Accelerated intimal hyperplasia and increased endogenous inhibitors for NO synthesis in rabbits with alloxan-induced hyperglycaemia

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1 We examined whether endogenous inhibitors of NO synthesis are involved in the augmentation of intimal hyperplasia in rabbits with hyperglycaemia induced by alloxan.

2 Four weeks after the endothelial denudation of carotid artery which had been performed 12 weeks after alloxan, the intimal hyperplasia was greatly augmented with hyperglycaemia. The degree of hyperplasia was assessed using three different parameters of histopathological findings as well as changes in luminal area and intima:media ratio.

3 There were positive and significant correlations between intima:media ratio, plasma glucose, and concentrations of N^G-monomethyl-L-arginine (L-NMMA) and N^G, N^G-dimethyl-L-arginine (ADMA) in endothelial cells, that is, the intima:media ratio became greater as plasma glucose and endothelial L-NMMA and ADMA were increased. Furthermore, endothelial L-NMMA and ADMA were increased in proportion to the increase in plasma glucose.

4 In contrast, there were inverse and significant correlations between cyclic GMP production by carotid artery strips with endothelium and plasma glucose, between cyclic GMP production and endothelial L-NMMA and ADMA, and between the intima:media ratio and cyclic GMP production.

5 Exogenously applied L-NMMA and ADMA inhibited cyclic GMP production in a concentration-dependent manner. IC₅₀ values were determined to be 12.1 µM for the former and 26.2 µM for the latter. The cyclic GMP production was abolished after the deliberate removal of endothelium from the artery strips.

6 These results suggest that the augmentation of intimal hyperplasia with hyperglycaemia is closely related to increased accumulation of L-NMMA and ADMA with hyperglycaemia, which would result in an accelerated reduction in NO production/release by endothelial cells.

Keywords: Hyperglycaemia; alloxan; accelerated intimal hyperplasia; L-NMMA; ADMA; NO production; endothelial denudation

Abbreviations: ADMA, asymmetric dimethylarginine; IBMX, 3-isobutyl-1-methylxanthine; LCA, left carotid artery; L-NMMA, N^G-monomethyl-L-arginine; RCA, right carotid artery; SMC, smooth muscle cell; SDMA, symmetric dimethylarginine

Introduction

Diabetes mellitus is associated with several cardiovascular complications, predisposing the patient to diseases such as hypertension, atherosclerosis and thrombosis (Christlieb *et al.*, 1976). These diseases have been studied in streptozotocin-induced hyperglycaemic rats or alloxan-induced hyperglycaemic rabbits as diabetic animal models (Taylor *et al.*, 1992; Tesfamariam *et al.*, 1989). It has been reported that atherosclerotic changes, brought about by the accelerated growth of smooth muscle cells (SMCs) are observed in the aorta of alloxan-induced diabetic rabbits (Miller & Wilson, 1984), and that cultured aortic SMCs from alloxan-induced diabetic rabbits grow faster than control SMCs (Alipui *et al.*, 1991). As one of the mechanisms of accelerated cell growth, Kawano *et al.* (1993) and Kanzaki *et al.* (1994) reported that the overexpression of platelet-derived growth factor (PDGF) β -receptors in SMCs of the medial layer played an important role.

Nitric oxide (NO) is reportedly generated in endothelial cells from L-arginine by constitutive NO synthase (Moncada & Higgs, 1993; Förstermann *et al.*, 1994). NO is well known as a potent vasodilator (Furchgott & Zawadzki, 1980), an anti-

aggregatory substance (Azuma *et al.*, 1986; Radomski *et al.*, 1987) and an inhibitor of SMC proliferation (Garg & Hassid, 1989). Azuma and coworkers (1990, 1992; Niimi *et al.*, 1994) have suggested that the neointima formation which follows endothelial denudation of the rabbit carotid artery might be caused, at least partly, by impairment of the ability of endothelial cells to produce/release NO. On the other hand, it has been reported that hyperglycaemia causes endothelial dysfunction (Kamata *et al.*, 1989; Cameron & Cotter, 1992; Taylor *et al.*, 1992). However, few studies have investigated the relationship between accelerated intimal hyperplasia and endothelial dysfunction in diabetes mellitus (Poston & Taylor, 1995).

Vallance *et al.* (1992) have obtained evidence that N^G-monomethyl-L-arginine (L-NMMA) and N^G, N^G-dimethyl-L-arginine (ADMA) play a role as endogenous inhibitors of NO synthesis. Recently, it has been reported that the accumulation of endogenous inhibitors in regenerated endothelial cells is associated with decreased NO production/release and occurrence of intimal hyperplasia after endothelial denudation of the rabbit carotid artery (Azuma *et al.*, 1995) and that the concentration of these inhibitors were increased in plasma of patients with peripheral arterial occlusive disease including diabetes mellitus (Böger *et al.*, 1997).

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Thus, the present experiments were designed to investigate whether hyperglycaemia modifies intimal hyperplasia after endothelial denudation of rabbit carotid artery and whether endogenous inhibitors for NO synthesis contribute to the modification.

Methods

Experimental animals

Thirty-three Japanese White male rabbits were used. These rabbits were purchased at 8 weeks of age and housed in a temperature ($23 \pm 1^\circ\text{C}$) and humidity ($50 \pm 20\%$) controlled room and were fed regular chow (RC-4, Oriental Yeast, Tokyo, Japan) throughout the experimental periods. A single intravenous bolus of alloxan (100 mg kg^{-1}) in $0.9\% \text{ w v}^{-1}$ saline were given *via* the marginal ear vein at 10 weeks of age. Twelve weeks after alloxan, nine normoglycaemic rabbits and 24 hyperglycaemic rabbits were distinguishable. Experiments were performed on these two groups. Animals underwent a unilateral endothelial denudation 12 weeks after alloxan as described below. This study complied with the Animal Welfare Regulation of Tokyo Medical and Dental University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Endothelial denudation

Endothelial denudation of the rabbit carotid artery was performed according to the method described previously (Azuma *et al.*, 1990, 1992, 1994; Niimi *et al.*, 1994). In brief, the right common carotid artery was exposed under anaesthesia with sodium pentobarbitone (25 mg kg^{-1} , i.v.) by aseptic surgery. An embolectomy catheter (12-040-3F/40cm/3F; Baxter Healthcare Corporation, Santa Ana, CA, U.S.A.) was then inserted into the artery in retrograde fashion through a small nick made near the carotid bifurcation. The luminal surface of the artery from the origin to the balloon entrance was gently rubbed back and forth with the balloon filled with 0.15 ml of air. This method of removing endothelial cells did not cause deep injury of the vascular wall (Azuma *et al.*, 1992, 1994; Niimi *et al.*, 1994; Hamasaki *et al.*, 1997). After the balloon was removed, the arterial incision was sutured with 10-0 nylon thread, keeping patency of the artery. The left carotid artery (LCA) underwent a sham operation and served as a control. Four weeks after the balloon denudation, animals were anaesthetized with sodium pentobarbitone (25 mg kg^{-1} , i.v.) and exsanguinated *via* the femoral arteries.

Determination of plasma glucose

Blood (0.9 ml) was withdrawn *via* the central ear artery by using a syringe containing 0.1 ml sodium citrate solution ($3.8\% \text{ w v}^{-1}$) once a week for 12 weeks after alloxan and for 4 weeks after endothelial denudation of the carotid artery. Plasma glucose was determined using the OTB (O-Toluidine Boric acid) method (Glucose-Test Wako kit, Wako Pure Chemicals, Tokyo, Japan).

Morphological examination

Approximately 5-mm pieces of the control left (LCA) and hyperplastic right carotid arteries (RCA) which had been isolated from normoglycaemic and hyperglycaemic rabbits were fixed in a $10\% \text{ v v}^{-1}$ neutral solution of formaldehyde

and embedded in paraffin after dehydration with ethanol. Thin sections were stained with hematoxylin-eosin or by the Elastica Van Gieson method for microscopic analysis (Azuma *et al.*, 1990, 1992, 1994; Niimi *et al.*, 1994). To provide a semiquantitative parameter for assessing intimal hyperplasia, the luminal area, intimal area and medial area were determined by means of an image analyzer (Quadra-950, Macintosh) and image scanner (ScanJet IIC, Hewlett Packard) (Obayashi *et al.*, 1996). The ratios (%) of the luminal area to whole area surrounded by the border between medial layer and adventitia, and of the intimal area to the medial area (intima:media ratio) (Obayashi *et al.*, 1996) were calculated.

Determination of L-NMMA and ADMA

The concentrations of N^G -monomethyl-L-arginine (L-NMMA) and N^G , N^G -dimethyl-L-arginine (ADMA) in endothelial cells were determined by means of automated high-performance liquid chromatography according to the method described previously (Azuma *et al.*, 1995, 1997; Hamasaki *et al.*, 1997). To estimate intracellular concentrations of L-NMMA and ADMA, we obtained the equation between the number of endothelial cells (y) and DNA concentration (x) by the least-square method as follows (Hamasaki *et al.*, 1997):

$$y = (14.3x + 6) \times 10^4 \quad (r = 0.9840)$$

From a determination of the DNA concentration in endothelial cells, the number of endothelial cells was calculated. If the intracellular water space is assumed to be 2 pl endothelial cell $^{-1}$ (Baydoun *et al.*, 1990), intracellular concentrations of L-NMMA and ADMA can be calculated.

Determination of cyclic GMP

Ring preparations weighing approximately 4 mg with intact endothelium were cut off from the carotid artery with a razor blade. Cyclic GMP level was determined according to the method described previously (Masuda *et al.*, 1997; Kamikawatoko *et al.*, 1998). In brief, preparations which had been processed without damaging the luminal surface were preincubated in modified Krebs solution for 20 min at 37°C , transferred into fresh Krebs solution and followed by a further 30 min incubation. Preparations were then rapidly transferred into $10\% \text{ w v}^{-1}$ trichloroacetic acid (TCA) with liquid nitrogen in order to stop the reaction. Noradrenaline (NA, 10^{-6} M) and acetylcholine (ACh, $3 \times 10^{-6} \text{ M}$) were added immediately and 15 min after transferring the preparations into the fresh Krebs solution, respectively. All experiments were performed in the presence of 10^{-4} M 3-isobutyl-1-methylxanthine (IBMX) as a non-selective inhibitor of phosphodiesterases. The net production of cyclic GMP was expressed as the difference between the production with NA (10^{-6} M) plus ACh ($3 \times 10^{-6} \text{ M}$) and that with NA (10^{-6} M) plus ACh ($3 \times 10^{-6} \text{ M}$) plus N^G -nitro-L-arginine (10^{-4} M) as an inhibitor of nitric oxide synthase (Kobayashi & Hattori, 1990). The basal cyclic GMP level was taken as the value without any agonist and antagonist expect for IBMX. The protein concentration of the sample was determined by use of the protein assay reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The composition of the modified Krebs solution was as follows (mM): NaCl 118.0, KCl 4.7, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5, KH_2PO_4 1.2, NaHCO_3 25.0 and glucose 10.0 (pH 7.4).

In order to examine the inhibitory activities of exogenously applied L-NMMA and ADMA, cyclic GMP production

stimulated by NA (10^{-6} M) plus ACh (3×10^{-6} M) was determined in the presence of L-NMMA or ADMA in concentrations of 10^{-6} M– 10^{-4} M using carotid artery strips from an additional three normal rabbits without any treatment. In order to remove endothelial cells, the luminal surface was gently rubbed with a wet cotton swab (Niimi *et al.*, 1994).

Determination of DNA

The microassay of DNA was performed according to the fluorometric method described by Kissane & Robins (1958).

Chemicals

The following chemicals were used in the present experiments: alloxan (Tokyo Kasei Kogyo Co., Tokyo, Japan), acetylcholine chloride (ACh) (Daiichi Pharmaceutical Co., Tokyo, Japan), N^G -nitro-L-arginine (Research Biochemicals Inc., Natick, MA, U.S.A.), and –noradrenaline bitartrate (NA), 3-isobutyl-1-methylxanthine (IBMX), N^G -monomethyl-L-arginine (L-NMMA) and N^G , N^G -dimethyl-L-arginine (ADMA) (all from Sigma Chemical Co. St. Louis, MO, U.S.A.).

Statistical analysis

Results are given as means $\pm$ s.e.mean. The statistical analysis was carried out by Student's *t*-test. Correlation was examined by Pearson's rank correlation coefficient (*r*) and Fisher's *z*-translation (analysis of statistical significance). Regression equation was obtained by least square method.

Results

Baseline data

As shown in Table 1, plasma glucose levels 12 and 16 weeks after the intravenous bolus injection of alloxan at a dose of 100 mg kg^{-1} were not different from the level before alloxan in nine rabbits out of 33 (normoglycaemic group). On the other hand, the remaining 24 rabbits showed significant ($P < 0.005$) hyperglycaemia (hyperglycaemic group), in which plasma glucose was approximately 3 fold higher than the corresponding value in normoglycaemic group and the value prior to alloxan injection. The body weight gain was significantly ($P < 0.005$) decreased in the hyperglycaemic group.

Table 1 Body weight and plasma glucose levels in experimental rabbits immediately before (0) and after alloxan

Group	Weeks	Body weight (kg)	Plasma glucose (mg dl ⁻¹)
NG	0	1.78 ± 0.04 (9)	113 ± 3 (9)
	12	2.75 ± 0.06 (9)	117 ± 11 (7) [†]
	16	2.87 ± 0.07 (9)	104 ± 6 (9)
HG	0	1.82 ± 0.02 (24)	116 ± 3 (24)
	12	$1.88 \pm 0.08^*$ (24)	$379 \pm 21^*$ (24)
	16	$2.00 \pm 0.09^*$ (24)	$373 \pm 17^*$ (24)

Results are given as means $\pm$ s.e.mean of 7–9 determinations for normoglycaemia (NG) and 24 for hyperglycaemia (HG).

*Significant difference at $P < 0.005$ vs corresponding value in the NG group. [†]Two determinations were omitted because of assay trouble.

Intimal hyperplasia

At week 16 (4 weeks after the endothelial denudation), rabbits were killed by exsanguination under anaesthesia with sodium pentobarbitone (25 mg kg^{-1} , i.v.) for the following experiments. A significant intimal hyperplasia, assessed by comparing the percentage luminal area and intima:media ratio (%) between the sham-operated left carotid artery (LCA) and the previously denuded right carotid artery (RCA), and by histopathological examination, was produced after endothelial denudation. The luminal area of RCA in normoglycaemic group was $49.1 \pm 1.1\%$ ($n = 5$), which was significantly ($P < 0.005$) different from the corresponding value in the LCA ($60.0 \pm 2.4\%$, $n = 5$). In the hyperglycaemic group, the reduction of luminal area in RCA was more pronounced ($44.9 \pm 1.1\%$, $n = 5$, $P < 0.05$) than the corresponding value in the normoglycaemic group, although the value of LCA ($56.7 \pm 1.9\%$, $n = 5$) remained unaffected. As shown in Figure 1, the intima:media ratios in LCA and RCA were significantly ($P < 0.005$) greater in the hyperglycaemic group compared with corresponding values in the normoglycaemic group. There were no changes in the medial thickness of both arteries from the two groups.

In the sham-operated LCA, no noticeable histopathological changes could be detected in the normoglycaemic group. However, light microscopy revealed a diffuse and slight proliferation of smooth muscle cells in the intima of LCA from the hyperglycaemic group. In contrast, the proliferation of smooth muscle cells and elastic fibres in the intima was observed clearly in the previously denuded RCA of normoglycaemic group. These changes in RCA became severe in the hyperglycaemic group.

Figure 1 Effect of hyperglycaemia on the intimal hyperplasia after endothelial denudation of rabbit carotid artery. Intimal hyperplasia after endothelial denudation was assessed by comparing the intima:media ratio (%). Results are given as mean of five determinations and vertical bars show s.e.mean. ^aSignificant difference at $P < 0.005$ vs corresponding value in LCA. ^bSignificant difference at $P < 0.005$ vs corresponding value in the normoglycaemia group. LCA, Sham operated left carotid artery; RCA, Previously denuded right carotid artery.

Concentrations of L-NMMA and ADMA in endothelial cells

Concentrations of L-NMMA and ADMA were 5.2 ± 0.1 and $5.0 \pm 0.3 \mu\text{M}$, respectively in the control endothelial cells of the normoglycaemic group. The concentrations in the regenerated endothelial cells (Azuma *et al.*, 1990) from hyperplastic carotid arteries were approximately 2 fold higher than the corresponding values in the control endothelial cells ($P < 0.005$). In hyperglycaemic rabbits, concentrations of L-NMMA and ADMA were significantly increased even in the control endothelial cells. Although the concentrations in the regenerated endothelial cells from hyperplastic arteries tended to be higher than the corresponding values in the normoglycaemic group, there was no significant difference between the corresponding two values. L-NMMA and ADMA were detectable at similar concentrations in endothelial cells of rabbit carotid arteries. These results are shown in Table 2.

Cyclic GMP levels in the carotid artery

In normoglycaemic rabbits, basal levels and stimulated production of cyclic GMP with NA and ACh were 66.9 ± 8.6 and 83.6 ± 12.0 pmoles mg^{-1} protein, respectively. The stimulated production of cyclic GMP was significantly ($P < 0.05$) decreased in the hyperplastic artery strips of this group. In hyperglycaemic rabbits, on the other hand, both basal level and stimulated production of the nucleotide were significantly ($P < 0.005$) decreased even in the sham-operated LCA strips compared with corresponding values in normoglycaemic rabbits. Basal level in the hyperplastic artery strips (RCA) of hyperglycaemic group was significantly ($P < 0.05$) more decreased compared with corresponding value in LCA of the same group (Table 3).

Inhibitory effects of exogenously applied L-NMMA and ADMA on the cyclic GMP production

Cyclic GMP production when stimulated by NA (10^{-6} M) and ACh (3×10^{-6} M) was assessed in the presence of L-NMMA (10^{-6} – 10^{-4} M) or ADMA (10^{-6} – 10^{-4} M). Both L-NMMA and ADMA inhibited cyclic GMP production in a concentration-dependent manner (Figure 2). The 50% inhibitory concentrations (IC_{50}) were $12.1 \mu\text{M}$ for the former and $26.2 \mu\text{M}$ for the latter. In addition, the basal levels and stimulated

production of cyclic GMP with NA and ACh were greatly decreased or abolished after the deliberate removal of endothelium from the artery strips (data not shown).

Relationships between plasma glucose concentration, intima:media ratio, endothelial ADMA or L-NMMA concentration and cyclic GMP production

When there are four different parameters, six combinations to estimate correlation between two parameters should be considered. Results are shown in Figure 3. On the one hand, there were positive and significant correlations between intima:media ratio and plasma glucose, between endothelial ADMA and plasma glucose, and between intima:media ratio and endothelial ADMA, that is, the intima:media ratio became greater as plasma glucose, and endothelial ADMA concentration increased. Furthermore, the endothelial ADMA concentration was increased in proportion to the

Table 3 Basal level and net production of cyclic GMP in the carotid artery (CA) strips from normoglycaemic (NG) and hyperglycaemic (HG) rabbits following alloxan

Group	Blood vessel	Basal level	Net production
NG	LCA	66.9 ± 8.6	83.6 ± 12.0
	RCA	44.5 ± 4.4	$43.6 \pm 3.1^*$
HG	LCA	$17.9 \pm 2.2^\dagger$	$24.8 \pm 1.5^\dagger$
	RCA	$8.3 \pm 3.1^{*\dagger}$	22.7 ± 10.7

Results (pmoles mg^{-1} protein) are given as means $\pm$ s.e.-mean of four determinations. Net production of cyclic GMP was expressed as the difference between the production with 10^{-6} M adrenaline (NA) plus 3×10^{-6} M acetylcholine (ACh) and that with NA (10^{-6} M) plus ACh (3×10^{-6} M) plus N^G -nitro-L-arginine (10^{-4} M) as an inhibitor of nitric oxide synthase. The basal level was given as the value without any agonist and antagonist except for 10^{-4} M 3-isobutyl-1-methylxanthine (IBMX) as a non-selective inhibitor of phosphodiesterases. *Significant difference at $P < 0.05$ vs corresponding value in LCA. † Significant difference at $P < 0.005$ vs corresponding value in the NG group. LCA, Sham operated left carotid artery; RCA, Previously denuded right carotid artery.

Table 2 Concentrations of L-NMMA and ADMA in the endothelial cells from carotid arteries of normoglycaemic (NG) and hyperglycaemic (HG) rabbits

Group	Blood vessel	Concentration (μM)	
		L-NMMA	ADMA
NG	LCA	5.2 ± 0.1	5.0 ± 0.3
	RCA	$11.4 \pm 0.6^*$	$11.0 \pm 0.6^*$
HG	LCA	$12.1 \pm 1.1^{\dagger\dagger}$	$12.1 \pm 1.4^\dagger$
	RCA	13.6 ± 1.7	14.1 ± 2.3

Results (μM) are given as means $\pm$ s.e.-mean of three determinations. The concentrations of methylarginine derivatives were estimated from the equation between DNA concentration and the number of endothelial cells (Hamasaki *et al.*, 1997), and the intracellular water space reported by Baydoun *et al.* (1990). *Significant difference at $P < 0.005$ vs corresponding value in LCA. † and †† Significant difference at $P < 0.01$ and $P < 0.005$ vs corresponding value in the NG group. LCA, Sham operated left carotid artery; RCA, Previously denuded right carotid artery.

Figure 2 Inhibitory activities of L-NMMA and ADMA on the cyclic GMP production. Results are given as means $\pm$ s.e.-mean of five determinations in the rabbit carotid artery strips with intact endothelium. Cyclic GMP production was stimulated by NA (10^{-6} M) plus ACh (3×10^{-6} M) in the presence of IBMX (10^{-4} M). Control production of cyclic GMP in the absence of NOS inhibitors was determined to be 55.9 ± 1.8 pmoles mg^{-1} protein ($n = 6$).

increase in plasma glucose concentration. There were, on the other hand, inverse and significant correlations between cyclic GMP production and plasma glucose, between cyclic GMP production and endothelial ADMA, and between intima:media ratio and cyclic GMP production, that is, cyclic GMP production was decreased as plasma glucose and endothelial ADMA concentration elevated. In addition, intima:media ratio was increased as cyclic GMP production

was decreased. These correlations could be observed in the previously denuded RCA and sham-operated LCA.

There were also positive and significant ($P < 0.05$ and $P < 0.005$) correlations between endothelial L-NMMA concentration and plasma glucose concentration, and between intima:media ratio and endothelial L-NMMA concentration. In contrast, correlation between cyclic GMP production and endothelial L-NMMA concentration was inverse and sig-

Figure 3 Relationships among four parameters of plasma glucose, intima:media ratio, endothelial ADMA concentration and cyclic GMP production in normoglycaemic and hyperglycaemic rabbits. Results are given as the values at 16 weeks after alloxan (4 weeks after the endothelial denudation). Each value was obtained from an individual rabbit except for ADMA concentration, which was determined in endothelial cells collected from carotid arteries of three rabbits each. The mean values of plasma glucose and intima:media ratio in corresponding three rabbits were used for assessing the correlations to ADMA. Correlation was examined by Pearson's rank correlation coefficient (r) and Fisher's z -translation. LCA, Sham operated left carotid artery; RCA, previously denuded right carotid artery.

nificant ($P < 0.05$ and $P < 0.005$) as in the case of ADMA (data not shown).

Discussion

An intravenous bolus injection of alloxan (100 mg kg^{-1}) induced hyperglycaemia in 24 rabbits out of 33. The remaining nine rabbits showed normoglycaemia, indicating variability in the susceptibility of rabbits to alloxan (Zhao *et al.*, 1987). These two groups of rabbits were used in the present experiments to investigate the effect of hyperglycaemia on the intimal hyperplasia after endothelial denudation of carotid artery.

Intimal hyperplasia was significantly augmented in alloxan-induced hyperglycaemic rabbits (Figure 1). Similar results have been reported by Miller & Wilson (1984), Kawano *et al.* (1993) and Kanzaki *et al.* (1994). Some of these investigators (Kawano *et al.*, 1993; Kanzaki *et al.*, 1994) reported that augmentation of intimal hyperplasia due to hyperglycaemia was accompanied by an overexpression of platelet-derived growth factor (PDGF) β -receptors in the medial smooth muscle cells. However, the mechanism of the augmentation of intimal hyperplasia associated with hyperglycaemia remains obscure.

We have demonstrated that intimal hyperplasia after endothelial denudation may be caused, at least partly, through impairment of the ability of endothelial cells to produce/release NO and that the accumulation of endogenous L-NMMA and ADMA as inhibitors of NO synthesis is associated with decreased NO production/release and, in turn, with the occurrence of intimal hyperplasia (Azuma *et al.*, 1990, 1992, 1994, 1995; Niimi *et al.*, 1994). In contrast, there are many reports that hyperglycaemia causes an endothelial dysfunction in the production of NO (Kamata *et al.*, 1989; Cameron & Cotter, 1992; Taylor *et al.*, 1994). As far as we know, there are no reports investigating whether the impaired endothelial function which might be brought about by increased L-NMMA and ADMA in endothelial cells is involved in the occurrence of the accelerated intimal hyperplasia with hyperglycaemia. In the present experiments, it was found that there were positive and significant correlations between intima:media ratio, plasma glucose, and concentrations of L-NMMA and ADMA in endothelial cells, that is, the intima:media ratio became greater as plasma glucose, and concentrations of L-NMMA and ADMA in endothelial cells increased. Furthermore, the concentrations of L-NMMA and ADMA were increased in proportion to the increase in plasma glucose concentration. In addition to these findings, there were significant and inverse correlations between cyclic GMP production and concentrations of L-NMMA and ADMA in endothelial cells, and between intima:media ratio and cyclic GMP production. Thus, it may be suggested that accelerated intimal hyperplasia associated with hyperglycaemia is closely related to increased concentrations of ADMA and L-NMMA in endothelium and decreased cyclic GMP production resulting from the increase in plasma glucose concentration.

Exogenously applied L-NMMA and ADMA inhibited cyclic GMP production in a concentration-dependent manner (Figure 2), which presumably reflects decreased NO biosyn-

thesis with these inhibitors. It is well established that NO binds the haem group of soluble guanylate cyclase (Ignarro, 1990a; Chinkers & Garbers, 1991), thereby stimulating the production of cyclic GMP. Consequently, the generation of cyclic GMP is widely used as an index of NO biosynthesis (Moncada *et al.*, 1988; 1991; Ignarro, 1990b; Lüscher *et al.*, 1990). Indeed, the basal level and the stimulated cyclic GMP production with NA and ACh were greatly decreased or abolished after the deliberate removal of endothelium from the artery strips in the present experiments. NO inhibits platelet adhesion and aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987) which are considered as an initiating event for the intimal hyperplasia (Ross, 1986; Schwartz *et al.*, 1981). It has also been demonstrated that NO inhibits vascular smooth muscle cell proliferation (Garg & Hassid, 1989). If all of these findings are considered together, they strongly suggest that an increased accumulation of L-NMMA and ADMA as endogenous inhibitors of NO synthesis with hyperglycaemia results in the accelerated decrease in the NO production/release by endothelial cells, which, in turn, brings about the augmentation of intimal hyperplasia.

The mechanisms which regulate the concentrations of L-NMMA and ADMA in endothelial cells are not clarified in the present experiments, although several possibilities can be considered. It has been reported that the transmembrane transport (system y^+) of endogenous methylarginines might alter their concentrations in NO-generating cells (Bogle *et al.*, 1995). Therefore, it may be suggested that hyperglycaemia may increase the uptake of L-NMMA and ADMA by endothelial cells, which, in turn, increases the intracellular concentration of the methylarginines and effectively inhibit NO biosynthesis. This speculation seems to be supported in part by the demonstration that system y^+ activity is increased by high glucose in umbilical vein endothelial cells cultured from gestational diabetic pregnancies (Sobrevia *et al.*, 1998). However, further experiments will be necessary to confirm this hypothesis. Dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes L-NMMA and ADMA, is localized in endothelial cells (MacAllister *et al.*, 1996). Hyperglycaemia may inhibit the activity of this enzyme, resulting in a high level of L-NMMA and ADMA in endothelial cells. This possibility may be supported partly by findings that the concentration in endothelial cells of N^G , N^G -dimethyl-L-arginine (SDMA), which is not a substrate for the metabolizing enzyme (Ogawa *et al.*, 1989), remained unaltered in all groups examined in the present experiments (data not shown).

In conclusion, the augmentation of intimal hyperplasia associated with hyperglycaemia seems to be closely related to the increased accumulation of endothelial endogenous inhibitors of NO synthesis which may result in reduced NO production/release by endothelial cells.

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References

- ALIPUI, C., TENNER, T.E. & RAMOS, K. (1991). Rabbit aortic smooth muscle cell culture. A model for the pharmacological study of diabetes-induced alterations in cell proliferation. *J. Pharmacol. Methods*, **26**, 211–222.
- AZUMA, H., FUNAYAMA, N., KUBOTA, T. & ISHIKAWA, M. (1990). Regeneration of endothelial cells after balloon denudation of the rabbit carotid artery and changes in responsiveness. *Jpn. J. Pharmacol.*, **52**, 541–552.
- AZUMA, H., HAMASAKI, H., NIIMI, Y., TERADA, T. & MATSUBARA, O. (1994). Role of endothelin-1 in neointima formation after endothelial removal in rabbit carotid arteries. *Am. J. Physiol.*, **267**, H2259–H2267.
- AZUMA, H., ISHIKAWA, M. & SEKIZAKI, S. (1986). Endothelium-dependent inhibition of platelet aggregation. *Br. J. Pharmacol.*, **88**, 411–415.
- AZUMA, H., MASUDA, H., SATO, J., NIWA, K. & TOKORO, T. (1997). A possible role of endogenous inhibitor for nitric oxide synthesis in the bovine ciliary muscle. *Exp. Eye Res.*, **64**, 823–830.
- AZUMA, H., NIIMI, Y. & HAMASAKI, H. (1992). Prevention of intimal thickening after endothelial removal by a nonpeptide angiotensin II receptor antagonist, losartan. *Br. J. Pharmacol.*, **106**, 655–671.
- AZUMA, H., SATO, J., HAMASAKI, H., SUGIMOTO, A., ISOTANI, E. & OBAYASHI, S. (1995). Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells. *Br. J. Pharmacol.*, **115**, 1001–1004.
- BAYDOUN, A.R., EMERY, P.W., PEARSON, J.D. & MANN, G.E. (1990). Substrate-dependent regulation of intracellular amino acid concentration in cultured bovine aortic endothelial cells. *Biochem. Biophys. Res. Commun.*, **173**, 940–948.
- BÖGER, R.H., BODE-BÖGER, S.M., THIELE, W., JUNKER, W., ALEXANDER, K. & FRÖLICH, J.C. (1997). Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation*, **95**, 2068–2074.
- BOGLE, R.G., MACALLISTER, R.J., WHITLEY, G.ST.J. & VALLANCE, P. (1995). Induction of N^G-monomethyl-L-arginine, uptake: a mechanism for differential inhibition of synthase? *Am. J. Physiol.*, **269**, C750–C756.
- CAMERON, N.E. & COTTER, M.A. (1992). Impaired contraction and relaxation in aorta from streptozotocin-diabetic rats: role of polyol pathway. *Diabetologia*, **35**, 1011–1019.
- CHINKERS, M. & GARBERS, D.L. (1991). Signal transduction by guanylyl cyclase. *Ann. Rev. Biochem.*, **60**, 553–575.
- CHRISTLIEB, A.R., JANKA, H., KRAUS, B., GLEASON, R.E., ICASASCABRAL, E.A., AIELLO, L.M., CABRAL, B.V. & SOLANO, A. (1976). Vascular reactivity to Angiotensin II and to norepinephrine in diabetic subjects. *Diabetes*, **25**, 268–274.
- FÖRSTERMANN, U., CLOSS, E.I., POLLOCK, J.S., NAKANE, M., SHWARZ, P., GATH, I., KLEINERT, H. (1994). Nitric oxide synthase isoenzymes: characterization, purification, molecular cloning, and functions. *Hypertension*, **23**, 1121–1131.
- FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial muscle by acetylcholine. *Nature*, **288**, 373–376.
- GARG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.*, **83**, 1774–1777.
- HAMASAKI, H., SATO, J., MASUDA, H., TAMAOKI, S., ISOTANI, E., OBAYASHI, S., UDAGAWA, T. & AZUMA, H. (1997). Effect of nicotine on the intimal hyperplasia after endothelial removal of the rabbit carotid artery. *Gen. Pharmacol.*, **28**, 653–659.
- IGNARRO, L.J. (1990a). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Ann. Rev. Pharmacol. Toxicol.*, **30**, 535–560.
- IGNARRO, L.J. (1990b). Nitric oxide: A novel signal transduction mechanism for transcellular communication. *Hypertension*, **16**, 477–483.
- KAMATA, K., MIYATA, N. & KASUYA, Y. (1989). Impairment of endothelium-dependent relaxation and changes in levels of cGMP in aorta from STZ-induced diabetic rats. *Br. J. Pharmacol.*, **97**, 614–618.
- KAMIKAWATOKO, S., TOKORO, T., ISHIDA, A., MASUDA, H., HAMASAKI, H., SATO, J. & AZUMA, H. (1998). Nitric oxide relaxes bovine ciliary muscle contracted by carbachol through elevation of cyclic GMP. *Exp. Eye Res.*, **66**, 1–7.
- KANZAKI, T., SHINOMIYA, M., UEDA, S., MORISAKI, N., SAITO, T. & YOSHIDA, S. (1994). Enhanced arterial intimal thickening after balloon catheter injury in diabetic animals accompanied by PDGF β -receptor overexpression of aortic media. *Eur. J. Clin. Invest.*, **24**, 377–381.
- KAWANO, M., KOSHIKAWA, T., KANZAKI, T., MORISAKI, N., SAITO, Y. & YOSHIDA, S. (1993). Diabetes mellitus induces accelerated growth of aortic smooth muscle cells: association with overexpression of PDGF β -receptors. *Eur. J. Clin. Invest.*, **23**, 84–90.
- KISSANE, J.M. & ROBINS, E. (1958). The fluorometric measurement of deoxyribonucleic acid in animal tissue with special reference to the central nervous system. *J. Biol. Chem.*, **233**, 184–188.
- KOBAYASHI, Y. & HATTORI, K. (1990). Nitroarginine inhibits endothelium-derived relaxation. *Jpn. J. Pharmacol.*, **52**, 167–169.
- LÜSCHER, T.F., BOCK, H.A., YANG, Z. & DIEDERICH, D. (1990). Endothelium-derived relaxing and contracting factors: Perspective in nephrology. *Kidney Int.*, **39**, 575–590.
- MACALLISTER, R.J., PARRY, H., KIMOTO, M., OGAWA, T., RUSSELL, R.J., HODSON, H., WHITLEY, G.ST.J. & VALLANCE, P. (1996). Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. *Br. J. Pharmacol.*, **119**, 1533–1540.
- MASUDA, H., TAMAOKI, S., GOTO, M., ISHIDA, A., KAMIKAWATOKO, S., TOKORO, T. & AZUMA, H. (1997). Nitric oxide-induced relaxation during the contraction with endothelin-1 is mediated through elevation of cyclic GMP. *Curr. Eye Res.*, **16**, 1245–1251.
- MILLER, R.A. & WILSON, R.B. (1984). Atherosclerosis and myocardial ischemic lesions in alloxan-diabetic rabbits fed a low cholesterol diet. *Arteriosclerosis*, **4**, 586–591.
- MONCADA, S. & HIGGS, E.A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.*, **329**, 2002–2012.
- MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1988). The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension*, **12**, 365–372.
- MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.
- NIIMI, Y., AZUMA, H. & HIRAKAWA, K. (1994). Repeated endothelial removal augments intimal thickening and attenuates EDRF release. *Am. J. Physiol.*, **266**, H1348–H1356.
- OBAYASHI, S., ASO, T., SATO, J., HAMASAKI, H. & AZUMA, H. (1996). Intimal hyperplasia in human uterine arteries accompanied by impaired synergism between prostaglandin I₂ and nitric oxide. *Br. J. Pharmacol.*, **119**, 1072–1078.
- OGAWA, T., KIMOTO, M. & SASAOKA, K. (1989). Purification and properties of a new enzyme, N^G, N^G-dimethylarginine dimethylaminohydrolase, from rat kidney. *J. Biol. Chem.*, **264**, 10205–10209.
- POSTON, L. & TAYLOR, P.D. (1995). Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. *Clin. Sci.*, **88**, 245–255.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). The antiaggregating properties of vascular endothelium: interaction between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 639–646.
- ROSS, R. (1986). The pathogenesis of atherosclerosis—an update. *N. Engl. J. Med.*, **314**, 488–500.
- SCHWARTZ, S.M., GAJDUSEK, C.M. & SELDEN, S.C. (1981). Vascular wall growth control: the role of endothelium. *Arteriosclerosis*, **1**, 107–171.
- SOBREVIA, L., YUDILEVICH, D.L. & MANN, G.E. (1998). Elevated D-glucose induces insulin insensitivity in human umbilical endothelial cells isolated from gestational diabetic pregnancies. *J. Physiol.*, **506**, 219–230.
- TAYLOR, P.D., MCCARTHY, A.L., THOMAS, C.R. & POSTON, L. (1992). Endothelium-dependent relaxation and noradrenaline sensitivity in mesenteric resistance arteries of streptozotocin-induced diabetic rat. *Br. J. Pharmacol.*, **107**, 393–399.
- TAYLOR, P.D., WICKENDEN, A.D., MIRRLEES, D.J. & POSTON, L. (1994). Endothelial function in the isolated perfused mesentery and aorta of rats with streptozotocin-induced diabetes: effect of treatment with the aldose reductase inhibitor ponalrestat. *Br. J. Pharmacol.*, **111**, 42–48.

- TESFAMARIAM, B., JAKUBOWSKI, J.A. & COHEN, R.A. (1989). Contraction of diabetic rabbit aorta due to endothelium-derived $\text{PGH}_2/\text{TXA}_2$. *Am. J. Physiol.*, **257**, H1327–H1333.
- VALLANCE, P., LEONE, A., CALVER, A., COLLIER, J. & MONCADA, S. (1992). Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *J. Cardiovasc. Pharmacol.*, **20**, S60–S62.
- ZHAO, Z.H., WATZCHINGER, B., BROWN, C.D., BEYER, M.M. & FRIEDMAN, E.A. (1987). Variations of susceptibility to alloxan induced diabetes in the rabbit. *Horm. Metabol. Res.*, **19**, 534–537.

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