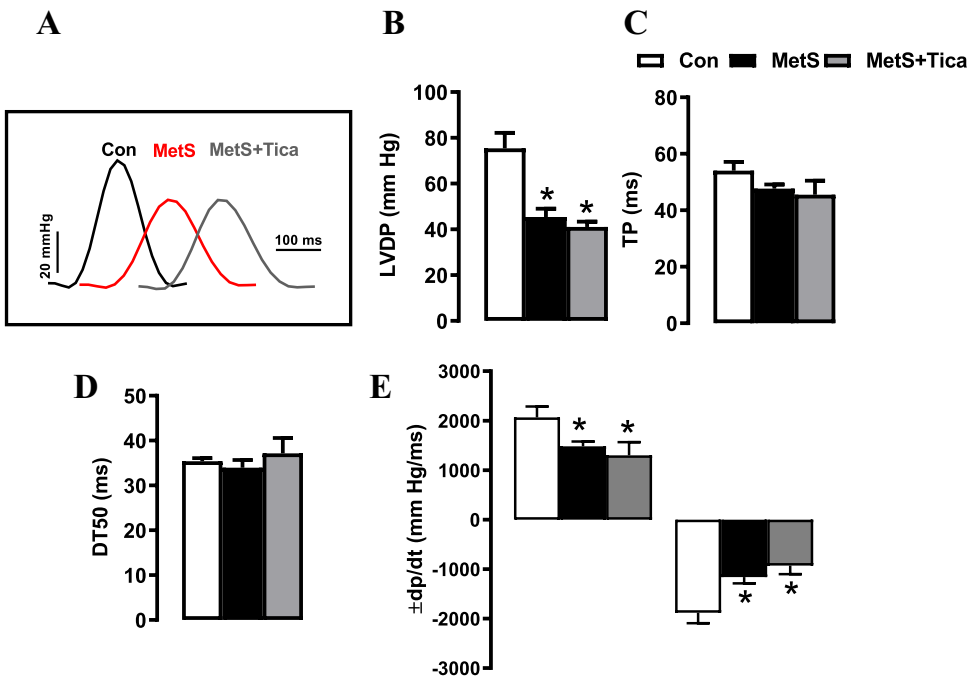

Fig. 1 Ticagrelor treatment and the depressed contractile activity of MetS rat heart. Representative left ventricular developed pressure (LVDP) changes for control (Con), metabolic syndrome (MetS), and ticagrelor (Tica)-treated MetS groups (**A**) and their average LVDP values (**B**). The time course of maximum LVDP changes, as TP representing the time to peak LVDP changes (**C**) and DT50 representing the time of 50% relaxation from peak LVDP changes (**D**). (**E**) Calculated velocity of LVDP changes (± dP/dt; mmHg/ms) for the experimental groups. Data presenting as mean (± SEM) values. The total number of rats per group; $n = 4–5$. Statistical significance, $^*p < 0.01$ vs. Con, with Kruskal–Wallis test

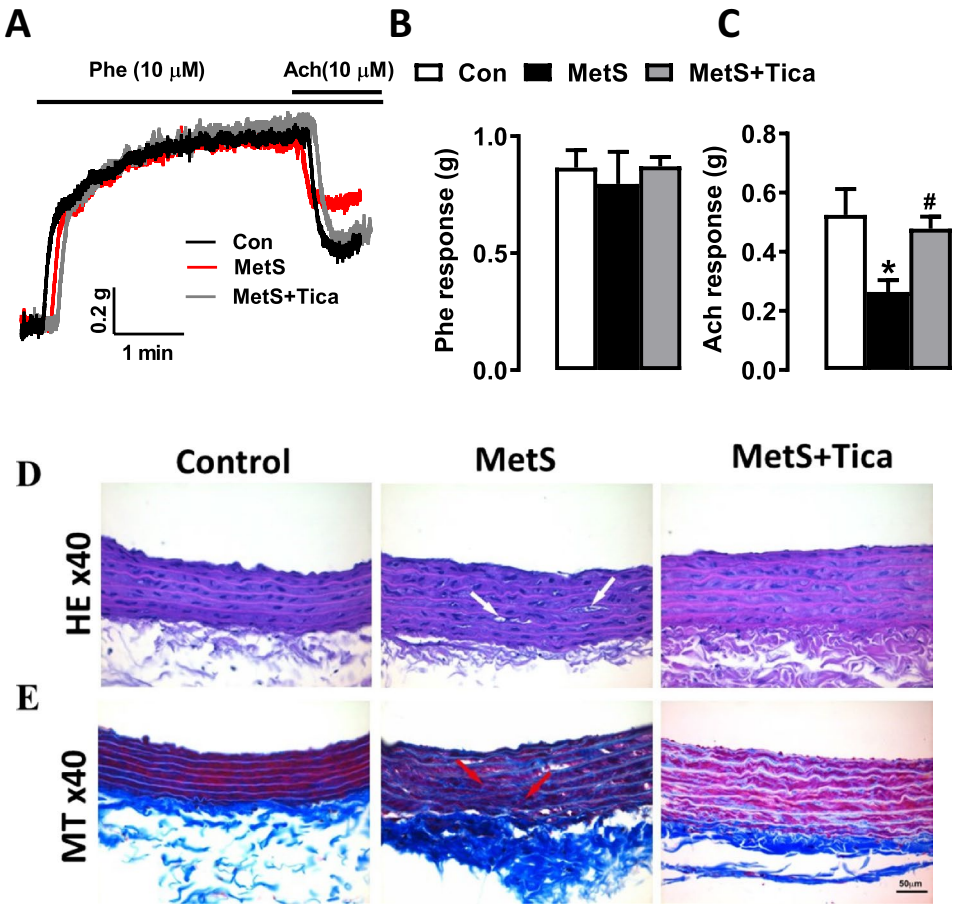

Fig. 2 Ticagrelor treatment reversed the relaxation activity of aortic rings in MetS rats. (**A**) The original recording of contraction-relaxation activity of aortic rings. The maximum contractile responses of aortic rings were obtained by phenylephrine (Phe; 10–5 M) stimulation, while they were relaxed with acetylcholine (Ach; 10–5 M) stimulation. The average values for contraction (**B**) and relaxation (**C**) of control (Con), metabolic syndrome (MetS), and ticagrelor (Tica)-treated MetS groups. Data presenting as mean (±SEM) values. The total number of aortic rings per group; $n=7$–8 from 4 to 5 rats. Statistical significance, $^{*}p<0.01$ vs. Con, $^{\#}p<0.01$ vs. MetS, with Kruskal–Wallis test. Representative light microscopic images of the aorta were obtained from the control, MetS, and Tica + MetS groups. In its group, Tunica media thickness, foamy cells (white arrow), and irregular elastic lamellae (red arrow) were seen. After Tica treatment, there are slight improvements in aortic sections. Examination of the aortic rings in light microscopy with HE staining (**D**) and Masson's trichrome (MT) staining (**E**). Magnification, ×40

Effects of Tica Treatment on the Morphological Structure and Extracellular Matrix Remodeling in the Heart

We examined the left ventricular tissue of hearts from Tica-treated MetS rats by light microscopy compared to that from untreated MetS mice or controls. Compared to MetS rats, we observed slight improvements in the histology of the myocardium of MetS rats treated with Tica, including relatively less cytoplasmic vacuolization, some reduction in increased collagen fiber around the cells, and improvement in pale staining compared to those of the untreated MetS group (in H&E-stained samples; Fig. 3A, upper part). In samples taken from Tica-treated MetS rats and undergoing Masson's trichrome staining, a slight reversal was observed in the cytoplasm of tissues compared to those taken from MetS rats, while a decrease in the density of collagen fibers was observed. Therefore, Tica treatment showed a small decrease in the content of collagen fiber accumulation in the MetS rat heart and a slight recovery in cytoplasmic disorganization (Fig. 3A lower part).

We also performed histological analysis of the aortic rings using a light microscope. Light microscopic images of the MetS group (Fig. 2D–E; H&E, upper part, Masson's Trichrome staining, lower part) drew attention to the presence of foamy cells in the tunica environment as well as irregularities in the elastic lamellae. A slight thickening was observed in the tunica environment. Some regain of these changes in aortic sections compared to MetS rats was observed with Tica treatment.

Since studies have shown the high glucose-associated decreases in the protein levels of MMPs and the important

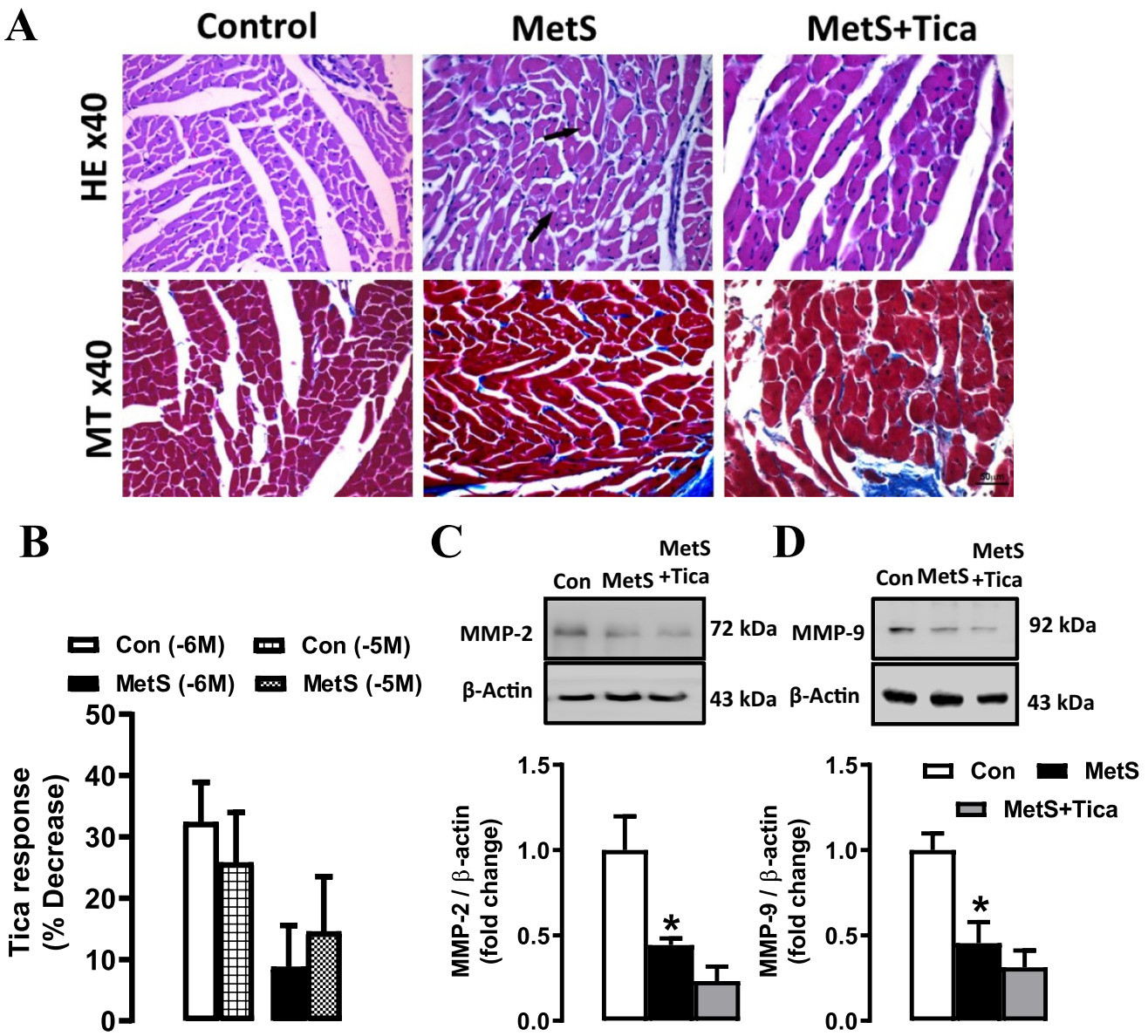

Fig. 3 Effects of ticagrelor treatment on structure and function as well as extracellular matrix remodeling in the left ventricular preparations. (**A**) Representative light microscopic images of the left ventricle were obtained from the control, MetS, and Tica + MetS groups. In the MetS group, cytoplasmic vacuolization (arrow) and enhanced collagen fiber (blue color) around the cardiomyocytes were seen. After Tica treatment, the left ventricular sections were slightly improved. (**B**) Contractile (tension) responses of papillary muscle strips isolated from left ventricles to acute Tica exposures (1 μM or 10 μM) in the MetS group compared to that of the Con group. The protein expression levels of extracellular matrix proteins, matrix metalloproteinases (MMPs) for MMP-2 (**C**), and MMP-9 (**D**) given for β-actin. Data presenting as mean (± SEM) values. The total number of rat hearts per group; $n = 4$–5. Statistical significance: $^{*}p < 0.05$ vs. Con, with Kruskal–Wallis test. Magnification, × 40

contribution of extracellular matrix proteins into the contractile activity of the heart (in process of cardiac remodeling), we examined MMP-2 and MMP-9 protein expression levels in heart tissue homogenates. Western images of groups for MMP-2 and MMP-9 are illustrated in the upper panels of Fig. 3C and D, respectively. In comparison to controls, MMP-2 and MMP-9 protein expression levels were significantly reduced in the MetS group (Fig. 3C), whereas Tica treatment had also no effects on these remodeling (Fig. 3D).

Assessment of $[Zn^{2+}]_i$ Levels in MetS-Mimicked Insulin-Resistant H9c2 Cell line

It is known that MMPs belong to the family that is structurally related to Zn^{2+}- and Ca^{2+}-dependent endopeptidases,

involved in the cleavage of extracellular matrix proteins [35]. We tested $[Zn^{2+}]_i$ levels in MetS-mimicked insulin-resistant H9c2 cell line. To obtain MetS-mimicked insulin-resistant cells, we used our previously published protocols [25, 32]. Confocal images of FluoZin-3-loaded cells are demonstrated in Fig. 4A. MetS-mimicked insulin-resistant H9c2 cell line depicted a significant increase $[Zn^{2+}]_i$ levels compared to vehicle-treated controls. Pretreatment of the cells with ticagrelor (Tica; 1 μM for 24 h) [25, 36] slightly but not significantly reduced the elevated $[Zn^{2+}]_i$ level in the insulin-resistant group.

Discussion

The findings of the present study provided important information on the bimodal effects of $P2Y_{12}$ antagonism with ticagrelor (Tica) on matrix metalloproteinase–associated contractile dysfunction in insulin-resistant metabolic syndrome (MetS) rat heart. Briefly, here, our data demonstrated that Tica treatment of MetS rats could not provide significant benefits on the depressed contractile activity of Langendorff-perfused hearts. On the other hand, this treatment provided a significant recovery in the reduced relaxation activity of the aortic preparations from the same MetS rats. Those observed neutral effects of Tica treatment in MetS rat heart were further in line with the findings from light microscopy examinations. The Tica treatment of MetS rats could also not induce any significant recovery in the degraded MMP-2 and MMP-9 levels. Moreover, the Tica treatment could not reverse significantly the elevated $[Zn^{2+}]_i$ in insulin-resistant cardiomyocytes. Taken into consideration the relationship between the contractile activity of the heart and the status of MMPs as well as the role of labile Zn^{2+} (as the catalytic component of proteins) in the regulation of MMPs, the observed lack of benefits with Tica treatment may arise via its lack of effects on these couples. An elevation of $[Zn^{2+}]_i$ can precipitate in the cleavage of extracellular matrix, which further leads to contractile dysfunction in the heart, because the extracellular matrix (ECM) is a highly dynamic structure and continuously undergoes controlled remodeling in many tissues including the heart mediated by MMPs responsible for ECM degradation [37].

Metabolic syndrome is a complex disorder, defined by a cluster of interconnected factors which can lead to a bunch of cardiovascular risk factors [38, 39] as well as increased susceptibility to oxidative stress and altered MMP expression, leading to degradation of collagen and fibronectin [40–42]. On the other hand, Tica allosterically inhibits $P2Y_{12}$ receptors and also exhibits clinically relevant "off-target" pleiotropic effects including preventing cardiac disorders with a lack of exact mechanism, yet. In the present study, Tica treatment showed a marked preventive effect on vascular relaxation tone, at least, by providing a significant improvement in structural disorganizations in the MetS rat heart. Supporting our data, Wang et al. demonstrated that

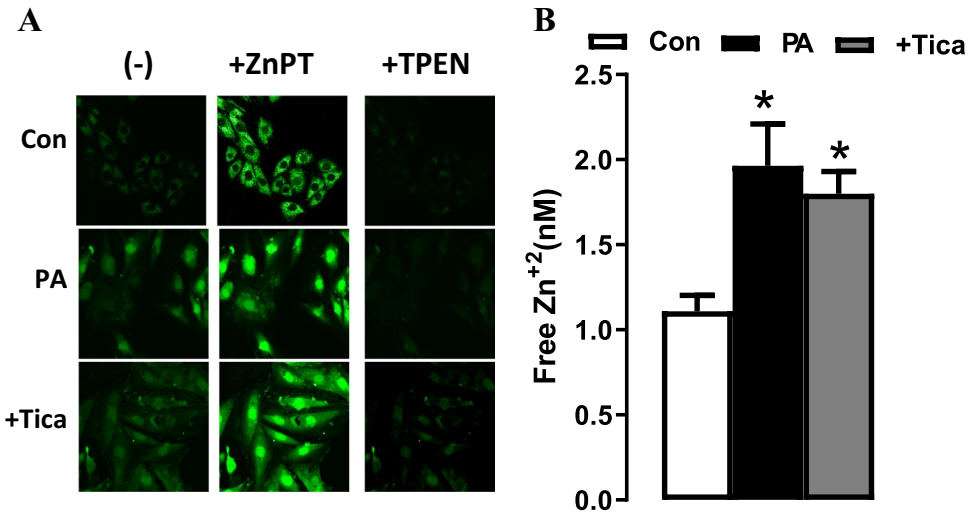

Fig. 4 Ticagrelor treatment could not reverse the increased level of intracellular labile Zn^{2+} in insulin-resistant H9c2 cell line. (**A**) Representative confocal images of cells for three experimental groups to determine the level of intracellular labile Zn^{2+} ($[Zn^{2+}]_i$). Here, the embryonic rat ventricular cells, H9c2 cell line, were incubated with palmitic acid (PA, 50 μM) in the presence and absence of ticagrelor (Tica, 1 μM) for 24 h to induce insulin resistance for mimicking of metabolic syndrome. The $[Zn^{2+}]_i$ measurement protocol was assessed with a Zn^{2+}-selective fluorescence dye FluoZin-3 and the maximum and minimum fluorescence signals obtained by application of a zinc ionophore, Zn^{2+}-pyrithione (ZnPT, 5-μM) and a zinc-chelator, TPEN (50-μM), respectively. The bar graph represents the calculated $[Zn^{2+}]_i$ for the groups. Data presenting as mean (±SEM) values. The total number of cells used per group; $n = 14–15$. Statistical significance, $^*p < 0.05$ vs. Con, with Kruskal–Wallis test

Tica-treated male rats for 14 days protected against AngII-induced endothelial dysfunction via preventing increased ROS production and eNOS phosphorylation and ameliorating ER stress [43]. Further supports were also provided with our previous study with Tica-treated insulin-resistant H9c2 cells. In that study, we have shown that the Tica treatment could abolish oxidative stress by hindering autophagosome-associated apoptosis in insulin-resistant H9c2 cells [25]. Therefore, this group of our present results provided important information related to the beneficial effects of Tica on the depressed relaxation responses in the aortic rings from insulin-resistant MetS rats. Although we did not determine any parameter related to endothelial function, these benefits on relaxation activity of aorta from MetS rats also seem to depend on the endothelial recovery of aortic preparations with Tica treatment. Our statement is further supported with a randomized, prospective, controlled study, in which authors demonstrated that clopidogrel, prasugrel, and Tica treatment have some important effect on endothelial function, besides other effects such as inflammatory effects and effects on oxidative stress parameters and platelet function, in patients undergoing coronary artery stenting for an acute coronary syndrome [44].

To demonstrate a direct heart-target effect of Tica, we performed in vitro studies with papillary muscle strips from the left ventricle of MetS rats compared to those from controls. Acute Tica application with 2 different concentrations did induce significant depression in the contractile activity of the control group with no further effect on the depressed activity of the MetS group. Our present observation on the depressive effect of Tica application in the papillary muscle strips can be supported with the previous data by Kucuk and co-workers [36]. They have shown that acute Tica application exerted significant influences on contractile properties via decreasing the voltage-gated Ca^{2+} channel currents of left ventricular myocytes from male rats.

Zinc is being a multipurpose element for the mammalian body and plays important role in the regulation of several cellular signaling mechanisms [45, 46]. It has been shown that the elevated $[Zn^{2+}]_i$ deleterious for various organ functions are mainly associated with increased oxidative stress, and mishandling in cellular Ca^{2+} homeostasis, as well as changes in the redox states, eventually culminates in disrupting the excitation–contraction coupling in cardiomyocytes [47–49]. There is also a well-established and interplay between matrix remodeling with elevated $[Zn^{2+}]_i$ in MetS hearts [11, 50]. We have shown that a depressed MMP-2 and MMP-9 protein expression in the heart homogenates from MetS rat heart and significant $[Zn^{2+}]_i$ elevations in insulin-resistant cells, while Tica treatment depicted no effects in these sets of experiments. These data could indicate that $[Zn^{2+}]_i$ is precipitating in cleavage of extracellular matrix thereby modulates MMP remodeling and contractility in cardiac dysfunction in MetS mammals. Supporting those statements, previously, we have shown that increased $[Zn^{2+}]_i$ can lead to depression in the contractile activity of the heart, at most due to increases in the production of reactive oxygen and nitrogen species (ROS/RNS) [51, 52]. In addition, our in vitro studies also demonstrated significant increases in the phosphorylation levels of proteins in contractile machinery such as ryanodine receptors and phospholamban as well as their mediators such as PKA and CamKII [52]. More importantly, some experimental results are showing an influence of aspirin on MMP-2 and MMP-9 in platelets [53]. In a pilot study, aspirin provided positive responses to the modulation of MMPs in the heart of diabetic patients [14]. More, interestingly, clopidogrel, a platelet $P2Y_{12}$ receptor inhibitor, reduces vascular inflammation and angiotensin II–induced abdominal aortic aneurysm progression, in part, affecting the production of MMPs [26].

Ticagrelor is a $P2Y_{12}$ receptor antagonist with already known clinical benefits in patients with vascular dysfunction. Apart from its principal antiplatelet action, pleiotropic effects have been implicated in the clinical profile of Tica, including a potentially beneficial impact on both heart and endothelial function [54]. However, several clinical studies have investigated the postulated effect of Tica on cardiovascular function, yielding conflicting results in terms of both experimental animal studies and clinical patient trials. Among them, limitations of the relevant studies as well as substantial differences in the patient population and animal-dependent variations, study design, and methods may account for these controversial findings. To elucidate the current controversial findings, further research efforts should aim to clarify how quickly does cardiovascular function responds to Tica, how sustained this response is during the dosing intervals and in the long term, which mechanisms are implicated, and whether this pleiotropic action is clinically significant. Future studies must include larger animal populations and longer application periods and should be used multiple methods of function measurement. Furthermore, reliable cardiovascular function assessment greatly depends on strict methodology, with the elimination of factors that might interfere with measurements. Still this study has discrepancies, our data offer important insights into its effect on the cardiovascular system under insulin resistance. Therefore, and because of the contradictory results of performed studies, further research is warranted on this issue. Future studies attempting to investigate the effect of Tica on the cardiovascular function in insulin-resistant mammals should aim to clarify how quickly does this system respond to Tica, how sustained this response is during the dosing intervals and in the long term, which mechanisms are implicated, and whether this pleiotropic action is clinically significant. Nevertheless, the extent of the clinical benefit of

Tica attributable to actions beyond its potent and consistent antiplatelet effect remains uncertain.

Author Contribution BT designed and supervised the research and provided the final approval of the version to be published; YO and ET contributed and performed the experiments and analyzed the data; DB performed all light and electron microscopic analysis. All authors discussed the results and commented on the manuscript.

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Data Availability All data and data materials are available if required.

Declarations

Ethics Approval and Consent to Participate All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Ankara University. All animals received humane care under an institutionally approved experimental animal protocol with an ethical license in Turkey.

Research İnvolving Human Participants and/or Animals Research involved no human data.

Consent for Publication None.

Competing İnterests The authors declare no competing interests.

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