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Synergistic effects of conjugated linoleic acid and chromium picolinate improve vascular function and renal pathophysiology in the insulin-resistant JCR:LA-*cp* rat

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Aims: Conjugated linoleic acid (CLA) is a natural constituent of dairy products, specific isomers of which have recently been found to have insulin sensitizing and possible antiobesity actions. Chromium is a micronutrient which, as the picolinate (CrP), has been shown to increase insulin sensitivity in animal models, including the JCR:LA-*cp* rat. We tested the hypothesis that these agents may have beneficial synergistic effects on the micro- and macrovasculopathy associated with hyperinsulinaemia and early type 2 diabetes.

Methods: Insulin-resistant *cp/cp* rats of the JCR:LA-*cp* strain were treated with mixed isomers of CLA (1.5% w/w in the chow) and/or CrP at 80 µg/kg/day (expressed as Cr) from 4 weeks of age to 12 weeks of age. Plasma insulin, lipid and adiponectin levels, aortic vascular function, renal function and glomerular sclerosis were assessed.

Results: CLA administration reduced food intake, body weight and fasting insulin in JCR:LA-*cp* rats. Plasma adiponectin levels were significantly elevated in rats treated with both CLA and CrP. Aortic hypercontractility was reduced and the relaxant response to the nitric oxide-releasing agent acetylcholine (ACh) was increased in CrP-treated rats. Striking reductions were also observed in the level of urinary albumin and the severity of glomerular sclerosis in rats treated specifically with CLA.

Conclusions: CLA and CrP have beneficial effects ameliorating several of the pathophysiologic features of an insulin-resistant rat model. These supplements may be useful adjuncts in the management of patients with the metabolic syndrome and warrant further study.

Keywords: adiponectin, chromium, conjugated linoleic acid, glomerular sclerosis, insulin resistance, JCR:LA-*cp* rat, metabolic syndrome, prediabetes, vasculopathy

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Introduction

The metabolic syndrome, characterized by abdominal obesity, insulin resistance, dyslipidaemia and endothelial/vascular dysfunction, is a prediabetic state [1,2]. The chronic sequelae of the metabolic syndrome are macro- and microvascular (particularly renal vascular) diseases

[3]. Recently, there has been a renewed focus on the capacity of bio-active dietary components to reduce the metabolic and pathological complications of type 2 diabetes, particularly in the early prediabetic phase.

Conjugated linoleic acid (CLA) is a collective term for a group of conjugated dienoic isomers of linoleic acid (18:2, ω -6), formed by bacterial transformation of

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linoleic acid [4]. The *cis*-9, *trans*-11 isomer is the common component found in dairy products, whereas *trans*-10, *cis*-12 is a product of synthetic alkaline isomerization of linoleic acid found in commercial CLA supplements. CLA has been shown to improve glucose tolerance and insulin resistance in rodent models and humans, possibly via a thiazolidinedione-like action [5–7]. There is some evidence that CLA may protect against atherogenesis [8], possibly by increasing agonist-mediated nitric oxide (NO) release and reducing levels of inflammatory mediators within vascular endothelial cells [9]. Ogborn *et al.* [10] showed that glomerular haemostatic regulation, including eicosanoid and prostaglandin activity, can be improved by supplementation with CLA. The expression and secretion of adipokines have also been implicated in the regulation of glomerular filtration rate as well as endocrine effects on insulin sensitivity [11–13].

By contrast, chromium is a transition metal element that, like others in the same elemental series, has potential metabolic effects and is classified as a micronutrient with accepted minimum daily intake [14]. Cr⁺⁺⁺, as chromium picolinate (CrP), has been shown to increase insulin sensitivity in animal models of insulin resistance, including the JCR:LA-*cp* rat [15]. There is also evidence of insulin-sensitizing action of chromium in cultured muscle cells possibly due to modulation of intracellular tyrosine kinase activity and Ca²⁺ concentrations [16]. CrP has been shown not to produce any measurable toxic effects in rats or mice, suggesting that it may be an effective therapeutic option [17]. We proposed that selective combination of both CLA and CrP treatment represents a unique therapeutic and synergistic approach to the prevention of chronic complications of type 2 diabetes. To investigate this hypothesis, we used an established rat model of the metabolic syndrome, hyperinsulinaemia and cardiovascular disease, the JCR:LA-*cp* rat.

The JCR:LA-*cp* rat is a unique strain that exhibits marked obesity and hyperinsulinaemia, resulting from a defect in the leptin receptor gene [18], with associated micro- and macrovascular disease [19,20]. This strain mimics the pathophysiology of the obese prediabetic human and has become a model-of-choice for study of prediabetes and complications of the metabolic syndrome [19–28]. Reduction of insulin concentrations in the *cp/cp* rat is associated with corresponding reduction in vasculopathy and cardiac dysfunction and can prevent ischaemic lesions of the heart [24–28]. Evidence from this study suggests that co-supplementation with CLA and CrP results in synergistic and striking functional improvements in the macro- and microvascular system of the *cp/cp* rat.

Materials and Methods

Animals and Treatment

Male rats of the JCR:LA-*cp* strain, obese (*cp/cp*) and lean [*+/?*, a 2:1 mix of heterozygous (*cp/+*) and homozygous normal (*+/+*)], were raised and housed as described previously [20,21]. The strain has recently been rederived and established at Charles River Laboratories (Wilmington, MA, USA) with the designation CrI:JCR(LA)-*Lepr*^{cp}. From 4 weeks of age, *cp/cp* animals were provided food containing (i) mixed isomers of CLA (Tonalin[®], Cogniz Corp. USA, Cincinnati, OH, USA) at a concentration of 1.5% (w/w); (ii) CrP at a concentration to deliver a dose of 80 µg/kg/day (expressed as elemental Cr); or (iii) both additives. Groups not receiving CLA, including controls, received 1.5% canola oil in the food as a lipid balance control. Food was formulated with Lab Diet 5001 (PMI Nutrition International, Brentwood, MO, USA) [27]. Rats were treated to 13 weeks of age, with a meal tolerance test performed 1 week prior to killing.

All animals were weighed and their food consumption measured twice weekly in order to maintain the desired dose of CrP on a mg/kg body weight basis. Rats were killed in the fed state under halothane anaesthesia by exsanguination from the heart, and collection of blood and urine samples. Care of animals and experimental procedures were in conformity with the guidelines of the Canadian Council on Animal Care and subject to prior review and approval by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta.

Meal Tolerance Test

Insulin sensitivity was assessed at 12 weeks of age through a meal tolerance test developed in our laboratory [21,28]. Tests were performed following a standardized, conscious, non-restraint protocol, a challenge of 5 g of rat chow and blood samples taken fasting (0 time) and 30 and 60 min after consumption of the meal.

Vascular Function

Vascular function of aortic rings with intact endothelium was determined as previously described [28,29]. Contractile concentration–response curves for phenylephrine (PE) (0.1 nmol/l to 100 µmol/l, Sigma Chemicals, Oakville, Ontario, Canada) were determined. NO-mediated relaxation of aortic rings (precontracted with PE to 80% of maximal contraction) was determined

using the endothelial NO-releasing agent, acetylcholine (ACh) (Sigma Chemicals), and the NO donor sodium nitroprusside (SNP) (Sigma Chemicals). Direct assessment of NO-mediated effects was also determined through addition of *N*^c-nitro-L-arginine methyl ester (L-NAME) (Sigma Chemicals), at 10^{-4} mol/l, in order to inhibit NO synthase (NOS) activity [29].

Analytical Methods

Plasma glucose was measured using a rapid glucose oxidase technique (Beckman Instruments, Brea, CA, USA), and insulin was assayed by a double antibody radioimmunoassay technique (Kabi Pharmacia, Uppsala, Sweden). Adiponectin was measured by radioimmunoassay (LINCO Research, St Louis, MO, USA). The plasma lipid profile was measured using the gas chromatographic technique of Kuksis *et al.* [30]. Urine albumin and creatinine concentrations were measured using immuno-turbidimetric and Jaffé methods, respectively.

Renal Histology

Kidneys were divided along the long axis, fixed, sectioned and stained with H&E. Sections were examined to quantify glomerular sclerosis by the method of Schäfer *et al.* [31]. Ten random fields of view of each kidney were recorded digitally using a $\times 10$ objective. All complete glomeruli in each field of view were scored, blinded, as sclerotic (mild to severe glomerular sclerosis) or normal (minimal sclerosis or normal). The fraction of sclerotic glomeruli was calculated for each kidney.

Statistical Analysis

Results are expressed as mean $\pm$ s.e.m. Data were plotted using SIGMAPLOT (SPSS, Chicago, IL, USA) and PRISM (Graphpad, San Diego, CA, USA) and analysed using one-way analysis of variance (ANOVA) followed by multiple comparison tests (SigmaStat, Jandel Scientific, San Rafael, CA, USA). Concentration–response curves were analysed using the program ALLEFIT [32], which fits the entire data set to the logistic equation and permits independent testing of differences between individual parameters.

Results

Food Intake and Body Weight

The body weight curves of *cp/cp* and *+/?* rats differ from an early age (figure 1). During the initial 2 weeks

of the treatment period (4 and 6 weeks of age), there was no detectable difference in food intake or body weight between the CLA-treated (alone or with CrP) *cp/cp* rats and the *+/?* controls. By 6 weeks of age, when these animals rapidly develop the phenotype of hyperinsulinaemia and the metabolic syndrome, the food intake and body weight of the CLA-treated rats diverged from the *+/?* controls towards values for the *cp/cp* control animals. At 12 weeks of age, food intake was not significantly reduced in CLA-treated rats (30.0 ± 1.7 vs. 34.1 ± 1.4 g/day, $p = 0.0906$) and was greater than that of the *+/?* controls (22.5 ± 0.4 g/day). Similarly the body weights, at 12.5 weeks of age, were reduced in CLA-treated *cp/cp* rats (412 ± 8 vs. 476 ± 12 g), but still greater than *+/?* animals (323 ± 6 g). In contrast to CLA, CrP administered alone or in combination had no effect on food intake or body weight.

Insulin Sensitivity

The *cp/cp* rats supplemented with CLA (with or without CrP) had significantly lower fasting insulin levels compared with untreated *cp/cp* rats ($p < 0.002$) (figure 2). Following the meal challenge, animals supplemented with combination therapy (CLA + CrP) showed a reduction in the insulin response at 30 min ($p < 0.002$) that was not evident with either agent alone. There was no difference between the fasting plasma concentrations of glucose (or at the 30-min time point) for the four groups of *cp/cp* rats (data not shown).

Biochemical Parameters

All lipid classes are moderately to markedly elevated in *cp/cp* compared with *+/?* animals (figure 3). The CrP-treated rats had a significant decrease in non-esterified cholesterol, but not in esterified cholesterol or other lipids. CLA-treated rats (with or without CrP) showed significant reduction in cholesterol ester levels ($p < 0.01$ and $p < 0.005$). Rats supplemented with both CLA and CrP demonstrated reduction in triglyceride and phospholipid fractions (37 and 19%, respectively, $p < 0.05$).

Plasma adiponectin concentrations did not differ between untreated *cp/cp* animals and those supplemented with either CLA or CrP alone (5.22 ± 0.57 , 6.00 ± 0.41 and 5.17 ± 0.52 μ g/ml, respectively). However, plasma adiponectin concentrations increased by approximately 54% (8.02 ± 0.42 μ g/ml, $p < 0.01$) in rats treated with both CLA and CrP.

Fig. 1 Food intake and body weight of JCR:LA-*cp* rats. Values are mean $\pm$ s.e.m., 10 rats in each group. Significant differences between groups are discussed in the text.

Fig. 2 Plasma insulin concentrations of JCR:LA-*cp* rats, fasted (0 min) and 30 and 60 min following a 5-g meal challenge. Values are mean $\pm$ s.e.m., 8–10 rats in each group. ** p < 0.01, *** p < 0.001 vs. *cp/cp* control.

Fig. 3 Plasma lipid concentrations of 13-week-old JCR:LA-*cp* rats. Values are mean $\pm$ s.e.m., 8–10 rats in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. *cp/cp* control.

Vascular Function Studies

Contractile response of aortae from +/? rats was lower than that of aortae from *cp/cp* rats (figure 4). The maximum contractile response of aortae from CrP-treated rats was significantly lower ($p < 0.02$). Furthermore, there was a significant decrease in the EC_{50} for ACh-mediated relaxation of aortae from CrP-treated rats. By contrast, there was no difference in the contractile response to PE, or relaxant response to ACh, of aortae from rats treated with CLA (with or without CrP). There was no difference in the contractile response to PE in the

presence of L-NAME between any of the groups nor any difference in the relaxant response to SNP (data not shown).

Renal Effects

Urinary albumin concentrations are elevated in *cp/cp* animals at 12 weeks of age (figure 5). The data showed a greater variance when expressed as the albumin/creatinine ratio, and albumin data only are shown. Supplementation with CLA (either alone or combined

Fig. 4 Contractile/relaxation function of aortic rings from JCR:LA-*cp* rats: dose response to PE in the absence (left panel) and presence (middle panel) of the NOS inhibitor L-NAME (10^{-4} mol/l; relaxant dose response to ACh (right panel). Values are mean $\pm$ s.e.m., 8–10 rats in each group. The maximum contractile response of +/? aortae was lower than that of *cp/cp* aortae (1.47 ± 0.202 and 2.15 ± 0.128 g, respectively, $p < 0.01$). The maximum contractile response of aortae of CrP-treated rats was lower (1.78 ± 0.062 g, $p < 0.02$). There was no difference in the contractile response of aortae from rats treated with CLA (either with or without CrP). EC_{50} for ACh-mediated relaxation of aortae from CrP-treated rats was decreased ($1.84 \pm 0.42 \times 10^{-6}$ to $5.6 \pm 1.07 \times 10^{-7}$ M, $p < 0.0001$). The aortae of rats treated with CLA (with or without CrP) showed no change in the relaxant response to ACh compared with untreated *cp/cp* rats.

with CrP) resulted in significant reduction in urinary albumin levels (75 and 85%, respectively, $p < 0.05$). Treatment with CrP alone resulted in an approximately 50% decrease in albumin levels (statistically insignificant due to the high variance in the control group).

The *cp/cp* rat exhibits a greater severity of glomerulosclerosis than the *+/?* rat, despite the early age of 12 weeks (figure 5). There was a striking and distinctive reduction in the fraction of sclerotic glomeruli of CrP-treated rats ($p < 0.0001$) and even further in CLA-treated rats ($p < 0.0001$). Rats treated with the combination of both CLA and CrP showed an intermediate but also highly significant reduction ($p < 0.0001$).

Discussion

The progression of the metabolic syndrome is accompanied by vascular dysfunction and culminates in type 2 diabetes, ischaemic coronary disease and/or end-stage renal injury. The process is complex and not well understood. We report here, for the first time, that dietary supplementation with CLA and CrP, in combination, can contribute significantly to the reduction of vascular dysfunction and renal sequelae in an

established animal model of the metabolic syndrome and prediabetic state.

Food Intake and Weight Gain

CLA – administered from 4 weeks of age, i.e. before the onset of the development of insulin resistance in the *cp/cp* rat [19] – temporarily normalized food intake and the rate of weight gain. However, the effect waned rapidly after 6 weeks of age. Regression analysis of the body weight data at the end of the treatment period, between 9.5 and 12 weeks of age, revealed that the rate of weight gain of the CLA-treated rats was equivalent to that of the *cp/cp* control rats (4.11 ± 0.44 and 5.06 ± 0.74 g/day, respectively, $p = 0.294$) with *+/?* rats having a much lower rate (1.73 ± 0.36 g/day, $p = 0.002$ vs. *cp/cp*). These results suggest that CLA may be most beneficial when administered at an early age, prior to the development of frank obesity and insulin resistance. By contrast, CrP appeared to exert its beneficial metabolic effects when obesity and insulin resistance were fully established.

Metabolism of Glucose and Insulin

Insulin resistance in the *cp/cp* rat is evident in the fasting hyperinsulinaemia with euglycaemia [21]. The meal tolerance test provides a well-documented physiological index of insulin sensitivity through both fasting and postprandial insulin levels [21,25–28]. Treatment with CLA reduced fasting plasma insulin concentrations, which is consistent with improved insulin sensitivity found in many other CLA-related studies. It appears that many of these endocrine/adipogenic effects are modulated by CLA's ability to stimulate the proliferator-activated receptor (PPAR) pathway in the adipocyte [33]. By contrast, CrP, which had no effect on body weight gain, did not reduce fasting insulin levels, despite the relatively high pharmacological dose given in this study. It is possible that the potential mechanism(s) of action of CrP (thought to be elicited through chromodulin) may be masked by the extreme hyperinsulinaemia in these animals. However, the combination group that received both CLA and CrP were the only animals to show a significantly reduced insulin response 30 min after the meal challenge, suggesting a synergistic effect. The absence of changes in plasma glucose levels for any of the treatment groups is consistent with the observation that the *cp/cp* rat maintains relative normoglycaemia, albeit at the expense of very high circulating insulin concentrations [21].

Fig. 5 Urine albumin concentrations and proportion of sclerotic glomeruli in 13-week-old JCR:LA-*cp* rats. Values are mean $\pm$ s.e.m., 8–10 rats in each group. * $p < 0.05$, *** $p < 0.001$ vs. *cp/cp* control.

Lipid Metabolism

When CrP and CLA were supplemented individually, the unesterified or esterified fractions of cholesterol were modestly reduced, respectively, supportive of differential mechanisms of lipid modulation. It has been suggested that CLA reduces both the production and secretion of apoB100 (an obligatory marker for VLDL assembly) by human hepatocytes [34]. Decreased intracellular concentrations of cholesterol esters are thought to facilitate a greater rate of intracellular proteolytic degradation of apoB100 (consistent with observations from this study). Other studies have shown that CLA can increase LDL receptor protein and mRNA expression in HepG2 cells, furthering the potential cholesterol-lowering effects of CLA [35]. By contrast, the mechanism(s) of action for CrP are less well understood. Previous studies have been based on the hypothesis that beneficial effects of CrP on lipid/cholesterol levels are an indirect and secondary consequence of its insulin-sensitizing properties [15]. Recent reports have provided evidence of a functional role for chromodulin, a small oligopeptide that can tightly bind up to four Cr⁺⁺⁺ ions [36]. Chromodulin is thought to activate tyrosine kinase activity of the insulin receptor and may explain (at least in part) the independent insulin-sensitizing actions of CrP observed in this study. Collectively, these distinct independent mechanisms of action, for both CLA and CrP, are indicative of synergistic bioactivity, particularly with respect to insulin sensitization and lipid lowering.

Adiponectin

Adiponectin is secreted by adipocytes and considered to be a marker of inflammation with lower plasma concentrations in both obese humans and rodent models of obesity/insulin resistance [37–39]. While adiponectin levels in obese *cp/cp* rats supplemented with either CLA or CrP alone were not significantly different from those in untreated *cp/cp* animals, adiponectin was increased by approximately 54% by the combination of the two supplements. This suggests a reduction in inflammation and is consistent with the reduced glomerular sclerosis seen in the treated rats. Nagao and colleagues [12,40] have reported that CLA increased mRNA expression for adiponectin in adipose tissue from *fa/fa* ZDF rats, consistent with activation of the PPAR pathway. It is also possible that CrP-associated chromodulin has intracellular stabilizing effects on the tyrosine-kinase pathway that may add to the apparently enhanced secretion of adiponectin observed.

Adiponectin is known to circulate in multimeric aggregates of different molecular weight [41]. Treatment with thiazolidendione agonists of PPAR γ , which have insulin-sensitizing effects in the *cp/cp* rat [27], increases the ratio of the higher molecular weight to the lower weight forms and can be predictive of improved insulin sensitivity [41].

Vascular Function

Improvements in vascular function were limited to vessels from animals treated with CrP alone. The observed reduction in maximum contractile response to PE is physiologically significant and suggests a direct role for CrP in modulation of vascular tone. The absence of an effect in the presence of L-NAME indicates that the functional improvement involves endothelial NO metabolism. The threefold reduction in the EC₅₀ for ACh-mediated relaxation in the CrP-treated rats is also highly significant and together with the lack of effect on SNP-mediated relaxation supports the concept of a direct beneficial effect on the endothelial cell. There are limited data indicating direct effects of CrP on endothelium. Moore and colleagues [42] have shown that CrP, in the presence of insulin, has a synergistic effect on the recovery rate of intracellular Ca²⁺ in vascular smooth muscle cells and can improve Ca²⁺-ATPase expression. We speculate that together with the insulin-sensitizing effects of chromodulin, CrP may play some part in improving endothelial Ca²⁺ flux, in turn improving vascular tone.

Diabetic Complications and Glomerulosclerosis

The end stages of the metabolic syndrome and type 2 diabetes include pathological complications such as myocardial ischaemia and glomerulosclerosis, evident in the JCR:LA-*cp* model [20,23,26,28]. Urinary albumin is an indicator of renal microvascular damage, resulting in increased glomerular permeability and inability to retain albumin. The damage is evident physically as glomerular sclerosis and is a major cause of end-stage renal failure in diabetic patients. Rats fed CLA (with or without CrP) had significant reduction in urinary albumin concentration. Consistent with this, but more striking, treatment with CLA markedly reduced the incidence of glomerulosclerosis. We show here, for the first time, that supplementation with CLA can markedly reduce the severity of glomerular sclerosis in an animal model of early stage prediabetic complications. Few studies have addressed the mechanisms of kidney damage with respect to lipid deposition profile and nutritional fatty acid status. However, Ogborn

et al. [10] concluded that CLA may have anti-inflammatory and antifibrotic effects in the kidney mediated via prostaglandin E₂. Given this and our findings, CLA, alone or in combination with CrP, has a potential therapeutic role in the amelioration of renal disease associated with type 2 diabetes.

In conclusion, the findings of this study offer novel and striking evidence of the benefit of co-supplementation of CLA and CrP in the JCR:LA-*cp* model of insulin resistance and vascular disease. CLA and CrP, in combination, offer improvements to markers of disease risk, including lipid profile, hyperinsulinaemia and circulating adipokines, which are not apparent for either alone. Moreover, this study provides insight into the potential of these supplements to reduce macrovascular dysfunction and protect against glomerular sclerosis and renal disease, the most important complications of the prediabetic state. The findings are sufficiently encouraging to support clinical trial of these promising agents.

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