Role of cyclooxygenase-2 induction by transcription factor Sp1 and Sp3 in neuronal oxidative and DNA damage response

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ABSTRACT **Cyclooxygenase-2 (COX-2) has been implicated in neuronal survival and death. However, the precise regulatory mechanisms involved in COX-2 function are unclear. In the present study we found that COX-2 is induced in response to glutathione depletion-induced oxidative stress in primary cortical neurons. Two proximal specific Sp1 and Sp3 binding sites are responsible for the COX-2 promoter activity under normal as well as oxidative stress conditions through enhanced Sp1 and Sp3 DNA binding activity. Site-directed mutagenesis confirmed that –268/–267 positions serve as specific Sp1 and Sp3 recognition sites under oxidative stress. Enforced expression of Sp1 and Sp3 using HSV vectors increased the promoter activity, transcription, and protein level of COX-2 in cortical neurons. The dominant negative form of Sp1 abrogated the oxidative stress-induced promoter activity and expression of COX-2. We also demonstrated that adenovirus-mediated COX-2 gene delivery protected neurons from DNA damage induced by oxidative, genotoxic, and excitotoxic stresses and by ischemic injury. Moreover, COX-2$^{-/-}$ cortical neurons were more susceptible to DNA damage-induced cell death. These results indicate that in primary neurons Sp1 and Sp3 play an essential role in the modulation of COX-2 transcription, which mediates neuronal homeostasis and survival by preventing DNA damage in response to neuronal stress.—Lee, J., Kosaras, B., Aleyasin, H., Han, J. A., Park, D. S., Ratan, R. R., Kowall, N. W., Ferrante, R. J., Lee, S. W., Ryu, H. Role of cyclooxygenase-2 induction by transcription factor Sp1 and Sp3 in neuronal oxidative and DNA damage response. *FASEB J.* 20, E1657–E1669 (2006)**

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Cyclooxygenase (COX), also known as prostaglandin H synthease, is the rate-limiting enzyme that converts arachidonic acid into prostaglandin H2, thromboxanes, and prostacyclins. Two isoforms of the COX enzyme have been characterized as COX-1 and COX-2. COX-1 protein is expressed constitutively in most cell types, but COX-2 protein is an inducible isoform that can be rapidly and transiently induced by inflammatory cytokines, mitogens, and a variety of stimuli (9, 14, 17, 28, 35). However, COX-2 has been found to be expressed constitutively in the brain and cerebral blood vessels of the newborn and appears to be developmentally and functionally regulated (43). The elevated expression of COX-2 in the tissue of neurodegenarative diseases such as Alzheimer's, amyotrophic lateral sclerosis (ALS), PD, and stroke has suggested that this protein is involved in the pathophysiological process of neurodegenerative diseases (7, 13, 15, 20, 21, 28, 41, 44).

It is well known that oxidative stress and inflammation are key components in the COX-2 expression as well as in the onset and progression of neurodegenerative diseases. But it has not been fully determined how early in the pathological cascade these processes become involved or which specific molecular components are important (2, 16). It has been shown that the structure of the transcription factors is determined by redox. For instance, the cysteine residues within a transcription factor may sensitize it to reactive oxygen intermediates (ROIs). NF-κB is one of the well-known transcription factors, and is involved in both the oxidative stress signaling pathway and the activation of neuroinflammatory genes. COX-2 is also under transcriptional control by NF-κB, CREB, and C/EBP (5, 27,

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36, 40, 42). Deletion analysis of COX-2 promoters indicates that the upstream region of NF-κB element also maintains a certain level of induced transcription. It reveals that the binding of NF-κB is a necessary but not a sufficient step in the induction of COX-2 by hypoxic signaling (36). A previous report shows that oxidative stress generated by reactive oxygen species (ROS) (ROSs) and interleukin (IL)-1-β induces COX-2 gene transcription in neuronal cell lines (15). However, the transcriptional regulation of COX-2 has not been precisely examined in primary neurons under oxidative stress conditions. In addition, it is intriguing whether COX-2 expression leads to a prosurvival or a proapoptotic effect in primary neurons under oxidative stress. Recent studies have shown that COX-2 overexpression protects against apoptosis induced by NGF withdrawal of differentiated PC12 cells (22). Genetic disruption (COX-$2^{-/-}$) or chemical inhibition of COX-2 decreases the recovery of cardiac function after ischemia (4, 6, 38, 37). It has also shown that oxidative stress increases the COX-2 protein level in cultured cardiomyocytes and COX-2 specific inhibition enhances oxidative stress-induced injury (1). These findings suggest that up-regulation of COX-2 may have beneficial effects and that inhibition of COX-2 may be associated with enhanced ischemia and oxidative stress-induced cell damage (3, 19, 24, 30).

We recently found that glutathione depletion-induced oxidative stress regulates DNA binding activity of Sp1 and Sp3 in cortical neurons (34). In primary neurons Sp1 appears likely to mediate a prosurvival role in response to oxidative stress and in the case of neurodegeneration such as Huntington's disease (18, 33). We undertook this study to determine whether the regulation of COX-2 expression is mediated by Sp1 and/or Sp3 activation under oxidative stress. We also examined the role of COX-2 as a downstream pathway in relation to the known prosurvival role of Sp1 in neurons subjected to various stress. We determined that the two Sp1 and Sp3 binding sites located within the proximal region of the human COX-2 promoter are critical for both basal and oxidative stress-induced COX-2 promoter activity. Moreover, overexpression of COX-2 protected neurons against stress-induced DNA damage *in vitro* and *in vivo*. These results show that COX-2 may play a protective role in primary neurons by nullifying oxidative stress-induced DNA damage as a downstream target gene of Sp1.

MATERIALS AND METHODS

Rat primary neuron culture

Cell cultures were obtained from the cerebral cortex of Sprague Dawley rats (day 17 of gestation) as described previously (33, 34). All experiments using immature cortical neurons were initiated 24–72 h after plating. Under these conditions the cells are not susceptible to glutamate-mediated excitotoxicity. For cytotoxicity studies, cells were rinsed with warm PBS, then placed in minimum essential medium (MEM, Gibco BRL) with 5.5 g/L glucose (Glc), 10% FCS, 2 mM L-glutamine. HCA was diluted from 100-fold concentrated solutions that were adjusted to pH 7.5. Cortical neurons were kept in MEM containing 10% FCS during the course of any treatment. Cells were either stimulated with indicated agonists and antagonist or treated with the same volume of the appropriated diluents for the indicated periods at 37°C with 5% CO_2. Cytoplasmic extracts were prepared essentially as described with some modifications (33). Cells were harvested and washed with ice-cold PBS, then resuspended (100 μl/10^7 cells) in an ice-cold cell extraction buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 1 μM pepstatin, 1 mM *N*-ethylmleimide (NEM), 2 mM Na_3VO_4, 20 mM sodium pyrophosphate, and 50 mM NaF). Lysates were centrifuged at 15,000 rpm at 4°C for 30 min. The clear cytosol was separated from the insoluble pellet fractions and immediately used for Western blot analysis.

COX-$2^{-/-}$ mice primary neuron culture

All knockout neurons were obtained from embryos derived from heterozygous breeding (the COX-2 knock-out colony was a generous gift from Dr. Robert Langenbach, National Institute of Environmental Health Sciences). Cortical neurons were obtained from 14.5 day mouse embryos (knockout breeding as described above) and plated. Cortical culture method has been described (10). Briefly, neurons were plated into 24-well dishes (~300,000 cells/well) coated with poly-D-lysine (100 μg/ml) in serum-free medium [MEM/F12 (1:1) supplemented with 6 mg/ml D-glucose, 100 μg/ml transferrin, 25 μg/ml insulin, 20 nM progesterone, 60 μM putrescine, 30 nM selenium]. Forty-eight hours after initial plating, the plated cells were treated with serum-free medium supplemented with camptothecin; 16 h after exposure to camptothecin, the cells were lysed and the numbers of viable cells were evaluated. Briefly, cells were lysed in 200 μl of cell lysis buffer (0.1 × PBS, pH 7.4, containing 0.5% Triton X-100, 2 mM $MgCl_2$, and 0.5 g/100 ml ethylhexadecyldimethylammonium bromide), which disrupts cell membranes but leaves the nuclei intact. Then 10 μl of sample from each culture was loaded onto a hemacytometer and the number of healthy intact nuclei was evaluated by phase microscopy

Construction and transduction of wild-type (WT) COX-2 adenovirus in primary neurons

Human COX-2 and GFP cDNA were subcloned into pShuttle-cytomegalovirus (pTRACK-cytomegalovirus vector), and recombination was performed in a BJ5180 bacterial cell line containing pAdEasy-1 vector. The linearized recombinant plasmid was transfected into adenopacking 293 cells. The recombinant virus was isolated, amplified, and analyzed for protein expression. Cells were infected with COX-2 or GFP viruses at a multiplicity of infection (MOI) of 50, or 100 for designated cells. After adenovirus infection, COX-2 expression was analyzed by Western blot analysis.

Construction of COX-2 promoter and site-directed mutagenesis

The human COX-2 promoter construct containing 3.92 kbp of the 5′ region was a generous gift from Dr. Stephen M. Prescott (University of Utah). The truncation mutant containing putative Sp1 site in the COX-2 promoter were generated by polymerase chain reaction (PCR). PCR fragments containing two restriction digestions, *Sma*I (5′ end) and *Bgl*II (3′

end), were digested and subcloned into pGL3-basic vector. Mutations in the Sp1 binding site were generated using the Quick Change site-directed mutagenesis kit (Stratagene, La-Jolla, CA, USA). The following primers were used, with mutations in italics: mutation –243/–244 sense primer, AGGAGAG*AA*AGGG-ATCAGACAG; mutation –268/–267 sense primer, TGTGCGCCTGG*AA*CGGTGGAA-computed tomography.

Immunofluorescence staining and confocal microscopy

Indirect labeling methods were used to determine the levels of COX-2 and COX-1, and neurofilament (NF; 200 kDa) in cortical neuronal cultures (33, 34). Dissociated cells from the cerebral cortex ($3–5\times10^5$) were seeded onto poly-D-lysine-coated 8-well culture slides (Becton Dickinson, Bedford, MA, USA) and treated with HCA as described above for 1–5 h. The cells were washed with warm PBS and fixed at room temperature for 15 min with 4% paraformaldehyde. After washing with PBS, fixed cells were incubated with blocking solution containing 0.3% Triton X-100, 5% BSA, and 3% goat serum for 1 h, followed by incubation with rabbit anti-COX-2, COX-1, and nNOS antibodies (1:200 dilution) overnight at 4°C. After three washes with PBS, the cells were incubated for 1 h with FITC-conjugated goat anti-rabbit IgG antibody (Ab) (1:200 dilution). The nuclear was counterstained with 4′,6′-diam idino-2-phenylidole (DAPI). All antibodies were diluted in PBS. Slides were washed three times with PBS and mounted with fluorochrome mounting solution (Vector Laboratories). Images were analyzed using a confocal microscope (MRC-1024; Bio-Rad, Hercules, CA, USA). Control experiments were performed in the absence of primary Ab.

Immunogold labeling and transmission electron microscopy (transmission electron microscopy, EM)

Labeling procedures for cryosections were as follows. Samples were submerged into 1% BSA in PBS for 10 min, then incubated with COX-2 Ab in 1% BSA for 30 min. After rinsing the samples four times with PBS, protein A-gold (10 nm) in 1% BSA was added for 20 min. Next, samples were rinsed four times with PBS and six times with distilled water. Contrasting stain procedures were undergone by using 2% methyl cellulose: 3% uranyl acetate (9:1) for 10 min on ice. To dry the samples, grids were picked up with a loop and excess liquid was removed with filter paper.

Western blot analysis

To measure the protein level by Western blot, cell lysates were prepared as described above. The supernatants were removed carefully and the protein concentration was quantified by Bradford method. Lysates were mixed with 2 × or 5 × boiling Laemmli's buffer (1 × is 100 mM Tris-HCL, pH 6.8, 4% SDS, 200 mM dithiothreitol, 20% glycerol, 2% SDS, 0.2% bromphenol blue, 10 μg/ml aprotinin, 10 μg/ml leupeptin. The samples were boiled for 10 min at 100°C, then spun at 15,000 rpm for 10 s. In general, 30 μg of protein were electrophoresed on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked in 5% skim milk in TBST (Tris, pH 7.4; 150 mM NaCl; 0.05% Tween 20) for 30 min at room temperature. Blots were probed with primary antibodies overnight at 4°C. This was followed by incubation with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad) for 1 h. Signals were detected by using the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL, USA) (33, 34).

RNA isolation and RT-polymerase chain reaction (RT-PCR)

Total RNA was extracted using a kit (RNAeasy; Qiagen, Valencia, CA, USA). Primers used were acidic ribosomal phosphoprotein (PO) 5′-TGTGTTCACCAAGGAGGACC-3′ (sense) and 5′-CATTGATGTGGAGTGAGGC-3′ (antisense); COX-1 5′-TCACAAGAGTACAGCTATGAGCAGT-3′ (sense) and 5′-TGGGCTGGCACTTCTCCAGCATCAG-3′ (antisense); COX-2 5′-CCCTGCTGGTGGAAAAGCCTCGTCC-3′ (sense) and 5′-TACTGTAGGGTTAATGTCATCTAG-3′ (antisense). Both cDNA synthesis and PCR were performed in a single tube using One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). The PCR amplification cycles were denatured at 94°C for 15 s, annealed at 55°C for 30 s, and extended at 72°C for 1 min.

Promoter activity assay

Transfection mixture was prepared by adding the reporter expression vector (pGL3-COX-2, firefly Luc plasmid) with a combination of pRL-cytomegalovirus vector (containing *Renilla* Luc gene) into DMRIE-C solution (Invitrogen). After 24 h of transfection, cells were treated with HCA or infected with pHSV-Flag-Sp1, Sp3, and ZnF viral vectors. After 24 h of treatment, cells were lysed with Luc assay buffer. The cell particulate was removed by brief centrifugation and the protein concentration was measured using a protein assay kit (Bio-Rad). Lysate (20 μl) was used for both the firefly and *Renilla* Luc readings. Firefly and *Renilla* Luc activities were measured using a Dual-Luc Reporter assay system (Promega, Madison, WI, USA) and a model TD-20/20 Luminometer, firefly Luc values were standardized to *Renilla* values or to protein concentration.

Electrophoretic mobility shift assays (EMSA) and supershift analysis

Double-stranded oligonucletides from four putative Sp binding sites (–775/–756, –472/–453, –280/ –261, and –253/–235) within the human COX-2 promoter (–3930 to +50) were fill-in labeled with [^{32}P]dCTP using Klenow fragment of DNA polymerase (33, 34). We performed EMSAs on nuclear extracts from cortical neurons using a ^{32}P-labeled oligonucleotide containing a Sp1 binding site. Supershifts were performed with Sp1 and Sp3 specific Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Neuronal DNA damage detection and TUNEL assay

Nuclear fragmentation was assessed by staining nuclear DNA using DAPI (33, 34). TUNEL assay was performed according to a standard protocol (Roche Diagnostics, Mannheim, Germany).

Viral injection and global ischemia

Neonates (1-day-old Sprague Dawley rat) were anesthetized with hypothermia according to Harvard IACUC protocols. A pulled glass microelectrode was inserted through the skull into the neocortex to a depth 250–300 μm using a Kite micromanipulator (WPI Instruments, Sarasota, FL, USA). Ad-Track and Ad-COX-2 virus (< 1 μl of each) were slowly injected using a Sutter positive displacement injector (Sutter Instruments, Foster City, CA, USA). After a 5 day survival period, global ischemia was induced on the injected animals. Pups were anesthetized with isofluorane and placed on their back. A median incision was made in the neck to expose the

left and right common carotid artery (CCA). Both CCAs were occluded using clips and removed after 10 min. Carotid blood flow restoration was verified with the aid of a microscope and neck incisions were then closed. During the surgery, body temperature was maintained at 37°C. 12h after the global ischemia animals were perfused transcardially with PBS, followed by 4% PFA in 0.1 M PBS (pH 7.4). Brains were immediately removed and postfixed for 3 h in the same fixative solution, then soaked for cryoprotection in 30% sucrose in PBS. Coronal sections were cut through the brain at a 30–50 μ m thickness on a cryostat, and serial sections were collected on slide.

Data analysis

Data are expressed as the mean and SE. Differences were compared using Student's *t* test. A significant level was designated at the 95% level ($P<0.05$). Statistical calculations were completed using the Stat View 512 software package (Abacus, Berkley, CA, USA).

RESULTS

COX-2 is involved in neuronal viability, and its transcripts and protein levels are regulated by oxidative stress in primary neurons

To explore the mechanism by which oxidative stress regulates COX-2 expression and to examine the role of COX-2 in neuronal survival and death, we used an established *in vitro* glutathione depletion-induced oxidative stress model in primary neurons. In the first series of experiments, we determined whether NS-398, a COX-2 specific inhibitor, is also capable of regulating cell viability in primary cortical neurons. NS-398 (100 μM) was not cytotoxic by itself in primary neurons (**Fig. 1*A***). Surprisingly, NS-398 accelerated and exacerbated glutathione depletion-induced cortical neuronal death. Cell viability, as measured by MTT reduction, revealed that cortical neurons treated with NS-398 were more sensitive to oxidative stress-induced death over a range of time in which cells were not yet committed to die by homocysteic acid (HCA) (0.5 mM) (Fig. 1*A*). To observe morphological changes, DAPI labeling was used along with immunfluorescence for NF-200 in cortical neurons. Cells exposed to HCA and NS-398 showed significant changes, with greatly enhanced DAPI fluorescence and enlarged nuclei (Fig. 1*B*). Unlike the case with glutathione depletion-induced apoptosis, NS-398-exacerbated neuronal death was associated with cytolysis and swelling of the entire nucleus and rapid neurite shrinkage (Fig. 1*B, C*). The NS-398-exacerbated neuronal death under oxidative stress featured characteristic morphological symptoms of necrosis. EM showed the collapse of plasma and nuclear membranes (Fig. 1*C*). These changes are distinguishable from the apoptotic cell death observed in cortical neurons exposed to glutathione depletion. The level of COX-2 protein was increased dose dependently (1 and 5 mM) in response

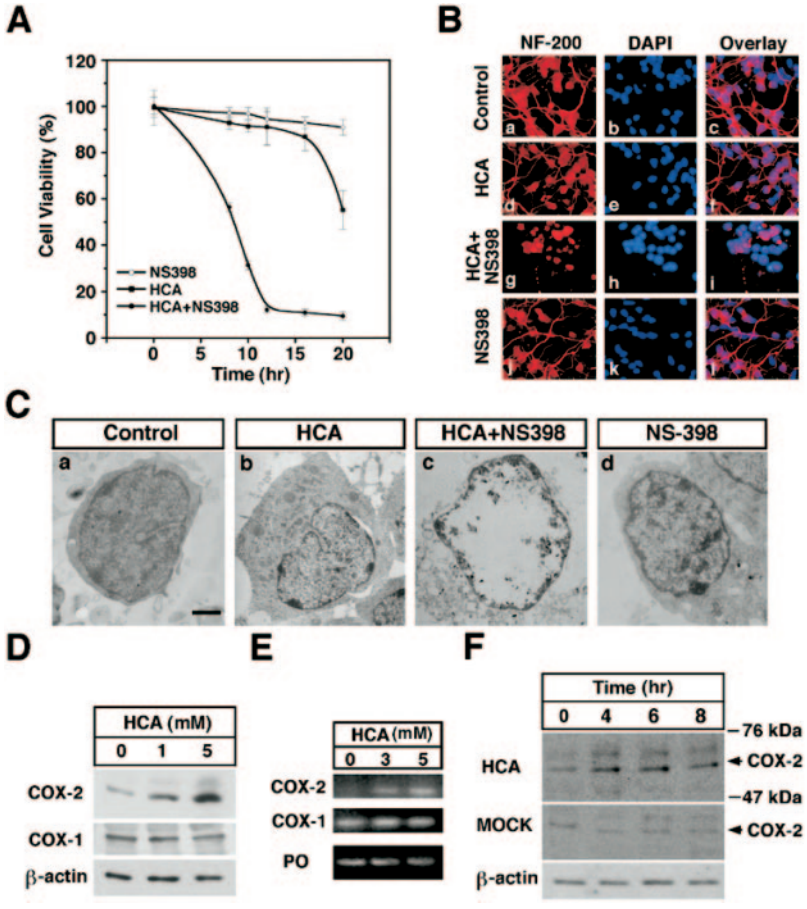

Figure 1. Cyclooxygenase-2 (COX-2) inhibition is involved in neuronal death under oxidative stress and COX-2 expression is induced by oxidative stress in primary neurons. *A)* COX-2 inhibition by NS-398, a COX-2-specific inhibitor, accelerates oxidative stress-induced neuronal cell death. The error bars indicate the SE of 4 experiments. *B)* Immunocytochemical analysis of neuronal cell death. Cells were stained with NF-200 (*a, d, g, j*), a neuronal specific marker, and DAPI (*b, e, h, k*) for nuclei staining. *c, f, i, l*) Images are an overlay view of NF-200 and DAPI fluorescence. Control (*a–c*); HCA (0.5 mM) (*d–f*); HCA+NS398 (100 mM) (*g–i*); NS-398 (*j–l*). Magnification, 400×. *C)* Transmission electron photomicrographs of cortical cell culture. *a)* control; *b)* HCA (0.5 mM); *c)* HCA+NS398; *d)* NS-398. Scale bar (black), 1 μm. *D)* COX-2 protein levels were dose-dependently increased by HCA. *E)* HCA increases the message level of COX-2 in primary neurons. COX-2 mRNA levels were elevated but not COX-1 and phosphoriboprotein (PO) by HCA treatment. *F)* COX-2 protein levels were quickly increased in response to HCA-induced oxidative stress.

to HCA-induced oxidative stress in cortical neurons (Fig. 1*D*). RT-PCR analysis was used to detect COX-2, COX-1, and acidic ribosomal phosphoprotein (PO) transcripts. We found that the transcripts of COX-2 were also clearly increased by HCA-induced oxidative stress in embryonic cortical neurons after 3 h (Fig. 1*E*). Either HCA (3 mM) treatment did not change the transcripts level of COX-1 or PO. COX-2 protein was induced 4 h after oxidative stress and its level lasted for 8 h (Fig. 1*F*).

COX-2 immunoreactivity is increased in the nuclear membrane of primary neurons in response to oxidative stress

Cellular localization of the COX-2 protein in response to HCA-induced oxidative stress was also examined by immunofluorescence staining and confocal microscopy. Cortical neurons were treated by HCA (3 mM) for 5 h and fixed by 4% PFA for staining. Increased immunoreactivity of COX-2 in the nuclear membrane of neurons was shown in HCA-treated cells (*e–h*, *i–l*) compared with control cells (*a–d*) (**Fig. 2*A***). Basal levels of COX-2 immunoreactivity were seen in embryonic cortical neurons *in vitro* (Fig. 2*A*). Neurons were monitored using antineurofilament 200 kDa (NF-200) Ab, a neuronal specific marker (Fig. 2*A*). COX-2 was mainly localized in the nuclear membrane region of the neurons. Immunogold labeling of COX-2 and EM confirmed that COX-2 localizes on the lumenal surfaces of the nuclear membrane (Fig. 2*D*) (39). The localization of COX-2 in the nuclear membrane of primary neurons concurred with the previous study using non-neuronal cells (39). It was also clear that the expression of COX-2 in the nuclear membrane was strongly enhanced after HCA-induced oxidative stress. The change of immunofluorescent intensity was consistent with Western and RT-PCR analysis, which showed the induction of COX-2 mRNA and protein in cortical neurons. On the other hand, COX-2 also partially localized to the dendrite and growth cone of cortical neurons in the presence of HCA (3 mM) (Fig. 2*B*). The white arrowhead in Fig. 2*B* indicates focal sites of a dendrite and a growth cone. In contrast to COX-2 immunoreactivity, COX-1 immunoreactivity was not changed in HCA (3 mM) -treated neurons (*u–x*) or the control (*q–t*) (Fig. 2*C*). COX-1 localization was mainly found in the cytosol and plasma membranes of primary neurons and was clearly distinguishable from COX-2. We also confirmed not only that the basal level of COX-2 immunoreactivity is found in both the contralateral and ipsilateral hemispheres but also that COX-2 immunoreactivity strongly increased in cortical layers 2 and 3 and around blood vessels 2 h after ischemia (Fig. 2*E*).

Two poximal Sp1-like sites are responsible for the basal and oxidative stress-induced COX-2 promoter activity in primary neurons

Using the MacPlasmap *pro*(CGC Scientific, Inc., Ballwin, MO, USA) software program, we found four putative Sp binding sites (–775 /–756, –472/–453, –280/–261, and –253/–235) within the human COX-2 promoter (–3930/+50). To investigate the regulation of COX-2 expression by Sp1 and Sp3 and to identify the transcriptional regulatory regions in the human COX-2 promoter, we constructed a nested series of proximal COX-2 promoter fragments containing various lengths of the 5′-flanking region using PCR (**Fig. 3*A***). The parental plasmid employed in this study spanned nucleotides –3930 to + 100. Each deletion construct was generated by inserting DNA fragments of various lengths into the luciferase (Luc) gene, then analyzed

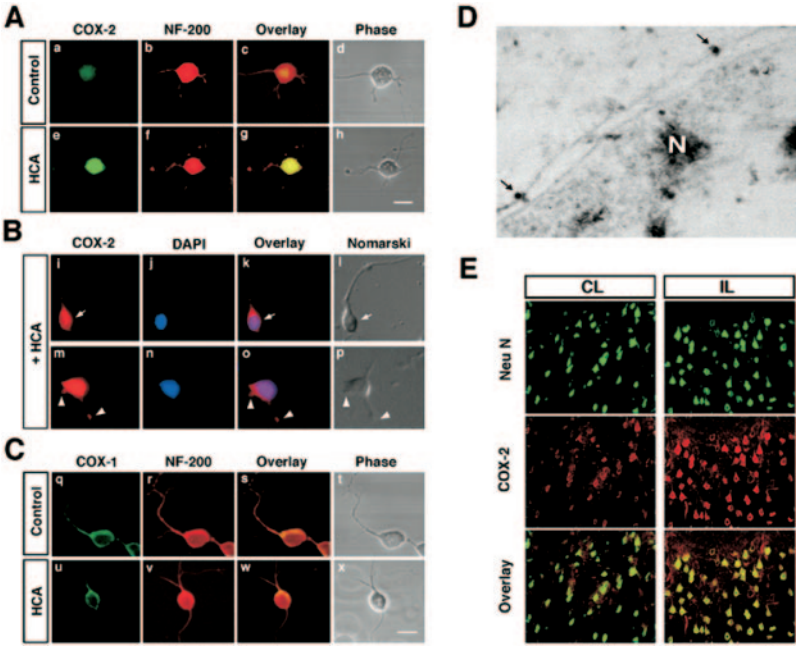

Figure 2. COX-2 immunoreactivity is increased in the nuclear membrane of neurons in response to oxidative stress. *A)* Increased immunoreactivity of COX-2 in the nuclear membrane of neurons is shown in HCA-treated cells (*e–h*, *i–l*) compared with control (*a–d*). Neurons were monitored using antineurofilament 200 kDa (NF-200) Ab, a neuronal specific marker. The white arrow indicates nuclear membrane localization of COX-2. Nuclei were counterstained with DAPI. *B)* COX-2 also localizes to dendrites and growth cone of cortical neurons in the presence of HCA (*m–p*). The white arrowheads indicate focal sites of a dendrite and a growth cone. *C)* COX-1 immunoreactivity is not changed in HCA-treated neurons (*u–x*) compared with control (*q–t*). Scale bar, 10 μm. *D)* Immunogold particles of COX-2 apposed to nuclear envelope of cortical neurons. N, nucleus. *E)* The protein level of COX-2 in ipsilateral (interleukin) penumbra region of brain was increased by focal ischemia compared with contralateral (CL) region. COX-2 immunoreactivity was colocalized with neuronal marker (NeuN).

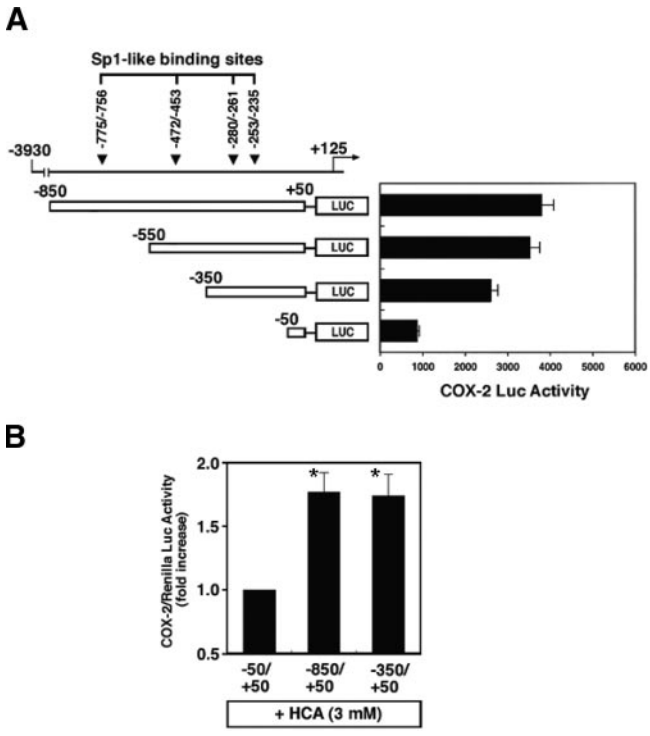

Figure 3. Two proximal Sp1-like sites are responsible for both the basal and oxidative stress-induced promoter activity of COX-2 in primary cortical neurons. *A)* Human COX-2 promoter activity was determined using a series of deletion reporter constructs which include Sp1-like sites (–850/+50, –550/+50, –350/+50, –50/+50. –280/–261, and –253/–235). Basal Luc activity was measured 24 h after transfection. Luc activity was normalized with protein concentration of neuronal extract. *B)* Oxidative stress by HCA increases COX-2 promoter (–850/+50 and –350/+50) activity. Luc activity was normalized with Renilla Luc activity. The results are mean ± SE of three separate experiments. *Significantly different at $P < 0.05$.

for promoter activity as described in Materials and Methods. Deletion constructs –850/+50, –550/+50, and –350/+50 showed a 3- to 4-fold increase in Luc activity compared with –50/+50, which has no Sp1-like sites (Fig. 3*A*). Then we measured the COX-2 promoter activity of deletion constructs in response to oxidative stress induced by HCA (3 mM) (Fig. 3*B*). The Luc activity of the –850/+50 and –350/+50 was significantly increased in response to oxidative stress in comparison with the –50 to + 50 construct. These results show that the proximal region of COX-2 promoter harbors oxidative stress-responsible elements.

Mutation of Sp1-like sites abolishes oxidative stress-induced COX-2 promoter activity

To confirm the functional Sp sites in proximal COX-2 promoters, we introduced mutations by site-directed mutagenesis into two GC element sites, –268/–267 and –244/–243, which were showing evident Sp1 and Sp3 DNA binding activity (**Fig. 5*A***). As expected, mutations in Sp1 DNA binding sites of the COX-2 promoter abolished the promoter activity (**Fig. 4*A***). A mutation of GC element at –268/–267 in the COX-2 promoter markedly decreased the basal Luc activity. In addition, double mutations in both –268/–267 and –244/–243 elements abrogated the basal activity of the COX-2 promoter, which is close to the basal expression level of pGL3-vector. Because our focus was to know whether Sp1 and/or Sp3 are involved in COX-2 expression in primary neurons subjected to oxidative stress, we further determined the promoter activity of mutated Sp sites upon HCA treatment (Fig. 4*B*). WT –350/+50 construct showed a 1.8-fold increase in promoter activity in response to HCA compared with its own control without HCA. Mutations in element –268/–267 reduced COX-2 promoter activity by 60% upon HCA treatment whereas element –244/–243 retained functionality. We found that mutations in element –244/

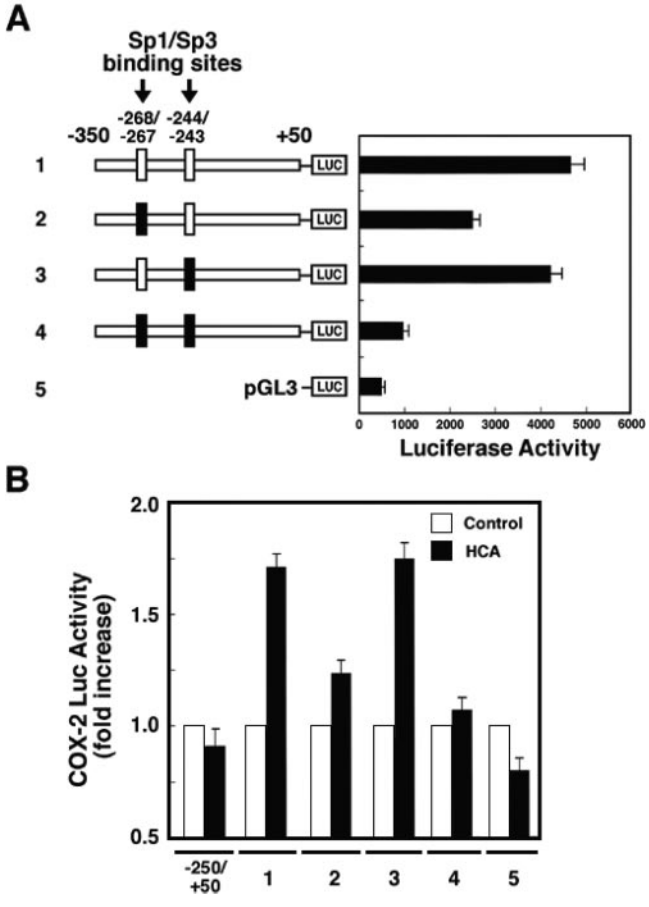

Figure 4. Mutations in two Sp1 DNA binding sites of COX-2 promoter abolish the basal and oxidative stress-induced promoter activity. *A)* Mutations in GC element –268/–267 and –244/–243 in COX-2 promoter abrogate the basal activity of COX-2 promoter. Mutations by site-directed mutagenesis were introduced into two GC element –268/–267 and –244/–243 sites. *B)* Oxidative stress-induced COX-2 promoter activities are reduced with mutations in element –268/–267 and –244/–243. Luc activity was normalized with protein concentration of neuronal extract. The error bars indicate the SEM of 3 experiments. 1, WT –350/+50 construct; 2, –268/–267 mutant; 3, –244/243 mutant; 4, double –268/–267 and –244/–243 mutant; 5, –50/+50 construct.

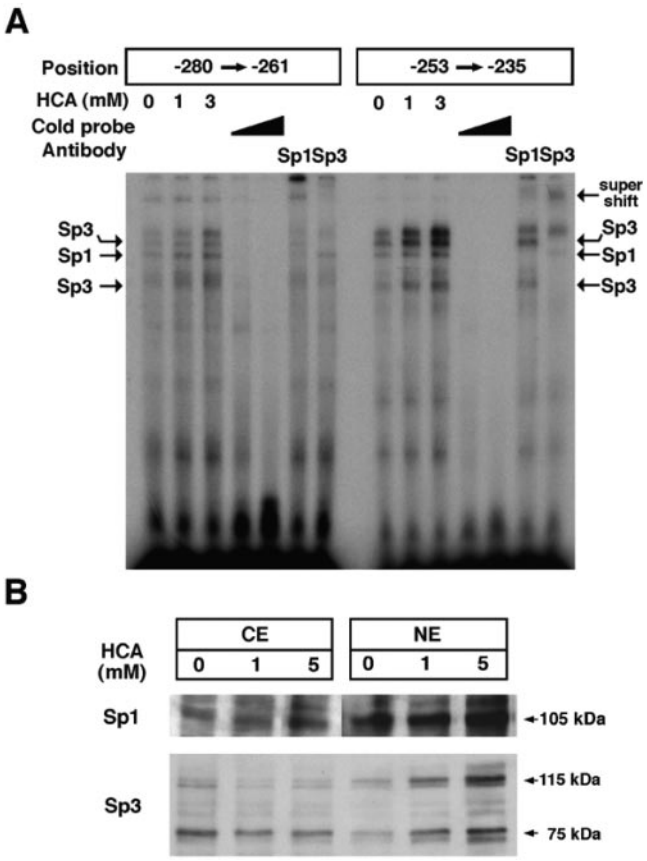

Figure 5. Oxidative stress increases Sp1 and Sp3 DNA binding to human COX-2 promoter sequence. *A)* DNA binding activity of Sp1 and Sp3 to two specific regions (–280/–261 and –253/–235) of human COX-2 promoter are enhanced by HCA. For the competition experiments 10- to100-fold molar excess of unlabeled Sp1 consensus sequence oligonucleotides were added to the reaction mixture of nuclear extracts and ^{32}P-labeled Sp1 consensus sequence of COX-2 promoter (–280/–261 and –253/–235). Identification of Sp1 and Sp3 complexes were examined by supershift analysis using anti-Sp1 and Sp3 Ab. *B)* Sp1 and Sp3 are increased in the nuclear fraction in response to HCA. Subcellular fractions were prepared from rat cortical neurons (1×10^7cells/100 mm dish) 3–5 h after HCA treatment. CE, cytoplasmic extracts; NE, nuclear extracts.

–243 increased Luc activity by > 1.8-fold upon HCA treatment (Fig. 4*B*). Deletion of-268/–267 elements in the COX-2 promoter construct (–250/+50) having only the –244/–243 Sp1 binding region functional indirectly supports the notion that –244/–243 does not respond to oxidative stress induced by HCA in primary neurons (Fig. 4*B*). These data indicate that the –268/–267 position serves as a specific Sp1 and Sp3 recognition site on oxidative stress. Therefore, we could conclude that both the –268/–267 and –244/–243 positions are required for the basal expression of COX-2. However, the –268/–267 element, but not the –244/–243, functions as an activating region of COX-2 expression when neurons are exposed to oxidative stress. Although oxidative stress-induced COX-2 promoter activity was clearly reduced by >70% in double mutations on element –268/–267 and –244/–243 compared with the control, 30% of the Luc activity remained. This basal Luc activity in the absence of Sp sites means that unknown factors apart from Sp1 and Sp3 may also modulate the transcriptional activity of COX-2, particularly in response to oxidative stress.

Oxidative stress increases Sp1 and Sp3 interaction with putative Sp1 binding sites within the human COX-2 promoter

We earlier found that the DNA binding activity of Sp1 and Sp3 is regulated by HCA-induced oxidative stress (34). Since oxidative stress regulates COX-2, we further characterized the binding of Sp1 and Sp3 to human COX-2 promoter sequences. To confirm the functional binding of Sp proteins with four putative binding sites (–775/–756, –472/–453, –280/–261, and –253/–235) *in vitro*, we performed electric mobility shift assay (EMSA) with radiolabeling of synthesized oligonucleotides and nuclear extracts from cortical neurons (Fig. 5*A*). EMSA revealed that three fragments, –775/–756, –280/–261, and –253/–235, interact with nuclear extracts. Two fragments, 280/–261 and –253/–235, showed strong DNA binding activity (Fig. 5*A*). However the –775/–756 fragment showed very weak DNA binding activity (data not shown). Nuclear extracts formed three specific complexes [complex (C) 1, 2, and 3] with labeled oligonucleotides containing a putative Sp1 binding site in the COX-2 promoter (Fig. 5*A*). Incubation of 10- to100-fold excess of unlabeled double-stranded oligonucleotides corresponding to three putative Sp1 binding sites competed with nuclear extracts and resulted in the abrogation of all three complexes. Supershift analysis using specific Sp1 and Sp3 antibodies showed that all three complexes were retarded by antibodies. Disruption and slow migration of C1 and C3 complexes by the Sp3 Ab indicated that these two complexes are yielded by the Sp3 protein. The Sp1 Ab also retarded the mobility of C2 complex. The supershift assays indicated that both Sp1 and Sp3 interact with Sp binding sites within the COX-2 promoter and play a role in the regulation of COX-2 expression. Figure 5*A* further shows that oxidative stress by HCA affected Sp1 and Sp3 DNA binding activity to Sp binding sites in the COX-2 promoter. Nuclear extracts from HCA-treated primary neurons showed considerably enhanced binding with three labeled oligonucleotide fragments (–280/–261 (lane 1 to 3), and –253/–235 (lanes 8–10) compared with the basal level of the constitutive binding of Sp1 and Sp3 (lane 1 and 8) (Fig. 5*A*). Enhanced Sp1 and Sp3 binding to Sp1 consensus sequences were HCA (1 and 3 mM) dose-dependent (Fig. 5*A*). To confirm whether oxidative stress by HCA regulates the translocation and accumulation of Sp1 and Sp3 from the cytosol to the nucleus, neuronal lysates were fractionated 3 h after HCA (1 to 5 mM) treatment (Fig. 5B). The level of Sp1, 105 kDa, was increased dose dependently in the nuclear fraction.

The level of the two forms of Sp3 molecules, 115 kDa and 75 kDa, were also markedly elevated in the nuclear fraction (Fig. 5*B*). These data concurred with the finding that Sp1 and Sp3 immunofluorescence intensity are mainly localized in nucleus (Supplementary Fig. 1) and are increased in nuclei of neurons subject to HCA-induced oxidative stress (34).

Dominant-negative form of Sp1 (Sp1-ZnF) nullifies oxidative stress-induced COX-2 promoter activity

To examine the relative contribution of Sp isoforms Sp1 and Sp3 to COX-2 promoter activity, primary neurons were transiently transfected with two reporter plasmids, including –350/+50 and –50/+50. Then Sp1 and Sp3 HSV viral vectors were used to infect cells 18 h after the transfection of reporter genes (**Fig. 6*A***). The transcriptional activity of –350/+50 reporter was increased 1.5- to 3.5-fold by Sp1 and Sp3 but not –50/+50 reporter when the Luc activity value was normalized with the value obtained from β-gal expression. These data indicated that both Sp1 and Sp3 act as positive activators of COX-2 promoters in cortical neurons. With increasing amounts of expression vectors for the dominant negative (DN) form of Sp1 (pHSV-Flag-Sp1-ZnF), the levels of COX-2 promoter activity induced by Sp1 and Sp3 were suppressed near to basal levels or lower than basal levels (Fig. 6*B*). These results indicate that the DN form of Sp1 effectively reduces Sp1 and Sp3 transcriptional activity by replacing the DNA binding activity in both transcription factors. In addition, the levels of COX-2 promoter activity induced by oxidative stress were nullified by Sp1-ZnF (Fig. 6*C*). Thus, the data provide evidence that COX-2 promoter activity induced by oxidative stress is mediated by a Sp1-dependent mechanism.

Enforced expression of Sp1 and Sp3 up-regulates COX-2 mRNA and protein levels in primary cortical neurons

To assess the COX-2 protein level in response to Sp1 and Sp3 overexpression using HSV recombinant vectors, primary neurons were infected with a range of MOI. Enforced expression of Sp1 and Sp3 for 16 to 18 h increased COX-2 mRNA without a change in transcripts level of COX-1 or PO protein (**Fig. 7*A***). HCA-induced COX-2 promoter activity was abolished by the DN form of Sp1 (pHSV-Flag-Sp1-ZnF) (Fig. 7*A*). Figure 7*B* illustrated that the overexpression of both WT Sp1 and Sp3 increases the level of COX-2 protein. A low MOI (1 MOI) of coinfection with Sp1 and Sp3 showed a synergistic elevation of COX-2 protein levels in primary cortical neurons (Fig. 7*B*). The patterns of synergistic effect of Sp1 and Sp3 on the expression of COX-2 proteins were consistent with COX-2 promoter activity (data not shown). With immunofluorescence staining and confocal microscopy, we further studied the pattern of COX-2 expression in cortical neurons after infection with HSV recombinants (Fig. 7*C*). Double immunofluorescence detection of expression vector (green) and COX-2 (red) showed that Sp1 and Sp3 increase COX-2 protein *in situ*. In sharp contrast, cells that were infected with WT Sp1 and Sp3 showed an increase in COX-2 immunofluorescence intensity in the nucleus of primary neurons (Fig. 7*C*). The DN form of Sp1 (pHSV-Flag-Sp1-ZnF) did not influence the COX-2 immunoreactivity in cortical neurons.

COX-2 expression using adenovirus gene delivery prevents staurosporine-induced neuronal DNA damage

To address the question of whether COX-2 is a prosurvival or prodeath protein in neurons under several

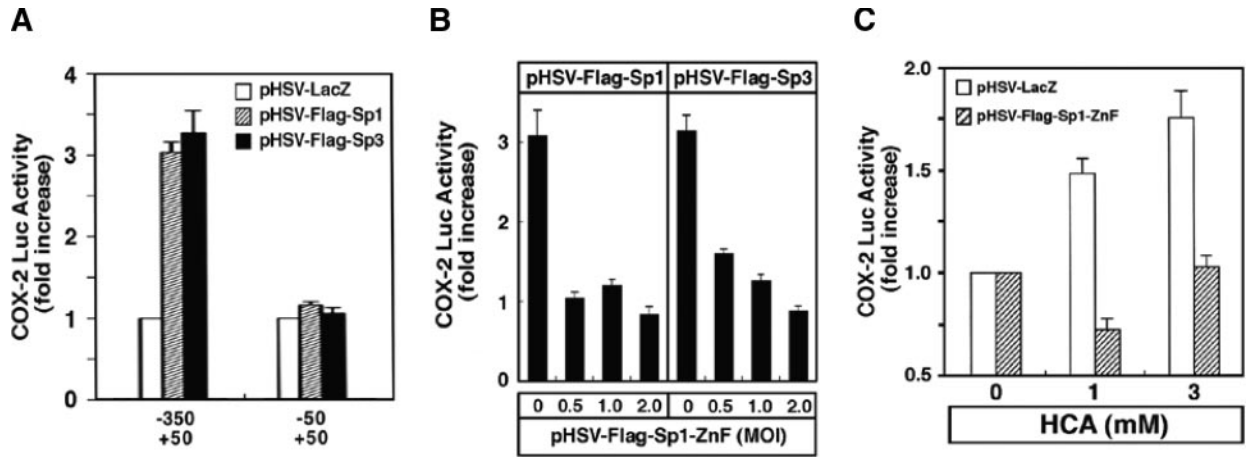

Figure 6. Sp1 and Sp3, but not Sp1-ZnF, up-regulates COX-2 promoter activity in primary cortical neurons. *A)* COX-2 promoter (–350/+50) is up-regulated by pHSV-Flag-Sp1 and Sp3 but not by pHSV-LacZ in cortical neurons. *B)* Dominant negative form of Sp1 (pHSV-Flag-Sp1-ZnF) down regulated COX-2 promoter (–350/+50) activity induced by pHSV-Flag-Sp1 and Sp3. *C)* HCA-induced COX-2 promoter activity is down regulated by pHSV-Flag-Sp1-ZnF. Viral vectors were infected into the same cells into which the COX-2 promoter constructs were transiently transfected for 18 h. Luc activity was normalized with protein concentration of neuronal extract. The error bars indicate the SEM of 3 experiments.

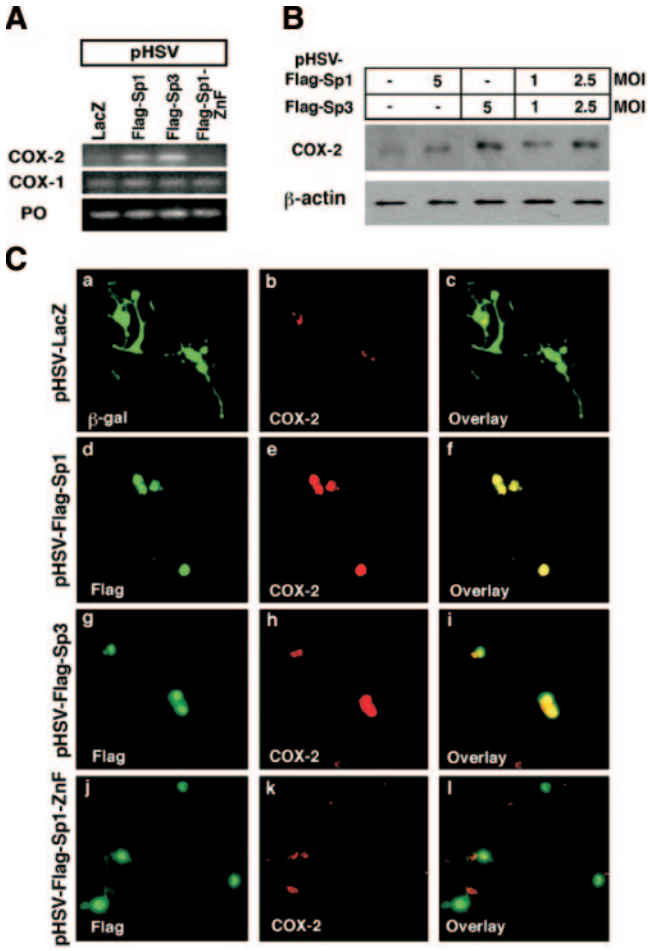

Figure 7. Enforced expression of Sp1 and Sp3, but not Sp1-ZnF, up-regulates COX-2 mRNA and protein levels in primary cortical neurons. *A)* RT-PCR analysis of COX-2 mRNA level from cells that were infected with HSV vectors. Enforced expression of Sp1 and Sp3 increases COX-2 mRNA in cortical neurons. *B)* Western blot analysis COX-2 protein level from cells that were infected with HSV vectors. *C)* Double immunofluorescence detection of COX-2 in HSV vectors infected cortical neurons. Cells were infected with pHSV-LacZ (*a–c*), pHSV-Flag-Sp1 (*d–f*), pHSV-Flag-Sp3 (*g–i*), and pHSV-Flag-Sp1-ZnF (*j–l*). pHSV-LacZ infected cells were monitored with anti-β-gal Ab (*a*) and the others were monitored with anti-Flag Ab (*d, g, j*). COX-2 was monitored with anti-COX-2 Ab (*b, e, h, k*) and its expression levels were overlaid with β-gal (*c*) or Flag staining (*f, i, l*). Magnification, 400×.

stress conditions, we established COX-2 transient expression in 10 days *in vitro* (10DIV) cultured neurons using the adenovirus gene delivery system. The Ad-Track control virus was used to determine the efficiency of transduction as well as the effect of viral infection on neuronal viability (**Fig. 8*B***). Our data showed that neurons could be infected with 5 to 10 MOI without altering neuronal viability. Western blot analysis confirmed that COX-2 expression in neurons transduced with Ad-COX-2 was significant and that MOI dependently increased but not in neurons transduced with Ad-Track (Fig. 8*A*). There was no change in the levels of α-tubulin (Fig. 8*A*). Next, we determined cell viability in response to a well-known stress signal, staurosporine. Neurons transduced with Ad-Track and exposed to staurosporine (500 nM) for 16 h showed apparent changes in nuclear structure (chromatin condensation and nuclear fragmentation), indicated by significantly enhanced DAPI fluorescence (Fig. 8*B*). In contrast, cells transduced with Ad-COX-2 retained normal and compact nuclei in the presence of staurosporine (Fig. 8*B*). COX-2 transduced cells were 58% more resistant to DNA damage induced by staurosporine. These data

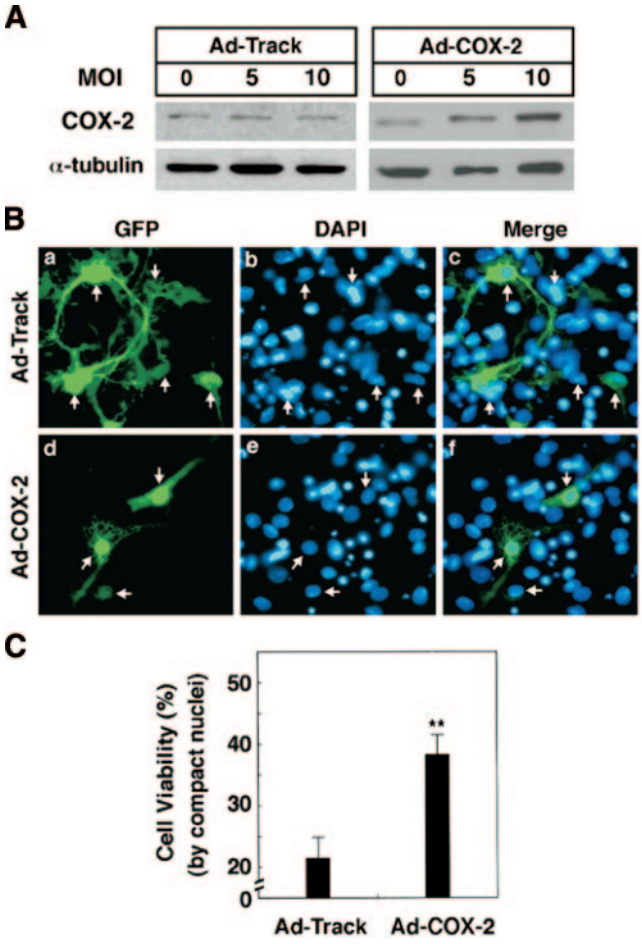

Figure 8. Enforced expression of COX-2 using adenovirus gene delivery in cortical neurons inhibits staurosporine-induced cell death. *A)* MOI-dependent expression of COX-2 protein in cortical neurons. *B)* Ad-COX-2 (*d–f*), but not the control vector alone (Ad-Track, *a–c*), protect staurosporine-induced DNA damage in cortical neurons. White arrows indicate Ad-Track or Ad-COX-2 infected positive cortical neurons. *a, d)* GFP fluorescence, markers for Ad-Track and Ad-COX-2 infected cells (*a, d*). *b, e)* DAPI fluorescence for nucleus staining. Magnification, 400 × (*b, e*). *C)* COX-2 expression increases neuronal cell viability against staurosporine-induced cell death. Cells with diffuse DAPI staining in compact nuclei were regarded as normal. Cells with chromatin condensation and nuclear fragmentation that display stronger DAPI fluorescence were regarded as DNA-damaged cells. At least 150 GFP- and DAPI-positive cells/chamber were counted at each experiment. The results are mean ± SE of three separate experiments. Significant at $*P < 0.05$; $**P < 0.01$.

suggested that COX-2 induction could rescue primary neurons from DNA damage (Fig. 8 C).

COX-2 protects neurons from genotoxic and exitotoxic-induced cell death *in vitro*

To determine whether the prosurvival effect by COX-2 against genotoxic, and excitotoxic stress-induced neuronal death, we used transient overexpression of COX-2 in 10 DIV cultured neurons using adenoviral vectors. First, we examined the chromatin condensation and nuclear fragmentation (karyorrhexis) that displays stronger DAPI fluorescence and characteristic of apoptotic cells after transduction of Ad-Track or Ad-COX-2 and treatment of camptothecin (CPT) (DNA topoisomerase I inhibitor), etoposide (ETP) (DNA topoisomerase II inhibitor), and kainic acid (KA) (**Table 1**). CPT and EPT produced significant neuronal cell death and nuclear fragmentation in the absence of COX-2. Cells transduced with Ad-COX-2 were protected from DNA damage by >50% compared with control neurons transduced with Ad-Track. It is well-known that neurons are susceptible to kainate (KA)-induced excitotoxicity. KA-induced neuronal death is linked to calcium influx and the activation of enzyme cascades that generate reactive oxygen intermediates (ROSs), subsequently damaging neuronal DNA (3). Therefore, we also tested whether COX-2 could similarly protect neurons against the KA-activated cell death pathway. Approximately 35% of neurons transduced with Ad-COX-2 were more resistant to KA-induced DNA damage and excitotoxicity (Table 1). However, COX-2 induction could not prevent DNA damage and cell death induced by hydrogen peroxide (data not shown).

COX-2$^{-/-}$ cortical neurons are more susceptible to DNA damage

To confirm whether COX-2 is implicated in the pathway of DNA damage-induced neuronal death, we tested the susceptibility of COX-2 knockout (COX-2$^{-/-}$) cortical neurons to camptothecin. COX-2$^{-/-}$ cells were significantly more susceptible to CPT-induced DNA damage than WT (COX-2$^{+/+}$) and heterogygous (COX-2$^{\pm}$) cells (**Fig. 9**). These data indicate that neurons are more vulnerable to genotoxic stress in the absence of functional COX-2.

TABLE 1. *COX-2 protects neurons from genotoxic and excitotoxic stress-induced DNA damage and cell death in vitro*[a]

Treatment	Ad-Track	Ad-COX-2
CPT	27.74 ± 3.20(%)	43.32 ± 2.29*
ETP	31.50 ± 4.55	48.55 ± 4.73**
KA	65.20 ± 1.75	87.02 ± 3.02*

[a] Cortical neurons (10DIV) were infected with Ad-Track and Ad-Track-COX-2 (10 MOI) for 24 h and treated with camptothecin (CPT) (10 μM), etoposide (ETP) (1 μM), kainate (KA) (100 μM), and medium control (Cont) for 18 h. Cells with diffuse DAPI staining in compact nuclei were regarded as normal cells. Cells with chromatin condensation and nuclear fragmentation were regarded as DNA-damaged cells. At least 150 GFP and DAPI positive cells/chamber were counted at each experiment. Data are mean ± SE of 3 independent experiments. Significant at $*P < 0.05$; $**P < 0.01$.

COX-2 expression protects ischemia-induced neuronal DNA damage *in vivo*

The *in vivo* effect of the transient expression of COX-2 on the ischemic injury-induced neuronal DNA damage was determined by transduction of Ad-COX-2 into the brain by microinjection. Ad-COX-2 infected cells were resistant to DNA damage induced by global ischemia (**Fig. 10*A***, *e–h*), but Ad-Track infected cells were not (Fig. 10*A*, *a–d*). Western blot analysis confirmed that COX-2 was overexpressed in cortex tissue injected with Ad-COX-2 compared with the tissue transduced with Ad-Track (Fig. 10*B*). Surprisingly, a comparison of TUNEL-positive cells in the region of adenovirus transduction showed that neurons with COX-2 overexpression were 3-fold more resistant to DNA damage than ischemic-induced neurons with Ad-Track transduction.

DISCUSSION

The COX-2 gene is an immediate-early gene and its 5′-flanking region has shown several potential transcription regulatory sequences including Sp1 binding regions as well as activating protein (AP)-2, C/EBP, cAMP response element (CRE), and NF-κB. NF-κB is known to be activated by reactive oxygen intermediates (ROIs) and is one transcription factor that links various stimuli to altered COX-2 expression (9, 36). Recently, functional analysis and DNA binding assays of the COX-2 promoter demonstrated that the Sp1 site, rather than the NF-κB sites, is required for the transcriptional induction by hypoxic stimuli in non-neuronal cell lines (42). This study showing the regulatory role of Sp1 in the hypoxic induction of COX-2 suggests that Sp1 is able to be an important transcription factor for the mechanistic link that exists between oxidative stress signals and the inflammatory processes associated with neuronal cell survival and death. Sp1 is a 95 to 105 kDa protein that binds DNA through C-terminal zinc finger motifs (18, 33, 34). Sp1 has been known as a positive transcriptional activator through interactions with Sp1 family members such as Sp2, Sp3, and Sp4, TFIID and with other transcriptional complexes. In contrast, Sp3, both 75 kDa and 115 kDa forms of the protein, show bifunctional roles as activators or repressors of Sp1-mediated transactivation. Contribution of Sp3 to Sp1-regulated gene transcription is dependent on the cell type and the promoter composition. In the present study, we found that the role of Sp1 and Sp3 in the neuronal induction of COX-2 is a sufficient requirement and mediates a high basal level of transcription. On the other hand, both Sp1 and Sp3 activity is required for efficient COX-2 reporter activation (Sup-

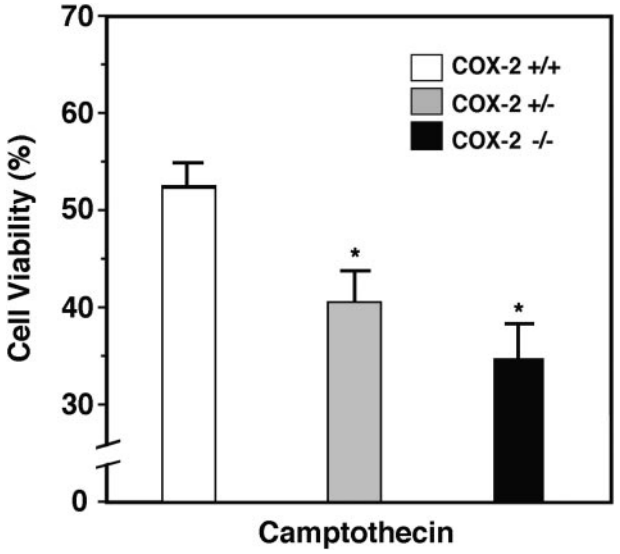

Figure 9. COX-2$^{-/-}$ primary neurons are more vulnerable to camptothecin-induced DNA damage and cell death. Cells were treated with serum-free medium supplemented with camptothecin (5 μM) for 16 h. The number of healthy intact nuclei was evaluated by phase microscopy. The results are mean ± SE of 3 separate experiments. Significant at $*P < 0.05$; $**P < 0.01$.

plementary Fig. 2), COX-2 mRNA transcription, and COX-2 protein levels in HCA-treated primary neurons (Fig. 7). Our results demonstrate that both Sp1 and Sp3 bind in several GC-rich regions of human COX-2 promoter and induce transcriptional activation of COX-2 in cortical neurons. It is noteworthy that the Sp3 synergistically increases Sp1-induced COX-2 expression. One of most striking results is that a putative recognition motif for Sp1 located in the –280/–261 region of the human COX-2 promoter sequence increased transcription in response to oxidative stress in primary neurons. This result suggests that this putative Sp1 motif represents a *bona fide* oxidative stress-response element that apparently plays an important role as a transcription activator on oxidative stress stimuli in primary neurons (Fig. 9*D*). Thus, our finding that Sp1 and Sp3 sites determine COX-2 expression in basal conditions and in response to oxidative stress provides a novel insight into molecular mechanisms underlying the regulation of the COX-2 gene in primary neurons.

We recently found that enhanced neuronal protection by histone deacetylase inhibitors in response to oxidative stress is mediated by Sp1 acetylation in primary neurons and in an animal model of Huntington's disease (34). The fact that acute oxidative stress induces Sp1 acetylation represents the protective role of Sp1 as the frustrated attempt of neurons to protect themselves from oxidative stress-induced cell death (18, 34). These studies strongly imply that Sp1 promotes neuronal survival in response to oxidative stress (Fig. 9*D*). However, the mechanism of neuronal protection and the downstream target of Sp1 in response to oxidative stress remain to be examined. In the present study we demonstrated the role of the COX-2 gene as a downstream target of Sp1 that mediates the protective role of Sp1 in primary neurons under oxidative stress. It has been known that the up-regulation of COX-2 is both protective and deleterious, depending on stimuli and many factors which are important determinants of the pathophysiological fate of COX-2 (8, 11, 12, 25, 31). For example, overexpression of COX-2 in endothelial cells up-regulates the vasoactive prostaglandin I_2 (PGI_2). PGI_2 suppresses monocyte activation and its adhesion to endothelial surface (26). These data convincingly show that the increased level of COX-2 in endothelial cells is linked to its protective role in vascular injury. Moreover, PGs produced by COX-2 are important for the early steps of liver regeneration after partial hepatectomy (32). Other lines of data also suggest that COX-2 can reduce cell death in cardiac tissue, and that long-term inhibition of COX-2 can enhance ishemic injury in hearts (6).

COX-2 levels are increased dramatically after N-methyl-D-aspartate (NMDA) -mediated activity in neurons but are not related to neural cell damage (23). Thus, COX-2 may be an important modulator of neuronal activity-dependent plasticity and ischemic or hypoxic injury response, such as stroke. Associations between COX-2, ischemic injury, and oxidative stress-induced cell death suggest that COX-2 plays some role in the onset and the progress of disease. Although there have been many studies that COX-2 is an important determinant of cytotoxicity related to ischemic injury, the mechanisms of its pathogenic function have not been clearly defined. Our current data showing that COX-2 expression diminishes TUNEL-positive cells in the cerebral region of ischemic brain suggests that COX-2 directly mediates neuronal cell survival by preventing DNA damage induced by ischemia. Furthermore, our finding that COX-2 protects neuronal cell damage induced by KA *in vitro* agrees with a previous study that specific COX-2 inhibitors aggravated KA-induced neuronal DNA damage in an *in vivo* model (3). These findings suggest that enhanced COX-2 induction can contribute to postischemic signaling response in neurons and regulate an adaptive neuronal response to altered environmental conditions (29).

In summary, we anticipated that the oxidative stress-induced Sp1 and Sp3 activation is involved in the expression of the COX-2 gene in primary neurons. The promoter activity, mRNA, the protein levels of COX-2, and nuclear Sp1 and Sp3 protein accumulation were increased in oxidative stress-induced neurons. We found that COX-2 promoter activity and mRNA levels are directly up-regulated by Sp1 and Sp3 but not by LacZ or the DN form of Sp1. These data demonstrate that both Sp1 and Sp3 play a critical role in oxidative stress-induced transcription regulation of the COX-2 gene in primary neurons. We also found that COX-2 expression correlates with neuronal cell survival by preventing DNA damage in response to genotoxic, excitotoxic, oxidative stress, and ischemic injury (Fig. 10*D*). Collectively, our results suggest that the oxidative stress induces COX-2 protein as a downstream target of

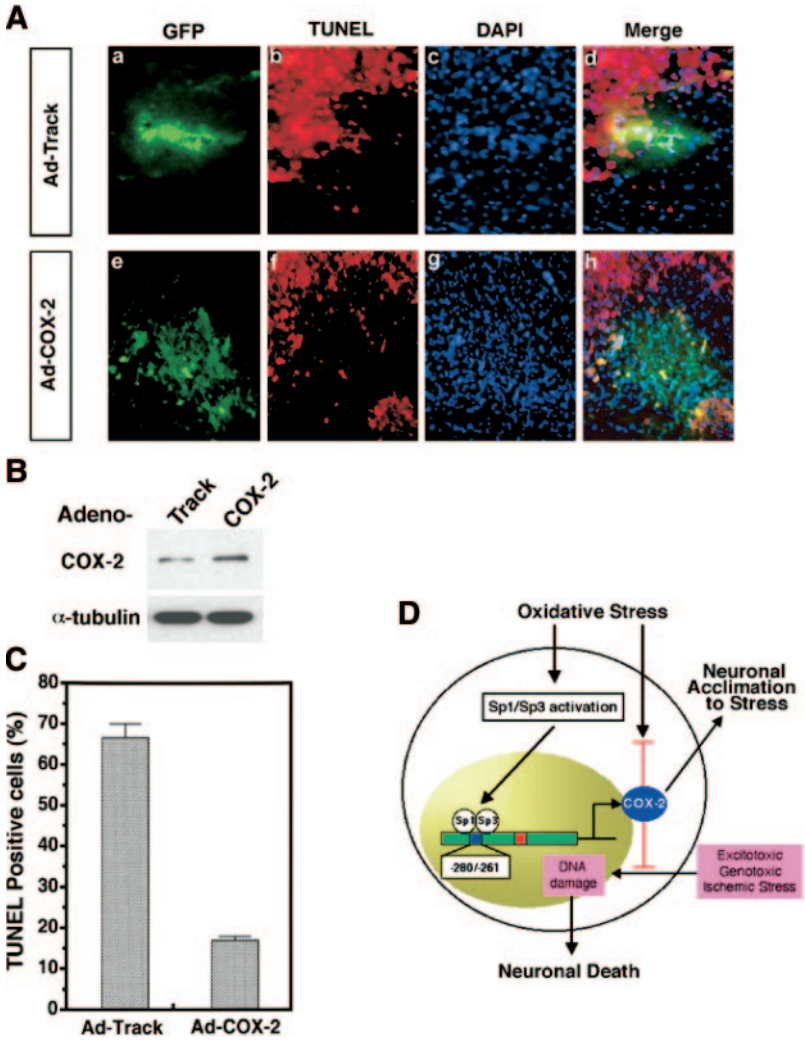

Figure 10. COX-2 expression prevents neuronal DNA damage induced by ischemia. *A)* Ad-COX-2 infected cells are more resistant to DNA damage induced by global ischemia (*e–h*) compared with Ad-Track infected cells (*a–d*). *a, e)* GFP fluorescence protein for Ad-Track infected cells in cortical layer II-V. *b, f)* TUNEL-stained cells; *c, g)* DAPI fluorescence for the nuclear counterstaining. *d, h)* The merge of three colored images, *a–c* and *e–g*, respectively. *B)* Western blot analysis of COX-2 protein in Ad-COX-2-infected cortex tissue. *C)* Comparison of cell viability between Ad-Track and Ad-COX-2 injected brain based on TUNEL-positive cells in panel *A*. Error bars indicate mean ± SE of three animals. *D)* Scheme of COX-2 regulation by Sp1 in response to acute oxidative stress. Acute oxidative stress leads to enhanced translocation of Sp1 and Sp3 molecules to nucleus. Oxidative-associated complexes can then recruit Sp1 and Sp3 to the promoter region (specifically at –280/–261 position) of the COX-2 gene and increase expression of its mRNA and protein. Increased levels of COX-2 permits neurons to protect them from excitotoxic, genotoxic, ischemic, and oxidative stress-induced DNA damage and promotes cell survival. Thus, COX-2 protein can play an adaptation role of neurons to oxidative stress.

Sp1 and Sp3 to compensate cellular level of stress (1, 11, 19, 22). Therefore, COX-2 is likely to be a homeostatic protein that mediates neuronal survival by preventing DNA damage under oxidative stress. FJ

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REFERENCES

1. Adderley, S. R., and Fitzgerald, D. J. (1999) Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. *J. Biol. Chem.* **274,** 5038–5046
2. Anderson, I., Adinolfi, C., Doctrow, S., Huffman, K., Joy, K. A., Malfroy, B., Soden, P., Rupniak, H. T., and Barnes, J. C. (2001) Oxidative signalling and inflammatory pathways in Alzheimer's disease. *Biochem. Soc. Symp.* **67,** 141–149
3. Baik, E. J., Kim, E. J., Lee, S. H., and Moon, C. (1999) Cyclooxygenase-2 selective inhibitors aggravate kainic acid induced seizure and neuronal cell death in the hippocampus. *Brain Res.* **843,** 118–129
4. Bolli, R., Shinmura, K., Tang, X. L., Kodani, E., Xuan, Y. T., Guo, Y., and Dawn, B. (2002) Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc. Res.* **55,** 506–519
5. Caivano, M., Gorgoni, B., Cohen, P., and Poli, V. (2001) The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. *J. Biol. Chem.* **276,** 48693–48701
6. Camitta, M. G., Gabel, S. A., Chulada, P., Bradbury, J. A., Langenbach, R., Zeldin, D. C., and Murphy, E. (2001) Cyclooxygenase-1 and -2 knockout mice demonstrate increased cardiac ischemia/reperfusion injury but are protected by acute preconditioning. *Circulation* **104,** 2453–2458
7. Chang, J. W., Coleman, P. D., and O'Banion, M. K. (1996) Prostaglandin G/H synthase-2 (cyclooxygenase-2) mRNA expression is decreased in Alzheimer's disease. *Neurobiol. Aging* **17,** 801–808
8. Dowd, N. P., Scully, M., Adderley, S. R., Cunningham, A. J., and Fitzgerald, D. J. (2001) Inhibition of cyclooxygenase-2 aggravates doxorubicin-mediated cardiac injury in vivo. *J. Clin. Invest.* **108,** 585–590
9. Feng, L., Xia, Y., Garcia, G. E., Hwang, D., and Wilson, C. B. (1995) Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor- and lipopolysaccharide. *J. Clin. Invest.* **95,** 1669–1675

10. Giovanni, A., Keramaris, E., Morris, E. J., Hou, S. T., O'Hare, M., Dyson, N., Robertson, G. S., Slack, R. S., and Park, D. S. (2000) E2F1 mediates death of B-amyloid-treated cortical neurons in a manner independent of p53 and dependent on Bax and caspase 3. *J. Biol. Chem.* **275,** 11553–11560
11. Han, J. A., Kim, J. I., Ongusaha, P. P., Hwang, D. H., Ballou, L. R., Mahale, A., Aaronson, S. A., and Lee, S. W. (2002) p53-mediated induction of Cox-2 counteracts p53- or genotoxic stress-induced apoptosis. *EMBO J.* **21,** 5635–5644
12. Hao, C. M., Komhoff, M., Guan, Y., Redha, R., and Breyer, M. D. (1999) Selective targeting of cyclooxygenase-2 reveals its role in renal medullary interstitial cell survival. *Am. J. Physiol.* **277,** F352–F359
13. Ho, L., Pieroni, C., Winger, D., Purohit, D. P., Aisen, P. S., and Pasinetti, G. M. (1999) Regional distribution of cyclooxygenase-2 in the hippocampal formation in Alzheimer's disease. *J. Neurosci. Res.* **57,** 295–303
14. Hoozemans, J. J., Veerhuis, R., Janssen, I., Rozemuller, A. J., and Eikelenboom, P. (2001) Interleukin-1beta induced cyclooxygenase 2 expression and prostaglandin E2 secretion by human neuroblastoma cells: implications for Alzheimer's disease. *Exp. Gerontol.* **36,** 559–570
15. Hoozemans, J. J., Rozemuller, A. J., Janssen, I., De Groot, C. J., Veerhuis, R., and Eikelenboom, P. (2001) Cyclooxygenase expression in microglia and neurons in Alzheimer's disease and control brain. *Acta Neuropathol. (Berlin)* **101,** 2–8
16. Iadecola, C., and Ross, M. E. (1997) Molecular pathology of cerebral ischemia: delayed gene expression and strategies for neuroprotection. *Ann. N.Y. Acad. Sci.* **835,** 203–217
17. Iadecola, C., Forster, C., Nogawa, S., Clark, H. B., and Ross, M. E. (1999) Cyclooxygenase-2 immunoreactivity in the human brain following cerebral ischemia. *Acta Neuropathol. (Berlin)* **98,** 9–14
18. Li, S. H., Cheng, A. L., Zhou, H., Lam, S., Rao, M., Li, H., and Li, X. J. (2002) Interaction of Huntington disease protein with transcriptional activator Sp1. *Mol. Cell. Biol.* **22,** 1277–1287
19. Lin, M. T., Lee, R. C., Yang, P. C., Ho, F. M., and Kuo, M. L. (2001) Cyclooxygenase-2 inducing Mcl-1-dependent survival mechanism in human lung adenocarcinoma CL1.0 cells. Involvement of phosphatidylinositol 3-kinase/Akt pathway. *J. Biol. Chem.* **276,** 48997–49002
20. Lukiw, W. J., and Bazan, N. G. (1997) Cyclooxygenase 2 RNA message abundance, stability, and hypervariability in sporadic Alzheimer neocortex. *J. Neurosci. Res.* **50,** 937–945
21. Mattammal, M. B., Strong, R., Lakshmi, V. M., Chung, H. D., and Stephenson, A. H. (1995) Prostaglandin H synthetase-mediated metabolism of dopamine: implication for Parkinson's disease. *J. Neurochem.* **64,** 1645–1654
22. McGinty, A., Chang, Y. W., Sorokin, A., Bokemeyer, D., and Dunn, M. J. (2000) Cyclooxygenase-2 expression inhibits trophic withdrawal apoptosis in nerve growth factor-differentiated PC12 cells. *J. Biol. Chem.* **275,** 12095–12101
23. Miettinen, S., Fusco, F. R., Yrjanheikki, J., Keinanen, R., Hirvonen, T., Roivainen, R., Narhi, M., Hokfelt, T., Koistinaho, J. (1997) Spreading depression and focal brain ischemia induce cyclooxygenase-2 in cortical neurons through N-methyl-D-aspartic acid-receptors and phospholipase A2. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6500–6555
24. Morham, S. G., Langenbach, R., Loftin, C. D., Tiano, H. F., Vouloumanos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., and Lee, C. A. (1995) Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* **83,** 473–482
25. Mukherjee, D., Nissen, S. E., and Topol, E. J. (2001) Risk of cardiovascular events associated with selective COX-2 inhibitors. *J. Am. Med. Assoc.* **286,** 954–959
26. Mukutmoni, M., Hubbard, N. E., and Erickson, K. L. (2001) Prostaglandin E(2) modulation of vascular endothelial growth factor production in murine macrophages. *Prostaglandins Leukot. Essent. Fatty Acids* **65,** 123–131
27. Ogasawara, A., Arakawa, T., Kaneda, T., Takuma, T., Sato, T., Kaneko, H., Kumegawa, M., and Hakeda, Y. (2001) Fluid shear stress-induced cyclooxygenase-2 expression is mediated by C/EBP beta, cAMP-response element-binding protein, and AP-1 in osteoblastic MC3T3–E1 cells. *J. Biol. Chem.* **276,** 7048–7054
28. Ohtsuki, T., K. Kitagawa, K. Yamagata, K. Mandai, T. Mabuchi, K. Matsushita, T. Yanagihara, and Matsumoto, M. (1996) Induction of cyclooxygenase-2 mRNA in gerbil hippocampal neurons after transient forebrain ischemia. *Brain Res.* **736,** 353–356
29. Planas, A. M., Soriano, M. A., Justicia, C., and Rodriguez-Farre, E. (1999) Induction of cyclooxygenase-2 in the rat brain after a mild episode of focal ischemia without tissue inflammation or neural cell damage. *Neurosci. Lett.* **275,** 141–144
30. Reuter, B. K., Asfaha, S., Buret, A., Sharkey, K. A., and Wallace, J. L. (1996) Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. *J. Clin. Invest.* **98,** 2076–2085
31. Rocha, J. L., and Fernandez-Alonso, J. (2001) Acute tubulointerstitial nephritis associated with the selective COX-2 enzyme inhibitor, rofeCoxib. *Lancet* **357,** 1946–1947
32. Rudnick, D. A., Perlmutter, D. H., and Muglia, L. J. (2001) Prostaglandins are required for CREB activation and cellular proliferation during liver regeneration. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8885–8890
33. Ryu, H., Lee, J., Olofsson, B. A., Mwidau, A., Deodoglu, A., Escudero, M., Flemington, E., Azizkhan-Clifford, J., Ferrante, R. J., and Ratan, R. R. (2003a) Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via a Sp1-dependent pathway. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4281–4286
34. Ryu, H., Lee, J., Zaman, K., Ferrante, R. J., Ross, B. D., Neve, R., and Ratan, R. R. (2003b) Sp1 and Sp3 are oxidative stress-inducible, anti-death transcription factors in cortical neurons. *J. Neurosci.* **23,** 3597–3606
35. Sairanen, T., Ristimaki, A., Karjalainen-Lindsberg, M. L., Paetau, A., Kaste, M., and Lindsberg, P. J. (1998) Cyclooxygenase-2 is induced globally in infarcted human brain. *Ann. Neurol.* **43,** 738–747
36. Schmedtje, J. F., Jr., Ji, Y. S., Liu, W. L., DuBois, R. N., and Runge, M. S. (1997) Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J. Biol. Chem.* **272,** 601–608
37. Shinmura, K., Xuan, Y. T., Tang, X. L., Kodani, E., Han, H., Zhu, Y., and Bolli, R. (2002) Inducible nitric oxide synthase modulates cyclooxygenase-2 activity in the heart of conscious rabbits during the late phase of ischemic preconditioning. *Circ. Res.* **90,** 602–608
38. Shinmura, K., Tang, X. L., Wang, Y., Xuan, Y. T., Liu, S. Q., Takano, H., Bhatnagar, A., and Bolli, R. (2000) Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10197–10202
39. Spencer, A.G., Woods, J. W., Arakawa, T., Singer, I. I., and Smith, W. L. (1998) Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J. Biol. Chem.* **273,** 9886–9893
40. Tang, Q., Chen, W., Gonzales, M. S., Finch, J., Inoue, H., and Bowden, G. T. (2001) Role of cyclic AMP responsive element in the UVB induction of cyclooxygenase-2 transcription in human keratinocytes. *Oncogene* **20,** 5164–5172
41. Tocco, G., Freire-Moar, J., Schreiber, S. S., Sakhi, S. H., Aisen, P. S., and Pasinetti, G. M. (1997) Maturational regulation and regional induction of cyclooxygenase-2 in rat brain: implications for Alzheimer's disease. *Exp. Neurol.* **144,** 339–349
42. Xu, Q., Ji, Y. S., and Schmedtje, J. F., Jr. (2000) Sp1 increases expression of cyclooxygenase-2 in hypoxic vascular endothelium: implications for the mechanisms of aortic aneurysm and heart failure. *J. Biol. Chem.* **275,** 24583–24589
43. Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A., and Worley, P. F. (1993) Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* **11,** 371–386
44. Yermakova, A., and O'Banion, M. K. (2000) Cyclooxygenases in the central nervous system: implications for treatment of neurological disorders. *Curr. Pharm. Des.* **6,** 1755–1776

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Role of cyclooxygenase-2 induction by transcription factor Sp1 and Sp3 in neuronal oxidative and DNA damage response

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SPECIFIC AIMS

Cox-2 is expressed constitutively in the brain and cerebral blood vessels of the newborn, and appears to be developmentally and functionally regulated. The elevated expression of COX-2 in the tissue of neurodegenarative diseases such as Alzheimer's, amyotrophic lateral sclerosis (ALS), PD, and stroke has implicated that this protein is involved in the pathophysiological process of neurodegenerative diseases. COX-2 is under transcriptional control by NF-κB, CREB, and C/EBP. It reveals that the binding of NF-κB is a necessary but not a sufficient step in the induction of COX-2 by hypoxic signaling. Despite reports showing that oxidative stress generated by reactive oxygen species (ROSs) induces COX-2 gene transcription in neuronal cell lines, the transcriptional regulation of COX-2 has not been precisely examined in primary neurons. It is intriguing whether COX-2 expression leads to a prosurvival or a proapoptotic effect in primary neurons under oxidative stress. We recently found that glutathione depletion-induced oxidative stress regulates DNA binding activity of Sp1 and Sp3 in cortical neurons. In primary neurons, Sp1 appears likely to mediate a prosurvival role in response to oxidative stress. We wanted to determine whether the regulation of COX-2 expression is mediated by Sp1 and/or Sp3 activation under oxidative stress. We also examined the role of COX-2 as a downstream pathway in relation to the known prosurvival role of Sp1 in primary neurons subjected to various stress.

PRINCIPAL FINDINGS

1. COX-2 transcripts and protein levels are regulated by oxidative stress in primary neurons

To explore the mechanism by which oxidative stress regulates COX-2 expression and examine the role of COX-2 in neuronal survival and death, we used an established *in vitro* glutathione depletion-induced oxidative stress model in primary neurons. The level of COX-2 protein was increased dose-dependently (1 and 5 mM) in response to HCA-induced oxidative stress in cortical neurons. RT-polymerase chain reaction (RT-PCR) analysis detected COX-2, COX-1, and acidic ribosomal phosphoprotein (PO) transcripts. We found that the transcripts of COX-2 were also clearly increased by HCA-induced oxidative stress in embryonic cortical neurons after 3 h. HCA (3 mM) treatment did not change the transcripts level of COX-1 or PO. COX-2 protein was induced 4 h after oxidative stress, and its level lasted for 8 h.

2. Two proximal Sp1-like sites are responsible for the basal and oxidative stress-induced COX-2 promoter activity in primary neurons

To investigate the regulation of COX-2 expression by Sp1 and Sp3 and to identify the transcriptional regula-

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tory regions in the human COX-2 promoter, we constructed a nested series of proximal COX-2 promoter fragments containing various lengths of the 5′-flanking region using polymerase chain reaction (PCR). The parental plasmid employed in this study spanned nucleotides –3930 to + 100. Each deletion construct was generated by inserting DNA fragments of various lengths into the luciferase (Luc) gene, then analyzed for promoter activity. Deletion constructs –850/+50, –550/+50, and –350/+50 showed a 3- to 4-fold increase in Luc activity compared with –50/+50, which has no Sp1-like sites. Then we measured the COX-2 promoter activity of deletion constructs in response to oxidative stress induced by HCA. Under oxidative stress, Luc activity of the –850/+50 and –350/+50 was similar and increased by 1.8-fold in comparison with –50 to +50. These results show that the deletion construct (–350/+50) harbors a region necessary not only for basal transcriptional activation but also for oxidative stress-dependent expression of COX-2 through Sp1 sites.

3. Mutation of Sp1 sites abolishes oxidative stress-induced COX-2 promoter activity

To confirm the functional Sp sites in proximal COX-2 promoters, we introduced mutations by site-directed mutagenesis into two GC element sites, –268/–267 and –244/–243, which showed evident Sp1 and Sp3 DNA binding activity by EMSA. As expected, mutations in Sp1 DNA binding sites of the COX-2 promoter abolished the promoter activity. Mutations in GC elements –268/–267 and –244/–243 in the COX-2 promoter decreased basal Luc activity by 2-fold. In addition, double mutations in both –268/–267 and –244/–243 elements abrogated the basal activity of the COX-2 promoter, which is close to the basal expression level of pGL3 vector.

4. Dominant-negative form of Sp1 (Sp1-ZnF) nullifies oxidative stress-induced COX-2 promoter activity

With increasing amounts of expression vectors for the dominant negative (DN) form of Sp1 (pHSV-Flag-Sp1-ZnF), COX-2 promoter activity levels induced by Sp1 and Sp3 were suppressed near to or lower than basal levels. These results indicate that the DN form of Sp1 reduces Sp1 and Sp3 transcriptional activity by replacing the DNA binding activity in both transcription factors. Levels of COX-2 promoter activity induced by oxidative stress were nullified by Sp1-ZnF. Thus, the data provide evidence that COX-2 promoter activity induced by oxidative stress is mediated by a Sp1-dependent mechanism.

5. Enforced expression of Sp1 and Sp3 up-regulates COX-2 mRNA and protein levels in primary cortical neurons

To assess the COX-2 protein level in response to Sp1 and Sp3 overexpression using HSV-recombinant vectors, primary neurons were infected with a range of MOI. Enforced expression of Sp1 and Sp3 for 16 to 18 h increased COX-2 mRNA without a change in transcripts level of COX-1 or PO protein. HCA-induced COX-2 promoter activity was abolished by the DN form of Sp1 (pHSV-Flag-Sp1-ZnF). The overexpression of wild-type (WT) Sp1 and Sp3 increased the level of COX-2 protein. A low MOI (1 MOI) of coinfection with Sp1 and Sp3 showed a synergistic elevation of COX-2 protein levels in primary cortical neurons.

6. COX-$2^{-/-}$ cortical neurons are more susceptible to DNA damage

To confirm whether COX-2 is implicated in the pathway of DNA damage-induced neuronal death, we testedthe susceptibility of COX-2 knockout (COX-$2^{-/-}$) cortical neurons to camptothecin. COX-$2^{-/-}$ cells were significantly more susceptible to CPT-induced DNA damage than WT (COX-$2^{+/+}$) and heterogygous (COX-$2^{\pm}$) cells (**Fig. 1**). These data indicate that neurons are more vulnerable to genotoxic stress in the absence of functional COX-2.

7. COX-2 expression protects ischemia-induced neuronal DNA damage *in vivo*

The *in vivo* effect of the transient expression of COX-2 on the ischemic injury-induced neuronal DNA damage was determined by transduction of Ad-COX-2 into the brain by microinjection. Ad-COX-2 infected cells were resistant to DNA damage induced by global ischemia

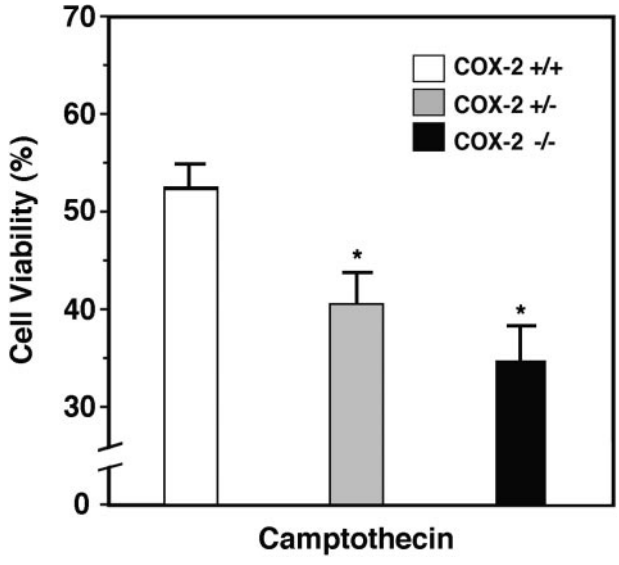

Figure 1. COX-$2^{-/-}$ primary neurons are more vulnerable to camptothecin-induced DNA damage and cell death. Cells were treated with serum-free medium supplemented with camptothecin (5 μM) for 16 h. The number of healthy intact nuclei was evaluated by phase microscopy. The results are mean ± SE of 3 separate experiments. Significant at $*P < 0.05$; $**P < 0.01$.

but Ad-Track infected cells were not. Surprisingly, a comparison of TUNEL-positive cells in the region of adenovirus transduction showed that neurons with COX-2 overexpression were 3-fold more resistant to DNA damage than ischemic-induced neurons with Ad-Track transduction.

CONCLUSIONS AND SIGNIFICANCE

In the present study we found that the role of Sp1 and Sp3 in the neuronal induction of COX-2 is a sufficient requirement and mediates a high basal level of transcription. On the other hand, both Sp1 and Sp3 activity are required for efficient COX-2 reporter activation, COX-2 mRNA transcription, and COX-2 protein levels in HCA-treated primary neurons. Our results demonstrate that both Sp1 and Sp3 bind in several GC-rich regions of human COX-2 promoter and induce transcriptional activation of COX-2 in cortical neurons. Sp3 synergistically increases Sp1-induced COX-2 expression. One of the most striking results is that a putative recognition motif for Sp1 located in the –280/–261 region of the human COX-2 promoter sequence increased transcription in response to oxidative stress in primary neurons. This suggests that this putative Sp1 motif represents a *bona fide* oxidative stress response element that apparently plays an important role as a transcription activator on oxidative stress stimuli in primary neurons (**Fig. 2**). Thus, our finding that Sp1 and Sp3 sites determine COX-2 expression in basal conditions and in response to oxidative stress provides a novel insight into molecular mechanisms underlying the regulation of the COX-2 gene in primary neurons.

We recently found that enhanced neuronal protection by histone deacetylase inhibitors in response to oxidative stress is mediated by Sp1 acetylation in primary neurons and in an animal model of Huntington's disease. The fact that acute oxidative stress induces Sp1 acetylation represents the protective role of Sp1 as the frustrated attempt of neurons to protect themselves from oxidative stress-induced cell death. These studies strongly imply that Sp1 promotes neuronal survival in response to oxidative stress. However, the mechanism of neuronal protection and the downstream target of Sp1 in response to oxidative stress remain to be examined. In the present study, we demonstrated the role of the COX-2 gene as a downstream target of Sp1 that mediates the protective role of Sp1 in primary neurons under oxidative stress (Fig. 2). The up-regulation of COX-2 is both protective and deleterious, depending on stimuli and many other factors that determine the pathophysiological fate of COX-2. For example, overexpression of COX-2 in endothelial cells up-regulates the vasoactive prostaglandin I_2 (PGI_2). PGI_2 suppresses monocyte activation and its adhesion to endothelial surface. These data show that the increased level of COX-2 in endothelial cells is linked to its protective role in vascular injury. Moreover, PGs produced by COX-2 are important for the early steps of liver regeneration after partial hepatectomy. Other lines of data also suggest that COX-2 can reduce cell death in cardiac tissue and that long-term inhibition of COX-2 can enhance ishemic injury in hearts.

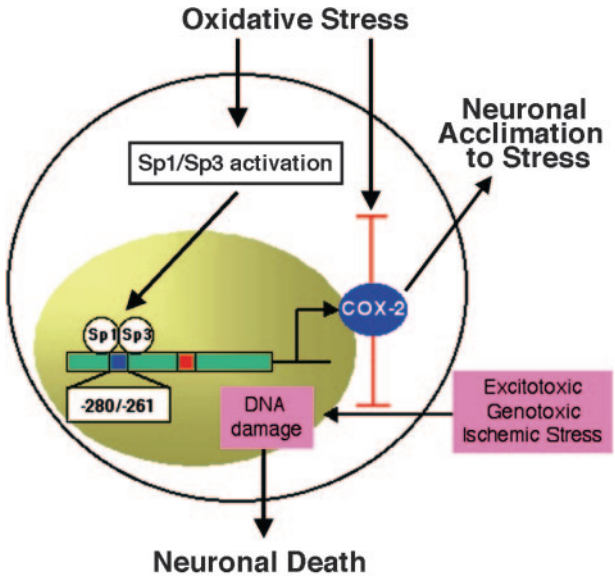

Figure 2. Scheme of COX-2 gene regulation by Sp1 and Sp3 in response to acute oxidative stress: COX-2 expression acclimatizes neuron to stress. Acute oxidative stress leads to enhanced translocation of Sp1 and Sp3 molecules to nucleus. Oxidative-associated complexes can then recruit Sp1 and Sp3 to the promoter region (specifically at –280/–261 position) of COX-2 gene and increase expression of its mRNA and protein. Increased levels of COX-2 permits neurons to protect them from excitotoxic, genotoxic, ischemic, and oxidative stress-induced DNA damage and promotes cell survival. Thus COX-2 protein can play an adaptation role of neurons to oxidative stress.

Associations between COX-2, ischemic injury, and oxidative stress-induced cell death suggest that COX-2 plays a role in the onset and progress of disease, but the mechanisms of the pathogenic function of COX-2 have not been clearly defined. Our current data showing that COX-2 expression diminishes TUNEL-positive cells in the cerebral region of ischemic brain suggest that COX-2 directly mediates neuronal cell survival by preventing DNA damage induced by ischemia. Furthermore, our finding that COX-2 protects neuronal cell damage induced by KA *in vitro* agrees with an earlier study that specific COX-2 inhibitors aggravated KA-induced neuronal DNA damage in an *in vivo* model. These findings suggest that enhanced COX-2 induction can contribute to postischemic signaling response in neurons and regulate an adaptive neuronal response to altered environmental conditions. Therefore, COX-2 is likely to be a homeostatic protein that mediates neuronal survival by preventing DNA damage under oxidative stress. FJ