

Animal Model

Potentiation of Excitotoxicity in Transgenic Mice Overexpressing Neuronal Cyclooxygenase-2

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In this study we describe the generation of a transgenic mouse model with neuronal overexpression of the human cyclooxygenase-2, h(COX)-2, to explore its role in excitotoxicity. We report that overexpression of neuronal hCOX-2 potentiates the intensity and lethality of kainic acid excitotoxicity in coincidence with potentiation of expression of the immediate early genes *c-fos* and *zif-268*. *In vitro* studies extended the *in vivo* findings and revealed that glutamate excitotoxicity is potentiated in primary cortico-hippocampal neurons derived from hCOX-2 transgenic mice, possibly through potentiation of mitochondrial impairment. This study is the first to demonstrate a cause-effect relationship between neuronal COX-2 expression and excitotoxicity. This model system will allow the systematic examination of the role of COX-2 in mechanisms of neurodegeneration that involve excitatory amino acid pathways. (Am J Pathol 1999, 155:995–1004)

Cyclooxygenase (COX) is the rate-limiting enzyme in the production of prostaglandins and, as such, a key target for anti-inflammatory drugs. Indeed, aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are COX inhibitors.^{1,2} There are two known isoforms, COX-1 and COX-2, which have quite distinct expression patterns and biological activities. COX-1 is a constitutively expressed protein found in most tissues, whereas COX-2 expression can be induced by a variety of mitogens, including cytokines, hormones, and phorbol esters.^{3,4} Inflammatory stimuli have little effect on COX-1 expression, but lead to

a rapid rise in COX-2 expression, suggesting a primary role for COX-2 in inflammation.^{4,5}

Recent studies of the effects of NSAIDs have revealed several possible health benefits of these COX inhibitors. Regular use of NSAIDs appears to reduce the risk of colorectal cancer^{6–9} and, more important for the current study, may slow the course of dementia in Alzheimer's disease (AD).¹⁰ Although it is unclear whether inflammation plays a role in the progression of AD, several lines of evidence indicate that regulation of COX-2 but not COX-1 expression may be involved in some of the neuropathology associated with AD.¹¹

In rodents, the response to kainic acid (KA)-mediated excitotoxicity, which may be a model for aspects of the hippocampal and cortical neurodegeneration observed in AD, includes a marked up-regulation of COX-2, although COX-1 remains unaffected.¹² The induction of COX-2 expression parallels the appearance of neuronal apoptotic features in cell types affected by KA, and excitotoxic neuronal death *in vitro* is accompanied by a selective elevation in COX-2 mRNA, indicating that COX-2 may be involved in pathways leading to neuronal death.¹² This information may have important implications for AD in view of the evidence that excitotoxicity may contribute to the widespread pattern of neurodegeneration in the AD brain.¹³ Recently we¹⁴ and others¹⁵ found elevation of expression of COX-2, but not COX-1, in neurons of the AD brain.

For the present study, we prepared transgenic mice with neuronal overexpression of the human COX-2 gene, hCOX-2, under the regulation of the rat neuron-specific enolase (NSE) promoter. We used this model to explore the role of neuronal COX-2 expression in KA-mediated excitotoxicity. Moreover, because KA excitotoxicity has profound effects on gene regulation associated with long-lasting cellular responses in rodent brain,¹⁶ we examined the expression of representative immediate early

Supported by National Institute on Aging grants AG13799, AG14239, and AG14766 to G. M. P.

Accepted for publication May 22, 1999.

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genes (IEG) as an index of cellular response to synaptic activation. We report that overexpression of neuronal COX-2 potentiates the intensity and lethality of KA-mediated seizures in heterozygous hCOX-2 transgenic mice coincident with the potentiation of hippocampal *c-fos* and *zif-268* mRNA expression. Finally, *in vitro* studies revealed that glutamate excitotoxicity was also potentiated in primary cortico-hippocampal neuron cultures derived from hCOX-2 transgenic mice.

Materials and Methods

Preparation of Transgene Construct

A 1950-bp *Hind*III cDNA fragment containing the entire coding region for hCOX-2¹⁷ was inserted into a unique *Hind*III site located within the second exon of the rat neuron-specific enolase (rNSE) gene. This rNSE/hCOX-2 (NHC) transgene contains approximately 2600 bp of rNSE promoter and 5' flanking region, all of exon 1 (non-translated sequence), intron 1, and 6 bp of exon 2 (without an ATG starting codon) from the rat NSE gene.¹⁸ In addition, the NHC transgene utilizes a polyadenylation signal from the SV40 late region (see Figure 1A).

Purification of Transgene Fragment and Microinjection

A 6.95-kb *Sal*I fragment containing the NHC transgene was purified from plasmid sequences by electrophoresis through low-melting-point agarose. The transgene was further purified by centrifugation through CsCl, followed by dialysis against 10 mmol/L Tris, pH 7.4, and 0.2 mmol/L EDTA. The concentration was adjusted to 5 Fg/ml for microinjection. The purified transgene fragment was used for microinjection of one-cell mouse eggs as previously described.¹⁹ C57Bl/6 × C3H (B6C3) F1 hybrid mice (Taconic Farms, Germantown, NY) were used as the source of fertilized eggs at the pronuclear stage.

Genotyping

Genomic DNA was isolated from tail skin as previously described.¹⁹ Transgenic mice produced from injection of the NHC *Sal*I fragment were identified by dot blot hybridization of tail skin DNA samples with a random-primed, 930-bp *Eco*RI fragment, which contains the entire SV40 sequence present in the NHC transgene (see Figure 1A). The transgenic founder mice identified in this fashion were mated with nontransgenic, B6C3 F1 hybrid mice to produce heterozygous transgenic offspring for establishment of the individual lines, as well as analyses of expression.

Northern Blot Analysis of hCOX-2 Transgene Expression

For the studies of hCOX-2 expression, half of the brain was frozen for extraction of total RNA²⁰ and the other half

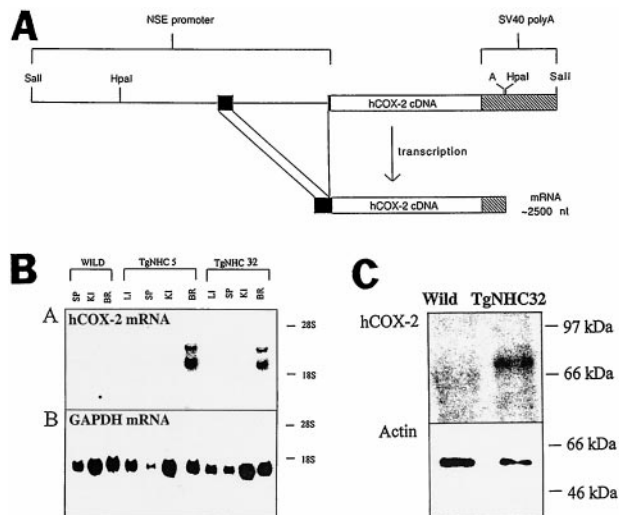


Figure 1. NSE/hCOX-2 (NHC) transgene for neuronal overexpression of human COX-2. **A:** Construction of the NHC transgene is detailed in the Methods section. A schematic representation of the hybrid NHC transgene is presented in which a 4-kb promoter fragment (thin line and dark boxes) from the rat NSE gene was fused to the 1.9-kb hCOX2 cDNA fragment (open box). The NSE promoter includes 5' flanking sequences, exon 1, a 1.2-kb intron in the 5' UTR, and 6 bp of exon 2; the exon sequences are represented by dark boxes. A 930-bp fragment containing the polyadenylation signal (A) from the SV40 late region (cross-hatched box) was fused to the 3' end of the hCOX-2 cDNA. The location of two *Hpa*I restriction endonuclease sites, which can be used to examine whether or not transgenic mice carry intact copies of the transgene, are indicated. A predicted mRNA of approximately 2500 nucleotides (not including the poly-A tail) is produced from this hybrid gene after splicing of the NSE intron (bottom line of figure). **B:** Northern analysis of transgenic mice from two independent NHC mouse lines, NHC5 and NHC32. hCOX-2 mRNA expression (top panel) was assessed by northern analysis of total RNA from a WT nontransgenic littermate, a NHC5, and a NHC32 transgenic animal using the entire hCOX-2 cDNA as a probe. The same blot was subsequently reprobbed for the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for sample loading (bottom panel). In each of the panels, the positions of 18S and 28S ribosomal RNA are as indicated. LI, liver; KI, kidney; BR, brain; SP, spleen. **C:** Western blot analysis of hCOX-2 expression in the brain of NHC32 transgenic mice. The expression of hCOX-2 protein in NHC32 mouse brain was assessed by Western blot immunoassay (see Methods). The same blot processed with anti-hCOX-2 was subsequently reacted to anti- β -actin antibody to control for sample loading. The position of molecular weight standards (97, 66, and 46 kD) are as indicated. The anti-hCOX-2 antibody used in this assay recognized an approximately 70-kD protein species from the NHC32 brain homogenate sample.

was processed for *in situ* hybridization (ISH) assay as described below. For gel blot Northern hybridization assay, total RNA was resolved electrophoretically through 1.1% agarose, 2.2 mol/L formaldehyde gel as previously described.²¹ After transfer to nitrocellulose, expression of the hCOX-2 transgene was determined by hybridization with a random-primed, 1950-bp *Hind*III fragment containing the entire hCOX-2 cDNA fragment.

ISH Assay

Brain frozen coronal tissue sections (10 μ m) were mounted on polylysine-coated slides and stored at -70°C . For ISH, tissue sections were postfixed in PBS containing 4% paraformaldehyde (30 minutes, room temperature), rinsed in phosphate-buffered saline (PBS), and incubated in acetic anhydride (0.25% v/v) in 0.1 mol/L triethanolamine, pH 8.0, for 10 minutes. After acetic anhydride treatment, tissue sections were hybridized with

[³⁵S]-cRNA probes (0.3 μg/ml, 2 × 10⁹ dpm μg⁻¹); hCOX-2 (1.9 kb hCOX-2 derived from the NHC construct (Figure 1A), *c-fos*, *zif-268*,^{22,23} and mouse (m) COX-2 and mCOX-1¹² were transcribed from linearized cDNA probes. After hybridization (3 hours, 50°C), stringency washes (0.1 × SSC, 60°C), and dehydration, slides were exposed to X-ray film for 5 days for quantification. Slides were then exposed to NTB-2 emulsion (Kodak, Rochester, NY) for approximately 12 days for microscopic analysis of mRNA distribution; after emulsion development, tissue sections were Nissl-counterstained. Hematoxylin and eosin histochemistry was used to assess morphological features of apoptosis on semiadjacent tissue sections.²⁴ Film autoradiograms were analyzed by computer-assisted video densitometry (Biometrics, Nashville, TN).

Combined ISH and Immunocytochemistry (ICC)

The neuronal expression of hCOX-2 transgene was verified by combining ISH for hCOX-2 mRNA with ICC for NSE on the same brain tissue section as previously described.²⁵ Following ISH, brain tissue sections were incubated for 30 minutes in PBS containing 10% methanol and 3% H₂O₂, rinsed, and permeabilized for 30 minutes at 4°C in PBS containing 0.2% Triton X-100, 0.2% normal goat serum, and 20 mg/ml L-lysine (Sigma, St. Louis, MO). Tissue sections were immunoreacted with a rabbit anti-human NSE antibody (1:1000, Dako, Carpinteria, CA). Immunoreactivities were visualized using the ABC method (Vector). Tissue samples were then dehydrated and coated with Kodak NTB2 emulsion as discussed above.

Western Blot Analysis

Brain samples were homogenized in an ice-cold buffer of Tris, pH 7.2, containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% lauryl sulfate, 100 μg/ml phenylmethylsulfonyl fluorid, 3 μg/ml aprotinin, 3 μg/ml pepstatin A, 15 μg/ml propionyl-Leupeptin, and 600 μg/ml Perfabloc (Boehringer Mannheim, Indianapolis, IN), boiled, centrifuged, and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.²⁶ Proteins were transferred to nylon Transblot membrane (Biorad, Hercules, CA) and immunoreacted with a polyclonal goat anti-hCOX-2 antibody (1:500 dilution, 3 hours, room temperature) in Superblock blocking solution (Pierce, Rockford, IL). The anti-hCOX-2 antibody was raised against a synthetic peptide derived from the carboxyl region of hCOX-2 sequence (Cayman, Ann Arbor, MI). In control studies we found that anti hCOX-2 antibodies do not cross-react with COX-1 (not shown). Immunoreactivity was visualized autoradiographically using a chemiluminescence detection kit (SuperSignal, Pierce). β-actin immunoreactivity (anti-β-actin, Sigma 1:5000) controlled for selectivity of changes.

KA-Mediated Seizures

KA-induced seizures were induced by intraventricular (ICV) infusion of KA (Sigma) in 4-month-old heterozygous

hCOX-2 - NHC32 transgenic mice and control wild-type (WT) littermates, as previously described.²⁷ KA, 1.15 nmoles/2 μl, or saline was injected in the lateral ventricle of anesthetized mice (Metofan) using a 5-μl Hamilton syringe at a rate of 1 μl/minute. Behavioral changes were assessed using a method described by Kondo et al,²⁸ modified for seizure assessment in mice. Seizures were classified into two groups: mild seizures, in which mice showed staring posture, occasional wet-dog shakes, or automatism without any myoclonic twitches; and severe seizures, in which mice showed myoclonic twitches of the forelimbs with standing posture or generalized tonic-clonic seizures with standing postures or generalized tonic-clonic seizures with falling or wild seizure jumps.

Because death occurred within 10 minutes after ICV KA infusion in the hCOX-2 transgenic mice (see Results), for comparative analysis of changes in gene expression or neuropathology, the control WT mice were sacrificed at the same time postlesioning. After decapitation, brains were rapidly removed, immediately frozen in methylbutane cooled to -25°C, and then stored at -70°C.

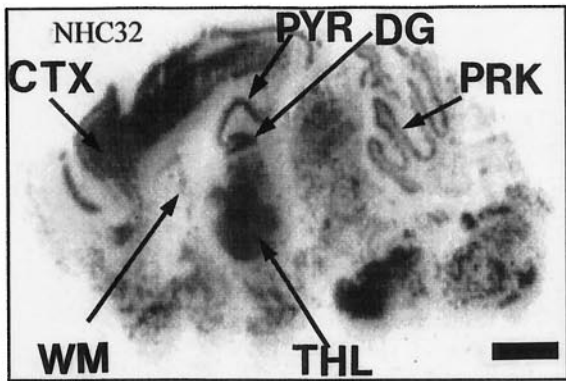
Primary Neuronal Cultures

Cortico-hippocampal primary neuron cultures derived from heterozygous hCOX-2 transgenic and WT mouse embryos (embryonic day 14–16) were prepared as previously described.²⁶ Briefly, after brain dissection, mechanical trituration, and centrifugation, neurons were seeded onto poly-D-lysine-coated 96-well plates at a density of 2 × 10⁵ cells per well. For ISH and/or ICC studies, primary neuron cultures were plated on glass chamber slides precoated with poly-D-lysine. For glutamate neurotoxicity, primary neuron cultures were aged 10 days and then treated with 50 μmol/L glutamate (Sigma) for 24 hours. Neuronal cultures were derived from each independent embryo; treatment and analysis were made in blind. Genotyping was used for identification of hCOX-2 transgenic or WT littermate cultures. Neuronal cultures were treated with glutamate for the indicated time, and parallel cultures derived from the same embryos were simultaneously treated with drug vehicle (control). Glutamate neurotoxicity was assessed by 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay;¹⁴ changes in MTT activity for each independent embryo culture was expressed as a percentage of its own control (identical drug vehicle-treated cultures).

Statistics

Analysis used statistical analysis software (CSS, Statsoft). *P* values <0.05 were considered to indicate statistically significant differences (*t*-test). KA-induced seizures studies used *n* = 6–8 mice per group and the studies were replicated in 3 independent experiments. Fisher's exact test was used for statistical analysis of mortality rate associated with KA-mediated seizures. ISH studies used *n* = 4–5 mice per group; changes in mRNA expression were computed from two tissue sections (bilaterally) en-

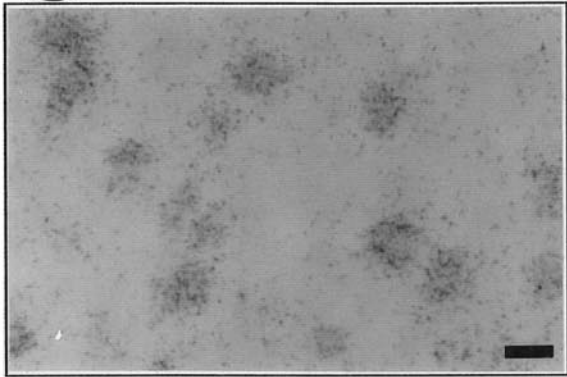
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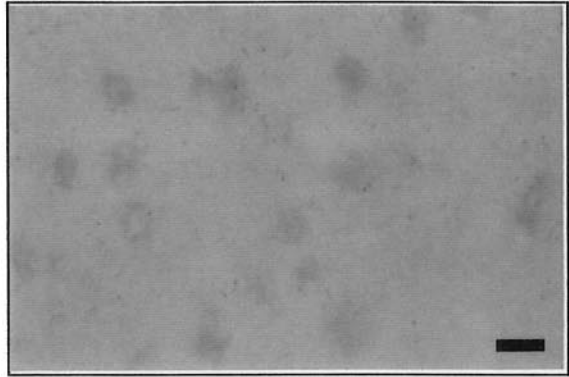
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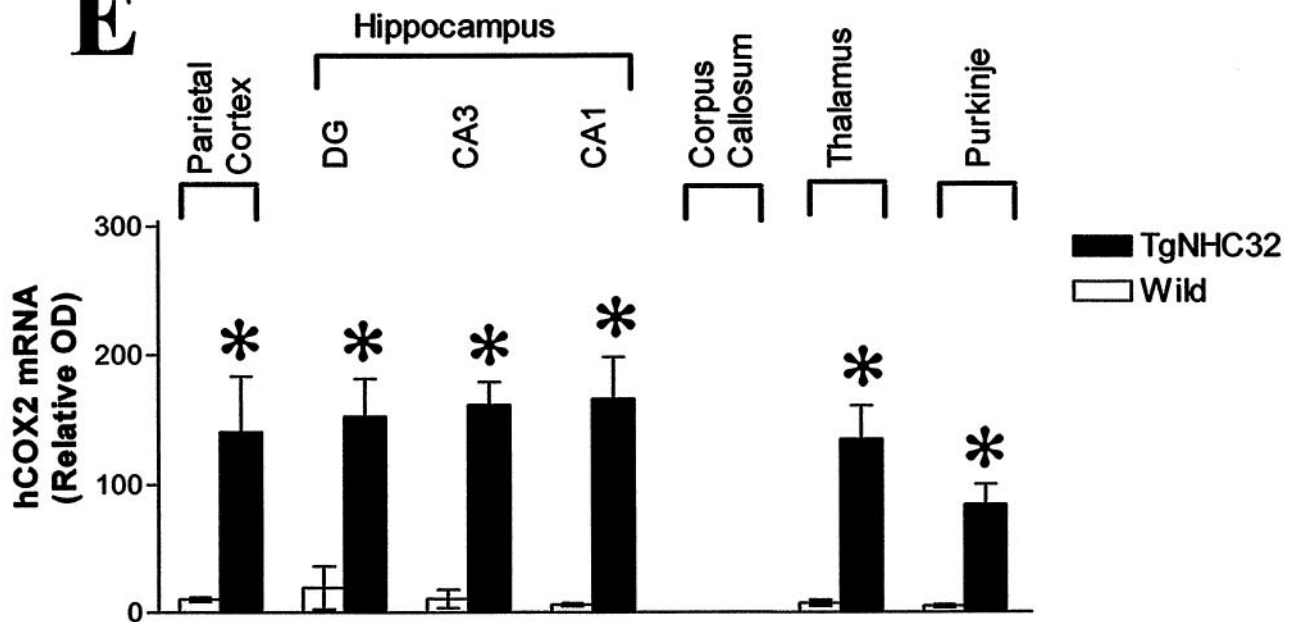
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compassing the hippocampal formation. Data from *in vitro* studies were averaged from 3 to 4 independent studies.

Results

Production of Human hCOX-2-Overexpressing Transgenic Mice

Forty-four offspring were born from eggs microinjected with the NSE-hCOX-2 transgene and DNA samples prepared from tail skin biopsies were examined to identify founder transgenic mice. A dot blot containing tail DNA samples from all of the mice was hybridized with an SV40 probe (not shown), resulting in the identification of 5 founder transgenic mice (NHC1, NHC5, NHC30, NHC32, and NHC34) that carry the randomly integrated transgene.

Establishment of Transgenic Lines

All 5 founder transgenics were mated with WT mice to obtain transgenic offspring for establishment of the transgenic lines and for analysis of hCOX-2 expression. Offspring from these matings were genotyped by tail DNA dot blot analysis using an SV40 probe to detect the NHC transgene. Only the NHC5 and NHC32 founders transmitted the transgene to multiple offspring. In contrast, transmission from the NHC1 and NHC30 founders was much lower than expected for mendelian transmission of the transgene sequences (not shown).

Tissue-Specific Expression of hCOX-2 Transgene

Total RNA was prepared from various organs isolated from NHC5 and NHC32 transgenic offspring, as well as a WT nontransgenic littermate. Northern blot hybridization of total RNA indicated that, as expected, NHC5 and NHC32 transgenic mice express the hCOX-2 transgene mRNA only in the brain (Figure 1B). In addition, the hCOX-2 hybridization signal indicates that the size of the major transcript in both lines is as predicted for the NHC gene (approximately 2500 nucleotides). A second, higher molecular weight transcript is also observed in both lines (Figure 1B). Because this analysis was performed on total cellular RNA, the most likely interpretation is that the larger RNA represents hnRNA in which the 1200-bp NSE intron has not been spliced out, whereas the smaller major transcript represents the mature mRNA.

Expression of hCOX-2 protein in the NHC32-hCOX-2 mouse brain was assessed by Western blot immunoassay (Figure 1C). The anti-hCOX-2 antibody used in this assay recognized an approximately 70-kd protein spe-

cies as assessed by Western blot assay of whole NHC32-hCOX-2 brain homogenate (Figure 1C, top panel). β -actin immunoreactivity controlled for equal loading (Figure 1C, bottom panel). This result indicates that the NHC transgene is specifically expressed in the brain and that the mRNA transcribed from the transgene is of sufficient length to encode the entire hCOX-2 protein.

Cell Type Expression of hCOX-2

The regional distribution of hCOX-2 mRNA expression in the mouse brain of the NHC32-hCOX-2 transgenic mice was assessed by ISH using a [³⁵S]-hCOX-2 antisense cRNA probe containing the entire hCOX-2 cDNA fragment and visualized by X-ray film autoradiography (Figure 2A). ISH for hCOX-2 mRNA combined with NSE ICC on the same tissue section confirmed the neuronal expression of hCOX-2 mRNA in the brain of hCOX-2 transgenic mice (parietal cortex, NHC32 line; Figure 2C); no detectable hCOX-2 signal was found in NSE-immunopositive neurons of WT mouse brain (Figure 2D).

Quantitative ISH autoradiography in the brain of NHC32-hCOX-2 transgenic mice (Figure 2E) revealed greater than eightfold hCOX-2 mRNA expression in parietal cortex (CTX), the pyramidal CA3-CA1 (PYR) and dentate gyrus (DG) granule neuron layers of the hippocampal formation, thalamus (THL), cerebellar Purkinje cell layer (PRK) of NHC32-hCOX2 transgenic mice (Figure 2A), relative to hCOX-2 hybridization signal in the brain of WT control littermates (Figure 2B). No detectable expression of hCOX-2 was found in corpus callosum white matter (Figure 2A; $n = 6-8$ per group, 4-month-old mice; $P < 0.001$). The intensity of hCOX-2 mRNA hybridization signal in the brain of WT control littermates (Figure 2B) approximated the intensity of the [³⁵S]-hCOX-2 sense-strand hybridization signal on brain tissue sections of NHC32-hCOX-2 transgenics and WT controls (not shown).

hCOX-2 mRNA Expression Relative to Endogenous COX-2 mRNA and Expression of Endogenous COX-1 mRNA

Because of the constitutive expression of COX-2 mRNA in rodent brain, we examined the relative levels of hCOX-2 mRNA expression over endogenous murine (m) COX-2 mRNA; mCOX-2 mRNA expression was assessed by ISH using a murine [³⁵S]-COX-2 antisense cRNA probe. Quantitative ISH revealed greater than three- to fourfold hCOX-2 mRNA expression in the DG granule and pyramidal (CA3-CA1) neuron layers of the hippocampal formation, parietal cortex, and thalamus over the mCOX-2 mRNA hybridization signal (Figure 3A). In control studies,

Figure 2. *In situ* hybridization (ISH) analysis of human COX-2 in transgenic and WT mice. **A:** Regional distribution of hCOX-2 in the brain of NHC32-hCOX-2 transgenic mouse. **B:** WT control littermate (4 months old), as assessed by ISH assay for hCOX-2 mRNA and visualized by X-ray autoradiography, respectively. **C:** Colocalization of hCOX-2 mRNA hybridization signal with NSE-immunopositive neurons in hCOX-2 transgenic mouse brain. **D:** Lack of hCOX-2 mRNA hybridization signal in NSE-positive neurons of WT control brain. **E:** Optical densities were quantified by computerized video densitometry from film autoradiograms using a Bioquant image analysis system (Biometrics). Values are expressed as mean \pm SE, * $P < 0.001$, hCOX-2 (NHC32) versus WT controls, $n = 6-8$ per group. CTX, parietal cortex; PYR, pyramidal layer of the hippocampal formation; DG, dentate gyrus of the hippocampal formation; THL, thalamus; PKK, cerebellar Purkinje's layer. Scale bars, 1 mm (A and B) and 8 μ m (C and D).

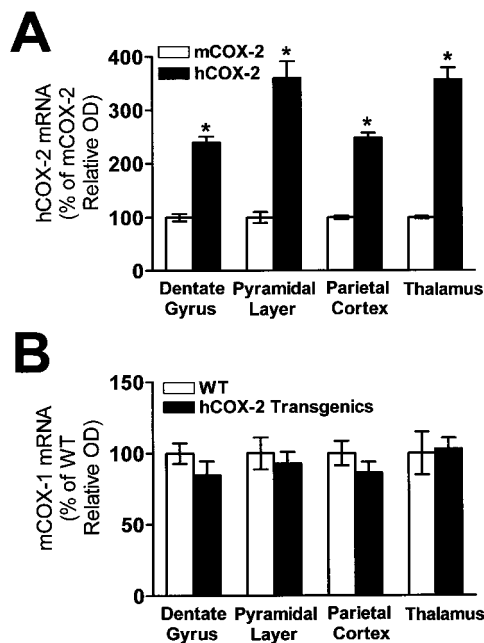


Figure 3. hCOX-2 mRNA expression relative to mouse COX-2 mRNA in hCOX-2 transgenic mice and endogenous mCOX-1 mRNA expression. **A:** hCOX-2 mRNA is expressed as a percentage of the endogenous mCOX-2 mRNA as assessed by ISH assay in adjacent brain tissue sections of the same brain (NHC32). Under the stringency conditions used in this study, we found negligible cross-hybridization of the hCOX-2 [³⁵S]-cRNA probe with the endogenous mCOX-2 mRNA (see Figure 2B). **B:** mCOX-1 mRNA expression in brain of NHC32-hCOX-2 transgenics relative to WT controls. Optical densities were quantified by computerized video densitometry from film autoradiograms using a Bioquant image analysis system (Biometrics). Values are expressed as mean ± SE, in **A** **P* < 0.01, *n* = 5–6 per group.

we found that mCOX-2 mRNA expression did not differ in control WT littermates and NHC32-hCOX-2 transgenics (not shown).

In parallel control studies, we also explored the regulation of the endogenous mCOX-1 mRNA and found no detectable change of expression in any brain region examined of the NHC32-hCOX-2 transgenics compared to WT controls (Figure 3B).

Neuronal Overexpression of hCOX-2 Potentiates KA-Induced Seizures in NHC32-hCOX-2 Transgenic Mice

Neuronal hCOX-2 overexpression potentiated seizures and increased mortality during response to ICV (lateral ventricle) KA infusion. There was marked increase in the intensity of KA-mediated seizures in NHC32 hCOX-2 transgenic mice compared to WT control littermates (Table 1). Vigorous status epilepticus, leading to accelerated death within 10 minutes after ICV infusion of 1.25 nmoles of KA, occurred in each of the hCOX-2 transgenic mice. Mild seizures but no death occurred in WT control littermates within 10 minutes after ICV infusion of an equal dose of KA (*n* = 6–8 per group, *P* < 0.004, Fisher's exact test; Table 1). For further comparative analysis of changes in gene expression or neuropathology, the control WT mice were sacrificed within 10 minutes after lesioning. No detectable seizures were found in either

hCOX-2 transgenics or WT controls following ICV infusion of an equal volume of vehicle (2 μl, saline).

Severe seizures in NHC32-hCOX-2 transgenic mice were characterized by intense myoclonic twitches of the forelimbs associated with generalized tonic-clonic seizures with wild seizure jumps. In contrast, seizures in WT littermates ICV injected with the same dose of KA were in general associated with staring posture and wet-dog shake behavior without any myoclonic twitches (Table 1).

In pilot titration studies, potentiation of KA-mediated seizures in NHC32-hCOX-2 transgenic mice was found after ICV infusion of KA at doses between 0.75 and 3 nmoles. Mild or no detectable seizures were found in both hCOX-2 transgenics and WT control littermates after ICV infusion of KA at doses below 0.75 nmoles (not shown). Similar potentiation of KA-induced seizures occurred in NHC5-hCOX-2 transgenic mice (data not shown).

Potentiation of KA-Mediated Induction of *c-fos* and *zif-268* mRNA Expression in NHC32-hCOX-2 Transgenic Mice

In NHC32-hCOX-2 transgenic mice, generalized status epilepticus, characterized by intense tonic-clonic seizures and death by 10 minutes after KA (1.25 nmoles), coincided with potentiation of *c-fos* mRNA expression in neuronal layers the hippocampal formation, and parietal cortex relative to KA-injected control WT littermates. Quantitative ISH autoradiography revealed greater than three- to fourfold (*n* = 4, *P* < 0.05) potentiation of *c-fos* mRNA expression in the granule neuron layer of the DG ipsilateral to the lesion side compared to WT controls (Figure 4, A and B; Figure 5A, left panel). Similarly, there was >1.5-fold potentiation (*P* < 0.05) of *c-fos* mRNA expression in the CA3-CA1 pyramidal layer of the hippocampal formation (Figure 4, A and B; Figure 5B, left panel) and in the parietal cortex (Figure 4, A and B Figure 5C, left panel), ipsilateral to the lesion side, compared to WT controls.

Similar potentiation of *zif-268* mRNA expression (approximately twofold, *P* < 0.05) in NHC32-hCOX-2 transgenic mice versus WT littermates was found in the granule neuron layer of the DG (Figure 4, C and D; Figure 5A, right panel). On the contrary, no detectable potentiation of *zif-268* mRNA expression was found in the CA3-CA1 pyramidal neuron of the hippocampal formation, compared to WT control littermates (Figure 4, C and D; Figure 5B, right panel), as assessed by ISH assay on tissue sections adjacent to those used for *c-fos* mRNA. No detectable potentiation of *zif-268* mRNA hybridization signal was found in parietal cortex of hCOX-2-NHC32 transgenic mice compared to control WT littermates (Figure 4, C and D; Figure 5C, right panel).

In response to ICV infusion of drug vehicle (saline) in NHC-32-hCOX-2 transgenic mice, we found no detectable potentiation of hippocampal and cortical *c-fos* and *zif-268* mRNA hybridization signal relative to control WT littermates (not shown). Moreover, hematoxylin and eosin histochemistry was used to identify neurons with pyknotic condensed nuclei in neuronal layer of the hippocampal

Table 1. Potentiation of KA-Induced Seizures in hCOX-2 Transgenic Mice Compared to Wild-Type Littermates

	<i>n</i>	Mortality	Remarks
Wild-type littermates	6	0%	Seizures were associated with staring posture and mild wet-dog shake behavior without any myoclonic twitches.
hCOX-2 transgenics	8	100%	Seizures characterized by intense myoclonic twitches of the forelimbs associated with generalized tonic-clonic seizures with wild seizure jumps.

Fisher's exact test, $P < 0.004$.

formation and cerebral cortex. No overt sign of neurodegeneration was found in either hCOX-2 transgenics or WT control littermates within 10 minutes after ICV KA (not shown).

Neuronal hCOX-2 Overexpression Potentiates Glutamate-Mediated Excitotoxicity in Vitro

Ten-day-old primary cortico-hippocampal neuron cultures generated from control WT embryos resulted in $31 \pm 2\%$ impairment of redox activity after exposure to glutamate ($50 \mu\text{mol/L}$, 24 hours) relative to vehicle-treated control cultures, as assessed by MTT assay. However, glutamate treatment in parallel cortico-hippocampal neuronal cultures generated from hCOX-2 (NHC32) transgenic embryos resulted in $46 \pm 3\%$ impairment of redox impairment relative to vehicle-treated control cultures. Thus, overexpression of COX-2 in cortico-hippocampal neurons potentiated glutamate-mediated redox impairment by approximately 50% relative to WT controls ($n = 5-6$ independent cultures per group, $P < 0.004$; Figure 6A)

In control studies, ISH for hCOX-2 mRNA combined with NSE ICC confirmed neuronal hCOX-2 expression in the cortico-hippocampal neuronal cultures generated from hCOX-2 (NHC32) transgenic embryos (Figure 6B).

Discussion

In this study we report that overexpression of neuronal hCOX-2 in NHC32 transgenic mice potentiated the intensity of KA-mediated seizures, leading to increased mortality coincident to increased expression of the transcription factors *c-fos* and *zif-268*. Moreover, glutamate neurotoxicity *in vitro*, as assessed by MTT assay, was potentiated in primary cortico-hippocampal neuron cultures derived from NHC32 transgenic mice compared to cultures derived from WT littermates. Our conclusion that potentiation of KA-induced excitotoxicity resulted from overexpression of hCOX-2 in neurons, rather than from disruption of another gene in the NHC32 transgenic line, is supported by similar results obtained with NHC5-hCOX-2 transgenics, a second line with neuronal overexpression of hCOX-2.

To generate our transgenic mice, a modified rat NSE promoter was used to regulate expression of hCOX-2 in neurons. The rat NSE promoter has been successfully used by multiple groups to selectively direct the overexpression of heterologous proteins in neurons of trans-

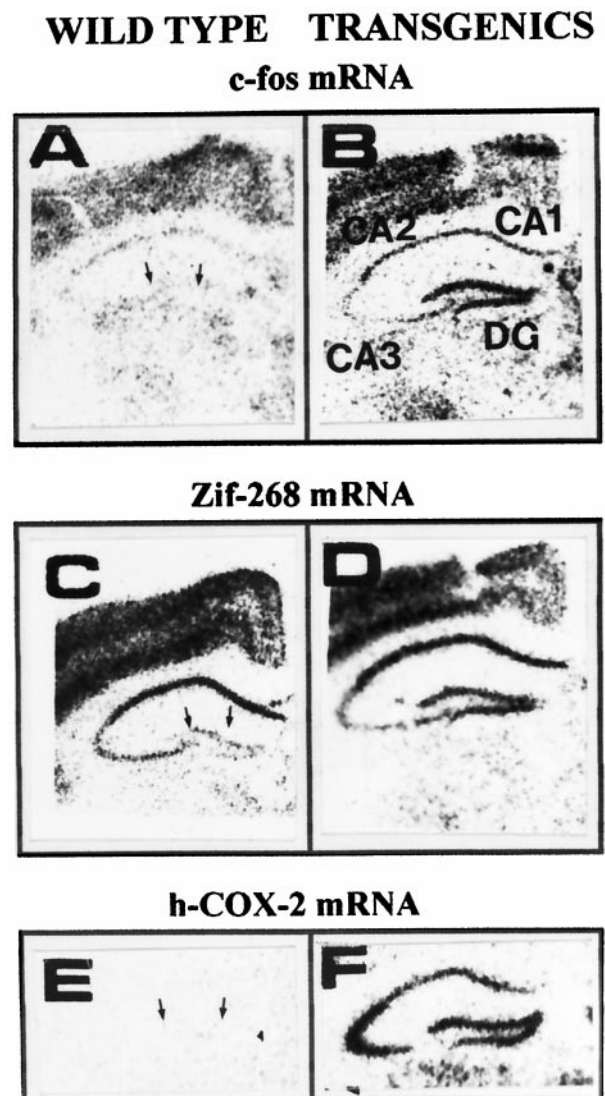


Figure 4. Potentiation of hippocampal *c-fos* and *zif-268* mRNA expression in hCOX-2 transgenic mice compared to WT littermates in response to KA-induced seizures (10 minutes after lesioning). A and B: *c-fos* mRNA. C and D: *zif-268* mRNA expression in WT controls (A, C) and in hCOX-2 transgenic mice (B, D) 10 minutes after ICV KA infusion, respectively. E: hCOX-2 mRNA expression in the hippocampal formation of WT (negative control). F: hCOX-2 transgenics, respectively. Hybridization signals from mouse brain sections are visualized by X-ray film autoradiography. In A, C, and E, arrows point toward the granule neuron layer of the hippocampal dentate gyrus (DG). In B, CA1 CA2, CA3 pyramidal layers of the hippocampal formation.

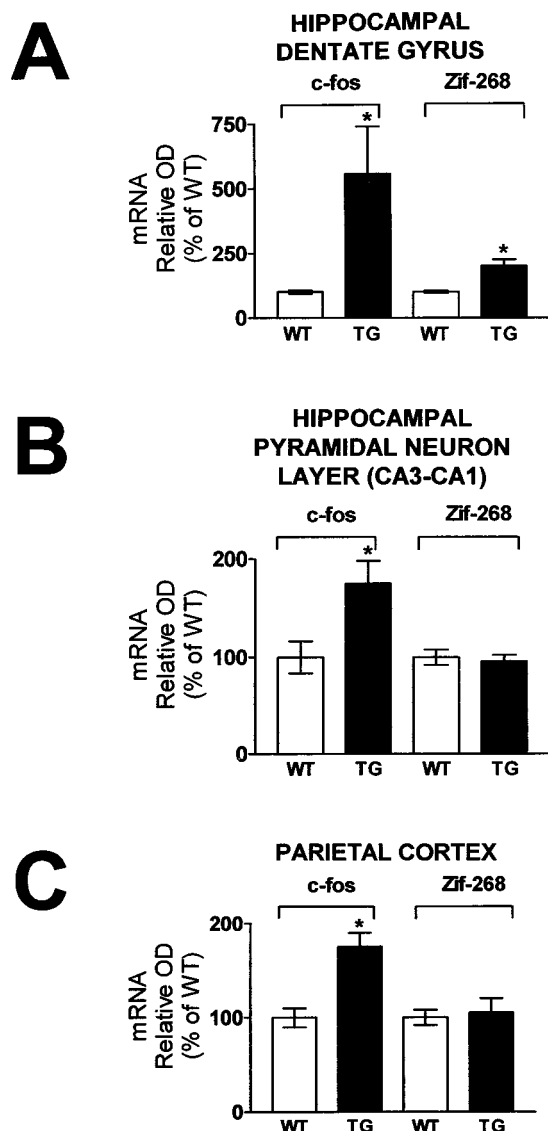


Figure 5. Potentiation of *c-fos* and *zif-268* mRNA expression in the hippocampal dentate gyrus, pyramidal neuron layer, and parietal cortex of hCOX-2 transgenic mice compared to WT littermates in response to KA-induced seizures. Changes in *c-fos* and *zif-268* mRNA expression in the granule cell layer of the dentate gyrus (A), in the pyramidal neuron layer of the hippocampal formation (B), and in the parietal cortex (C), respectively. Relative optical densities were quantified by computerized video densitometry from film autoradiograms using a Bioquant image analysis system (Biometrics). Control studies confirmed that the hippocampal level of *c-fos* and *zif-268* mRNA expression did not differ between control WT littermates and hCOX-2 transgenics following ICV infusion of saline (not shown). Values are expressed as mean \pm SE, $n = 4$ per group, * $P < 0.05$, WT versus transgenic.

genic mice.^{18,29–37} Previous studies reported the use of a rat NSE promoter consisting only of a 1.8-kb sequence located 5' end to the first exon; this 1.8-kb promoter fragment has been found to regulate selective neuron-specific expression, but also with some minor expression in other tissues.^{29–35}

In this study, in addition to a longer segment of the promoter and 5' flanking region (2.6 kb), the rat NSE regulatory fragment also included exon 1, intron 1, and 6 bp of exon 2 (Figure 1A). Intron 1 contains a putative neuronal regulatory element,³⁸ and this larger rat NSE

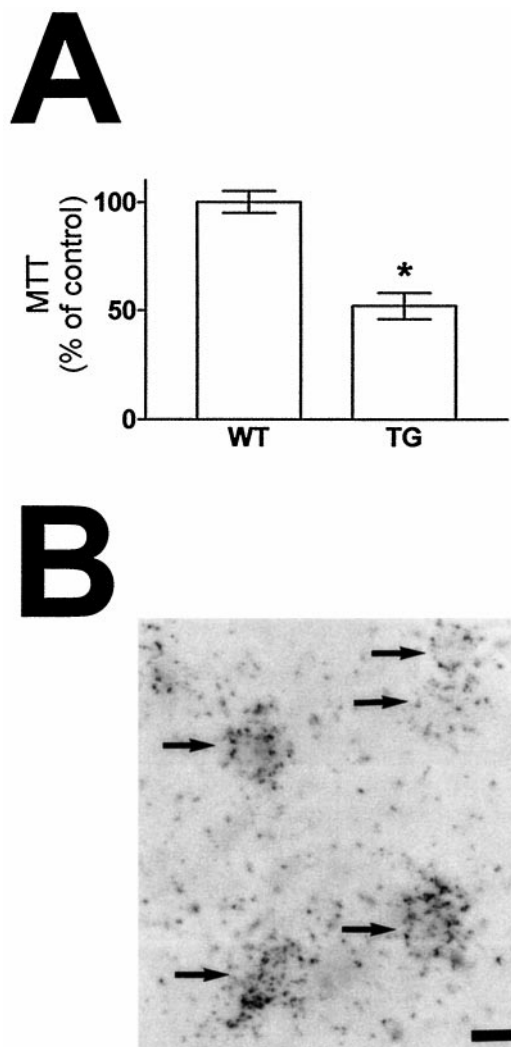


Figure 6. Potentiation of glutamate-mediated impairment of redox activity in primary cortico-hippocampal neurons derived from hCOX-2 transgenic mice compared to WT control mice. **A:** Primary cortico-hippocampal neuron cultures were derived from 14-embryonic-day-old transgenic NHC32-hCOX-2 or control WT embryos. Ten days after plating, neurons were treated with 50 μ mol/L glutamate or vehicle for 24 hours' treatment and glutamate toxicity was assessed by MTT assay. Changes in MTT are expressed as percentage of identical cultures treated with drug vehicle. **B:** hCOX-2 mRNA expression was visualized by combined ISH and NSE ICC using bright field illumination. Values are expressed as mean \pm SE, $n = 4–8$ per group in $n = 5$ independent studies (* $P < 0.05$) transgenic versus WT. Scale bar in C, 10 μ m. Arrows point toward NSE-immunopositive neurons expressing hCOX-2 (silver grains).

promoter construct was previously used to direct specific expression of heterologous proteins to neurons in transgenic mice.^{18,36,37} The results presented in this study confirm the cell type specificity determined by the larger NSE promoter, which includes intron 1; transgene expression in the NHC32-hCOX-2 line examined was restricted to the brain, whereas ISH revealed that the detectable brain expression is essentially only neuronal.

One possible mechanism by which neuronal COX-2 overexpression may influence the neuronal response to KA-induced seizures and glutamate excitotoxicity *in vitro* may involve the control of transcription factor DNA-binding activities. We found that neuronal hCOX-2 overexpression in NHC32-hCOX-2 transgenic mice potentiate

the expression of the IEG transcription factors *c-fos* and *zif-268* in subsets of hippocampal neurons in response to KA, compared to WT littermates. This regulation of gene expression might occur through paracrine and autocrine signaling mediated by prostaglandins (PGs) through PG receptors, which are members of the G-protein-coupled family of receptors. In non-neuronal cells, the signals depend on the specific agonist and the receptor, but include changes in cyclic AMP, turnover of phosphatidylinositol, and activation of both protein kinase C (PKC) and mitogen-activated protein (MAP) kinase.³⁹ Thus, it is possible that overexpression of hCOX-2 mRNA in neurons may increase sensitivity or lower the threshold level of excitation to KA through changes in gene expression. We also note that there is evidence suggesting a direct link between COX-2 and *c-fos* and *zif-268* expression in excitotoxicity. All three are rapidly transcribed during the response to KA-induced seizures⁴⁰ and inhibition of intracellular platelet activating factor (PAF),⁴¹ whereas limiting KA-mediated induction of *zif-268* expression also attenuates COX-2 induction.⁴⁰

The lack of potentiation of *zif-268* mRNA expression in the hippocampal CA3-CA1 pyramidal layers and parietal cortex of hCOX-2 transgenic mice versus WT littermates in response to KA-induced seizures is puzzling; it may indicate a regional variation in the time course of changes in gene expression in this model system. In support of this hypothesis we note that a common feature of these responses to excitotoxic insult is the concerted induction of IEG with a distinct time course of activation.^{42,43}

COX-1 and COX-2 are key enzymes in the generation of prostanoids including PGs. Both enzymes catalyze two functional reactions: a cyclooxygenase reaction to form the endohydroperoxide PGG₂, and an iron heme-mediated peroxidase activity to reduce PGG₂ to PGH₂.¹¹ PGH₂ is the precursor to all other PG. The primary source of arachidonate for COX is thought to be membrane phospholipid; arachidonic acid (AA) is released from esterified phospholipid by the action of phospholipase A₂. Substances that increase intracellular Ca²⁺, such as glutamate,⁴⁴ also activate phospholipase A₂⁴⁵ to make available the substrate AA for COX reactions. Thus, agents increasing neuronal intracellular free Ca²⁺ in presence of high levels of COX-2 may lead to higher levels of prostanoid synthesis. Because several of the prostanoid products of AA metabolism generated by COX may also control the activity of excitatory and inhibitory amino acid pathways,⁴⁶ it is plausible that overexpression of neuronal COX-2 might also modulate glutamate mediated responses through excitotoxic mechanisms. In support of this hypothesis there is evidence that (N-methyl-D-aspartate (NMDA)-mediated neuronal death is diminished in a dose-dependent manner by COX-2 inhibitors in primary neuronal cultures.⁴⁷ However, we note that there is also evidence that PGs may protect from glutamate toxicity in cortical neurons under certain conditions.⁴⁸

Free radicals are intermediate products in COX-mediated PG synthesis, and lipid peroxides influence the activity of COX.⁴⁹ Moreover, the role of free radicals in NMDA receptor-mediated glutamate excitotoxicity is well

established in various experimental conditions.^{50,51} Thus, given that oxidative stress seems to be involved in mechanisms of excitotoxicity,^{52,53} it is not unexpected that neuronal COX-2 activity may contribute to neurodegeneration via oxidative mechanisms¹¹; this COX-2 influence may not necessarily be closely linked to PG production.⁵⁴ Oxidative stress resulting from COX-2 activity may have important implications for neurodegenerative diseases such as AD, in which an elevated glutamatergic tone, also defined as disinhibition syndrome, may be responsible for a widespread pattern of neurodegeneration.¹³ The evidence of potentiation of glutamate-mediated redox impairment in primary neuron cultures from the hCOX-2 transgenic mice suggests that COX-2 overexpression indeed may be involved in mechanisms of oxidative stress *in vitro*. Future studies will clarify the role of neuronal COX-2 expression in neurodegenerative pathways such as caspase activation and apoptosis *in vitro* and *in vivo*.

In conclusion, this study demonstrated a possible causative role for neuronal COX-2 expression in excitotoxicity. This model system will allow a systematic examination of the role of COX-2 in mechanisms of neurodegeneration that involve excitatory amino acid pathways. Ongoing laboratory studies exploring the role of selective and nonselective COX-2 inhibitors on KA-mediated responses in NHC32-hCOX-2 transgenics will clarify the causative role of COX-2 in mechanisms of excitotoxicity.

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