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Effects of 3-*n*-butylphthalide on production of vasoactive substances by cerebral and aortic endothelial cells[1]

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KEY WORDS butylphthalides; endothelin-1; cerebral cortex; aorta; cultured cells; tumor necrosis factor; 6-ketoprostaglandin F_1 alpha; epoprostenol; nitric oxide

ABSTRACT

AIM: The effects of *dl*-3-*n*-butylphthalide (*dl*-NBP), *l*-3-*n*-butylphthalide (*l*-NBP), and *d*-3-*n*-butylphthalide (*d*-NBP) on the production of nitric oxide (NO), epoprostenol (Epo) and endothelin-1 (ET-1) were investigated in cerebrovascular and aortic endothelium in culture. **METHODS:** Bovine cerebral endothelial cells (BCEC) and bovine aortic endothelial cells (BAEC) were cultured in Medium 199 *in vitro*. After incubation with *dl*-, *l*-, and *d*-NBP for 24 h, the release of NO, Epo, and ET-1 were analyzed by using spectrometry assay and radioimmunoassay (RIA) respectively. **RESULTS:** Low concentrations of *dl*- and *l*-NBP ($0.1 - 10\ \mu mol \cdot L^{-1}$) enhanced nitrite and 6-ketoprostaglandin $F_{1\alpha}$ (6-keto$PGF_{1\alpha}$) production in both BAEC and BCEC after a 24-h incubation, and *l*-NBP has a potent effect on promoting Epo production in BCEC. The production of ET-1 secreted by BCEC and BAEC was increased after TNFα stimulation, this enhancement was not blunted by the simultaneous addition of *dl*-, *l*-, and *d*-NBP. **CONCLUSION:** 1) *dl*-NBP and *l*-NBP increase NO production in both BCEC and BAEC. 2) *l*-NBP increases more Epo production in BCEC than that in BAEC, and *dl*-NBP has selective effect on increasing Epo production in BCEC.

INTRODUCTION

In recent years, it has been demonstrated that the blood vessel wall is an important source of vasoactive substances, which acts in the autocrine or paracrine manner on endothelial or smooth muscle cells to maintain and regulate the vascular tone. Nitric oxide (NO), a potent vasodilator, is synthesized in endothelial cells by a constitutive Ca^{2+}- and calmodulin-dependent NO synthase. Epoprostenol (Epo), another important vasodilator, is possibly released by phospholipase A_2 from endothelial cells[1]. The release of NO and Epo is an important mechanism by which the endothelial cells influence themselves and the cells surrounding them. Microvascular endothelial cells play a critical role in the regulation of vascular tone through the secretion of a variety of vasoactive factors, and the brain microvessel endothelial cells were also found to be able to release these vasoactive substances. Endothelial-dependent relaxation is mediated by the production of Epo, the major product of the metabolism of arachidonic acid by cyclo-oxygenase, and by another factor called endothelium-derived relaxing factor, which has been identified as NO. Endothelial cells also produce a potent vasoconstrictor, endothelin-1 (ET-1), a member of the endothelin/sarafotoxin peptide family. Interestingly, NO, Epo, and ET-1 possess a wide range of biological activities within the cardiovascular system and in other organs, including the brain. The regulation of NO, Epo, and ET-1 secretion has mostly studied *in vivo* or *in vitro* with endothelial cells in recent years.

3-*n*-Butylphthalide (NBP), a promising anti-cerebral ischemia drug, has been shown to reduce the infarct size after middle cerebral artery occlusion

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(MCAO) in rats[2], attenuate delayed neuronal damage after cerebral ischemia[3], increase regional cerebral blood flow[4], improve cerebral microcirculation[5]. Previous study showed that NBP markedly enhanced extracellular NO production and intracellular cGMP formation in primary cultured neurons, and significantly increased the ratio of Epo/TXA_2 in rat brain during focal ischemia and reperfusion, which may have beneficial effects on improving cerebral microcirculation[6,7]. However, the mechanisms accounted for the effects of NBP on increasing cerebral blood flow and improving cerebral microcirculation deserve further study. The present study was designed to clarify its mechanism by exploring the abilities of endothelial cells to produce vasoactive substances and the effect of NBP on these functions. More and more experiments demonstrated that bovine aortic endothelial cells (BAEC) and bovine cerebral endothelial cells (BCEC) have remarkable heterogeneity in terms of character and function. Accordingly, the present study was performed with BAEC and BCEC.

MATERIALS AND METHODS

Calves Newborn calves were provided by Beijing Red Star Biochemical Company.

Materials *dl*-, *l*-, and *d*-NBP were synthesized by Department of Medicinal Synthetic Chemistry of our institute, purity > 96 %. Medium 199 (M199, high glucose, Gibco, BRL) was supplemented with streptomycin 100 $g \cdot L^{-1}$, benzylpenicillin 100 $kU \cdot L^{-1}$ and 10 % heat-inactived fetal bovine serum (FBS) (Gibco, BRL). *N*-(*l*-naphthyl) enthylenediamine dihydrochloride (NEDA), phosphoric acid and sulfanilamide were domestic AR grade products. Griess reagent was composed of 1 % sulfanilamide and 0.1 % NEDA in 5 % phosphoric acid. The [^{125}I]-6-ketoprostaglandin F_1 alpha ([^{125}I]-6-keto$PGF_{1\alpha}$) and [^{125}I]-ET-1 kits were purchased from General Hospital of PLA, Beijing. Aspirin was purchased from Xin Hua Pharmaceutical Factory, Shandong. TNF_α was supplied by Biotinge Biomedicine Company, Beijing.

Cell cultures BCEC were isolated from cortical gray matter as previously described[8]. BAEC were harvested from aortic cords by 0.1 % collagenase treatment at 37 ℃ for 20 min. The cells were plated in M199, supplemented with 10 % heat-inactived FBS, and the culture were incubated in a humidified atmosphere of 5 % CO_2 + 95 % air. Cells were removed by trypsinization between the fifth to seventh passage and seeded into 24-well tissue culture plates (Costar), and used for the experiments when cells grew to confluent. Endothelial cells were identified by their characteristic cobblestone morphology and positive labeling with mouse anti-human factor Ⅷ.

Nitrite assay The measurement of nitrite was based on the Griess reaction[9] by adding 500 μL of aliquot of cell-free supernatants to 500 μL Griess reagent (1 % sulfanilamide and 0.1 % NEDA in 5 % phosphoric acid), followed by incubation at 25 ℃ for 10 min with shaking. Nitrite concentration, proportional to A_{540}, was determined using the Model 450 Bio-Rad ELISA plate reader (Bio-Rad Company, USA).

6-Keto$PGF_{1\alpha}$ assay The stable metabolite of Epo, was measured by RIA. BCEC or BAEC conditioned medium (200 μL) were incubated at 4 ℃ for 6 h with rabbit anti-6-keto$PGF_{1\alpha}$ antibody in total volume of 300 μL. The next day, 100 μL of [^{125}I]-6-keto$PGF_{1\alpha}$ was added and incubated at 4 ℃. After 24 h, the precipitant was added and incubated at 25 ℃ for 15 min. Then the precipitate was centrifuged at 800 × *g* at 4 ℃ for 30 min, and the radioactivity was measured in LKB Wallac 1271 automatic gamma counter (Finland).

ET-1 assay The formation of ET-1 in culture supernatants were determined with a newly developed RIA kit. Briefly, aliquots of supernatants (100 μL) were transferred, together with 100 μL of ET-1 monoclonal antibody into the plastic tubes. After reaction for 24 h, [^{125}I]-ET 100 μL was added. After the next 24 h, the precipitate after centrifuged at 4 ℃ was used for radioimmunometric assay. The lower limit of detection was 6 $ng \cdot L^{-1}$.

Experimental protocols BCECs or BAECs were seeded in 24-well dishes at a density of 1×10^6 $cells \cdot cm^{-2}$. Three days later, the confluent cells were washed by phosphate buffered saline. Corresponding drugs or TNF_α or both were added in the culture, 24 h later, the cell-conditioned medium were centrifuged and used for nitrite, epoprostenol and ET-1 determinations.

Statistical analysis Data are expressed as $\bar{x} \pm s$ (*n* = number of cell culture wells), and compared with paired *t* test.

RESULTS

Effects of *dl*-, *l*-, *d*-NBP on the production of NO released from BCEC and BAEC BAEC growing to confluence spontaneously produced nitrites, the stable oxidation products of NO, which accumulated in the culture medium for up to 24 h, reaching concentrations similar to those simultaneously measured in the supernatant of BAEC. *dl*-NBP and *l*-NBP (0.1 – 10 $\mu mol \cdot L^{-1}$) could concentration-dependently augment the amount of NO-derived nitrite in BAEC and BCEC supernatants. The similar effect on BAEC was evident at the different concentration of *d*-NBP (0.1 – 10 $\mu mol \cdot L^{-1}$). However, *d*-NBP showed no effect on nitrite production in BCEC (Tab 1).

Effects of *dl*-, *l*-, *d*-NBP on the production of Epo secreted by BCEC and BAEC BCEC and BAEC constitutively secreted Epo, but the basal productions of Epo in BAEC and BCEC were quite different. BAEC released more Epo than BCEC released (11.0 ± 3.1 *vs* 7.8 ± 2.8 ng per well per 24 h). Furthermore, application with Aspirin (10 $\mu mol \cdot L^{-1}$) could inhibit 6-keto$PGF_{1\alpha}$ accumulated in the incubation medium of both BCEC and BAEC, while *dl*-NBP and *l*-NBP (0.1 – 10 $\mu mol \cdot L^{-1}$) could produce an enhancement of 6-keto$PGF_{1\alpha}$ accumulated in BCEC culture. On the contrary, 6-keto$PGF_{1\alpha}$ in the culture of BAEC was not modified by *dl*-NBP, and only high concentration of *l*-NBP had weaker effect of increasing the level of 6-keto$PGF_{1\alpha}$ produced by BAEC. In addition, *d*-NBP had no effect on 6-keto$PGF_{1\alpha}$ production in both BAEC and BCEC (Tab 2).

Effects of *dl*, *l*, *d*-NBP on the production of ET-1 released from BAEC BAEC spontaneously produced ET-1 under basal conditions which accumulated in the culture medium. ET-1 production was enhanced by incubation with TNFα for 24 h. The augment of ET-1 production was not attenuated by simultaneous treatment with *dl*-, *l*-, and *d*-NBP. Additionally, *l*-NBP (10 $\mu mol \cdot L^{-1}$) was found to exacerbate this increasing of ET-1 production in BAEC (Tab 3).

DISCUSSION

To identify factors that lead to vasodilate effect of NBP, we compared the changes in NO, epoprostenol, ET-1 accumulated in supernatant of cultured EC

Tab 1. Effect of 24 h incubation with NBP on nitrite production accumulated in the culture media of BCEC and BAEC. $n = 6$ samples. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* vehicle group.

Dose/$\mu mol \cdot L^{-1}$	Nitrite production (nmol per well in 24 h)							
	BAEC				BCEC			
	Vehicle	*dl*-NBP	*d*-NBP	*l*-NBP	Vehicle	*dl*-NBP	*d*-NBP	*l*-NBP
	125 ± 22				130 ± 27			
0.1		145 ± 39	158 ± 12^b	173 ± 32^b		136 ± 39	152 ± 19	173 ± 10^b
1		171 ± 19^b	192 ± 15^b	219 ± 6^c		163 ± 24	147 ± 10	192 ± 29^b
10		211 ± 16^c	189 ± 5^c	256 ± 51^c		170 ± 15^b	157 ± 10	250 ± 28^c

Tab 2. Effect of 24 h incubation with NBP or aspirin (Asp) on 6-ketoprostaglandin $F_{1\alpha}$ production in the culture media of BAEC and BCEC. $n = 6$ samples. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* vehicle group.

Dose/$\mu mol \cdot L^{-1}$	6-Ketoprostaglandin $F_{1\alpha}$ production (nmol per well in 24 h)									
	BAEC					BCEC				
	Vehicle	Asp	*dl*-NBP	*d*-NBP	*l*-NBP	Vehicle	Asp	*dl*-NBP	*d*-NBP	*l*-NBP
	10 ± 4					8 ± 3				
0.01			8 ± 1	12 ± 2	15 ± 2					
0.1			11 ± 2	12 ± 8	13 ± 4			28 ± 17^c	8 ± 7	17 ± 9^b
1			15 ± 6	8 ± 6	17 ± 3^b			38 ± 32^b	12 ± 9	29 ± 28^b
10		3 ± 3^b	8 ± 4	11 ± 3	18 ± 5^b		4 ± 1^b	28 ± 17^c	8 ± 4	34 ± 24^b

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Tab 3. Effect of a 24-h incubation with NBP on TNF_{α}-induced ET-1 production in BAEC. BAEC were pretreated with NBP 0.5 h before TNF_{α} (200 $ku \cdot L^{-1}$) added. $\bar{x} \pm s$. $n=6$ samples. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control; $^{e}P<0.05$ *vs* vehicle group.

Dose/ $\mu mol \cdot L^{-1}$	ET-1 production (pg per well in 24 h) control	veh	*dl*-NBP	*d*-NBP	*l*-NBP
	23 ± 6	56 ± 32^{b}			
0.01			42 ± 19^{a}	52 ± 30^{b}	74 ± 27^{c}
0.1			43 ± 25^{b}	38 ± 10^{c}	76 ± 47^{b}
1			48 ± 25^{b}	46 ± 12^{c}	61 ± 41^{b}
10			44 ± 42^{b}	63 ± 36^{b}	106 ± 33^{ce}

pretreated by NBP. The results showed that both BAEC and BCEC had a basal release of NO, as shown by the accumulation of nitrites in the culture supernatant, and the basal productions of NO released from BCEC and BAEC were very similar. The formation of NO could be enhanced by *dl*-, *l*-, and *d*-NBP in dose-dependent fashion, and *l*-NBP had more potential effect than *dl*-NBP and *d*-NBP. We also furthered our study by determining Epo production stimulated by *dl*-, *l*-, and *d*-NBP. To our surprise, *dl*-NBP and *l*-NBP at very low concentration (0.1 $\mu mol \cdot L^{-1}$) enhanced the level of Epo released from BCEC, while *d*-NBP had no effect on Epo production. In addition, the formation of Epo in BAEC was not affected by *dl*-NBP and *d*-NBP, and *l*-NBP had more potent effect on Epo production in BCEC, suggesting that *l*-NBP might act on BCEC selectively.

ET-1, a potent vasoconstrictor peptide with pleiotropic activities, is also secreted by endothelial cells, and this local vasoactive substance may mediate part of the effects of TNF_{α}, which has been implicated in the pathogenesis of several inflammatory disease[9]. TNF_{α} produces a variety of alterations in blood vessel function, including overproduction of ET-1 in EC, together with a cytotoxic effect. ET-1 was constitutively secreted by EC, and TNF_{α} could enhance its production[10]. Therefore, we analysed the directe effect of *dl*-, *l*-, and *d*-NBP on endothelial cells with or without stimulation by TNF_{α}. Radioimmunoassay found that *dl*-, *l*-, and *d*-NBP could not blunt the increase of ET-1 production elicited by TNF_{α}. So we could conclude that ET-1 was not the target of NBP in its mechanism of vasodilate effect.

The previous experiments[6,7] found that NO and Epo produced by neurons were promoted by administration of *dl*-NBP, which suggested that NO and Epo were the most important mediators for the activities of NBP. In the meantime, the result of the later study demonstrated that *l*-NBP and *d*-NBP had counteractive effects on pial arterioles in focal cerebral ischemia rats[5]. The results of the present study tested the hypothesis that NO and Epo might play an important role in the mechanism of the vasodilate effect of NBP.

In summary, the results implicated that NO and Epo not ET-1 produced by EC were mediators of vasodilate effects of NBP. These beneficial effects of NBP on EC might account for its anti-ischemic activity.

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丁基苯酞对牛脑微动脉及胸主动脉内皮血管活性物质生成的影响[1]

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关键词 丁基苯酞；内皮素-1；大脑皮质；主动脉；培养的细胞；肿瘤坏死因子；6-酮前列腺素 $F_{1\alpha}$；依前列醇；一氧化氮

目的：观察消旋、左旋、右旋丁基苯酞（*dl*，*l*，*d*-NBP）对体外培养的脑血管内皮细胞（BCEC）及主动脉内皮细胞（BAEC）合成一氧化氮（NO）、前列环素（epoprostenol，Epo），内皮素-1（ET-1）的影响．**方法**：自牛大脑灰质及胸主动脉分离培养内皮细胞，并用分光光度法和放免法测定细胞培养液中 NO，Epo，ET-1 的含量．**结果**：BCEC 及 BAEC 可持续分泌 NO，Epo，ET-1．*dl*，*l*-NBP 可以剂量依赖性地明显促进 BAEC 及 BCEC 中 NO 的合成，不同浓度的 *d*-NBP 均能增加 BAEC 中 NO 的产生，而对 BCEC 无明显作用．低浓度的 *dl*，*l*-NBP（0.1 – 10 $\mu mol \cdot L^{-1}$）即可显著升高 BCEC 中 Epo 的产生，而对 BAEC 无明显影响．另外，*dl*，*l*，*d*-NBP 对 TNF_{α} 诱导的 BAEC 中 ET-1 增加均无显著影响．**结论**：1）*dl*-和 *l*-NBP 均可显著增加 BAEC 及 BCEC 中 NO 的释放．2）*l*-NBP 可升高 BCEC 和 BAEC 中 Epo 的释放，且对 BCEC 具有选择性．*dl*-NBP 选择性增加 BCEC 中 Epo 的释放．

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