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Pharmacological characterization of guanidinoethyldisulphide (GED), a novel inhibitor of nitric oxide synthase with selectivity towards the inducible isoform

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1 Guanidines, amidines, S-alkylisothioureas, and recently, mercaptoalkylguanidines have been described as inhibitors of the generation of nitric oxide (NO) from L-arginine by NO synthases (NOS). We have recently demonstrated that guanidinoethyldisulphide (GED), formed from the dimerisation of mercaptoethylguanidine (MEG), is a novel inhibitor of nitric oxide synthases. Here we describe the pharmacological properties of GED on purified NOS isoforms, various cultured cell types, vascular ring preparations, and in endotoxin shock.

2 GED potently inhibited NOS activity of purified inducible NOS (iNOS), endothelial NOS (ecNOS), and brain NOS (bNOS) enzymes with K_i values of 4.3, 18 and 25 μM, respectively. Thus, GED has a 4 fold selectivity for iNOS over ecNOS at the enzyme level. The inhibitory effect of GED on ecNOS and iNOS was competitive vs. L-arginine and non-competitive vs. tetrahydrobiopterin.

3 Murine J774 macrophages, rat aortic smooth muscle cells, murine lung epithelial cells, and human intestinal DLD-1 cells were stimulated with appropriate mixtures of pro-inflammatory cytokines or bacterial lipopolysaccharide to express iNOS. In these cells, GED potently inhibited nitrite formation (EC_{50} values: 11,9, 1 and 30 μM, respectively). This suggests that uptake of GED may be cell type- and species- dependent. The inhibitory effect of GED on nitrite production was independent of whether GED was given together with immunostimulation or 6 h afterwards, indicating that GED does not interfere with the process of iNOS induction.

4 GED caused relaxations in the precontracted vascular ring preparations (EC_{50}: 20 μM). Part of this relaxation was endothelium-dependent, but was not blocked by methylene blue (100 μM), an inhibitor of soluble guanylyl cyclase. In precontracted rings, GED enhanced the acetylcholine-induced, endothelium-dependent relaxations at 10 μM and caused a slight inhibition of the relaxations at 100 μM. The vascular studies demonstrate that the inhibitory potency of GED on ecNOS in the ring preparations is considerably lower than its potency against iNOS in the cultured cells. These data suggest that the selectivity of GED towards iNOS may lie, in part, at the enzyme level, as well as differential uptake by cells expressing the various isoforms of NOS.

5 In a rat model of endotoxin shock *in vivo*, administration of GED, at 3 mg kg^{-1} bolus followed by 10 mg kg^{-1} h^{-1} infusion, starting at 90 min after bacterial lipopolysaccharide (LPS, 15 mg kg^{-1}, i.v.), prevented the delayed fall in mean arterial blood pressure, prevented the development of the vascular hyporeactivity to noradrenaline of the thoracic aorta *ex vivo* and protected against the impairment of the endothelium-dependent relaxations associated with this model of endotoxaemia. The same bolus and infusion of the inhibitor did not alter blood pressure or *ex vivo* vascular reactivity in normal animals over 90 min.

6 Administration of GED (10 mg kg^{-1}, i.p.) given at 2 h after LPS (120 mg kg^{-1}, i.p.) and every 6 h thereafter caused a significant improvement in the survival rate in a lethal model of endotoxin shock in mice between 12 and 42 h.

7 In conclusion, we found that GED is a competitive inhibitor of iNOS activity. Its selectivity towards iNOS may lie both at the enzyme level and at the level of cell uptake. GED has beneficial effects in models of endotoxin shock that are driven by iNOS. GED or its derivatives may be useful tools in the experimental therapy of inflammatory conditions associated with NO overproduction due to iNOS expression.

Keywords: Nitric oxide; tetrahydrobiopterin; L-arginine; mercaptoalkylguanidines; isothioureas; blood pressure; shock; endotoxin; vasoconstriction; vasodilatation

Introduction

The inducible isoform of nitric oxide (NO) synthase (iNOS), expressed in macrophages and vascular smooth muscle cells, has been implicated in the development of cellular energetic failure and vascular hyporesponsiveness during inflammation and circulatory shock (Nathan, 1992; Szabó, 1995). In shock induced by bacterial endotoxin (lipopolysaccharide, LPS), NO produced by iNOS results in a fall in blood pressure, a decrease in peripheral vascular resistance and vascular hyporesponsiveness (Szabó, 1995). Using pharmacological tools (Szabó *et al.*, 1994; Wu *et al.*, 1995; Worrall *et al.*, 1995) and genetically engineered animals (MacMicking *et al.*, 1995; Wei *et al.*, 1995), it has been demonstrated that selective inhibition of iNOS activity provided

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therapeutic benefit in shock and inflammation. Selective inhibition of iNOS is important in order to leave the important physiological roles of the other NOS isoforms undisturbed.

The generation of NO by NOS can be inhibited by analogues of the substrate, L-arginine. However, the most commonly used inhibitors of NOS, namely N^G monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) and its methyl ester, L-NAME, inhibit ecNOS at least as strongly as they inhibit iNOS (Gross *et al.*, 1990; Lambert *et al.*, 1991). Recently, compounds that are not amino acids, such as guanidines (Hasan *et al.*, 1993), S-alkylisothioureas (Szabó *et al.*, 1994; Garvey *et al.*, 1994; Southan *et al.*, 1995b) and amidines (Southan *et al.*, 1995a) have also been reported to inhibit NOS, some of them with selectivity towards iNOS (Southan & Szabó, 1996). Recently, we investigated the mechanism of action of S-aminoethylisothiourea (AETU) and found that pharmacological actions of AETU and related aminoalkylisothioureas can be explained by their rearrangement to yield mercaptoalkylguanidines, a new class of potent inhibitors of NOS (Southan *et al.*, 1996). Some of these mercaptoalkylguanidines, such as mercaptoethylguanidine (MEG) and its dimeric form, guanidinoethyldisulphide (GED) were found to be potent inhibitors of nitrite production in immunostimulated macrophages (Southan *et al.*, 1996). Here we describe the results of the pharmacological characterization of GED on purified NOS isoforms, on various cultured cell types, on vascular ring preparations and in endotoxin shock.

Methods

Purification and characterization of NOS isoforms

iNOS from murine macrophages was prepared as previously described (Wolff & Gribin, 1994; Wolff & Lubeskie, 1995). A typical preparation exhibited a specific activity of 0.8–1.6 μmol citrulline formed mg^{-1} min^{-1} as measured at 100 μM L-arginine and co-factors. Bovine pulmonary arterial endothelial NOS (ecNOS) was prepared and characterized as previously described (Wolff *et al.*, 1994). These preparations of endothelial NOS typically possessed a specific activity of 0.3 ± 0.1 μmol citrulline formed mg^{-1} min^{-1} at 100 μM L-arginine. A NOS preparation which is physically, kinetically and immunologically identical to bovine bNOS (but contains substoichiometric quantities of bound BH_4 (0.15 mol BH_4 mol^{-1}) was prepared as previously described (Wolff & Lubeskie, 1995) from extracts of GH3 cells by adsorption to ADP-agarose and elution with NADPH.

Measurements of NOS activity

NOS activity of iNOS, ecNOS and bNOS was measured by conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline using a modification of the procedure of Bredt & Snyder (1990) as described previously (Wolff & Datto, 1992). Standard incubations for the measurement of citrulline formation by ecNOS or bNOS contained 30 mM HEPES, pH 7.4, 1 mM dithiothreitol, 120 nM [^{3}H]-L-arginine (a sub-saturating concentration), 1 mM EGTA, 0.85 mM Ca^{2+}, 6 μM calmodulin, 100 μM NADPH and 100 μM BH_4. Standard incubations for the measurement of iNOS contained 30 mM HEPES, pH 7.4, 1 mM dithiothreitol, 120 nM [^{3}H]-L-arginine, 1 mM EGTA, 100 μM NADPH and 300 μM BH_4. Incubations were conducted at 30°C for 30 min in duplicate and the mean values were calculated. The effect of various concentrations (6, 10 and 60 μM) of GED were tested on NOS activity. For the Dixon plots, the following arginine concentrations used were employed: 1, 2, 5, 10, and 25 μM. Variability of values around the mean routinely averaged ±3% of the mean. Routinely, assays were conducted at dilutions of enzyme that provided 5–10% of total substrate consumption. Under these conditions, product formation was linear over time. Reactions were stopped by dilution with 0.5 ml of ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline was measured by scintillation counting.

In separate assays, iNOS activity was determined by the measurement of NO formation as described by Noack *et al.* (1992). Standard incubations contained 50 mM HEPES, pH 7.4, 10 μM L-arginine, 1 mM EGTA, 100 μM NADPH and 15 μM oxyhaemoglobin. Reactions were initiated with ADP-agarose purified iNOS and the difference in light absorbance at 401 and 411 nm determined. The NO produced converts oxyhaemoglobin to methaemoglobin. Methaemoglobin formation is quantified using an extinction coefficient for methaemoglobin of 38 mM^{-1} cm^{-1}. This assay is suitable for the detection of NO formation over shorter time periods (up to 10 min in our studies).

For the studies on NOS activity, data shown in the Results section represent pooled data from at least 3 different experimental days.

Cell culture

The mouse macrophage cell line J774.2, and DLD-1 cells, a human cell line derived from an intestinal adenocarcinoma, were cultured in Dulbecco's modified Eagle's medium (DMEM) and rat aortic smooth muscle (RASM) cells were cultured in RPMI medium, both media with 4 mM L-glutamine, penicillin (10,000 ul^{-1}), streptomycin (10,000 ul^{-1}) and 10% foetal calf serum (Szabó *et al.*, 1993; 1994; Salzman *et al.*, 1996). Murine lung epithelial cells (MLE cells; Wikenheiser *et al.*, 1993) were grown in Hite's medium in the presence of 10% foetal calf serum.

Cells were cultured in 96-well plates with 200 μl culture medium until they reached 60–80% confluence. To induce iNOS in J774 and RASM cells, fresh culture medium containing *E. coli* LPS (10 μg ml^{-1}) and murine γ-interferon (IFN, 50 u ml^{-1}) was added. To induce iNOS in MLE cells, a mixture of murine IL-1, TNF and IFN (1 ng ml^{-1} each) was added. To induce iNOS in DLD cells, a mixture of cytokines (human recombinant IL-1β at 0.5 ng ml^{-1} and IFN at 100 u ml^{-1}) was added. Accumulation of nitrite (J774, RASM, MLE cells) or nitrite/nitrate (in the DLD cells, where nitrate is the major degradation product of NO; Salzman *et al.*, 1996) in the cell culture medium (in the absence or presence of various inhibitors) was measured after 24 h. NOS inhibitors were added to the cells together with immunostimulation or 6 h thereafter to investigate whether they interfere with the induction of iNOS (see: Szabó *et al.*, 1994).

Nitrite/nitrate production

Nitrite production, an indicator of NO synthesis, was measured in the cell culture supernatant as previously described (Szabó *et al.*, 1994) by the Griess reaction using the Spectramax microplate reader. The nitrate in the supernatant of the DLD-1 cells was first converted to nitrite by incubation with 60 mu nitrate reductase and 25 μM NADPH for 120 min, and then nitrite was measured by the Griess reaction. A standard curve was obtained with sodium nitrate (1–100 μM).

Cell respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5 - dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] to formazan (Szabó *et al.*, 1994). The inhibitory effects on nitrite production presented in the figures and tables were not accompanied by significant changes in cell viability, except as noted otherwise.

Haemodynamic measurements

All animal experiments have been performed in accordance with NIH guidelines and with the approval of the institutional review board of the Children's Hospital Research Foundation. Male Wistar rats (Charles River Laboratories, Wilmington, MA, U.S.A.) were anaesthetized with sodium thiopentone (120 mg kg^{-1}, i.p.) and instrumented as described (Southan *et al.*, 1995b). The trachea was cannulated to facilitate respiration and temperature was maintained at 37°C using a homeothermic blanket. The right carotid artery was cannulated and connected to a pressure transducer for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate which were digitalized using a Maclab A/D converter (AD Instruments, Milford, MA, U.S.A.). Renal cortical blood flow was measured with a laser doppler surface probe and a transonic laser-doppler blood flow system (Transonic Systems Inc. Model T206) and was expressed as a percentage of the initial flow. The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 10 min. For the dose-response curves with NOS inhibitors, cumulative doses of L-NMMA or GED (0.1, 0.3, 1, 3, 10, and 30 mg kg^{-1} bolus injections) were injected 5 min apart and readings were taken at 4.5 min after the injection of each dose. When investigating the effect of GED on MAP, in separate experiments, only one bolus injection of GED (10 mg kg^{-1}, i.v.; $n=4$ or 30 mg kg^{-1}, i.v., $n=4$) was tested, in order to avoid the potential development of tachyphylaxis.

Endotoxic shock protocol

Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 10 min. Endotoxic shock was induced by injection of *E. coli* LPS (15 mg kg^{-1}, i.v.) at time 0. At 90 min saline was infused at a rate of 0.2 ml kg^{-1} for a further 90 min (vehicle-treated LPS-rats), or at 90 min, GED was injected (at 3 mg kg^{-1}, i.v. in 0.1 ml kg^{-1}, saline) and then infused (10 mg kg^{-1} h^{-1} in 0.2 ml kg^{-1}) for a further 90 min (GED-treated LPS-rats). In the control groups of rats, animals were injected with vehicle (saline, 0.1 ml kg^{-1}, i.v.) at time 0. At 90 min GED was injected (at 3 mg kg^{-1}, i.v. in 0.1 ml kg^{-1} saline) and then infused (10 mg kg^{-1} h^{-1} in 0.2 ml kg^{-1}) for a further 90 min (GED-treated control-rats). At 180 min, animals were killed and thoracic aortae taken for the measurements of *ex vivo* vascular reactivity.

Organ bath experiments

Rings of thoracic aortae from rats were mounted in organ baths (5 ml) filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7, in the presence of indomethacin (10 μM). Isometric force was measured with isometric transducers (Kent Scientific Corp. Litchfield, CT, U.S.A.). A tension of 1 g was applied and the rings were equilibrated for 60 min, changing the Krebs solution every 15 min (Zingarelli *et al.*, 1994). In endothelium-denuded vessels, after incubation and wash-outs, the vessels were first precontracted with 100 nM noradrenaline, and then the effect of acetylcholine (1 nM – 10 μM) was tested. Lack of a detectable acetylcholine-induced relaxation was taken as evidence that endothelial cells had been removed.

In control vessels (endothelium-intact or endothelium-denuded) the relaxant effect of GED (1 – 100 μM) was studied in rings precontracted with noradrenaline (100 nM) in the presence or absence of a 30 min pretreatment with methylene blue (100 μM), an inhibitor of guanylyl cyclase activation.

In addition, in endothelium-intact rings, the effect of 30 min pretreatment with various concentrations of GED (1 μM, 10 μM, 100 μM) was studied on the relaxations in response to acetylcholine (1 nM – 10 μM) in rings precontracted with noradrenaline (100 nM). Since, in this protocol, 100 μM GED caused a near-complete reduction of the contractility, the relaxant effect of acetylcholine could not be investigated. Therefore, in the presence of 100 μM GED, the concentration of noradrenaline used for precontraction was increased to 1 μM to achieve tone similar to that generated by 100 nM noradrenaline in control rings.

To investigate the effect of GED on the endotoxin-induced alterations in vascular reactivity, concentration-response curves to noradrenaline (10^{-9} – 10^{-5} M) were obtained in endothelium-denuded aortic rings taken from control rats or rats injected with LPS (with or without GED treatment) and killed 180 min later. In addition, relaxations to acetylcholine (1 nM – 10 μM) were tested in endothelium-intact rings obtained from the various groups of animals (control; control + GED, LPS, LPS + GED).

Survival studies

Swiss albino mice (26-30 g, Charles River Laboratories, Wilmington, MA, U.S.A.) were injected with *E. coli* LPS (120 mg kg^{-1}, i.p.); 2 h later and every 6 h thereafter, animals were treated with GED (10 mg kg^{-1}, i.p.) or with a vehicle control.

Materials

GED was prepared by the following route: To a solution of cystamine dihydrochloride (0.50 g, 2.2. mmol) in H_2O (25 ml) was added 10 ml of Amberlite IRA 402 (OH^-) resin followed by 1H-pyrazole-1-carboxamidine HCl (0.98 g, 6.6 mmol). After stirring the reaction mixture at room temperature for 16 h, the resin was removed by filtration and washed with H_2O (25 ml). The aqueous solution (filtrate) was extracted with EtOAc (4 × 50 ml) and the organic layer discarded. The aqueous layer was acidified with 2N HCl to pH 2 and lyophilized to afford 0.56 g (82%) of GED dihydrochloride as a white solid. GED was characterized by ^{1}H-n.m.r. and mass spectroscopy and was also homogeneous by thin layer chromatography.

DMEM, RPMI, Hite's Medium and foetal calf serum were from Gibco (Grand Island, NY, U.S.A.). N^G monomethyl-L-arginine monoacetate (L-NMMA) was from Calbiochem (La Jolla, CA, U.S.A.). Amberlite IRA 402 (OH^-) resin was from Rohm and Haas (Philadelphia, PA, U.S.A.). 1H-pyrazole-1-carboxamidine HCl was synthesized by the method of Bernatowitz *et al.* (1992). Sodium pentothal was obtained from Abbott Laboratories (Chicago, IL, U.S.A.). Murine IFN was from Genzyme (Cambridge, MA, U.S.A.). Murine TNF and IL-1 were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Tetrahydrobiopterin was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). S-aminoethyl-isothiourea was obtained from Aldrich (St. Louis, MO, U.S.A.). [2,3,4,5-^{3}H]-L-arginine hydrochloride was obtained from DuPont/NEN (Boston, MA, U.S.A.). Human recombinant IL-1β and IFN were kind gifts from Amgen (Thousand Oaks, CA, U.S.A.). Bacterial lipopolysaccharide (*E. coli*, serotype No. 0127:B8) and all other reagents were obtained from Sigma. (St. Louis, MO, U.S.A.).

Statistical evaluation

EC_{50} values were calculated by linear regression after logit-log transformation of concentration-response curves. For the evaluation of the NOS measurements, data were plotted in Dixon formats. All values in the figures and text are expressed as mean ± standard error of the mean (s.e.mean) of n observations. Student's unpaired t test was used to compare means between groups. For comparisons in the survival rate in the murine studies, the Chi-square test was used. A P value less than 0.05 was considered statistically significant.

Results

Effects of GED on the activity of the purified iNOS, ecNOS and bNOS enzymes: characterization of the inhibition

Data depicted in Figure 1 show the effect of GED on the L-arginine-dependent formation of L-citrulline by purified iNOS in Dixon format (i.e. 1/v versus GED concentration at a series of fixed L-arginine concentrations). The series of lines intersect at a common point above the x axis providing a K_i value of 4.3 μM for GED with a pattern of inhibition competitive versus L-arginine substrate.

The data depicted in Figure 2 represent an identical study employing affinity purified ecNOS in Dixon format, providing a K_i value of 18 μM. Thus GED is also a competitive inhibitor of ecNOS (versus L-arginine substrate), and has a 4 fold selectivity for iNOS over ecNOS. In addition, we found GED is an inhibitor of bNOS activity, with a K_i value of 25 μM (Table 1, data not shown).

The data depicted in Figure 3 show the effect of GED on the BH_4 concentration-dependence of iNOS. The data are plotted in the Dixon format and reveal a noncompetitive pattern of inhibition, i.e. the lines intersect at a common point on the x axis to give a K_i value of 4 μM, a value similar to that determined independently in the experiment depicted in Figure 1. Similarly, examination of the effect of GED on the BH_4 concentration-dependence of ecNOS demonstrates non-competitive pattern and a K_i value of 18 μM, identical to the value determined with L-arginine (Figure 4). Thus, while the inhibitory effect of GED on ecNOS and iNOS was competitive vs. L-arginine, the inhibition was non-competitive vs. BH_4.

The data depicted in Figure 5 were obtained from an independent assay of NOS activity, based on the reaction of NO with oxyhaemoglobin to form methaemoglobin. Initially, at 10 μM L-arginine substrate without added GED, the reaction rate is linear with time and exhibits an activity of 240 nmol mg^{-1} protein min^{-1}. At 100 s post-initiation, the incubation was adjusted to contain 50 μM GED. GED immediately reduced activity to 60 nmol mg^{-1} min^{-1} (i.e. by 75%). Cumulative product formation in the presence of GED continued at a diminished but linear rate. At 330 s post initiation, the concentration of L-arginine substrate was raised to 400 μM. The rate of NO formation immediately increased to 320 nmol mg^{-1} min^{-1} and sustained this linear rate of product formation. These data support the assertion that GED is a reversible, competitive antagonist of iNOS at the L-arginine site.

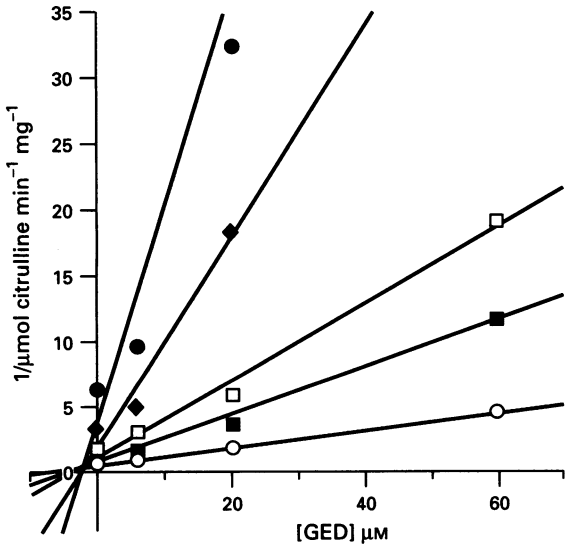

Figure 1 The effect of GED on the L-arginine concentration-dependence of L-citrulline formation by iNOS. Incubations were initiated with 2 μg of macrophage iNOS and L-citrulline formation was measured. Data are plotted in the Dixon format for incubations conducted in the presence of 300 μM BH_4 at 1 (●), 2 (◆), 5 (□), 10 (■), and 25 (○) μM L-arginine at the indicated concentrations of GED.

GED is a potent inhibitor of NO production in immunostimulated macrophages, smooth muscle cells, and epithelial cells

GED, when applied 6 h after immunostimulation, inhibited nitrite accumulation in J774 cells, RASM cells and MLE cells with EC_{50} values of 11, 9 and 1 μM, respectively (Figure 6a-c). Thus, GED was approximately 20–30 times more potent than L-NMMA in these cells, since EC_{50} values for L-NMMA were 150, 110 and 100 μM in the J774, RASM and MLE cells, respectively. The most potent inhibition of nitrite production was observed in the epithelial cells from the three murine cell types studied (Figure 6a-c). In contrast, in the human epithelial DLD-1 cells, GED appeared to be a weaker, whereas L-NMMA appeared to be a more potent inhibitor than in the rodent cell types. Thus, L-NMMA and GED were approximately equipotent (EC_{50} 30 μM) in inhibiting nitrite production in DLD-1 cells (Figure 6d), despite the fact that GED was 10

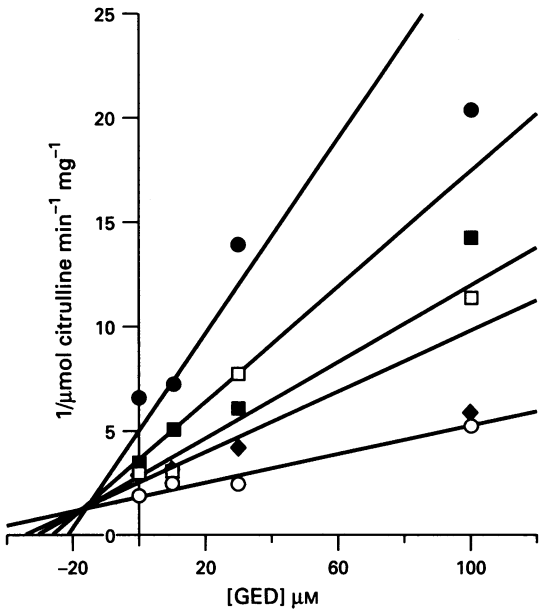

Figure 2 The effect of GED on the L-arginine concentration-dependence of citrulline formation by the ecNOS. Incubations were initiated with 0.5 μg of ecNOS and L-citrulline formation was measured. Data are plotted in the Dixon format for incubations conducted in the presence of 100 μM BH_4 at 2 (●), 5 (■), 6.7 (□), 10 (◆), and 25 (○) μM L-arginine at the indicated concentrations of GED.

Table 1 Inhibition of nitric oxide synthase isoforms by guanidinoethyldisulphide

NOS isoform	*Inhibition of citrulline formation* (IC_{50}, μM)	*Inhibition pattern versus* *Arginine*	*BH_4*
Macrophage iNOS	4.3	C	NC
Endothelial eNOS	18.0	C	NC
Neuronal bNOS	25.0	ND	ND

C – competitive; NC – noncompetitive; ND – not determined.

times more potent than L-NMMA on iNOS activity in homogenates of immunostimulated DLD-1 cells. In these crude homogenates (where iNOS activity was measured as described in Salzman *et al.*, 1996), GED (as well as MEG) exhibited an EC_{50} value of 20 μM ($n=3$) (the EC_{50} of L-NMMA was 200 μM in the same experimental system, $n=3$).

GED did not affect mitochondrial respiration at concentrations up to 10 μM in any of the cell types studied. At 100 μM, it caused a slight, but non-significant inhibition in the J774 cells (by $8\pm3\%$, $P>0.1$), an enhancement (by $20\pm2\%$, $P<0.01$) in the RASM cells, and a $9\pm1\%$ ($P<0.05$) inhibition in the MLE cells ($n=6-12$). In the DLD-1 cells, GED did not affect mitochondrial respiration at concentrations up to 1 mM ($n=6$).

In all the cell types studied, the potency of GED or L-NMMA remained unaltered when the agents were applied prior to immunostimulation, as opposed to 6 h later (not shown), indicating that these agents do not interfere with the process of iNOS induction.

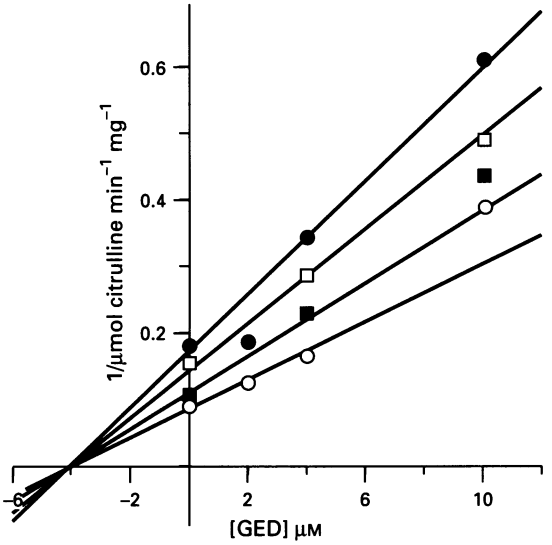

Figure 3 The effect of GED on the BH_4 concentration-dependence of L-citrulline formation by iNOS. Standard incubations containing 120 nM arginine were constructed with 0.3 (●), 1 (□), 3 (■), and 333 (○) μM BH_4 and the indicated concentrations of GED. Incubations were initiated with 0.8 μg of iNOS and L-citrulline formation was measured. Data are plotted in the Dixon format as the reciprocal of nmol L-citrulline formed $min^{-1}mg^{-1}$ at a series of fixed concentrations of BH_4.

Vascular effects of GED

GED caused a dose-dependent vasodilatation in precontracted vascular rings (Figure 7), with an EC_{50} value of approximately 20 μM. Removal of the endothelium attenuated, but did not abolish, the GED-induced relaxations (Figure 7). These observations support our previous findings demonstrating transient hypotensive effects of GED in anaesthetized rats (Southan *et al.*, 1996). The relaxant effect of GED was not affected by a 30 min pretreatment with methylene blue (100 μM), an inhibitor of guanylyl cyclase. Pretreatment with methylene blue abolished acetylcholine-induced relaxations (not shown). Thus, the endothelium-dependent component of the GED-induced relaxations does not appear to be related to activation of soluble guanylyl cyclase within the vascular smooth muscle.

In precontracted rings, at 1 μM, GED did not alter contractions to 100 nM noradrenaline (Figure 8a) and did not affect relaxant responses to acetylcholine (Figure 8b). At 10 μM, GED failed to affect contractions to noradrenaline (Figure 8a) but enhanced the acetylcholine-induced relaxations (Figure 8b), decreasing the EC_{50} for acetylcholine from 100 nM (control) to approximately 30 nM. Since virtually no contractions were achieved with 100 mM noradrenaline in the presence of 100 μM GED (Figure 8a), the concentration of the noradrenaline was increased to 1 μM to achieve a precontractile tone comparable to the control. Under these conditions, GED (at 100 μM) increased the EC_{50} of acetylcholine to approximately 500 nM, but did not inhibit the magnitude of the maximal relaxant response in response to 10 μM acetylcholine (Figure 8b).

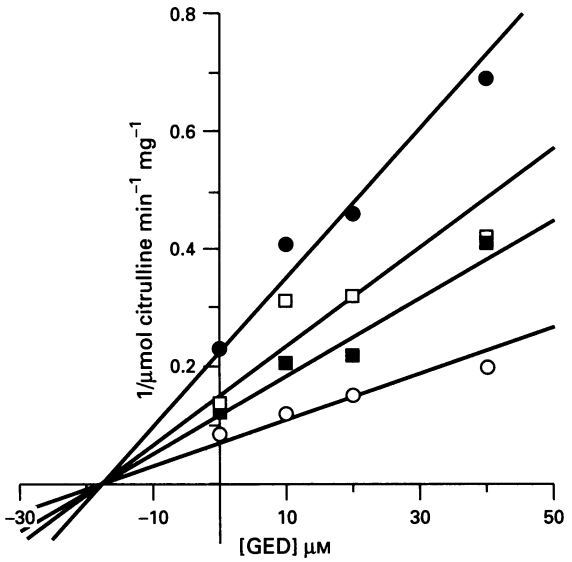

Figure 4 The effect of GED on the BH_4 concentration-dependence of citrulline formation by the endothelial nitric oxide synthase. Standard incubations containing 120 nM arginine were constructed with 0.3 (●), 1 (□), 10 (■), and 100 (○) μM BH_4 and the indicated concentrations of GED. Incubations were initiated with 0.3 μg of ecNOS and L-citrulline formation was measured. Data are plotted in the Dixon format as the reciprocal of nmol L-citrulline formed $min^{-1}mg^{-1}$ at a series of fixed concentrations of BH_4.

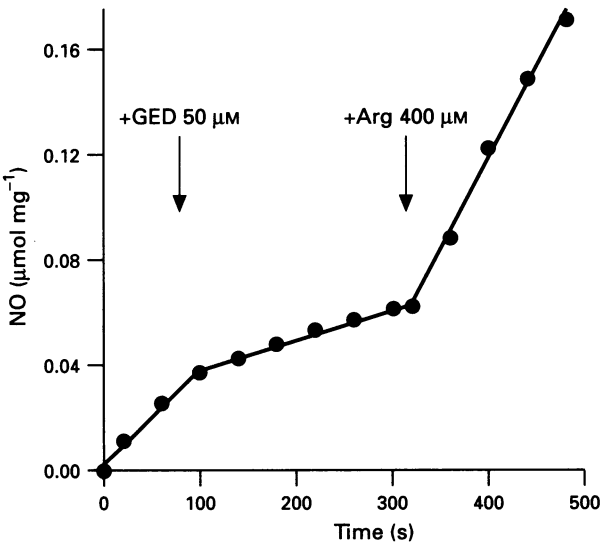

Figure 5 The effect of GED on the time-dependence of NO formation by iNOS. NO formation was measured by the conversion of HbO_2 to MetHb over time. Standard incubations contained 50 mM HEPES, pH 7.4, 1 mM EGTA, 100 μM NADPH, 100 μM BH_4, 10 μM arginine, and 15 μM HbO_2. Reactions were initiated at zero time by addition of 20 μg of affinity purified iNOS. At 100 s after the start the incubations were adjusted to contain 50 μM GED. At 320 s post initiation the incubations were adjusted to 400 μM arginine.

Effect of GED on blood pressure and renal cortical blood flow in anaesthetized rats

Administration of GED (0.1–30 mg kg^{-1}) starting 90 min after LPS (15 mg kg^{-1}) caused a biphasic change in blood pressure, with an initial hypotensive effect of 20–30 mmHg (see: Southan *et al.*, 1996), which returned to near-control levels within 2 min. The sustained MAP valued at 5 min after GED administration were not significantly different from the control MAP values, although at 10–30 mg kg^{-1} a tendency for hypotension was observed (Figure 9). In order to investigate, whether the lack of pressor effect was due to the development of tachyphylaxis, higher doses of GED were injected i.v. into separate groups of rats. Under these conditions,

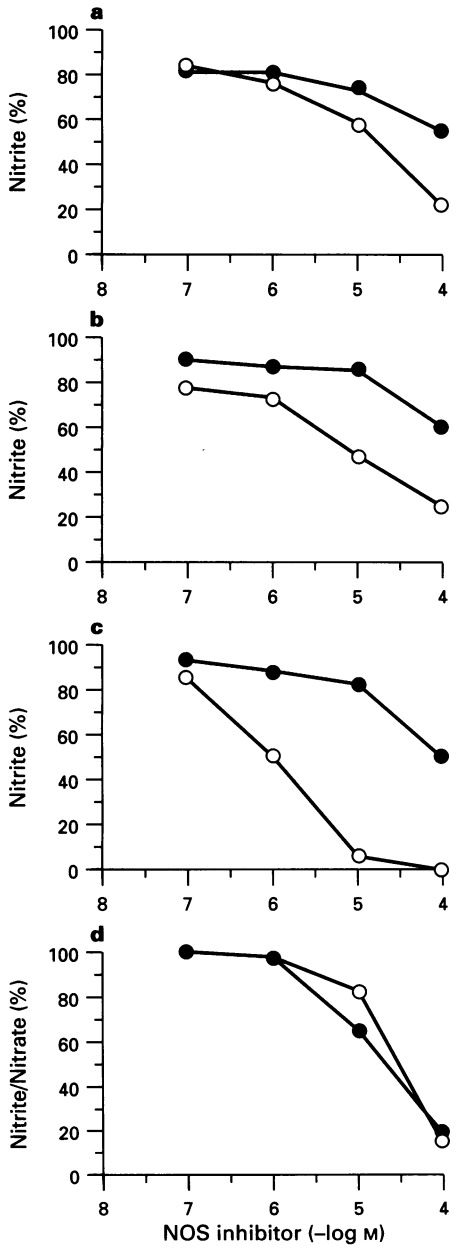

Figure 6 Effect of 0.1 μM–100 μM of GED (○) or L-NMMA (●) applied at 6 h after the stimulus of iNOS induction, on nitrite (a-c) or nitrite/nitrate (d) production of immunostimulated J774 cells (a), RASM cells (b), MLE cells (c) and DLD-1 cells (d). Error bars are contained within the symbols. Nitrite concentration in the wells of J774 cells was 89±7 μM, RASM cells: 84±6 μM; MLE cells: 3.6±0.1 μM and DLD-1 cells (nitrite/nitrate): 41±4 μM at 24 h after immunostimulation. Data represent means±s.e.mean from $n=6-12$ wells.

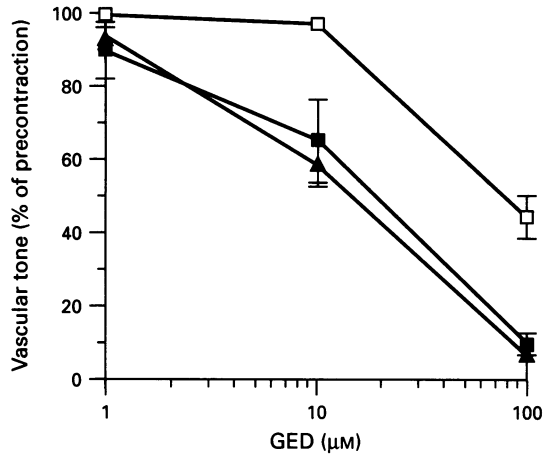

Figure 7 Effect of GED (1 μM–100 μM) on the vascular tone of precontracted rat thoracic aortic rings: (▲) relaxations in the presence of endothelium; (□) relaxations in the absence of endothelium; (■) relaxations after a 30 min pretreatment with methylene blue (100 μM) in rings in the presence of endothelium. Data represent means±s.e.mean from $n=6-8$ rings.

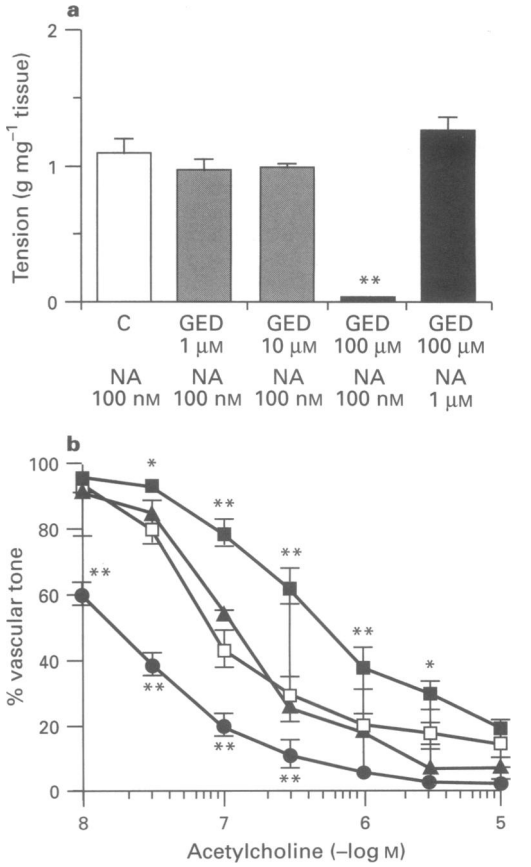

Figure 8 Effect of GED (1 μM–100 μM) on the contractions elicited by 100 nM noradrenaline (a) and on the relaxations elicited by acetylcholine (10 nM–10 μM) (b) in intact rat thoracic aortic rings in the presence of endothelium. Acetylcholine-induced relaxations were studied under control conditions (□), in the presence of 1 μM GED (▲), 10 μM GED (●) and 100 μM GED (■). As GED at 100 μM inhibited the contractions, the concentrations of noradrenaline was increased to 1 μM to induce a precontraction comparable to the tone produced by 100 nM noradrenaline in the absence of GED. *$P<0.05$; **$P<0.01$ represent significant difference from control. Data represent means±s.e.mean from $n=6-8$ rings.

10 or 30 mg kg^{-1} GED ($n=4$ each) caused hypotensive effects that were similar to those observed when cumulative dose-response curves were obtained (not shown).

GED caused a significant renal cortical vasoconstriction at 10–30 mg kg^{-1} (Figure 9). Administration of L-arginine (300 mg kg^{-1}) after 30 mg kg^{-1} GED reversed the renal vasoconstriction and caused a significant fall in MAP (Figure 9). L-NMMA, as expected, caused a dose-dependent increase in MAP and a decrease in renal cortical flow. This was significant even at 3 mg kg^{-1}, became pronounced at 10–30 mg kg^{-1} and was reversed by L-arginine (Figure 9).

Effect of GED on haemodynamic parameters, vascular changes and survival rate in rodent models of endotoxin shock

Administration of GED (3 mg kg^{-1} bolus and 10 mg kg^{-1} h^{-1} infusion) starting 90 min after LPS (15 mg kg^{-1}) prevented the delayed fall in mean arterial blood pressure (Figure 10), completely restored the vascular hyporeactivity to noradrenaline of the thoracic aorta *ex vivo* (Figure 11a), and provided partial protection against the impairment of the endothelium-dependent relaxations in a rat model of endotoxaemia (Figure 11b). The same bolus and infusion of the inhibitor did not alter MAP in normal animals over 90 min (control MAP: 117±7 mmHg, MAP at 90 min: 122±2 mmHg, $n=6$) and did not alter the *ex vivo* vascular reactivity (Figure 11). In agreement with our previous observations (Southan *et al.*, 1996), bolus injection of GED (3 mg kg^{-1}, i.v.) caused a transient hypotensive effect of approximately 30 mmHg which fully recovered over 5 min (not shown).

Intraperitoneal injection of LPS (120 mg kg^{-1}) into mice resulted in approximately 60% mortality over 42 h. Administration of GED (bolus injections 2 h after LPS and every 6 h thereafter) at the dose of 10 mg kg^{-1} resulted in a reduction of LPS-induced mortality (Figure 12) at 6–42 h after LPS ($P<0.05$).

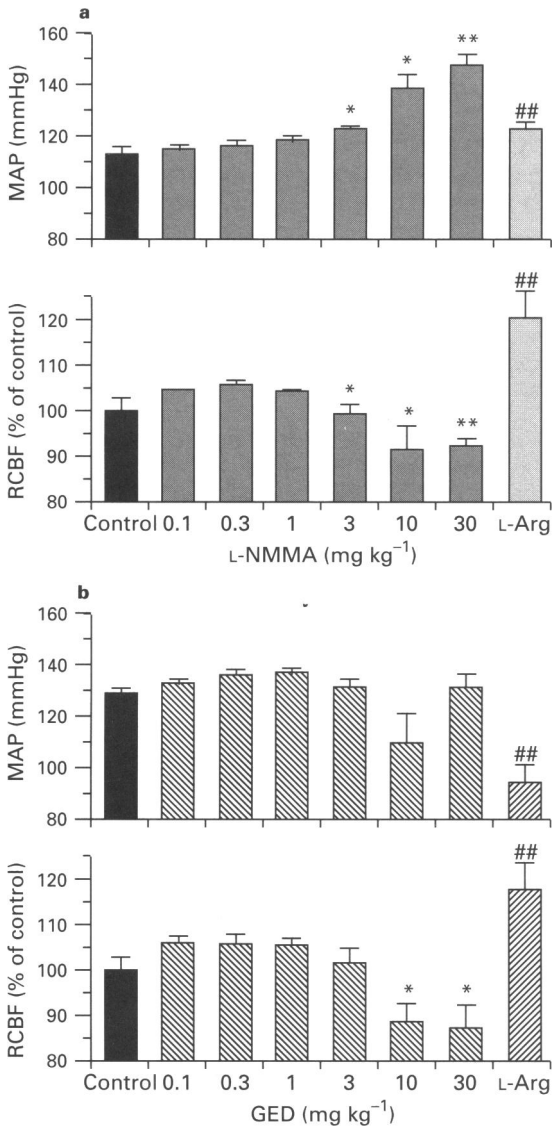

Figure 9 Effect of L-NMMA (a) or GED (b) (at 0.1–30 mg kg^{-1} bolus doses) on mean arterial blood pressure (MAP) and renal cortical blood flow (RCBF) in anaesthetized rats. Readings were taken at 4.5 min after the administration of each dose of the inhibitors. At the end of the study, L-arginine was injected at 300 mg kg^{-1} and readings were taken at 5 min afterwards. *$P<0.05$; **$P<0.01$ represent significant changes in MAP, ##$P<0.01$ represents significant change in MAP or RCBF in response to L-arginine. Data represent means±s.e.mean of 6 rats in each group.

Discussion

We have recently demonstrated that mercaptoalkylguanidines represent a novel class of NOS inhibitors, some of them with selectivity towards iNOS (Southan *et al.*, 1996). In particular, we have identified MEG and its 'dimer', GED, as potent NOS inhibitors in crude cell and tissue homogenates and in immunostimulated macrophages. Here we have characterized the pharmacological actions of GED.

GED was found to be a reversible, time-independent competitive antagonist of L-arginine at the substrate site of both iNOS and ecNOS, while L-NOARG is a slowly reversible (time-dependent) competitive antagonist of L-arginine. L-NMMA (isoform non-selective) and aminoguanidine (iNOS isoform-selective), by contrast, are alternate substrate, mechanism-based, irreversible inactivators of NOS that produce a time-dependent loss of enzyme activity (Olken & Marletta, 1993; Furfine *et al.*, 1993; Wolff & Lubeskie, 1995, 1996; Reif & McGreedy, 1995). Interestingly, the inhibition by GED of ecNOS and iNOS activity was found to be non-competitive versus BH_4. Only a few NOS inhibitors have been studied thus far with respect to their effects on the BH_4 dependence of NOS activity. The substrate analogue inhibitors (aminoacid and non-aminoacid) that bind to the L-arginine site appear to be noncompetitive inhibitors versus BH_4. By contrast 7-nitroindazole is competitive versus BH_4 and L-arginine substrate

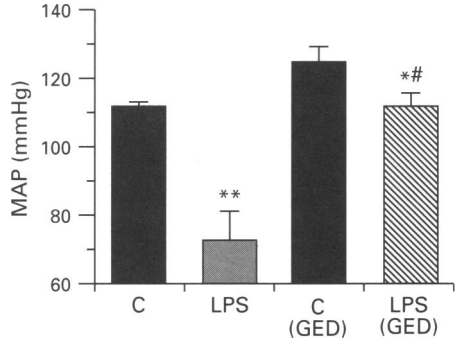

Figure 10 Effect of GED, 3 mg kg^{-1} bolus and 10 mg kg^{-1} h^{-1} infusion, starting at 90 min after LPS on the MAP at 180 min after LPS injection. 'C' represents MAP in the LPS treated group prior to the injection of LPS; 'LPS' represents MAP at 180 min after LPS injection in the LPS treated group. 'C(GED)' represents MAP in the LPS+GED-treated group prior to LPS injection and 'LPS(GED)' represents MAP at 180 min after LPS injection in the LPS+GED-treated group. *$P<0.05$; **$P<0.01$ represent significant fall in MAP at 180 min after LPS, #$P<0.05$ represents significant protection by GED against the fall in MAP at 180 min. Data represent means± s.e.mean of 6 rats in each group.

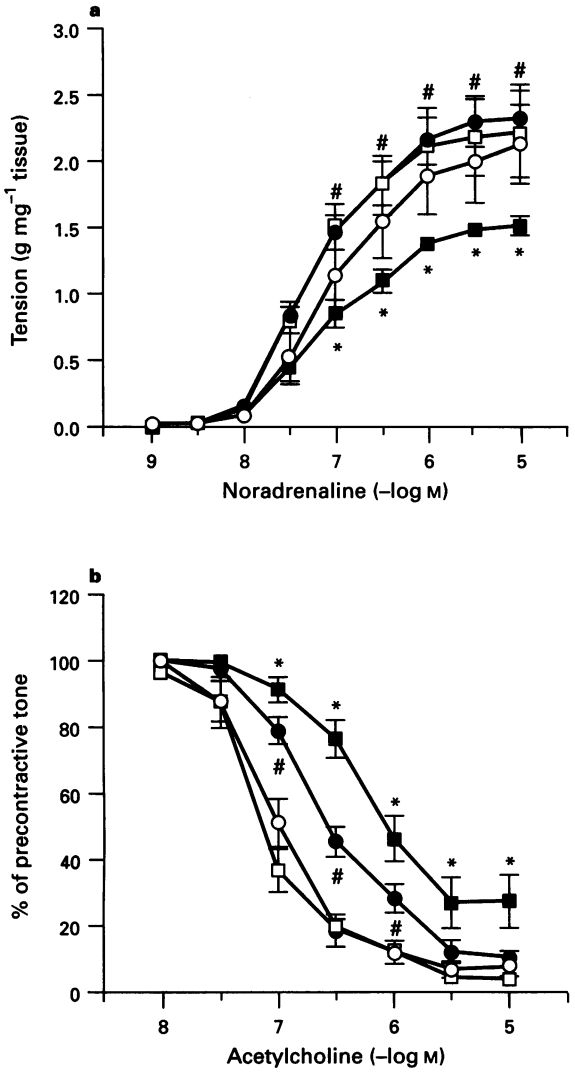

Figure 11 Effect of GED (3 mg kg^{-1} bolus and 10 mg kg^{-1} h^{-1} infusion for 90 min) on the *ex vivo* contractile responses to noradrenaline (1 nM – 10 μM) (a) and on the *ex vivo* relaxant responses to acetylcholine (10 nM – 10 μM) (b) of the thoracic aorta in control rats and in rats treated with LPS; (□) control responses without GED infusion; (○) responses after 90 min of GED infusion in control animals; (■) responses at 180 min after LPS injection and (●) responses at 180 min after LPS injection in animals in which GED (3 mg kg^{-1} bolus and 10 mg kg^{-1} h^{-1} infusion) had been infused at 90 – 180 min after LPS. Note that there was a reduction in contractility and relaxant responsiveness after LPS, which was ameliorated by GED. GED, however, did not alter contractile or relaxant responses in control rings. $n = 6-8$ rings; $^*P < 0.05$ represents significant reduction in the contractile or relaxant responses in LPS-treated rats; #significant improvement of the contractions or the relaxations by GED in the LPS-treated rats. Data represent means ± s.e.mean of 6 – 8 rings in each group.

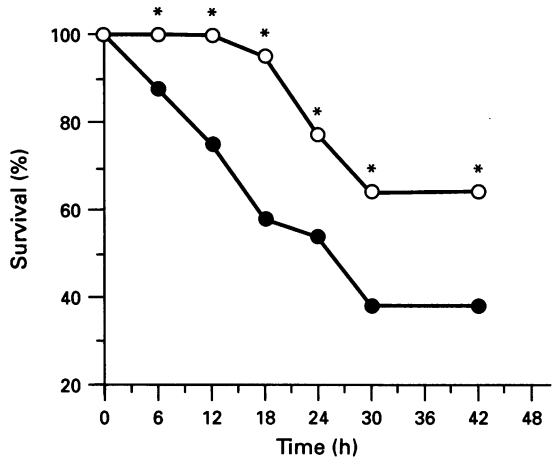

Figure 12 Effect of endotoxic shock (120 mg kg^{-1} LPS i.p. at time 0) on survival rate in vehicle-treated mice (●) and in mice treated with GED (10 mg kg^{-1} at 2 h after LPS and every 6 h thereafter, ○). $^*P < 0.05$ represents significant improvement in survival by GED. Data are expressed as % of initial survival rate (100%); $n = 24$ and 22 animals were used in the vehicle-treated and in the GED-treated groups, respectively.

(Klatt *et al.*, 1994). This may imply that the haeme group and the BH_4 site are very closely aligned at the 'active site' of NOS. Since bound L-arginine must be brought into proximity to the haeme-bound oxygen in order to be oxidized, it is possible that sterically large ligands bound to the L-arginine site might overlap the BH_4 site and act as competitors vs. the BH_4 co-factor.

We have found that GED has a 4 fold selectivity for iNOS over ecNOS in affinity purified NOS preparations. Thus, the selectivity of GED for iNOS on the enzyme level is similar to that of methylguanidine (EC_{50} values: 90 and 240 μM for iNOS and ecNOS), greater than that of L-NMMA (EC_{50} values: 2.6 and 0.7 μM for iNOS and ecNOS) or L-NOARG (EC_{50} values: 8 and 0.02 μM for iNOS and ecNOS), but less than that of aminoguanidine (EC_{50} values: 16 and >8000 μM for iNOS and ecNOS) (Wolff & Lubeskie, 1995; 1996; Reif & McGreedy, 1995).

The EC_{50} values for most of the NOS inhibitors tested were approximately 10 times lower in the present study than the ones previously reported in crude organ and cell homogenates (see Southan *et al.*, 1995b; 1996), although the relative orders of potencies are similar in most cases. One explanation for this difference is that in the present study, we conducted our kinetics at 120 nM L-arginine and hence there was minimal competition with L-arginine substrate. The IC_{50} values follow the Cheng-Prusoff relationship $IC_{50} = K_i\ (1 + [S]/K_m)$, and, therefore, our [S] is far below the K_m so that $IC_{50} = K_i$. The values provided in our present study are K_i values and hence are those obtained by extrapolation to infinitely dilute L-arginine. The experiments with the organ and cell homogenates were conducted at 10 μM L-arginine which raises the IC_{50} values.

The relatively high potency of GED on iNOS in the *in vitro* assays was in agreement with the findings in cultured cells. The finding that the inhibition was weaker (and similar to L-NMMA in potency) in the human DLD-1 cells may be due to poor uptake of non-arginine based NOS inhibitors by human cells. We did not observe marked differences in the inhibitory potencies of L-NMMA, L-NOARG, S-methyl-isothiourea, and aminoethylisothiourea (i.e. MEG) in immunostimulated DLD-1 cells (Salzman *et al.*, 1996), which contrasts with the findings in murine cells (e.g. Southan *et al.*, 1995b; 1996). Garvey *et al.* (1994) have also proposed that DLD-1 cells have poor uptake characteristics for isothiourea NOS inhibitors.

Differences in cellular uptake may be responsible for the relatively weak inhibitory effect of GED on ecNOS activity in the vascular ring preparations. In these preparations, inhibition started to occur at 100 μM. We even observed a slight enhancement of the endothelium-dependent relaxant responses at 10 μM GED (a dose which is near the K_i value of affinity purified ecNOS and inhibits nitrite formation by more than 50% in cultured murine cells). Thus, it appears that the selectivity of GED towards iNOS *in vivo* may be due to two components: (1) a modest selectivity on the enzyme level and (2) a preferential uptake into cells that express iNOS. A primary example of isoform-selective NOS inhibition due to differential cellular uptake is the inhibition of bNOS by 7-nitroindazole (Southan & Szabó, 1996).

GED, up to 3 mg kg^{-1} did not affect blood pressure or

renal cortical flow (suggestive of little interference with ecNOS activity *in vivo*), and, therefore, was selected for the endotoxin shock studies. However, the renal vasoconstriction at 10–30 mg kg^{-1} GED, and the hypotensive effect of L-arginine in the presence of higher concentrations of GED (30 mg kg^{-1}) suggest that at higher doses, GED inhibits ecNOS activity, which is, in part, masked by its direct vasodilator action. Inhibition of ecNOS, and the consequent vasoconstriction and decrease in end-organ perfusion may become a limiting factor in *in vivo* experiments using GED.

In addition to its effects on NOS, GED clearly has additional actions. Such actions include a transient hypotensive effect (Southan *et al.*, 1996) and endothelium-independent vasodilator action (as shown in this study). The mechanism of this action (and the mechanism of the enhancement of the acetylcholine-induced relaxations by 10 μM GED) requires further investigations, but, based on our data, does not appear to be related to cyclicGMP-dependent processes. A wide variety of amidines, guanidines, and isothioureas also possess direct smooth muscle dilator activities (Fastier & Smirk, 1947). The vasodilator action of GED may be a property of the disulphide compound, as the 'monomer' MEG does not relax precontracted rat aortic rings (Southan *et al.*, 1996).

In endotoxin shock, typical changes in vascular reactivity include a loss of vascular smooth muscle contractility and loss of endothelium-dependent relaxant responsiveness. The vascular hyporeactivity is due to expression of iNOS within the vascular smooth muscle cells (Szabó, 1995). The impairment of the endothelium-dependent relaxations may be related to the expression of iNOS in the endothelial cells and a consequent 'isoform-switch', i.e. inhibition of ecNOS expression or activity by high levels of NO produced by iNOS (Walter *et al.*, 1994). An additional trigger leading to the impairment of the endothelium-dependent relaxations may be the generation of peroxynitrite (from superoxide and NO) in the vicinity of the endothelium (Villa *et al.*, 1994; Szabó *et al.*, 1995; 1996). The present data with GED support the view that iNOS contributes to the pathogenesis of both vascular hyporeactivity and endothelial dysfunction in endotoxin shock. The improvement of the endothelium-dependent relaxations by GED in endotoxaemia is unlikely to be due to the intrinsic relaxant effect of GED (seen at higher concentrations *in vitro*), inasmuch as we did not observe any enhancement of the relaxations in control rats (without LPS) after infusion of the same dose of GED.

In addition to the protective vascular actions, as expected from studies with other iNOS-selective inhibitors (Szabó *et al.*, 1994; Wu *et al.*, 1995), GED also prevented the delayed fall in MAP and the delayed mortality associated with endotoxin shock. The cumulative dose of GED in the mortality studies was 60 mg kg^{-1} over 42 h. According to previous data, GED has an LD_{50} of approximately 300–400 mg kg^{-1} (i.p.) (Doherty *et al.*, 1957; Schwartz & Shapiro, 1960).

There is a substantial amount of literature on the pharmacology and metabolism of mercaptoalkylguanidines, including MEG and GED, as these agents have been previously described as radioprotective agents (Doherty *et al.*, 1957; Schwartz & Shapiro, 1960; Kollmann *et al.*, 1963; Shapiro *et al.*, 1963; Kozak & Arient, 1973). The radioprotective effects may reside at the level of oxyradical scavenging properties of the free thiol groups. Many radioprotective agents that contain free thiols are not inhibitors of NOS (Southan & Szabó, unpublished data).

The possibility that GED, in addition to its potent inhibitory effect on NOS, may have additional beneficial pharmacological effects, which contribute to its beneficial effects in shock, requires further investigations. One of these effects may be the direct vasodilator effect of the compound. The potential scavenging effect by GED (or MEG) of NO, superoxide or peroxynitrite should be the subject of future investigations.

The questions arise as to whether MEG or GED is predominant *in vivo* and whether MEG or GED is the active NOS inhibitory molecule. Previous studies have demonstrated the formation of MEG in animals treated with GED and the formation of GED in animals treated with MEG or AETU (Kollmann *et al.*, 1963; Kozak & Arient, 1973). We have previously demonstrated that S-ethyl- and S-methyl-MEG are potent inhibitors of iNOS activity and these compounds are not susceptible to oxidation of the thiol group. Thus, we believe that dimerisation is not an absolute requirement for iNOS inhibition by mercaptoalkylguanidines (Southan *et al.*, 1996). Nonetheless, administration of GED to animals may have advantages over the administration of AETU or MEG, for the following reasons: (1) AETU spontaneously rearranges into MEG as well as small amounts of 2-aminothiazoline, a non-isoform selective potent NOS inhibitor (Southan *et al.*, 1996); (2) partial, spontaneous dimerisation to GED may occur during the preparation and storage of pure MEG; (3) the studies in J774 cells suggest that GED may have a preferential cellular uptake over MEG (Southan *et al.*, 1996).

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