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VASCULOPROTECTIVE MECHANISMS OF RESVERATROL IN HIGH GLUCOSE-INDUCED VASCULAR ENDOTHELIAL DYSFUNCTION: AN IN VITRO MODEL OF DIABETES

YÜKSEK GLUKOZA BAĞLI VASKÜLER ENDOTELYAL DİSFONKSİYONDA RESVERATROLÜN VAZOPROTEKTİF MEKANİZMALARI: BİR İN VİTRO DİYABET MODELİ

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ABSTRACT

Objective: Diabetes mellitus is characterized by hyperglycemia, which is associated with micro- and macrovascular complications. This study aimed to evaluate the effects of high glucose on the mechanisms of vascular endothelial dysfunction (VED) in diabetes mellitus using an in vitro diabetes model, and to elucidate the mechanisms by which resveratrol, previously shown to improve vascular responses in vivo, exerts its beneficial effects in a high glucose environment.

Methods: Isolated rat thoracic aortic rings were incubated for 2 hours in normal glucose (NG), high glucose (HG,44 mM), HG +10^{-5} M resveratrol (HG+Resv), or high sucrose (HS) buffers. Endothelium-dependent (carbachol) and -independent (sodium nitroprusside, SNP) relaxation, and phenylephrine (Phe)-induced contraction responses were assessed. To investigate the mechanisms of resveratrol, endothelium-dependent relaxations were recorded separately following a 2-hour incubation with L-NAME, methylene blue, indomethacin, tetraethylammonium, glibenclamide, and 4-aminopyridine.

Results: While responses to KCl and SNP were similar in all groups, HG significantly impaired carbachol-induced endothelium-dependent relaxation and enhanced Phe-induced contraction. Resveratrol co-incubation significantly restored both carbachol relaxation and Phe contraction towards NG levels. HS incubation yielded responses similar to NG, indicating impaired relaxation was independent of hyperosmolality. Furthermore, L-NAME and methylene blue significantly suppressed carbachol relaxation in the HG+Resv group, as did tetraethylammonium and 4-aminopyridine. Conversely, indomethacin and glibenclamide had no significant effect, suggesting limited roles for PGI_2 and K_{ATP}.

Conclusion: Resveratrol prevents high glucose-induced VED by modulating the NO-sGC-cGMP pathway and activating K_{Ca} and K_V channels, while also diminishing enhanced contractile responses. These findings highlight resveratrol's potential as a protective agent against diabetic vascular complications.

Keywords: Resveratrol, high glucose, hyperglycemia, thoracic aorta, vascular endothelial dysfunction

ÖZ

Amaç: Diyabetes mellitus, mikro ve makrovasküler komplikasyonlarla ilişkili hiperglisemi ile karakterizedir. Bu çalışma, yüksek glukozun diyabetes mellitustaki vasküler endotelyal disfonksiyon (VED) mekanizmaları üzerindeki etkilerini bir in vitro diyabet modeli kullanarak değerlendirmeyi ve daha önce in vivo olarak vasküler yanıtları iyileştirdiği gösterilen resveratrolün, yüksek glukoz ortamında faydalı etkilerini hangi mekanizmalarla gösterdiğini aydınlatmayı amaçlamıştır.

Yöntem: İzole sıçan torasik aort halkaları, normal glukoz (NG), yüksek glukoz (HG, 44 mM), HG + 10^{-5} M resveratrol (HG+Resv) veya yüksek sükroz (HS) tamponlarında 2 saat inkübe edildi. Endotele bağımlı (karbakol) ve -bağımsız (sodyum nitroprussid, SNP) gevşeme ile fenilefrin (Phe) kaynaklı kasılma yanıtları değerlendirildi. Resveratrolün mekanizmalarını araştırmak için, endotele bağımlı gevşemeler, L-NAME, metilen mavisi, indometasin, tetraetilamonyum, glibenklamid ve 4-aminopiridin ile 2 saatlik inkübasyon sonrası ayrı ayrı kaydedildi.

Bulgular: KCl ve SNP'ye verilen yanıtlar tüm gruplarda benzerken, HG karbakol kaynaklı endotele bağımlı gevşemeyi anlamlı ölçüde bozdu ve Phe kaynaklı kasılmayı artırdı. Resveratrol ile birlikte inkübasyon, hem karbakol gevşemesini hem de Phe kasılmasını NG seviyelerine yakınlaştırdı. HS inkübasyonu NG'ye benzer yanıtlar verdi, bu da bozulmuş gevşemenin hiperozmolaliteden bağımsız olduğunu gösterdi. Ayrıca, L-NAME ve metilen mavisi, HG+Resv grubundaki karbakol gevşemesini anlamlı ölçüde baskıladı; tetraetilamonyum ve 4-aminopiridin de benzer etki gösterdi. Buna karşılık, indometasin ve glibenklamidin anlamlı bir etkisi olmadı, bu da PGI_2 ve K_{ATP} için sınırlı roller olduğunu düşündürdü.

Sonuç: Resveratrol, NO-sGC-cGMP yolunu modüle ederek ve K_{Ca} ile K_V kanallarını aktive ederek yüksek glukoza bağlı VED'i önlerken, artan kasılma yanıtlarını da azaltmaktadır. Bu bulgular, resveratrolün diyabetik vasküler komplikasyonlara karşı potansiyel bir koruyucu ajan olduğunu vurgulamaktadır.

Anahtar Kelimeler: Resveratrol, yüksek glukoz, hiperglisemi, torasik aorta, vasküler endotel disfonksiyon

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Introduction

Diabetes mellitus (DM) is a complex metabolic disorder fundamentally characterized by hyperglycemia, which results from either insufficient insulin secretion or compromised insulin action.[1,2] This systemic condition is associated with widespread involvement across multiple organ systems. The World Health Organization (WHO) classifies DM into distinct categories, including Type 1 Diabetes, Type 2 Diabetes (T2DM), hybrid forms, and other specific types.[3] Although these classifications differ in their underlying pathophysiology, prognosis, and management strategies, sustained hyperglycemia remains the definitive common hallmark of all DM phenotypes.[3]

Hyperglycemia is a potent risk factor strongly implicated in the pathogenesis and progression of both microvascular and macrovascular complications of diabetes.[4,5] The heightened risk observed in individuals with T2DM is often attributed to the silent nature of hyperglycemia, which can lead to extended periods of undiagnosed disease progression.[3,6] Furthermore, DM significantly predisposes patients to increased susceptibility to cardiovascular and cerebrovascular events, thus representing a major contributor to diabetes-related morbidity and mortality.[7,8] Consequently, elucidating the precise mechanisms underlying these complications is critical for developing effective prevention strategies.

Specifically, acute or postprandial hyperglycemia has been rigorously identified as a causative factor in both micro- and macrovascular diabetic complications.[8,9] Experimental models designed to simulate glycemic variability have demonstrated that alternating periods of hyperglycemia and normoglycemia induce more severe endothelial dysfunction and greater oxidative stress when compared to persistent, stable hyperglycemia.[10] Consistent evidence from both clinical observations in diabetic patients and established experimental animal models further confirms that diabetes and hyperglycemia directly induce endothelial dysfunction *in vivo*.[11–15] The molecular mechanisms contributing to acute hyperglycemia-induced endothelial dysfunction are multifaceted, involving a reduction in nitric oxide (NO) bioavailability, enhanced formation of advanced glycation end-products, and elevated states of oxidative stress and inflammation.[5,8,11,12,16,17] Earlier animal studies have specifically documented a marked attenuation of endothelium-dependent relaxation responses alongside an increase in phenylephrine (Phe)-mediated contraction responses within diabetic models.[13,18,19]

Resveratrol, a natural polyphenolic phytoalexin stilbene compound highly abundant in grapes and wine, has become a subject of intense scientific scrutiny.[20] Pre-clinical and clinical investigations have revealed a broad spectrum of properties, including antioxidant, anti-inflammatory, vasodilator, anti-proliferative, and anti-hypertensive effects relevant to cardiovascular pathology.[21–24] Significantly, chronic administration of resveratrol has been proven to ameliorate diabetes-induced vascular endothelial dysfunction *in vivo*.[14,24] This protective action is primarily attributed to mechanisms involving the upregulation of endothelial nitric oxide synthase (eNOS) levels and the reduction of free radical production.[14,24] Furthermore, studies investigating the vasorelaxant properties of resveratrol in isolated aortic preparations (independent of diabetic pathology) have shown its capacity to stimulate the release of NO and/or prostaglandin (PGI_2). Its mechanisms of action also encompass the opening of various potassium (K^+) channels, inhibition of intracellular calcium (Ca^{2+}) release from the sarcoplasmic reticulum, and blockade of calcium channels, collectively contributing to both endothelium-dependent and -independent relaxation responses.[25–27]

Given the established causative link between acute hyperglycemia and endothelial dysfunction, coupled with the documented efficacy of resveratrol in mitigating diabetic vascular endothelial dysfunction in *in vivo* settings, there is a compelling scientific rationale to investigate resveratrol's effects and underlying mechanisms within an acute hyperglycemic state relevant to diabetic vascular complications. The *in vitro* diabetes model represents a reliable and validated experimental approach for studying the direct impact of hyperglycemia or glucose fluctuations on vascular function—a critical component in the etiopathogenesis of all DM types.[28,29] This model typically involves the prolonged incubation of isolated smooth muscle tissue in a high-glucose medium to monitor functional alterations. Therefore, the primary objective of this study was to evaluate the effects of high glucose on the mechanisms mediating vascular endothelial dysfunction in diabetes mellitus using an *in vitro* model. Specifically, the study aimed to elucidate the mechanisms by which resveratrol, previously shown to improve vascular responses through chronic *in vivo* administration, exerts its beneficial effects within an acute *in vitro* high glucose environment. The investigation was particularly focused on determining the definitive role of nitric oxide and specific potassium channels in mediating resveratrol's ameliorative effect on high glucose-induced vascular dysfunction. The anticipated findings from this research are expected to provide critical insights for the future development of novel therapeutic strategies aimed at the prevention and management of diabetes-induced cardiovascular complications

Methods

Preparation of the Thoracic Aorta and *in vitro* Evaluation of Vascular Reactivity

Experiments were conducted on adult male Wistar albino rats (weighing 250-350 g), sourced from the Experimental Medical Research and Application Center at Kocaeli University, Turkey. Rats were housed under standard laboratory conditions with ad libitum access to food and water. All experimental procedures received ethical approval from the Kocaeli University Animal Experiments Local Ethics Committee (11/9-2017).

The experimental design adhered to the principles of the 3R rule (Replacement, Reduction, Refinement) for animal use. Specifically, the total number of animals was minimized (Reduction) by maximizing the utilization of the tissue obtained; an average of approximately 3 individual thoracic aortic rings were consistently harvested from each rat. This comprehensive tissue use resulted in the utilization of a minimal number of 20 rats (N=20) to achieve the necessary statistical power, yielding a total of 60 rings (n=60) for all experimental groups and inhibitor studies. To maximize data efficiency and ensure methodological consistency, the concentration-response curve data obtained for the HG + Resv group's cumulative carbachol relaxation response serves as a common control across multiple figures. Specifically, the HG + Resv relaxation responses presented in Figure 1A are the identical dataset used for pairwise comparisons against the inhibitor groups in Figure 3 (L-NAME and Methylene Blue) and Figure 4 (Indomethacin). This approach ensures that all inhibitor effects are assessed against the same, standardized protective control response.

Rats were euthanized with deep ketamine/xylazine anesthesia. The descending thoracic aortas, extending from the aortic arch to the diaphragm, were immediately excised and placed in cold, oxygenated modified Krebs buffer (composition: NaCl 118 mM, KCl 4.7 mM, $MgSO_4.7H_2O$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.5 mM, and glucose 11.1 mM).[28] After careful purification from surrounding connective tissue, aortas were dissected into 3-5 mm rings.

Aortic rings were then mounted in 20 mL organ bath chambers between a stainless-steel tissue holder and a hook connected to the force transducer. The buffer was maintained at 37 °C by a thermoregulated water circuit and continuously aerated with a 95% O_2-5% CO_2 mixture, ensuring a pH of 7.4. Rings underwent a 2-hour equilibration period in the organ baths, during which the buffer was refreshed every 30 minutes.

During the incubation phase, aortic rings were exposed for 2 hours to modified Krebs buffer containing: 11 mM normal glucose (NG), 44 mM high glucose (HG), 44 mM high glucose + 10^{-5} M resveratrol (HG + Resv), or 33 mM high sucrose+11mM glucose (HS).[28–30] A constant 1 gram pre-tension was maintained throughout the experiment. Isometric tensions of each aortic ring were measured using a MAY-COM FDT 10A force-displacement transducer and recorded via a computer-based data acquisition system (MP30B-CE; Biopac Systems, Santa Barbara, CA, USA) with Biopac BSL Pro 3.7 software.

Agonist-Induced Contractions and Relaxations

Agonist-induced contractions

The viability of aortic rings was initially confirmed by exposure to an 80 mM high K+ solution, where NaCl was equimolarly replaced with KCl. Once the maximal KCl-induced contraction plateau was reached, rings were thoroughly washed and allowed to return to their pre-contracted tension. Following this initial viability assessment and washout period, cumulative contraction curves were then generated using Phe at concentrations ranging from 10^{-9} M to 10^{-4} M. Each Phe concentration was added incrementally after the preceding concentration achieved a steady-state response.

Agonist-induced relaxations

Each aortic ring was submaximally pre-contracted with Phe ($3x10^{-6}$-10^{-5} M). Upon reaching a stable plateau contraction, cumulative concentration-dependent relaxation curves were obtained for carbachol (10^{-9}-10^{-5}M) or SNP (10^{-10}-10^{-4} M) across all buffer conditions. Agonist concentrations were increased only after the previous concentration reached a plateau. Between successive cumulative concentration-response curves, aortic rings were washed twice with fresh buffer and allowed to restore their basal tension.

Vasculoprotective Effects of Resveratrol and Mechanistic Investigations

Vasculoprotective effects of resveratrol

To assess the protective role of resveratrol on vascular reactivity following high glucose exposure, endothelium-dependent vascular relaxations to carbachol were recorded in a separate set of experiments. These recordings were conducted in the presence of 10^{-5} M resveratrol,[29] which was incubated for 2 hours in all types of modified Krebs buffer previously described.

Identifying the possible endothelial mediators associated with the protective effects of resveratrol on vascular reactivity

To elucidate the potential mechanisms underlying resveratrol's protective effect against high glucose-induced vascular endothelial dysfunction, the relaxant responses to cumulative carbachol were re-evaluated in the presence of various pharmacological inhibitors. These agents included: endothelial nitric oxide synthase (eNOS) inhibitor Nω-nitro-L-arginine-methylester (L-NAME; (10^{-4} M), cyclic guanosine monophosphate (cGMP) inhibitor methylene blue (MB; 10^{-5} M), cyclooxygenase (COX) inhibitor indomethacin (10^{-5} M), a nonselective calcium-activated K^+ channel (K_{Ca}) blocker tetraethylammonium (TEA; 6 mM), a voltage-dependent K^+ channel (K_V) blocker 4-aminopyridine (4-AP; 3 mM), and a non-specific ATP-sensitive K^+ channel (K_{ATP}) blocker glibenclamide (10^{-5} M).[25,29,31]

Drugs and Reagents

All chemicals and drugs, including resveratrol, phenylephrine hydrochloride (Phe), carbamylcholine chloride (Carbachol), sodium nitroprusside dihydrate (SNP), papaverine hydrochloride, indomethacin, L-NAME, 4-AP, TEA, Gli, MB, ethanol, and dimethyl sulfoxide (DMSO), were procured from Sigma-Aldrich (St. Louis, MO, USA).

All drugs and solutions were freshly prepared and dissolved in distilled water, with specific exceptions.

Indomethacin and resveratrol stock solutions were prepared in absolute ethanol, while the glibenclamide stock solution was dissolved in DMSO. Subsequent serial dilutions for all drugs were made using distilled water. The vehicle final concentration was 0.1 % (v/v) DMSO and 0.1 % (v/v) ethanol. Under these conditions, vehicles alone did not alter baseline tension or agonist-induced responses. The choice of 0.1 % DMSO is supported by prior work in which low concentrations of DMSO (≤0.1 %) are generally considered non-perturbing in pharmacological assays.[32] On the ethanol side, concentrations up to 0.1 % (v/v) are widely used as vehicle levels in *in vitro* studies without appreciable cytotoxic or functional effects.[33]

Statistical Analysis

All collected data are presented as the mean ± standard error of the mean (SEM). For contraction measurements: KCl-induced contraction was quantified as milligrams of developed tension. Cumulative Phe-induced contractile force was expressed as a percentage of the maximal contraction induced by KCl. For relaxation measurements: Agonist-induced relaxation was expressed as a percentage of the pre-contraction induced by $3x10^{-6}$-10^{-5} M Phe.

Student's t-test was utilized to determine significant differences between two experimental groups. For comparisons involving three or more groups, one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test was employed. A *P*-value of <0.05 was considered to indicate statistical significance. All statistical computations were performed using GraphPad Prism 9.3.0 (GraphPad Software Inc., CA, USA).

Results

Effects of High-Glucose, High-Sucrose, and Resveratrol Incubations on Vascular Relaxation Responses

Cumulative concentration-dependent relaxation responses to carbachol (10^{-9}-10^{-5} M) were evaluated in isolated thoracic aortas from NG, HG, HG + Resv, and HS groups, all pre-contracted with submaximal concentrations of Phe (Figure 1A).

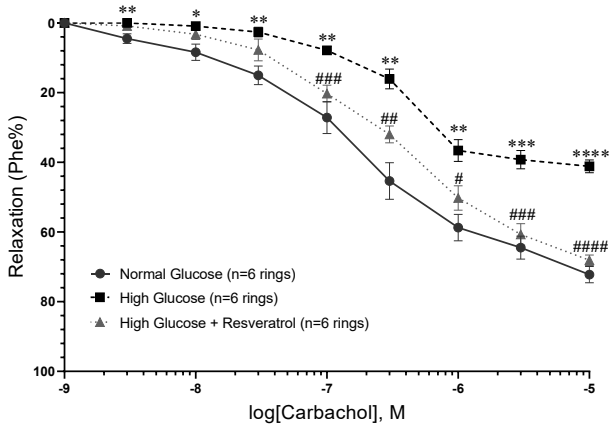
Endothelium-dependent relaxation responses to carbachol were significantly reduced in aortic rings incubated in HG compared to those in NG (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001). This finding demonstrates that exposure to a high glucose medium causes a significant impairment of endothelium-dependent relaxation responses in the thoracic aorta. However, the co-incubation with resveratrol during high glucose (HG + Resv) significantly restored these responses, increasing carbachol-induced relaxation compared to the HG group and bringing them closer to normoglycemic levels (#*P*<0.05, ##*P*<0.01, ###*P*<0.001, ####*P*<0.0001).

Conversely, HS incubation did not significantly alter carbachol-induced relaxation responses compared to NG (*P* > 0.05; Supplementary Figure 1). This suggests that the observed decrease in relaxation responses was specifically attributable to high glucose, rather than hyperosmolarity. Consequently, the high sucrose incubation series was excluded from subsequent analyses.

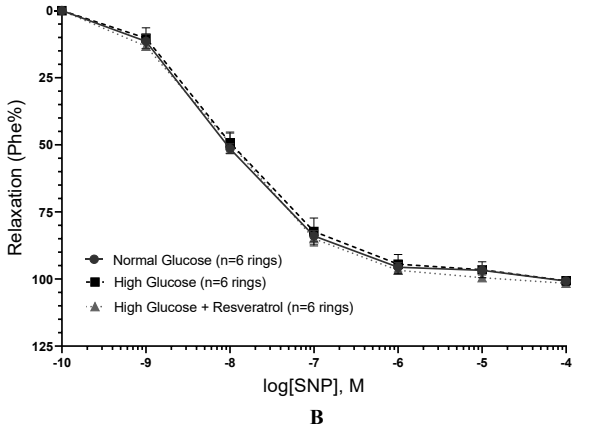
Effects of High-Glucose and Resveratrol Incubations on Endothelium-Independent Relaxation Responses

Endothelium-independent relaxation responses induced by SNP (10^{-10}-10^{-4} M) showed no significant differences among the NG, HG, and HG + Resv groups (Fig. 1B). Group differences in relaxation at each cumulative dose of SNP were determined by one-way ANOVA followed by Tukey's post-hoc test; at the concentration of 10^{-9} M the inter-group difference was F (2, 15) = 0.3175 value, *P*=0.7328; at 10^{-8} M F (2, 15) = 0.1460 value, *P*=0.8654; at 10^{-7} M F (2, 15) = 0.1841 value, *P*=0.8337; at 10^{-6} M F (2, 15) = 0.2269 value, *P*=0.7997; at 10^{-5} M F (2, 15) = 0.7272value, *P*=0.4995; at 10^{-4} M F (2, 15) = 0.2548 value, *P*=0.7784.

Figure 1. High glucose-induced endothelial dysfunction and protection by resveratrol



A



B

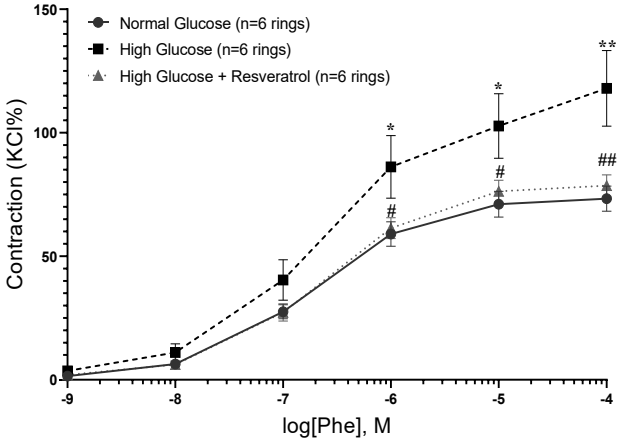
*****Concentration-response curves of similarly pre-contracted thoracic aortic rings (Phe $3x10^{-6}$-10^{-5} M) incubated in Normal Glucose (NG), High Glucose (HG), or High Glucose + Resveratrol (HG+Resv) **A.** Endothelium-dependent relaxation responses to cumulative Carbachol (10^{-9}-10^{-5} M) **B.** Endothelium-independent relaxation responses to cumulative SNP (10^{-10}-10^{-4} M). Data are expressed as mean ± SEM. Statistical Analysis: One-way ANOVA followed by Tukey's post-hoc test was used for inter-group comparisons. Six isolated thoracic rings were examined from different rats for each group (* *P*<0.05, ** *P*<0.01, ****P*<0.001, **** *P*<0.0001 vs. NG; # *P*<0.05, ## *P*<0.01, ### *P*<0.001, #### *P*<0.0001 vs. HG).

Effects of High-Glucose and Resveratrol Incubations on Contraction Responses

The maximum contractile response (E_{max}) induced by 80 mM KCl was assessed across all experimental groups to determine if high glucose or resveratrol incubation directly affected basal vascular smooth muscle contractility. One-way ANOVA revealed no statistically significant difference in the KCl-induced E_{max} values among the Normal Glucose, High Glucose, High Sucrose, and High Glucose + Resveratrol groups [F (3,20) = 0.07103, *P*=0.9748]. This finding indicates that neither the high glucose exposure nor the resveratrol co-incubation altered the maximal contractile capacity of the vascular smooth muscle, confirming that the subsequent changes in receptor-mediated responses are likely due to modulations in endothelial function or specific signaling pathways.

Cumulative addition of Phe (10^{-9} M–10^{-4} M) induced concentration-dependent contractile responses in all groups. Compared to the NG group, the HG group exhibited significantly enhanced contractile responses across the concentration range of 10^{-6} M to 10^{-4} M Phe. This difference was confirmed by the student's t-test at each concentration (**P*<0.05; **P*<0.05; ***P*<0.01, respectively; Figure 2). Specifically, the most pronounced difference was observed at the 10^{-4} M concentration (*P*=0.0059). Crucially, this observed increase was fully prevented in the HG + Resveratrol (HG + Resv) group, which displayed responses statistically indistinguishable from the NG control (*P*>0.05) across the concentration range of 10^{-6} M to 10^{-4} M Phe (*P* values are 0.7277, 0.4666, and 0.4496, respectively). Furthermore, contractile responses in the HG + Resv group were significantly reduced compared to the HG group (#*P*<0.05; #*P*<0.05; ##*P*<0.01, respectively).

Figure 2. Effect of high glucose and resveratrol on phenylephrine-induced contractility



Concentration-dependent contraction responses to cumulative Phenylephrine (10^{-9}-10^{-4}M) in aortic rings incubated in Normal Glucose (NG), High Glucose (HG), and High Glucose + Resveratrol (HG + Resv). Contractile force is expressed as a percentage of the 80 mM KCl-induced maximal contraction. Statistical Analysis: Student's t-test was used for pairwise comparisons between groups at each concentration. Data are expressed as mean ± SEM. Six isolated thoracic rings were examined from different rats for each group (*P*<0.05, ** *P*<0.01 vs. NG; # *P*<0.05, ## *P*<0.01 vs. HG).

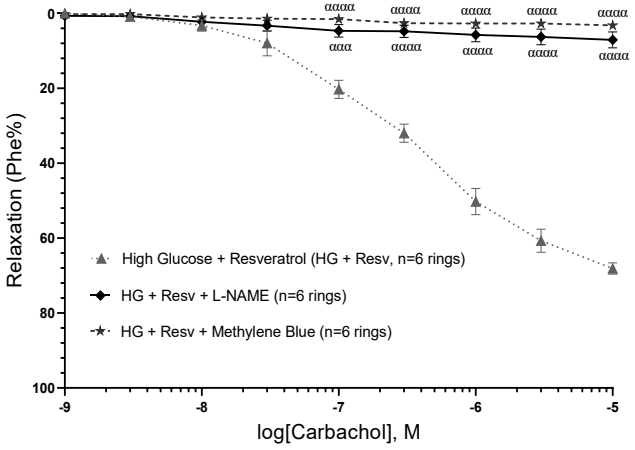
Role of Endothelial Mediators in Resveratrol's Vasculoprotective Effects

Role of eNOS and cGMP

To evaluate the potential role of the endothelium-dependent NO-sGC-cGMP pathway in resveratrol's vasculoprotective effects, concentration-dependent relaxation responses to carbachol (10^{-9}-10^{-5} M) were separately assessed in submaximally Phe-contracted thoracic aortic rings. This assessment was performed in the presence of the eNOS inhibitor L-NAME (10^{-4} M) and the cGMP blocker MB (10^{-5} M).

Compared to aortic rings incubated in high glucose with resveratrol alone (HG + Resv), the addition of both L-NAME (HG + Resv + L-NAME) and methylene blue (HG + Resv + MB) resulted in a significant and marked reduction in carbachol-induced relaxation responses. This reduction began at carbachol concentrations of 10^{-7} M and continued through the maximal concentration (10^{-5} M). Specifically, Student's t-tests comparing the HG + Resv group against the inhibitor groups demonstrated highly significant differences (ααα*P*<0.001 αααα*P*<0.0001 compared with HG + Resv, Figure 3). These findings strongly indicate that the NO-sGC-cGMP pathway is of critical importance in mediating the protective effect of resveratrol against high glucose-induced endothelial dysfunction.

Figure 3. Role of NO-sGC-cGMP pathway in resveratrol's protective effect



*Concentration-response curves to Carbachol (10^{-9}-10^{-5} M) in HG + Resv rings incubated with the eNOS inhibitor L-NAME (10^{-4} M) or the sGC inhibitor Methylene Blue (10^{-5} M). Statistical Analysis: Student's t-test was used for pairwise comparisons between HG + Resv and inhibitor groups at each concentration. Data are expressed as mean ± SEM. Six isolated thoracic rings were examined from different rats for each group. (ααα*P*<0.001, αααα *P*<0.0001 vs. HG + Resv).

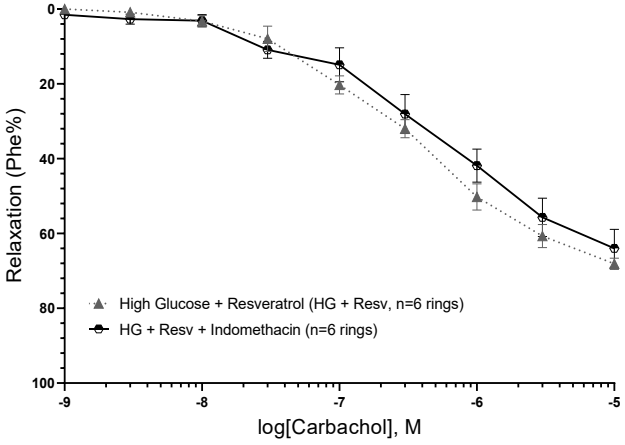
Role of PGI_2

To examine the possible role of PGI_2, another endothelium-dependent relaxing factor, in the vasculoprotective effects of resveratrol, carbachol-induced (10^{-9}-10^{-5} M) relaxation responses were obtained in Phe-precontracted thoracic aortic rings in the presence

of indomethacin (10^{-5} M), an inhibitor of COX, the enzyme that synthesizes PGI_2.

While a modest reduction in the carbachol-induced relaxation response was observed with indomethacin incubation at carbachol concentrations of 10^{-8} M and higher, this reduction was not statistically significant when compared to responses from aortic rings co-incubated with high glucose and resveratrol alone. Student's t-tests confirmed this finding, showing no significant difference between the HG + Resv and HG + Resv + Indomethacin groups across the entire concentration range (P>0.05, Figure 4). This observation indicates that the involvement of PGI_2 is limited in mediating the protective effect of resveratrol against high glucose-induced endothelial dysfunction in this specific *in vitro* context.

Figure 4. Role of PGI_2 in resveratrol's protective effect



*Concentration-response curves to Carbachol (10^{-9}-10^{-5} M) in HG + Resv rings incubated with the COX inhibitor Indomethacin (10^{-5} M). Statistical Analysis: Student's t-test was used for pairwise comparisons between HG + Resv and the Indomethacin group at each concentration. Data are expressed as mean ± SEM. Six isolated thoracic rings were examined from different rats for each group.

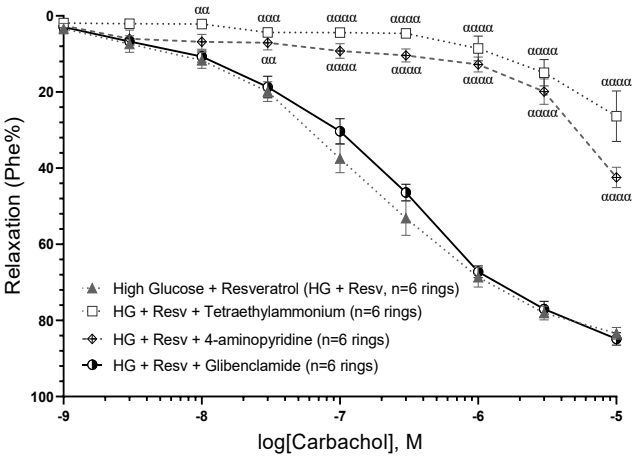
Role of hyperpolarizing K^+ channels

To investigate the possible involvement of K^+ channels in resveratrol's vasculoprotective effects, carbachol-induced (10^{-9}-10^{-5} M) concentration-dependent relaxation responses were studied in Phe-precontracted thoracic aortic rings. This was performed in the presence of TEA (6 mM), 4-AP (3 mM), and glibenclamide (Gli; 10^{-5} M).

Cumulative carbachol relaxation responses were significantly suppressed in the presence of both TEA and 4-AP in the high glucose and resveratrol co-incubated medium (Figure 5). Student's t-tests comparing the HG + Resv group with the TEA and 4-AP groups, the suppression was significant, starting at lower concentrations and intensifying dose-dependently (ααP<0.01, αααP<0.001, ααααP<0.0001). This indicates that both calcium-activated K^+ channels (K_{Ca}) and voltage–dependent K^+ channels (K_V) are critical mediators of resveratrol's ameliorative effect.

Conversely, the addition of glibenclamide (a K_{ATP} inhibitor) to the HG + Resv medium resulted in no statistically significant change in carbachol-induced vasorelaxant responses compared to HG + Resv alone. This lack of difference was confirmed by the Student's t-test across the entire concentration range (P>0.05, Figure 5). Therefore, the present findings indicate that K_{Ca} and K_V channels are essential, while ATP-sensitive K^+ channels do not play a significant role in this protective mechanism.

Figure 5. Role of hyperpolarizing K^+ channels in resveratrol's protective effect



*Concentration-response curves to (10^{-9}-10^{-5} M) in HG + Resv rings incubated with K+ channel inhibitors: TEA (6 mM), 4-AP (3mM), and Glibenclamide (10^{-5} M). Statistical Analysis: Student's t-test was used for pairwise comparisons between HG + Resv and each inhibitor group at each concentration. Data are expressed as mean ± SEM. Six isolated thoracic rings were examined from different rats for each group. (ααP<0.01, ααα P<0.001, αααα P<0.0001 vs. HG + Resv).

Discussion

Our primary findings collectively demonstrate that *in vitro* exposure of endothelium-intact thoracic aortic preparations to high glucose (HG) significantly impairs endothelium-dependent vascular relaxation and enhances phenylephrine (Phe)-mediated contractile responses. Importantly, endothelium-independent responses (SNP-induced) were unaffected. Crucially, we further show that resveratrol co-incubation effectively ameliorates this HG-induced vascular dysfunction. This vasculoprotective effect is primarily mediated by modulating the nitric oxide (NO)-sGC-cGMP pathway and specific calcium-activated (K_{Ca}) and voltage-dependent (K_V) potassium channels. Conversely, PGI_2 and ATP-sensitive potassium channels (K_{ATP}) appear to play limited or negligible roles in this context.

This study employed a well-established *in vitro* hyperglycemia model, incubating isolated thoracic aortic rings from healthy Wistar rats in 44mM HG buffer for two hours. This duration models postprandial high glucose levels,[2,28] and was specifically chosen to isolate and precisely control the hyperglycemic condition. This approach allowed for a focused investigation into the immediate, direct impact of HG on vascular function, independent of systemic confounding factors present in

in vivo models. This aligns with the literature suggesting that acute hyperglycemia, even at sub-symptomatic levels[34], can induce rapid vascular changes.[35] The consistent maximum contractile response to 80 mM KCl across all groups confirms that neither HG nor resveratrol incubation, nor the osmotic changes (confirmed by high sucrose), significantly impacted voltage-dependent calcium channels or basal smooth muscle contractility. This finding, combined with the observation that endothelium-independent relaxation induced by sodium nitroprusside was also unaffected, strongly suggests that the observed HG-induced vascular changes are likely mediated primarily through receptor-mediated or endothelium-dependent pathways, rather than direct effects on smooth muscle function.

Our findings that HG incubation significantly increased concentration-dependent contractile responses to Phe is consistent with previously reported enhanced Phe-induced contractile responses in streptozotocin-induced diabetic rat aortas.[19,36,37] Crucially, the HG-induced increase in contractile responses to Phe was returned to control levels in the HG + Resv group. This result is in line with studies indicating that long-term resveratrol treatment can reduce the sensitivity of diabetic rabbit aortas to contractile responses.[38] Furthermore, our observations that HG-incubated aortic rings exhibited suppressed carbachol-induced endothelium-dependent relaxation (i.e, vascular endothelial dysfunction VED), while endothelium-independent SNP relaxation was unaffected, are compatible with numerous *in vitro*[28,29,39,40] and *in vivo*[14,19,41,42] studies examining the effects of hyperglycemia on the aorta. For example, Pieper (1999)[43] reported that VED became evident in in vivo rat models of diabetes at 8 weeks post-streptozotocin (STZ) injection, following an initial phase of unchanged responses. Similarly, our prior *in vivo* studies[14,44] demonstrated diminished carbachol-induced relaxation responses at four weeks post-STZ injection. The synthesis of these findings suggests that acute exposure to hyperglycemia, as demonstrated here, can indeed rapidly lead to VED that subsequently becomes chronic and evident in the later stages of diabetes *in vivo*.

Crucially, the co-incubation of resveratrol effectively prevented this HG-induced VED, corroborating the vasculoprotective effects of resveratrol observed in various experimental models of diabetes.[14,24] This result is consistent with Pektaş et al. (2018),[29] who also reported that acute HG exposure significantly decreased acetylcholine-mediated relaxation responses linked to Akt/eNOS pathway suppression, which was ameliorated by resveratrol co-incubation. Although our study used the same resveratrol dose, our experimental design focused on its preventive potential by co-incubating it throughout the HG exposure, whereas Pektaş et al.[29] investigated its therapeutic potential by introducing resveratrol only in the final 30 minutes.

We further investigated the role of the NO-sGC-cGMP pathway. The significant suppression of carbachol responses by L-NAME (an eNOS inhibitor) and methylene blue (a cGMP blocker) in the HG+Resv group strongly suggests that resveratrol protects endothelium-dependent responses by improving the synthesis or bioavailability of nitric oxide. These findings are supported by our previous *in vivo* study, which reported increased eNOS mRNA expression in diabetic rats chronically treated with resveratrol.[14] Similar observations, including increased eNOS mRNA expression and phosphorylated-eNOS/total-eNOS ratio, have also been reported in *in vitro* aortic rings co-incubated with resveratrol and HG.[29]

Regarding PGI_2, the only arachidonic acid metabolite with a known vasodilator effect,[45] its involvement in resveratrol-induced vasodilation has been noted in normoglycemic buffers.[25] However, direct comparison with this literature (Ref. 25) must consider key methodological distinctions: In the study by (Ref. 25), a cumulative concentration-response curve for resveratrol was generated following a 20-minute indomethacin incubation. Our methodology differed significantly, as our study focused on the cumulative carbachol response following a 2-hour high glucose and fixed resveratrol co-incubation, with indomethacin added only during the final 30 minutes of this 2-hour period. Furthermore, differences in experimental animal strains (Sprague Dawley vs. our Wistar male rats) and incubation duration may influence the basal activity of the COX/PGI_2 pathway. In our present study, although carbachol-dependent relaxation showed a modest, non-significant reduction in the presence of the cyclooxygenase inhibitor indomethacin, this observation did not reach statistical significance. This suggests that while resveratrol may potentially modulate cyclooxygenase enzymatic activity and compensate for the PGI_2-reducing effects of hyperglycemia,[46] its overall contribution to resveratrol's protective effect may be limited in this specific acute context. The lack of statistical significance for indomethacin's effect biologically suggests that the primary compensatory mechanism employed by resveratrol against acute HG-induced VED does not rely on the COX/PGI_2 pathway. Instead, it is highly likely that the dominant protective actions occur either through the NO-sGC-cGMP pathway or the hyperpolarization mechanisms via K^+ channels, as confirmed by our inhibition studies. This pathway's diminished role may be inherent to the rat thoracic aorta model or may indicate that the severe oxidative stress induced by HG completely overshadows any subtle PGI_2-related compensatory effect.

A notable finding is the demonstrated involvement of K_{Ca} and K_V, potassium channels, in addition to endothelium-mediated NO, in the protective effect of resveratrol on HG-induced VED. Conversely, K_{ATP} potassium channels appeared to play a negligible role. The identified involvement of K^+ channels aligns with previous in vitro studies on resveratrol's vasorelaxant effects. For instance, Tan et al.[25] and Shen et al.[26] observed the involvement of K_{Ca}, K_V, and K_{ATP} channels in promoting vascular hyperpolarization. However, direct comparison is complicated by distinct methodological approaches: These prior studies examined resveratrol's vasorelaxant

effect in healthy, normoglycemic Sprague Dawley rats using short inhibitor incubation times (10–20 minutes) during either cumulative or single-dose resveratrol administration. Our work, conversely, focuses on the 30-minute inhibitor effect following a 2-hour fixed-dose resveratrol pre-incubation specifically against high glucose-induced pathology in Wistar rats. This difference in K_{ATP} contribution might be attributed to two main factors: 1. Dose and Incubation Protocol: Our experimental setup involved adding glibenclamide (a non-specific K_{ATP} blocker) to the medium before obtaining endothelium-dependent carbachol cumulative responses, differing from protocols that test glibenclamide during cumulative resveratrol administration. The distinct incubation times and dose formats may alter K_{ATP} channel sensitivity. 2. Pathophysiological Context: Our study specifically investigated resveratrol's protective effect against high glucose-induced VED, while other researchers evaluated resveratrol's vasorelaxant effects in normoglycemic conditions. Given that diabetes mellitus has been reported to reduce K_{ATP} channel expression without affecting signaling pathways[47], it is plausible that resveratrol does not play a role in increasing the number of K_{ATP} channels, which are likely already reduced by hyperglycemia. The necessity of K_{Ca} and K_V in our model thus highlights a compensatory shift in the hyperpolarizing mechanism under diabetic stress. Further studies are warranted to explore the complex interplay between NO and K^+ channels in different endothelial integrity contexts.

In conclusion, this study successfully employed an *in vitro* diabetes model to investigate the effects of high glucose on the mechanisms of VED and examined the potential of resveratrol to attenuate these dysfunctions. Our findings strongly suggest that resveratrol effectively prevents HG-induced endothelial dysfunction primarily through the NO-sGC-cGMP pathway, K_{Ca} and K_V potassium channels. Additionally, resveratrol significantly diminished the enhanced Phe-mediated contraction caused by HG. These results highlight resveratrol's potential as a protective agent against acute HG-induced vascular disorders, mainly mediated via the NO-sGC-cGMP pathway and specific K^+ channels.

This study has a few inherent limitations that should be acknowledged. Firstly, the use of an acute, two-hour HG exposure may not fully replicate the chronic, sustained metabolic dysregulation observed in clinical diabetes. Secondly, the use of aortic tissue provides insights into large-vessel function but may not reflect changes in the microcirculation. Finally, the use of male Wistar rats restricts the generalizability of these specific findings to female subjects or other species. Future studies should explore the long-term *in vivo* implications of these mechanisms and their translational potential.

Ethical Approval

This study complies with the regulation on the welfare and protection of animals used for experiments and other scientific purposes, publicized in Turkey. Ethical approval was granted by the Kocaeli University Animal Experiments Local Ethics Committee with decision number 11/9-2017 and approval date 30/11/2017.

Conflict of Interest

All authors declare that there is no conflict of financial or non-financial interests in the content of this article.

Author Contributions

GC: Design, Data collection, Literature search, Data analysis, Manuscript writing, review& editing. SSG: Design, Project development, Literature search, Data analysis, Funding, Manuscript writing, review& editing. ES: Data collection, Data analysis, Manuscript review& editing. MEE: Data collection, Data analysis, Manuscript review& editing. TU: Design, Project development, Literature search, Funding, Critical review, Manuscript review& editing.

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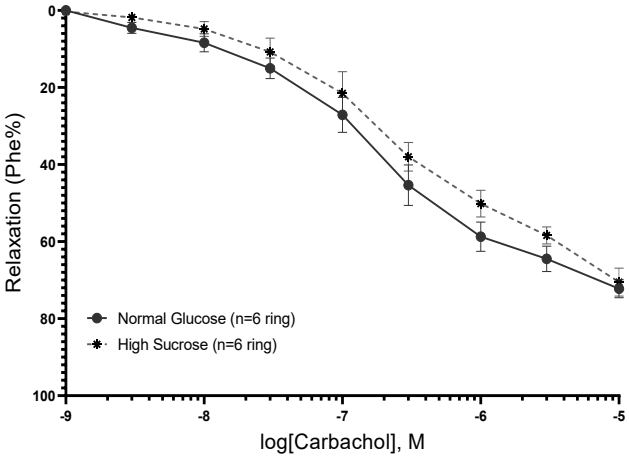
Supplementary Figure 1. The effect of hyperosmolarity induced by high sucrose on endothelium-dependent relaxation responses to carbachol in rat thoracic aorta.



*Concentration-response curves illustrating endothelium-dependent relaxation induced by cumulative additions of Carbachol (from 10^{-9} M to 10^{-5} M) in rat thoracic aortic rings pre-contracted with Phenylephrine (Phe). The rings were incubated for two hours in either Normal Glucose (11 mM glucose, solid line with circles) or High Sucrose (HS, 44 mM sucrose, dashed line with asterisks) buffer to confirm that the changes observed in the High Glucose group were not due to osmotic effects. Relaxation responses are expressed as a percentage of the maximal contraction induced by Phenylephrine. Data are presented as Mean ± SEM (n=6 rings per group). No significant difference was observed between the two groups (Student's t test, $P>0.05$), confirming that hyperosmolarity alone does not impair endothelium-dependent relaxation.