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Dinoprostone potentiates cytokines and lipopolysaccharides inducing nitric oxide production in cultured rat hepatocytes[1]

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KEY WORDS nitric oxide; dinoprostone; indomethacin; forskolin; cyclic AMP; dibutyryl cyclic GMP; cytokines; lipopolysaccharides; cultured cells; liver.

ABSTRACT

AIM: To study the effects of dinoprostone (Din) on nitric oxide (NO) production induced by lipopolysaccharides (LPS) and cytokines mixture (CM) in cultured rat hepatocytes, and the role of cAMP signaling pathway in the induction process. **METHODS**: Rat hepatocytes were incubated with indometacin (Ind), Din, forskolin (For), or dibutyryl cyclic GMP (db cGMP) in a medium containing CM (LPS plus TNF-α, IL-1β, and IFN-γ) for 24 h. NO production in the cultured supernatant was measured with the Griess reaction. Intracellular cAMP level was measured with radioimmunoassay. **RESULTS**: NO production stimulated by CM was markedly attenuated by Ind in the cultured supernatant, whereas Din and For increased nitrite concentration in the medium. In the presence of CM, both Din and For increased intracellular cAMP level, and had no effect on cAMP level in absence of the stimuli; db cGMP had no influence on the cAMP and NO production both in the presence and absence of same stimuli. **CONCLUSION**: Din potentiates cytokines and LPS to induce NO production in rat hepatocytes, and the induction may be regulated via intracellular second messenger cAMP signaling pathway.

INTRODUCTION

Nitric oxide (NO) produced by inducible nitric-oxide synthase (iNOS) has been recognized as one of important effector molecule in immune and inflammatory responses[1]. Attention has been paid to identify the extracellular stimuli and the intracellular signaling mechanisms that regulate the iNOS activity and to evaluate the contribution of NO synthesized by iNOS to the development and the prevention of various diseases. The inflammatory cytokines such as IL-1β and TNF-α increased the synthesis of prostanoids, in particular dinoprostone (Din) in vascular smooth muscle cells[2]. Elevation of intracellular cAMP is an important intracellular signaling mechanism involved in the regulation of the expression of many proteins[3], and this cAMP also acts as a second messenger for same inflammatory mediators such as epoprostenol and Din[4]. NO production required the induction of multiple inflammatory cytokines, including IL-1β, TNF-α, IFN-γ, and LPS in the primary cultured rat hepatocytes[5]. However, whether a classical inflammatory mediator Din affects NO production in the hepatocytes ramains unknown. It also remains undetermined whether cAMP is involved in the process of this induction. The purpose of this study was to examine the effects of Din on cytokines-stimulated NO production in cultured rat hepatocytes, and the role of cAMP

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signaling pathway in the induction process.

MATERIALS AND METHODS

Materials Wistar rats (♂, 180 – 220 g. Certificate № 01-3056 conferred by Beijing Medical Experimental Animal Management Committee) were purchased from Experimental Animal Center of Beijing Medical University. Collagenase (type Ⅳ), bovine insulin, lipopolysaccharides (LPS) from *E coli* 0111 : B4, forskolin (For), dibutyryl cyclic GMP (db cGMP), dinoprostone (Din), and indometacin (Ind) were purchased from Sigma Chemical Co. Human recombinant (rh) tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interferon-gamma (IFN-γ) were from Academy of Military Medical Sciences (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) from Gibco BRL. ^{3}H-radioimmunoassay kit for cAMP was bought from China Institute of Atomic Energy, Beijing. All reagents were diluted in medium before use.

Isolation and culture of hepatocytes Rat hepatocytes were harvested using an *in situ* collagenase perfusion technique[6]. Viability of cells exceeded 90 % as determined by trypan blue exclusion. Hepatocytes were placed onto 6-well tissue culture plates (1×10^9 cells·L^{-1}, 1 mL/well). Medium consisted of DMEM with *L*-arginine 0.5 mmol·L^{-1}, insulin 1 μmol·L^{-1}, HEPES 15 mmol·L^{-1}, *L*-glutamine, benzylpenicillin sodium, streptomycin sulfate, and 10 % low-endotoxin newborn calf serum. After overnight incubation, the medium was changed with a cytokines mixture (CM) containing LPS 10 mg·L^{-1}, IL-1β 10 kU·L^{-1}, TNF-α 500 kU·L^{-1}, and IFN-γ 100 kU·L^{-1}[5]. Other experimental conditions included addition of CM with Ind, For, Din, or db cGMP. After the primary cultures were maintained at 37 ℃ in 95 % air + 5 % CO_2 for 24 h, hepatocytes or cultured supernatants were collected for NO and cAMP assays.

Determination of NO production NO was measured as its stable oxidative product, nitrite[7]. At the end of the incubation, 100 μL of the culture supernatant was mixed with an equal volume of Griess reagent (1 part 0.1 % naphthylethylenediamine dihydrochloride and 1 part 1 % sulfanilamide in 5 % phosphoric acid). The absorbance at 540 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

Determination of cAMP levels The intracellular levels of cAMP were determined using a ^{3}H-labeled radioimmunoassay (RIA) kit[3]. The reaction was terminated by adding 2 mL of ice-cold 5 % trichloroacetic acid, and the cells were scraped from the dishes into the medium. The acidified medium containing the scraped cells was frozen, thawed, and centrifuged at 1500 × *g* for 10 min. Trichloroacetic acid in the supernatant was then removed by extraction with 5 volumes of water-saturated diethyl ether, and the combined extracts were dried at 75 ℃. The cAMP content of the dried cell extracts were processed for RIA determination of cAMP.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared by *t* test

RESULTS

Effects of Ind and N^{ω}-nitro-*L*-arginine (*L*-NNA) on cytokines- and LPS-stimulated NO production Accumulation of NO was inhibited by Ind 0.1 mmol·L^{-1}, following 24-h incubation with cytokines mixture (CM). *L*-NNA had more potent inhibition of NO production stimulated by CM than that of Ind under the similar condition (Tab 1).

Effects of Din, For, and db cGMP on cytokines- and LPS-stimulated NO production To examine the role of intracellular second

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Tab 1. Effect of indometacin and N^{ω}-nitro-*L*-arginine on NO production of rat hepatocytes in the presence of cytokines and LPS mixture (CM) for 24 h. $n = 6$ rats (3 wells for each treatment in each experiment, 1×10^9 cells·L^{-1}, 1 mL/well). $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^eP < 0.05$, $^fP < 0.01$ *vs* CM.

Treatment	Nitrite /μmol·L^{-1}	/% of CM
Control	6.2 ± 1.1	53.4
Cytokines mixture (CM)	11.7 ± 2.3^c	100
CM + Indometacin 0.1 mmol·L^{-1}	7.8 ± 2.5^{ae}	66.6
CM + *L*-NNA 0.1 mmol·L^{-1}	6.6 ± 2.0^{af}	56.4

messenger cAMP in the NO induction period, hepatocytes were incubated with media containing For or Din in the presence and absence of CM for 24 h. Din (1 – 100 μmol·L^{-1}) increased NO production in the culture media under the presence of CM condition in a concentration-dependent fashion. Similarly, adenylate cyclase activator For also enhanced NO production induced by CM. Din and For appeared to slightly potentiate NO production in the absence of cytokines stimuli ($P > 0.05$). db cGMP, a membrane-permeable cGMP derivative, had only slightly influence on NO formation induced by inflammatory stimuli. (Tab 2)

Effects of Din, For, and db cGMP on intracellular cAMP levels in cultured rat hepatocytes In the presence of CM stimuli conditions, Din and For enhanced intracellular cAMP level by 3- and 5-fold above control levels, respectively. On the other hand, db cGMP failed to affect the levels of intracellular cAMP both in the presence and absence of cytokine stimuli in rat hepatocytes (Tab 3).

Tab 3. Effect of forskolin, db cGMP, and Din on intracellular cAMP levels in the presence (CM +) and absence (CM –) of cytokines mixture (CM) in cultured rat hepatocytes after 24 h. $n = 4$ or 5 rats (4 wells for each treatment in each experiment, 1×10^9 cells·L^{-1}, 1 mL/well). $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* control of CM (–). $^dP > 0.05$, $^fP < 0.01$ *vs* control of CM (+).

Concentration/μmol·L^{-1}	n	cAMP/nmol per well CM (–)	CM (+)
Control	4	7.5 ± 0.8	10.8 ± 2.7
Forskolin 100	4	18.3 ± 6.5^b	54.5 ± 12.3^f
Control	5	9.7 ± 1.8	11.2 ± 2.4
db cGMP 100	4	7.4 ± 1.8^a	9.8 ± 2.1^d
Control	5	9.7 ± 2.5	8.4 ± 2.4
Dinoprostone 100	5	10.3 ± 0.8^a	24.8 ± 2.8^f

DISCUSSION

The present study has demonstrated that Ind attenuated NO production stimulated by CM. Whereas, exogenous application of Din increased nitrite concentration in the media, suggesting that prostaglandins, such as Din may be involved with

Tab 2. Effect of forskolin, db cGMP, and Din on NO production in the presence/absence of cytokines mixture (CM) for 24 h. $n = 6$ rats (3 wells for each treatment in each experiment, 1×10^9 cells·L^{-1}, 1 mL/well). $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* CM.

Treatment /μmol·L^{-1}	Nitrite /μmol·L^{-1}	Treatment /μmol·L^{-1}	Nitrite /μmol·L^{-1}	Treatment /μmol·L^{-1}	Nitrite /μmol·L^{-1}
Control	5.7 ± 1.2	Control	5.8 ± 1.3	Control	6.5 ± 1.3
Cytokines mixture (CM)	9.1 ± 3.1^b	Cytokines mixture (CM)	9.4 ± 2.9^b	Cytokines mixture (CM)	9.3 ± 2.5^b
Forskolin 100	6.9 ± 1.2^a	db cGMP 100	6.2 ± 1.3^a	Dinoprostone 100	6.7 ± 2.0^a
CM + Forskolin 1	8.4 ± 2.1^{bd}	CM + db cGMP 1	9.4 ± 3.2^{bd}	CM + Dinoprostone 1	9.7 ± 2.3^{bd}
CM + Forskolin 10	9.3 ± 2.6^{bd}	CM + db cGMP 10	10.4 ± 3.2^{cd}	CM + Dinoprostone 10	11.8 ± 1.1^{cd}
CM + Forskolin 100	13.2 ± 3.0^{ce}	CM + db cGMP 100	11.2 ± 2.4^{cd}	CM + Dinoprostone 100	14.3 ± 2.6^{cf}

the upregulating cytokines-induced NO production in the hepatocytes. Both LPS and TNF-α stimulated the release of Din, one of several eicosanoids synthesized by Kupffer cells[8], this release is sensitive to ambient arginine concentration to the extent that a reduction in arginine availability can augment Din production to downregulate the LPS-stimulated release of TNF-α[9]. The modulation of Din by ambient arginine may be relevant particularly in the liver, the primary organ of the urea cycle, because *L*-arginine is a substrate not only for NO synthase but also for arginase, an enzyme of the urea cycle, and arginine levels are lowered within the liver by high hepatic arginase activity and by inducible NOS[10]. Thus, it is possible that Din synergistics with cytokines to act as a feedback regulate loop in the process of NO formation in hepatocytes, conversely, as a negative feedback mechanism in Kupffer cells. Furthermore, recent work prompted that *L*-NNA might affect NO synthesis by modifying electron transfer through iron centers. On the other hand, arachidonic acid is known to accelerate electron transfer[11], thus it is conceivable that through inhibition of arachidonic acid release, NO synthesis is also indirectly inhibited.

The present study has also shown that in the presence of CM, both Din and For enhanced intracellular cAMP level, and in parallel with this reaction, NO production was also increased in culture supernatant, but db cGMP neither increased cAMP level nor affected NO formation, sugessting that cAMP as a second messenger, might be an important factor to participate in *induction mechanism of cytokines and Din for NO* production in rat hepatocytes. Binding of Din to a specific cell surface receptor led to activation of adenylate cyclase via a family of membrane-associated G proteins[12], then, cAMP mediated its hormonal induction of numerous genes through a specific protein kinase C (PKC) that termed "cAMP response element binding protein"[13], to initiate gene transcription. On the other hand, inducible NOS was mainly regulated in transcription level[14]. Thus, although the question as to whether iNOS gene contains cAMP response element sequence in the promoter region needs to be determined by more precise experiment, the present results lend strong support to above hypothesis.

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地诺前列酮增强细胞因子及脂多糖对培养大鼠肝细胞一氧化氮生成的诱导作用[1]

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关键词 一氧化氮; 地诺前列酮; 吲哚美辛; 弗司扣林; 环腺苷一磷酸; 双丁酰环鸟苷一磷酸; 细胞因子类; 脂多糖; 培养的细胞; 肝细胞

目的: 探讨地诺前列酮(Din)对细胞因子(IL-1β, TNF-α, IFN-γ)及脂多糖(LPS)诱导的原代培养大鼠肝细胞一氧化氮(NO)生成的影响, 以及环腺苷一磷酸(cAMP)信号通路在诱导过程中的作用. **方法**: 将吲哚美辛(Ind), Din, 弗司扣林(For)及双丁酰环鸟苷一磷酸(db cGMP)分别加入含细胞因子 TNF-α, IL-1β, IFN-γ 及 LPS 的培养液中. 原代培养肝细胞 24 h 后, 采用 Griess 反应法测定细胞培养上清中 NO 含量, 放射免疫法测定细胞内 cAMP 水平. **结果**: Ind 明显抑制 LPS 与细胞因子刺激所致 NO 生成, 反之, Din 及 For 促进 NO 生成; 在同样刺激条件下, Din 与 For 使细胞内 cAMP 水平增高, 而在对照组细胞未观察到上述反应. db cGMP 则无论炎性刺激存在与否均无此作用. **结论**: Din 协同细胞因子及 LPS 诱导肝细胞 NO 生成, 细胞内第二信使 cAMP 通路参与此诱导过程.

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