of glutathione conjugate metabolism and cysteine conjugate β-lyase in the mechanism of S-cysteine conjugate toxicity in LLC-PK$_1$ cells. *J Biol Chem* **261**: 3325–3332, 1986.
22. Montine TJ and Borch RF, Quiescent LLC-PK$_1$ cells as a model for *cis*-diamminedichloroplatinum(II) nephrotoxicity and modulation by thiol rescue agents. *Cancer Res* **48**: 6017–6024, 1988.
23. Hoeschele JD, Butler TA, Roberts JA and Guyer CE, Analysis and refinement of the microscale synthesis of the [^{195m}Pt]-labeled antitumor drug, *cis*-dichlorodiammineplatinum(II), *cis*-DDP. *Radiochim Acta* **31**: 27–36, 1982.
24. Baldew GS, Volkers KJ, de Goeij JJM and Vermeulen NPE, Determination of cisplatin and related platinum complexes in plasma ultrafiltrate and urine by high-performance liquid chromatography with on-line radioactivity detection. *J Chromatogr Biomed Appl* **491**: 163–174, 1989.
25. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
26. Safirstein R, Winston J and Guttenplan J, Cisplatin nephrotoxicity: physiological and biochemical aspects. In: *Biochemical Mechanisms of Platinum Antitumor Drugs* (Eds. McBrien DCH and Slater TF), pp. 271–306. IRL Press, Oxford, 1986.
27. Knox RJ, Friedlos F, Lydall DA and Roberts JJ, Mechanisms of cytotoxicity of anticancer platinum drugs: evidence that *cis*-diamminedichloroplatinum(II) and *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res* **46**: 1972–1979, 1986.
28. Roberts JJ and Thompson AJ, The mechanism of action of antitumor platinum compounds. *Prog Nucleic Acid Res Mol Biol* **22**: 71–133, 1979.
29. Baldew GS, McVie JG, Van der Valk MA, Los G, De Goeij JJM and Vermeulen NPE, Selective reduction of *cis*-diamminedichloroplatinum (II) nephrotoxicity by ebselen. *Cancer Res* **50**: 7031–7036, 1990.
30. Hori R, Yamamoto K, Saito H, Kohno M and Inui K, Effect of aminoglycoside antibiotics on cellular functions of kidney epithelial cell line (LLC-PK$_1$): a model system for aminoglycoside nephrotoxicity. *J Pharmacol Exp Ther* **230**: 742–748, 1984.
31. Bodenner DL, Dedon PC, Keng PC, Katz JC and Borch RF, Selective protection against *cis*-diamminedichloroplatinum(II)-induced toxicity in kidney, gut and bone marrow by diethyldithiocarbamate. *Cancer Res* **46**: 2751–2755, 1986.
32. Yuhas JM, Active versus passive absorption kinetics as the basis for selective protection of normal tissues by *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid. *Cancer Res* **40**: 1519–1524, 1980.
33. Baldew GS, Volkers KJ and van den Hamer CJA, Reduction of cisplatin nephrotoxicity by selenium: does metallothionein play a role? *Arch Toxicol Suppl* **12**: 171–174, 1988.
34. Baldew GS, van den Hamer CJA, Los G, Vermeulen NPE, de Goeij JJM and McVie JG, Selenium-induced protection against *cis*-diamminedichloroplatinum (II) nephrotoxicity in mice and rats. *Cancer Res* **49**: 3020–3023, 1989.
35. Naganuma A, Satoh M, Yokoyama M and Imura N, Selenium efficiently depressed toxic side effect of *cis*-diamminedichloroplatinum. *Res Commun Chem Pathol Pharmacol* **42**: 127–134, 1983.
36. Satoh M, Naganuma A and Imura N, Optimum schedule of selenium administration to reduce lethal and renal toxicities of *cis*-diamminedichloroplatinum in mice. *J Pharmacobio-Dyn* **12**: 256–258, 1989.
37. Baldew GS, Mol JGJ, de Kanter FJJ, van Baar B, de Goeij JJM and Vermeulen NPE, The mechanism of interaction between cisplatin and selenite. *Biochem Pharmacol* **41**: 1429–1437, 1991.

Biochemical Pharmacology, Vol. 44, No. 2, pp. 387–390, 1992.
Printed in Great Britain.

0006-2952/92 $5.00+0.00

Soybean trypsin inhibitor and β-amylase induce alveolar macrophages to release nitrogen oxides

(*Received* 24 *June* 1991; *accepted* 6 *April* 1992)

Abstract—Rat alveolar macrophages incubated with soybean trypsin inhibitor and β-amylase produced nitrite in a dose- and time-dependent manner. This production depends on the presence of L-arginine (L-arg) in the culture medium. The precursor of this nitrite was demonstrated as being nitric oxide by bleaching ferredoxin at 410 nm when added to the culture medium. N^G-Monomethyl-L-arginine and the tetrahydrobiopterin biosynthesis inhibitor 2,4-diamino-6-hydroxypyrimidine inhibited the release of nitrite in a dose-dependent manner. Dexamethasone was able to modulate this release. These data indicate that alveolar macrophages are capable of secreting L-arg-derived nitrogen oxides when stimulated with certain alimentary proteins.

Immunostimulated macrophages, either murine peritoneal macrophages [1, 2] or rat Kuppfer cells, mice peritoneal, wound and bone marrow macrophages [3–5] synthesize nitrite (NO_2^-) and nitrate (NO_3^-) from L-arginine (L-arg*) by oxidation of the intermediate nitric oxide (NO) [4]. The true biological role of nitrogen oxides produced by

* Abbreviations: STI, soybean trypsin inhibitor; L-arg, L-arginine; rIFN-γ, recombinant interferon-γ; LPS, lipopolysaccharide; DAHP, 2,4-diamino-6-hydropyrimidine; Fd, ferredoxin; L-NMMA, N^G-monomethyl-L-arginine; DMEM, Dulbecco's modified Eagle's medium.

macrophages remains unknown, but they are thought to play a role in the microbiostatic and tumoricidal activity of stimulated (murine) peritoneal macrophages [6, 7].

Alveolar macrophages, resident in the lung alveoli, play an important role in lung defense against injury [8] through the release of numerous secretory products such as enzymes, antiproteases, cytokines and oxygen radicals. Recent work in our laboratory showed that recombinant interferon-γ (rIFN-γ), opsonized zymosan and lipopolysaccharide (LPS) can stimulate rat alveolar macrophages in a dose- and time-dependent manner to release nitric oxide and subsequently nitrite [9].

A recent observation suggests that some food proteins, e.g. soybean trypsin inhibitor (STI), Kunitz Type and β-amylase, are potent stimulants of the macrophage cell line RAW 264.7 and can induce these cells to release nitrite [10]. Crude soybean extract containing trypsin (protease) inhibitors also has some anticarcinogenic properties, capable of partially blocking in mice the development of lung tumors following methylcholanthrene administration [11].

It is not known whether soybean proteins can stimulate alveolar macrophages to release inflammatory mediators. The purpose of the present study was therefore to investigate whether STI and β-amylase can stimulate alveolar macrophages to release nitrogen oxides.

Materials and Methods

Culture reagents and media. β-Amylase (sweet potato, EC 3.2.1.2), cortexolone (4-pregnene-17,21-diol-3,20 dione), dexamethasone, 2,4-diamino-6-hydropyrimidine (DAHP), ferredoxin (Fd) from *Clostridium pasteurianum*, polymyxin β-sulfate and STI (Type I-s) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Sitosterol and stigmasterol were obtained from Roth (Karlsrühe, F.R.G.). Coatest (limulus amoebocyte lysate endotoxin test) was purchased from Kabi diagnostica (Brussels, Belgium). N^G-Monomethyl-L-arginine (L-NMMA) (S. Moncada, Wellcome Research Foundation, Beckenham, U.K.) and rat rIFN-γ (P. H. van der Meide, TNO, Rijswijk, The Netherlands) were kindly provided by the persons mentioned in parentheses. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) without phenol red (with and without L-arg) supplemented with 5% heat inactivated, low endotoxin fetal calf serum (Gibco, Paisley, U.K.) and penicillin/streptomycin. L-arg-free DMEM without phenol red was manufactured by Gibco.

Culture and stimulation of alveolar macrophages. Alveolar macrophages from male Wistar specific pathogen-free rats (Proefdierencentrum, Leuven, Belgium, weight 200–250 g) were harvested according to standard procedures [12]. After washing, the macrophage populations were enriched by adherence (>98% pure) to plastic in 24-well sterile dishes (Nunc, Roskilde, Denmark) and adjusted to 0.5×10^6 macrophages in each well. Subsequently, 1050 μL medium (with or without L-arg) with or without the appropriate stimuli or modulating agents dissolved in 50 μL DMEM containing no L-arg were added to duplicate wells (incubation period 1–72 hr, 37° and 5% CO_2). Cell viability was assessed by trypan blue dye exclusion test and was always greater than 92%.

Nitrite assay. Nitrite (NO_2^-) was determined on cell free supernatant by a spectrophotometric assay based on the Griess reaction [13]. Briefly, 90 μL 6.5 M HCl and 90 μL 37.5 mM sulfanilic acid were added to 900 μL cell-free supernatant. After 10 min, 90 μL 12.5 mM *N*-(1-naphthyl)-ethylene diamine·HCl was added. Subsequently, the absorbance was measured at 540 nm after 30 min. Assays were performed on duplicate wells.

Fd assay. Per well, 2×10^6 macrophages were pretreated with either 100 μg/mL STI or 50 μg/mL β-amylase. After 12 hr, the supernatant was harvested and Fd (8.2 μM in DMEM with or without L-arg) was added to the wells. Loss of absorbance at 410 nm was measured after an additional 18 hr and compared to incubations under the same conditions without cells added.

Results

Concentration- and time-dependent production of nitrite. As shown in Table 1, the 48 hr production of nitrite was dependent on the amount of stimulus (STI, β-amylase) added to the culture medium. A minimum amount of 10 μg/mL of STI was necessary to detect any induction. Release of nitrite by stimulated macrophages was concentration-dependent from 5 up to 100 μg/mL β-amylase. Higher concentrations of this protein seemed to activate macrophages to a lesser extent. In a range of 1 pg–500 μg/mL, two lipid derivatives from soybean, i.e. sitosterol and stigmasterol, were not able to stimulate these macrophages to release nitrogen oxides (data not shown). A contact period of 3–6 hr between the alveolar macrophages and STI (100 μg/mL) and β-amylase (50 μg/mL) was required to detect any significant nitrite production during the 24 hr subsequent to removal of these stimuli.

Table 1. Production of nitrite by rat alveolar macrophages, with or without addition of L-NMMA, DAHP and polymyxin B

Addition	STI (100 μg/mL)	P value*	β-amylase (50 μg/mL)	P value*
—	28.98 ± 1.46	—	46.0 ± 1.06	—
L-NMMA 30 μM	13.79 ± 1.25	<0.05	25.67 ± 2.10	<0.01
300 μM	4.05 ± 0.19	<0.01	11.58 ± 1.79	<0.01
DAHP 10^{-8} M	29.97 ± 1.25	NS	46.1 ± 1.03	NS
10^{-7} M	28.88 ± 1.35	NS	43.51 ± 1.00	NS
10^{-6} M	24.15 ± 0.37	NS	30.3 ± 0.77	<0.01
10^{-5} M	18.61 ± 0.23	<0.01	21.9 ± 1.58	<0.01
10^{-4} M	6.12 ± 0.17	<0.01	10.34 ± 0.62	<0.01
Polymyxin 2.5 μg/mL	27.57 ± 0.57	NS	43.34 ± 2.79	NS

Results of six individual cultures are nmol/10^6 macrophages/48 hr, means ± SEM.

* As compared with alveolar macrophage cultures with the inducers (STI and β-amylase) alone.

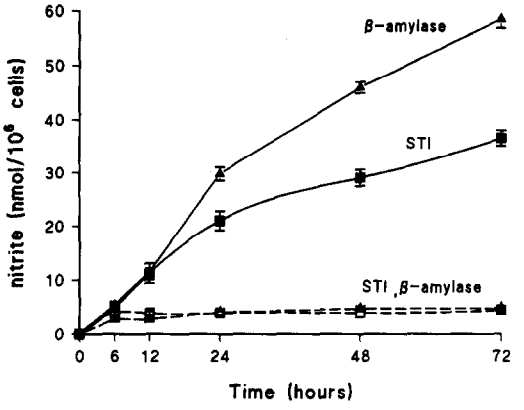

Fig. 1. Time-dependent release of nitrite from alveolar macrophages grown in L-arg-containing (—) and L-arg-free (----) medium and incubated with STI (100 μg/mL) and β-amylase (50 μg/mL). The time scale indicates hours in culture. The data are shown as the means ± SEM, N = 6.

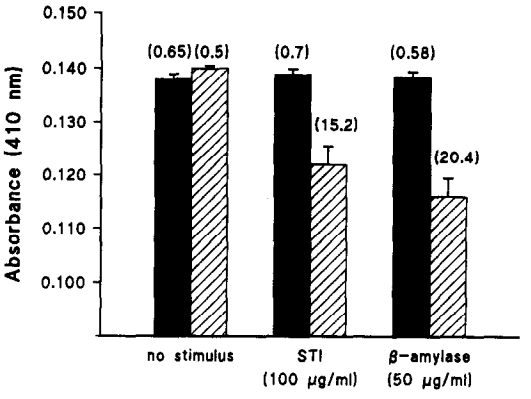

Fig. 3. Absorbance of Fd at 410 nm following production of nitric oxide during 18 hr. Cells were pretreated with STI (100 μg/mL), β-amylase (50 μg/mL) or no stimulus for 12 hr. Fd was dissolved in DMEM with (▨) or without (■) L-arg. Results are the means ± SEM for three similar experiments. The amount of nitrite produced is indicated in brackets.

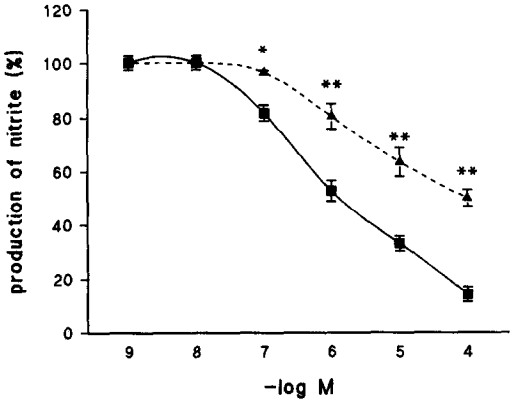

Fig. 2. Inhibition of the production of nitrite by 10^6 alveolar macrophages incubated with either STI (100 μg/mL) in the presence of dexamethasone (—) or dexamethasone and cortexolone (---). Results are expressed as % production of control situation (stimulus without added corticosteroids 28.98 ± 1.46 nmol/10^6 cells/48 hr), mean ± SEM, N = 6. **P < 0.01, *P < 0.05 (Student's *t*-test) as compared with incubations with dexamethasone.

As shown in Fig. 1, the production was time dependent. The secretion also depended on the presence of L-arg, since culture of these macrophages in L-arg-free medium resulted in almost no production of nitrite.

Modulation of nitrite release. L-NMMA and DAHP inhibited the release of nitrite by STI and β-amylase in a dose-dependent manner (Table 1). Addition of polymyxin β-sulfate (2.5 μg/mL) resulted in a significant reduction in the LPS-induced nitrite release (data not shown) but not in the release caused by STI and β-amylase. Cell viability was always greater than 92%, regardless of the modulator added to the culture medium. Figure 2 shows that co-incubation with dexamethasone (10^{-9}–10^{-4} M) caused a concentration-dependent inhibition of the STI (100 μg/mL)-induced nitrite formation. Addition of equimolar concentrations of cortexolone to incubations with dexamethasone partially reversed this inhibitory effect. Similar results were obtained for co-incubation of β-amylase (50 μg/mL) with the corticoids mentioned.

Limulus amoebocyte lysate test. The LPS content of the solutions of STI and β-amylase was <100 pg/mL, a concentration far below the minimum concentration of LPS required to stimulate alveolar macrophages to release nitrogen oxides.

Fd assay. The absorbance of Fd decreased when Fd was added to alveolar macrophages, previously stimulated with STI or β-amylase. No bleaching of Fd absorbance took place when cells were not pretreated or when pretreated cells were incubated with Fd dissolved in DMEM without L-arg (Fig. 3). No change was observed in the pH of the incubation medium during this 18-hr experiment.

Discussion

We have found previously that alveolar macrophages can produce nitrogen oxides when stimulated with rIFN-γ, LPS and opsonized zymosan [9]. The data presented here extend these results by showing that other proteins, i.e. STI and β-amylase, can provoke similar effects.

Dissolved in the medium at a concentration of more than 10 and 2 μg/mL respectively, STI and β-amylase similarly caused a dose- and time-related production of nitrite by alveolar macrophages. These threshold amounts are in the same range as required to stimulate mice peritoneal macrophages [10]. L-NMMA, known to inhibit nitrite production in macrophages [14], was also effective in inhibiting STI- and β-amylase-induced production. Addition of polymyxin B, the LPS-inactivating antibiotic, resulted in a significant reduction in LPS-induced nitrite secretion, but not in rIFN-γ-, STI- and β-amylase-induced secretion. These results, together with the low endotoxin content of the incubation fluid as determined by the limulus amoebocyte lysate test, prove that the observed results are not due to contamination of the STI and β-amylase with endotoxin.

The production of nitrite by NO-synthase was apparently dependent on tetrahydrobiopterin as a cofactor since the addition of DAHP, a known inhibitor of GTP-cyclohydrolase, i.e. 5,6,7,8-tetrahydrobiopterin *de novo* synthesis, inhibited this production in a dose-dependent manner. This effect of DAHP on NO-synthase activity has

until now only been reported on the cytokine-induced production of nitrite in mouse skin fibroblasts [15].

Glucocorticoids were able to block this induction of nitrite synthesis in alveolar macrophages in a comparable way to that reported for LPS-induced nitrite synthesis in a macrophage cell line [16]. This observed effect is thought to be receptor-mediated, since addition of the partial antagonist cortexolone partially reversed the inhibitory effect. Furthermore, through the bleaching of the chromophore Fd it was proven that nitric oxide (NO) was an intermediate of this nitrite biosynthesis. This spectral change is indeed characteristic for the interaction of nitric oxide with Fd [17].

Protease inhibitors have been shown to suppress neoplastic transformation *in vivo* and *in vitro* [18]. The mechanism for this action is not known but appears to involve multiple processes including suppression of oxygen radical formation, induction of poly(ADP)-ribose or other mechanisms (reviewed in Ref. 19). It remains to be determined by which mechanism STI and β-amylase induce NO-synthase activity in (alveolar) macrophages and if this *in vitro* observation may explain in part the previously observed but not elucidated anticarcinogenic effect of STI. Indeed, production of NO derived from L-arg is believed to play a key role in the metabolic inhibition and lysis of tumor cells by mouse and rat macrophages [2, 7, 17].

Departments of Respiratory Medicine and †Experimental Pharmacology
UIA, University of Antwerp
Universiteitsplein 1
B-2610 Wilrijk
Antwerp, Belgium

PHILIPPE G. JORENS*
FRANS J. VAN OVERVELD
HIDDE BULT†
PAUL A. VERMEIRE
ARNOLD G. HERMAN†

REFERENCES

1. Stuehr DJ and Marletta MA, Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. *J Immunol* **139**: 518–525, 1987.
2. Hibbs JB, Taintor RR and Vavrin Z, Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **235**: 473–476, 1987.
3. Billiar TR, Curran RD, Stuehr DJ, West MA, Bentz BG and Simmons RL, An L-arginine-dependent mechanism mediates Kuppfer cell inhibition of hepatocyte protein synthesis *in vitro*. *J Exp Med* **169**: 1467–1472, 1989.
4. Albina JE, Mills CD, Henry WL and Caldwell MD, Regulation of macrophage physiology by L-arginine: role of the oxidative L-arginine deiminase pathway. *J Immunol* **143**: 3641–3646, 1989.
5. Keller RM, Geiges M and Keist R, L-arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res* **50**: 1421–1425, 1990.
6. Liew FY, Millott S, Parkinson C, Palmer RM and Moncada SM, Macrophage killing of Leishmania parasite *in vivo* is mediated by nitric oxide from L-arginine. *J Immunol* **144**: 4794–4797, 1990.
7. Drapier JC, Wietzerbin J and Hibbs JB, Interferon-γ and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. *Eur J Immunol* **18**: 1587–1592, 1988.
8. Sibille Y and Reynolds HJ, Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* **41**: 471–501, 1990.
9. Jorens PG, Van Overveld FJ, Bult H, Vermeire PA and Herman AG, L-arginine-dependent production of nitrogen oxides by rat pulmonary macrophages. *Eur J Pharmacol* **200**: 205–209, 1991.
10. Mina M, Kong ZL, Shinohara K and Watanabe M, Soybean trypsin inhibitor and β-amylase stimulate macrophages. *Biochem Biophys Res Commun* **173**: 296–301, 1990.
11. Witschi H and Kennedy AR, Modulation of lung tumor development in mice with the soybean-derived Bowman–Birk protease inhibitor. *Carcinogenesis* **10**: 2275–2277, 1989.
12. Pauwels RA, Kips JC, Peleman RA and Van Der Straeten ME, The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats. *Am Rev Respir Dis* **141**: 540–545, 1990.
13. Schmidt HH, Nau H, Wittfoht J, Gerlach J, Prescher KE, Klein MM, Niroomand F and Böhme E, Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur J Pharmacol* **154**: 213–216, 1988.
14. Gross SS, Stuehr DJ, Aisaki K, Jaffe EA, Levi R and Griffith OW, Macrophage and endothelial nitric oxide synthesis: cell-type selective inhibition by N_G-aminoarginine, N_G-nitroarginine and N_G-methyl-arginine. *Biochem Biophys Res Commun* **170**: 96–103, 1990.
15. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G and Wachter H, Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. *J Exp Med* **172**: 1599–1607, 1990.
16. Di Rosa M, Radomski M, Carnuccio R and Moncada S, Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. *Biochem Biophys Res Commun* **172**: 1246–1252, 1990.
17. Stuehr DJ, Gross IS, Sakuma R, Levi R and Nathan CF, Activated murine macrophages secrete a metabolite of arginine with the bioactivity of EDRF and the chemical reactivity of NO. *J Exp Med* **169**: 1011–1020, 1989.
18. Troll W, Frenkel K and Weisner R, Protease inhibitors as anticarcinogens. *J Natl Cancer Inst* **73**: 1245–1250, 1984.
19. Troll W, Wiesner R and Frenkel K, Anticarcinogenic action of protease inhibitors. *Adv Cancer Res* **49**: 265–283, 1987.

* Corresponding author. Tel. (32) 3 820 25 73; FAX (32) 3 820 25 90.