Hydrogen Peroxide-induced Inhibition of Vasomotor Activity: Evaluation of Single and Combined Treatments with Vitamin A and Insulin in Streptozotocin-diabetic Rats

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A positive correlation has been established between increased oxidative stress and cardiovascular diseases in diabetes mellitus. We evaluated the effects of single or combined treatments with vitamin A (retinol acetate, 30 mg/kg/day, for 12-weeks) and insulin (8-10 IU/rat/day for the final 6-week) on vasomotor activity, oxidative stress and retinol metabolism in 12-week streptozotocin diabetic rats. The vasomotor activity was determined by measuring *in vitro* responsiveness of aorta rings to phenylephrine (PE) and acetylcholine (ACh) in the absence or in the presence of hydrogen peroxide (H_2O_2). Preincubation with H_2O_2 (10 µM) produced a significant decrease in PE (1 mM)-induced contraction in untreated-diabetic but not in control rats. Single treatment with insulin counteracted this effect of H_2O_2 and also reversed the increased contractile response of diabetic aorta to PE, while vitamin A was found to be ineffective. H_2O_2 (10 µM) also inhibited ACh (1 mM)-stimulated endothelium-dependent relaxation two fold more in diabetic than in control aorta. In the prevention of H_2O_2-induced inhibition of vascular relaxation to ACh, vitamin A alone was markedly effective while insulin alone was not. The combination of vitamin A plus insulin removed the inhibitory action of H_2O_2 in diabetic aorta. Diabetic animals displayed an increased level of

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aorta thiobarbituric acid reactive substance (TBARS) in association with decreased levels of plasma retinol and retinol-binding protein (RBP). Single treatment with insulin, in spite of allowing recovery of normal growth rate and improved glucose and retinol metabolism in diabetic rats, was unable to control TBARS production to the same extent as vitamin A alone. Our findings suggest that the maintenance of ACh-stimulated endothelium-dependent vasorelaxant tone in normal physiological levels depends largely on the prevention and/or inhibition of peroxidative stress, which is achieved by combined treatment with vitamin A plus insulin. The use of vitamin A together with insulin provides a better metabolic control and more benefits than use of insulin alone in the reduction of diabetes-induced vascular complications.

Key words: STZ-diabetes, rat aorta, hydrogen peroxide, vasomotor activity, vitamin A, insulin, retinol binding protein, peroxidative stress

INTRODUCTION

One of the most dangerous consequences of diabetes-associated "glucose toxicity" is oxidative stress resulting from vascular complications [1-3]. We consistently investigated the role of reactive oxygen species in abnormal vascular reactivity and endothelial function in experimental diabetes [4-7]. Our previous findings suggested that increased production of superoxide anion radical ($^{\bullet}O_2{}^{-}$) associates with increased vasoconstrictor response to α-adrenoceptor agonists and accompanies the enhanced transient endothelium-dependent vasorelaxation in diabetic animals [4,7,8]. In addition to $^{\bullet}O_2{}^{-}$, hydrogen peroxide (H_2O_2) is also an important candidate responsible for the development of diabetes-induced vascular dis-

ease since its increased vascular production and activity was reported in diabetic vessels [6,7]. Our investigations have also focused on exploring the benefits of various antioxidants in the prevention or recovery of oxidative stress-induced cardiovascular abnormalities in diabetes mellitus. We have reported that the treatment with vitamin E [4,5,9], probucol [10] and alpha-lipoic acid [11] serve as important therapeutic approach against diabetes-induced vascular complications.

Vitamin A (retinol), is a member of the lipid-soluble retinoid compounds, plays a central role in the maintenance of many essential biological processes, and shows a function as lipoperoxyl-radical scavenger [12,13]. It has been reported that long-term vitamin A deficiency and poorly controlled insulin-dependent diabetes mellitus (IDDM) share common complications such as blindness, impaired immune response and increased risk of ischemic heart disease [14]. Abnormal metabolism of vitamin A, as indicated by its decreased circulatory levels along with carrier protein, retinol-binding protein (RBP), has been reported to occur in IDDM [14,15] and STZ-induced diabetic rats [16] but not in non-insulin dependent diabetes mellitus (NIDDM) [17]. It has been also reported that in subnormal vitamin A status in poorly controlled diabetic subjects or diabetic animals there is no response to vitamin A supplementation [14]. I*n vivo* insulin treatment is able to reverse abnormal vitamin A metabolism [14] as well as impaired vascular reactivity associated with diabetes [8]. On the other hand, vitamin A supplementation has been reported to modulate the negative effects of dietary fat on aorta responses to acetylcholine (ACh) [18] and to preserve endothelial vasodilator function through a mechanism related to vascular tissue antioxidant content in atherosclerotic rabbits [19]. Although increased risk of cardiovascular disease at suboptimal plasma concentrations of vitamin A [20] and decreased plasma concen-

trations of vitamin A and RBP in IDDM have previously been identified, the effects of vitamin A supplementation or the comparable effects of vitamin A with insulin on abnormal vasomotor activity of STZ-diabetic rats have not been investigated previously. Our study was undertaken to compare the effects of single or combined treatments with insulin and vitamin A on STZ-diabetes-induced alterations in vasomotor activity by the assessment of responsiveness of aorta rings to phenylephrine (PE) and ACh in the absence or in the presence of exogenous H_2O_2. We also assessed the general characteristics, plasma retinol and RBP levels of all experimental animals used in this study.

MATERIALS AND METHODS

ANIMALS, INDUCTION OF DIABETES AND THE TREATMENT PROTOCOL

"The principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. Male Wistar rats weighing 250-300 g were used in the study. Diabetes was induced by 55 mg/kg, i.p. injection of STZ and confirmed by tail vein blood glucose levels two days later. Rats with blood glucose levels of 300 mg/dl or above were considered as diabetic. Two days after injection of either STZ or vehicle, rats were divided into following groups: 1) Untreated-diabetic rats (n = 15); 2) Diabetic rats treated with retinol acetate (30 mg/kg/day, orally) for 12 weeks (n = 12); 3) Diabetic rats untreated for 6 weeks and then treated with insulin (8-10 IU/rat/day, s.c.) for 6 weeks (n = 9); 4) Diabetic rats treated with both retinol acetate and insulin as same as protocols 2 and 3 (n = 9); 5) Untreated control rats (n = 15). The treatment dose of retinol acetate was chosen according to a previous study, and retinol acetate (2 mg) has been prepared into 0.1 ml corn oil as described previously [21]. Rats were maintained under standard housing conditions for 12 weeks before experiments were conducted.

ISOLATION OF AORTIC RINGS AND ASSESSMENT OF VASOMOTOR ACTIVITY

Details of the method have been described previously [4-7]. Before the aorta was removed, samples of blood were obtained by cardiac puncture. Thoracic aorta was carefully cleaned from fat and connective tissue and was sectioned into 3-4 mm-long rings. In all rings, extreme care was taken to avoid stretching and contact with the luminal surface of endothelium during isolation. The rings were suspended between parallel hooks in 5 ml tissue baths filled with Krebs-Henseleit bicarbonate buffer solution, which were thermoregulated at 37 °C. The buffer consisted of (mM): NaCl 118, KCl, 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_2 1.2, $NaHCO_3$ 25 and glucose 11. Changes in isometric tension were recorded on an Ugo Basile recorder via Ugo Basile-7004 transducer (Varese, Italy). All rings were equilibrated for 60 min under a resting tension of 2 g before experiments were begun. During this period, the rings were washed every 15 min.

At the end of the equilibration period, concentration-response curves to increasing concentrations of PE were performed on each ring. Rings were then contracted with a submaximal concentration of phenylephrine, which produced approximately 80% of the maximum response. This concentration was usually 1 μM, but was occasionally varied between 1–3 μM to obtain equieffective agonist activity. After reaching plateau of contraction, cumulative concentration response curves to ACh (0.01–10 μM) were obtained to evaluate endothelium-dependent relaxation. In some rings, the vascular effect of a single dose of PE (1 μM) or ACh (1 μM) was evaluated in the absence or in the presence of H_2O_2 (1 μM - 1 mM, 30 min preincubation).
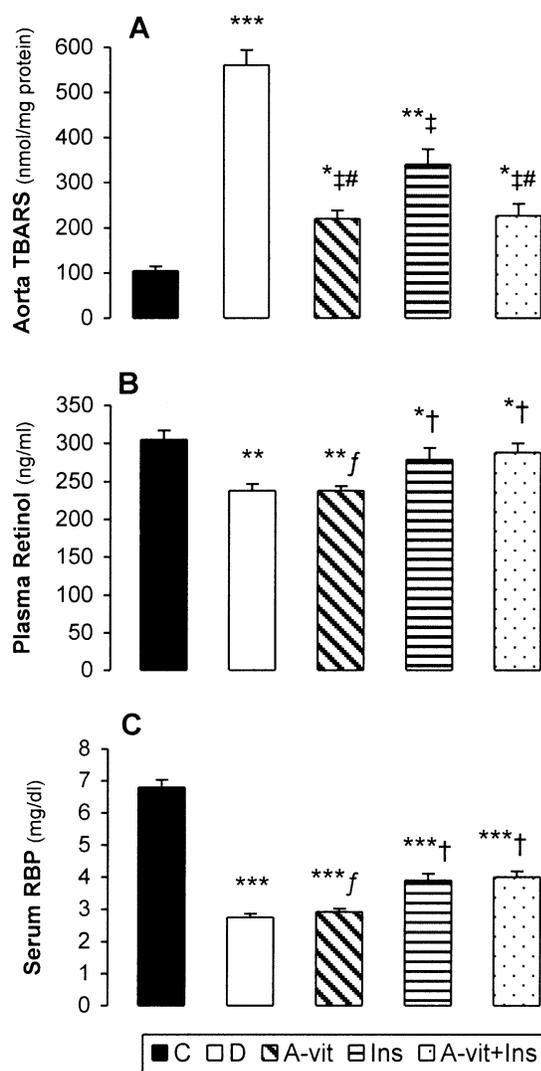
FIGURE 1
Thiobarbituric acid reactive substance in aorta homogenates (**A**), plasma retinol (**B**) and serum retinol binding protein (RBP) (**C**) levels in untreated-control (C), untreated-diabetic (D), vitamin A treated-diabetic (A-vit), insulin treated-diabetic (Ins.) and vitamin A plus insulin treated-diabetic (A-vit+Ins.) rats. Data (mean ± SEM) were analysed by ANOVA and between groups differences for each variable were tested using Newman-Keul's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ *vs.* control; †$P<0.05$, ‡$P<0.001$, *vs.* untreated-diabetic; *f*$P<0.05$, #$P<0.01$ *vs.* insulin-treated diabetic rats.

FOOD AND WATER CONSUMPTIONS, BODY WEIGHTS, BLOOD AND TISSUE ANALYSIS

Food and water consumptions were measured daily over a 24-h period for each cage of three rats, but data were transformed to give values/rat/day. Body weights of animals were recorded at least once a week. Blood glucose concentrations were measured weekly by an Ames glucometer (Glucometer III, Bayer Diagnostics, France).

Plasma retinol concentrations were determined by a high-performance liquid chromatography as previously described [22]. For measuring serum retinol binding protein (RBP), a "radial immunodiffusion assay" method was used [23].

Thiobarbituric acid reactive substances (TBARS), as an index of lipid peroxidation hence oxidative stress were measured by a previously described method [24] in aorta homogenates.

DRUGS AND STATISTICAL ANALYSIS

All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). Food and water intake, although expressed per rat, were calculated per cage, so that statistical analysis was not legitimate. Contractile response to phenylephrine was expressed as gram tension/mg tissue wet weight. Relaxations to cumulative doses of ACh were expressed as a percentage decrease of the maximum contractile response. The sensitivity to the agonist was evaluated as the pD_2 ($-\log EC_{50}$). Data are expressed as mean ±SE. They were first subjected to Bartlett's test for homogeneity of variances and were given a log transformation if necessary. One-way analysis of variance was then performed, followed by the Student-Newman-Keuls test to estimate the significance of differences for individual between-group comparisons. For the interaction study, differences within group before and after H_2O_2 incubation were analysed using unpaired Student's t tests. A value of P less than 0.05 was considered statistically significant.

TABLE 1
Table shows daily food and water consumptions, body weights and blood glucose levels of untreated-control (**Control**), untreated-diabetic (**Diabetic**), vitamin A treated-diabetic (**Diabetic + vitamin A**), insulin treated-diabetic (**Diabetic + insulin**) and vitamin A plus insulin treated-diabetic (**Diabetic + vitamin A and insulin**) animals. The maximum contractions to phenylephrine, maximum relaxation to acetylcholine and pD_2 values for phenylephrine or acetylcholine are also demonstrated in this table. pD_2 values calculated from phenylephrine or acetylcholine concentration-response curves. Data (mean ± SEM) were analysed by ANOVA and between groups differences for each variable were tested using Newman-Keul's test. Mean daily food and water consumptions although expressed per rat, were calculated per cage, so that statistical analysis was not legitimate. 1-week: 1-week after STZ injection. 6-week: 6-week after STZ injection. 12-week: 12-week after STZ injection; at the end of the treatment period. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ *vs.* control; [d]$P<0.05$, [e]$P<0.01$, [f]$P<0.001$ *vs.* diabetic.

	Control	Diabetic	Diabetic + Vitamin A	Diabetic + Insulin	Diabetic + Vitamin A and Insulin
	(n=15)	(n=15)	(n=12)	(n=9)	(n=9)
Daily food consumption (g)					
1-week	23.4 ± 0.54	31.2 ± 0.40	31.2 ± 0.45	32.4 ± 0.44	30.8 ± 0.34
6-week	25.1 ± 0.69	40.1 ± 0.68	36.2 ± 0.71	39.1 ± 0.22	37.3 ± 0.31
12-week	25.3 ± 0.50	47.6 ± 1.40	37.8 ± 0.43	31.1 ± 0.80	27.2 ± 0.90
Daily water consumption (ml)					
1-week	25.4 ± 1.0	109 ± 20	112 ± 18	100 ± 14	105 ± 10
6-week	30.0 ± 0.9	250 ± 13	210 ± 5.0	250 ± 6.0	229 ± 3.0
12-week	42.3 ± 6.3	260 ± 14	152 ± 12	118 ± 11	85.0 ± 9.0
Body weight (g)					
1-week	277 ± 17	248 ± 5.3	272 ± 15	250 ± 8.3	273 ± 6.7
6-week	344 ± 15	219 ± 14 [c]	220 ± 9.0 [c]	217 ± 4.7 [c]	218 ± 9.3 [c]
12-week	367 ± 10	152 ± 8.0 [c]	188 ± 4.0 [c,d]	230 ± 6.5 [c,e]	266 ± 4.1 [c,f]
Blood glucose (mM)					
1-week	5.1 ± 0.1	17.9 ± 0.2 [c]	16.4 ± 0.4 [c]	18.3 ± 0.5 [c]	17.0 ± 0.5 [c]
6-week	4.9 ± 0.1	26.3 ± 0.3 [c]	21.9 ± 0.7 [c]	25.2 ± 0.6 [c]	21.4 ± 0.6 [c]
12-week	4.8 ± 0.1	26.6 ± 0.8 [c]	20.0 ± 0.8 [c,d]	12.4 ± 1.5 [b,f]	8.1 ± 1.3 [a,f]
Maximum contraction to phenylephrine (g tension/mg tissue)	0.143 ± 0.01	0.266 ± 0.01 [c]	0.204 ± 0.008 [b,e]	0.158 ± 0.01 [f]	0.155 ± 0.01 [f]
pD_2 values for phenylephrine	6.90 ± 0.16	7.05 ± 0.21	7.03 ± 0.28	6.89 ± 0.33	7.10 ± 0.25
Maximum relaxation to acetylcholine (%)	91.2 ± 0.99	87.6 ± 1.38	88.2 ± 2.70	92.1 ± 3.26	90.9 ± 1.24
pD_2 values for acetylcholine	7.33 ± 0.34	7.14 ± 0.25	7.31 ± 0.31	7.25 ± 0.24	6.99 ± 0.40

A

□ in the absence of hydrogen peroxide
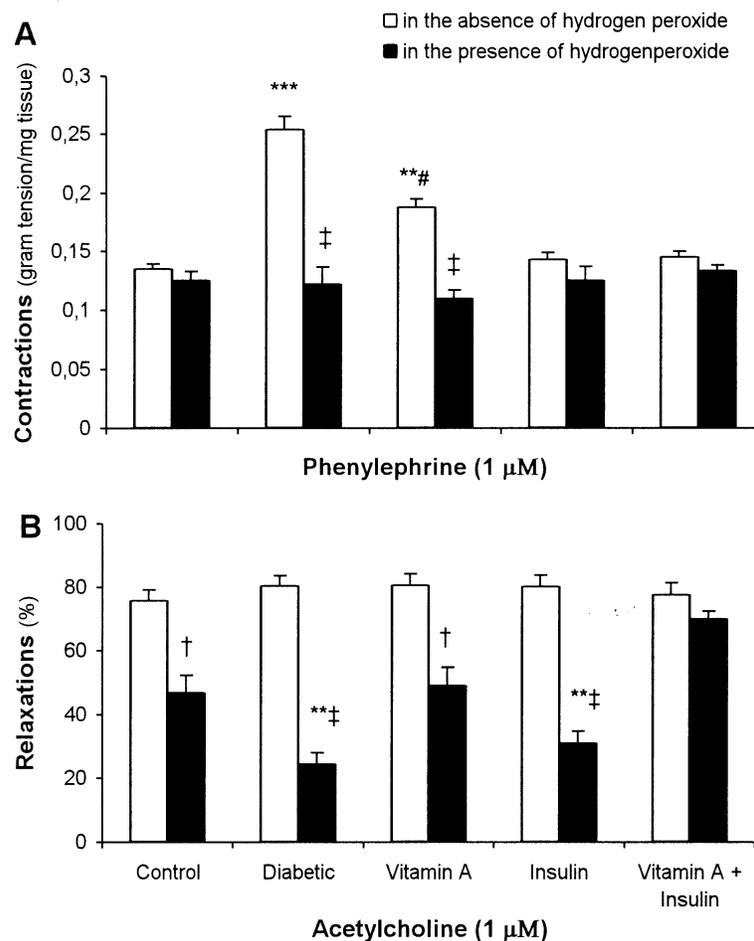■ in the presence of hydrogenperoxide

FIGURE 2
The contractile response of aorta to 1μM concentration of phenylephrine (**A**) and the relaxant response of aorta to 1μM concentration of acetylcholine (**B**) in the presence (dark) or in the absence (light) of H_2O_2 (10 μM) in untreated-control (control, n=10), untreated-diabetic (diabetic, n=10), vitamin A treated-diabetic (vitamin A, n=9), insulin treated-diabetic (insulin, n=9) and vitamin A plus insulin treated-diabetic (vitamin A + insulin, n=8) rats. Data (mean ± SEM) were analysed by ANOVA and between groups differences for each variable were tested using Newman-Keul's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control; #$P<0.01$ vs. untreated-diabetic. †$P<0.01$, ‡$P<0.001$ express statistically significant difference before and after H_2O_2 within each group.

RESULTS

FOOD AND WATER CONSUMPTION, BODY WEIGHTS, AND BLOOD GLUCOSE LEVELS

As expected, the food and water intake was increased in untreated-diabetic animals (Table 1). The single treatment with insulin was more effective than vitamin A treatment in the recovery of food and water intake of diabetic animals. However, combined treatment with vitamin A plus insulin almost entirely controlled feeding regimen of diabetic animals (Table 1). The effects of both treatments were progressive. The body weight loss was more effectively controlled by single insulin treatment when compared with the effect of single vitamin A treatment. Although the combined treatment

The percentage inhibition in PE-induced contraction in the presence of H_2O_2 was $17.9 \pm 4.57\%$ in insulin-treated diabetic animals, and $15.5 \pm 4.07\%$ in insulin plus vitamin A treated-diabetic animals for both $P>0.05$ *vs.* non-diabetic control rats; $P<0.001$ *vs.* untreated-diabetic rats.

Preincubation of rings with H_2O_2, at 10 μM, inhibited 1 μM ACh-induced endothelium-dependent relaxation in non-diabetic control rats ($38.6 \pm 6.01\%$) (Figure 2B). This effect of H_2O_2 significantly increased in untreated-diabetic rats ($74.5 \pm 4.07\%$; $P<0.001$ *vs.* non-diabetic control rats). In rats treated with vitamin A alone, H_2O_2–induced inhibition in ACh response was prevented and the degree of H_2O_2–induced inhibition was observed very similar with the corresponding parameter from the non-diabetic control animals ($34.1 \pm 5.00\%$; $P>0.05$ *vs.* non-diabetic control group; $P<0.01$ *vs.* untreated-diabetic group). Single treatment with insulin did not change the inhibitory action of H_2O_2 ($69.04 \pm 4.61\%$; $P<0.001$ *vs.* non-diabetic control group; $P>0.05$ *vs.* untreated-diabetic group). The inhibitory action of H_2O_2 on endothelium-dependent relaxations disappeared when diabetic animals were treated with vitamin A plus insulin ($18.41 \pm 4.75\%$; $P>0.05$ *vs.* non-diabetic control and $P<0.001$ *vs.* untreated-diabetic animals) (Figure 2B).

A preliminary study of normal age-matched rats was carried out to determine the effects of treatments with vitamin A. Since no change in vascular responses or blood and tissue analysis was observed, data are not shown. Normal control animals were not treated with insulin because of hypoglycemia risk.

DISCUSSION

Our confirmed previous investigations showing that the STZ-diabetes results in increased vascular reactivity to α-adrenoceptor agonists [2,4,8,25-27]. The diabetes-induced increased vasoconstrictor activity has been linked with excess production of reactive oxygen species [2-4,7]. *In vivo* treatments with insulin [8,27,28] or other antioxidants [4,11,29] were demonstrated to prevent or reverse the abnormal contractile response in diabetic vessels. Previously, we found that *in vivo* treatment with a low dose of insulin (4 IU/kg/day, for 15 days) is able to reverse the increased vasoconstrictor response to norepinephrine in 6 weeks alloxan-diabetic rats [8]. Accordingly, present study demonstrated that the increased PE-induced tone is completely reversed by a higher dose of insulin (8-10 IU/rat/day, for 6 weeks, started after 6-week of untreated-diabetes) in 12 weeks STZ-diabetic rats. This insulin regimen also well controlled hyperglycemia and vitamin A metabolism, and partly decreased oxidative stress in diabetic animals. Other investigators showed that insulin treatment improved the increased protein kinase C activity and diacylglycerol levels [30] that are responsible for abnormal vascular reactivity to α-adrenoceptor agonists in diabetic vessels. In our study, single treatment with vitamin A throughout 12-week showed less effectiveness than insulin monotreatment in controlling vasoconstrictor activity and hyperglycemia in diabetic rats. In addition, antioxidant treatment with vitamin A, started two days after STZ injection was unable to maintain the normal metabolism of endogenous retinol; however it markedly reduced the oxidative stress in diabetic vessels. Our findings indicate that the replacement of insulin hence the management of hyperglycemia as well as other metabolic events are essential to achieve normal contractile response of vessels to the stimulants of α-adrenoceptors in an insulin-dependent model of diabetes. To our knowledge, the effects of treatment with vitamin A alone or combination of vitamin A with insulin on vaso-

motor activity in diabetic vessels have not been studied previously. Nevertheless, dietary vitamin A supplementation has been shown to reduce the maximal vessel tension in response to PE in rats fed different oil diets [18].

Another important finding of this study is that exogenous H_2O_2 at 10 mM inhibited PE-induced contraction in diabetic but not in control rats. When the incubation dose of H_2O_2 was increased to 1 mM, this inhibition was also observed in some non-diabetic control vessels. Although there is no previous report indicating depressor effect of H_2O_2 on PE-induced contraction in diabetic vessel, our findings are consistent with some previous studies showing that incubation with H_2O_2 at 1 mM, but not at 0.1 mM, is able to inhibit PE-induced contraction in non-diabetic normal rats [31,32]. These investigators explained the mechanism responsible for inhibitory action of H_2O_2 via impairment of the cellular signalling reactions initiated by the PE. In addition to these studies, recently, addition of 0.5 mM H_2O_2 to organ bath has been reported to cause a decrease in KCl-induced contraction in pig coronery artery [33]. The reason for increased destructive effect of H_2O_2 on PE-induced contraction in diabetic vessels could be related to its increased production and effectiveness in diabetic vessels. Previously, we reported that the biological availability and the vasodilator action of H_2O_2 are increased in diabetic aorta [6,7]. The increased responsiveness of diabetic vessels to the inhibitory actions of exogenous H_2O_2 may reflect an important facet of H_2O_2 linked to its specific modulatory role that may function to compensate increased vascular reactivity in response to α-adrenoceptor stimulation under oxidative stress. It is well known that H_2O_2 produces vasorelaxation in PE-precontracted artery rings, and the relaxant effect of H_2O_2 requires formation of cyclic guanosine monophosphate (cGMP), and is mediated by nitric oxide (NO) [6,32,34,35]. In addition,

H_2O_2 also augments the secretion of other endothelium-derived relaxing peptides, adrenomodulin and C-type natriuretic peptide, that has been reported to function to compensate the vasoconstriction in situations by which oxidative stress are increased, as in the case of hypertension and atherosclerosis [36].

Our study showed that the maximum endothelium-dependent relaxations of aortic rings to ACh are unaffected from of STZ-diabetes, insulin or vitamin A therapy. This is in accord with previous studies showing that 8-12 weeks STZ diabetes [7,34] or an antioxidant, α-lipoic acid, [11] did not change endothelium-dependent vascular relaxations of rat aorta. Parallel with findings of other investigators [32], the incubation with H_2O_2 in our study produced a decrease in ACh-stimulated endothelium-dependent relaxation in the rat aorta. Additionally, we also demonstrated that the inhibitory action of H_2O_2 on ACh-induced relaxation significantly increased in diabetic animals. This effect of H_2O_2 may appear when the vessels are exposed long-term to the high concentrations of H_2O_2 as in the case of diabetes [6,7]. Some studies demonstrated the inhibitory actions of high concentrations of H_2O_2 on soluble guanylate cyclase [37] and endothelial nitric oxide synthase (eNOS) [38]. Also, a long-term treatment with H_2O_2 by luminal perfusion or in the organ bath was reported to result in loss of endothelium in arteries [32,33]. Long-term exposure of vascular smooth muscle cells [39] and endothelial cells [40] to H_2O_2 were reported to lead to sustained increase in intracellular calcium levels. It is well known that the final outcome of a sustained elevation of intracellular calcium causes tissue injury via lipid peroxidation and activation of calcium-dependent proteases [39].

In our study, insulin treatment of diabetic animals did not change the inhibitory action of H_2O_2 on ACh-induced relaxation. In contrast, vitamin A treatment alone prevented the

inhibitory action of H_2O_2 on ACh-induced relaxation in diabetic animals. This is in agreement with a previous report showing the protective effect of vitamin A against H_2O_2–induced tissue toxicity [41]. Surprisingly, the inhibitory action of H_2O_2 disappeared and the ACh-induced relaxation normalized when insulin and vitamin A were given together to the diabetic animals, suggesting that insulin increases effectiveness of vitamin A against H_2O_2-induced destruction on eNOS activity / NO bioavailability. The underlying mechanism for the interaction of vitamin A with insulin is not completely known with total certainty, nevertheless previous studies have demonstrated that Type-1 diabetic patients or diabetic rats exhibit an impaired metabolic availability of vitamin A, and insulin treatment reverses to normal the diabetes-induced changes in vitamin A metabolism [14,16,42]. Accordingly, our study showed that *in vivo* insulin treatment improves abnormal metabolism of vitamin A. Vitamin A treatment also produced partially low blood glucose in untreated-diabetic animals. This finding is parallel with the previous reports showing that 1) some retinyl esters and vitamin A protect beta cells against STZ- and alloxan induced toxicity [43,44], and 2) vitamin A deficiency leads to an inhibition in insulin secretion *in vivo* and *in vitro* [43].

In our study, there was no significant difference in vessel TBARS levels between vitamin A-treated and vitamin A plus insulin treated diabetic animals. The TBARS lowering effect of insulin alone was moderate when compared with the effect of vitamin A alone. Although vitamin A plus insulin treatment did not provide a complete inhibition in TBARS levels, our findings suggest that the maintenance of ACh-stimulated endothelium-dependent vasorelaxant tone in normal level depends largely on the prevention and/or inhibition of peroxidative stress, which is achieved only by combined treatment with vitamin A plus insulin.

Insulin treatment alone ameliorated the endogenous retinol metabolism, better controlled blood glucose and weight gain and normalized PE-induced contractility in diabetic animals However, vitamin A treatment alone largely inhibited diabetes-induced oxidative/peroxidative stress and its effects on endothelium-dependent relaxation. Combination of two agents especially is needed to maintain of ACh-stimulated endothelium-dependent vasorelaxant tone in normal physiological level. In conclusion, when considering the achievements of the two agents in different steps of controlling diabetes-induced metabolic and vascular abnormalities, the combination of insulin with an antioxidant, vitamin A, appears to be a more appropriate strategy than insulin monotherapy in the treatment of diabetic complications.

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has more beneficial, the body weights of diabetic rats were still significantly lower than that in control rats at the end of treatment periods (Table 1).

The insulin or vitamin A treatment alone resulted in a significant fall in blood glucose levels of diabetic animals. However, the effect of vitamin A was fairly modest when compared with the effect of insulin alone. Combination of insulin with vitamin A was more effective in the controlling of hyperglycemia, but was unable to produce a normoglycemia in diabetic animals (Table 1).

THIOBARBITURIC ACID REACTIVE SUBSTANCES, RETINOL AND RETINOL BINDING PROTEIN LEVELS

STZ-diabetes led to an increase in TBARS levels of aorta (Figure 1A). Single treatment with vitamin A significantly prevented TBARS augmentation; however a complete normalization was not achieved by this treatment. There was no significant difference in aorta TBARS levels between single vitamin A treatment and vitamin A plus insulin treatment. Single treatment with insulin was able to decrease TBARS levels in diabetic aorta, but its effect was moderate when compared with the effect of vitamin A alone or combined treatment with the two agents (Figure 1A).

Plasma retinol levels were significantly lower in untreated-diabetic animals than in non-diabetic control animals (Figure 1B). Insulin treatment either alone or in combination with vitamin A produced a significant increase in plasma retinol levels of diabetic animals. The effect of combination was very similar to the effect of insulin alone (Figure 1B). Plasma retinol levels was not changed when diabetic animals were treated with only vitamin A (Figure 1B).

Serum retinol binding protein (RBP) levels were profoundly decreased in untreated-diabetic rats (Figure 1C). Vitamin A treatment alone failed to affect serum RBP levels of diabetic rats. Diabetes-induced diminish in RBP was

gently restored by insulin treatment. There was no evidence for an additive effect of two agents when applied together (Figure 3B).

VASOMOTOR ACTIVITY MEASUREMENTS

The contractile response to cumulatively increasing concentrations and a single concentration (1 µM) of phenylephrine (PE) was significantly increased in untreated-diabetic rats (Table 1). Neither STZ-diabetes nor each treatment protocol altered the sensitivity (pD_2) to PE (Table 1). Insulin treatment alone normalized the contractile response of diabetic aorta to PE (Table 1, Figure 1A). Vitamin A treatment showed a similar trend to insulin treatment decreasing vasoconstriction in diabetic animals, but its effect was moderate.   There was no significant difference between single insulin treatment and combined treatment in the recovery of PE-induced response in diabetic aorta (Table 1, Figure 1A).

The endothelium-dependent vasodilator effect of acetylcholine (ACh) did not seriously alter in aortic rings from diabetic animals (Table 1, Figure 1B). The maximum relaxations to ACh were similar in all experimental groups. No change in pD_2 values for ACh among the experimental groups was detected in the present study (Table 1).

Addition of H_2O_2 to the bath medium, at 10 µM, inhibited PE (1 µM)-induced contraction in diabetic rats (Figure 2A). When aortic rings incubated with 1 mM H_2O_2, slight inhibition was also observed in some control animals (data not shown). With respect to percentage changes in 1 µM PE-induced response, H_2O_2 produced inhibition was 12.8 ± 4.57% in control animals. This value was not significantly different from the percentage changes in the absence of H_2O_2, and was increased to 51.5 ± 6.42% by 12 weeks of STZ-diabetes (P<0.001). Vitamin A monotherapy did not significantly alter the inhibitory action of H_2O_2 (46.6 ± 7.04%; P>0.05 vs. untreated-diabetic group).

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