

Original Article**NORMAL VASCULAR REACTIVITY IS RESTORED BY APIGENIN IN DIABETIC RATS****HANY M. EL-BASSOSSY^{1,2}, NORA DESOKY², ABDULRAHMAN M ALAHDAL³, AHMED FAHMY²**

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Received: 15 Dec 2016 Revised and Accepted: 22 Nov 2017

ABSTRACT

Objective: Diabetes is a disease whose complications have serious implications for the health of sufferers; one of the most serious such complications is the deterioration of vascular reactivity. Apigenin is a natural flavonoid with PKC inhibiting and antioxidant properties. In this study, the impact of apigenin on vascular reactivity deterioration was investigated.

Methods: Insulin resistance (IR) and insulin deficiency (ID) were induced by fructose and streptozotocin respectively. The isolated aortae vasoconstriction response to phenylephrine (PE) and potassium chloride (KCl) in addition to the vasodilation response to acetylcholine (ACh) and sodium nitroprusside (SNP) were tested.

Results: IR and ID were associated with significantly exaggerated vasoconstriction to KCl and PE while significantly impaired vasodilation to ACh. Response to SNP was not significantly affected by both IR and ID. *In vitro* incubation with apigenin (7.7 μM) for 20 min restored normal responses to PE, KCl and ACh in aortae isolated from insulin-resistant or insulin-deficient rats. Incubation for one hour with the PKC stimulant, phorbol 12-myristate 13-acetate (PMA, 800 nM) resulted in aortic impairment similar to that seen in aortae isolated from IR and ID animals. Incubation with both apigenin prevented PMA-induced exaggerated vasoconstriction response to both PE and KCl.

Conclusion: Apigenin alleviates vascular exaggerated vasoconstriction and impaired dilation associated with diabetes or PKC activated.

Keywords: Apigenin, Diabetes, PMA, Isolated aorta, Vascular reactivity

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DOI: <http://dx.doi.org/10.22159/ijpps.2018v10i1.16621>

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases resulting either from insulin resistance or insulin deficiency [1]. The former manifests as a failure of insulin to perform its intended biological function of inducing cellular absorption of glucose during its circulation [2]. In an attempt to compensate for this, hyper-insulinaemia ensures, with the eventual result that β-cells are incapable of secreting sufficient insulin, leading to type 2 diabetes [3].

Complications of diabetes have serious, deleterious effects on patients' clinical status as they generally involve major organs. However, while significant progress has been made in the treatment of the symptoms of diabetes itself, the complications remain a major cause of considerable concern. The importance of preventing the complications [4] is particularly great in the case of changes in the structure and function of the vascular system, which themselves lead to further complications [5].

Flavonoids, considered to be important in the human diet, are phenylpropanoids found in many foods. Apigenin, a member of a non-mutagenic and less toxic [6] subclasses of flavone is found naturally in many plants. It has been reported [7] that apigenin is a powerful protein kinase C (PKC) inhibitor and antioxidant, with a range of pharmacological applications, many related to hyperlipidaemia [8] and hypertension [9].

While apigenin has reportedly induced vasodilation in the thoracic aorta of rats [10], its impact on the aorta of diabetic animals in isolated vascular preparations has yet to be confirmed. Therefore, the purpose of the current study was to investigate the effect of apigenin on the diabetes-associated exaggerated vasoconstriction and impaired vasodilation. In addition, the effect of apigenin on PKC induced vascular deterioration has been studied to investigate the apigenin effect on a major pathway of vascular complication in diabetes.

MATERIALS AND METHODS**Chemicals and drugs**

Fructose was obtained from El-Nasr Chemical Company (Cairo, Egypt) and apigenin, PE, ACh and sodium nitro-prusside (SNP) from Sigma-Aldrich (Munich, Germany). ACh, SNP and PE were dissolved in distilled water. Apigenin and phorbol 12-myristate 13-acetate (PMA) were first dissolved in DMSO and then diluted to a concentration of 10 % in the KHB.

Animals

In keeping with the standards of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, clear cages made from polypropylene were each used to house 3-4 male albino rats with a weight of 120-130g and obtained from Zagazig University, Zagazig, Egypt.

The rats were kept in stable environmental conditions with proportionate light-dark cycles and rodent-pellet food and purified water were supplied freely to them. Approval for the experimental protocol was obtained from the Zagazig Ethical Committee for Animal Handling. Ethical approval number is P6/4/2012.

Study protocol

24 rats were divided into experimental groups: insulin-resistant (IR) (n = 8), insulin-deficient (ID) (n = 8) and a control group (n = 8). In IR rats, insulin resistance was induced by the provision of drinking water containing 10 % fructose. Insulin resistance was confirmed by the presence of hyper insulinaemia (defined as 12–16 mg/l) after six weeks. Insulin deficiency in ID rats was induced by streptozotocin (STZ, 50 mg/kg, ip). Insulin deficiency was confirmed by the presence of stable hyperglycaemia (300-350 mg/dl) two weeks after STZ injection. The IR rats were left for 12 w after fructose administration; ID rats were left for 10 w after STZ injection.

The descending aorta was isolated twelve hours after the final injection and placed into a cold Krebs-Henseleit buffer (KHB). The KHB contained NaCl 118.1 mmol, NaHCO₃ 25.0 mmol, glucose 11.7 mmol, KCl 4.69 mmol, CaCl₂ 2.5 mmol, KH₂PO₄ 1.2 mmol and MgSO₄ 0.5 mmol. After clearing the aorta of connective tissue and adherent fat, it was divided into three rings, each approximately 3 mm in length. In order to investigate the vascular reactivity, one aortic ring was mounted in the organ bath. The impact of apigenin on vascular resistivity was investigated by first incubating the aortic rings from the rats in the three groups in apigenin 7 μ M for 20 min (conditions previously demonstrated to significantly inhibit protein kinase C [11]).

Vascular reactivity

The isolated artery technique comprehensively addressed in [12-18] was applied to undertake vascular reactivity of isolated thoracic aortas. The rise in tension caused by increasing addition of phenylephrine (PE, 10⁻⁹ to 10⁻⁵ M) or KCl (10 to 100 nmol) formed the basis for the evaluation of aortic ring contraction. Meanwhile, to generate similar pre-contraction reactions in every group, the aortic rings were incubated with submaximal PE concentrations, which permitted assessment of aorta vasodilation. The organ bath was subsequently enriched with increasing concentrations of acetylcholine ACh, 10⁻⁹ to 10⁻⁵ M or sodium nitroprusside (SNP, 10⁻⁹ to 10⁻⁵ M), with thorough documentation of responses.

For studying the effect of PMA and apigenin on vascular reactivity, PMA (800 nM) and apigenin (7 μ M) were added to the organ bath during the 60 min equilibration period and during the addition of vasoactive agents.

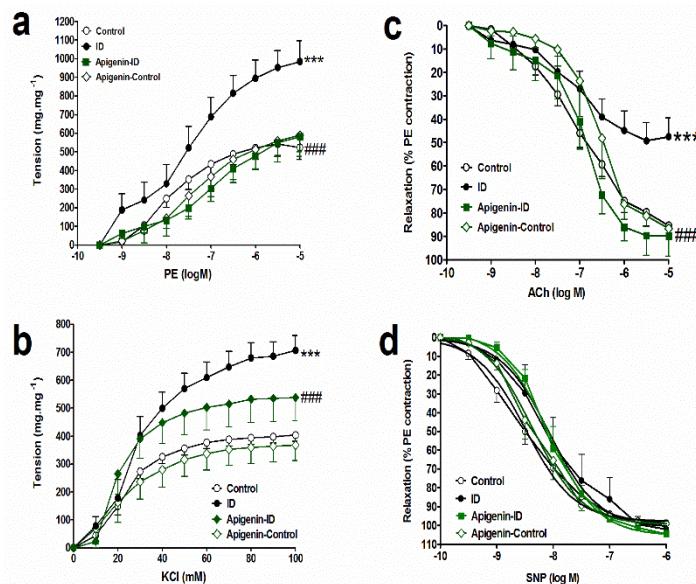


Fig. 1: Effect of apigenin incubation (7 μ M, 20 min) on the vasoconstriction response to (a) phenylephrine, (b) and KCl and the vasodilation response to ACh and (d) SNP in isolated insulin deficiency (streptozotocin-induced) aortae (ID; 50 mg/dl, for 10 w). Symbols indicate mean \pm SEM for N=6-8 animals; *p<0.001, compared with the corresponding control group values #p<0.001 compared with the corresponding insulin-deficient group values; by one-way analysis of variance (ANOVA) and Newman-Keuls post hoc test**

Table 1: Effect of apigenin incubation (7 μ M, 20 min) on the maximal response (E_{max}) and pD_2 (-Log EC₅₀) values of phenylephrine, KCl, ACh and SNP dose-response curves of aortae isolated from streptozotocin-induced insulin deficiency (ID; 50 mg/dl, for 10 w)

Treatment	Control	Apigenin-control	ID	Apigenin-ID
PE	E_{max}	503.5 \pm 23.5	554.5 \pm 51.0	936.0*** \pm 50.2
	pD_2	8.0 \pm 0.2	7.4 \pm 0.2	7.5 \pm 0.2
KCl	E_{max}	363.7 \pm 8.4	326.6 \pm 19.8	649.7** \pm 23.9
	pD_2	-20.2 \pm 0.1	-20.1 \pm 0.3	-39.7*** \pm 0.3
ACh	E_{max}	82.6 \pm 2.5	90.0 \pm 5.7	48.3*** \pm 4.0
	pD_2	7.1 \pm 0.1	6.6 \pm 0.1	7.1 \pm 0.2
SNP	E_{max}	97.9 \pm 1.9	99.5 \pm 2.3	101.1 \pm 3.9
	pD_2	8.4 \pm 0.1	8.3 \pm 0.1	8.1 \pm 0.1

Values are expressed as the mean \pm SE mean; N=8 animals; ***P<0.001, compared with the corresponding control group values; #P<0.001 compared with the corresponding insulin deficient group values; by One Way ANOVA and Bonferroni post hoc test.

Statistical analysis

Quantified values took the form of mean \pm standard error of the mean (SEM). The Prism 5 computer-based fitting program (GraphPad, CA, USA) was employed to determine the agonist maximum response (Emax) with the help of a non-linear regression. Furthermore, one-way analysis of variance (ANOVA) alongside Newman-Keuls' post-hoc test enabled estimation of statistical significance.

RESULTS AND DISCUSSION

Short-term *in vitro* incubation with the natural flavonoid apigenin was found to restore normal vascular reactivity in the thoracic aorta of both insulin-resistant and insulin dependent rats. This is a novel discovery. Moreover, such incubation was found to protect from the exaggerated vasoconstriction response that is induced by protein kinase C (PKC) stimulation.

This study investigated the effects of apigenin with regard to insulin resistance and deficiency—two separate processes associated with diabetes—through two well-established models [19, 20]. Insulin deficiency was found to be associated with a reduction in vascular reactivity. The results suggest that apigenin, a flavonoid that is well-tolerated by *in vivo*, is associated with a novel biological activity of potential use in the management of vascular complications of diabetes.

Enhanced vasoconstriction in response to both phenylephrine (PE) and KCl was seen in the isolated aortas of diabetic rats. This was reflected in significant rises in apparent E_{max} (p<0.01) in both situations (fig. 1a and 1b respectively and table 1).

In insulin-deficient rats, a significant decrease in apparent E_{max} ($p<0.001$) indicated impaired relaxation in response to ACh in the aortas (fig. 1c and table 1). Insulin dependency did not impact upon the *in vitro* response of the aortas to SNP, although its resultant hyperglycaemia did cause an exaggerated response to vasoconstriction agents [21] and impaired vasodilation [22].

Fig. 2 and table 2 indicate the results for the insulin-resistant rats. Significant increases in the apparent E_{max} ($p<0.001$) indicated large responses both to PE and to KCl (fig. 2a and 2b respectively). Conversely, there was a reduction in aortic response to ACh at the same significance level, as can be seen in table 2 and fig. 2c, in insulin-resistant rats. No connection between insulin resistance and *in vitro* aortic responsiveness to SNP was observed.

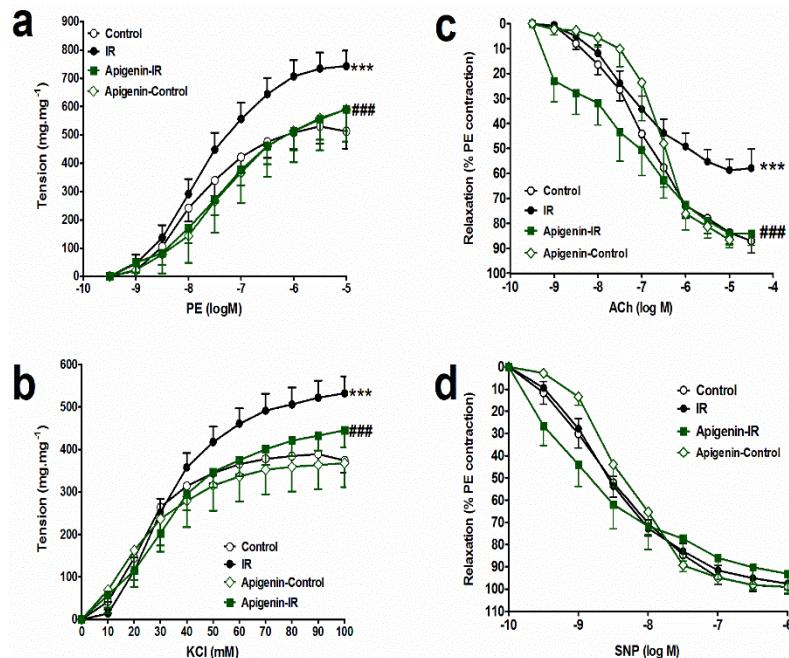


Fig. 2: Effect of apigenin incubation (7 μ M, 20 min) on the vasoconstriction response to (a) phenylephrine, (b) KCl and the vasodilation response to ACh and (d) SNP in isolated insulin resistance (fructose-induced) aortae (IR; 10% in drinking water, for 12 w). Symbols indicate mean \pm SEM for N=6-8 animals; * $p<0.001$, compared with the corresponding control group values; ### $p<0.001$ compared with the corresponding insulin-resistant group values; by one-way ANOVA and Newman-Keulspost *hoc* test**

Table 2: Effect of apigenin incubation (7 μ M, 20 min) on the maximal response (E_{max}) and pD_2 (-Log EC₅₀) values of phenylephrine, KCl, ACh and SNP dose-response curves of aortae isolated from fructose-induced insulin resistance (IR; 10% in drinking water, for 12 w)

Treatment	Control	Apigenin-control	IR	Apigenin-IR
PE	E_{max} 491.8 \pm 23.1	554.5 \pm 51.0	731.5*** \pm 25.9	551.1### \pm 31.5
	pD_2 7.9 \pm 0.2	7.4 \pm 0.3	7.4 \pm 0.2	6.5 \pm 0.1
KCl	E_{max} 352.0 \pm 8.7	326.6 \pm 19.8	469.8*** \pm 13.4	363.6### \pm 13.9
	pD_2 -20.2 \pm 0.2	-20.1 \pm 0.4	-30.0*** \pm 0.15	-20.5### \pm 0.3
ACh	E_{max} 81.7 \pm 2.5	89.9 \pm 5.8	57.3*** \pm 2.2	79.9### \pm 3.9
	pD_2 7.0 \pm 0.1	7.6 \pm 0.1	7.3 \pm 0.1	7.2 \pm 0.2
SNP	E_{max} 98.1 \pm 1.9	99.6 \pm 2.4	95.9 \pm 1.2	88.9 \pm 3.4
	pD_2 8.5 \pm 0.1	8.4 \pm 0.1	8.6 \pm 0.1	8.9 \pm 0.2

Values are expressed as the mean \pm S.E. mean; N=8 animals; *** $p<0.001$, compared with the corresponding control group values; ### $p<0.001$ compared with the corresponding insulin resistant group values; by One Way ANOVA and Bonferroni *post hoc* test.

As described in [3], a considerable body of evidence indicates that vascular occlusive diseases and hypertension are mediated by disease-associated deteriorated vascular reactivity in insulin resistance. Previous *in vitro* research has demonstrated exaggerated responses to KCl and PE [23] and impaired dilation in response to ACh [24] in the aortas of rats fed fructose. This has been reflected in reports of impaired endothelium-dependent dilation in insulin-independent humans [25]. As previously mentioned, an increased influx of extracellular calcium may mediate exaggerated vasoconstriction [21]; this may explain the results seen in this study. However, vasoconstriction modulation can be the result of Ca²⁺sensitization, the inhibition of myosin light chain phosphatase activity, and so independent of changes in Ca²⁺within cells [26]. The inhibition of NO generation may be result in the impairment of

endothelial-dependent dilation; [27] showed that NO synthase uncoupling and stimulation of reactive oxygen species (ROS) generation was associated with impaired pulmonary artery endothelial-dependent relaxation.

Fig. 1a and table 3 show a reduction in E_{max} ($p<0.001$), indicating that an exaggerated response to PE-induced by insulin deficiency was prevented by incubating the rat aorta with 7 μ M apigenin for 20 min. Similarly, this incubation protocol prevented an exaggerated response to KCl—see the significant ($p<0.001$) decrease in apparent E_{max} in fig. 1b and table 3. The impairment of the response to ACh induced by insulin resistance was alleviated ($p<0.001$) by incubation with apigenin, shown in fig. 2c, table 2, although the responsiveness to SNP was affected, as indicated in fig. 2d.

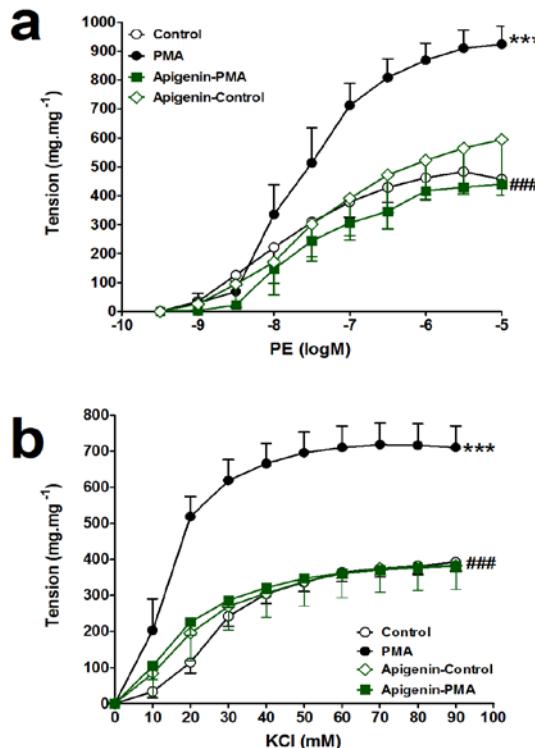


Fig. 3: Effect of apigenin incubation (7 μ M, 20 min) on the vasoconstriction response to (a) phenylephrine, (b) and KCl in injured aortae induced by PMA (800 nM, 1 hour) incubation. Symbols indicate mean \pm SEM for n=6-8 animals; *p<0.001, compared with the corresponding control group values; ###p<0.001 compared with the corresponding PMA group values; by one-way ANOVA and Newman-Keuls post hoc test**

Table 3: Effect of apigenin incubation (7 μ M, 20 min) with or without PMA (800 nM, 1h) on the maximal response (E_{max}) and pD₂ (-Log EC₅₀) values of phenylephrine and KCl dose-response curves of isolated aortae

Treatment	Control	Apigenin-control	PMA	Apigenin-PMA
PE	E_{max}	442.2 \pm 21.8	554.3 \pm 57.9	895.8 \pm 35
	pD ₂	8.0 \pm 0.2	7.5 \pm 0.3	7.7 \pm 0.1
KCl	E_{max}	381.2 \pm 13.8	384.7 \pm 48	705.7 \pm 25
	pD ₂	24.7 \pm 3.1	20.6 \pm 3.4	12.4 \pm 4.4

Values are expressed as the mean \pm SE mean; N=8 animals; ***P<0.001, compared with the corresponding control group values; ###P<0.001 compared with the corresponding PMA group values; by One Way ANOVA and Bonferroni post hoc test.

In this study, the apigenin was used at that concentration at which it has previously been reported to inhibit PKC [11]. This appears to explain its direct effects on the exaggerated diabetes-related vascular contractility. The similar significant increases seen in apparent E_{max} in aortic responsiveness to both KCl and PE through incubation for one hour with PMA (800 nM) to those seen in insulin resistance and insulin deficiency supports this. However, it was also seen that protection against PMA-induced hyperresponsiveness to KCl or PE by incubation with apigenin was seen. Aortic responsiveness to ACh was unaffected by PMA and apigenin incubation (data is not shown); similarly, as illustrated by table 1 to 3 and fig. 1 to 3, responsiveness to any of the vasoactive agents was unaffected by AP incubation. As apigenin has strong antioxidant properties [7], it will reduce the quenching of NO by suppressing ROS; apigenin is likely to mediate its effect on impaired vasodilation in diabetes, thereby restoring normal vascular dilation.

CONCLUSION

Apigenin was able to restore normal vasoconstriction and vasodilation in the thoracic aorta isolated from insulin-resistant and insulin-deficient rats. In addition, apigenin prevented PKC induced exaggerated vasoconstriction and impaired relaxation. These results point to the beneficial effect of apigenin in preventing vascular complications in diabetes most likely through protein kinase C inhibition.

AUTHORS CONTRIBUTIONS

HM El-Bassossy, raised the idea, designed the experiments and participated in statistical analysis and revising the manuscript; N Desoky, carried out laboratory experiments, animal handling procedures, data and statistical analysis and participated in drafting the manuscript; an Alahdal, participated in the study conception and manuscript revision; A Fahny, participated in study design and manuscript revision.

CONFLICT OF INTERESTS

All authors have none to declare

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