Hyperoxia induces the neuronal differentiated phenotype of PC12 cells via a sustained activity of mitogen-activated protein kinase induced by Bcl-2

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We previously reported that rat pheochromocytoma PC12 cells express the neuronal differentiated phenotype under hyperoxia through the production of reactive oxygen species (ROS). In the present study, we found that in this phenotype, Bcl-2, an apoptosis inhibitor, affects mitogen-activated protein (MAP)-kinase activity, which is known as a key enzyme of the signal-transduction cascade for differentiation. When PC12 cells were cultured under hyperoxia, a rapid increase in MAP-kinase activity, including that of both p42 and p44, was observed. Although the activity level then decreased quickly, activity higher than the control level was observed for 48 h. PD98059, an inhibitor of MAP kinase, suppressed the hyperoxia-induced neurite extensions, suggesting the involvement of MAP-kinase activity in the mechanism of differentiation induced by ROS. An elevation of Bcl-2 expression was observed after culturing PC12 cells for 24 h under hyperoxia. This Bcl-2 elevation was

not affected by treatment with PD98059, suggesting that it did not directly induce neurite extension under hyperoxia. However, the blockade of the Bcl-2 elevation by an antisense oligonucleotide inhibited the sustained MAP-kinase activity and neurite extensions under hyperoxia. Further, in PC12 cells highly expressing Bcl-2, the sustained MAP-kinase activity and neurite extensions under hyperoxia were enhanced. These results suggested that MAP kinase is activated through the production of ROS, and the subsequent elevation of Bcl-2 expression sustains the MAP-kinase activity, resulting in the induction of the neuronal-differentiation phenotype of PC12 cells under hyperoxia.

Key words: antisense oligonucleotides, apoptosis, neurite extension, PD98059, reactive oxygen species.

INTRODUCTION

Reactive oxygen species (ROS) have been recently suggested as an induction signal for neuronal differentiation. Kamata et al. [1] reported that a signal for the nerve growth factor (NGF)-induced differentiation of rat pheochromocytoma PC12 cells is inhibited by activators of glutathione peroxidase, which is an endogenous reactive oxygen scavenger. Although it is not known where ROS act in the induction mechanism of differentiation, an induction of the early genes *c-fos* and *c-jun* was observed in PC12 cells [2,3]. These observations suggest that ROS play an important role in the mechanism of inducing differentiation in PC12 cells. The differentiation of neuronal cells induced by neurotrophic factors is thought to be mediated by a sustained activation of the mitogen-activated-protein (MAP)-kinase cascade; MAP kinase is a typical phosphorylase for serine/threonine residues [4,5]. These observations indicate the presence of an activation system of the MAP-kinase cascade through the production of ROS.

ROS are harmful to cells and are known to induce apoptosis in neuronal cells [6,7]. This suggests that the ROS-related mechanism of inducing differentiation is activated simultaneously with some type of apoptosis-inhibiting mechanism. Alternatively, the apoptosis-inhibiting mechanism may act directly, triggering the mechanism that induces differentiation. Bcl-2 is a proto-oncogene that inhibits various types of apoptosis, representing the typical system of inhibiting differentiation [8,9]. Lasorella et al. [10] reported that Bcl-2 expression was elevated in the retinoic acid-induced differentiation of a neuroblastoma and that the expression was not increased in cells which did not differentiate.

Eves et al. [11] performed experiments using neuronal cell lines and rat hippocampus neurons and suggested that Bcl-2 is involved in neuronal cell differentiation, regulating apoptosis. These findings indicate that Bcl-2 is involved not only in regulating apoptosis but also in the induction of neuronal cell differentiation.

We reported recently that hyperoxia induces the expression of a differentiated neuronal phenotype in PC12 cells [12]. It is also known that hyperoxia induces the production of ROS [13]. The induction of a differentiated phenotype under hyperoxia was inhibited by scavengers of ROS, and differentiation was induced by the generation of ROS [12]. These findings suggest that ROS act as induction-signal molecules for the neuronal differentiation of PC12 cells. Thus, the expression of a differentiated neuronal phenotype in PC12 cells under hyperoxia is an appropriate experimental system for examining the hypothesis described above, that the MAP-kinase cascade and apoptosis-inhibiting mechanism involving Bcl-2 are activated simultaneously by ROS. The purpose of the present study was to investigate this hypothesis.

MATERIALS AND METHODS

Cell culture and neurite outgrowth under hyperoxia

Rat pheochromocytoma PC12 cells were obtained from the RIKEN Cell Bank (Ibaraki, Japan) and maintained as described previously [14]. PC12 cells transfected with human-Bcl-2-DNA-inserted pUC-CAGGS vector or empty vector were kindly provided by Professor Y. Tsujimoto (Osaka University, Osaka,

Abbreviations used: ROS, reactive oxygen species; MAP, mitogen-activated protein; NGF, nerve growth factor; NAC, *N*-acetylcysteine.

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Japan). The cells were cultured in a mixture (1:1, v/v) of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Cosmo Bio Co., Tokyo, Japan) containing 15 mM Hepes buffer, pH 7.4, 30 nM selenium, 50 units/ml penicillin G and 0.1 mg/ml streptomycin sulphate (DF medium) supplemented with 5% horse serum and 5% fetal calf serum in 35-mm collagen-coated dishes (Iwaki Glass, Chiba, Japan) in a 5% CO₂ humidified atmosphere. After a 16 h incubation, the cells were incubated in an N₂/O₂/CO₂ incubator (BNP-110M, Tabai, Osaka, Japan) with a 9:1:10 N₂/CO₂/O₂ mixture in serum-free DF medium containing 5 µg/ml human insulin, 5 µg/ml human transferrin and 50 ng/ml sodium selenite. Cells with neurites longer than the cell body were counted as neurite-bearing cells.

Treatment with reagents

To study the involvement of ROS in the differentiation of PC12 cells, the cells were exposed to hyperoxia in the presence of 10 µM ascorbic acid or 1 mM *N*-acetylcysteine (NAC; Sigma, St. Louis, MO, U.S.A.). To inhibit MAP-kinase activity, the cells were treated with 100 µM 2'-amino-3'-methoxyflavone (PD98059; 513000-S, Calbiochem, La Jolla, CA, U.S.A.), a MAP-kinase inhibitor [15], 30 min prior to exposure of PC12 cells to hyperoxia.

Oxidative stress with the xanthine/xanthine oxidase system

For oxidative stress using a xanthine/xanthine oxidase system, cells were incubated with DF medium containing 0.25 mM xanthine and 1 m-unit/ml xanthine oxidase (Sigma).

Kinase assay for MAP kinase

The cell monolayer was washed once with ice-cold PBS. Cellular proteins were solubilized for 15 min on ice in 50 mM Hepes, pH 7.5, containing 150 mM NaCl, 10 mM EDTA, 10 mM Na₂O₇, 2 mM sodium orthovanadate, 100 mM NaF, 1% Triton X-100, 10 µg/ml aprotinin and 10 µg/ml leupeptin. MAP kinase was immunoprecipitated with rabbit anti-MAP-kinase (p42 and p44) polyclonal antibody (442704-S; Calbiochem) from the cell extracts, and MAP-kinase activities were assayed in the immune complexes using a p42/p44 MAP-kinase enzyme assay system (RPN84; Amersham, Bucks., U.K.).

Immunoblotting

After being washed twice with PBS, the cells were harvested with a Cell Scraper (Sumitomo Bakelite Co., Osaka, Japan) in 100 µl of lysis buffer containing 20 mM Tris, pH 8, 150 mM NaCl, 100 µM 4-amidinophenylmethanesulphonyl fluoride hydrochloride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 2 mM EDTA. After a mild homogenization of the cells, insoluble materials were collected by centrifugation at 14000 *g* at 4 °C for 5 min. The soluble fractions were used as immunoblotting samples for MAP kinase. Insoluble materials were further homogenized in lysis buffer containing 1% Triton X-100 and 10% glycerol, and centrifuged at 14000 *g* at 4 °C for 5 min. The mixture of the soluble fractions and the supernatants obtained from insoluble materials was used to provide immunoblotting samples for Bcl-2. A rabbit anti-MAP-kinase (p42 and p44) polyclonal antibody (442704-S, Calbiochem) and a rabbit anti-Bcl-2 polyclonal antibody (PC68-C, Calbiochem) were used as the primary antibody for the detection of MAP kinase and Bcl-2 (dilution, 1:100), respectively. MAP kinase or Bcl-2 was visualized by a ProtoBlot II AP System (Promega, Madison, WI, U.S.A.).

Treatment with Bcl-2-antisense oligonucleotides

Antisense and sense 20-mer phosphorothioate oligodeoxynucleotides directed to the translation initiation site of Bcl-2 transcripts were purchased from Geneset SA (Paris, France: antisense, 5'-CAGCGTGCGCCATCCTTCCC-3'; and sense, 5'-GGGAAGGATGCGCACGCTG-3'). After the incubation of PC12 cells with 30 µM antisense or sense oligonucleotides for 3 days in serum-free media, the cells were exposed to hyperoxia. The media containing antisense or sense oligonucleotides were exchanged every 2 days.

RESULTS

Neurite extensions and MAP-kinase activity under hyperoxia

When PC12 cells were cultured under 50% oxygen, the cell morphology changed to a flat shape after 24 h (results not shown). After 5 days, some cells extended neurites (Figure 1a). The proportion of cells showing neurite extensions increased to approximately 70% after 10 days (Figure 1b). The proportion of cells showing chromatin condensation, a typical feature of apoptosis, was approximately 10% as assessed by nuclear staining with Hoechst 33258 [14] after 10 days (results not shown). A rapid increase in MAP-kinase activity, including that

Figure 1 Induction of neurite extension under hyperoxia in PC12 cells

(a) Phase-contrast micrograph showing the morphology of PC12 cells. Magnification $\times 60$. PC12 cells were incubated under 21 (a, c) or 50% (b, d) O₂ for 5 (a, b) or 10 (c, d) days. (b) Percentage of cells with neurites. PC12 cells were incubated under 21 (○) or 50% (●) O₂ for the indicated time periods. Each point is the mean $\pm$ S.D. of three or four samples.

Figure 2 Hyperoxia increases MAP-kinase activity and neurite extension by producing ROS

(a) MAP-kinase activity in PC12 cells. The cells were incubated with 10 μ M ascorbic acid (AA, Δ), 1 mM NAC ($\square$), xanthine/xanthine oxidase (X/XOD, $\blacktriangle$) or 100 μ M PD98059 ($\times$) under 21 ($\circ$, $\blacktriangle$) or 50% ($\bullet$, Δ , $\square$, $\times$) O_2 for the indicated time periods. After the incubation, MAP-kinase activities were assayed as described in the Materials and methods section. Each point is the mean $\pm$ S.D. of three samples. (Inset) Amount of MAP kinase (p44, p42) in PC12 cells at 1 (lane 1), 24 (lane 2), 48 (lane 3) and 72 (lane 4) h after exposure to hyperoxia. (b) Percentage of cells with neurites. The cells were incubated with AA, NAC, X/XOD or PD98059, as in (a), under 21 or 50% O_2 . Cells with neurites were counted after 10 days. Each bar is the mean $\pm$ S.D. of three or four samples.

of p42 and p44, was observed after exposure to hyperoxia, and the activity started to decrease after 3 h (Figure 2a). However, this MAP-kinase activity was sustained at a higher level than that in the control group for 48 h (Figure 2a). The immunoblotting experiments indicated that this increase did not result from an increase in the amount of MAP kinase (Figure 2a).

Figure 3 Hyperoxia induces an elevation of Bcl-2 expression by producing ROS

PC12 cells were incubated without (lanes 1–6 and 10) or with (lane 7) 10 μ M ascorbic acid (AA), 1 mM NAC (lane 8), xanthine/xanthine oxidase (X/XOD, lane 11) or 100 μ M PD98059 (lane 9) under 21 (lanes 10 and 11) or 50% (lanes 1–9) O_2 for the indicated time periods. After the incubation, the cells were collected, 30 μ g of cellular protein was applied to SDS/PAGE, and the expression of Bcl-2 was assayed as described in the Materials and methods section. Similar results were obtained in three separate experiments.

Involvement of ROS in neurite extension and MAP-kinase activity under hyperoxia

The hyperoxia-induced increase in MAP-kinase activity was not detected in the presence of reducing agent, either ascorbic acid or NAC (Figure 2a). In this case, the hyperoxia-induced neurite extensions were suppressed (Figure 2b). Further, when ROS were produced by adding xanthine/xanthine oxidase, even though the cells were incubated under normoxia, both an increase in MAP-kinase activity and an induction of neurite extensions similar to those under hyperoxia were observed (Figure 2). When the cells were treated with PD98059, an inhibitor of MAP kinase (p42 and 44), the hyperoxia-induced increases in MAP-kinase activity and neurite extensions were suppressed (Figure 2).

Elevation of Bcl-2 expression under hyperoxia

There was no change in Bcl-2 expression immediately after exposure to hyperoxia (Figure 3). However, the expression was clearly elevated at 24 h and thereafter. This elevated expression persisted for at least 48 h (Figure 3), and then decreased to the baseline level after 72 h (results not shown). In contrast, when cells were cultured under normoxia, no change in Bcl-2 expression was observed (Figure 3). The elevated Bcl-2 expression under hyperoxia was inhibited by ascorbic acid and NAC. Further, the Bcl-2 expression was elevated 48 h after treatment with xanthine/xanthine oxidase, as under hyperoxia. The inhibition of MAP-kinase activity by PD98059 did not affect the hyperoxia-induced induction of Bcl-2 expression (see Figure 3).

Influence of Bcl-2 antisense oligonucleotides on neurite extension and MAP-kinase activity under hyperoxia

After treatment with Bcl-2 antisense oligonucleotides for 3 days, PC12 cells were cultured under hyperoxia. The hyperoxia-induced increase in Bcl-2 expression after 24 h was inhibited in these cells (Figure 4a). The induction of neurite extension in these

Figure 4 Bcl-2 antisense oligonucleotides decrease hyperoxia-induced neurite extension and the sustained MAP-kinase activity

(a) Expression of Bcl-2 in PC12 cells. The cells were incubated without (lanes 1 and 2) or with (Bcl-2-AS, lane 3) 30 μ M Bcl-2 antisense oligonucleotides, 30 μ M Bcl-2 sense oligonucleotides (Bcl-2-S, lane 4) under 21% O_2 for 3 days. After the incubation, the cells were further incubated under 21 (lane 1) or 50% O_2 (lanes 2–4). Immunoblotting for Bcl-2 was performed after 24 h. (b) Percentage of PC12 cells with neurites. The cells were treated with Bcl-2 antisense or sense oligonucleotides, as in (a), and further incubated under 21 or 50% O_2 . Cells with neurites were counted after 10 days. Each bar is the mean $\pm$ S.D. of three samples. (c) MAP-kinase activity of PC12 cells. The cells were treated with the same Bcl-2 antisense or sense oligonucleotides, as in (a), and further incubated without oligonucleotides (○, ●), or with Bcl-2 antisense (△, ▲) or sense (□, ■) oligonucleotides, under 21 (○, △, □) or 50% (●, ▲, ■) O_2 . MAP-kinase activities were assayed at the indicated time periods. Each point is the mean $\pm$ S.D. of three samples.

Figure 5 High expression of Bcl-2 enhances the sustained MAP-kinase activity and neurite extensions under hyperoxia

(a) MAP-kinase activity of PC12HB and PC12V cells. PC12HB (△, ▲, ×) and PC12V (○, ●) cells were incubated under 21 (○, △) or 50% (●, ▲, ×) O_2 in the absence (○, ●, △, ▲) or presence (×) of PD98059. MAP-kinase activities were assayed at the indicated time periods. (Inset) Amount of MAP kinase (p44, p42) in PC12V (lanes 1 and 2) and PC12HB cells (lanes 3 and 4) at 1 (lanes 1 and 3) and 48 h (lanes 3 and 4) after exposure to hyperoxia. Each point is the mean $\pm$ S.D. of three samples. (b) Percentages of PC12HB and PC12V cells with neurites. The cells were incubated as in (a). Cells with neurites were counted at the indicated time periods. Each point is the mean $\pm$ S.D. of three or four samples.

antisense oligonucleotide-treated cells was clearly inhibited after 10 days (Figure 4b). In this case, the proportion of cells showing apoptosis was approximately 75% (results not shown). In contrast, in the sense oligonucleotide-treated cells, the hyperoxia-induced Bcl-2 elevation and neurite extensions were observed, as in the control group (Figures 4a and 4b). The MAP-kinase activity in the cells treated with Bcl-2 antisense oligonucleotides showed no rapid increase under hyperoxia (Figure 4c). However, the sustained MAP-kinase activity for 24 h after the initiation of culture under hyperoxia was inhibited by Bcl-2 antisense oligonucleotides (Figure 4c). In contrast, the MAP-kinase activity was not influenced in the sense oligonucleotide-treated cells.

Influence of high Bcl-2 expression on neurite extension and MAP-kinase activity under hyperoxia

We next performed experiments using PC12HB cells, which are PC12 cells that highly express human Bcl-2, established by Shimizu et al. [16]. PC12V cells transfected with an empty vector were used as a control. High Bcl-2 expression further enhanced the increase in MAP-kinase activity (Figure 5a). This increase in the activity clearly persisted longer than that in the control cells (Figure 5a). In contrast, the hyperoxia-induced increase in MAP-kinase activity in PC12V cells was at the same level as that in the wild-type PC12 cells (Figures 2a and 5a). These changes in MAP-kinase activity did not result from changes in the amounts of MAP kinase (Figure 5a). Further, the high Bcl-2 expression did not influence the morphology of PC12 cells under normoxia (Figure 5b). However, the induction of neurite extension in PC12HB cells under hyperoxia was clearly enhanced compared with that in control cells (Figure 5b). Few PC12HB cells under hyperoxia showed typical apoptotic features after 10 days (results not shown). The inhibition of MAP kinase by PD98059 suppressed the hyperoxia-induced neurite extensions in PC12HB (Figure 5b).

DISCUSSION

The reducing agents inhibited both the rapid increase and the sustained increases of MAP kinase activity observed in PC12 cells under hyperoxia. Further, artificially produced ROS induced an increase of MAP-kinase activity as under hyperoxia, suggesting that ROS activates MAP kinase. When the hyperoxia-induced increase in MAP-kinase activity was blocked by PD98059, the hyperoxia-induced neurite extensions were also suppressed. These results suggest that an increase in MAP-kinase activity is essential for hyperoxia-induced neurite extension. An elevation of Bcl-2 expression was observed after the rapid increase in MAP-kinase activity. Bcl-2 antisense oligonucleotides suppressed a sustained increase in MAP-kinase activity but did not effect a rapid increase. This suggests that the hyperoxia-induced Bcl-2 elevation contributes to the sustainment of MAP-kinase activity. Further, Bcl-2 antisense oligonucleotides clearly suppressed the hyperoxia-induced neurite extension, suggesting that sustained MAP-kinase activity is necessary for neurite extension under hyperoxia. These concepts were confirmed by the enhancements of the sustained MAP-kinase activity and neurite extension under hyperoxia in PC12 cells highly expressing Bcl-2.

Chen et al. [17] reported that ROS induced a rapid increase in MAP kinase in Hela cells. They suggested that ROS contributed to the MAP-kinase cascade. The present study's findings suggest that a rapid increase in MAP-kinase activity under hyperoxia was directly induced by ROS. The differentiation of neuronal cells induced by neurotrophic factors is thought to be mediated

by a sustained activation of the MAP-kinase cascade [4,5]. Therefore, under hyperoxia, ROS may induce a rapid increase in MAP-kinase activity, and the elevated Bcl-2 may sustain the increase in MAP-kinase activity, resulting in the induction of neurite extensions of PC12 cells. However, the rapid increase in MAP-kinase activity of the PC12HB cells was to a higher level compared with that of the PC12V cells. This may be a result of an artificial enhancement of the rapid increase in MAP-kinase activity by the putative MAP-kinase-sustaining mechanism with Bcl-2.

The MAP-kinase inhibitor PD98059 inhibited the hyperoxia-induced neurite extensions but not the elevation of Bcl-2 expression. Further, PC12HB cells did not show neurite extensions in the presence of PD98059 under hyperoxia or normoxia. These results indicate that an elevation of Bcl-2 expression alone cannot induce the neurite extensions. Bcl-2 has a sequence called BH4 that is known to bind with various proteins within the molecule [18–20]. A serine/threonine kinase, raf-1, is known to bind to BH4 [17,21]. Raf-1 is a signal molecule of the NGF-induced mechanism of neuronal differentiation in PC12 cells, and is located upstream of the MAP-kinase cascade [22–24]. Wang et al. [19] reported that the serine/threonine kinase activity of raf-1 is activated when raf-1 binds with Bcl-2 at BH4. Therefore, Bcl-2 expression induced by hyperoxia may contribute to the mechanism that induces differentiation by activating raf-1 through this site, which contributes to sustained MAP-kinase activation. Further investigation on this point is underway.

We reported previously that NGF elevates the Bcl-2 expression in PC12 cells [24,25]. We also found that Bcl-2 antisense oligonucleotides inhibited the induction of the differentiated neuronal phenotype by NGF [14,25]. However, these experiments were performed under conditions of cellular stress, such as hyperoxia or a serum-free state [14,25]. Therefore, it is unclear whether the same Bcl-2 response is observed when cells are stimulated by differentiation inducers under normal conditions. Sato et al. [26] reported that a high expression of Bcl-2 does not directly influence the NGF-induced differentiation of PC12 cells, but under conditions that induce apoptosis, Bcl-2 induces neuronal differentiation. When cells are exposed to critical conditions and differentiate to escape from this crisis, Bcl-2, an inhibitor of apoptosis, may be involved.

In this study, it was shown that the Bcl-2 molecule actively contributes to the hyperoxia-induced neuronal differentiation of PC12 cells. This finding may provide important information for research on neuronal differentiation.

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