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Effect of Septicaemia on the Plasma Levels of Biopterin and Nitric Oxide Metabolites in Rats and Rabbits

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ABSTRACT. Live *Escherichia coli* decreased mean arterial blood pressure in rabbits from 67 to 20 mmHg. *E. coli* did not affect blood pressure in rats but did significantly increase heart rate by 29%. To relate the cardiovascular effects with putative relevant biochemical pathways, the plasma levels of nitrate + nitrite (NOx) and biopterin, representing the main metabolites of nitric oxide and tetrahydrobiopterin, respectively, were determined in conscious rats and rabbits after treatment with live *E. coli*. In rats, *E. coli* induced a rapid 43% increase in the plasma level of biopterin preceding the 7- to 26-fold increase in NOx level. In rabbits, no increase in the NOx level was observed despite a 3- to 5-fold increase in the biopterin level at 6–10 hr posttreatment. It is concluded that the synthesis of tetrahydrobiopterin precedes nitric oxide synthesis after induction of septicaemia in the rat. After the induction of septicaemia, rabbits show a clear hypotensive response and an increase in biopterin level but no concomitant increase in NOx. Biopterin apparently represents a more appropriate biochemical marker of septic shock than does NOx. BIOCHEM PHARMACOL 52;9:1447–1451, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. NO synthase; biopterin, tetrahydrobiopterin, nitric oxide; septic shock

Interest in NO‡ released from L-arginine through the action of NOS has increased exponentially since the discovery that NO is identical with EDRF and responsible for the maintenance of vascular tone, platelet inhibition and bronchodilatation. iNOS is induced within various pathophysiological conditions by factors such as LPS, γ-IFN, TNF and a combination of cytokines (for review, see [1, 2]).

These factors not only induce NOS but also GTP cyclohydrolase I (EC 3.5.4.16), the enzyme catalyzing the initial step of BH_4 synthesis [3–9]. A major role of cytokine-induced pteridine synthesis is to provide cells with the active cofactor BH_4 required for the NOS reaction [10, 11]. This notion is supported by the observation that the biosynthesis of both NO and neopterin are inhibited by corticosteroids [12] and the GTP-cyclohydrolase I inhibitor DAHP [13].

Both neopterin and biopterin are metabolites of GTP, the substrate of the inducible enzyme GTP-cyclohydrolase. Depending on the species, an increase in the plasma level of biopterin or neopterin reflects the induction of BH_4 synthesis. In nonprimate mammals, BH_4 is metabolized to dihydrobiopterin and biopterin; in humans, the induction of GTP-cyclohydrolase I leads to an increase in the plasma neopterin level [14]. Enhanced neopterin serum levels have been observed in patients with bacterial [14] or viral [15] infection, but by far the most excessive enhancement of neopterin synthesis was observed in patients with septic shock syndrome [16, 17]. In addition, plasma levels of NO metabolites are elevated in septic patients [18] and in rodents (including urine) after treatment with *Escherichia coli* or LPS [19–24]. An increase in the urinary nitrate level was recently shown to be a sensitive indicator of NOS activity *in vivo* [24].

The aim of this study was first to determine whether the production of BH_4 would precede the enhancement in NO production in two species differing considerably in sensitivity to septic stimuli, and second whether plasma levels of biopterin and NOx are useful parameters to follow the course of septic shock in the selected species.

Materials and Methods

Cardiovascular Measurements and Blood Sampling

Blood pressure in Wistar rats (175–250 g) was performed after heparinization (1000 IE/kg, i.p.), anaesthesia with urethane (1.5 g/kg i.p.) and intravenous (i.v.) treatment with live *E. coli* at a dose of 15×10^9 CFU/kg. Elco rabbits

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‡ *Abbreviations:* NOx, nitrate + nitrite; NO, nitric oxide; iNOS, inducible NO synthase; EDRF, endothelium-derived relaxation factor; LPS, lipopolysaccharide; BH_4, tetrahydrobiopterin; DAHP, 2,4-diamino-6-hydroxypyridine; GTP, guanosine triphosphate; L-NMMA, N^G-monomethyl-L-arginine; γ-IFN, gamma-interferon; TMF, tumor necrosis factor.

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(200–300 g) were anaesthetised with initial intramuscular (i.m.) injections of 20 mg/kg ketamine and 6 mg/kg xylazine (repeated after 15 min), followed by 20 mg/kg every half hour and 6 mg/kg xylazine every hour. Rabbits were treated with 12×10^9 CFU/kg i.v. live *E. coli*. Left carotis artery (rat) and femoral artery (rabbit) were cannulated and blood pressure recorded by a cardiotachometer. Heart rate (beats/min; bpm) was derived from the blood pressure signal.

The time course of septic shock was studied in rats and in rabbits (both conscious) after i.v. treatment with live *E. coli* (24×10^9 and 2.4×10^9 CFU/kg, respectively; doses producing 75–100% are lethal within 24 hr). Six animals were used per time point, and blood was obtained once at the time point indicated after heparinisation (1000 IE/kg, i.p.) by venapuncture (rats) or heart punction under anesthesia (rabbits, thiopental 25 mg/kg i.v.). After centrifugation (1500*g*, 5 min), plasma samples were frozen and stored at −20°C. Animals treated with saline and killed at time point 0 hr served as the control group.

Reduction of Plasma Nitrate to Nitrite

In the circulation, NO is metabolised to nitrite and nitrate. To allow its assay as Griess chromophore, nitrate was reduced to nitrite by *Klebsiella pneumoniae* as previously described [25]. Because the method refers to reduction of urinary nitrate, it was validated for plasma nitrate by using pooled plasma spiked with increasing amounts of nitrate. Bacteria were grown at 30°C in stagnant culture in air on Oxoid bovine broth supplemented with 30 mM KNO_3 and 5 μM ammonium molybdate as described elsewhere [25]. After washing, cells were resuspended in phosphate buffered saline and stored at −70°C. Activity is retained for at least 12 months.

Plasma samples in a volume of 0.2 mL were deproteinised with 20 μL 1 M NaOH and 20 μL 1 M $ZnSO_4$. After standing for 15 min on ice, samples were centrifuged (5 min, 24,000*g*), and 100 μL of the supernatant was incubated with *K. pneumoniae* (0.3 mg/mL protein) suspended in 50 μL TES buffer (80 mM, pH 7.0) containing 80 mM sodium formate for 30 min *in vacuo* at room temperature [25]. After adding 0.5 mL of water, samples were centrifuged at 24,000*g* for 5 min, and nitrite in supernatant was assayed by absorbance of the Griess chromophore. The sum of nitrite originally present and that obtained after reduction of nitrate is designated as NOx.

Radioimmunoassay of Biopterin

Total plasma biopterin was assayed after acidic oxidation of BH_4 and dihydrobiopterin to biopterin [26]. Thus, 100-μL samples and standards were mixed with 20 μL MnO_2 suspension (400 mg/mL of 1 M HCl) and shaken for 15 min in the dark. After centrifugation at 10,000*g* (5 min), the pH of the supernatant was adjusted to 6.5, and total biopterin was determined by radioimmunoassay using the double antibody technique (Henning GmbH, Berlin, Germany). Precision of the assay was 3–5% and the detection limit was 3 nM.

Materials

Escherichia coli 055:B5 (ATCC 12014) was suspended in pyrogen-free saline containing 20% glycerol. N-tris-[hydroxymethyl]methyl-2-aminoethane sulfonic acid, N-[1-naphthyl]-ethylenediamine, bovine serum albumin (fraction V) and sulphanylamide were obtained from Sigma (St. Louis, MO, USA) and heparin from LEO (Weesp, The Netherlands). All other chemicals were of analytical grade.

Statistical Analysis

Values are expressed as means ± SEM. Level of significance was evaluated by analysis of variance. $P < 0.05$ was considered significant.

Results

Table I shows that *E. coli* did not decrease mean arterial blood pressure in rats but did significantly increase heart rate to 129% of basal level at 4 hr posttreatment. In rabbits, *E. coli* strongly decreased mean arterial blood pressure from

TABLE 1. Cardiovascular effects of *E. coli* in rats and rabbits

	Mean Arterial Pressure (mmHg)		Heart Rate (bpm)	
	Predose	Posttreatment	Predose	Posttreatment
Rat				
Saline	85 ± 5	84 ± 2	367 ± 16	390 ± 14
E. coli	90 ± 4	95 ± 9$^{N.S.}$	381 ± 20	490 ± 12*
Rabbit				
Vehicle	78 ± 4	51 ± 4$^{N.S.}$	162 ± 27	183 ± 20$^{N.S.}$
E. coli	67 ± 4	20 ± 7**	53 ± 8	231 ± 13**

Cardiovascular responses refer to 4 hr posttreatment for rats (15×10^9 CFU/kg, i.v.) and 3 hr posttreatment for rabbits (12×10^9 CFU/kg, i.v.). N = 5–8. N.S., not significant; *$P < 0.05$ compared with saline at 4 hr and *E. coli* at t = 0 hr; **$P < 0.001$ compared with predose value.

67 to 20 mmHg within 3 hr. As in rats, a compensatory 50% increase in heart rate was observed.

To relate the modulatory effects of *E. coli* on the cardiovascular system with putative relevant biochemical mediators, the time course of the *E. coli*-induced increase in the plasma level of total biopterin and NOx (representing the sum of the main metabolites of NO nitrate + nitrite) was determined in conscious rats and rabbits. The results (Figs. 1, 2) show that treatment of rats with live *E. coli* induced a significant and rapid (within 1–3 hr) but modest (+43%) initial increase in the biopterin level, which gradually rose further to become maximal at 5 hr posttreatment. The maximal increase of 76% is, however, not statistically significant. Over the same time span, the plasma NOx level increased although with some delay; the increase in the NOx level reached a statistically significant level at 5 hr posttreatment and steeply increased thereafter. At 6 and 10 hr posttreatment, the NOx plasma level was increased 7 and 26 times, respectively.

In rabbits and rats, baseline plasma levels of biopterin and NOx were roughly the same: 239 and 224 nM (biopterin) and 61 and 34 μM (NOx). However, in rabbits the pattern of *E. coli*-induced increase in biopterin and NOx levels was quite different compared with rats (cf. Fig 2). In rabbits, *E. coli* induced a large increase in the plasma biopterin level, with a lag time of approximately 5 hr, which at 10 hr posttreatment had increased fivefold. In the same period, the plasma NOx level hardly increased (nonsignificant increase of 38%). Similarly, the plasma NOx level did not increase after treatment of anaesthetised rabbits with 700 μg/kg, i.v. LPS (increase from 55 to 76 μM at 2–6 hr posttreatment; not shown).

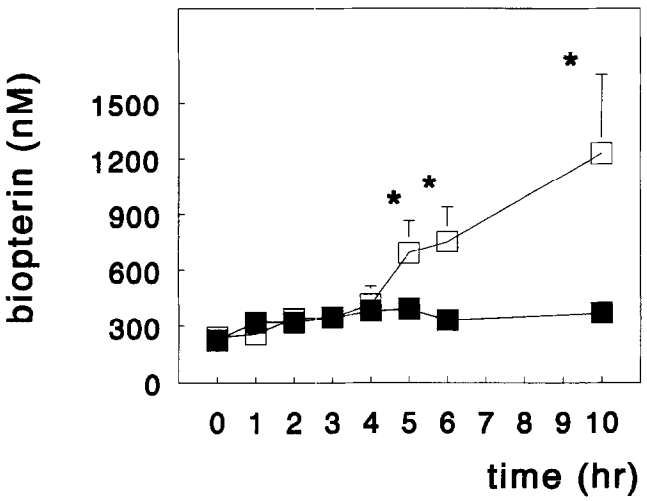

FIG. 1. Total biopterin plasma level (mean ± SEM) of conscious rats (closed symbols) and rabbits (open symbols) treated with *E. coli* at a dose of 24 × 10^9 and 2.4 × 10^9 CFU/kg i.v., respectively, with six animals per time point. Two rabbits died at t > 6 hr and were not evaluated. In rats, there was a significant increase at t = 3 and 4 hr. *P < 0.05 compared with the level at t = 0 hr.

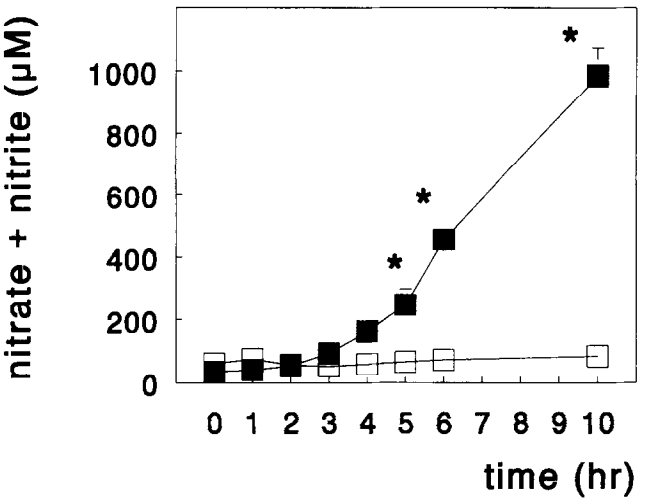

FIG. 2. NOx plasma level (μM ± SEM) of conscious rats (closed symbols) and rabbits (open symbols) treated with *E. coli* at a dose of 24 × 10^9 and 2.4 × 10^9 CFU/kg i.v., respectively, with six animals per time point. Two rabbits died at t > 6 hr and were not evaluated. *P < 0.05 compared with the level at t = 0 hr.

Discussion

Expression of iNOS has been suggested to be responsible for hypotension and hyporesponsiveness during septic shock [27, 28]. Inhibition of iNOS by L-NMMA reverses these symptoms in rodents and improves the clinical condition of septic patients [29]. In addition, *in vitro* studies have shown that inhibition of *de novo* BH_4 biosynthesis reduced iNOS activity in cytokine-stimulated human fibroblasts [13], vascular smooth cells [31], endothelial cells [32, 33] and murine macrophages [34], suggesting that induction of NO synthesis is limited by BH_4 availability [13]. To determine whether *de novo* BH_4 synthesis is required for and precedes increased NO synthesis by iNOS in septic shock syndrome, the time course of the plasma level of NOx and total biopterin in response to live *E. coli* was determined in two species (rats and rabbits) differing considerably in sensitivity to septic stimuli.

Escherichia coli induces a profound decrease in mean arterial pressure in rabbits (cf. Table I) but not in rats. In the rat, however, heart rate increases by 50%, probably to compensate for a decrease in blood pressure. Septic stimuli have been reported to decrease blood pressure in the rat.

In agreement with previous studies, *E. coli* increases the NOx level in the rat [21–23]. In the rabbit, however, at 3 hr posttreatment, mean arterial blood pressure declined to 30% of predose value, whereas the plasma NOx level was and remained unaffected up to 10 hr posttreatment. This observation does not rule out a functional role for NO, which retains vasodilating properties [1, 2], but it does clearly show that plasma NOx is not an appropriate parameter to study the course of septicaemia, at least not in rabbits. In addition, this observation shows that in the two species different effects of *E. coli* on the NOx level are

obtained. Leaf *et al.* [35] showed that LPS treatment of rats, but not of ferrets, induced an increase in NO production. Furthermore, Schneemann *et al.* [36] showed that, in contrast with mice and rats, resident and elicited monocytes/macrophages from rabbits (and humans) do not secrete nitrite upon *in vitro* stimulation with LPS. Assuming that monocytes/macrophages are the main source of NOx in the circulation, this observation is consistent with the difference between rats and rabbits regarding the NOx response *in vivo*. However, the increase in the total biopterin plasma level induced by *E. coli* suggests that monocytes/macrophages are actually activated in rabbits.

In rats, *E. coli* increases both total biopterin and NOx levels. Werner-Felmayer *et al.* [37] observed a similar increase in the plasma level of NOx at 7 hr after induction of septicaemia by using LPS (6 mg/kg, i.v.). BH_4 might be kept effectively within the tissues because levels of biopterin were increased in tissues but not in plasma.

Although the time course obtained in *E. coli*-treated rats shows that the production of BH_4 precedes the production of NO, the results obtained in the rabbits clearly demonstrate that a relation between stimulation of GTP-cyclohydrolase I and NOS cannot be easily established by using the level of typical metabolites such as biopterin and NOx in plasma. Considering the increase in biopterin, which coincides with the decrease in blood pressure, biopterin may represent a more appropriate parameter than NOx to study the course of experimental septic shock. By analogy, determination of the plasma neopterin level will provide a valuable tool in monitoring the development of septic shock syndrome in humans.

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