COX-2 Regulates the Insulin-Like Growth Factor I–Induced Potentiation of Zn$^{2+}$-Toxicity in Primary Cortical Culture

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ABSTRACT

The pretreatment of cultured cortical neurons with neurotrophic factors markedly potentiates the cytotoxicity induced by low concentrations of Zn$^{2+}$ or excitotoxins. In the current study, we investigated the mechanism underlying the insulin-like growth factor I (IGF-I)-induced Zn$^{2+}$ toxicity potentiation. The pretreatment of primary cortical cultures for more than 12 h with 100 ng/ml of IGF-I increased the cytotoxicity induced by 80 μM Zn$^{2+}$ by more than 2-fold. The IGF-I–enhanced cell death was blocked by the COX-2–specific inhibitors N-[2-(cyclohexyloxyl)-4-nitrophenyl]-methane sulfonamide (NS-398; 10–100 μM) and 1-[(4-methylsulfonyl)phenyl]-3-trifluoro-methyl-5-[(4-fluoro)phenyl]pyrazole (SC58125; 10 μM) and by the antioxidant trolox (30 μM). In addition, it was observed that COX-2 expression was increased 12 to 24 h after IGF-I treatment. Preincubation of cortical cultures with IGF-I increased arachidonic acid (AA)-induced cytotoxicity, and AA increased Zn$^{2+}$ toxicity, which suggested the involvement of COX activity in these cellular responses. Moreover, enhanced COX-2 activity led to a decrease in the cell’s reducing power, as indicated by a gradual depletion of intracellular GSH. Cortical neurons pretreated with IGF-I and then Zn$^{2+}$ showed consistently enhanced reactive oxygen species production, which was repressed by NS-398 and SC58125. Cortical neurons treated with Zn$^{2+}$ and then AA displayed the increased ROS production, which was also suppressed by NS-398 and SC58125. These results suggest that COX-2 is an endogenous factor responsible for the IGF-I–induced potentiation of Zn$^{2+}$ toxicity and that enhanced COX-2 activity leads to a decrease in the cell’s reducing power and an increase in ROS accumulation in primary cortical cultures.

Neurotrophic factors such as nerve growth factor, BDNF, NT-3, and IGF-I play a critical role in cell proliferation, survival, and neurite outgrowth during neuronal development. They also produce neuroprotective effects against various neural injuries, including ischemia, trauma, and Alzheimer’s disease (Wu and Pardridge, 1999; Nabeshima and Yamada, 2000; Namiki et al., 2000; Guan et al., 2001). Under certain conditions, however, neurotrophic factors produce cytotoxic effects. Pretreatment for 24 h with various neurotrophic factors, such as BDNF and NT-4/5, markedly potentiates the necrotic cell death of primary cortical neurons induced by oxygen-glucose derivation or NMDA (Koh et al., 1995; Behrens et al., 1999; Lobner and Ali, 2002). Moreover, the preincubation of cultured cortical neurons with BDNF increases their sensitivity to excitotoxicity through the activation of phosphoinositide-3 kinase and the induction of nNOS or TrkB (Samdani et al., 1997; Fryer et al., 2000; Kim et al., 2003b). BDNF increases the excitotoxic cell death of hippocampal neurons by inducing NMDA receptor subunits (Prehn, 1996; Small et al., 1998; Glazner and Mattson, 2000) and potentiates free radical-mediated injury or NO donor-induced apoptosis in primary cortical cultures (Gwag et al., 2003b).
As is required for BDNF-induced potentiation, IGF-I potentiation requires new protein synthesis. IGF-I pretreatment aggravates Fe\(^{2+}\)- or BSO-mediated injury in cortical neurons, a process that involves the up-regulation of phosphoinositide-3 kinase and/or extracellular signal-regulated kinase activation (Gwag et al., 1995; Fryer et al., 2000). Thus, IGF-I–induced potentiation seems to have features distinct from those of BDNF and NT-4/5. Although IGF-I–induced potentiation might occur through a mechanism that involves the altered activations and/or enhanced expression of genes, which make neuronal cells highly vulnerable to subsequent or concurrent cytotoxic stress, the detailed mechanism underlying IGF-I–induced potentiation remains ill-defined.

A number of previous studies have reported that COX-2 activation leads to neuronal cell death via neuroinflammation (Vane et al., 1998; Kyrkanides et al., 2002). The COX-2–selective inhibitors, NS398 and SC58125, reduced the production of prostaglandin E2 and the neuronal loss in the models of middle cerebral artery occlusion (Nogawa et al., 1997) and global ischemia (Nakayama et al., 1998). Nonetheless, there is a growing body of evidence, albeit indirect, suggesting that COX-2 might be involved in ROS generation. For example, KA-induced damage in the rat hippocampus suggests that COX-2 might be involved in ROS generation. As is required for BDNF-induced potentiation, IGF-I pretreatment with varying doses of IGF-I for 6, 12, 18, and 24 h or for the designated times. For Zn\(^{2+}\) treatment, cortical cultures were washed three times with HEPES-buffered salt solution (120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 20 mM HEPES, 10 mM NaOH, and 15 mM glucose) and then incubated with HEPES-buffered salt solution with varying doses of ZnCl\(_2\) for 30 min at room temperature. Exposure to Zn\(^{2+}\) was terminated by washing cultures three times with serum free medium. Cultures were then maintained in serum-free medium until required for analysis. Cell death was determined 24 h after Zn\(^{2+}\) treatment unless otherwise indicated.

**Assessment of Cell Death.** Cell death was determined using the lactate dehydrogenase (LDH) assay, as described previously (Cho et al., 2003). In brief, 25 \(\mu\)l of culture medium was transferred to a microplate and 100 \(\mu\)l of NADH solution (0.3 mg/ml NADH and 0.1 M potassium phosphate, pH 7.4) was added to the medium. After 2 min, 25 \(\mu\)l of pyruvate solution (22.7 mM pyruvate and 0.1 M potassium phosphate, pH 7.4) was added. The immediate change of NADH to NAD\(^{+}\) after this pyruvate addition was determined by measuring absorbance reduction at 340 nm on a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). LDH activity was normalized such that sham-treated cultures and complete cell death were counted as 0% and 100%, respectively, and normalized LDH activity was regarded to indicate cell death. Complete cell death was accomplished by treatment with 300 \(\mu\)M glutamate for 24 h. In some experiments, trypan blue staining was used to visualize cell death. In this case, culture medium was removed by aspiration and cells were immersed in 0.04% trypan blue solution for 1 min, washed with HEPES-buffered salt solution, and subjected to microscopic analysis. To visualize the morphological changes of cells during cell death, cortical cultures were stained with anti-MAP-2 (Upstate Biotechnology, Lake Placid, NY).

**Immunoblotting.** Western blot analysis was carried out as described previously (Lee et al., 1999). In brief, cultures were lysed with ice-cold 150 mM NaCl and 1% Nonidet P-40 in 20 mM Tris-HCl. Protein samples were resolved on 10% SDS-PAGE, and specific signals on the blots were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ). Immunoblotting was performed using monoclonal anti-COX-2 antibody (1:80; BD Transduction Laboratories, Lexington, KY).

**Immunocytochemistry.** Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 to 20 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS containing 5% goat serum (blocking solution) for 1 h, and then reacted with...
and 5'-CCA CCA GTG CCT CAA CC-3'/H11032-dichlorodihydrofluorescein diacetate (CM-
/H11032-6)-chloromethyl-2-min, media were collected to determine PGE 2 release by using the
RT kit; Stratagene, La Jolla, CA). PCR reactions were set up in a
Moloney murine leukemia virus reverse transcriptase (First Strand
GTC TGG GAT GGA-3 quantified using a Bioimaging Analyzer System (Fuji Film, Tokyo,
arated on 5% polyacrylamide gel, visualized by autoradiography, and
cycles necessary to detect the PCR products was 23 to 25 under the
madzu).
lecular Probes, OR), washed with PBS, and lysed in 0.2% Triton
/x/H11003 incubated for 30 min at 37
°C with 100
scribed previously (Greenlund et al., 1995). In brief, cultures were
/scription purposes, fluorescence levels were measured at three ad-
tion. Total RNA was isolated from a cortical culture using Tri re-
[44x652]with 0.01% hydrogen peroxide.
/h11032-peroxidase reaction product was visualized with 0.05% 3-
diaminobenzidine (DAB) in the presence of 0.01% hydrogen peroxide.
/86x542]/H9262-AGG GAC GTC CGT GTT GA-3
/106x532]-[32-P]dCTP (3000 Ci/mmol), 1 μM concentration of each primer, 1× Taq buffer, 1 unit of Taq (QIAGEN, Valencia, CA), and 1% of the synthesized cDNA. The primer sequences used were 5'-GGG ACA TGG ACT CAC TGC
/H11032 and 5'-GA CAA GCC GTC CGT GA-3' and 5'-CAT CCA CCA CCT CAA CC-3' for COX-1; and 5'-AGG GCA TCT TGG GCT ACA CTA AGG-3' and 5'-GGT ATT ATG GGG
/H11032 GTC GAG GA-3' for GAPDH. The minimum number of PCR cycles necessary to detect the PCR products was 23 to 25 under the
given conditions. After PCR amplification, PCR products were se-
ated on 5% polyacrylamide gel, visualized by autoradiography, and
ified using a Bioimaging System (Fuji Film, Tokyo, Japan).
Cytochrome oxygenase activity was assayed by measuring prostaglandin E2 (PGE 2) release, the major product of prostaglandin synthesis. Cultures were exposed to IGF-I for 24 h. After incubating with 30 μM arachidonic acid (AA) for 30 min, media were collected to determine PGE 2 release by using the
PGE 125I assay system (RPA530; Amersham Biosciences).
ROS Visualization. ROS visualization was performed as de-
scribed previously (Greenlund et al., 1995). In brief, cultures were incu-
bated for 30 min at 37°C in medium containing 10 μM 5-(and-
6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-
/H11032-DCFDA; Molecular Probes, Eugene, OR); CM-H 2DCFDA is a cell membrane-permeable, redox-sensitive fluorescent dye that is not fluorescein in the reduced state. However, when oxidized by hydro-
gen peroxide, it is converted into its fluorescent form (Mahadev et al., 2001; Hool and Arthur, 2002). The cultures were washed twice with
HEPES buffer and subjected to fluorescence microscopic analysis.
Dichlorofluorescein fluorescence was visualized under a Nikon Eclipse inverted microscope (Nikon, Japan) equipped with a 75-W Xenon lamp, a 450- to 490-nm excitation filter, and a 520-nm emission filter; excitation was measured at 504 nm and emission at 529
nm using a spectrofluorometer (Shimadzu, Kyoto, Japan). For quant-
ification purposes, fluorescence levels were measured at three ad-
acent spots that roughly covered the diameter of each well; these
three measurements were averaged and regarded as a data point.
GSH Measurement. GSH concentration was determined as de-
scribed previously (Kim et al., 2003a). In brief, cortical cultures were
incubated for 30 min at 37°C with 100 μM monochlorobimane (Mo-
olecular Probes, OR), washed with PBS, and lysed in 0.2% Triton
X-100 in PBS. The changes in fluorescence caused by the formation of the GSH-monochlorobimane adduct were determined at 380 nm
/402x512dx/H9262 excitation and 478 nm emission using a spectrofluorometer (Shi-
madzu).

Results
The Potentiation of Zn 2+ Toxicity by IGF-I in Cortical Neuronal Culture. We observed that pretreatment of the primary cortical culture for 24 h with 100 ng/ml IGF-I
notably enhanced Zn 2+ -induced cytotoxicity. To explore the
mechanism involved, we first determined the dose- and time-
dependent effects of IGF-I and Zn 2+ treatments on cell death
using the LDH assay (Fig. 1, A–C). The results of obtained
generally agreed with the trypan blue staining results (data
not shown) and with the observed morphological changes.
Pre-exposing the cortical culture to 30 to 100 ng/ml IGF-I for more than 12 h greatly aggravated Zn 2+ -induced neuritic
degeneration and somatic cell loss versus the neurons
treated with IGF-I or Zn 2+ alone (Fig. 1D). In essence, the
pretreatment of primary cortical culture for 12 to 24 h with
100 ng/ml IGF-I increased the cytotoxic effect of 80 μM Zn 2+
more than 2-fold (Fig. 1, B–D). Continuous treatment with
100 ng/ml IGF-I proved slightly cytotoxic (Fig. 1B). Unless
otherwise indicated, IGF-I was applied at 100 ng/ml for 12 to
24 h and 80 μM Zn 2+ was treated for 30 min throughout this
work.

Increased ROS Was Associated with the IGF-I–In-
duced Potentiation of Zn 2+ Toxicity. IGF-I–enhanced
Zn 2+ toxicity was significantly inhibited by 30 μM trolox,
indicating that oxidative stress is involved in this process

Fig. 1. Characterization of the IGF-I–induced potentiation of Zn 2+ toxicity in primary cortical neurons. A, cytotoxicity induced by Zn 2+ was aggravated when cortical cultures were pretreated with 100 ng/ml IGF-I. Zn 2+ was applied briefly for 30 min throughout this work as described under Materials and Methods. Cell death was measured at 24 h after Zn 2+ treatment. B, IGF-I dose-dependently potentiated Zn 2+ toxicity. Zn 2+ (80 μM) was treated for 30 min and cell death was measured 24 h later. C, the strong potentiation of Zn 2+–induced cell death necessitated long-term incubation with IGF-I (100 ng/ml). Cell death was measured 24 h after Zn 2+ treatment. D, photomicrographs of cortical cultures stained with anti-MAP-2 antibody 24 h after Zn 2+ challenge. Cortical cultures were incubated for 12 h with 30 ng/ml IGF-I followed by treat-
ment for 30 min with 80 μM Zn 2+ (IGF-I + Zn). Sister cultures treated with 80 μM Zn 2+ only (Zn) or 30 ng/ml IGF-I only (IGF-I) were prepared. Sham, sham control.  *, p < 0.05 and **, differences at the p < 0.01 levels, respectively, compared with the corresponding control. n = 8 to 12 in all cases.
(Fig. 2A). This result prompted us to search for ROS-generating cellular factors induced by IGF-I pretreatment. As summarized in Fig. 2B, the COX-2–specific inhibitors NS398 (10–100 μM) and SC58125 (1–10 μM), substantially attenuated the IGF-potentiated Zn$^{2+}$ toxicity, whereas the COX-1 inhibitor SC560 (1–10 μM) did not. Neither the xanthine dehydrogenase inhibitor allopurinol (1 mM), the NADPH oxidase inhibitor DPI (1 μM), nor the NOS inhibitors Nω-monomethyl-L-arginine (1 mM) and Nω-nitro-L-arginine methyl ester (5 mM) repressed IGF-I–enhanced Zn$^{2+}$ toxicity, despite the fact that these inhibitors except DPI were applied at millimolar levels (Fig. 2, C and D). Based on these results, we focused on the role of COX-2 in IGF-I–enhanced Zn$^{2+}$ toxicity.

COX-2 Is a ROS-Generating Factor Induced by IGF-I Pretreatment. The need for a long-term (>12 h) preincubation with IGF-I to produce strong potentiation (Fig. 1C) suggested that cellular reprogramming, including new protein synthesis, is required for the IGF-I–induced potentiation of Zn$^{2+}$ toxicity. In fact, cotreatment with cycloheximide or anisomycin during IGF-I pretreatment completely blocked the potentiating effect of IGF-I (Fig. 3, A and B). In contrast, the IGF-I–potenti ated Zn$^{2+}$ toxicity was barely affected when these inhibitors were applied after Zn$^{2+}$ treatment (Fig. 3C).

We tested whether COX-1 and COX-2 were candidate ROS-generating factors induced by IGF-I pretreatment. Semiquantitative RT-PCR using $^{[32P]}$cytosine indicated that the transcription levels of COX-1 and -2 were gradually increased after treatment with 100 ng/ml IGF-I, although the induction of COX-1 was delayed and occurred at a relatively lower level than induction of COX-2. The increased expression of the COX-2 transcript peaked 10 to 12 h after the IGF-I treatment start and decreased gradually thereafter. At its peak, the expression level of COX-2 reached $\sim$2.4-fold that of the control (Fig. 3D). The expression of the other ROS-generating enzymes, including xanthine dehydrogenase, NADPH oxidase (p47$^{phox}$, gp91$^{phox}$), and nNOS, were not significantly altered in the corresponding tests (data not shown).

In accordance with these results, Western blot analysis revealed that the level of COX-2 protein increased and peaked 12 to 24 h after treatment with 100 ng/ml IGF-I (Fig. 4A). In primary cortical cultures treated with 100 ng/ml IGF-I for 24 h, PGE$_2$ release was $\sim$2.7-fold the basal level (Fig. 4B), indicating that the COX activity was enhanced after IGF-I treatment. Consistent with these results, immunocytochemical staining also revealed increased COX-2 expression in neuronal cells 24 h after the treatment with 100 ng/ml IGF-I (Fig. 4C), whereas COX-1 expression remained unchanged until 24 h after treatment with IGF-I (data not shown).
Arachidonic Acid Metabolism Is Involved in the IGF-I-Induced Potentiation of Zn\(^{2+}\) Toxicity. To further substantiate the hypothesis that increased COX-2 activity increases Zn\(^{2+}\) toxicity, we tested whether the exogenous supply of the COX-2 substrate AA affects Zn\(^{2+}\) toxicity. We found that pretreatment of cortical cultures with 100 ng/ml IGF-I doubled the cytotoxicity induced by different doses of AA alone (Fig. 5A), supporting the notion that the increased COX-2 expression was important. Furthermore, the intracellular GSH level was effectively blocked by NS-398 in a dose-dependent manner (Fig. 5C). However, PGE$_2$ or 15-deoxy-12,14-PGJ$_2$, even at 30 \(\mu M\), did not potentiate Zn\(^{2+}\)-induced cell death (Fig. 5D).

Next, we examined whether the COX-2-mediated potentiation of Zn\(^{2+}\)-toxicity is accompanied by increased oxidative stress; this was suggested by the observed protective effect of trolox (Fig. 2A). First, to monitor the intracellular ROS pressure, we chose GSH as a biochemical marker. At 3 h after treatment with 80 \(\mu M\) AA, the level of intracellular GSH reduced to 78% of the control \((p < 0.05, \text{Newman-Keuls test})\). Likewise, Zn\(^{2+}\)-treated primary cortical cultures pretreated with IGF-I showed markedly enhanced ROS production compared with neurons treated with IGF-I or Zn\(^{2+}\) alone. Moreover, this increased ROS production was effectively blocked by 100 \(\mu M\) NS-398 (Fig. 7, A–E). We next tested whether the AA metabolism itself could cause ROS production. Cortical

![Fig. 4. Increases of COX-2 protein and COX activity after IGF-I pretreatment. A, Western blot analysis for COX-2 protein level in cortical culture at 6, 12, and 24 h after 100 ng/ml IGF-I treatment. B, COX activity was determined by measuring PGE$_2$ production in cortical cultures 24 h after treatment with 100 ng/ml IGF-I. C, photomicrographs showing anti-COX-2 immunoreactivity of the cortical culture 24 h after treatment with 100 ng/ml IGF-I (left) and paired phase contrast images (right). The large granule-like cells in the phase contrast images are neurons. Astrocytes lying on the culture plate are not clearly visible in these phase contrast images. *\(, \text{ difference at the } p < 0.05 \text{ level compared with the sham control. } n = 3 \text{ for A and C, and } n = 8 \text{ for B.}

![Fig. 5. Arachidonic acid mimicked the potentiating effect of IGF-I pre-treatment. A, the cytotoxic effect of AA doubled when cortical cultures were exposed to 100 ng/ml of IGF-I for 12 h before treating AA. B, the addition of AA (20 \(\mu M\)) after treatment for 30 min with 80 \(\mu M\) Zn\(^{2+}\) (Zn + AA) strongly potentiated the cytotoxicity induced by 80 \(\mu M\) Zn\(^{2+}\) alone. C, the AA-induced potentiation of Zn\(^{2+}\)-toxicity was blocked by NS-398 in a dose-dependent manner. D, PGE$_2$ (30 \(\mu M\)) and 15-deoxy-12,14-PGJ$_2$ (30 \(\mu M\)) did not mimic the potentiating effect of IGF-I on Zn\(^{2+}\)-toxicity. * and **, differences at the \( p < 0.05 \) and \( p < 0.01 \) levels, respectively, compared with the paired control (A), Zn\(^{2+}\) (B) or Zn\(^{2+}\) + AA (C). n = 8 to 12 in all cases.}
cultures treated with 80 μM Zn2+ for 30 min, followed by incubation with 20 μM AA showed markedly higher intracellular ROS levels, and this was also blocked by 100 μM NS-398 (Fig. 7, F–H). Cortical cultures challenged with 100 μM H2O2, a level that produces severe neuronal death 18 h after treatment (Cho et al., 2003), showed high ROS signs, and this was not suppressed by 100 μM NS-398 (Fig. 7, J and K). Therefore, the possibility that NS-398 has a nonspecific ROS-quenching effect can be excluded. Quantitative measurements of accumulated ROS in cortical neurons indicated that the level of ROS after treatment with IGF-1 + Zn2+ reached >2.3-fold that of the sham control, which was returned to the control level by trolox (100 μM), NS-398 (100 μM), or SC58125 (10 μM) (Fig. 7L). As a whole, ROS generation in neurons challenged with 80 μM Zn2+ and then 20 μM AA is reminiscent of ROS generation in neurons insulted with 100 ng/ml IGF-1 and then 80 μM Zn2+, which supports the hypothesis that AA metabolism and ROS generation are involved in the IGF-1 enhancement of Zn2+ toxicity.

**Discussion**

This study demonstrates that COX-2 is responsible for the IGF-1 induced potentiation of Zn2+ toxicity in primary cortical culture. The following findings support this. First, the COX-2–specific inhibitors, NS398 (10–100 μM) and SC58125 (10 μM), blocked IGF-1 potentiated cell death (Fig. 2B). Second, COX activity was notably enhanced after exposure to IGF-1 (Fig. 4B). The pretreatment of cortical cultures consistently with IGF-1 markedly exacerbated the cytotoxicity induced by AA, the COX-2 substrate (Fig. 5A). Third, COX-2 expression was increased 12 to 24 h after IGF-1 exposure (Figs. 3D and A and C), which is in agreement with the need for new protein synthesis (Fig. 3, A–C) and the long-term (>12 h) incubation with IGF-1 to produce its potentiation effects (Fig. 1C). Taken together, these results strongly support the hypothesis that COX-2 is responsible for the IGF-1–induced potentiation of Zn2+ toxicity in primary cortical culture.

We found that the Zn2+ treatment in the presence of increased COX-2 activity produced a substantial increase in the level of intracellular ROS (Figs. 6 and 7). To explain the results of our study, we propose the following model. COX-2 enzyme has both oxygenase activity, which uses molecular oxygen and peroxidase activity, which reduces hydroperoxides (Eling et al., 1986; Kukreja et al., 1986; Lu et al., 1999). The conversion of one molecule of AA to PGs by the sequential action of the oxygenase and peroxidase activities of COX-2 requires two oxygen molecules and two electrons (Smith et al., 1996). Therefore, as COX-2 activity increases, cells require more cellular NAD(P)H or GSH, which results in the gradual consumption of the cell’s reducing power, unless new reducing factors are introduced. The concepts incorporated into this model are summarized in Fig. 8. As this model explains, cortical cells with enhanced COX-2 activity may accumulate intracellular ROS, even when the cells are challenged by weak ROS-generating conditions, such as treatment with 80 μM Zn2+ for 30 min. In agreement with this speculation, our biochemical data indicate that the level of intracellular GSH rapidly reduced in cortical cultures pretreated with IGF-1 and followed by Zn2+ challenge and in cortical cultures treated with Zn2+ and followed by AA (Fig. 6A). Cortical neurons challenged with 10 μM Fe2+ and 20 μM AA greatly increased ROS production, which was suppressed by NS-398 (data not shown). To the best of our knowledge, this model is the first to explain how enhanced COX-2 activity directly contributes to the generation of ROS stress. Cells with low reducing power may be vulnerable to subsequent ROS stress, as is the case for cellular processes exposed to low doses of free Zn2+. Accumulated ROS in cells oxidizes cellular substrates, such as proteins, lipids, and DNA, which may result in cell death (Kim et al., 1999a,b). In the case of a Zn2+ overload, GSH depletion might be accelerated, and the proper maintenance of thiol homeostasis is likely to be disrupted (Kim et al., 2003a). On the other hand, reactive oxygen radicals may interact with thiols and generate cytotoxic thiol radicals and thiol free radical metabolites (Eling et al., 1986; Takeuchi et al., 1991).

IGF-I–induced potentiation of Zn2+ toxicity was completely blocked by the protein synthesis inhibitors cycloheximide or anisomycin only when they were applied during the IGF-1 pretreatment (Fig. 3, A–C). Thus, de novo protein

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**Fig. 6.** Increased COX-2 activity reduced the intracellular GSH level. A, intracellular GSH levels in cortical cultures at 3 h after treatment with 80 μM Zn2+ only (Zn) or with 80 μM Zn2+ and 20 μM AA (Zn + AA) and at 3 h after a 12-h pretreatment with 100 ng/ml IGF-1 alone (IGF-I) or after a 12-h pretreatment with 100 ng/ml IGF-1 followed by treatment with 80 μM Zn2+ (IGF-I + Zn) or with Zn + AA, are presented (3 h). Intracellular GSH levels for similar sets were also measured at 6 h after treatment (6 h). B, 12 h of treatment with the GSH-depleting agent BSO (1 mM) greatly exacerbated the cytotoxicity induced by 80 μM Zn2+. NS398 (10–100 μM) still afforded protection, although its effect was completely blocked by the protein synthesis inhibitors cycloheximide or anisomycin only when they were applied during the IGF-1 pretreatment (Fig. 3, A–C). Thus, de novo protein...
synthesis was involved in the IGF-I–induced potentiation, as it was in BDNF-induced processes (Gwag et al., 1995; Ryu et al., 1999; Fryer et al., 2000). However, unlike BDNF-induced potentiation, NOS inhibitors did not block the IGF-I–induced potentiation of Zn^{2+} toxicity (Fig. 2D), and IGF-I pretreatment barely changed the expression of nNOS in cortical cultures (data not shown). Kim et al. (2002) reported that the prolonged incubation of primary cortical cultures with BDNF rapidly and substantially induced the expression of NADPH oxidase, which caused superoxide-mediated neuronal injury. Our semiquantitative RT-PCR study using [32P]cytosine indicated that the level of p47phox and gp91phox expression in cortical cultures, treated for 18 h with 100 ng/ml IGF-I, was barely changed (data not shown). These results suggest that the detailed mechanism underlying the IGF-I–induced potentiation of Zn^{2+} toxicity is distinct from that involved in BDNF-induced potentiation.

NS398 (10 μM) and SC58125 (10 μM) significantly, but partially, suppressed IGF-potentiated Zn^{2+} toxicity, although 100 μM NS398 strongly suppressed this potentiated cell death (Fig. 2B). Several possibilities are conceivable. First, although COX-2 is a critical factor for the process, additional factors that are induced by IGF-I may play a role in IGF-potentiated Zn^{2+} toxicity. Second, Zn^{2+} influx into neurons caused by treating with 80 μM Zn^{2+} may elicit multiple arrays of cytotoxic signals along with the activation of the COX-2–related pathway. There is a wealth of evidence that free Zn^{2+} disrupts various aspects of the intracellular physiology, including the induction and activation of NADPH oxidase and energy failure (Noh and Koh, 2000; Sheline et al., 2000). Third, COX-1 could add functionally to COX activity enhancement. The IC_{50} for the inhibition of COX-2 by NS-398 is 1 to 2 μM in cell free assays. In general, however, much higher doses of this inhibitor are used in studies with intact cells. The IC_{50} for the inhibition of COX-1 by NS-398 is reported to be ~100 μM (DeWitt, 1999). NS-398 is known to

**Fig. 7.** Visualization by CM-H$_2$DCFDA of ROS generation involved in IGF-I potentiation and AA metabolism. A–E, ROS generation in cortical cultures at 4 h after a 12-h pretreatment with 100 ng/ml IGF-I (IGF-I; C), 80 μM Zn^{2+} (for 30 min) (Zn; B), or a 12-h pretreatment with 100 ng/ml IGF-I followed by 80 μM Zn^{2+} treatment (IGF-I + Zn; D). NS-398 (100 μM) blocked the ROS rise in cortical neurons challenged with IGF-I + Zn (IGF-I + Zn + NS398; E). F–I, ROS levels in cortical neurons at 4 h after treatment with 20 μM AA (F) or treatment with 80 μM Zn^{2+} followed by 20 μM AA (Zn + AA; G). The rise of ROS accumulation in Zn + AA was inhibited by 100 μM NS-398 (Zn + AA + NS398; H), which was comparable with that observed for IGF-I + Zn + NS398 (E). NS-398 (100 μM) application for 30 min before CM-H$_2$DCFDA application did not suppress ROS generation (Zn + AA + NS398-post; I). J–K, ROS levels in cortical neurons challenged with 100 μM H$_2$O$_2$ (J). Coincubation of 100 μM H$_2$O$_2$ with NS-398 did not suppress the rise in ROS levels (H$_2$O$_2$ + NS398; K). These experiments (A–K) were repeated more than three times, and representative photomicrographs are presented. L, ROS levels in cortical cultures prepared in 24-well plates were quantified after staining with CM-H$_2$DCFDA. ROS levels in IGF-I (100 ng/ml)-pretreated cortical cultures at 4 h after treatment with 80 μM Zn^{2+} reached a level that was ~2.3-fold higher than that in the sham control. This rise in ROS level was brought back to the control level after treating with 100 μM trolox, 100 μM NS-398, or 10 μM SC58125 (p < 0.05, n = 24 spots from eight wells in each group). ***, difference at the p < 0.01 level compared with the sham control.
exhibit a 1000-fold selectivity for the inhibition of COX-2 over COX-1 (Giere et al., 1995). Despite these properties of NS-398 and the failure of the COX-1-selective inhibitor SC560 (10 μM) to suppress IGF-potentiated Zn2+ toxicity (Fig. 2B), the results of our study do not exclude the possibility that 100 μM NS398 partially inhibits COX-1 and thus contributes to the inhibition of potentiated cell death.

In summary, IGF-I pretreatment makes primary cortical neurons highly vulnerable to subsequent weak cytotoxic insults. The underlying mechanism involves the induction of COX-2, and the loss of the cell’s reducing power. Subsequent weak ROS insults may be highly detrimental to viability. The results of our study raise the possibility that appropriately controlled intervention may be necessary to avoid the potential exacerbating effects of IGF-I in the treatment of neuropathological conditions.

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Fig. 8. A hypothetical model for the role of COX-2 in ROS generation and potentiated cell death. COX-2, which has both oxygenase and peroxidase activities, uses two oxygen molecules and two electrons to convert one molecule of AA to PGs. NADH, NADPH, or GSH may donate the required electrons as a result of their oxidation to NAD+, NADP+, or GSSG, respectively. Therefore, the increase in COX-2 activity caused by either enhanced COX-2 expression or an increase in the supply of its substrate, AA, depletes the cellular reservoirs of NADH, NADPH, or GSH, resulting in a decrease in the cell’s reducing power. In this cellular context, neurons may not be able to cope with weak ROS stress such as a Zn2+ insult, and thus cell death is enhanced. This model emphasizes not only that the induction of COX-2 by IGF-I pretreatment potentiates cell death but also that enhanced COX-2 activity contributes to the accumulation of ROS. This model does not necessarily eliminate the possibility that IGF-I affects the cell death induced by other cytotoxic molecules. Selected steps are indicated in the diagram: (1) Sensi et al., 1997; (2) Noh et al., 1999; (3) Noh and Koh, 2000; Kim et al. 2003a; (4) Ryu et al., 1999; (5) Smith et al., 1996; (6) Kim et al., 1999a; (7) Kim et al., 1999b. VSCC, voltage gated Ca2+ channel; PLA2, phospholipase A2; GSSG, oxidized glutathione (GSH); PI3-K, phosphatidylinositol 3-kinase.

References


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