A CLA enriched diet improves organ damage associated with the metabolic syndrome in spontaneous hypertensive rats

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RESUMEN

Una dieta enriquecida con CLA mejora el daño a órganos asociados al síndrome metabólico en ratas hipertensas espontáneas.

El objetivo del presente estudio fue proporcionar evidencia que el CLA dietario puede evitar la patogénesis del síndrome metabólico en la estructura tisular, lo que sugiere beneficios potenciales en el desarrollo de este síndrome. Dos grupos de ratas Wistar macho espontáneamente hipertensas (SHR) fueron alimentadas con una dieta normal por 8 semanas: una dieta contenía 7.5% de aceite de girasol (grupo VS-HR), y el otro contenía 6% de aceite de girasol y 1.5% de CLA (grupo CLA-SHR). A un grupo testigo sano de ratas macho Kyoto-Wistar les fue proporcionada una dieta normal con 7.5% de aceite de girasol. Los animales fueron sacrificados y se tomaron secciones de hígado, riñones y aorta, las cuales fueron fijadas en formaldehído al 10% y teñidas con Hematoxilina y Eosina. Las tinciones de aorta mostraron morfología irregular del endotelio en la aorta solo en el grupo V-SHR; el parénquima hepático se caracterizó por infiltración de neutrófilos inflamatorios, fibrosis y engrosamiento del epitelio de la vena porta, hiperplasia hepática y esteatosis. El tejido renal de este mismo grupo evidenció hiperplasia en las células del endotelio de la cápsula de Bowman. No se observaron cambios anormales histológicos tanto en el grupo testigo como en el alimentado con CLA, lo que sugiere un papel protector del CLA en el desarrollo del síndrome metabólico.

PALABRAS CLAVE: Ácido linoléico conjugado – Disfunción endotelial – Esteatosis – Síndrome metabólico – Resistencia a la insulina.

SUMMARY

A CLA enriched diet improves organ damage associated with the metabolic syndrome in spontaneous hypertensive rats.

The purpose of this study was to provide evidence that dietary CLA can prevent the pathogenesis of metabolic syndrome in tissue structure, suggesting potential benefits in the onset of this syndrome. Wistar male spontaneous hypertensive rats (SHR), were classified into two groups that were fed a standard diet for eight weeks: one with 7.5% sunflower oil (V-SHR group), and the other with 6% sunflower oil and 1.5% CLA (CLA-SHR group). A control healthy group consisted of Kyoto-Wistar male rats fed the standard diet with 7.5% sunflower oil. The animals were sacrificed, and sections of liver, kidneys and aorta were fixed in 10% formaldehyde, and then stained with Hematoxylin and Eosin. Only in the V-SHR group, the stain of the aorta indicated irregular endothelial morphology; liver parenchyma was characterized by an infiltration of inflammatory neutrophils, fibrosis, thickening of the portal vein epithelium, hepatocyte hyperplasia and steatosis. The renal tissue of this group evidenced hyperplasia in the cells of the endothelial of Bowman´s capsule. Abnormal histological changes were not observed in either the control group or the rats fed with CLA, suggesting a protective role of CLA in the onset of metabolic syndrome.

KEY-WORDS: Conjugated linoleic acid – Endothelial dysfunction – Insulin resistance – Metabolic syndrome – Steatosis.

1. INTRODUCTION

Reaven (1998) first introduced the concept of syndrome X or metabolic syndrome (MS); which he described as a cluster of abnormal glucose tolerance or diabetes, dyslipidemia (low HDL cholesterol, high total triglycerides), obesity, insulin resistance and elevated blood pressure. Recently, several other metabolic disturbances that are more frequently found in combinations rather than isolated in individuals with MS have been included (Lamarche & Desroches 2004). Thus, an increased number of reduced size LDL particles, impaired fibrinolytic activity, a pro-inflammatory state, impaired postprandial metabolism, abdominal obesity, and a pro-oxidative state were all considered as important features of MS (Reaven, 2002).

It has been established that insulin is an anti-inflammatory hormone, so an impairment causing an insulin resistance promotes the activation of some pro-inflammatory transcription molecules such as the intra-nuclear factor (NF)-kB, early growth response-1 (Egr-1) and activating protein-1 (AP-1) (Aljada *et al.*, 2002). Similarly, obesity has been associated to inflammation. This association was first proposed by Hotamisligil and co-workers (1993), who found that the tumor necrosis factor-α (TNF-α) was over expressed in the adipose tissue of obese mice. TNF-α is a pro-inflammatory cytokine that can activate various cascades, including many of the pathways that control insulin activity.

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Other factors are also associated with the increased incidence of MS; for example, the amount of food that is consumed by a single person today is far greater than before; furthermore, large portions of the average diet of humans consist of fast foods and may not contain enough fiber (Dandona *et al.*, 2005). Because resistance to insulin also results in the relative non-suppression of adipocyte hormone–sensitive lipase, there is a further enhancement of lipolysis and increased FFA concentration. Thus, a vicious circle of lipolysis, increased FFA, insulin resistance, and inflammation is formed (Dandona *et al.*, 2005; Roche, 2005; Phillips *et al.*, 2008).

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid (octadeca-9:12-dienoic acid). The major dietary sources of CLA for humans are beef and dairy products; beef tallow contains ~0.5% of fatty acids as CLA (Ohnuki *et al.*, 2001). CLA has attracted considerable attention over the past few years because of its beneficial biological effects, including protective roles against several types of cancers, atherosclerosis, and obesity (Chin *et al.*, 1992; Nicolosi *et al.*, 1997; Terpstra, 2004; Mullen *et al.*, 2007). One of the major risk factors for metabolic syndrome is diabetes, which has been tested in animal models. In this regard the antidiabetic effects of CLA have been examined in animal models like Zucker diabetic fatty (ZDF), fa/fa rats, which exhibit obesity and hyperglycemia at an age of 7-8 weeks. Feeding with 1.5% of CLA for 14 days normalized impaired glucose tolerance and attenuated fasting hyperinsulinemia and free fatty acid concentrations (Nagao *et al.*, 2003).

The purpose of this study was to provide evidence that dietary CLA prevents the pathogenesis of MS on tissue structure, suggesting potential benefits in the onset of this condition.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals were of analytical grade, obtained from Sigma (St. Louis, MO), Bayer (Mexico City) and J.T. Baker (Mexico City). Dietary components were purchased from Harlan Teklad Inc. (Mexico City).

2.2. Experimental design

Ten male Kyoto-Wistar (KW) and 16 SHR (spontaneously hypertensive rats), all nearly 21 days old, were purchased from Harlan Teklad Inc. (México City). Animals were individually housed in stainless-steel cages and maintained in 12-h light/dark cycles at 25°C. Animal maintenance and handling were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1985). The rats were divided into two groups: the KW rats as the normotensive control group (n =10) and the SHR as the MS group (n = 16). Both groups received a standard diet (Lab Diet 2001, Harlan Teklad) for 2 weeks. Then, the spontaneously hypertensive rats (SHR) were divided into two groups: a sunflower oil group (V-SHR; n = 8), and a CLA mix group (CLA–SHR; n = 8). The V-SHR group received a diet with 7.5% sunflower oil (Patrona brand, from the local market) and the CLA–SHR group received a diet with 6 % sunflower oil and 1.5 % of CLA (Lipid Nutrition; Clarinol A-95) as lipid source. The control group (KW; n=10) received the sunflower oil diet; the three groups received the experimental diets depicted in Table 1 for 8 weeks. Diets were prepared including 0.02% BHT as antioxidant and stored under refrigeration until used.

2.3. Blood pressure measurement

At the end of the experimental diet period, body weight and systolic blood pressure were measured by a tail-cuff method (IITC) noninvasive blood pressure system, model 29; Life Science Instruments (Woodland Hills, CA).

2.4. Analytical Methods

Blood samples from 18-hour fasted animals were carefully collected from the tail tip, and centrifuged at 1086xg for 10 min. Serum samples were kept at –20°C for further analysis. Serum glucose concentration was measured by the glucose oxidase method (Barham & Trinder 1972). Total cholesterol and triglycerides were quantified using an enzymatic assay (Assmann, 1979).

2.5. Microscopic analysis of tissues

To assess tissue damage, slices of each organ were fixed for 24 h in 10% neutral buffered formalin. Sections (4-6 µm) of the tissues were embedded in paraffin, and stained with hematoxylin and eosin (H-E) prior to microscopic examination (Bayliss, 1990) Images were obtained using an Olympus BX51 microscope equipped with a Camedia C3040ZOOM digital camera (Olympus America, Melville, NY). All the images were taken at 50X enlargement.

Table 1
Composition of the experimental diets fed to the rats

Ingredients	KW and V-SHR Diet (g)	CLA–SHR Diet (g)
Casein	440	440
DL-Methionine	3.1	3.1
Cellulose	40	40
Corn starch	658	658
Vitamin mixture	20	20
Mineral mixture	80	80
BHT	0.4	0.4
Sunflower oil	100	80
CLA mix	–	21.5

2.6. Data analysis

Data are presented as the mean ±SD. Statistical significance was determined by ANOVA, and Tukey's multiple range test was used for mean comparison (p<0.05).

3. RESULTS

3.1. Body weight, biochemical measurements and blood pressure

Table 2 shows the differences in body weight as well as food intake at the start and after the eight weeks of experimental treatment with sunflower oil and conjugated linoleic acid (CLA-SHR). The rats with sunflower oil (V-SHR) administration showed an increase in body weight and in food consumption (378.30±16.67 g and 9.61±0.30 g/day/100 g bw, respectively) (p<0.05) with respect to the control group Kyoto-Wistar (KW) (312.09±17.61 g and 5.18±0.70 g/day/100 g bw, respectively) (p<0.05). On the other hand, the rats with (CLA-SHR) only showed a significant difference in body weight (345.36±11.70 g) (p<0.05) with respect to the control group (KW).

Regarding the profile of serum parameters, Table 3 depicts the concentrations of glucose, cholesterol and triglycerides in the three experimental groups. The values between groups were no different before the experiment. However, after 8 weeks, in the CLA-SHR rats the concentration of serum glucose (91.05±1.13 mg/dL), cholesterol (42.68±1.77 mg/dL) and triglycerides (33.00±2.14 mg/dL) were significantly lower than in the KW (glucose, 122.0±5.27 mg/dL; cholesterol 75.67±4.46 mg/dL and triglycerides 108.25±4.24 mg/dL) and V-SHR (glucose 165.03±1.86 mg/dL; cholesterol 94.46±1.99 mg/dL and triglycerides 136.39±2.08 mg/dL) groups (see Table 4).

Blood pressure was measured in animals before and after the experiment to assess the effect of the diets. At the beginning of the experiment, the systolic blood pressure value was similar in the three groups (see Table 3). However, at the end of the 8 weeks of treatment, statistical differences were found. The CLA-SHR rats showed a decrease in systolic blood pressure with respect to the group of rats fed only sunflower oil diet (see Table 4).

3.2. Microscopic tissue analysis

Figure 1, panels A through C, depicts the changes in the aorta in the groups KW, V-SHR and CLA-SHR. The H-E stain suggests proliferation of elastic fibers and collagen in the intimae and middle layers in the V-SHR group, compared to the control group. It was observed that samples from the CLA-SHR group had a positive change in the endothelium. Figure 2, panels D to G, shows liver parenchyma from the KW and CLA-SHR group, respectively. An infiltration of inflammatory neutrophils, the presence of globose hepatocytes and steatosis, characterized by fat-filled hepatocytes (panels E and F) were clearly noted in the V-SHR group.

The cortical portion of the renal tissue can be identified in Figure 3, panels H to J. It is characterized by the presence of renal glomeruli (rg) surrounded by the endothelium forming Bowman's capsule (bc). In Figure 3, panel I, the renal tissue of all animals from group V-SHR showed hyperplasia in the cells of the endothelial of Bowman's capsule.

4. DISCUSSION

This study showed that a daily supplementation of the diet with sunflower oil lead to a significant elevation of glucose, cholesterol and triglyceride

Table 2
Body weight and food intake in rats fed experimental diets for eight weeks

	KW	V-SHR	CLA-SHR
Food consumption (g/day/100 g bw.	5.18±0.70[a]	9.61±0.30[b]	5.50±0.04[a]
Weight after eight weeks/g)	312.09±17.61[a]	378.30±16.67[b]	345.36±11.70[c]

bw=body weight. Values represent the mean ±S.D. Means in the same row with the same superscript are statistically similar (p<0.05).

Table 3
Biochemical parameters and blood pressure in rats before the experiment

Animal group	KW n=10	SHR* n=16
glucose (mg/dL)	136.15±8.96[a]	135.96±14[a]
cholesterol (mg/dL)	49.30±4.21[a]	40.97±4.09[b]
triglycerides (mg/dL)	85.74±3.07[a]	109.30±9.50[b]
systolic blood pressure (mm Hg)	133.4±9.52[a]	196.8±12.78[b]

KW=Kyoto Wistar; *Refers to rats to be fed both the sunflower diet and (V-SHR) and conjugated linoleic acid (CLA-SHR) diets. Values represent the mean ±S.D. Means in the same row with the same superscript are statistically similar (p<0.05).

Table 4
Biochemical parameters and blood pressure in rats after the experiment

Animal group	KW n=10	V-SHR n=8	CLA-SHR n=8
glucose (mg/dL)	122.00±5.27[a]	165.03±1.86[b]	91.05±1.13[c]
cholesterol (mg/dL)	75.67±4.46[a]	94.46±1.99[b]	42.68±1.77[c]
triglycerides (mg/dL)	108.25±4.24[a]	136.39±2.08[b]	33.00±2.14[c]
systolic blood pressure (mm Hg)	142.00±6.32[a]	222.88±12.32[b]	160.00±8.16[c]

KW=Kyoto Wistar; sunflower diet and (V-SHR) and conjugated linoleic acid (CLA-SHR) diets. Values represent the mean ±S.D. Means in the same row with the same superscript are statistically similar ($p<0.05$).

concentrations. These findings clearly support the atherogenic properties of saturated fatty acid which were previously demonstrated by Bayindir and co-workers (2002). The morphological examination of the aorta of the V-SHR group revealed major alterations related to hyperlipidemia, including irregular endothelium morphology and growth of fibrous tissue. It has been recognized that the endothelial cells release nitric oxide that relaxes the muscle cell of the blood vessel wall, by allowing blood to flow more freely (Cohen, 1995; Wilson *et al.*, 2006). Fatty acids such as eicosa-5:8:11:14-tetraenoic acid (arachidonic acid) regulates and reduces nitric oxide synthesis, thereby fostering an inadequate transit of substances through the endothelium. This may explain the presence of fibrous tissue in the endothelium of the aorta of the group V-SHR (see Figure 1, panel B).

It has been shown that an intake of polyunsaturated fatty acids such as CLA in the diet of animals with MS exerted a positive effect on the symptoms of the MS. Lee and co-workers (1994) reported that CLA increased the functionality and decreased the cellular state for insulin resistance. This assertion suggests the absence of an atherogenic process in the aorta of the animals fed CLA, as shown in the CLA-SHR group in Figure 1 (panel C). Similarly, Alexander and co-workers (2004) reported that n-3 PUFA induced structural changes that improved the physico-chemical properties of the membrane of the adipocites by increasing its fluidity and viscosity in hypertensive rats. These structural changes of the membrane, could account for the limited

development of atheroma in animals of the CLA-SHR group (see Figure 1 panel C).

Moreover, the pathogenesis observed in liver parenchyma of the experimental animals fed with vegetable oil (Figure 2, panels E and F) was characterized by an infiltration of inflammatory neutrophils. Different etiological factors have been proposed, so that an increase in liver mitochondrial oxidation by free fatty acids may cause oxidative stress (Kumar *et al.*, 2006). This process is characterized by the production of nitric oxide by the inflammatory cells present in liver parenchyma that enlarge the endothelium and promote smooth muscle relaxation. This event decreases blood circulation into the liver cells. In the same way, the oxidative stress promotes the formation of peroxides in the lipids of the cellular membrane, thus affecting its viscosity (Karninski, 2004). In the rest of the parenchyma it is possible to assess the presence of globose hepatocytes (gh) (panel E). Additionally, fibrosis, thickening of the portal vein epithelium (pv), hepatocyte hyperplasia and steatosis (s), characterized by fat-loaded hepatocytes (panels E and F) were noted.

Histological analysis performed on samples of animals fed with CLA (Figure 2, panel G) shows the absence of steatosis in liver tissue. Recent data obtained by Moloney and co-workers (2007a) showed that the intake of dietary CLA normalized insulin levels, and suggested an alternative mechanism for reduced levels of triglycerides. This was probably due to a decline in insulin production to prevent the re-esterification of free fatty acids into triglycerides
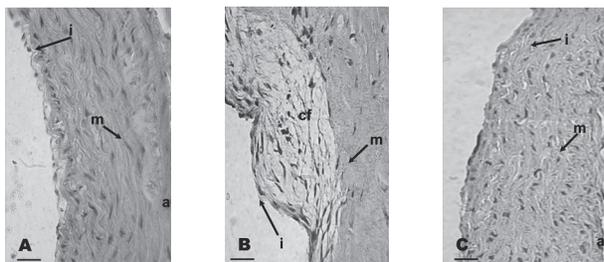

Figure 1
Effects of experimental diets on aorta. (A): normal cytoarchitecture of aorta in the control group (KW); (B): proliferation of collagen fibers (cf) in the intimal (i) and media (m) layers in the V-SHR group; (C): absence of pathological alterations in the intimal (i), media (m) and adventitious (a) layers of the CLA-SHR group. Staining was done with hematoxylin-eosin and enlargement was 50X (bar= 60 μm).
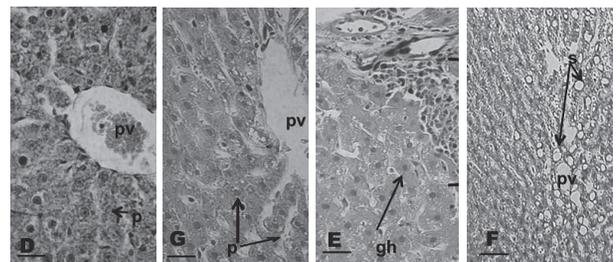
Figure 2
Effects of experimental diets on liver. (D): normal cytoarchitecture of the portal vein (pv) and the rest of parenchyma (p) of the hepatic tissue in the KW group; (G): normal parenchyma (p) in the CLA-SHR group. (E): an inflammatory process (ip) as well as globose hepatocytes (gh) in the V-SHR group; (F): steatosis and hyperplasia of the hepatocytes in the same V-SHR group. Staining was done with hematoxylin-eosin, and enlargement was 50X (bar = 60 μm).

in the liver. This phenomenon could explain the absence of excessive fat in the hepatocytes of animals that received dietary CLA. Similarly, studies at the molecular level suggest that there are regulatory genes that can be triggered by dietary fatty acids (Belury *et al.*, 2002; Phillips *et al.*, 2008). Probably the lack of hepatic steatosis in the parenchyma of the animals that received the CLA was caused by regulatory genes activated in the presence of polyunsaturated fatty acids, such as p65 and NFkBα (Belury *et al.*, 2002; Chen 2004; Phillips *et al.*, 2008); however, it is necessary to pursue additional studies to support this.

Our data revealed significant changes in renal tissue; the cortical portion of renal tissue can be identified in Figure 3 panels H-J. It is characterized by the presence of renal glomeruli surrounded by the endothelium forming Bowman's capsule. In Figure 3 panel I, the renal tissue of all animals from group V-SHR showed hyperplasia in the cells of Bowman`s capsule endothelium. Epidemiological evidence, as well as biochemical studies, have shown that high levels of insulin may be related to high blood pressure (Monoley, 2007b). Insulin stimulates the peripheral nervous system and facilitates sodium absorption, thereby promoting additional changes in the cell membrane system of the ion transport responsible for cell hyperplasia (Weir, 2007). Similarly, insulin levels induce lipolysis, thus activating triacylglycerol synthesis, which form the abdominal adipose tissue and then lead to obesity. With obesity the amount of pro- and anti-inflammatory adipokines released is increased within the fat tissue. These molecules are implicated in many clinical expressions of this pathology such as diabetes, arterial hypertension or cardiovascular disease (Rodríguez *et al.*, 2009).

Rats that received the CLA-SHR diet showed a recovery in the integrity of glomerular epithelium because hyperplasia was only observed in less than half of the experimental animals (p <0.05) (see Figure 3, panel J). This suggests that CLA participates in the regulation of blood pressure and reduces renal damage, which consequently improves the vascular tone in the endothelium of Bowman's capsule. This has been attributed to a shift in eicosanoid production away from the 2-series prostaglandins derived from arachidonic acid, which are potent vasoconstrictors (Alexander *et al.*, 2004).
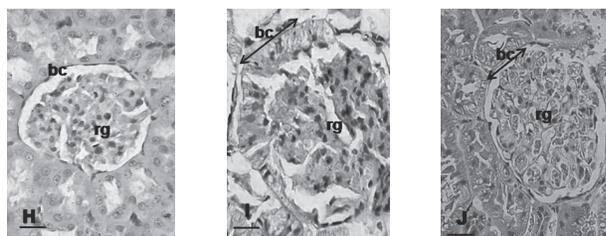

Figure 3
Effects of the experimental diets on kidneys. (H): renal glomeruli (rg) in the (KW) group. (I): hyperplasia in the endothelial cells of the Bowman's capsule (bc) in the V-SHR group. (J): recovery of the cells in the Bowman's capsule in the CLA-SHR group. Staining was done with hematoxylin-eosin and enlargement was 50X (bar= 60 μm).

5. CONCLUSION

This study demonstrated that intervention with a CLA enriched diet significantly reduced biochemical parameters, improved irregular endothelial morphology, reduced liver damage and prevented the hyperplasia of renal cells in rats with MS.

ACKNOWLEDGMENTS

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