A dietary conjugated linoleic acid treatment that slows renal disease progression alters renal cyclooxygenase-2-derived prostanoids in the Han: SPRD-cy rat

Andrew P. Wakefield⁎, Malcolm R. Ogborn, Naser Ibrahim, Harold M. Aukema

Abstract

A mixture of dietary conjugated linoleic acid (CLA) isomers reduces inflammation and mitigates disease progression in the Han:SPRD-cy rat model of chronic kidney disease. Since cyclooxygenase (COX) activities and prostanoid levels are higher in diseased kidneys in this rat, and dietary CLA can inhibit COX2 and prostanoid production in other tissues, the effects of dietary CLA were investigated. Kidney homogenates from normal and diseased Han:SPRD-cy rats were analyzed for prostanoid levels under various conditions: endogenous levels, steady-state levels (60-min incubations) and produced by COX isoforms. Thromboxane B2 (TXB2; TXA2 metabolite), 6-keto-prostaglandin F1α (6-keto-PGF1α; PGF2α metabolite) and PGE2 levels under these conditions were two- to ninefold higher in diseased kidneys. Dietary CLA resulted in ~32%–33% lower levels of prostanoids produced by total COX and COX2 activities in normal and diseased kidneys and partially mitigated alterations in COX2 protein levels associated with disease. The COX1 protein and activity were higher in renal disease, resulting in increased production of TXB2 and 6-keto-PGF1α, but not PGE2. Dietary CLA had no effect on COX1, however. Disease resulted in up to twofold higher ratios of TXB2/6-keto-PGF1α, TXB2/PGE2 and 6-keto-PGF1α/PGE2, and dietary CLA partially mitigated these increases under several conditions. Elevated levels of renal membrane associated cytosolic phospholipase A2 in diseased kidneys also were reduced by 50% with CLA feeding. The effects of CLA feeding on COX2 protein levels and activity indicate that the beneficial effect of dietary CLA in this renal disorder is mediated in part via effects on COX2-derived prostanoids.

Keywords: Conjugated linoleic acid; Kidney disease; Prostanoid; Cyclooxygenase 2; Phospholipase A2

1. Introduction

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. The two most studied isomers are the trans-10, cis-12 (t10, c12) and cis-9, trans-11 (c9, t11) isomers, the latter of which is found predominantly in dairy products and meats of ruminant animals. The t10, c12 isomer is produced commercially for use in nutritional supplements. Conjugated linoleic acid has been explored as a potential dietary therapy to reduce inflammation in several chronic diseases through inhibition of cyclooxygenase-2 (COX2) and the modification of prostanoid formation. The mechanisms behind this protective effect remain to be elucidated, but CLA may interfere with prostanoid production by reducing the level of arachidonic acid (ARA) in tissues and competing with its metabolism to prostanoids, or by altering enzymes in prostanoid biosynthesis [1–6].

Physiologic and pathophysiologic stimuli activate cytosolic phospholipase A2 (cPLA2) that triggers the release of ARA from cell membrane phospholipids [7,8]. Cyclooxygenase is thought to be the rate-limiting enzyme for the biosynthesis of prostanoids via the conversion of ARA and O2 to the intermediate prostaglandin H2 (PGH2) [9,10]. Prostaglandin H2 is further metabolized to the individual bioactive prostaglandins and thromboxanes by specific prostanoid synthases [11]. Although it initially was thought that COX1 was expressed constitutively and COX2 only was induced in response to inflammatory and mitogenic stimuli, it is now known that both isoforms can be constitutively expressed or be induced in several tissues, including the kidney [12–16].

Renal prostanoids are abundant and have numerous roles in renal physiology including maintenance of glomerular hemodynamics, regulation of sodium and water balance, and renin secretion. In pathologic states, these lipid-derived mediators can influence renal inflammatory events and vascular hypertension. Prostacyclin (PGI2) is a potent vasodilator that can increase renal and tubular blood flow and potassium excretion [17]. Depending on the specific receptor present in tissue, PGE2 is generally a less potent vasodilator and regulates sodium reabsorption, but can also cause vasoconstriction [18]. Thromboxane (TX) A2 is a potent vasoconstrictor and decreases renal blood flow and filtration in the kidney [19]. In addition, PGE2...
plays a role in inflammation that in kidney disease leads to a progressive loss of renal function.

Inhibition of COX activity and the resulting prostanoid synthesis reduces renal injury in experimental models of diabetes and hypertension, the metabolic syndrome, cystic kidney disease, induced nephropathy and renal ablation [20–26]. In a previous study, we reported that CLA reduces renal steady-state in vitro production of one prostanoid (PGF_{2\alpha}) and has significant renal anti-inflammatory and antifibrotic effects in the Han:SPRD-cy rat model of chronic kidney disease [27]. In subsequent work, we demonstrated that renal cytosolic PLA2 (cPLA2) and COX1 protein levels are elevated and COX2 is reduced in the diseased kidneys of Han:SPRD-cy rats [28]. In this model, lower renal COX2 levels are associated with increased enzymatic activity in disease, and pharmacological COX2 inhibition increases COX2 levels while decreasing COX2 activities, prostanoid production and disease progression [16,21]. Therefore, the effect of dietary CLA on prostanoid production and COX isoforms was examined to determine whether the protective effects of dietary CLA in this model of renal disease could be mediated by alterations in COX activity and prostanoid production.

2. Materials and methods

2.1. Experimental animals and diet

Renal tissues for the analyses described herein were obtained from a study in which the protective effects of dietary CLA in chronic kidney disease were demonstrated. Details of the study design, diets and animal model are described as described [27]. To summarize, male Han:SPRD-cy rats with renal cystic disease were randomly assigned to CLA or control groups at weaning at 3 weeks of age. Diets were based on the AIN 93G diet [29] and were identical except for the source of lipid. The control diet contained an oil source (Biooriginal Food and Science Corp, Saskatoon, SK, Canada) that contained an oil source (Biooriginal Food and Science Corp, Saskatoon, SK, Canada) that contributed 0.67% CLA isomers to the diet with a concomitant reduction in corn oil. After 8 weeks, animals were anesthetized with sodium pentobarbital (65 mg/kg intraperitoneal), weighted and exsanguinated by cardiac puncture. Kidneys were then immediately removed and snap frozen in liquid nitrogen to minimize potential postmortem changes. Kidney tissue was stored at –80°C until analysis.

2.2. Renal prostanoid levels and COX activities

Prostanoid levels and production were determined as described [16,21]. Briefly, right kidneys were lyophilized and homogenized in fresh Tyrode’s buffer. After adding Triton X-100 and incubating on ice for 30 min, duplicate aliquots containing vehicle (1% ethanol) or the selective COX1 inhibitor SC560 (Cayman Chemical Company, Ann Arbor, MI, USA) for 60 min of incubation. The level of SC560 used was determined from previous studies demonstrating that 0.1 μM SC560 inhibits >90% of COX1 activity under these conditions, while not inhibiting COX2 at all [16].

At the end of each incubation period, ice-cold 5 mM L-ascorbic acid-saline buffer (Sigma, St. Louis, MO, USA) was added to stop further COX activity. Samples were vortexed and centrifuged, and the supernatant was removed and stored at –80°C until analysis using commercial enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, USA) for PGE_{2}, and the stable metabolites of TXA_{2} (TXB_{2}) and PG_{2} (6-keto-PGF_{1α}). Samples were diluted between 100× and 1500×. An aliquot of the homogenate was set aside for protein determination using the Bradford assay [30].

2.3. Western immunoblotting of cPLA2, COX1 and COX2

Steady-state levels of cPLA2, COX1 and COX2 protein levels were determined as described [16,21]. Briefly, kidneys were lyophilized, and a representative sample was homogenized in 100 vol of ice-cold homogenization buffer containing protease inhibitors. Homogenates were centrifuged at 100,000g for 30 min at 4°C, and the supernatant, which represents the cytosolic fraction, was collected. The remaining pellet was resuspended in 15 vol of homogenization buffer containing 1% Triton X-100 (Sigma, St. Louis, MO, USA), incubated on ice for 10 min and centrifuged again at 100,000g for 30 min at 4°C. The resulting supernatant was collected as the particulate extract and represents the Triton-soluble fractions of plasma and intracellular membranes. Protein concentrations of both fractions were determined by protein assay as described by Bradford [30], with bovine serum albumin as the standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was as described [16,21], using primary antibodies for COX1 and COX2 (Cayman Chemical Company, Ann Arbor, MI, USA) and cPLA2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). COX1 and COX2 are present in particulate fractions only, while cPLA2 is present in both fractions [28]. Following SDS-PAGE, transfer to PVDF, blocking and incubation with primary and secondary antibodies, immunoblots were incubated with ChemiGlow (Alpha Innotech, San Leandro, CA, USA). Image analysis and quantitation of immunoreactive bands were performed using the Fluorochrome FC digital imaging system (Alpha Innotech, San Leandro, CA, USA). A range of protein amounts was loaded onto gels for each antibody to ensure that quantitative comparisons could be made for results within each isoform. The amount of protein that was in the middle of the linear response (14 μg) was used for analysis. A reference kidney homogenate was loaded on each gel in duplicate so that results could be compared across gels. Data are expressed as arbitrary density units per microgram protein, with the data from kidneys obtained from normal rats on the control diet being assigned a value of 100.

2.4. Statistical analyses

Results were analyzed by two-way analysis of variance (diet-genotype) using SAS software (SAS Institute, Cary, NC, USA). Where interactions were significant (P<.05), simple effects were tested using the Protected LSMeans test. Data were tested for normality using the Shapiro–Wilks statistic and normalized by log transformation if necessary. Where W–W.01 was not achieved even by transforming the data, differences were confirmed using nonparametric analysis (Wilcoxon rank sum test, P<.10). All data are expressed as geometric means±S.E.M.

3. Results

Dietary CLA resulted in lower endogenous and in vitro steady-state levels of prostanooids in both diseased and normal kidneys. Endogenous and steady-state levels of TXB_{2} were ~53% and ~33% lower, respectively. For 6-keto-PGF_{1α}, the endogenous and steady-state levels between 0 and 10 min and the steady-state levels of prostanooid production is achieved at 60 min of incubation. The level of SC560 used was determined from previous studies demonstrating that 0.1 μM SC560 inhibits >90% of COX1 activity under these conditions, while not inhibiting COX2 at all [16].

Table 1

| Kidney levels of TXB_{2}, PGE_{2} and 6-keto-PGF_{1α} in normal and diseased Han:SPRD-cy rats that consumed control or CLA diets for 8 wk |

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>CLA diet</th>
<th>P values</th>
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<tbody>
<tr>
<td></td>
<td>Normal rats</td>
<td>Diseased rats</td>
<td>Diet</td>
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<tr>
<td>Endogenous levels</td>
<td></td>
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</tr>
<tr>
<td>TXB_{2}</td>
<td>0.24±0.04</td>
<td>1.32±0.13</td>
<td>&lt;.0001</td>
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<tr>
<td>PGE_{2}</td>
<td>1.07±0.22</td>
<td>2.70±0.27</td>
<td>&lt;.0001</td>
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<tr>
<td>6-keto-PGF_{1α}</td>
<td>1.42±0.30</td>
<td>4.58±0.49</td>
<td>&lt;.0001</td>
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<tr>
<td>In vitro steady-state levels</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TXB_{2}</td>
<td>0.79±0.10</td>
<td>6.78±0.04</td>
<td>&lt;.0001</td>
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<tr>
<td>PGE_{2}</td>
<td>4.52±0.70</td>
<td>7.74±0.71</td>
<td>&lt;.0001</td>
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<tr>
<td>6-keto-PGF_{1α}</td>
<td>13.13±3.52</td>
<td>41.67±4.45</td>
<td>&lt;.0001</td>
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</tbody>
</table>

Values are means±S.E.M. (n=5–9 for each value). There were no significant (P>.05) diet×genotype interactions.
Table 2

Kidney COX activity levels in normal and diseased Han:SPRD-cy rats that consumed control or CLA diets for 8 weeks

<table>
<thead>
<tr>
<th>Control diet</th>
<th>CLA diet</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>Diseased rats</td>
<td>Normal rats</td>
</tr>
<tr>
<td>COX activity</td>
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<tr>
<td>TXB2</td>
<td>0.07±0.01</td>
<td>0.49±0.06</td>
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<tr>
<td>PGE2</td>
<td>0.36±0.10</td>
<td>0.43±0.03</td>
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<tr>
<td>6-keto-PGF1α</td>
<td>0.53±0.08</td>
<td>1.95±0.36</td>
</tr>
</tbody>
</table>

Values are means±S.E.M. (n=5–9 for each value). There were no significant (P>.05) diet×genotype interactions.

1tr, trace; <0.01 ng/min/mg protein.

Values with different letters denote significant differences within each assay condition.

Fig. 1. Ratio of TXB2 to 6-keto-PGF1α in kidneys (endogenous levels), in 60-min incubations (steady-state levels) and produced by renal cyclooxygenase activities in normal and diseased Han:SPRD-cy rats that consumed control or CLA diets for 8 weeks. Bars represent mean±S.E.M. values. Letter superscripts indicate significant (P<.05) diet×genotype interactions, and values with different letters denote significant differences within each assay condition.

were ~36% and ~33% lower, and for PGE2, these levels were ~43% and 32% lower, respectively, in kidneys from rats offered CLA (Table 1).

Similar to the prostanoid levels, dietary CLA resulted in lower COX and COX2 activities in the kidneys of diseased and normal animals. Total COX and COX2 activities when measured by TXB2, 6-keto-PGF1α or PGE2 production, respectively, were ~52% and 42%, ~41% and 34%, and ~45% and 47% lower in rats given dietary CLA compared to CO. In contrast to COX2, dietary CLA did not alter COX1 activities as measured by each prostanoid (Table 2).

These effects of dietary CLA on renal prostanoids and COX activities were in the opposite direction to the effect of disease on these parameters. In diseased kidneys, the endogenous and steady-state levels of prostanoids were ~6 and ~9 times higher for TXB2, ~4 times higher for 6-keto-PGF1α and ~2 and 3 times higher for PGE2 than in normal kidneys (Table 1). Renal COX activities also were higher in disease, with differences in concert with the effects on prostanoid levels. With renal disease, total COX and COX2 activities that produced TXB2, 6-keto-PGF1α and PGE2 were ~6.7 times, ~4 times and ~1.3 times higher, respectively, compared to normal rats (Table 2).

As with dietary effects, the effects of renal disease on COX1 activity differed from effects on COX2 activity. When measured using TXB2 or 6-keto-PGF1α production, COX1 activity was more than 10 times higher in diseased compared to normal kidneys, but when using PGE2 production, the difference (~2 times) was not significant. Hence, the presence of disease induces COX1 activity that selectively produces TXB2 and 6-keto-PGF1α. Overall, COX1 made up ~6% of total COX activity in normal kidneys, compared to ~16% in diseased kidneys (Table 2).

To determine which prostanoids were most affected by dietary CLA, ratios of the prostanoids were calculated and compared (Figs. 1-3). While all three prostanoids were higher in diseased kidneys, comparison of prostanoid ratios indicated that the relative difference was greatest for TXB2. Treatment with CLA had no effect on the ratio of steady-state levels of TXB2/6-keto-PGF1α, which was ~1.8 times higher in diseased kidneys (Fig. 1). On the other hand, there were diet by genotype interactions for the endogenous and COX activity conditions. In rats given the control diet, renal disease resulted in similarly increased (~1.6 to 1.9 times higher) TXB2/6-keto-PGF1α ratios for endogenous levels and COX and COX2 activities compared to normal kidneys. Dietary CLA abrogated the alterations as evidenced by the lower TXB2/6-keto-PGF1α ratios that were not different from the values observed in normal kidneys for these conditions. For the TXB2/PGE2 ratio, kidneys from diseased rats had ~2 to 4 times higher ratios for endogenous levels, steady-state levels, total COX and COX2 activity (Fig. 2). Dietary CLA resulted in a significantly lower value for this ratio only for the COX2 activity measurement. With respect to the ratio of 6-keto-PGF1α/PGE2, it was ~2 to 3 times higher in the steady-state levels, total COX and COX2 activities of diseased kidneys, with no apparent interactions, and values with different letters denote significant differences within each assay condition.
effect of diet on these parameters (Fig. 3). For endogenous levels, this ratio was higher in diseased kidneys given the CLA diet only, due to a diet by genotype interaction for this parameter. Hence, dietary CLA partially normalized some of the prostanoid ratios in diseased kidneys.

Protein levels of renal COX1 reflected the higher levels of COX1 activity in diseased compared to normal kidneys, with COX1 being ∼2.7 times higher in diseased kidneys (Fig. 4). Consistent with the lack of dietary effect on COX1-derived prostanoids, CLA did not exert a dampening effect on renal COX1 protein levels. For COX2, protein levels were in the opposite direction as activity, as previously observed [16,21,31]. While renal COX2 activities were higher with disease, protein levels of COX2 were ∼85% lower. Dietary CLA improved the level of COX2 in both normal and diseased kidneys, although the levels in diseased kidneys were still lower than normal (Fig. 5).

Since substrate fatty acids for COX are released from membrane phospholipids in kidney by cPLA2, the protein levels of this enzyme also were determined. As previously observed [28], levels of renal cPLA2 protein in the cytosolic and particulate fractions were ∼3 times higher in diseased rats (Figs 6 and 7). Dietary CLA resulted in lower levels of cPLA2 only in the particulate fraction (by ∼50%) in diseased kidneys, but levels were still higher than in normal kidneys (Fig. 7).

4. Discussion

We have previously demonstrated that COX2 inhibition (with NS398) in the Han:SPRD-cy rat reduces cyst growth, interstitial inflammation, macrophage infiltration and oxidant injury [21]. In the present findings, dietary CLA lowered renal COX2 activity in both normal and diseased rats. Previous analysis of these rats demonstrated a protective effect of dietary CLA on kidney disease progression [27]. Therefore, CLA may be a dietary alternative or adjunct to COX2 inhibitors in the amelioration of renal disease.

The COX2 inhibitory effect of dietary CLA and its protective effect in renal disease will be important if the findings in this model are applicable to human renal diseases since there are concerns regarding the nephrotoxicity of COX inhibitory drugs. The COX1 inhibitory activity of nonsteroidal anti-inflammatory drugs is believed to be related to the nephrotoxic effects of these analgesics [32]. Hence, there has been a shift to the use of COX2 selective inhibitors. Yet, questions remain as to whether these inhibitors also are unsafe for the kidneys. Reports in the literature indicate that COX2 inhibitors may cause nephrotoxicity via acute renal failure in high-risk patients [17,33,34]. On the other hand, use of the selective COX2 inhibitor SC58236 has been shown to retard the progression of progressive renal injury in rats [20] and decrease glomerular and tubulointerstitial injury in streptozotocin-induced diabetic rats [22], indicating its potential benefit in specific renal pathologies. The COX2 inhibitory effect of dietary CLA therefore may allow the use of lower doses of these drugs and possibly reduce nephrotoxic effects.

Thromboxane A2 acts as a vasoconstrictor, platelet aggregator and cell proliferator in remnant kidneys and is an important contributor in the progressive nature of kidney disease [25,35–38]. In the advanced stages of coronary heart failure, the presence of increased levels of TXA2 markedly worsens renal function, and inhibition of its production improves renal hemodynamics and function [39]. At the same time, prostanoids such as PGI2 are important protective...
mediators that counter the pathological effects of disease in the kidney by modulating sodium secretion, maintaining glomerular filtration rate and renin release as well as increasing renal blood flow [34,37]. In the current study, the balance between the metabolites of TXA2 and PGI2 in the kidneys is worse in diseased kidneys, but is improved when CLA is given to the rats. Similar implications can be applied to the TXA2/PGE2 ratio since PGE2 also is involved in maintaining normal renal function in the face of pathological challenges. However, PGE2 also can contribute to the inflammatory response, so the lower PGE2 levels in kidneys from CLA-fed rats may confer an anti-inflammatory effect as well. In this regard, the higher endogenous levels of the 6-ketoPGF1α/PGE2 ratio with CLA feeding may reflect a greater vasodilatory and anti-inflammatory condition.

The effect of CLA on prostanoid production observed herein is consistent with reports on its effects in cells and tissues. Conjugated linoleic acid inhibits PGE2 in a variety of scenarios, including 12-O-tetradecanoylphorbol-13 acetate (TPA)-induced cultures of murine keratinocytes [40], UV-radiation-exposed human keratinocytes and CCD922SK fibroblasts [41], epidermis of TPA-treated mice [42], mouse lungs [43], brains of pregnant mice [44], trachea of guinea pigs challenged with antigens [45] and human macrophages [46]. In endothelial cells, individual CLA isomers appear to stimulate, while mixtures inhibit, prostanoïd production [47,48]. Conjugated linoleic acid also attenuates COX-dependent PGI2 synthesis in bovine aortic endothelial cells [49].

A mechanism by which dietary CLA could alter prostanoïd metabolism would be via its effects on COX enzymes, the putative rate-limiting step in prostanoïd formation. In murine and human macrophages, CLA lowers PGE2 release and suppresses cPLA2 and COX2 [5,46,50]. In vitro and in vivo studies with Raw264.7 macrophage cells and BALB/c mice showed that t10, c12 CLA down-regulates COX2 protein and mRNA expression and decreases PGE2 production [43]. In hairless mice topically administered c9, t11 CLA in the presence of TPA, COX2 expression was significantly inhibited [51]. In canine mammary cells, administration of c9, t11 CLA decreased COX2 protein levels in cancerous and normal cells, while t10, c12 CLA was only effective on this COX isoform in malignant cells [52]. Similar attenuation of COX2 transcription has been demonstrated in MCF-7 breast cancer cells treated with a mix of CLA or the c9, t11 and t10, c12 isomers independently in the presence of TPA [53].

It also has been suggested that the displacement of ARA in tissues by CLA and its elongation and desaturation products may be another mechanism behind the inhibitory effect of CLA on prostanoïd production [1,54]. In the current study, higher levels of membrane-associated cPLA2 are present in the kidneys of diseased rats, and this is
lower in rats given dietary CLA, suggesting that less ARA is available for prostanoid synthesis. However, the renal content of ARA and linoleic acid was not altered by dietary CLA intervention [27], a finding that has also been observed in other tissues [45,55,56].

In summary, prostanoid levels were elevated in diseased rat kidneys and were lower in both control and diseased rats given dietary CLA. The reduction in prostanoid levels coincides with a reduction in renal damage by dietary CLA [27]. The COX2 activity and protein levels were altered by dietary CLA in both normal and diseased kidneys, suggesting that the beneficial effect of dietary CLA is mediated in part by inhibiting the COX2 activity. Whether specific individual CLA isomers would have similar effects remains to be elucidated.

Acknowledgments

The authors are grateful for the technical assistance of Laurie Evans, Lori Warford-Woolgar, Evan Nitschmann, Neda Bankovic-Calic and Romi Banerjee.

References


