Original

Influence of G1359A polymorphism of the cannabinoid receptor gene (CNR1) on insulin resistance and adipokines in patients with non alcoholic fatty liver disease

R. Aller1,2, D. A. de Luis2, D. Pacheco2, M. C. Velasco2, R. Conde1, O. Izaola2 and M. González Sagrado2


Abstract

Background: Considering the evidence that endogenous cannabinoid system plays a role in metabolic aspects of body weight and metabolic syndrome components such as non alcoholic fatty liver disease (NAFLD). The aim of our study was to investigate the influence of this polymorphism on insulin resistance, liver histological changes, anthropometric parameters and adipocytokines in patients with NAFLD.

Material and methods: A population of 71 patients with NAFLD was recruited in a cross sectional study. A biochemical analysis of serum was measured. Genotype of G1359A polymorphism of CB1 receptor gene CB1 receptor was studied. Forty one patients (36.9%) had the genotype G1359G (wild type group) and twenty nine (26.1%) patients G1359A or A1359A (mutant type group).

Results: Twenty four 24 patients (32.3%) had a Brunt grade > 4 and 12 patients (17%) had a significant fibrosis (F = 2). HOMA values were higher in wild type group than mutant type group. Adiponectin and visfatin levels were higher in mutant type group. Moreover, TNF-alpha and resistin levels were higher in wild type group than mutant type group. Patients with mutant genotype showed less frequently elevated levels of AST. AST > 40 U/L was detected in 28.5% of patients in the mutant vs. 53% of patients with wild genotype, p < 0.05. Patients with mutant type group presented a percentage of Brunt grade > 4 less frequently than patients with wild type group (28.5% vs 7.1%).

Conclusion: A variant of the polymorphism G1359A CB1 is associated with lower levels of HOMA, TNF-alpha, resistin and higher levels of adiponectin than patients with the wild variant of this polymorphism. Besides, patients with A allele variant show lower Brunt grade in liver biopsy.

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Key words: Adipokines. Cannabinoid receptor gene. Steatosis. Polymorphism. Insulin resistance.

Correspondence: R. Aller.
Professor Associated of Gastroenterology.
C/los perales, 16.
47130. Simancas. Valladolid. Spain
E-mail: dadluis@yahoo.es
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Resumen

Antecedentes: Teniendo en cuenta la evidencia de que el sistema cannabinoide endógeno juega un papel importante en aspectos metabólicos, peso corporal y componentes del síndrome metabólico como la enfermedad hepática NO alcohólica (EHNA). El objetivo de nuestro estudio fue investigar la influencia de este polimorfismo en la resistencia a la insulina, cambios en la histología hepática, parámetros antropométricos y adipocitoquinas en pacientes con hígado graso no alcohólico.

Material y métodos: Una población de 71 pacientes con hígado graso no alcohólico fue reclutado en un estudio de corte transversal. Se realizó un análisis bioquímico de suero. El genotipo del polimorfismo G1359A del gen del receptor CB1 se ha estudiado en todos los pacientes. Cuarenta y un pacientes (36.9%) tenían el genotipo G1359G (grupo de tipo salvaje) y veintinueve (26.1%) de los pacientes o G1359A A1359A (grupo mutante).

Resultados: Veinticuatro 24 pacientes (32.3%) tenían un grado de Brunt > 4 y 12 pacientes (17%) tenían una fibrosis significativa (F = 2). Los valores de HOMA fueron mayores en el grupo con genotipo salvaje que el grupo mutante. Los niveles de adiponectina y visfainá fueron mayores en el grupo con genotipo mutante. Por otra parte, el TNF-alfa y los niveles de resistina fueron más altos en el grupo con genotipo salvaje que el grupo mutante. Los pacientes con genotipo mutante mostraron niveles elevados de menor frecuencia de AST. AST > 40 U/L se detectó en el 28.5% de los pacientes con el genotipo mutante frente a 53% de los pacientes con genotipo salvaje, p < 0.05. Los pacientes con genotipo mutante presentaron un porcentaje de grado de Brunt > = 4 con menos frecuencia que los pacientes con genotipo salvaje (28.5% vs 7.1%).

Conclusión: Una variante del polimorfismo G1359A CB1 se asocia con menores niveles de HOMA, TNF-alfa, resistina y mayores niveles de adiponectina que los pacientes con la variante salvaje de este polimorfismo. Además, los pacientes con una variante del alelo muestra menor grado de Brunt en la biopsia hepática.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a liver disease characterized by elevated serum aminotransferase levels and accumulation of fat in liver accompanied by inflammation and necrosis resembling alcoholic hepatitis in the absence of heavy alcohol consumption.1,2

The endocannabinoid system comprises receptors, CB1 and CB2, their endogenous lipidic ligands and machinery dedicated to endocannabinoid synthesis and degradation. An overactive endocannabinoid system appears to contribute to the pathogenesis of several diseases, including liver diseases. Endogenous activation of peripheral CB1 receptors is a key mediator of insulin resistance and enhances liver lipogenesis in experimental models of NAFLD. Moreover, some authors have demonstrated that adipose tissue CB2 receptors are markedly upregulated and promote fat inflammation, thereby contributing to insulin resistance and liver steatosis.3 Also, tonic activation of CB1 receptors is responsible for progression of liver fibrosis, whereas CB2 receptors display anti-fibrogenic properties. Moreover, preliminary data derived from clinical trials strongly suggest that selective CB1 receptor antagonism improves insulin resistance and reduces liver fat. The current view of adipose tissue is that of an active secretor organ, sending out and responding to signals that modulate appetite, insulin sensitivity, energy expenditure, inflammation and immunity.4 In this scenario, the important role played by endocannabinoid system is emerging: it controls food intake, energy balance and lipid and glucose metabolism through both central and peripheral effects, and stimulated lipogenesis and fat accumulation.

Cannabis (Cannabis Sativa, marijuana) has been used for medicinal and ritual purposes for over 3 millennia, and remains the most commonly used recreational drug in the western world.5 The identification of the cannabinoid receptor 1 (CB1) in human brain some twenty years ago6 and the subsequent discovery of endogenous cannabinoids, has led to an understanding of the importance of the endocannabinoid system in health and disease. There are two G protein-coupled cannabinoid receptors; CB1 and CB2.7

Emerging evidence suggests that cannabinoids play an important role in the modulation of fatty liver. The endocannabinoid system is primarily comprised of three components: endocannabinoids, endocannabinoid receptors, and endocannabinoid-metabolizing enzymes. Endocannabinoids (endogenous cannabinoids) are lipid mediators that interact with cannabinoid receptors to produce effects similar to those of delta 9-tetrahydrocannabinol (THC), which is the main psychoactive component of cannabis. The two main endocannabinoids discovered are arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG), and two main cannabinoid receptors identified to date are cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). CB1 receptors are expressed at high levels in the brain, but they are also present in peripheral tissues, though at much lower concentrations in most of the peripheral tissues.8

A silent intragenic biallelic polymorphism (1359 G/A) (rs1049353) of the CB1 gene resulting in the substitution of the G to A at nucleotide position 1359 in codon 435 (Thr), was reported as a common polymorphism in the German population,9 reaching frequencies of 24-32% for the rarer allele (A). Considering the evidence that endogenous cannabinoid system plays a role in metabolic aspects of body weight and metabolic syndrome components such as NAFLD,8 we decide to investigate the physiological implications of this CB1 receptor polymorphism in patients with NAFLD.

The aim of our study was to investigate the influence of the missense polymorphism (G1359A) of CB1 receptor gene on insulin resistance, liver histological changes, anthropometric parameters and adipocytokines in patients with NAFLD.

Subjects and methods

Subjects

A population of 70 Caucasian subjects were recruited for this study. The exclusion criteria were alcohol consumption, diabetes mellitus, intolerance fasting glucose, hepatitis B, C, cytomegalovirus, Epstein Barr infections, nonorgan-specific autoantibodies, medication (blood-pressure lowering medication and statins) and hereditary defects (iron and copper storage diseases and alpha 1-antitrypsin deficiency). The study was approved by the institutional ethics committee. These patients signed an informed consent.

Liver biopsies

The diagnosis of NAFLD was confirmed by percutaneous liver biopsy performed in all subjects with a 1.6 mm Menghini-type biopsy needle. Liver samples were routinely processed, sectioned, and stained with hematoxinil-eosin and Manson’s trichome. All biopsies were studied by the same liver pathologist. Histology was analysed using the Brunt classification:10 Steatosis was graded as mild (< 33% of hepatocytes affected); moderate-severe (≥ 33% of hepatocytes affected). The Brunt system also includes as grading: portal inflammation, ballooning and lobular inflammation. Brunt grade was graded as follows: mild (< 4) and moderate-severe (≥ 4) and stating fibrosis: stage 1: zone 3 perivenular perisinusoidal/pericellular fibrosis, focal or extensive; stage 2: as above with focal or extensive perportal fibrosis; stage 3: bridging fibrosis, focal or extensive; stage 4: cirrhosis. In our study, fibrosis variable was divided as absent or presence Weight, blood pressure, basal glucose, c-reactive protein (CRP),
insulin, insulin resistance (HOMA), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides blood and adipocytokines (leptin, adiponectin, resistin, TNF alpha, and interleukin 6) levels were measured at basal time. A tetrapolar bioimpedance, an indirect calorimetry and a prospective serial assessment of nutritional intake with 3 days written food records were realized. Genotype of CB1 receptor gene polymorphism was studied.

Genotyping of CB1 gene polymorphism

Oligonucleotide primers and probes were designed with the Beacon Designer 4.0 (Premier Biosoft International®, LA, CA). The polymerase chain reaction (PCR) was carried out with 50 ng of genomic DNA, 0.5 uL of each oligonucleotide primer (primer forward: 5’-TTC ACA GGA CCG CAG AAA G-3’ and reverse 5’-GAG GCA TCA GGC TCA CAG AG-3’), and 0.25 uL of each probe (wild probe: 5’-Fam-ATC AAG AGC GAG GCA TCA GGC TCA CAG AG-3’), and 0.25 uL of each oligonucleotide primer (primer forward: 5’-TTC ACA GGG CCG CAG AAA G-3’ and reverse 5’-CCG TGC GCC TAC TAC TCT GCT-3’). The PCR was run in a 25 uL final volume containing 12.5 uL of IQTM Supermix (Bio-Rad®, Hercules, CA) with hot start Taq DNA polymerase. Hardy Weinberger equilibrium was assessed.

Assays

Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5 mU/L (normal range 0.5-30 mU/L) (11) and the homeostasis model assessment for insulin sensitivity (HOMA) was calculated using these values.12 CRP was measured by immunoturbimetry (Roche Diagnostics GmbH, Mannheim, Germany), with a normal range of (0-7 mg/dl) and analytical sensitivity 0.5 mg/dl. Lipoprotein (a) was determined by immunonephelometry with the aid of a Beckman array analyzer (Beckman Instruments, Calif., USA).

Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y., USA), while HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate-magnesium. LDL cholesterol was calculated using Friedewald formula.

Alanine amino transferase, aspartate aminotransferase activity, bilirubin and gamaglutamine trans-

ferase were determined by enzymatic colorimetric assay Hitachi 917 (Roche Diagnostics, Geneve, Switzerland).

Adipocytokines

Resistin was measured by ELISA (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/ml with a normal range of 4-12 ng/ml.13 Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., Texas, USA) with a sensitivity of 0.05 ng/ml and a normal range of 10-100 ng/ml.14 Adiponectin was measured by ELISA (R&D systems, Inc., Mineapolis, USA) with a sensitivity of 0.246 ng/ml and a normal range of 8.65-21.43 ng/ml.15 Interleukin 6 and TNF alpha were measured by ELISA (R&D systems, Inc., Mineapolis, USA) with a sensitivity of 0.7 pg/ml and 0.5 pg/ml, respectively. Normal values of IL6 was (1.12-12.5 pg/ml) and TNFalpha (0.5-15.6 pg/ml).16-17

Anthropometric measurements

Body weight was measured to an accuracy of 0.5 kg and body mass index computed as body weight/ (height²). Waist (narrowest diameter between xiphoid process and iliac crest) and hip (widest diameter over greater trochanters) circumferences to derive waist-to-hip ratio (WHR) were measured, too. Tetrapolar body electrical bioimpedance was used to determine body composition with an accuracy of 5 g.18 An electric current of 0.8 mA and 50 kHz was produced by a calibrated signal generator (Biodynamics Model 310e, Seattle, WA, USA) and applied to the skin using adhesive electrodes placed on right-side limbs. Resistance and restance were used to calculate total body water, fat and fat-free mass.

Blood pressure was measured twice after a 10 minutes rest with a random zero mercury sphygmo-

manometer, and averaged.

Statistical analysis

Sample size was calculated to detect differences over 1 units of HOMA with 90% power and 5% signifi-

cance (n = 70). The results were expressed as average ± standard deviation. The distribution of variables was analyzed with Kolmogorov-Smirnov test. Quantitative variables with normal distribution were analyzed with a two-tailed Student’s-t test. Non-parametric variables were analyzed with the U-Mann-Whitney test. Qualitative variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher’s test.

The statistical analysis was performed for the combined G1359A and A1359A as a group and wild type G1359G as second group, with a dominant model.
A p-value under 0.05 was considered statistically significant.

Results

Seventy patients gave informed consent and were enrolled in the study. The mean age was 42.9 ± 11.5 years and the mean BMI 36.1 ± 10.1. All subjects were weight stable during the 2 weeks period preceding the study (body weight change, 0.25 ± 0.2 kg). Forty seven were men and 23 women.

Forty one patients (36.9%) had the genotype G1359G (wild type group) and twenty nine (26.1%) patients G1359A or A1359A (mutant type group). Age was similar in both groups (wild type: 42.95 ± 16.6 years vs mutant group: 45.1 ± 16.8 years:ns). In wild type group, 29 were men and 12 were women and in the mutant type group 18 were men and 11 were women.

Twenty four patients (32.3%) had a Brunt grade > 4 and 12 patients (17%) had a significative fibrosis (F > = 2).

Table I shows the anthropometric variables. No statistical differences were detected between genotype groups.

Table II shows the classic cardiovascular risk factors. HOMA values were higher in wild type group than mutant type group.

Table III shows levels of adipocytokine levels. Adiponectin and visfatin levels were higher in mutant type group. Moreover, TNF-alpha and resistin levels were higher in wild type group than mutant type group.

Patients with mutant genotype did not show significant differences in liver biochemistry parameters (serum aminotransferase levels) with respect to patients with wild genotype: Alanine amino transferase (ALT:67.7 ± 25.1 UI/L vs. 60.4 ± 30.4 UI/L; p > 0.05), aspartate aminotransferase activity (AST:41.3 ± 15.5 UI/L vs. 35.6 ± 10.4 UI/L; p > 0.05) and gamaglutamine transferase levels (GGT:118.2 ± 63.1 UI/L vs. 107.7 ± 60.8 UI/L; p > 0.05). Only patients with mutant genotype showed less frequently elevated levels of AST. AST > 40 UI/L was detected in 28.5% of patients in the mutant vs. 53% of patients with wild genotype, p < 0.05.

Table IV shows the histological lesions in relation to both genotypes. Patients with mutant type group presented a percentage of Brunt grade > = 4 less frequently than patients with wild type group. We observed similar ratios of absent/presence of fibrosis in both genotype groups.

Discussion

Metabolic syndrome, leading to liver steatosis, has emerged as an important and frequent cause of chronic liver injury, ranging from simple steatosis to steatohepatitis, which is accompanied by inflammatory reaction and progressive fibrosis of liver tissue.
The main finding of our study is the association of the A variant of the polymorphism G1359A CBR1 with lower levels of HOMA, TNF-alpha, resistin and higher levels of adiponectin than patients with the wild variant of this polymorphism. Besides, patients with A allele variant shown lower Brunt grade in liver biopsy. These above-mentioned relationships have an unclear explanation. However, the literature supports the notion that endocannabinoid system is positioned for regulation of endocannabinoid levels that could influence craving and reward behaviors through the relevant neuronal circuitry and metabolic parameters. Osei-Hyiaman et al. have observed that endocannabinoid activation of hepatic CB1 receptors contributes to diet-induced steatosis and associated hormonal and metabolic changes. This study suggests that peripheral CB1 receptors could be selectively targeted for the treatment of fatty liver, impaired glucose homeostasis and dyslipidemia.

Otherwise, a reduction in the CB1-mediated endocannabinoid system activity in visceral fat is associated with a normalization of adipocyte metabolism, which may be a determining factor in the reversion of liver steatosis induced by treatment with the CB1-specific antagonist (SR141716).

The involvement of the endocannabinoid system in the pathogenesis of fatty liver disease has been shown recently. Since endocannabinoids are essential in regulation of energy balance, food intake and lipogenesis, impairment of this homeostasis results in various metabolic disturbances. Apart from central control of energy homeostasis via CB1 receptors localized in the brain, endocannabinoids seem to exert, as well CB1-receptor-dependent, peripheral effects on lipid metabolism in adipocytes, liver tissue and skeletal muscle. Moreover, fat-rich diet has been shown to contribute to enhanced hepatic expression of CB1 in liver tissue and increased levels of endocannabinoids, thus increasing the metabolic imbalance. This association might be related with our recent results, because patients with the A variant of rs1049353 SNP had higher percentage of low Brunt’s grades and a lower frequency of AST under 40 U/l than patients with wild type genotype.

Ravinet et al. found that CB-1 gene-deficient mice were lean and resistant to diet-induced obesity and showed reduced plasma insulin and leptin levels. In our patients, HOMA index was higher in patients carrying the wild type CB1 allele than in heterozygous subjects (G/A and A/A) as shown by Gazeerro et al. This metabolic relationship between the polymorphism and metabolic profile has been described by other previous study. In this cross sectional study, an association of the mutant type group G1359A and A1359A with a better cardiovascular profile (triglyceride, HDL cholesterol, insulin and HOMA levels) than wild type group was detected.

In our study we do not observed an association between genotype of CBR1 and liver fibrosis. Others authors have observed that an antifibrogenic effects results were obtained either by pharmacological inactivation with rimonabant (SR141716), a selective antagonist of CB1 receptor, or via genetic inactivation in homozygous CB1-deficient mice. Decreased progression of fibrosis was accompanied by reduced hepatic TGF-β expression and growth inhibition and increased apoptosis of myofibroblasts. These effects seemed to result from reduced phosphorylation of protein kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERK), thus affecting the pathways responsible for cell proliferation and survival. A possible explanation of these discrepancies is that our study is not a prospective study and this is an important limitation to assess.

In our study, patients with mutant group type presented higher resistin and TNF-alpha levels and lower adiponectin levels. Some investigators have indicated that increased serum resistin levels are associated with increased obesity, visceral fat, and type 2 diabetes mellitus, whereas other groups have not observed such associations. Visfatin expression is regulated by cytokines that promote insulin resistance, such as TNF-alpha.26 De Luis et al. have showed that visfatin is related in a negative way to insulin resistance and a relation was detected with inflammatory markers such as TNF-alpha. Perhaps, the associations of these adipokines levels with the polymorphism (1359 G/A) (rs1049353) of the CB1 gene could explain the relationship of this SNP with liver histology. However, the cross sectional design of our study could not response to this association with a hypothesis of causality.

In conclusion, A variant of the polymorphism G1359A CBR1 is associated with lower levels of HOMA, TNF-alpha, resistin and higher levels of adiponectin than patients with the wild variant of this polymorphism. Besides, patients with A allele variant show lower Brunt grade in liver biopsy. Further prospective studies are need to clarify the association of this polymorphism and non alcoholic fatty liver disease.

References


