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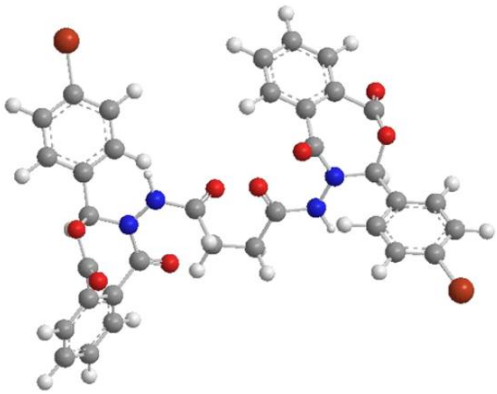

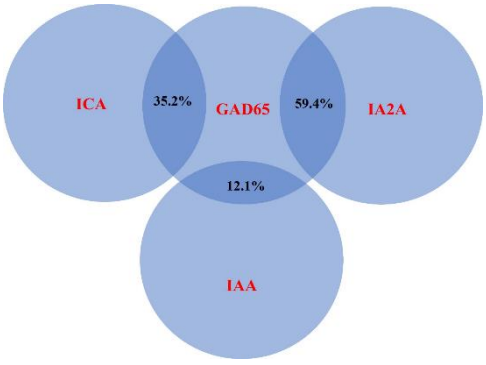

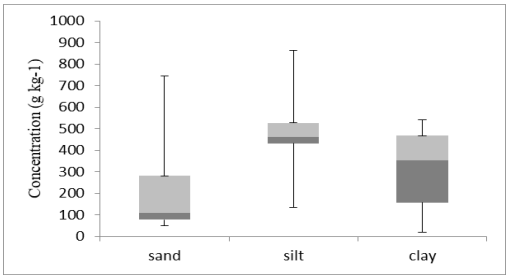

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The roles of NADPH oxidase and PKC pathways in the modulation of GLP-1 induced-vasorelaxation in diabetic rat aorta

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Abstract

A hormone of theincretin system known as glucagon-like peptide-1 (GLP-1) is important for a number of glucoregulatory functions. This study aimed to investigate the effect of inhibitors on the vasorelaxant response of GLP-1 in T1D rats. A single subcutaneous injection of 50 mg/kg of streptozotocin (STZ) was used to develop diabetes mellitus. Eighty aorticrings from twenty male albino rats were prepared. An Automatic Organ Bath was used. In our study, apocynin increased vascular response to GLP- 1 in the non-diabetic group with (Emax:72.40±0.350) and (pD2: - 9.923±0.444). Ininduced diabetes, the NADPH oxidase inhibitor decreased GLP-1 vasodilatation property with (Emax; 68.91±2.002) and (pD2: -9.480±0.138). The results show that bisindolylmaleimide IX (RO31-8220) has an increased effect on GLP-1 vasodilation in non-diabetic groups with (Emax:88.45±38.18) and (pD2: - 10.81±0.281). While in induced diabetic rat's vascular relaxation of GLP-1 decreased with (Emax: 77.73±2.801) and (pD2: - 10.28±0.203). The data analysis demonstrated that rotenone with (Emax: 63.69±35.10) (pD2: - 9.612±0.246), and in diabetic rats with (Emax:69.98±22.94) and (pD2: -9.612±0.246). In our study oxypurinol with (Emax: 82.16±16.10) and (pD2: - 9.434±0.443), and in diabetic rats with (Emax: 58.03±8.350) and (pD2: -9.612±0.246). We concluded that inhibitors could increase the vasorelaxant response of GLP-1 in non-diabetic rats, while this vasorelaxant response of GLP-1 diminished in diabetic-induced rats.

Introduction

Diabetes, which is expected to impact 693 million individuals globally by 2045, is one of the diseases with the greatest rate of growth [1]. The complex hormone glucagon-like peptide-1 (GLP-1) has a wide range of therapeuticapplications. GLP-1 has many metabolic effects, one of which is the stimulation of insulin secretion in a glucose-dependent manner [2]. It has been hypothesized that diabetes impairs endothelial function, causing both micro-and macrovascular problems, and that conduit and resistance arteries, including the aorta, are nonfunctional in diabetes [3]. Reactive oxygen speciesare often described as superoxide radicals (O2•−) hydrogen peroxide (H2O2), hydroxyl radicals (•OH), and singlet oxygen (1O2) [4]. The mitochondrial electron transport system, xanthine oxidase, cytochrome p450, NADPH oxidase, uncoupled NO synthase (NOS), and myeloperoxidase are some of the enzymes that produce reactive oxygen species (ROS) [5]. Inadult blood arteries, endothelium creates electrical impulses and releases vasodilators called EDRF (NO, PGI2, H2O2, and AA metabolites). EDRF is essential for managing vascular tone [6]. Endothelial nitric oxide (NO)generation and vasodilation may both be mediated by the phosphokinase C pathway. This route may also mediate the release of endothelium-derived constricting factors like endothelin-1 (ET-1) and induce vasoconstriction [7]. Potassium (K+) ion channel activity

controls cell membrane potential (MP) and plays a significant role in determining vascular tone. GLP-1 appears to impact a variety of physiological processes, including blood sugar and metabolic management as well as the direct involvement of multiple cardiovascular pathways in atherogenesis ***[8]***. This article describes the vasorelaxant response of GLP-1 focusing on the mechanism of NADPH oxidase, PKC pathways, NADH–ubiquinone oxidoreductase and xanthine oxidoreductase with the role of inhibitors in blocking these pathways.

Materials and methods

T1DM induction in rats

A single subcutaneous injection of 50 mg/kg of streptozotocin (STZ), dissolved in an ice-cold citrate buffer (PH = 4.5), was used to develop T1DM in fasting rats for an overnight period. Only citrate buffer was given to the animals under control [9]. To avoid a catastrophic drop in blood glucose caused by a huge release of insulin after STZ injection, STZ-treated rats were given access to 5% glucose solution (Merck KGG, A Darmstadt, Germany) for the first 24 hours [10].

Preparation of aortic rings

Eighty aortic rings from 20 male albino rats were used. The rats were intraperitoneally treated with a combination of xylazine (10 mg/kg) and ketamine (60 mg/kg). In a cold, fresh Krebs solution, 12 mm of the proximal descending thoracic aorta was placed next to the left subclavian branch. After removing the excess surrounding lipids and tissues from the aorta, 4 aortic rings of 3 mm length were produced (11).

Vascular reactivity

Krebs bicarbonate solution (mM/L: 119 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 25 $NaHCO_3$, 11 glucoses, pH = 7.4) was put into each glass chamber of the organ bath (Automatic Organ Bath-Panlab Harvard Apparatus-USA, AD instrument Power Lab 8/35-Australia). Stainless steel hooks were used to suspend and hold the aortic rings. Using a 2GS mixer, the solution's temperature was maintained at 37°C while bubbling with a combination of roughly 95% O_2 and 5% CO_2. The aortic ring was filled with 2 g of tension and given at least 60 minutes to stabilize. Every 15 minutes, the Krebs solution was changed, and the tension was continuously readjusted to the ideal force. Before using any vasoactive chemicals, the produced aortic rings were cleaned and stabilized at the ideal tension for around 30 minutes before adding 60 mM KCL to the chamber solution to verify their functional integrity. The aortic ring lumen was gently scraped with tiny forceps tip to remove the endothelial layer, and the denuded rings were created by adding acetylcholine (Ach) (10 µM) to pre-contracted aortic rings using phenylephrine [12].

Enzyme inhibitors

I- Non-induced-T1DM: Cumulative doses of GLP-1 (10-13-10-7 M) were applied and the findings were used as a control group to assess the vascular response to GLP-1.

i- Apocynin (n=4): The cumulative doses of GLP-1 were added after apocynin (0.09 M), NADPH-oxidase inhibitor was added to the aortic segments and incubated for 20 minutes.

ii- (RO31-8220) (n=8): The cumulative doses of GLP-1 were performed after Ro31-8220 (1 mM), PKC inhibitor, was added to the aortic segments and incubated for 20 minutes.

iii- Rotenone (n=4): The cumulative doses of GLP-1 were added after rotenone (0.06 M), an enzyme NADH ubiquinone reductase inhibitor, was added to the aortic segments and incubated for 20 minutes.

iv- Oxypurinol (OXP) (n=4): The cumulative doses of GLP-1 were added after the aortic rings were incubated for 20 minutes with oxypurinol (OXP) (100 µM), the xanthine oxidase inhibitor.

II- Induced-DM group

The same above groups and testes were repeated for induced-DM aorta rings of GLP-1 accumulative doses.

Statistical analysis

Results were expressed as the mean± standard error. Statistical analyses were performed with one-way analysis of variance (ANOVA) with Dunnett post hoc testof multiple comparison tests. For comparison between groups and control two-wayANOVA was used. An Independent t-test was used for creating dAUC graph, which is independent t-test was used. for calculating two different groups like diabetic and non-diabetic groups. The $p<0.05$ was considered statistically significant. All data were analyzed using Prism(Graphpad version 8). The maximum response efficacy (Emax) found by increasing dose response curve when applying the doses from the lowest to the highest concentrations, the lowest point on the curve is the maximum response efficacy (Emax).

Results and Discussion

Glucagon like peptide -1 control group

The roles of cumulative concentration doses of glp-1 on vascular responses in control and induced T1DM groups were studied. Glp-1 (10-13–10-7M), was added to the endothelium-intact segment at the plateau of a maximal contraction to 1µM PE which induced relaxation, this group was set as a comparison reference against experimental groups (Figure 1). Furthermore, glp-1 was added to denuded aortic rings, to evaluate the role of the endothelium in the vascular response of the aortic rings to glp-1 hormone. The data are expressed as Mean ±SE (Figure 1). In our study the vascular response was increased in DM rats in compare to non-DM rats. Maximum response efficacy (Emax) and potency (Pd2) were increased in DM in compare to non-dm rats

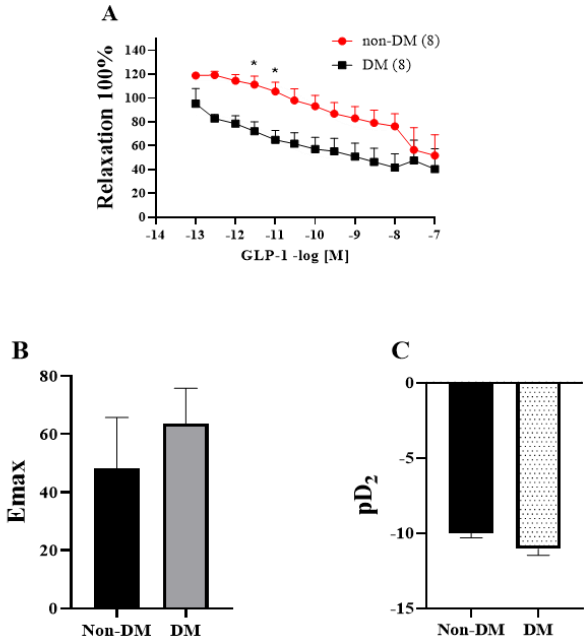

Figure 1: Glp-1 dose response curve (DRC) (10-13 – 10-7 M) in control and induced- T1DM groups of endothelial intact segments of proximal thoracic rat aorta. Parentheses indicate the number of aortic rings used. The vascular responses to glp-1 were calculated as a percentage vascular response to 1µM PE. The inset graphs show differences in area under the concentration-response curve (dAUC).

The role of NADPH oxidase on vascular response to GLP-1 in bothdiabetic and non-diabetic groups

To find out the role of the NADPH oxidase on vascular response to GLP-1, apocynin,a NADPH oxidase inhibitor (0.09M) was used which highly increasing effect on GLP-1 efficacy (Emax:72.40±0.350), while GLP-1 potency remains nearly unchanged (pD2: -9.923±0.444) in non-diabetic groups (Table1). The results exhibit increasing GLP-1efficacy (Emax; 68.91±2.002) and very highly significantly decreased GLP-1 potency (pD2: -9.480±0.138) in diabetic groups ($P<0.001$). The dAUC graph shows the effect of apocynin on vascular response of GLP-1 between non-diabetic and diabetic groups (Figure 2).

Table 1: The potency (pD2) and the maximum response (Emax) from the thoracicaortic rings' responses to GLP-1 in non-diabetic and STZ-induced diabetes.

Groups	Non-diabetic mellitus			Induced diabetes mellitus		
	N	Emax (%)	PD2	n	Emax (%)	PD2
Control	8	48.21±17.55	-9.999±0.302	8	63.70±12.10	-11.6±0.407
Apocynin	4	72.40±0.350	-9.923±0.444	4	68.91±2.002	- 9.480±0.14***
Ro31–8220	8	88.45±38.18	-10.81±0.281	4	77.73±2.801	-10.28±0.203*
Rotenone	4	63.69±35.10	-9.434±0.443	4	69.98±22.94	-9.612±0.24**
Oxypurinol	4	82.16±16.10	-8.358± 0.555*	4	58.03±8.350	-10.07±0.20*

Data were presented as mean± standard error of the mean. All groups in each column were compared with their control. One-way ANOVA and post hoc Dunnett test was used. *, **, *** means significant differences between compared group at $P<0.05$, $P<0.01$, $P<0.001$, respectively. pD2= potency; Emax= maximum response.

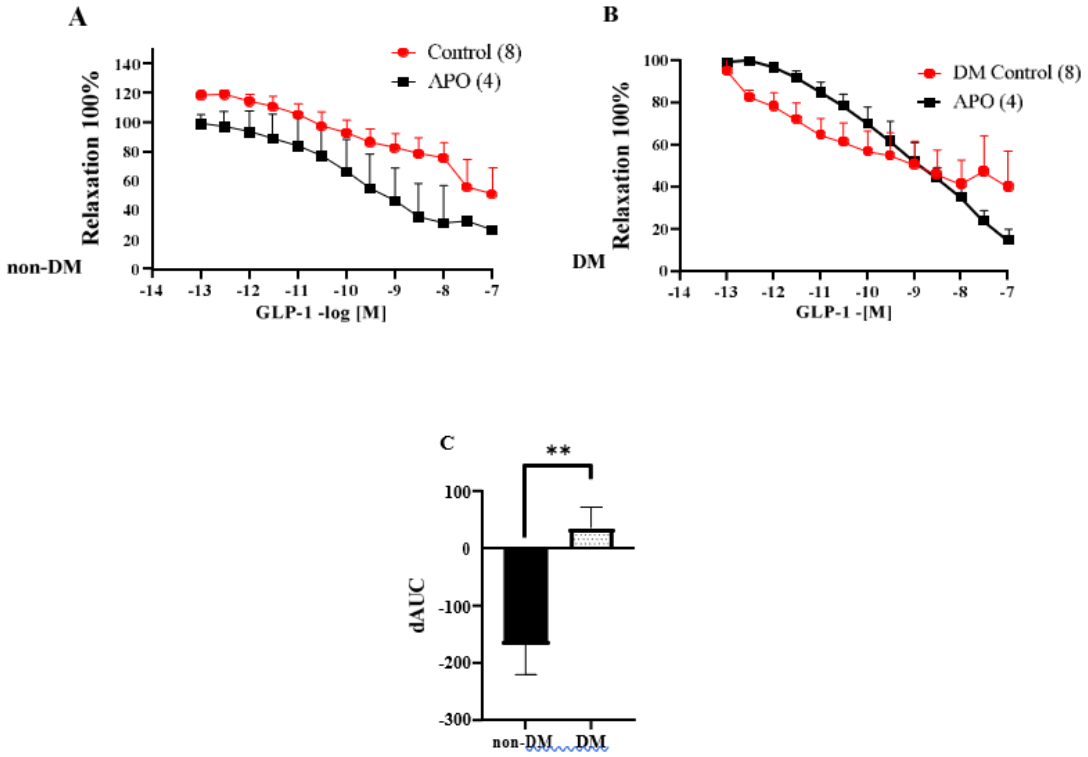

Figure 2: The effect of apocynin (0.09M) on the vasorelaxant responses to GLP-1 in non-induced andinduced-T1DM aortic rings.

The role of PKC pathway on vascular response to GLP-1 in diabetic and non-diabetic groups

The data analysis of the present study showed the role of PKC pathway in alteringthe vascular reactivity of GLP-1 in diabetic and non-diabetic groups. As shown in(Figure 3) inhibiting of PKC pathway by RO31-8220 (1 mM) exhibited maximum response in non- diabetic groups (Emax: 88.45±38.18) and in diabetic groups (Emax:77.73±2.801). The increased potency of GLP-1(pD2: -10.81±0.281) in non-diabetic groups but a significant decrease in potency (pD2: -10.28±0.203) in diabetic groups *(P*<0.05). The dAUC graph shows the effect of RO31-8220 on vascular response of GLP-1 between non-diabetic and diabetic groups.

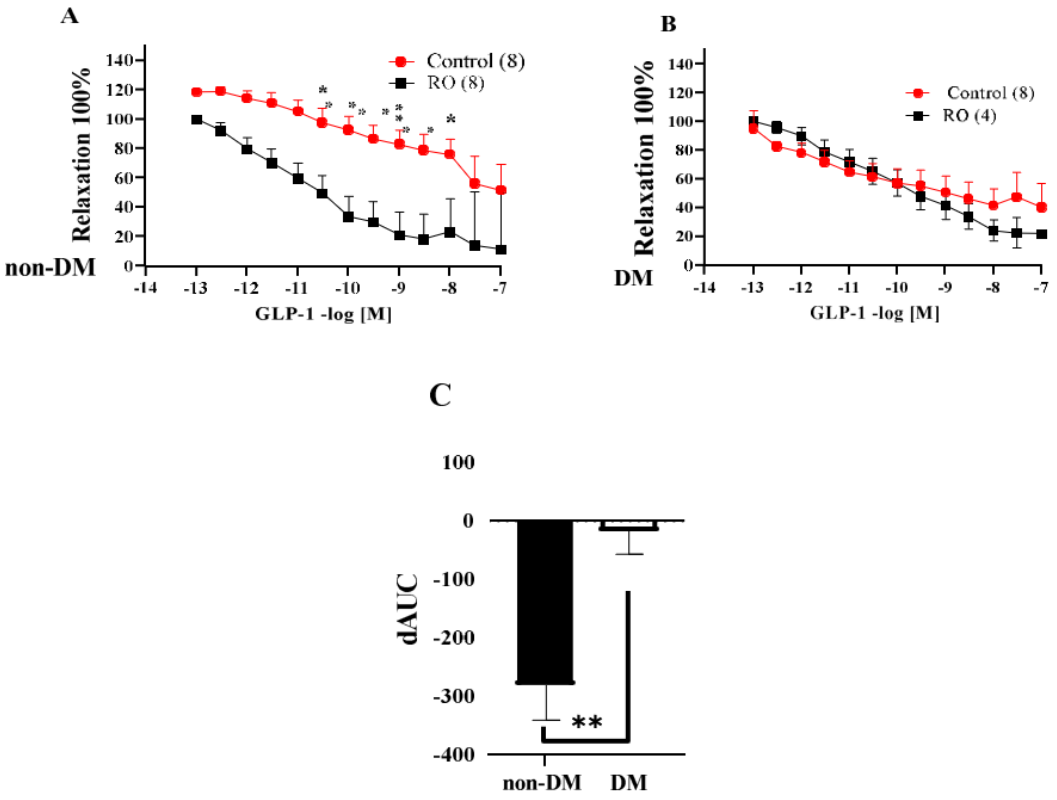

Figure 3: The effect of RO 31-8220 (1mM) on the vasorelaxant responses to GLP-1 non-induced and induced-T1DM aortic rings.

The effect of enzyme NADH ubiquinone on vascular responsiveness to GLP-1 in both diabetic and non-diabetic groups

To investigate the role of enzyme NADH ubiquinone in vascular response to GLP-1, rotenone (0.06M), enzyme NADH ubiquinone reductase was used. Our results showed that reducing enzyme NADH ubiquinone is significantly increased vascular maximum response (Emax) to GLP-1 in both diabetic and non-diabetic groups. In non-diabetic group (Emax: 63.69±35.10) and in diabetic group (Emax: 69.98±22.94), and altered potency (pD2: -9.434±0.443) in non-diabetic groups and highly significantly decreased potency (pD2: -9.612±0.246) in diabetic groups (P<0.01). The dAUC graph shows the effect of rotenone on vascular response of GLP-1 between non-diabetic and diabetic groups (Figure 4).

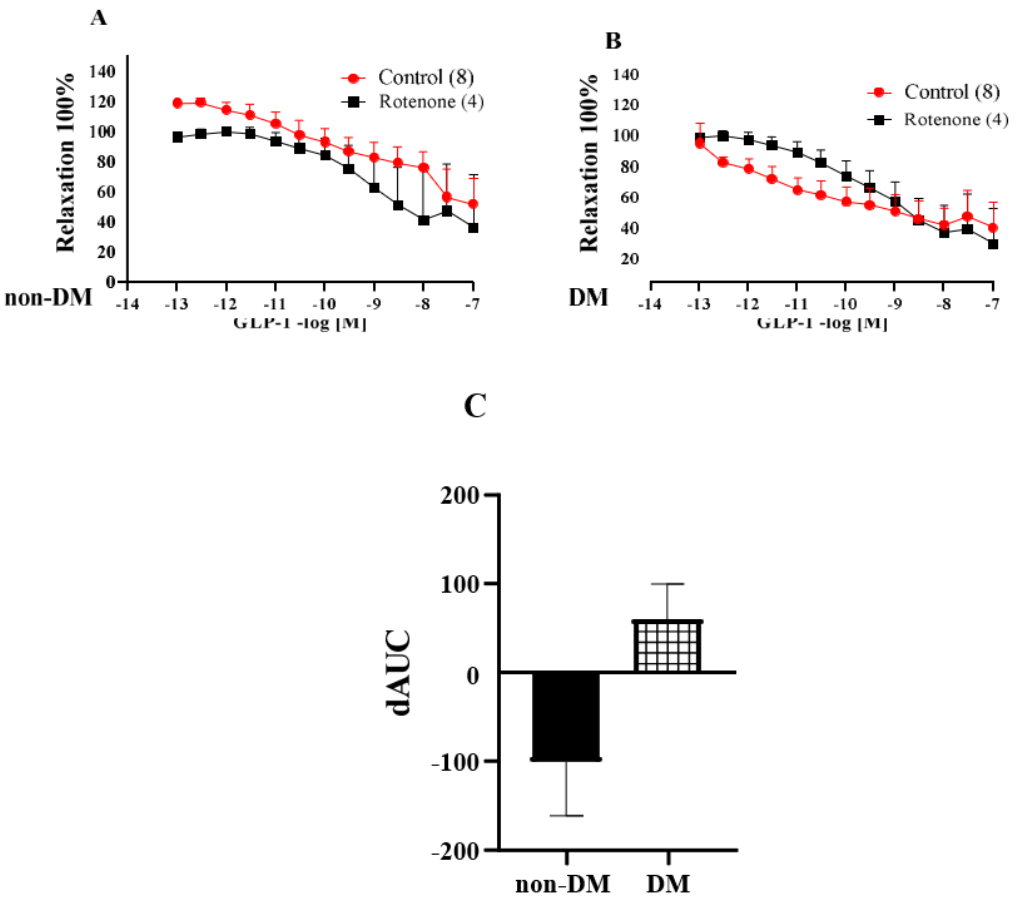

Figure 4: The effect of rotenone (0.06 M) on the vasorelaxant responses to glp-1In non-induced and induced-T1DM aortic rings

The role of xanthine oxidase on vascular response to GLP-1 in both diabetic and non-diabetic groups

To investigate the role of xanthine oxidase on vascular response to GLP-1,oxypurinol (100 μM), xanthine oxidase inhibitor was used. The results show that inhibiting xanthineoxidase increased efficacy (Emax: 82.16±16.10) in non-diabetic group and decreased efficacy (Emax: 58.03±8.350) in diabetic groups, while a significant decreased potency in both diabetic and non-diabetic groups (P<0.05). The dAUC graph shows the effect of oxypurinol on vascular response of GLP-1 between non-diabetic and diabetic groups (Figure 5).

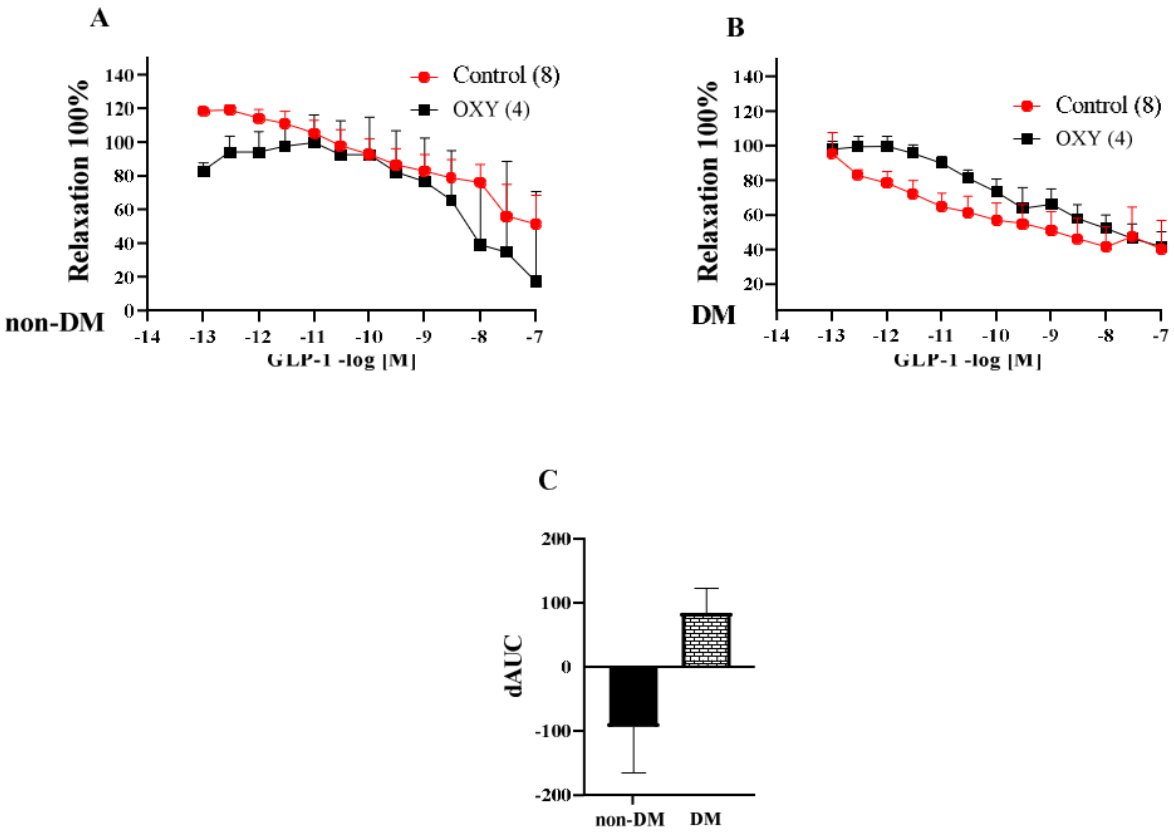

Figure 5: The effect of OXp (100µM) on the vasorelaxant responses to glp-1 in non-induced and induced-T1DM aortic rings.

The effect of NADPH oxidase on vascular response to GLP-1 in both diabetic and non-diabetic groups

Since NAD(P)H oxidase is the main source of ROS in the vasculature, it is logicalto assume that lowering NAD(P)H oxidase activity will alleviate endothelial dysfunction. Apocynin, an NAD(P)H oxidase inhibitor, was used for revealing the vasorelaxant effect of NADPH oxidase to GLP-1, our study demonstrated that apocynin increased vascular response to GLP-1 in the control group, which may be related to that by inhibiting nitric oxide, NADPH oxidase-derived superoxide contributes to the impairment of endothelium- dependent vasodilation [13]. Apocynin's capacity to decrease oxidative stress and increase NO bioavailability helps endothelial function [14]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is inhibited by the naturally occurring acetophenone known as apocynin. Apocynin may have pharmacological effects that are NADPH oxidase independent, according to recent research. Vasorelaxant characteristics have been mentioned among them[15]. Interestingly in induced diabetes the NADPH oxidase inhibitor inverted GLP-1 vasodilatation property, in which maximum response increased and the potency of glp-1 highly significantly decreased, the result may be due to that apocynin restores serum antioxidant enzyme activity in diabetic rats [16]. In the diabetic rat aorta, NADPH oxidase expression is enhanced. Endothelial dysfunction is a result of NADPH oxidase-mediated oxidative stress, which is associated by decreased eNOS and elevated iNOS levels. Apocynin successfully breaks the loop that results in the increased NADPH oxidase expression in diabetic aorta and recovers the altered NOS expression, preventing diabetes-related endothelial damage [17]. The dAUC graph clearly shows the difference is highly significant between non- diabetic and diabetic groups.

The effect of PKC pathway on vascular response to GLP-1 in diabetic and non- diabetic groups.

In our study, we used RO31-8220 for inhibiting the PKC pathway that the results showed increased

efficacy and potency in non-diabetic groups and increased effectof GLP-1 vasodilation, this may be due to that endothelin-1 (ET-1) and other endothelium-derived contracting factors may be released by PKC, which would then encourage vasoconstriction [18]. Besides having avasoconstrictive effect, ET-1 also induces vascular cell fibrosis and increases the formation of reactive oxygen species [19]. Vasoconstriction, vascular remodeling, inflammation, and fibrosis are all caused by increased oxidative stress [20]. Another reason is that previous studies haveshown that PKCα is a direct regulator of Nox5 phosphorylation and activity, and Nox5 phosphorylation is PKC-dependent [21]. A key regulator of platelet granule production, integrin activation, aggregation, spreading, and procoagulant activity is the protein kinase C (PKC) family. The PKC family is typically regarded as a positive regulator of platelet activation since broad-spectrum PKC inhibitors decrease secretion and aggregation [22]. Endothelial dysfunction is linked to platelet aggregation [23]. While in induced diabetic rats' vascular relaxation of GLP-1 decreased, the reason for thatmay be high quantities of reactive oxygen species (ROS) are induced in diabetic mellitus. The vascular problems of diabetes are exacerbated by this. Recent research has demonstrated that a glycolytic metabolic shift in diabetic monocytes and macrophages causes an increase in ROS production. Another reason may be due to that patients who suffer from diabetes have higher circulating levels of ET-1, which may be a factor in the disease's associated endothelial dysfunction [24]. Additionally, it appears that hyperglycemic oxidative stress caused by NADPH oxidase (Nox), the only knownenzyme specifically designed to produce reactive oxygen species, is involved [25]. The dAUC graph shows the difference is highly significant between non-diabetic and diabetic groups.

The effect of enzyme NADH–Ubiquinone Oxidoreductase on vascular responsiveness toGLP-1 in both diabetic and non-diabetic groups.

In this study, rotenone was used for inhibiting NADH–ubiquinone oxidoreductase; the data analysis demonstrated that rotenone increased the vasorelaxant effect of GLP-1 in non- diabetic rats, the possible reason is that mitochondria produce superoxide complex I (NADH: ubiquinone oxidoreductase) [26]. Rotenone, a complex I inhibitor, can mimic this elevated ROS generation [27]. Another reason is that asa vasodilator, nitric oxide (NO) primarily causes vasodilation through the eNOS/NO/cGMP pathway [11]. The cytochrome c oxidase of the mitochondrial respiratory chain is one of the numerous suggested pathways forthe cellular inactivation of endogenous NO. Physiological levels of NO are inactivated by cytochrome c oxidase when it is in turnover and in its oxidized state, according to pharmacological treatment of this enzyme [28]. However, the vasorelaxant effect of rotenone has been decreased in diabetic rats, this may be due to that the balance between NADH and NAD+ can be drastically disturbed in diabetes and its consequences. On the one hand, the activation of the polyol pathway and the input of hyperglycemia into theglycolytic and Krebs cycle pathways cause an excess of NADH to be generate [29]. On the other hand, NO bioavailability is decreased in diabetes, which causes endothelial dysfunction. Hyperglycemia-inducedoverproduction of NO is linked to oxidative stress and tissue damage [30]. The dAUC graph shows a change between non-diabetic and diabetic groups.

The effect of xanthine oxidoreductase on vascular response to GLP-1 in bothdiabetic and non-diabetic groups

In our study we used oxypurinol for inhibiting xanthine oxidoreductase that oxypurinol could increase the vasorelaxant response of GLP-1, there are some reasons that in addition to producing ROS, xanthine oxidoreductase (XOR) catalyzes the oxidative hydroxylation of hypoxanthine to xanthine to uric acid [31]. The biomarker for the activation of xanthine oxidase, which releases oxidants during the production of UA, is known as hyperuricemia. Increased generation of oxidants, especially superoxide, encourages endothelial dysfunction. Additionally,the urate transporter GLUT 9/URATv1 (voltage-driven urate efflux transporter 1) is expressed in human blood vessels and is crucial for UA absorption into blood vessels,

which results in inflammation, oxidative stress, and dephosphorylation of eNOS (endothelial nitric oxide synthase) in the vasculature. NO is crucial for controlling vascular tone and arterial stiffness. Endothelial dysfunction is encouraged by an imbalance or a reduction in NO production [32]. However, the vasorelaxant effect of oxypurinol has been decreased in diabetic rats, reasons for that may be due to chronic or acute high glucose levels in diabetesboost ROS generation and trigger apoptosis in –cells [33]. Another reason is that in the early phases of poor glucose metabolism, uric acid levels rise. Additionally, hyperuricaemia in diabetic individuals has been connectedto both micro- and macrovascular complications [34]. The dAUC graph shows non-significant change between non-diabetic and diabetic groups.

Conclusions

The present study concluded that GLP-1 provoked dramatic vasodilatory effect in STZ induced diabetes mellitus in rats' aortic rings through attenuation following inhibitory impact, the possible modulatory effect of GLP-1 has seen in maximum efficacy (Emax) of apocynin, RO31-8220, rotenone and oxypurinol in non-diabetic and diabetic groups except oxypurinol in diabetic group. While, the previous vasotonic action of GLP-1 was also confirmed in its potency higher than those parameters in non-diabetes mellitus induced rat's aortic rings. Therefore, GLP-1may give vasculoprotection action through different cellular signaling pathways in modeled aorta.

Conflict of interest

The authors confirm that they are not affiliated with or involved in any organization or entity with financial interests.

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