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# Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: a possible role for protein tyrosine kinase

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**1** Here we compared the effects of various inhibitors of the activity of protein tyrosine kinase on (i) the expression of the activity of the inducible isoform of nitric oxide (NO) synthase (iNOS) caused by endotoxin (lipopolysaccharide, LPS) in cultured macrophages, (ii) the induction of iNOS and cyclo-oxygenase 2 (COX-2) protein and activity in rats with endotoxaemia, and (iii) the circulatory failure and organ dysfunction caused by LPS in the anaesthetized rat.

**2** Activation of murine cultured macrophages with LPS (1 $\mu$g ml$^{-1}$) resulted, within 24 h, in a significant increase in nitrite (an indicator of the formation of NO) in the cell supernatant. This increase in nitrite was attenuated by the tyrphostins AG126, AG556, AG490 or AG1641 or by genistein in a dose-dependent fashion ($IC_{50}$: $\sim$15 $\mu$M). In contrast, tyrphostin A1 (an analogue of tyrphostin AG126) or daidzein (an analogue of genistein) had no effect on the rise in nitrite caused by LPS.

**3** Administration of LPS (*E. coli*, 10 mg kg$^{-1}$, i.v.) caused hypotension and a reduction of the pressor responses elicited by noradrenaline (NA, 1 $\mu$g kg$^{-1}$, i.v.). Pretreatment of rats with the tyrphostins AG126, AG490, AG556, AG1641 or A1 attenuated the circulatory failure caused by LPS. Although genistein attenuated the vascular hyporeactivity to NA, it did not affect the hypotension caused by LPS. Daidzein did not affect the circulatory failure caused by LPS.

**4** Endotoxaemia for 360 min resulted in rises in the serum levels of (i) urea and creatinine (indicators of renal failure), (ii) alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and $\gamma$-glutamyl transferase ($\gamma$GT) (indicators of liver injury/dysfunction), lipase (an indicator of pancreatic injury) as well as lactate (an indicator of tissue hypoxia). None of the tyrosine kinase inhibitors tested had a significant effect on the rise in the serum levels of urea, but the tyrphostins AG126, AG556 or A1 significantly attenuated the rises in the serum levels of creatinine caused by LPS. In addition, all tyrphostins and genistein attenuated the liver injury/failure, the pancreatic injury, the hypoglycaemia and the lactic acidosis caused by LPS. In contrast, daidzein did not reduce the organ injury/dysfunction or the lactic acidosis caused by LPS.

**5** Injection of LPS resulted (within 90 min) in a substantial increase in the serum level of tumour necrosis factor $\alpha$ (TNF$\alpha$), which was attenuated by pretreatment of LPS-rats with any of the tyrphostins used. Genistein, but not daidzein, also reduced the rise in the serum levels of TNF$\alpha$ caused by LPS. Endotoxaemia for 6 h also resulted in a substantial increase in the expression of iNOS and COX-2 protein and activity in the lung, which was attenuated by pretreatment of LPS-rats with the tyrphostins AG126, AG556 or genistein, but not by daidzein.

**6** Thus, tyrphostins (AG126, AG490, AG556, AG1641 or A1) and genistein, but not daidzein (inactive analogue of genistein), prevent the (i) circulatory failure, (ii) the multiple organ dysfunction (liver and pancreatic dysfunction/injury, lactacidosis, hypoglycaemia), as well as (iii) the induction of iNOS and COX-2 protein and activity in rats with endotoxic shock

**Keywords:** Nitric oxide; tyrosine kinase; tyrphostin; genistein; endotoxin shock; liver injury; multiple organ failure; cyclo-oxygenase

## Introduction

Phosphorylation of proteins on tyrosine residues by protein tyrosine kinases plays an important role in the regulation of cell proliferation, cell differentiation and signalling processes in cells of the immune system. The receptor tyrosine kinases participate in transmembrane signalling, whereas the intracellular tyrosine kinases take part in the signal transduction to the nucleus. Enhanced activity of tyrosine kinases has been implicated in the pathophysiology of many diseases associated with local (atherosclerosis, psoriasis) or systemic inflammation, including sepsis and septic shock (see Levetzki & Gazit, 1995 for review).

Endotoxin (lipopolysaccharide, LPS) causes the phosphorylation of tyrosine kinases in macrophages (and other target cells) (Weinstein *et al*., 1991), resulting in the release of proinflammatory cytokines including tumour necrosis factor $\alpha$ (TNF$\alpha$), interleukin-1 (IL-1) and interferon-$\gamma$. In human monocytes activated with LPS, inhibition of tyrosine kinase activity with genistein or herbimycin A attenuates the expression of mRNAs for IL-1, TNF$\alpha$ and IL-6 (Geng *et al*., 1993). TNF$\alpha$ and IL-1 also induce the phosphorylation of tyrosine in target cells (Evans *et al*., 1990; Kohno *et al*., 1990; Guy *et al*., 1991; Munoz *et al*., 1992) and when given to animals mediate many of the effects of LPS (see Dinarello, 1996). Inhibition of the activity of tyrosine kinases by tyrphostin AG126 prevents (when given 2 h before LPS) the mortality caused by LPS in mice, but is less effective when given together with LPS (Novogrodsky *et al*., 1994). Tyrphostin AG556, which is more lipophilic than AG126, prevents the mortality caused by endotoxin in mice when given as late as 2 h after injection of endotoxin (Vanichkin *et al*., 1996). The mechanism(s) of these beneficial effects of tyrosine kinase inhibitors in shock is largely unknown. There is now good evidence that an enhanced for-

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mation of nitric oxide (NO) by the inducible isoform of NO synthase (iNOS) contributes to the circulatory failure (hypotension and vascular hyporeactivity to vasopressor agents), multiple organ dysfunction (e.g. liver injury/failure) and death caused by endotoxin in rodents (see Szabo *et al*., 1994; Thiemermann *et al*., 1995). The expression of iNOS caused by wall fragments of gram-negative (endotoxin) or gram-positive (lipoteichoic acid, peptidoglycan) bacteria in cultured cells (e.g. macrophages) involves the phosphorylation of tyrosine residues in proteins, and is prevented by the tyrosine kinase inhibitors genistein, erbstatin and tyrphostin AG126 (Dong *et al*., 1993; Marczin *et al*., 1993; Akarasereenont *et al*., 1995; Kengatheran *et al*., 1996; Salzman *et al*., 1996). In addition to iNOS, activation of tyrosine kinases also mediates the expression of the inducible isoform of cyclo-oxygenase (COX-2) caused by LPS in murine cultured macrophages (Akarasereenont *et al*., 1995) or by IL-1 in a human epithelial cell line (A549; Akarasereenont & Thiemermann, 1996). Induction of COX-2 result in an enhanced formation of metabolites of arachidonic acid (e.g., prostacyclin), and elevated plasma levels of certain prostaglandins have been documented in animals and man with septic shock (see Feuerstein & Hallenbeck, 1987).

Here we investigated the effects of various chemically distinct inhibitors of the activity of protein tyrosine kinase, which inhibit this enzyme by different mechanisms, on (i) the expression of iNOS activity caused by LPS in cultured macrophages, (ii) the induction of iNOS and COX-2 protein and activity in rats with endotoxaemia, and (iii) the circulatory failure (hypotension and vascular hyporeactivity to noradrenaline) and organ dysfunction (liver, kidney, pancreas) caused by LPS in the anaesthetized rat, and (iv) the rise in the plasma levels of TNF$\alpha$ and lactate (an indicator of tissue hypoxia) as well as the hypoglycaemia caused by LPS in the rat. The following tyrosine kinase inhibitors were used in this study: genistein (Ullrich & Schlessinger, 1990; Marscin *et al*., 1993), tyrphostin AG126 (Novogrodzky *et al*., 1994), tyrphostin AG556 (Kaur *et al*., 1994; Vanichkin *et al*., 1996), tyrphostin AG490 (Levitzki & Gazit, 1995) and tyrphostin AG1641 (Levitzki & Gazit, 1995). Genistein inhibits a wide range of tyrosine kinases, as this isoflavone acts as a competitive inhibitor of the binding of ATP and as a non-competitive inhibitor of the substrate binding to protein tyrosine kinases (Ullrich & Schlessinger, 1990). The tyrphostins on the other hand act as competitive inhibitors of the substrate binding to protein tyrosine kinases (Levitzki & Gazit, 1995). In addition, we have evaluated the effects of (i) daidzein, an isoflavone-analogue of genistein, which does not inhibit tyrosine kinase activity, and (ii) tyrphostin A1, a tyrphostin which is only a very weak inhibitor of tyrosine kinase activity *in vitro* (Negrescu *et al*., 1995; Levitzki & Gazit, 1995).

## Methods

### *Induction of nitrite formation by endotoxin in cultured macrophages*

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum (Szabo *et al*., 1993). Cells were cultured in 96-well plates with 200 $\mu$l culture medium until they reached confluence. To induce iNOS in macrophages, fresh culture medium containing *Escherichia coli* lipopolysaccharide (LPS, 1 $\mu$g ml$^{-1}$, serotyp: 0127:B8) was added. Nitrite accumulation in the cell culture medium was measured after 24 h. To assess the effects of tyrphostins AG126, AG490, AG556, AG1641 or A1 as well as of genistein and its inactive analogue daidzein (all 1–100 $\mu$M) on the formation or nitrite, these compounds were added to the cells 30 min before LPS. The amount of nitrite, an indicator of NO synthesis, in the supernatant of J774.2 was measured by the Griess reaction (Green *et al*., 1981) by adding 100 $\mu$l of Griess reagent to 100 $\mu$l samples of unfiltered serum or supernatant. The optical density at 550 nm ($OD_{550}$) was measured by a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculated by comparison with $OD_{550}$ of standard solution of sodium nitrite prepared in control serum or culture medium. 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 (Mosmann 1983). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml$^{-1}$ for 60 min). Culture medium was removed by aspiration and cells solubilized in dimethyl sulphoxide by measurement of $OD_{550}$ with a Molecular Devices microplate reader (Richmond, CA., U.S.A.). Formazan production was expressed as a percentage of the values obtained from untreated cells.

### *Measurement of haemodynamic changes*

This study was carried out on 68 male rats (Wistar, Glaxo Laboratories Ltd., Greenford, Middlesex, U.K.) weighing 240–320 g receiving a standard diet and water *ad libitum*. All rats were anaesthetized with thiopentone sodium (Trapanel; 120 mg kg$^{-1}$, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Statham, Oxnard, CA., U.S.A.) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA., U.S.A.). The femoral vein and jugular vein were cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 15 min. After baseline haemodynamic parameters had been recorded, animals were given noradrenaline (NA, 1 $\mu$g kg$^{-1}$, i.v.) and 10 min later animals received vehicle for LPS (1 ml kg$^{-1}$, i.v., saline) or *E. coli* lipopolysaccharide (LPS, 10 mg kg$^{-1}$, i.v. in 0.3 ml of saline) as a slow injection over 10 min. The pressor responses to NA were reassessed at 1 h, 3 h and 6 h after LPS injection. At 6 h after LPS, blood was taken to measure the changes in the serum levels of various biochemical marker enzymes of multiple organ dysfunction/failure (see below). Animals were divided into 12 different groups. The dose regimen for each individual animal group as well as the number of animals studied are provided in Table 1.

### *Quantification of liver, renal, or pancreatic injury*

At 6 h after the injection of LPS, 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from a catheter placed in the carotid artery. The blood sample was centrifuged (6,000 r.p.m. for 3 min) to separate serum. All serum samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, U.K.). The following marker enzymes were measured in the serum as biochemical indicators of multiple organ dysfunction/failure: (1) liver dysfunction and failure were assessed by measuring the rises in serum levels of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury); aspartate aminotransferase (AST, a non-specific marker for hepatic injury), $\gamma$-glutamyl transferase ($\gamma$GT; a non-specific indicator of hepatic injury) and bilirubin (an indicator of hepatic excretory function and predictor of the development of liver failure (Baue, 1993; Hewett *et al*., 1993). (2) Renal dysfunction and failure were assessed by measuring the rises in serum levels of creatinine (an indicator of reduced glomerular filtration rate, and hence, failure) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism). (3) Pancreatic injury was assessed by measuring the rises in the serum levels of lipase (Thiemermann *et al*., 1995; Ruetten *et al*, 1996).

**Table 1** Animal groups

| *Treatment* | n | *Dose* |
|---|---|---|
| Vehicle + vehicle | 4 | DMSO/PBS (1 ml $kg^{-1}$, i.p.) + saline (1 ml $kg^{-1}$, i.v.) |
| Vehicle + LPS | 12 | DMSO/PBS (1 ml $kg^{-1}$, i.p.) + 10 mg $kg^{-1}$, i.v. |
| Tyrphostin AG126 + vehicle | 4 | 5 mg $kg^{-1}$, i.p. 2 h before saline (1 mg $kg^{-1}$, i.v.) |
| Tyrphostin AG126 + LPS | 5 | 3 mg $kg^{-1}$, i.p. 2 h before LPS |
| Tyrphostin AG126 + LPS | 6 | 5 mg $kg^{-1}$, i.p. 2 h before LPS |
| LPS + tyrphostin AG126 | 4 | 5 mg $kg^{-1}$, i.p. 2 h before LPS |
| Tyrphostin AG490 + LPS | 5 | 5 mg $kg^{-1}$, i.p. 2 h before LPS |
| Tyrphostin AG556 + LPS | 6 | 5 mg $kg^{-1}$, i.p. 2 h before LPS |
| Tyrphostin AG1641 + LPS | 6 | 5 mg $kg^{-1}$, i.p. 2 h before LPS |
| Tyrphostin A1 + LPS | 5 | 5 mg $kg^{-1}$, i.p. 2 h before LPS |
| Genistein + LPS | 6 | 10 mg $kg^{-1}$, i.p. 2 h before LPS |
| Daidzein + LPS | 5 | 10 mg $kg^{-1}$, i.p. 2 h before LPS |

### *Measurement of glucose and lactate*

Glucose and lactate concentrations were measured spectrophotometrically (Glucose and Lactate Kit, Sigma Chemical Co., Poole, Dorset, UK) according to the protocol provided by the manufacturer.

### *Measurement of nitric oxide synthase activity*

NOS activity was measured as the ability of tissue homogenates to convert L-[$^3$H]-arginine to L-[$^3$H]-citrulline (Szabo *et al*., 1993). Lungs and livers were removed at 6 h after LPS and frozen in liquid nitrogen. Frozen organs were homogenized on ice with an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen i. Br., Germany) in a buffer composed of: Tris-HCl 50 mM, EDTA 0.1 mM, EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethylsulphonyl fluoride 1 mM (pH 7.4). Briefly, tissue homogenetes (30 $\mu$l, approx. 60 $\mu$g protein) were incubated in the presence of [$^3$H]-L-arginine (10 $\mu$M, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 $\mu$M) and calcium (2 mM) for 25 min at 25°C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 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 activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA., U.S.A.). Experiments performed in the absence of NADPH determined the extent of [$^3$H]-L-citrulline formation independent of a specific NOS activity. Experiments in the presence of NADPH, without calcium and with 5 mM EGTA, measured the calcium-independent iNOS activity, which was taken to represent iNOS activity. Protein concentration was measured spectrophotometrically in 96-well plates with Bradford reagent (Bradford, 1976), with bovine serum albumin as standard.

### *Western (immuno) blot analysis*

Lungs and livers were homogenized on ice in an extraction buffer (pH = 7.4) as previously described (De Kimpe *et al*., 1995; Bryant *et al*., 1995). The homogenates were centrifuged (500 *g*) for 15 min at 4°C and the supernatant was boiled for 10 min with gel-loading buffer in a ratio of 1:1 (v/v). Protein concentrations of the supernatants were determined as above, and total protein equivalents for each sample were separated by one dimensional gel electrophoresis (7.5% SDS gel for iNOS and 10% SDS gel for COX-2) with molecular weight markers (SDS-7B; Sigma). After transfer to nitrocellulose by electrophoresis, nonspecific IgGs were blocked with 5% dried milk protein and incubated with specific antibodies to COX-2 (Cayman Chemicals, Ann Arbor, MI., U.S.A.) or iNOS (a gift from Dr C. Bryant) used at a concentration of 1:5000. The blots were then incubated with a horseradish peroxidase-conjugated secondary antibody and developed by means of an enhanced horseradish peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents, Amersham International plc. Buckinghamshire, U.K.) and exposed to X-ray film for 30–60 s. Subsequently, the relative expression of iNOS or COX-2 protein in each tissue was quantified by densitometric scanning of the Western blots with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst Macintosh).

### *Measurement of the serum concentrations of tumour necrosis factor α*

The content of TNFα in serum samples (50 $\mu$l) was determined by ELISA (Mouse TNF-α ELISA kit, Genzyme, Cambridge, MA., U.S.A.) in 96-well plates. Binding was detected by a peroxidase-conjugated polyclonel anti-mouse TNFα antibody with tetramethylbenzidine as a substrate. Following acidification (sulphuric acid, 0.5 M final) the absorbance of each well was measured at 450 nm by a Molecular microplate reader (Anthos Labtec Instruments, Richmond, CA., U.S.A.).

### *Measurement of 6-keto $PGF_{1\alpha}$*

At 6 h after the injection of LPS, 0.5 ml of blood was collected from the catheter placed in the carotid artery. The blood sample was centrifuged (10,000 r.p.m. for 3 min) to prepare serum. The concentration of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) present in the serum was measured by radioimmunoassay (Salmon, 1978).

### *Materials*

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotyp 0.127:B8), NADPH, noradrenaline bitartrate, sulphanilamide, naphtyleneethylenediamide, prostaglandin H synthase 2 (cyclo-oxygenase), phosphoric acid, sulphuric acid, L-glutamine, foetal calf serum, Bradford reagent, bovine serum albumin, Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Tyrphostins AG126 ((3-hydroxy-4-nitrobenzylidine)malononitrile or α-cyano-(3-hydroxy-nitro)cinnamonitrile), tyrphostins AG556 (N-(4-phenylbutyl)-3,4-dihydroxybenzylidene cyanoacetamide or α-cyano-(3,4-dihydroxy)-N-(4-phenylbutyl) cinnamamide), tyrphostin AG490 (N-benzyl-3,4-dihydroxy-benzylidene cyanoacetamide or α-cyano-(3,4-dihydroxy)-N-benzylcinnamamide), tyrphostin A1 ((4-methoxybenzylidene) malonitrile or α-cyano-(4-methoxy) cinnamonitrile) as well as genistein (4,5,7-trihydroxyisoflavone) and daidzein (4,7-dihydroxyisoflavone) were from Calbiochem (Nottingham, U.K.). Tyrphostin AG1641 was a generous gift from Prof. A. Levetzki (Dept. of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel). L-[2,3,4,5-$^3$H]-arginine hydrochloride and radiolabelled 6-keto $PGF_{1\alpha}$ were obtained from Amersham (Buckinghamshire, U.K.). Tetrahydrobiopterin (6**R**-L-erythro-5,6,7,8-tetra-hydrobiopterin) was obtained from Dr B. Schirks Laboratories (Jona, Switzerland) and sodium thiopentone (intraval Sodium) was obtained from Rhone Merieux Ltd. (Harlow, Essex, U.K.).

### *Statistical analysis*

A one-way analysis of variance (ANOVA) followed by, if appropriate, a Dunnett's *post hoc* test was used to compare means between groups (*in vivo* study). Student's unpaired *t* test was used to compare means between groups (*in vitro* study). A $P$ value $<0.05$ was taken as significant.

## Results

### *Effects of inhibitors of protein tyrosine kinase on the formation of nitrite by cultured macrophages activated with endotoxin*

Activation of J774.2 macrophages with endotoxin resulted within 24 h in a significant increase in nitrite in the cell supernatant from $1.1 \pm 0.4$ $\mu$M (control) to $43 \pm 3$ $\mu$M ($n=12$). The tyrphostins AG126, AG490, AG556 or AG1641 or the isoflavone genistein caused dose-dependent inhibitions of the formation of nitrite elicited by LPS ($IC_{50}$: $\sim 15$ $\mu$M) (Figure 1). In contrast, A1 (analogue of tyrphostin AG126) or daidzein (analogue of genistein), did not prevent the increase in nitrite formation in the supernatant of macrophages activated with LPS (Figure 1). Incubation of the cells with LPS alone caused a small ($\sim 20\%$) reduction in cell viability. Incubation of the cells with the drugs used (but without LPS) did not result in a significant reduction in cell viability. None of the drugs used attenuated the reduction in cell viability caused by LPS (data not shown).

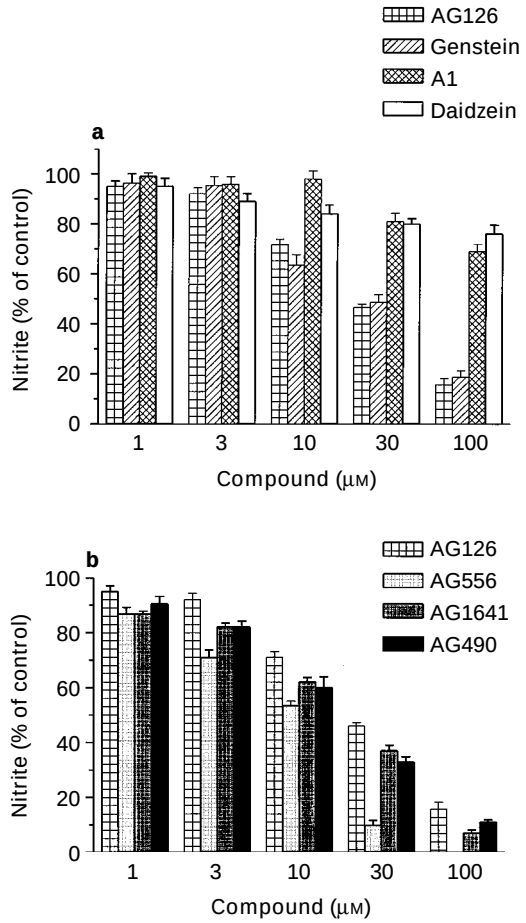


**Figure 1** Effect of (a) tyrphostin AG126, genistein, tyrphostin A1, daidzein, or (b) tyrphostin AG126, tyrphostin AG556, tyrphostin AG1641 or tyrphostin AG490 on the increase in the concentration of nitrite in the supernatant of cultured J774.2 macrophages activated with LPS for 24 h. Data are expressed as mean $\pm$ s.e.mean from triplicate determinations (well) from 4 separate experimental days ($n=12$). Nitrite concentration in cultured medium of J774.2 cells activated with LPS at 24 h was $43 \pm 3$ $\mu$M.

### *Effects of inhibitors of tyrosine kinase on the circulatory failure caused by LPS in the anaesthetized rat*

Baseline values for MAP and heart rate of the animal groups studied ranged from $116 \pm 4$ mmHg to $123 \pm 3$ mmHg (Table 2) and from $357 \pm 13$ to $386 \pm 11$ beats $min^{-1}$ (data not shown), respectively, and were not significantly different between groups. Administration of LPS (10 mg $kg^{-1}$) caused a rapid (within 15 min), but transient fall in MAP which had partly recovered by 120 min. After 180 min, there was a second, further fall in MAP from $103 \pm 4$ mmHg to $75 \pm 4$ mmHg at 360 min (Figure 2a). This delayed, but not the early fall in MAP, was abolished by pretreatment of rats with AG126 (Figure 2a, and b), AG556 (Figure 2b), AG490, AG1641 or A1 (Table 2), but not by genistein (Figure 2b) or the lower dose of AG126 (3 mg $kg^{-1}$) (Table 2). The hypotension caused by LPS was not affected by administration of tyrphostin AG126 (5 mg $kg^{-1}$) at 2 h after LPS (late administration) or by daidzein (Figure 2a). The mean baseline values for the pressor responses to NA (1 $\mu$g $kg^{-1}$, i.v.) ranged from $39 \pm 4$ to $43 \pm 4$ mmHg and were not significantly different between any of the experimental groups studied. Injection of LPS resulted, within 360 min, in a more than 50% reduction in the pressor response elicited by NA (Figure 2c, Table 2). This vascular hyporeactivity to NA was attenuated by the tyrphostins AG126 (3 or 5 mg $kg^{-1}$, Figure 2c and Table 2), AG556 (Figure 2c), AG490, AG1641 or A1 (Table 2) or by the isoflavine genistein (Figure 2c). However, the vascular hyporeactivity to NA caused by LPS was not affected by administration of tyrphostin AG126 (5 mg $kg^{-1}$) at 2 h after LPS (late administration) or by daidzein (Figure 2c). The administration of AG126 (5 mg $kg^{-1}$, i.p., 2 h before LPS) to rats which had received saline rather than LPS had no significant effect on any of the haemodynamic parameters studied ($n=4$, Table 2).

### *Effects of tyrosine kinase inhibitors on the rise in the serum levels of tumour necrosis factor α elicited by LPS in the rat*

Injection of LPS resulted in an increase in the serum level of TNFα from below the detection limit (35 pg $ml^{-1}$) to $5658 \pm 404$ (pg $ml^{-1}$) at 90 min (Figure 3, Table 2). The tyrphostins AG126, AG556 (Figure 3), AG490, AG1641 and A1 (Table 2) attenuated the rise in the serum level of TNFα caused

**Table 2** Effect of the tyrosine kinase inhibitors AG126, AG490, AG1641 or A1 on the delayed fall in mean blood pressure (MAP) and vascular hyporeactivity (at 360 min) to noradrenaline (NA) and the rise in the serum concentration of tumour necrosis factor α (TNFα) (at 90 min after LPS) elicited by LPS

| *Animal groups* | *MAP (mmHg)* 0 min | 360 min | *NA* (mmHg) 360 min | *TNFα* (pg $ml^{-1}$) |
|---|---|---|---|---|
| C (vehicle) | $118 \pm 3$ | $111 \pm 3$* | $44 \pm 3$* | 0 |
| AG126 | $120 \pm 4$ | $112 \pm 4$* | $46 \pm 4$* | 0 |
| LPS | $120 \pm 4$ | $75 \pm 4$ | $19 \pm 4$ | $5658 \pm 404$ |
| +AG126 | $117 \pm 2$ | $88 \pm 4$ | $35 \pm 4$* | $4426 \pm 254$ |
| +AG490 | $119 \pm 4$ | $98 \pm 4$* | $37 \pm 3$* | $3156 \pm 298$* |
| +AG1641 | $121 \pm 3$ | $96 \pm 2$* | $36 \pm 3$* | $3445 \pm 348$* |
| +A1 | $123 \pm 3$ | $101 \pm 2$* | $41 \pm 3$* | $3253 \pm 452$* |

Different groups of animals received vehicle (50% DMSO/PBS, 1 ml $kg^{-1}$, i.p.; $n=4$), vehicle plus AG126 (5 mg $kg^{-1}$, i.p.; $n=3$), vehicle plus LPS (10 mg $kg^{-1}$, i.v.; $n=12$), or LPS plus AG126 (3 mg $kg^{-1}$, i.p.; $n=5$), AG490 (5 mg $kg^{-1}$, i.p.; $n=6$), AG1641 (5 mg $kg^{-1}$, i.p.; $n=5$) or A1 (5 mg $kg^{-1}$, i.p.; $n=6$). Data are expressed as mean $\pm$ s.e.mean of $n$ observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

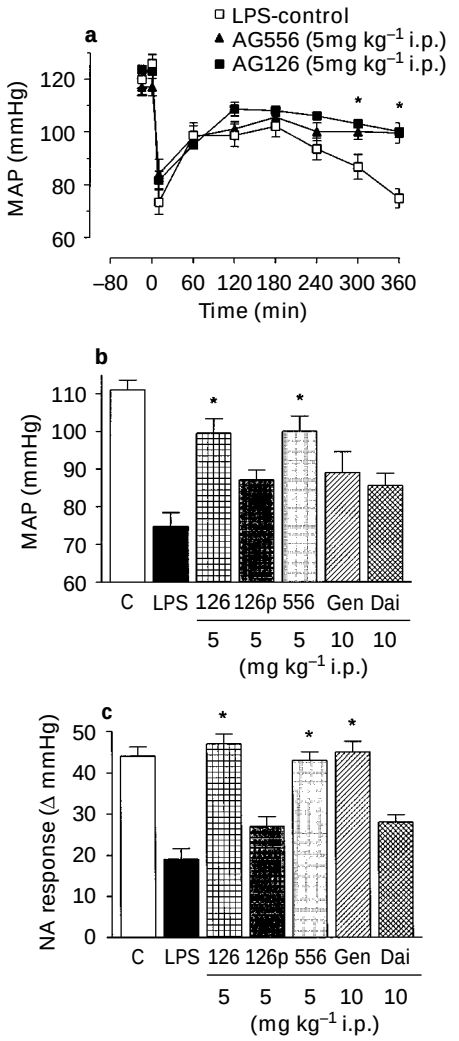


**Figure 2** (a) Effect of tyrphostin AG126 ($n=6$) or tyrphostin AG556 ($n=6$) on the fall in mean arterial blood pressure (MAP) caused by LPS (10 mg kg$^{-1}$, i.v.) in the anaesthetized rat. The alterations in MAP (over time) of rats that were pretreated with vehicle (for the drugs) and then received LPS are also shown (LPS-control, $n=12$). *$P<0.05$, AG556 or AG126 vs LPS control. (b) Comparison of MAP values and (c) the pressor responses to noradrenaline (NA; 1 $\mu$g kg$^{-1}$, i.v.) in rats treated with (i) vehicle (saline) rather than LPS (C, $n=4$), (ii) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.p.) plus LPS (LPS, $n=12$), (iii) LPS plus tyrphostin AG126 (126; 5 mg kg$^{-1}$, i.p.; $n=6$), (iv) LPS plus tyrphostin AG556 (556; 5 mg kg$^{-1}$, i.p.; $n=6$), (v) LPS plus genistein (Gen, 10 mg kg$^{-1}$, i.p.; $n=6$), (vi) LPS plus daidzein (Dai; 10 mg kg$^{-1}$, i.p.; $n=5$), or (vii) LPS plus a delayed treatment at 2 h after LPS with tyrphostin AG126 (126p; 5 mg kg$^{-1}$, i.p., 2 h after LPS; $n=4$) at 360 min after injection of LPS. Data are expressed as mean$\pm$s.e.mean of $n$ observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

by LPS, while the lower dose of AG126 (3 mg kg$^{-1}$) had no significant effect (Table 2). Genistein also reduced the rise in the serum levels of TNF$\alpha$ caused by LPS, while daidzein had no significant effect (Figure 3). The administration of AG126 (5 mg kg$^{-1}$, i.p., 2 h before LPS) to rats which had received saline rather than LPS had no significant effect on the serum levels of TNF$\alpha$ (Table 2).

### *Effects of tyrosine kinase inhibitors on the multiple organ dysfunction syndrome caused by LPS in the rat*

Endotoxaemia for 6 h was associated with a significant rise in the serum levels of urea, creatinine (indicators of renal failure), bilirubin, ALT and $\gamma$GT (all indicators of liver injury or failure) as well as lipase (an indicator of pancreatic injury)

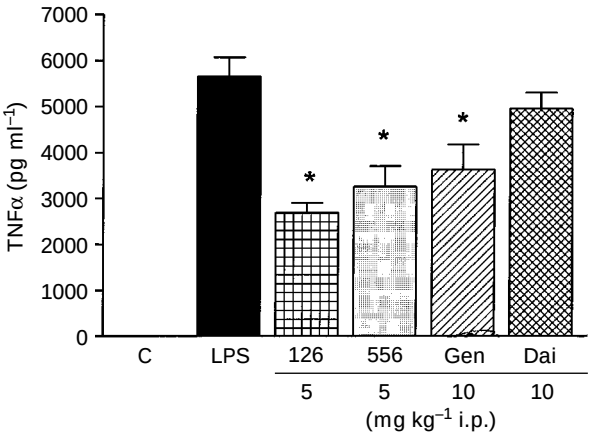


**Figure 3** Effect of tyrosine kinase inhibitors on the increase in the plasma levels of TNF$\alpha$ caused by LPS (at 90 min after injection of LPS) in the rat. Different groups of rats received (i) vehicle (saline) rather than LPS (C, $n=4$), (ii) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.p.) plus LPS (LPS; $n=6$), (iii) LPS plus tyrphostin AG126 (126; $n=6$), (iv) LPS plus tyrphostin AG556 (556; $n=6$), (v) LPS plus genistein (Gen; $n=6$), or (vi) LPS plus daidzein (Dai; $n=5$). Please note that levels of TNF$\alpha$ in the serum of rats treated with vehicle (C) were below the detection limit. Data are expressed as mean$\pm$ s.e.mean of $n$ observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

($P<0.05$; see Figure 4 and Table 3). None of the tyrosine kinase inhibitors tested had a significant effect on the rise in the serum levels of urea caused by endotoxin (Figure 4a and Table 3). The tyrphostins AG126, AG556 (Figure 4b–f) or A1 (Table 3) significantly attenuated the rises in the serum levels of creatinine, bilirubin, ALT, lipase and $\gamma$GT. The tyrphostins AG1641 and AG490 also attenuated the rises in the serum levels of bilirubin, ALT and lipase, but did not affect the rise in the serum levels of creatinine or $\gamma$GT (Table 3). Pretreatment of rats with genistein attenuated the rises in the serum levels of ALT and lipase, but did not affect the rises in the serum levels of creatinine, bilirubin or $\gamma$GT (Figure 4), while its analogue daidzein had no significant effect. The delayed administration of AG126 did not attenuate any of the parameters of organ dysfunction/injury measured (Figure 4a–f). The administration of AG126 (5 mg kg$^{-1}$, i.p., 2 h before LPS) to rats which had received saline rather than LPS had no significant effect on any of the parameters of organ dysfunction/injury studied (Table 3).

### *Effects of tyrosine kinase inhibitors on the hypoglycaemia and lactic acidosis caused by LPS in the rat*

Endotoxaemia for 6 h resulted in a substantial reduction in the serum levels of glucose ($P<0.05$; Figure 5a and Table 3). The hypoglycaemia caused by LPS was attenuated by pretreatment of rats with the tyrphostins AG126, AG556 (Figure 5a), AG490, AG1641 or A1 (Table 3), but not by the lower dose of AG126 tested (Table 3). Similarly, genistein (but not daidzein) reduced the degree of hypoglycaemia caused by LPS (Figure 5a). In contrast, the delayed administration of tyrphostin AG126 did not affect the hypoglycaemia caused by LPS (Figure 5a). Endotoxaemia for 6 h also caused a significant rise in the serum levels of lactate (Figure 5 and Table 3), which was reduced in LPS-rats pretreated with the tyrphostins AG126, AG556 (Figure 5b), AG490, AG1641 or A1 (Table 3). Genistein, but not its inactive analogue daidzein, also reduced the increase in the serum levels of lactate caused by LPS (Figure 5b). In contrast, the delayed administration of tyrphostin AG126 did not affect the hypoglycaemia caused by LPS (Figure 5b). The administration of AG126 (5 mg kg$^{-1}$, i.p., 2 h before LPS) to rats which had received saline rather than

LPS had no significant effect on the serum levels of glucose or lactate (Table 3).

*Tyrosine kinase inhibitors inhibit the expression of inducible nitric oxide synthase in the lung of rats with endotoxaemia*

Endotoxaemia for 6 h was associated with a substantial increase in iNOS activity in the lung ($P<0.05$, Figure 6a and Table 3). This increase in iNOS activity caused by LPS was attenuated by pretreatment of rats with the tyrphostins AG126, AG556 (Figure 6a), AG490, AG1641 or A1 (Table 3), but not by the lower dose of AG126 tested (Table 3). Genistein, but not its inactive analogue daidzein, also reduced the increase in iNOS activity caused by LPS in the lung (Figure 6a). In contrast, the delayed administration of tyrphostin AG126 did not cause a significant reduction in iNOS activity in lungs of rats with endotoxaemia (Figure 6a). In addition, en-

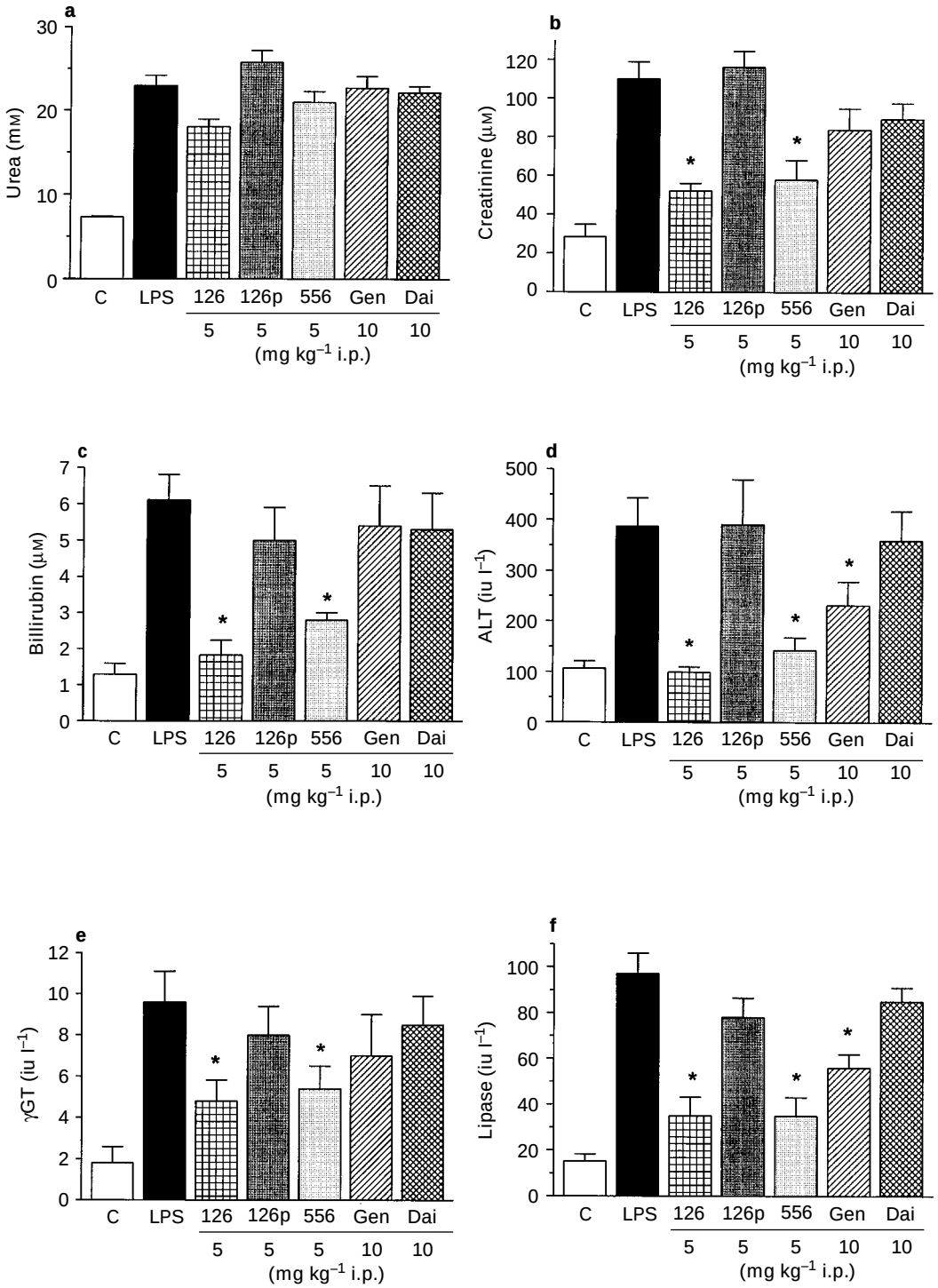


**Figure 4** Effect of tyrosine kinase inhibitors on the LPS-induced increases in the serum concentrations of (a) urea, (b) creatinine, (c) bilirubin, (d) alanine aminotransferase (ALT), (e) $\gamma$-glutamyl transferase ($\gamma$GT) and (f) lipase at 6 h after the injection of LPS (10 mg kg$^{-1}$). Different groups of rats received (i) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.p.) rather than LPS (C, $n=4$), (ii) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.p.) plus LPS (LPS; $n=6$), (iii) LPS plus tyrphostin AG126 (126; $n=6$), (iv) LPS plus delayed administration of tyrphostin AG126 (126p; $n=4$), (v) LPS plus tyrphostin AG556 (556; $n=6$), (vi) LPS plus genistein (Gen; $n=6$) or (vii) LPS plus daidzein (Dai; $n=5$). Data are expressed as mean$\pm$s.e.mean of $n$ observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

**Table 3** Effects of the tyrosine kinase inhibitors AG126, AG490, AG1641 or A1 on the liver and renal dysfunction as well as the increases in the serum concentrations of glucose, lactate and 6-keto-$PGF_{1\alpha}$ caused by LPS

| *Animal groups* | *Urea* (mM) | *Creatine* ($\mu$M) | *Bilirubin* ($\mu$M) | *ALT* (iu $ml^{-1}$) | $\gamma$GT (iu $ml^{-1}$) | *Lipase* (iu $ml^{-1}$) | *Glucose* (mg $dl^{-1}$) | *Lactate* (mg $dl^{-1}$) | *iNOS activity* (pmol $min^{-1}$ $mg^{-1}$) | *6-Keto-$PGF_{1\alpha}$* (pmol $mlu^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| C (vehicle) | 7±1* | 28±11* | 1.3±0.4* | 109±17* | 1.8±0.7* | 17±3* | 121±5* | 13±1* | 0.04±0.05* | 1.0±0.2* |
| Vehicle + AG126 | 5±2* | 25±8* | 0.9±0.7* | 98±21* | 1.4±0.6* | 14±4* | 117±7* | 12±2* | 0.002±0.03* | 1.2±0.4* |
| LPS | 23±3 | 114±6 | 6.1±0.7 | 389±58 | 9.6±1.4 | 97±12 | 56±4 | 37±6 | 8.2±0.3 | 8.1±1.2 |
| + AG126 | 21±3 | 82±5 | 2.3±0.7* | 267±39 | 6.3±0.4 | 72±15 | 64±6 | 31±5 | 5.8±1.1 | 3.8±0.8* |
| + AG490 | 22±2 | 89±11 | 3.1±0.8* | 223±17* | 5.9±0.8 | 58±10* | 89±7* | 21±3* | 3.5±0.6* | 2.8±0.5* |
| + AG1641 | 21±1 | 101±10 | 3.4±0.9* | 258±18* | 7.8±0.5 | 60±8* | 83±6* | 23±4* | 3.9±0.5* | 2.4±0.4* |
| + A1 | 20±1 | 65±8* | 2.4±0.3* | 142±14* | 4.8±0.3* | 52±10* | 98±5* | 22±4* | 2.9±0.5* | 1.5±0.5* |

The serum concentrations of urea, creatine, bilirubin, alamine aminotrasferase (ALT), lipase, $\gamma$-glutamyl transferase ($\gamma$GT), glucose, lactate, 6-keto-$PGF_{1\alpha}$ or the iNOS activity in lung homogenates were measured at 6 h after injection of LPS (10 mg $kg^{-1}$, i.v.). Different groups of animals received vehicle (50% DMSO/PBS, 1 ml $kg^{-1}$, i.p.; $n=4$), vehicle plus AG126 (5 mg $kg^{-1}$, i.p.; $n=3$), vehicle plus LPS ($n=12$), or LPS plus AG126 (3 mg $kg^{-1}$, i.p.; $n=5$), AG490 (5 mg $kg^{-1}$, i.p.; $n=6$), AG1641 (5 mg $kg^{-1}$, i.p.; $n=5$) or A1 (5 mg $kg^{-1}$, i.p.; $n=6$). Data are expressed as mean±s.e.mean of $n$ observations. *$P<0.05$ represents significant difference when compared to LPS-controls. AG126, AG490, AG1641 and A1 inhibited the increase in the iNOS activity in lung homogenates elicited by LPS.

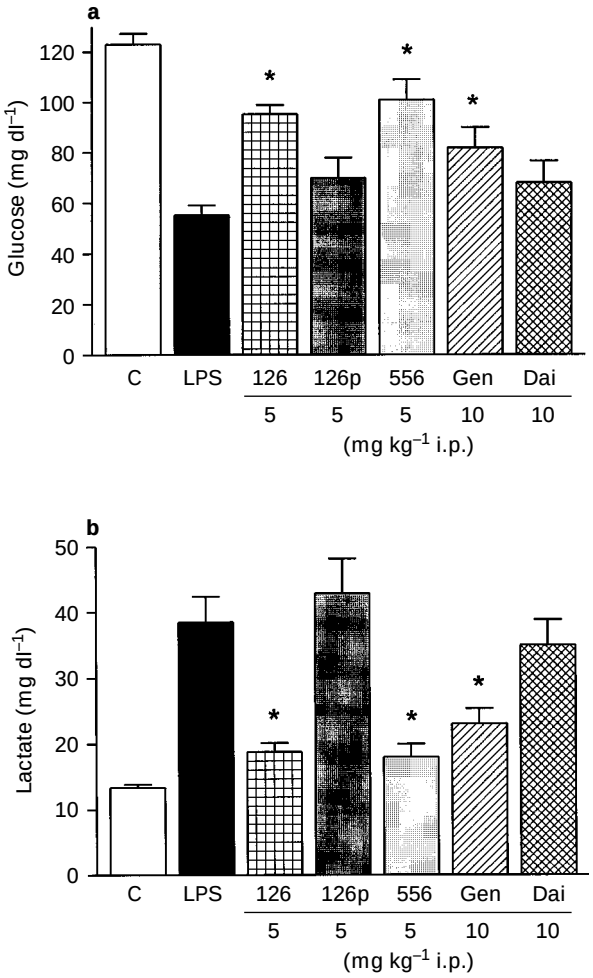


**Figure 5** Effect of tyrosine kinase inhibitors on the rises in serum concentrations of (a) glucose and (b) lactate caused by LPS in the rat. Different groups of rats received (i) vehicle (saline) rather than LPS (C, $n=4$), (ii) LPS plus drug-vehicle (50% DMSO/PBS, 1 ml $kg^{-1}$, i.p.) (LPS; $n=6$), (iii) LPS plus tyrphostin AG126 (126; $n=6$), (iv) LPS plus a delayed administration of tyrphostin AG126 (126p; $n=4$), (v) LPS plus tyrphostin AG556 (556; $n=6$), (vi) LPS plus genistein (Gen; $n=6$) or (vii) LPS plus daidzein (Dai; $n=5$). *$P<0.05$ represents significant difference when compared to LPS-controls.

dotoxaemia for 6 h also caused an increase in the expression of iNOS protein in lung homogenates (Figure 6), which was significantly reduced by pretreatment of LPS-rats with tyrphostins AG126 (3 or 5 mg $kg^{-1}$) and AG556 (Figure 6b). Genistein, but not daidzein, also reduced the expression of iNOS protein caused by LPS in the lung (Figure 6b). In contrast, the delayed administration of tyrphostin AG126 did not cause a significant reduction in iNOS protein expression in lungs of rats challenged with LPS for 6 h (Figure 6b). The administration of AG126 (5 mg $kg^{-1}$, i.p., 2 h before LPS) to rats which had received saline rather than LPS had no significant effect on iNOS activity (Table 3) or iNOS protein expression (data not shown) in the lung.

### *Tyrosine kinase inhibitors inhibit the increase in the serum levels of 6-keto-$PGF_{1\alpha}$ and the expression of COX-2 protein in the lung of rats with endotoxaemia*

Endotoxaemia for 6 h was associated with a 8 fold increase in the serum levels of 6-keto-$PGF_{1\alpha}$ from 1.0±0.2 to 8.1±1.7 (pmol $ml^{-1}$) ($P<0.05$, Figure 7a and Table 3). The tyrphostins AG126, AG556 (Figure 7a), AG490, AG1641 or A1 (Table 3) abolished the increase in the serum levels of 6-keto-$PGF_{1\alpha}$ caused by LPS (Figure 7a and Table 3). Similarly, the delayed administration of AG126 attenuated the rise in the serum levels of 6-keto-$PGF_{1\alpha}$ caused by LPS (Figure 7a). Genistein, but not its inactive analogue daidzein, also reduced the increase in the serum levels of 6-keto-$PGF_{1\alpha}$ caused by LPS (Figure 7a). The rise in the serum levels of 6-keto-$PGF_{1\alpha}$ caused by LPS was associated with an increase in the expression of COX-2 protein in the lung (Figure 7b). The expression of COX-2 protein was abolished in LPS-rats which had been pretreated with the tyrphostins AG126 or AG556 (Figure 7b). Genistein, but not daidzein, also abolished the expression of COX-2 caused by LPS in the lung (Figure 7b). Moreover, the late administration of tyrphostin AG126 also caused a significant reduction of the expression of COX-2 protein caused by LPS (Figure 7b). The administration of AG126 (5 mg $kg^{-1}$, i.p., 2 h before LPS) to rats which had received saline rather than LPS had no significant effect on the serum levels of 6-keto-$PGF_{1\alpha}$ (Table 3) or the expression of COX-2 protein in the lung (data not shown).

## Discussion

The tyrphostins AG126 and AG556, which are known to inhibit the activity of tyrosine kinase (see Levitzki & Gazit, 1995 for review), improve survival of mice challenged with endotoxin (Novogrodsky *et al*., 1994; Vanichkin *et al*., 1996). The mechanism of this beneficial effect of these tyrphostins is largely unknown. This study provides the first evidence that the tyrphostins AG126, AG556, AG490 AG1641 and A1 prevent the circulatory failure (hypotension and vascular hyporeactivity to noradrenaline) caused by endotoxin *in vivo*. Genistein also attenuated the delayed vascular hyporeactivity to noradrenaline, but failed to reduce the hypotension elicited by en-

dotoxin in the rat. The progression of shock to multiple organ failure (or multiple organ dysfunction syndrome; MODS) is associated with an increase in mortality such that with the number of organs failing (from 1–4), mortality progressively increases from 30% (in the absence of MODS) to 100% (see Baue, 1993). In the rat model of endotoxic shock used here, six hours of endotoxaemia resulted in a substantial increase in the plasma levels of bilirubin, ALT and $\gamma$GT indicating the development of acute liver injury/dysfunction. Pretreatment of rats with the tyrphostins AG126, AG556, AG490, AG1641 or A1 attenuated the acute liver injury/dysfunction caused by endotoxaemia. Like the tyrphostins, genistein also attenuated the rise in the plasma levels of ALT caused by LPS, while its inactive (with respect to the inhibition of tyrosine kinase activity) analogue daidzein was without effect. Endotoxaemia also resulted in (i) an increase in the plasma activity of lipase,

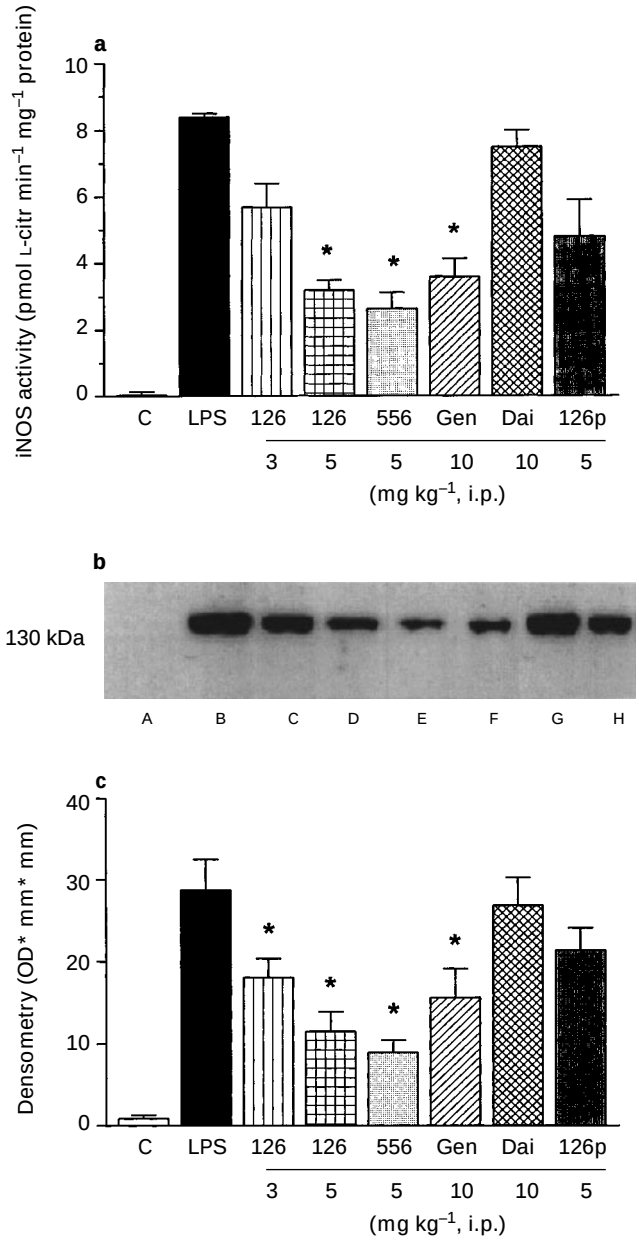


**Figure 6** Effect of tyrosine kinase inhibitors on (a) iNOS activity and (b) the expression of iNOS protein in lung homogenates from rats 6 h after treatment with LPS (10 mg kg$^{-1}$). Lungs were obtained from animals, which had received (i) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.p.; C or lane A), (ii) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.v.) plus LPS (LPS or lane B), (iii) LPS plus low dose tyrphostin AG126 (126 or lane C), (iv) LPS plus high dose of tyrphostin AG126 (126 or lane D), (v) LPS plus tyrphostin AG556 (556 or lane E), (vi) LPS plus genistein (Gen or lane F), (vii) LPS plus daidzein (Dai or lane G), or (viii) LPS plus delayed treatment with tyrphostin AG126 (126p or lane H). Data are expressed as mean$\pm$s.e.mean of $n=3$ observations. Similar results of the Western blots were seen with tissue extracts from two other animals with the same treatment. *$P<0.05$ represents significant difference when compared to LPS-controls.

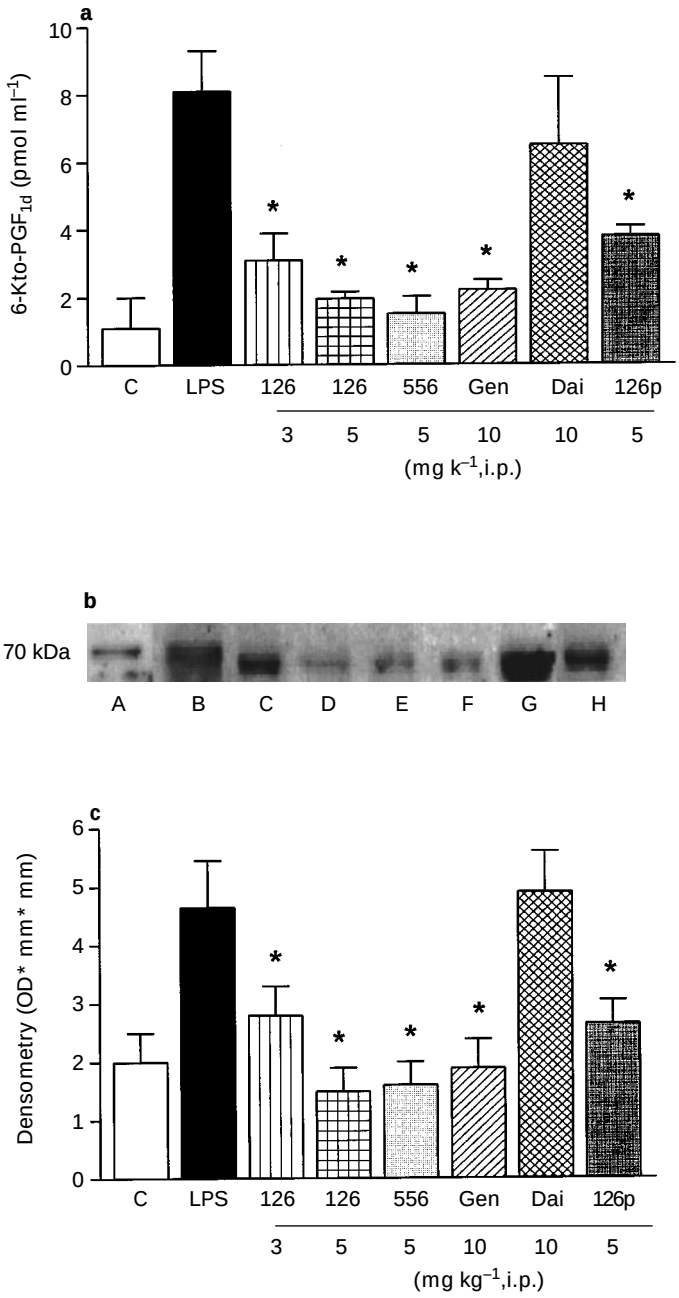


**Figure 7** Tyrosine kinase inhibitors inhibit (a) the increase in the serum levels of 6-keto-PGF$_{1\alpha}$ and (b) the expression of COX-2 protein in lung homogenates of rats with endotoxaemia. Different groups of animals received (i) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.p.; C or lane A), (ii) vehicle (50% DMSO/PBS, 1 ml kg$^{-1}$, i.v.) plus LPS (LPS or lane B), (iii) LPS plus low dose of tyrphostin AG126 (126 or lane C), (iv) LPS plus high dose of tyrphostin AG126 (126 or lane D), (v) LPS plus tyrphostin AG556 (556 or lane E), (vi) LPS plus genistein (Gen or lane F), (vii) LPS plus daidzein (Dai or lane G), or (viii) LPS plus delayed treatment with tyrphostin AG126 (126p or lane H). Data are expressed as mean$\pm$s.e.mean of $n=3$ observations. Similar results of the Western blots were seen with tissue extracts from two other animals with the same treatment. *$P<0.05$ represents significant difference when compared to LPS-controls.

an indicator of pancreatic injury, (ii) a fall in glucose (hypoglycaemia) and most importantly, in (iii) an increase in the plasma levels of lactate demonstrating the development of tissue hypoxia, presumably secondary to an impairment in tissue oxygen extraction (Baue, 1993). Pretreatment of rats with any of the tyrphostins or with genistein attenuated the pancreatic injury, the hypoglycaemia as well as the rise in lactate (tissue hypoxia) associated with endotoxaemia. Six hours of endotoxaemia also resulted in an increase in the serum levels of urea and creatinine, indicating the development of acute renal dysfunction/failure. None of the tyrosine kinase inhibitors used affected the rise in urea, but the tyrphostins AG126, AG556 and A1 attenuated the rise in creatinine caused by endotoxin in the rat. The following paragraphs discuss the mechanism(s) contributing to the reduction by genistein and the tyrphostins of the circulatory failure and organ dysfunction/injury caused by endotoxin in the rat.

### *Mechanism of the beneficial effect of tyrphostins and genistein in shock: inhibition of TNFα formation*

An enhanced formation of TNFα mediates both the circulatory collapse (Mozes *et al*., 1991; Thiemermann *et al*., 1993) as well as the liver injury and dysfunction (De La-Mata *et al*., 1990; Hewett *et al*., 1993) caused by LPS in the rat. We demonstrate here that all of the tyrphostins (AG126, AG490, AG556, AG1641 and A1), which reduced the circulatory failure and the multiple organ dysfunction/injury caused by endotoxin in the rat, also significantly attenuated the rise in the serum levels of TNFα caused by LPS. In contrast, a lower dose of AG126, which failed to reduce the rise in the serum levels of TNFα, did not diminish the detrimental effects of LPS on haemodynamics and organ function. Thus, it is possible that the beneficial effects of the tyrphostins used in this study are secondary to their ability to reduce the formation of TNFα *in vivo*. Like the tyrphostins, genistein also reduced the rise in the serum levels of TNFα as well as the circulatory failure and the organ injury/dysfunction caused by LPS, while daidzein had no effect on either parameter. Thus, it is possible that the inhibition of the increase in the serum levels of TNFα contributes to the beneficial effects of the tyrphostins and genistein in rats with endotoxic shock. The expression by LPS of the mRNA for TNFα in (human) monocytes is prevented by genistein and herbimycin A (Geng *et al*., 1993), suggesting that the expression of the TNFα gene caused by endotoxin involves the activation of protein tyrosine kinase.

### *Mechanism of the beneficial effect of tyrphostins and genistein in shock: inhibition of the activation of NFκB*

Although there is good evidence that TNFα (and other proinflammatory cytokines) cause the activation and translocation of nuclear factor-κB (NF-κB) into the nucleus, there is also evidence that (i) tyrosine phosphorylation itself plays an important role in the activation of NF-κB and (ii) that tyrosine kinase inhibitors diminish the activation of NF-κB. For instance, higher concentrations of genistein (100 μM) attenuate the translocation of NF-κB in rat pancreatic β cells activated with interleukin-1 (IL-1) (Kwon *et al*., 1995). Herbimycin A also suppresses the activation of NF-κB and the phosphorylation of JAK2 caused by LPS and interferon-γ in C6 glial cells, respectively (Nishiya *et al*., 1995). Herbimycin A also reduces the activation of NF-κB caused by IL-1 and phorbol 12-myristate 13-acetate (PMA) in thymoma cells or by PMA in Jurkat T cells. However, the inhibition of the activation of NFκB by herbimycin A is secondary to a modification of the P50 subunit on cysteine 62 in the NF-κB complex, but independent of the inhibition of tyrosine kinase activity (Mahon & O'Neill, 1995). The expression of matrix metalloproteinase-9 (MMP-9) caused by IL-1 in glomerular mesangial cells is also (at least in part) due to tyrosine kinase-mediated activation of NF-κB (Yokoo & Kitamura, 1996). The prevention by genistein and herbimycin A of the expression of COX-2 in rat mesangial cells is not due to inhibition of the activation of NF-κB, suggesting that an upstream tyrosine kinase pathway is not required for the IL-1-induced activation of NF-κB in these cells (Tetsuka *et al*., 1996). Most notably, stimulation of Jurkat T cells with the protein tyrosine phosphatase inhibitor and T cell activator, pervanadate, leads to activation of NF-κB due to tyrosine phosphorylation, but not degradation of I-κBα. It has therefore been suggested (Impert *et al*., 1996) that the tyrosine phosphorylation of I-κBα represents a proteolysis-independent mechanism of NF-κB activation that directly couples NF-κB to cellular tyrosine kinase. Thus, it is possible that the tyrosine kinase inhibitors used in our study prevent the activation of NFκB either by an indirect (e.g. prevention of the formation of TNFα, see above) or by a direct effect. Suppression of the activation of NF-κB by the tyrosine kinase inhibitors used in this study may well result in a reduced expression of enzymes (e.g. iNOS, COX-2, $cPLA_2$ etc), cytokines (TNFα, IL-1β, IL-6 etc), chemokines (IL-8, RANTES etc) or adhesion molecules (ICAM-1, VCAM-1, E-selectin) known to play an important role in the pathophysiology of endotoxin shock (see Barnes & Adcock, 1987).

### *Mechanism of the beneficial effect of tyrphostins and genistein in shock: inhibition of the expression of iNOS protein*

This study demonstrates that several tyrphostins (AG126, AG490, AG556, AG1641) and the isoflavone genistein prevent the formation of nitrite and, hence, the induction of iNOS activity (Radomski *et al*., 1990; Southan *et al*., 1995; Kengatharan *et al*., 1996) in cultured macrophages activated with LPS. Tyrphostin A1, which is less potent inhibitor of tyrosine kinase activity than other tyrphostins *in vitro* (Negrescu *et al*., 1995; Levitzki & Gazit, 1995), did not affect the increase in the formation of nitrite by activated macrophages. The isoflavone daidzein also did not reduce the increase in nitrite caused by LPS in cultured macrophages. As daidzein differs from genistein only in one hydroxyl-group in position 5, but does not inhibit the activity of tyrosine kinases (Negrescu *et al*., 1995; Levitzki & Gazit, 1995), it is likely that the signal transduction events leading to the induction of iNOS activity in macrophages challenged with LPS involves the activation of tyrosine kinases (see Akarasereenont *et al*., 1995; Paul *et al*., 1995). The expression of iNOS caused (i) by lipoteichoic acid in macrophages (Kengatharan *et al*., 1996), (ii) by IL-1 in pancreatic β-cells of the rat (Kwon *et al*., 1995) and (iii) by LPS and interferon-γ in C6 glioma cells (Nishiya *et al*., 1995) involves the activation of tyrosine kinases as well as the activation of NF-κB.

In the rat, endotoxin causes an overproduction of NO due to induction of iNOS (Knowles *et al*., 1990) which contributes to the circulatory failure (Rees *et al*., 1990; Szabo *et al*., 1993), the liver injury and dysfunction as well as the pancreatic dysfunction caused by LPS (Thiemermann *et al*., 1995; Ruetten *et al*., 1996). We demonstrate here that pretreatment of LPS-rats with several tyrphostins significantly attenuated the increase in iNOS activity (AG126, AG556, AG490, AG1641, A1) or protein (AG126, AG556) caused by endotoxin in the lung of the rat. Genistein also reduced the expression of iNOS protein and activity caused by LPS in the lung, while daidzein was without effect. Thus, we propose that the reduction of the expression of iNOS protein and activity, caused by genistein and the tyrphostins studied, contributes to the reduction by these agents of the circulatory failure and the organ dysfunction and injury caused by LPS in the rat. This conclusion is supported by the finding that the tyrosine kinase inhibitors used in this study – like several chemically distinct inhibitors of iNOS activity (Thiemermann *et al*., 1995; Ruetten *et al*., 1996) or other agents which prevent the expression of iNOS protein such as dexamethasone or calpain inhibitor I (Ruetten & Thiemermann, 1997) – attenuate the dysfunction of liver and pancreas as well as the hypoglycaemia and lactic acidosis caused by LPS in the rat.

### *Mechanism of the beneficial effect of tyrphostins and genistein in shock: inhibition of the expression of COX-2 protein*

This study also demonstrates that the tyrphostins (AG126, AG490, AG556, AG1641, A1) and genistein prevent the increase in 6-keto-$PGF_{1\alpha}$ caused by LPS in the rat. This effect is likely to be due to the prevention of the expression of COX-2, as (i) the tyrphostins AG126 or AG556 and the isoflavone genistein prevented the expression of COX-2 protein caused by LPS in the lung (this study), and (ii) inhibition of tyrosine kinase activity with genistein or tyrphostin-25 does not reduce phospholipase $A_2$ activity (Glaser *et al*., 1993). The prevention of the expression of COX-2 by the tyrosine kinase inhibitors used in this study is associated with beneficial haemodynamic effects. However, it is unclear whether the formation of arachidonic metabolites (by COX-2) contributes importantly to the pathophysiology of septic shock. Although there is some evidence that prostaglandins contribute to the haemodynamic alterations and the liver injury associated with endotoxic shock (see Feuerstein & Hallenbeck, 1987 for review), the effects of selective inhibitors of COX-2 activity (Griswold & Adams, 1996) on circulatory failure or MODS have not been investigated. Moreover, there are no studies in which the effects of antisense oligonucleotides against COX-2 mRNA were evaluated in animal models of shock. Although we can, therefore, not exclude the possibility that an enhanced formation of arachidonic acid metabolites by COX-2 contributes to the observed pathophysiology, it is impossible to predict if, and to what degree, prevention of the expression of COX-2 protein and activity contributes to the beneficial effects of the tyrosine kinase inhibitors in rats with endotoxic shock.

### *Mechanism of the beneficial effect of tyrphostins and genistein in shock: inhibition of the activity of tyrosine kinase*

We have not investigated the effects of the tyrphostins or the isoflavones used in this study on the LPS-induced activation of protein tyrosine kinase activity in murine macrophages or in tissues/organs of the rat. We can therefore not clearly demonstrate that inhibition of tyrosine kinase activity accounts for the observed beneficial effects of tyrphostins or genistein in rats with endotoxic shock. Thus, it is possible that (some of) the beneficial effects of e.g. genistein in shock are unrelated to the inhibition of the activity of tyrosine kinases. Indeed, the inhibition by genistein of the activity of L-type calcium channels in rat ventricular myocytes appears to be independent of the inhibition of tyrosine kinases, as daidzein (which has little or not inhibitory effect on tyrosine kinase activity of the EGF receptor) also inhibited L-type calcium channels to a similar degree (Yokoshiki *et al*., 1996). Similarly, the inhibition by genistein (and dadzein) of the fast sodium current in uterine leiomyosarcoma cells is also independent of the inhibition of tyrosine kinase activity (Kusaka & Sperelakis, 1996). In our study, daidzein had no significant effect on the circulatory failure, organ dysfunction/failure, increase in the serum levels of TNF$\alpha$ or the induction of iNOS or COX-2 protein and activity caused by LPS in the rat. This finding suggests that the beneficial effects of genistein in shock are more likely to be due to inhibition of tyrosine kinase activity than due to other (non-specific) effects. Tyrphostin A1 has been shown to be a much weaker inhibitor of tyrosine kinase activity than other tyrphostins (e.g. AG126) and, hence, has often been used to differentiate between those effects of the tyrphostins which are due to inhibition of tyrosine kinase activity from other (non-specific) effects. We showed, in the present study, that the effects of tyrphostin A1 on the LPS-induced circulatory failure, organ dysfunction/failure, increase in the serum levels of TNF$\alpha$ or the induction of iNOS or COX-2 activity were very similar to those elicited by any of the other tyrphostins tested (AG126, AG490, AG556, AG1641). This finding can be interpreted in two possible ways, namely (i) that all of the observed effects of tyrphostins are independent of the inhibition of tyrosine kinase (e.g. inhibition of the generation of TNF$\alpha$ by a tyrosine kinase-independent mechanism) or (ii) that the dose of tyrphostin A1 used in this study (*in vivo*) was sufficient to cause a significant inhibition of the activity of tyrosine kinases. There is evidence (in addition to the results provided here) that tyrphostins (including A1) and genistein exert effects, which are not seen with daidzein. For instance, tyrphostin A1, tyrphostin 23 and genistein, but not daidzein, inhibit voltage-operated calcium channels in vascular smooth muscle cells of the rabbit (Wijetunge *et al*., 1992).

Thus, most of the inhibitors of tyrosine kinase activity used today exert (non-specific) effects which are unrelated to the inhibition of tyrosine kinase activity. It is therefore impossible to prove the involvement of the phosphorylation of protein tyrosine kinases in a biological process without (i) measuring the degree of inhibition of tyrosine kinase activity afforded by one (or preferably several distinct inhibitors of tyrosine kinase activity) and (ii) correlating the degree of inhibition of tyrosine kinase activity observed to the measured biological response. Clearly, more specific inhibitors of tyrosine kinase activity will be needed to elucidate if, and which of, the beneficial effects elicited by tyrphostins or genistein in rats with endotoxin shock are due to inhibition of tyrosine kinase activity.

### *Summary and conclusion*

This study demonstrated that the tyrphostins AG126, AG490, AG556, AG1641 or A1 or the isoflavone genistein prevent the (i) circulatory failure, (ii) the multiple organ dysfunction (liver and pancreatic dysfunction/injury, lactacidosis, hypoglycaemia), as well as (iii) the induction of iNOS and COX-2 protein and activity in rats with endotoxic shock. The mechanism(s) by which tyrphostins or genistein exert these beneficial effects in shock warrants further investigation, but may involve the prevention of the (i) formation of TNF$\alpha$. (ii) the expression of iNOS protein and (iii) the expression of COX-2 protein. We propose that tyrosine kinase inhibitors such as tyrphostins or genistein may be useful in the therapy of circulatory shock or of disorders associated with local or systemic inflammation.

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