Role of glucagon-like peptide-1 in vascular endothelial dysfunction

Sandeep Goyal* & Suresh Kumar
Department of Pharmacology, S D College of Pharmacy, Barnala 148 101, India
and
Krishnareddy V Bijjem & Manjeet Singh
Cardiovascular Pharmacology Division, ISF College of Pharmacy, Moga 142 001, India

Received 12 June 2009; revised 24 September 2009

The present study has been undertaken to investigate the effect of exendin-4 (a glucagon-like peptide-1 agonist) in diabetes mellitus (DM) and hyperhomocysteinemia (HHcy)-induced vascular endothelial dysfunction (VED). Streptozotocin (55 mg kg\(^{-1}\), iv, once) and methionine (1.7% w/w, po, 4 weeks) were administered to rats to produce DM (serum glucose >200 mg dl\(^{-1}\)) and HHcy (serum homocysteine >10 \(\mu\)M) respectively. VED was assessed using isolated aortic ring preparation, microscopy of thoracic aorta, and serum nitrite/nitrate concentration. Serum TBARS concentration was estimated to assess oxidative stress. Atorvastatin has been employed as standard agent. Exendin-4 (1 \(\mu\)g kg\(^{-1}\), ip) and atorvastatin (30 mg kg\(^{-1}\), po) treatments significantly attenuated increase in serum glucose and homocysteine but their concentrations remained markedly higher than sham control value. Exendin-4 and atorvastatin treatments markedly prevented DM and HHcy-induced (i) attenuation of acetylcholine-induced endothelium-dependent relaxation, (ii) impairment of vascular endothelial lining, (iii) decrease in serum nitrite/nitrate concentration, and (iv) increase in serum TBARS. However, this ameliorative effect of exendin-4 has been prevented by L-NAME (25 mg kg\(^{-1}\), ip), an inhibitor of NOS. It may be concluded that exendin-4 may activate eNOS due to activation of GLP-1 and consequently reduce oxidative stress to improve vascular endothelial dysfunction.

Keywords: Diabetes mellitus, Exendin-4, Hyperhomocysteinemia, Vascular endothelial dysfunction

Vascular endothelial dysfunction (VED) has been reported to be implicated in congestive heart failure\(^1\), secondary complications of cardiovascular disorders due to diabetes mellitus (DM)\(^2\), hyperhomocysteinemia (HHcy)\(^3\), essential hypertension\(^4\) and hypercholesterolemia\(^5\). VED has been characterized as partial or complete loss of balance between vasorelaxation and vasoconstriction\(^6,7\), thrombosis and thrombolysis\(^8\), growth promotion and growth inhibition\(^9\). Streptozotocin-induced DM\(^2,10\) and methionine-induced HHcy\(^11,12\) have been noted to produce experimental vascular endothelial dysfunction. Reduced NO bioavailability, production and its increased metabolism are documented for producing vascular endothelial dysfunction\(^1,13\). Glucagon-like peptide-1 (GLP-1) is an insulinotropic peptide having incretin character and possesses antidiabetic properties\(^14,15\). GLP-1 expression is downregulated in diabetes mellitus\(^16\) and hyperhomocysteinemia\(^17\). Exendin-4 is a structural analogue of GLP-1 having dipeptidylpeptidase- IV (DPP-IV) resistant and mimetic activity\(^18,20\). DPP- IV is an enzyme which inactivates GLP-1\(^21\). GLP-1 has been documented to activate Akt\(^22-24\) which is known to stimulate eNOS\(^25-27\). GLP-1 inhibits apoptosis\(^28\) and reduces oxidative stress\(^29\). This may be suggested that GLP-1 is implicated in VED. Therefore, the present study has been undertaken to investigate the effect of exendin-4, a GLP-1 agonist, in diabetes mellitus and hyperhomocysteinemia induced vascular endothelial dysfunction.

Materials and Methods

Drugs and chemicals—Streptozotocin (Sigma Chemicals, St. Louis, USA) was used to induce diabetes mellitus in rats. Exendin-4 (Cadila Research Laboratories, Ahmedabad) was diluted with saline. Atorvastatin (Dr. Reddy’s Laboratories, Hyderabad) was suspended in 0.5% sodium CMC. Methionine (Loba Chemie, Mumbai) was mixed with normal...
chow diet (Ashirwad Industries Ltd., Chandigarh). All other chemicals used were of AR grade (E. Merck, Mumbai). All drug solutions were freshly prepared before use.

**Animals**—Age matched young Wistar albino rats of either sex weighing about 200–250 g housed in animal house, included in this study were provided 12 h light and 12 h dark cycle. They were fed on standard chow diet and provided water ad libitum. The experimental protocol was approved by Institutional Animal Ethics Committee and experiments conducted in accordance with guidelines of CPCSEA, Chennai, India on use and care of experimental animals. In diabetic group, rats were administered streptozotocin (55 mg kg\(^{-1}\) iv, once only) dissolved in 0.1 M citrate buffer (pH 4.5)\(^{30}\). The animals were maintained hyperglycemic (>200 mg dl\(^{-1}\)) for 8 weeks. Methionine (1.7% w/w) was mixed with standard diet and was re-pelleted. In hyperhomocysteinemic group, rats were fed on methionine-containing diet for 4 weeks\(^{11,12}\).

**Assessment of diabetes mellitus and hyperhomocysteinemia**—Blood samples were collected after 1 week of administration of streptozotocin and 4 weeks of administration of methionine diet in clean dry centrifuge tubes from 8 h fasted rats and were allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 3000 rpm for 25 min, aliquoted and stored at \(-20^\circ\text{C}\) until analysis was carried out. Serum homocysteine concentration was analyzed using HPLC (Varian Inc., CA, USA) technique maintaining flow rate of 2 ml min\(^{-1}\) and retention time of 8.5 min\(^{31}\). Serum glucose was measured spectrophotometrically using enzymatic kit (Vital Diagnostics Ltd., Thane).

**Assessment of vascular endothelial dysfunction**

Isolated rat aortic ring preparation—The rats were decapitated, thoracic aorta was removed, cut into a ring of 4–5 mm width and mounted in organ bath containing Krebs-Henseleit solution (NaCl, 119 mM; KCl, 4.7 mM; NaHCO\(_3\), 25 mM; MgSO\(_4\), 1.0 mM; glucose, 11.1 mM; K\(_2\)HPO\(_4\), 1.2 mM and CaCl\(_2\), 2.5 mM), bubbled with carbonated oxygen (95% O\(_2\) and 5% CO\(_2\)) and maintained at 37°C. The preparation was primed three times with 80 mM KCl to check its functional integrity and to improve its contractility. The cumulative dose responses of acetylcholine (ACh; 10\(^{-8}\) to 10\(^{-4}\) M) were recorded in phenylephrine (3 \times 10\(^{-6}\) M) precontracted preparation\(^3\). The intimal layer of aortic ring was removed, cut into a ring of 4–5 mm width and mounted in organ bath containing Krebs-Henseleit solution (NaCl, 119 mM; KCl, 4.7 mM; NaHCO\(_3\), 25 mM; MgSO\(_4\), 1.0 mM; glucose, 11.1 mM; K\(_2\)HPO\(_4\), 1.2 mM and CaCl\(_2\), 2.5 mM), bubbled with carbonated oxygen (95% O\(_2\) and 5% CO\(_2\)) and maintained at 37°C. The preparation was allowed to equilibrate for 90 min under 1.5 g tension. The isometric contractions were recorded\(^{10}\) with a force-displacement transducer (FT-2240) connected to physiograph (INCO, Ambala).

The preparation was primed three times with 80 mM KCl to check its functional integrity and to improve its contractility. The cumulative dose responses of acetylcholine (ACh; 10\(^{-8}\) to 10\(^{-4}\) M) were recorded in phenylephrine (3 \times 10\(^{-6}\) M) precontracted preparation\(^3\). The intimal layer of aortic ring was removed, cut into a ring of 4–5 mm width and mounted in organ bath containing Krebs-Henseleit solution (NaCl, 119 mM; KCl, 4.7 mM; NaHCO\(_3\), 25 mM; MgSO\(_4\), 1.0 mM; glucose, 11.1 mM; K\(_2\)HPO\(_4\), 1.2 mM and CaCl\(_2\), 2.5 mM), bubbled with carbonated oxygen (95% O\(_2\) and 5% CO\(_2\)) and maintained at 37°C. The preparation was allowed to equilibrate for 90 min under 1.5 g tension. The isometric contractions were recorded\(^{10}\) with a force-displacement transducer (FT-2240) connected to physiograph (INCO, Ambala).

The preparation was primed three times with 80 mM KCl to check its functional integrity and to improve its contractility. The cumulative dose responses of acetylcholine (ACh; 10\(^{-8}\) to 10\(^{-4}\) M) were recorded in phenylephrine (3 \times 10\(^{-6}\) M) precontracted preparation\(^3\). The intimal layer of aortic ring was removed, cut into a ring of 4–5 mm width and mounted in organ bath containing Krebs-Henseleit solution (NaCl, 119 mM; KCl, 4.7 mM; NaHCO\(_3\), 25 mM; MgSO\(_4\), 1.0 mM; glucose, 11.1 mM; K\(_2\)HPO\(_4\), 1.2 mM and CaCl\(_2\), 2.5 mM), bubbled with carbonated oxygen (95% O\(_2\) and 5% CO\(_2\)) and maintained at 37°C. The preparation was allowed to equilibrate for 90 min under 1.5 g tension. The isometric contractions were recorded\(^{10}\) with a force-displacement transducer (FT-2240) connected to physiograph (INCO, Ambala).

Estimation of serum nitrite/nitrate concentration—The serum obtained was incubated with 0.15 g of Cu-Cd filings for 1 h at room temperature to reduce nitrate to nitrite. The reaction was stopped by addition of 100 µL of 0.35 M sodium hydroxide. The zinc sulfate solution (400 µL of 120 mM) was added to deproteinate the serum samples, allowed to stand for 10 min and then centrifuged at 4000 rpm for 10 min. Greiss reagent (250 µL of 1.0% sulfanilamide and 250 µL of 0.1% N-naphthylethylenediamine) was added to aliquots (500 µL) of the clear supernatant and serum nitrite/nitrate was measured spectrophotometrically (UV-1-100 Spectrophotometer, Thermo Electron Spectroscopy Ltd., UK) at 545 nm\(^{34}\). The standard curve of sodium nitrite (0.1-25 µM) was plotted to calculate serum concentration of nitrite/nitrate.

Histological study—Aorta preserved in 10% formalin was immersed in 70, 80, 90% and absolute alcohol for one hour in each case. After alcohol treatment, aorta was placed in xylene for 3 h replacing with fresh xylene every 1 h. The aorta was kept overnight in molten paraffin wax maintained at 60°C. Paraffin block of the tissue sample was prepared and 10 parallel cefalocaudal thoracic aorta artery (Ao) of 4 mm thickness cross-sliced incisions were performed every 1 mm using microtome. The sections were fixed on a slide smeared with a mixture of equal volumes of glycerine, distilled water and egg white. The slide was placed in xylene for 5 min followed by two successive treatments of 1 min each with fresh absolute alcohol. The slide was rinsed with water and stained with haematoxylin for 3 min and excess haematoxylin was washed with water. The slide was then counter stained with 1% solution of eosin for 3 min, followed by washing
with water for 1 min. The slide was dipped thrice in fresh absolute alcohol, for dehydration and each time dipping the slide alcohol was discarded. The slide was cleared with xylene twice and the slide was mounted with DPX.

Assessment of oxidative stress

Estimation of serum thiobarbituric acid reactive substances (TBARS)—Trichloracetic acid (1000 µl of 20%) was added to 100 µl serum in a test tube, and then 1000 µl of 1% TBARS reagent (mixture of equal volume of 1% TBA aqueous solution and glacial acetic acid) was added, mixed, and incubated at 100°C for 30 min. After cooling on ice, samples were centrifuged at 1000 rpm for 20 min, and absorbance was noted spectrophotometrically at 532 nm against prepared blank solution. A standard curve using 1,1,3,3-tetramethoxypropane was plotted to calculate the concentration of TBARS.

Experimental protocol—Rats (54) were divided in 9 groups of 6 rats each. Group I (sham control) rats were not given any treatment and the assessment of vascular endothelial function and serum TBARS were carried out. Group II (hyperhomocysteinemic control) rats were administered methionine (1.7% w/w, 4 weeks) and after 4 weeks of methionine administration, assessment of VED and serum TBARS were carried out. In Group III (diabetic control) rats were administered streptozotocin (55 mg kg\(^{-1}\), iv) and after 8 weeks of streptozotocin administration, assessment of VED and serum TBARS were carried out. In group IV (exendin-4, 1 \(\mu\)g kg\(^{-1}\) day\(^{-1}\), ip) and group V (exendin-4, 1 \(\mu\)g kg\(^{-1}\), ip in diabetic rats) rats were administered exendin-4 daily for 7 days in fourth week of methionine administration, assessment of VED and serum TBARS were carried out. In Group VI (exendin-4, 1 \(\mu\)g kg\(^{-1}\), ip + L-NAME, 25 mg kg\(^{-1}\) in hyperhomocysteinemic rats) and Group VII (exendin-4, 1 \(\mu\)g kg\(^{-1}\), ip + L-NAME, 25 mg kg\(^{-1}\) in diabetic rats) rats were administered exendin-4 daily for 7 days in fourth week of methionine administration and seventh week of streptozotocin administration. Then, assessment of VED and serum TBARS were carried out. In Group VIII (atorvastatin, 30 mg kg\(^{-1}\), po in hyperhomocysteinemic rats) and group IX (atorvastatin, 30 mg kg\(^{-1}\), po in diabetic rats) rats were administered atorvastatin daily for 7 days in fourth week of methionine administration and seventh week after administration of streptozotocin. At the end of treatments, assessment of VED and serum TBARS were carried out.

Statistical analyses—All values were expressed as mean±SE. Statistical analyses were performed using Sigma Stat software. Data for isolated aortic ring preparation were statistically analyzed using one way ANOVA followed by Newmann Keul’s Test. The data for serum nitrite/nitrate concentration and TBARS were statistically analysed using one way ANOVA followed by Tukey’s multiple range Test. \(P<0.05\) was considered to be statistically significant.

Results

Endothelium dependent and independent relaxations—Acetylcholine (ACh) and sodium nitroprusside (SNP) produced endothelium-dependent relaxation and endothelium-independent relaxation dose dependently in phenylephrine (3×10\(^{-6}\) M) precontracted isolated rat aortic ring preparation respectively. Diabetes mellitus and hyperhomocysteinemia markedly attenuated ACh-induced endothelium-dependent relaxation but there is no marked affect on SNP-induced endothelium-independent relaxation. The administration of exendin-4 (1 \(\mu\)g kg\(^{-1}\) day\(^{-1}\), ip) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), po) significantly prevented diabetes mellitus and hyperhomocysteinemia induced attenuation of ACh-induced endothelium-dependent relaxation. SNP-induced endothelium-independent relaxation was not modulated by treatment of exendin-4 (1 \(\mu\)g kg\(^{-1}\) day\(^{-1}\), ip) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), po) in diabetic and hyperhomocysteinemic rats. However, L-NAME (25 mg kg\(^{-1}\), ip) significantly prevented exendin-4 (1 \(\mu\)g kg\(^{-1}\))- induced increase in ACh evoked endothelium dependent relaxation (Figs 1 and 2)

Serum nitrite/nitrate concentration—DM and HHcy markedly reduced serum nitrite/nitrate concentration. Exendin-4 (1 \(\mu\)g kg\(^{-1}\) day\(^{-1}\), ip) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), po) significantly prevented DM and HHcy-induced decrease in serum nitrite/nitrate concentration. However, L-NAME administration significantly prevented exendin-4 induced increase in serum nitrite/nitrate concentration in DM and HHcy rats (Fig. 3).

Serum thiobarbituric acid reactive substances (TBARS)—DM and HHcy significantly increased
serum TBARS concentration. Exendin-4 (1 μg kg\(^{-1}\) day\(^{-1}\), ip) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), po) treatment significantly prevented DM and HHcy-induced increase in serum TBARS. However, L-NAME administration significantly prevented exendin-4 (1 μg kg\(^{-1}\)) induced decrease in serum TBARS concentration in DM and HHcy rats (Fig. 4).

Vascular endothelium integrity—Diabetes mellitus and hyperhomocysteinemia impaired integrity of vascular endothelium lining of thoracic aorta.

Exendin-4 (1 μg kg\(^{-1}\) day\(^{-1}\), ip) or atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), po) treatments prevented DM and HHcy-induced impairment of vascular endothelium (Fig. 5).

Serum concentration of glucose and homocysteine—Exendin-4 (1 μg kg\(^{-1}\) day\(^{-1}\), ip) and atorvastatin (30 mg kg\(^{-1}\) day\(^{-1}\), po) treatments have significantly reduced concentration of blood glucose
in diabetic rats and serum homocysteine in hyperhomocysteinemic rats. However, L-NAME administration did not markedly modulate the effect of exendin-4 (1 μg kg$^{-1}$) in diabetes mellitus and hyperhomocysteinemic rats (Table 1).

### Discussion

The present study provides evidence that GLP-1 may be implicated as important target site to ameliorate diabetes mellitus and hyperhomocysteinemia induced experimental vascular endothelial dysfunction. Thus, streptozotocin induced diabetes mellitus$^{10}$ and hyperhomocysteinemia$^{11}$ have been shown to produce VED as revealed from previous studies. Estimation of blood glucose and serum homocysteine concentration have been used as markers to confirm diabetes mellitus and hyperhomocysteinemia respectively.

Atorvastatin has been used as standard agent in present study to improve vascular endothelial
dysfunction in diabetic and hyperhomocysteinemic rats. GLP-1 has been reported to activate Akt\textsuperscript{22-24}. Akt increases GLUT-4-mediated glucose transport\textsuperscript{35,36}. Moreover, Akt stimulates the conversion of homocysteine to methionine\textsuperscript{37,38} and cystathione\textsuperscript{39}. Therefore, the observed effect of exendin-4 or atorvastatin to decrease serum glucose and homocysteine concentration in diabetic mellitus and hyperhomocysteinemic rats may be due to activation of Akt. DM\textsuperscript{16,40} and HHcy\textsuperscript{17,41} are noted to inhibit GLP-1 and Akt. Therefore, it may be suggested that DM- and HHcy-induced inhibition of GLP-1 may produce vascular endothelial dysfunction. This contention is further supported by the noted ameliorative effect of exendin-4 in DM- and HHcy-induced vascular endothelial dysfunction. Akt activation also stimulates expression and activity of eNOS\textsuperscript{42,43} and consequently increase the formation.

Fig. 5—Effect of exendin-4 on integrity of vascular endothelial lining. (A) sham control, (B) diabetic control, (C) hyperhomocysteinemic control (D) exendin-4 treatment in diabetic rats, (E) exendin-4 treatment in hyperhomocysteinemic rats
of NO. Therefore, the observed increase in serum nitrite/nitrate concentration, ACh-induced endothelium-dependent relaxation and improvement in the integrity of endothelial lining in exendin-4 treated diabetic mellitus and hyperhomocysteinemic rats, may be due to activation increase in expression and activity of eNOS. This contention is further supported by the observation that L-NAME, being an inhibitor of NOS, has attenuated the effect of exendin-4 on above mentioned parameters. Previous reports also suggest that exendin-4 has endothelium-dependent action on vascular endothelium. The effect of exendin-4 may be endothelium dependent because it has not modulated SNP-induced endothelium-independent relaxation. DM and HHcy have been documented to produce oxidative stress. Exendin-4 has been noted to reduce concentration of serum TBARS as supported from previous reports that GLP-1 agonists decrease oxidative stress may be due to GLP-1 activation.

Previous studies performed on vascular endothelial dysfunction suggests effect of exendin-4 on diabetes-induced VED, but no work was carried out on HHcy-induced VED. In the present study, data on diabetes-induced VED has been substantiated and also confirmed role of exendin-4 in management of HHcy-induced VED.

It may be concluded that exendin-4 may increase expression and activity of eNOS and reduce oxidative stress through activation of GLP-1, thereby, subsequent improvement in vascular endothelial dysfunction. GLP-1 may be implicated as pivotal pharmacological target site to improve DM- and HHcy-induced vascular endothelial dysfunction.

Acknowledgement

The authors are grateful to Mr. Parveen Garg, Chairman, ISF College of Pharmacy, Moga for research facilities and to Mr. Ajay Sharma, Manager, Cadila Research Laboratories, Ahmedabad for test drug, exendin-4.

References


endothelium derived nitric oxide production by the protein kinase Akt, *Nature*, 400 (1999) 792.


