SPECIAL REPORT

Inhibition by sodium nitroprusside of the expression of inducible nitric oxide synthase in rat neutrophils

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A well-known nitric oxide (NO)-releasing compound, sodium nitroprusside (SNP), decreases in a dose-dependent manner NO synthase (NOS) activity induced in rat neutrophils by treatment with lipopolysaccharide (LPS). This inhibitory action of SNP seems not to be due to its direct effect on the enzyme activity. The strong nitrosonium ion (NO⁺) character of SNP could be responsible for its inhibition of NOS induction in neutrophils.

Keywords: Nitric oxide; nitric oxide synthase; sodium nitroprusside; neutrophil; nitrite/nitrate

Introduction The expression of inducible nitric oxide synthase (iNOS), present in various cells including macrophages, neutrophils and hepatocytes, is induced by lipopolysaccharide (LPS) and some lymphokines such as interferon gamma. The promoter region of iNOS gene has been shown to contain a number of regulatory sequences. Recently a feedback inhibition of NOS by NO[•] has been also reported in the macrophage (Assreuy *et al.*, 1993). There is little information available on a possible effect of NO on the induction of iNOS gene expression.

In this work we present data suggesting that a NO-releasing compound, sodium nitroprusside (SNP), acts as an inhibitor of iNOS expression in neutrophils of LPS-treated rats.

Methods *Animal treatment* Female Sprague-Dawley rats (Charles River, Italy) weighing 220–240 g, which had been fasted overnight but allowed free access to water, were treated with SNP (1, 5 and 10 mg kg⁻¹ body weight) suspended in saline solution and administered orally in a volume of 1 ml 100 g⁻¹ body weight; control rats received the vehicle. LPS (5 mg kg⁻¹ body weight) was given via the tail vein 1 h after the drug administration. Four hours later, rats were anaesthetized with sodium pentobarbitone (60 mg kg⁻¹ body weight) i.p. and blood samples were collected in EDTA-containing tubes.

Separation of neutrophils Neutrophils were separated by Ficoll-Hypaque density gradient centrifugation and erythrocytes removed by dextran sedimentation followed by lysis with NaCl 0.2% and then with NaCl 1.6%. Cell viability was 97%, as determined by Trypan blue exclusion, and the suspension contained less than 2% contaminating leukocytes.

Assay of NOS activity in the neutrophils NOS activity was estimated by measuring the conversion of L-[2,3,4,5-³H]-arginine to L-[2,3,4,5-³H]-citrulline according to a modification of the method described by Bredt & Snyder (1990).

Protein determination Protein concentration was measured by the method of Bradford (1976).

Quantitation of nitrite/nitrate Nitrite was assayed colorimetrically after reaction with the Griess reagent (Green *et al.*,

1982); nitrate was reduced to nitrite using a bacterial nitrate reductase according to a modification of the method described by Bartholomew (1984).

Chemicals L-[2,3,4,5-³H]-arginine monohydrochloride (specific activity: 60 Ci/mmol⁻¹; 1 Ci = 37 GBq) was supplied by Amersham Life Science. (6R)-5,6,7,8-tetrahydro-1-biopterin was purchased from Dr B. Schircks Lab., Jona, Switzerland. Lipopolysaccharide from *E. coli* (serotype 026:B6) and sodium nitroprusside were Sigma products.

Statistical analysis The statistical analysis of data was performed by one-way analysis of variance followed by Dunnett's multicomparison test. *P* < 0.05 or less was considered as indicative of significance.

Results As shown in Figure 1, in control rats NOS activity was not detectable under our assay conditions. LPS treat-

Figure 1 Changes in nitric oxide synthase (NOS) activity in rat neutrophils after treatment with sodium nitroprusside (SNP) or lipopolysaccharide (LPS) or both. The values are expressed as mean $\pm$ s.e. (*n* = 6). **P* < 0.005 significance compared to values obtained from LPS-treated rats.

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ment induced a consistent increase of NOS activity in purified neutrophils. The co-administration to rats of LPS and SNP caused a dose-dependent decrease of NOS activity, while SNP alone did not induce NOS activity. SNP, at least up to a concentration of 100 μM , had no effect on the neutrophil NOS activity under the assay conditions used in this work (data not shown).

Plasma $\text{NO}_2^-/\text{NO}_3^-$ levels are shown in Figure 2. LPS administration produced a 7 fold increase of $\text{NO}_2^-/\text{NO}_3^-$ concentrations. No significant changes were observed in rats treated with SNP before LPS administration. On the contrary, plasma $\text{NO}_2^-/\text{NO}_3^-$ levels increased in a dose-dependent manner in rats to which only SNP was administered.

Discussion In the present study, SNP has been shown to inhibit, in a dose-dependent manner, neutrophil NOS activity induced by LPS. In animals treated with SNP alone, no modification in NOS activity could be detected, while a dose-dependent increase in plasma $\text{NO}_2^-/\text{NO}_3^-$ levels was observed, suggesting the gastrointestinal absorption of the drug. The decrease in NOS activity in neutrophils of rats co-treated with LPS and various doses of SNP should not be due to a direct inhibitory effect of the drug on NOS, since 100 μM SNP added to the assay mixture did not affect the neutrophil NOS activity (data not shown). This is in contrast with the data of Assreuy *et al.* (1993), that suggest a direct inhibitory effect of NO-releasing compounds on macrophage NOS. The results reported here raise the question how SNP could decrease NOS activity in the neutrophils. A possible answer could be found in a recent paper of Lipton *et al.* (1993) on the different effects shown by nitrogen monoxide when it was used in different redox states. SNP is a nitroso-compound with a strong nitrosonium ion (NO^+) character. Although it can also readily generate $\text{NO}^\bullet$, a reductive activation is required for this. An increased level of NOS activity induced by LPS treatment and the consequent increase of plasma $\text{NO}_2^-/\text{NO}_3^-$ concentration suggest that endogenous NO did not exert, at least under our experimental conditions, an inhibitory effect on the induction of neutrophil NOS. In this context, the data presented here seem to support the hypothesis that NO exogenously released from SNP could act because of its strong NO^+ character, as an inhibitory agent on iNOS expression, while endogenous NO might not

Figure 2 Plasma $\text{NO}_2^-/\text{NO}_3^-$ levels in rats to which lipopolysaccharide (LPS) and sodium nitroprusside (SNP) had been administered. The values are expressed as mean $\pm$ s.e. ($n = 6$). * $P < 0.005$ significance compared to control rats.

have a significant effect on this event. The absence of significant differences between plasma $\text{NO}_2^-/\text{NO}_3^-$ levels in rats treated with LPS alone and in those treated also with the drug could be explained in the following two ways: (a) a decrease in endogenous $\text{NO}^\bullet$ production due to an inhibition of iNOS expression in the neutrophils caused by NO^+ which derives from SNP or, (b) an increase in $\text{NO}^\bullet$ plasma concentration, which could be released directly from SNP under appropriate physiological conditions.

In conclusion, the results obtained by us in an *in vivo* system suggest an inhibitory action of the NO-releasing compound SNP on the iNOS expression in rat neutrophils; this inhibitory action should be ascribed to a strong nitrosonium ion character.

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(Received November 21, 1994
Accepted December 15, 1994)