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SHORT REPORT

Antilipolytic effect of calcimimetics depends on the allelic variant of calcium-sensing receptor gene polymorphism rs1042636 (Arg990Gly)

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Calcium-sensing receptor polymorphism rs1042636 (Arg990Gly) affects the response to the calcimimetic cinacalcet, used to treat hypercalcemia in secondary hyperparathyroidism (sHPT) or parathyroid carcinoma. Carriers of the Arg allelle, show less parathyroid hormone secretion suppression in response to the drug. This effect was reproducible in transfected cultured human embryonic kidney cells, supporting a causal relationship on the protein level. We previously established that cinacalcet has an antilipolytic effect in isolated human adipocytes; however, there were a number of samples that did not respond to the treatment. The present work aimed to investigate whether the variable antilipolytic response to cinacalcet in adipocytes was consistent with the effect reported for the rs1042636 polymorphism. Lipolysis was assessed by measuring glycerol release after exposure to cinacalcet (10 μM) or vehicle in adipocytes isolated from 38 donors. Responsiveness was defined as lipolysis suppression (cinacalcet *vs* vehicle control) greater than 20%. Genotype analysis showed that 23 adipocyte donors were homozygous for Arg at position 990, 14 heterozygous and 1 homozygous Gly–Gly. Among the Arg homozygotes, one was responsive to cinacalcet, whereas five Gly carriers responded to the calcimimetic. In all, 83% of adipocytes showing response to cinacalcet carried the glycine allele, whereas in 96% of Arg–Arg individuals adipocytes did not respond to the calcimimetic (*P*=0.027, Fisher's exact test). Confirming sHPT observations, adipocytes from rs1042636 Gly-allele carriers show higher sensitivity to the antilipolytic action of cinacalcet. The potential benefit of cinacalcet as a suppressor of basal lipolysis and free fatty acid release in uremic patients needs to consider the rs1042636 single-nucleotide polymorphism.
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INTRODUCTION

The calcium-sensing receptor (CASR) is a seven transmembrane domain, G-protein-coupled protein, first described in parathyroid glands as the main regulator of parathyroid hormone (PTH) secretion and circulating calcium.[1] Upon an increase in extracellular calcium, the receptor triggers intracellular signals that suppress PTH secretion. As an allosteric modulator, which enables the CASR to sense lower concentrations of extracellular calcium, the calcimimetic cinacalcet has been used in clinical practice to treat secondary hyperparathyroidism (sHPT), a common complication of chronic kidney disease (CKD), allowing for a decrease in the release of PTH with lower endogenous calcium levels.

The presence of the CASR has been observed in numerous other cell types, with many roles differing from that of calcium homeostasis. We reported its expression[2] and a functional role inhibiting lipolysis (cytosolic triglyceride breakdown to yield free fatty acids (FFAs) and glycerol) in human adipocytes.[3] The lipolysis-induced release of FFA from adipocytes provides an energy source; however, an elevation in their circulating levels may induce a lipotoxic effect in target organs, contributing to the metabolic syndrome, that is, visceral obesity, dyslipidemia, hypertension and impaired insulin sensitivity. Numerous studies have determined that the development of CKD is associated with obesity-related metabolic syndrome[4] and central fat has been linked to renal abnormalities in obese and lean subjects.[5] Considering that elevated FFA-induced lipotoxicity may result in worsening CKD,[6] our studies showing that cinacalcet decreases lipolysis[3] suggest that sPTH treatment in patients with renal disease may have this added beneficial effect.

Activating and inactivating mutations in the *CASR* gene have been described, causing several disorders of calcium homeostasis. Although activating mutations lead to various degrees of hypocalcemia, inactivating mutations lead to hypercalcemia; ranging from an heterozygous benign form with elevated PTH levels, to the homozygous form with severe hypercalcemia, hyperparathyroidism and failure to thrive.[7] In addition, single-nucleotide polymorphisms (SNPs) have been identified, one in intron 5 and five in the coding region of exon 7. One of the few SNPs for which causal effects have been demonstrated on the protein level is rs1042636. This SNP has been associated with primary hypercalciuria,[8] lower circulating calcium in healthy individuals[9] and with lower plasma PTH in patients with primary or sHPT.[10,11] Patients with the glycine allele show a more pronounced suppression of PTH after administration of cinacalcet, as compared with homozygous arginine carriers.[12] Transfected cells expressing CASR with glycine in position 990 respond more strongly to a calcimimetic added to the culture medium than those with arginine.[13]

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During our studies on the antilipolytic effect of cinacalcet in human adipocytes, we observed in a considerable number of samples a null response to the calcimimetic (unpublished data). On the basis of the above-mentioned evidence, we investigated whether the phenotype of an antilipolytic response to cinacalcet could be explained by the genotype of rs1042636.

MATERIALS AND METHODS

Isolation of adipocytes

Human omental adipose tissue was obtained from 38 Hispanic subjects without end-stage kidney disease, undergoing elective abdominal surgery (either gastric bypass, gynecological or gastrointestinal). Subjects were 56 ± 7 years (range 17–84 years) with body mass index 30.9 ± 8.9 kg/m^2 (range 17–54 kg/m^2). The protocol was approved by the Institutional Review Board at INTA, University of Chile and informed consent was signed by the donors. Adipose tissue was processed as previously described[3] and adipocytes were isolated by incubation with 1 g/l collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37 °C for 60 min.[14] The resulting cell suspension was filtered, and floating adipocytes were recovered. An aliquot of washed adipocytes was immediately used for lipolysis studies, and the rest frozen for genomic DNA isolation. The lipolytic responsiveness to β-adrenergic stimulus (10 μM isoproterenol) was evaluated as evidence for the presence of viable and metabolically responsive adipocytes after tissue digestion (data not shown).

Lipolysis

Lipolysis was assessed by the measurement of glycerol release during 3 h. Briefly, a 10% adipocyte suspension was incubated with 10 μM cinacalcet or vehicle at 37 °C with gentle swirling, and then stopped by cooling the tubes at 4 °C. The incubation medium under the cell layer was recovered and frozen until analyzed. Glycerol was measured using a colorimetric assay (Sigma, St Louis, MO, USA) on a microplate reader at 540 nm (EL-808, BioTek Instruments Inc., Winooski, VT, USA). Glycerol release was expressed as a percentage of each subject's control (vehicle) condition.

Isolation of genomic DNA, PCR analysis and DNA sequencing

Isolated adipocytes were placed in Chomzynski-phenol solution (Winkler Ltd, Santiago, Chile) for genomic DNA isolation, according to the manufacturer's instructions. Amplification by PCR was performed at a final concentration of 1.5 mM $MgCl_2$, 0.2 mM each dideoxyribonucleotide, 1 μM of each primer and 0.025 U/μl of GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). The sequences of the primers[8] were 5′-CAGAAGGTCATCTTTGGCAGCG GCA-3′ (forward) and 5′-TGCAGACCTGTTTCCTGGACGGTC-3′ (reverse). The following PCR protocol was used in a MJ Research PT100 thermal cycler (MJ Research Inc., Watertown, MA, USA): initial 5-min denaturation step at 95 °C followed by 40 cycles of amplification (30-s denaturation at 96 °C, 1-min annealing at 61 °C, and 30-s extension at 72 °C). The reaction was completed with a single cycle at 72 °C for 10 min to allow completion of extension. PCR products from at least three independent reactions per subject were pooled and resolved by electrophoresis in a 2% agarose gel in 0.04 M Tris-acetate, 0.001 M EDTA buffer and stained with ethidium bromide. Bands of DNA of the expected size were excised from the gel and recovered using an Ultrafree DA centrifugal filter device (Millipore, Bedford, MA, USA). Sequence analysis was carried out by a commercial laboratory (Macrogen, Rockville, MD, USA).

Statistics

Fisher's exact test was used to evaluate the significance of the association between the categorical variables 'presence/absence of the polymorphism' and 'responsiveness to cinacalcet'. A P-value <0.05 was considered significant.

RESULTS

CASR rs1042636 genotype

Among the 38 subjects evaluated, 23 were homozygous for Arg at codon 990, whereas 14 were heterozygous. As expected for a non-Asian population, only a minor proportion of our studied subjects showed the homozygous genotype for Gly (2.6%; n=1).

CASR rs1801725 genotype

As another variant related to calcium handling, we evaluated the rs1801725 (Ala986Ser) polymorphism. In our sample, most subjects were homozygous for the Ala genotype, and only 2 of the 38 volunteers (5%) were heterozygous, with no homozygotes for the Ser variant. Both heterozygous were non-responders to cinacalcet.

Responsiveness to cinacalcet

Based on our previous work,[3] subjects were considered responders to the antilipolytic effect of cinacalcet if their basal lipolysis was reduced by more than 20% as compared with the vehicle-treated controls during the 180-min incubation. Six adipocyte samples met these criteria (with a response of 34 ± 4% (mean ± SE), Figure 1), five of which (83%) were from donors with the Gly allele. Of the 32 non-responders (-2 ± 2% response), 22 (70%) were homozygous for Arg at position 990 of the CASR (Figure 2). Table 1 shows the number and percentage of responders and non-responders for each genotype.

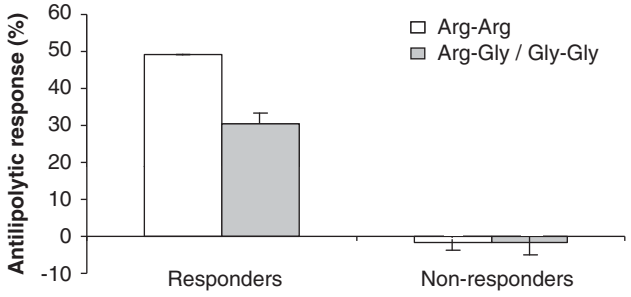

Figure 1 Percent antilipolytic effect according to genotype in responders and non-responders to cinacalcet. Bars represent the mean ± SEM for the antilipolytic effect in adipocytes exposed to the calcimimetic agent, as described in the Materials and methods section. The number of subjects were: responders—1 (AA), 5 (AG/GG); non-responders—22 (AA) and 10 (AG/GG).

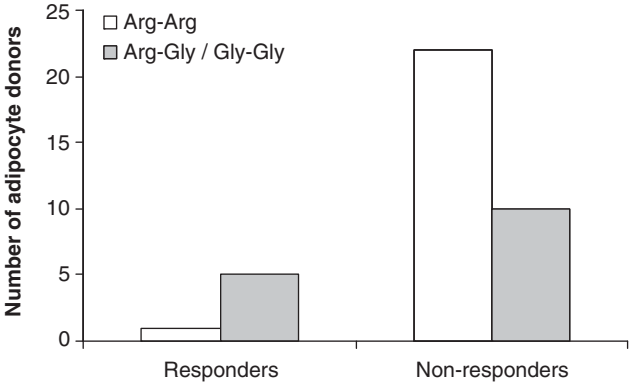

Figure 2 Adipocytes from 990 Gly carriers respond in a greater proportion to the antilipolytic action of cinacalcet. Number of adipocyte samples classified according to their antilipolytic response (>20%) to treatment with 10 μM cinacalcet, and according to their rs1042636 genotype. Fisher's exact test $P<0.05$.

Table 1 Number (% within the genotype) of adipocyte samples considered responder and non-responder to cinacalcet antilipolytic effect

Genotype	*Responders*	*Non-responders*
AA	1 (4.3)	22 (95.7)
AG	5 (35.7)	9 (64.3)
GG	0 (0)	1 (100)

DISCUSSION

We previously showed that the CASR gene polymorphism rs1042636 affects the PTH-lowering dose–response to the calcimimetic cinacalcet in patients on hemodialysis.[12] Recent registry data[15] support the idea that cinacalcet therapy target levels of PTH should depend on the patients' rs1042636 status: survival of Japanese end-stage kidney disease patients (95% carry the glycine allele) is best at PTH levels between 80–160 pg/ml, whereas Blacks and Whites (glycine allele 5–10%) need levels between 150–300 pg/ml. Our present *in-vitro* evidence is consistent with the clinical observations, showing a genotype–phenotype relationship where this polymorphism influences the antilipolytic response of CASR stimulation in adipocytes.

As other CASR variants have also been reported to be frequent and associated with serum calcium handling,[9] we also evaluated the rs1801725 (Ala986Ser) polymorphism. Most subjects were homozygous for the Ala genotype, only 5% were heterozygous, and there were no homozygotes for the Ser variant. Even though both heterozygous were non-responders to cinacalcet, when considering that in our entire sample more than 80% of the subjects were non-responders, we cannot speculate or draw any conclusions based on this information.

There are several links between kidney function and lipolysis, with the known harmful consequence of excess circulating FFA. Recently, obesity was described as a possible independent risk factor for CKD.[16] The underlying mechanism could be overstimulated lipolysis, leading to high levels of circulating FFA, which may elevate kidney damage.[6] Decreasing kidney function leads to the elevation of PTH levels,[17] which may be linked to greater extracellular calcium concentrations needed to activate the CASR. It may be speculated that this impairment in CASR function may also induce the suppression of its antilipolytic function,[3] thus elevating basal adipose lipolytic activity.

It is relevant to note that, unlike most studies evaluating lipolytic responses, this work was focused on basal (non-stimulated) lipolysis. We previously observed that *in-vitro* basal lipolysis is very low in obese subjects,[18] thus making an antilipolytic effect more difficult to assess, particularly for the present study, which included many obese subjects. In addition, several metabolic and other subject-related factors may influence lipolysis, increasing the chances for undetermined variability in the ability to respond to cinacalcet. In our study, only 6 subjects of 38 (16%) respond to cinacalcet with an antilipolytic effect. However, the antilipolytic effect of CASR stimulation is supported by our previous studies in isolated human adipocytes[3] and more recently by the studies of He *et al*[19] in the adipocyte cell line SW872. These authors observed that the CASR-elicited decline in lipolysis was associated with decreased cyclic AMP, protein kinase A activity, hormone sensitive lipase and adipose triglyceride lipase, all of which are key players in the lipolytic process.

We also recognize that the low number of subjects is as a limitation of our study. Taken all this into account, it is striking that the influence of rs1042636 reached the statistical significance that we report, and larger clinical studies are warranted to study *in vivo* the impact of our findings, focusing on the genotype-dependent impact of cinacalcet therapy in circulating FFAs and the clinical consequences that may ensue.

A pitfall of polymorphism studies is that they often show associations with a certain response not because the evaluated allele is the actual cause, but because of a linkage disequilibrium with another unknown polymorphism, which is the real causal agent. For this particular SNP, the causal effect has been demonstrated at the protein level, as evidenced by the influence on the response to the calcimimetic cinacalcet in patients[12] and in cells transfected with CASR expressing each of the two genotypes.[13]

In summary, our data suggest that the antilipolytic effect of cinacalcet depends on the CASR gene polymorphism rs1042636 (Arg990Gly). As a suppressor of FFA release from adipocytes, cinacalcet treatment of CKD patients may be additionally beneficial preventing excess circulating FFA levels, and clinical studies evaluating this novel potential benefit are warranted. It would be relevant to take into account the allelic variant of the subjects though, as different therapy targets might be necessary for Arg–Arg patients and those carrying the Gly allele.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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