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cGMP 介导脑室注入 L-精氨酸诱导的大鼠血管加压素释放效应¹

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关键词 一氧化氮; 精氨酸加压素; 精氨酸; cGMP; 亚甲蓝; 鸟苷酸环化酶

目的: 探讨 cGMP 是否介导 L-精氨酸(一氧化氮合

酶底物)引起的血管加压素(AVP)释放增多效应。
方法: 用放射免疫法测定大鼠血浆中 AVP 水平。
结果: 侧脑室分别注射 L-精氨酸和 8-溴-cGMP (一种可透过膜的 cGMP 衍生物)能刺激血浆 AVP 水平增加[分别从(3.2 ± 0.5)升至(5.8 ± 1.4) ng·L⁻¹, 从(2.6 ± 0.3)升至(6.6 ± 0.4) ng·L⁻¹, P < 0.01], 同时注射 L-精氨酸和亚甲蓝(鸟苷酸环化酶抑制剂)对血浆 AVP 的水平没有影响。
结论: cGMP 介导 L-精氨酸引起的 AVP 释放增加效应。

Up-regulation of LPS-induced iNOS activity in dibutyryl cyclic AMP-differentiated rat astrocytes

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KEY WORDS lipopolysaccharides; nitric-oxide synthase; cyclic AMP; cell differentiation; up-regulation (Physiology); astrocytes; immunohistochemistry

AIM: To study the effect of dBcAMP on bacterial endotoxin LPS-induced NOS activity.
METHODS: Microscopic changes were observed. Nitrite levels were measured by fluorometric assay. NOS activity was measured by citrulline assay.
RESULTS: Within 3-4 h after the addition of dBcAMP 1 mmol·L⁻¹ to culture medium, a morphological transformation reminiscent of *in vivo* differentiation occurred. Coincubation with LPS and dBcAMP 1 mmol·L⁻¹ resulted in a marked increase in the nitrite production as compared with LPS alone. This increase was concentration- and time-dependent with a maximal effect after 24 h treatment. Nitrite production stimulated by LPS is parallel to the degree of cell differentiation. After a 24-h costimulation with LPS and dBcAMP, L-citrulline formation assay revealed a 3-fold increase in

NOS activity over LPS treatment alone. Simultaneous incubation with L-NAME, completely inhibited the stimulation effect of LPS/dBcAMP on nitrite production. Cycloheximide and dactinomycin also suppressed enhancement of NOS activity stimulated by LPS/dBcAMP, both in nitrite production and citrulline assay, indicating that the enhancement of NOS activity was due to the expression of inducible NOS (iNOS) gene and protein.
CONCLUSION: Inflammatory signals can trigger astrocytes to express substantially different levels of iNOS depending on their degree of differentiation.

NO, a potent and pleiotropic mediator of many biological functions, is synthesized from L-arginine by NOS. Three forms of NOS have been characterized^[1]. The constitutive endothelial and neuronal types are calcium-dependent. The third form of the enzyme is the calcium-independent inducible NOS (iNOS), which is only expressed in the presence of bacterial toxins or cytokines. This form of the enzyme produces high levels of NO which is used by the macrophage to kill tumor cells as well as invasive microorganisms including bacteria and viruses^[2]. The excessive production of NO by iNOS has also been implicated in the

Abbreviations used: cAMP = cyclic AMP; dBcAMP = N⁶,2'-O-dibutyryl cyclic AMP; GFAP = glial fibrillary acidic protein; LPS = lipopolysaccharide; L-NAME = N^G-nitro-L-arginine methyl ester; NO = nitric oxide; NOS = nitric-oxide synthase; PBS = phosphate-buffered saline.

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pathogenesis and tissue destruction of a growing number of immunologic and inflammatory diseases, including neurodegenerative disease^[3,4]. Recent *in vitro* studies demonstrated that astrocytes, known to play a prominent role in the initiation and maintenance of immunoinflammatory responses in the central nervous system, could also be induced by LPS or combinations of the cytokines to express an iNOS which was similar to those in other cell types^[5].

The incorporation of dBcAMP into the culture medium resulted in a pronounced morphological and functional differentiation which resembled the mature astrocytes found *in vivo*^[6], providing a useful system to study differentiation-correlated changes of astrocytes *in vivo*. The present study was to examine the difference of LPS-stimulated iNOS activity between undifferentiated and differentiated astrocytes.

MATERIALS AND METHODS

Materials Newborn Sprague-Dawley rats (1-day-old, grade II, Certificate No 02-49-2) were provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences. Dulbecco's modified Eagle's medium, F-12 nutrient mixture, fetal bovine serum, and tissue culture plastics were purchased from Gibco-BRL. *L*-[2, 3, 4, 5-³H] arginine was purchased from Amersham. *L*-NAME, cycloheximide, actinomycin D, dBcAMP, LPS (*E. Coli* 055 : B5), Dowex-50W, monoclonal antibodies against GFAP were obtained from Sigma. The ABC immunocytochemical Kit was from Sino-American Biotechnology Company (Shanghai, China).

Astrocyte cultures Cultures of rat cortical type I astrocytes were obtained^[7]. The resultant cell suspension was filtered through Nitex mesh (40 μm) and plated in a 75-cm² flask. Before experiment, cultures were processed by trypsinization and replanted in 12-well plates at a seeding of 5×10^5 cells/well. These treatments yielded a highly purified culture of astrocytes, consisting of > 98 % astrocytes, as determined by immunostaining for the astrocyte-specific marker for GFAP.

Morphological observation and immunocytochemistry Changes in cell morphology were assessed by microscopic examination. The number of stellate astrocytes (rounded cell bodies

and several extended processes) was quantified under phase-contrast microscopy^[8]. Some of the cultures were fixed with 4 % paraformaldehyde, permeabilized with 0.2 % Triton X-100 and used for immunocytochemical examination. After overnight incubation at 4 °C with monoclonal antibodies to GFAP, cells were stained with an ABC Kit. Differentiation of astrocytes was quantified by calculating the percentage of stellate cells^[8].

Nitrite assay After the desired incubation time, media were assayed for nitrite by the fluorometric assay which based upon the reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form the fluorescent product, 1-(H)-naphthotriazole. In brief, to 500 μL of sample, an equal volume of double-deionized water was added. To this, 100 μL of freshly prepared DAN ($0.05 \text{ g} \cdot \text{L}^{-1}$ in $\text{HCl } 0.62 \text{ mol} \cdot \text{L}^{-1}$) was added. After a 10-min incubation at 20 °C, the reaction was terminated with 50 μL of $\text{NaOH } 2.8 \text{ mol} \cdot \text{L}^{-1}$. Formation of 1-(H)-naphthotriazole was measured using a fluorescence spectrophotometer (650-10 S, Hitachi, Japan) at $\lambda_{\text{ex}} = 365 \text{ nm}$ and $\lambda_{\text{em}} = 450 \text{ nm}$ with a gain setting at 100 %. Fresh culture media was used as the blank and solution of sodium nitrite was used as standard. The detection limit for nitrite measurement in culture medium was $0.1 \mu\text{mol} \cdot \text{L}^{-1}$.

Citrulline assay NOS activity was measured directly by production of *L*-[³H]citrulline from *L*-[³H] arginine^[5]. In brief, cells cultured in 35-mm Petri dish were washed 3 times with PBS. After equilibration, the cells were incubated in the presence of PBS 0.5 mL with *L*-[2, 3, 4, 5-³H] arginine monohydrochloride $74 \text{ MBq} \cdot \text{L}^{-1}$ at 37 °C for 30 min. The reaction was halted by adding 1 mL of perchloric acid $0.3 \text{ mol} \cdot \text{L}^{-1}$. Cell scrapings were neutralized with 250 μL of $\text{K}_2\text{CO}_3 \text{ } 1 \text{ mol} \cdot \text{L}^{-1}$, and centrifuged at $1400 \times g$ for 10 min. The supernatant 0.5 mL was applied to a 1 mL Dowex AG 50WX-8 column (Na^+ form, 100 - 200 mesh). *L*-[³H] arginine was retained on the Dowex resin, whereas *L*-[³H] citrulline was recovered in the eluate and determined by liquid scintillation. [³H] Citrulline formation was expressed as % of the total radioactivity incorporated into the cells.

RESULTS

Effect of dBcAMP on astrocytic morphology In 5 % FBS-containing medium, the majority of astrocytes remained flat and polygonal and possessed few process. Within 3–4 h after the addition of dBcAMP $1 \text{ mmol} \cdot \text{L}^{-1}$, a morphological transformation reminiscent of *in vivo* differentiation occurred. The flat and polygonal astrocytes differentiated to process-bearing stellate cells with enhanced GFAP immunoreactivity. The continued presence of dBcAMP was needed for the maintenance of the fibrous morphology. After dBcAMP was removed, the cells returned to their flat and polygonal morphology (Fig 1, Plate 1). LPS alone had no effect on the morphology or GFAP expression.

Effects of dBcAMP and LPS stimulation on nitrite production Incubation of primary astrocyte cultures with LPS resulted in a marked accumulation of nitrite in the culture medium in a time-dependent manner. The nitrite accumulation stimulated by LPS was augmented in dBcAMP-differentiated astrocytes, in a time-dependent manner. After 24-h incubation, the levels of LPS-stimulated nitrite accumulation in the presence of dBcAMP were about 4-times those with LPS alone. (Fig 2)

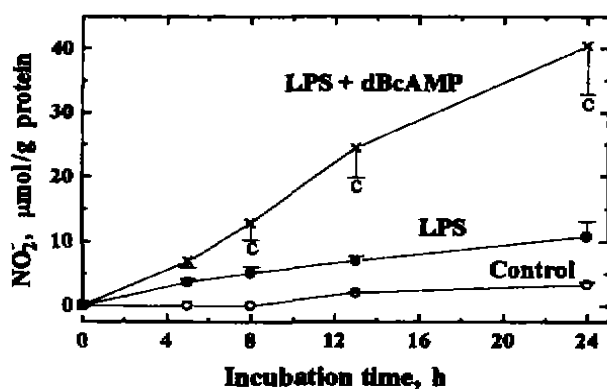


Fig 2. NO_2^- production in primary culture of astrocytes induced by LPS ($1 \text{ mg} \cdot \text{L}^{-1}$) or LPS + dBcAMP ($1 \text{ mmol} \cdot \text{L}^{-1}$). $n = 3$ experiments from 3 rats. $\bar{x} \pm s$. $^c P < 0.01$ vs LPS alone.

Simultaneous incubation with *L*-NAME ($1 \text{ mmol} \cdot \text{L}^{-1}$), an NOS inhibitor, for 24 h completely inhibited dBcAMP/LPS-induced nitrite production. The NO biosynthesis induced by LPS/dBcAMP was also prevented by

coincubation with dactinomycin ($10 \text{ mg} \cdot \text{L}^{-1}$) or cycloheximide ($10 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$). RNA and protein synthesis were required for the induction of NOS activity (Tab 1).

Tab 1. Effects of dBcAMP on LPS-stimulated nitrite formation in primary astrocyte cultures. Confluent cells were incubated with indicated agents for 24 h. $n = 3$ experiments from 3 rats (each in 3 wells from one rat respectively). $\bar{x} \pm s$. $^c P < 0.01$ vs control. $^d P < 0.01$ vs LPS alone.

Addition of agents	Nitrite ($\mu\text{mol/g protein}$)
Control	3.2 ± 0.3
LPS $1 \text{ mg} \cdot \text{L}^{-1}$	11.7 ± 1.3^c
dBcAMP $1 \text{ mmol} \cdot \text{L}^{-1}$	3.3 ± 0.4
LPS + dBcAMP	42.3 ± 5.6^d
LPS + dBcAMP + <i>L</i> -NAME $1 \text{ mmol} \cdot \text{L}^{-1}$	4.1 ± 0.7
LPS + dBcAMP + dactinomycin $10 \text{ mg} \cdot \text{L}^{-1}$	4.0 ± 0.6
LPS + dBcAMP + cycloheximide $10 \text{ mmol} \cdot \text{L}^{-1}$	3.9 ± 0.7

Effects of dBcAMP and LPS stimulation on NOS activity Unstimulated astrocyte had a very low basal level of NOS activity. Addition of dBcAMP to cultures for 24 h showed no effect on the NOS activity, while addition of LPS increased the NOS activity ($P < 0.01$ vs control). Furthermore, coincubation of LPS and dBcAMP for 24 h greatly augmented NOS activity as compared with those induced by LPS alone ($P < 0.01$ vs LPS alone). Both dactinomycin ($10 \text{ mg} \cdot \text{L}^{-1}$) and cycloheximide ($10 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$) almost completely suppressed the increase of NOS activity stimulated by LPS plus dBcAMP. (Tab 2)

Correlation between enhanced nitrite production with induction and differentiation Effect of differentiation on the induction of NOS activity by LPS, the change of nitrite in the medium was measured as a function of dBcAMP. With the increase of the concentration of dBcAMP, the proportion of stellate astrocytes raised and maximized nearly 100 % at dBcAMP $1 \text{ mmol} \cdot \text{L}^{-1}$. Meanwhile, as the number of differentiated cells rose, the LPS-induced nitrite production increased in parallel (Fig 3).

DISCUSSION

Evidence has accumulated for the induction of iNOS in astrocytes *in vitro* by inflammatory agents such as LPS and cytokines^[5]. But, what

Tab 2. Effects of dBcAMP on LPS-stimulated NOS activity in primary astrocyte cultures. Confluent cells were incubated with indicated agents for 24 h. $n = 3$ experiments from 3 rats (each in 3 wells from one rat respectively). $\bar{x} \pm s$. * $P < 0.01$ vs control. [†] $P < 0.01$ vs LPS alone. [‡] $P < 0.01$ vs LPS + dBcAMP.

Addition of agents	L-citrulline (% total radioactivity)
Control	2.15 ± 0.22
dBcAMP 1 mmol·L ⁻¹	2.57 ± 0.29
LPS 1 mg·L ⁻¹	5.28 ± 0.46 [*]
LPS + dBcAMP	23.4 ± 4.15 [‡]
LPS + dBcAMP + L-NAME + dactinomycin 10 mg·L ⁻¹	3.12 ± 0.33 [†]
LPS + dBcAMP + cycloheximide 10 mmol·L ⁻¹	3.26 ± 0.39 [†]

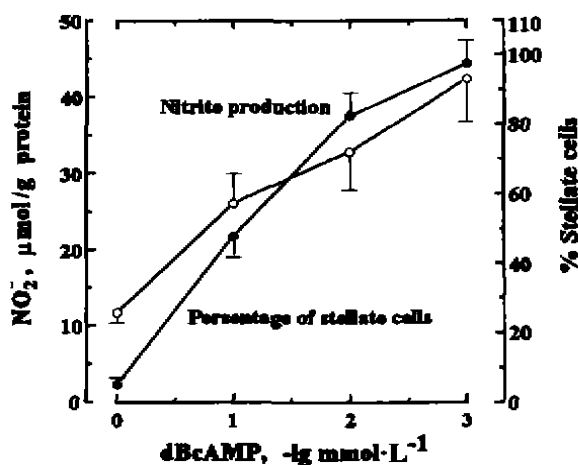


Fig 3. LPS-induced production of nitrite from dBcAMP-differentiated astrocytes. Cells were stimulated with LPS 1 mg·L⁻¹ in the presence of dBcAMP. The number of astrocytes under each field of vision is 20 ± 4 cells from 20 independent observation. $n = 3$ experiments from 3 rats. $\bar{x} \pm s$.

is the situation *in vivo*? In order to gain more information for the regulation of iNOS expression in astrocytes *in vivo*, we used dBcAMP as an inducer of differentiation to minimize the mature astrocytes *in vivo*. In the experiment, dBcAMP-differentiated astrocytes yielded a higher level of iNOS activity when induced by LPS than undifferentiated astrocytes did. Furthermore, the LPS-induced iNOS activity was closely parallel to the differentiation degree of astrocytes. These results strongly suggested that mature astrocytes *in vivo* may be more sensitive to

inflammatory signal to express iNOS.

Although enhancement of iNOS activity by cAMP has been shown in vascular smooth muscle cell, glomerular mesangial cells and cardiac myocytes^[10], the results was controversial in astrocytes. Burgher *et al* showed that the addition of 8-bromo-cAMP enhanced the expression of iNOS activity induced by cytokines^[11]. Whereas Pahan K *et al* found that forskolin, 8-bromo-cAMP and (S_p)-cAMP inhibited LPS- and cytokine-mediated production of NO as well as the expression of iNOS^[12]. However, both of them had not used dBcAMP, the only cAMP-elevating agents with the differentiation effect on primary astrocyte cells^[13]. Studies showed that the exposure to dBcAMP led to the morphological differentiation of the primary astrocyte cells, resulting in a close resemblance to the morphology of the mature astrocytes found *in vivo*^[13]. These changed characters include the formation of multiple cellular processes, the promotion of the polymerization of intermediate filaments containing GFAP, and ultrastructural characteristic changes of astrocytes found *in vivo*. Besides, dBcAMP also induced specific biochemical events, eg, a significant increase in glutamate uptake^[14], carbonic anhydrase, and Na⁺, K⁺-ATPase, all of which were also the character of the mature astrocytes^[15]. Taken together, the dBcAMP differentiated astrocytes may be a good model for studying the biological events of mature astrocytes *in vivo*. Our results provided the first evidence for the up-regulation of iNOS activity stimulated by LPS in dBcAMP-differentiated rat cortical astrocytes.

NO is emerging as an important regulator of a variety of physiologic processes; however, under conditions of excessive formation, NO is emerging as an important mediator of neurotoxicity in a variety of disorders of the nervous system^[3]. As astrocytes is the predominant cell in CNS, the iNOS system in astrocytes may be the most potential reservoir of NO in the CNS. Uncovering and understanding the regulation of iNOS in astrocyte will hopefully lead to a better understanding of basic pathological process in such conditions as inflammatory disorders of the CNS and the development of selective therapeutic agents.

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462-466

dBcAMP 诱导的细胞分化上调大鼠星状胶质细胞
内脂多糖诱导的一氧化氮合酶活力 R9724宋伟, 朱兴族¹

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关键词 脂多糖类; 一氧化氮合酶; 环一磷酸腺苷; 细胞分化; 上调(生理学); 星状胶质细胞; 免疫组织化学

目的: 研究 dBcAMP 诱导分化成熟后的星状胶质细胞内脂多糖诱导型一氧化氮合酶活力的诱导表达情况。方法: 采用荧光分光光度法和胍胺酸生成实验考察星状胶质细胞内一氧化氮合酶的活力, 并通过免疫细胞化学和光学显微镜考察细胞的分化情况。结果: 经 dBcAMP 诱导后, 星状胶质细胞分化成星型细胞, GFAP 表达增强。dBcAMP 显著增强 LPS 诱导产生的亚硝酸盐水平的提高; dBcAMP/LPS 共同诱导 24 h 后, 使 NOS 活力比 LPS 单独诱导提高 4 倍。L-NAME, 环己亚胺或放线菌素 D 都可分别抑制 dBcAMP/LPS 的共同诱导作用。结论: dBcAMP 可以上调 LPS 对星状胶质细胞内 iNOS 的诱导作用。

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