Diabetes and insulin-induced stimulation of L-arginine transport and nitric oxide synthesis in rabbit isolated gastric glands

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1. The properties of L-arginine transport have been characterized and correlated with cGMP production (index of nitric oxide (NO)) in whole gastric glands isolated from non-diabetic and alloxan-diabetic rabbits.
2. In non-diabetic and diabetic glands, transport of L-arginine was stereoselective, Na^+ and pH independent and inhibited by other cationic amino acids. L-Arginine transport was slightly inhibited by L-leucine and L-phenylalanine, but unaffected by other neutral amino acids.
3. Diabetes enhanced the V_{max} for saturable L-arginine transport from $10{\cdot}7 \pm 1{\cdot}0$ to $17{\cdot}7 \pm 0{\cdot}5$ pmol (mg protein)$^{-1}$ s^{-1}, with negligible changes in K_m.
4. Accumulation of the membrane potential-sensitive probe tetra[^{3}H]phenylphosphonium (TPP^+) was increased 2-fold in diabetic compared with non-diabetic gastric glands, suggesting a membrane hyperpolarization.
5. Basal intracellular cGMP levels were elevated 2-fold in diabetic gastric glands, and in non-diabetic glands histamine, vasoactive intestinal peptide, and bradykinin increased cGMP levels. The NO synthase inhibitor N^G-nitro-L-arginine methyl ester (100 μM) abolished basal cGMP accumulation.
6. Addition of extracellular L-arginine induced a concentration-dependent increase in cGMP levels in gastric glands isolated from non-diabetic rabbits, but had no effect on elevated cGMP levels in diabetic glands.
7. Insulin induced a rapid (5 min) concentration-dependent increase in cGMP levels in non-diabetic gastric glands, but reduced elevated cGMP levels in diabetic gastric glands.
8. The present study has identified a specific transport system for L-arginine in gastric glands which resembles the classical system y^+. Our findings also provide the first direct evidence that diabetes increases the basal activity of system y^+ and NO synthase in gastric glands. The differential modulation of L-arginine transport by insulin and L-arginine identified in non-diabetic and diabetic glands, may be of importance in protecting the gastric mucosa from injuries associated with diabetes.

Limited information is available about the pathways mediating cationic amino acid transport in mammalian gastric epithelia. In a previous study in rabbit isolated gastric glands, both Na^+-independent (system y^+) and Na^+-dependent (system ASC) transport pathways were suggested to mediate L-lysine influx (Barahona & Bravo, 1993). The cationic amino acid L-arginine is the physiological precursor for the potent vasodilator nitric oxide (NO; Knowles & Moncada, 1994), which is synthesized by gastric mucosal cells (Brown, Tepperman, Hanson, Whittle & Moncada, 1992; Tepperman & Soper, 1993; Kluger & Drenckhahn, 1994) and plays an important role in the maintenance of gastric mucosal integrity and function (Schubert, 1994). As synthesis of NO has been shown to be dependent on

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availability of extracellular L-arginine (Assreuy & Moncada, 1992; Bogle, Baydoun, Pearson, Moncada & Mann, 1992), membrane transport of L-arginine may represent a rate-limiting step in local mucosal NO production and modulate the protective role of NO in gastric tissue.

Diabetes mellitus is often accompanied by abnormalities in gastric motility and metabolic activity (Jebbink, Bravenboer, Akkermans, van Berge-Henegouwen & Smout, 1993; Horowitz & Fraser, 1994), and a high occurrence of gastritis is frequently found in patients with Type I or Type II diabetes (DeAngelis *et al.* 1993). Inhibition of NO synthesis in the gastric mucosa leads to gastric mucosal injuries and a role for L-arginine transport has been suggested (Whittle, Lopez-Belmonte & Moncada, 1990). Although diabetes has been shown to increase transport of cationic amino acids in rat hepatocytes (Handlogten & Kilberg, 1984), peritoneal macrophages (Wu & Flynn, 1993) and human endothelium (Sobrevia, Cesare, Yudilevich & Mann, 1995*a*), there is no such information for isolated gastric tissue of diabetic origin. In view of the potential role of L-arginine in the local synthesis of NO in gastric tissue, we have characterized L-arginine transport and NO synthesis in the gastric mucosal epithelium isolated from non-diabetic and alloxan-diabetic rabbits.

Part of this work has been published in abstract form (Sobrevia & Fuentes, 1995; Sobrevia, Fuentes & Mann, 1995*b*).

METHODS

Diabetic rabbits

Diabetes was induced in adult male rabbits (1–2 kg) by injecting alloxan (125 mg kg^{-1}, I.V.). Blood glucose was determined in whole blood samples using glucose oxidase reagent strips (Haemoglucotest; Boehringer Mannheim, FRG). Urine volume was determined over 24 h periods for 2–3 days after injection of alloxan. Animals were weighed initially and then prior to the isolation of gastric glands. After removal of the stomachs, the rabbits were killed by cervical vertebrae extension.

Isolation of gastric glands

Gastric glands were isolated from the non-stimulated stomach of anaesthetized rabbits (sodium pentobarbitone, 30 mg kg^{-1}, I.V.; Bravo & Sobrevia, 1990; Sobrevia, Medina, Reinicke & Bravo, 1992). Briefly, the mucosa (5–8 g) of the corpus was digested with collagenase (1 mg ml^{-1}, 37 °C, 40 min) in Ringer medium of the following composition (mM): NaCl, 132; KCl, 10; $CaCl_2$, 1; $MgCl_2$, 0·8; Na_2HPO_4, 5; NaH_2PO_4, 1·2; pyruvic acid, 1; glucose, 11·1; and, additionally, 1 mg ml^{-1} bovine serum albumin (pH 7·4). The glands were filtered (nylon cloth: pore size, 230 μm) and the viability and integrity of the isolated gastric glands were determined by Trypan Blue dye exclusion and by light and electron microscopy (see Bravo & Sobrevia, 1990). About 98% of the glands excluded Trypan Blue. Endothelial cell contamination of isolated gastric glands was assayed by measuring binding of the endothelial cell marker lectin *Ulex europaeus* agglutinin I (UEA I; Jackson, Garbett, Nissen & Schrieber, 1990). Human umbilical vein endothelial cells served as positive controls for UEA I binding. UEA I binding to endothelial cells was 96–98%, but only 5–9% in both normal and diabetic gastric glands. Protein determinations were made using the Bradford reagent (Bradford, 1976).

Measurement of L-arginine transport

L-Arginine transport was initiated by addition of 200 μl of Ringer medium containing unlabelled L-arginine (100 μM) and L-[^{3}H]arginine (1 μCi ml^{-1}) to 50 μl (20–30 μg protein) of isolated gastric glands. The mixtures were filtered through Whatman GF/B glass fibre filters (pore size, 0·25 μm). Filters were washed twice with 5 ml of ice-cold Ringer medium containing 10 mM L-arginine and dried overnight. In kinetic studies, the gastric glands were exposed for 30 s to L-[^{3}H]arginine and increasing concentrations of L-arginine (0·012–2·5 mM). In cross-inhibition experiments, gastric glands were exposed simultaneously to 100 μM L-[^{3}H]arginine (1 μCi ml^{-1}, 30 s) and the putative competitor amino acids L-lysine, D-lysine, L-ornithine, L-alanine, L-leucine, and L-phenylalanine (final concentration, 25 mM). The concentration-dependent inhibition of L-arginine transport by these inhibitors was then examined over concentrations ranging from 0·03 to 50 mM. Control measurements of L-[^{3}H]arginine transport were unaffected by equimolar concentrations of D-mannitol (data not shown). All influx values for L-[^{3}H]arginine were corrected for extracellular ^{14}C counts (D-[^{14}C]mannitol) adhering to the filters.

To assay the Na^+ and pH dependence of L-arginine transport, 100 μM L-[^{3}H]arginine transport (1 μCi ml^{-1}, 30 s) was measured in gastric glands incubated in medium in which Na^+ was isosmotically replaced with choline chloride, K_2HPO_4 and KH_2PO_4. L-Arginine transport was also measured after incubating the glands for 15 min in Ringer medium adjusted to pH 6 or 8 by titration with HCl (0·5 N) or NaOH (0·5 N), respectively.

Measurement of tetra[^{3}H]phenylphosphonium (TPP^+) influx

Gastric glands were incubated with Ringer medium containing 100 μM L-arginine and [^{3}H]TPP^+ (5 μCi ml^{-1}) in normal (5·5 mM) or elevated (131 mM) extracellular KCl, and initial rates of [^{3}H]TPP^+ influx were then measured at various times (0–120 s), as described previously (Sobrevia *et al.* 1995*a*). Tracer uptake was terminated by centrifugation (12 000 r.p.m.) and radioactivity associated with the gastric glands was determined.

Radioimmunoassay for cGMP

Synthesis of NO was monitored by measuring intracellular levels of cGMP. Gastric glands were pre-incubated for 15 min in 0·5 mM isobutylmethylxanthine (IBMX) and cellular accumulation of cGMP was measured over a 5 min incubation period (37 °C) in a phosphate buffer Ringer medium containing 0·5 mM IBMX and 100 μM L-arginine. cGMP in HCl extracts (0·1 N, 4 °C, 12–24 h) of gastric glands was determined by radioimmunoassay (see Sobrevia *et al.* 1995*a*). Agonist-stimulated NO synthesis was determined by incubating the gastric glands for 5 min with histamine (100 μM), bradykinin (1 μM), vasoactive intestinal peptide (VIP, 1 μM) or sodium nitroprusside (SNP, 100 μM). The effects of human insulin (0·1–100 nM) and L-arginine (0·01–10 mM) on the synthesis of NO (5 min, 37 °C) was studied in gastric glands isolated from non-diabetic or diabetic rabbits. NO synthesis was determined in the absence and presence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM).

Conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline

Gastric glands were incubated with 100 μM L-arginine containing 1 μCi ml^{-1} L-[^{3}H]arginine for 30 min (37 °C) in the absence and presence of 100 μM histamine (last 5 min of a 30 min incubation period). Aliquots of 100 g of the cation ion-exchange resin Dowex 50W (50X8-200) in its protonated form were converted into

the sodium ion form by incubation with 200 ml 1 N NaOH. After calibration of the Dowex column, 300 μl of gastric gland suspension was digested in formic acid (95%), passed through the column and eluates of H_2O and NaOH were collected (Murthy, Jin & Makhlouf, 1994). Radioactivity as a measure of [^{3}H] was determined in 200 μl aliquots of H_2O or NaOH eluates. The amount of L-[^{3}H]citrulline produced after 30 min incubation with L-[^{3}H]arginine was determined in the H_2O eluate and expressed as d.p.m. (mg protein)$^{-1}$ (30 min)$^{-1}$.

Paired samples were analysed by thin-layer chromatography (TLC) to determine radioactive L-arginine and L-citrulline levels in gastric glands. Aliquots of gastric glands (200 μl, 80–120 μg protein) were digested with formic acid (98%; 12 h, room temperature), desiccated by evaporation (37 °C) and resuspended in 100 μl water. Aliquots of 10 μl samples and standards (L-arginine, L-citrulline, L-ornithine and ATP) were chromatographed on silica gel-coated plates (Whatman, 150A) using the following running solvent: chloroform, methanol, ammonium hydroxide and water, in the proportions 5 : 45 : 20 : 10, respectively (Iyengar, Steuhr & Marletta, 1987). Amino acids were localized using 0·2% ninhydrin (in methanol : acetone, 1 : 1) : 12·5 M pyridine (200 : 1, v/v). After drying, the plates were scanned using a Berthold TLC linear scanner (LB2760) and relative band speed (R_f) values for L-[^{3}H]-arginine, L-[^{3}H]-ornithine and L-[^{3}H]-citrulline were 0·30, 0·58 and 0·77, respectively.

Intracellular contents of L-citrulline and L-arginine were determined by high-performance liquid chromatography (HPLC) in gastric gland extracts. Aliquots of gastric gland suspension (1 ml) were pelleted and extracted with 0·5 ml methanol (96%) for 30 min under constant shaking. Samples were then exposed to three cycles of freeze-down on liquid nitrogen and thawed at 37 °C, centrifuged (1500 r.p.m., 2 min), and the supernatant then evaporated to dryness under a stream of nitrogen gas and resuspended in 100 μl methanol. Aliquots of 20 μl samples or standards were prepared as described by Baydoun, Emery, Pearson & Mann (1990) and injected onto a Hypersil Ultratechsphere ODS-5μ reversed-phase HPLC column (Jones Chromatography, Mid-Glamorgan, UK) in a Kontron 400 Series gradient HPLC system (Kontron Instruments Ltd, Watford, Herts, UK). The amino acid concentrations were calculated from the peak areas by reference to the area of the internal standard homoserine peak. L-Citrulline and L-arginine contents were expressed as nmol (mg protein)$^{-1}$.

Materials

The radioactive materials L-[2,3,-^{3}H]arginine (58·4 Ci mmol^{-1}) and D-[1-^{14}C]mannitol (56 Ci mmol^{-1}) and tetraphenylphosphonium bromide[phenyl-^{3}H] (37 Ci mmol^{-1}) were purchased from New England Nuclear (Dreieich, Germany). 3′,5′-cyclic GMP-TME [tyrosine-^{125}I] was from ICN (UK). All other chemicals used were obtained from Merck (FRG) and Sigma (USA). Collagenase Type II from *Clostridium histolyticum* was from Boehringer Mannheim (FRG). Human insulin, a gift from Dr K. C. Pedley (King's College, London, UK), was a 1st International Standard (1986, NIBSC, UK) and Bradford protein reagent was from BioRad Laboratories (Herts, UK).

Statistics

L-Arginine transport data were fitted to a single-site model (goodness-of-fit index, 0·81). Data were analysed using the equations described previously (Sobrevia *et al.* 1995*b*) and the computer programs Enzfitter and Ultra Fit (Elsevier, Biosoft). Values are expressed as means ± S.E.M., where n is the number of animals with three replicate measurements per experiment. Statistical analysis of the data was carried out on raw data by use of the Peritz F multiple means comparison test (Harper, 1984). Student's t test was applied for unpaired raw data. $P < 0·05$ was considered statistically significant.

RESULTS

Diabetic rabbits

Rabbits were diabetic within 7–9 days of injection of alloxan. In alloxan-induced diabetic rabbits blood glucose (non-fasting) concentration and urine excretion were 3·8- and 3·1-fold higher ($P < 0·01$) compared with non-diabetic rabbits, respectively (Table 1). At the time of gastric gland isolation (10–12 days after injection) body weight was lower ($P < 0·05$) in diabetic compared with non-diabetic rabbits.

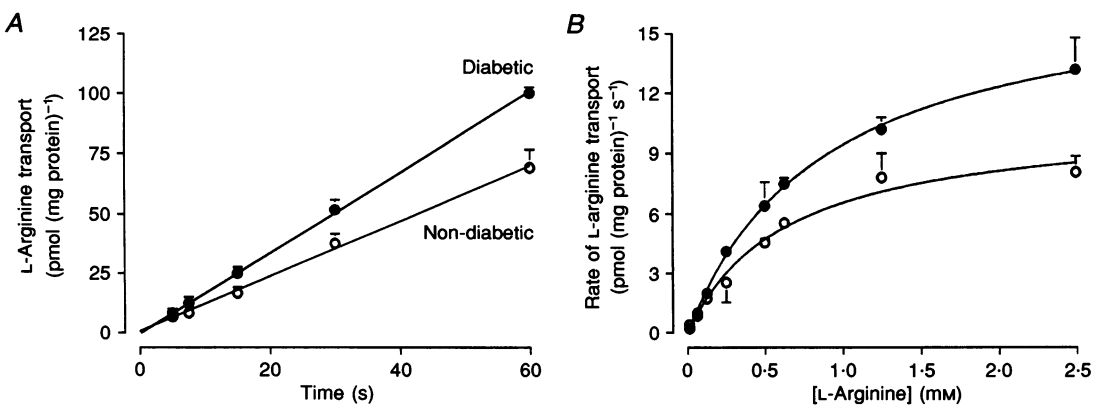

Figure 1. L-Arginine transport in rabbit gastric glands

Gastric glands were isolated from non-diabetic (○) and alloxan-diabetic (●) rabbits. *A*, time course of 100 μM L-arginine transport in isolated gastric glands. Data were fitted with non-linear least squares regression analysis. *B*, kinetics of L-arginine transport (30 s, 37 °C) in glands incubated with increasing concentrations of L-arginine (0·012–2·5 mM). Mean influx values were weighted for the reciprocal standard error and fitted by a single Michaelis–Menten equation (see Methods). Values are means ± S.E.M., $n = 8$ rabbits.

Table 1. Characteristics of non-diabetic and alloxan-diabetic rabbits

Rabbits	Blood glucose (mM)	Body weight (kg)	Urine (ml h^{-1})
Non-alloxan treated	4·1 ± 0·2	1·4 ± 0·2	2·5 ± 0·3
Alloxan treated	15·8 ± 0·4**	1·0 ± 0·1*	7·8 ± 1·5**

Values are means ± S.E.M., $n = 16$ rabbits. * $P < 0·05$ and ** $P < 0·01$ relative to values in non-alloxan-treated rabbits.

Table 2. Kinetic parameters for saturable L-arginine transport in isolated gastric glands from non-diabetic and alloxan-diabetic rabbits

Rabbits	K_m (mM)	V_{max} (pmol (mg protein)$^{-1}$ s^{-1})	K_i (mM) L-Lysine	L-Ornithine	L-Leucine	L-Phenyl-alanine	L-Alanine	D-Lysine
Non-diabetic	0·6 ± 0·2	10·7 ± 1·0	1·0 ± 0·1	1·4 ± 0·1	5·7 ± 1·2	3·1 ± 0·9	No inhibition	No inhibition
Diabetic	0·9 ± 0·2	17·7 ± 0·5*	1·3 ± 0·2	1·6 ± 0·2	5·4 ± 1·1	8·1 ± 2·4	No inhibition	No inhibition

The apparent inhibition constants (K_i) were calculated using the expression $K_i = IC_{50}/(1 + [Arg]/K_m)$, where K_m is the apparent K_m value for L-arginine transport, [Arg] is L-arginine concentration (100 μM) and IC_{50} is the half-maximal inhibitory concentration of the inhibitors. Values are means ± S.E.M., $n = 4$–8 rabbits. * $P < 0·02$ relative to values in non-diabetic rabbits.

Selectivity and kinetics of L-arginine transport

Initial rates of 100 μM L-arginine transport were linear for 60 s and significantly higher ($P < 0·05$) in diabetic (151 ± 15 pmol (mg protein)$^{-1}$ (60 s^{-1})) compared with non-diabetic (98 ± 16 pmol (mg protein)$^{-1}$ (60 s^{-1})) gastric glands (Fig. 1*A*). L-Arginine transport was unaffected by changes in external Na^+ or pH (not shown). As shown in Fig. 1*B*, L-arginine transport was saturable and mediated by a single entry site. The maximum velocity (V_{max}) for L-arginine transport in diabetic gastric glands was 1·7-fold higher ($P < 0·02$) than in gastric glands isolated from non-diabetic rabbits (Table 2). In contrast, no significant differences were

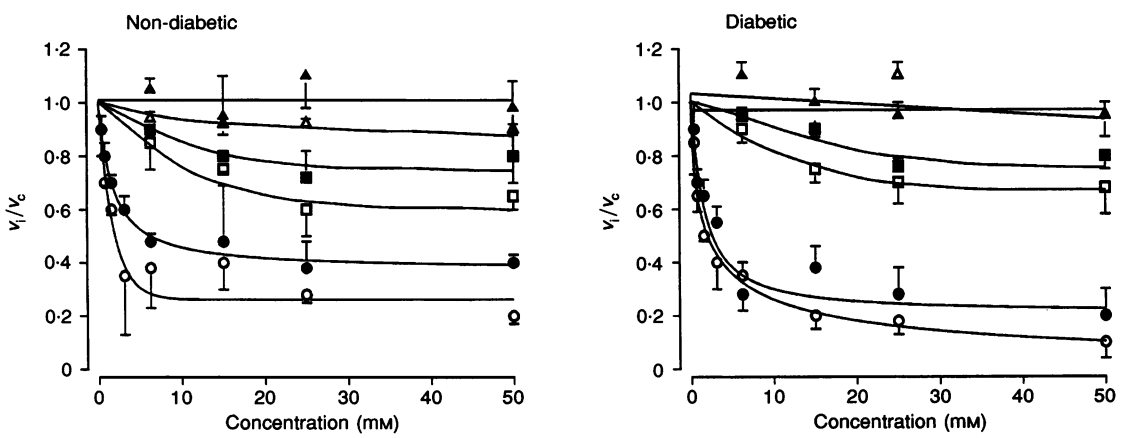

Figure 2. Inhibition of L-arginine transport by other amino acids

Transport of 100 μM L-arginine (30 s, 37 °C) in non-diabetic and diabetic gastric glands was measured in the presence of increasing concentrations (0·03–50 mM) of L-lysine (○), L-ornithine (●), L-alanine (△), L-leucine (□), L-phenylalanine (■), and D-lysine (▲). Values are expressed as the ratio between control transport (v_c) and transport measured in the presence of unlabelled inhibitor (v_i). Basal rates of L-arginine transport in non-diabetic and diabetic gastric glands were 1·3 ± 0·2 and 2·5 ± 0·2 pmol (mg protein)$^{-1}$ s^{-1}, respectively. Values denote means ± S.E.M., $n = 6$ rabbits.

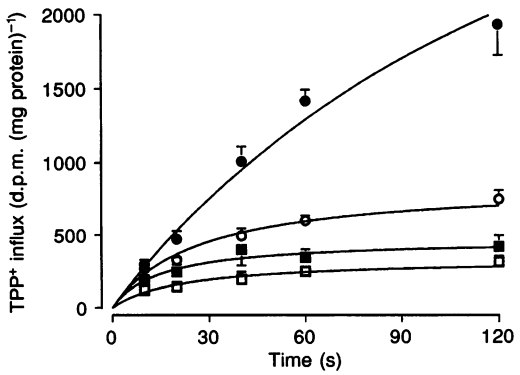

Figure 3. Effects of diabetes and elevated K^+ on influx of the membrane potential-sensitive probe TPP^+

The time course of [^{3}H]TPP^+ influx was determined in non-diabetic (○, □) and diabetic (●, ■) gastric glands incubated in normal (5·5 mM; ○, ●) or elevated (131 mM; □, ■) K^+. Values are means ± S.E.M., $n = 4$ rabbits.

observed in the apparent K_m values for non-diabetic and diabetic rabbits. Eadie–Hofstee transformations confirmed that L-arginine transport was mediated by a single saturable pathway (data not shown).

L-Arginine transport was inhibited by other cationic amino acids (L-lysine and L-ornithine, $P < 0.01$) in both non-diabetic and diabetic gastric glands (Fig. 2). The apparent K_i values for the inhibitors were in the same range as the corresponding apparent K_m values for L-arginine transport in non-diabetic and diabetic glands (Table 2), and values in non-diabetic and diabetic gastric glands were not significantly different. L-Leucine and L-phenylalanine inhibited L-arginine transport significantly ($P < 0.05$), but only in the presence of Na^+. L-Arginine transport was unaffected by D-lysine or L-alanine (Table 2).

Influx of tetraphenylphosphonium (TPP^+)

Initial rates of [^{3}H]TPP^+ influx in diabetic gastric glands were significantly higher ($P < 0.02$) than in non-diabetic gastric glands (Fig. 3). Elevated extracellular K^+ (131 mM) inhibited influx to similar values in both non-diabetic and diabetic gastric glands, confirming the dependence of TPP^+ influx on membrane potential.

Intracellular levels of cGMP

Basal accumulation of intracellular cGMP was 2-fold higher in diabetic compared with non-diabetic gastric glands ($P < 0.05$, Fig. 4). Intracellular cGMP levels were also increased by histamine (2·6-fold, $P < 0.05$), bradykinin (2·5-fold, $P < 0.04$) and VIP (3·2-fold, $P < 0.04$), and these increases in cGMP levels were abolished by co-incubation of gastric glands with 100 μM L-NAME. The NO donor sodium nitroprusside (SNP) increased basal levels of cGMP 21-fold in non-diabetic and 8-fold in diabetic glands, reaching similar maximal values in both preparations. As shown in Fig. 5, formation of L-citrulline from L-arginine was higher in diabetic ($P < 0.02$) compared with non-diabetic gastric glands. Radioactivity detected by thin-layer chromatography associated with L-[^{3}H]citrulline (R_f, 0·59) was increased by 65% in diabetic compared with non-diabetic gastric glands. Moreover, the intracellular content of L-citrulline determined

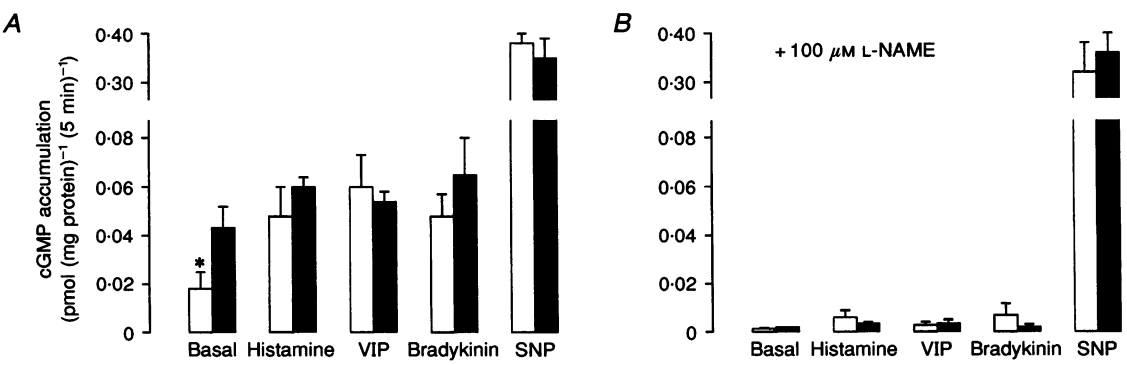

Figure 4. Accumulation of cGMP in rabbit gastric glands

□, non-diabetic and ■, diabetic gastric glands. *A*, gastric glands were initially pre-incubated in Ringer solution containing 100 μM L-arginine and 0·5 mM IBMX (15 min, 37 °C), and HCl cell extracts were then stored at −20 °C for radioimmunoassay of cGMP levels. cGMP accumulation was measured in the absence or presence (5 min) of histamine (100 μM), vasoactive intestinal peptide (VIP, 1 μM), bradykinin (1 μM), or sodium nitroprusside (SNP, 100 μM). *$P < 0.05$ compared with all other values. *B*, basal and agonist-stimulated cGMP levels were measured in the presence of 100 μM L-NAME. Values are means ± S.E.M., $n = 5$ rabbits.

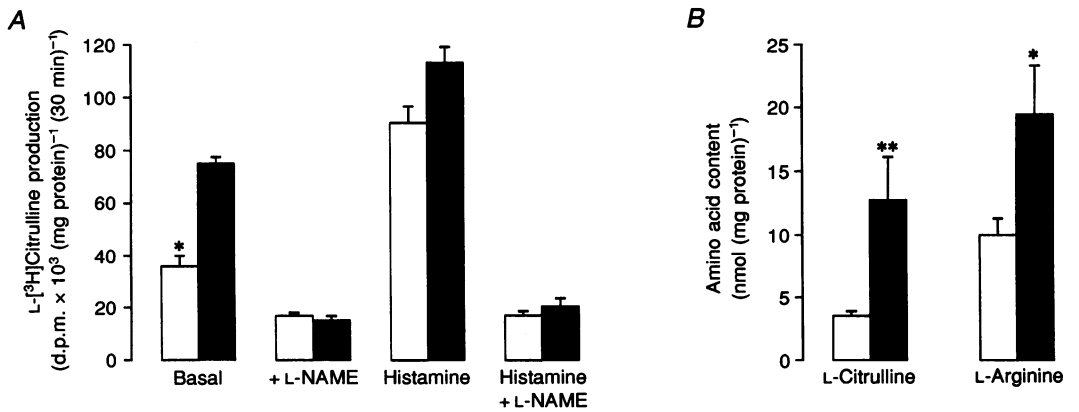

Figure 5. Basal and histamine-stimulated formation of L-citrulline from L-arginine in rabbit gastric glands

□, non-diabetic and ■, diabetic gastric glands. *A*, gastric glands were incubated with 100 μM L-arginine containing 1 μCi ml^{-1} L-[^{3}H]arginine for 30 min (37 °C) in the absence and presence of 100 μM histamine (last 5 min of a 30 min incubation period). Aliquots of 300 μl formic acid-digested gastric glands were passed through a cation ion-exchange resin Dowex 50W (50X8-200) and radioactivity as a measure of L-[^{3}H]citrulline was determined in 200 μl aliquots of H_2O eluates. *B*, basal content of L-citrulline and L-arginine in non-stimulated gastric glands was measured by HPLC. In *A*, $*P < 0{\cdot}02$ relative to all other values; in *B*, $*P < 0{\cdot}02$ and $**P < 0{\cdot}01$ relative to corresponding control values in non-diabetic gastric glands. Values are means ± S.E.M., $n = 3$–5 rabbits.

by high-performance liquid chromatography in non-stimulated gastric glands was also found to be higher in diabetic (12·8 ± 3·3 nmol (mg protein)$^{-1}$, $P < 0{\cdot}01$) compared with non-diabetic gastric glands (3·5 ± 0·4 nmol (mg protein)$^{-1}$).

Effects of insulin and L-arginine on intracellular cGMP levels

Insulin caused a concentration-dependent increase in cGMP levels in gastric glands from non-diabetic rabbits (Fig. 6*A*), with a half-maximal ($K_{½}$) effect detected at 18 ± 2 nM. In contrast, insulin reduced the elevated basal cGMP content in gastric glands from diabetic rabbits ($K_{½} = 1{\cdot}4 \pm 0{\cdot}4$ nM). In both preparations the effect of insulin was abolished by L-NAME. When gastric glands were exposed to increasing concentrations of L-arginine in the incubation medium, cGMP levels in non-diabetic glands increased from 0·012 ± 0·004 pmol (mg protein)$^{-1}$ (5 min)$^{-1}$ to a maximal value of 0·05 ± 0·005 pmol (mg protein)$^{-1}$ (5 min)$^{-1}$ ($P < 0{\cdot}01$) at 300 μM L-arginine (Fig. 6*B*), but subsequently decreased to basal values in the presence of 3–10 mM L-arginine. In diabetic gastric glands, extracellular L-arginine had no effect

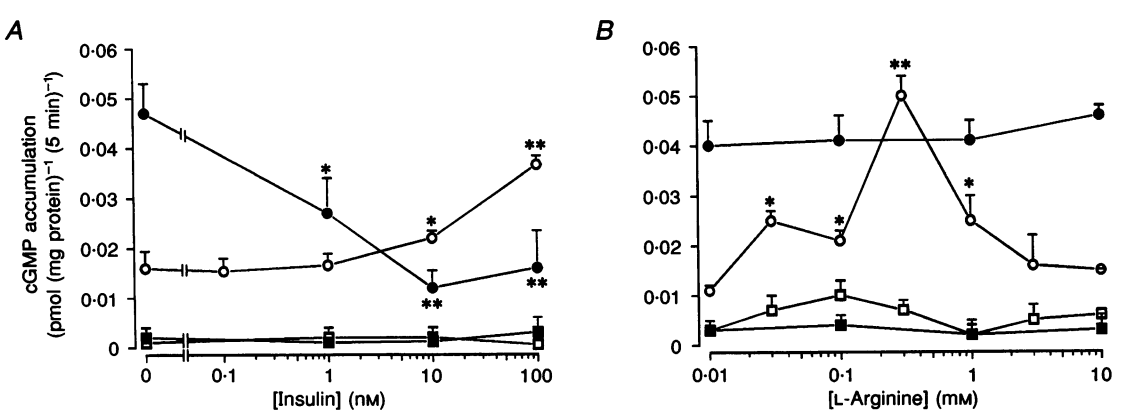

Figure 6. Effect of human insulin and L-arginine on cGMP levels in gastric glands

Intracellular cGMP was measured as described in the legend to Fig. 4. Non-diabetic (○) and diabetic (●) gastric glands were exposed for 5 min to Ringer medium containing increasing concentrations of human insulin (0·1–100 nM, *A*) or L-arginine (0·01–10 mM, *B*). cGMP accumulation was also determined in non-diabetic (□) and diabetic (■) gastric glands in the presence of 100 μM L-NAME . $*P < 0{\cdot}02$ and $**P < 0{\cdot}01$ relative to corresponding control values. Values are means ± S.E.M., $n = 5$ rabbits.

on cGMP levels. L-NAME abolished basal and L-arginine-stimulated changes in cGMP levels.

DISCUSSION

The present study in rabbit isolated gastric glands has established that (i) L-arginine transport is probably mediated by the classical system y^+, (ii) diabetes increases the basal activity of system y^+ and NO synthase, and (iii) extracellular insulin and L-arginine were associated with an increased transport of L-arginine and NO synthesis in non-diabetic but not diabetic glands.

In gastric glands isolated from non-diabetic rabbits transport of L-lysine is mediated by both Na^+-dependent and Na^+-independent transport systems (Barahona & Bravo, 1993). In this study, L-arginine transport was mediated only by the Na^+-independent transport system y^+. In many cell types cationic amino acid transport occurs preferentially by a single saturable transport system with apparent K_m values ranging between 0·03 and 1 mM (see reviews by White, 1985; Malandro & Kilberg, 1996). In this study we found that L-arginine transport in gastric glands was saturable with apparent K_m values within the range reported for system y^+ and similar to the Na^+-independent L-lysine transport pathway described previously in rabbit gastric glands (K_m, 1·42 mM; Barahona & Bravo, 1993). Moreover, the K_m for L-arginine was similar to the determined aggregate concentration of cationic amino acids in plasma (see Brosnan, Man, Hall, Colbourne & Brosnan, 1983). These results contrast with some previous reports suggesting that cationic amino acid transport occurs via a very low affinity system with K_m values of 20 mM or higher (see White, 1985). In cultures of human endothelium, L-arginine transport is mediated by both saturable and non-saturable components (up to 70% of total transport at 1 mM L-arginine, Sobrevia *et al.* 1995*b*). No evidence was found for a non-saturable component for L-arginine transport in either non-diabetic or diabetic gastric glands. In addition, apparent K_i values for L-lysine and L-ornithine, inhibitors of L-arginine transport, were close to the apparent K_m for L-arginine transport, suggesting that these amino acids may use the same transport system as L-arginine. In rabbit gastric glands transport of 68 μM L-lysine in the presence of Na^+ was shown to be completely inhibited by 5 mM L-leucine (Barahona & Bravo, 1993), although in the present study L-arginine transport was only inhibited by about 30% by L-leucine (K_i, ~5 mM) or L-phenylalanine (K_i, ~3–8 mM). Devés, Chavez & Boyd (1992) have described a very high affinity transport system y^+L for L-lysine (half-saturation constant, K_m, ~0·01 mM) in human erythrocytes, which is shared with neutral amino acids. Our kinetic studies suggest that system y^+L is unlikely to be operative in gastric glands, since the apparent K_m values determined were 60- to 90-fold higher than that of system y^+L. In addition, the K_m values for the family of cationic amino acid transporters (CAT-1, CAT-2A, CAT-2) recently cloned (see review by Malandro & Kilberg, 1996) fall within the range of those found in this study for system y^+.

Effect of diabetes on L-arginine transport in gastric glands

In gastric glands isolated from diabetic rabbits, the V_{max} for L-arginine transport was, on average, 1·7-fold higher than values in non-diabetic rabbits (Fig. 1). This finding agrees with previous reports showing that experimental diabetes increased the activity of cationic amino acid transport in rat hepatocytes (Handlogten & Kilberg, 1984), peritoneal macrophages (Wu & Flynn, 1993) and human umbilical vein endothelial cells (Sobrevia *et al.* 1995*a*). Expression of system y^+/CAT-1 mRNA level is increased in liver cells isolated from rats fed with a diet rich in carbohydrates (Wu, Robinson, Kung & Hatzoglou, 1994), which is consistent with our preliminary findings of an increased activity of system y^+/CAT-1 detected in human umbilical vein endothelial cells exposed to elevated glucose (25 mM) for 24 h *in vitro* (Sobrevia, Nadal, Yudilevich & Mann, 1996). These results suggest a possible direct effect of glucose or glucose metabolites on cationic amino acid transporter activity in gastric glands isolated from diabetic rabbits. Another possible mechanism for the increased L-arginine transport in diabetes is an increased synthesis or derepression of the L-arginine transporter in the plasma membrane (see White, 1985; Malandro & Kilberg, 1996).

Studies performed in human fibroblasts demonstrated that the activity of system y^+/CAT-1 is dependent on the membrane potential, and influx of L-arginine has been considered an indicator of membrane potential (Bussolati *et al.* 1989). We have recently shown, using whole-cell patch and measuring the accumulation of the lipophilic cation tetraphenylphosphonium (TPP^+), that elevated rates of L-arginine transport in human fetal endothelial cells isolated from gestational diabetic patients were associated with a membrane hyperpolarization (Sobrevia *et al.* 1995*b*). In the present study accumulation of TPP^+ was also higher in diabetic compared with non-diabetic gastric glands (Fig. 3), suggesting that hyperpolarization of the basolateral membrane may in part be responsible for the elevated rates of L-arginine transport.

Effect of diabetes on nitric oxide synthesis in gastric glands

Synthesis of NO via constitutive Ca^{2+}-dependent NO synthase in the mucosal fraction of the gastric mucosa has been postulated as an important self-defence mechanism regulating epithelial cell integrity and secretion (Brown *et al.* 1992; Middleton *et al.* 1995). In the present study, we have demonstrated that basal synthesis of NO in gastric glands isolated from alloxan-diabetic rabbits is elevated compared with non-diabetic animals, suggesting that the activity of NO synthase in the gastric glands may be increased by diabetes. This observation was confirmed by an elevated conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline

in diabetic gastric glands, resulting in a higher intracellular concentration of L-citrulline.

Diseases such as gastritis, oesophagitis and duodenitis in diabetic patients are correlated with elevated glucose, duration of diabetes, and vascular complications (Faigel & Metz, 1996), and risk of chronic gastritis may be due to increased levels of anti-gastric parietal cell antibodies in these patients (DeAngelis *et al.* 1993). The elevated synthesis of NO in gastric glands from diabetic rabbits may provide a self-defence mechanism against the deleterious effects of diabetes, such as ulcer formation or HCl hypersecretion (Jebbink *et al.* 1993; Horowitz & Fraser, 1994). Furthermore, a sustained synthesis of NO may be required by gastric tissues in conditions of oxidative stress such as is encountered in diabetes (Wolff, 1993; Jaap & Tooke, 1995).

In the present study we have found that extracellular L-arginine, the substrate for NO synthase, increased the accumulation of cGMP only in non-diabetic gastric glands, but had no effect in tissue isolated from diabetic animals. Inhibition of mucosal NO synthesis by administration of N^G-monomethyl-L-arginine (L-NMMA) has been reported to increase the occurrence of gastric mucosal injuries, which are attenuated after administration of L-arginine in rats (Whittle *et al.* 1990) suggesting that availability of L-arginine may be a limiting factor for mucosal NO synthesis. Thus, exogenous L-arginine and its incorporation into mucosal cells may play a role in protecting the integrity of gastric mucosa. A similar finding has been reported for human umbilical vein endothelial cells isolated from gestational diabetic pregnancies (Sobrevia *et al.* 1995*a*). In endothelial cells, diabetes increased L-arginine transport but transport rates were not further elevated when cells were exposed for 24–48 h to elevated D-glucose. In endothelial cells isolated from normal pregnancies, both L-arginine transport and NO synthesis were increased by elevated glucose (Sobrevia *et al.* 1996). It seems plausible that hyperglycaemia associated with diabetes may alter protein functions, such as non-enzymatic glycation of intracellular proteins (see Jaap & Tooke, 1995). The possibility that cGMP levels detected in our study are due to contaminating endothelial cells is unlikely because binding of the lectin *Ulex europaeus* I (UEA I), a well-known marker for endothelial cells (Jackson *et al.* 1990), was low in both non-diabetic and diabetic gastric gland suspensions. However, we have not ruled out the possibility that other cell types present in the lamina propria such as smooth muscle, lymphocytes, polymorphonuclear leucocytes, eosinophils, mast cells and nerves, may contribute to the accumulation of cGMP and/or L-arginine transport determined in the present study.

Insulin-like growth factors have been shown to act on the gastrointestinal tract as growth promoters, and an increased release of insulin induced by the polypeptide proglucagon(78–108) reduced gastric acid secretion in rats (Utthenthal & Blazquez, 1990). Insulin induced an increase in cGMP accumulation in gastric glands from non-diabetic animals which was blocked by co-incubation of gastric glands with the NO synthase inhibitor, L-NAME, suggesting that the effect of insulin was due to an increased NO synthesis. We have recently shown a similar effect of this islet hormone in human umbilical vein endothelial cells (Sobrevia *et al.* 1996), but insulin reduced the elevated cGMP accumulation detected in endothelial cells isolated from gestational diabetic pregnancies. These paradoxical results for insulin were also found in this study using gastric glands from diabetic rabbits, suggesting that this may reflect a generalized effect of insulin in diabetic tissues involving guanylyl cyclase activity stimulated by NO. A higher activity of protein kinase C (PKC) has been described in the majority of diabetic tissues, and activation of PKC has been shown to reduce NO synthase activity in dispersed gastric muscle cells (Murthy *et al.* 1994). Insulin stimulation of phosphoinositide hydrolysis could exert opposing effects on NO synthase activity with an increase in activity caused by the rise of cytosolic free Ca^{2+} and a decrease in activity caused by activation of PKC (Bredt, Ferris & Snyder, 1992). It has been shown that inositol trisphosphate formation and PKC translocation is impaired in gastric muscle from diabetic BB/W rats (Takahashi *et al.* 1996). Thus, occupancy of insulin receptors by insulin may result in a reduced or increased synthesis of NO, depending on the predominant pathway for the effects of insulin in gastric glands.

Vasoactive intestinal peptide (VIP) is known to increase guanylyl cyclase leading to relaxation of gastric myocytes (Desai, Warner, Bishop, Polak & Vane, 1994). VIP, as well as bradykinin and histamine, increased cGMP levels in gastric glands from non-diabetic rabbits and their effects were completely blocked by L-NAME, suggesting that the effect of these agonists increased the activity of NO synthase in gastric glands. The heterogeneous nature of the cell types and functions in gastric tissue and gastric glands (i.e. parietal cells, chief cells, mucus neck cells) makes it difficult to ascribe the results to only one type of mucosal cell. However, parietal cells are the main cell type present in gastric glands (about one-third of corpus mass) and are activated by histamine through H_2 receptors (Schubert, 1994). These cells may well account for the histamine-elevated cGMP levels detected in this study. It is well known that VIP is a potent inhibitor of gastrointestinal motility, and VIP-increased NO synthesis in gastric mucosa may contribute to the altered gastric emptying commonly associated with diabetes mellitus Type I and Type II (Jebbink *et al.* 1993; Horowitz & Fraser, 1994). The VIP-induced increase in gastric mucosal NO synthesis may result from a higher hydrolysis of phosphoinositides rather than inhibition of PKC activity (see Murthy *et al.* 1994), and an imbalance of these intracellular pathways may be altered in diabetic gastric glands.

Our findings establish that alloxan-induced diabetes mellitus in rabbits is associated with a hyperpolarization of the basolateral membrane in gastric glands and an increase in the basal activity of the L-arginine transport system y^+/NO synthase. Differential modulation of the

L-arginine–NO signalling pathway in non-diabetic and diabetic gastric glands by insulin and L-arginine itself may have important implications for the protection of the gastric mucosa from injury in diabetes. Since endothelial cells are known to be the major source of NO, the finding that NO is synthesized in gastric glands and appears to be differentially modulated in mucosal tissue of diabetic origin, is novel and this experimental model may provide a valuable tool for further studies of the regulation of the L-arginine–NO pathway in gastric tissue.

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