Gene Expression of Cyclooxygenase in the Aging Heart

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Cyclooxygenase (COX) is the key rate-limiting enzyme in the prostaglandin synthetic pathway. Two isoforms of COX have been identified: a constitutive COX-1 and an inducible COX-2, which is activated in response to various stimuli. We investigated the changes of COX-1 and COX-2 in rat heart during aging. We measured the age-related changes in the mRNA and protein levels of COX by using reverse-transcription polymerase chain reaction and Western blotting, respectively. COX-2 mRNA and protein levels increased with age, whereas those of COX-1 showed no change. The COX activity determined by prostaglandin E_2 production increased with age. Because the COX-catalyzed arachidonate cascade is an important source of reactive oxygen species (ROS) generation, changes in ROS generation and lipid peroxidation were also assessed. The amount of ROS generated by the COX pathway increased with age, as did the total ROS generation and lipid peroxidation. These results show that COX-2 activity increases with age, partially because of elevated transcriptional expression and protein content, and they suggest that increased COX-2 can play a role in oxidative alterations in the aged heart.

T HE oxidative stress theory of aging proposes that the progressive deterioration and time-associated changes observed during aging are the cumulative result of continual reactive oxygen species (ROS) production occurring in the course of normal cellular metabolism (1,2). Under normal conditions, the mitochondria are considered the major loci for free radical production, because most of the oxygen-derived species arise during mitochondrial respiration (3). Although less explored, the arachidonate cascade, in which cyclooxygenase (COX) is the key rate-limiting enzyme, could be a potentially important intracellular source of ROS, particularly under various pathological conditions (4).

Prostaglandins (PGs), produced by diverse cell types, modulate a variety of physiological and pathophysiological processes (5). COX is the key rate-limiting enzyme in the conversion of arachidonic acid to PGs (6). COX exists in two isoforms: cyclooxygenase-1, or COX-1 (7–9), a constitutive isoform located in the endoplasmic reticulum that is responsible for normal prostaglandin production, and a mitogen-inducible cyclooxygenase-2, or COX-2, located both in the endoplasmic reticulum and in the nuclear membrane (10). COX-2 is expressed highly upon appropriate stimulation with proinflammatory agents, such as lipopolysaccharides (11) and cytokines (12).

The arachidonate cascade catalyzed by COX contributes to ROS generation in two ways: first, oxygen radical generation during the catalytic conversion of prostaglandin G_2 to H_2 (13) and second, PG-facilitated oxidative alterations of the inflammatory process (14). Previous work from this laboratory (15) and others (16) showed that aging leads to elevated oxidative damage in the heart. The source and oxidative processes of the ROS have not been defined. One possibility that has not been investigated is that an age-related activation of COX may contribute to the appearance of oxidation stress in the aging heart. The aim of the present study was to determine whether changes occur in the COX activity of the two isoforms that could contribute to the age-related oxidative damage to the heart.

MATERIALS AND METHODS

Tissue Preparation

Hearts from male, specific pathogen free, Fischer 344 rats that were raised in the barrier facilities at the Department of Physiology in San Antonio, TX, were used in the study. Complete descriptions of the housing, care, and feeding of the animals have been reported elsewhere (17). At 6, 12, 18, and 24 months of age, the rats were decapitated, their chests were opened, and their hearts were quickly excised and immersed in ice-cold isotonic saline. Six rats per age group were used in the study. The atria were removed. The ventricles were rapidly frozen between aluminum tongs cooled to the temperature of liquid nitrogen and stored at that temperature until assayed.

The ventricles were individually homogenized with a polytron homogenizer in 7 vol (vol/wt) of ice-cold homogenization solution of the following composition: 50 mM phosphate buffer (pH 7.4), 0.5 mM phenylmethylsulfonyl

fluoride (PMSF), 1 mM ethylenediamine tetra-acetic acid (EDTA), 80 mg/l of trypsin inhibitor, and 1 μ M leupeptin. In order to obtain a mitochondrial fraction, the homogenate was centrifuged at 900× g for 15 minutes at 4°C, and the supernatant was centrifuged at 12,000× g for 15 minutes at 4°C. The supernatant was regarded as a postmitochondrial fraction, and the pellet was resuspended in homogenization buffer as a mitochondrial fraction.

Cyclooxygenase Activity

The COX activity was measured in the postmitochondrial fraction. Forty microliters (~ 0.3 mg of protein) of the supernatant fraction were incubated in the presence of 0.6 μ M arachidonate at 37°C for 30 minutes. At the end of the incubation period, the mixture was boiled for 3 minutes and centrifuged for 15 minutes at 10,000× g. The prostaglandin E₂ content of the supernatant was assayed by using a commercially available enzyme immunoassay kit (Amersham, Bucks, UK) (18).

Reverse-Transcription Polymerase Chain Reaction

Preparation of RNA.-RNA samples were isolated from three rat tissues per each age. Tissue samples were homogenized in the presence of RNAzol B (2 ml/100 mg of tissue; Tel-Test, Inc., Friendwood, TX) with a Polytron homogenizer. Chloroform (0.2 ml) was added to 2 ml of homogenate. The samples were covered tightly, shaken vigorously for 15 seconds, and placed on ice for 5 minutes. The suspension was centrifuged at $12,000 \times g$ at 4°C for 15 minutes. The aqueous phase was transferred to a fresh tube, an equal volume of isopropanol was added, and the sample was cooled to 4°C for 15 minutes. Samples were centrifuged a second time as above. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing. After a final centrifugation at $7500 \times g$ at 4°C for 8 minutes, the pellet was dried for 10-15 minutes and dissolved in diethylpyrocarbonate (DEPC)-treated water.

Reverse transcription.—The first strand cDNA was synthesized from 2 μ g total RNA. DEPC-treated water and 250 ng of random primer were added, and the mixture was incubated at 75°C for 5 minutes. The mixture was placed on ice for 5 minutes. Aliquots containing 2 μ l of 0.1 M dithiothreitol (DTT), 4 μ l of 5× buffer, 4 μ l of 2.5 mM deoxynucleoside triphosphate (dNTP), 100 U of reverse transcriptase (Gibco-Bethesda Research Laboratory, Gaithersburg, MD) and 16.5 U of ribonuclease (RNase) inhibitor were added and incubated at 37°C for 2 hours. The reaction was stopped by boiling at 100°C for 2 minutes, and the cDNA was stored at -20°C until use.

Preparation of radiolabeled primer.—One microgram of sense primer, 1 µl of 10× T4 kinase buffer, 10 U of T4 kinase, 2.5 µl of γ -³²P-adenosine triphosphate (10 µCi/µl, 3000 Ci/mmol), and distilled water were mixed, incubated at 37°C for 1 hour, and then left at 75°C for 10 minutes. DEPC-treated water, 8 M ammonium acetate, and ethanol were added and left at -80°C for 1 hour. The mixture was centrifuged at 12,000× g for 25 minutes and the supernatant was discarded. Ethanol (75%) was added to the pellet and the mixture was centrifuged for an additional 5 minutes. The supernatant was discarded; the pellet was dried for 20 minutes and then dissolved in DEPC-treated water.

Polymerase chain reaction.—The primer pairs for COX-2 (19) were as follows: sense, 5'-CAAGCAGTGGCAAAG-GCCTCCATT-3'; antisense, 5'-TAGTCTGGAGTGGGA-GGCACTTGC-3'. For COX-1, the primer pairs were as follows: sense, 5'-CTGCATGTGGCTGATGTCATC-3'; antisense, 5'-AGGACCCGTCATCTCCAGGGTAATC-3'. The expected product sizes for COX-1 and COX-2 are 441 and 474 base pairs (bp), respectively.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used in separate polymerase chain reactions to control for the efficiency of cDNA synthesis in each sample. The primer pairs were as follows: sense, 5'-GGGT-GATGCTGGTGCTGAGTATGT-3'; antisense, 5'-AAGA-ATGGGAGTTGCTGTTGAAGTC-3'. The GAPDH primer set yields a polymerase chain reaction (PCR) product of 700 bp in length.

As a way to carry out the PCR, 25 μ l of PCR master mix was added to each tube directly. Fifty nanograms of sense primer and 35 ng of antisense primer were used per reaction. Deoxynucleotides were added to a final concentration of 0.2 mM; 10× PCR buffer (Perkin Elmer, Gaithersburg, MD) was diluted to a ratio of 1:10. Taq DNA polymerase (Promega, Madison, WI; 1.25 U) and ³²P-labeled primer (0.25 μ l) was added to each tube. Reaction conditions consisted of 38 cycles for rat COX-2, 33 cycles for rat COX-1, and 20 cycles for GAPDH of 94°C for 30-second denaturation, 54°C for 30-second annealing, and 72°C for 1-minute extension. Electrophoresis was performed in 5% polyacrylamide gel. After suitable separation was achieved, the gel was vacuum dried for autoradiography and exposed to Fuji x-ray film.

Western Blotting

Samples were boiled for 5 minutes with gel loading buffer, consisting of 0.125 M Tris-Cl, 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, pH 6.8, and 0.2% Bromphenol Blue, at a ratio of 1:1. Total protein equivalents for each sample were separated on a 10% SDSpolyacrylamide minigel by using a Laemmli buffer system and were transferred to a polyvinylidene difluoride membrane (Millipore, Hertfordshire) at 15 V for 1 hour in a semidry transfer system. The membrane was immediately placed in blocking buffer (1% nonfat milk) in 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20. The blot was allowed to block at room temperature for 1 hour. The membrane was incubated with specific rabbit polyclonal anti-COX-2 antibody (Oxford Biomedical, Oxford, MI; 1:2,000) or goat polyclonal anti-COX-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) for 1 hour at 25°C followed by a horseradish peroxidase-conjugated donkey antirabbit antibody (Amersham; 1:2,000) or antigoat antibody (Serotec, Kidlington, Oxford, England; 1:3,000) for 1 hour at 25°C. Antibody labeling was detected by using enhanced chemiluminescence (Amersham) per the manufacturer's instructions. Prestained blue protein markers were used for molecular weight determination (20).

Measurement of ROS

For total ROS generation in the postmitochondrial fractions, the various oxygen species, including superoxide, hydrogen peroxide, hydroxyl radicals, and lipid hydroperoxides, were quantified by using previously published methods (21–23), using the probe, 2',7'-dichlorofluorescein-diacetate (DCFDA). One hundred micrograms of protein were added to ice-cold potassium phosphate buffer (50 mM, pH 7.4) containing 25 μ M DCFDA. The final volume of the mixture was 250 μ l. The changes in fluorescence intensity were measured at 10, 15, 20, 25, and 30 minutes on a Fluorescence Microplate Reader (FL500, Bio-Tek Instruments), with excitation and emission wavelengths set at 485 nm and 530 nm, respectively.

For COX-derived ROS generation, quantitation of reactive species generation was carried out as described above, except that 1 mM indomethacin was included in the reaction mixture. The difference in ROS generation in the presence and absence of indomethacin was considered the COXderived ROS generation.

Lipid Peroxidation

Aliquots of the supernatant fraction were incubated in the presence of 0.5 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) for 30 minutes at 37°C. To the incubate was added 0.5 ml of the assay mixture containing 1.2% thiobarbituric acid (TBA) solution, 8.1% SDS solution, and 20% acetic acid at a ratio of 20:4:30, respectively. The reaction mixture was boiled at 94°C for 30 minutes and cooled. The TBA reactive substance (TBARS) was extracted by butanol and the entire mixture was centrifuged for 10 minutes at 900× g. The fluorescence intensity of the butanol fraction was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm (24).

Protein Assay

The concentration of protein was measured by Lowry's method; bovine serum albumin was used as a standard (25).

Materials

The DCFDA was from Molecular Probes, Inc. (Eugene, OR). The γ -³²P-ATP (250 μ Ci) and enzyme immunoassay kit for the thromboxane B₂, prostaglandin E₂, and leukotriene B₄ were purchased from Amersham. RNAzol B was obtained from Tel-Test. Primers for reverse-transcription PCR (RT-PCR) were synthesized by Bioneer (Daejeon, Korea). Antibodies of COX-1 and COX-2 for Western blotting were obtained from Santa Cruz Biotechnology and Oxford Biomedical, respectively. Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Bedford, MA). Other chemicals were purchased from Sigma (St. Louis, MO).

Statistical Analysis

Results were analyzed statistically by a one-factor analysis of variance. Differences between the means of individual groups were assessed by the Fischer's Protected LSD post hoc Test.

RESULTS

COX activity, measured as the production of prostaglandin E_2 , increased with age (Figure 1). The activity at 24 months of age was significantly higher than that observed at 6 months of age. As a way to determine whether the increase in COX activity was due to enhanced gene expression of COX-2 or of COX-1, their mRNA and protein levels were examined, using RT-PCR and Western blotting, respectively. Aging was associated with an increase in the mRNA for COX-2 (Figure 2A) and an elevation in the COX-2 protein content (Figure 2B). At 6 and 12 months of age, very low levels of COX-2 mRNA were present, but significant levels were detected at 18 and 24 months of age. The mRNA and protein levels of COX-1 remained unaffected by aging (Figures 3A and 3B), indicating that the age-related increase in COX activity is due to higher COX-2 expression.

The effect of age on total ROS generation measured by the DCF method is shown in Figure 4. ROS production significantly increased from 85.4 ± 12.0 nmol DCF (formed/ min)/mg protein at 6 months of age to 160.3 ± 12.3 nmol DCF (formed/min)/mg protein at 24 months of age. COXderived ROS production contributes to the age-related increase in the overall ROS production. COX-derived ROS production (Figure 5) increased from 23.92 ± 2.55 nmol DCF (formed/min)/mg protein at 6 months of age to $32.84 \pm$ 1.71 nmol DCF (formed/min)/mg protein at 24 months of age.

Comparable results were obtained for lipid peroxidation. The lipid peroxidation of the cardiac postmitochondrial fraction increased with age (Figure 6). The concentration of AAPH-induced TBARS rose from 2851 ± 246 (pmol/30 min)/mg protein at 6 months of age to 3740 ± 180 (pmol/30 min)/mg protein at 24 months of age.

DISCUSSION

COX plays a crucial role in the arachidonic acid cascade in a number of tissues and cells. However, little is known

Figure 1. Effects of age on cyclooxygenase activity in rat heart. Each value represents the mean \pm standard error for six rats. *p < .05 vs 6 months of age.





Figure 2. **A**, Effects of age on cardiac mRNA levels of COX-2. **B**, Changes of cardiac protein levels of COX-2. Rats of 6, 12, 18, and 24 months of age were used. COX-2 = cyclooxygenase-2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. **p < .01 vs 6 months of age.

Figure 3. **A**, Cardiac mRNA levels of COX-1 with age. **B**, Cardiac protein levels of COX-1. Rats of 6, 12, 18, and 24 months of age were used. COX-1 = cyclooxygenase-1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

about the expression and regulation of COX in the heart during aging. We observed a significant increase in COX-2 mRNA (Figure 2A), COX-2 protein (Figure 2B), and COX enzyme activity (Figure 1) in the heart with age. This indicates that the basis for the age-related changes in COX is at the level of gene expression. The possibility that the elevated concentration of COX-2 could also arise from a decline in the removal of the molecule cannot be ruled out. Because the mRNA for COX-1 did not change with age, in our study, it is reasonable to assume that changes in the expression of the mRNA for COX-1 appear not to play any significant role in normal aging process.

One salient finding of our study is that a substantial amount (\backsim 25%) of the total ROS generated in the supernatant fraction arises from COX activity (Figures 4 and 5). ROS generation in the heart increases with age (Figure 4) and a significant fraction of that increase arises from enhanced COX activity. As the aging heart suffers a substantial amount of oxidative damage (15,16) and according to the oxidative theory of aging (1,2), this damage that was ex-

acerbated by the increased ROS generated by means of a COX reaction could contribute significantly to the functional decline observed in aging organisms (26). The increase in the levels of lipid peroxidation shown in this study (Figure 6) further confirms this notion of enhanced oxidative stress in the aging heart (15,16). Much of the previous work in the aging heart utilized a mitochondria fraction. The salient finding in this study is that a nonmitochondrial fraction can contribute substantially to the increase in oxidative damage seen in the aged heart.

It is noteworthy that COX activity is regulated by lipid peroxides (27), the products of oxidative stress. Consequently, an increase in the concentration of lipid peroxides should enhance COX activity. From the point of view of a positive feedback phenomenon, the stimulatory role of the lipid peroxidation in inflammatory process and other tissue damage can be substantial in the aged organisms. Results from this study (Figure 6) and others (2,15) have shown that the degree of lipid peroxidation increases with age. It is possible that this increase leads to elevated COX activity, en-

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Figure 4. Effects of age on ROS generation in heart postmitochondrial fraction. Each value represents the mean \pm standard error for six rats. **p < .01 vs 6 months of age. DCF = 2'7'-dichlorofluorescein.

hanced ROS production, and finally greater lipid peroxidation—thus completing the positive feedback loop.

In summary, the present experiments demonstrate that the heart exhibits an increase in the COX-2 with age and this increase is associated with elevations in ROS. An age-related elevation in COX-2 activity may be due to an increased mRNA expression and higher levels of the enzyme itself.



Figure 5. Effects of age on cyclooxygenase-derived ROS generation in heart postmitochondrial fraction. Each value represents the mean \pm standard error for six rats. *p < .05 vs 12 months of age. DCF = 2'7'-dichlorofluorescein.



Figure 6. Effects of age on AAPH-induced lipid peroxidation in heart postmitochondrial fraction. TBARS, thiobarbituric acid reactive substance; AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride). Each value represents the mean \pm standard error for six rats. *p < .05 vs 6 months of age.

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References

- Yu BP. Aging and oxidative stress: modulation by dietary restriction. Free Radic Biol Med. 1996;21:651–668.
- 2. Harman D. The aging process. Proc Natl Acad Sci USA. 1981;78: 7124–7128.
- Yu BP, Yang R. Critical evaluation of the free radical theory of aging. A proposal for the oxidative stress hypothesis. *Ann NY Acad Sci.* 1996; 786:1–11.
- Demopoulos HB, Flamm ES, Pietronigro DD, Seligman ML. The free radical pathology and the microcirculation in the major central nervous system disorders. *Acta Physiol Scand.* 1980;492(suppl):91–119.
- Ristimaki A, Garfinkel S, Wessendorf J, Maciag T, Hla T. Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. *J Biol Chem.* 1994;269:11,769–11,775.
- Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem. 1996;271: 33,157–33,160.
- DeWitt DL, Smith WL. Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc Natl Acad Sci USA*. 1988;85:1216–1412.
- Merlie JP, Fagan D, Mudd J, Needleman P. Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). *J Biol Chem.* 1988;263:3550–3553.
- Yokoyama C, Takai T, Tanabe T. Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. *FEBS Lett.* 1988;231:347–351.
- 10. Morita I, Schindler M, Regier MK, et al. Different intracellular loca-

tions for prostaglandin endoperoxide H synthase-1 and -2. J Biol Chem. 1995;270:10,902-10,908.

- Fu JY, Masferrer JL, Seibert K, Raz A, Needleman P. The induction and suppression of prostaglandin H₂ synthase (cyclooxygenase) in human monocytes. *J Biol Chem.* 1990;265:16,737–16,740.
- Feng L, Xia Y, Garcia GE, Hwang D, Wilson CB. Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide. J Clin Invest. 1995;95:1669–1675.
- Demopoulos HB, Flamm ES, Pietronigro DD, Seligman ML. The free radical pathology and the microcirculation in the major central nervous system disorders. *Acta Physiol Scand.* 1980;492(suppl):91–119.
- 14. Nielsen OH. In vitro studies on the significance of arachidonate metabolism and other oxidative processes in the inflammatory response of human neutrophils and macrophages. With special reference to chronic inflammatory bowel disease. *Scand J Gastroenterol.* 1988; 150(suppl):1–21.
- Kim JD, Yu BP, McCarter RJ, Lee S, Herlihy JT. Exercise and diet modulate cardiac lipid peroxidation and antioxidant defenses. *Free Radic Biol Med.* 1996;20:83–88.
- Lee J, Yu BP, Herlihy JT. Modulation of cardiac mitochondrial membrane fluidity by age and calorie intake. *Free Radic Biol Med.* 1999; 26:260–265.
- Yu BP, Masoro EJ, McMahan CA. Nutritional influences on aging of Fischer 344 rats: I. Physical, metabolic, and longevity characteristics. *J Gerontol.* 1985;40:657–670.
- Bos ES, van der Doelen AA, van Rooy N, et al. 3,3',5,5'-tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. J Immunoassay. 1981;2:187– 204.
- 19. Feng L, Sun W, Xia Y, et al. Cloning of two isoforms of rat cyclooxy-

genase: differential regulation of their expression. Arch Biochem Biophys. 1993;307:361–368.

- Habib A, Creminon C, Frobert Y, Grassi J, Pradelles P, Maclouf J. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. *J Biol Chem.* 1993;268:23,448–23,454.
- Cathcart R, Schwiers E, Ames BN. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Biochem.* 1983;134:111–116.
- Cathcart R, Schwiers E, Ames BN. Detection of picomole levels of lipid hydroperoxides using a dichlorofluorescein fluorescent assay. *Methods Enzymol.* 1984;105:352–358.
- LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol*. 1992;5:227–231.
- Laganiere S, Yu BP. Antilipoperoxidation action of food restriction. Biochem Biophys Res Comm. 1987;145:1185–1191.
- Lowry DH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193:265–275.
- Kim SW, Yu BP, Sanderford M, Herlihy JT. Dietary restriction modulates the norepinephrine content and uptake of the heart and cardiac synaptosomes. *Proc Soc Exp Biol Med.* 1994;207:43–47.
- Hemler ME, Cook HW, Lands WE. Prostaglandin biosynthesis can be triggered by lipid peroxides. *Arch Biochem Biophys.* 1979;193:340– 345.

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