Free Radical Research, 2002 Vol. 36 (5), pp. 545–554

Taylor & Francis
healthsciences

Increased Resistance to Nitric Oxide Cytotoxicity Associated with Differentiation of Neuroblastoma-Glioma Hybrid (NG108-15) Cells

KOICHI KAWAHARA[a,*], MUNETAKA SAITOH[a], TAKAYUKI NAKAJIMA[a], HIDEOMI SATO[a], MOTOKI TANAKA[a], TAKURO TOJIMA[b] and ETURO ITO[b]

[a]*Laboratory of Biomedical Control, Research Institute for Electronic Science, Hokkaido University, Sapporo 060-0812, Japan;* [b]*Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*

Accepted by Professor V. Darley-Usmar

(Received 17 June 2001; In revised form 7 November 2001)

Nitric oxide (NO), synthesized by the enzyme nitric oxide synthase (NOS), acts as an intercellular messenger associated with various physiological and pathological events. In this study, we investigated whether there exits a difference in the vulnerability to NO-induced cytotoxicity between undifferentiated and differentiated NG108-15 cells, and if so, the mechanisms responsible for the difference. Following a 7- to 8-day exposure to dibutyryl cAMP (dbcAMP), NG108-15 cells exhibited a neuron-like morphology associated with the expression of the neuronal protein, synaptophysin, and with increased NADPH-d activity. Neuron-like differentiated NG108-15 cells acquired resistance to exogenously applied NO. This increased resistance to NO toxicity in differentiated cells was almost completely cancelled out by inhibiting the activity of superoxide dismutase (SOD), but not by inhibiting the activity of NOS. The present study suggested that the activity of SOD increased in parallel with the activity of NOS associated with differentiation and was crucial for the acquired resistance to NO toxicity in differentiated cells.

Keywords: NG108-15 cells; Differentiation; Nitric oxide; Cytotoxicity; Superoxide dismutase

INTRODUCTION

Nitric oxide (NO) is generated in various mammalian tissues, and acts as an intracellular messenger associated with various physiological and pathological events.[1] In neuronal cells, NO is synthesized by neuronal Ca^{2+}/calmodulin-dependent nitric oxide synthase (NOS), and is released in response to the activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors.[2] Inhibiting neuronal NOS either pharmacologically or genetically renders cultured neurons resistant to NMDA-induced death,[3,4] also reduced infarct volume in rodent models of transient focal ischemia.[5] Therefore, NO has been supposed to be involved in the glutamate-induced neurotoxicity. However, recent studies have revealed that NO inhibits the activation of caspase-3, an enzyme crucial for apoptosis, via S-nitrosylation of the active-site cysteine of the enzyme,[6,7] and prevents neurons from undergoing apoptosis.[8] In addition, increased NOS activity is required in the ischemic preconditioning of neurons.[9,10] Thus, the pathological as well as functional roles of NO in the brain are not fully understood.

Although NO is possibly involved in the neurotoxicity, NO-synthesizing neurons have been reported to be resistant to NO- and glutamate-induced neurotoxicity.[3] In addition, NO-synthesizing neurons are selectively spared in patients with Alzheimer's disease, even in severely affected regions of the brain such as the hippocampal formation.[11] NO-synthesizing neurons in the brain of patients with Huntington's disease are also spared despite a massive loss of neurons in the corpus

*Corresponding author. Tel./Fax: +81-11-706-2893. E-mail: kawahara@bmc.es.hokudai.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290025924

striatum.[12] But why NO-synthesizing neurons are selectively resistant to various kinds of neurotoxicity remains largely unknown.

In this study, we have tried to elucidate whether there exist differences in the vulnerability to cytotoxicity induced by exogenously applied NO between undifferentiated and differentiated NG180-15 cells following treatment with dibutyryl cAMP (dbcAMP).[13–15] We then tried to clarify the mechanisms responsible for any changes in the resistivity to NO toxicity. NG108-15 cells were selected, because they have been widely used in the study of changes in neuronal functions dependent on differentiation,[16] and thus their morphologic and physiologic features are well described.[17,18]

MATERIALS AND METHODS

Cell Cultures

NG108-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing HAT (10 mM hypoxantine, 0.1 mM aminoproterin and 1.6 mM thymidine), and 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 90% air, 10% CO_2. To induce neuron-like differentiation in NG108-15 cells, the cells were treated with 1 mM dibutyryl cAMP (dbcAMP; Wako, Tokyo, Japan)[13–15] for 7–8 days. For immunocytochemical staining, the cells were cultured on collagen-coated glass slides under identical conditions.

Immunocytochemical Staining

Rabbit anti-synaptophysin polyclonal antibody and mouse anti-nitrotyrosine monoclonal antibody was purchased from Progen Biotechnik (Heidelberg, Germany) and Upstate Biotechnology (Lake Placid, NY), respectively. For synaptophysin and nitrotyrosine labeling, the cells were fixed with 4% paraformaldehyde for 5 min at 4°C followed by 95% methanol in PBS for 10 min at −20°C. The cells were then incubated with a primary antibody for 45 min using 1:100 dilutions for synaptophysin, and for 24 h using 1:200 dilutions for nitrotyrosine. After being washed with PBS, the cells were incubated with a secondary antibody containing 1.5% goat serum for 30 min. For labeling, a 1:500 dilution of biotinylated goat antibody against rabbit IgG (Vector Laboratories, Burlingame, CA) was used. Bound antibodies were detected by the ABC method,[19] following incubation with avidin–biotin-peroxidase complex (Vector Laboratories) for 30 min. Peroxidase activity was visualized by incubation with 0.1% 3,3′-diaminobenzidine (DAB) in 50 mM Tris–HCl buffer (pH 7.4) supplemented with 0.02% H_2O_2. The cells were dehydrated in 70–100% ethanol, cleared in xylene, and mounted on glass cover slips in Permount (Fischer Scientific, Fair Lawn, NJ) for light microscopic observation.

Diaphorase Staining

NG108-15 cells were fixed for 30 min at 4°C in a 4% paraformaldehyde (PF); 0.1 M phosphate buffer (PB). The PF solution was washed away with Tris-buffered saline (TBS); 50 mM Tris–HCl, 1.5% NaCl, pH 7.4. The reaction solution containing 1 mM NADPH, 0.2 mM nitroblue tetrazolium, 0.2% Triton X-100 (TX-100), 1.2 mM sodium azide, and 0.1 mM Tris–HCl, pH 7.2, was applied to the fixed cell culture for 1 h at 37°C.

Cell Death Estimation

Cell death was analyzed following visualization of nuclear morphology with the fluorescent DNA-binding dyes, bis-benzimide (Hoechst 33342) (Sigma, St. Louis, MO) and propidium iodide (PI) (Wako). Cells were incubated with these dyes for 15 min at 37°C. Individual nuclei were visualized using fluorescent microscopy (IX70; Olympus, Tokyo, Japan) and analyzed; PI was used to identify nonviable cells. An average of 45–500 cells from random fields were analyzed for one experiment. The cell death rate was calculated as (dead cells/total cells). At least four independent experiments were performed and analyzed.

Other Chemicals

Nitric oxide donors, S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), 1-hydroxy-2-ox-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC 7), and peroxynitrite ($ONOO^-$) were obtained from Dojindo Chemical (Kumamoto, Japan). Decomposed SNAP (deSNAP) was prepared by incubating SNAP at room temperature for 48 h to completely liberate NO. An antioxidant, reduced glutathione (GSH), was obtained from Calbiochem (San Diego, CA). A nonspecific inhibitor of NOS, N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of superoxide dismutase (SOD), diethyldithiocarbamic acid (DDC), a scavenger of nitric oxide, hemoglobin (Hb), and a superoxide anion scavenger, 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron) were obtained from Sigma.

Statistics

The data are expressed as the mean ± SD. Each experimental group consists of at least 4 independent experiments. Inter-group comparisons were performed using the one-way analysis of variance

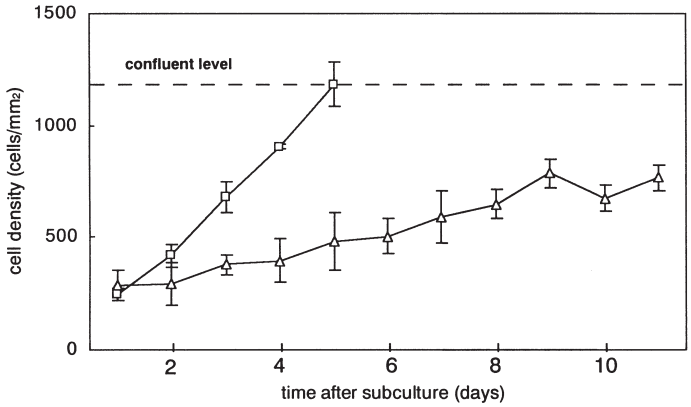


FIGURE 1 Growth rates of undifferentiated and differentiated NG108-15 cells. Open squares and open triangles indicate undifferentiated and differentiated cells, respectively. Cells not treated with dbcAMP became almost confluent at about 5 days. In contrast, cell proliferation reached a plateau at around 8–9 days after differentiation induced by dbcAMP treatment. Data are expressed as the mean ± SD ($n = 4$ for each cell type).

(ANOVA) followed by a paired *t*-test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Growth Rates of Undifferentiated and Differentiated NG108-15 Cells

At first, we analyzed the growth rates of undifferentiated and differentiated NG108-15 cells as an index of cellular differentiation induced by 1 mM dbcAMP treatment (Fig. 1). Cells were seeded in culture flasks at an initial density of 5.0×10^4 cells/ml and counted every 24 h. The growth rate of differentiated cells was markedly lower than that of differentiated cells. Proliferation of differentiated cells gradually increased but reached a plateau at about 8–9 days after treatment with dbcAMP.

Neuron-like Differentiation of NG108-15 Cells

We then analyzed the morphological characteristics of undifferentiated and differentiated cells. Figure 2(A1) and (A2)show photomicrographs of undifferentiated and differentiated cells, respectively. Undifferentiated cells were flat and lacked neurites (Figure 2(A1)). Following 7–8 days treatment with dbcAMP, most cells had long extending neurites showing neuron-like morphology (Figure 2(A2)). To confirm that the differentiated cells had neuronal characteristics, we examined whether the neuronal protein synaptophysin, a synaptic vesicle membrane protein, was expressed in the differentiated cells (Fig. 2(B)). Differentiated cells were positively stained for synaptophysin (Fig. 2(B2)), but undifferentiated cells were negatively or weakly stained (Fig. 2(B1)). Immunoreactivity was prominent at the varicosities in differentiated cells, supporting the idea that synaptophysin is involved in the exocytosis of transmitters.

NG108-15 cells showed the cytostatic activity at about 8–9 days after dbcAMP treatment (Fig. 1). Previous studies have demonstrated that NO plays a crucial role in triggering a switch for growth arrest and differentiation in PC12 cells[20] and in other neuronal cells, such as neural precursor cells and a human neuroblastoma cell line.[21] Therefore, we examined whether the activity of NOS varied depending on the differentiation in NG108-15 cells (Fig. 2(C)).

After 8-day treatment with dbcAMP, NADPH-d-negative undifferentiated cells (Fig. 2(C1)) showed intensive NADPH-d staining (Fig. 2(C2)), suggesting that the activity of NOS increased in NG108-15 cells associated with neuron-like differentiation.

Differentiation and Resistance to NO Toxicity

NO-producing cells, in general, are resistant to NO-induced cytotoxicity, if not, they would die. Therefore, we then tried to elucidate whether there exists a difference in the resistance to exogenously applied NO between undifferentiated and differentiated cells.

The exposure of undifferentiated NG108-15 cells to 1 mM SNAP, a NO donor, for 24 h resulted in massive cell death (Fig. 3(A) and (C)). In contrast, 1 mM SNAP-treatment of differentiated cells for 24 h did not produce significant cell death (Fig. 3(B) and (C)). Differentiated NG108-15 cells became markedly resistant to exogenously applied NO. SNAP (1 mM) preincubated in solution to exhaust all the NO (deSNAP) did not produce significant death of undifferentiated NG108-15 cells (Fig. 3(C)). In addition, a different kind of NO donor, NOC 7 (1 mM) also induced massive death of undifferentiated cells (Fig. 4(A) and (C)), but did not produce

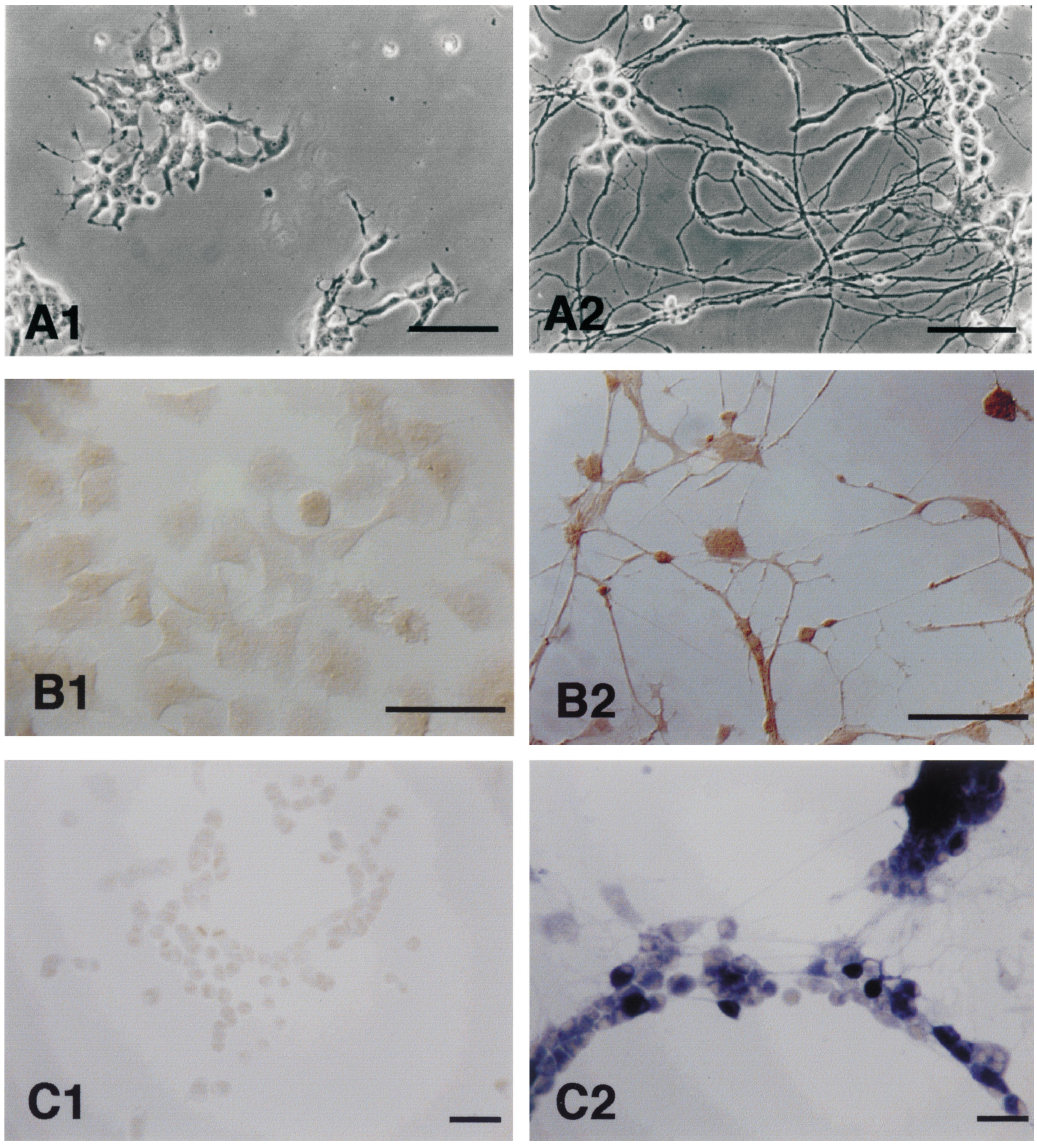


FIGURE 2 Neuron-like differentiation of NG108-15 cells. Figures A1 and A2 indicate phase-contrast photomicrographs of undifferentiated and differentiated cells, respectively. Differentiated cells exhibited a neuron-like morphology and developed extended neurites (A2). Immunocytochemical analysis revealed that differentiated NG108-15 cells were positively stained for neuronal protein, synaptophysin (B2), but undifferentiated cells were negatively or weakly stained (B1). NADPH-d staining of undifferentiated (C1) and differentiated (C2) cells, respectively. Differentiated cells showed strong NADPH-d activity, but undifferentiated cells showed weak activity. Scale bars in (A)–(C) show 100, 50, and 50 μm, respectively.

significant death of differentiated cells (Fig. 4(B) and (C)). These results suggested that NO liberated from NO donors was responsible for the cytotoxicity of undifferentiated cells, and that NG108-15 cells acquired resistance to NO toxicity on differentiation.

Differentiated cells showed strong reactivity to NADPH-d (Fig. 2(C2)). Therefore, the question arises as to whether the increased NOS activity itself was involved in the increased resistance to NO toxicity in differentiated cells. Exposure of differentiated NG108-15 cells to 1 mM SNAP while inhibiting the activity of NOS caused by co-treatment with 1 mM L-NMMA, a nonspecific NOS inhibitor, did not produce significant cell death (Fig. 4(D), NM and SNAP). When differentiated cells were pretreated with 1 mM L-NMMA for 24 h, SNAP exposure also did not result in significant cell death (Fig. 4(D), NM24 and SNAP). These results suggested that the activity of NOS itself had nothing to do with the resistance to NO-induced cytotoxicity in differentiated NG108-15 cells.

Superoxide and NO Toxicity

NO reacts with superoxide *in vivo* by the near-diffusion-limited process,[22] and then peroxynitrite is generated. Peroxynitrite is a strong biological oxidant that directly and rapidly attacks targets such as thiols[23] and iron–sulfur centers,[24] and induces tissue injuries.[25,26] Therefore, in order to make NO a

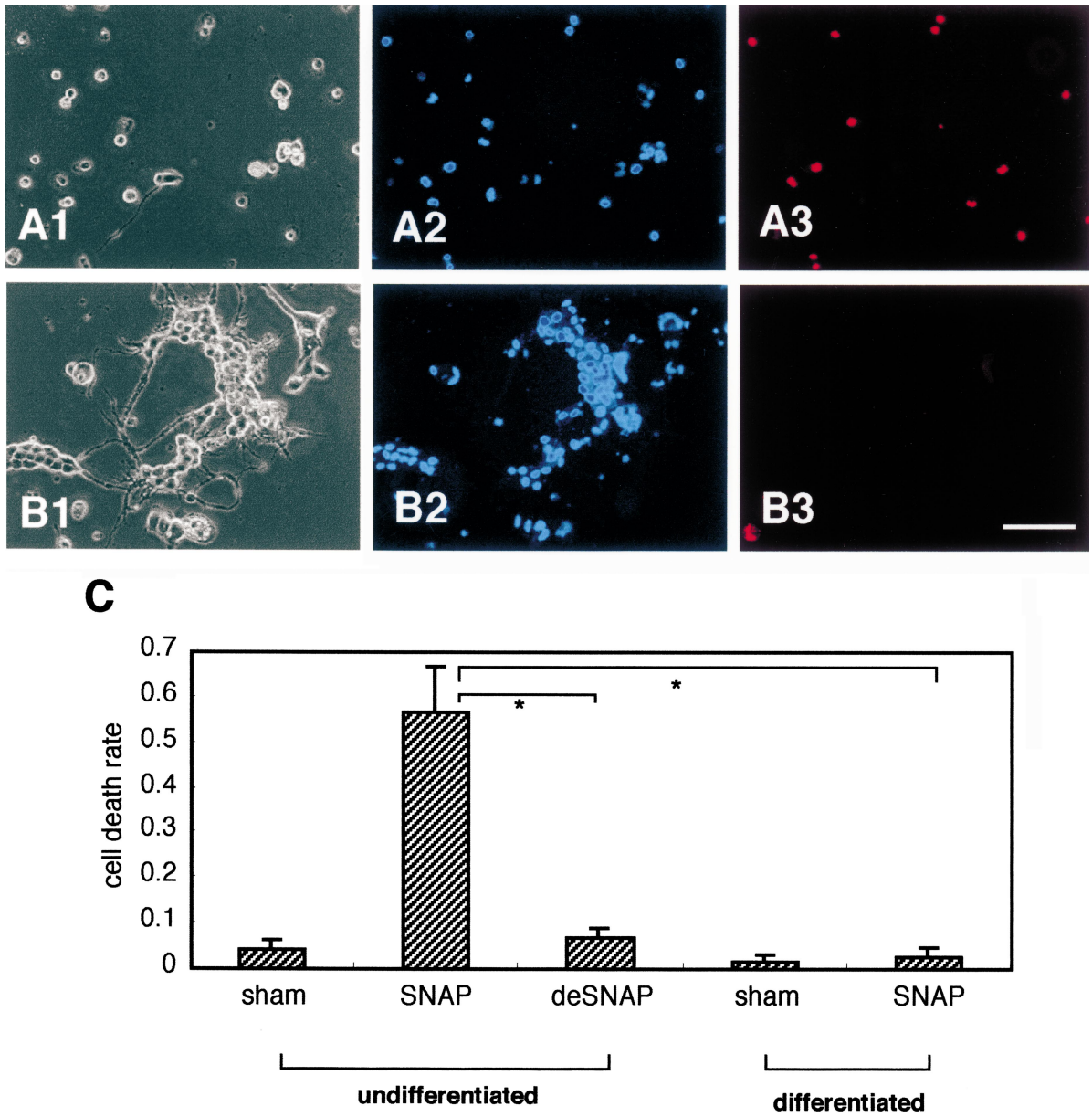
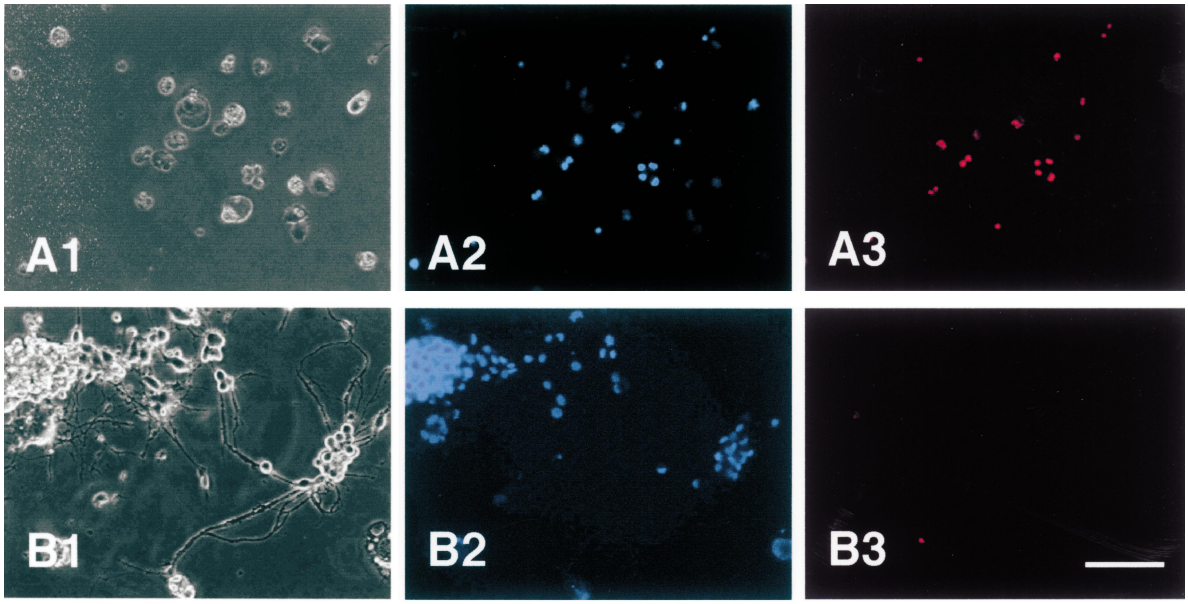
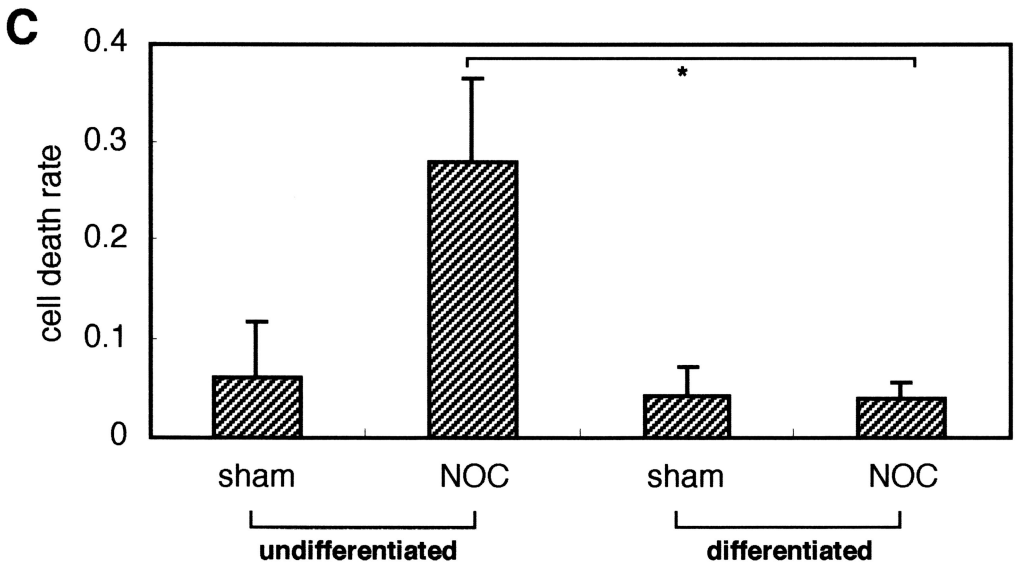


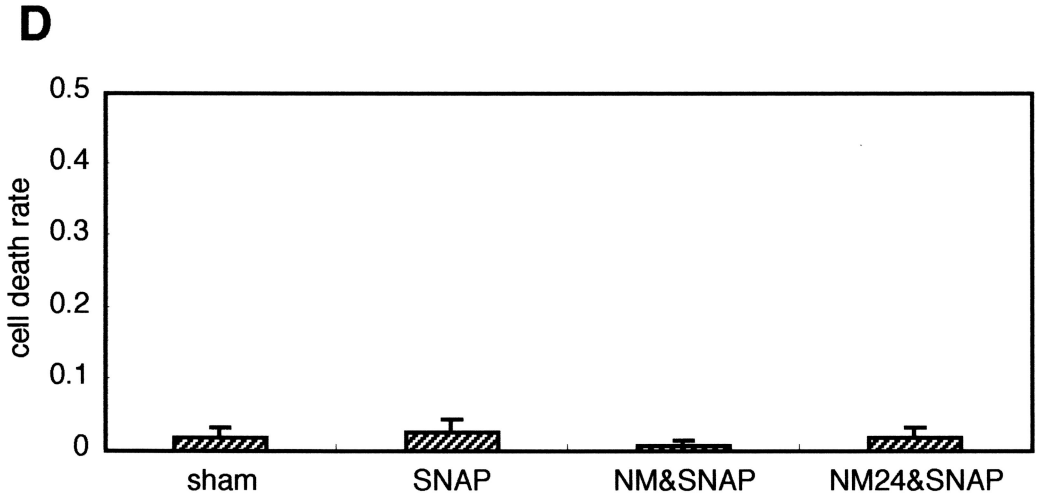
FIGURE 3 Increased resistance to NO-induced toxicity in differentiated NG108-15 cells. Figures A1 and B1 show phase-contrast photomicrographs of undifferentiated and differentiated cells treated with 1 mM SNAP for 24 h, respectively. Figures A2 and B2 indicate photomicrographs of undifferentiated and differentiated cells stained with Hoechst 33342 (bisbenzimide) at 24 h after SNAP treatment, respectively. Figures A3 and B3 show photomicrographs of undifferentiated and differentiated cells stained with propidium iodide (PI) at 24 h after SNAP treatment, respectively. The treatment of undifferentiated cells with 1 mM SNAP for 24 h resulted in massive cell death (A1–A3, C). In contrast, the same dose did not result in significant cell death in differentiated cells (B1–B3, C). Decomposed SNAP (deSNAP) did not produce significant death of undifferentiated cells (C). SNAP was dissolved in DMSO and the final concentration of DMSO (1%) was added to the sham control. DMSO (1%) itself had no effect on cell viability in either undifferentiated or differentiated cells (C; sham). The scale bar (100 μm) is the same in (A) and (B). Data are expressed as the mean ± SD ($n = 4–8$). An asterisk indicates statistical significance ($P < 0.05$).

functional signaling molecule in differentiated NG108-15 cells, NO-producing differentiated cells must acquire the capability to scavenge superoxide. This idea led to the suggestion that the activity of SOD might increase in parallel with the increase in NOS activity in differentiated NG108-15 cells. We then tested this possibility.

Exposure of differentiated cells to 1 mM SNAP for 24 h resulted in massive cell death when the cells were co-treated with 150 μM DDC, an inhibitor of cytosolic (Cu, Zn-SOD) and extracellular SOD[27] (Fig. 5). DDC loading itself at the concentration used here did not produce significant cell death. The most likely explanation of this result was that superoxide was overproduced on inhibition of SOD by DDC and reacted with NO to produce peroxynitrite, a strong biological oxidant. If this was the case, SNAP-induced cytotoxicity in differentiated cells, observed when the activity of SOD was inhibited with DDC, might be prevented by scavenging either one of the







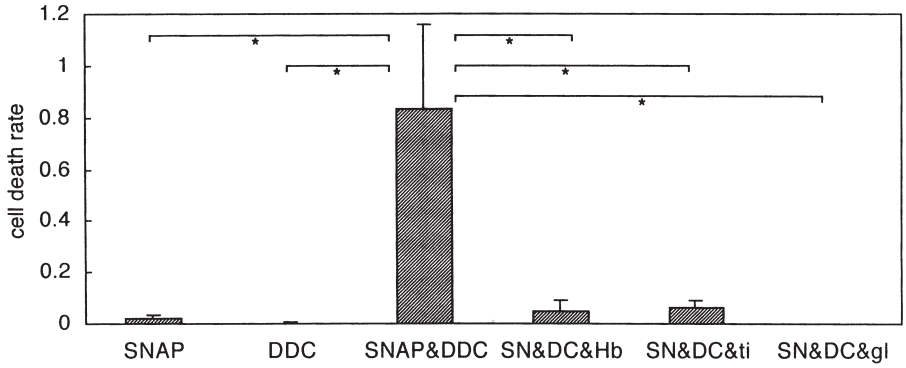


FIGURE 5 Increased resistance to NO toxicity and SOD activity in differentiated NG108-15 cells. The treatment of differentiated cells with 1 mM SNAP resulted in massive cell death when the SOD activity was inhibited by DDC (150 μM) treatment. The cell death was markedly reduced by co-treatment either with oxy-hemoglobin (2 mg/ml), tiron (5 mM), or glutathione (5 mM). Treatment of cells with DDC itself at the concentration used did not induce significant cell death. Data are expressed as the mean ± SD ($n = 4–6$). Asterisks indicate statistical significance ($P < 0.05$). *Abbreviations*: SN and DC and HB; co-treatment with SNAP, DDC, and oxy-hemoglobin, SN and DC and ti; co-treatment with SNAP, DDC, and tiron, SN and DC and gl; co-treatment with SNAP, DDC, and glutathione.

following molecules; NO, superoxide, or peroxynitrite. Expectedly, massive death of differentiated NG108-15 cells caused by co-treatment with SNAP and DDC was almost completely suppressed by treatment with an NO scavenger, Hb, Tiron (a nonenzymatic superoxide scavenger[28]), or reduced glutathione (GSH; a scavenger of peroxynitrite[29]).

To confirm further the possible involvement of peroxynitrite in the SNAP-induced cytotoxicity in differentiated NG108-15 cells, we then investigated whether the exposure of differentiated cells to SNAP resulted in the extensive tyrosine nitration when SOD activity was inhibited. Tyrosine nitration is a widely used marker of peroxynitrite produced from the reaction of nitric oxide with superoxide.[30,31] Immunocytochemical analysis demonstrated that SNAP treatment markedly increased nitrated tyrosine when the activity of SOD was inhibited with DDC (Fig. 6(C)) in differentiated cells. However, immunoreactivity for nitrated tyrosine was not clearly observed in differentiated cells with SNAP treatment only (Fig. 6(B)) and in cells with sham-treatment (Fig. 6(D)). These results further supported our proposition that peroxynitrite formation was involved in the SNAP-induced cytotoxicity in differentiated NG108-15 cells observed when the activity of SOD was inhibited.

DISCUSSION

This study revealed that differentiated, neuron-like NG108-15 cells acquired resistance to exogenously applied NO. The SOD activity in the differentiated cells was crucial to this increased resistance to NO toxicity, because such resistance was almost perfectly cancelled out by inhibiting the activity of SOD.

Since the first report by Peunova and Enikolopov,[20] many studies supporting their idea that NO is involved in the regulation of cell growth and differentiation in neuronal[21,32–34] and non-neuronal cells[35,36] have been published. In the present study, the activity of NOS increased in association with the neuron-like differentiation of NG108-15 cells on treatment with dbcAMP (Fig. 2(C)). This raised the question of whether cAMP and cAMP-dependent protein kinase (PKA) directly contributed to the increase in NOS activity in differentiated cells. Previous studies have demonstrated that the increased NOS activity associated with differentiation is independent of the methods for inducing differentiation; that is, treatment of cells with a nerve growth factor (NGF),[20,32] all-trans retinoic acid (ATRA),[21] or with other molecules[35] results in the increased NOS activity. Therefore, cAMP itself seems not to be directly involved in the increased NOS

FIGURE 4 Increased resistance to NO-induced toxicity in differentiated NG108-15 cells and NOS activity. Figures A1 and B1 show phase-contrast photomicrographs of undifferentiated and differentiated cells treated with 1 mM NOC 7 for 24 h, respectively. Figures A2 and B2 indicate photomicrographs of undifferentiated and differentiated cells stained with Hoechst 33342 (bisbenzimide) at 24 h after NOC 7 treatment, respectively. Figures A3 and B3 show photomicrographs of undifferentiated and differentiated cells stained with propidium iodide (PI) at 24 h after NOC 7 treatment, respectively. The treatment of undifferentiated cells with 1 mM NOC 7 for 24 h resulted in significant cell death (A1–A3, C). In contrast, the same dose did not result in significant cell death in differentiated cells (B1–B3, C). This increased resistance to NO toxicity in differentiated cells was unchanged after the inhibition of NOS by 1 mM L-NMMA (D). Since NOC 7 immediately starts degradation and release NO, cells were treated with NOC 7 immediately after the dilution of a stock solution (100 mM in 0.01N NaOH). The scale bar (100 μm) is the same in (A) and (B). Data are expressed as the mean ± SD ($n = 4–6$). An asterisk indicates statistical significance ($P < 0.05$). *Abbreviations*: NOC, NOC 7 treatment; NM and SNAP, co-treatment with L-NMMA and SNAP; NM24 and SNAP, pretreatment with L-NMMA for 24 h.

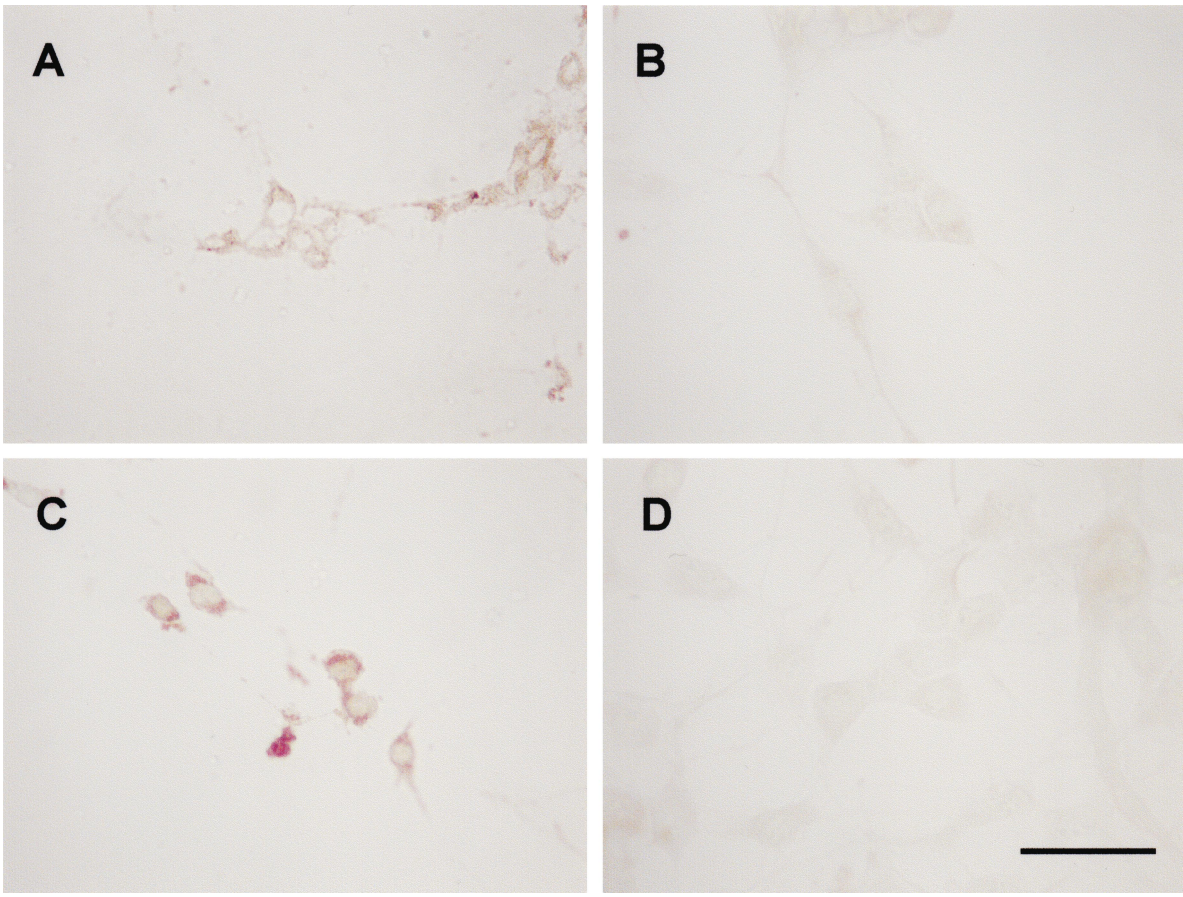


FIGURE 6 Nitrotyrosine immunoreactivity in differentiated NG108-15 cells. Differentiated NG108-15 cells showed immunoreactivity for nitrotyrosine when the cells were co-treated with 1 mM SNAP and 150 μM DDC (C) for 7 h, but no specific immunostaining was observed when the cells were treated with 1 mM SNAP only (B) or with 1% DMSO (D; sham) for the same duration. As a positive control, differentiated NG108-15 cells were treated with 24 mM peroxynitrite (A). The scale bar (50 μm) is the same in (A)–(D).

activity in differentiated NG108-15 cells. Rather, treatment with dbcAMP caused the cells to differentiate, and resulted in the increased activity of NOS.

In this study, the endogenous NOS activity in NG108-15 cells was estimated from the reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) activity (Fig. 2(C)). In the nervous system, NOS immunoreactivity has been consistently co-localized with NADPH-d activity, reflecting the two functions carried out by the same molecule.[37] In spite of some limitations,[38] it is now generally accepted that the histochemical detection of NADPH-d activity is one of most useful ways of identifying putative NOS-containing neuronal cells.[39–41]

This study demonstrated that differentiated NG108-15 cells became markedly resistant to NO-induced toxicity (Fig. 3). This increased resistance was correlated with SOD activity, but not with NOS activity (Figs. 3 and 4). This finding seems to support the suggestion by Lei *et al.*[42] that NO is necessary, but not sufficient for neuronal injury and is toxic only in the presence of other factors, such as the superoxide anion. In addition, findings similar to those of the present study have been reported; PC12 cells that differentiated on treatment with NGF for more that 3 days acquired resistance to NO toxicity in association with increased NOS activity.[43] However, the mechanisms responsible for the increased resistance to NO toxicity were not clarified in that report. A recent study by Yabuki *et al.*[44] has demonstrated that NO-resistant HL-60 variant cells (HL-NR6) are associated with the increase in Cu, Zn-SOD/catalase, and that NO-mediated apoptosis in HL-60 cells is correlated with the generation of reactive oxygen species (ROS) such as superoxide and molecules like peroxynitrite. All these previous findings support our notion that the activity of SOD increased in parallel with that of NOS and is crucial for the strengthened resistance to NO-induced toxicity in differentiated NG108-15 cells.

Although the present study has made it clear that the activity of SOD, not of NOS itself, is crucial to the acquired resistance to NO toxicity in differentiated NG108-15 cells, our preliminary immunocytochemical and western blot analyses using antibodies for Cu, Zn-SOD and Mn-SOD failed to demonstrate a clear difference in the expression level of these

enzymes between undifferentiated and differentiated cells. Therefore, there is a possibility that the activity of SOD is regulated at the level of enzymatic activity, not at the level of expression. In fact, Lièvre *et al.*[45] have recently demonstrated that in some cases, increased SOD activity is not in parallel with the increase of SOD mRNA and protein synthesis, suggesting that the prominent SOD induction is not necessarily accompanied by SOD protein accumulation, and thus by increased enzymatic activity. Further study will be needed to clarify this.

Acknowledgements

The authors thank Dr Osamu Inanami, Laboratory of Radiation Biology, Graduate School of Veterinary Medicine, Hokkaido University, for helpful discussion of the results.

References

[1] Nathan, C. (1992) "Nitric oxide as a secretory product of mammalian cells", *FASEB J.* **6**, 3501–3604.
[2] Garthwaite, J. (1991) "Glutamate, nitric oxide and cell–cell signaling in the nervous system", *Trends Neurosci.* **14**, 60–67.
[3] Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S. and Snyder, S.H. (1991) "Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures", *Proc. Natl Acad. Sci. USA* **88**, 6368–6371.
[4] Lee, J.-M., Grabb, M.C., Zipfel, G.J. and Choi, D.W. (2000) "Brain tissue responses to ischemia", *J. Clin. Investig.* **106**, 723–731.
[5] Samdani, A.F., Dawson, T.M. and Dawson, V.L. (1997) "Nitric oxide synthase in models of focal ischemia", *Stroke* **28**, 1283–1288.
[6] Kim, Y.-M., Kim, T.-H., Seol, D.-W., Tanalian, R.V. and Billiar, T.R. (1998) "Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome c release", *J. Biol. Chem.* **273**, 31437–31441.
[7] Mannick, J.B., Hausladen, A., Liu, L., Hess, D.T., Zeng, M., Miano, Q.X., Kane, L.S., Gow, A.J. and Stamler, J.S. (2000) "Fas-induced caspase denitrosylation", *Science* **284**, 651–654.
[8] Jensen, M.S., Nyborg, N.C.B. and Thomsen, E.S. (2000) "Various nitric oxide donors protect chick embryonic neurons from cyanide-induced apoptosis", *Toxicol. Sci.* **58**, 127–134.
[9] Gonzalez-Zulueta, M., Feldman, A.B., Klesse, L.J., Kalb, R.G., Dillman, J.F., Parada, L.F., Dawson, T.M. and Dawson, V.L. (2000) "Requirement of nitric oxide activation of $p21^{RAS}$/extracellular regulated kinase in neuronal ischemic preconditioning", *Proc. Natl Acad. Sci. USA* **97**, 436–441.
[10] Nandagopal, K., Dawson, T.M. and Dawson, V.L. (2001) "Critical role for nitric oxide signaling in cardiac and neuronal preconditioning and tolerance", *Pharmacol. Exp. Ther.* **297**, 474–478.
[11] Hyman, B.T., Marzloff, K., Wenninger, J.J., Dawson, T.M., Bredt, D.S. and Snyder, S.H. (1992) "Relative sparing of nitric oxide synthase-containing neurons in the hippocampal formation in Alzheimer's disease", *Ann. Neurol.* **32**, 818–820.
[12] Li, X.J., Sharp, A.H., Li, S.H., Dawson, T.M., Snyder, S.H. and Ross, C.A. (1996) "Huntingtin-associated protein (HAP1): discrete neuronal localizations in the brain resemble those of neuronal nitric oxide synthase", *Proc. Natl Acad. Sci. USA* **93**, 4839–4844.
[13] Daniels, M.P. and Hamprecht, B. (1974) "The ultrastructure of neuroblastoma-glioma somatic cells hybrids", *J. Cell Biol.* **63**, 691–699.
[14] Tojima, T., Yamabe, Y., Takahashi, M. and Ito, E. (2000) "Acquisition of neuronal proteins during differentiation of NG108-15 cells", *Neurosci. Res.* **37**, 153–161.
[15] Tojima, T. and Ito, E. (2001) "A cyclic AMP-regulated negative feedforward system for neutritogenesis revealed in a neuroblastoma × glioma hybrid cell line", *Neuroscience* **104**, 583–591.
[16] Morimoto, Y., Morimoto, Y., Kemmotsu, O. and Alojado, E.S. (1997) "Extracellular acidosis delays cell death against glucose-oxygen deprivation in neuroblastoma–glioma hybrid cells", *Crit. Care Med.* **25**, 841–847.
[17] Hamprecht, B. (1977) "Structual, electrophysiological, biochemical and pharmacological properties of neuroblastoma–glioma cell hybrids in cell culture", *Int. Rev. Cytol.* **49**, 99–170.
[18] Puro, D.G. and Nirenberg, M. (1976) "On the specificity of synaptic formation", *Proc. Natl Acad. Sci. USA* **73**, 3544–3548.
[19] Hsu, S.M., Raine, L. and Fanger, H. (1981) "Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody PAP procedures", *J. Histochem.* **27**, 577–580.
[20] Peunova, N. and Enikolopov, G. (1995) "Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells", *Nature* **375**, 68–73.
[21] Ogura, T., Nakayama, K., Fujisawa, H. and Esumi, H. (1996) "Neuronal nitric oxide synthase expression in neuronal cell differentiation", *Neurosci. Lett.* **204**, 89–92.
[22] Huie, R.E. and Padmaja, S. (1993) "The reaction rate of nitric oxide with superoxide", *Free Radic. Res. Commun.* **18**, 195–199.
[23] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) "Peroxynitrite-mediated sulfhydryl oxidation: the cytotoxic potential of superoxide and nitric oxide", *J. Biol. Chem.* **266**, 4244–4250.
[24] Castro, L., Rodrigue, M. and Radi, R. (1994) "Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide", *J. Biol. Chem.* **269**, 29409–29415.
[25] Estevez, A.G., Radi, R., Barbeito, L., Shin, J.T., Thompson, J.A. and Beckman, J.S. (1995) "Peroxynitrite-induced cytotoxicity in PC12 cells: evidence for an apoptotic mechanism differentially modulated by neurotrophic factors", *J. Neurochem.* **65**, 1543–1550.
[26] Bailly, F., Zoete, V., Vamecq, J., Catteau, J.-P. and Bernier, J.L. (2000) "Antioxidant actions of ovothiol-derived 4-mercaptoimidazoles: gluthathione peroxidase activity and protection against peroxynitrite-induced damage", *FEBS Lett.* **486**, 19–22.
[27] Heikkila, R.E. and Cohen, G. (1977) *The inactivation of copper-zinc superoxide dismutase by diethyldithiocarbamate Superoxide and Superoxide Dismutases*, (Academic Press, London), pp. 367–373.
[28] Siwik, D.A., Tzortzis, J.D., Pimental, D.R., Chang, D.L.-F., Pagano, P.J., Singh, K., Sawyer, D.B. and Colucci, W.S. (1999) "Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myoeytes *in vitro*", *Circul. Res.* **85**, 147–153.
[29] Quijano, C., Alvarez, B., Gatti, R.M., Augusto, O. and Radi, R. (1997) "Pathways of peroxynitrite oxidation of thiol groups", *Biochem. J.* **322**, 167–173.
[30] Reiter, C.D., Teng, R.-J. and Beckman, J.S. (2000) "Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite", *J. Biol. Chem.* **275**, 32460–32466.
[31] Radi, R., Peluffo, G., Alvarez, M.N., Naviliat, M. and Cayota, A. (2001) "Unraveling peroxynitrite formation in biological system", *Free Radic. Biol. Med.* **30**, 463–488.
[32] Phung, Y.T., Bekker, J.M., Hallmark, O.G. and Black, S.M. (1999) "Both neuronal NO synthase and nitric oxide are required for PC12 cell differentiation: a cGMP independent pathway", *Mol. Brain Res.* **64**, 165–178.
[33] Moritz, G.C., Tenorib, F., Allodi, S. and Mendez-Otero, R. (1999) "Expression of nitric oxide synthase in the developing rat hippocampus", *Neurosci. Lett.* **263**, 89–92.
[34] Hilbig, H., Franke, H., Bidmon, H.-J. and Illes, P. (2001) "Nitric oxide synthase isozymes during *in vitro* development of rat neuronal and human non-neuronal cells", *Neurosci. Lett.* **297**, 9–12.
[35] Morcos, E., Jansson, O.T., Adolfsson, J., Kratz, G. and Wiklund, N.P. (1999) "Endogonously formed nitric oxide modulates cell growth in bladder cancer cell lines", *Urology* **53**, 1252–1257.
[36] Bloch, W., Fleischmann, B.K., Lorke, D.E., Andressen, C., Hops, B., Hescheler, J. and Addicks, K. (1999) "Nitric oxide

synthase expression and role during cardiomyogenesis", *Cardiovasc. Res.* **43**, 675–684.
[37] Hope, B.T., Micheal, G.J., Knigge, K.M. and Vincent, S.R. (1991) "Neuronal NADPH-diaphorase is a nitric oxide synthase", *Proc. Natl Acad. Sci. USA* **88**, 2811–2820.
[38] Matsumoto, T., Nakanem, M., Pollock, J.S., Kulk, J.E. and Fostermann, U. (1993) "A correlation between soluble brain nitric oxide synthase and NADPH-d activity is only seen after exposure of the tissue to fixative", *Neurosci. Lett.* **155**, 61–64.
[39] Spanggord, H., Sheldon, R.A. and Ferriero, M. (1996) "Cysteamine eliminates nitric oxide synthase activity but is not protective to the hypoxic-ischemic neonatal rat brain", *Neurosci. Lett.* **213**, 41–44.
[40] Kanda, K. (1996) "Expression of neuronal nitric oxide synthase in spinal motoneurons in aged rats", *Neurosci. Lett.* **219**, 41–44.
[41] Bruning, G. and Mayer, B. (1996) "Localization of nitric oxide synthase in the brain of the frog, *Xenopus laevis*", *Brain Res.* **741**, 331–343.
[42] Lei, S.Z., Pan, Z.-H., Aggarwal, S.K., Chen, H.-S.V., Sucher, N.J. and Lipton, S.A. (1992) "Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex", *Neuron* **8**, 1087–1099.
[43] Wada, K., Okada, N., Yamamura, T. and Koizumi, S. (1996) "Nerve growth factor induces resistance of PC12 cells to nitric oxide cytotoxicity", *Neurochem. Int.* **29**, 461–467.
[44] Yabuki, M., Kariya, S., Ishikawa, R., Yasuda, T., Yoshioka, T., Horton, A.A. and Utsumi, K. (1999) "Resistance to nitric oxide-mediated apoptosis is associated with increased activities of Cu, Zn-superoxide dismutase and catalase", *Free Radic. Biol. Med.* **26**, 325–332.
[45] Lièvre, V., Becuwe, P., Bianchi, A., Koziel, V., Franck, P., Schroeder, H., Nabet, P., Dauça, M. and Daval, J.-L. (2000) "Free radical production and changes in superoxide dismutases associated with hypoxia/reoxygenation-induced apoptosis of embronic rat forebrain neurons in culture", *Free Radic. Biol. Med.* **29**, 1291–1301.