ORIGINAL ARTICLE

Alphacalcidol Supplementation Improves Acetylcholine-Mediated Relaxation in Aorta of Diabetic Rats on Vitamin D-Deficient Diet

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ABSTRACT

Introduction: Vitamin D deficiency has been implicated as one of the factors involved in endothelial dysfunction associated with diabetes. This study aimed to evaluate the effects of active vitamin D (alphacalcidol) supplementation on aortic endothelial function in diabetic rats receiving vitamin D-deficient diet. **Methods:** Streptozotocin-induced diabetic rats were fed with standard diet (D) or vitamin D-deficient diet (DD and DDS) for 10 weeks. Group DDS was then supplemented with 0.2 µg/kg alphacalcidol at the last four weeks of the study duration. Non-diabetic rats were fed with standard diet (N) or vitamin-D deficient diet (ND). At the end of the experiment, the rats were sacrificed, and their aortic rings were harvested for endothelial functional study. **Results:** Acetylcholine-induced relaxation in aorta of diabetic rats (D and DD) were significantly lower compared to non-diabetic rats (N). In the presence of endothelial nitric oxide synthase blocker (L-NAME), maximal relaxation induced by acetylcholine in aorta of D and DD groups were significantly higher compared to N, ND and DDS groups, indicating involvement of non-nitric oxide (NO) relaxation pathways in diabetes. Four weeks supplementation with alphacalcidol in DDS group significantly improved acetylcholine-induced relaxation and reduced the reliance on non-NO relaxation pathways. **Conclusion:** The present study suggests that impairment of acetylcholine-induced relaxation in aorta of diabetes and diabetes with vitamin D-deficient diet was largely due to a decrease in NO related pathways, and this was compensated by non-NO pathways. Supplementation with alphacalcidol alleviated endothelial impairment in aorta of diabetic rats with vitamin D-deficient diet.

Keywords: Alphacalcidol, Aorta, Diabetes, Endothelial dysfunction

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INTRODUCTION

The vascular endothelium is a single layer of endothelial cells which forms the inner lining of blood vessels. The endothelium controls the tone of the underlying vascular smooth muscle through the production of various endothelial-derived relaxing factors (EDRF), including nitric oxide (NO), prostacyclin and endothelium-dependent hyperpolarising factor (EDHF), in addition of endothelium-derived contracting factors (EDCF). Endothelial dysfunction, defined as impaired endothelium-mediated relaxation is characterised by the imbalance in the availability of EDRF and EDCF. Nitric oxide (NO) is the major and potent EDRF in most vessels. Besides its role in maintaining vascular tone, it helps to maintain the quiescent endothelium by inhibiting inflammation and thrombosis (1). Endothelial nitric oxide synthase (eNOS) enzyme catalyzes the production of NO from L-arginine in the endothelium. The released NO leads to vasorelaxation. It also results in S-nitrosylation of a wide range of proteins which include nuclear factor kappa β (NF-Kß), cell cycle controlling proteins and proteins involved in the generation of tissue factor. These help to silence cellular processes which would otherwise activate the endothelium. Endothelial activation reflects a switch from a quiescent state, mostly regulated by NO, to an activated endothelium mediated by redox signalling. Chronic hyperglycemia in diabetes can cause endothelial activation by triggering molecular mechanisms in the endothelium that results in expression of chemokines, cytokines and adhesion molecules contributing to inflammation. Endothelial dysfunction is associated with many cardiovascular disorders such as hypertension and diabetes (2).

Diabetes mellitus is a complex metabolic disease associated with increased risk of developing cardiovascular diseases, a major cause of morbidity and mortality in diabetic patients (3,4). A number of

growing evidence suggested that endothelial function is attenuated in diabetes mellitus and plays a critical role in the pathogenesis of diabetic vascular complications (5-7). Moreover, endothelial dysfunction was seen in both diabetic patients as well as in healthy individuals with family history of diabetes, suggesting impaired endothelial function predates vascular complications (8).

Vitamin D is known to have vascular protective effects by maintaining the quiescent state of endothelium as well as modulating certain stages during endothelial activation (9). It is a fat-soluble vitamin produced endogenously in the skin after exposure to ultraviolet radiation, followed by conversion into biological active form, 1,25-hydroxycholecalciferol. Vitamin D exerts its action via vitamin D receptor (VDR) expressed in various tissues including the vasculature. Physiologically, vitamin D plays an important role in calcium and phosphorous homeostasis. In addition to its well-known implications on bone health, vitamin D also affects endothelium homeostasis (10, 11).

Vitamin D enhances eNOS activity and formation of NO (1). It interacts with the VDR causing the phosphorylation of p38, Akt and extracellular signal-regulated kinase (ERK) leading to eNOS activation, thus increased NO formation. Vitamin D also improves the bioavailability of endothelial NO via enhancement of the transcriptional regulator of eNOS (12) and/or affecting phosphatidylinositol 3 kinase in the endothelium cell, which activates eNOS to catalyse NO production (10, 13). These will be useful in diabetes, as hyperglycemia has been reported to be associated with reduced eNOS protein expression and NO production (14).

Diabetes is associated with increased oxidative stress and reactive oxygen species (ROS), a contributor to the development of diabetes related vascular complications (15). ROS have the ability to inactivate EDRF, reducing their availability, thus impairing vasorelaxation and leads to endothelial dysfunction (16). Vitamin D acts as an antioxidant; it can reduce the formation of ROS by suppressing the gene expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; one of the main sources of ROS (17). It can also elicit antioxidant effects through upregulating expression of antioxidative enzymes which include superoxide dismutase (SOD), copper-zinc superoxide dismutase, glutathione peroxidase (GPx), catalase and glutathione (GSH) that scavenges free radicals (10, 18).

Diabetics exhibit reduced 1,25-hydroxycholecalciferol (19). It is due to reduce 1α-hydroxylase activity (20). Alphacalcidol (1α-hydroxycholecalciferol) is a non-endogenous analogue of vitamin D and does not require renal 1α-hydroxylase activity to form the physiologically active form of vitamin D (1,25-hydroxycholecalciferol). Thus, it is often used in patients needing vitamin D who have impaired renal function (21). Therefore, the aim of this study was to evaluate the effects of alphacalcidol supplementation on the endothelial function of diabetic rats receiving vitamin D-deficient diet.

MATERIALS AND METHODS

Materials

The standard diet (AIN93G) and vitamin D-deficient diet (SF03-009) were purchased from Specialty Feeds (Glen Forrest, Western Australia). Acetylcholine hydrochloride, alphacalcidol, phenylephrine hydrochloride, sodium nitroprusside and streptozotocin were purchased from Sigma Chemical (St Louis, MO, USA). Indomethacin and L-NAME hydrochloride were purchased from Cayman Chemical Company (Ann Arbor, MI, USA)

Animals and induction of diabetes

The experimental protocol used in this study was approved by the Animal Ethics Committee of Universiti Sains Malaysia [No. USM/Animal Ethics Approval/2013/(82)(450)]. Male Sprague Dawley rats (6-8 weeks old) weighing between 250-300 g were used. After one week of acclimatisation period, 21 rats were randomly chosen to be induced with diabetes while 14 rats formed the nondiabetic group. Diabetes was induced by an intraperitoneal injection of streptozotocin (55 mg/kg) prepared in citrate buffer (pH 4.5). Control rats were injected with the citrate buffer alone and kept under identical conditions. After 72 hours and overnight fasting, blood glucose was determined from blood samples obtained from the tail vein using a one-touch glucometer (Accu-check, Roche Diagnostics, IN, USA). Rats with fasted blood glucose level higher than 11 mM were considered diabetic. Fasting blood glucose levels and body weight measured at the day of successful diabetes induction were recorded as the baseline value.

The control and diabetic rats were then divided into the following groups:
Group N: normal rats fed with standard diet for 10 weeks.
Group ND: normal rats fed with vitamin D-deficient diet for 10 weeks.
Group D: diabetic rats fed with standard diet for 10 weeks.
Group DD: diabetic rats fed with vitamin D-deficient diet for 10 weeks.
Group DDS: diabetic rats fed with vitamin D-deficient diet for 10 weeks, with four weeks of oral alphacalcidol supplementation (0.2 μg/kg, daily) starting from week 7 of diabetes induction until the end of the study period.

At the end of 10 weeks after diabetes induction, all rats were fasted overnight and anaesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). FBG level and body weight measured on the day of sacrifice were taken as the final value for the parameters. Blood samples were

collected via cardiac puncture for the measurements of calcium level by sending to certified laboratory (BP Lab, Malaysia). The thoracic aorta tissues were isolated to be used in endothelial functional study.

Measurement of endothelial function

The thoracic aortas were placed in Kreb's solution (composition in mM: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.18, $NaHCO_3$ 25, D-glucose 5.5 and $CaCl_2$ 2; oxygenated with 95% O_2-5% CO_2; pH 7.4) and cleaned from fats and surrounding tissues. The aortas were cut into ring segments (3 mm long) and suspended vertically in an organ bath (ADInstruments Pty. Ltd., Australia) containing 10 ml Kreb's solution. The bath temperature was maintained at 37°C and continuously bubbled with 95% O_2 and 5% CO_2. The aortic rings were equilibrated at a baseline value of 1 gram for 60 minutes and then exposed twice to potassium chloride (60 mM). Afterwards, the aortic rings were exposed to phenylephrine (1 μM) and after a steady contraction was obtained, acetylcholine (1 μM) was added to assess for functional endothelial cells.

In order to assess endothelium-mediated relaxations, the aortic rings were first pre-contracted with phenylephrine (1 μM) and exposed to cumulative concentrations of acetylcholine (1 nM to 10 μM). To assess the contribution of NO and prostacyclin to endothelium-dependent relaxation, the aortic rings were exposed to pharmacological inhibitors, L-NAME (inhibitor of eNOS, 100 μM) or indomethacin (inhibitor of cyclooxygenase, 10 μM), respectively (22, 23). The rings were incubated with the inhibitors for 30 minutes before the administration of phenylephrine. The aortic rings were also exposed to cumulative concentrations of sodium nitroprusside (10 nM to 100 nM) after pre-contracted with phenylephrine to assess endothelial-independent relaxation.

Statistical analysis

Statistical analysis were performed using GraphPad Prism version 6 for Windows (GraphPad software, San Diego California, USA). The data were presented as mean ± standard deviation (SD). Relaxation is expressed as a percentage relative to the tension generated by 1 μM phenylephrine. The maximal relaxation (R_{max}) in each experiment was the greatest relaxation to either acetylcholine or sodium nitroprusside. Difference between groups were compared using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. P values less than 0.05 were considered significantly different.

RESULTS

Baseline parameters

The body weight for the experimental animals were comparable at week 0. Body weight at week 10 was lower for diabetic rats (include those put on a vitamin D deficient diet and those receiving supplementation). Blood glucose taken at the end of 10-week study period was above 11 mmol/L for all diabetic rats and significantly different from non-diabetic rats ($p<0.001$). Serum calcium was not significantly different between groups (Table I).

Table I: Body weight, blood glucose and calcium levels for all experimental groups.

Parameters	Groups				
	N	ND	D	DD	DDS
Weight, W0 (g)	289.17 (11.89)	291.61 (14.12)	289.60 (18.02)	285.67 (17.81)	290.82 (13.57)
Weight, W10 (g)	417.83 (28.95)	421.20 (25.24)	333.80 (18.99) [a,b]	329.33 (20.32) [a,b]	341.40 (16.53) [a,b]
Blood glucose, W0 (mmol/L)	5.43 (0.59)	5.44 (0.53)	20.32 (4.34) [a,b]	22.52 (1.88) [a,b]	21.66 (2.15) [a,b]
Blood glucose, W10 (mmol/L)	5.38 (0.74)	5.48 (0.82)	25.40 (6.91) [a,b]	28.15 (6.13) [a,b]	27.86 (5.30) [a,b]
Serum calcium (mmol/L)	2.49 (0.09)	2.50 (0.10)	2.53 (0.15)	2.35 (0.17)	2.52 (0.17)

N, normal rats fed with standard diet; ND: control rats fed with vitamin D-deficient diet; D, diabetic rats fed with standard diet; DD: diabetic rats fed with vitamin D-deficient diet; DDS, diabetic rats fed with vitamin D-deficient diet and supplemented with alphacalcidol; W0, baseline; W10, week 10.

[a]$p<0.001$ vs. N, [b]$p<0.001$ vs. ND

Endothelium-dependent relaxation

Control responses

The maximal relaxation to acetylcholine in aortic rings of normal rats receiving vitamin D-deficient diet (group ND) was similar to that of normal rats (group N) (Table II, Fig. 1A). The relaxation was significantly reduced by 13.2% and 17.0% in group D and DD, respectively when compared to that of normal group. There was no difference in the endothelium-dependent relaxation between diabetic rats fed with standard diet and vitamin D-deficient diet. The diabetic group supplemented with alphacalcidol (group DDS) showed significantly higher endothelium-dependent relaxation compared to groups D and DD; with the increment of 14.5% and 18.3%, respectively.

Table II: Percentage of maximal relaxation to acetylcholine in the rat aorta

Groups	Maximal relaxation, %		
	Control solution	L-NAME	Indomethacin
N	86.5±6.5	17.2±6.6	90.1±13.6
ND	82.1±3.3	13.5±4.7	81.7±4.5
D	73.3±11.3[a,b]	40.7±9.7[a,b,c]	85.9±11.4
DD	69.5±11.2[a,b]	50.0±15.3[a,b,c]	76.2±3.7
DDS	87.8±10.3	19.9±10.1	87.2±15.3

N, normal rats fed with standard diet; ND: control rats fed with vitamin D-deficient diet; D, diabetic rats fed with standard diet; DD: diabetic rats fed with vitamin D-deficient diet; DDS, diabetic rats fed with vitamin D-deficient diet and supplemented with alphacalcidol; L-NAME, NG-nitroarginine methyl ester

[a]$p<0.05$ vs. N, [b]$p<0.05$ vs. DDS, [c]$p<0.05$ vs. ND

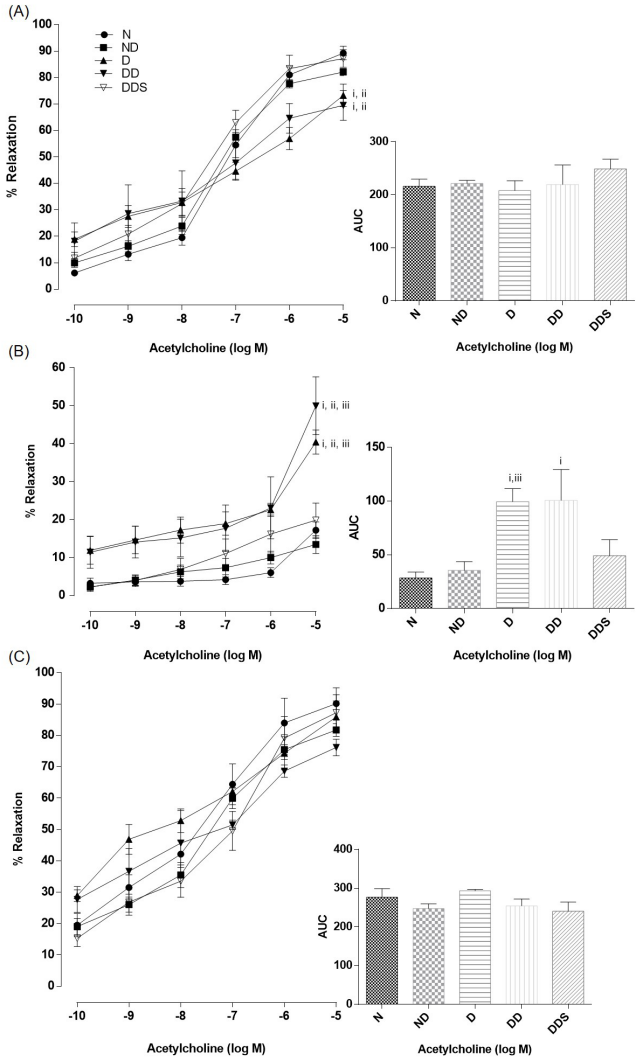

Figure 1: Concentration response curves and area under the curve (AUC) to acetylcholine in the rat aorta in (A) control solution (absence of blocker), (B) L-NAME and (C) indomethacin. Relaxations are expressed as a percentage of the contraction induced by phenylephrine. ($^{i}p < 0.05$ vs. N; $^{ii}p < 0.05$ vs DDS; $^{iii}p < 0.05$ vs ND, n=7)

Table III: Percentage of maximal relaxation to sodium nitroprusside in the rat aorta.

Groups	Maximal relaxation, %
N	102.6±1.9
ND	102.1±1.4
D	103.5±0.7
DD	102.7±2.9
DDS	97.4±6.2

N, normal rats fed with standard diet; ND: control rats fed with vitamin D-deficient diet; D, diabetic rats fed with standard diet; DD: diabetic rats fed with vitamin D-deficient diet; DDS, diabetic rats fed with vitamin D-deficient diet and supplemented with alphacalcidol; L-NAME, NG-nitroarginine methyl ester

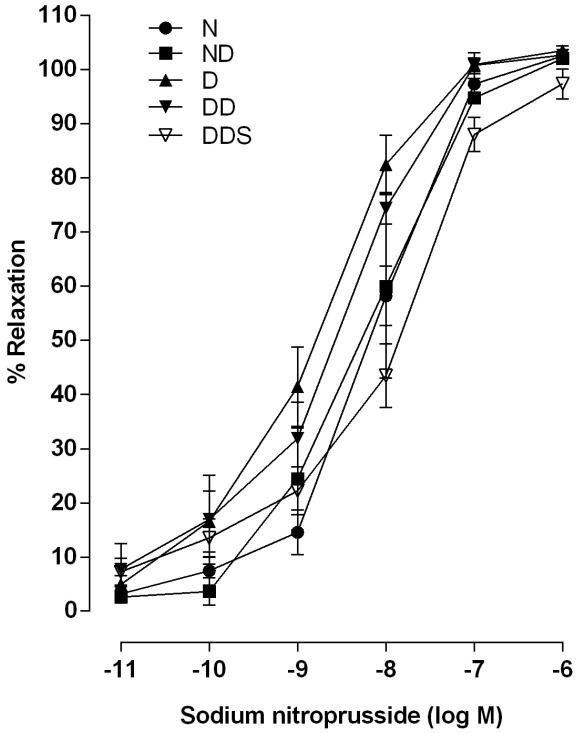

Figure 2: Concentration response curves to sodium nitroprusside in the rat aorta. Relaxations are expressed as a percentage of the contraction induced by phenylephrine (n=7).

Endothelial response after incubation with pharmacological blockers

Incubation with L-NAME generally caused reductions in maximal relaxation in all experimental groups and particularly marked in groups N, ND and DDS (69.3%, 68.6% and 67.9%, respectively). The maximal relaxation of aorta in diabetic groups (D and DD) were significantly greater compared to other experimental groups (Table II, Fig. 1B). In the presence of indomethacin, the maximal relaxation to acetylcholine was comparable in all experimental group (Table II, Fig. 1C).

Endothelium-independent relaxation

Endothelium-independent relaxation to sodium nitroprusside was comparable in all experimental groups (Table III, Fig. 2).

DISCUSSION

The present study showed that endothelium-dependant relaxation was impaired in diabetic group when compared to control group. Vitamin D-deficient diet did not significantly alter the endothelial function in both normal and diabetic rats. However, supplementation with alphacalcidol significantly improved the endothelial function in diabetic rats receiving vitamin D-deficient diet and the response is comparable to normal rats. On the other hand, endothelium-independent relaxation to sodium nitroprusside was comparable across all experimental groups, indicating that aorta smooth muscle was relatively unaffected during 10 weeks of diabetes, even in the presence of vitamin D deficiency.

Vascular tone is controlled via the release of various vasodilators and vasoconstrictors. The NO, released mainly from the endothelium, acts as the major contributor to vascular relaxation. This is supported by our findings whereby reduction of endothelial relaxation was evidenced across all groups when the NO synthase actions were inhibited by the presence of L-NAME. Diminished NO bioavailability, whether due to a decrease in NO synthase expression, impaired NO synthase activity or oxidative stress (which inactivates NO synthase activity) eventually leads to impaired NO-mediated vasodilation. This has been reported in various disease models including diabetes (24, 25). For instance, ROS can react quickly with NO to generate reactive nitrogen species (RNS) and reduces NO bioactivity.

Both ROS and RNS cause oxidative damage to nucleic acids, proteins and lipids. Moreover, persistent oxidative and nitrosative stress also cause depletion of the essential cofactor, tetrahydrobiopterin (BH_4) which leads to the uncoupling of NO synthase, thereby limiting the production of NO and contributes to impaired insulin action in the vasculature (26,27). Another possible mechanism is that oxidative stress enhanced asymmetric dimethylarginine (ADMA) activity which inhibit eNOS enzyme, thus disrupts endothelial NO production and bioavailability (28,29).

Regardless of the cause, diabetic models have consistently demonstrated NO impairment as a key factor to endothelial dysfunction, so it is unsurprising that the endothelium turns to non-NO forms as a compensatory mechanism to maintain vasodilatation. In the present study, maximal relaxation in the presence of L-NAME in diabetic rats was higher when compared to the control group, suggesting an increased dependence on other non-NO-mediated pathways for vasodilatation. For instance, Mokhtar et al. (22) showed impaired NO-mediated vasodilation in diabetic patients may partly be compensated via enhanced EDHF-mediated relaxation. The fact that the EDHF-type response becomes apparent only in the presence of impaired NO availability is supported by experimental evidence in the animal study indicating that endothelial NO dampens EDHF-type responses under physiological conditions and that the latter become more prominent when the production of NO is curtailed (30, 31).

Prostacyclin-mediated endothelium-dependent relaxation is another possible pathway involved. Under physiological condition, prostacyclin counteracts the vasoconstrictor effects of thromboxane (TXA_2) and the balance between both prostanoids plays an important role in the regulation of vascular tone. In this study, endothelium-dependent prostacyclin-mediated relaxation was relatively preserved in all groups in the presence of indomethacin, suggesting that prostanoids may have relatively insignificant role in regulating the vascular tone. However, the diabetic groups demonstrate slightly improved relaxation (approximately 85% in the presence of indomethacin as compared to 73% in control solutions) in the present study, possibly indicating that the activity of vasoconstrictor prostanoids becomes enhanced in disease conditions. Moreover, increased lipid peroxidation in diabetes enhances cyclooxygenases activity and inhibits prostacyclin synthase, causing higher thromboxane/prostacyclin ratio which favours to vasoconstriction (32-34).

In this study, endothelial function does not seem to be impaired in thoracic aorta of both control and diabetic rats receiving vitamin D-deficient diet compared groups on normal diets. Similarly, it has been reported that vitamin D-deficient diet for 10 weeks in diabetic rats did not alter aortic endothelial function (35). This finding is in contrast with Wee et al. (36) which reported that endothelium-dependent relaxation was reduced in mesenteric arteries of diabetic rats with vitamin D deficiency compared to diabetic control rats. One possible explanation for this discrepancy is due to different type of vascular tissue used in the study. Wee at al. (36) performed the study using mesenteric arteries, while this study used thoracic aorta. The vitamin D-deficient diet for 10 weeks might be sufficient to cause an impairment on endothelial function in mesenteric arteries, as it is a microvasculature which is the initial site for vasculopathy, whereas the duration was not enough to introduce impairment in conduit arteries such as aorta.

Supplementation with 0.2 μg/kg alphacalcidol for four weeks significantly reverted the impaired endothelial function of diabetic rats receiving vitamin D-deficient diet comparable to that in the control group. In addition, supplementation with this dosage did not cause any changes to serum calcium level compared to normal rat, which was in accordance with previous reported literatures (37,38). Improved endothelial function may be explained by the multiple actions of vitamin D in regulating NO bioavailability. For instance, vitamin D enhances the activity of antioxidant enzymes and improves the overall antioxidant capacity, thereby reducing oxidative stress-mediated impairment in NO bioavailability. This is supported by a recent study which demonstrated that calcitriol supplementation improves oxidative status and upregulate the expression of endothelial NO synthase in vitamin D-deficient diabetic rats (36). Vitamin D also reduces proinflammatory cytokine production and release, suppress NF-κB signaling suppression, thereby protect against proinflammatory-mediated endothelial dysfunction and damage (39).

The present study has some limitations. Firstly, the level of serum vitamin D was not determined in this study. As we aimed to determine the involvement of vitamin D on endothelial function, the level of vitamin D in the experimental animal should be measured to better

illustrate the efficacy of the vitamin D deficient diet and alphacalcidol supplementation. This study has used similar brand of vitamin D-deficient diet to that of Wee et al. (36), and it was reported that 10 weeks treatment with this diet have successfully reduced serum level of vitamin D. Unfortunately, we could not provide the serum level of vitamin D after alphacalcidol supplementation. Secondly, NO level was not determined in this study; however, the contribution of NO in endothelium-mediated relaxation is usually measured by inhibiting the synthesis of NO using L-NAME. This is in view of NO being reported as the major endothelium-derived relaxing factor in large conduit vessels such as aorta (39,40).

CONCLUSION

This study showed that impaired endothelial-dependant relaxation in the aorta of diabetic and diabetic rats on vitamin D-deficient diet is largely due to a marked decrease in NO-related pathways. This phenomenon appears to be compensated by non-NO pathways. Vitamin D (alphacalcidol) supplementation to diabetic rats on vitamin D-deficient diet improves endothelial function to a level that is comparable to the non-diabetic control group.

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