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COX-2 expression in cystic kidneys is dependent on dietary n-3 fatty acid composition☆

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Abstract

Dietary n-3 fatty acids generally attenuate elevated cyclooxygenase-2 (COX-2) levels in disease states. However, models of renal cystic disease (RCD) exhibit reduced renal COX-2 expression. Therefore, the in vivo regulation of COX-2 expression by dietary n-3 fatty acids was examined. In archived tissues from dietary studies, COX-2 protein and gene expression was up-regulated in diseased *pcy* mouse and Han:SPRD-*cy* rat kidneys when given diets containing eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA), but not those containing α-linolenic acid (ALA), compared to control diets with linoleic acid (LA). The presence of disease was necessary to elicit these effects as COX-2 expression was unaltered by diet in normal kidneys. The effects were specific for COX-2, since COX-1 levels were unaltered by these dietary manipulations in either model. Thus, in RCD, diets containing EPA and DHA but not ALA appear to specifically up-regulate renal COX-2 gene and protein levels in vivo.

Keywords: COX-2; Cystic kidney disease; n-3 fatty acids

1. Introduction

Cyclooxygenase (COX)-2 is one of the COX isoforms involved in the conversion of 20-carbon polyunsaturated fatty acids to physiologically active prostanoids. COX-1 and COX-2 have similar structures but distinct physiologic functions [1]. Initially, COX-1 was considered to be constitutively expressed and to have general "housekeeping" functions, while COX-2 was believed to be inducible and involved in inflammatory responses. However, evidence of both constitutive and inductive roles for both isoforms has been observed in the kidney [2].

The role of n-3 fatty acids in the modulation of COX-2 expression in vivo as well as in vitro has been studied in a number of experimental models of disease. Both long chain [α-linolenic acid (ALA), 18:3 n-3] and very long chain [eicosapentaenoic acid (EPA), 20:5 n-3; and docosahexaenoic acid (DHA), 22:6 n-3] n-3 fatty acids attenuate elevated COX-2 levels and activity in various cancer cell lines as well as animal models [3–9], in mice with mycotoxin-induced IgA nephropathy [10,11] and in Long-Evans Cinnamon rats with acute hepatitis [12]. In these models, attenuation of the disease-induced COX-2 elevation by n-3 fatty acids is associated with slower disease progression.

COX-2 expression is generally up-regulated with disease, as seen in inflammatory conditions, cancer and some nephritic models. However, in models of renal cystic disease (RCD), COX-2 levels are either down-regulated or not increased with renal disease [13,14]. Renal cystic disease is the most frequently inherited nephropathy affecting approximately 1 in 1000 people [15] and is characterized by abnormal cyst growth and renal interstitial inflammation and

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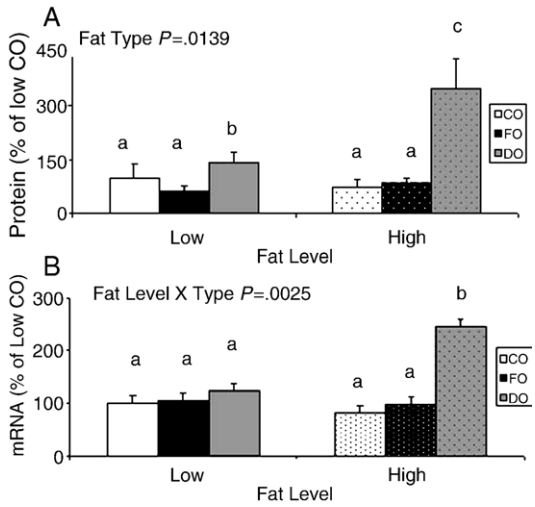

Fig. 1. Relative renal COX-2 protein, (A) and mRNA (B) levels in *pcy* mice given low (7 g fat/100 g diet) and high (20 g fat/100 g diet) CO, FO and DO diets for 8 weeks. Values are means (as percentage of low CO)±S.E.M. ($n=6$). Means with different alphabets denote significant differences ($P<.05$).

fibrosis which eventually lead to renal failure. Animal models of RCD are useful as experimental models of chronic kidney disease because they exhibit the pathology of chronic renal disease without the metabolic, autoimmune or endocrine alterations present in other models of renal disease. In addition, the effects of dietary n-3 fatty acids on disease progression in models of RCD vary according to the type of n-3 fatty acid and the model. Therefore, since RCD models exhibit reduced COX-2 expression with disease and distinct effects of diets containing different fatty acids, these models provide a unique opportunity to examine the regulation of COX-2 expression by dietary n-3 fatty acids in vivo.

2. Materials and methods

For this study, archived tissues from several previous studies conducted in our laboratory were utilized. The animal and experimental protocols were in accordance with the standards of the Canadian Council on Animal Care and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional committees on animal care and ethics. All animals were housed under temperature-, humidity- and light-controlled conditions. Diet ingredients, with the exception of flax (Omega Nutrition, Vancouver, BC, Canada for

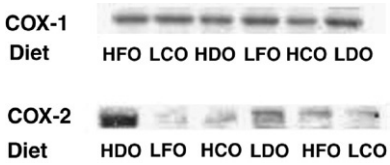

Fig. 2. Representative Western immunoblots of renal COX-1 and COX-2 protein expression in *pcy* mice given low (7 g fat/100 g diet) and high (20 g fat/100 g diet) CO, FO and DO diets for 8 weeks.

Study 3; Bioriginal Food Science and Corp., Saskatoon, SK, Canada for Study 1) and algal oils, were purchased from Dyets, Inc. (Bethlehem, PA, USA) and Harlan Teklad (Madison, WI, USA). Algal oil was a generous gift from Martek Biosciences Corporation (Winchester, KY, USA). All diets were based on AIN 93 guidelines for rodent diets and varied only in fat source and level in each study. The protocols of these studies are briefly described below.

2.1. Study 1

Weanling CD1-*pcy/pcy* (*pcy*) mice were obtained from our breeding colony that was established from mice provided to us by V.H. Gattone II [16]. *pcy* mice were randomly assigned to one of six experimental diets (4 weeks of age, $n=8$/diet group) for 8 weeks as described [17]. The diets contained three types of dietary oils, namely, corn (rich in n-6 LA), flaxseed (rich in n-3 ALA) and DHASCO (algal oil rich in n-3 DHA), at high (20 g of fat/100 g diet) and low (7 g of fat/100 g diet) levels in a 2×3 design. Diets are indicated as CO, containing corn oil, FO, containing flaxseed/corn oil (4:1, g/g), and DO, containing DHASCO/corn oil (4:1, g/g). The FO diet contained 45 g of ALA, while the DO diet contained 0.23 g of ALA and 31 g of DHA per 100 g of total fat. The CO diet contained 0.9 g of ALA and no EPA or DHA per 100 g of total dietary fat.

2.2. Study 2

Weanling Han:SPRD-*cy* rats [also known as PKD/Mhm (*cy*/+) rats] were obtained from our breeding colony that was established from rats provided to us by Dr. B.D. Cowley [18]. Rats were randomly assigned to either a high- or a low-fat diet containing 20 or 5 g of fat/100 g of diet using cottonseed oil (CSO, rich in n-6 LA) and menhaden oil+soybean oil (4:1, g/g) (MO, rich in n-3 EPA and DHA) for 6 weeks as described [19]. The n-3 fatty acid composition of the MO diet was as follows: 2.1 g of ALA, 13 g of EPA and 4.2 g of DHA per 100 g of total dietary fat. The CSO diet contained 0.5 g of ALA and no EPA or DHA per 100 g of total dietary fat.

Table 1
Renal COX-1 protein and mRNA levels in *pcy* mice fed low (7 g fat/100 g diet) and high (20 g fat/100 g diet) CO, FO and DO diets for 8 weeks

	Low fat ($n=6$)			High fat ($n=6$)			Significant effects
	CO	FO	DO	CO	FO	DO	
COX-1 Protein	100±12	111.2±13.2	81.6±9.2	107.1±21	79.8±13.9	108.1±8.8	None
COX-1 mRNA	100±19.0	117±19.0	106±19.0	75±20.4	90±20.4	147±20.4	None

Values are means (as percentage of low CO)±S.E.M. ($n=6$).

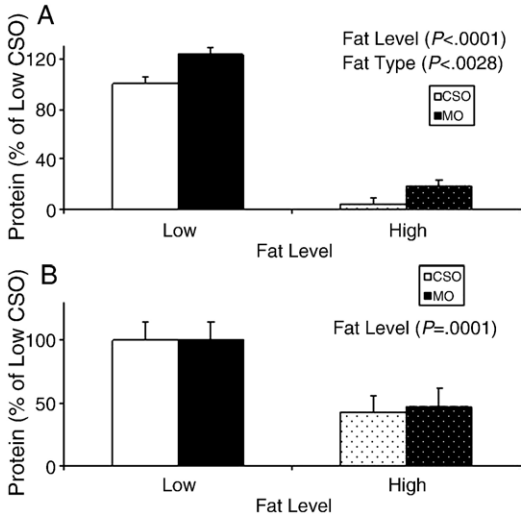

Fig. 3. Relative renal COX-2 protein levels in diseased (A) and in normal (B) Han:SPRD-*cy* rats given low (5 g fat/100 g diet) and high (20 g fat/100 g diet) CSO and MO diets. Values are means (as percentage of low CSO)±S.E.M. ($n=7$).

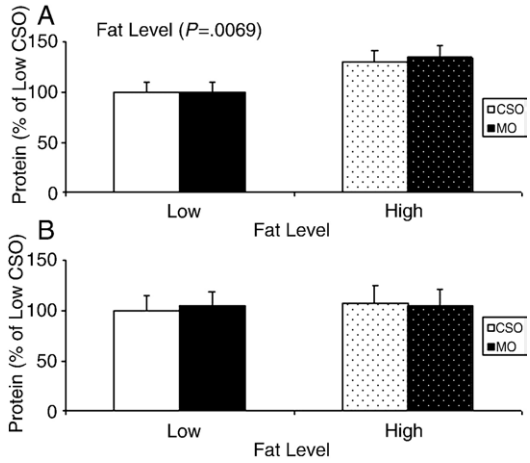

Fig. 5. Renal COX-1 protein expression in diseased (A) and in normal (B) Han:SPRD-*cy* rats given low (5 g fat/100 g diet) and high (20 g fat/100 g diet) CSO and MO diets for 8 weeks. Values are means (as a percentage of low CSO)±S.E.M. ($n=7$).

2.3. Study 3

Weanling male Han:SPRD-*cy* rats were given either a 7% CO diet (rich in n-6 LA) or a 7% FO diet (rich in n-3 ALA) for 12 weeks. The FO diet contained 52.3 g compared to 0.9 g of ALA in the CO diet per 100 g of total dietary fat [20].

In all three studies, kidneys were removed, weighed, snap frozen in liquid nitrogen and stored at −80°C until mRNA and protein analyses.

2.4. Immunoblotting

Half of the kidney was lyophilized and homogenized in ice-cold homogenization buffer containing protease inhibitors as described [13,14]. After centrifugation of the kidney homogenate, the resulting pellet was resuspended in buffer containing 1% Triton X-100 and recentrifuged. The resulting supernatant contains the COX proteins. Protein concentrations of these fractions were determined using the Bradford method. After SDS-PAGE, detection of COX-1 and COX-2 was carried out with primary antibodies (1:250 dilution, Cayman Chemical, Ann Arbor, MI, USA) followed by incubation with a peroxidase-conjugated secondary antibody. ChemiGlow (Alpha Innotech, San Leandro, CA, USA) substrate was used to visualize the immunoreactive bands which were then analyzed and quantified on the Fluorchem FC digital imaging system (Alpha Innotech).

2.5. Quantitative RT-PCR

Total RNA was extracted from 10 to 20 mg of lyophilized kidneys using TRIzol as described [14]. DNA was removed by treatment with DNase I (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature. One-step RT-PCR was performed on 0.5 μg of total RNA using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Mississauga, Canada). PCR primers were chosen using Primer 3 software as described [14]. Oligonucleotide sequences for the rat COX-1 and -2 primers have been described [14]. Mouse primers for COX-1 and -2 were as follows: COX-1 sense 5′-CACAACACTTCACCCACCAG-3′, COX-1 antisense 5′-AGAGCCGCAGGTGATACTGT-3′,COX-2 sense 5′-GCTGTACAAGCAGTGGCAAA-3′, COX-2 antisense 5′-TTCTGCAGCCATTTCCTTCT-3′. Quantitative, real-time RT-PCR was performed on a Cepheid SmartCycler II (Cepheid, Sunnyvale, CA, USA) using the following protocol: reverse transcription at 50°C for 30 min, PCR activation at 95°C for 15 min, 40 PCR cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. Relative amounts of mRNA were determined by comparing cycle threshold (CT) numbers for equal amounts of RNA subjected to RT-PCR and calculating differences in gene expression using the formula $2^{\Delta CT}$ as described [14].

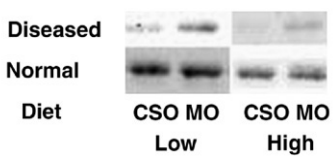

Fig. 4. Representative Western immunoblots of renal COX-2 protein expression in diseased and in normal Han:SPRD-*cy* rats given low (5 g fat/100 g diet) and high (20 g fat/100 g diet) CSO and MO diets for 8 weeks.

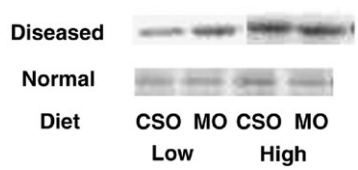

Fig. 6. Representative Western immunoblots of renal COX-1 protein expression in diseased and in normal Han:SPRD-*cy* rats given low (5 g fat/100 g diet) and high (20 g fat/100 g diet) CSO and MO diets for 8 weeks.

2.6. Statistical analysis

Data were analyzed using a general linear model ANOVA using SAS software (SAS, Cary, NC, USA). Normality of the data was assessed using a plot of actual residuals vs. predicted residuals, and the Shapiro–Wilk's W statistic and data were normalized by logarithmic transformation if necessary. Post hoc t-tests were performed only if the overall main or interaction effect was significant at $P<.05$.

3. Results

3.1. Effect of diets rich in DHA and ALA on renal COX-2 protein and gene expression in pcy mice

To examine the effect of different types of dietary n-3 PUFA on the renal COX isoforms in the *pcy* mouse model of renal cystic disease, mRNA and protein expression of these isoforms were determined in *pcy* mice given high and low CO, FO and DO. Mice given high-DO diets (rich in DHA) had approximately 4.3 times more renal COX-2 protein than mice fed high-FO and -CO diets (Figs. 1A and 2). Also, low DO-fed mice had nearly twice as much renal COX-2 protein than their low FO- and CO-fed counterparts (Figs. 1A and 2). The main effect of fat level on COX-2 protein did not attain statistical significance ($P=.0690$). However, since the P value was almost significant, and because the pattern of protein expression was similar to the gene expression in which there was a significant fat level effect in the DO-fed mice, post hoc analyses were performed on these groups of mice. The analyses revealed that high DO-fed mice had 143% more COX-2 immunoreactive bands ($P<.05$) compared to those fed low DO (Figs. 1A and 2). Neither level of dietary FO (rich in ALA), compared to CO, altered renal COX-2 gene expression (Fig. 1B). The relative increases in COX-2 protein in the (high) DO group were reflected in the COX-2 mRNA levels (Fig. 1B). Kidneys from mice fed a high level of DO had up to 200% higher COX-2 mRNA levels than kidneys from mice in any of the other diet groups (Fig. 1B). In contrast, neither fat level nor type altered protein and gene levels of renal COX-1 (Table 1, Fig. 2).

3.2. Effect of diets rich in EPA+DHA and ALA on renal COX-2 protein and gene expression in Han:SPRD-cy rats

To further probe this regulatory effect of very long chain n-3 fatty acid diets on COX-2 in vivo, we examined COX protein expression in Han:SPRD-*cy* rats fed high- or low-MO or -CSO diets. Diseased rats given high-MO diets (rich in EPA and DHA) had nearly five times more (Figs. 3A and 4), while those on low-MO diets had 1.2 times more COX-2 protein levels (Figs. 3A and 4), compared to their counterparts given high- and low-CSO diets, respectively. However, this effect of dietary fat type was only observed in diseased and not normal kidneys (Figs. 3B and 4). Similar to the effects of dietary very long chain n-3 PUFA in the *pcy* mice, dietary MO did not significantly alter COX-1 enzyme levels in the Han:SPRD-*cy* rats (Figs. 5A,B and 6).

Rats given low-fat diets had 2–10 times more renal COX-2 immunoreactive protein levels compared to their high fat-fed counterparts in both genotypes, indicating that this effect is independent of disease (Fig. 3A and B). On the other hand, high-fat diets resulted in greater renal COX-1 immunoreactivity in diseased rats (Fig. 5B) compared to normal rats (Fig. 5A). Gel analyses revealed that the quality of the mRNA from these archived tissues was compromised, precluding its use for determination of gene expression (data not shown).

The expression of these enzymes in Han:SPRD-*cy* rats given FO compared to CO diets was also examined to determine whether dietary FO (rich in ALA) affected COX isoform expression in this model. In contrast to the effects of MO on COX-2 in this model shown in Study 2, FO diets did not alter COX-2 protein or gene expression (Table 2, Fig. 7). Renal COX-1 protein and gene expression also remained unaltered by FO diets, consistent with the observed lack of an effect of n-3 fatty acids on COX-1 expression in Studies 1 and 2 (Table 2, Fig. 7).

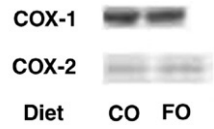

Fig. 7. Representative immunoblots of renal COX-1 and COX-2 protein expression in diseased Han:SPRD-*cy* rats given CO and FO diets for 12 weeks.

Table 2
Renal COX-1 and COX-2 protein and mRNA expression in diseased Han:SPRD-*cy* rats given CO and FO diets for 12 weeks

	CO ($n=8$)	FO ($n=8$)	Significant effects
COX-1 Protein	100±9.2	98±9.6	None
COX-1 mRNA	100±24.2	127±22.5	None
COX-2 Protein	100±13.4	79.8±13.4	None
COX-2 mRNA	100±14.0	112±13.1	None

Values are means (as a percentage of CO)±S.E.M. ($n=8$).

4. Discussion

The current study provides evidence that dietary oils enriched in the very long chain n-3 fatty acids, EPA and DHA, specifically up-regulate COX-2 mRNA and protein levels in diseased kidneys. In contrast, dietary oils enriched in the long-chain n-3 fatty acid, ALA, do not exert this effect. The highly unsaturated n-3 fatty acids, EPA and DHA, alter COX-2 expression at the gene and protein level in in vitro as well as in some in vivo feeding studies [6,7,10,11,21–23]. Dietary enrichment with 18:3n-3 ALA alters COX-2 mRNA levels in a mouse hepatoma model [5]. However, ALA did not alter COX-2 protein levels in a breast cancer cell line, while stearidonic acid (18:4n-3) significantly reduced COX-2 expression even at low

concentrations [8], indicating that regulation of COX-2 expression by n-3 fatty acids appears to be dependent on the degree of unsaturation in some cases. Therefore, the observation in the current study that diets containing EPA and DHA or DHA alone up-regulate, while those containing ALA do not alter COX-2 expression in both rodent models of inherited chronic kidney disease, supports the premise that COX-2 regulatory effect of n-3 fatty acids depends on the number of double bonds in the fatty acid chain.

Interestingly, although the n-3 fatty acid-containing diets elicit similar responses in COX-2 in both rodent models of RCD, their effects on renal disease progression in these models are not similar. In *pcy* mice, the FO diet (rich in ALA) compared to the n-6-enriched CO diet had beneficial effects on renal injury, while the DO diet (containing DHA) worsened renal injury [17]. The detrimental effect of dietary oils containing very long chain n-3 fatty acids is supported by the results of a previous study in which *pcy* mice given dietary fish oil compared to an LA-enriched diet had lower survival rates and exhibited worsened renal function as well as a deterioration of renal architecture [24]. On the contrary, in diseased Han:SPRD-*cy* rats, both ALA-enriched FO and EPA- and DHA-enriched MO diets, compared to diets rich in n-6 fatty acids, significantly attenuated markers of renal injury such as cyst expansion, renal fibrosis and inflammation [19,20]. Therefore, it is likely that in these models of RCD, dietary n-3 fatty acids alter COX-2 expression independent of their effects on renal disease progression. However, the presence of kidney disease is important, as COX-2 expression was not altered by dietary MO in normal rats. Thus, the induction of COX-2 by diets containing EPA and DHA or DHA alone appears to be independent of their effects on disease progression, although the presence of disease appears to be necessary to elicit these effects.

COX-2 expression has been shown to be modulated by a number of transcription factors which appear to be cell specific. For example, COX-2 expression in the liver [5] and in macrophages [25] is tightly regulated by members of CCAAT enhancer binding proteins (C/EBP), while in cultured glomerular (including mesangial) cells nuclear factor (NF) κB modulates transcriptional regulation of COX-2 [26,27]. Supplementation with n-3 fatty acids has been shown to mitigate induction of COX-2 mRNA and protein levels in a number of inflammatory and cancer models [5–8,10–12,21,22]. One of the mechanisms by which n-3 fatty acids mediate their effects on COX-2 expression is likely via transcriptional regulation of COX-2 since n-3 fatty acids have been shown to alter NFκB and C/EBP β [5,8,22,28–30]. Thus, in the current study, modulation of transcription factors that regulate COX-2 expression is a potential mechanism by which diets containing EPA and DHA or DHA alone exert their effects on renal COX-2 levels.

Notably, n-3 fatty acids did not alter COX-1 expression in both *pcy* mice and Han:SPRD-*cy* rats, although expression of this enzyme was modulated by the presence of renal disease and by fat level [13,14]. This is consistent with the fact that COX-2 metabolizes a wider range of fatty acids as substrates and oxygenates EPA and ALA more efficiently than COX-1 [1,31].

In a number of models of chronic renal disease, renal injury increases the expression of COX protein and mRNA and this parallels increased enzyme activity [32–36]. However, in models of RCD, the presence of renal disease results in reduced COX-2 expression and elevated enzyme activity [13,14]. Thus, in the current study, the increase in renal COX-2 expression with diets containing EPA and DHA or DHA alone may reflect a decrease in renal COX-2 activity. This premise is supported by a number of in vivo and in vitro studies that demonstrate a decrease in COX-2 activity after supplementation with EPA and/or DHA [4,9,28,37,38]. This inverse relationship between COX-2 expression and its activity in models of RCD has also been reported in 5/6 nephrectomized rats in which celecoxib administration resulted in a tripling of immunoreactive COX-2 in the macula densa while in vivo COX-2 activity was reduced [39]. A reduction in enzyme level with a concomitant increase in activity suggests that feedback inhibition by prostanoids is likely occurring, and this may be one of the regulatory mechanisms controlling the protein levels of COX-2. Depletion of macula densa cells in Han:SPRD-*cy* rats [40] and focal cyst development in the cortex, extending to the medulla, only occasionally [18] suggest that the observed increased renal COX-2 activity in diseased rats may originate from the relatively preserved medulla.

The results of the current study indicate that the level of fat in the diet modulates the effect of n-3 fatty acid on COX-2 expression. Diets containing high levels of n-3 fatty acids appear to exaggerate the effect of the n-3 fatty acids on renal COX-2 expression. *pcy* mice given high-DO diets had a greater elevation in renal COX-2 expression compared to mice given low-DO diets. Although fat level effects in Han:SPRD-*cy* rats were such that high fat-fed rats had lesser renal COX-2 immunoreactivity compared to those given low-fat diets, this effect was mitigated to a greater extent in diseased rats given a high level of MO in the diet.

In conclusion, the in vivo modulation of renal COX-2 expression in diseased kidneys by diets containing n-3 fatty acids appears to be dependent on the chain length of the fatty acid. In murine models of RCD, diets containing oils rich in EPA and DHA up-regulate COX-2 expression, while diets containing oils rich in ALA do not alter COX-2 in vivo.

Acknowledgments

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