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Age-Related Increase of Brain Cyclooxygenase Activity and Dietary Modulation of Oxidative Status

Bong Sook Baek,[1,2] Jung Won Kim,[1,2] Ji Hyeon Lee,[2] Hyun Joo Kwon,[1,2] Nam Deuk Kim,[1,2] Ho Sung Kang,[2] Mi Ae Yoo,[2] Byung Pal Yu,[3] and Hae Young Chung[1]

[1]College of Pharmacy and [2]Research Institute of Genetic Engineering, Pusan National University, Pusan, South Korea.
[3]The University of Texas Health Science Center at San Antonio, Texas, USA.

Several studies have demonstrated that inhibitors of cyclooxygenase (COX) attenuate various neuronal injuries and age-dependent demented conditions. From these findings, we proposed to test the effect of age on COX activity and its possible suppression by the antiaging action of dietary restriction in the rat brain. The status of reactive oxygen species (ROS) was also assessed to correlate with COX activity to delineate the underlying mechanism of the altered COX activity during aging. These results showed that COX activity significantly increased in 24-month-old rats compared with 6-month-old rats in an ad libitum group. Interestingly, mRNA and protein levels of COX-2 showed little corresponding age-related change. The formation of ROS was found to increase gradually with age in ad libitum fed rats. However, dietary restriction suppressed the increase at the age of 24 months. To substantiate the relationship between ROS and COX activity when the rats were 24 months of age, we conducted in vitro experiments with a C6 glioma cell line. Together, it is concluded that increased COX activity with age is due to the activation of COX catalytic reaction by ROS without increased gene expression of COX-2 and that it is related to the increased pro-oxidant status in aged rats.

CYCLOOXYGENASE (COX) is the rate-limiting enzyme in prostaglandin (PG) synthetic pathway, which converts arachidonic acid to various PG metabolites (1). Two isoforms of COX have been identified in eukaryotic cells: constitutively expressed COX-1 (2,3) and inducible COX-2 (4,5). COX-1 is expressed in most tissues and is responsible for maintaining prostanoids at physiological levels (1). In contrast, COX-2 that shows undetectable levels under normal physiological conditions can be induced by lipopolysaccharides and cytokines (6). COX-2 catalyzes high levels of prostanoid synthesis under various pathological conditions. For example, in the brain, the induction of COX-2 is pathologically responsible for an increased prostanoid production (7). In astrocytes and microglial cells, COX-2 has often been implicated in neurodegenerative diseases (7–9) and cerebral inflammatory processes (10,11).

Several studies have shown that COX is activated by hydroperoxides for the initiation of the arachidonic acid cascade pathway (12,13), and recently nonradical peroxynitrite has been reported to be an effective initiator of COX activity (14); this suggests that a variety of oxidants may be physiological activators of COX.

Recent studies on the modulation of reactive oxygen species (ROS) reactions by dietary restriction (DR) as an antiaging measure have established that this dietary intervention can attenuate the age-related inflammatory process (15) and oxidative stress (16–18). For instance, the production of superoxide, hydroxyl radicals, and H_2O_2 and the lipid peroxidation of mitochondrial and microsomal membranes are all suppressed by DR (16). In addition, the decline of antioxidant defense and detoxification systems during aging has been effectively modulated by DR (17,18). From these findings, Yu (19) proposed the broad antioxidative action exhibited by DR as the underlying mechanism of its life-prolonging effect.

However, to date, it is not known whether DR attenuates the production of ROS and PG, and whether it modulates COX activity in the brain. Thus, the aim of this study was to investigate changes in COX activity with age and the modulation by DR. We quantified ROS generation to correlate with COX activity and its mRNA gene expression in the cerebrums of aged rats. To substantiate these findings in brain tissue, we further examined the in vitro effect of various oxidants on COX activity in C6 glioma cells.

Materials and Methods

Materials and Chemicals

2′,7′-dichlorofluorescein diacetate (DCFDA) was purchased from Molecular Probes, Inc. (Eugene, OR). ^{32}P-adenosine triphosphate (250 μCi) and an enzyme immunoassay (EIA) kit for prostaglandin E_2 were purchased from Amersham (Bucks, UK). RNAzol B was obtained from TEL-TEST, Inc. (Friendwood, TX). Primers for reverse transcription polymerase chain reaction (RT-PCR) were synthesized by Bioneer (Daejeon, Korea). Primary and secondary antibodies for Western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Serotec

(Oxford, England), respectively. Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Bedford, MA).

Animals and Diet

Specific pathogen-free (SPF) male Fischer 344 rats from the colony of the National Institute on Aging, part of the National Institutes of Health, were raised at the University of Texas Health Science Center at San Antonio and were fed a diet of the following composition: 21% soybean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15% L-methionine, 0.2% choline chloride, 5% salt mix, 2% vitamin mix, and 3% Solka-Floc (International Fiber Co., North Tonawanda, NY). All rats were fed ad libitum (AL) until 6 weeks of age, and then they were divided into two groups: an AL-fed control group and a DR group. Rats in the DR group were restricted by 40% of the food intake of the AL control group. Cerebrums from rats of 6, 12, 18, and 24 months of age were used in this study. Detailed information and procedures on dietary modulation and the maintenance of the SPF status of animals have been published by Yu and colleagues (20).

Tissue Preparation

Rats were sacrificed by decapitation, and the brains were immediately dissected to isolate the cerebrums. Isolated rat cerebrums were homogenized in seven volumes of ice-cold 50mM phosphate buffer at a pH of 7.4; the buffer contained 0.5mM phenylmethylsulfonyl fluoride, 1mM ethylenediamine tetra-acetic acid, 80 mg/L trypsin inhibitor, and 1μM leupeptin. It was centrifuged at 900 × *g* at 4°C for 15 minutes. The supernatants were centrifuged at 12,000 × *g* at 4°C for 15 minutes to yield pelletized mitochondrial fractions, and these supernatants were referred to as postmitochondrial fractions.

Cyclooxygenase Activity

COX activity was measured by a modified method of Bos and colleagues (21). The activity was determined by the quantification of prostglanden E_2 (PGE_2). Briefly, postmitochondrial fractions were incubated in 50mM phosphate buffer (pH 7.4) containing 0.6μM arachidonate at 37°C for 30 minutes. Then, the mixture was boiled for 3 minutes to terminate the reaction, followed by centrifugation at 10,000 × *g* for 15 minutes. PGE_2 in supernatant was assayed for the measurement of COX activity with an EIA kit.

Measurement of COX mRNA

Total RNA was prepared by using RNAzol B (2 ml/100 mg of tissue) according to the manufacturer's instruction. After the first strand cDNA was synthesized from 2 μg of total RNA by reverse transcription, polymerase-chain reaction was performed with the cDNA. The primer pairs used for COX-1 detection (22) were sense, 5′-CTGCATGTGGCTGATGTCATC-3′ and antisense, 5′-AGGACCCGTCATCTCCAGGGTAATC-3′, and those for COX-2 (22) were sense, 5′-CAAGCAGTGGCAAAGGCCTCCATT-3′ and antisense, 5′-TAGTCTGGAGTGGGAGGCACTTGC-3′. The expected product sizes for COX-1 and COX-2 were 441 and 474 base pairs, respectively. The mRNA levels of constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as positive control. The primer pairs for GAPDH were sense, 5′-GGGTGATGCTGGTGCTGAGTATGT-3′ and antisense, 5′-AAGAATGGGAGTTGCTGTTGAAGTC-3′. The GAPDH primer set yields a PCR product of 700 base pairs in length. PCR conditions were 33 cycles for COX-1, 35 cycles for COX-2, and 20 cycles for GAPDH, respectively, consisting of 30-second denaturation at 94°C, 30-second annealing at 54°C, and 1-minute extension at 72°C. Electrophoresis was performed in 1.5% agarose gel. After the gel was stained with ethidium bromide, the band was visualized and quantified under a transilluminator.

Western Blotting

Western blotting was carried out by the method of Habib and colleagues (23). Briefly, total protein equivalents (60 μg) for each sample were separated by sodium dodecyl sulfate (SDS)-polyacrylamide minigel electrophoresis and transferred to a PVDF membrane at 15 V for 1.5 hours in a semidry transfer system. Nonspecific binding to the membrane was blocked by 1% nonfat milk blocking buffer of 10mM Tris (pH 7.5), 100mM NaCl, and 0.1% Tween 20 at room temperature for 1 hour. The membrane was washed and then probed with the polyclonal goat antibody against COX-1 or COX-2 (Santa Cruz Biotechnology, 1:200) overnight at 4°C. Bands were visualized by using horseradish peroxidase-conjugated donkey antigoat antibody (Serotec, 1:1000) and enhanced chemiluminescence assay (Amer-

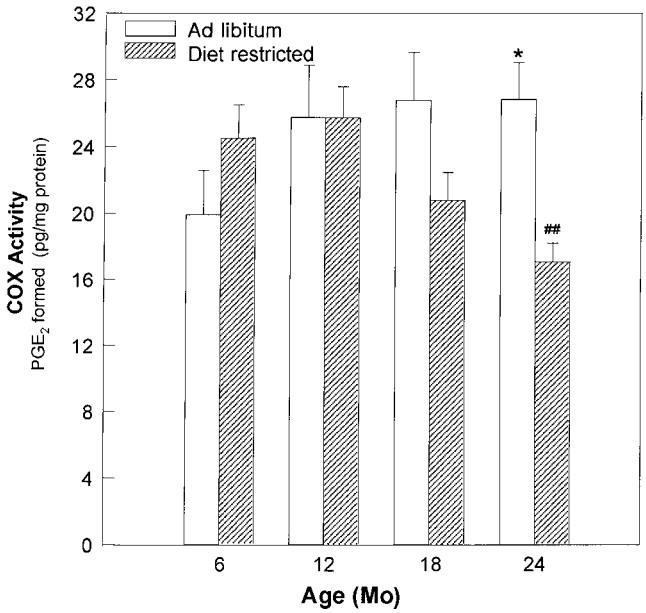

Figure 1. Effects of age and dietary restriction on cyclooxygenase (COX) activity in cerebrums. Each value is the mean ± SE of six rats. Statistical significance: *$p < .05$ vs 6 month-old rats of the ad libitum group, ##$p < .01$ vs 24-month-old rats of the ad libitum group. PGE_2 = prostaglandin E_2.

sham). Prestained blue protein markers (Biorad, Hercules, CA) were used for molecular weight determination.

Measurement of ROS

ROS generation was measured by the method of Thomas and colleagues (24) as previously described. Briefly, 25μM DCFDA was mixed with postmitochondrial fractions isolated from cerebrums and incubated at 37°C. Changes in fluorescence intensity were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by the Fluorescence Plate Reader (BIO-TEK Instruments, Winooski, VT) for 30 minutes. The concentrations of dichlorofluorescein (DCF) formed were calculated according to a DCF standard calibration curve.

PGE_2 Production by Glioma Cells

Glioma cells were plated in six-well plates and incubated with various oxidants, H_2O_2, $\cdot O_2^-$, *t*-butyl hydroperoxide (*t*-BHP), hydroxynonenal (HNE), hydroxyhexenal (HHE), and malondialdehyde (MDA) for 6 hours. The generation of $\cdot O_2^-$ was done by the use of pyrogallol (25). The collected supernatants from the culture medium were applied for determination of PGE_2 with an EIA kit (21).

Statistical Analysis

Results were analyzed statistically by post hoc tests of the statistical package Super ANOVA. Values of $p < .05$ were considered statistically significant.

Results

Effects of Age and DR on COX Activity

The COX activity of AL-fed rats showed a significant and age-related increase, more pronouncedly in 24-month-old rats than in 6-month-old rats (Figure 1). It is worth noting that the increase of COX activity in AL-fed rats was in parallel to the increased ROS generation at 24 months of age, as shown Figure 4 below. In contrast, DR suppressed the increase at 24 months of age.

Modulation of COX Gene Expression by Age and DR

As a way to measure mRNA levels of COX, both COX-1 and COX-2, total RNA extracts were analyzed by the RT-PCR method. As shown in Figure 2, the mRNA expression of the constitutive COX-1 and inducible COX-2 showed little change either with age or by DR.

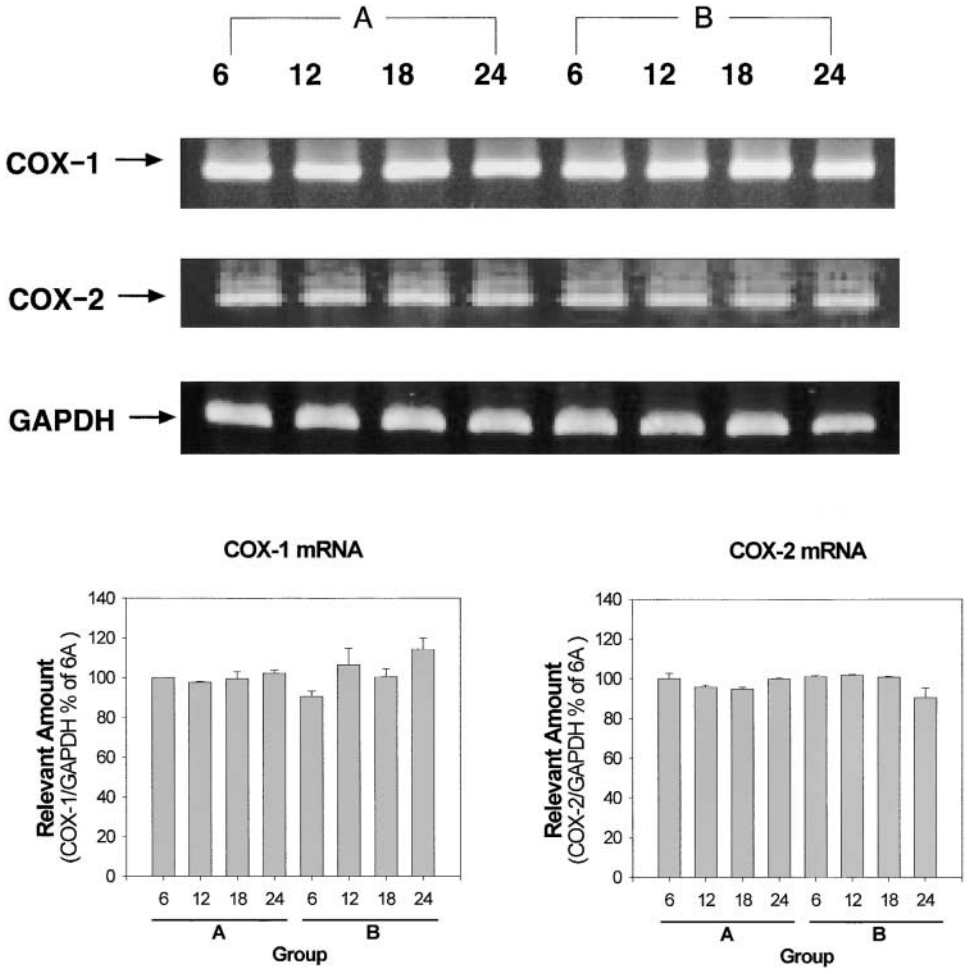

Figure 2. Effects of age and dietary restriction on the mRNA levels of cyclooxygenase (COX)-1 and COX-2; A is the ad libitum fed group and B is the diet-restricted group. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; 6A = 6-month-old rats of ad libitum group.

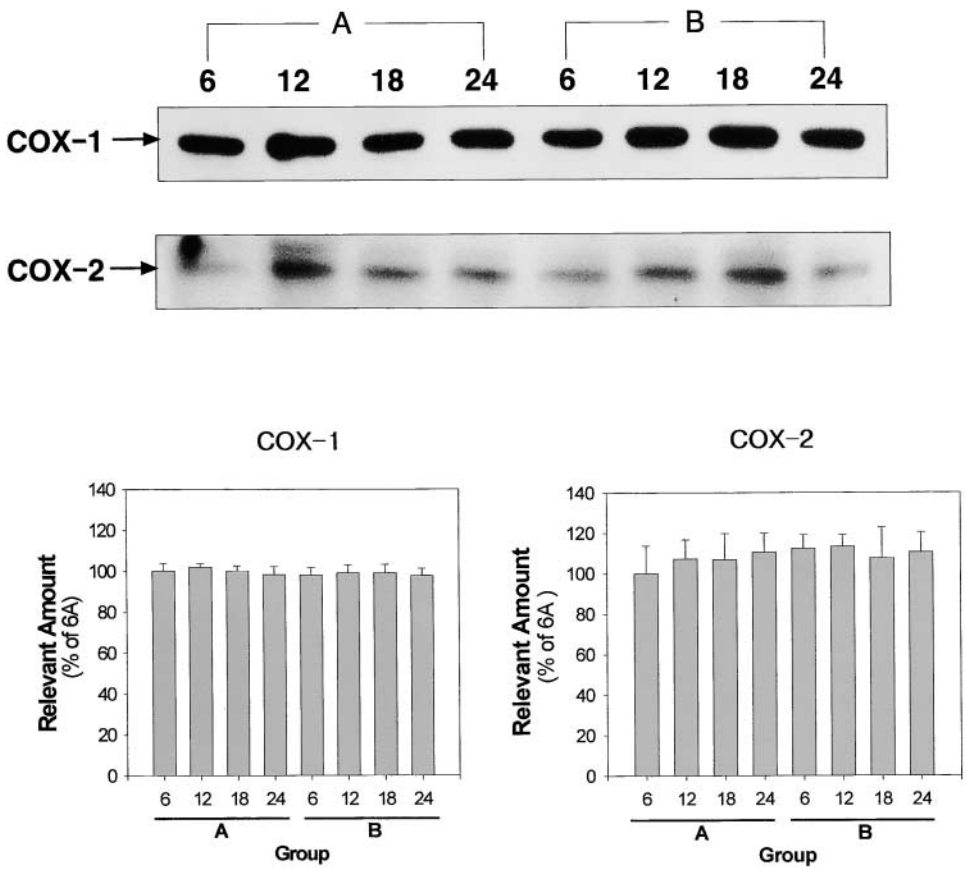

Figure 3. Effects of age and dietary restriction on the protein level of cyclooxygenase (COX)-1 and COX-2; A is the ad libitum fed group and B is the diet-restricted group. 6A = 6-month-old rats of ad libitum group.

As a way to examine whether COX-2 activity was modulated at the protein synthesis step, Western blotting was carried out. The COX-2 protein levels were similar to mRNA levels (Figure 3), indicating that the age-associated increase of COX-2 activity was not accompanied by newly synthesized COX protein. Therefore, it is concluded that the age-related increase in COX activity is most likely due to post-translational modulation, the catalytic activation of the COX-2 molecule per se rather than the involvement of increased COX-2 gene expression.

ROS Generation in the Aging Process

Because the increase of COX activity with age may be affected by changes in oxidative status, we examined ROS generation with age and its suppression by DR (Figure 4). Data clearly showed that the ROS formation increased with age in AL-fed rats, whereas the antioxidative DR consistently suppressed this age-dependent increase (Figure 4).

Effect of Oxidants on COX Activity in C6 Glioma cells

As a way to obtain further clues on the nature of ROS on the COX activation observed in the rat brain tissue, C6 glioma cells were challenged in vitro with various pro-oxidant reactive species. For this purpose, H_2O_2, $\cdot O_2^-$, *t*-BHP, HNE, HHE, and MDA were used. As shown in Figure 5, the COX activity measured by PGE_2 production was significantly stimulated by H_2O_2 (two-fold), *t*-BHP (six-fold), and $\cdot O_2^-$ (1.5-fold), whereas HNE, HHE, and MDA provided no discernable stimulation. These results are consistent with evidence, albeit indirect, that the age-related increase of COX activity might closely be related to the increased levels of oxygen-derived ROS during aging.

Discussion

Increased COX activity and PGE_2 production have been implicated as active participants in the pathogenesis of several age-related diseases, such as cancer, Alzheimer's disease, and autoimmune diseases (26). More specifically, COX-2 is shown to be associated with acute exudative inflammation, granuloma formation, bone resorption, and pain in rheumatoid arthritis (27). These evidences clearly indicate a significant role of COX-2 in these age-related pathophysiological processes.

In the present study, we obtained data for the first time that cerebral COX activity significantly increases in 24-month-old rats compared with 6-month-old rats. More significantly, we were able to show that DR rats suppress the increases found at 24 months. As a way to further define the molecular insights on whether this increased activity was modulated at a gene level, COX mRNA and protein levels were analyzed. Our data showed no indication that the amounts of either COX mRNA or protein were involved.

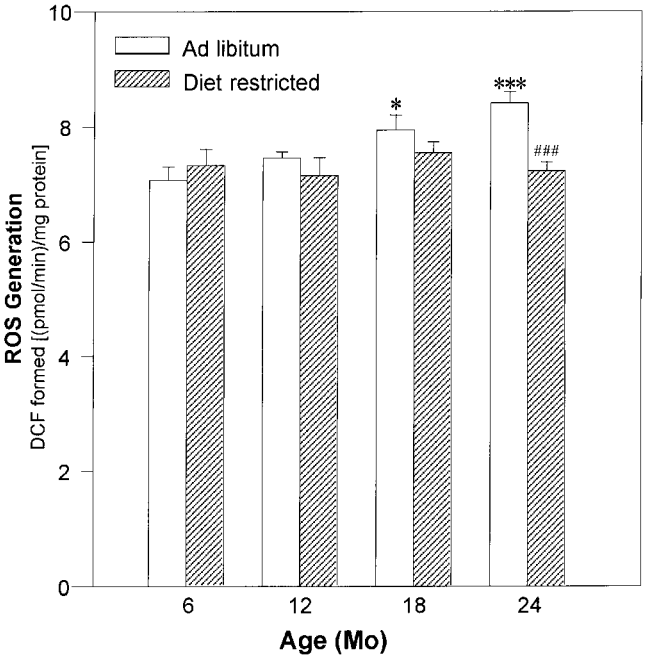

Figure 4. Effects of age and dietary restriction on reactive oxygen species (ROS) generation in postmitochondrial fraction of cerebrum. Each value is the mean ± SE of six rats. Statistical significance: *$p < .05$; ***$p < .001$ vs 6-month-old rats of the ad libitum group; ###$p < .001$ vs 24-month-old rats of the ad libitum group. DCF = dichlorofluorescein.

Thus, it is concluded that the increase of COX activity and its suppression by DR observed is due to the posttranslational modulation responsible for the increase in COX activity rather than the involvement of increased COX-2 gene expression. Our results are in line with those of Wu and colleagues (28), who showed the age-associated increase in COX activity and PGE_2 production of macrophage without a change of COX gene expression. The influence of redox status including various pro-oxidants on COX activity has been shown by several studies (29). For instance, some antioxidants and antioxidant enzymes such as glutathione peroxidase can inhibit COX activity (29). These studies indicated that the full activation of COX under pathophysiological conditions may require the presence of pro-oxidants as possible regulators. In this context, it is worth pointing out that Kulmacz and Wang (30) showed that COX-2 activation is more receptive to ROS activation than COX-1. Thus, it can be speculated, based on our present data and others, that prostanoid hydroperoxides synthesized from the arachidonate cascade can exacerbate the inflammatory condition by further activation of COX-2. This possibility is consistent with previous findings on age-related increased proinflammatory processes often observed with age and the attenuation by the antiaging action of DR (31,32).

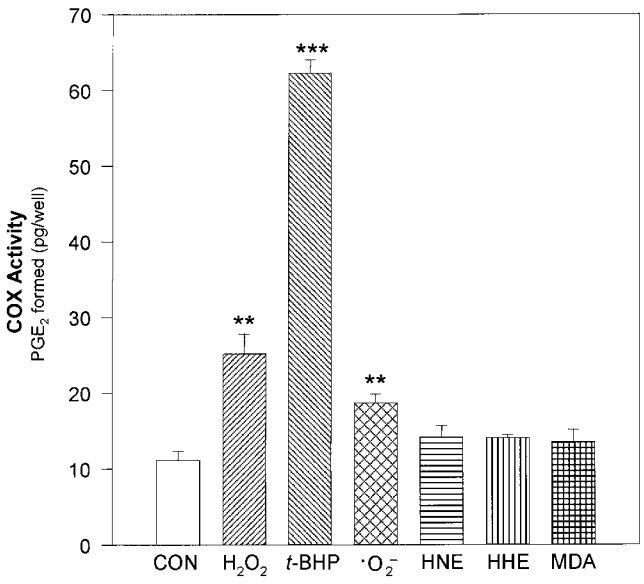

Figure 5. Effects of various reactive oxygen metabolites on cyclooxygenase (COX) activity in C6 glioma cells. Each value is the mean ± SE of three samples. Statistical significance: **$p < .01$; ***$p < .001$ vs control. PGE_2 = prostaglandin E_2; CON = control; *t*-BHP = *t*-butyl hydroperoxide; HNE = hydroxynonenal; HHE = hydroxyhexenal; MDA = malondialdehyde.

In summary, our results showed that COX activity increase with age, and this age-associated increase in COX activity is due to COX activation by ROS rather than induced gene expression of COX-2. This was confirmed in vitro by showing the stimulating effect of ROS on COX activity in C6 glioma cells. Furthermore, we were able to establish that DR can reverse the increase found when the rats were at 24 months of age, suggesting a mechanistic basis for the DR's known anti-inflammatory efficacy.

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Address correspondence to Hae Young Chung, Department of Pharmacy, College of Pharmacy, Pusan National University, Gumjung-ku, Pusan 609-735, Korea. E-mail: hyjung@hyowon.pusan.ac.kr

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