Induction and Activation by Zinc of NADPH Oxidase in Cultured Cortical Neurons and Astrocytes

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Zinc overload may be a key mechanism of neuronal death in acute brain injury. We have demonstrated previously that zinc overload neurotoxicity involves protein kinase C (PKC)-dependent rises in intracellular levels of reactive oxygen species (ROS). However, the cascade linking PKC activation to ROS generation in cultured cortical neurons has been unknown. A recent study has demonstrated that ROS-generating NADPH oxidase is present in sympathetic neurons and contributes to NGF deprivation-induced cell death. Because NADPH oxidase is activated by PKC, in the present study, we examined the possibility that NADPH oxidase is the effector for oxidative stress in zinc-overloaded cortical cells.

Reverse transcription-PCR and Western blot analyses revealed that naive cultured cortical cells express subunits of NADPH oxidase at low levels. Exposure to zinc substantially increased levels of NADPH oxidase subunits in both neurons and astrocytes. In addition, zinc exposure induced transloca-tion of the $p47^{PHOX}$ and $p67^{PHOX}$ subunits to the membrane, a signature event for NADPH oxidase activation. Addition of a selective PKC inhibitor, GF109203X, blocked both the induction and the membrane translocation of NADPH oxidase by zinc. Supporting the role for NADPH oxidase in zinc-triggered oxidative injury, NADPH oxidase inhibitors attenuated ROS production and cortical neuronal death induced by zinc. In addition, Cu/Zn-superoxide dismutase and catalase attenuated zinc-induced cortical neuronal death.

Our results have demonstrated that zinc overload induces and activates NADPH oxidase in cortical neurons and astrocytes in a PKC-dependent manner. Thus, NADPH oxidase may be an enzyme contributing to ROS generation in zinc-overloaded cortical neurons and astrocytes.

Key words: oxidative stress; protein kinase C; superoxide; astrocyte; calcium; neuronal death

Recent evidence indicates that endogenous zinc may play a key role in neuronal death after acute brain insults such as ischemia, seizures, and trauma (Frederickson et al., 1989; Tonder et al., 1990; Koh et al., 1996; Choi and Koh, 1998; Suh et al., 2000). In these brain injury paradigms, intracellular accumulation of zinc correlates well with neuronal death at the single cell level. Furthermore, chelation of zinc prevents both intracellular zinc accumulation and neuronal death in those brain injury models. Cytotoxic mechanisms of intracellular zinc overload may involve diverse processes such as mitochondrial damage, nicotinamide adenine dinucleotide (NAD)-positive (NAD^+) degradation, ATP depletion, and caspase activation (Lobner et al., 1997; Manev et al., 1997; Sheline et al., 2000). In addition, evidences suggest that oxidative stress plays a significant role in zinc neurotoxicity in cortical culture. First, zinc influx increases the levels of reactive oxygen species (ROS) in neurons (Kim et al., 1999; Sensi et al., 1999). Second, various neuroprotective measures effective against zinc toxicity, such as protein kinase C (PKC) inhibitors, attenuate zinc-induced increases in ROS in parallel (Noh et al., 1999). Finally, antioxidants attenuate zinc neurotoxicity (Kim et al., 1999). Although the evidence indicates that oxidative stress is a significant mechanism of zinc-induced neuronal death, the effector protein(s) directly responsible for the generation of ROS in zinc-injured cells is unknown.

NADPH oxidase is a superoxide-producing enzyme consisting of the membrane ($gp91^{PHOX}$ and $p22^{PHOX}$) and the cytosolic ($p47^{PHOX}$, $p67^{PHOX}$, and $p40^{PHOX}$) components (DeLeo and Quinn, 1996; Babior, 1999). In addition, small G-proteins such as rac1, as well as kinases including PKC, regulate its activity (Heinecke et al., 1990; Benna et al., 1997; Reeves et al., 1999; Ozaki et al., 2000). Although NADPH oxidase is mainly expressed in phagocytic cells, an increasing body of evidence suggests that various subunits of NADPH oxidase are also expressed in nonphagocytic cells such as mesangial cells, endothelial cells, vascular smooth muscles, and fibroblasts (Fukui et al., 1995; Jones et al., 1995, 1996; Thannickal and Fanburg, 1995). However, functions of NADPH oxidase subunits in nonphagocytic cells are not yet clearly delineated. Recently, all of the subunits of NADPH oxidase are to be expressed by sympathetic ganglion neurons and to contribute to NGF-deprivation-induced ROS generation and cell death, suggesting the possibility that neurons in general might express NADPH oxidase (Tammariello et al., 2000). Because NADPH oxidase can be activated by PKC and because PKC

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activation appears to be a key step in zinc-induced oxidative injury, we hypothesized that NADPH oxidase may be the effector enzyme mediating the PKC-dependent oxidative injury in zinc-overloaded cultured cortical neurons. This possibility was examined in the present study.

MATERIALS AND METHODS

Cortical cell culture and assessment of cell death. Mixed mouse cortical cultures containing both neurons and astrocytes, and pure astrocyte cultures, were prepared from fetal (15 d of gestation) and neonatal (1–3 postnatal days) mice, respectively, as described previously (Kim et al., 1999). Near-pure neuronal cultures were prepared from fetal mice (Sheline and Choi, 1998). Immunocytochemical staining with anti-Macrophage (Mac-1-antigen) antibody (Boehringer Mannheim, Mannheim, Germany) revealed that <3% of cells were microglia in all three types of cultures.

Brief (15 min) exposure of cortical cultures (10–13 d *in vitro*) to zinc was performed in serum-free HBSS at room temperature. After exposure, cultures were incubated in serum-free MEM and placed back into the incubator. In addition to morphological assessment under a phase-contrast microscope, in most experiments, overall neuronal cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) (Koh and Choi, 1987) released by damaged cells into the bathing medium 24 hr after exposure to zinc. Each LDH value, after subtracting background LDH value in sham-washed controls, was scaled to the maximal neuronal LDH release (of 100) produced by 24 hr exposure of sister cultures to 300 μM NMDA, which produces near-complete neuronal death with no glial damage.

Reverse transcription-PCR. RNA was prepared with TRIZOL (Life Technologies, Gaithersburg, MD) and reverse-transcribed to cDNA using oligo-dT 14 primers (Promega, Madison, WI). Specific cDNAs were amplified for 30 cycles. Signals for amplified cDNAs, after separation on 1.7% agarose gels, were visualized with ethidium bromide and quantitatively measured with an image analyzer (Alpha Imager 2000; Alpha Innotech, San Leandro, CA). Primer sequences were as follows: 5′-CTTGGATGATAGCACTGCAC-3′ and 5′-CTTCATCTGAAGCTC-AATGG-3′ for p91^{PHOX} (626 bp); 5′-AGAGCGACTTTGAG-CAGCTT-3′ and 5′-TGTGGAGACACACCCTTGAT-3′ for p40^{PHOX} (460 bp); 5′-AGCCTGAGACATACCTGGTG-3′ and 5′-AGACTTCTG-CAGATACATGG-3′ for p47^{PHOX} (446 bp); 5′-CAAGGCTACGGTTG-TAGCAT-3′ and 5′-ACCTTGAGCATGTAAGGCAT-3′ for p67^{PHOX} (465 bp); and 5′-CTCAAGATTGTCAGCAATGC-3′ and 5′-CACT-GAGGACCAGGTTGTCT-3′ for glyceraldehyde-3-phosphate dehydrogenase (415 bp).

Cell lysis, fractionation, and immunoblotting

Cortical cells were lysed in lysis buffer and centrifuged at 14,000 rpm for 20 min. The pellet was discarded, and the supernatant was used for protein quantification. Fractionation of cytosol and membrane was performed as described previously (Noh et al., 1999). Equal amounts of protein from total cell lysates or membrane and cytosolic fraction were electrophoresed on 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were then incubated with respective primary antibodies (anti-p67^{PHOX}, anti-p47^{PHOX}, rac-1, and anti-gp91) overnight at 4°C The enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL) protocol was used to visualize the immunoreactive bands.

Immunohistochemistry. After fixation (4% paraformaldehyde) and blocking, cells were double-labeled with anti-p67^{PHOX} antibody (Transduction Laboratories, San Diego, CA) and either anti-glial fibrillary acidic protein (GFAP) or anti-microtubule-associated protein-2 (MAP2) antibody (Sigma, St. Louis, MO). After washes, signals were visualized by appropriate secondary antibodies coupled to either fluorescein isothiocyanate (FITC) or rhodamine (Jackson ImmunoResearch, West Grove, PA).
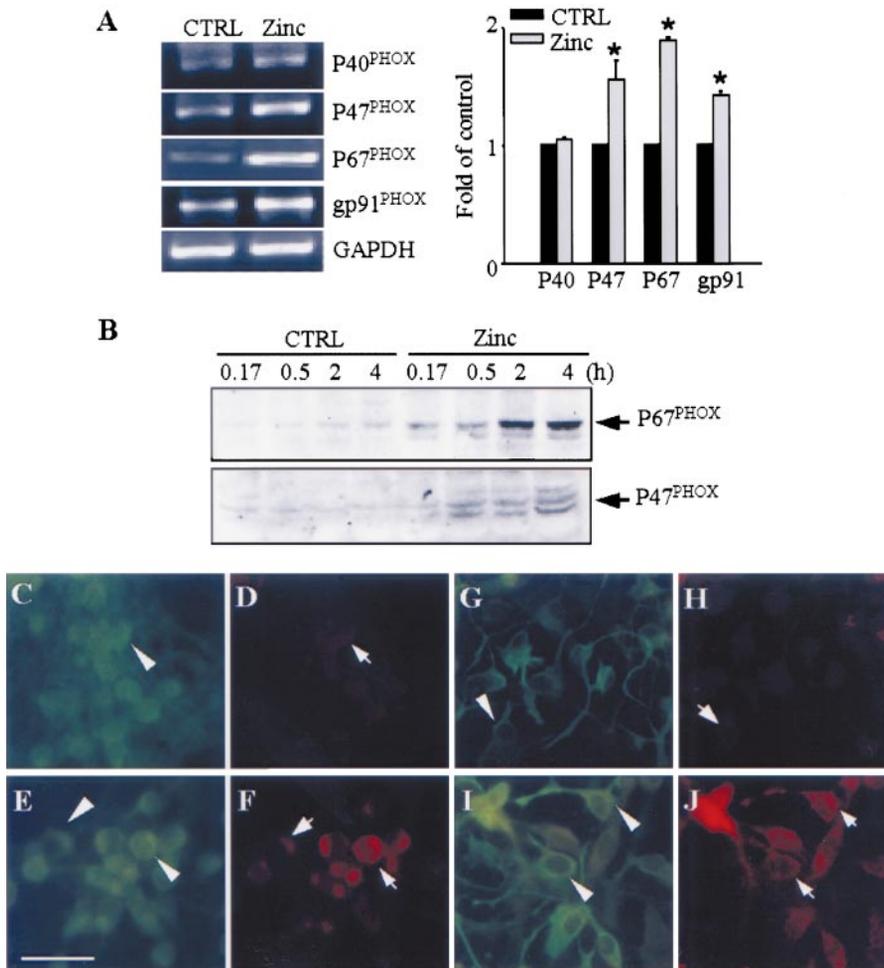

Figure 1. Induction of NADPH oxidase subunits in cortical cells exposed to zinc. *A*, RT-PCR for p40^{PHOX}, p47^{PHOX}, p67^{PHOX}, and gp91^{PHOX} in sham-washed control cultures (*CTRL*) and in sister cultures 2 hr after 15 min exposure to 400 μM zinc (representative of 3 experiments). Bars denote densitometer readings of RT-PCR signals expressed as folds of respective control values (mean ± SEM; n = 3). *Asterisks* denote difference from respective controls (p < 0.05; two-tailed t test). *B*, Western blots for p67^{PHOX} and p47^{PHOX}. Fifteen minute exposure to 400 μM zinc increased both p67^{PHOX} and p47^{PHOX} protein levels in cortical culture beginning 0.5–2 hr after zinc exposure. *C–F*, Fluorescent photomicrographs of near-pure neuronal cultures, sham-washed control (*C, D*), or 4 hr after 15 min exposure to 400 μM zinc (*E, F*), stained with anti-MAP2 antibody (*C, E*, FITC) and then with anti-p67^{PHOX} antibody (*D, F*, rhodamine). *Arrows* and *arrowheads* denote identical landmark cells in matched sets. *G–J*, Fluorescent microphotographs of astrocyte-rich cultures, sham-washed control (*G, H*), or 4 hr after 15 min exposure to 400 μM zinc (*I, J*), stained with anti-GFAP antibody (*G, I*) and anti-p67^{PHOX} antibody (*H, J*). Scale bar, 50 μm.
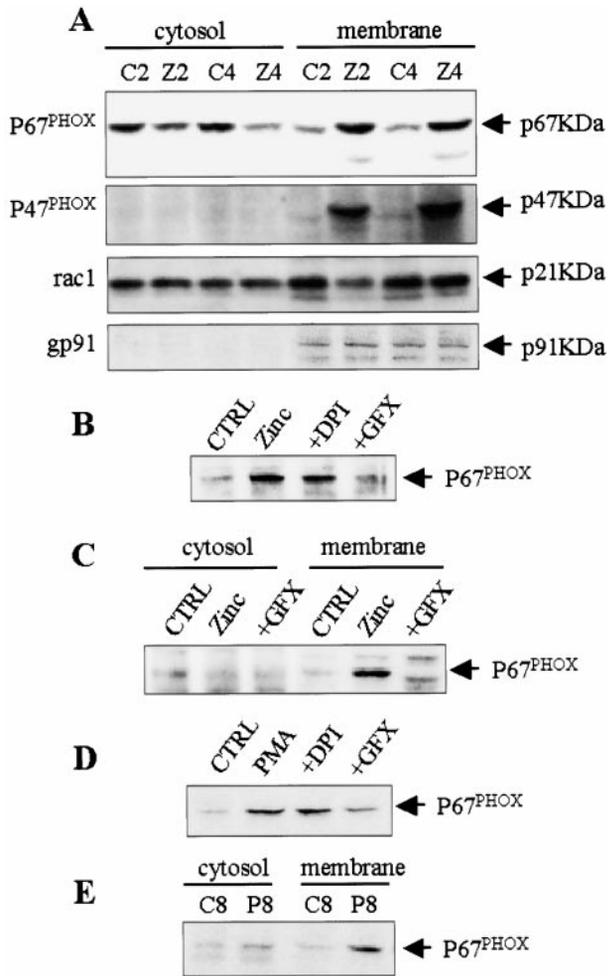
Figure 2. Mediation by PKC of translocation and induction of NADPH oxidase. *A,* Western blots for indicated subunits of NADPH oxidase in the cytosolic fraction and the membrane fraction of cortical cultures, 2 or 4 hr after sham wash or 15 min zinc exposure (representative of 5 experiments). Whereas levels of rac1 in each fraction did not change much with zinc exposure, levels of p67^{PHOX} appeared to be decreased in the cytosolic fraction and to be increased in the membrane fraction. Also, the membrane levels of p47^{PHOX} markedly increased upon zinc exposure. The level of the membrane-anchored subunit gp91^{PHOX} did not change much with zinc exposure. *B,* GF109203X (*GFX*) blocks NADPH oxidase induction by zinc. Western blots show levels of p67^{PHOX} in cortical cultures 4 hr after sham wash (*CTRL*) or 15 min exposure to 400 μM zinc without (*Zinc*) or with addition of DPI (100 nM) or GF109203X (3 μM) during and continuously after the zinc exposure (representative of 4 experiments). GF109203X markedly attenuated the induction of p67^{PHOX} by zinc, whereas DPI had no effect. *C,* GF109203X blocks NADPH oxidase activation by zinc. Western blots of the membrane fractions with anti-p67^{PHOX} antibody after 4 hr exposure to 400 μM zinc (representative of 5 experiments). Addition of 3 μM GF109203X during and after the zinc exposure decreased membrane translocation of p67^{PHOX} by zinc. *D,* PMA increases p67^{PHOX} levels. Western blots show levels of p67^{PHOX} in sham-washed control or cultures after 8 hr exposure to 200 nM PMA (representative of 4 experiments). The increase in p67^{PHOX} levels by PMA was completely blocked by 3 μM GF109203X, whereas 100 nM DPI had little effect. *E,* PMA induces translocation of p67^{PHOX}. Membrane p67^{PHOX} levels markedly increased with 8 hr exposure to 200 nM PMA (*P8*) (representative of 4 experiments) compared with cultures 8 hr after sham wash (*C8*).

Measurement of ROS generation. Two independent methods were used to measure ROS production in cortical cultures. 2,7-Dichlorodihydrofluorescein diacetate (DCF) fluorescence was used to visualize intracellular superoxides (Greenlund et al., 1995). H$_2$O$_2$ accumulation in media was measured using the Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) Hydrogen Peroxide Assay kit (Molecular Probes, Eugene, OR).
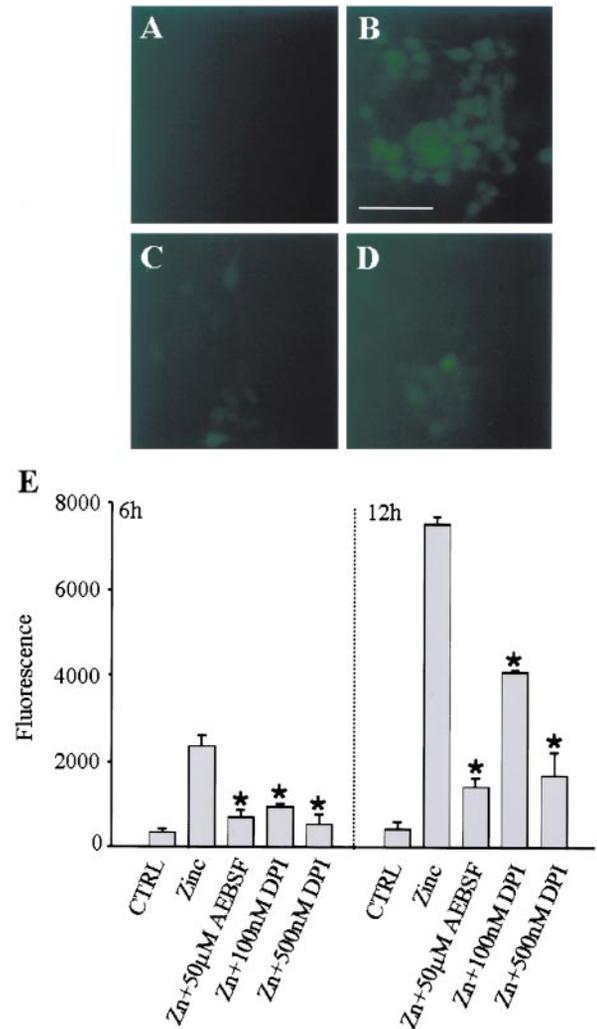

Figure 3. NADPH oxidase inhibitors attenuate ROS increases by zinc. *A–D,* DCF fluorescence in cortical cultures 3 hr after sham wash (*A*) or 15 min exposure to 400 μM zinc without (*B*) or with (*C*) addition of 50 μM AEBSF or 500 nM DPI (*D*) during and after the zinc exposure. *E,* Levels of H$_2$O$_2$ in the bathing media of cortical cultures (mean $\pm$ SEM; $n = 3$ cultures) as estimated by the Amplex Red fluorimetry (arbitrary units of fluorescence). Media samples were obtained from sham-washed control cultures (*CTRL*) or from cultures 6 hr (*left bars*) or 12 hr (*right bars*) after 15 min exposure to 400 μM zinc without (*Zinc*) or with addition of AEBSF (50 μM) or DPI (100 or 500 nM). *Asterisks* denote difference from *Zinc* ($p < 0.05$; two-tailed t test with Bonferroni correction for 3 comparisons). Scale bar, 100 μm.

RESULTS

Expression and induction of NADPH oxidase in cultured cortical cells

Subunits of NADPH oxidase were examined at the mRNA level by reverse transcription (RT)-PCR analysis. Whereas cortical cultures expressed low levels of mRNA for NADPH oxidase subunits (Fig. 1*A*), 15 min exposure to 400 μM zinc substantially increased the mRNA levels for p47^{PHOX}, p67^{PHOX}, and gp91^{PHOX} in cortical cultures with little change in that for p40^{PHOX} (Fig. 1*A*). Western blot assay confirmed that zinc exposure increased the expression of both p47^{PHOX} and p67^{PHOX}, beginning ~30 min to 2 hr after the exposure (Fig. 1*B*). Because mixed cortical cultures contain both neurons and astrocytes, we examined in which cell type NADPH oxidase expression increases in response to zinc exposure. Immunocytochemical stain-
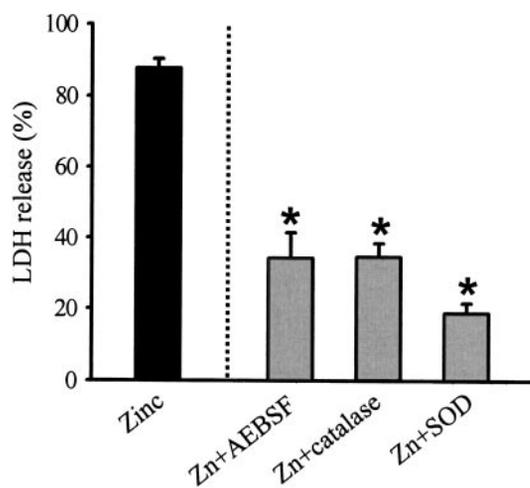
Figure 4. An NADPH oxidase inhibitor as well as Cu/Zn-SOD and catalase attenuate zinc neurotoxicity. LDH release in cortical cultures (mean ± SEM; $n = 3$) 24 hr after 15 min exposure to 400 μM zinc without (*Zinc*) or with addition of 50 μM AEBSF, catalase (300 mU/ml), or Cu/Zn-SOD (50 U/ml). *Asterisks* denote difference from *Zinc* ($p < 0.05$; two-tailed t test with Bonferroni correction for 3 comparisons).

ing of neuron-rich cultures with neuron-specific anti-MAP2 antibody revealed that >90% of cells were indeed neurons (92.8 ± 1.2% of Hoechst 33342$^+$ cells; $n = 4$) (Fig. 1*C,E*). In control cultures, immunoreactivity to p67^{PHOX} was present at low level (Fig. 1*D*). However, 4 hr after 15 min zinc exposure, immunoreactivity to p67^{PHOX} markedly increased in the majority of MAP2$^+$ neurons (Fig. 1*F*). On the other hand, in astrocyte-rich cultures, almost all cells exhibited immunoreactivity to anti-GFAP antibody (97.8 ± 1.4% of Hoechst 33342$^+$ cells; $n = 4$) (Fig. 1*G,I*). Again, baseline expression of p67^{PHOX} appeared quite low in astrocytes (Fig. 1*H*). However, exposure to zinc substantially increased p67^{PHOX} levels in most astrocytes (Fig. 1*J*). Hence, zinc exposure increased p67^{PHOX} expression in both neurons and astrocytes.

PKC mediates induction and membrane translocation of NADPH oxidase

Next, we examined whether zinc exposure leads to the functional activation of NADPH oxidase. The necessary step for NADPH oxidase activation is the translocation of cytosolic subunits, including p47^{PHOX} and p67^{PHOX}, to the membrane (Leusen et al., 1996; Han et al., 1998; Johnson et al., 1998). Consistent with the membrane translocation, at 2 and 4 hr after 15 min zinc exposure, levels of p47^{PHOX} and p67^{PHOX} in the membrane fraction markedly increased (Fig. 2*A*). Because p47^{PHOX} was barely detectable in the cytosol in both control and zinc-exposed cultures (Fig. 2*A*), markedly increased p47^{PHOX} in membrane of zinc-exposed cells may signify the fairly rapid recruitment of newly synthesized protein to the membrane. It is well known in phagocytic cells that levels and activity of NADPH oxidase are regulated by protein kinase C (Heinecke et al., 1990; Benna et al., 1997; Reeves et al., 1999). Because zinc activates PKC (Murakami et al., 1987; Csermely et al., 1988; Noh et al., 1999), we examined whether the effect of zinc on NADPH oxidase induction and activation is mediated by PKC. Indeed, a selective PKC inhibitor, GF109203X, completely blocked the induction of p67^{PHOX} by zinc, whereas a selective inhibitor of NADPH oxidase, diphenyleneiodonium (DPI), did not (Fig. 2*B*). Furthermore, the membrane translocation of p67^{PHOX} by zinc was also inhibited by GF109203X (Fig.

2*C*). Conversely, addition of a PKC activator, PMA, alone was sufficient for the induction and translocation of p67$_{PHOX}$ in cortical cultures (Fig. 2*D,E*). Induction of NADPH oxidase by PMA was blocked by GF109203X but not by DPI (Fig. 2*D*).

NADPH oxidase contributes to ROS generation induced by zinc

We have shown previously that zinc exposure leads to the activation of PKC, which leads to increases in superoxide generation in cultured cortical neurons (Noh et al., 1999). Having demonstrated that PKC mediates the induction and activation of NADPH oxidase by zinc, we examined whether NADPH oxidase really contributes to superoxide generation after zinc exposure. Exposure of mixed cortical cultures to zinc markedly increased cellular DCF fluorescence (Fig. 3*B*), an indicator for superoxide, compared with controls (Fig. 3*A*). NADPH oxidase inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and DPI (Diatchuk et al., 1997; Li and Trush, 1998) markedly reduced the DCF fluorescence (Fig. 3*C,D*). This finding was confirmed independently by the Amplex Red fluorimetry assay; gradual H_2O_2 accumulation in the media was observed at 6 and 12 hr after zinc exposure. NADPH oxidase inhibitors AEBSF and DPI markedly reduced H_2O_2 buildup in the media of zinc-exposed cultures at both times (Fig. 3*E*).

NADPH oxidase contributes to oxidative neuronal death induced by zinc

The next question was whether ROS generated by NADPH oxidase contributes to zinc-induced neuronal death. Neurons exhibited marked cell body swelling several hours after 15 min zinc exposure, followed by release of LDH into the bathing medium (Fig. 4), as described previously (Kim et al., 1999). Addition of AEBSF significantly reduced neuronal death and resultant LDH release induced by the zinc exposure (Fig. 4); we could not use another NADPH oxidase inhibitor, DPI, because even at low concentrations (100 nM), it had significant cytotoxicity (data not shown). Addition of Cu/Zn-superoxide dismutase (SOD), which lowers superoxide levels, also attenuated zinc-mediated neuronal death (Fig. 4).

DISCUSSION

Although oxidative stress has been proposed as one of the major mechanisms of ischemic brain injury (Uyama et al., 1992; Chan et al., 1998), detailed information about the cascades connecting ischemic events to increased ROS generation is currently lacking. Combined with the evidence that zinc overload is a key mechanism of ischemic neuronal death (Choi and Koh, 1998), the present study suggests that the induction and activation of NADPH oxidase in intrinsic brain cells by zinc overload may play such a role in ischemic brain injury.

Although the presence of NADPH oxidase in sympathetic ganglion neurons has raised the possibility that other neurons may also express NADPH oxidase (Tammariello et al., 2000), functional NADPH oxidase expression in central neurons and astrocytes has not been directly documented. The present study has demonstrated that NADPH oxidase subunits, albeit at low levels, are indeed expressed in central neurons and astrocytes. More interestingly, certain subunits of NADPH oxidase were rapidly induced after injurious zinc exposure. Furthermore, in addition to being induced, NADPH oxidase appears to be functionally activated after zinc exposure.

The next question was which event links between zinc overload and NADPH oxidase induction–activation. Whereas several

known activators of NADPH oxidase are known, we focused on PKC in this study, because zinc has been shown to directly activate PKC (Noh et al., 1999). Supporting this possibility, addition of a selective PKC inhibitor blocked the induction and membrane translocation of p67^{PHOX} after zinc exposure. Conversely, addition of a PKC activator (PMA) was sufficient to induce and activate NADPH oxidase. Together, these results suggest a critical role for PKC in both induction and activation of NADPH oxidase by zinc.

In the present study done in cortical cultures, the contribution by NADPH oxidase to zinc-induced oxidative injury was directly supported by the findings that inhibitors of NADPH oxidase attenuate both superoxide generation and neuronal death after zinc exposure. This again supports the idea that oxidative stress is a significant mechanism of zinc overload neurotoxicity. Consistently, Cu/Zn-SOD and catalase, enzymes reducing free radical levels, significantly attenuated zinc-induced neuronal death. Together, the present results suggest that, in brain injury conditions in which zinc neurotoxicity contributes, PKC activation and the resultant NADPH oxidase induction–activation may play a significant role in causing oxidative neuronal injury. Consistent with this idea, the PKC inhibitor staurosporine and antioxidative measures have been shown to attenuate the death of CA1 neurons after transient ischemia (Hara et al., 1990; Tagami et al., 1999; Sheng et al., 2000) in which zinc neurotoxicity may play a significant role (Koh et al., 1996).

The present study, for the first time, has demonstrated the expression and activation of NAPDH oxidase in cultured cortical neurons and astrocytes are under control of PKC, which is activated by zinc influx. Additional studies seem warranted to elucidate the potential role for NADPH oxidase in oxidative injury *in vivo* as well, because oxidative stress is likely a common mechanism for cellular damage in various neurological diseases.

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