Journal of Neurochemistry, 2004, **90**, 1036–1049 doi:10.1111/j.1471-4159.2004.02577.x

Arachidonic acid-induced carbon-centered radicals and phospholipid peroxidation in cyclo-oxygenase-2-transfected PC12 cells

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Abstract

Cyclo-oxygenase-2 (COX-2) is believed to induce neuronal oxidative stress via production of radicals. While oxygen radicals are not directly involved in COX-2-catalytic cycle, superoxide anion radicals have been repeatedly reported to play a critical role in COX-2-associated oxidative stress. To resolve the controversy, we characterized production of free radicals in PC12 cells in which COX-2 expression was manipulated either genetically or by direct protein transfection and compared them with those generated by a recombinant COX-2 in a cell-free system. Using spin-traps α-(4-pyridyl-1-oxide)-*N*-t-butylnitrone, 5,5-dimethyl-1-pyrroline-*N*-oxide and 4-((9-acridinecarbonyl) amino)-2,2,6,6- tetramethylpiperidine-1-oxyl (Ac-Tempo), we observed arachidonic acid (AA)-dependent production of carbon-centered radicals by heme-reconstituted recombinant COX-2. No oxygen radicals or thiyl radicals have been detected. COX-2 also catalyzed AA-dependent one-electron co-oxidation of ascorbate to ascorbate radicals. Next, we used two different approaches of COX-2 expression in cells, PCXII cells which express isopropyl-1-thio-β-D-galactopyranoside inducible COX-2, and PC12 cells transfected with COX-2 using a protein delivery reagent, Chariot. In both models, COX-2-dependent AA-induced generation of carbon-centered radicals was documented using spin-traps and Ac-Tempo. No oxygen radical formation was detected in COX-2-transfected cells by either spin-traps or fluorogenic probe, dihydroethidium. In the presence of ascorbate, AA-induced COX-2-dependent ascorbate radicals were detected. AA caused a significant and selective oxidation of one of the major phospholipids, phosphatidylserine (PS). PS was not a direct substrate for COX-2 but was co-oxidized in the presence of AA. The radical generation and PS oxidation were inhibited by COX-2 inhibitors, niflumic acid, nimesulide, or NS-398. Thus, COX-2 generated carbon-centered radicals but not oxygen radicals or thiyl radicals are responsible for oxidative stress in AA-challenged PC12 cells overexpressing COX-2.

Keywords: carbon-centered radical formation, cyclo-oxygenase-2 inhibitor, cyclo-oxygenase-2 transfection, PC12 cells, phosphatidylserine oxidation, radical spin-trapping.
J. Neurochem. (2004) **90,** 1036–1049.

Received March 9, 2004; accepted April 12, 2004.

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Abbreviations used: AA, arachidonic acid; AA-PS, 1-palmitoyl-2-arachidonoyl-phosphatidylserine; Ac-Tempo, 4-((9-acridinecarbonyl) amino)-2,2,6,6-tetramethylpiperidine-1-oxyl; 2-AG, 2-arachidonyl glycerol; COX, cyclo-oxygenase; DHE, dihydroethidium; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; EPR, electron paramagnetic resonance; GSH, glutathione; hSA, human serum albumin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NA, niflumic acid; NS-398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; PCXII, PC12 cell line expressing IPTG-inducible COX-2; PCMT, mock transfected PC12 cells; PGE2, prostaglandin E2; PGH2, prostaglandin H2; PGs, prostaglandins; PnA, *cis*-parinaric acid; POBN, α-(4-pyridyl-1-oxide)-*N*-t-butylnitrone; ROS, reactive oxygen species.

Cyclo-oxygenase (COX) is a bifunctional enzyme that catalyzes the committed step in the conversion of arachidonic acid (AA) to prostaglandins (PGs). A constitutive form of COX, COX-1, and an inducible form of COX, COX-2, have been identified as protein products encoded by distinct genes but exhibiting significant structural and enzymatic similarities (Hla and Neilson 1992). Both enzymes function in a cycle that includes catalysis of:

1. a COX reaction in which AA is converted into prostaglandin G2 (PGG2) as a result of stereospecific addition of two molecules of O_2
2. a peroxidase reaction in which PGG2 is reduced to prostaglandin H2 (PGH2) in a two-electron process (Smith and Dewitt 1996).

PGH2 is then converted by isomerases to PGs and thromboxane (Dietz *et al*. 1988; Karthein *et al*. 1988), which exert numerous physiological and pathophysiological effects. COX-1 is expressed in nearly all cell types at a constant level and accounts for the generation of low levels of prostaglandins involved in physiological functions (Smith and Dewitt 1995). In contrast, COX-2, normally undetectable in most tissues (Smith and Dewitt 1995), is encoded by an immediate–early response gene induced by a variety of agonists (including cytokines, mitogens, reactive oxygen species (ROS), hormones, and pro-inflammatory substances) and is the main form expressed during inflammation (Appleton *et al*. 1996; Herschman 1996). Low amounts of COX-2 are constitutively expressed in brain but can be greatly upregulated by diverse stimuli, including synaptic activity and cerebral ischemia (Yamagata *et al*. 1993; Breder *et al*. 1995; Kaufmann *et al*. 1997).

Neuronal injury in neurodegenerative diseases, including Parkinson's disease (Beal 2003), Alzheimer's disease (Perry *et al*. 2002), and amyotrophic lateral sclerosis (Coyle and Puttfarcken 1993), as well as by brain ischemia/reoxygenation injury and trauma (Love 1999; Chan 2001), is often associated with oxidative stress, whose origin is not fully understood. There is accumulating evidence that COX-2 is a likely candidate enzyme that contributes to oxidative stress during neuronal injury (Nogawa *et al*. 1997; Iadecola *et al*. 2001; Candelario-Jalil *et al*. 2002). Elevation of COX-2 expression has been shown during cerebral ischemia in humans (Nogawa *et al*. 2002), focal cerebral ischemia following permanent middle cerebral artery occlusion (Kinouchi *et al*. 1999), and global ischemia in animal models (Nakayama *et al*. 1998). Moreover, administration of COX-2-specific inhibitors reduced the volume of the infarct after cerebral ischemia in rat (Nogawa *et al*. 1997) and death of hippocampal neurons after global ischemia (Nakayama *et al*. 1998). It has also been demonstrated that COX-2-deficient mice have reduced susceptibility to ischemic brain injury and *N*-methyl-D-aspartate mediated neurotoxicity than wild-type mice (Iadecola *et al*. 2001). Therefore, it is tempting to speculate that COX-2-catalyzed free radicals may contribute significantly to oxidative stress during brain injury.

However, reported specific mechanisms of COX-2-induced neuronal oxidative stress are elusive if not confusing. Current understanding of COX-2 reaction pathways suggests that two major types of radicals – a tyrosyl radical on the protein and a carbon-centered radical of its substrate, AA – are essential for the COX-2-catalytic cycle (Karthein *et al*. 1988; Marnett 2000). However, a number of publications, starting from early works in the 1970–1980s (O'Brien and Hawco 1978; Kukreja *et al*. 1986), proposed that one-electron reduction intermediates of oxygen, i.e. oxygen radicals (or so called ROS) are involved in COX-2-dependent oxidative stress (Nelson *et al*. 1992; Chan 1996, 2001; Armstead *et al*. 1998; Busija *et al*. 1998; Armstead 2003). Paradoxically, despite the lack of any direct evidence for the involvement of oxygen radicals in COX-2-mediated neuronal damage, it became customary in neurobiology literature to consider ROS as the primary source of COX-2-generated radicals and oxidative stress. As a result, several attempts have been made to employ oxygen radical scavenging antioxidant enzymes – SOD and catalase – to protect against COX-2-dependent oxidative stress during neuronal injury (Horakova *et al*. 1997; Armstead 2003). While co-oxidation of some physiologically relevant reductants, e.g. NAD(P)H, accompanied by intermediary production of NAD(P)* during COX-2-catalyzed reactions can, indeed, yield catalytic generation of superoxide radicals due to NAD(P)*-driven reduction of molecular oxygen (Kukreja *et al*. 1986), oxygen radicals are not directly involved in catalytically competent COX-2 mechanisms (Marnett 2000). To effectively regulate COX-2-induced oxidative stress in the brain it is imperative that COX-2-generated radicals be identified not only in simple cell-free biochemical systems but also in physiologically relevant environments containing essential complements of intracellular reductants. To this end, we conducted a comparative study to characterize production of free radicals by a recombinant COX-2 in a cell-free system, with that in rat pheochromocytoma PC12 cells in which COX-2 expression was manipulated either genetically or by direct protein transfection. By using spin-traps, α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) and 4-((9-acridinecarbonyl) amino)-2,2,6,6- tetramethylpiperidine-1-oxyl (Ac-Tempo), we assessed the generation of carbon-, sulfur-, and oxygen radicals. Because ascorbate and glutathione (GSH) are two major antioxidant radical scavengers in the brain, we also determined ascorbate radical and thiyl radical production. Furthermore, we utilized metabolic labeling of phospholipids with oxidation-sensitive fluorescent *cis*-parinaric acid (PnA) to perform measurements of peroxidation of different classes of phospholipids in COX-2-transfected PC12 cells. Our results demonstrate that generation of carbon-centered radicals but not of oxygen radicals are likely responsible for oxidative stress associated with catalytic activity of

the enzyme in AA challenged PC12 cells overexpressing COX-2.

Materials and methods

Materials

Tissue culture medium Ham's F-12K (DMEM medium) and horse serum were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Chariot protein transfection kit was from ActiveMotif (Carlsbad, CA, USA). AA was purchased from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS), DMPO niflumic acid (NA), human serum albumin, nimesulide, microperoxidase, and POBN were from Sigma (St Louis, MO, USA). Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), PnA, DHE (dihydroethidium) and Ac-Tempo were obtained from Molecular Probes (Eugene, OR, USA). 2-Arachidonyl glycerol (2-AG), human recombinant COX-2, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398), and STAT-PGE2 EIA kit were purchased from Cayman (San Diego, CA, USA). 1-palmitoyl-2-arachidonoyl-phosphatidylserine (AA-PS) was from Avanti Polar Lipids (Alabaster, AL, USA).

Detection of COX-2-catalyzed production of free radicals in a cell-free system

Human recombinant COX-2 was reconstituted with hemin (dissolved in 0.1 N NaOH) at the ratio of 1 : 5 for 5 min at room temperature.

EPR detection of AA-induced COX-2 tyrosyl radicals

Reaction was initiated on ice upon addition of 100 μM AA or 25 μM H_2O_2 to a solution containing 80 mM Tris-HCl buffer (pH 8.0), 0.1% Tween-20 and 5 μM reconstituted COX-2. The samples were frozen in liquid nitrogen 10 s later. EPR measurements were performed on a JEOL-RE1X spectrometer (Kyoto, Japan). Spectra were acquired using following instrumental settings: 3235 G, center field; 100 G, sweep width; 3.2 G, field modulation; 10 mW microwave power; 0.3 s, time constant; 2 min, time scan; 77°K temperature.

EPR detection of ascorbate radicals

Spectra of ascorbate radicals were recorded upon addition of AA (20 μM) to the reaction mixture containing 10 mM Tris-HCl (pH 7.4), 100 μM DTPA, 0.2 μM reconstituted COX-2, 400 μM ascorbate. Spectra were obtained by repeated scanning of the magnetic field using the internal mode of recording: at 3357 G, center field; 5 G sweep width; 0.04 G, field modulation; 10 mW, microwave power; 0.3 s, time constant; 10 s, time scan.

EPR detection of carbon-centered, oxygen, and thiyl radicals using spin-trap, POBN

Recording of EPR spectra commenced 30 s after addition of AA (100 μM) to reconstituted COX-2 (0.75 μM) in the presence of POBN (100 mM). In experiments with COX-2 inhibitors, NA was incubated with COX-2 before addition of AA. As a control, EPR spectra were recorded in the absence of COX-2. Spectra of POBN adducts were obtained at the following conditions: 3350 G, center field; 80 G, sweep width; 1 G, field modulation; 10 mW, microwave power; 0.1 s, time constant. Spectrum recorded at 10 min were presented as a representative trace.

Detection of carbon-centered radicals using Ac-Tempo fluorescence assay

A Shimadzu spectrofluorimeter RF-5301PC was employed for fluorescence measurements in a 0.2 mL quartz cuvette. Time course of Ac-Tempo fluorescence (λ_{Ex} = 360 nm, λ_{Em} = 440 nm; excitation slit of 1.5 nm, and an emission slit of 3 nm) was monitored after addition of AA (20 μM) to the reaction buffer (10 μM Ac-Tempo, 75 nM reconstituted COX-2 in PBS). As a control, fluorescence was measured under the same condition without COX-2. In experiment with a COX-2 inhibitor, 100 μM NA was added to the reaction mixture.

Cell culture

The PCXII (PC12 cell line expressing IPTG-inducible rat COX-2) and PCMT (mock transfected PC12 cells) cell lines were obtained as previously described (McGinty et al. 2000). PC12 cell line was purchased from ATCC. All cell lines were cultured in the presence of rat tail collagen type I in Ham's F12K medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 15% horse serum, 2.5% fetal bovine serum in a humidified atmosphere at 37°C.

For PCXII and PCMT cells, geneticin (G418; 0.8 mg/mL) (Invitrogen, Carlsbad, CA, USA), and hygromycin B (0.08 mg/mL; Invitrogen) were added into the culture medium. To induce COX-2 expression, PCXII cells were coincubated with 2.5 mM IPTG for 24 h. As control, PCMT cells were also treated with IPTG under the same conditions.

Human recombinant COX-2-transfection in PC12 cells with Chariot

Human recombinant COX-2 was delivered into PC12 cell using Chariot protein transfection kit. In brief, 1 μg [in 100 μL phosphate-buffered saline (PBS)] of COX-2 protein was added to Chariot (6 μL in 100 μL H_2O), incubated at room temperature for 30 min to form complex. Cells were washed with serum free medium once, Chariot/COX-2 complex was then added to cells, and another 400 μL serum free medium was added to achieve the final transfection concentration. Cells were incubated with complex for 2 h to allow COX-2 internalization.

Determination of COX-2 in cells

Western blot analysis and PGE2 production measurements were conducted to evaluate COX-2 level in cells

1. Western blot analysis. Cells protein extracts were prepared by homogenization of 1×10^6 cells in lysis buffer. Total of 20 μg of protein from each sample was loaded onto an 8% sodium dodecyl sulfate (SDS)/polyacrylamide gel. Protein was transferred to nitrocellulose and probed with the appropriate polyclonal antibody. Human recombinant COX-2 protein was used as a positive control.

2. PGE2 determination. To determine COX-2 activity, cells were incubated with AA (30 μM) in serum-free medium for 30 min at 37°C. Levels of PGE2 released into the medium of treated cells were measured by a STAT-PGE2 EIA kit according to the manufacturer's instructions. Amounts of PGE2 are expressed as pg of PGE2 produced/mL of medium.

Inhibition of COX-2 activity

Niflumic acid, NS-398, or nimesulide were used in the subsequent experiments interchangeably to ensure the effects observed were indeed due to COX-2 inhibition. These inhibitors display similar competitive and time-dependent COX-2 inhibition profile by non-covalently binding to the AA binding site. Because in the current study we were particularly interested in AA-induced COX-2-dependent generation of radicals and subsequent oxidative stress, we inferred that this mode of inhibition is particularly appropriate. To determine the concentrations of COX-2 inhibitors to study AA-induced COX-2-dependent generation of radicals and phospholipid peroxidation in cells, preliminary experiments were conducted to test the effectiveness of the inhibitors towards PGE2 formation.

Detection of free radicals in cells

EPR detection of ascorbate radicals

Freshly prepared cell homogenates (5×10^6 cells/mL) were used for the EPR assay of ascorbate radical production. Spectra of ascorbate radicals were recorded as described above upon addition of AA (100 μM) to cell homogenate in the presence of ascorbate (500 μM). NA (100 μM) was added in the experiments when COX-2 inhibition was studied.

EPR detection of radicals in cells using a spin-trap, POBN

For PCXII and PCMT cells, cells (1.5×10^6) were collected at the end of IPTG induction, and then washed with PBS and resuspended in 60 μL of 500 mM POBN (in PBS) for 5 min. After addition of AA (600 μM), the samples were immediately loaded into Teflon tubing for EPR measurements. For PC12 cells, cells were washed and collected at the end of transfection, then resuspended in POBN (60 μL, 100 mM in PBS) at the density of 1.5×10^6/60 μL. AA (100 μM) was added 5 min later, and EPR spectra were recorded immediately. In experiment with COX-2 inhibitor, cells were preincubated with NA or nimesulide for 5 min before addition of AA. Spectra of radicals were recorded under the following conditions: 3350 G, center field; 1 G, field modulation; 50 G, sweep width; 10 mW, microwave power; and 0.03 s time constant.

Detection of superoxide anion using DHE

Generation of superoxide anion in cells was assessed by determining the oxidation of DHE as previously reported (Sharikabad *et al*. 2001; Zhao *et al*. 2003). Briefly, cells (0.8×10^6) were incubated with DHE (2.5 μM) for 30 min at 37°C in buffer (pH 7.4) containing 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 25 mM HEPES, supplemented with 12.5% FBS. DHE-loaded cells were then treated with AA (30 μM) for 30 min. At the end of incubation, cells were harvested and then analyzed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). Oxidation of DHE was assessed by monitoring the red fluorescence using a 585 nm, 42 nm band-pass filter (FL2 Channel). Ten thousand events were collected and analyzed using the CellQuest software (Becton-Dickinson).

Detection of carbon-centered radicals using Ac-Tempo with florescence microscope

PCXII and PCMT cells (5×10^6 cells/mL) were detached and incubated in PBS with 100 μM Ac-Tempo, 20 μM AA (and 10 μMNA when COX-2 inhibition was studied). After 10 min incubation, cells were spun down and resuspended in PBS, and then applied on a slide for an immediate fluorescence microscopy analysis. Fluorescence was evaluated using a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu CCD camera (C4742-95–12NRB). Data were analyzed using MetaImaging Series software version 4.6 (Universal Imaging Corp.). For each sample, at least 200 cells were analyzed.

Measurements of lipid peroxidation using *cis*-PnA-labeled cells

To assess peroxidation of major membrane phospholipids, *cis*-PnA was incorporated into cells by addition of PnA-human serum albumin (hSA) complex at a final concentration of 4.5 μg of PnA/mL in serum-free DMEM medium without phenol red for 2 h as described previously (Kagan *et al*. 1998). Oxidative stress was initiated by adding 30 μM AA. After 30 min incubation, total lipids were extracted and separated by normal phase HPLC using a 5 μm Microsorb-MV Si column (4.6 mm × 250 mm) and an ammonium acetate gradient. The separations were performed using a Shimadzu HPLC system (LC-600) (Kyoto, Japan) equipped with an RF-10AxL fluorescence detector. The fluorescence of PnA was measured at 420 mm (emission) after extraction at 324 mm. Lipids were extracted and the amount of lipid phosphorus was determined using a micro method (Folch *et al*. 1957).

Detection of lipid hydroperoxides

Lipid hydroperoxides were determined by fluorescence HPLC of products formed in peroxidase-catalyzed reaction of specific lipid hydroperoxides with a fluorogenic substrate, Amplex Red. HPLC separation of the reaction mixture with fluorescent detection of resorufin (an Amplex Red oxidation product) is conducted as follows. The assay is started by addition of 1 μL of reaction mixture containing 50 μM Amplex Red to 100 μL of basic reaction mixture containing 25 mM NaH_2PO_4, 0.5 mM EDTA (pH 7.4 at 4°C) and aliquot (1–2 μL) of lipid samples dissolved in ethanol. The reaction was initiated by addition of 1 μL of microperoxidase solution (0.25 μg/μL). The samples were incubated at 4°C for 40 min. The reaction is terminated by addition of 100 μL of stop solution (10 mM HCl, 4 mM butylated hydroxytoluene in ethanol). The samples are centrifuged at 15 000 *g* for 5 min and the supernatant is used for HPLC analysis. Aliquots (5 μL) were injected into a C-18 reverse phase column (Eclipse XDB-C18, 5 μm, 150 × 4.6 mm). The column is eluted by mobile phase composed of 25 mM NaH_2PO_4 (pH 7.0)/methanol (60 : 40 v/v) at 1 mL/min of flow rate. The resorufin fluorescence was measured at 590 nm after excitation at 560 nm. Shimadzu LC-100AT vp HPLC system equipped with fluorescence detector (model RF-10Axl) and autosampler (model SIL-10AD vp) was used. Chromatograms are processed and stored in digital form with Class-VP software.

Statistics

Data are presented as means ± SD and significance of differences are assessed by Student's *t*-test. Differences are considered significant at $p < 0.05$.

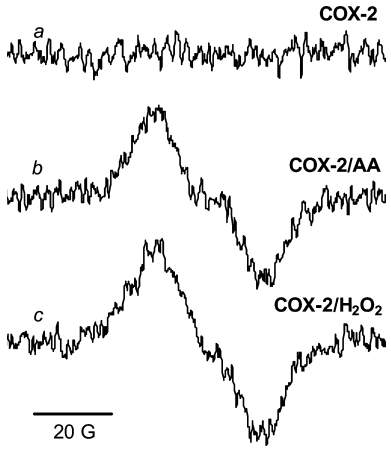

Fig. 1 EPR spectra of COX-2 tyrosyl radical generated during the catalytic cycle initiated with AA or H_2O_2 in a cell-free system. EPR spectra were recorded 10 s after addition of AA (100 μM, b) or H_2O_2 (25 μM, c) to reconstituted COX-2 (5 μM) under following conditions: 3235 G, center field; 100 G, sweep width; 3.2 G, field modulation; 10 mW microwave power; 0.3 s, time constant; 2 min, time scan; 77 K temperature.

Results

COX-2-generated radicals in a cell-free system

During the catalytic cycle, a tyrosyl residue of COX-2 is oxidized to the corresponding phenoxyl radical (Gunther *et al*. 1997; Xiao *et al*. 1997). Indeed, EPR signal in the region g = 2 with peak-to-trough width of ~29 G and visible splitting which has been assigned to COX-2 tyrosyl radical was recorded in the presence of 5 μM human recombinant COX-2 upon addition of either AA or H_2O_2 (Fig. 1).

Ascorbate is one of the major endogenous reductants readily interacting with oxidizing radicals to produce ascorbate radicals in one-electron redox reactions (Njus and Kelley 1991). Ascorbate concentrations are particularly high in the brain (0.9–2.0 mM, and may be as high as 10 mM in some regions; Grunewald 1993; Rice and Russo-Menna 1998). Therefore, we assessed ascorbate radical generation by COX-2. Incubation of reconstituted COX-2 with AA and ascorbate produced characteristic doublet EPR signal of ascorbate radical with $a^H = 1.7$ G (Fig. 2). The magnitude of EPR signal was 12-fold lower in the absence of AA, and it did not differ from the signal from ascorbate alone in control samples. The background signals are generated due to catalytic oxidation of ascorbate on metal impurities present in the buffer solutions. Substitution of COX-2 with complex of hSA and hemin resulted in EPR signal that was three times lower than that in the presence of COX-2. It is important to note that ascorbate radicals are unstable and disproportionate with rate constant of ~ 10^7/M/s (Bartlett *et al*. 1995). The magnitude of the ascorbate radicals EPR signal corresponds to their steady-state concentrations in the solution and is proportional to the rate of ascorbate oxidation. Thus, significant increase of COX-2-induced EPR signal of ascorbate radical over hemin-induced and control signals clearly demonstrates COX-2-catalyzed ascorbate oxidation.

Detection and identification of short-lived radicals in biological systems is feasible through use of spin-traps, molecules reacting with highly reactive radicals to produce relatively stable radical adducts with distinct EPR features. Heme-reconstituted COX-2 was incubated with POBN, a spin-trap that is known to give specific adducts with carbon-, oxygen-, and sulfur-centered radicals, and EPR spectra were recorded upon the addition of exogenous AA (100 μM). A spectrum with six lines and splitting constants of

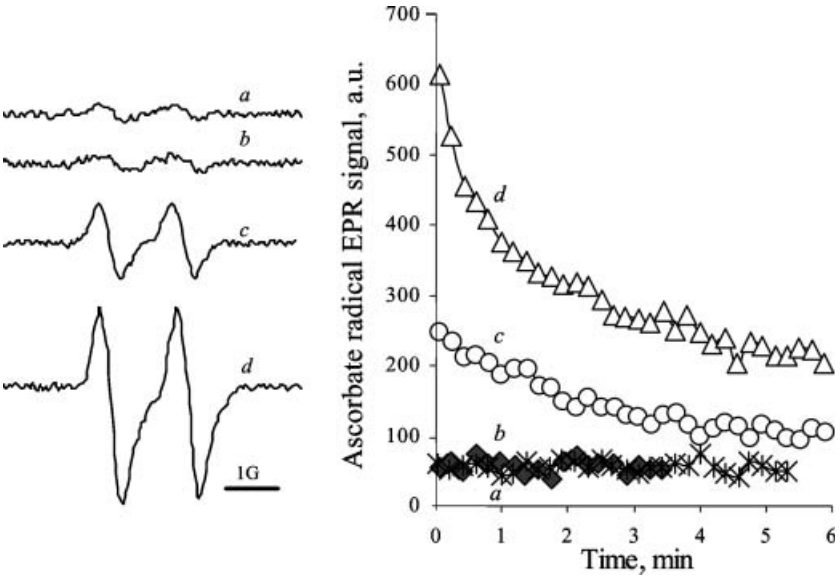

Fig. 2 COX-2-dependent AA-induced generation ascorbate radicals in a cell-free system. (a) ascorbate in PBS; (b) a plus hemin and COX-2 (c) a plus AA, hemin and human serum albumin; (d) *b* plus AA. (a) EPR spectra of ascorbate radicals were recorded 30 s after addition of AA (20 μM) to the reaction buffer containing 10 mM Tris–Cl (pH 7.4), 100 μM DTPA, 0.2 μM reconstituted COX-2, and 400 μM ascorbate under the following condition: 3357 G, center field; 5 G, sweep width; 0.4 G, field modulation; 10 mW, microwave power; 0.3 s, time constant; 10 s, time scan. (B) Time course of ascorbate radicals.

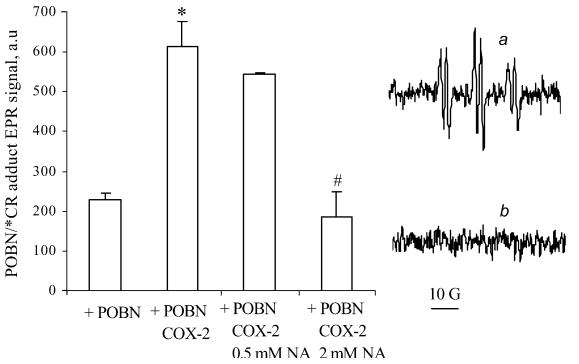

Fig. 3 COX-2-dependent AA-induced generation of carbon-centered radicals in a cell-free system. EPR spectra of POBN adducts were recorded 10 min after addition of AA (100 μM) to PBS containing 100 mM POBN and 0.75 μM reconstituted COX-2 at the following conditions: 3350 G, center field; 80 G, sweep width; 1 G, field modulation; 10 mW, microwave power; 0.1 s, time constant. As a negative control, EPR spectra were recorded in the absence of COX-2. NA was applied under indicated condition. Data are mean ± SD ($n = 3$), *$p < 0.01$ versus POBN, #$p < 0.01$ versus POBN/COX-2. Inserts shows typical EPR spectra of COX-2-generated adducts of POBN with (a) carbon-centered radicals and (b) inhibitory effect of 2 mM NA.

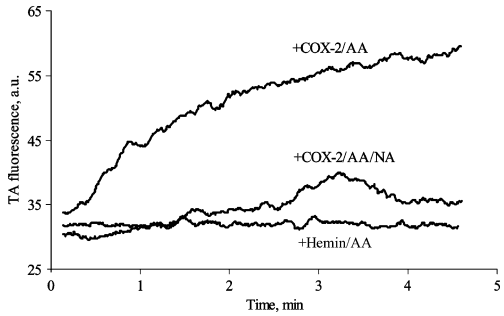

Fig. 4 COX-2-dependent AA-induced fluorescence response of Ac-Tempo in a cell-free system. Time course of Ac-Tempo (10 μM) fluorescence was recorded in the presence of reconstituted COX-2 (75 nM) and AA (20 μM). As a control, fluorescence was measured under the same conditions without COX-2. Note that NA (10 μM) completely inhibited Ac-Tempo fluorescence response. Data shown are representative of three independent experiments.

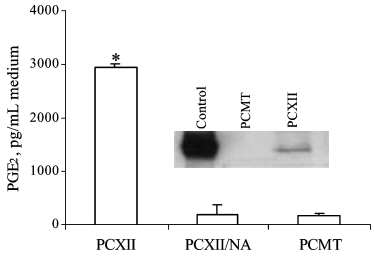

Fig. 5 Expression of COX-2 protein in IPTG-induced PCXII and PCMT cells. COX-2 activity was assessed by PGE2 production using a STAT-PGE2 EIA kit according to the manufacturer's instructions. PCXII and PCMT cells were incubated with AA (30 μM) in serum-free medium for 30 min at 37°C after IPTG-induction, levels of PGE2 released into the medium of treated cells were measured. NA (10 μM) was applied 30 min before AA addition. Amounts of PGE2 are expressed as pg of PGE2 produced/mL of medium. Data are mean ± SD ($n = 3$). *$p < 0.01$ compared with PCMT cells or PCXII cells treated with NA. Insert is the western blot analysis of COX-2 protein. Equal amounts of protein (20 μg) were loaded to each lane. Recombinant COX-2 protein was used as a positive control.

$a^N = 15.8/15.4$ G and $a^H = 2.7$ G characteristic of POBN adducts with carbon-centered radicals (POBN/°CR) (Buettner 1987) was observed (Fig. 3). EPR signal did not emerge when AA and POBN were incubated with either apo-enzyme or hemin (data not shown). In addition, a specific COX-2 inhibitor, NA, completely blocked formation of carbon-centered (pentyl) radical-POBN adducts, indicating that this radical was generated exclusively due to the COX-2 enzymatic activity. Formation of oxygen radicals, superoxide anion radicals or hydroxyl radicals, has not been detected using POBN. Qualitatively similar results were obtained using another spin-trap, DMPO, instead of POBN: only adducts of carbon-centered radicals were observed upon incubation of heme-reconstituted COX-2 with AA (data not shown). Moreover, coincubation of COX-2 and AA with GSH, one of the major intracellular reductants, did not yield any POBN adducts with sulfur-centered (thiyl) radicals.

These findings were further confirmed by the results obtained with a fluorescence-based assay of radicals using Ac-Tempo, a conjugate of fluorescent acridine and a stable nitroxide radical Tempo. Ac-Tempo is a non-fluorescent compound, as its stable radical Tempo moiety quenches fluorescence of the acridine moiety. The fluorescence can be regained upon adduct formation between the nitroxide moiety (Tempo) and carbon-centered radicals, which eliminates the quenching effect, as it was described previously for similar compound proxyl fluorescamine (Blough and Simpson 1988). We found that incubation of Ac-Tempo with reconstituted COX-2 and AA induced pronounced fluorescence response while hemin and AA did not produce fluorescence (Fig. 4). This effect was significantly diminished by NA in much the same fashion as it was observed with POBN.

COX-2-associated free radical production and oxidative stress in PCXII and PCMT cells

COX-2 expression in PCXII and PCMT cells

PCXII cell line stably expressing COX-2 and PCMT cell line transfected with empty vector were treated with 2.5 mM IPTG for 24 h to determine the changes of COX-2 protein expression. Western blots showed that a significant amount of COX-2 protein was expressed in IPTG-pre-treated PCXII cells while essentially no COX-2 protein was present in

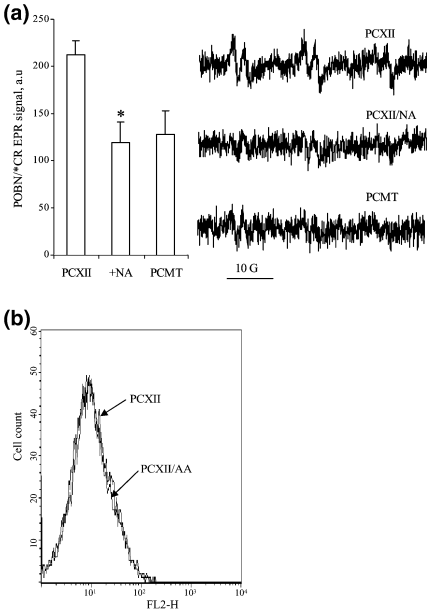

Fig. 6 (a) COX-2-dependent AA-induced production of carbon-centered radicals in PCXII and PCMT cells. PCXII and PCMT cells were collected at the end of IPTG-induction, and then resuspended in 60 μL (1.5×10^6 cells) of 500 mM POBN (in PBS) for 5 min. The samples were immediately used for EPR measurements after addition of AA (600 μM). Spectra were recorded under the following instrumental conditions: 3350 G, center field; 50 G, sweep width; 1 G, field modulation; 10 mW, microwave power; 0.03 s, time constant. Data are mean ± SD ($n = 3$). *$p < 0.05$ compared with control PCXII cells. Spectra demonstrated are representative of three independent experiments. (b) Detection of superoxide anion in PCXII cells. Cells were pre-incubated with 2.5 μM of DHE for 30 min at 37°C before addition of 30 μM AA. Cells were harvested after additional 30 min incubation. Oxidation of DHE was determined by monitoring the fluorescence with a 585-nm bandpass filter (FL2 Channel) using FACScan. Data shown are representative of four independent experiments.

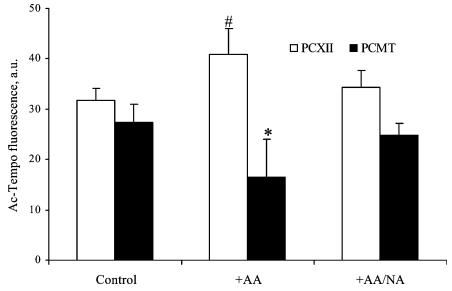

Fig. 7 COX-2-dependent AA-induced fluorescence of Ac-Tempo in PCXII (□) and PCMT (■) cells. IPTG pre-treated cells were incubated in PBS with Ac-Tempo (100 μM) and AA (20 μM). To inhibit COX-2 activity, NA (10 μM) was applied. Control cells were incubated with Ac-Tempo in the absence of AA. An immediate fluorescence microscopy analysis was performed after 10 min incubation. For each sample, at least 200 cells were analyzed. Data are mean ± SD ($n = 3$), # $p < 0.05$ compared with control PCXII cells. *$p < 0.05$ compared with PCXII cells under the same condition.

PCMT cells (Fig. 5, insert). PGE2 is a primary product of the COX-2-catalyzed reaction, and its accumulation reflects the activity of the enzyme. After 30 min exposure to AA (30 μM), the level of produced PGE2 was remarkably higher in IPTG-treated PCXII cells (2937.9 ± 135 pg/mL medium) than in PCMT cells (156.1 ± 39 pg/mL medium; Fig. 5). The difference could be completely eliminated by NA (176.3 ± 63.4 pg/mL medium).

Detection of COX-2-generated free radicals in PCXII and PCMT cells

To evaluate COX-2-catalyzed production of free radicals in cells, PCXII and PCMT cells pretreated with IPTG (24 h) were incubated with the spin-trap, POBN, and exposed to AA. Addition of exogenous AA (600 μM) to PCXII cells caused appearance of EPR signal corresponding to a carbon-centered POBN adduct with the same characteristics as those observed with reconstituted COX-2 in the cell-free system (Fig. 6a). Carbon-centered radical production in PCXII cells was sensitive to a specific COX-2 inhibitor – only trace EPR signal emerged in the presence of NA. In PCMT cells, AA-induced EPR signal was hardly detectable. As in the cell-free system, no oxygen radicals or thiyl radicals were detected in AA-stimulated PCXII cells. To further attempt to detect oxygen radical production, we utilized a sensitive fluorogenic probe, DHE, which has been recently recommended as a specific reagent for superoxide anion. Generation of superoxide anion was assessed by monitoring the red fluorescence of DHE oxidation products using flow cytometry. No fluorescence increase was observed in either AA-challenged PCXII (Fig. 6b) or PCMT cells (data not shown).

Detection of free radicals in cells by spin-trapping technique required relatively high concentrations of AA. To detect radicals at biologically more relevant conditions we employed a more sensitive fluorescence method. Cells were loaded with a fluorogenic conjugate of a stable nitroxide radical Tempo with acridine (Ac-Tempo) which is reactive towards carbon-centered radicals (Blough *et al.* 1988), and analyzed by fluorescence microscopy. Relatively weak fluorescence was observed from both PCXII and PCMT cells after treatment with Ac-Tempo alone (31.7 ± 2.5 a.u and 27.4 ± 3.6 a.u., respectively; Fig. 7). Presumably, non-specific reduction of nitroxide-functionality of Tempo to corresponding hydroxylamine accounts for this fluorescence. Co-incubation of PCXII cells with Ac-Tempo and AA (20 μM) increased fluorescence to a level of 40.8 ± 5.2 a.u., that was significantly higher than that of PCXII cells incubated with Ac-Tempo alone ($p < 0.05$) or that of PCMT cells under the same conditions (16.5 ± 7.6 a.u). Treatment with NA resulted in a decreased fluorescence response (34.3 ± 3.4 a.u) from AA-treated PCXII cells suggesting the

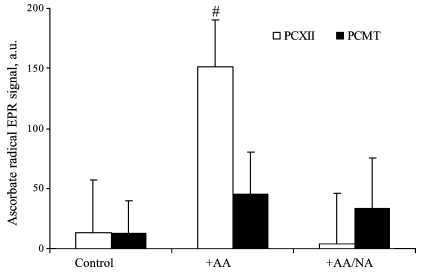

Fig. 8 COX-2-dependent AA-induced generation of ascorbate radicals in PCXII (□) and PCMT (■) cells. Spectra of ascorbate radicals were recorded upon addition of AA (100 μM) to cell homogenates (5×10^6 cells/mL) in the presence of ascorbate (500 μM). Spectra recorded from samples without addition of AA were used as controls. Note that production of ascorbate radicals in PCXII cells was completely inhibited by NA (100 μM). Data are means ± SD ($n = 3$), #$p < 0.05$ compare with control PCXII cells.

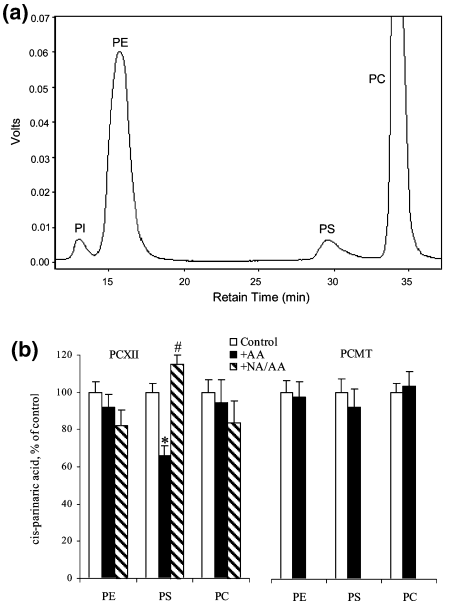

Fig. 9 (a) A typical HPLC chromatography. (b) COX-2-dependent AA-induced membrane phospholipid peroxidation in PCXII and PCMT cells. Cells were labeled with *cis*-PnA after IPTG-induction, and then exposed to AA (30 μM, 30 min). Total lipids were extracted and resolved by fluorescence-HPLC. In an experiment with COX-2 inhibitor, cells were pre-incubated with NA (10 μM) for 30 min before AA addition. PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. Data are means ± SD ($n = 3$). *$p < 0.05$ compared with control PCXII cells, #$p < 0.05$ compared with AA-treated PCXII cells.

involvement of COX-2. In contrast, fluorescence response from Ac-Tempo-treated PCMT cells was not significantly different from that elicited by Ac-Tempo/AA-treated PCMT cells or Ac-Tempo/AA/NA-treated PCMT cells.

Generation of ascorbate radical

Because cell culture media do not usually contain ascorbate we added exogenous ascorbate to freshly prepared homogenates of PCXII and PCMT for assessment of ascorbate radical production using EPR assay. Ascorbate radical EPR signals of equal magnitudes were detected in PBS in the absence and in the presence of cell homogenates. This background signal originated from the radicals formed during oxidation of ascorbate catalyzed by metal impurities. Incubation of ascorbate with PCXII cell homogenates in the presence of AA resulted in a pronounced increase of EPR signal amplitude (151.3 ± 39.4 vs. 13.2 ± 44.4 a.u. in control, $p < 0.05$) after subtraction of background (Fig. 8). NA completely abolished AA-induced radical generation (4 ± 42.4 a.u., $p < 0.05$). Only a slight statistically insignificant increase in AA-induced ascorbate radical production was observed in PCMT cell homogenates.

COX-2-enhanced membrane phospholipid oxidation

To examine whether COX-2 activity contributes to oxidative stress, we studied the effect of exogenous AA on membrane phospholipid peroxidation in PCXII and PCMT cells. Cell phospholipids were metabolically labeled with oxidation-sensitive fluorescent fatty acid, *cis*-PnA, and the content of fluorescently labeled individual phospholipid classes was determined by HPLC with a fluorescence detector (Fig. 9a). Lipid peroxidation was calculated as oxidation-induced loss of fluorescence compared to control. As demonstrated in Fig. 9(b), addition of AA to PCXII cells induced oxidation of one of the aminophospholipids, phosphatidylserine (PS), which was significantly different from control cells ($p < 0.05$). Importantly, PS oxidation was completely blocked by NA. In contrast, AA did not exert any significant oxidation of membrane phospholipids in PCMT cells.

Although our assessments of PnA-labeled phospholipid oxidation sensitively and specifically detected that PnA-PS underwent AA-induced COX-2-catalyzed oxidation, our measurements did not provide any information on whether PS was actually a direct substrate of the reaction. To further address this issue, we compared human recombinant COX-2-dependent accumulation of hydroperoxides in the presence of AA, 2-AG, and AA-PS all containing the same oxidizable arachidonyl residue. The lipid hydroperoxides are quantified by a fluorometric assay using Amplex Red reagent based on their quantitative microperoxisase-catalyzed reaction. After incubation of lipids with COX-2, we found that both AA and 2-AG acted as COX-2 substrates and caused increased accumulation of lipid hydroperoxides (2.9-fold and 1.8-fold higher than that in the absence of COX-2, respectively; Fig. 10a). Interestingly, formation of hydroperoxides in PS was dramatically decreased when incubated with COX-2 ($p < 0.001$ compared with PS alone; Fig. 10a). However, when AA-PS was co-incubated with free AA in the presence of COX-2, formation of hydroperoxides was 4.2-fold

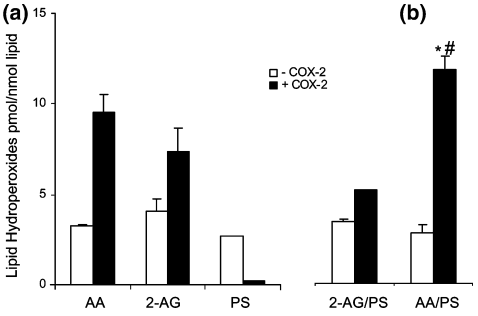

Fig. 10 Human recombinant COX-2-catalyzed co-oxidation of PS. (a) Effect of COX-2 (0.2 μM) on the formation of lipid hydroperoxides in AA (82.5 μM), 2-AG (39.6 μM), and AA-PS (39.6 μM). (b) Effect of COX-2 (0.2 μM) on the formation of lipid hydroperoxides in AA-PS in the presence AA or 2-AG. Lipid hydroperoxides were assessed by determining the formation of resorufin in peroxidase-catalyzed reaction of specific lipid hydroperoxides with a fluorogenic substrate, Amplex Red, using HPLC. Data are mean ± SD ($n = 3$). *$p < 0.01$ compared with AA/PS in the absence of COX-2, #$p < 0.05$ compared with AA/COX-2; □, – COX-2, ■, + COX-2.

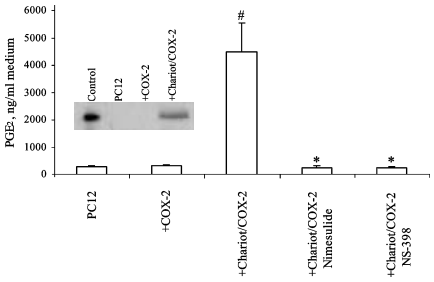

Fig. 11 Transfection of PC12 cells with heme-reconstituted COX-2 protein using Chariot. PC12 cells (0.4×10^6/well) were incubated with Chariot (6 μL)/COX-2 (1 μg) complex according to the manufacturer's instructions. Cells were washed with serum-free F12 medium twice and collected at the end of transfection. As a negative control, cells were also incubated with COX-2 (without Chariot) under the same conditions. COX-2 activity in cells was determined by PGE2 production as described above. Specific COX-2 inhibitor – nimesulide (100 μM) or NS-398 (10 μM) – was applied 30 min before AA addition. Data are means ± SD ($n = 3$), #$p < 0.01$ compared with control PC12 cells. *$p < 0.01$ compared with chariot/COX-2-transfected PC12 cells. Insert is the western blot analysis of COX-2 protein in PC12 cells. Reconstituted COX-2 was used as a positive control.

($p < 0.01$) higher that in the absence of COX-2 (Fig. 10b), and importantly, 1.3-fold ($p < 0.05$) higher than AA/COX-2. Overall, these results clearly demonstrate that PS is not effectively utilized by COX-2 as its substrate but is rather co-oxidized in the presence of AA.

Free radicals production and oxidative stress in PC12 cells transfected with Chariot/COX-2

To further confirm the contribution of COX-2 to free radical generation and oxidative stress, we developed a COX-2-transfection model in which we delivered reconstituted COX-2 protein into PC12 cells using Chariot, a protein transfection reagent. It should be noted that the efficiency of protein transfection varied considerably from one experiment to another. In a representative successful experiment, Western blot analysis demonstrated the presence of COX-2 in transfected PC12 cells (incubated with Chariot and COX-2 protein), but not in control PC12 cells or PC12 cells that were incubated with reconstituted COX-2 protein in the absence of Chariot (Fig. 11, insert). We next examined COX-2 activity by evaluating the PGE_2 production. Figure 9 shows that PGE2 production was more than 10 times higher in transfected cells compared to control cells, which was inhibitable by COX-2 inhibitor nimesulide or NS-398. There was no significant difference in PGE2 production between PC12 control cells and cells incubated with reconstituted COX-2 protein in the absence of Chariot.

Despite variations in the efficiency of transfection, we were able to detect formation of carbon-centered radicals in PC12 cells successfully transfected with COX-2, similarly to PCXII cells. The results of one of such experiments are presented in Fig. 12. No EPR signal was detected when AA was added to PC12 cells in the presence of POBN. Addition

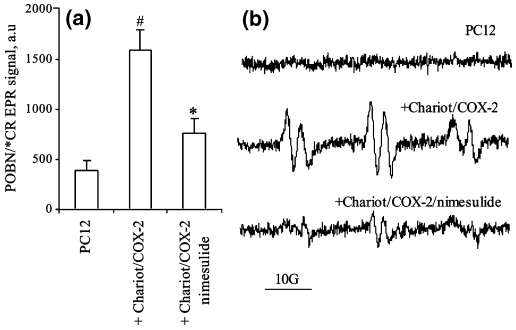

Fig. 12 COX-2-dependent AA-induced generation of carbon-centered radicals in PC12 cells transfected with Chariot/COX-2. PC12 cells were washed and collected at the end of transfection, then resuspended in POBN (60 μL, 100 mM in PBS) at the density of 1.5×10^6/60 μL. AA (100 μM) was added 5 min later, and EPR spectra were recorded immediately. In experiment with COX-2 inhibitor, cells were pre-incubated with nimesulide (100 μm) for 5 min before addition of AA. (a) Data are means ± SD ($n = 3$), #$p < 0.05$ compared with control PC12 cells, *$p < 0.05$ compared with COX-2-transfected cells; (b) EPR spectra of carbon-centered radicals in PC12 cells transfected with Chariot/COX-2 obtained in one of representative successful transfection experiments.

of AA to Chariot/COX-2-transfected PC12 cells induced a distinct EPR signal of POBN adducts with carbon-centered (pentyl) radicals. This signal was approximately twofold lower in the presence of nimesulide, a specific inhibitor of COX-2. Cells incubated with COX-2 without Chariot or cells treated with Chariot but without COX-2 did not

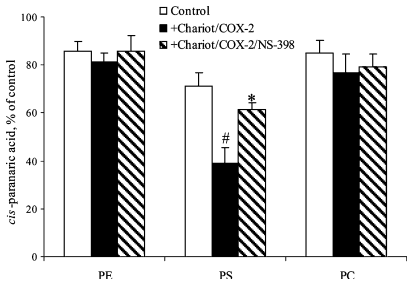

Fig. 13 COX-2-dependent AA-induced phospholipid peroxidation in PC12 cells transfected with Chariot/COX-2. PC12 cells were first labeled with *cis*-PnA, labeled cells were then transfected with Chariot/COX-2. At the end of transfection, AA (30 μM) was added to labeled cells (30 min). Total lipids were extracted and resolved by fluorescence-HPLC. As a control, PC12 cells were incubated with AA under the same conditions. In an experiment with COX-2 inhibitor, transfected cells were incubated with NS-398 (10 μM) for 30 min before AA addition. Data are means ± SD ($n = 3$), #$p < 0.05$ compared with control PC12 cells, *$p < 0.05$ compared with Chariot/COX-2-transfected cells.

generate any EPR signal under the same conditions (data not shown).

Membrane phospholipid oxidation was then evaluated as a biomarker of oxidative stress in COX-2-transfected PC12 cells. As shown in Fig. 13, AA stimulation of COX-2-transfected cells caused remarkable oxidation of PS (down to 40% of its level in control cells, $p < 0.01$). No AA-induced PS oxidation was observed in non-transfected cells. Moreover, AA-induced lipid peroxidation could be attenuated by a COX-2 inhibitor, NS-398, suggesting that AA induced oxidative stress was associated, at least in part, with COX-2 enzymatic activity.

Discussion

Excessive generation of free radicals and subsequent oxidative stress are believed to be a common feature of the brain cell injury during a number of neurodegenerative diseases as well as brain trauma and ischemia–reoxygenation (Love 1999; Chan 2001; Perry *et al*. 2002; Beal 2003). The specific mechanisms predominantly responsible for the massive production of ROS, however, may differ significantly in these disease conditions. Understanding these specific mechanisms as the major source of oxidative stress is critical to development of effective mechanism-based antioxidant strategies and interventions. Among the most common mechanisms for excessive free radical generation, nonenzymatic iron-catalyzed Fenton-type reactions, as well as enzymatic mechanisms involving lipoxygenases, COX, peroxidases, NADPH oxidase, and xanthine oxidase are said to be important for brain injury (Mishra and Delivoria-Papadopoulo 1999).

Cyclo-oxygenases catalyze the conversion of AA to prostaglandins and thromboxane via a cycle including oxygenation of AA to the hydroperoxy endoperoxide (PGG2; cyclo-oxygenase part of the cycle) followed by the reduction of PGG2 to the alcohol (peroxidase part of the cycle). Detailed mechanistic studies have identified a carbon-centered pentadienyl radical at C-11 and a carbon-centered radical at C-8 as the two radical species involved in endoperoxide formation during the cyclo-oxygenase part of the cycle and a tyrosyl radical derived from Tyr385 during the peroxidase part of the cycle (Marnett 2000). According to proposed schemes of the COX catalytic cycle, oxygen is not activated by the enzyme and oxygen radicals are not formed during COX-catalyzed reactions (Marnett 2000). Paradoxically, a common fallacy in the neurobiology literature is the notion that COX-catalyzed metabolism is accompanied by the production of oxygen radicals (Chan and Fishman 1980; Kontos *et al*. 1980; Armstead *et al*. 1998) and that oxygen radical scavenging enzymes (SOD, catalase) can be protective against COX-induced oxidative stress (Horakova *et al*. 1997; Armstead 2003). Partly, these misconceptions are due to possible co-oxidation of intracellular reductants on radical centers generated during COX catalytic cycle. For example, co-oxidation of NADH, NADPH, and GSH during COX-catalyzed reaction may lead to the secondary production of oxygen radicals and thiyl radicals (Kukreja *et al*. 1986; Schreiber *et al*. 1989). In the current study, we attempted to identify free radical species generated by COX-2-catalyzed metabolism of AA in physiologically relevant conditions using PC12 cells in which we manipulated COX-2 levels by either stably transfecting them with a vector expressing IPTG-inducible COX-2 or transiently transfecting them with heme-reconstituted COX-2 recombinant protein. We utilized different versions of spin-trapping techniques to identify free radical species, as well as highly sensitive and specific fluorescence HPLC protocol to characterize oxidative stress in phospholipids induced in these cells upon activation of COX-2 by AA.

In our initial experiments, we used a spin-trap, POBN, known to form specific adducts with carbon-, sulfur-, and oxygen radicals. In a cell-free system containing heme-reconstituted COX-2 and AA, only characteristic six line spectra of POBN adducts with carbon-centered (pentyl) lipid radicals were detected, while no oxygen or thiyl radicals were produced under these conditions. Association of the adduct formation with the COX-2-catalyzed metabolism of AA was confirmed by the inhibitory effect of NA, a known COX-2 inhibitor. These results are in line with previous reports from several laboratories on the production of carbon-centered radicals by prostaglandin synthase-catalyzed metabolism of AA in cell-free systems (Mason *et al*. 1980; Schreiber *et al*. 1986; Tsai *et al*. 1998).

Using another spin-trap, DMPO, similar results were obtained thus further demonstrating that carbon-centered lipid (pentyl) radicals were the only radical species generated in COX-2/AA system. Finally, generation of carbon-centered

radicals was corroborated by the results obtained by a fluorescence-based assay with Ac-Tempo. COX-2/AA induced pronounced fluorescence response inhibitable by NA in much the same fashion as it was observed with POBN providing strong evidence for the production of carbon-centered radicals in the system. A significant increase of COX-2-induced EPR signal of ascorbate radical observed upon addition of ascorbate to COX-2/AA over hemin-induced and control signals clearly demonstrated that COX-2-catalyzed carbon-centered radicals can co-oxidize intracellular reductants such as ascorbate potentially yielding secondary radicals. Therefore, we further determined whether these secondary radicals are formed in physiologically relevant environments. To this end, we established two different COX-2-transfected rat pheochromocytoma PC12 cell models, PCXII cells, stably expressing IPTG inducible COX-2, and PC12 cells, transiently transfected with a recombinant and heme-reconstituted COX-2 protein using a protein delivery reagent, Chariot.

Although the COX-2 tyrosyl radical was EPR detectable in our cell-free system, EPR detection of AA-induced tyrosyl radicals is not feasible under physiological conditions due to its relatively short half life [$\sim$ 1 min at 0°C (Tsai *et al*. 1999)], low intracellular concentrations of COX-2, and the presence of reductants capable of reducing the tyrosyl radicals in cells.

Using spin-trapping with POBN, we were able to detect COX-2/AA-catalyzed production of carbon-centered lipid (pentyl) radicals in PCXII cells, but not in PCMT cells (lacking COX-2). Incubation with a COX-2 inhibitor, NA, blocked the radical generation, indicating that its production was associated with COX-2-catalyzed metabolism of AA. Most importantly, neither oxygen radical adducts nor sulfur-centered radical adducts were observable in PCXII cells treated with AA. Fluorescence measurements of superoxide using DHE also failed to detect any AA-induced oxygen radical production in PCXII cells. Increase of Ac-Tempo fluorescence in PCXII cells, but in PCMT cells, further confirmed the AA-induced generation of carbon-centered radicals which was sensitive to a COX-2 inhibitor, NA. We next took advantage of a protein transfection reagent, Chariot, to deliver recombinant COX-2 protein into PC12 cells within a short period of time (2 h) to cause only minimal (if any) effects on the gene expression profile in the cells to identify COX-2/AA-generated radical species. In this case too, we found that carbon-centered radical POBN-adducts were detected only in Chariot/COX-2-transfected PC12 cells after AA stimulation. Notably, no oxygen radicals or S-centered radicals have been observed in COX-2-transfected cells.

To determine whether secondary radicals could be generated by COX-2/AA-dependent mechanisms, we added exogenous ascorbate to PCXII cell homogenates and observed marked COX-2/AA-dependent production of ascorbate radicals. These data suggest that interaction of physiological reductants with carbon-centered radicals generated by COX-2/AA can be accompanied by their co-oxidation resulting in the formation of the secondary radicals. However, in spite of high levels of reductants in PC12 cells [e.g. millimolar concentrations of GSH (Rice and Russo-Menna 1998) and high micromolar concentrations of reduced pyridine nucleotides, NADH + NADPH (Tsunawaki and Nathan 1986)] they do not effectively interact with COX-2/AA-generated radicals. The importance of these results is emphasized by the known formation of oxygen radicals and enhancement of oxidative stress during co-oxidation of NADPH and GSH. One-electron oxidation of NADPH is known to result in autocatalytic production of oxygen radicals (Griendling *et al*. 2000). GSH is also a potential co-oxidation substrate of COX-2 that can be oxidized in one-electron fashion to GS which in turn, reacts with another GSH molecule to form GSSG, a reducing radical readily and effectively donating electron to molecular oxygen to generate superoxide (Taylor *et al*. 2003). Importantly, no thiyl-radicals or superoxide radicals have been detected when GSH was added to the COX-2/AA model system. Most notably, neither of these radicals was detected in both of our PCXII and COX-2 protein-transfected PC12 cells. Combined, these results indicate that carbon-centered radicals were likely the only radical species generated in PC12 and PCXII cells by COX-2 in the presence of AA.

In contrast to PC12 cell cultures grown *in vitro*, brain contains a large pool of ascorbate where its concentration can be as high as 10 mM. These levels of ascorbate may be sufficient to quench the reactive COX-2 intermediates and prevent the release of radicals and propagation of oxidative stress. In fact, our experiments demonstrated that ascorbate, one of the most potent sacrificial antioxidants in cells and biological fluids can, indeed, fulfill this role. AA-induced production of ascorbate radicals provides direct evidence for the ability of ascorbate to regulate COX-2-dependent oxidative stress as has been previously reported by others (Buettner and Jurkiewicz 1993) and us (Tyurin *et al*. 2000). Hypoxic conditions have been reported to decrease ascorbate levels in the brain (Awasthi *et al*. 1997). This suggests that ascorbate protection of COX-2-induced oxidative stress may be compromised under hypoxic conditions.

We further investigated whether COX-2/AA-generated radicals were able to cause oxidative stress in cells without involvement of oxygen radical or thiyl radical formation. Because carbon-centered radicals were the only radical species detected, we were particularly interested in studying phospholipid peroxidation as polyunsaturated fatty acid residues of membrane phospholipids may be particularly susceptible to damage by these radicals. High content of polyunsaturated fatty acid residues in brain phospholipids makes them likely substrates of oxidative reactions (Halliwell and Chirico 1993; Markesbery 1997). Using our

sensitive fluorescence HPLC-based technique with metabolic labeling of phospholipids with oxidation-sensitive polyunsaturated *cis*-PnA, we were able to demonstrate that phospholipids in COX-2-transfected PCXII cells (but not in COX-2-negative PCMT cells) underwent significant oxidation upon treatment with AA. Interestingly, the oxidation was not random among different classes of phospholipids but mainly and most significantly involved PS. This PS oxidation was almost completely eliminated by a COX-2 inhibitor, NA, revealing its dependence on COX-2. Similarly, in PC12 cells transiently transfected with heme-reconstituted COX-2, AA also induced phospholipids peroxidation. Again, PS was the only significantly peroxidized phospholipid, and its peroxidation was preventable by COX-2 inhibitor, NS-398.

Because PS in PC12 cells is represented by numerous individual molecular species different in their fatty acid composition making mass spectrometry assessments ambiguous, we opted to utilize a different approach in which we compared COX-2-dependent accumulation of hydroperoxides in the presence of AA, 2-AG, and AA-PS all containing the same oxidizable arachidonoyl residue. After incubation of the lipids with COX-2, we utilized a new protocol that we have developed for analysis of lipid hydroperoxides based on their quantitative microperoxidase-catalyzed reaction with Amplex Red, whose oxidation product, resorufin, was analyzed by fluorescence HPLC. We demonstrated that PS was not effectively utilized by COX-2 as its substrate but was rather co-oxidized in the presence of good substrates such as AA in cell-free system. These results suggest that oxidation of PnA-PS in cells was likely due to its co-oxidation during AA-induced COX-2-dependent metabolism. Apoptosis of neuronal cells is the major cause of their massive damage and death during neurodegenerative diseases as well as after trauma and ischemia/reoxygenation episodes. Interestingly, our previous work has established that selective peroxidation of PS is characteristic of intrinsic apoptosis induced by a number of exogenous and endogenous toxic compounds (Kagan *et al*. 2003). This PS oxidation acts as an important signaling mechanism facilitating externalization of PS on the surface of apoptotic cells and acting as an 'eat-me-signal' for phagocytes thus contributing to effective clearance of apoptotic cells (Kagan *et al*. 2003). Selective PS oxidation during intrinsic apoptosis is catalyzed by peroxidase activity of cytochrome *c* released from mitochondria into the cytosol (Jiang *et al*. 2003). It is temping to speculate that COX-2 may play a similar role in catalyzing selective PS oxidation as a part of execution of apoptotic program. It has been shown, however, that COX-2 functioned as an antiapoptotic protein and suppressed caspase-3 activation in PC12 cells when apoptosis was induced by the removal of nerve growth factor following differentiation (McGinty *et al*. 2000). It should be noted that PS oxidation and externalization signaling has not been characterized in a model of trophic withdrawal apoptosis and may function separately from the final common pathway for apoptosis. Further studies are necessary to elucidate the role of COX-2/AA selective PS oxidation in apoptotic neuronal signaling and its role in clearance of apoptotic neuronal cells by microglial cells.

Generation of ROS is believed to be characteristic of neuronal cell damage, particularly during hypoxia/reoxygenation, and ROS are considered to be one of the major determinants of ischemic brain death (Chan 2001; Kontos 2001; Adibhatla *et al*. 2002; Kirkland *et al*. 2002). A number of potential intracellular sources for ROS during ischemia/reoxygenation have been proposed (Ikeda and Long 1990). It has been demonstrated that the oxygen-dependent conversion of AA to prostanoids by COX concomitantly forms superoxide radicals (McGowan *et al*. 1994; Armstead *et al*. 1998). For instance, Armstead *et al*. (1998) has reported indomethacin-inhibitable postischemic generation of superoxide anion in newborn pig brain; McGowan *et al*. (1994) found that indomethacin decreased superoxide formation and lipid peroxidation during reperfusion following cerebral hypoxia-ischemia. These data are obviously in conflict with the present results. One possible explanation is that ROS production may be realized secondarily through the reaction products of the COX-2 enzymatic pathway. Takadera *et al*. (2002) reported that one of the COX-2 reaction products, PGE2, acting via an EP2-like receptor, induces apoptosis in neurons that may contribute to the progression of the neuronal injury (Li *et al*. 1995; Du *et al*. 1996; Endres *et al*. 1998). Disruption of mitochondria during COX-2-associated apoptosis is a likely source of ROS production as has been established for a number of different cells (Cai and Jones 1998). Indeed, triggering of intrinsic apoptotic pathways in AA-challenged cells is likely to be accompanied by downstream ROS generation due to disruption of mitochondrial electron transport and dissipation of membrane potential (Toborek *et al*. 1999; Garrido *et al*. 2001). These important consequences of COX-2-dependent AA-triggered secondary events, usually occurring at later time points (hours later after AA challenge) were not in the immediate focus of the current study. In fact, the only apoptosis-related marker that we reported was AA-induced oxidation of PS that occurred 30 min after treatment of COX-2-transfected cells. It should be noted, however, that PS signaling is not always directly associated with the major common pathway of apoptosis and can be dissociated from it without affecting successful execution of other parts of apoptotic program (Tyurina *et al*. 2004).

Our data demonstrate that production of oxygen radicals is not necessary for the induction of oxidative stress via COX-2/AA-dependent pathway. Generation of carbon-centered radicals is sufficient to cause phospholipid peroxidation. Additional experiments are needed to reveal the link between COX-2-mediated mechanisms of oxidative stress and other damaging pathways including mitochondrial injury.

Acknowledgements

This study was supported by grants NS 37459 and NS 30318 from National Institute of Health, the International Neurological Science Fellowship Program (F05 NS 43922) administered by National Institute of Health, National Institute of Neurological Disorders and Stroke in collaboration with World Health Organization, and Russian Foundation for Basic Research (010448844).

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