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Inhibition of PKCβ Protects Against Diabetes-Induced Impairment in Arachidonic Acid Dilation of Small Coronary Arteries

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Running Title: PKCβ mediated vascular dysfunction in Type 1 diabetes

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Abbreviations: AA, arachidonic acid; LY, LY333531; PKC, protein kinase C; SOD, superoxide dismutase; CYP, cytochrome P450; COX, cyclooxygenase; LOX, lipoxygenase; ROS, reactive oxygen species; NOS, nitric oxide synthase; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; ET-1, enodthelin-1; BK channel, large conductance Ca^{2+}-activated K^+ channel; K_{ATP}, ATP-sensitive K^+ channel; DHE, dihydroethidium; EET, epoxyeicosatrienoic acid; LY333531, (*S*)-13[(dimehtylamino)methyl]-10,11-14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[e,k]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione.

Abstract

To test the hypothesis that PKCβ-induced reactive oxygen species (ROS) underlie the vascular dysfunction in diabetes, we examined the effects of LY333531 (LY), a specific PKCβ inhibitor, on arachidonic acid (AA)-mediated dilation in small coronary arteries from streptozotocin-induced diabetic rats. This study was designed to determine whether diabetes impairs AA-induced vasodilation of small coronary arteries and whether this defect could be blunted by dietary treatment with LY. Coronary diameter was measured using video-microscopy in isolated pressurized vessels. In controls, AA dose dependently dilated coronary arteries, with 1 μM producing 54.7±3.1% and 30 μM 72.0±3.0% dilation (n=9). In diabetic rats, 1 μM AA only produced 31.4±3.8% (n=8, $p<0.01$ vs. control) and 30 μM 43.8±3.7% dilation (n=8, $p<0.001$ vs. control). Nitroprusside-mediated vasodilations were similar in control and diabetic rats. In contrast, in diabetic rats receiving LY, AA-mediated coronary dilations were normal. In controls, AA-mediated vasodilation was inhibited by miconazole (an inhibitor of cytochrome P450 epoxygenase) and by iberiotoxin (IBTX, an inhibitor of the large conductance Ca^{2+}-activated K^+ channel) but miconazole and IBTX had no effects in diabetic vessels. In diabetic rats receiving LY, the effects of miconazole and IBTX were similar to control. Superoxide dismutase (SOD) restored responses to AA in diabetic vessels but had no effect in vessels from control or diabetic rats on LY. These results suggest that AA-mediated vasodilation in rat coronary arteries are impaired in diabetic rats due to increases in generation of ROS. LY protects against these defects in diabetes through inhibition of PKCβ-mediated production of ROS.

Introduction

Diabetes mellitus has become a disease of epidemic proportions with cardiovascular disease the leading cause of death in diabetic patients (Geiss et al., 1995) and patients with diabetes have a 2- to 4-fold increase in the risk of coronary artery disease (Beckman et al., 2002a). Metabolic, humoral, and hemodynamic factors all contribute to the development of vascular dysfunction (Cooper et al., 2001). Endothelial dysfunction with impaired activity of various endothelial-derived factors plays an integral role in diabetic vasculopathy (De Vriese et al., 2000; Brownlee, 2001). The process by which hyperglycemia produces endothelial and vascular dysfunction is complex, but several major mechanisms have been proposed (Nishikawa et al., 2000; Brownlee, 2001; Cooper et al., 2001; Gutterman, 2002), including overactivity of the polyol pathway, accumulation of advanced glycation end products, and activation of protein kinase C (PKC). Each of these would result in an enhanced generation of ROS. Activated PKC mechanisms have received increasing attention (Way et al., 2001). Hyperglycemia stimulates PKC through the action of diacylglycerol and nonesterified fatty acids (Cooper et al., 2001; Egan et al., 2001). In particular, the β isoform is increased in diabetic vascular tissues (Inoguchi et al., 1992), and administration of LY333531 (LY), a highly specific inhibitor of PKCβ, attenuates the various vascular abnormalities in streptozotocin-induced diabetic rats (Inoguchi et al., 1992; Ishii et al., 1996). Recently, oral administration of LY in humans has been shown to prevent impaired endothelium-dependent vasodilation caused by hyperglycemia (Beckman et al., 2002b). These findings position PKCβ as a key participant in the development of vascular dysfunction in diabetes mellitus.

Arachidonic acid (AA) is an important precursor for many vasoactive metabolites that are crucial for the regulation of vascular function. AA is metabolized by cyclooxygenase (COX) into prostaglandins and thromboxane, by lipoxygenase (LOX) into leukotrienes, lipoxins and intrachain hydroxyeicosatetraenoic acids (HETEs), and by cytochrome P450 (CYP) epoxygenase into epoxyeicosatrienoic acids (EETs) and chain terminal HETEs (Foegh and Pamwell, 2002). AA produces potent dilation in human coronary arterioles that is dependent on the CYP pathway (Miura and Gutterman, 1998), whereas the dilation produced in rat mesenteric microvessels is mediated mainly through the LOX pathway (Miller et al., 2003; Zhou et al., 2005). However, the role of AA in the vascular dysfunction of diabetes mellitus is not fully known.

Enhanced PKC activities could produce vascular dysfunction through different mechanisms but the common denominator appears to be increase in ROS {Gutterman, 2002 #6}. PKC could induce ROS production through activation of NAD(P)H in vascular endothelial cells {Inoguchi, 2003 #45}. In addition, nitric oxide synthase (NOS) in diabetic vessels may become uncoupled, resulting in the generation of superoxide rather than nitric oxide (NO) {Hink, 2001 #19}. Increase ROS is known to affect the COX {Zou, 2002 #21}, LOX {Zhou, 2005 #42}, and CYP {Lin, 2005 #44} enzymes, and could significantly modulate AA metabolism and the vascular effects of its bioactive products. The goal of this study is to determine whether the AA-mediated dilation of small coronary arteries is impaired in streptozotocin-induced diabetic rats, and to determine the role of PKCβ in such impairment.

Materials and Methods

Animals

Diabetes mellitus was produced in male Sprague-Dawley rats (200 to 250 g) by injection of streptozotocin (60 mg/kg, ip). Control rats received vehicle injection. Blood glucose levels in excess of 300 mg/dl were considered diabetic. At induction of diabetes, control and diabetic rats received regular rat chow, but some animals in each group received chow containing LY (10 mg/kg/day) (Inoguchi et al., 1992; Ishii et al., 1996). LY333531, (*S*)-13[(dimehtylamino)methyl]-10,11-14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[e,k]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione, is a highly specific inhibitor of PKCβ, and the LY containing chow was specially prepared by Eli Lilly and Company (Indianapolis, IN). Handling and care of animals, and all animal procedures were approved by the Institutional Animal Care and Use Committee, Mayo Foundation.

Vasoreactivity Measurements

Two to four weeks following induction of diabetes and administration of LY, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). Hearts were rapidly excised and placed in ice-cold Krebs solution that contained (in mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4, 1.2, $NaHCO_3$ 25, and dextrose 11.1. The secondary and tertiary branches (50-200 μm in intraluminal diameter) of the right and left coronary arteries from the epicardial surface as well as branches of the septal coronary arteries were carefully dissected and isolated free of surrounding myocardium and connective tissue under a dissecting microscope (Olympus SZ4045 Stereo Microscope, Olympus America Inc., Melville, NY). Isolated small coronary arteries (1-2 mm in length) were

transferred to a custom-made vessel chamber filled with Krebs solution. The arteries were mounted and secured between two borosilicate glass micropipettes (30 μm diameter tips) with 10-O ophthalmic suture. The lumen of the vessel was filled with Krebs solution through the micropipettes and maintained at a constant pressure (no flow) of 60 mmHg. The vessel chamber was transferred to an inverted light microscope stage (Olympus CK40) coupled to a video measurement system (VIA-100, Boeckeler Instruments, Inc., AZ) equipped with a video camera, monitor and calibrated video calipers for visualization and recording the intraluminal diameter as described previously (Zhou et al., 2005). Vessels were equilibrated for at least 30 min in oxygenated (20% O_2, 5% CO_2, balanced with N_2, 37^{o}C) Krebs solution, which was continuously circulated through the vessel bath. Responses to cumulative additions of each compound were determined at 5 min intervals. The average diameter of the vessels used was 137±5 μm for controls, 137±8 μm for control on LY diet, 122±6 μm for diabetic rats, and 123±5 μm for diabetic rats on LY diet (p=N.S. among groups). Vessels were unacceptable for experiments if they demonstrated leaks, failed to produce >30% constriction to 60 mM KCl or to graded doses of endothelin-1 (ET-1), or failed to produce an 80% dilation with nitroprusside (10^{-4} M).

To assess the role of endothelium in responses, endothelium was removed by passing an air bubble (1 ml volume) through the isolated vessels. Vessels were used only if they did not relax with acetylcholine (10^{-4} M, <10% relaxation) but had normal response to nitroprusside (10^{-4} M, >80% dilation of constriction by endothelin-1) and to KCl (60 mM, >30% constriction of baseline resting diameter).

Pharmacologic Interventions

All compounds were added abluminally, and the cumulative concentration-responses were determined at 3 to 5 min intervals between doses. Vessels were constricted to 30-60% of baseline diameter with endothelin-1 (doses used were 3.6±0.3 to 6.6±0.6 nM). Concentration-response curves to acetylcholine (ACh, 10^{-11} to 10^{-4} M, endothelium-dependent), sodium nitroprusside (10^{-11} to 10^{-4} M, endothelium-independent), AA (1×10^{-10} to 3×10^{-5} M) were determined.

To determine the mechanisms responsible for mediating dilation to AA, small coronary arteries were pre-incubated for 30 min with miconazole (10^{-5} M) to inhibit the CYP epoxygenase pathway, or with iberiotoxin (IBTX 10^{-7} M) to block the large conductance Ca^{2+}-activated K^{+} (BK) channels, prior to dose response experiments.

To determine the effects of ROS in vascular dysfunction, vessels were treated with superoxide dismutase (SOD, 150 U/ml, 30 to 45 min) before measuring vasodilator response to ACh and AA.

To determine the effects of acute PKCβ inhibition in vascular dysfunction, vessels were treated with LY333591 (30 nM) for 30 min before measuring vasodilation response to AA.

Fluorescent Microscopy of Oxidative Stress

The oxidative fluorescent dye, dihydroethidium (DHE), was used to evaluate the production of superoxide in coronary arteries as previously described (Miller et al., 1998). DHE is a chemically reduced ethidium derivative that is permeable to viable cells. DHE exhibits blue fluorescence in cytoplasm but can be oxidized in cells, reacting with superoxide to form ethidium, which intercalates DNA to produce bright red fluorescence

(Munzel et al., 2002). Unfixed frozen ring segments of rat coronary arteries from control, diabetic, and LY-treated diabetic animals were cut into 30-µm-thick sections and placed on a glass slide. DHE (2 µM) was topically applied to each tissue section and incubated in a light-protected humidified chamber at 37°C for 30 min. Slides were then coverslipped and images were obtained with a confocal laser microscope (LSM 510, Zeiss, Germany) with a 63X water immersion lens. DHE was excited at 488 nm and fluorescence emission was detected with a 585-615 nm band-pass filter. In addition, autofluorescence intrinsic to the internal elastic lamina, which separates the endothelium from smooth muscles and is present in small arteries, was detected using a 505-550 nm band-pass filter (green fluorescence) (Wong and Langille, 1996; Burnham et al., 2002), and transmitted light micrographs of the same sections were also obtained. Laser settings were identical for acquisition of images, and vessels from control, diabetic, and diabetic rats on LY were processed in parallel. The light micrograph and fluorescent images for DHE signals and internal elastic lamina were digitally merged to demonstrate anatomic distribution of ROS. The DHE signals were further analyzed densitometrically using Scion Image software (Scion Corp.) and the results were expressed as relative densitometric units per unit area.

Materials

DHE was purchased from Molecular Probes (Eugene, OR). LY333531 was a generous gift from Eli Lilly and Company and was solubilized in DMSO as a 20 mM stock solution. All other chemicals were obtained from Sigma-Aldrich Corporation (St. Louis, MO). AA, ACh, and nitroprusside were solubilized in deionized water, and stored

under nitrogen at –20°C. Iberiotoxin was freshly prepared in Krebs solution at 10^{-7}M. Streptozotocin was freshly prepared in sterile water prior to injection into the animals.

Statistical Analysis

Data are presented as mean ± SEM. N represents the number of vessels used in each experiment. All concentration-response relationships were analyzed using one-way ANOVA with repeated measures. Pair-wise comparisons among the groups were performed using Tukey test with SigmaStat software (Systat Software, Inc., Point Richmond, CA). Statistical significance was defined as $p<0.05$.

Results

Rats treated with streptozotocin had higher plasma glucose (571±6 mg/dl vs. 167±7 mg/dl in control, $p<0.05$) and lower body weights (223±5 g vs. 316±6 g in control, $p<0.05$) compared to controls. Diabetic animals did not lose weight (230±6 g and 223±5 g respectively before and after induction of diabetes, p=N.S.), but they failed to gain weight as in control rats (199±5 g to 316±6 g during same period of time). LY had no effect on plasma glucose (570±9 mg/dl) or body weights (215±8 g) of streptozotocin-treated rats compared to diabetic rats on normal diet. Likewise, control rats on LY diet had similar blood glucose levels (157±8 mg/dl) and body weights (335±8 g) compared to their counterparts on normal diet.

Coronary Vasoreactivity

Coronary arteries from diabetic rats dilated to sodium nitroprusside similar to controls (Figure 1A), suggesting intact vascular smooth muscle function. However, dilation to ACh was significantly reduced in diabetic coronary arteries, with 1 μM ACh producing only 44% the dilation of control (29.1±1.7% dilation in diabetic, n=6 vs. 66.4±1.5% in control n=8, $p<0.001$). Decreased responses to ACh suggest endothelial dysfunction in diabetic rats (Figure 1B). However, in diabetic rats that received LY, ACh-induced dilation was preserved, with 1 μM ACh producing 55.9±4.6% dilation (n=9, $p<0.001$ vs. diabetic group). Control animals on LY diet also had normal responses to nitroprusside and to ACh (Figure 1). These results suggest that inhibition of PKCβ by LY was protective against the development of endothelial dysfunction in diabetic animals.

AA-Mediated Vasodilation

AA produced dose-dependent dilation of coronary arteries from control rats, where 1 μM AA produced 54.7±3.1% and 30 μM 72.0±3.0% dilation (n=9, Figure 2A). In diabetic rats, 1 μM AA only produced 31.4±3.8% and 30 μM 43.8±3.7% dilation (n=8, $p<0.001$ vs. control for both), indicating AA-mediated dilation in coronary arteries is impaired in diabetes. In contrast, in diabetic rats on LY diet, dilation to AA was preserved, with 1 μM AA producing 54.5±4.9% (n=8, $p<0.01$ vs. diabetic rats) and 30 μM 70.3±3.7% dilation (n=8, $p<0.001$ vs. diabetic rats, p=N.S. vs. control). These results suggest that AA-mediated dilation is impaired in diabetic rat coronary arteries but the administration of LY is protective. In comparison, LY had no effect on the vasodilation response to AA in control rats. Responses to AA are primarily endothelial-mediated since the majority (>70%) of the response was abolished following removal of the endothelium in both control and diabetic vessels (Figure 2B).

To determine the effect of acute PKCβ inhibition, control and diabetic vessels were exposed to 30 nM LY333531 for 30 min prior to determination of AA-mediated vasodilation. Short-term inhibition of PKCβ had no protective effects against abnormal AA-mediated vasodilation in isolated diabetic vessels (Figure 3A and 3B). These results suggest that vascular dysfunction in diabetes is produced by events downstream of PKCβ signaling and are not mitigated by acute PKCβ inhibition.

Role of CYP Epoxygenase

In control arteries, AA-mediated dilation was significantly reduced by preincubation with miconazole (10^{-5} M), suggesting that CYP epoxygenase metabolites are important contributors of AA-mediated vasodilation (Figure 4A). In contrast, miconazole had no effect in diabetic coronary arteries (Figure 4B), suggesting that there

is diminished ability for diabetic vessels to produce dilation by the AA products of CYP epoxygenase. Similar to controls, diabetic rats on LY diet showed significant reduction in AA-mediated vasodilation after pre-incubation with miconazole (Figure 4C). These results suggest that the enhanced PKCβ activities in diabetic vessels may underlie the derangements of AA metabolism by CYP epoxygenase and LY prevents the development of such abnormalities.

Role of BK Channels

Since BK channels are important targets of AA vasoactive metabolites, we examined the role of BK channels on the abnormal AA-mediated dilation in diabetic coronary arteries by pre-incubation with IBTX (10^{-7} M). In control coronary arteries, IBTX produced significant inhibition of AA-mediated dilation (Figure 5A), suggesting that BK channels were important targets of the vasoactive metabolites of AA. In contrast, IBTX had no effect in diabetic coronary arteries (Figure 5B), suggesting that BK channels do not play a significant role either due to the lack of channel activating vasodilators or abnormal channel function. However, in diabetic rats on LY diet, sensitivity to IBTX was preserved (Figure 5C), and the coronary arteries from these rats behaved similarly as in control rats. These results suggest that inhibition of PKCβ preserves the role of BK channels and protects against the impairment of AA-mediated vasodilation in diabetes.

Effect of SOD on ACh- and AA-Mediated Vasodilation in Diabetic Coronary Arteries

Since the enhanced PKCβ activity in diabetes has been shown to be associated with an increased generation of ROS (Cooper et al., 2001; Inoguchi et al., 2003), we examined the effects of superoxide dismutase (SOD) on ACh-mediated relaxation in

diabetic coronaries. Treatment with SOD did not affect the vasodilation produced by ACh in vessels from control rats (Figure 6A). However, in diabetic vessels that showed impairment to ACh-mediated vasodilation, SOD significantly improved the effects of ACh (Figure 6A). SOD had no effects on vessels from diabetic rats on LY diet, similar to that observed in control vessels (Figure 6B). These results suggest that ROS is involved in the impairment in ACh-mediated vasodilation, and treatment with SOD could effectively ameliorate such dysfunction.

Similarly, treatment with SOD had no effect on AA-mediated vasodilation in coronary arteries from control rats (Figure 7A). However, SOD normalized the impaired vasodilation observed in diabetic coronaries to AA (Figure 7A). Similar to controls, vessels from diabetic rats on LY diet were not affected by treatment with SOD (Figure 7B). These results suggest that the impairment of AA-mediated vasodilation in diabetic vessels could be caused by over-production of ROS, and treatment with SOD was able to maintain normal vessel function.

The results from Figures 6 and 7 together indicate that vascular endothelial dysfunction in diabetic rats might be due to enhanced ROS generated by the increase in PKCβ activities.

Measurement of Oxidative Stress Level in Coronary Arteries

To confirm that ROS is elevated in diabetic vessels, we assessed and compared the level of ROS in coronary arteries from control, diabetic, and diabetic rats on LY diet by using fluorescence microscopy with DHE (Figure 8). The light micrograph, the DHE fluorescence image, the internal elastic lamina autofluorescence image, and the digitally merged composite image for each vessels from control, diabetic, and diabetic rats on LY

diet are displayed (Figures 8A, 8B, and 8C) Vessels from control rats showed only a low level of red fluorescence, suggesting the presence of low ROS (Figure 8A). In contrast, under identical imaging settings and conditions, vessels from diabetic rats showed marked bright red fluorescence indicating an elevated level of oxidative stress (Figure 8B). Also, the DHE signals were enhanced both in endothelial and smooth muscle layers of the vessel. In comparison, vessels from diabetic rats on LY diet showed a low level of red fluorescence, similar to that observed in control vessels (Figure 8C). Group data showing the DHE signals per unit vessel area are summarized in Figure 8D. DHE fluorescence in control, diabetic and diabetic + LY vessels showed that the level of superoxide in diabetic vessels was almost doubled compared to controls. LY administration prevented the increase in ROS in diabetic vessels, suggesting PKCβ activation produces oxidative stress, which leads to endothelial dysfunction in diabetes. These results indicate that administration of LY protects against the development of endothelial dysfunction in diabetes through the suppression of ROS generation by inhibition of PKCβ.

Discussion

In this study, we reported several important findings. First, in streptozotocin-induced diabetic rats, AA-mediated dilation in small coronary arteries was impaired. Second, oral administration of LY prevented the development of endothelial dysfunction, including those mediated by ACh and AA. Third, AA-mediated vasodilation through the CYP epoxygenase pathway and through activation of BK channels was impaired in diabetic vessels but not in diabetic rats on LY. Fourth, the level of ROS is elevated in diabetic coronary arteries and treatment with SOD restored the vessel sensitivity to ACh and AA. Fifth, the enhanced production of ROS in diabetic vessels was suppressed by oral administration of LY. These results suggest that the elevated PKCβ activity in diabetic vessels is a central mechanism that promotes endothelial dysfunction. Generation of superoxide from enhanced PKCβ activity appears to be the final common course that produces impairment in vasodilator responses in diabetic coronary arteries.

We found that AA is a potent vasodilator in small coronary arteries in rats and this effect requires an intact endothelium. These results are in agreement with previous reports (Miura and Gutterman, 1998; Lu et al., 2005). In human coronary arterioles, the AA-mediated dilation was dependent on CYP and BK channel activities (Miura and Gutterman, 1998). AA metabolism in coronary arteries is ostensibly different from that in mesenteric arteries in which the LOX pathway produces 12-HETE as the predominant AA-derived vasodilator (Miller et al., 2003; Zhou et al., 2005). However, with the development of diabetes mellitus, AA lost its ability to produce endothelial mediated relaxation in the rat small coronary arteries, similar to the mesenteric arteries in ZDF rats (Zhou et al., 2005). In normal rat coronary arteries, AA-mediated vasodilation is

dependent on the products of CYP epoxygenase and on BK channel activation. However, these mechanisms of AA-mediated vasodilation are no longer effective in diabetic coronary arteries (Figure 4 and 5). In contrast, in diabetic rats on LY diet, function of the CYP epoxygenase pathway and BK channels remain intact. It is important to point out that diabetic rats on LY diet are hyperglycemic, similar to diabetic rats on normal diet. These results suggest that the pathophysiological consequences of PKCβ elevation are central to the development of diabetic endothelial dysfunction, because inhibition of PKCβ by LY is able to maintain normal vascular function. However, acute inhibition of PKCβ did not restore normal vessel function (Figure 3), suggesting that the events downstream of PKCβ are important in causing vascular dysfunction in diabetes mellitus.

Endothelial dysfunction in streptozotocin-induced diabetic rats was quite extensive, involving multiple sites and multiple pathways. The major culprit appears to be generation of ROS associated with diabetes. Indeed, acute treatment with SOD was effective in reversing the endothelial defects and restoring normal function. We found that acute exposure to SOD restored the diabetic coronary artery's ability to respond to ACh and AA (Figures 6 and 7). These findings suggest that the pathophysiological mechanisms are dynamic, modulating the target proteins in a time course of minutes, suggesting a post-translational modification of existing proteins and enzymes, rather than involving changes in gene expressions. These results are consistent with findings from other laboratories (Erdos et al., 2004). The exogenous SOD appeared to exert its effects extracellularly. Recently, it has been reported that extracellular SOD (ecSOD) is a major form of SOD in the vessel wall playing a critical role in protecting the bioavailability of NO, and reduced ecSOD is associated with abnormal vascular reactivity in cardiovascular

diseases including arteriosclerosis (Fukai et al., 2002) and diabetes (Ciechanowski et al., 2005). Indeed, ecSOD polymorphism is associated with insulin resistance and susceptibility to type 2 diabetes (Tamai et al., 2006), and gene transfer of ecSOD improves endothelial function in rats with heart failure (Iida et al., 2005). We believe one of the major mechanisms through which ecSOD restores vascular function is by extracellular scavenging of ROS so that the availability of NO and other vasodilators is preserved.

Enhanced generation of ROS in diabetes is well established but its cause is less well defined. Recent evidence suggests that elevated levels of PKC might be an important contributor to this process. Activation of PKC is known to cause endothelial dysfunction (Tesfamariam et al., 1991). Hyperglycemia increases diacylglycerol, a potent activator of PKC, with PKCβ being preferentially elevated in the aorta and heart of diabetic rats (Inoguchi et al., 1992). In this study, the dietary administration of LY to diabetic rats had no effect on hyperglycemia but significantly prevent the development of endothelial dysfunction. In diabetic rats on LY diet, vasodilation to ACh and AA were either normal or close to normal with significant improvement compared to their counterparts on normal diet. These results suggest that enhanced PKCβ might underlie the development of diabetic vasculopathy. Enhanced PKC activity is known to inhibit BK channel (Shipston and Armstrong, 1996) and vascular K_{ATP} channel functions (Hayabuchi et al., 2001; Chrissobolis and Sobey, 2002), but the sequelae of elevated PKCβ in diabetes appears to have a much wider impact than its kinase effects. Indeed, PKC has been shown to enhance production of ROS in diabetes through activation of superoxide producing enzymes including NADPH oxidase (Inoguchi et al., 2003). In addition,

diabetic rats are found to have a PKC-dependent upregulation of a dysfunctional, superoxide-producing, uncoupled endothelial nitric oxide synthase (NOS III) (Hink et al., 2001). The PKC-mediated production of superoxide may interact with NO, reducing NO availability, and produce the highly reactive peroxynitrite. Peroxynitrite has been shown to tyrosine nitrate important proteins and enzymes such as prostaglandin I_2 synthase, reducing its activity and resulting in diminished bioavailability of prostaglandin I_2 (Zou et al., 2002). We have also reported that in ZDF rat mesenteric arteries, tyrosine nitration of LOX is enhanced, resulting in reduced LOX activity and 12-S-HETE production (Zhou et al., 2005). Cytochrome P450 enzymes are also known targets of peroxynitrite-mediated nitrotyrosine formation and inactivation of the enzyme (Lin et al., 2005). Hence, all three pathways of AA metabolism can be modulated by superoxide/peroxynitrite, and may account for their functional impairment in diabetes. Recently, peroxynitrite has been shown to cause nitration and functional loss of voltage-gated K^+ channels in rat coronary microvessels exposed to high glucose (Li et al., 2004). BK channels are also known to be inhibited by ROS, through direct effects of peroxynitrite (Liu et al., 2002) and oxidation of specific cysteine residues on the channel by hydrogen peroxide (Tang et al., 2004). These mechanisms may contribute to our observation that BK channel mediated vasodilation is impaired in diabetic vessels (Figure 5). Hence, the enhanced PKCβ activity promotes formation of ROS, inactivating key enzymes and proteins that produce vasodilators and inhibit target effector function.

Our results demonstrated that dietary administration of LY, a PKCβ inhibitor, could prevent the development of endothelial dysfunction and maintain AA-mediated vasodilation, suggesting this strategy could have important therapeutic implications in the

treatment of diabetes. LY has been shown to have protective effects against vascular dysfunction (Ishii et al., 1996). LY prevented the impairment of endothelium-dependent vasodilation by hyperglycemia (Beckman et al., 2002b), attenuated leukocyte entrapment in retinal microcirculation (Nonaka et al., 2000), corrected the neurovascular dysfunction (Cameron and Cotter, 2002), normalized glomerular hyperfiltration, reduced albumin excretion, and improved renal function in diabetes (Tuttle and Anderson, 2003). In our experiments, LY did not improve blood glucose regulation but was able to restore endothelial function in diabetic rats and its effects are similar to treating vessels with SOD. With diabetic rats on LY, SOD has no further beneficial effects (Figures 6 and 7), suggesting LY was efficacious in suppressing the production of ROS in diabetes. These results are supported by fluorescent microscopy using DHE (Figure 8), showing that coronary arteries from diabetic rats had an elevated level of oxidative stress but not those from diabetic rats receiving LY. Our results support the notion that inhibition of PKCβ together with antioxidant therapy could be beneficial to vascular function in patients with diabetes. Indeed, it is important to note that ruboxistaruin (LY333531) mesylate is in the process of undergoing phase III clinical trials in patients with type 1 and type 2 diabetes mellitus to determine its efficacy in preventing the development of diabetic microvascular complications.

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Footnotes

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Legends for Figures

Figure 1. (A) Concentration dependent dilation to sodium nitroprusside in small coronary arteries from control rats, control rats on LY diet, streptozotocin-induced diabetic rats, and diabetic rats on LY diet. (B) Concentration dependent dilation to ACh in small coronary arteries from control rats, control rats on LY diet, diabetic rats, and diabetic rats on LY diet. * represents $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs. control rats; ++ represents $p<0.01$, and +++ $p<0.001$ vs. diabetic rats.

Figure 2. (A) Concentration dependent dilation to AA in small coronary arteries from control rats, control rats on LY diet, diabetic rats, and diabetic rats on LY diet. ** represents $p<0.01$ and *** $p<0.001$ vs. control rats; ++ represents $p<0.01$ and +++ $p<0.001$ vs. diabetic rats. (B) Effect of removal of endothelium on AA-mediated vasodilation in small coronary arteries from control rats and diabetic rats. *** represents $p<0.001$ vs. control rats; ++ represents $p<0.01$ vs. diabetic rats.

Figure 3. Concentration dependent dilation to AA in small coronary arteries from control rats (A) and diabetic rats (B) with and without a 30 min pretreatment with 30 nM LY333531. Short-term inhibition of PKCβ did not alter AA-mediated vasodilation in control and diabetic vessels.

Figure 4 Effects of miconazole (10^{-5} M), an inhibitor of CYP epoxygenase, on AA-mediated dilation in small coronary arteries from control (A), diabetic (B), and diabetic

rats on LY diet (C). Vessels were incubated with miconazole for 30 min prior to the experiment. * represents $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs. vessels not exposed to miconazole.

Figure 5 Effects of iberiotoxin, an inhibitor of the large conductance Ca^{2+}-activated K^+ (BK) channels, on AA-mediated dilation in small coronary arteries from control (A), diabetic rats (B), and diabetic rats on LY diet (C). Vessels were incubated with iberiotoxin (IBTX, 10^{-7} M) for at least 30 min prior to the determination of concentration-response with AA. * represents $p<0.05$ vs. vessels not exposed to IBTX.

Figure 6. Effects of SOD on dilation by ACh in small coronary arteries from control and diabetic rats (A), and diabetic rats on LY 333531 diet (B). * represents $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs. control; + represents $p<0.05$, ++ $p<0.01$, and +++ $p<0.001$ vs. diabetic rats

Figure 7. Effects of SOD on dilation by AA in small coronary arteries from control and diabetic rats (A), and diabetic rats on LY 333531 diet (B). ** represents $p<0.01$ and *** $p<0.001$ vs. control; + represents $p<0.05$ and +++ $p<0.001$ vs. diabetic rats.

Figure 8 Fluorescence microscopy of oxidative stress in rat coronary arteries. Microscopic sections of the vessels were incubated with DHE. At identical laser and photomultiplier settings, coronary artery sections from control rats (A), diabetic rats (B), and diabetic rats on LY diet (C) were processed and imaged in parallel. Four different

images are displayed for each vessel section, including: a DHE fluorescence image (red), an internal elastic lamina autofluorescence image (green), a transmitted light micrograph (no color), and a digitally merged image. Autoflourescence of the internal elastic lamina was used to locate the endothelium and outline the vessel lumen. The vessel sections from diabetic rats showed marked increase in red fluorescence compared with vessels from control and diabetic rats on LY diet, indicating that the level of ROS was elevated in diabetic rat coronary arteries but LY diet prevented this increase in oxidative stress. The results are representative of multiple vessel sections from two animals from each group. (D) Group data in bar graphs showing the densitometric analysis of the DHE signals in coronary arteries from control, diabetic, and diabetic rats on LY diet (n=3 for each group). Results are expressed as relative densitometric units per unit area of vessel cross-section. *represents $p<0.05$ vs. control.

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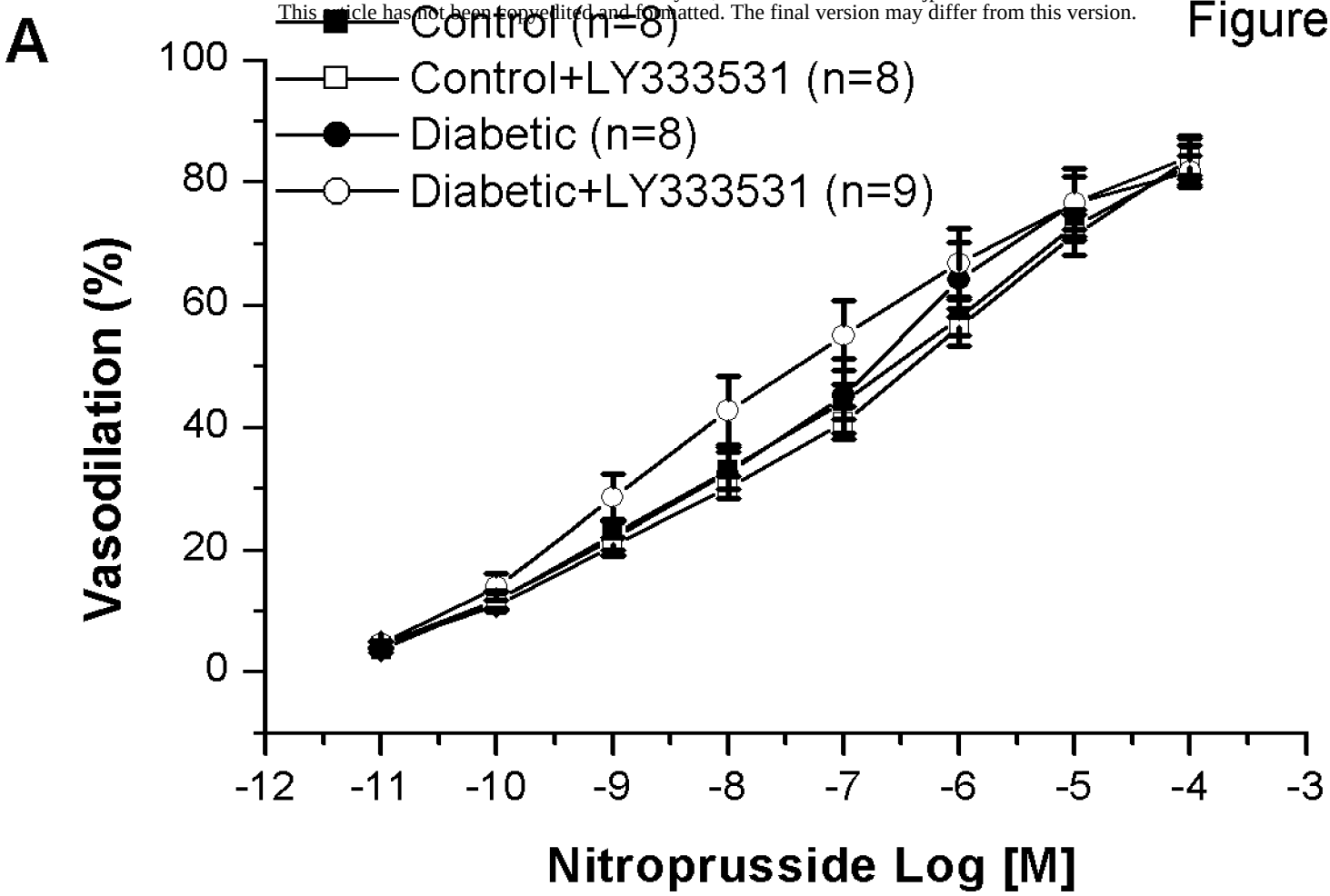
Figure 1



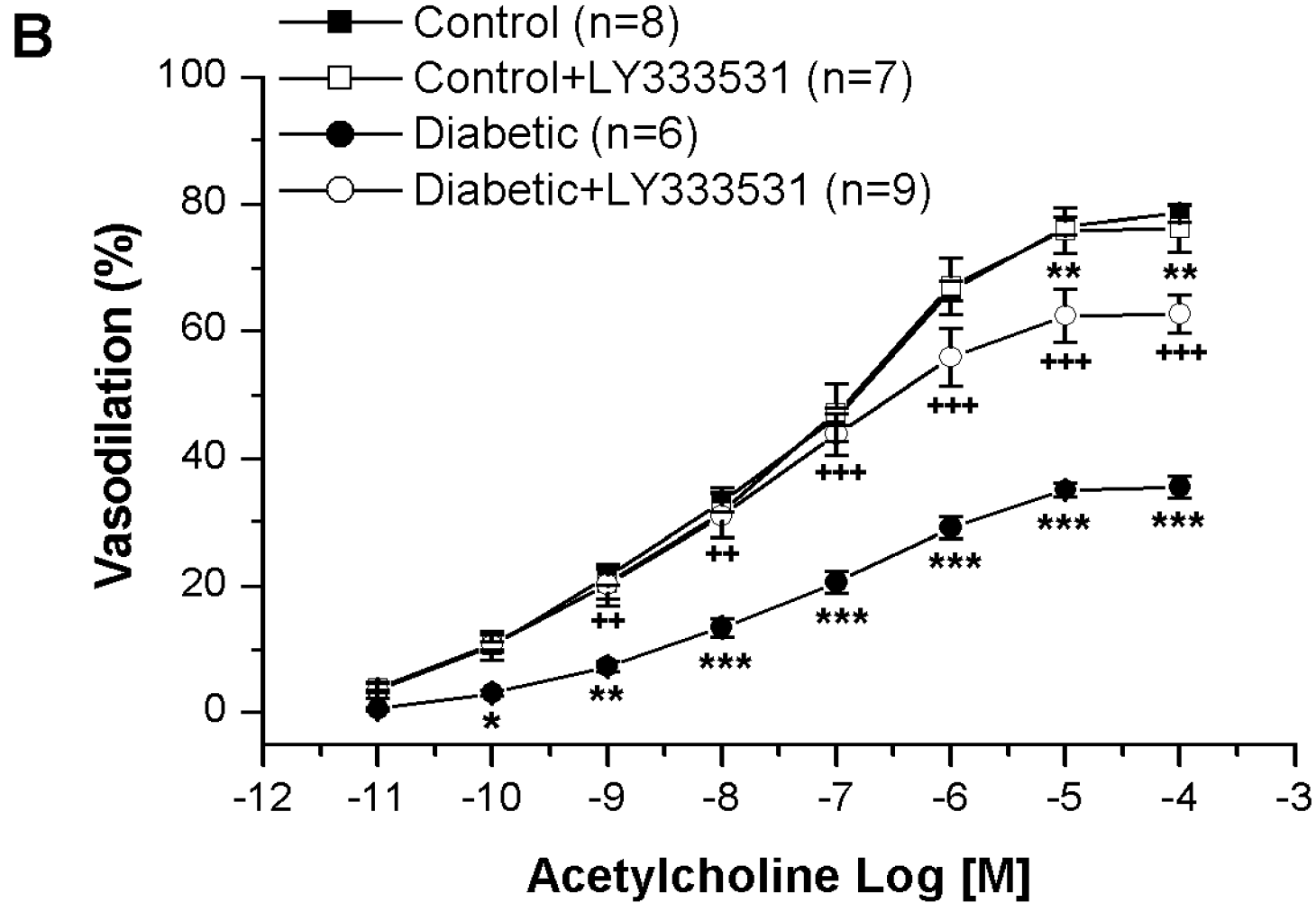


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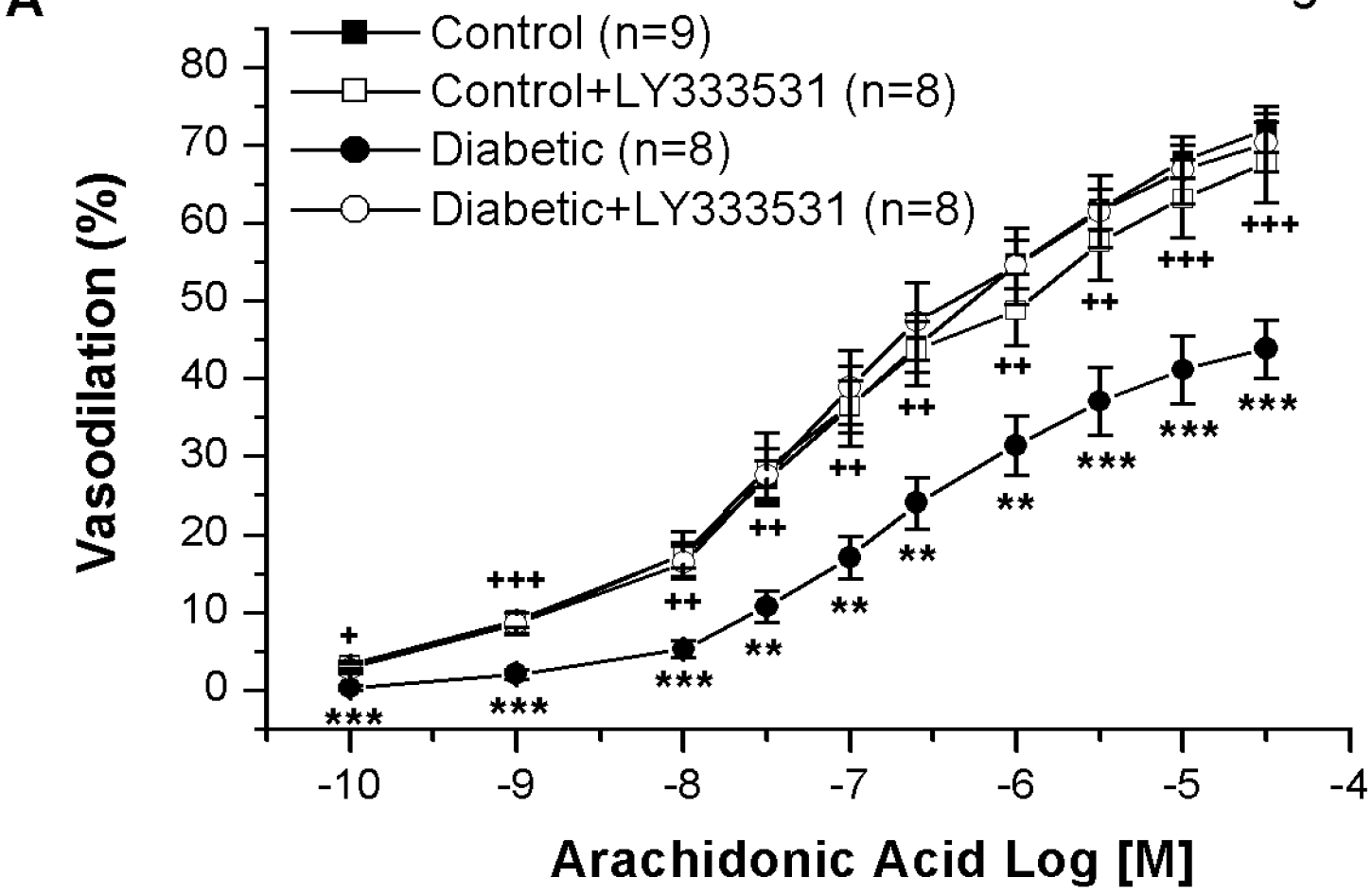
Figure 2



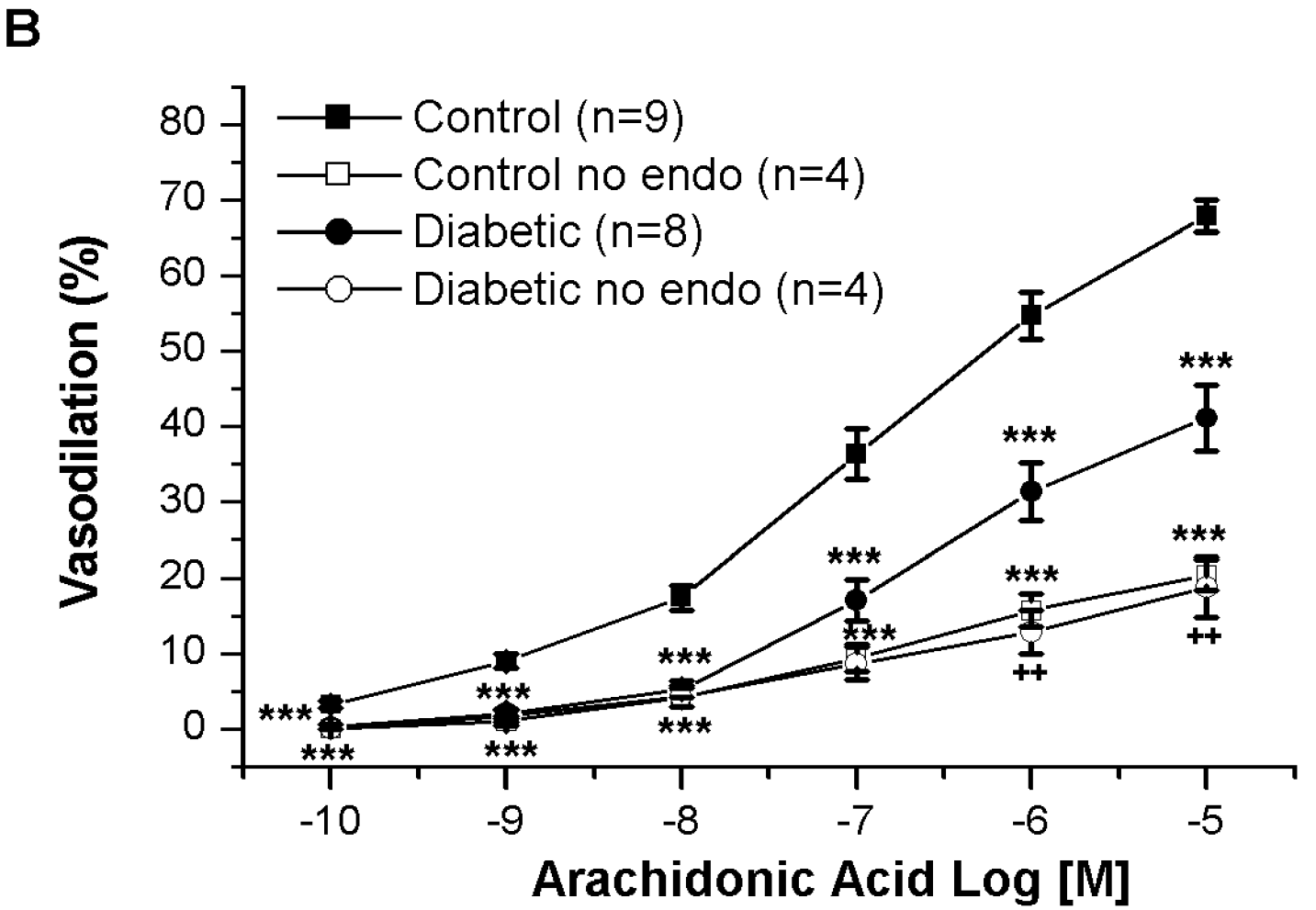


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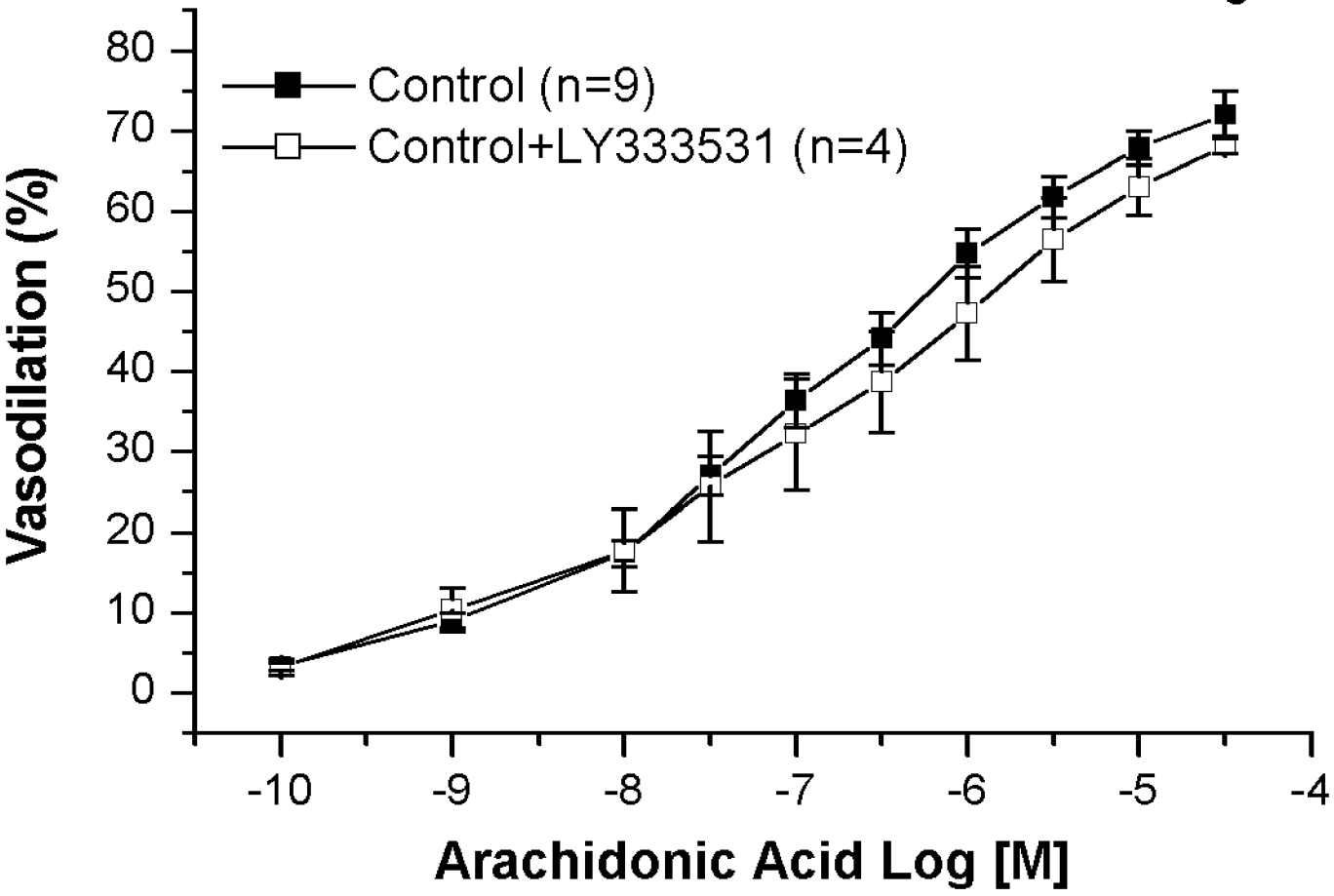
Figure 3

A



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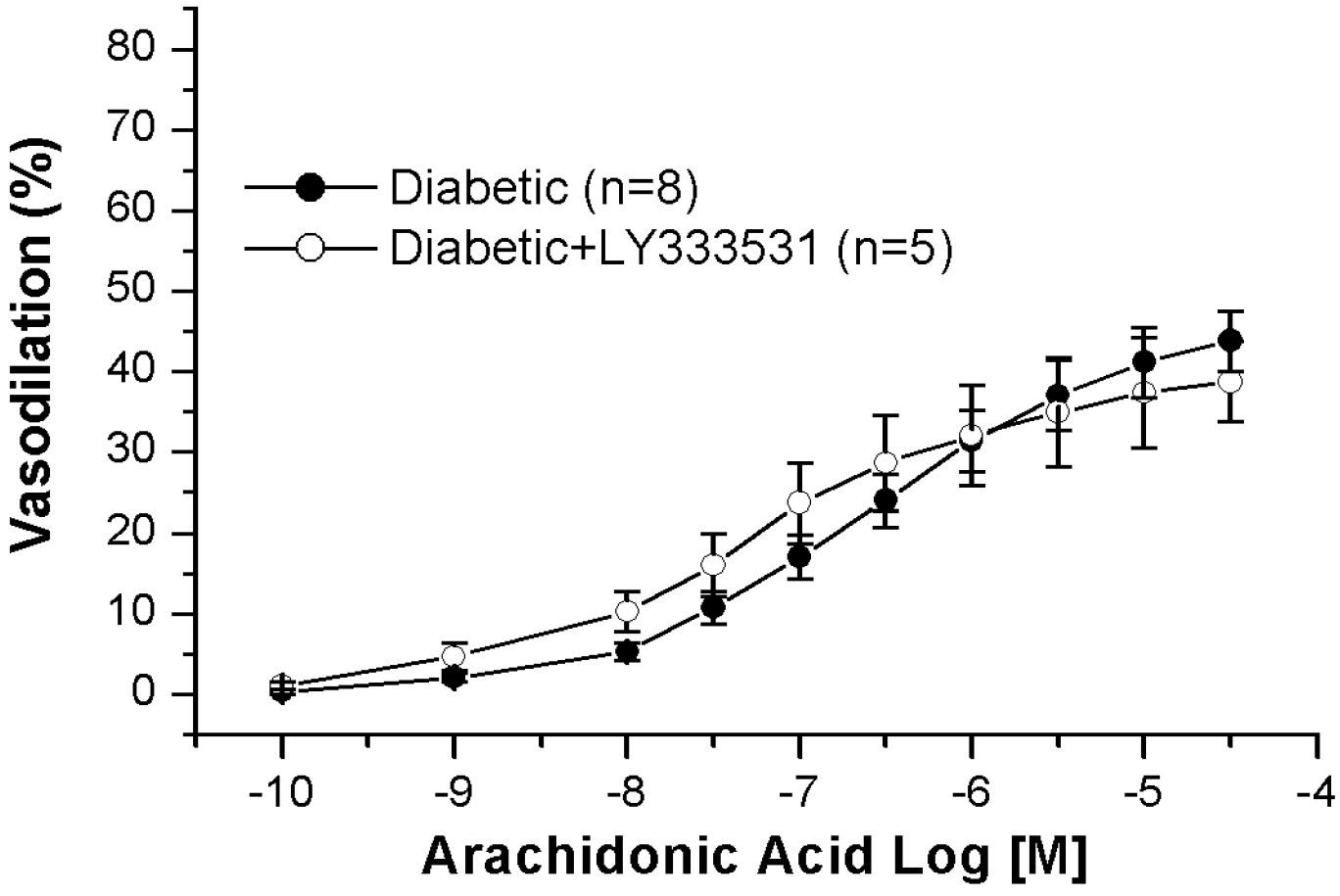

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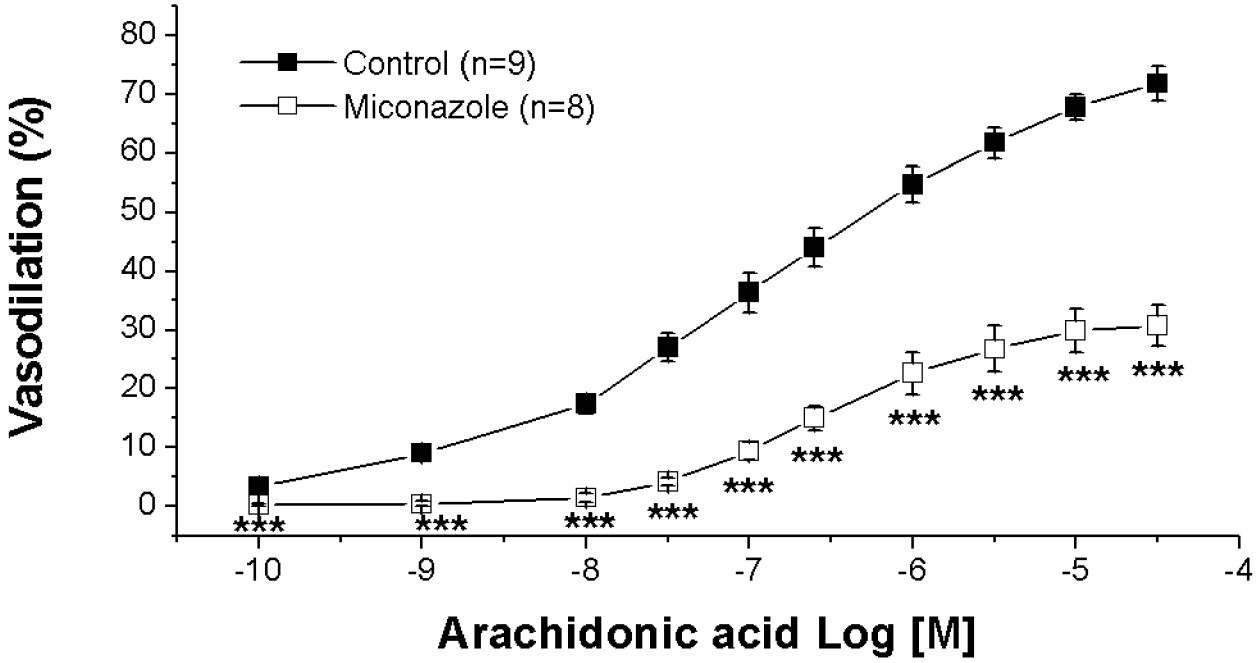
Figure 4

A



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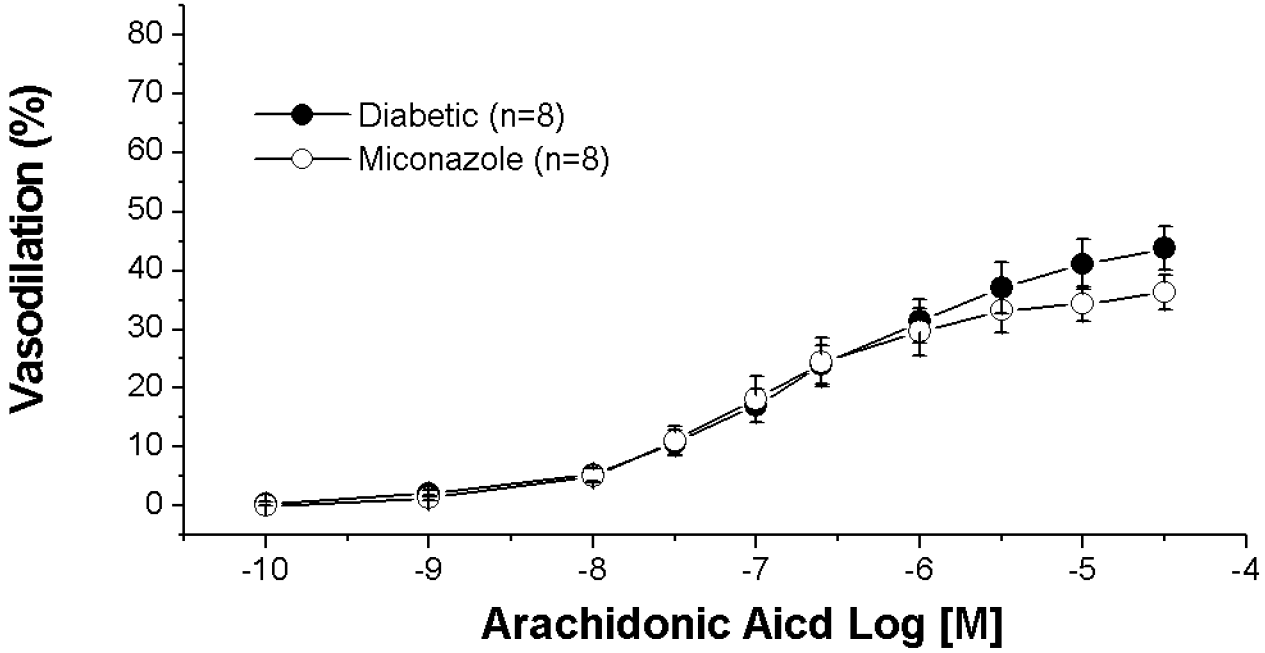

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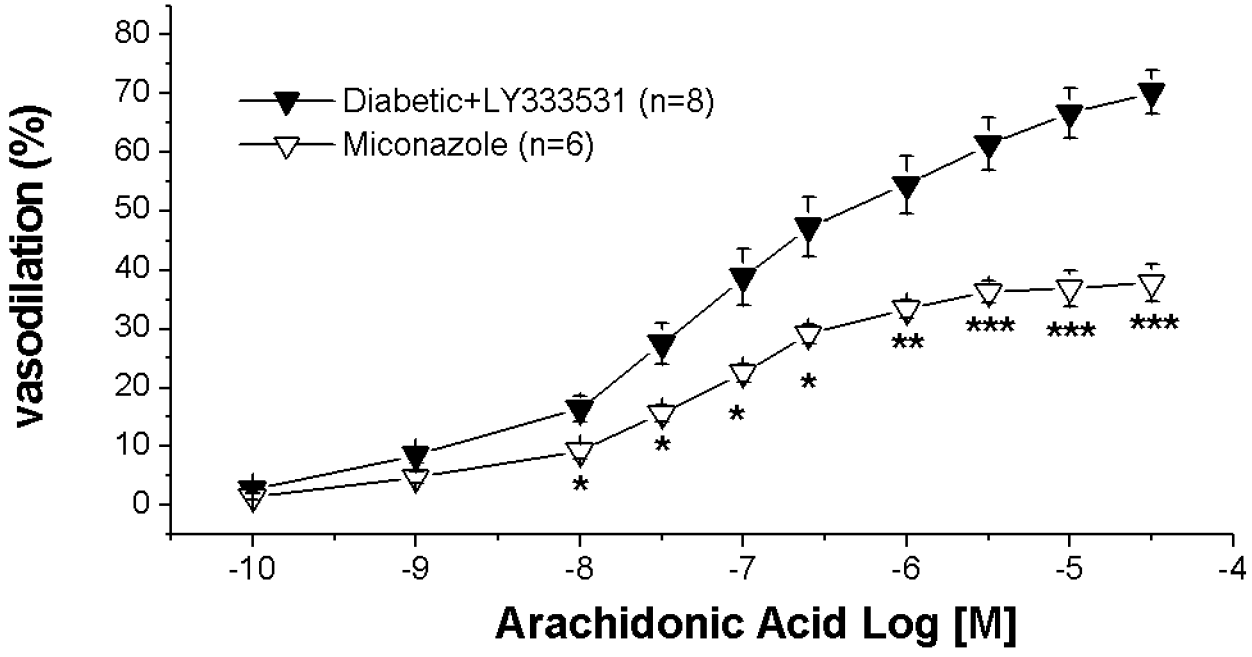

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Figure 5

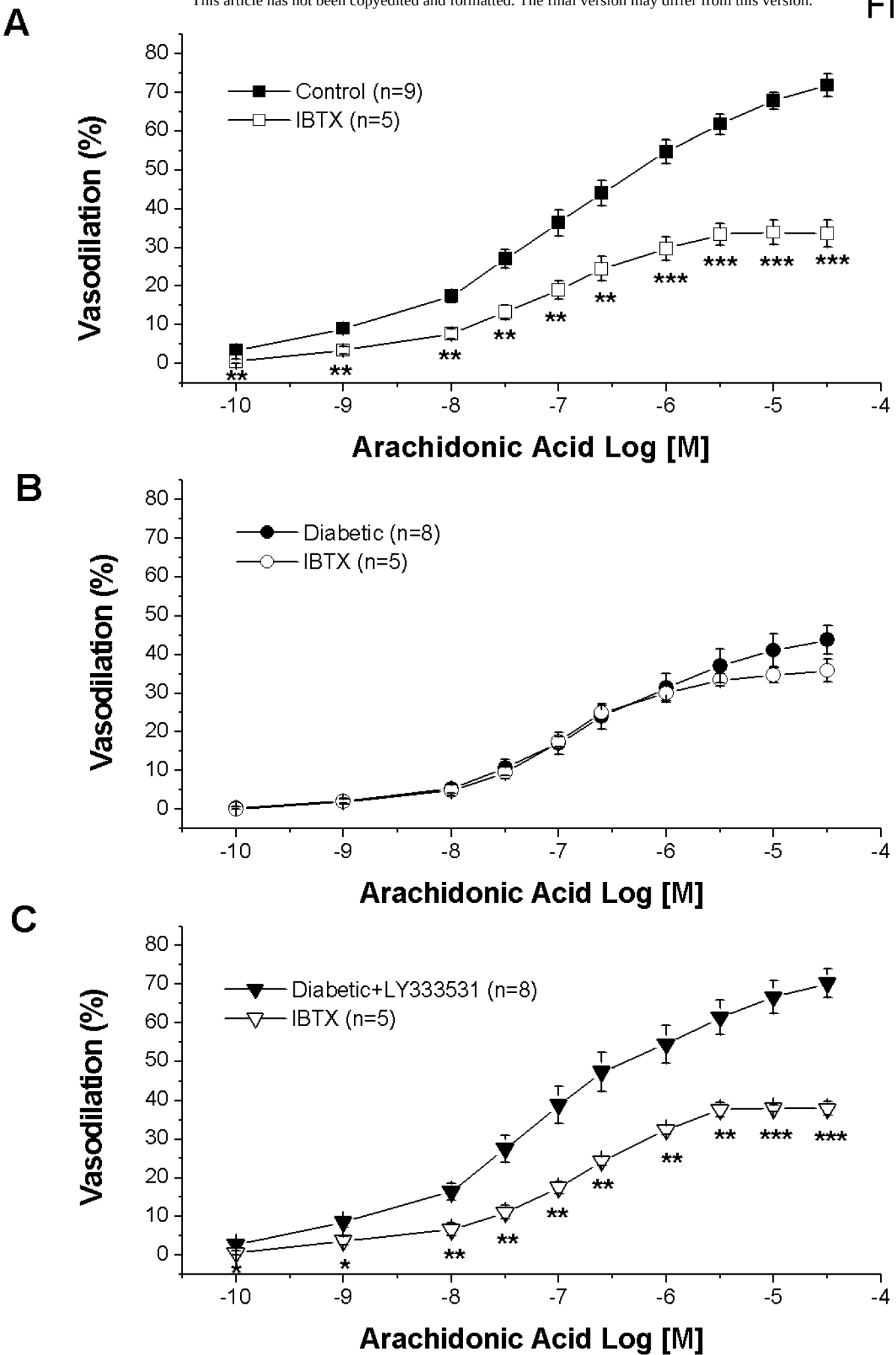

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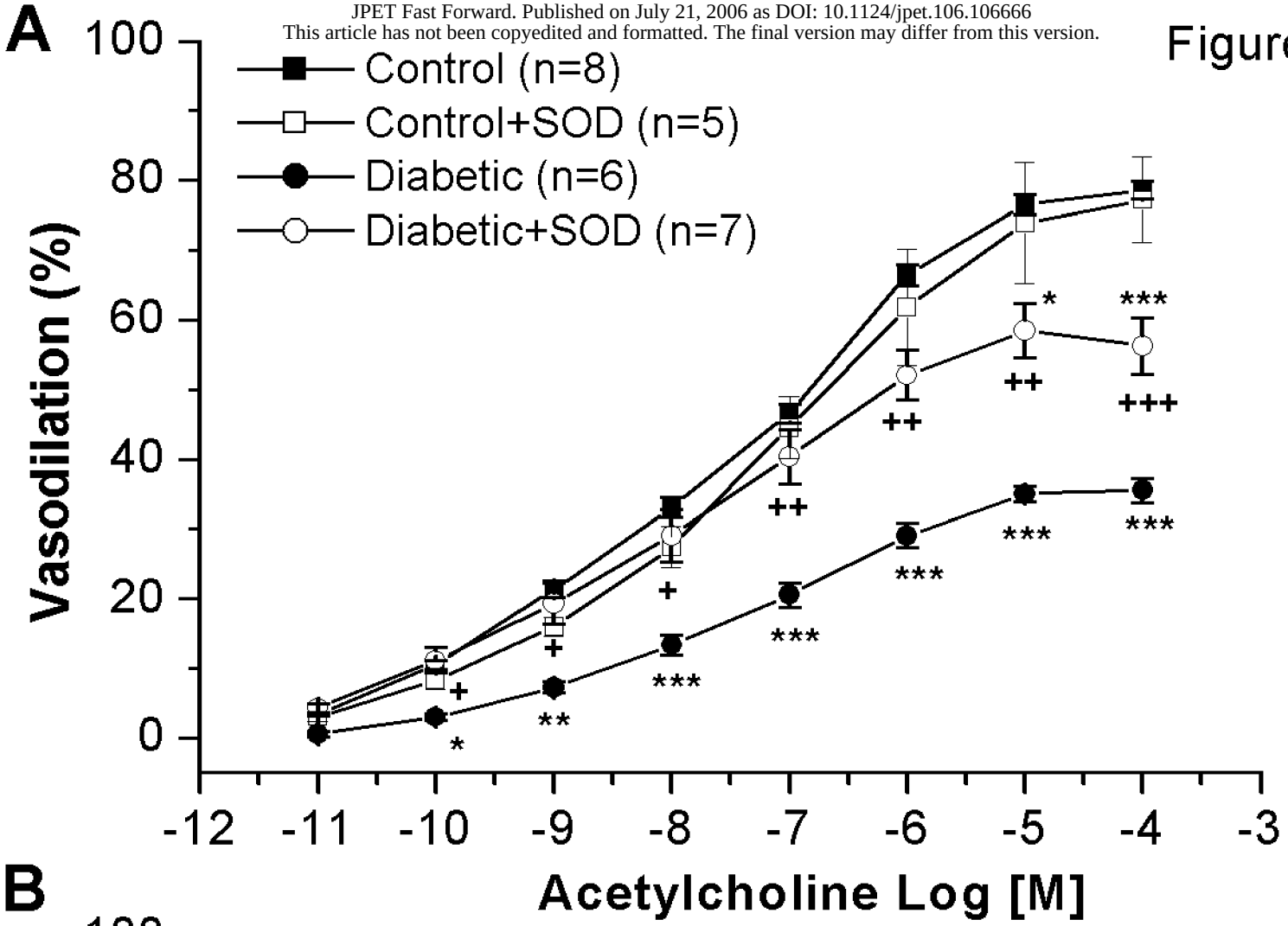
Figure 6



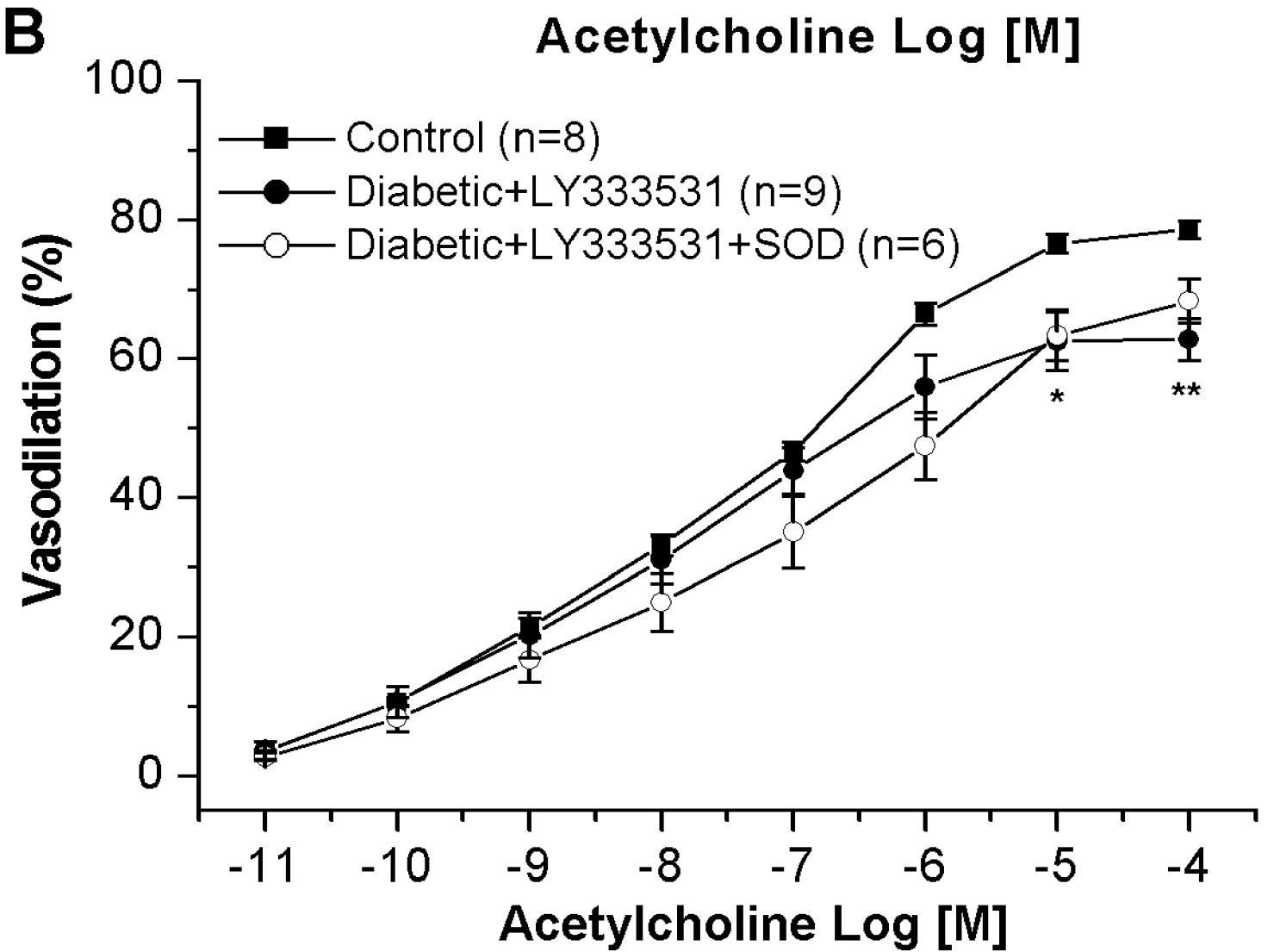


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Figure 7