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Apolipoprotein A5 and Hypertriglyceridemia in Prague Hypertriglyceridemic Rats

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Summary

High plasma triglyceride (TG) level is a major independent risk factor of coronary heart disease. A newly identified *Apolipoprotein A5* (*Apoa5*) gene has been shown to play an important role in determining plasma TG concentrations in humans and mice. Prague hereditary hypertriglyceridemic (HTG) rats are a useful model of human hypertriglyceridemia and other symptoms of metabolic syndrome. Thus, the variation of *Apoa5* gene and its expression were studied in this strain under normal conditions and after chronic fructose loading. Lewis and Wistar rats served as normotriglyceridemic controls. Plasma TG were significantly higher in HTG rats in comparison with both control strains. Sequence analysis of the rat *Apoa5* gene revealed the existence of two introns. However, screening of the coding regions and intron-exon boundaries of *Apoa5* gene did not indicate any mutation of this gene in HTG rats in comparison with Lewis and Wistar ones. Under the basal conditions the expression of *Apoa5* was lower in all age groups of HTG rats compared to Wistar animals. Furthermore, during chronic fructose loading there were no significant changes of *Apoa5* expression in HTG rats, although plasma TG levels rose 3-4 times within first two days of fructose loading and were increased during the whole period of fructose treatment. In conclusion, *Apoa5* does not seem to be a genetic determinant of hypertriglyceridemia in HTG rats. The absence of significant changes in *Apoa5* gene expression during chronic fructose-induced TG elevation excludes its major role in mechanisms compensating severe hypertriglyceridemia.

Key words

Hypertriglyceridemia • Metabolic syndrome • Apolipoprotein A5 • Genetic analysis • Gene expression • Chronic fructose loading • Rat

Introduction

Hypertriglyceridemia is a common metabolic disorder in the human population. It has been demonstrated that both genetic and environmental factors determine plasma triglyceride (TG) levels – a major independent risk factor for coronary heart disease (Genest *et al.* 1992, Jeppesen *et al*. 1998). Thus, understanding the factors that control plasma TG levels and the genes that primarily regulate circulating concentrations of TG-rich lipoproteins is of major importance. This may provide new opportunities for therapeutic intervention in

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dyslipidemia and atherosclerosis.

Apolipoprotein A5 gene has been recently identified by comparative sequencing of human and mouse DNA (Pennacchio *et al.* 2001). It has been demonstrated that this gene is located within well-defined *APOA1/C3/A4* gene cluster on human chromosome 11 and on mouse chromosome 9 (Bruns *et al*. 1984, Karathanasis 1985, Majesky 2002). The cluster of these genes has been thoroughly studied and has been linked to defects in lipid metabolism in humans and some other species (Groenendijk *et al*. 2001, Šeda and Šedová 2003). Recently, it has been demonstrated that increased levels of plasma TG were observed in *Apoa5* knock-out mice while transgenic mice overexpressing human *APOA5* gene have considerably reduced plasma TG levels (Pennacchio *et al*. 2001). Because of such significant results, the role of *Apoa5* in the metabolism of TG was also studied in humans. Extensive sequencing of the *APOA5* interval in humans revealed common polymorphisms, which were used for subsequent genetic association studies (for reviews see Pennacchio and Rubin 2003, Hubáček 2005). Out of the more than 10 variants detected so far, not all polymorphisms are common or associated with plasma TG levels (Pennacchio *et al*. 2002, Hubáček *et al.* 2004a, Hubáček 2005). Polymorphism discovery and haplotype analysis in Caucasians defined three common haplotypes in the *APOA5* interval. Three major haplotypes were found in about 97 % of the individuals in the populations (Pennacchio and Rubin 2003). It was demonstrated in many population studies that the less common *APOA5* alleles are associated with elevated triglycerides, despite the fact that some ethnic specific effects were observed (Endo *et al*. 2002, Nabika *et al.* 2002, Ribalta *et al.* 2002, Evans *et al.* 2003, Talmud *et al.* 2002, Hubáček *et al.* 2004b, Hubáček 2005). The role of the *APOA5* in determination of other biochemical and anthropometrical parameters (CRP, BMI and some others) is suggested but remains unclear (Jang *et al.* 2004, Hubáček *et al.* 2005, Aberle *et al.* 2005).

Prague hereditary hypertriglyceridemic rats (HTG) were developed as an animal model of human hypertriglyceridemia and insulin resistance (Vrána and Kazdová 1990). We have later demonstrated that HTG rats have mild hypertension and that their blood pressure correlates positively with plasma TG (Štolba *et al.* 1992). This positive correlation was also documented in F_2 hybrids obtained from HTG and normotensive Lewis progenitors (Kuneš *et al*. 1995). We have recently reported several QTL loci for plasma triglycerides in this intercross (Ueno *et al.* 2004). Moreover, the locus on chromosome 2 overlapped with the rat syntenic region of the human locus for metabolic syndrome and for small LDL. The loci on chromosome 13 overlapped with the syntenic region of loci for human familial combined hyperlipidemia suggesting the usefulness of HTG for the comparative studies of human dyslipidemia.

The purpose of this study was to investigate the structure of *Apoa5* gene in the rat, to search for its possible polymorphisms and/or mutations in HTG rats in comparison with normotriglyceridemic controls and finally, to evaluate the expression of this gene under chronic fructose loading when plasma TG level is considerably elevated.

Methods

Animals

Male Prague hereditary hypertriglyceridemic (HTG) rats of different ages and age-matched Lewis and Wistar normotriglyceridemic controls were obtained from our breeding colonies at the Institute of Physiology AS CR. Animals were housed in a temperature- and light-controlled (12:12 dark:light cycle) room and had *ad libitum* access to standard chow (ST1) and water. The Institutional Ethical Committee approved all experimental procedures, which conform to the European Convention on Animal Protection.

The relationship between plasma TG level and the expression of *Apoa5* gene was studied i) during the ontogeny and ii) in adult rats chronically loaded with 10 % fructose in a drinking fluid. At the end of the experiment, the animals were sacrificed, livers removed, frozen in liquid nitrogen and used for DNA and RNA isolation. Plasma TG concentrations were measured by using of commercial kit (TG L4x100, BIO-LA-TEST, Pliva-Lachema, Brno).

DNA and RNA preparation

DNA was prepared from the liver by standard methods. PCR amplification was performed in Eppendorf PCR System under the following conditions: preheating of the mixture at 95 °C for 2 min., followed by 35 cycles of denaturation for 40 s at 95 °C, annealing for 30 s at 67 °C, and extension for 50 s at 72 °C, with final extension for 10 min at 72 °C. Specific PCR primers for *Apoa5* were designed based on the nucleotide sequence for mouse gene: RAV1, forward primer 5´-

GCATCGTGGAAAGCATGGCTGCCGTCATC-3′ and reverse primer, 5′-GTTGAAACCCTACACGGTCGA GCTGAT-3′, RAV2, forward primer, 5′-AGCA GGTCGGCTGGAACCTGGAGGGC-3′ and reverse primer, 5′-CCGTGTCAGCACAGACGGGGCAGAC AA-3′, RAV3, forward primer, 5′-ATCCAACGCAA CCTGGATCAGCTGCG-3′, and reverse primer, 5′-CCC TGAGCCTTCAGCATGGCCTAAT-3′, RAV4, forward primer, 5′-AGACTGACTGCAAGCCAGTACTTGA CCG-3′, and reverse primer, 5′-ATGTGTGCA TCCCTGCAGTGGTCCTGCT-3′. The PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet transilluminator.

RNA was isolated from liver tissue using the RNeasy Midi Kit (Qiagen). To remove DNA, the extracted RNA was treated with Deoxyribonuclease I (Fermentas). The reverse transcription (RT) reaction was performed on 5 μg total RNA with Transcriptase reagents (Invitrogen) in a total volume of 20 μl using the oligo(dT) primer system, 0.5 μg; reaction buffer (1x); M-MLV reverse transcriptase, 200 units; dNTP mix, 0.5 mM; DTT, 0.01 M, RNase OUT, 40 units. RNA with oligo(dT) primer was heated to 70 °C for 10 min and after cooling the remaining chemicals were added. The incubation continued 60 min at 37 °C and was stopped by heating at 95 °C for 5 min.

Real-time quantitative polymerase chain reaction was carried out with the Light Cycler Instrument (Roche Molecular Biochemicals) using the DNA binding dye SYBR Green I (LightCycler-Fast Start DNA Master SYBR Green I kit, Roche) for detection of PCR products. Gene-specific PCR primers for apoA5, RAV1c forward primer, 5′-AAAGCTGGGACCCTTGAGAGA-3′ and reverse primer, 5′-ATCAGCTCGACCGTGTAGG GTTTCAAC-3′ and the house-keeping gene beta-actin forward primer, 5′-CCGTAAAGACCTCTATGCCA-3′ and reverse primer, 5′-AAGAAAGGGTGTAAAAC GCA-3′ were synthesized in VBC-GENOMIC. PCR was performed in a total volume of 10 μl containing 1 μl twofold diluted cDNA, 4 mM (RAV1c) and 5 mM (beta-actin) $MgCl_2$, 0.5 μM of each primer and (1x) PCR reaction mix. PCR amplification in the LightCycler was run under the following conditions: preincubation and denaturation of the template cDNA at 95 °C for 10 min, followed by 45 cycles amplification: 95 °C for 15 s, 55 °C for 10 s (RAV1c, beta-actin) and 72 °C for 15 s. The melting curve analysis was performed at 95 °C for 0 s, 65 °C for 60 s and 99 °C for 0 s. For quantification, we prepared standard curves for each pair of primers from serial dilution of liver cDNA. The expression levels of a*poA5* were evaluated by the ratio of the target mRNA to that of beta-actin mRNA.

Data analysis

Statistical significance was assessed by ANOVA or unpaired Student´s t-test. Data are presented as means ± S.E.M. $P<0.05$ value was considered as statistically significant.

Results

Body weight of all three strains studied was not different (data not shown). There was no substantial difference in plasma TG levels between both normotriglyceridemic control strains in either age (Table 1). However, HTG rats had significantly higher plasma TG level in comparison with both control strains and this difference was more pronounced at the age of 14 weeks than is younger animals.

Table 1. Plasma triglycerides (mmol/l) in 4-week-old, 11-week-old and 14-week-old Prague hypertriglyceridemic (HTG) rats and normotriglyceridemic Lewis (LEW) or Wistar (W) controls.

	4-week-old	11-week-old	14-week-old
HTG	1.10±0.16	1.24±0.12	2.20±0.10**
LEW	0.46±0.13$^{\#\#}$	0.56±0.14$^{\#\#}$	0.66±0.08$^{\#\#}$
W	0.70±0.03$^{\#}$	0.78±0.05$^{\#\#}$	0.79±0.02*$^{\#\#}$

Data are means ± S.E.M. ** $p<0.01$, * $p<0.05$ adult vs. younger rats, $^{\#\#}$ $p<0.01$, $^{\#}$ $p<0.05$ vs. HTG rats.

Sequence analysis of the rat *Apoa5* gene revealed the existence of two introns (Fig. 1). However, PCR analysis of the *Apoa5* gene by using specific primers (RAV1, RAV2, RAV3, RAV4) did not show any polymorphism between HTG and LEW rats (Fig. 2). Detailed sequencing of the coding regions and intron-exon boundaries of *Apoa5* gene did not reveal any mutation in HTG rats in comparison with Lewis or Wistar rats (data not shown).

Under the basal conditions the expression of *Apoa5* gene was lower in HTG than in Wistar rats in all age groups studied. Production of mRNA in liver of

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   1 ATGGCTGCCGTCATCACCTG GGCACTCGCCCTCCTCTCAG gtgggtcttccggctgactt
  61 gggtgtggtcttggaatgga ggatgggctgaggggcctgg ctcttaactgtctcatcctc
 121 tctgcagTGTTTGCAACTGT ACAGGCGAGGAAGAGCTTCT GGGAGTACTTCGGCCAGAAC
 181 AGCCAGGGCAAAGGCATGAT GGGCCAGCAGCAGAAGCTGG CACAGGAgtaagtagcctgg
 241 gcaacagtgctgtgagggta cgagacagcgctactgtgga atgattcctcagctccgtag
 301 ttaacacagagctccactga gggatagaatggcaaggtgg tgtgactattggcatgcgta
 361 tgacaccttcttggatgggg tgacagcttcttggatggtg ctgtaaggccaaatgccttt
 421 ttgtgccctgaatgtaatga tgcagttggttggggtcagg actaaagaccctagctctgg
 481 agccattcttcctgccctgg tttctgcagagtattggtgt cctacagcgatagggcaagg
 541 gagatttcaaggctagccgt ctaatggcctggttctgtct ttctccttctccagGAGCCT
 601 GAAAGGTAGCTTGGAGCAAG ACCTCTACAATATGAACAAT TTCCTAGAAAAGCTGGGACC
 661 CTTGAGAGAGCCTGGGAAGG AGCCTCCTCGGCTGGCACAG GATCCAGAAGGCATTCGGAA
 721 GCAGTTGCAGCAAGAGCTGG AGGAAGTGAGCACACGCCTG GAGCCCTACATGGCTGCAAA
 781 GCACCAGCAGGTCGGCTGGA ACCTGGAGGGCTTGAGGCAG CAGTTGAAACCCTACACGGT
 841 CGAGCTGATGGAGCAGGTAG GCCTGAGCGTGCAGGATCTG CAAGAACAGCTGCGCATGGT
 901 GGGAAAAGGCACCAAGGCCC AGCTCCTGGGGGGCGTGGAT GAGGCGATGAGCCTGCTGCA
 961 GGATATGCAAAGTCGAGTGC TGCACCATACGGACCGAGTC AAAGAACTCTTCCACCCTTA
1021 TGCAGAACGCTTGGTGACTG GAATTGGGCACCATGTGCAG GAGCTGCACCGGAGTGTTGC
1081 TCCTCACGCAGTTGCCAGCC CCGCGAGACTCAGTCGCTGC GTGCAGACCCTGTCCCACAA
1141 ACTCACACGTAAGGCGAAGG ACTTGCACACCAGCATCCAA CGCAACCTAGATCAGCTGCG
1201 AGATGAGCTCAGTACCTTCA TCCGTGTCAGCACAGACGGG GCAGACAACAGAGACTCCCT
1261 GGACCCTCAAGCTCTCTCTG ACGAGGTCCGCCAGAGACTC CAGGCTTTTCGACATGACAC
1321 CTACCTGCAGATCGCTGCAT TCACTCAGGCCATTGACCAG GAGACCGAGGAAATCCAGCA
1381 CCAGCTGGCACCACCCCCGC CTAGCCACAGCGCCTTCGCT CCAGAGTTGGGACACTCAGA
1441 CAGTAATAAGGCCCTGAGCA GACTGCAGAGCCGGCTGGAC GACCTCTGGGAAGATATTGC
1501 CTATGGCCTTCATGACCAGG GCCATAGTCAGAATAACCCT GAGGGTCACTCAGGTTAA
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Fig. 1. Sequence analysis of the rat *Apoa5* gene. Sequence starts with the first coding triplet. Intron sequences are in lower letters.

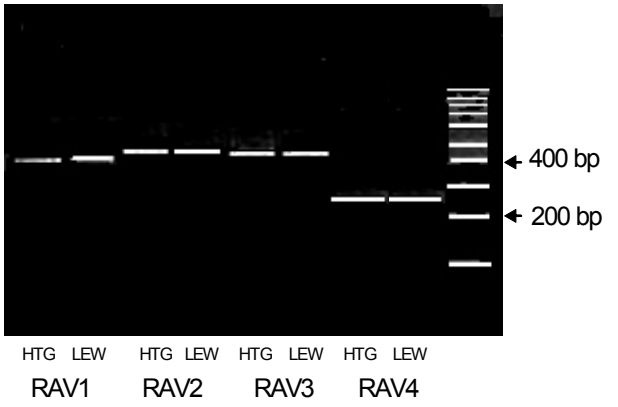

Fig. 2. Representative PCR analysis of genomic DNA by using of specific primers RAV1 (409 bp), RAV2 (453 bp), RAV3 (429bp), RAV4 (269 bp), which amplified segments of *Apoa5* gene in both HTG and LEW strains.

Table 2. Plasma triglyceride levels (mmol/l) in adult Prague hypertriglyceridemic (HTG) rats and normotriglyceridemic Lewis (LEW) and Wistar (W) rats during fructose loading which started at the age of 11 weeks.

	Days of fructose loading				
	0	**2**	**5**	**10**	**20**
HTG	1.24±0.12	5.70±0.39**	5.00±0.53**	5.70±0.81**	7.40±0.38**
LEW	0.56±0.14$^{\#\#}$	0.57±0.04$^{\#\#}$	0.70±0.02$^{\#\#}$	n.d.	0.70±0.04$^{\#\#}$
W	0.78±0.05$^{\#\#}$	0.74±0.08$^{\#\#}$	0.85±0.14$^{\#\#}$	n.d.	0.68±0.02$^{\#\#}$

Data are means ± S.E.M. ** $p<0.01$ vs. baseline (days 0 of fructose loading), $^{\#\#}$ $p<0.01$, $^{\#}$ $p<0.05$ vs. HTG rats, n.d. – not detected.

immature Lewis rats was comparable to that of age-matched HTG animals, but became significantly elevated in 14-week-old Lewis rats (Fig. 3).

Fructose loading for four weeks had only a small effect on plasma TG levels in both control strains, but in HTG rats this regimen increased plasma TG level 3-4 fold (Table 2), indicating the development of severe hypertriglyceridemia in HTG rats. However, the expression of *Apoa5* gene had no direct relationship to the TG changes occurring after chronic fructose loading. Fructose loading, which increased significantly TG level in HTG rats, had no effect on *Apoa5* expression in this strain (Fig. 4) (ANOVA F=0.699, df 5/29).

Discussion

The exchangeable apolipoproteins play a critical role in plasma lipoprotein metabolism. It is generally accepted that alterations in the concentration or structure of exchangeable apolipoproteins can lead to dyslipidemia in humans. Recently, a new member of apolipoproteins family, apolipoprotein Apoa5, was discovered (Pennacchio *et al.* 2001). It was demonstrated that mice overexpressing human *APOA5* gene had plasma TG levels that were about one-third of those in controls. On the other hand, *Apoa5* knock-out mice had about four times higher plasma TG levels in comparison with wild-type littermates (Pennacchio *et al.* 2001). This suggested an important role of *Apoa5* gene in TG metabolism.

In the present study we have analyzed the structure and function of *Apoa5* gene in Prague hereditary hypertriglyceridemic rats. The sequence screening did not disclose any important difference between mouse and rat *Apoa5* genes in the coding regions and there was no mutation of this gene in HTG rats. Under the basal conditions the expression of *Apoa5* was lower in all age groups of HTG rats compared to Wistar rats. Nevertheless, chronic fructose loading did not induce any

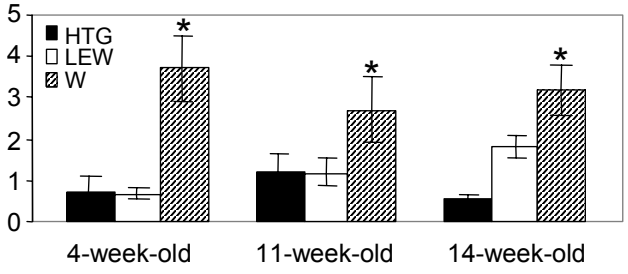

Fig. 3. Expression of *Apoa5* gene in 4-week-old, 11-week-old and 14 week-old Prague hypertriglyceridemic (HTG) rats and normotriglyceridemic Lewis (LEW) and Wistar (W) controls under basal conditions. *significantly different (P<0.01) from HTG rats.

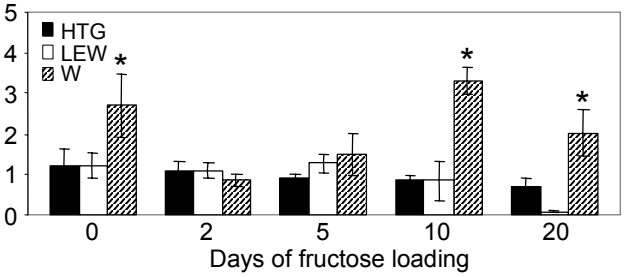

Fig. 4. Expression of *Apoa5* gene in Prague hypertriglyceridemic (HTG) rats and normotriglyceridemic Lewis (LEW) and Wistar (W) rats after fructose loading which started at the age of 11 weeks. *significantly different (P<0.01) from HTG rats.

significant changes of *Apoa5* expression in HTG rats, although plasma TG levels rose 3-4 times within first two days of fructose loading.

In spite of the fact that HTG rats are hypertriglyceridemic and this hypertriglyceridemia can be further increased by high fructose intake, no structural changes (either mutations or polymorphisms) were found in *Apoa5* gene of HTG rats. Several single nucleotide polymorphisms in human *APOA5* gene have been identified and shown to constitute three distinct haplotypes, two of which exhibit a very strong association with elevated fasting triglycerides in several populations (Pennacchio *et al.* 2002, Talmud *et al.* 2002, Ribalta *et al.* 2002). However, not all hypertriglyceridemic individuals did exhibit critical APOA5 alleles, which should be responsible for increased plasma TG in the population (Hořínek *et al*. 2003, Vráblík *et al.* 2003).

Recently published results showed that apolipoprotein A5 binds to and enhances the activity of lipoprotein lipase. In mice expressing human *APOA5*, this leads to the reduction of TG levels in VLDL particles. Additionally, the treatment with apolipoprotein A5 leads in mice to a reduction of VLDL-TG production rate, but the concentration of the VLDL particles is the same as in normal mice (Schaap *et al.* 2004, Fruchart-Najib *et al.* 2004). It could be concluded that apolipoprotein A5 serves like an activator of lipoprotein lipase.

The results of our study have indicated that the role of apolipoprotein A5 in TG metabolism might be species-specific. At least in the rat TG metabolism seems to be more dependent on other mechanisms, which might be different from those in human and mouse. We can conclude that *Apoa5* gene is not a major genetic determinant of hypertriglyceridemia in HTG rats.

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