

ARTICLE

Effect of DPP-4 inhibitor sitagliptin against ischemia-reperfusion (I/R) injury in hyperlipidemic animals

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ABSTRACT Hyperlipidemia is a major risk factor associated with increased risk of myocardial infarction. Dipeptidyl peptidase-4 (DPP-4) inhibitors such as sitagliptin are a class of oral anti-diabetic drugs with secondary pleiotropic effects on metabolic and cardiovascular parameters. This study aimed to determine the possible cardioprotective effects of sitagliptin on ischemia-reperfusion (I/R) injury in animals kept on high-fat diet. Male Wistar rats were fed with high-fat diet (HF) for 12 weeks, to induce hyperlipidemia. During the last two weeks of the feeding period, animals were orally treated with different doses of sitagliptin (Sitg: 25, 50, 100, and 150 mg/kg/day), or saline as a control. Heart tissues were then isolated and subjected to two different I/R-injury protocols for infarct size (IS) measurement and biochemical analysis. To test the role of NOS enzyme, NOS inhibitor (L-NAME) was injected intraperitoneally for IS evaluation. As an effective dose, Sitg (50 mg) exhibited a significant impact on IS. NOS activity increased significantly in the Sitg (50 mg) treated groups; however this protective effect was abolished in the presence of L-NAME. The protective effect of Sitg that was mediated by TRP channels in our previous study on normolipidemic animals was abrogated in animals fed with high-fat diet.

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INTRODUCTION

Hyperlipidemia is considered a major risk factor for ischemic heart disease (Kupai et al. 2009) and myocardial infarction (MI) (Wu et al. 2013), due to its contribution in atherosclerotic plaque formation in coronary vessels, accompanied by increase in infarct size (IS) and cardiac apoptotic and necrotic death (Ma et al. 2013).

Experimental studies mostly addressed cardioprotective effects against ischemia-reperfusion (I/R) injury in healthy animal models, in which I/R is imposed in the absence of other diseases or risk factors such as hyperlipidemia (Kupai et al. 2009). However, studies using diseased animal models with high-fat and high-cholesterol diets showed a high susceptibility of rodent hearts to I/R injury and diminished cardioprotective outcomes (Lauzier et al. 2009). As the incidence of cardiovascular disorders with an increased risk of myocardial infarction continues to grow due to dyslipidemia and obesity, and finding new and novel therapeutic targets is still a challenge.

Most of the drugs failed to find their way into clinical practice, and no drug has been approved for infarct

size limitation in patients with acute coronary syndrome (Downey and Cohen 2009). Additionally, commonly used anti-diabetic drugs against diabetes including dipeptidyl peptidase-4 (DPP-4) inhibitors, were found to be independently associated with pleiotropic effects in cardiovascular diseases (Nissen and Wolski 2007). DPP-4 inhibitors (DPP-4i) are incretin-based drugs that act by inhibiting the DPP-4 enzyme, resulting in prolonged action of glucagon-like peptide-1 (GLP-1) (Apaijai et al. 2013). In addition to their glycemic control effect, DPP-4i has been reported to exert cardioprotective actions in high-fat diet (HFD) animal models (Apaijai et al. 2013). To the best of our knowledge, only one study addressed the effect of sitagliptin on cardiovascular complications after long-term of HFD consumption (Apaijai et al. 2013), without addressing its effect against I/R injury. Consequently, the ability of these therapies to reduce the deleterious effects of high-fat diet on the cardiovascular system are still equivocal (Torekov et al. 2011).

Endothelial nitric oxide synthase (e-NOS) is constitutively expressed in cardiomyocytes, while hypercholesterolemia was found to be associated with impaired endothelial function in coronary circulation (Kuo et

al. 1992), lower nitric oxide (NO) production (White et al. 1994). Moreover, reduced e-NOS expression was observed in heart tissues of hypercholesterolemic rabbit model (Onody et al. 2003). The correlation between DPP-4 inhibitors and nitric oxide synthase (NOS) system was depicted in few studies; however, the potential role of NOS remains unclear in ischemic heart. Transient receptor potential (TRP) channels including the Canonical (TRPC) and Vanilloid (TRPV) subfamilies are non-selective calcium (Ca^{2+}) permeable ion channels (Nilius and Droogmans 2001) vastly expressed in sensory nerve fibers that innervates the heart, blood vessels and vascular endothelial cells (Shenton and Pyner 2014). These channels became the subject of growing research interest over the last decades, due to their disparate effects in cardiovascular complications, but their protective effects against myocardial I/R injury were scarcely reported (Vemula et al. 2014).

Whether sitagliptin-induced cardioprotection against I/R injury can be mediated by NOS and/or TRP channels in hyperlipidemia is still unknown. Therefore, in this study we aimed to elucidate the possible protective mechanisms of sitagliptin against I/R injury and myocardial infarct size (IS) in a hyperlipidemic rat model, in comparison with the results from normolipidemic animals in our previous study (Al-Awar et al. 2018).

We have previously tested the hypothesis in normolipidemic animals (Al-Awar et al. 2018), and we found that NOS and transient receptor potential (TRP) channels are potent mediators in sitagliptin-induced cardioprotection.

MATERIALS AND METHODS

Drug preparations

Sitagliptin filmtablets (Januvia 100 mg, Merck, Hertfordshire, UK) were purchased and dissolved in physiological saline solution (0.9%) before each oral treatment and according to different intended doses. Tiopental (Tiobarbital Braun, 0.5 g, B. Braun Medical USA) was dissolved in saline (0.9%) and used as anesthetic agent. NOS-inhibitor ($\text{N}\omega$ -nitro-L-arginine methyl ester hydrochloride; L-NAME), purchased from Sigma-Aldrich and dissolved in physiological saline (0.9%).

Animals and experimental design

Our study falls within the standards of the European Community guidelines for the Care and Use of Laboratory Animals. All procedures were performed according to the protocols approved by the Institutional Ethical Animal Care and Use Committee of University of Szeged, with the project identification code and date of approval (XX/4801/2015, 15 December 2015). Six to eight-week-

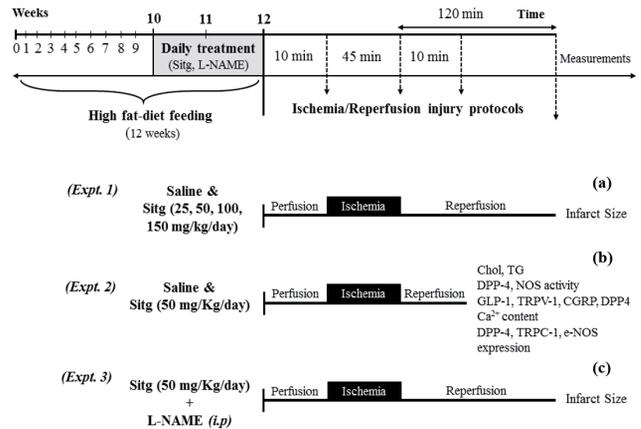


Figure 1. Summary of 3 different experimental protocols. (a) Heart tissues subjected to 45 min ischemia and 120 min of reperfusion, after two weeks of oral treatment with saline and different doses of Sitg, for infarct size measurements. (b) Hearts subjected to 45 min ischemia and 10 min brief reperfusion for biochemical measurements. (c) Infarct size measurement from heart tissues exposed to prolonged ischemia-reperfusion injury, after 2-weeks co-treatment with saline or Sitg (50 mg), and intraperitoneal injection of NOS-inhibitor (L-NAME).

old male Wistar rats (body weight 200-300 g; Toxi-Coop, Dunakeszi, Hungary) were obtained and acclimatized for one week before the onset of treatments. All animals were housed in temperature-controlled animal facility (23 °C) that belongs to our lab and maintained with a 12h-12 h light-dark cycle with food and water provided *ad libitum*. Animals were fed with standard rat chow mixed with fats (High fat = HF) for 12 weeks to induce hyperlipidemia.

Animals were assigned into 3 different experiments:

Experiment 1. To determine the effective dose (kg^{-1}day) of sitagliptin (Sitg), animals were randomly divided into different groups: (Control (saline), Sitg (25 mg), Sitg (50 mg), Sitg (100 mg) and Sitg (150 mg)/kg/day; $n = 5-10$). Daily oral treatment with the different doses of Sitg or its vehicle (Saline) started at the last two weeks of the high fat diet (2.5%) feeding. At the end of the treatment, the whole-heart preparation and ischemia-reperfusion (I/R) injury protocol was performed. Rats were anesthetized with thiopental (i.p. 100 mg/kg), heart tissues were rapidly excised, placed in ice-cold saline (0.9%), mounted and ligated through the aorta into the cannula (*ex vivo*) of a modified Langendorff Apparatus, and perfused with Krebs buffer (118 mM NaCl, 4.70 mM KCl, 2.50 mM CaCl_2 , 1.18 mM MgSO_4 , 1.18 mM KH_2PO_4 , 5.50 mM glucose and 25 mM NaHCO_3) than gassed with 95% O_2 and 5% CO at 37 °C. Hearts were exposed to 10 min perfusion, 45 min prolonged regional ischemia by the occlusion of the left anterior descending (LAD) coronary artery, and 120 min reperfusion. At the end of reperfusion, the LAD

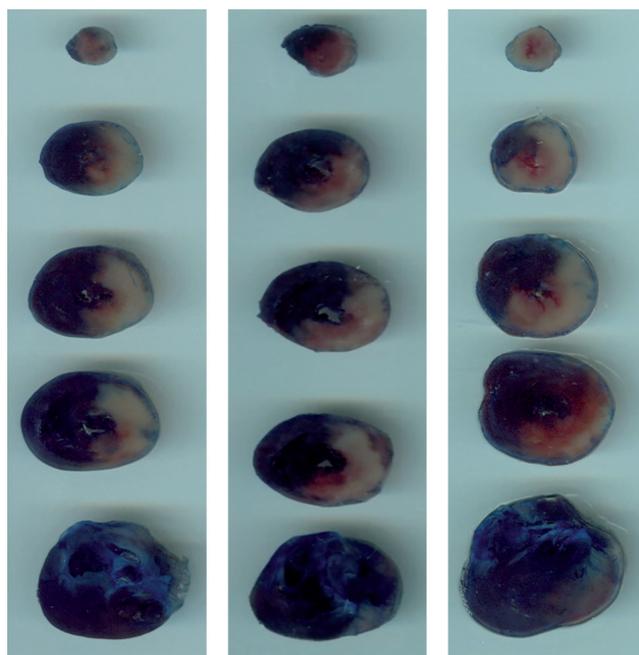


Figure 2. Representative photographs of transversely-sectioned, Evans-blue perfused and TTC-stained heart tissues outlining the area at risk (AAR; sum of white and red area); blue, healthy viable tissue; and pale white, infarcted tissue. Myocardial infarct area (IS; white) was measured post-myocardial ischemia-reperfusion and TTC staining, in treated and control groups.

coronary artery was religated, and the area at risk (AAR) was stained with Evans blue dye via the aortic root. Hearts were weighed and stored at -20°C for further staining with triphenyltetrazolium chloride (TTC). In the clinical setting, the importance lies in the dose- protective effect of any drug; therefore, we considered the 50 mg dose of sitagliptin as the effective dose for biochemical measurements (Fig. 1a).

Experiment 2. For *in vitro* laboratory measurements, another set of experiments was carried out, by assigning only two animal groups (Control (Saline) and Sitg (50 mg), $n = 10$). At the end of the treatment, same anesthetization procedure and whole-heart preparation process were carried out as in *Experiment 1*. Cannulated hearts were exposed to 10 min perfusion, 45 min prolonged regional ischemia by occluding the LAD coronary artery, followed by 10 min brief reperfusion. At the end of the experiment, heart tissues were weighed, clamped and stored at -80°C for further biochemical analyses (Fig. 1b).

Experiment 3. To confirm the involvement of NOS in Sitg-mediated (50 mg) cardioprotection against IR injury, four different animal groups (Control (Saline), Sitg (50 mg), Control (Saline) + L-NAME, and Sitg (50 mg) + L-NAME, $n = 6-8$) were included. The Control (Saline) and Sitg (50 mg) animal groups received the same daily oral

treatment as in *Experiment 1*, while the other two groups were co-treated intraperitoneally (i.p.) with the NOS inhibitor (L-NAME, 25 mg/kg/day) (Jaarin et al. 2015), three hours' post-oral administration of Sitg (50 mg) and its vehicle (saline). At the end of the treatment, the same anesthetization procedure, whole-heart preparation process, and I/R injury protocol (10 min perfusion, 45 min prolonged regional ischemia and 120 min reperfusion, *ex vivo*), coronary artery re-ligation, and tissue staining procedure were carried out as in *Experiment 1* (Fig. 1c).

Tissue staining and infarct size (IS) measurements

At the end of each prolonged reperfusion phase (120 min), the LAD coronary artery was re-ligated, and the risk zone was stained with Evans blue dye via the aortic root. Hearts were frozen, transversely sectioned (5-6 slices, 2-mm thickness) from the apex to the base, and incubated in 1% TTC for 10 min at 37°C . After incubation, tissue sections were fixed for 10 min in 10% formalin and placed for 30 min in phosphate buffer (pH 7.4). All sections were mounted on glass slides, images were captured with a digital camera, and infarcted areas were analyzed using an ImageJ 1.34 software. The infarction was analyzed in each section by an investigator who was blinded to the identity of the sections (Fig. 2).

Serum cholesterol and triglyceride measurements

After removal of heart tissues, blood samples were taken from the abdominal aorta, centrifuged, and serum samples were collected in Eppendorf tubes and stored at -20°C for cholesterol (Chol) and triglyceride (TG) measurements. Chol and TG reagent kits (Diagnosticum, Hungary) were used for both measurements. Quantitative determination of cholesterol and triglyceride concentration in serum was based on enzymatic colorimetric method (PAP). Standard and sample (10 μl) measurements at wavelength 490-550 nm were carried out in 96-well plates, after 5 min incubation at 37°C and according to the protocols provided in the kit's manual. Results of both measurements were expressed in (mmol/l).

Hepatic cholesterol and triglyceride measurements

At the end of the treatment and after the animals were anesthetized, liver tissues were harvested, rapidly clamped in liquid nitrogen and stored at -80°C . Measured samples from liver were homogenized in ice-cold modified phosphate buffer saline (PBS) by Ultra-Turrax T25 (13 500 rpm). Liver supernatants were collected, and the same Chol- and TG-kits (Diagnosticum, Hungary) were used with some modifications in the provided protocols regarding dilutions and sample volume. Obtained results were expressed in mmol/l.

DPP-4 activity test

Cardiac DPP-4 activity was measured in Control (Saline) and Sitg (50 mg) treated groups, using a DPP-4 activity assay kit and according to the manufacturer's guidelines (Sigma-Aldrich, St. Louis, USA). Ten mg of heart tissue were homogenized in ice-cold DPP-4 Assay Buffer, centrifuged at 13 000 g for 10 min (4 °C), after which supernatants were collected. Standard and sample fluorescence intensity (FLU) measurements ($\lambda_{ex} = 360 \text{ nm} / \lambda_{em} = 460 \text{ nm}$) were carried out after 5-min incubation at (37 °C) in 96-well black plates designed for fluorescence assays, using a fluorescence multi-well plate reader. Incubation and measuring cycles were repeated until the most active sample was near to or greater than the value of the highest standard (100 pmol/well). Results were expressed as microunit/ml.

Nitric oxide synthase (NOS) activity

NOS activity was measured by quantifying the conversion of [^{14}C]-labeled L-arginine to citrulline by a previously described method with some minor modifications (Boughton-Smith et al. 1993). Heart tissues were homogenized with Ultra-Turrax T25 (13 500 rpm; twice for 30 sec) in ice-cold buffer containing 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 32 mM sucrose, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 10 $\mu\text{g/ml}$ soybean trypsin inhibitor, 10 $\mu\text{g/ml}$ leupeptin, and 2 $\mu\text{g/ml}$ aprotinin, at pH 7.4 (all chemicals were from Sigma-Aldrich, Budapest, Hungary). Supernatants were collected after centrifugation (20 000 g, 30 min, 4 °C). Samples (40 μl) were incubated for 10 min at 37 °C with 100 μl of assay buffer (50 mM KH_2PO_4 , 1.0 mM MgCl_2 , 50 mM L-valine, 0.2 mM CaCl_2 , 1.0 mM dithiothreitol (DTT), 1.0 mM L-citrulline, 15.5 nM L-arginine, 30 μM flavin adenine dinucleotide, 30 μM flavin mononucleotide, 30 μM tetrahydro-L-biopterin dihydrochloride, 450 μM β -nicotinamide adenine dinucleotide phosphate (β -NADPH), and 12 pM [^{14}C]-L-arginine monohydrochloride (all chemicals were from Sigma-Aldrich). Reactions were terminated by the addition of 0.5 ml of a 1:1 (v/v) suspension of ice-cold DOWEX (Dowex 50WX8 hydrogen form 100-200 mesh, Sigma Aldrich) prepared in distilled water. The mixture was resuspended by adding 850 μl of ice-cold distilled water, and 970 μl of supernatant was removed for determination of radioactivity by scintillation counting. Ca^{2+} dependence of NOS activity was determined by adding 10 μl of ethylene glycol-bis (β -aminoethyl ether) tetraacetic acid (EGTA; 1 mM, Sigma-Aldrich). NOS activity was confirmed by inhibition with 10 μl of N ω -nitro-L-arginine methyl ester (L-NNA; 3.7 mM, Sigma-Aldrich). The level of inducible NOS (i-NOS) was defined as the extent of citrulline formation that

was inhibited by L-NNA, but not by EGTA. Constitutive NOS (cNOS) activity was calculated from the difference between the extent of citrulline formation inhibited by EGTA and the total activity. As the nature of the cNOS isoform (e-NOS or n-NOS) was not determined, this activity is referred to as cNOS. NOS activity was expressed as pmol/min/mg protein.

ELISA measurements (GLP-1, TRPV-1 and CGRP)

Double-antibody sandwich ELISA kits for rat GLP-1, TRPV-1 and CGRP measurements were purchased from SunRed Biotechnology (Shanghai, China). The same homogenization procedure (Ultra-Turrax T8, 20 min centrifugation at 2000-3000 rpm) was followed, using the PBS homogenization buffer (pH 7.2-7.4). Tissue sample preparation procedure was done on ice. The three parameters were measured according to the manufacturer's protocols, and optical densities (OD) were determined at 450 nm wavelength. Results are expressed in ng/ml for GLP-1 and TRPV-1 and ng/mg protein for CGRP.

Calcium (Ca^{2+}) content test

A colorimetric Calcium Detection Assay Kit (Abcam, Cambridge, UK) was used to determine the calcium (Ca^{2+}) concentration. Samples were homogenized on ice using PBS + 0.1% NP-40 and centrifuged at a maximum speed (15 300 rpm) for 2-5 min at 4 °C. Measurements from supernatants were performed according to the provided procedure. OD were measured ($\lambda = 575 \text{ nm}$) and results were expressed in ng/mg protein.

CD26 (DPP-4), TRPC-1 and e-NOS protein expression by Western blotting normalized to β -actin

Measured heart tissues were homogenized by Ultra-Turrax T25 (13 500/s; twice for 30 sec) with ice-cold radio immunoprecipitation assay (RIPA) buffer (containing a protease inhibitor and TRITON-X-100), for CD26 (DPP-4) and TRPC-1 proteins, and Homo-buffer (containing phosphatase inhibitor, vanadate (1:50)), for e-NOS. Homogenates were centrifuged (12 000 rpm, 10-15 min, 4 °C). Proteins were separated by an 8% and 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE, 1 mm gel cassette), and transferred into nitrocellulose membranes. Blots were probed overnight (4 °C, and 1% milk) with anti-TRPC-1 rabbit primary antibody (1:500, ab192031, Abcam), and anti-eNOS mouse primary antibody (1:250, ab 76198, Abcam), respectively, 2 hours at room temperature with anti-CD26 rabbit primary antibody (1:500, ab129060, Abcam), and anti-beta actin mouse primary antibody (1% BSA, 1:4000, ab 8226, Abcam). Membranes were then incubated for 1 h at room temperature with secondary anti-rabbit antibody (1:1000, sc-2370, Santa Cruz, TX, USA), secondary anti-mouse an-

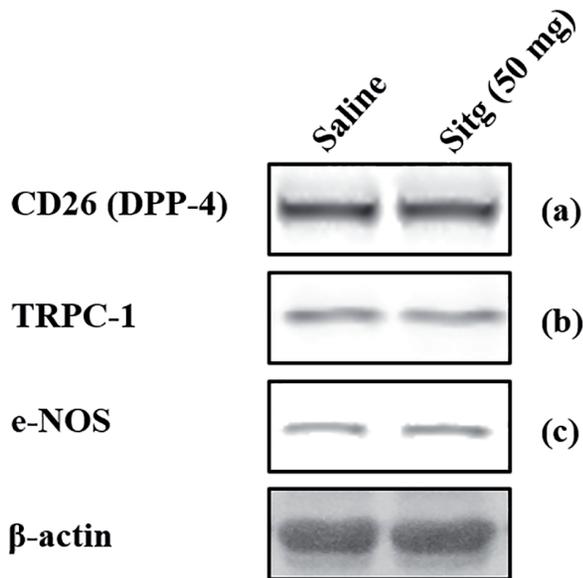


Figure 3. Protein expression of CD26 (DPP-4), TRPC-1 and e-NOS (a, b and c, respectively) in heart tissues from Sitg-treated (50 mg) group, compared to the Control (saline) group.

tibody (1:5000, A9044, Santa Cruz), secondary anti-rabbit (1:5000, sc-2370, Santa Cruz), and secondary anti-mouse antibody (1:2000, A9044, Santa Cruz) for TRPC-1, e-NOS, CD26 and β -actin, respectively. Secondary antibodies were conjugated with horseradish peroxidase (HRP) enzyme. Signals were developed using an enhanced chemiluminescent substrate for detection of HRP (ECL Western Blotting Substrate, Thermo Scientific, Rockford, USA) and exposed to Hyperfilm. Films and protein band densities were analyzed using the Image Quant Software (Amersham Pharmacia, Buckinghamshire, UK) after scanning with Gel Analyst 3.01 Software (Iconix, Toronto, Canada), and were normalized to housekeeping protein β -actin (Fig. 3).

Protein determination

Aliquots (20 μ l) from diluted samples (15- or 25-fold with distilled water) were mixed with 980 μ l of distilled water and 200 μ l of Bradford reagent was added to each sample. After mixing and 10-min of incubation, samples were assayed spectrophotometrically in a microplate reader at 595 nm with a commercial protein assay kit (Bio-Rad, Budapest, Hungary). Protein levels were expressed as mg protein/ml.

Statistical analysis

All data are shown as Mean \pm standard error of mean (SEM). Statistical comparisons were performed with Student's two-tailed unpaired t test, one-way ANOVA

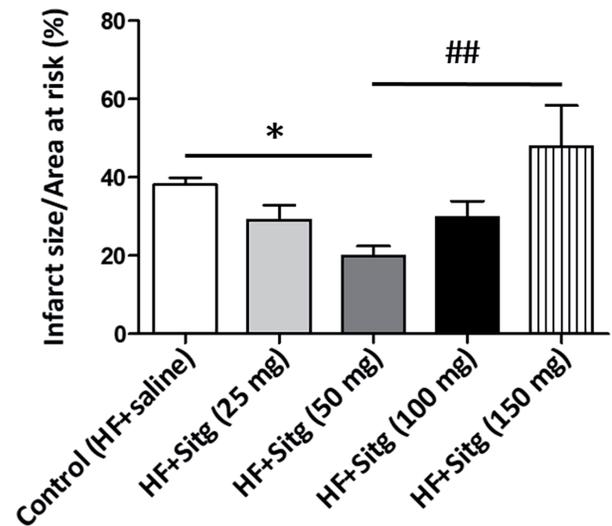


Figure 4. Effect of different doses of Sitg on infarct size (expressed in %). Results are shown as Mean \pm SEM; n = 5-10 animals/group. Statistical significance is represented as: *P < 0.05 compared to the Control group, and ##P < 0.01 comparing HF + Sitg (50 mg) and HF + Sitg (150 mg) groups together. Sitg (50 mg) exhibited a significant decrease in infarct size, while no significance was observed in other doses (25, 100, and 150 mg).

multiple comparison test (Bonferroni), and two-way ANOVA, when necessary. Differences were considered significant when P < 0.05.

RESULTS

DPP-4i decreased infarct size (IS) in heart tissues of Sitg (50 mg) group

Two weeks following the daily oral administration of different doses of the same DPP-4 inhibitor (Sitg), Sitg treatment (50 mg/kg) resulted in a significant decrease in IS (19.99 \pm 2.44%) compared to the control group (38.11 \pm 1.82%), after 45 min of regional ischemia and 120 min of reperfusion. The area of infarction is expressed as the percentage of infarct size over the area at risk (Fig. 4).

Serum cholesterol and triglycerides concentration

Cholesterol measurements from serum samples revealed significant increase in high-fat diet animals (HF + saline; 2.72 \pm 0.15 mmol/l) compared to normal control animals (Saline; 1.95 \pm 0.17 mmol/l). Sitagliptin administration (HF + Sitg (50 mg)) failed to reduce cholesterol levels (2.75 \pm 0.10 mmol/l) when compared to control animal group kept on high-fat diet (HF + saline; 2.72 \pm 0.15 mmol/l). Results are shown in Fig. 5a.

Similarly, serum triglyceride level was significantly increased in HF + saline vs. Control (saline); 2.12 \pm 0.13

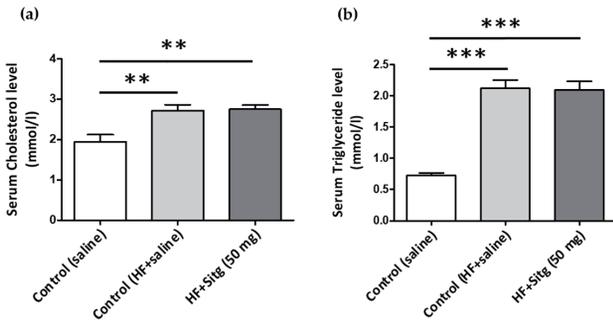


Figure 5. Serum cholesterol (a) and triglyceride (b) levels (expressed in mmol/l) in control animals kept on normal or high-fat diet, and high-fat diet animals treated with sitagliptin. Data are presented as Mean \pm SEM; n = 5-8 animals/group. One-way ANOVA multiple comparison test (Bonferroni) was used for statistical analysis, and results were considered significant when *P < 0.05.

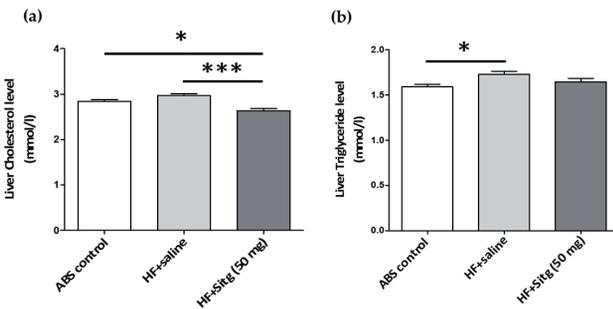


Figure 6. Liver cholesterol (a) and triglyceride (b) levels expressed in mmol/l. Results are considered significant when *P < 0.05. Data are presented as Mean \pm SEM (n = 4-5 animals/group).

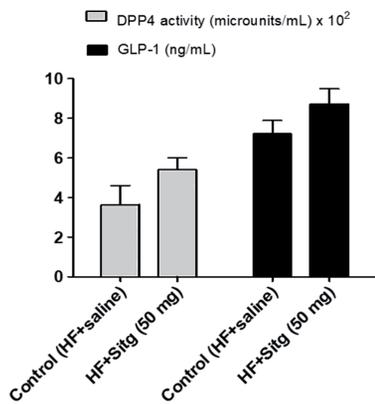


Figure 7. Ischemic cardiac tissue GLP-1 level and DPP-4 activity after two weeks oral treatment with Sitg (50 mg). Results are expressed in ng/ml and microunits/ml $\times 10^2$, for GLP-1 and DPP-4 activity, respectively. Presented data are presented as Mean \pm SEM (n = 4-10 animals/group).

vs. 0.72 ± 0.04 mmol/l. In addition, no significant decrease was observed in triglyceride level in sitagliptin-treated group kept on high-fat diet (HF + Sitg (50 mg); 2.09 ± 0.14 mmol/l) (Fig. 5b).

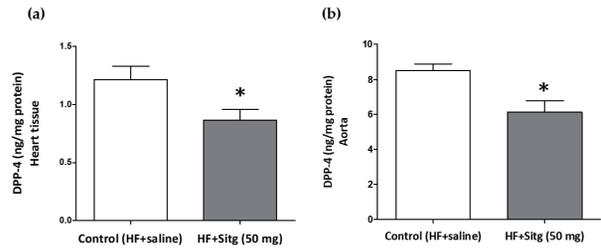


Figure 8. Changes in DPP-4 level (expressed in ng/mg protein) in heart tissues (a) and aorta (b) of Sitg (50 mg) treated animal group (HF + Sitg (50 mg)), compared to Control (HF + saline). Data are presented as Mean \pm SEM (n = 5-7 animals/group). Statistical significance: *P < 0.05 compared to the control group.

Serum cholesterol and triglyceride concentration

Measurements from liver homogenates showed a significant decrease in cholesterol level in HF + Sitg (50 mg) group compared to both the HF + saline group (2.64 ± 0.03 vs. 2.94 ± 0.04 mmol/l), and the absolute (ABS) control group (2.64 ± 0.03 vs. 2.86 ± 0.04), respectively. However, no change in cholesterol profile was reported when comparing the HF + saline group (2.924 ± 0.044 mmol/l) to the ABS control group (2.982 ± 0.146 mmol/l) (Fig. 6a). On the contrary, liver triglyceride exhibited a significant increase in animals kept on high-fat diet (HF + saline; 1.73 ± 0.03 mmol/l), compared to the absolute control group (ABS control; 1.59 ± 0.03 mmol/l). On the other hand, no significant difference in hepatic triglyceride was observed in high-fat diet group treated with sitagliptin (HF + Sitg (50 mg)) compared to high-fat diet control group (HF + saline), (1.65 ± 0.04 mmol/l vs. 1.73 ± 0.03 mmol/l) (Fig. 6b).

Effect of Sitg on heart tissue DPP-4 activity and GLP-1 level

Measurements from heart tissues subjected to brief reperfusion (10 min) showed no significant change in GLP-1 levels (8.72 ± 0.76 ng/ml vs. 7.21 ± 0.67 ng/ml) and DPP-4 activity (5.41 ± 0.95 microunits/ml $\times 10^2$ vs. $3.64 \pm 0.95 \times 10^2$), in sitagliptin-treated groups compared to Controls (Fig. 7).

Sitg (50 mg) normalized high DPP-4 level in heart tissues and aortas of control group

Heart tissues (Fig. 8a) and aortas (Fig. 8b) exhibited a significant reduction in DPP-4 level (0.87 ± 0.09 vs. 1.22 ± 0.12 ng/mg protein) and (6.13 ± 0.55 vs. 8.52 ± 0.34 ng/mg protein), respectively, in Sitg (50 mg) group vs. Control (saline).

Effect of DPP-4i treatment on DPP-4 protein expression

No significant difference in DPP-4 (CD26) protein expression was noticed in HF + Sitg (50 mg) group (554.17

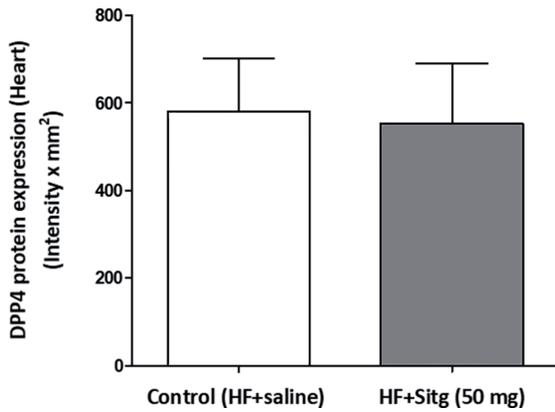


Figure 9. DPP-4 protein expression level (expressed in intensity x mm²) in heart tissues of HF + Sitg (50 mg) group (n = 6), compared to control group (HF + Saline; n = 5) group. Data are presented in term of Mean ± SEM.

± 136.91 intensity x mm²) vs. HF + Control (saline) group (581.03 ± 121.71 intensity x mm²), after normalization to β-actin (Fig. 3a and 9).

DPP-4i increased CGRP but not TRPV-1 levels

No marked change in heart TRPV-1 level was observed in Sitg-treated (50 mg) group (4.08 ± 0.28 ng/ml x 10) compared to the Control (5.23 ± 0.31 ng/ml x 10). CGRP level was augmented in Sitg (50 mg) group (16.65 ± 1.04 ng/mg protein) vs. Control group (10.93 ± 1.84 ng/mg protein) (Fig. 10).

Enhanced cardiac calcium (Ca²⁺) content in Sitg-treated (50 mg) group

To determine whether sitagliptin impacts calcium concentration of the ischemic myocardium in hyperlipidemic state, a colorimetric calcium detection assay was

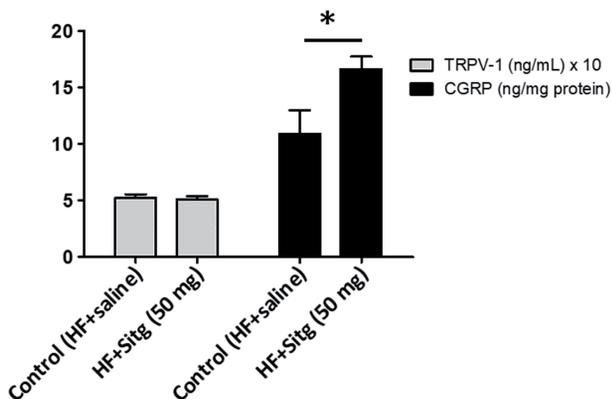


Figure 10. Effect of 50 mg dose of Sitg on cardiac TRPV-1 and CGRP levels, after two weeks oral treatment. Data are illustrated as Mean ± SEM; (n= 4-9 animals/group).

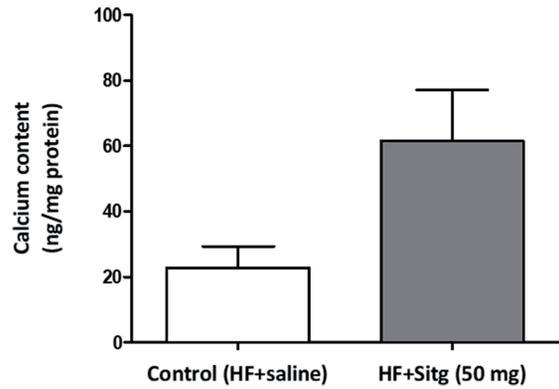


Figure 11. Changes in cardiac calcium content of the Sitg (50 mg) treated animals, compared to the control ones. Values are in terms of Mean ± SEM.

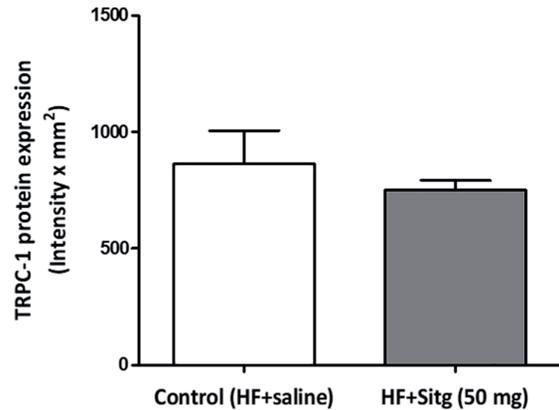


Figure 12. Unaffected TRPC-1 protein expression level (intensity x mm²) in heart tissues of HF + Sitg (50 mg) treated group (n = 8) vs. Control group (HF + saline; n = 8). Data are presented as Mean ± SEM.

used. Results showed non-significant change in calcium content in heart tissues subjected to drug therapy (61.52 ± 13.51 ng/mg protein) vs. Controls (22.79 ± 6.53 ng/mg protein) (Fig. 11).

TRPC-1 protein expression level

The difference in TRPC-1 protein expression level between the Control (HF + saline) and HF + Sitg (50 mg) treated groups is presented in Figures 12 and 3b. Unlike the results from normolipidemic animals (Al-Awar et al. 2018), Sitg-treated (50 mg) group exhibited a slight non-significant decrease (752.19 ± 40.11 intensity x mm²) in TRPC-1 expression in comparison with the Control group (862.77 ± 143.44 intensity x mm²).

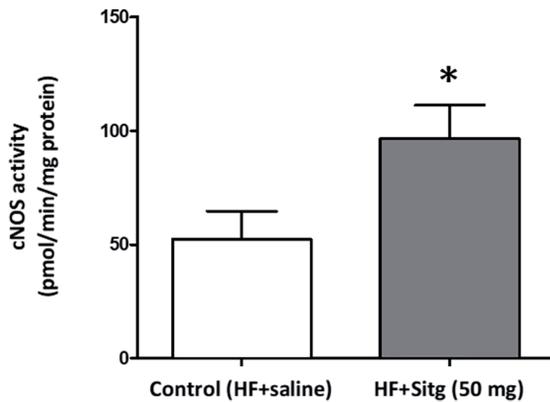


Figure 13. Significant increase (* $P < 0.05$) in constitutive nitric oxide synthase (cNOS) activity in heart tissues of HF + Sitg (50 mg) group ($n = 7$), compared to Control group (HF + saline; $n = 7$). Values are expressed in pmol/min/mg protein. Data are presented as Mean \pm SEM.

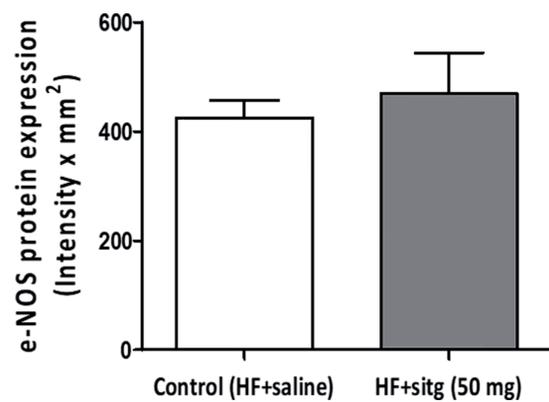


Figure 14. Unaffected level of e-NOS expression (expressed in Intensity x mm²) in heart tissues of HF + Sitg (50 mg) group ($n = 8$), compared to Control group (HF + saline; $n = 6$). Data are presented as Mean \pm SEM.

DPP4-i upregulated cNOS activity and e-NOS protein expression in heart tissues of Sitg (50 mg)

cNOS activity

Two weeks of daily treatment with Sitg (50 mg), followed by excision of heart tissues and brief reperfusion (45 min occlusion and 10 min reperfusion) of the coronary artery, lead to a significant increase in cNOS activity (96.51 ± 13.75 pmol/min/mg protein), compared to the Control group (52.38 ± 11.56 pmol/min/mg protein) (Fig. 13).

e-NOS protein expression

Expression of endothelial nitric oxide synthase (e-NOS) as determined by Western blot is shown in Figures 3c and 14. The level of expression was insignificantly changed in HF + Sitg (50 mg) treated animals (470.32 ± 73.79 intensity x mm²), compared to the Controls (425.26 ± 31.77 intensity x mm²).

Effect of L-NAME on NOS-mediated cardioprotection against infarct size

The implication of NOS in DPP-4 inhibition-induced cardioprotection was confirmed by treating the animals with NOS-inhibitor (L-NAME) and measuring the size of infarction. The current results are in line with the previous work in normolipidemic animals, showing a significant reduction ($18.47 \pm 1.22\%$) in Sitg (50 mg) group, compared to Control (HF + saline) group ($43.33 \pm 1.86\%$). However, this protective effect was lost in HF + Sitg (50 mg) + L-NAME group ($59.17 \pm 6.09\%$) when compared to the results obtained from animals treated with Sitg (50 mg) without L-NAME (HF + Sitg (50 mg); $18.47 \pm 1.22\%$) (Fig. 15).

DISCUSSION

DPP-4 inhibitors were extensively studied in healthy animal models as a remedy for cardiovascular disorders; however, the interventional mechanisms of the latter drugs were poorly addressed in diseased models, such as hyperlipidemia. This study was carried out with the aim of extending the results of previous work in normolipidemic animals (Al-Awar et al. 2018). In this HF rat model, the effect of hyperlipidemia on the development of myocardial infarction (MI) following a temporary coronary occlusion (ischemia-reperfusion injury) was studied. We hypothesized that hypercholesterolemia can be associated with increased infarct size, and sitagliptin can decrease its detrimental effect on the heart, clarifying mechanisms underlying this protection.

Treatment with Sitg (50 mg) showed a significant decrease in infarct size and increase in cNOS activity in comparison with the control group, while this infarct size-limiting effect was abolished after NOS-inhibition by L-NAME, similarly as in normolipidemic animals (Al-Awar et al. 2018). L-NAME is a non-selective NOS inhibitor that inhibits the 3 NOS isoforms: endothelial NOS (e-NOS), inducible NOS (i-NOS) and neuronal NOS (n-NOS). According to a previous study, NOS inhibition also blocked its protective effect against myocardial infarct size in high-fat diet-fed animal model (Ding et al. 2015).

Sitagliptin exhibited a lowering effect in liver cholesterol, but not in liver triglycerides. Moreover, no significant change was observed in serum Chol and TG levels in animal groups treated with Sitg (50 mg), compared to the controls. According to previous clinical studies, treatment with 50 mg dose of sitagliptin was effective in lowering lipid profile and glucose levels, and reducing DPP-4 activity by approximately 80% in patients with type 2 diabetes (Herman et al. 2005; Shigematsu et al. 2014)

In the contrary of obtained findings from normolipidemic animals (Al-Awar et al. 2018), the expected decrease in DPP-4 activity and increase in GLP-1 was not observed in high-fat diet condition. However, treatment with sitagliptin exhibited a significant decrease in DPP-4 protein level measured from heart tissues and aortas. Taking into consideration the dietary factor alone, a significant increase in GLP-1 level can be observed in hyperlipidemic (Control (HF + saline)) group compared to the normolipidemic (Control (Saline)) group, making high-fat diet a suspicious factor in blocking the protective effect of sitagliptin.

On the level of protein expression, results revealed no remarkable change in CD26, e-NOS and TRPC-1 protein levels, comparing the Sitg-treated groups to Control ones. On the other hand, a significant increase in e-NOS and TRPC-1 expression was observed in animals kept on normal diet and treated with sitagliptin (Al-Awar et al. 2018).

It was previously suggested that hypercholesterolemia can partially block the ion channels and its membrane receptor downstream signaling by reducing membrane fluidity, leading to cardiomyocyte dysfunction (Wu et al. 2017). This blockage might be also evident in the current study, because the protective effect of Sitg shown to be mediated by TRPV/TRPC upregulation in normolipidemic animals, was lost after a long-term consumption of high

fat-diet. Obtained results from CGRP measurement are in line with the results obtained from animals fed with standard diet (Al-Awar et al. 2018). This suggests that Sitg can have a direct effect on CGRP in hyperlipidemic condition, independently from TRP channels.

Based on the obtained results of infarct size analysis, we extended our experiments to study the mechanisms and biochemical markers underlying the Sitg (50 mg) dose. We studied the effect of sitagliptin in an *ex vivo* I/R injury model. However, investigating the same drug using an *in vivo* I/R injury model can support the protective effects and clinical relevance of sitagliptin.

Conclusions

Sitagliptin was effective in lowering the infarct size (IS) and upregulating nitric oxide synthase (NOS). However, its protective effect mediated by transient receptor potential (TRP) channels in normolipidemic animals was abolished under hyperlipidemic condition.

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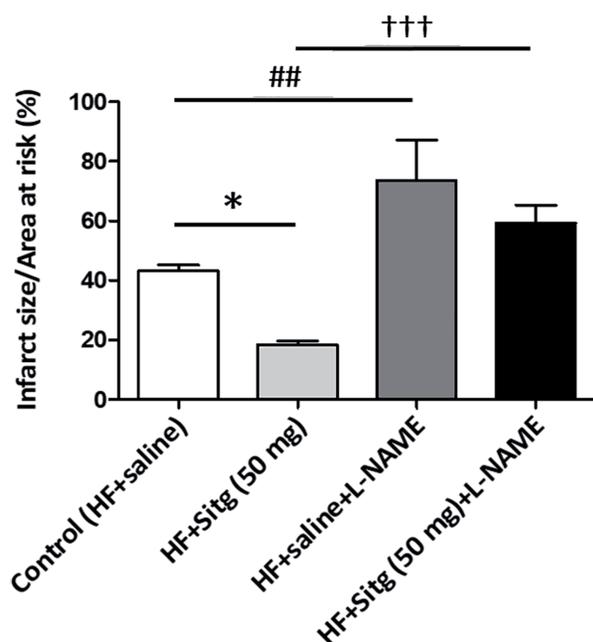


Figure 15. Loss of Sitg-induced (50 mg) cardioprotection mediated by NOS, translated by increase in infarct size (%), after intraperitoneal injection of NOS inhibitor (L-NAME). Statistical analysis was performed using One-way ANOVA, as well as two-way ANOVA when necessary. Data plotted as Mean \pm SEM.

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