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# CONTRIBUTIONS OF NO SYNTHASE AND HEME OXYGENASE TO cGMP FORMATION BY CYTOKINE AND HEMIN TREATED BRAIN CAPILLARY ENDOTHELIAL CELLS

Paul VIGNE (1), Erick FEOLDE (1), Annie LADOUX (1), Danièle DUVAL (2) and Christian FRELIN* (1)

(1) Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, Université de Nice-Sophia Antipolis, 660 route des Lucioles, 06530 Valbonne, France

(2) Laboratoire de Chimie Physique Organique, Université de Nice-Sophia Antipolis, Parc Valrose 06034 Nice Cedex, France

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Two mechanisms contribute to cGMP formation by soluble guanylyl cyclase (i) NO production by NO synthase and (ii) CO production by heme oxygenase. We analyze here the contributions of these two pathways to IL1, TNF, lipopolysaccharide and hemin treated brain capillary endothelial cells. Cytokines and LPS induced cGMP formation in manners that were completely prevented by LY 83,583, methylene blue and by cyclosporin A. They were partially inhibited by inhibitors of NO synthase. Cyclosporin A acts by a posttranscriptional mechanism. Cells constitutively expressed mRNAs for heme oxygenase-1. Expression was enhanced by hemin but not by IL1 or lipopolysaccharide. Induction of heme oxygenase-1 and its inhibition by Sn protoporphyrin IX had no effect on cGMP levels. 

Cyclic GMP is well known to decrease the permeability of the blood brain barrier (BBB) (1,2). Its formation in cultured rat brain capillary endothelial cells (BCEC) which *in vivo* form the anatomical and functional basis of the BBB is controlled by ANPB receptors for natriuretic peptides (3) and by soluble guanylyl cyclase (sGC) (4), the natural target for NO produced by NO synthase (NOS) (5) and CO produced by heme oxygenase (HO) (6). Less is known about cGMP production under conditions of endotoxic or hemorrhagic shock that are associated to severe alterations of the BBB permeability. Brain inflammatory diseases are associated to local productions of IL1 and TNFα (7). Hemorrhagic shocks are associated to a perivascular accumulation of hemoglobin degradation products such as hemin (8). Knowing that, in other cell types, inflammatory cytokines and hemin are strong inducers of iNOS and HO-1 respectively (5,9), we analyze in this paper the regulation of the expressions of iNOS and HO-1 in cultured rat BCEC and their contributions to the formation of cGMP.

## MATERIALS AND METHODS

LPS (E. coli) and hemin were from Sigma. NG-monomethyl-L-arginine and NG-nitro-L-arginine were from Calbiochem. Recombinant hIL1 and hTNFα were from Roussel Uclaf

* Corresponding author . Fax: (33) 93 95 77 08.

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(Romainville, France) and Immunex (Biotrans, Los Angeles, CA) respectively. LY-83,583 was from RBI. Sn protoporphyrin IX was synthesized as described (10). Cyclosporin A (CsA) was from Sandoz.

Rat BCEC was prepared and grown as previously described (11). Cells were exposed to cytokines or LPS for 24 hours and then to 0.1 mM IBMX for 25 min.. Cellular cGMP was quantitated by radioimmunoassay (Amersham) and normalized to cell protein content (12). Dose response curves were fitted with the Sigma plot software.

Total RNA was isolated as previously described (13). RNA (20 μg) was submitted to denaturing electrophoresis on 1.3 % agarose formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell) prior to hybridization with a $^{32}P$ labelled rat HO-1 probe corresponding to nucleotides 436-595 of the coding sequence (9) or a GAPDH probe. Hybridization was performed at 42°C in a 30% formamide, 5X SSPE, 5X Denhardt's solution supplemented with 0.1 % SDS and 100 μg/ml denatured salmon sperm DNA. Blots were washed twice for 15 minutes in 2X SSPE at 42°C.

cDNA was synthesized from 1 μg of total RNA using the MMLV reverse transcriptase (GIBCO BRL, 200 units/assay) in the presence of 1 μg oligo dT, dNTPs (0.1 mM each), and 40 units RNasin (Promega). After a 5 min. denaturation at 85°C, dNTPs and the enzyme were added, the reaction was allowed to proceed for 1 h at 37°C and stopped by heating to 95°C for 5 min. Amplification was performed using 100 ng of iNOS primers, 400 μM dNTPs and 1 units of Goldstar DNA polymerase (Eurogentec) using a PHC-2 Techne cycler programmed with the following conditions: 3 min denaturation at 98°C followed by 10 cycles of 40 s at 94°C, 120 s at 62°C, 90 s at 72°C. $P_{2U}$ purinoceptor primers (100 ng) were then added and the reaction allowed to proceed for 30 additional cycles. The sense primer for $P_{2U}$ receptors was 5'-CACSTGCATMAGCGTGCA-3'. The antisens primer was 5'-CTCTACTTCCTGGCMGGG-3'. The sense primer for rat iNOS was 5'-ATGGCTTGCCCCTGGAAGTTTCTC-3'. The antisens primer for iNOS was 5'-CCTCTGATGGTGCCATCGGGCATCTG-3'. PCR products, resolved on an agarose gel, were stained with ethidium bromide. The identity of amplification products was confirmed by Southern blot analysis using rat iNOS and rat $P_{2U}$ probes and by partial sequencing.

## RESULTS AND DISCUSSION

IL1α, IL1β, TNFα and LPS increased cellular cGMP levels in BCEC (Table 1). Concentrations for half maximum stimulations were 0.2 ± 0.1 ng/ml for IL1α, 0.8 ± 0.3 ng/ml for IL1β and 20 ± 5 ng/ml for LPS. TNFα was only active at concentrations > 1 ng/ml. All these actions were potentiated by IBMX and were completely inhibited by LY-83,583 (IC50 = 0.1 μM) and by 0.1 mM methylene blue, two inhibitors of sGC. They were partially inhibited by 0.1 mM NG-monomethyl-L-arginine or by 0.1 mM NG-nitro-L-arginine (Table 1) and insensitive to 1 μM dexamethasone.

Table 1: Inflammatory cytokines and LPS increase cGMP levels

| Conditions | cGMP (pmol/mg of protein) | | |
|---|---|---|---|
| | -IBMX | +IBMX | +L-NMA |
| Control | 0.34 ± 0.03 | 1.81 ± 0.17 | |
| Na nitroprusside (0.1 mM) | 16.8 ± 3.4 | 49.5 ± 4.1 | |
| IL1α (10 ng/ml) | 2.32 ± 0.15 | 13.84 ± 1.81 | 9. 87 ± 1.01 |
| IL1β (10 ng/ml) | 5.98 ± 1.53 | 18.64 ± 2.50 | 13.42 ± 1.79 |
| LPS (50 μg/ml) | 2.51 ± 0.12 | 7.56 ± 1.79 | 4.34 ± 1.02 |
| TNFα (50 ng/ml) | 0.90 ± 0.07 | 3.26 ± 0.27 | 2.39 ± 0.19 |

IBMX and L-NMA were used at 0.1 mM. Means ± SE (n = 3-20) are indicated.

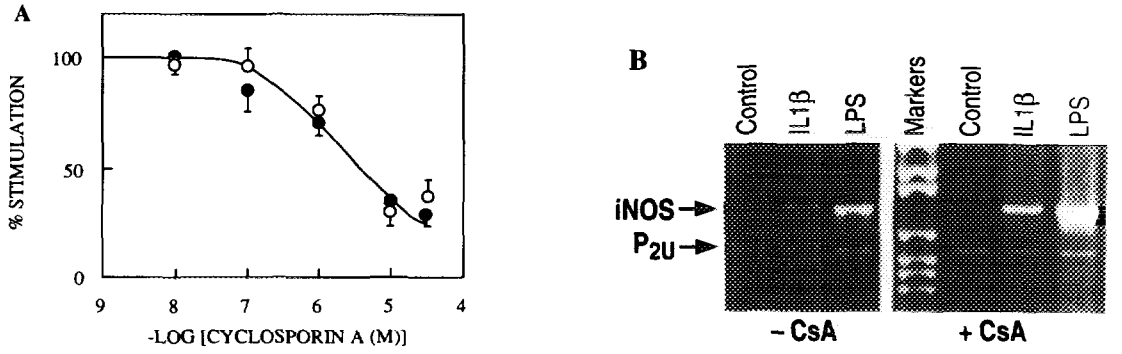


Figure 1. Cyclosporin A inhibits IL1 and LPS induced formation of cGMP.
Panel A: cGMP measurements. Cells were exposed to the indicated concentrations of CsA, 10 ng/ml IL1β (○) or 50 μg/ml LPS (●) for 24 hours and cGMP measured. Means ± SE (n > 3).
Panel B: Ethidium bromide stained agarose gel of PCR amplification products corresponding to iNOS and P2U purinoceptor (used as a control) sequences. Concentrations used were 30 ng/ml IL1, 50 μg/ml LPS and 30 μM CsA. Cells were exposed to agonists for 24 hours.

Figure 1A shows that CsA inhibited most of IL1 or LPS induced cGMP formations (IC50 = 2 μM). It had no effect on Na nitroprusside (0.1 mM) induced formation of cGMP indicating that it did not decrease sGC activity. Figure 1B further presents the results of PCR experiments showing (i) that IL1 and LPS induced iNOS mRNA expression and (ii) that CsA had no effect or slightly enhanced it, hence suggesting a posttranscriptional inhibitory mechanism.

Figure 2 presents a typical Northern blot showing (i) that BCEC constitutively expressed low levels of HO-1 mRNA, (ii) that expression was strongly increased by its substrate hemin and (iii) that it was unaffected by IL1α, IL1β or TNFα. HO-1 expression was not accompanied by a production of cGMP. Sn protoporphyrin IX (1 μM), a potent inhibitor of HO-1 (6) had no action on basal, IL1β (10 ng/ml) or LPS (50 μg/ml) stimulated cGMP formation. Taken together, these suggested that, although BCEC can be induced to express HO-1, the enzyme does not contribute to cGMP formation.

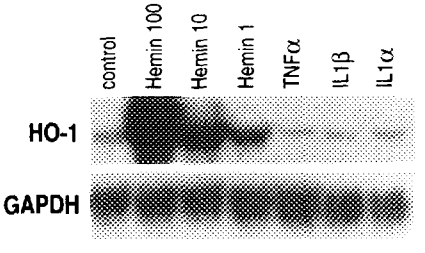


Figure 2. Regulation of HO-1 mRNA expression.
Cells were treated for 4 hours with 1, 10 or 100 μM hemin or for 24 hours with 10 ng/ml IL1 or 30 ng/ml TNF. The Northern blot was hybridized with a HO-1 probe (upper panel) or a GAPDH probe (lower panel) to control for loading errors.

In conclusion, rat BCEC can be induced to express iNOS (after treatment with inflammatory cytokines or LPS) and HO-1 (after treatment with hemin). Only expression of the former is accompanied by a measurable production of cGMP. Cytokine induced iNOS expression has been documented in a large variety of cells (5). Two features appear specific for the cells used. First, cGMP formation was only partially prevented by NOS inhibitors, which could suggest that NO was not the only activator of sGC produced in response to cytokines. Data presented clearly rule out however an involvement of CO in the action of cytokines. It could be that BCEC maintained high intracellular L-arginine concentrations that prevented the action of NOS inhibitors. In agreement, we observed that a sustained production of cGMP by BCEC did not require exogenous L-arginine. The poor sensitivity of BCEC to NOS inhibitors may further provide an explanation for the observation that in a rat model of experimental meningitis induced by LPS, NOS inhibitors only partially prevented the alterations in blood cerebrospinal fluid barrier (14). Obviously drugs such as LY 83,583 and CsA are probably better tools than NOS inhibitors to evaluate the role of the nitridergic pathway in brain inflammatory diseases. The second unique feature of rat BCEC is the fact that CsA did not inhibit iNOS mRNA expression as described in other cell types (15,16). This suggests that CsA inhibits the nitridergic pathway by more than one mechanism.

Cyclic GMP being a major permeabilizing effector of the BBB (1,2), it is tempting to propose that the actions described in this paper contribute to the alteration in the BBB observed in inflammatory brain diseases. They are probably of less importance in hemorrhagic diseases.

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