INTRODUCTION

Polyamines, such as putrescine (PUT), spermidine (SPD) and spermine (SPM), are polycationic compounds known to be ubiquitously distributed in the living organisms, and speculated to be implicated in cell proliferation and other biological processes. Previously, polyamine biosynthesis has been shown to be highly regulated, but the physiological functions of these amines have not yet been fully elucidated at the cellular and molecular levels. Previous studies have suggested that polyamines can preferentially bind to the GC-rich regions of DNA and RNA, proposing the possibility that the interaction of these compounds with the GC-rich region of the DNA in a cell-free system may contribute to their biological activities in vivo [1-3]. On the other hand, polyamines have been suggested to be implicated in mental disorders [4], and also reported to protect neuronal cells against mechanical injuries, neurotoxic insults and ischemic damage in the brain [5-7]. Particularly, the genetic variants in the polyaminergic genes have been suggested to be closely related to the psychiatric conditions, thereby proposing a possible connection between polyamine metabolism and affective disorders, such as anxiety, depression and attempted suicide [4]. Recent studies have provided evidence for suggesting the possibility that polyamines may be able to contribute to the adult neurogenesis, the aged-related hippocampal neurogenesis and the learning and memory functions [8,9].
suggesting that polyamines may be involved in the modulation of chromatin structures, the gene transcription and translation and the DNA stabilization as well as the functions of specific cellular proteins has also been provided [10,11].

Polyamines and their metabolizing enzymes have previously been reported to be localized in different regions of the brain and different types of the cells, and it, therefore, seems reasonable to consider that polyamine biosynthesis and storage may be conducted at the different locations in the brain [12]. On the other hand, polyamines have been shown to be preferentially accumulated in astrocytes, thus suggesting a possible involvement of polyamines in the regulation of the glial network under the normal and pathological conditions [13]. These observations are considered to propose the possibility that polyamines may be able to cause the modulation of neuronal cell function as a consequence of acting on glial cells in the brain, but little is known about the biological or the physiological actions of polyamines on the functions and metabolism of glial cells. On the other hand, we have investigated the direct effects of various neurotransmitters and neuroactivators on glial cell functions and metabolism in vitro, and suggested that these agents may be able to stimulate the biosynthesis of neuroactive 5α-reduced steroids in the glioma cells, resulting in the enhancement of their ability to produce brain-derived neurotrophic factor probably through the promotion of glial cell differentiation, thus suggesting a possible role of glial cells in protecting and reviving the functions of neuronal cells as well as maintaining the integrity of neural network in the brain [14,15]. Furthermore, the neurosteroid-mediated differentiation of the glioma cells has also been suggested to induce the enhancement of glutamate transporter-1 gene expression, and therefore speculated to reduce the excitotoxic damage to neuronal cells as a consequence of facilitating the removal of glutamate from the space of brain tissue [16]. Based on these previous findings, we have suggested that various neuroactive agents may be able to act directly on glial cells, thus modulating the neuronal cell functions through the intercellular communication between neuronal and glial cells in the brain. Furthermore, the former study has provided evidence for suggesting that ornithine decarboxylase (ODC) can be induced in rat brain during the recovery from various injuries [17,18], thereby allowing us to propose the hypothesis that glial cells may play a potential role in the recovery of neuronal cell function from degenerative damage in the brain through the intercellular communication mediated by polyamines produced in glial cells. Then, as the first step to verify this hypothetical idea, chemical hypoxia was selected as a noxious insult, and the effects of cobalt- and azide-induced hypoxia on polyamine contents and ODC mRNA levels were examined using C6 glioma cells as an in vitro model system.

MATERIALS AND METHODS

Materials

Rat C6 glioma cells (CCL-107) were purchased from the American Type Culture Collection (Rockville, MD, USA). Polyamines, such as PUT, SPD and SPM, and DL-α-difluoromethylornithine (DFMO) hydrochloride hydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). M-MLV reverse transcriptase was obtained from Promega Corp. (Madison, WI, USA). Taq DNA polymerase was from LabGene Scientific (Chatel-St-Denis, Switzerland). Cobalt sulfate (CoSO₄) heptahydrate and sodium azide (NaN₃) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were commercially available reagent grade or ultrapure grade.

The PCR primers for rat ODC and rat Hif-1α genes and the phosphorothioate-capped scrambled and antisense oligodeoxynucleotides (ODNs) for rat Hif-1α gene were commercially synthesized by Sigma Genosys Japan (Hokkaido, Japan) according to their sequences reported previously. The sequences of ODNs were: 5'-AGCAGACCCCGCTTCCGACAT-3' (forward) and 5'-AGACATGGCCAGAGTCCCAA-3' (reverse) for ODC (426 bp) [19]; 5'TGCTTGTGTGTTGATTGTGAGA3'-3' (forward) and 5'-GGTCAGATGATCAGAGTCCA-3' (reverse) for Hif-1α [210 bp] [20]; 5'-CTACATGAAGCTTGTGTTGGCC-3' (forward) and 5'-CAGTCGACACGCCGAG-ATGCCG3'- (reverse) for small-size β-actin [271 bp]; 5'-TGCCTGCAACAGCCTTCCGGCATG-3'- (forward) and 5'-CCAGCAGGTCCAGACGCAGAT-3' for large-size β-actin [520 bp]. The antisense and scrambled ODNs were: 5'-CsCsTCCATGCCGAATCGTGCGTG3' (antisense) and 5'-AsCsTCTGACCCGGCAGTTTsC3' (scrambled) for Hif-1α [21].

Cell Culture and Drug Treatment

Rat C6 glioma cells were seeded onto a 60-mm culture dish at a density of 1 × 10⁶ cells/dish, and then maintained for 72 h in 3 ml of the growth medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 2 μM L-glutamine, 5% heat-inactivated bovine serum, 5% heat-inactivated equine serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 50 μg/ml of gentamicin sulfate at 37°C in a humidified incubator containing 95% air and 5% CO₂ atmosphere. After replacing the medium with the serum-free medium containing 5 μg/ml of insulin, 5 μg/ml of transferrin and 5 ng/ml of sodium selenite instead of sera, the cells were maintained for additional 24 h, and then exposed to CoSO₄ or NaN₃ at various concentrations for different time periods.

High-Performance Liquid Chromatography (HPLC) Analyses of Cellular Polyamines

The glioma cells were exposed to various concentrations of either CoSO₄ or NaN₃ for 24 h in the serum-free medium, and the medium was removed by aspiration at the end of the exposure time. The cells were washed with 3 ml of ice-cold phosphate-buffered saline (PBS) for three times, and then scraped off with 1 ml of PBS into an Eppendorf tube. The tube was centrifuged at 1,000 × g for 5 min to precipitate the cells, and the pelleted cells were disrupted by suspending in 500 μl of distilled water followed by a freeze-thaw cycle. The lysate was centrifuged at 20,000 × g for 20 min, and soluble proteins...
contained in 400 μl of the cell extract was denatured by mixing with 200 μl of 20% trichloroacetic acid and keeping on ice for at least 30 min. The mixture was centrifuged at 20,000 × g for 20 min to remove denatured proteins, and the obtained supernatant solution was stored in a freezer until use.

The determination of polyamines contained in the cell extract was carried out using a HPLC system (Hitachi, Ltd.) with a fluorescence detector Hitachi L-7480 as reported previously with modifications [22,23]. Briefly, the acidified sample (100 μl) was applied onto a cation-exchange column Hitachi No. 2619F (2.6 × 50 mm, 5 mm particle size) kept at 70°C, and polyamines were successively eluted out from the column with a mobile phase consisting of the different ratio of A-buffer (0.045 M sodium citrate, 0.06 M citric acid, 0.06 M NaCl, 4 ml/l of benzyl alcohol) and B-buffer (0.2 M sodium citrate, 2 M NaCl, 4 ml/l of benzyl alcohol, 50 ml/l of n-propyl alcohol) at a flow rate of 0.25 ml/min. The elution schedule was as follows: (1) the column was washed with the mobile phase in which the percentage of B-buffer was elevated from 0 to 50% during 0-4 min, (2) B-buffer was raised from 50% to 85% during 4-10 min, (3) from 85 to 90% during 10-15 min, (4) elevated from 90% to 100% during 15-20 min, (5) finally eluted with 100% B-buffer for 20-60 min. Polyamines flowing out the column were reacted with α-phthalaldehyde, and the fluorescence of the reaction products was then measured at the excitation at 340 nm and the emission at 435 nm using the on-line reaction-detection system. Then, the column was rinsed with Buffer-B containing 0.2 M NaOH for 5 min to revive the resin after completing the elution process.

One-step Reverse Transcription Polymerase Chain Reaction (RT-PCR) Determination of ODC and Hif-1α mRNA Levels

Total RNA was prepared from a single culture according to the method described previously with repeating the extraction step twice [24], and ODC and Hif-1α mRNA levels were determined by a one-step RT-PCR technique as described previously [25]. Total RNA (2-3 μg) was reverse transcribed and successively amplified using an automated thermal cycler in 25 μl of the first strand buffer containing 500 μM dNTPs, 400 μM dithiothreitol, 5% DMSO, 25 pmoles of the primers using 100 units of M-MLV reverse transcriptase and 2.5 units of recombinant Taq DNA polymerase. The reaction mixture was incubated at 48°C for 45 min to generate the first strand cDNA, and heated at 95°C for 2 min to inactivate reverse transcriptase, and the cDNA was then amplified by subjecting the mixture to 35 cycles of the DNA polymerase chain reaction with each cycle including denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min, primer extension at 72°C for 2 min, and a final extension step at 72°C for 7 min. The PCR products were separated on a 1.8% agarose gel, and the intensities of these bands were determined by a computerized densitometric analysis using Imagej 1.47 for Windows software. Rat β-actin mRNA levels were simultaneously determined as an internal standard, and the target mRNA levels were normalized by calculating the ratio of them to the internal standard mRNA levels.

Statistical Analysis

Results were presented as the mean ± standard error of mean, and the statistical assessment of the differences were carried out using a one-way Analysis of Variance followed by Tukey’s post hoc test. The P < 0.05 was regarded as indicating a statistically significant difference between two groups.

RESULTS

Effect of Chemical Hypoxia on Cellular Polyamine Contents

Previous studies have shown that the polyamine-biosynthetic enzyme ODC can be induced in the brain during the recovery from various toxic injuries, thereby proposing a possible implication of polyamines in the process restoring the damaged brain function [17,18]. Then, to demonstrate this putative process, hypoxia was employed as a typical noxious impact on the brain, and the effects of both cobalt- and azide-induced chemical hypoxia on the cellular contents of polyamines were examined using C6 glial cells as an in vitro model system. As shown in Figure 1, neither PUT nor SPD contents in the glial cells were significantly changed by exposing the cells to various concentrations of CoSO4 (100-400 μM) for 24 h, but SPM contents were substantially increased by CoSO4 at the concentrations of 200 μM or higher. By contrast, the exposure to 50 μM NaN3 significantly increased PUT contents without altering both SPD and SPM contents in the cells under the same experimental conditions, [Figure 2]. These results could be speculated to suggest that both CoSO4 and NaN3 might be able to cause an alteration in the biosynthesis of polyamines as a consequence of inducing chemical hypoxia. However, these two chemicals were shown to cause the different effects on the conversion of PUT, SPD, and SPM, it seemed possible to presume that the different effects of CoSO4 and NaN3 on the metabolism of polyamines might reflect their individual effect rather than their common effect to induce chemical hypoxia within the cells. Further study therefore seemed necessary to elucidate whether chemical hypoxia might be able to influence the biosynthesis and metabolism of polyamines in glial cells.

The exposure to CoSO4 was clearly shown to significantly increase SPM contents without any noticeable changes in the PUT and SPD contents, and it therefore seemed still doubtful whether CoSO4-increased SPM contents might actually reflect the biosynthesis of polyamines in the cells. Further study therefore seemed necessary to clarify this point.

The exposure to NaN3 was shown to increase both PUT and SPD contents, and it therefore seemed still doubtful whether NaN3-increased PUT and SPD contents might actually reflect the biosynthesis of polyamines in the cells.
necessary to confirm whether the hypoxic environment of the
cells could be achieved under the experimental conditions used
here. Then, the glioma cells were exposed to CoSO_{4} and NaN_{3}
for different time periods, and the effects of these chemicals
on Hif-1α gene expression was determined as an index for the
hypoxic cellular environment. As shown in Figure 4, both CoSO_{4}
(400 μM) and NaN_{3} (50 μM) caused the rapid elevation of
Hif-1α mRNA levels at the concentrations causing an increase in
the cellular contents of polyamines in the cells. In fact, an
approximately 2-fold increase in Hif-1α mRNA levels was
observed at 2 h after the exposure of the glioma cells to these
chemicals. Therefore, it seemed reasonable to consider that
the exposure to CoSO_{4} and NaN_{3} might be able to produce the
hypoxic cellular environment, proposing the possibility that the
induction of hypoxic conditions might contribute to an increase
in the cellular contents of polyamines observed here.

Figure 1: Effect of CoSO_{4} on polyamine contents in rat C6 glioma cells. Cells were exposed to various concentrations of CoSO_{4} (0 - 400 μM) for 24 h, and the cellular contents of putrescine, spermidine and spermine were then determined using a high-performance liquid chromatography method as described in the text. Results were expressed as a percent of the control. Values are the mean ± standard error of mean (*P < 0.05, n = 6)

Figure 2: Effect of NaN_{3} on polyamine contents in rat C6 glioma cells. Cells were exposed to various concentrations of NaN_{3} (0 - 50 μM) for 24 h, and the cellular contents of putrescine, spermidine and spermine were then determined using a high-performance liquid chromatography method as described in the text. Results were expressed as a percent of the control. Values are the mean ± standard error of mean (*P < 0.05, n = 6)
Effect of Chemical Hypoxia on ODC mRNA Levels

Chemical hypoxia was shown to increase the cellular contents of polyamines through the elevation of ODC activity in the glioma cells, and therefore seemed possible to anticipate that the elevation of this enzyme activity observed under the hypoxic conditions might be the result of enhancing the ODC gene expression. Then, the glioma cells were exposed to CoSO₄ or NaN₃ under the conditions in which the elevation of cellular polyamine contents was observed, and ODC mRNA levels were then determined to elucidate a possible effect of chemical hypoxia on the transcription of ODC gene in the glioma cells. As shown in Figure 5, both CoSO₄ and NaN₃ caused the significant elevation of ODC mRNA levels at 8 h after the exposure, which was observed following the enhancement of Hif-1α mRNA levels in the cells observed at 2-6 h after the exposure [Figure 4]. Furthermore, the concentrations of these chemicals required for the induction of ODC gene transcription were shown to be almost similar to those required for the increment

![Graph: Effect of Chemical Hypoxia on ODC mRNA Levels](image)

**Figure 3:** Effect of ornithine decarboxylase (ODC) inhibitor on CoSO₄-induced elevation of polyamine contents in rat C6 glioma cells. Cells were exposed to 400 μM CoSO₄ for 24 h in the presence and absence of 2.5 mM DL-α-di fluoromethylornithine, and the contents of putrescine, spermidine and spermine in the glioma cells were then determined using a high-performance liquid chromatography assay method as described in the text. Results were expressed as a percent of the control. Values are the mean ± standard error of mean (*P < 0.05, n = 6)

![Graph: Effect of Chemical Hypoxia on ODC mRNA Levels](image)

**Figure 4:** Effects of CoSO₄ and NaN₃ on Hif-1α mRNA levels in rat C6 glioma cells. Cells were exposed to 400 μM CoSO₄ or 50 μM NaN₃ for different time periods, and Hif-1α mRNA levels were determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± standard error of mean (*P < 0.05, n = 6)
of cellular polyamine contents [Figure 6] and the elevation of Hif-1α mRNA levels in the cells (data not shown). These results were considered to suggest a possible connection between the chemically-induced hypoxic conditions and the ODC gene transcription in the glioma cells, but there was no conclusive evidence obtained yet.

Generally, Hif-1α is a transcription factor induced by the hypoxic conditions and involved in the regulation of hypoxia-induced gene transcription in various cells and tissues. Then, to confirm the implication of hypoxia in cobalt-induced ODC gene transcription, the effect of CoSO₄ on ODC mRNA levels was examined in the glioma cells preloaded with the scramble or the antisense ODN targeting Hif-1α gene. In the cells preloaded with the scrambled ODN, CoSO₄ caused the elevation of both Hif-1α and ODC mRNA levels, similar in extent to those obtained in the naive cells. By contrast, CoSO₄ failed to cause any significant changes in Hif-1α and ODC mRNA levels in the cells preloaded with the antisense ODN [Figure 7], thus indicating that the cobalt-induced ODC gene transcription might be completely suppressed by silencing Hif-1α gene expression under the experimental conditions used here.

DISCUSSION

Polyamines are known as biologically active substances ubiquitously distributed in the living organisms and have been...
studies to elucidate their potential roles in a variety of the cellular metabolism and functions. Previously, the enhancement of ODC activity has been shown to be observed during the early phase of recovery from various toxic insults, such as chemical, mechanical, thermal and metabolic injuries to the brain, and the ODC products polyamines have therefore been presumed to play their potential roles in the protection and recovery of the brain function from noxious injuries [17,18]. Furthermore, polyamines have also been shown to be in preferentially accumulated in gial cells, and therefore allowed us to speculate a possible involvement of gial cells in the regulation of neuronal cell function via accumulated polyamines in the brain. In the present study, the influence of cobalt- and azide-induced chemical hypoxia on the cellular contents of polyamines were first investigated using C6 glioma cells as an in vitro model. Consequently, the exposure to CoSO₄ was shown to significantly increase the cellular SPM contents without causing any significant change in the PTU and SPD contents [Figure 1]. By contrast, the exposure to NaN₃ was shown to cause a significant increase in the PUT contents without any substantial increase in the SPD and SPM contents in the cells [Figure 2]. The results seemed to provide evidence for supporting the previous finding that hypoxia might be able to increase the biosynthesis of polyamines in gial cells. However, the profile of cobalt-induced polyamine formation observed here was different from the typical profile of polyamine biosynthesis, which is ordinary known to reveal the elevation of PUT contents without any remarkable increase in the contents of others, just like the profile of azide-induced PUT formation [Figure 2].

Generally, the elevation of PUT levels in the damaged cells and tissues is known to be observed following severe cellular stresses as a consequence of increasing ODC activity and decreasing S-adenosylmethionine decarboxylase, which can enhance the formation of PUT and reduce the conversion of PUT to SPD, and/or facilitating the conversion of SPD to PUT through the activation of SPD N-acetyltransferase and polyamine oxidase [26]. Therefore, it seems possible to speculate that the difference in the profile of cobalt-induced and azide-induced polyamine formation may be connected with the different extent of cobalt-induced and azide-induced damage to the cells. Practically, azide is more toxic and can be considered to cause the severe damage to the cells, thus resulting in an increase in cellular PUT contents not only by enhancing ODC activity but also by reducing the conversion of PUT to SPD in the cells. In contrast, cobalt is less toxic to the cells, and therefore can be speculated to cause the elevation of ODC activity without affecting the interconversion process, thus resulting in the elevation of SPD contents without any notable change in PUT and SPD contents. Therefore, this notion may be considered to account for the different profile of cobalt-induced and azide-induced polyamine formation observed here. Further study about the influence of cobalt- and azide-induced hypoxic conditions on the interconversion of polyamines is still necessary to obtain a conclusive answer.

Cobalt-induced chemical hypoxia was shown to cause the elevation of SPM contents without any noticeable change in PUT and SPD contents in the cells [Figure 1]. Therefore, it basically seemed still questionable whether the cobalt-induced elevation of SPM contents observed here could actually reflect the elevation of ODC activity within the cells. Then, the effect of an ODC inhibitor DFMO on cobalt-stimulated polyamine formation was examined to confirm that polyamine formation might be practically dependent on ODC activity. In fact, DFMO failed to cause any noticeable effect on the basal levels of cellular polyamines at the concentration of 2.5 mM, but the cobalt-induced elevation of SPM was almost completely abolished by the inhibitor. Therefore, it seems likely that the excess amount of PTU can be synthesized by ODC activated by cobalt exposure, and subsequently, PUT may be freely converted to SPM. On the other hand, azide exposure also produced a large amount of PUT by activated ODC, and newly formed PUT may be accumulated without converting to any other species of polyamines in the cells. However, to bring up this possibility, it seems absolutely necessary to demonstrate that substantial
changes in the polyamine-interconverting enzymes under the chemical hypoxic conditions.

The treatment of cultured cells with cobalt and azide is originally designed for the in vitro artificial process generating the chemically-induced hypoxic cellular environment. Therefore, it seems quite reasonable to expect that both cobalt- and azide-treatment can potentially exert almost similar effect on the cellular function, as far as they may be able to chemically bring about the hypoxic circumstances within the cells. Then, to confirm whether the cellular circumstances of the cobalt- and azide-treated cells might be hypoxic, the effects of cobalt- and azide-treatment on Hif-1α gene expression were examined, and the significant elevation (approximately 200%) of Hif-1α mRNA levels was observed in both cobalt- and azide-treated cells at 2 h after the exposure [Figure 4]. Therefore, it seems possible to consider that both cobalt- and azide-treatment may be able to produce the hypoxic state within the cells. On the other hand, the elevation of ODC mRNA levels was clearly shown to be observed in these cells under both cobalt- and azide-induced hypoxic conditions, and a significant increase in ODC mRNA levels (approximately 200%) was obtained by exposing these cells to the hypoxic conditions for at least 8 h [Figure 5], following to the elevation of Hif-1α mRNA levels observed at 2 h after the exposure [Figure 4]. Furthermore, the elevation of ODC mRNA levels was shown to be dependent on both CoSO₄ and NaN₃ concentrations [Figure 6], and the obtained dose-response curves for CoSO₄ and NaN₃ appeared to be a saturation curve-like, and might be possible to regard them as an atypical saturation curve. Thus, it may be possible to imagine that the elevation of ODC mRNA levels observed here seems to possess the fundamental characteristics of the biological response rather than those of the chemical response, thereby allowing us to speculate that chemical hypoxia-induced ODC expression and polyamine biosynthesis can be the intrinsic biological response of the glioma cells.

Generally speaking, Hif-1α, a hypoxia-inducible transcription factor-alpha, is known to regulate the transcription of ODC and other hypoxia-sensitive genes, thereby participating in the regulation of the hypoxia-induced gene transcription. Then, to elucidate a possible involvement of Hif-1α in cobalt-stimulated ODC gene transcription in the glioma cells, the antisense ODN targeting Hif-1α gene was preloaded into the cells, and the effect of cobalt treatment on both Hif-1α and ODC mRNA levels was examined. Consequently, there was no doubt that the cobalt-induced elevation of Hif-1α mRNA levels was completely suppressed by silencing Hif-1α gene [Figure 7a], and the stimulatory effect of cobalt treatment on ODC mRNA levels was abolished by silencing Hif-1α gene in the same cells as well [Figure 7b]. Therefore, it seems reasonable to consider that ODC gene expression may be tightly connected with Hif-1α expression in the glioma cells, or rather possible to speculate that ODC gene transcription can be directly regulated by Hif-1α activity. Thus, these findings can provide evidence for suggesting that ODC gene may be one of the target genes of Hif-1α transcription factor, thereby supporting a possible connection between hypoxia and polyamine biosynthesis reported previously [27-29]. The present studies attempted to investigate the previous findings that the elevation of ODC activity was observed during the recovery periods from various noxious injuries at the cellular and molecular levels. Cobalt- and azide-induced chemical hypoxia was applied to C6 glioma cells as a metabolic insult, and polyamine synthesis and ODC gene transcription in the glioma cells were determined. Consequently, both cobalt- and azide-treatment could increase the cellular contents of SPm and PUT, respectively, by enhancing ODC gene expression probably through the stimulation of Hif-1α expression in C6 glioma cells under the intracellular chemical hypoxic conditions. Moreover, the present study can give us a clue as to a hypothetical idea that there may be existing a possible intercellular communication system between glial and neuronal cells taking polyamines as a putative mediator in the brain.

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