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Protective effect of zingerone on increased vascular contractility in diabetic rat aorta

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Abstract:

The aim of the present study was to investigate the effect and possible mechanism of action of zingerone, the main constituent of ginger, on vascular reactivity in isolated aorta from diabetic rats. The results show that incubation of aortae with zingerone alleviates the exaggerated vasoconstriction of diabetic aortae to phenylephrine, as well as the impaired relaxatory response to acetylcholine in a concentration-dependent manner. Furthermore, zingerone stimulates aortic nitric oxide generation but has no effect on advanced glycation end product formation. The vasorelaxatory response is significantly attenuated by the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride and the guanylate cyclase inhibitor methylene blue but no effect of either the potassium channels blocker tetraethylammonium chloride, or the cyclooxygenase inhibitor indomethacin was observed. In conclusion, zingerone ameliorates enhanced vascular contraction in diabetic aortae which may be partially attributed to its ability to increase the production of NO and guanylate cyclase stimulation.

Key words: diabetes, zingerone, vasorelaxant, nitric oxide, advanced glycation end products, vascular complications.

1. Introduction

It is well established that vascular disease is a complicating feature in patients with diabetes mellitus and responsible for its morbidity and mortality (Christrieb 1973, Schalkwijk et al., 2005). These vascular complications may be partially attributed to impairment of vasomotor function of smooth muscles (Nugent et al., 1996). In this respect, the reactivity of vascular smooth muscles to contractile and vasorelaxant agents in diabetic rats has been previously studied (Kamata et al., 1989, Stanley et al, 2013). Many studies have investigated the mechanism of the enhanced contractile response of diabetic blood vessels but the mechanism of enhancement is still unknown. However, an impaired endothelial activity (MacLeod, 1985), increased response to Ca^{2+} (Buluc et al., 2006), and increased production of vasoconstrictor prostanoids prostaglandin F_2 alpha, prostaglandin H_2 or thromboxane A_2 due to increased superoxide anion (Kanie et al., 2000) might be responsible for the increased contractile responses in diabetic rat vessels. In addition, the generation of reactive oxygen species (ROS) within the vascular wall scavenges nitric oxide (NO), decreasing its ability to stimulate soluble guanylate cyclase (sGC) and hence produce cGMP (Guerci et al., 2001).

Herbal medicines have recently attracted the interest of scientific communities as alternative therapy. The rhizome of *Zingiber officinale* (ginger) is consumed worldwide as a spice and flavoring agent. Zingerone is a phenolic alkanone which is present in a significant amount of about 9 % in ginger (Zhang et al., 2012). Previous studies have showed that zingerone has anti-inflammatory and antioxidant effects (Kim et al., 2010). In addition, zingerone was found to inhibit contractile movements of isolated colonic segments (Iwami et al., 2011). Although these useful effects have been demonstrated, the molecular mechanism of zingerone on relaxation of smooth muscle was not fully studied and poorly understood. Therefore, the aim of this study is to examine the effect and potential mechanism of action of zingerone on aortae from diabetic rats.

2. Materials and Methods

2.1.Drugs and chemicals

Zingerone, Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), methylene blue (MB), tetraethylammonium chloride (TEA), indomethacin (INDO), aminoguanidine (AG), ribose, bovine serum albumin (BSA), acetylcholine (ACh) and phenylephrine (PE) were purchased from Sigma-Aldrich Chemical company (Munich, Germany). 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate was purchased from Molecular Probes (New York, USA). All chemicals were dissolved in ultrapure deionized water except for zingerone and DAF-FM diacetate, which was dissolved in dimethylsulphoxide (DMSO). The final DMSO concentration did not exceed 0.1%, which has no effect on vascular reactivity according to our preliminary studies.

2.2.Animals and grouping

Male Wistar rats (King Abdulaziz University, Saudi Arabia) weighing 120–140 g, 6 weeks age, were housed in clear polypropylene cages (3-4 rats per cage) and kept under constant environmental conditions with equal light–dark cycle. Rats had free access to commercially available rodent pellet diet and purified water. All the experimental procedures were performed in accordance with Saudi Arabia Research Bioethics and Regulations, which are consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experimental protocol was approved by the Unit of Biomedical Ethics, Faculty of Medicine, King Abdulaziz University. Animals were randomly divided into two experimental groups; control (C) and Diabetic (D) groups (6-8 rats in each group). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg). Glucose levels in tail blood were determined using a glucose meter (ACCU-CHEK, Roche, Mannheim, Germany) with noble metal electrode strips. Diabetes was confirmed by a

stable hyperglycemia (blood glucose levels of 250-350 mg/dl) after 2 weeks of STZ injection. Rats were left for an additional 8 weeks to develop vascular complications based upon results of recent work from our laboratories (El-Bassossy et al., 2012).

2.3.Vascular reactivity

Vascular reactivity was assessed using the isolated artery technique previously described in previous work of our laboratories (El-Bassossy et al., 2014). Briefly, animals were sacrificed by decapitation with a rodent guillotine. The thoracic aorta was then cleaned of fat and connective tissue and cut into 3 rings (2-3 mm length). Rings were suspended in an organ bath containing 10 ml Krebs–Henseleit buffer and temperature was maintained at 37∘ C. Krebs–Henseleit solution (pH 7.4) was composed of (mM) sodium chloride 118.1, glucose 11.7, magnesium sulphate 0.5, sodium bicarbonate 25.0, dihydrogen potassium phosphate 1.2, potassium chloride 4.69, and calcium chloride 2.5. In order to avoid anoxic condition, and a continuous supply of oxygen was maintained throughout experiment using carbogen (95% oxygen and 5% carbon dioxide). Isolated aortae from diabetic group were incubated with different concentrations of zingerone (1-100 µM) for 30 min before studying the vasoconstriction and vasodilatation responses, whereas the isolated aortae from the control group were incubated in DMSO. The suspended tissue equilibrated for 1 h under 1.5 g resting tone as previously described (Kesler et al. 2002). Tissues were then treated with cumulative phenylephrine concentrations (PE, 1 nM to 10 µM) to study the contractile response before. Cumulative concentrations of acetylcholine (ACh, 1 nM to 10 µM) were added to aortic rings precontracted with maximal concentrations of PE (10 µM) and the response was recorded as a percent of PE precontraction response. Changes in the isometric tension in control and treated conditions were measured with force-displacement transducer (AD Instruments) coupled with PowerLab data acquisition system (AD Instruments, Sydney, Australia) and data stored and

analyzed with LabChart 8 software (AD Instruments, Sydney, Australia) running on a personal computer.

2.4.Studying the possible mechanism of vasodilatory effect of zingerone

To clarify whether the direct vasorelaxant effect of zingerone is specific for diabetic aortae or not, it was tested on normal control aortae precontracted with PE or KCl using the isolated artery technique as follows. Cumulative concentrations of zingerone (1-100 µM) were added to the organ bath containing isolated aortae precontracted with PE (10 µM) or KCl (80 mM) and compared with non-zingerone treated ones and the decrease in tension was recorded. In other sets of experiments, the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 µM), the guanylate cyclase (GC) inhibitor methylene blue (MB, 5 µM), the calcium-activated potassium channels blocker tetraethylammonium chloride (TEA, 10 mM), or the cyclooxygenase (COX) inhibitor indomethacin (INDO, 5 µM) was added 30 minutes before investigating the vasorelaxant effect of zingerone as above.

2.5.Advanced glycation end-product (AGE) assay

The effect of zingerone on AGE formation was carried out and the formation of fluorescent AGE measured using a monochromator SpectraMax® M3 plate reader according to Adisakwattana et al. (2010).

2.6.Statistical analysis

Statistical analysis was performed by the one or two-way analysis of variance (ANOVA) followed by Dunnett's post hoc test using GraphPad prism version 5 software. All data are expressed as mean ± standard error of mean (SEM). In all the analyses $P < 0.05$ was considered significant.

3. Results

3.1.Effect of zingerone on vascular reactivity of diabetic aortae

The current study showed that diabetic aortae exhibited exaggerated vasoconstriction in response to PE (10^{-9} to 10^{-5} M) compared to control aortae (figure 1 A, B). This enhancement of vasoconstriction was highly significant ($P < 0.001$) at PE concentrations of $3x10^{-6}$ to 10^{-5} M (figure 1 C, D & E and figure 2). Thirty minutes incubation with zingerone (1–100 μM) alleviated the exaggerated vasoconstriction of diabetic aortae in a concentration-dependent manner. The inhibition of PE (10^{-5} M)–induced contraction was highly significant ($P < 0.001$) at the 3 concentrations of zingerone tested (1, 10, 100 μM) and the highest concentration alleviated the exaggerated response to PE to below the control value.

Diabetic aortae showed impaired vasodilation to ACh (10^{-9} to 10^{-5} M) compared to controls and this impaired relaxation was highly significant ($P < 0.001$) at an ACh concentration of 10^{-5} M. This impaired relaxation was alleviated after incubation with zingerone in a concentration dependent manner and this alleviation was highly significant with 100 μM zingerone concentration (figure 3).

3.2.Mechanism of vasorelaxant effect of zingerone

Addition of cumulative concentrations of zingerone (3×10^{-5} to 10^{-3} M) to the organ bath led to a concentration-dependent vasodilation of isolated normal aortae precontracted with PE or KCl that was statistically significant ($p<0.005$) compared to non-zingerone treated normal aortae precontracted with PE or KCl (figure 4A).

Moreover, addition of cumulative concentrations of zingerone (3×10^{-5} to 10^{-3} M) to the organ bath led to a concentration-dependent vasodilation of isolated diabetic aortae precontracted with PE that was statistically significant ($p<0.005$) at zingerone concentrations of 3×10^{-4} and 10^{-3} M. Thirty minutes pre-incubation with L-NAME or MB (at 100 and 5 μM) before the

cumulative addition of zingerone significantly inhibited the zingerone-induced vasodilation (both at $P<0.005$). In contrast, pre-incubation with TEA (10 mM), or INDO (5 µM) did not significantly affect the zingerone-induced vasodilation (figure 4B).

3.3.Effect of zingerone on AGE

Incubation of BSA with ribose increased AGE significantly ($P < 0.001$), as assessed by fluorimetry. This increased level of AGE was significantly ($P < 0.001$) ameliorated when incubated with AG (1 mM, as positive control) while there was no effect when incubated with different concentrations of zingerone (figure 5)

4. Discussion

Vascular complications of diabetes mellitus pose an extensive socioeconomic burden on public health. Approximately 50% of patients with diabetes die prematurely of a cardiovascular cause (van Dieren et al., 2010). The current study demonstrates, in agreement with previous studies, that STZ-induced DM caused increased vascular responsiveness to phenylephrine as well as impaired relaxation response to ACh in diabetic rat aorta (Abebe et al., 1990, Orie et al., 1993, Roghani et al., 2004). Hyperglycemia appears to contribute to endothelial dysfunction (Cagliero et al., 1991, Tesfamariam et al., 1992). Tesfamariam et al. (1991) found that hyperglycemia activates protein kinase C in endothelial cells, which increases the production of vasoconstrictor substances and vascular growth factors, which directly and indirectly enhance vasomotor reactivity and vascular remodeling and growth.

Ginger has been found to have antihypertensive effect (Ghayur and Gilani 2005, Manosroi et al., 2013, Akinyemi et al., 2014). Despite that previous studies demonstrated the protective effect of ginger on diabetes mellitus and its ameliorative effect on renal derangement in diabetes (Shidfar et al., 2015, Zhu et al., 2015, Kazeem et al., 2015), the present study is the first to show the protective effects of zingerone, a pungent component of ginger, on vascular

tissue in STZ-induced diabetic rats. In addition zingerone was found to have a relaxant effect on colon (Iwami et al., 2011). Previous pharmacokinetic studies of zingerone has revealed that administration of zingerone either orally or intraperitoneally results in oxidation of side chain at all available sites. During catabolism of zingerone, glucuronidation and sulfation occur which leads to excretion of glucuronide compounds and sulphate conjugates in urine within 24 hours of consumption (Ahmad et al, 2015). In addition, zingerone is insoluble in water and decompose under light (Shimoda eta l., 2007)

The current study showed that zingerone has significantly ameliorated the enhanced contractile response of diabetic aortae to PE at all concentrations. In addition, the impaired relaxation of diabetic aortae to ACh was alleviated after incubation with zingerone and this alleviation was highly significant at 100 μM zingerone concentration. This is in agreement with the reported antihypertensive effect of aqueous ginger extract and its phenolic constituents in rats through stimulation of muscarinic receptors (Ghayur et al., 2005). In addition, Ghayur and Gilani (2005) reported that the methanolic extract of fresh ginger exhibits hypotensive, endothelium-independent vasodilator property through its specific inhibitory action at the voltage-dependent calcium channels. Aside from ginger itself, some of ginger components, 6-gingerol and 6-shogaol have been studied for their effects on BP in laboratory animals (Suekawa et al., 1984) where both were found to produce a depressant effect, at lower doses, although a tri-phasic effect (consisting of an initial hypotensive followed by a sharp hypertensive and then a delayed hypotensive effect) was observed at higher doses. In addition, the present study revealed vasorelaxant effect of zingerone even in normal nondiabetic rat aortae precontracted with PE.

To further investigate the mechanism of action of this ameliorative effect, we have studied the effect of zingerone on normal aortae precontracted with KCl. The present results revealed significant relaxant effect of zingerone which indicate that the relaxant effect is

through direct vascular mechanism and not related to interfering with PE. Moreover, the effect of zingerone on AGE generation was studied as it is implicated in the development of cardiovascular complications in diabetics (Mukohda et al., 2012). In addition we have investigated the possible mechanism of action of zingerone on NOS, GC, COX and potassium channels. The present study revealed that the possible mechanism(s) by which zingerone alleviates vasoconstriction of diabetic aortae could be by direct vasodilation through NO generation and stimulation of GC. The vasodilator effect of zingerone was significantly inhibited by the NOS inhibitor (L-NAME) and the GC inhibitor (MB) while not affected by the COX inhibitor indomethacin or the calcium-activated potassium channel blocker TEA. It was previously found that 6-gingerol, 8-gingerol, and 10-gingerol have potent vasodilator effects on isolated rat aortae (Connell and McLachlan, 1972). The observed vasodilator effect of gingerols is insensitive to atropine pretreatment but considerable blockade is observed in the presence of L-NAME, a NO synthase inhibitor (Thorin et al., 1998). In addition, Imanishi et al (2004) found that ginger rhizome contributed to activation of macrophage-inducible NOS.

The current study found no evidence that zingerone affects AGE formation, suggesting that the observed alleviation of exaggerated vasoconstriction by zingerone is through inhibiting the effects of AGE on vessels rather than on AGE formation. Previous study by Rao and Rao (2010) found that zingerone has anti-apoptotic and anti-lipid peroxidative potency, probably due to its antioxidant/free radical scavenging ability and by the suppression of oxidative stress.

In conclusion, our data provides preliminary evidence that zingerone ameliorates enhanced vascular contraction in diabetic aortae and the possible mechanism of this vasodilatory effect may be attributed to the stimulation of aortic NO generation and GC stimulation. Further studies in this direction are warranted in addition to further investigations to evaluate the possible beneficial vascular effect of zingerone treatment in diabetic animals.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgment

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Figure legends

Figure 1: Representative racing showing the vasoconstrictor response to phenylephrine (PE) in control rats (A), the exaggerated vasoconstrictor response in diabetic rats (B) and the ameliorative effect of different concentrations of zingerone (C, D and E).

Figure 2: Concentration-response curve showing the effect of incubation with zingerone (1-100 μM) on the isolated aorta responsiveness to PE in STZ-induced diabetes (D) compared to control group (C). Values shown are mean ±SEM, *n* =8-10.

***Significantly (P < 0.001) different from the respective control values.

$^{###}$Significantly (P < 0.001) different from the respective diabetic values.

NOTE fig 2 and 3 – difficult to discriminate between symbols and colours for diabetic and controls (green versus black circles look quite similar). Suggest you increase point size on all symbols and use different symbol shapes to clarify differences – open circles in all figures for non-diabetic controls might be better, and filled circles for diabetics?

Figure 3: Concentration-response curve showing the effect of incubation with zingerone (1-100 μM) on the isolated aorta responsiveness to ACh (B) in STZ-induced diabetes (D) compared to control group (C). Values shown are mean ±SEM, *n* =8-10.

***Significantly (P < 0.001) different from the respective control values.

$^{###}$Significantly (P < 0.001) different from the respective diabetic values

Figure 4: Effect of cumulative doses of zingerone on normal control aortae precontracted with phenylephrine (PE) or potassium chloride (KCl) compared with nonzingerone precontracted ones (4A). Effect of the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM), the guanylate cyclase inhibitor methylene blue (MB, 5

µM), the calcium-activated potassium channels blocker tetraethylammonium chloride (TEA, 10 mM), and the cyclooxygenase inhibitor indomethacin (INDO, 5 µM) on the direct vasorelaxant effect of zingerone on diabetic aorta.

***Significantly ($P < 0.001$) different from the respective control values. Points show mean ± SEM of n=5-8 tissues.

Figure 5: Effect of zingerone (1 µM – 100 µM) on generation of advanced glycation end products (AGE) compared to bovine serum albumin (BSA) with glucose (Glu) and aminoguanidine (AG). Bars show mean ± SEM of n=6.

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Figures

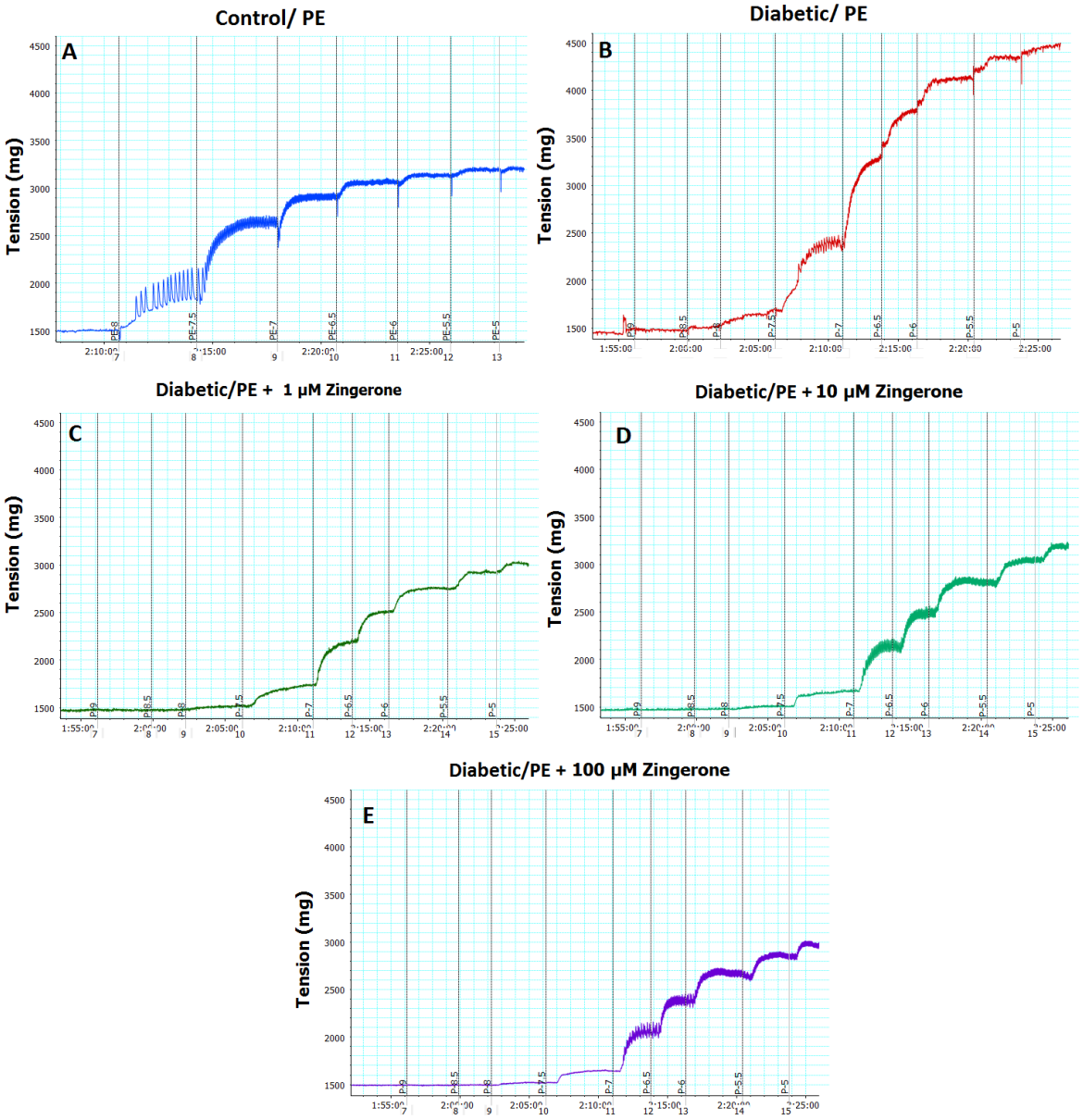

Figure 1

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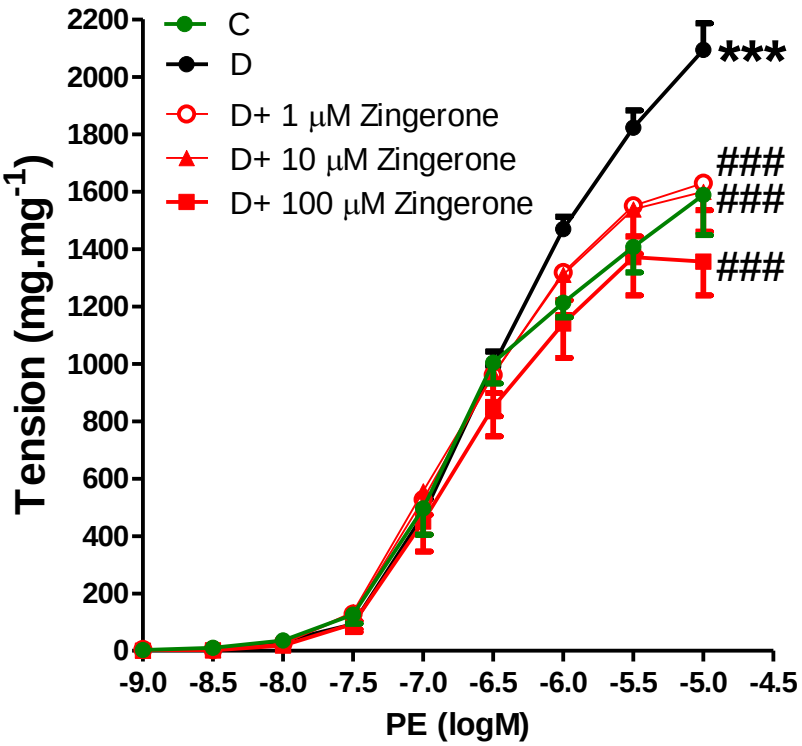

Figure 2

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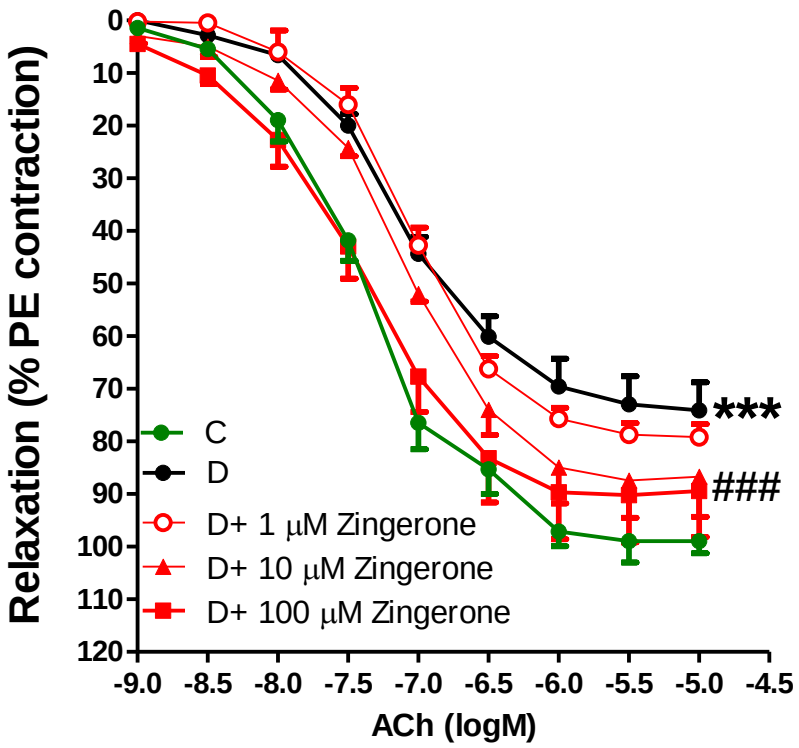

Figure 3

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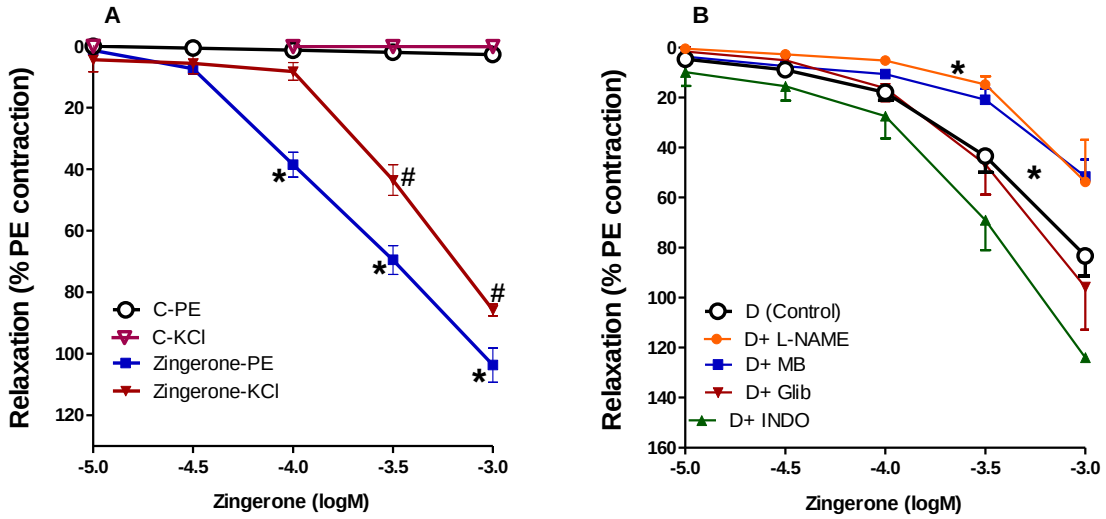

Figure 4

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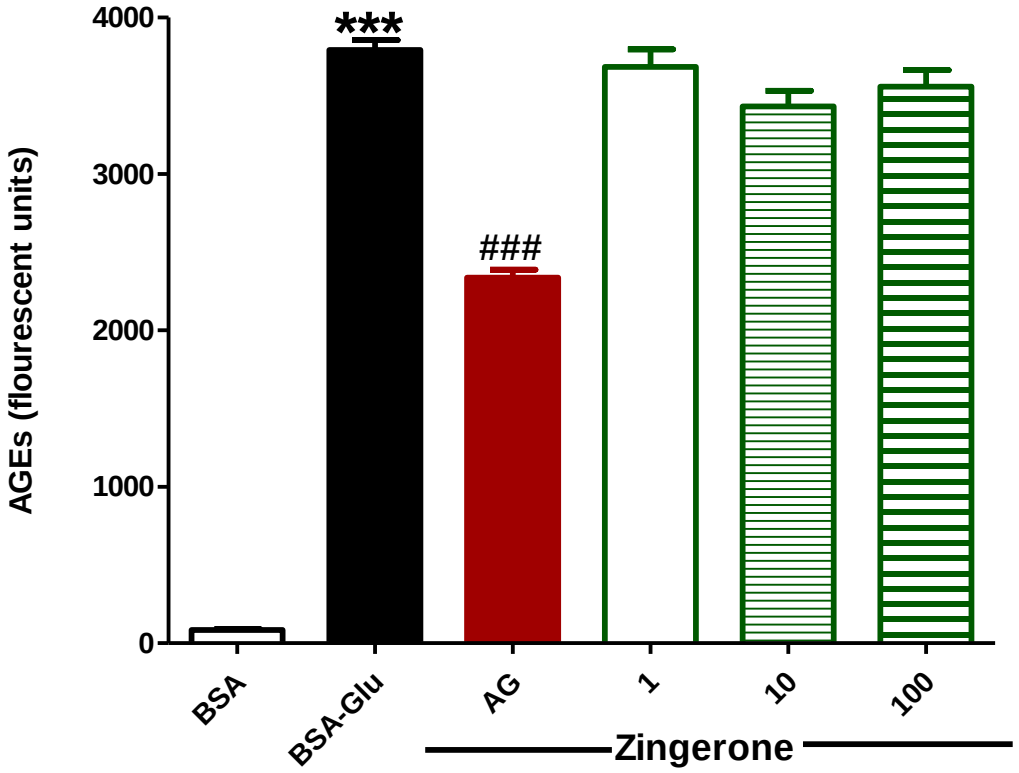

Figure 5