

## Possible involvement of tetrahydrobiopterin in the trophic effect of insulin-like growth factor-1 on rat pheochromocytoma-12 (PC12) cells

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### Abstract

Tetrahydrobiopterin (BH<sub>4</sub>) has a trophic effect on pheochromocytoma-12 (PC12) cells such as insulin-like growth factor-1 (IGF-1). We investigated involvement of BH<sub>4</sub> in the trophic effect of IGF-1 on PC12 cells. IGF-1 (10–300 ng/ml) increased cellular BH<sub>4</sub> content in a dose-related manner. Cellular BH<sub>4</sub> content increased after 6–36 h incubation with IGF-1. IGF-1-induced increase in the cellular BH<sub>4</sub> content was blunted by 0.3 mM 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor for BH<sub>4</sub> synthesis. IGF-1 protected PC12 cells from the cell death induced by depletion of serum and nerve growth factor, which was attenuated by DAHP. The effects of IGF-1 on the cellular BH<sub>4</sub> content and cell viability were eliminated by 0.2 μM wortmannin. These results suggest that BH<sub>4</sub> is involved in the trophic effect of IGF-1 on PC12 cells and that the effect of IGF-1 on BH<sub>4</sub> synthesis is mediated by phosphatidylinositol 3-kinase. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Insulin-like growth factor; Tetrahydrobiopterin; Pheochromocytoma 12 cells; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; Wortmannin; 2,4-Diamino-6-hydroxypyrimidine; Nitric oxide

Insulin-like growth factor-1 (IGF-1) is a growth promoting peptide which is important during development of the brain [3]. Recently, it was reported that IGF-1 has a trophic effect on pheochromocytoma-12 (PC12) cells like nerve growth factor (NGF) [5,13]. NGF and epidermal growth factor (EGF) were found to enhance synthesis of tetrahydrobiopterin (BH<sub>4</sub>) [1], a cofactor for nitric oxide synthase [10] and aromatic amino acid hydroxylases [6,11,12]. We have reported that BH<sub>4</sub> has a trophic effect on PC12 cells [8]. These results raised a possibility that the trophic effect of IGF-1 on PC12 cells is mediated by BH<sub>4</sub>. In the present study, we investigated whether BH<sub>4</sub> is involved in the trophic effect of IGF-1 on PC12 cells.

IGF-1 was a generous gift from Fujisawa Pharmaceutical Co., Osaka, Japan. Mouse NGF (7.0S) was purchased from Nacalai Tesque, Kyoto, Japan. All other chemicals were of the purest grade available from regular commercial sources. PC12 cells (Riken Cell Bank, Tsukuba, Japan) were maintained, subcultured and differentiated by 100 ng/ml NGF as reported previously [9]. Following 5–7 days of culture for differentiation, the cells were used for experiments. To examine the effect of IGF-1 on cellular BH<sub>4</sub> content, NGF

was depleted from the culture medium. Following incubation of PC12 cells with the culture medium containing drugs for the indicated period, the cellular content of total biopterin was measured as reported previously [15]. Since most of the cellular biopterin exists as BH<sub>4</sub> [4], the total biopterin content was used as an index for cellular BH<sub>4</sub> content. After a 3-day culture with drugs added every 48 h, cell viability was estimated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously reported [9]. To induce cell death, PC12 cells were cultured without serum and NGF. Protein content was measured using Bio-Rad protein assay with bovine serum albumin as the standard [2]. All results were expressed as means ± SEM. The significance of difference was evaluated with analysis of variance and Fisher's test. A probability level of  $P < 0.05$  was considered statistically significant.

First, the effect of IGF-1 on cellular biopterin content in PC12 cells was examined. When the cells were cultured with various concentrations of IGF-1 for 12 h, cellular biopterin content increased in a dose-related manner. The effect of IGF-1 on cellular biopterin content was maximal at 100 ng/ml (Fig. 1A). When PC12 cells were cultured with 100 ng/ml IGF-1 for varying periods, cellular biopterin content increased after 6, 12, 24 and 36 h. The effect of IGF-1 was maximal at 12 h (Fig. 1B). These results suggest that IGF-1

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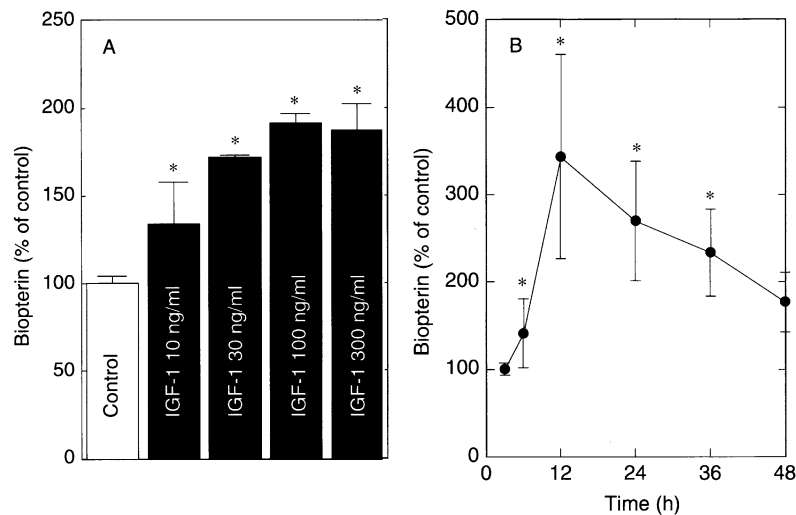


Fig. 1. Effect of IGF-1 on biopterin content in PC12 cells. (A) PC12 cells were cultured for 12 h with culture medium which was added with varying concentrations of IGF-1 and depleted of NGF. Then, cellular biopterin content was measured. Each experiment was carried out in triplicate. In each experiment, the examined value was expressed as a percentage of the mean value of the control group ( $61.78 \pm 2.53$  pmol/mg protein). Each column shows the mean  $\pm$  SEM of six experiments. \* $P < 0.05$  vs. the control group. (B) PC12 cells were cultured with 100 ng/ml IGF-1 for 3, 6, 12, 24, 36 and 48 h. Then, cellular biopterin content was measured. In each experiment, the examined value was expressed as a percentage of the mean value of the corresponding control group. Each point shows the mean  $\pm$  SEM of six experiments. \* $P < 0.05$  vs. the corresponding control group.

increased cellular BH<sub>4</sub> content within physiological concentrations. In non-differentiated PC12 cells, 12-h incubation with 100 ng/ml IGF-1 had no effect on cellular biopterin content (biopterin content of control cells ( $n = 6$ ) for  $60.17 \pm 4.51$  pmol/mg protein vs. that of IGF-1 loaded cells ( $n = 6$ ) for  $61.83 \pm 2.00$  pmol/mg protein). Thus, it is possible that the effect of IGF-1 on BH<sub>4</sub> synthesis depends on cell differentiation. When the cells were cultured in the presence of wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3 kinase), the IGF-1-induced increase in the cellular biopterin content was blunted (Fig. 2A). These data suggest that the stimulatory effect of IGF-1 on cellular BH<sub>4</sub>

content was mediated by PI3 kinase. When the cells were cultured with 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor for guanosine triphosphate cyclohydrolase 1 as a rate-limiting enzyme for BH<sub>4</sub> synthesis, the increase in the cellular biopterin content was eliminated (Fig. 2B). These results indicate that IGF-1 stimulated the synthesis of BH<sub>4</sub> in PC12 cells. Then, the trophic effect of IGF-1 on PC12 cells was examined in the presence of wortmannin. When the cells were cultured without serum and NGF for 3 days, cell viability was significantly decreased as reported previously [8,14]. IGF-1 (100 ng/ml) protected PC12 cells from cell death induced by depletion of serum and NGF.

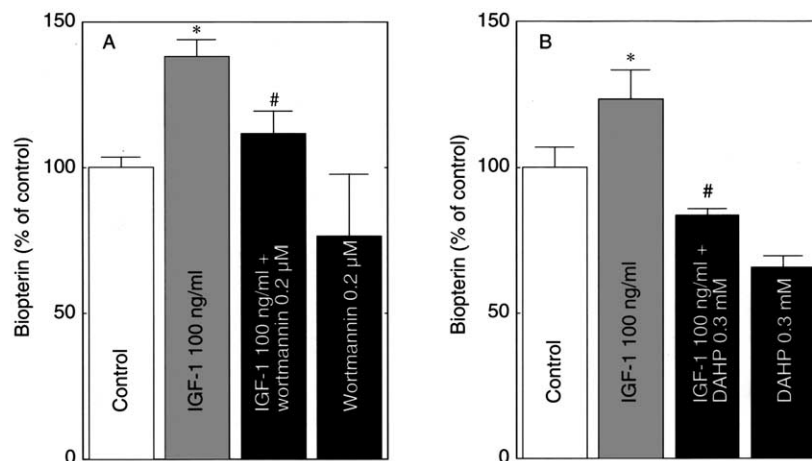


Fig. 2. Effect of wortmannin (A) and DAHP (B) on IGF-1-induced increase in cellular biopterin content. After PC12 cells were cultured with test drugs for 12 h, cellular biopterin content was measured. Each experiment was carried out in triplicate. In each experiment, the examined value was expressed as a percentage of the mean value of the control group. Each column shows the mean  $\pm$  SEM of six experiments. \* $P < 0.05$  vs. the control group. # $P < 0.05$  vs. the group cultured with IGF-1.

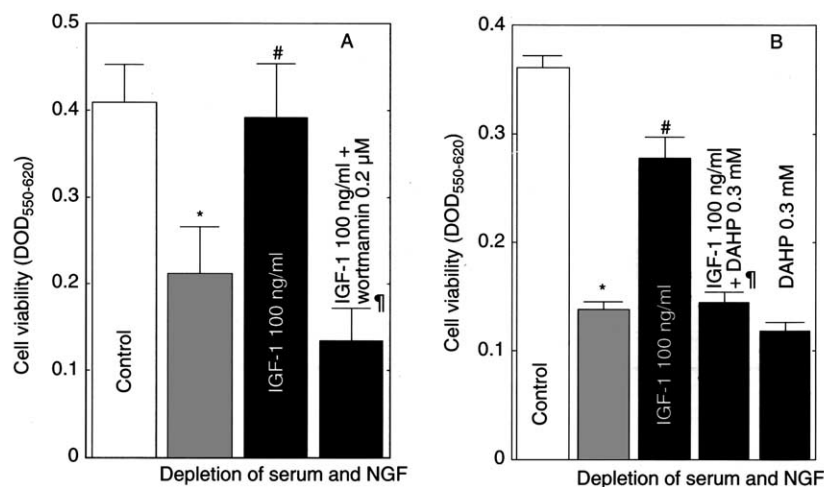


Fig. 3. Effect of wortmannin (A) and DAHP (B) on the trophic action of IGF-1 on PC12 cells. PC12 cells were cultured in the presence or absence of serum and NGF for 3 days. At 48 h intervals, test drugs were added to the culture medium which was depleted of serum and NGF. Then, cell viability was estimated by MTT assay. Each column shows the mean  $\pm$  SEM of six experiments. \* $P < 0.05$  vs. control group. # $P < 0.05$  vs. the group cultured without serum and NGF.  $\dagger P < 0.05$  vs. the group cultured with IGF-1.

The protective effect of IGF-1 was blunted by wortmannin (Fig. 3A), suggesting that the protective effect of IGF-1 was mediated by PI3 kinase. It is known that PI3 kinase is involved in diverse signalings. Although we observed that PI3 kinase was involved in the stimulatory effect of IGF-1 on BH<sub>4</sub> synthesis in PC12 cells, it is not clarified whether IGF-1–PI3 kinase signaling in the protective effect of IGF-1 is independent or dependent on BH<sub>4</sub> synthesis. Thus, we examined the protective effect of IGF-1 on PC12 cells in the presence of DAHP. As shown in Fig. 3B, the cell protective effect of IGF-1 was blunted by DAHP.

These data taken together lead to the possibility that the cell protective effect of IGF-1 is dependent on synthesis of BH<sub>4</sub> in PC12 cells. We have studied the effect of BH<sub>4</sub> on neuronal cells using PC12 cells. BH<sub>4</sub> protected PC12 cells from cell death induced by depletion of serum and NGF [8]. Erythropoietin (EPO) had a protective effect on PC12 cells cultured without serum and NGF [7]. The trophic effect of EPO was mediated by BH<sub>4</sub> [15]. BH<sub>4</sub> was reported to be involved in the trophic effects of NGF and EGF on PC12 cells [1]. Among diverse intracellular signalings for trophic factors such as NGF, EGF, IGF-1 and EPO, BH<sub>4</sub> is assumed to play a key role in the intracellular signalings for these trophic factors.

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