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ORIGINAL CONTRIBUTION

DPP-4 inhibition by linagliptin prevents cardiac dysfunction and inflammation by targeting the Nlrp3/ASC inflammasome

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Abstract

We compared the effects of linagliptin (Lina, a DPP4 inhibitor) and GLP-1 receptor activation by exenatide followed by exendin-4 in an infusion pump (EX) on infarct size (IS), post-infarction activation of the inflammasome and remodeling in wild-type (WT) and db/db diabetic mice. Mice underwent 30 min ischemia followed by 24 h reperfusion. IS was assessed by TTC. Additional mice underwent permanent coronary artery occlusion. Echocardiography was performed 2w after infarction. Activation of the inflammasome in the border zone of the infarction was assessed by rt-PCR and ELISA 2w after reperfusion. Further in vitro experiments were done using primary human cardiofibroblasts and cardiomyocytes exposed to simulated ischemia–reoxygenation. Lina and EX limited IS in both the WT and the db/db mice. Lina and EX equally improved ejection fraction in both the WT and the db/db mice. mRNA levels of ASC, NALP3, IL-1β, IL-6, Collagen-1, and Collagen-3 were higher in the db/db mice than in the WT mice. Infarction increased these levels in the WT and db/db mice. Lina more than EX attenuated the increase in ASC, NALP3, IL-1β, IL-6, Collagen-1 and Collagen-3, TNFα and IL-1β, and decreased apoptosis, especially in the db/db mice. In vitro experiments showed that Lina, but not EX, attenuated the increase in TLR4 expression, an effect that was dependent on p38 activation with downstream upregulation of Let-7i and miR-146b levels. Lina and EX had similar effects on IS and post-infarction function, but Lina attenuated the activation of the inflammasome and the upregulation of collagen-1 and collagen-3 more than direct GLP-1 receptor activation. This effect depends on p38 activation with downstream upregulation of miR-146b levels that suppresses TLR4 expression.

Keywords DPP4 inhibitor · GLP-1 · Myocardial infarction · Nlrp3/ASC inflammasome · TLR4 · microRNA

Introduction

Almost 26 million Americans (8.3% of the population) have diabetes mellitus (DM) [2]. It is estimated that in Europe, there are 55.4 million people with DM (6.9% of the population) [85]. The mortality from cardiovascular disease is about 2–4 times higher in adults with DM than in adults without DM [1]. In fact, heart disease and stroke account for 65% of deaths in patients with DM. Moreover, the short- and long-term prognosis after an acute myocardial infarction is worse for patients with DM [20, 34, 67, 88]. The effects of DM and hyperglycemia are wide ranging and include systemic inflammation [30, 65], endothelial dysfunction [83, 114], hypercoagulability with impaired fibrinolysis [69, 70] and increased platelet degranulation [98], reduced coronary collateral blood flow [47], and interference with myocardial protection by preconditioning, remote ischemic preconditioning, and postconditioning [102, 109].

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Inflammation plays an important role in the pathogenesis of insulin resistance and type 2 DM (T2D) [23, 94, 99, 100]. Activity of the inflammasome, an inflammation-induced adaptor complex, can be triggered by a variety of exogenous and endogenous noninfectious stimuli. Dysregulation of the inflammasome is associated with many diseases including metabolic disorders (obesity and diabetes) [23, 32] and cardiovascular disease (atherosclerosis, myocardial infarction) [77]. The NOD-like receptor 3 (Nlrp3) inflammasome has been linked to inflammation and is especially important for increased inflammation in atherosclerosis [4]. Nlrp3 inflammasome and increased production of mature interleukin (IL)-1β are also triggered by hyperglycemia [56, 64]. Upregulation of Nlrp3 inflammasome is seen in patients with T2D [22, 56]. Elimination of Nlrp3/ASC inflammasome protects the pancreatic beta-cells from cell death induced by high-fat feeding during obesity [112]. Nlrp3 interaction with apoptosis-associated speck-like protein (ASC) leads to activation of caspase-1 with subsequent increased production of the pro-inflammatory cytokines such as IL1β. Numerous pathways have been shown to contribute to the development of diabetic cardiomyopathy in animal models including altered substrate metabolism, mitochondrial dysfunction, impaired Ca^{2+} handling, lipotoxicity, increased oxidative stress, activation of the renin–angiotensin–aldosterone system, inflammation, increased levels of advanced glycation end products, post-translation modification of various proteins, ER stress, autophagy, and alterations in the levels of numerous noncoding RNAs, DNA methylation, and histone modifications [71].

Dipeptidyl-peptidase-4 (DPP-4) inhibitors are available as oral anti-hyperglycemic drugs for the treatment of T2D [26, 27]. The metabolic effect of DPP-4 inhibitor is mediated through sparing incretin hormones [glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP)] from the rapid degradation by DPP-4. DPP-4 has also several non-incretin substrates. DPP-4 cleaves cytokines, chemokines, and neuropeptides that are associated with inflammation, immunity, and vascular function [31]. Thus, the end results could be different from that of pure GLP-1 receptor agonists. Data from both animal and human studies suggest a beneficial effect of GLP-1 on the myocardium, endothelium, and vasculature, including potential anti-inflammatory effects. DPP-4 inhibitors [109], including linagliptin [41], attenuate ischemia–reperfusion injury and limit infarct size. However, the effects of DPP-4 inhibitors on the inflammasome in nondiabetic and diabetic hearts are unknown.

In this study, we focused on the effect of linagliptin on cardioprotection and regulation of the Nlrp3/ASC inflammasome complex and myocardial infarction. We found that T2D therapy by linagliptin eliminates inflammation, protects the myocardium from ischemic injury through inhibition of the Nlrp3/ASC inflammasome, an effect that is dependent on p38 activation with downstream attenuation of Toll-like receptor 4 (TLR4) expression via microRNA interference. Knowledge obtained from the present study unravels important insights into the dysregulated inflammatory pathways in T2D and the effects of DPP4 inhibition on post-infarction inflammation.

Methods

Animal models of diabetes and treatment

Male *db/db* mice with T2D and littermate wild-type (WT, C57BL/6J) non-diabetic mice were purchased from Jackson Laboratory. Mice at 10 weeks of age were used to compare the effects of linagliptin (Lina, a DPP4 inhibitor) and Exenatide (EX, a direct GLP-1 receptor agonist) on cardioprotection, post-infarction remodeling, and inflammation.

The experimental designs and animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch.

Ischemia–reperfusion (IR) model

Experiments were conducted according to recent published guidelines for experimental models of myocardial ischemia and infarction [59] and cardioprotection [14]. Ischemia–reperfusion (IR) was induced in *db/db* mice and WT non-diabetic mice as previously described by our group [106, 108]. Mice were randomly divided into the following pretreatment groups (1) WT + IR; (2) WT + 3 mg/kg/day Lina + IR; (3) WT + 1 μg/kg EX + IR; (4) *db/db* + MI; (5) *db/db* + Lina + IR; (6) *db/db* + EX + IR. Lina was mixed with chow (83 mg/kg of chow) [3, 119] and was given for 1 week before surgery. EX was administered once subcutaneously 1 h before infarction. The dose is based on our previous publication [110]. Mice were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated and ventilated. The depth of anesthesia was monitored by the respiratory rate and lack of withdrawal reflex upon toe pinching. A thoracotomy was performed in the left third intercostal space, and the left anterior descending coronary artery was occluded for 30 min. Ischemia was verified by the regional dysfunction and discoloration of the ischemic zone. Isoflurane (0.5–5% titrated to effect) was added after the beginning of ischemia to maintain anesthesia. At 30 min of ischemia, the snare was released, and myocardial reperfusion was verified by a change in the color of the myocardium. The chest was closed, and the mice were recovered from anesthesia. To provide analgesia, 0.1 mg/

kg buprenorphine was injected subcutaneously after the operation.

Infarct size Twenty-four hours after surgery, the mice were re-anesthetized, the coronary artery was reoccluded, Evans blue dye (3%) was injected into the right ventricle, and the mice were euthanized under deep anesthesia. The hearts were removed and the left ventricle (LV) was sectioned into seven transverse slices. Slices were incubated for 10 min at 37 °C in 1% buffered (pH 7.4) 2,3,5-triphenyltetrazolium chloride, fixed in a 10% formaldehyde, and photographed to identify the ischemic area at risk (AR) (uncolored by the blue dye), the infarct size (IS) (unstained by 2,3,5-triphenyltetrazolium chloride), and the nonischemic zones (colored by blue dye). The AR and IS in each slice were determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice, and the results were summed to obtain the weight of the myocardial AR and IS [106, 108].

The prespecified exclusion criteria were the lack of signs of ischemia during coronary artery ligation, the lack of signs of reperfusion after the release of the snare, a prolonged ventricular arrhythmia with hypotension, and an area at risk (AR) ≤ 10% of the left-ventricular weight.

The myocardial infarction (MI, permanent coronary artery occlusion) model

Additional mice were randomly allocated into the following groups (1) WT + sham-operation; (2) WT + MI; (3) WT + Lina +MI; (4) WT + EX +MI; (5) *db/db* + sham-operation; (6) *db/db* + MI; (7) *db/db* + Lina +MI; (8) *db/db* + EX + MI. Lina was mixed with chow (83 mg/kg of chow) and was given for 1 week before surgery and continued after surgery. Ex was administered once subcutaneously 1 µg/kg 1 h before infarction [110]. Exendin-4 (24 nmol/kg/24 h, Sigma-Aldrich, USA) was given via miniosmotic pump (ALZET Osmotic Pumps, USA) for 2 weeks after surgery. The dose is comparable to that used in the previous publications [39, 72]. Heart function was evaluated 2 weeks after infarction by echocardiography using a fully digitized Vevo 770 high-resolution ultrasound system (VisualSonics, Inc.) with a 25 MHz transducer-710B (Visualsonics-Toronto, Canada) designed for the examination of small rodents. Inhalational anesthetic gas (Isoflurane, 0.5–5%) was used to anesthetize the animals. The mice were examined in the supine position. The transducer was placed in the left parasternal position described previously [48, 105]. Standard M-mode images were recorded in the long-axis and short-axis position at the level of the papillary muscles for each animal. Left-ventricle internal diameters at end diastole (LVIDd) and systole (LVIDs) and left-ventricular fractional shortening (FS) were measured. Ejection fraction was calculated by a single-plane area length using 2D parasternal long-axis images.

Systolic blood pressures (SBP) were measured in prewarmed un-anesthetized mice using tail-cuff.

LV hypertrophy was evaluated by the ratio between left-ventricular weight (HW) and tibia length (TL) as previously described [24].

Glucose/insulin tolerance test

Mice were fasted for 4 h prior to the test. Glucose was injected intraperitoneally at 1 g/kg of body weight. Blood samples were collected from the tail vein before (0 min) and 30, 60, 90, and 120 min after the glucose administration. The blood glucose levels were determined using an auto kit glucose assay (Abcam, Cat# ab6533) [105].

Detection of apoptosis

The border zone of infarction was identified by visual inspection. Tissue sections from border zone of infarction were fixed and embedded in paraffin, and apoptotic cells were detected by labeling DNA-strand breaks using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (Abcam Cat# ab66110). Apoptosis was visualized using an Apop Tag Peroxidase In Situ Apoptosis Detection Kit, according to the manufacturer's instructions. The total number of positive nuclei was determined by counting all the labeled nuclei present in the entire area. Ten areas were randomly selected. The apoptotic index was expressed as the ratio of positively stained nuclei to total nuclei. Images were obtained using Olympus IX71 microscope at a magnification of 20× [105].

RT-PCR

Total RNA from the border zone of infarction was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol as per the manufacturer's instruction. 2 µg total RNA from each sample was reverse-transcribed into cDNA and equal amounts of the reverse transcriptional products were subjected to PCR amplification. The evaluation of mRNAs expressions of NALP3, ASC, IL-1β and IL-6, collagen-1, and collagen-3 was performed using the primers reported in Supplementary Table 1. The Ct (threshold cycle) is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Expression of the gene relative to the GAPDH was calculated as the difference between the threshold values of these two genes (2-Δct). Melting curve analysis was performed during real-time PCR to analyze and verify the specificity of the reaction. The values are given as the mean ± SE of

four independent experiments. Each sample was analyzed in triplicate and normalized by GAPDH.

Enzyme-linked immunosorbent assay (ELISA)

Myocardial samples from the border zone of infarction were homogenized and prepared in RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Roche Diagnostics). After centrifugation, the supernatant of tissue lysate was collected. Protein concentration was determined by the Bradford method. IL-1β, TNFα, MIP-2, and FGF-2 (R&D system, Cat# MLB00C, MTA00B, MM200 and MFB00) levels in serum were determined using ELISA kits according to the manufacturer's instructions. The absorbance was recorded at 450 nm with a plate reader.

In vitro studies

Simulated ischemia–reperfusion (SIR)

Experiment 1 Primary human cardiofibroblasts (HCF) and cardiomyocytes (HCM) were purchased from ScienCell Research Laboratories (USA). HCMs and HCF were cultured in their standard medium at 37 °C in a humidified incubator containing 5% CO_2 and 95% air, referred to as normoxic conditions. HCMs and HCF were exposed to Lina (80 nM) or EX (150 nM) with or without GLP-1 (10 nM). Compounds were added 2 h before the exposure to simulated ischemia–reperfusion (SIR) [13]. The concentration of Lina was based on our pilot study (Supplement, Figure S1). The concentrations of EX [110] and GLP-1 [107] were based on our previous publication.

Experiment 2 Human cardiomyocytes and cardiofibroblasts were in normoglycemic (glucose 5.5 mM) or hyperglycemic (glucose 25 mM) solution [17] with Lina, Lina + GLP-1, GLP-1 alone, EX or SB203580, a p38 inhibitor (Sigma-Aldrich; S8307; 20 µM). SIR was induced by replacing standard medium with RPMI-1640 (Invitrogen, USA) without glucose and serum, and then cultured under hypoxic conditions (94% N_2, 5% CO_2, and 1% O_2) for 2 h. Subsequently, RPMI-1640 was replaced by standard medium, and the cells were then incubated under normoxic conditions for 2 h of reoxygenation [13, 107]. Cell incubated for 4 h without drugs without exposure to SIR (NSIR) or with SIR served as controls.

Cell viability assays

Cell viability was estimated using a tetrazolium bromide (MTT)-based colorimetric method. Briefly, 10 µl of a stock solution of MTT (0.5 µg/µl) was added to each plate. After 4 h of incubation with MTT (at 37 °C in a humidified atmosphere, 5% CO_2), 100 µl of HCl 0.01 M in SDS was applied to solubilize the purple formazan for overnight. The OD value was measured with an ELISA reader at 550 nm. In viable cells, the mitochondrial enzyme succinate dehydrogenase can metabolize MTT into a formazan dye that absorbs light at 550 nm.

Caspase-3 activity

Caspase-3 activity, an early marker of apoptosis, was measured with an assay kit according to the manufacturer's instructions. The cells were harvested and then suspended in the cell lysis buffer to obtain cell lysate. Protein concentration was determined using Lowry Protein Assay. 200 µg protein of cell lysate was incubated in 100 µl of reaction buffer containing 5 µl of caspase-3 substrate (4 mM DEVD-pNA) in 96-well plates. The reaction buffer contains 1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM *N*-acetyl-cysteine, and 10% glycerol. The samples were incubated in the dark and Caspase-3 activity (Abcam, Cat# ab39401) was evaluated using a spectrophotometer at 405 nm.

DNA fragmentation

DNA fragmentation, a late marker of apoptosis, was measured to detect programmed cell death. The degree of intracellular DNA fragmentation (apoptosis) was determined using cell death detection ELISA kit (Sigma-Aldrich, Cat# 11544675001) according to the manufacturer's instructions.

Immunoblotting

HCF cells were lysed after reoxygenation, and then subjected to immunoblotting for NLRP3, ASC, Caspase-1, EGR-1, P-p38, T-p38, P-ERK 1/2, T-ERK 1/2, TLR2, and TLR4. Samples were homogenized in lysis buffer (in mMol): 25 Tris·HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, 1 Na_3VO_4, 1% Triton X-100, 2% SDS, and 1% protease inhibitor cocktail. The lysate was centrifuged at 10,000*g* for 15 min at 4 °C. The resulting supernatants were collected. Protein (50 µg) was fractionated by SDS-PAGE (4–20% polyacrylamide gels) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated overnight at 4 °C with primary antibodies. Bound antibodies were detected using the chemiluminescent substrate (NEN Life Science Products, Boston, MA, USA). The protein signals were quantified with an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin signal. Data are expressed as percent of the expression in the control group (NSIR).

Determination of miRNAs expression level

HCF were exposed to treatment group: (1) NSIR; (2) SIR-cont; (3) SIR-Lina; (4) SIR-Lina + GLP1; (5) SIR-GLP1; (6) SIR-EX; (7) SIR + SB. Compounds were added 2 h before the exposure to simulated ischemia–reperfusion (SIR). Cells were then harvested at and total RNA was extracted from cells by Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Small RNA enrichment was performed using *mir*Vana miRNA Isolation Kit and quantified using the *mir*Vana™ qRT-PCR miRNA Detection Kit as described before [106]. For real-time PCR, SYBR Green I was used for quantification of miRNA transcripts following the manufacturer's instructions. The appropriate cycle threshold (Ct) was determined using the automatic baseline determination feature. Reactions containing qRT-PCR primer sets were specific for human Let-7i. As an internal control, U6 was used for Let-7i template normalization. The relative gene expression was calculated by comparing cycle times for Let-7i.

Transfection and treatment groups

HCF were cultured in standard medium FM-2. At confluence, cells were split and cultured in 6-well plates for 24 h. Then, cells were exposed to various agents for the transfection experiments. 10 nM of the following mimics and inhibitors were placed in tube 1 and the transfection reagent placed in tube 2 with serum-free medium. The contents of tubes 1 and 2 were combined and incubated for 20 min at room temperature. After removing the growth medium, complete medium together with the mixture from the above were then added to the plate. Cells were incubated at 37 °C with 5% CO_2 for 24 h with antibiotic-free medium. The following groups were studied: (1) negative control for mimic (NC-mim); (2) negative control for inhibitor (NC-inh); (3) miR-21-mimic (miR-21-mim); (4) miR-21-inhibitor (miR-21-inh); (5) miR-146b-mimic (miR-146b-mim); (6) miR-146b-inhibitor (miR-146-inh); (7) let-7i-mimic (miR-let7i-mim); (8) let-7i-inhibitor (miR-let7i-inh). Twenty-four hours after, transfection cells were harvested and immunoblotting for TLR4 and TLR2 was performed.

Statistical analysis

Data are presented as mean ± standard error (SE). Analysis of variance (ANOVA) with Sidak correction for multiple comparisons was applied to compare the different groups. The differences in glucose levels during glucose tolerance test were compared using two-way repeated-measures ANOVA with Holm–Sidak multiple comparison procedures. Values of $p < 0.05$ were considered statistically significant. In each figure, the p value of the ANOVA is shown along with symbols for the individual comparisons between specific groups, as explained in the figures' legends.

Results

In vivo experiments

Infarct size

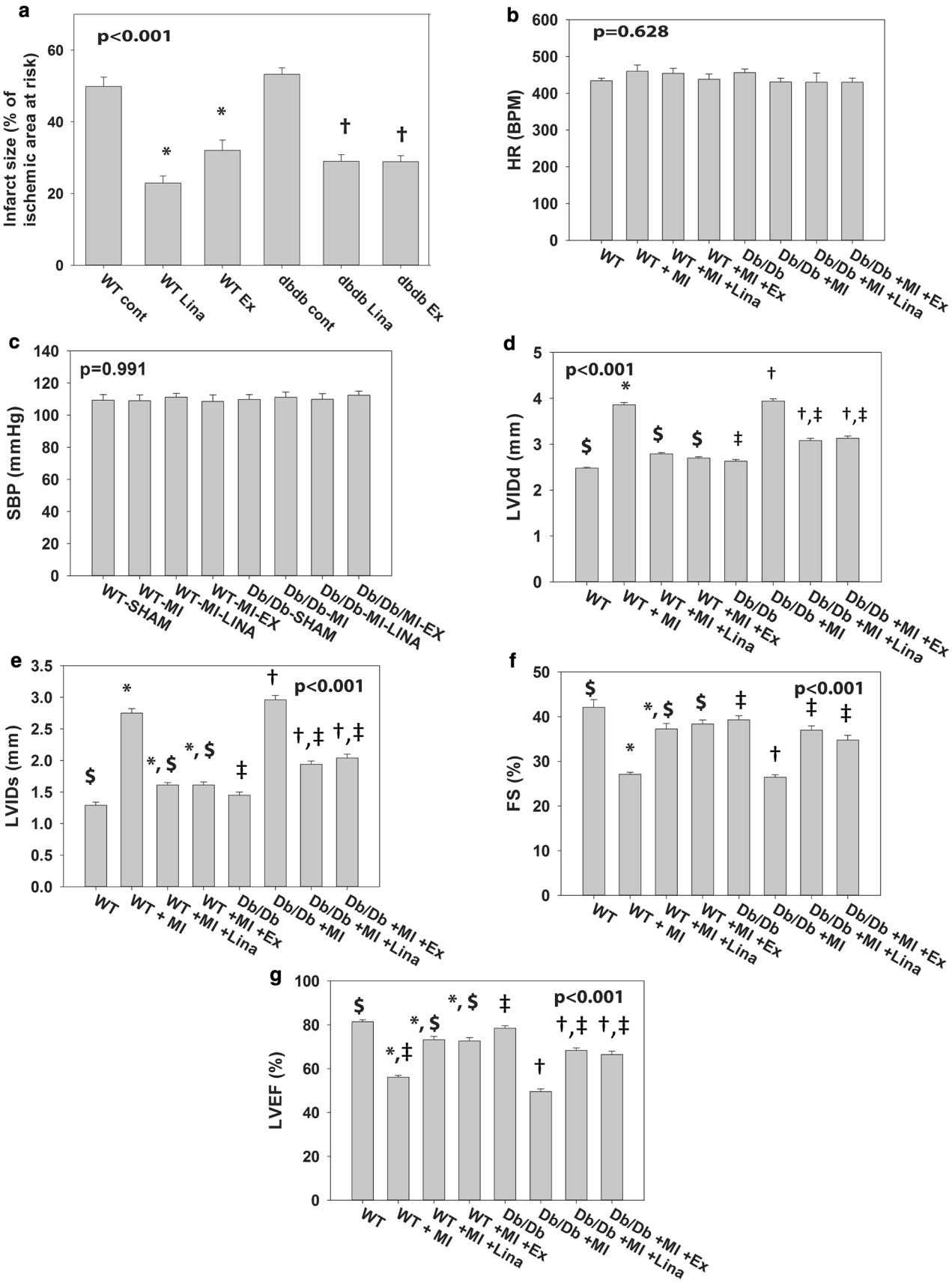
A total of 49 mice were subjected to the ischemia (30 min)–reperfusion (24 h) protocol, four animals died (one from the WT-infarct group during induction of anesthesia and one from the WT-infarct, one from the db/db-infarct, and one from the db/db-infarct + EX group during ischemia). Therefore, a total of 45 mice were included (22 WT and 23 db/db mice) (Table 1). Body weight, left-ventricular weight, and the size of the ischemic area at risk (Table 1) were comparable among groups. Lina and EX limited infarct size, expressed either as a percentage of the LV weight (Table 1) or percentage of the ischemic area at risk (Table 1, Fig. 1a) in both the WT and the db/db mice. IS tended to be smaller in the WT mice treated with Lina than in the WT treated with Ex ($p = 0.055$); however, there was no difference in IS in the db/db mice treated with Lina or Ex ($p = 1.0$).

Next, we assessed whether the protective effect translates into long-term improvement in function in a model of permanent coronary artery ligation. As there has been no published protocol for long-term EX administration in mice, we followed the initial EX injection with prolonged Exendin-4 administration via osmotic pump for 2 weeks in the WT-EX

Table 1 Body weight, LV weight, ischemic area at risk (AR), and infarct size (IS) of the animal subjected to 30 min myocardial ischemia/24 h reperfusion

Group	Body weight (g)	LV weight (mg)	AR (% of LV)	IS (% of LV)	IS (% of AR)
WT-Cont ($n = 6$)	25.9 ± 0.4	155 ± 5	38 ± 1	19 ± 1	49.9 ± 2.6
WT-Lina ($n = 8$)	26.0 ± 0.3	164 ± 6	40 ± 2	9 ± 1*	22.9 ± 2.0*
WT-EX ($n = 8$)	25.8 ± 0.3	163 ± 5	40 ± 1	13 ± 1*	32.0 ± 2.9*
db/db-Cont ($n = 8$)	54.8 ± 0.9	152 ± 5	39 ± 1	21 ± 1	53.3 ± 1.8
db/db-Lina ($n = 8$)	55.5 ± 1.4	155 ± 5	36 ± 1	10 ± 1†	29.0 ± 1.8†
db/db-EX ($n = 7$)	53.4 ± 0.7	155 ± 5	41 ± 1	12 ± 1†	28.9 ± 1.7†
p value	< 0.001	0.505	0.261	< 0.001	< 0.001

*$p < 0.002$ vs. WT-control; †$p < 0.001$ vs. db/db-control


a
p<0.001
Infarct size (% of ischemic area at risk)
WT cont
WT Lina
WT Ex
dbdb cont
dbdb Lina
dbdb Ex
b
p=0.628
HR (BPM)
WT
WT + MI
WT +MI +Lina
WT +MI +Ex
Db/Db
Db/Db +MI
Db/Db +MI +Lina
Db/Db +MI +Ex
c
p=0.991
SBP (mmHg)
WT-SHAM
WT-MI
WT-MI-LINA
WT-MI-EX
Db/Db-SHAM
Db/Db-MI
Db/Db-MI-LINA
Db/Db/MI-EX
d
p<0.001
LVIDd (mm)
e
p<0.001
LVIDs (mm)
f
p<0.001
FS (%)
g
p<0.001
LVEF (%)

◂**Fig. 1** **a** Infarct size (IS), expressed as a percentage of the ischemic area at risk (AR). Number of animals in each group as in Table 1. *$p < 0.002$ vs. WT- control; †$p < 0.001$ vs. db/db-control. **b** Heart rate (HR) 2 weeks after induction of infarct. There were eight animals in each group. **c** Systolic blood pressure (SBP). There were eight animals in each group. **d** Left-ventricular internal diameter in diastole (LVIDd). **e** Left-ventricular Internal diameter in systole (LVIDs). **f** Fractional shortening (FS). **g** Left-ventricular ejection fraction (LVEF). **d**–**g** There were eight animals in each group. *$p < 0.05$ vs. WT-sham; $\$p < 0.05$ vs. WT + MI; †$p < 0.05$ vs. db/db-sham; ‡$p < 0.05$ vs. db/db + MI

and db/db-EX groups, while the WT-Lina and db/db-Lina continued to receive the drug mixed with Chew. Infarct and the drugs had no effect on heart rate (Fig. 1b) or systolic blood pressure (Fig. 1c) 2 weeks after ischemia. Infarct significantly increased left-ventricular internal diameter diastolic (LVIDd) and systolic (LVIDs) and reduced fractional shortening (FS) and left-ventricular ejection fraction (LVEF) in both the WT and the db/db mice (Fig. 1d–g). Both Lina and EX equally attenuated remodeling and systolic dysfunction in the WT, as well as db/db mice.

Glucose tolerance test, performed 2 weeks after infarction, following 4-h fasting is presented in Fig. 2a. Glucose levels were significantly higher in the db/db mice than in the WT mice. Infarct, Lina and EX had no effect on glucose blood levels in the WT mice. Infarct did not affect glucose levels in the db/db mice. Both Lina ($p < 0.001$) and EX ($p < 0.001$) significantly reduced glucose levels in the db/db mice compared to the db/db-MI group. Lina decreased glucose levels more than EX ($p < 0.001$).

Body weight 2 weeks after infarction was greater in the db/db mice than in the WT mice. Infarct, Lina and EX had no effect on body weight in both the WT and db/db mice (Fig. 2b). Heart weight (expressed in mg or as a ratio with tibial length to control for obesity) [24] significantly increased after infarct in both the WT and db/db mice (Fig. 2c, d). Lina but not EX prevented this increase.

Rt-PCR showed that mRNA levels of ASC, NALP3, IL-1β, IL-6, Collagen-1, and Collagen-3 were higher in the db/db than in the WT mice (Fig. 3). Infarct increased these levels in the WT and db/db mice. Lina attenuated the increase in ASC, NALP3, IL-1β, IL-6, Collagen-1, and Collagen-3 more than EX, especially in the db/db mice.

Myocardial levels of TNFα (Fig. 4a), IL-1β (Fig. 4b) were significantly higher in the db/db-sham than in the WT-sham. Infarct increased the levels in both the WT and the db/db mice. Lina, but not EX attenuated the increase. Levels of MIP-2 (Fig. 4c) and FGF-2 (Fig. 4d) were comparable between the WT-sham and db/db-sham groups. Infarct increased their levels more in the db/db mice than in the WT mice. Again, Lina, but not EX attenuated the increase.

Apoptosis in the border zone was greater in the db/db-sham mice than in the WT-sham mice (Fig. 4e, f). Infarct increased the percentage of apoptotic cells in both the WT and db/db mice. Both Lina and EX attenuated apoptosis; however, the effect of Lina was significantly greater than that of EX.

In vitro studies

Human cardiomyocytes and cardiofibroblasts were exposed to 2 h hypoxia followed by 2 h reoxygenation. Hypoxia–reoxygenation increased Caspase-3 activity in both the cardiomyocytes and cardiofibroblasts (Fig. 5a, b). Lina and EX attenuated the increase in Caspase-3 activity. As in our in vitro model, there is no source of endogenous GLP-1; the effect of Lina is probably GLP-1 independent. Exogenous GLP-1 had no effect, as it was probably rapidly degraded by the endogenous DPP4. However, when GLP-1 and Lina were combined, attenuation of the increase in Caspase-3 activity augmented compared to Lina alone ($p = 0.745$ in cardiomyocytes and $p < 0.001$ in cardiofibroblasts) and EX alone ($p < 0.001$ in cardiomyocytes and $p < 0.001$ in cardiofibroblasts). Adding GLP-1 to EX did not augment the effect of EX.

As expected, hypoxia–reoxygenation increased apoptosis in the cardiofibroblasts and cardiomyocytes (Fig. 5c, d). Both Lina and EX equally attenuated apoptosis. Again, GLP-1 alone had no effect and did not significantly augment the effect of Lina when combined ($p = 0.998$ in cardiomyocytes and $p = 0.50$ in cardiofibroblasts), suggesting that the Lina is mainly GLP-1–independent.

Hypoxia–reoxygenation increased the activation of the inflammasome (Fig. 5e, f) and caspase-1 (Fig. 5g). Lina and EX equally attenuated the increase. GLP-1 alone had no effect and it did not alter the effect of EX. However, when added to Lina, the attenuation effect was augmented for NLRP3 ($p < 0.001$) and caspase-1 ($p < 0.001$), but not ASC ($p = 0.386$), suggesting that Lina has both GLP-1-independent and -dependent effects on NLRP3 and caspase-1.

In cells incubated in normoglycemic conditions, hypoxia–reoxygenation increased EGR1 (Fig. 6a, b), P-p38 (Fig. 6a, c), P-ERK 1/2 (Fig. 6a, d), TLR2 (Fig. 6a, e), and TLR4 (Fig. 6a, f) levels. Lina but not EX and GLP-1 attenuated the increase. GLP-1 added to Lina did not alter the effect of Lina alone, suggesting that these effects are GLP-1-independent. SB203580 (SB), a p38 inhibitor, attenuated p38 phosphorylation, and tended to attenuate the increase in EGR1 expression and ERK 1/2 phosphorylation, and significantly attenuated the increase in TLR2 and TLR4 expression, suggesting that the effects of Lina on TRL2 and TLR4 expression are p38-dependent. In cells incubated in high glucose solution, similar results were obtained (Fig. 7). High glucose without simulated hypoxia–reoxygenation significantly reduced cell viability (Supplement Figure S2). Hypoxia–reoxygenation decreased cell viability in cells

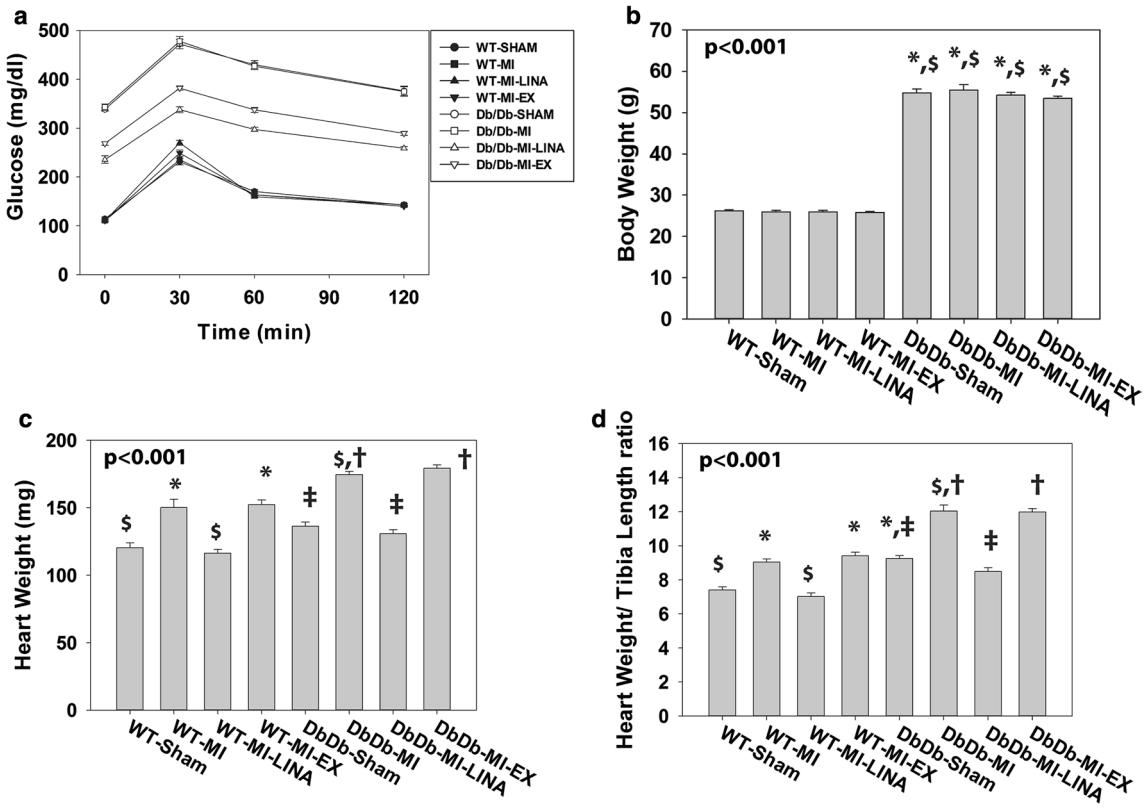


Fig. 2 **a** Glucose tolerance test: mice fasted for 4 h prior to glucose tolerance tests. Blood glucose levels were measured at the indicated time-points following i.p. administration of glucose (1 g/kg body weight). Two-way repeated-measures ANOVA: there was a significant group effect ($p<0.001$). There was a significant time effect ($p<0.001$). There is a statistically significant interaction between group and time ($p<0.001$). db/db-sham vs. db/db-MI $p=0.568$; db/db-MI vs. db/db-MI-LINA $p<0.001$; db/db-MI vs. db/db-MI-EX $p<0.001$; db/db-MI-LINA vs. db/db-MI-EX $p<0.001$. **b** Body weight (g) 2 weeks after infarct. There were seven animals in each group. $^{*}p<0.001$ vs. WT-sham; $^{\$}p<0.001$ vs. WT + MI. **c** Heart weight (mg) 2 weeks after infarct. There were seven animals in each group. $^{*}p<0.05$ vs. WT-sham; $^{\$}p<0.05$ vs. WT + MI; $^{\dagger}p<0.05$ vs. db/db-sham; $^{\ddagger}p<0.05$ vs. db/db + MI. **d** Heart weight/tibial length ratio 2 weeks after infarct. There were seven animals in each group. $^{*}p<0.05$ vs. WT-sham; $^{\$}p<0.05$ vs. WT + MI; $^{\dagger}p<0.05$ vs. db/db-sham; $^{\ddagger}p<0.05$ vs. db/db + MI

incubated in high glucose solution. Lina alone, Lina + GLP1 and SB equally improved cell viability after hypoxia–reoxygenation in cells incubated with high glucose. In contrast, both EX and GLP1 had no effect on cell viability in cells exposed to high glucose and hypoxia–reoxygenation.

High glucose significantly increased EGR1, P-p38, P-ERK 1/2, TLR2, and TLR4 levels. Exposure to hypoxia–reoxygenation increased these levels in cells incubated in high glucose. Lina and SB, but not EX and GLP-1, significantly attenuated the increase.

It has been reported that the expression of TRL4 is controlled by Let7i [9, 79, 117], miR-21 [104], miR27a [91], miR-145 [44], and miR-146b [92]. We assessed whether hypoxia–reoxygenation changes the expression of these microRNAs, and whether Lina, EX, GLP-1, and SB can affect the changes induced by hypoxia–reoxygenation in human cardiofibroblasts. Hypoxia–reoxygenation had no effect on Let 7i-3p levels (Fig. 8a), miR-27a levels (Fig. 8d), and miR-145 levels (Fig. 8e), but it decreased let-7i-5p levels (Fig. 8b), miR-21 levels (Fig. 8c), and miR-146b levels (Fig. 8f). Lina but not GLP-1 or EX increased the levels of Let-7i-3p, Let-7i-5p, miR-21, and miR-146b. SB increased miR-21 and miR-146b levels, similar to Lina; however, it had only a smaller effect on Let-7i-5p levels and no effect on Let-7i-3p levels. Thus, it seems that the effects of Lina on miR-21 and miR-146b levels are mediated by p38.

Next, we asked whether these microRNAs affect TLR4 expression in the human cardiofibroblasts. Mir-21-mimic and -inhibitor had no effect on TLR4 mRNA expression (Fig. 9a). On the other hand, miR-146b-mimic and Let7i-mimic decreased TLR4 expression, whereas miR-146b-inhibitor and Let-7i-inhibitor significantly increased TLR-4 expression. Both miR-146b-inh and Let7i-inh significantly increased TLR4 protein levels (Fig. 9b), while their mimics decreased TLR4 protein levels. Thus, Lina increases the expression of Let-7i and miR-146b with downstream suppression of TLR4 expression. The effect on miR-146b is

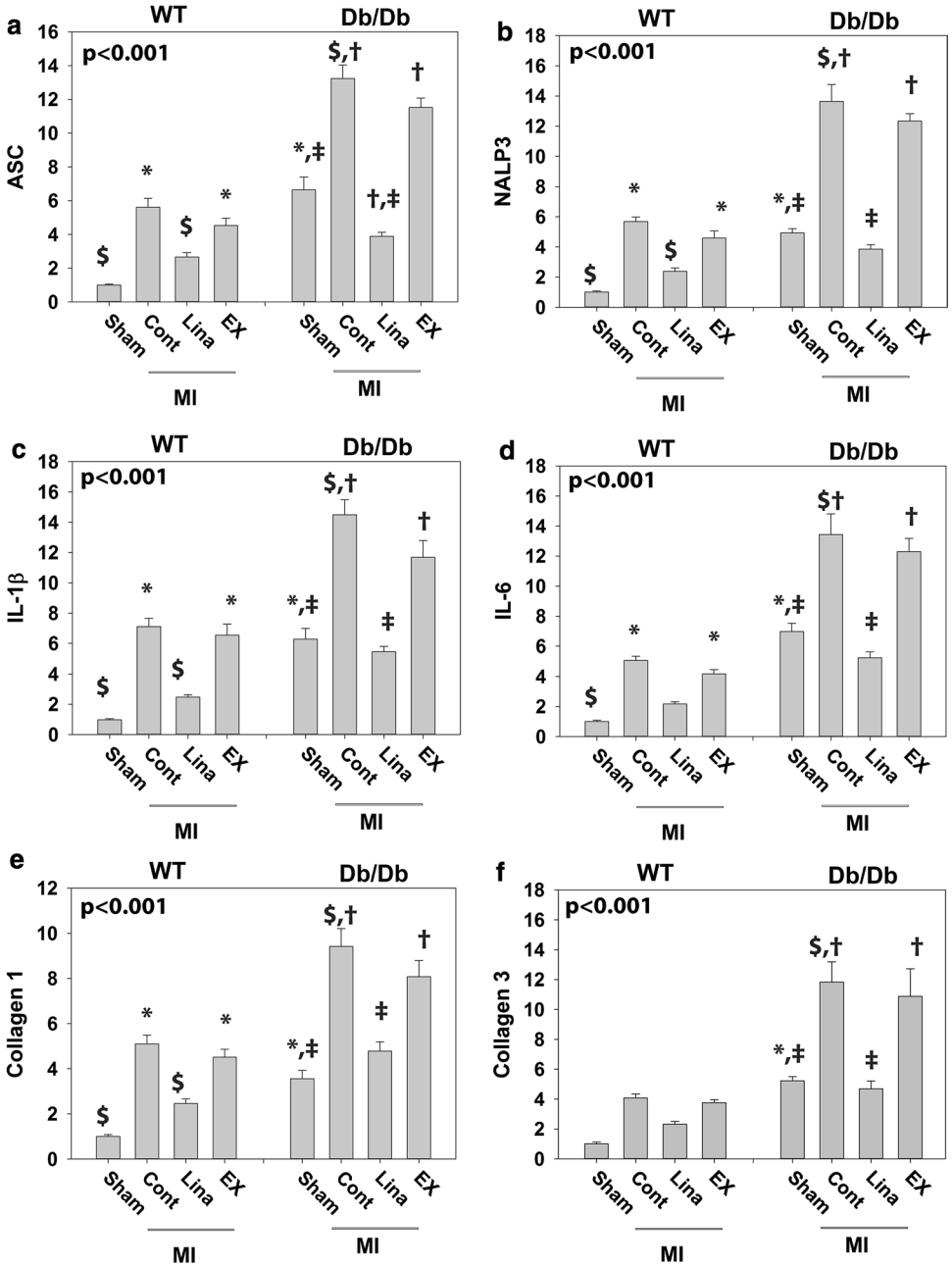


Fig. 3 In the border zone of the myocardial infarction, mRNA levels of ASC (**a**), NALP3 (**b**), IL-1β (**c**), IL-6 (**d**), Collagen-1 (**e**), and Collagen-3 (**f**) were higher in the db/db than in the WT mice. Infarct increased these levels in the WT and db/db mice. Lina attenuated the increase in ASC, NALP3, IL-1β, IL-6, Collagen-1, and Collagen-3 more than EX. There were four animals in each group. $^{*}p < 0.05$ vs. WT-sham; $^{\$}p < 0.017$ vs. WT + MI; $^{\dagger}p < 0.026$ vs. db/db-sham; $^{\ddagger}p < 0.003$ vs. db/db + MI

p38 dependent, whereas the effect on Let-7i is mostly p38 independent.

Discussion

Here, we found that both Lina and Ex limited infarct size in WT, as well as db/db mice with T2D in an ischemia–reperfusion model. We also found that both Lina and direct GLP-1 activation attenuated adverse remodeling and the decrease in left-ventricular systolic dysfunction, as assessed by echocardiography 2 weeks after permanent coronary ligation. This model assesses post-infarction remodeling rather than protection against ischemia–reperfusion injury. Yet, Lina, but not direct GLP-1 activation, attenuated the post-infarction increase in heart weight. Moreover, Lina attenuated the post-infarction activation of the inflammasome and the upregulation of collagen-1 and collagen-3 more than direct GLP-1 activation. The levels of Macrophage Inflammatory Protein 2 [MIP-2, also known as Chemokine (C-X-C motif) ligand 2 (CXCL2)], a small cytokine that is secreted by monocytes and neutrophils at sites of inflammation, increased after infarction in the db/db mice more than in the WT mice. Lina, more than

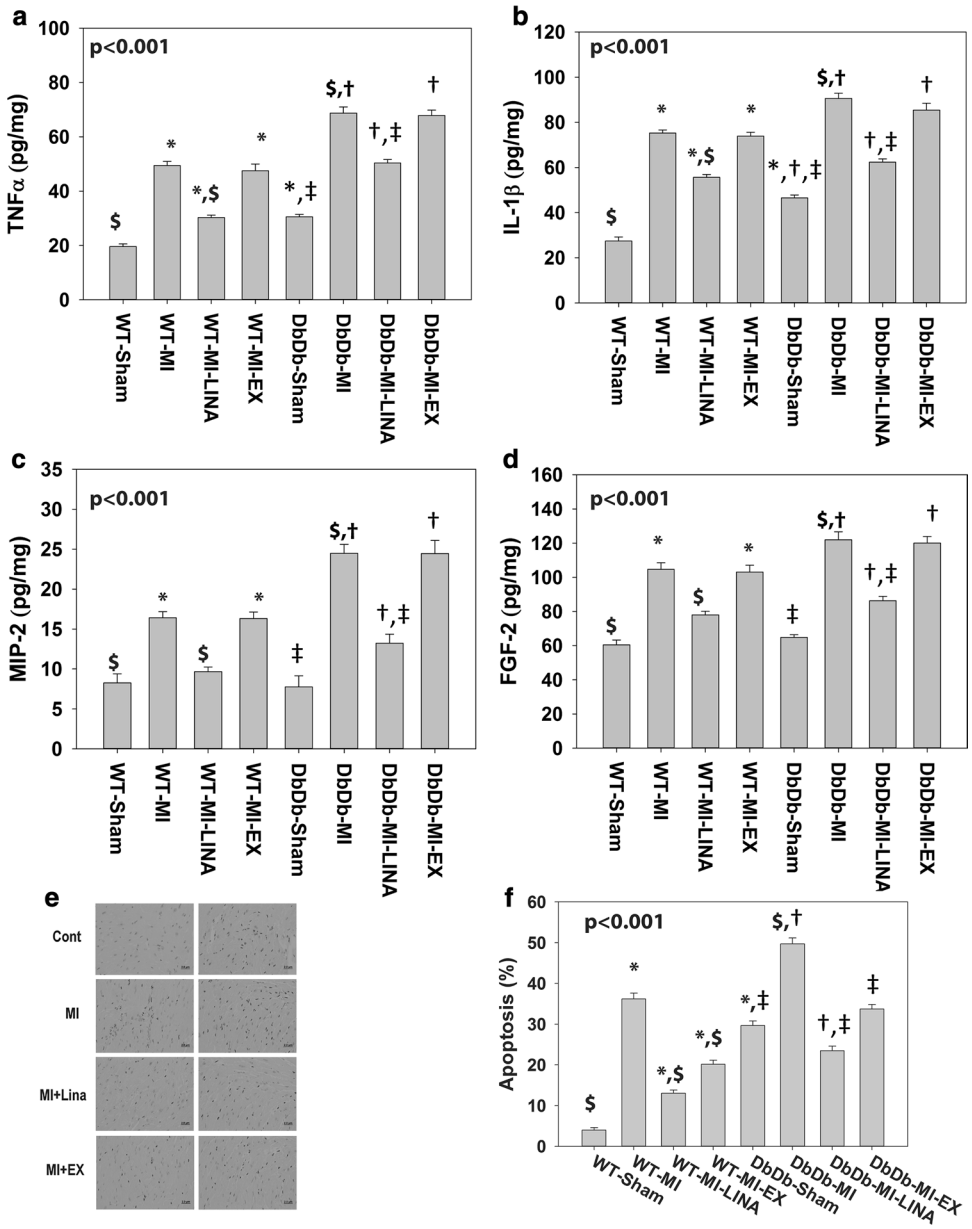


Fig. 4 In the border zone of the myocardial infarction, TNFα levels (**a**), IL-1β levels (**b**), MIP-2 levels (**c**), and FGF-2 levels (**d**). There were six animals in each group. $^{*}p<0.003$ vs. WT-sham; $^{\$}p<0.022$ vs. WT-MI; $^{\dagger}p<0.040$ vs. db/db + MI; $^{\ddagger}p<0.001$ vs. db/db + MI + Lina. **e** Samples of TUNNEL staining for apoptosis. **f** Percent of apoptotic cells. There were 10–12 samples for each group. $^{*}p<0.001$ vs. WT-sham; $^{\$}p<0.001$ vs. WT + MI; $^{\dagger}p<0.006$ vs. db/db-sham; $^{\ddagger}p<0.001$ vs. db/db + MI

direct GLP-1 activation, attenuated the increase. The same pattern was seen with Fibroblast Growth Factor 2 (FGF-2); Lina attenuated the increase after infarction more than direct GLP-1 activation. In addition, the effect of Lina on attenuation of apoptosis post-infarction was greater than that of direct GLP-1 activation.

Several studies have shown that both direct GLP-1 activators [36, 42, 68, 103, 109, 110] and DPP4 inhibitors [7, 109, 111] reduce infarct size in models of ischemia–reperfusion injury in non-diabetic animals. However, only few studies have shown an effect in animals with T2D [12, 18, 78, 120]. Moreover, one study suggested that liraglutide had no effect on remodeling after permanent coronary artery occlusion in non-diabetic rats [53].

Some [10, 61, 62] but not all studies [73] found that exenatide limits infarct size in patients with ST elevation myocardial infarction undergoing primary percutaneous coronary interventions. However, the majority of the patients included in these studies did not have diabetes. No similar effects have been reported with DPP4 inhibitors in the clinical setting.

We found that Lina attenuated the post-infarction activation of the inflammasome more that direct GLP-1 receptor agonists. The increase in myocardial levels of TNFα and IL-1β was suppressed only by Lina. The NLRP3 inflammasome has a major role in post-infarction inflammation, remodeling, and diabetic cardiomyopathy [60]. Previous studies reported that liraglutide, a GLP-1 receptor agonist,

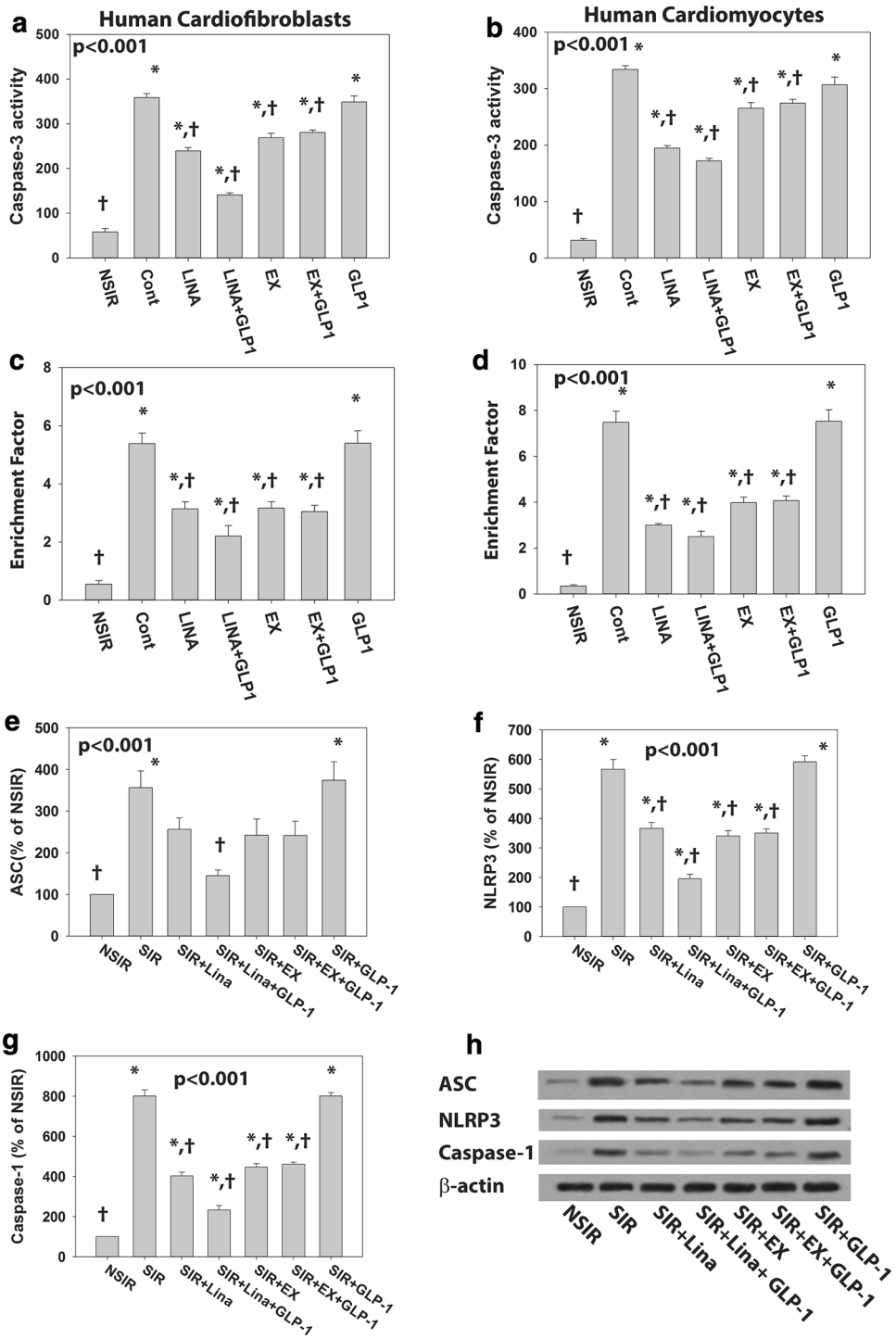


Fig. 5 **a** Caspase-3 activity in cardiofibroblasts. **b** Caspase-3 activity in cardiomyocytes. **c** DNA fragmentation (apoptosis) in cardiofibroblasts. **d** DNA fragmentation (apoptosis) in cardiomyocytes. Experiments were repeated six times. Densitometric analysis of the protein concentrations of ASC (**e**), NLRP3 (**f**), and Caspase-1 (**g**) in cardiofibroblasts. **h** Samples of immunoblots of ASC, NLRP3, and Caspase-1 in the cardiofibroblasts. Experiments were repeated four times. *$p < 0.049$ vs. control without simulated hypoxia–reoxygenation (NSIR); †$p < 0.004$ vs. control with simulated ischemia–reperfusion (SIR)

attenuates the activation of the inflammasome in the liver, lung, and H9c2 cardiomyoblasts [16, 116, 118]. Dai et al. reported that sitagliptin and NVPDPP728, both DPP4 inhibitors, suppressed the activation of the inflammasome in macrophages exposed to oxidized low-density lipoprotein by increasing the expression of GLP-1 receptors with downstream suppression of protein kinase C (PKC) phosphorylation [19]. Liraglutide also suppressed the inflammasome activation in their model. They concluded that the effect is mediated by GLP-1 receptor activation. Yet, there was no source of GLP-1 in their in vitro experiments. Moreover, while phorbol 12-myristate 13-acetate (PMA), a PKC activator, blocked the effects of DPP4 inhibition, it did not block the effect of liraglutide, suggesting different signaling pathways [19]. We have previously shown that saxagliptin, a DPP4 inhibitor, suppressed the activation of the

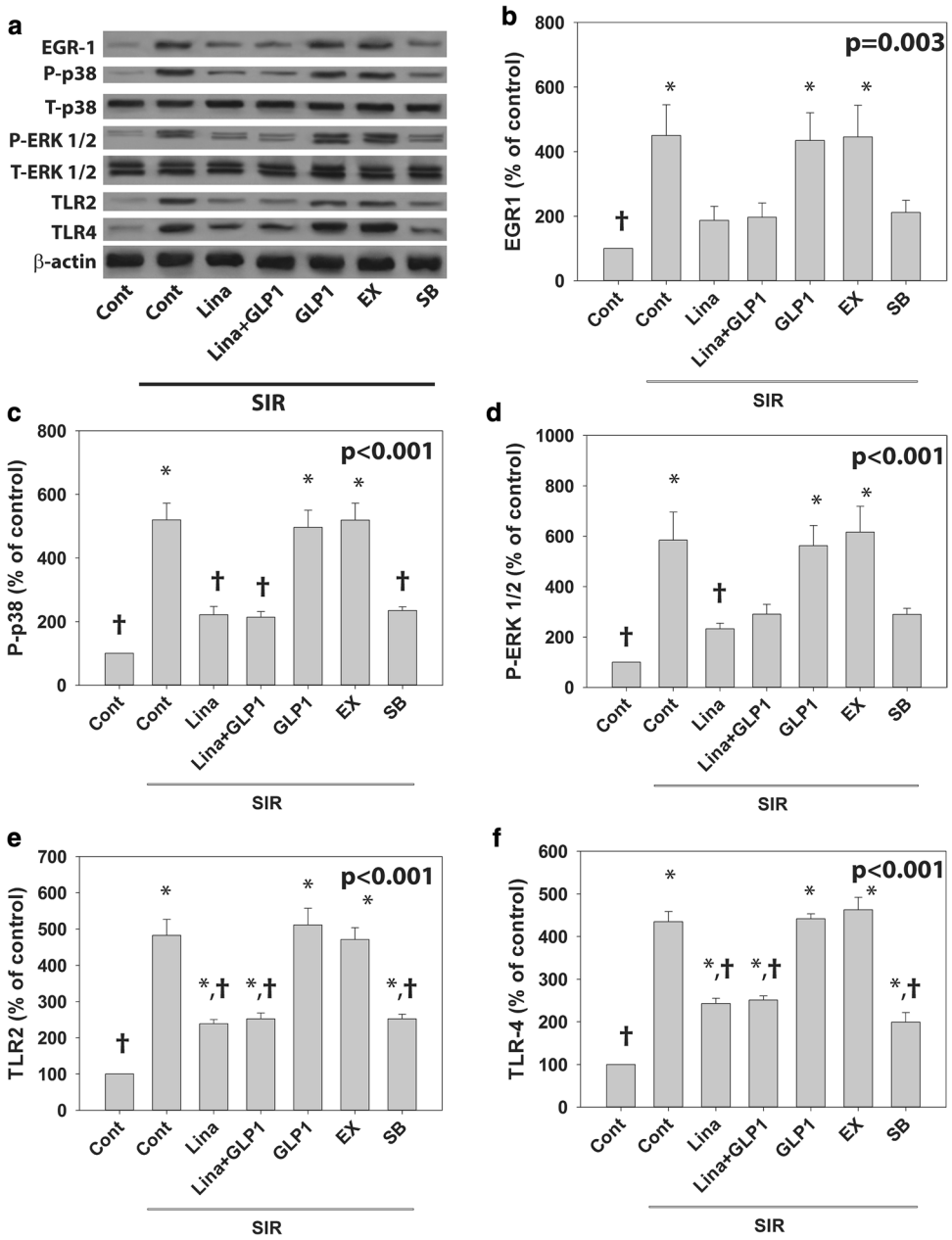


Fig. 6 **a** Samples of immunoblots and densitometric analysis of the protein concentrations of EGR-1 (**b**), P-p38 (**c**), P-ERK 1/2 (**d**), TLR2 (**e**), and TLR4 (**f**) in human cardiofibroblasts incubated in normoglycemic solution and exposed to 2 h hypoxia followed by 2 h reoxygenation. Experiments were repeated four times. *$p < 0.049$ vs. control without simulated hypoxia–reoxygenation; †$p < 0.030$ vs. control with simulated ischemia–reperfusion (SIR)

inflammasome in the kidney of mice with type-1 (Akita) and type-2 (BTBR) diabetes [11]. As glucose levels were not changed in the Akita mice, we concluded that the protective effect is glucose-lowering independent.

The DDP4 inhibitors inhibit the degradation of the incretin hormones (GLP-1, GIP, and GLP-2). However, DPP4 has additional non-incretin targets [31]. For example, Shi et al. found that DPP4 directly interacts with integrin β1 and that the beneficial effect of DPP4 inhibition on endothelial-to-mesenchymal transition in the kidney is dependent on integrin β1 suppression with downstream suppression of TGF-β2 and SMAD3 phosphorylation [86]. Connelly et al. also found that saxagliptin, a DPP4 inhibitor, but not liraglutide, a direct GLP-1 agonist, improved remodeling and attenuated the decrease in left-ventricular contractility 4 weeks after permanent coronary artery occlusion [18]. They attributed the protective effect to upregulation of stromal cell-derived factor-1α (SDF-1α) that occurred with saxagliptin, but not liraglutide, suggesting that the protective effect is DPP4-dependent, rather than mediated by GLP-1 receptor activation. On the other hand, Baba et al. suggested that the protective effect of alogliptin, a DPP4 inhibitor, in rabbits is solely dependent on GLP-1 receptor activation and that the protective effect involves nitric oxide production [7].

The in vivo models cannot fully separate between the incretin-dependent and independent effects. Moreover,

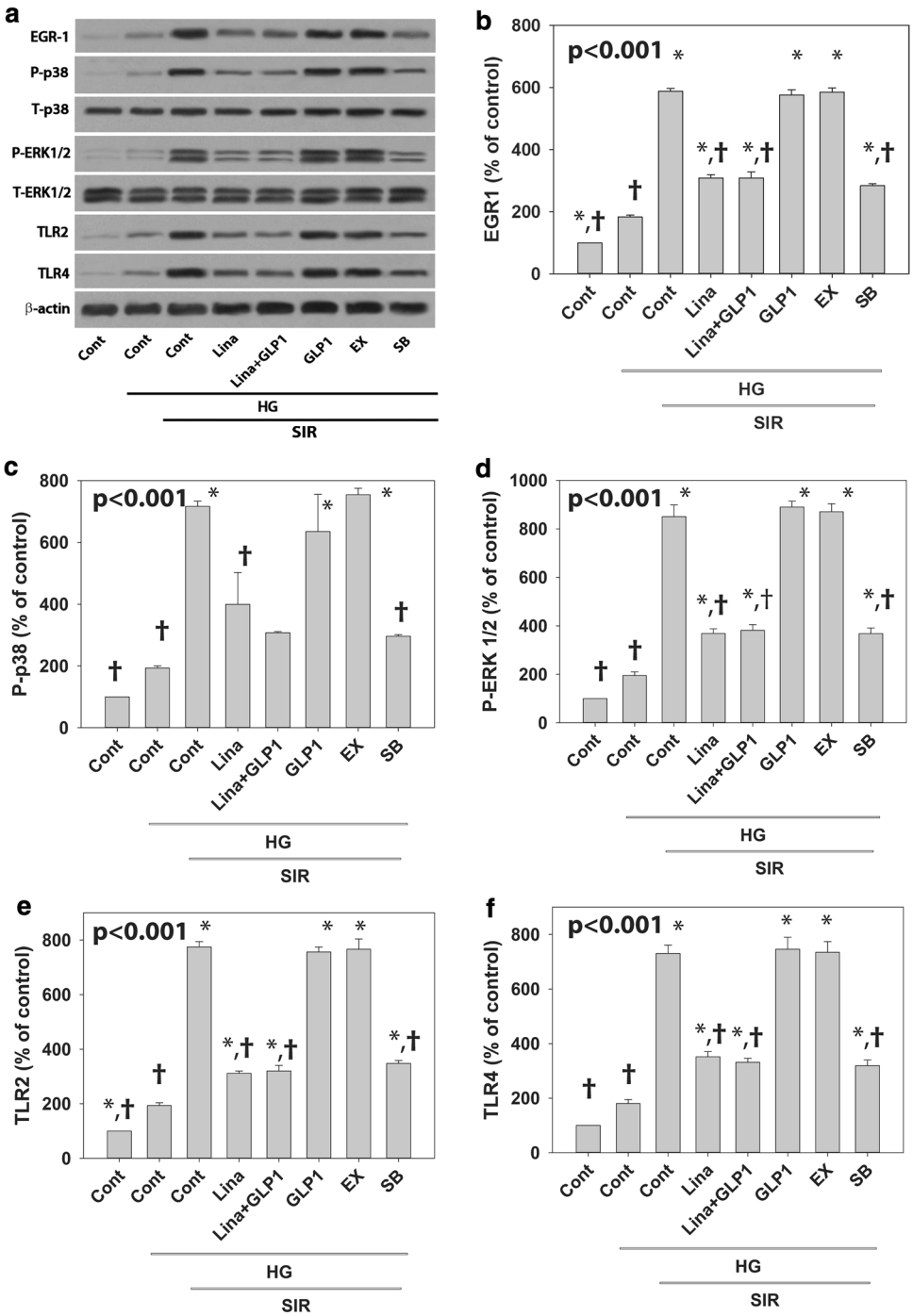


Fig. 7 **a** Samples of immunoblots and densitometric analysis of the protein concentrations of EGR-1 (**b**), P-p38 (**c**), P-ERK 1/2 (**d**), TLR2 (**e**), and TLR4 (**f**) in human cardiofibroblasts incubated in high glucose solution and exposed to 2 h hypoxia followed by 2 h reoxygenation. Experiments were repeated four times. $^{*}p < 0.050$ vs. control high glucose without simulated hypoxia–reoxygenation; $^{\dagger}p < 0.018$ vs. control high glucose with simulated ischemia–reperfusion (SIR)

it is difficult to separate between the glucose-lowering-dependent and independent effects of the DPP4-inhibitors and GLP-1 receptor agonists in T2D models. Therefore, we conducted a series of in vitro experiments. In these experiments, there is no change in glucose levels and as there is no endogenous source of incretins, if not added externally; therefore, the direct GLP-1 independent effects can be separated.

In vitro experiments

Using a model of simulated hypoxia–reoxygenation, we found that both Lina and EX equally attenuated the upregulation of Caspase-3, apoptosis, and activation of the inflammasome in cardiomyocytes and cardiofibroblasts. As there is no endogenous source of incretins in this model, the effects of Lina are GLP-1-independent.

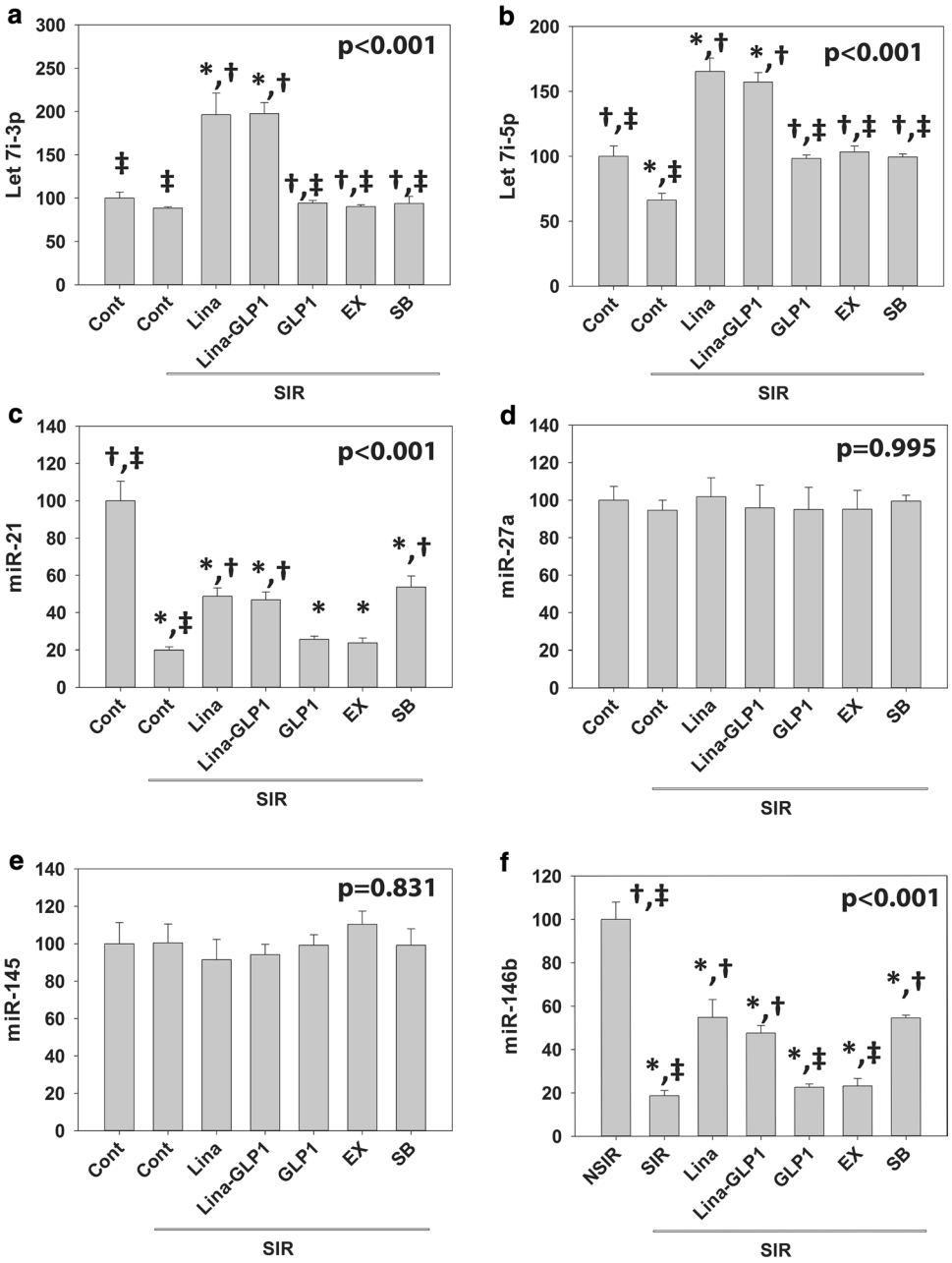


Fig. 8 Levels of Let7i-3p (**a**), Let7i-5p (**b**), miR-21 (**c**), miR-27a (**d**), miR-145 (**e**), and miR-146b (**f**) in cardiofibroblasts exposed to 2 h hypoxia–2 h reoxygenation and treated with Lina, EX, GLP-1 or SB. Experiments were repeated four times. $^{*}p < 0.024$ vs. control-no simulated ischemia–reperfusion; $^{\dagger}p < 0.037$ vs. control-simulated ischemia–reperfusion (SIR); $^{\ddagger}p < 0.019$ vs. Lina

However, some of the effects could be augmented when exogenous GLP-1 was added to Lina, suggesting that there might be additional GLP-1 dependent effects. However, there is a controversy whether the effects of the GLP-1 analogs against myocardial ischemia–reperfusion injury are GLP-1 receptor-dependent. For example, Wohlfart et al. reported similar protective effects of lixisenatide, a GLP-1 analog, on isolated cardiomyocytes of WT and GLP1 receptor knockout mice, suggesting that the effect is GLP-1 receptor independent [103]. Moreover, although GLP-1 receptor mRNA transcripts are found in all four human heart cardiac chambers, they could not be found in human cardiofibroblasts and the exact identity of the cells expressing the transcripts in the heart is unclear [8].

Interestingly, Lina and EX attenuated the activation of both Caspase-1 (a member of the inflammasome pathway) and Caspase-3 (a member of the apoptosis pathway), suggesting attenuation of two separate cell death pathways. The role of apoptosis in mediating myocardial ischemia–reperfusion injury has been questioned. It was shown that deletion of Caspase-3 and Caspase-7 had no effect on myocardial infarct size in mice exposed to 45 min coronary artery ligation followed by reperfusion [45].

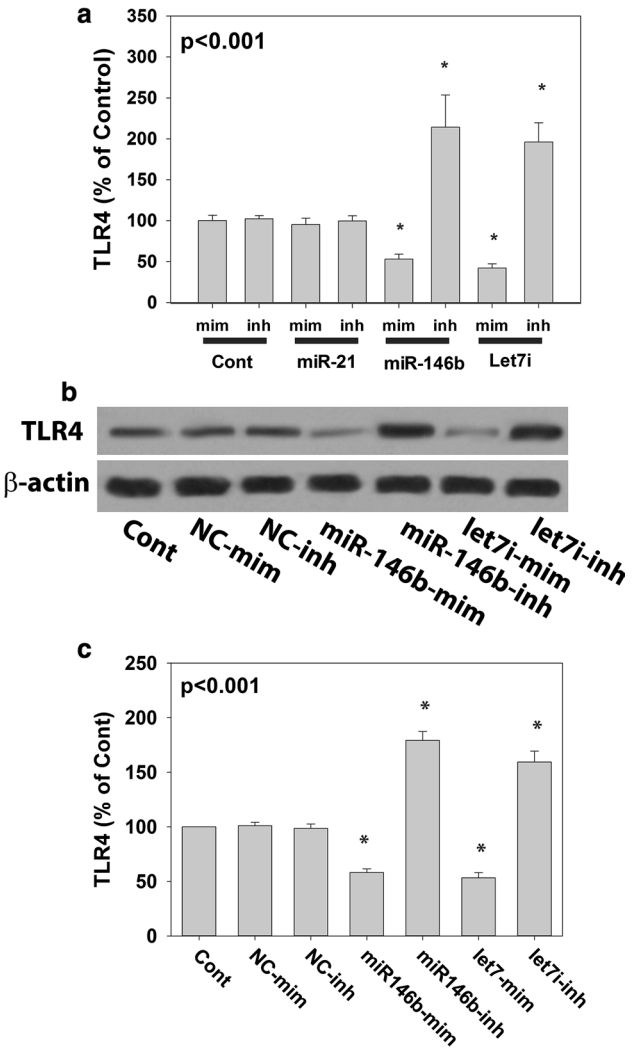


Fig. 9 **a** TLR4 mRNA levels following transfection with the various microRNA-mimics and inhibitors. Each experiment was repeated four times. *$p < 0.001$ vs. normal control (NC). **b** Samples of immunoblots of TLR4 protein levels following transfection with the various microRNA-mimics and inhibitors. **c** Densitometric analysis of the TLR4 protein levels. Each experiment was repeated six times. *$p < 0.001$ vs. control

While Lina and EX had similar effects on the suppression of the activation of the inflammasome and apoptosis, we found that Lina, but not EX, attenuated the activation of p38 and ERK 1/2 and the hypoxia–reoxygenation-induced increase in TLR2, TLR4, and EGR1 expression.

ERK 1/2 is a member of the Reperfusion Injury Salvage Kinase (RISK) pathway. Its phosphorylation before ischemia or during reperfusion mediates cardioprotection [54]. Our in vitro experiment showed that Lina and SB, but not EX attenuated ERK 1/2 phosphorylation 2 h after reoxygenation. It could be that if we checked P-ERK 1/2 levels during hypoxia or early after reperfusion, levels could be higher with Lina exposure. However, it has been shown that cardioprotection, especially by TNFα, can be mediated by alternative signaling pathway [Survivor Activating Factor Enhancement (SAFE)], even when ERK 1/2 and AKT are inhibited [54]. Moreover, others have also shown that anagliptin [29] and Lina [51] attenuate ERK 1/2 phosphorylation.

Early growth response-1 (EGR1) belongs to a family of zinc-finger transcription factors, "immediate-early response proteins". EGR1 induced rapidly by a wide spectrum of extracellular signals, including cytokines, growth factors, and numerous physiologic stimuli [93]. EGR1 is involved in cell growth, cell differentiation, and cell survival [21]. Decreasing EGR1 levels protects against renal inflammation and fibrosis [40] and protects the heart against ischemia–reperfusion injury [43, 66, 95]. Atorvastatin inhibits EGR1 expression in patients with acute coronary syndromes [84]. It was shown that EGR1 expression is dependent on ERK 1/2 activity [58, 95]. Interestingly, previous studies suggested that both direct GLP-1 analogs [49, 50] and Lina [89] increase the expression of EGR1. In contrast to the study by Suda et al. [89], here, we report that Lina, but not EX, decreased the levels of both P-ERK 1/2 and EGR1 in cells exposed to hypoxia–reoxygenation.

P38 has a central role in cell signaling [75]. Direct physical interaction and cross-talk between p38 and ERK 1/2 were described [115]. Here, we found that EX did not alter p38 phosphorylation in vitro, confirming the results of Kyhl et al. that liraglutide did not attenuate p38 phosphorylation after permanent coronary artery occlusion in non-diabetic rats [53]. It was even reported that Exendin-4 increases p38 phosphorylation in the non-diabetic mouse heart following permanent coronary ligation [28]. On the other hand, previous study also reported that DPP4 inhibitors attenuate p38 phosphorylation. For example, Lina decreased levels of P-p38 in the hearts of mice fed Western Diet [6]. Anagliptin attenuated the increase in P-p38 levels in cells exposed to lipopolysaccharides [87]. Sitagliptin attenuated p38 phosphorylation in the liver of rats exposed to porcine serum [46] and in atherosclerotic plaques of apolipoprotein-E knockout mice [113].

While we found that Lina decreased p38 phosphorylation and p38 inhibition by SB replicated most of the favorable effects of Lina in vitro, other have shown that inhibiting p38 with SB blocked the myocardial protective effects of ischemic preconditioning in pigs using an in vivo model [80, 81]. The reason for the differences between the models is unclear.

Interestingly, SB, a p38 inhibitors, replicated the effects of Lina in our model. It attenuated the increase in EGR1, P-ERK 1/2, TLR2, and TLR4 in cardiofibroblasts exposed to hypoxia–reoxygenation. This suggests that p38 is upstream of ERK 1/2, EGR1, TLR2, and TLR4. Previous studies have shown that both ERK 1/2 and p38 lead to upregulation of

TLR4 expression in murine immature dendritic cells exposed to lipopolysaccharide [5]. Vildagliptin attenuated lipopolysaccharide-induced increased expression of TLR4 and TLR2 in macrophages in vitro [55]. The increased expression of TLR4 in cardiofibroblasts exposed to lipopolysaccharide is dependent on p38 [15]. Zhou et al. reported that p38 and ERK 1/2 affect let-7i and let-7e microRNA expression that affects TLR4 expression in epithelial cells [117]. Searching the literature for potential microRNAs that could affect TLR4 expression, we found that the expression of TRL4 is controlled by Let7i [9, 79, 117], miR-21 [104], miR27a [91], miR-145 [44], and miR-146b [92]. Therefore, we assessed whether hypoxia–reoxygenation changes the expression of these microRNAs, and whether Lina, Ex, GLP-1, and SB can affect the changes induced by hypoxia–reoxygenation in human cardiofibroblasts. We found that Lina increases the expression of Let-7i and miR-146b with downstream suppression of TLR4 expression. The effect of Lina on miR-146b is p38 dependent, whereas the effect of Lina on Let-7i is mostly p38 independent.

Yet, others reported that TLR4 activation leads to p38 phosphorylation, putting TLR4 upstream, rather than downstream of p38 activation [25, 97]. A cross-talk between p38 and TLR4 is possible [35]. TLR4 activation by myeloid differentiation 2 (MD2) can activates inflammatory cascade [37].

Both EX and Lina attenuated the hypoxia–reoxygenation-induced increase in caspase-3 activity and apoptosis. The protective effects were augmented when Lina was combined with GLP-1. This suggests that in vivo, the effects of DPP4 inhibition are mediated by both GLP-1-dependent and GLP-1-independent signaling pathways. Yet, only Lina, but not EX, attenuated EGR1, P-ERK 1/2, P-p38, TLR2, and TLR4 levels. Moreover, GLP-1 added to Lina did not augment these changes, suggesting that this pathway is GLP-1 independent.

Thus, our in vitro experiments suggest that Lina leads to suppression of the inflammasome by downregulation of TLR2 and TLR4 expression via changes in microRNA expression. Some of the effects leading to TLR4 downregulation are p38 dependent (miR-146b) and some are only partially p38 dependent (Let-7i). Changes in the levels of non-coding RNAs, including microRNAs, have been reported in various animal models of diabetes [71]. A schematic representation of the signaling pathways involved is presented in Fig. 10.

There is a concern that numerous successful interventions in animal models of cardioprotection do not translate

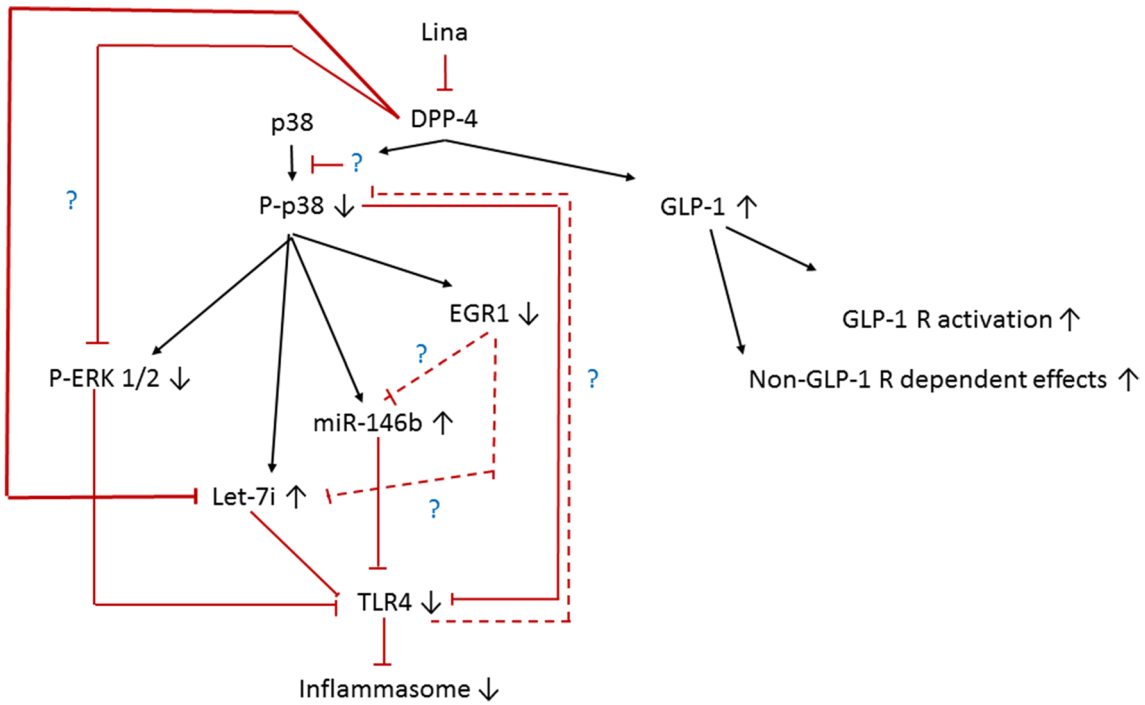


Fig. 10 Schematic presentation of the signaling pathway. Lina inhibits its DPP-4. This leads to: 1. Increased levels of the incretins, including GLP-1, leading to GLP-1 receptor (GLP-1 R) activation and to non-GLP-1 R mediated effects. 2. Decreased p38 phosphorylation. 3. Decreased ERK 1/2 phosphorylation (it is unclear if this is a direct effect of DPP-4 inhibition or dependent on p38). Levels of P-p38 decrease, levels of P-EK 1/2 and EGR1 decrease and levels of miR-146b and Let-7i increase. It seems that DPP4 inhibition partially increases Let-7i in a pathway that is independent of p38 inhibition. Let-7i and miR-146b suppresses the expression of TLR4 and, thus, attenuate the activation of the inflammasome

to meaningful benefit in the clinical setting [38]. Here, we followed recent recommendation by Heusch and included both wild-type as well as db/db mice with T2D. Moreover, we conducted both short- and long-term experiments and studied some of the pathways in an in vitro model [38]. It is unclear whether all DPP4 inhibitors share these GLP-1-independent effects and are capable to exert protective effects in vitro. For example, sitagliptin alone without added GLP-1 did not decreased cell death in mouse cardiomyocytes exposed to hypoxia–reoxygenation protocol [107]; however, it was reported to attenuate inflammation in H9c2 rat ventricular myoblast cells exposed to lipopolysaccharide [57]. Diprotin A, a DPP4 inhibitor, did not improve viability of cardiomyocytes exposed to H_2O_2 [52]. On the other hand, alogliptin inhibits IL-6 expression by mononuclear cells exposed in vitro to lipopolysaccharide [90]. Vildagiptin attenuated the inflammatory response of RAW264.7 macrophages exposed to lipopolysaccharide in vitro [55]. Lina suppressed profibrosis signals in isolated mouse cardiofibroblasts exposed to high glucose or angiotensin II [96] or to aldosterone [6]. Moreover, Lina, but not sitagliptin or vildagliptin reduced vascular superoxide production and improved relaxation of rat mesenteric artery rings exposed in vitro to high glucose [76].

It is also unclear whether these GLP-1 independent effects occur in the clinical setting in patients with T2D with the current recommended doses of the drugs; and if they occur, do they have significant impacts on clinical outcomes. So far, the results of four clinical long-term multicenter randomized trials with different DPP4 inhibitor agents, including saxagliptin [82], alogliptin [101], sitagliptin [33], and Lina [74], did not show reduction in cardiovascular events in patients with T2D. Yet, the results of the CARdiovascular Outcome Trial of LINAgliptin Versus Glimepiride in Type 2 Diabetes (CAROLINA) trial have not been published yet [63].

Further studies are needed to explore the effects of the different DPP4 inhibitors on EGR1, ERK 1/2, p38, and microRNA miR-146b, and Let-7 expression and activity and their downstream effects on TLR2 and TLR4 expression, the activation of the inflammasome, and their potential effects on inflammation and cardiovascular outcomes. Potential interactions between DPP4 inhibitors and other agents that are commonly used in diabetic patients (other anti-diabetic drugs, angiotensin converting enzyme inhibitors, statins, aspirin, etc.), effects of drug concentrations, etc. should be further explored.

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Compliance with ethical standards

Conflict of interest Dr. Ye received research grants from Astra Zeneca and Boehringer Ingelheim. Dr. Bajaj received research grants from AstraZeneca, Boehringer Ingelheim, Eli-Lilly, Sanofi Aventis, and Novo Nordisk. Dr. Birnbaum receives research grants from Astra Zeneca. Dr. Tran has no conflict of interest.

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