

# Control of hypercholesterolemia and atherosclerosis using a peptide containing the cholesterol recognition/interaction amino acid consensus VLNYVWR

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## INTRODUCTION

Coronary heart disease (CHD) is an important cause of mortality and morbidity in western countries and high serum levels of cholesterol are associated with atherosclerosis and an increased risk of CHD. Various clinical trials have demonstrated the usefulness of decreased LDL in reducing the risk to develop CHD and ameliorate the outcome of the disease. Over the years numerous compounds were developed to control hypercholesterolemia. Among the most widely used hypolipidemic drugs are fibrates and statins. However, although both these therapeutic classes were proven efficacious in lowering LDL cholesterol level, their efficacy is limited by their inability to increase HDL cholesterol level, which is acknowledged nowadays as a key factor to reduce CHD prevalence. In addition, fibrates and statins display significant side-effects.

Steroidogenesis begins with the transport of cholesterol from intracellular sources into mitochondria, a process mediated by the translocator protein (18 kDa) TSPO, previously known as the peripheral-type benzodiazepine receptor. TSPO is a high affinity cholesterol and drug binding protein located in the outer mitochondrial membrane. A domain in the carboxy-terminus of TSPO was identified and characterized as the cholesterol recognition/interaction amino acid consensus (CRAC). The CRAC sequence was further characterized to bind cholesterol with nanomolar affinities and the interaction of cholesterol with the CRAC domain of TSPO was further corroborated by NMR.

The ability of the CRAC domain to bind cholesterol led us to hypothesize that this peptide could be used as an intercalating agent to remove cholesterol from lipoproteins and areas of depot and thus a potential hypocholesterolemic agent acting through a new mechanism of action distinct to the mechanisms mediating the effects of fibrates and statins. We report herein the use of 8 amino acid CRAC sequence (VLNYVWR) to lower cholesterol in two different hypercholesterolemic animal models, the ApoE knock-out B6.129P2-Apoetm1Unc/J mice and the 2% cholesterol diet fed guinea pigs and the impact of the treatment on atherosclerosis.

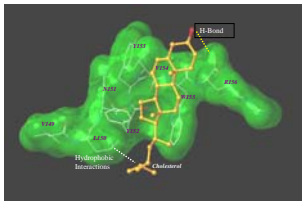
## MATERIALS AND METHODS

All experimental protocols involving animals were approved by the Georgetown University Animal Care and Use Committee. Experiments were performed according to the code of practice for animal experimentation of the Animal Welfare Act and the Public Health Service Policy on Laboratory Animal Care.

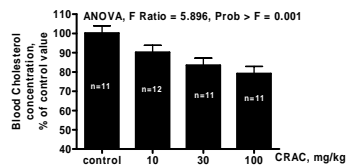
ApoE knock-out B6.129P2-Apoetm1Unc/J mice (Jackson Laboratory, Bar Harbor, MN) weighing 18-22 g at the study initiation received one daily intraperitoneal (i.p.) injection of CRAC solution at 10, 30 and 100 mg/kg (0.15 ml/kg) or vehicle for 14 days. At the end of the treatment period blood was withdrawn by cardiac puncture for cholesterol measurement.

Hartley male guinea pigs weighing 400 g at the study initiation were fed with standard or 2% cholesterol enriched diet for 14 weeks. CRAC treatment started at the end of week 8 until the end of the experiment. Guinea pig received one i.p. injection of CRAC solution at 3 and 30 mg/kg (2.5 ml/kg) or vehicle every other day. Blood was withdrawn under anesthesia (isoflurane 3%) at the study initiation, at the end of week 8 and week 14 to determine total cholesterol, free cholesterol, LDL, and HDL. Creatine kinase (CK) was measured at weeks 8 and 14. Creatine kinase isoforms MB was measured at the end of week 14 just before euthanasia. At the end of week 14, aorta and liver were collected for histology purposes as well as the gallbladder content for cholesterol measurement.

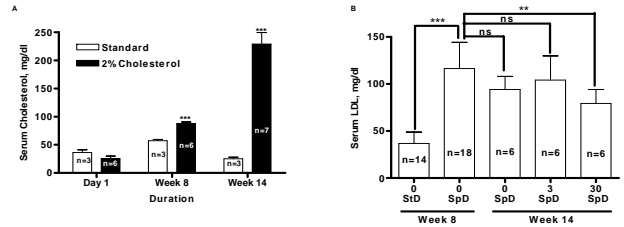
**Statistical analysis.** Data were analyzed by ANOVA followed by Dunnett's test or ANOVA followed by Student's t test. Results are presented as means  $\pm$  SD.



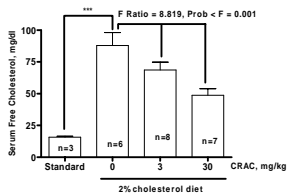
**Figure 1.** Molecular Modeling of cholesterol binding to the eight amino acid residue (VLNYVWR) CRAC sequence.



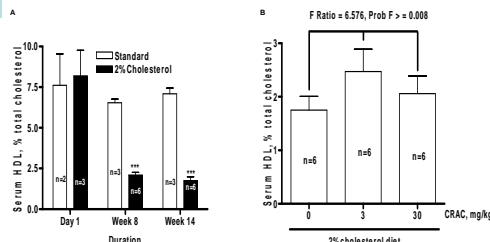
**Figure 2.** Effect of CRAC treatment on hypercholesterolemic ApoE knock-out B6.129P2-Apoetm1Unc/J mice.



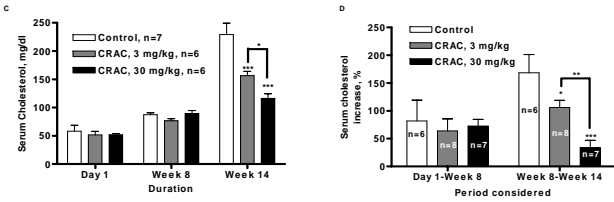
**Figure 3.** Effect of CRAC treatment on serum total and LDL cholesterol levels in guinea pigs fed with high cholesterol diet. **A.** Evolution of total cholesterol concentration measured at day 1, week 8 and week 14 in guinea pigs fed with standard- or 2% cholesterol diet. **B.** Evolution of LDL cholesterol concentration measured at week 8 in guinea pigs fed with standard- or 2% cholesterol diet and week 14 in guinea pigs fed with 2% cholesterol diet and treated with CRAC at 0, 3 or 30 mg/kg. **C.** Evolution of total cholesterol concentration measured at day 1, week 8 and week 14 in guinea pigs fed with 2% cholesterol diet and treated with CRAC 3, 30 mg/kg or its vehicle. **D.** Evolution of total cholesterol concentration from day 1 to week 8 and from week 8 to week 14 in percentage of individual variation measured in 2% cholesterol fed guinea pigs.



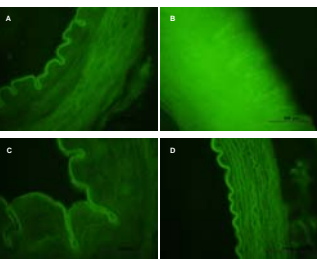
**Figure 4.** Effect of CRAC treatment on serum free cholesterol levels in guinea pigs fed with high cholesterol diet.



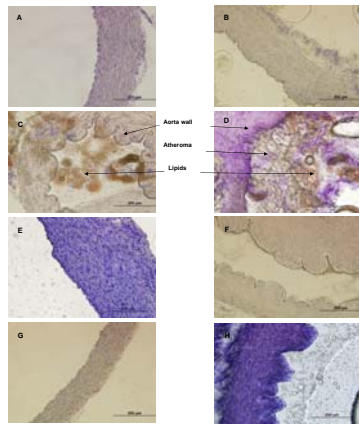
**Figure 5.** Effect of CRAC treatment on serum HDL in guinea pigs fed with high cholesterol diet. **A.** Evolution of HDL cholesterol concentration measured at day 1, week 8 and week 14 in guinea pigs fed with standard or 2% cholesterol diet. **B.** Evolution of HDL cholesterol concentration measured at week 14 in guinea pigs fed with 2% cholesterol diet and treated with CRAC 3, 30 mg/kg or its vehicle.



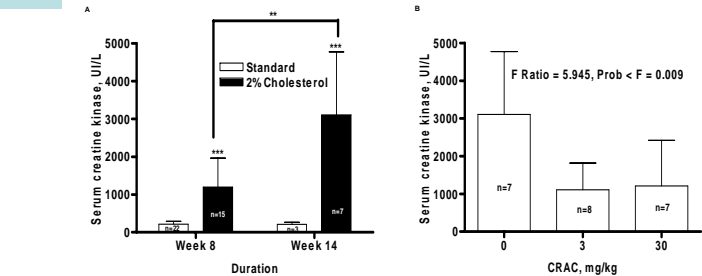
**Figure 9.** Effect of CRAC treatment on serum CK-MB isoforms levels in guinea pigs fed with high cholesterol diet.



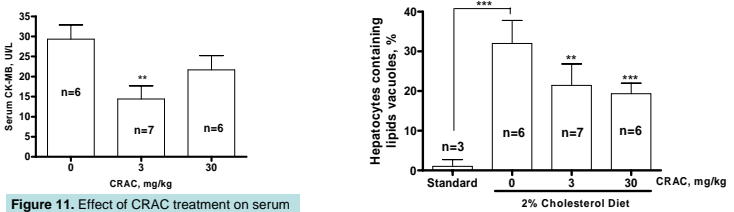
**Figure 12.** Effect of CRAC treatment on oxidative stress in aortas of guinea pigs fed with high cholesterol diet. At the end of week 14, aortas were collected for immunohistochemistry for nitrosylated proteins, a marker of oxidative stress, detected using an anti-nitrotyrosine antibody. **A.** Standard diet; **B.** 2% cholesterol diet; **C.** 2% cholesterol diet treated with CRAC 3 mg/kg; **D.** 2% cholesterol diet treated with CRAC 30 mg/kg.



**Figure 13.** Effect of CRAC treatment on aortic atheroma formation in guinea pigs fed with high cholesterol diet. At the end of week 14, aortas were collected for histology and lipid histochemistry. **A, B.** Standard diet; **C, D.** 2% cholesterol diet; **E, F.** 2% cholesterol diet treated with CRAC 3 mg/kg; **G, H.** 2% cholesterol diet treated with CRAC 30 mg/kg.



**Figure 10.** Effect of CRAC treatment on serum creatine kinase levels in guinea pigs fed with high cholesterol diet. Serum creatine kinase was measured at weeks 8 and 14 in guinea pigs fed with standard and 2% cholesterol diet (**A**) and at week 14 in 2% cholesterol diet fed guinea pigs treated or not with increasing doses of CRAC (**B**).



**Figure 11.** Effect of CRAC treatment on serum CK-MB isoforms levels in guinea pigs fed with high cholesterol diet.

**Figure 9.** Effect of CRAC treatment on the 2% cholesterol diet-induced liver lipid droplet content. To determine the percentage of hepatocytes that contain lipid droplets, 200 cells were counted on hematoxylin/eosin stained liver slices.

## CONCLUSION

In the spontaneously hypercholesterolemic ApoE knock-out B6.129P2-Apoetm1Unc/J mice, CRAC treatment reduced in a dose-dependent manner blood total cholesterol.

In hypercholesterolemic guinea pigs fed with 2% cholesterol, CRAC treatment:

- Reduced total and free cholesterol, decreased LDL cholesterol and increased HDL cholesterol
- Reduced atheroma plaques in aorta
- Reduced hypercholesterolemia-related oxidative stress in aortic wall
- Reduced cardiac suffering as shown by CK-MB decrease.

Taken together, these results suggests that an eight amino acids sequence of the CRAC domain of the cholesterol-binding protein TSPO represents a novel cholesterol lowering drug candidate.