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CHLORPROMAZINE INHIBITS BOTH THE CONSTITUTIVE NITRIC OXIDE SYNTHASE AND THE INDUCTION OF NITRIC OXIDE SYNTHASE AFTER LPS CHALLENGE

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The effects of chlorpromazine on either the activity of mouse brain nitric oxide synthase or the induction of lung nitric oxide synthase in mice and rats were studied.
Chlorpromazine inhibited the nitric oxide synthase activity in mouse brain cytosol. This effect could be reversed by adding an excess of calmodulin. In addition, chlorpromazine was able to inhibit the induction of lung nitric oxide synthase, in both species, after LPS administration. Furthermore, chlorpromazine also inhibited arginase activity in mouse lung cytosol.

Many of the changes occuring during endotoxic shock, such as hypotension, hyporeactivity to vasoconstrictor agents, vascular damage and disseminated intravascular coagulation leading to multiple organ failure and death, have been associated with the initial release of tumor necrosis factor alpha (TNF-α), since pretreatment with anti-TNFα antibodies abolished the hypotensive action of endotoxin and protects against its lethal effects (1).
It has also been demostrated that nitric oxide (NO) derived from L-arginine is a principal mediator of the TNF-induced shock in dog, since N^G-monomethyl-L-arginine

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(L-NMMA), a specific inhibitor of NO synthesis can restore he blood pressure to baseline (2).

Nitric oxide can be synthesized from L-arginine from L-arginine by at least two different NO synthases. The constitutive NO synthase (cNO synthase) is Ca^{+2}-dependent and the NO released by this type of enzyme activates the soluble guanylate cyclase, resulting in maintenance of the dilator tone in blood vessels as well as many other physiological effects (3). The other isoenzyme (iNO synthase) can be induced by immunological stimuli such as lipopolysccharide and cytokines (4). This iNO synthase, once expressed, generates NO for long periods and may account, at least in part, for the lethal effects of endotoxin. Because the dual role of NO as a physiological mediator and an effector cytotoxic radical during the endotoxic shock, the inhibition of both the cNO synthase and the iNO synthase during endotoxaemia. could be deleterious (5,6). In this study we have investigated whether the protective effect of chlorpromazine (7) against endotoxic shock is related to the induction of the iNO synthase and whether inhibition of either iNO synthase or cNO synthase could be involved in this protection.

Materials and Methods

Materials. Tricholoroacetic acid-extracted lipopolysaccharide (LPS) from *Salmonella typhimurium* was used. N^G-monomethyl-L-arginine (Wellcome Research Laboratories), L-[$U^{14}C$]-arginine and L-[$G^{14}C$] arginine (Amersham, UK), chlorpromazine hydrochloride and other chemicals (Sigma) were obtained as indicated.

Animals and treatments. Sprague Dawley rats were used from CENPALB, Havana. BAlb/c mice were from Charles River, UK. For the direct effect of chlorpromazine upon the cNO synthase, rat brain cytosol was used and for the iNO synthase, rat lung cytosol was obtained from rats 6 hours after treatment with LPS. The tissues were removed, freeze-clamped in liquid nitrogen and kept at -70 C.

Two groups of male mice (20 g) were treated with 20 mg/Kg LPS, one of which also received 4 mg/kg of chlorpromazine 15 minutes before the administraion of LPS. Three groups of male rats (200-300 g) were treated with 4 mg/kg of LPS, one of which was also treated with 4 mg/kg and another with 12 mg/kg of chlorpromazine.

Animals were killed at appropiated times for maximal induction of the enzyme in the lungs of both species: for mice 16 hours and for rats 6 hours after LPS

administration. In all cases both chlorpromazine and LPS were administered intraperitoneally. Control groups receiving chlorpromazine alone were also done in both species.

NO synthase activity. Ca^{+2}-dependent and Ca^{+2}-independent NO synthase activities were measured by ^{14}C-L-arginine conversion to ^{14}C-Citrulline as previously described (8), in cytosol (150 000 xg supernatants) from lungs and brains and calculated from the difference between control incubations and those containing 1mM EGTA (to remove free Ca) or 1 mM EGTA plus L-NNMA to inhibit all NO synthase activity. Some assays of NO synthase were also carried out by determination of L-NNMA-inhibitable oxyhemoglobin oxidation (8) in the presence or absence of EGTA.

Arginase activity. This was measured by the amount of radiolabelled urea formed from [G^{14}C]-arginine (9). Briefly, 20 ul of 100 000 x g supernatant from homogenized tissues was incubated for 1 hour at 37 C in 100 ul of pre-warmed buffer consisting of 12,5 mM MgCl2, 94 mM Hepes, 156 mM KCL at pH 7.5, 1.2 mM L-arginine and [G 14C] arginine. The reactions were stopped by adding 500 ul of 250 mM acetic acid, 7M urea, 10 mM L-arginine, 0.001 % methyl red, then 1 ml of 1:1 (v/v) Dowex 50W (200-400 mesh, H^+ form) in water was added. After 3 min of centrifugation (10 000 xg), 600 ul of supernatants were taken for liquid scintillation counting.

Determination of plasma nitrate. Plasma samples, taken at time of death, were ultracentrifuged (100 000 x g, 30 min) and nitrate was reduced to nitrite using nitrate reductase, before measurement of nitrite formed by chemiluminescence (10). Briefly, samples were injected into a reaction vessel containing refluxing 6 % aqueous iodide/glacial acetic acid (1:5, v/v). Under these conditions, nitrite was reduced to NO and removed in the gas phase by a constant stream of nitrogen. This was then mixed with ozone to form a chemiluminescent product measured in a detector coupled to an electronic integrator. The area under the peak of the chemiluminescent signal is proportional to the amount of nitrite in the samples, as calibrated using a standard solution of $NaNO_2$. Linearity was in the range of 5 to 100 uM.

Results and Discussion

In vitro effect of chlorpromazine on the formation of NO and citrulline by the constitutive and the inducible NO synthase.

Chlorpromazine inhibited NO formation by cNO synthase in rat brain cytosol, measured either by citrulline formation (Fig. 1A) or haemoglobin oxidation assays (data not shown). Chlorpromazine had no effect on the iNO synthase from rat lung cytosol in the range of

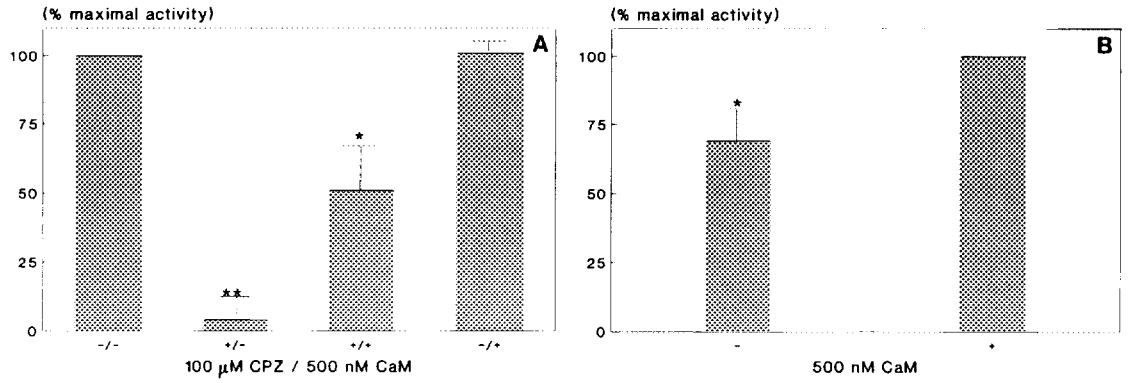

Fig. 1. NO synthase activity in rat brain cytosol, measured by citrulline formation and expressed as percentage of maximal activation. 1A, in vitro addition of 100 μM chlorpromazine in the presence or absence of 500 nM calmodulin (n=3). 1B, ex vivo effect of 500 nM calmodulin in the brain NO synthase from animals treated with 12 mg/kg chlorpromazine i.p. (n=3). Results are means +/- SEM. * $p < 0.05$, ** $p < 0.01$ by Student's t test.

concentrations between 1nM and 100uM in either assay (data not shown). The cNO synthase is a Ca^{+2} calmodulin-dependent enzyme, and calmodulin inhibitors such as trifluoperazine can inhibit the formation of citrulline and NO by this enzyme (11) with an IC_{50} around 10 μM. This effect can be reversed by an excess of calmodulin (12). When 500 nM calmodulin was added to brain cytosol NO synthase in the presence of an inhibitory concentration (100μM) of chlorpromazine, the inhibition was reversed by 54 % (Fig. 1A). The brain NO synthase from rats treated with 12 mg/kg chlorpromazine was inhibited by 25 % ex vivo, and this inhibition was also reversed by 500 mM calmodulin (Fig. 1B). Pharmacokinetic studies in rodents (13) have demostrated that the concentration of chlorpromazine in the brain is 40-fold higher than in blood. The concentration of chlorpromazine (approximately 10 μM) predicted to be present in brain at the dose used in these studies would be enough to inhibit the cNO synthase, but would not inhibit iNO synthase.

In vivo effect of chlorpromazine after LPS challenge. Clorpromazine inhibited the induction by LPS of NO synthase in the lungs of rats (Fig. 2) in a dose-dependent manner. Similarly in mice, chlorpromazine

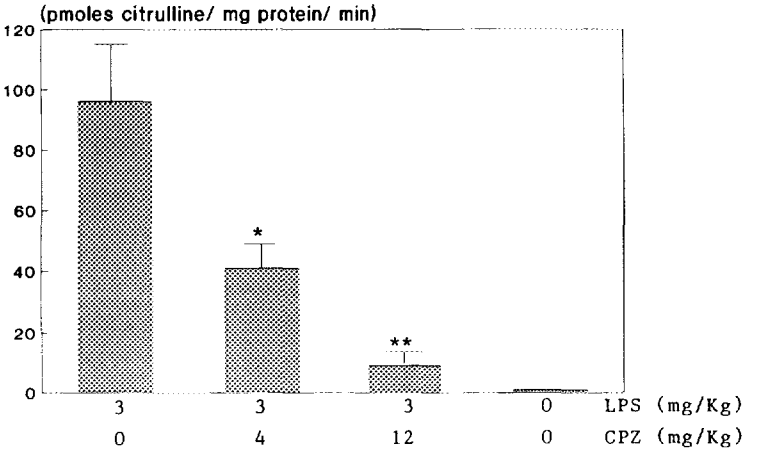

Fig. 2. Citrulline formation from inducible NO synthase in rat lung cytosol 6 hours after administration of 4 mg/kg i.p. and different doses of chlorpromazine given 15 min before LPS challenge (n=3) Results are means +/- SEM. * $p < 0.05$, ** $p < 0.01$ by Student's t test.

inhibited the induction of NO synthase in the lung after a lethal dose of LPS (Fig. 3). The plasma nitrite levels 16 hours after LPS administration in mice were also significantly reduced by chlorpromazine. The dose of chlorpromazine which protected animals against the lethal effect of LPS reduced the induction of NO synthase by 88 %. It has been demostrated that 4 mg/kg

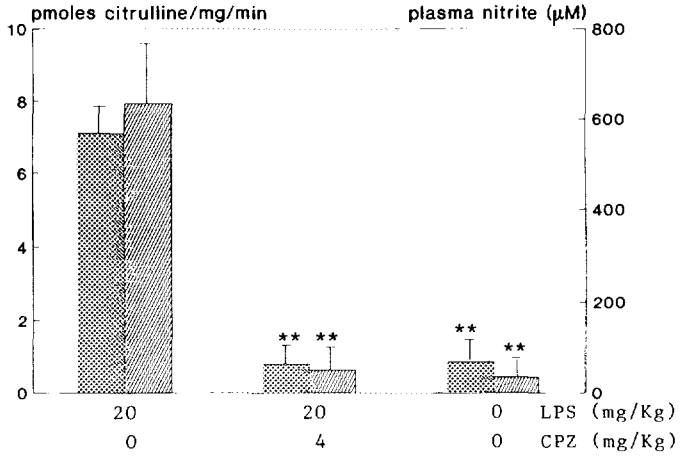

Fig. 3. Citrulline formation in lung cytosol(left-hand bars) and nitrite levels (right-hand bars) in plasma of mice after challenge with 20 mg/kg with and without 4 mg/kg i.p. Results are means +/- SEM (n=4). * $p < 0.05$, ** $p < 0.01$ by Student's t test.

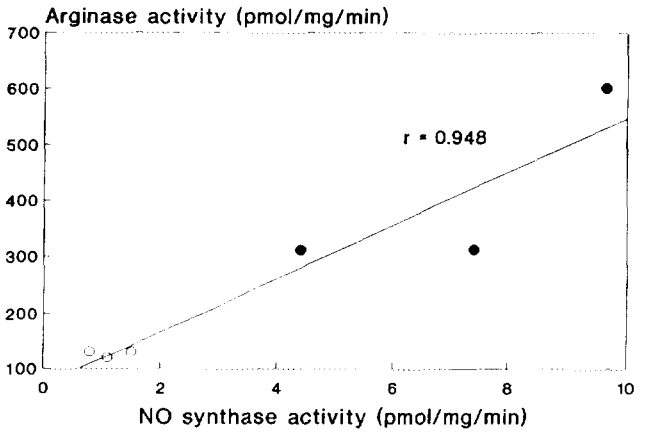

Fig. 4. Correlation between NO synthase and arginase activities in mouse lung cytosol. Enzyme activities after 16 hours of LPS challenge (closed circles). Enzyme activities of mice treated with chlorpromazine before LPS challenge (open circles).

chlorpromazine abolishes the rise in TNF levels in serum following LPS administration (7) and this may explain why the induction of NO synthase is inhibited. Furthermore, administration of 4 mg/kg chlorpromazine also inhibited the induction of arginase by LPS in mouse lung cytosol (Fig. 4).

This inhibition correlated with the inhibition of iNOS induction in the same cytosols (Fig. 4). Thus, chlorpromazine inhibits the induction of two distinct enzymes by LPS. Because these enzymes compete for the same substrate, L-arginine, it will be important to find out whether both enzymes are induced in the same cells or regions of the lung; the functional consequences of their co-induction are not known at present.

These findings may represent new ligths into the protective effects of chlorpromazine in endotoxic shock.

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