Identification of an Interactive Effect of β_3- and α_{2b}-Adrenoceptor Gene Polymorphisms on Fat Mass in Caucasian Women

Isabelle J. Dionne, Amy N. Turner, André Tchernof, Toni I. Pollin, Dionyssia Avrithi, Daniel Gray, Alan R. Shuldiner, and Eric T. Poehlman

Several adrenoceptor subtypes are expressed in adipocytes, which together exert their influence on adipocyte metabolism. Therefore, we specifically examined the interactive effect of Trp64Arg (β_3) and Glu^{12}/Glu^9 (α_{2b}) adrenoceptor (AR) polymorphisms on energy metabolism and body composition in healthy women with a wide range of body habitus. We genotyped 909 unrelated women (age 55 ± 12 [mean ± SD] years, range 19–87; body weight 88 ± 22 kg, range 40–167; and BMI 33 ± 8 kg/m^2, range 16–64) for Trp64Arg β3AR and Glu^{12}/Glu^9 α2bAR variants. We examined the independent effect of the Glu^{12}/Glu^9 α2bAR variant on body composition and energy balance, in a large cohort of Caucasian women (n = 909). A second goal was to examine the interaction effect of Glu^{12}/Glu^9 α2bAR and Trp64Arg β3AR on the same phenotypes. The obesity-related phenotypes studied were as follows: body weight, BMI, fat mass, visceral fat, fat-free mass, resting metabolic rate (RMR), $\dot{V}o_{2max}$, leisure time physical activity, and daily energy intake. Body composition and body fat distribution were measured by dual-energy X-ray absorptiometry and radiographic imagery, $\dot{V}o_{2max}$ by a treadmill test to exhaustion, and RMR by indirect calorimetry. An analysis of covariance indicated that in the entire cohort, there was no significant difference between Glu^{12}/Glu^9 α2bAR carriers and control subjects for any of the obesity-related phenotypes that were examined. However, we observed a significant interaction effect of the Trp64Arg and Glu^{12}/Glu^9 variants on fat mass (P = 0.009) and percent fat (P = 0.016). Age, height, body weight, BMI, fat-free mass, visceral fat, energy expenditure, respiratory quotient, physical fitness, and energy intake were not different among groups. Collectively, these findings support an interaction effect of the two adrenoceptor variants on body fatness in Caucasian women, although the physiological mechanism by which they exert this effect remains to be determined. *Diabetes* **50:91–95, 2001**

M ore than 50% of U.S. adults are overweight, with a BMI >25 kg/m^2 (1), suggesting a chronic imbalance between energy expenditure and energy intake. Because of the association of obesity with numerous metabolic comorbidities, it is of considerable public health importance to understand its genetic and environmental determinants. Associations have been demonstrated between several gene variants and obesity, but a simple Mendelian inheritance pattern, as observed in some inbred rodent strains (2), has not been found in humans with typical obesity.

Our laboratory has participated in a series of prospective interventional studies that examined the association between the Trp64Arg β3AR variant in the β_3-adrenoceptor gene, with several obesity-related phenotypes. We and others have shown reduced energy expenditure (3–6), insulin resistance (7), a reduced ability to mobilize visceral fat (8), a lower rate of glucose disposal (9), and greater visceral obesity (7) and BMI (6) in individuals harboring the Trp64Arg variant. On the other hand, others have found no meaningful effect of this variant on obesity-related phenotypes (10,11).

Because it is recognized that the genetic component of human obesity is complex and polygenic, it is reasonable to hypothesize that the examination of only one genetic variant provides insufficient information regarding genetic susceptibility to obesity. Moreover, we would suggest that discrepancies found in aforementioned studies regarding the effect of the Trp64Arg β3AR variant on obesity-related phenotypes may be partially attributed to the presence of other genetic variants that may interact with the Trp64Arg β3AR to influence obesity and obesity-related phenotypes. For instance, several studies have shown additive effects of the Trp64Arg β3AR variant and a variant in the uncoupling protein-1 gene on obesity-related phenotypes (12–15).

The β_3-adrenoceptor is known to activate lipolysis and energy expenditure. Because other adrenoreceptors are expressed in adipocytes and play a regulatory role in energy balance and lipolysis, the presence of other genetic variants affecting adrenergic function may be interactive with the Trp64Arg β3AR variant. Recently, a newly described variant, a three–amino-acid deletion in the α_{2b}-adrenoceptor (Glu^{12}/Glu^9 α2bAR), has been shown to be associated with a lower basal metabolic rate, but not increased BMI (16). The high frequency of this variant (16,17) and the potential of the α_{2b}-adrenoceptor variant to interact with the Trp64Arg β3AR variant make it a poten-

tial candidate to influence obesity-related phenotypes. The deletion of three glutamic acid residues occurs within a polyglutamic tract at codons 297–309, which is within the acidic domain of the third intracellular loop of this seven-membrane-spanning G-protein–coupled receptor. The functional consequence of this deletion is currently unknown. The purpose of this study was to examine the independent and interactive effect of the Glu[12]/Glu[9] α2bAR and Trp64Arg β3AR variants on body composition and energy balance in a large cohort of unrelated Caucasian women ($n = 909$).

RESEARCH DESIGN AND METHODS

In this study, 909 unrelated Caucasian women (age 55 ± 12 [mean ± SD] years, range 19–87; body weight 88 ± 22 kg, range 40–167; and BMI 33 ± 8 kg/m^{2}, range 16–64) from different protocols conducted at the University of Vermont over the course of the last 3 years were genotyped for Trp64Arg β3AR and Glu[12]/Glu[9] α2bAR variants. Among these 909 women, 244 were characterized using more direct assessments of phenotypes. A portion of the cohort ($n = 82$) was ascertained based on their genotype for Trp64Arg β3AR for the purpose of another study (3,8). Thus, because of a potential selection bias, we will not address the issue of the independent effect of this variant. Subjects were recruited by local advertisement, and inclusion criteria included the following: *1*) nonsmokers; *2*) not taking any medication that could affect cardiovascular function or metabolism; *3*) nondiabetic; and *4*) absence of cardiovascular diseases or hypertension (diastolic blood pressure >90 mmHg). Subjects gave their written consent to be genotyped and to be included in this study.

Genotyping

Detection of the Trp64Arg β3AR variant. The β3-adrenoceptor gene is located on chromosome 8. Polymerase chain reaction (PCR) was performed from ~20 ng genomic DNA with upstream primer 5′-CGCCCAATACCGC CAACAC-3′ and downstream primer 5′-CCACCAGGAGTCCCATCACC-3′ in the presence of 10% dimethylsulfoxide. The resulting 210-bp product was digested with the restriction endonuclease BST N1. The digested products were subjected to electrophoresis through a 4% agarose gel (Metaphor agarose; FMC Bioproducts, Rockland, ME). The gel was stained with ethidium bromide and DNA was visualized by ultraviolet (UV) transillumination. The expected sizes were 99, 62, 30, 12, and 7 bp for Trp64 homozygotes; 161, 30, 12, and 7 bp for Arg64 homozygotes; and 161, 99, 62, 30, 12, and 7 bp for heterozygotes.

Detection of the Glu[12]/Glu[9] α2bAR variant. The α2b-adrenoceptor gene is located on chromosome 2. PCR was performed from ~20 ng genomic DNA with upstream primer 5′-AGGGTGTTTGTGGGGCATCTCC-3′ and downstream primer 5′-CAAGCTGAGGCCGGAGACACTG-3′. The PCR product was subjected to electrophoresis through a 4% agarose gel (Metaphor agarose; FMC Bioproducts). The gel was stained with ethidium bromide and DNA was visualized by UV transillumination. The expected sizes were 112 bp for Glu[12] homozygotes, 103 bp for Glu[9] homozygotes, and 112 and 103 bp for heterozygotes.

Resting metabolic rate. Resting metabolic rate (RMR) was measured by indirect calorimetry for 45 min as previously described (3,18), using the ventilated hood technique, after a 12-h overnight fast at the University of Vermont General Clinical Research Center. Respiratory gas analysis was performed using a Deltatrac metabolic cart (Sensormedics, Yorba Linda, CA). RMR (kcal/d) was calculated from the equation of Weir (19). The test-retest correlation coefficient within 1 week is 0.90 for RMR in our laboratory.

Body composition

Dual-energy X-ray absorptiometry. Fat mass and fat-free mass were assessed in the supine position, using dual-energy X-ray absorptiometry (DEXA) at the University of Vermont (model DPX-L; Lunar Radiation, Madison, WI) as previously described (20,21).

Computed tomography. Visceral adipose tissue and subcutaneous adipose tissue were measured by computed tomography (CT) as previously described (3) using a High-Speed Advantage CT scanner (General Electric Medical Systems, Milwaukee, WI). The subjects were examined in the supine position with both arms stretched above their head. The position of the scan was established at the L4–L5 level using a scout image of the body. Visceral adipose tissue area was quantified by delineating the intra-abdominal cavity at the internal-most aspect of the abdominal and oblique muscle walls surrounding the cavity and the posterior aspect of the vertebral body. Adipose tissue was highlighted and computed using an attenuation range of –190 to –30 Hounsfield units. The subcutaneous adipose tissue area was quantified by highlighting adipose tissue located between the skin and the external-most aspect of the abdominal muscle wall.

***Vo₂max*.** Vo₂max was determined from an incremental exercise test to exhaustion on a treadmill, as previously described (22). Briefly, after an initial 3-min warm-up, the speed was set so that the heart rate would not exceed 70% of the age-predicted maximal heart rate (220 – age [years]). Thereafter, the speed was held constant and the grade was increased by 2.5% every 2 min. The criteria for achieving VO₂max were *1*) a respiratory exchange ratio >1.0, or *2*) a heart rate at or above the age-predicted workload. Test-retest conditions for nine individuals (on two occasions retested 1 week apart) yielded an intraclass correlation of 0.94 using Haggard's model (23) and a coefficient of variation of 3.8% in our laboratory.

Energy intake. Energy intake was measured as previously described (24). Briefly, total daily food intake was assessed using the 3-day food record (2 weekdays and 1 weekend day). Subjects were trained to measure, weigh, and record portion sizes. Food scales and measuring instruments were provided to all subjects. Nutritional assessment was conducted using the Nutritionist III software package (Version 4.0; N-Squared Computing, Salem, OR). Although the measurement of energy intake using the 3-day food record may provide an approximation of habitual energy intake, we have shown that this instrument underestimates true energy intake (20).

Leisure time physical activity. Leisure time physical activity (LTA) was measured by the Minnesota LTA questionnaire (25), a commonly used interviewer-administered questionnaire that assesses daily physical activity accumulated during leisure time and household physical activity over the past 12 months. Trained personnel administered the questionnaire during a 20-min interview. LTA was calculated based on the number of months spent completing the specific activity per year, the average number of times for the specific activity each month, the total time of each physical activity session, and the activity-specific intensity code. The test-retest correlation coefficient over a month has been shown to be 0.92 in older women and men (26).

Statistical analyses. All values are presented as means ± SE, unless otherwise specified. We performed an analysis of covariance to examine the independent effect of the Glu[12]/Glu[9] α2bAR variant on dependent variables, using appropriate covariates. Because age, aerobic fitness, and substrate oxidation influence body weight, BMI, and fat mass, we used age, Vo₂max, and respiratory quotient as covariates for body composition. Visceral fat was adjusted for age and fat mass and resting energy expenditure was adjusted for age, fat mass, and fat-free mass. We then performed a univariate analysis of variance using a general linear model procedure to examine the interactive effect of the two variants on dependent variables using the aforementioned covariates. All analyses were performed using Statistical Package for Social Science software, version 9.0.0 (Chicago).

RESULTS

The frequency of the Glu[12]/Glu[9] α2bAR variant was 0.33, which is somewhat lower than in a previous study of Finnish Caucasians (frequency = 0.45) (16). Altogether, 44% were Glu[12] homozygotes, 43.4% were Glu[12]/Glu[9] heterozygotes, and 11.9% were Glu[9] heterozygotes. The frequency of the Trp64Arg β3AR variant allele in our cohort was 0.06, with 0.3% of our population homozygous and 11.4% heterozygous for the variant allele, comparable to previous studies in Caucasians (allele frequencies of 0.04–0.012) (4,5,7,11,27,28). The genotype frequencies for both the Trp64Arg β3AR and Glu[12]/Glu[9] α2bAR variants were consistent with Hardy-Weinberg equilibrium. The α2b- and β3-adrenoceptor genes are located on different chromosomes (chromosomes 2 and 8, respectively) and therefore sort independently of each other. The frequency of the combinations of genotypes was within expectations of independent assortments.

Table 1 shows the individual effect of the Glu[12]/Glu[9] α2bAR variant on age, height, body weight, and BMI in the entire cohort ($n = 909$). There was no independent association of the variant with any of these variables. Furthermore, in the subset of subjects ($n = 214$) for whom detailed phenotypes were obtained, there was no association of the Glu[12]/Glu[9] α2bAR variant with fat mass, fat-free mass, visceral fat, RMR, respiratory quotient, physical fitness, or energy intake (Table 2).

Because a subgroup of obese subjects ($n = 82$) was selected based on their Trp64Arg β3AR genotype for the purpose of a weight-loss intervention study (3,8), the independent effect of this variant on obesity-related phenotypes is confounded by a selection bias and therefore not reported. However, the

TABLE 1
Age, height, body weight, and BMI in carriers and noncarriers of
Glu^{12}/Glu^9 α2bAR of the entire cohort

Phenotypes	Control subjects	Glu^{12}/Glu^9	P
n	406	503	—
Age (years)	55.4 ± 0.6	54.7 ± 0.6	0.390
Height (cm)	161.8 ± 0.15	162.6 ± 0.29	0.065
Body weight (kg)	86.5 ± 1.1	88.7 ± 0.9	0.114
BMI (kg/m^2)	33.0 ± 0.4	33.6 ± 0.4	0.271

Data are means ± SE. All variables were adjusted for age.

increase in the number of subjects with the Trp64Arg β3AR variant enhanced our power to examine the interactive effect of the Trp64Arg β3AR and Glu^{12}/Glu^9 α2bAR variants on obesity-related phenotypes. For these analyses, because the number of homozygotes was small, and previous studies suggest that heterozygosity for each of the variants is associated with their respective phenotypes, heterozygotes and homozygotes for each of the variants were combined. We found a significant interaction effect between the two variants on fat mass (P = 0.009) and percent fat (P = 0.016) (Table 3). Although the Glu^{12}/Glu^9 α2bAR variant did not associate with fat mass or percent fat when examined individually (Tables 1 and 2), subjects with both the Glu^{12}/Glu^9 α2bAR and Trp64Arg β3AR variants had 9.3 kg and 4.8% greater fat mass and percent fat, respectively, than subjects who carried only the Trp64Arg β3AR variant. Age, height, body weight, BMI, fat-free mass, visceral fat, energy expenditure, respiratory quotient, physical fitness, and energy intake were not different between groups. Collectively, our findings support an interaction effect of the two variants on body fatness.

DISCUSSION
To our knowledge, this is the first study to examine the interactive effects of the Trp64Arg β3AR and the Glu^{12}/Glu^9 α2bAR variants on several obesity-related phenotypes. We

found an interaction effect of the two variants on fat mass and percent fat, because carriers of both variants displayed a significantly greater fat mass and percent fat than carriers of Trp64Arg β3AR only. We also examined the independent effect of the Glu^{12}/Glu^9 α2bAR variant on obesity-related phenotypes in a large cohort of unrelated Caucasian women, since Heinonen et al. (16) previously reported association of this variant with lower RMR. Contrary to their findings, using a dominant model, we did not find any association of RMR or other obesity-related phenotypes with this variant (Tables 1 and 2). In fact, when examined in a recessive model, subjects homozygous for the Glu^{12}/Glu^9 α2bAR variant had higher RMR than heterozygotes or Glu^{12} homozygotes (P < 0.01; data not shown). The reason for this discrepancy is not clear and warrants further study.

Controversies exist among studies pertaining to the singular effect of the Trp64Arg β3AR polymorphism on obesity-related phenotypes (3,6–11,28,29). This controversy is likely due to the moderate effect of the variant and the failure to use highly controlled experimental conditions to control for important covariates (e.g., diet, fluctuations in body weight, sex, ethnicity, and even obesity itself) that may obscure the effect of the variant on obesity-related phenotypes. Another potential reason for discrepancies among investigators is that the Trp64Arg β3AR variant may interact with other variants to influence body fatness. We specifically examined the interaction between the Glu^{12}/Glu^9 α2bAR and Trp64Arg β3AR variants on body composition in a relatively large cohort of well-characterized women with a wide range of body habitus. The Glu^{12}/Glu^9 α2bAR variant was selected because of its previously reported association with a lower RMR and its potential association with obesity-related variables (16).

Since both the β3AR and α2bAR are expressed in adipocytes and reciprocally regulate lipolysis, we hypothesized that the simultaneous existence of the Trp64Arg β3AR and Glu^{12}/Glu^9 α2bAR variants would be associated with an unfavorable metabolic profile when compared with individuals who carry only one of the variants. To address this issue, we performed direct measurements of body fatness, energy

TABLE 2
Body composition, energy metabolism, and physical activity phenotypes in well-characterized carriers and noncarriers of Glu^{12}/Glu^9 α2bAR

Phenotypes	Control subjects (n)	Glu^{12}/Glu^9 (n)	P
Age (years)	51.5 ± 1.8 (97)	51.0 ± 1.6 (117)	0.813
Height (cm)	161.9 ± 0.7 (97)	163.5 ± 0.6 (117)	0.065
Body weight (kg)	72.8 ± 1.8 (97)	75.8 ± 1.6 (117)	0.215
BMI (kg/m^2)	27.7 ± 0.6 (97)	28.4 ± 0.6 (117)	0.455
Fat mass (kg)	27.1 ± 1.2 (97)	29.6 ± 1.1 (117)	0.138
Fat-free mass (kg)	41.5 ± 0.6 (97)	42.2 ± 0.6 (117)	0.436
Percent fat (%)	35.4 ± 0.9 (97)	36.8 ± 0.8 (117)	0.246
Visceral fat (cm^2)	126 ± 7 (58)	115 ± 6 (71)	0.230
Resting energy expenditure (kcal/day)	1,411 ± 12 (97)	1,439 ± 11 (117)	0.083
Resting respiratory quotient	0.85 ± 0.004 (97)	0.85 ± 0.004 (114)	0.724
LTA (kcal/day)	335 ± 24 (91)	321 ± 23 (103)	0.668
Vo$_{2max}$ (ml · kg^{-1} · min^{-1})	29.1 ± 0.7 (77)	29.9 ± 0.7 (88)	0.437
Energy intake (kcal/day)	1,728 ± 49 (58)	1,793 ± 43 (77)	0.322

Data are means ± SE (n). All variables were adjusted for age. In addition, body weight, BMI, fat mass, and percent fat were adjusted for respiratory quotient and Vo$_{2max}$; visceral fat was adjusted for fat mass; and resting energy expenditure was adjusted for fat-free mass and fat mass.

TABLE 3
Body composition, energy metabolism, and physical activity phenotypes in Trp64Arg β3AR carriers only and in carriers of both Glu^{12}/Glu^9 α2bAR and Trp64Arg β3AR

Phenotypes	Trp64Arg only (n)	Trp64Arg + Glu^{12}/Glu^9 (n)	P
Age (years)	56.8 ± 1.8 (45)	54.0 ±1.6 (61)	0.233
Height (cm)	162.2 ± 0.9 (45)	163.1 ± 0.8 (61)	0.463
Body weight (kg)	88.4 ± 3.1 (45)	90.8 ± 2.6 (61)	0.555
BMI (kg/m^2)	34.0 ± 1.2 (45)	34.0 ± 1.0 (61)	0.966
Fat mass (kg)	31.2 ± 2.5 (24)	40.5 ± 2.2 (31)	0.009
Fat-free mass (kg)	43.2 ± 1.5 (24)	45.7 ± 1.3 (31)	0.223
Percent fat (%)	37.9 ± 1.4 (24)	42.7 ± 1.2 (31)	0.016
Visceral fat (cm^2)	165 ± 13 (21)	137 ± 12 (26)	0.123
Resting energy expenditure (kcal/day)	1,532 ± 28 (24)	1,521 ± 25 (31)	0.766
Resting respiratory quotient	0.84 ± 0.009 (24)	0.85 ± 0.008 (31)	0.272
LTA (kcal/day)	272 ± 44 (22)	317 ± 37 (31)	0.447
Vo_{2max} (ml · kg^{-1} · min^{-1})	24.7 ± 1.7 (16)	27.0 ± 1.8 (15)	0.383
Energy intake (kcal/day)	1,770 ± 87 (17)	1,846 ± 70 (26)	0.501

Data are means ± SE (n). All variables were adjusted for age. In addition, body weight, BMI, fat mass, and percent fat were adjusted for respiratory quotient and Vo_{2max}; visceral fat was adjusted for fat mass; and resting energy expenditure was adjusted for fat-free mass and fat mass.

expenditure, and energy intake under inpatient conditions, using state-of-the-art methods. We found an interactive effect of both variants on fat mass in which carriers of both variants displayed significantly higher fat mass. That is, we observed a difference of ~9.3 kg fat mass between the group harboring only the Trp64Arg β3AR variant and the group harboring both variants. These results suggest an interaction effect of the Trp64Arg β3AR and Glu^{12}/Glu^9 α2bAR on body fatness and suggest the occurrence of a chronic energy imbalance at some point in these women's lives.

As with most population-based association studies, the present study has potential statistical biases. First, the issue of multiple comparisons in the interpretation of P values is widely debated (30). Allison and Beasley (30) have suggested the use of an approach for multiple comparisons that utilizes simulations to take into account correlations between the variables. With use of this approach, the corrected P values for associations between fat mass and percent fat, and the interactive effects of the gene variants were $P = 0.09$ (from an uncorrected P value of 0.009) and $P = 0.15$ (from an uncorrected P value of 0.016), respectively. However, the selection of the phenotypes that we examined was based on specific mechanism-based hypotheses. That is, we selected the major determinants of energy balance, and examined whether each phenotype was specifically influenced by the two variants. Of course, it should be pointed out the method used to account for the multiple comparisons in adjusting our P value, as well as all classical frequentist P values approaches, do not take into account the fact that the hypotheses tested were based on specific postulated mechanistic/functional relationship. Therefore, the likelihood of the genetic variants observed having effects on the phenotypes studied should be viewed in light of both the P values obtained and the a priori nature of the hypotheses. Second, population admixture can be another source of bias leading to false-positive associations. However, the present study included only Caucasian women of European ancestry, thus minimizing this possibility.

One potential mechanism by which the Trp64Arg β3AR variant may influence adiposity is an impairment of the β_3-adrenoceptor–mediated lipolysis. Indeed, several studies have shown that the Trp64Arg substitution results in decreased signaling and impaired lipolysis (15,31–38). The Glu^{12}/Glu^9 α2bAR variant has also been suggested to influence the rate of lipolysis. Indeed, it has been suggested that the variant observed in the α_{2b}-adrenoceptor gene may impair receptor desensitization in response to prolonged agonist exposure (39). Because of this receptor's inhibitory role in lipolysis, this variant may potentially cause an increase in the α_{2b}-adrenoceptor–mediated lipolysis inhibition. Thus, it is possible that the presence of both variants favors a pattern of fat storage that is greater than lipolysis and fat oxidation.

We examined several physiological mechanisms by which the Trp64Arg β3AR and Glu^{12}/Glu^9 α2bAR variants may influence energy balance by measuring energy intake, energy expenditure, and fasting substrate oxidation. Our study, however, did not provide evidence of an interactive effect of Trp64Arg β3AR and Glu^{12}/Glu^9 α2bAR variants on these phenotypes. Therefore, the results of the present study do not permit us to identify which component of energy balance may have been altered to cause a greater fat mass in women carrying both variants. It is possible that the experimental design of our study may have limited our ability to detect differences among genotypes in energy expenditure, energy intake, and substrate oxidation that had the potential to impact on body fat accumulation. That is, by definition, the subjects who were eligible for the present study were weight stable, suggesting that individuals were in energy (energy intake = energy expenditure) and macronutrient balance. We thus assume that the energy imbalance that led to an accumulation of fat mass in individuals with both variants had already taken place and was not detectable when these phenotypes were actually measured. Experimental studies using a weight loss/regain design would be needed to investigate the effect of the combination of Trp64Arg β3AR and Glu^{12}/Glu^9 α2bAR variants on dynamic changes in body weight and fat mass during a period of energy and macronutrient imbalance.

In conclusion, although the physiological mechanisms remain to be determined, we found an interaction effect of

Glu[12]/Glu[9] α2bAR and Trp64Arg β3AR variants on body fatness. These results suggest that the combined effect of both variants on fat mass is significantly greater than the separate effects of the polymorphisms. Further studies are needed to confirm and extend these findings.

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