

# Involvement of nitric oxide in glucose toxicity on differentiated PC12 cells: prevention of glucose toxicity by tetrahydrobiopterin, a cofactor for nitric oxide synthase

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## Abstract

Effects of high concentrations of glucose on cell survival of differentiated PC12 cells were examined. Seven day-culture with D-glucose (9.0–27.0 mg/ml as 2–6-fold of the optimal level) induced cell death in a dose-related manner but 3-day culture with high concentrations of glucose had no effect on cell viability. L-glucose had no effect on viability of PC12 cells, suggesting that D-glucose toxicity was independent of its osmolarity effect. Seven-day culture with D-glucose (13.5 mg/ml as 3-fold of the optimal level) increased nitric oxide metabolites (NO<sub>x</sub>) in the culture medium. Glucose-induced increase in NO<sub>x</sub> was eliminated by 0.1 mM L-nitro-arginine methylester (L-NAME), a nitric oxide synthase (NOS) inhibitor. Intracellular Ca<sup>2+</sup> concentration was increased by D-glucose in a dose-related manner, suggesting that D-glucose activated NOS by increasing intracellular Ca<sup>2+</sup> concentration in PC12 cells. Glucose-induced cell death was blunted by 0.1 mM L-NAME, showing that nitric oxide (NO) was involved in the glucose toxicity to PC12 cells. Tetrahydrobiopterin (BH<sub>4</sub>), a cofactor for NOS, attenuated both glucose-induced cell death and NO<sub>x</sub> production at 1 μM but not at 10 μM. The effects of BH<sub>4</sub> on glucose-induced cell death and NO<sub>x</sub> production were not mimicked by reducing agents such as ascorbate and cysteine. These results taken together suggest that high concentrations of glucose induced cell death via NO production and that low concentration of BH<sub>4</sub> had a protective effect against glucose neurotoxicity in differentiated PC12 cells. © 2002 Elsevier Science Ireland Ltd and the Japan Neuroscience Society. All rights reserved.

*Keywords:* PC12; Glucose; Cell death; Nitric oxide (NO); Intracellular Ca<sup>2+</sup> concentration; Tetrahydrobiopterin (BH<sub>4</sub>)

## 1. Introduction

Recently, there are increasing population of people suffered from diabetes mellitus (DM) (Paterson, 1999). DM is a disorder of glucose metabolism where insulin secretion from β cells and glucose uptake into insulin-consuming cells are deteriorated (Shepherd and Kahn, 1999). Hyperglycemia, which occurs under diabetic condition, induces serious diabetic complications such as neuropathy, nephropathy and retinopathy (Tooke, 1996; McMillan, 1997; Yuan et al., 1999). The mechanisms of diabetic complications remain to be fully clarified. Several lines of evidence indicate that hyperglycemia induces atherosclerosis (Schmidt et al., 1999;

Yuan et al., 1999). Insufficiency of blood flow to neuronal cells is considered to be a major problem in diabetic neuropathy (Ewing, 1996; Harati, 1996; Mooradian, 1997). Since neuronal cells have no storage of glucose, glucose depletion induces neuronal cell death (Small et al., 1997; Nath et al., 1998; Kalda et al., 2000). In contrast to low glucose stress to neuronal cells, little is known about the direct toxic effect of high concentrations of glucose on neuronal cells. In the present study, the effect of high concentrations of glucose on cellular viability was examined using differentiated PC12 cells as a model of neuronal cells.

It is well known that nitric oxide (NO) is involved in neuronal degeneration (Dawson et al., 1991; Chao et al., 1992; Lipton et al., 1993). We have observed neurotoxicity of NO in differentiated PC12 cells (Koshimura et al., 1998). Thus, in the present study, possible involvement of NO in the glucose toxicity was investigated.

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Furthermore, we investigated the effect of tetrahydrobiopterin (BH<sub>4</sub>), which is a cofactor for NO synthase (NOS) and is reported to attenuate toxicity of NO (Mayer et al., 1995; Koshimura et al., 1998), on the glucose toxicity to PC12 cells.

We demonstrate that high concentrations of glucose had a direct toxic effect on PC12 cells, which was mediated by NO and attenuated by BH<sub>4</sub>.

## 2. Materials and methods

### 2.1. Drugs

BH<sub>4</sub> · 2HCl is a generous gift from Suntory Biomedical Research Center, Osaka, Japan. Mouse nerve growth factor (NGF, 7.0S) was purchased from Funakoshi (Tokyo, Japan). All other chemicals are of the purest grade available from regular commercial sources.

### 2.2. Cell culture

PC12 cells were obtained from Riken Cell Bank (Tsukuba, Japan), maintained and subcultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 10% horse serum, 4 mM glutamine, 500 U/ml penicillin and 500 mg/ml streptomycin. The concentration of glucose in DMEM was 4.5 mg/ml. At 90% of confluency ( $2 \times 10^6$ /ml), cells were plated at  $10^5$ /ml on culture plates and were cultured for 3–5 days. At this point, confluency of the cells was about 60%. Then, the cells were differentiated by administration of 100 ng/ml NGF at 48 h intervals to DMEM which was supplemented with 0.5% fetal calf serum, 0.5% horse serum, 4 mM glutamine, 500 U/ml penicillin and 500 mg/ml streptomycin. Following 5–7 day culture for differentiation, when the cells were grown at about 70% of confluency, the cells were used for experiments.

### 2.3. MTT assay

After culture with drugs added every 48 h for indicated period, cell viability was estimated with MTT assay as previously reported (Tanaka et al., 1997). Specific optical density ( $\Delta OD_{550-620}$ ) was obtained by subtracting optical density (OD) at 620 nm from OD at 550 nm. Viable cell number cultured with varying concentrations of glucose was measured using trypan blue and the results were compared with those of MTT assay.

### 2.4. Measurement of intracellular Ca<sup>2+</sup> concentration

Intracellular Ca<sup>2+</sup> concentration was measured with fura-2 as described previously (Miyake et al., 1996). At

the end of each experiment, cells were depolarized with 50 mM KCl to estimate the cell viability. Maximum fluorescence ( $F_{max}$ ) was obtained by lysing cells with Triton X-100 (0.1%) and minimum fluorescence ( $F_{min}$ ) by adding EGTA (5 mM). Intracellular Ca<sup>2+</sup> concentration ( $[Ca^{2+}]_i$ ) was calculated from the relationship;  $[Ca^{2+}]_i = Kd \times (F - F_{min}) / (F_{max} - F)$  using 220 nM as Kd for the calcium complex of fura-2.

### 2.5. Nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) assay

After drugs were added to the incubation medium every 48 h for 7 days, concentrations of such NO metabolites (NOx) as nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) in the incubation medium were measured as previously reported (Koshimura et al., 1998). Briefly, the incubation medium was collected, mixed with equal volume of ethanol and centrifuged at 15000 rpm for 10 min. Aliquot of the supernatant was applied on NOx analyzing HPLC system (ENO-10, EICOM, Kyoto, Japan). NaNO<sub>2</sub> and NaNO<sub>3</sub> were used as authentic standards. NOx levels were expressed as nmol/ml.

### 2.6. Protein content

Protein content was measured using Bio-Rad protein assay with bovine serum albumin as the standard (Bradford, 1976).

### 2.7. Statistical analysis

All results were expressed by means ± S.E.M. The significance of difference was evaluated with ANOVA and Fisher's test. A probability level of  $P < 0.05$  was considered statistically significant.

## 3. Results

The effects of high concentrations of glucose on viability of PC12 cells were examined using MTT assay. According to the manufacturer's instruction, PC12 cells were maintained with culture medium containing 4.5 mg/ml glucose. Compared with human serum glucose concentration, this value is remarkably high. Thus, first, the optimal glucose concentration for differentiated PC12 cells was investigated. After 1-, 3-, 5- and 7-day culture with varying concentrations of glucose, cell viability was estimated by MTT assay. As shown in Fig. 1, cell viability increased in accordance with glucose concentrations. We also measured viable cell number using trypan blue and obtained similar results (data not shown). The cell viability reached the maximal at the concentration of 4.5 mg/ml in each culture point. From these data, the optimal glucose concentration for PC12 cells was assumed to be around 4.5 mg/ml as indicated

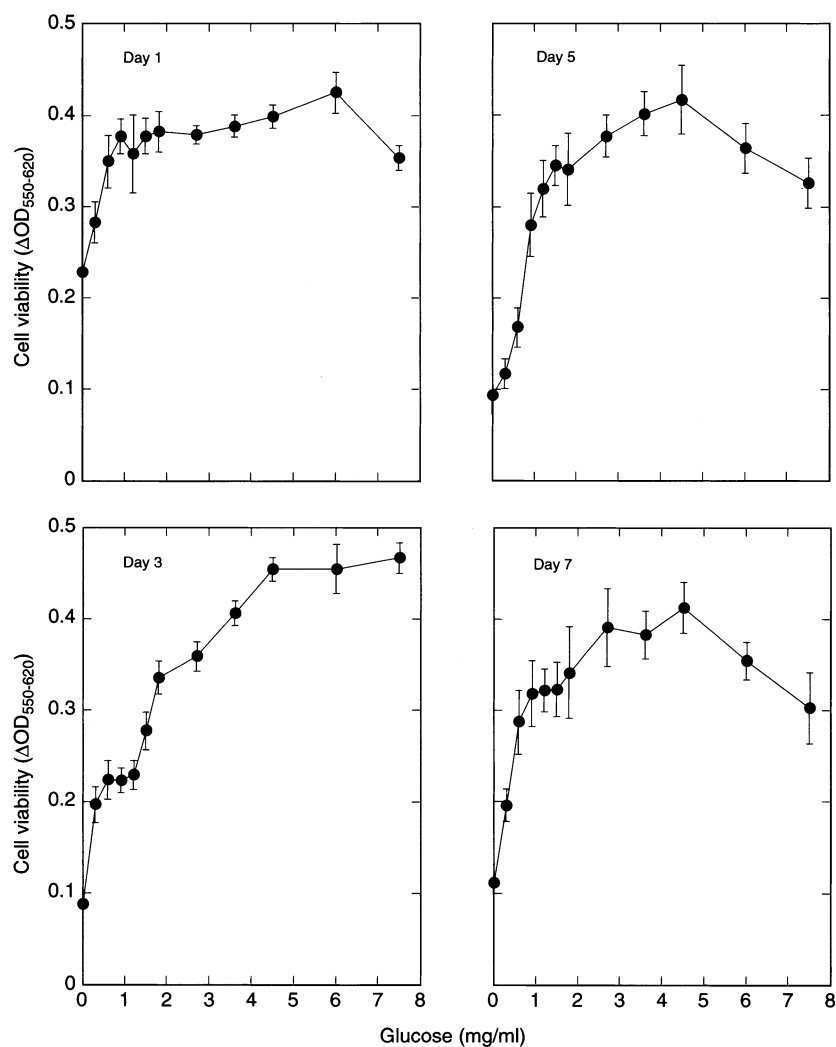


Fig. 1. Effect of glucose concentrations in the culture medium on viability of PC12 cells. After differentiated, PC12 cells were cultured with the differentiating medium with varying concentrations of glucose for 1, 3, 5 or 7 days. Glucose was added on Day 0. Cell viability was estimated on days 1, 3, 5 and 7. Each point shows the mean  $\pm$  S.E.M. of six experiments.

by the manufacturer. Therefore, in the following examination, the optimal glucose concentration for PC12 cell-culture was set at 4.5 mg/ml. When the cells were cultured with various concentrations of glucose (4.5–27.0 mg/ml) for 3 or 7 days, cell viability decreased in a dose-related manner after 7-day (Fig. 2B) culture but not 3-day culture (Fig. 2A).

Then, the osmolarity effect of the medium containing a high concentration of glucose on the cell viability was examined. Unless specified otherwise, glucose used in the present study was D-glucose, a natural form of glucose. For PC12 cell-culture, 4.5 mg/ml D-glucose was added to the culture medium as a critical nutritional element. When 9.0 mg/ml D-glucose or L-glucose was added to the culture medium containing 4.5 mg/ml D-glucose, cell viability decreased in the D-glucose-loaded group after 7-day culture as shown above, whereas 7-

day incubation with the medium containing 9.0 mg/ml L-glucose did not reduce cell viability (Fig. 3).

To investigate the mechanism of high concentrations of glucose-induced toxicity to PC12 cells, the effect of glucose on NO production was examined by measuring the levels of NOx as nitrite plus nitrate in the incubation medium (Fig. 4). When the cells were cultured with 9.0 or 13.5 mg/ml glucose for 7 days, NOx levels in the incubation medium increased in a dose-related manner. The glucose-induced increase in NOx levels in the culture medium was eliminated by 0.1 mM L-nitro-arginine methylester (L-NAME), an inhibitor of NO synthase (NOS) (Fig. 4A). To clarify the involvement of NO in the glucose toxicity, the effect of L-NAME on the glucose-induced cell death was studied. When the cells were cultured with 0.1 mM L-NAME in the presence of 13.5 mg/ml glucose for 7 days, the glucose-induced cell

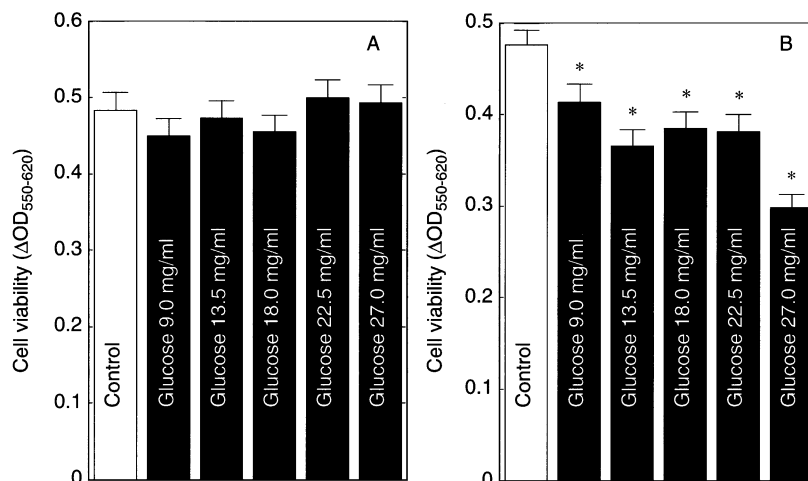


Fig. 2. Effect of glucose on viability of PC12 cells. The control culture medium was added with 4.5 mg/ml glucose. PC12 cells were cultured with high concentrations of glucose for 3 days (A) or 7 days (B) prior to MTT assay. Each column shows the mean  $\pm$  S.E.M. of ten experiments.

death was blunted. L-NAME alone had no effect on the cell viability (Fig. 4B).

It is considered that neuronal NOS (nNOS) is predominantly induced in NGF-treated PC12 cells (Schonhoff et al., 2001). Since NOS is activated by elevation of intracellular  $Ca^{2+}$  concentrations (Nathan and Xie, 1994), the effect of glucose on intracellular  $Ca^{2+}$  concentrations was examined using fura-2. After the cells were added with glucose at the concentration of 9.0 or 13.5 mg/ml, intracellular  $Ca^{2+}$  concentration was increased in a dose-related manner (Fig. 5). In contrast, intracellular  $Ca^{2+}$  concentration in the control group was unchanged until the end of the experiments (data not shown).

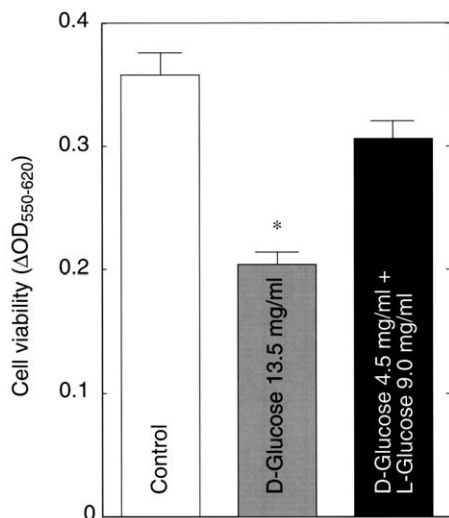


Fig. 3. Comparison of the effects of D-glucose and L-glucose on viability of PC12 cells. The control culture medium was added with 4.5 mg/ml D-glucose. High concentration (9.0 mg/ml) of D-glucose or L-glucose was added to the control medium. The cells were cultured in the test medium for 7 days prior to MTT assay. Each column shows the mean  $\pm$  S.E.M. of six experiments. \*  $P < 0.05$  vs. control group.

We have reported that toxicity of NO donors to PC12 cells was prevented by  $BH_4$ , a cofactor for NOS (Koshimura et al., 1998). Therefore, the effects of  $BH_4$  on the glucose-induced increase in  $NO_x$  levels in the culture medium and the glucose toxicity to PC12 cells were examined (Fig. 6). When the cells were cultured with 1, 10 or 30  $\mu M$   $BH_4$  in the presence of 13.5 mg/ml glucose for 7 days,  $NO_x$  levels in the incubation medium were decreased by 1  $\mu M$   $BH_4$ , but not by 10  $\mu M$  or 30  $\mu M$   $BH_4$  as compared with those in the incubation medium containing 13.5 mg/ml glucose alone (Fig. 6A). Cell viability increased when the cells were cultured with 1  $\mu M$   $BH_4$  in the presence of 13.5 mg/ml glucose as compared with the cells cultured with 13.5 mg/ml glucose alone. However, the cell protective effect of  $BH_4$  against glucose toxicity was not observed when the cells were cultured with 10 or 30  $\mu M$   $BH_4$  (Fig. 6B). Since  $BH_4$  has a reducing action, effects of reducing agents on glucose-induced cell death and NO production were examined. To avoid toxicity of reducing agents to PC12 cells, PC12 cells were cultured with varying concentrations of such reducing agents as ascorbate and cysteine and cell viability was monitored with MTT assay. Ascorbate and cysteine had no toxicity to PC12 cells below 0.1 and 10  $\mu M$ , respectively. When the cells were cultured with 0.1  $\mu M$  ascorbate or 10  $\mu M$  cysteine together with 13.5 mg/ml glucose, glucose-induced NO production was not blunted (Fig. 7A). The protective effect of  $BH_4$  on glucose-induced cell death was not mimicked by the reducing agents (Fig. 7B).

#### 4. Discussion

Present study demonstrates that high concentrations of glucose induced neuronal death. The glucose con-

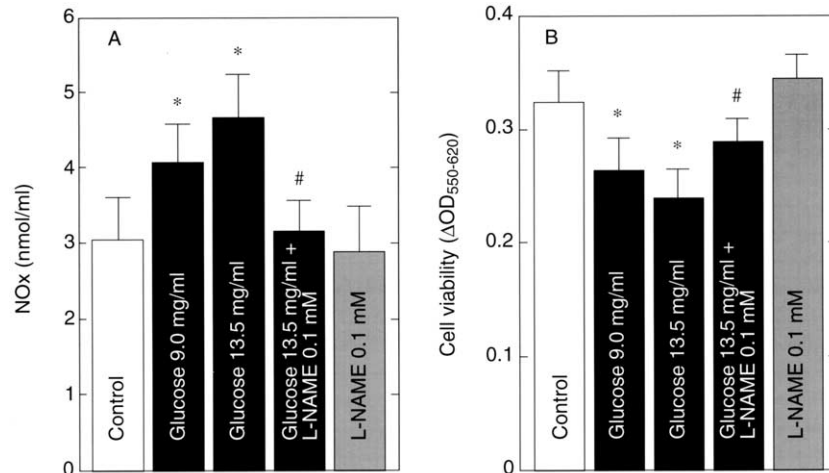


Fig. 4. Effects of glucose and L-NAME on NO<sub>x</sub> levels in the medium (A) and cellular viability (B). The control culture medium was added with 4.5 mg/ml glucose. PC12 cells were cultured with test drugs for 7 days prior to NO<sub>x</sub> measurement. Each column shows the mean  $\pm$  S.E.M. of nine experiments. \*  $P < 0.05$  vs. the control cells. #  $P < 0.05$  vs. the cells cultured with 13.5 mg/ml glucose.

centrations examined in the present study were markedly high as compared with human serum glucose concentrations. As shown in Fig. 1, the optimal glucose concentration for PC12 cell-culture was 4.5 mg/ml, which was much higher than the serum glucose concentrations. Therefore, to simulate the condition of high concentrations of glucose for PC12 cells, we used 9.0–27.0 mg/ml glucose as 2–6-fold of the optimal glucose concentration. It is reported that glucose toxicity on neuronal cells is mediated by insufficient blood flow

(Ewing, 1996; Harati, 1996; Mooradian, 1997). High concentrations of glucose is considered to induce atherosclerosis (Schmidt et al., 1999; Yuan et al., 1999). Our data indicate that besides the atherogenic effect, glucose had direct toxic effects on neuronal cells. Although D-glucose, a natural form of glucose, had neurotoxicity, L-glucose, which is not utilized in the cells, had no effect on neuronal viability. These data suggest that neurotoxicity of glucose was not due to the osmolarity effect of the medium but to the metabolic effect of glucose in the neuronal cells.

Since NOS is activated by glucose (Sobrevia et al., 1996), the effect of a high concentration of glucose on NO production was examined in PC12 cells. NO<sub>x</sub>, a marker of NO production, in the culture medium was increased after the incubation with 9.0 or 13.5 mg/ml glucose. When the cells were cultured in the presence of L-NAME, a NOS inhibitor, the glucose-induced increase in NO<sub>x</sub> levels in the culture medium was blunted. Furthermore, the glucose-induced cell death was also blunted by L-NAME. These results taken together suggest that the glucose toxicity to PC12 cells was mediated by NO.

In the PC12 cells differentiated with NGF, nNOS is assumed to be predominantly induced (Schonhoff et al., 2001). It is well known that nNOS activity is stimulated by intracellular Ca<sup>2+</sup> levels (Nathan and Xie, 1994). Intracellular Ca<sup>2+</sup> concentration monitored by fura-2 was increased after the cells were added with high concentrations of glucose. Thus it is suggested that NOS activity was enhanced via increase in the intracellular Ca<sup>2+</sup> concentration under high concentrations of glucose. The mechanism of the elevation of the intracellular Ca<sup>2+</sup> levels by high concentrations of glucose remains to be clarified. It may be possible that high concentrations of glucose induces production of ATP, a

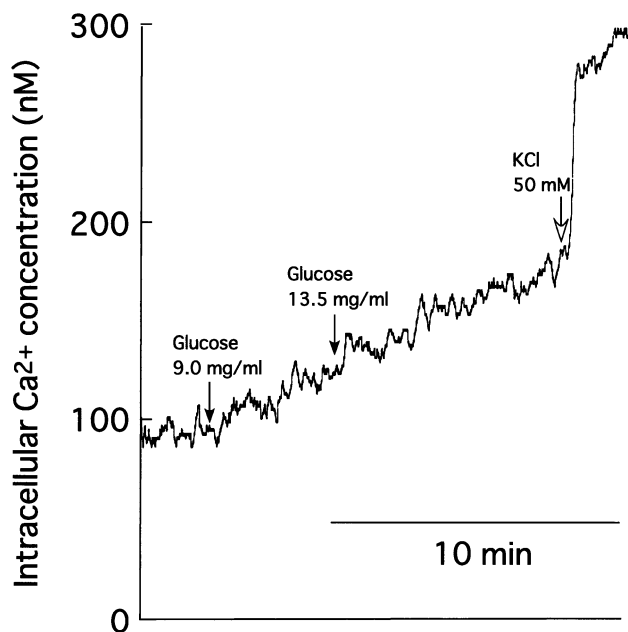


Fig. 5. Effect of glucose on intracellular Ca<sup>2+</sup> concentration in PC12 cells. PC12 cells were added with test drugs at the final concentrations indicated. Representative results were shown. Similar results were obtained from six experiments.

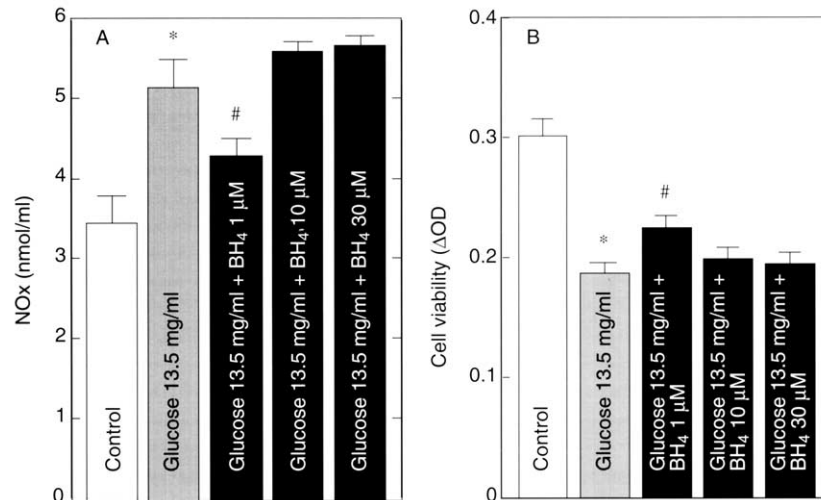


Fig. 6. Effect of tetrahydrobiopterin (BH<sub>4</sub>) on NO<sub>x</sub> levels in the medium (A) and cell viability (B) of PC12 cells cultured with 13.5 mg/ml glucose for 7 days. The control culture medium was added with 4.5 mg/ml glucose. Each column shows the mean  $\pm$  S.E.M. of six experiments. \*  $P < 0.05$  vs. the control cells; #  $P < 0.05$  vs. the cells cultured with glucose alone.

substrate for adenylate cyclase which catalyzes cAMP synthesis, resulting in Ca<sup>2+</sup> channel phosphorylation by cAMP-dependent protein kinase (Sculptoreanu et al., 1993; Wang et al., 1993; Koshimura et al., 1999).

It is reported that NO is degraded by BH<sub>4</sub>, a cofactor for NOS (Mayer et al., 1995). We have observed that BH<sub>4</sub> blunted the cytotoxic effect of NO to PC12 cells by degrading NO (Koshimura et al., 1998). These results lead to a possibility that the glucose toxicity to PC12 cells is prevented by BH<sub>4</sub>. When the cells were cultured with BH<sub>4</sub> (1, 10 or 30 μM) in the presence of a high concentration (13.5 mg/ml) of glucose, NO<sub>x</sub> levels in the culture medium was reduced by a low concentration (1 μM) of BH<sub>4</sub>. The glucose-induced cell death was also

attenuated by 1 μM BH<sub>4</sub>. These results supported our speculation. At higher concentrations (10 and 30 μM), BH<sub>4</sub> had no effect on either glucose-induced NO<sub>x</sub> levels in the culture medium or glucose toxicity. Since BH<sub>4</sub> is a cofactor for NOS, it is likely that BH<sub>4</sub> acts mainly as an NO scavenger at low concentrations and as a cofactor for NOS at high concentrations. Although BH<sub>4</sub> has a reducing activity, such reducing agents as ascorbate and cysteine had no effect on glucose-induced NO production and cell death. These results suggest that the effects of BH<sub>4</sub> was independent on its reducing activity. As reported previously, BH<sub>4</sub> had trophic effect on PC12 cells even cultured under control conditions (Fig. 8). However, trophic effect of BH<sub>4</sub> under normal glucose

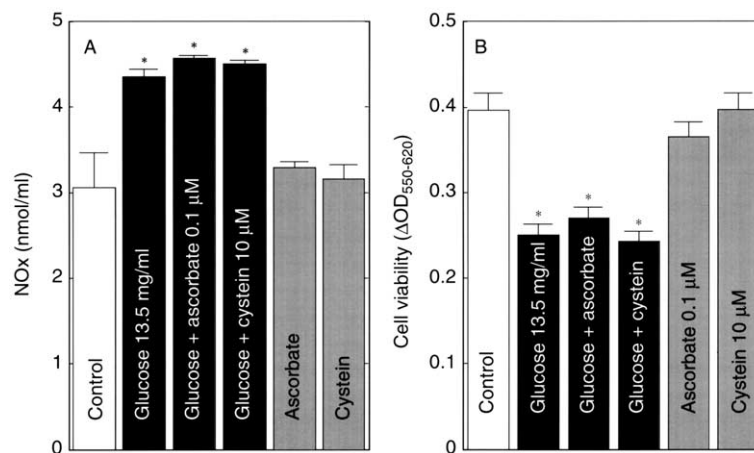


Fig. 7. Effect of reducing agents on NO<sub>x</sub> levels in the medium (A) and cell viability (B) of PC12 cells cultured with 13.5 mg/ml glucose for 7 days. The control culture medium was added with 4.5 mg/ml glucose. Each column shows the mean  $\pm$  S.E.M. of six experiments. \*  $P < 0.05$  vs. the control cells.

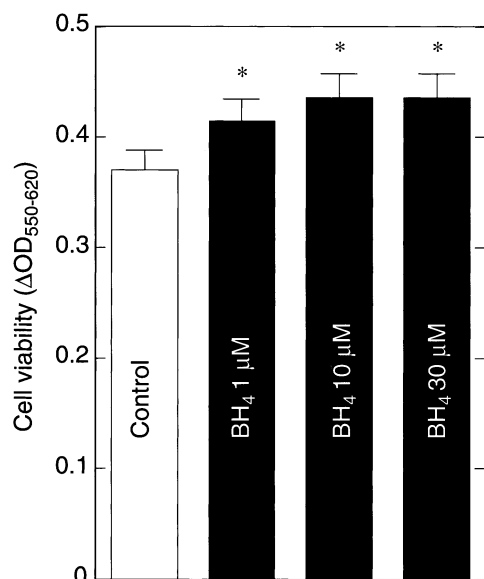


Fig. 8. Effect of tetrahydrobiopterin (BH<sub>4</sub>) on viability of PC12 cells cultured under control conditions. The cells were cultured under differentiated conditions. Then varying concentrations of BH<sub>4</sub> was added to the culture medium every 48 h, and cell viability was estimated by MTT assay after 7-day culture. Each column shows the mean  $\pm$  S.E.M. of six experiments. \*  $P < 0.05$  vs. the control cells.

concentration was blunted by high concentrations of glucose.

In addition to the glucose-induced neurotoxic effect shown in the present study, NO is reported to be involved in insulin secretion from  $\beta$  cells (Coiro et al., 1997; Henningsson and Lundquist, 1998) or insulin resistance (Steinberg et al., 1996; Shinozaki et al., 1999; Kurioka et al., 2000). Thus it is important for the strategy of DM therapy to investigate the action of NO under high concentrations of glucose.

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