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Esculetin attenuates alterations in Ang II and acetylcholine mediated vascular reactivity associated with hyperinsulinemia and hyperglycemia

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ABSTRACT

Esculetin (6, 7- dihydroxycoumarin) was found to be protective against hepatic and renal damage associated with Streptozotocin (STZ) induced type 1 diabetes, because of its radical scavenging property. However, there are no reports regarding its effect on vascular dysfunction under hyperinsulinemic and hyperglycemic conditions. Hence, the present study aimed to investigate the effect of esculetin on vascular dysfunction under these conditions. Non-genetic model of hyperinsulinemia and hyperglycemia were developed by high fat diet (HFD) feeding and HFD + Streptozotocin (STZ, 35 mg/kg, *I.P*) treatment in Wistar rats, respectively. Esculetin was administered at 50 and 100 mg/kg/day (*P.O*, 2 weeks) doses and biochemical, vascular reactivity and immunohistochemical experiments were performed to assess the effect of esculetin on vascular dysfunctions. Esculetin treatment significantly attenuates metabolic perturbations, alleviates insulin levels in hyperinsulinemic condition. Thoracic aorta of hyperinsulinemic and hyperglycemic rats showed hyper-responsiveness to Ang II mediated contraction and impaired acetylcholine mediated relaxation, and esculetin attenuates alterations in vascular reactivity to Ang II and acetylcholine challenges. In addition, immunohistochemical evaluations revealed that esculetin prevents increase in AT_1R, AT_2R, Keap1, TGF-β, and decrease in ACE2 expression in aorta of hyperinsulinemic and hyperglycemic rats.

1. Introduction

Among all macrovascular and microvascular complications associated with diabetes, vascular complication and thereby cardiovascular diseases (CVD) alone is the reason for more than 50% of diabetic patients' death [1]. Ang II, an integral component of renin angiotensin system (RAS) plays a pivotal role in the development of vascular complications. Ang II modulates a variety of physiological effects through Angiotensin II type 1 receptors (AT_1R) and Angiotensin II type 2 receptors (AT_2R) in numerous tissues including the vascular system [2]. Experimental and epidemiological studies suggest that hyperinsulinemia and hyperglycemia lead to activation of the RAS and up-regulation of AT_1R, which are considered to be the main culprit for the development of vascular complications associated with diabetes [2–4].

In the present study, we have used high fat diet (HFD) fed and HFD fed/Streptozotocin (STZ, 35 mg/kg, *I.P*) treated rats as non-genetic model of hyperinsulinemia and hyperglycemia which can mimic the altered metabolic events as seen in human type 2 diabetes [5]. HFD and STZ treated rats showed metabolic perturbations, hypertension, oxidative stress, over activation of renin-angiotensin system (RAS), and increased Ang II vascular reactivity which lead to vascular dysfunction [5]. Therefore, there is a need of novel therapeutic intervention which can prevent, reverse or delay these multiple alterations to address vascular dysfunction associated with hyperinsulinemia and hyperglycemia.

Esculetin a naturally occurring 6,7-dihydroxy derivative of coumarin was found to be protective against hepatic and renal dysfunction associated with diabetes in STZ induced type 1 diabetic rats [6–8]. Since ancient time esculetin containing plants are

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commonly used as a folk medicine to counteract various inflammatory, allergic and infectious diseases. Esculetin has been reported for inhibiting lipoxygenase and cycloxygenase [9], scavenging reactive oxygen species (ROS) [8], anti-fibrotic [7], and anti-proliferative [10,11] effects. However, effect of esculetin on diabetic vascular dysfunction is yet to be studied. A selective 5-lipoxygenase inhibitor (caffeic acid phenethyl ester) have been reported to reduce vascular hyper-responsiveness and vascular stiffness, thereby, alleviates diabetic atherosclerotic manifestations [12]. Further, metformin has been reported to restore endothelial function through inhibiting endoplasmic reticulum stress, oxidative stress and increasing NO bioavailability by activation of AMPK/PPARδ pathway in obese diabetic mice [13]. Recent report, suggest that esculetin has anti-adipogenic effects through modulation of PPARγ and C/EBPα via the AMPK signaling pathway [14]. Based upon these findings, we hypothesized that due to ROS scavenging, lipoxygenase inhibitory potential and AMPK modulating effect, esculetin may attenuate Ang II mediated vascular hyper-responsiveness, alleviate impaired acetylcholine induced relaxation and prevent vascular dysfunction associated with type 2 diabetes.

2. Methods

2.1. Animal studies

The male Wistar rats (160–180 g) were procured from the central animal facility of the institute, BITS Pilani, and all the animal experiments were performed in accordance with the CPCSEA guidelines (Government of India). Animals were maintained under standard environmental conditions and provided with feed and water *ad libitum*. All the animals were fed on a normal pellet diet (NPD) one week prior to the experimentation. Hyperinsulinemia and hyperglycemia were induced by High Fat Diet (HFD) and HFD/STZ (35 mg/kg, *I.P*) treatment respectively, as per the protocol described by Gaikwad et al., [15]. Briefly, rats were allocated to two dietary regimens either Normal Pellet Diet (NPD, n = 24) or HFD (n = 36) *ad libitum* respectively for an initial period of 2 weeks. After 2 weeks of dietary manipulation, the rats from the HFD-fed group were injected with a low dose of STZ (35 mg/kg, *I.P*) (n = 18), whereas the respective control rats were given vehicle citrate buffer (pH 4.4). After 4 weeks, rats fed with NPD, HFD and HFD + STZ were treated with esculetin (Sigma Aldrich) 50 and 100 mg/kg/day, *P.O* and respective control animals treated with vehicle (0.5% sodium carboxy methyl cellulose) for 2 weeks and the rats were allowed to feed on their respective diet till end of the study (n = 6) [7]. Body weight, biochemical estimations, and blood pressure measurements were performed at the end of 6 weeks.

2.2. Assessment of biochemical and hemodynamic changes

The blood samples were collected, plasma was separated and fasting plasma glucose (PGL), triglycerides (PTGs), and total cholesterol (PTC) were estimated as per manufacturer's instructions by using commercially available spectrophotometric kits (Accurex Biomedical Pvt. Ltd., Mumbai, India). Plasma insulin (PI) was estimated by ultra sensitive rat insulin kit (Crystal Chem, IL, USA) [16]. Systolic blood pressure (SBP) was recorded on the last day of the treatment in all groups using a tail cuff blood pressure recorder (AD Instruments, Australia) [17].

2.3. Vascular tissue experiments

After development of hyperinsulinemia and hyperglycemia, animals were sacrificed. The thoracic aorta (from the arch of aorta to the diaphragm) was quickly excised and placed in ice-cold oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit buffer (KHB). The aorta was cut into 5 mm segments after it was cleaned of adhering fat and adventitial tissues. This was freed from fat and connective tissue. Care was taken not to stretch the vessel. Aortic rings were made by cutting it with sharp micro scissors and were placed in 10 ml organ bath containing a modified Krebs Henseleit buffer (KHB) (NaCl – 119 mM, KCl – 4.75 mM, NaH_2PO_4 – 1.19 mM, $MgSO_4$ – 1.19 mM, $CaCl_2$ – 2.54 mM, $NaHCO_3$ – 25 mM, and glucose – 11 mM) of pH 7.4. The solution was continuously aerated with carbogen (95% O_2 + 5% CO_2) at 37 °C. Tissue was subjected to equilibration for 120 min under the tension of 2 g. The bath fluid was changed for every 15 min. Then, contraction was measured isometrically by using force transducer (77005F, UGO Basile, Italy). At the end of every experiment, tissue was subjected to drying. Then, dried weight of tissue was taken for the calculation of contraction in terms of tension [5,18].

2.4. Experimental design for vascular studies

Aortic ring was suspended by a pair of stainless steel hooks in water-jacketed organ bath [UGO Basile, Italy], filled with 10 ml of oxygenated Krebs-Henseleit buffer (KHB) maintained at 37 °C. A resting tension of 2 g was applied to the aortic rings, which were then allowed to equilibrate for 2 h and the buffer was changed every 15 min. The tissue was exposed to 80 mM KCl depolarizing solution. After two such challenges, cumulative contractile responses to increasing concentrations of Ang II (1 nM–30 μM) were recorded. Aortic rings were pre-contracted with sub-maximal concentration of phenylephrine (PE, 100 nM) to evaluate acetylcholine (1 nM–30 μM) induced vasodilatation [3,18]. The responses were normalized to cross sectional area of the tissue and the tension developed was calculated as described before [3,5,18].

2.5. Immunohistochemistry

Immunohistochemistry was performed as per the protocol described by Gaikwad et al., [17]. Briefly, from each rat, portion of aorta tissue was fixed in 10% (v/v) formalin in phosphate buffer solution (PBS) and embedded in paraffin after completing the routine processing. For immunohistochemistry, 5 μm sections were taken from paraffin blocks and deparaffinised with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L, pH-6). The following primary antibodies were used: anti- Angiotensin II type 1 receptors (AT_1R), anti- Angiotensin II type 2 receptors (AT_2R), anti- Angiotensin-converting enzyme 2 (ACE2) (rabbit, 1:50 dilution) (Santa Cruz Biotechnology, CA, USA), anti-kelch ECH Associated Protein 1(Keap1), anti- Transforming growth factor-beta (TGF-β) (rabbit, 1:50 dilution) (Cell Signaling Technology, MA, USA) and HRP linked anti-rabbit secondary antibody was used, followed by detection with diaminobenzidine (DAB) as a chromogen. Slides were counterstained with haematoxylin, dehydrated by using alcohols and xylene, and mounted in DPX (Sigma Aldrich). For each group, at least 25 aorta sections were observed under microscope (Olympus BX41, NY, USA) and images were captured. The intensity of the spots was graded from 1 to 4 (1, slight or no color; 2, very low color; 3, moderate brown color; and 4, very intense brown color) [18]. The immunohistochemistry score is expressed as means ± S.E.M. for each experimental group.

2.6. Statistical analysis

Experimental values were expressed as means ± S.E.M. Statistical comparison between different groups was performed using one way analysis of variance (ANOVA) and if F value was significant

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