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VO_2MAX IS UNCORRELATED WITH THE *PRKAA2* GENE METHYLATION, BUT INFLUENCES THE GLUCOSE-INSULIN CORRELATION

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ABSTRACT

Purpose. Cardiovascular fitness (maximal oxygen uptake [VO_2max]) is linked with health indicators and the α2 subunit of the AMP-activated protein kinase (AMPKα2), encoded by the *PRKAA2* gene, is an important metabolic sensor and can mediate part of exercise effect. It has been proposed that changes in the metabolic process bound with exercise might occur through epigenetic regulations. However, how VO_2max can influence the epigenetic mechanism and consequently health is still unknown. The aim of this study was to investigate the *PRKAA2* gene methylation profile and its relation to metabolic variables in normoglycemic monozygotic twins discordant for VO_2max.

Methods. Nine pairs of monozygotic twins were studied, with the intra-pair VO_2max difference ≥ 10 ml · kg^{-1} · min^{-1}. An oral glucose tolerance test was used, and blood samples were collected for biochemical and DNA methylation analyses.

Results. No DNA methylation differences were observed between the groups. The low-cardiorespiratory-fitness group demonstrated a positive correlation between the methylation profile and low-density lipoprotein (CpG1, r = 0.714) and total cholesterol (CpG1, r = 0.723; CpG3, r = 0.678). Only the high-cardiorespiratory-fitness group showed correlations between glucose and insulin variables.

Conclusions. Our data suggest a link between high cardiorespiratory fitness and glucose-insulin correlation. The results provide important insights for future studies about the mechanisms through which VO_2max can influence glucose metabolism.

Key words: exercise, glucose, methylation, *PRKAA2*, twins, VO_2max

Introduction

The AMP-activated protein kinase (AMPK) is an important metabolic sensor and can mediate part of the effect of exercise on glucose and lipid metabolism [1]. AMPK is a heterotrimeric protein [2] and the activity of the AMPKα2 subunit, encoded by the *PRKAA2* gene, has been related to exercise [3, 4].

In this regard, the maximal oxygen uptake (VO_2max) is the best indicator of cardiovascular fitness [5] and is linked to health indicators [6]. Low VO_2max is associated with an imbalance in fasting glucose [7] and regulation of glucose metabolism [8]. However, there are individual differences in exercise adaptation and response due to individual variability, which may reflect genetic diversity [9]. In pairs of twins, VO_2max response to standardized training showed 6-fold more inter-genotype variance (dizygotic twins) than intra-genotypes (monozygotic [MZ]) [10]. These differences in the response to exercise training can also depend on epigenetic signals and have an important role in the modulation of gene expression [11].

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Epigenetic processes are related to metabolic regulation and result from complex interactions between genes and the environment [12]. DNA methylation is currently the best characterized epigenetic modification, and it can affect genomic stability [13]. Furthermore, changes in the methylation profile also occur under pathological conditions and in metabolic disorders [14]. In turn, epigenetic imbalance is caused by environmental factors, such as physical activity, diet, and stress [15].

The relative importance of both genes and the environment for determining a phenotype can be verified by investigating the phenotype of MZ twins [16, 17]. In a sample of pairs of MZ twins, only those discordant for a particular characteristic are selected. The case-control analysis is considered the only well-established model by which the effect of environmental and physical factors on a trait can be quantified independently of genetic influences [17]. Nevertheless, studies involving MZ twins have investigated the influence of DNA on phenotypic variation [16].

In turn, how epigenetic mechanisms related to exercise can influence metabolic processes involving health is still unclear. In this study, we investigated the VO_2max changes on the *PRKAA2* gene methylation profile and its relation with metabolic variables in normoglycemic MZ twins who were discordant for VO_2max.

Material and methods

Model of study and subjects

The total of 38 healthy pairs of twins were involved, and in view of the design of this study, only MZ twins with the intra-pair difference ≥ 10 ml · kg^{-1} · min^{-1} (discordant) for VO_2max were included in the study (n = 9 pairs). The MZ twins and their parents and/or legal guardians were previously informed about the experimental procedures.

Measurement of anthropometric characteristics and cardiorespiratory fitness

Anthropometric measurements of body mass and height were used to calculate the body mass index (BMI). To determine VO_2max, a physical test was performed on an ATL Super Model® (Inbrasport, Porto Alegre, Brazil) treadmill with 1% inclination. Then the test started with the protocol involving an initial speed of 4 km/h, with progressive increments of 1 km/h per minute in workload until the respiratory quotient (RQ = VCO_2/VO_2) of at least 1.1 or 19–20/20 on the Borg scale for perceived exertion [18]. Verbal encouragement was employed. Minute volume (VE), oxygen uptake (VO_2), and carbon dioxide production (VCO_2) were continuously recorded (metabolic analyser MedGraphics VO2000®, Medical Graphics Corp., St. Paul, USA) at rest and during the physical test. The equipment was calibrated prior to the development of the study and at the beginning of each physical test. VO_2max was collected breath by breath, and the value adopted to analyse the data was recorded as the average oxygen uptake in the last 30 seconds before the physical test was terminated.

Blood samples, oral glucose tolerance test, and lipid profile measurement

One week after VO_2max assessment, the twins came to the clinical analysis laboratory, accompanied by their legal guardians. After overnight fasting (10–12 hours), a standard oral glucose tolerance test (OGTT) (1.75 g · kg^{-1} or a maximum of 75 g of glucose) was performed for all subjects. For glucose and insulin assessments, blood samples were obtained 0 and 120 min after glucose administration. The twins had blood samples taken from the antecubital vein, with the use of the vacuum blood collection system (Vacutainer™, Becton Dickinson Company, Plymouth, United Kingdom). Aliquots were dispensed into tubes with anticoagulant (fluoride associated with ethylenediaminetetraacetic acid [EDTA], 1 mg/ml blood) and heparin for analysis purposes, and 150 µl of the blood collected were directly pipetted onto QIAcard® (Qiagen, Valencia, USA) for further DNA analysis. Plasma was used to determine concentrations of glucose and insulin, triglycerides, total cholesterol, and high density lipoprotein cholesterol (HDL-C). Low density lipoprotein cholesterol (LDL-C) was estimated in accordance with Friedewald et al. [19]. Insulin resistance was determined with the homeostasis model assessment of insulin resistance (HOMA-IR) index as described by Matthews et al. [20].

Genotyping

Twins were categorized as MZ or dizygotic by genotyping genetic markers (DNA), such as minisatellite loci, which are also known as short tandem repeats (STRs). The analysis of 16 autosomal STRs (CSF1PO, D2S1338, D3S1358, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, D5S51, FGA, TH01, TPOX, VWA and the amelogenin locus) was performed in DNA samples with the use of polymerase

chain reaction (PCR) amplification with a commercial kit (Identifiler), in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, USA).

Methylation analysis

Blood samples were spotted onto QIAcard® FTA paper (Qiagen® GmbH, Hilden, Germany); 20 µl of blood from each participant was applied. A 10-mm punch of QIAcard-dried blood was used for DNA extraction with the QIAamp® DNA Micro Kit (Qiagen® GmbH, Hilden, Germany), as indicated in the protocol supplied by the manufacturer. Subsequently, DNA samples were treated with bisulfite to convert unmethylated cytosine residues into uracil with the use of the EpiTect® Bisulfite Kit (Qiagen® GmbH, Hilden, Germany). After the conversion, the DNA was amplified by PCR performed with the PyroMark PCR kit (Qiagen® GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The amplified DNA was pyrosequenced with the use of the PyroMark Gold Q24 kit (Qiagen® GmbH, Hilden, Germany). The PCR step and pyrosequencing involved 2 sets of primers designed by Qiagen® specific for the *PRKAA2* gene. The sequences analysed were: GCAGATGGGCGCGGAACCTG-GAACCCAGGACGC (Hs_PRKAA2_01_PM PyroMark CpG assay – antisense – cat. PM00004452) and TG-GACTCGTTCTGCGAGGCGC (Hs_PRKAA2_02_PM PyroMark CpG assay – sense – cat. PM00004459). The analysis of the methylation profile was performed with the PyroMark Q24 software, version 2.0.6 (© 2009 by Qiagen group).

Statistical analysis

The twins were arranged into 2 groups; siblings were separated depending on cardiorespiratory fitness. Co-twins with the highest VO_2max comprised the high cardiorespiratory fitness (HCF) group, while those with the lowest VO_2max created the low cardiorespiratory fitness (LCF) group, which was used to control for genomic effects.

With the consideration of the genetic similarity between siblings due to homozygosis and the methodological design of this study, Wilcoxon test was applied to assess the differences between discordant twins (higher and lower VO_2max), while the Spearman correlation was used to analyse statistical associations between VO_2max, the *PRKAA2* gene methylation, and anthropometric and biochemical variables. Analyses were performed with the SPSS statistical package, version 15.0, with the assumption of $\alpha = 0.05$ as the significance level.

Ethical approval

The research related to human use has been complied with all the relevant national regulations and institutional policies, has followed the tenets of the Declaration of Helsinki, and has been approved by the Research Ethics Committee of the São Paulo State University (protocol No. 3143 – UNESP).

Informed consent

Informed consent has been obtained from all individuals included in this study and their parents and/or legal guardians.

Results

The assessment of cardiorespiratory fitness in 38 pairs of MZ twins showed that 9 pairs aged 13 (11–17) years (4 male pairs and 5 female pairs) presented an intra-pair difference ≥ 10 ml · kg^{-1} · min^{-1} in VO_2max.

Significant differences in VO_2max values were observed between the groups, and the intra-pair difference ranged from 10.4 to 22.5 ml · kg^{-1} · min^{-1}. The relative difference between discordant twin pairs equalled 16.9–42.1%. We found no significant differences in anthropometric measurements (Table 1).

The HCF group had a significantly lower level of fasting glucose when compared with the LCF group. Furthermore, other biochemical variables (glucose: post-OGTT, insulin: fasting and post-OGTT, HOMA-IR, triglycerides, total cholesterol, HDL-C, and LDL-C) were similar between the twins (Table 1).

No difference was found between the co-twins with regard to the methylation profile in the promoter region of the *PRKAA2* gene (Figure 1).

Spearman correlation analysis showed no correlation between VO_2max and the other variables. However, analysis of the variables related to glucose metabolism in the HCF group revealed a strong correlation between glucose and insulin fasting ($r = 0.780$), and a moderate correlation between glucose and insulin post-OGTT ($r = 0.700$). For the LCF group, only a strong positive correlation was found between insulin fasting and HOMA-IR ($r = 0.983$) (Table 2).

In the HCF group, no correlation was observed between the variables related to lipid metabolism and the *PRKAA2* gene methylation. In turn, variables related to lipid in the LCF group correlated with CpG islands separately, triglycerides (CpG5, $r = 0.668$; $p < 0.05$), total cholesterol (CpG1, $r = 0.723$; CpG3, $r = 0.678$; $p < 0.05$), and LDL (CpG1, $r = 0.700$; $p < 0.05$).

Table 1. Anthropometric characteristics and metabolic parameters of 9 pairs of monozygotic twins discordant for VO_2max†

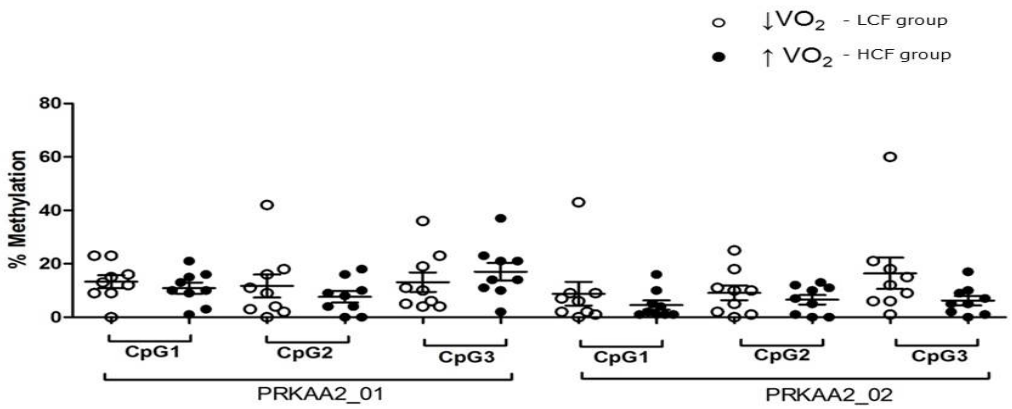
	HCF group	LCF group	*p*-value
Age (year)	13.9 ± 2.2		
VO_2max (ml · kg^{-1} · min^{-1})	45.9 ± 10.0	32.4 ± 10.6**	0.0039
Body mass (kg)	46.4 ± 9.0	46.2 ± 8.7	1.0000
Height (cm)	155.7 ± 11.5	156.4 ± 11.0	0.3738
BMI (kg/m^2)	18.9 ± 1.4	18.7 ± 1.5	0.6523
INSf (μU/ml)	5.5 ± 1.5	6.0 ± 1.6	0.8588
GLUf (mg/dl)	82.9 ± 7.3	86.7 ± 7.6*	0.0122
INS2h (μU/ml)	26.2 ± 16.6	22.3 ± 10.9	0.5469
GLU2h (mg/dl)	84.2 ± 18.9	80.5 ± 17.9	0.5534
HOMA-IR	1.2 ± 0.4	1.3 ± 0.4	0.8203
TC (mg/dl)	151.2 ± 42.1	161.7 ± 36.4	0.1232
HDL-C (mg/dl)	42.6 ± 5.3	44.2 ± 5.6	0.2463
LDL-C (mg/dl)	93.2 ± 40.1	102.5 ± 34.6	0.1548
TG (mg/dl)	77.2 ± 26.1	74.9 ± 27.1	0.6115

zVO_2max – maximal oxygen uptake, HCF – high cardiorespiratory fitness, LCF – low cardiorespiratory fitness, BMI – body mass index, INSf – fasting insulin, GLUf – fasting glucose, INS2h – insulin 2 hours after oral glucose tolerance test, GLU2h – glucose 2 hours after oral glucose tolerance test, HOMA-IR – homeostasis model assessment of insulin resistance, TC – total cholesterol, HDL-C – high density lipoprotein cholesterol, LDL-C – low density lipoprotein cholesterol, TG – triglycerides
† intra-pair difference for VO_2max ≥ 10 ml · kg^{-1} · min^{-1}
n = 9 pairs (GLU2h and INS2h, *n* = 8 pairs); data expressed as mean ± standard deviation
* $p < 0.05$, ** $p < 0.01$ (HCF group vs. LCF group)

Table 2. Relation between VO_2max and biochemical variables related to glucose metabolism for the groups of twins with high and low cardiorespiratory fitness

		INSf	INS2h	GLUf	GLU2h	HOMA-IR
HCF group	VO_2max	–0.167	–0.283	0.051	–0.217	–0.033
	INSf		0.683*	0.780*	0.750*	0.971**
	INS2h			0.220	0.700*	0.611
	GLUf				0.356	0.868**
	GLU2h					0.669*
	HOMA-IR					
		INSf	INS2h	GLUf	GLU2h	HOMA-IR
LCF group	VO_2max	–0.433	–0.150	0.067	–0.067	–0.400
	INSf		0.033	0.283	0.050	0.983**
	INS2h			0.450	0.617	0.133
	GLUf				–0.033	0.400
	GLU2h					0.067
	HOMA-IR					

VO_2max – maximal oxygen uptake (ml · kg^{-1} · min^{-1}), HCF – high cardiorespiratory fitness, LCF – low cardiorespiratory fitness, INSf – fasting insulin (mg/dl), INS2h – insulin 2 hours after oral glucose tolerance test (mg/dl), GLUf – fasting glucose (mg/dl), GLU2h – glucose 2 hours after oral glucose tolerance test (mg/dl), HOMA-IR – homeostasis model assessment of insulin resistance
Spearman's rank correlation tests
* $p < 0.05$; ** $p < 0.01$



Two sets of primers designed by Qiagen® (Hs_PRKAA2_01_PM PyroMark CpG assay – antisense – cat. PM00004452 and Hs_PRKAA2_02_PM PyroMark CpG assay – sense – cat. PM00004459).

Wilcoxon paired test and Mann–Whitney test for difference assessment between CpG islands; $p < 0.05$.

Figure 1. Methylation analyses for pyrosequencing – the *PRKAA2* gene methylation intra-pair profile

Discussion

This is the first study involving the *PRKAA2* gene methylation, VO_2max, and biochemical variables related to glucose metabolism and lipid profile. By studying MZ twins who are normoglycemic but discordant for cardiorespiratory fitness, we found no differences in the methylation profile between twins with high and low VO_2max values (HCF vs. LCF). Also, no difference in lipid metabolism variables was observed between the groups. Corroborating these results, a previous study conducted in our laboratory with the use of the same samples [21] proved a significantly lower level of fasting glucose in the HCF group when compared with the LCF group. In addition, we showed that the HCF condition correlated with blood glucose and insulin concentration, suggesting that cardiorespiratory fitness had a modulatory role on plasma glucose concentration, independent of genetic factors.

Indeed, previous studies have shown that cardiorespiratory fitness has an influence on glucose metabolism. Leite et al. [22] observed that a decrease in VO_2max in normoglycemic adults presenting a risk factor for developing type 2 diabetes correlated with a decline in insulin sensitivity, which in turn may lead to insulin resistance and type 2 diabetes. Nyholm et al. [23], as well as Eriksson and Lindgärde [8] also proved a significant association between insulin resistance and low cardiorespiratory fitness in non-diabetic individuals. Still, low cardiorespiratory fitness is associated with deterioration in fasting glucose [7], and the gradual decrease in VO_2max causes a decline in the regulation of glucose metabolism [8].

Given that the twins included in this study had no metabolic disorders, it was surprising to find discordance for cardiorespiratory fitness between young MZ twins. The fact that differences are more common over time, particularly when twins are separated, suggests that epigenetic changes increase with age [24, 25], when different patterns become more evident. Although this study included a small sample size, the clone-control model [26] is considered a powerful tool to investigate the relationship between variables (phenotype) of two genetically identical people [17] regardless of their genetic background.

In a previous study with MZ twins, global methylation of DNA in peripheral blood was significantly associated with insulin resistance, independently of its risk factors [27]. Moreover, a significant impact of physical activity on DNA methylation has been observed and a high risk for global hypomethylation has been bound with low levels of physical activity [28].

Therefore, these changes in metabolic processes related to exercise might occur through epigenetic regulations [11]. In addition, the regulation of carbohydrate and lipid metabolism via enzymatic activation of the AMPK signalling pathway has been suggested to be a key feature underlying distinct states of metabolic fitness [29]. In turn, the methylation profile in the *PRKAA2* gene showed no differences between the study groups, suggesting that neither was VO_2max able to cause changes nor it constituted a marker of the methylation profile this gene. In this regard, King-Himmelreich et al. [30] did not observe differences in the *PRKAA2* methylation either in adults (age, 46.1 ± 8.56 years; BMI, 29.9 ± 5.79 kg · m^{-2}) who practised recreational physical activity for 4 weeks or in mice that performed 4-week exercise on a treadmill. In this study, recreational activities were applied in humans; therefore, accurate data about exercise intensity, time

to sample collection after recreational activities, and BMI, which indicates overweight, need to be considered in the analysis.

Furthermore, the epigenetic mechanisms might be variable and change rapidly, besides depending upon the intensity and duration of the exercise stimulus [31]. Barrès et al. [32] showed that acute exercise caused increases in mRNA expression according to transient DNA hypomethylation of gene-specific promoter regions. Thus, pulses of elevated mRNA lead the adaptation to exercise training with gradual alteration in metabolic functions [33]. Moreover, it might be possible that the epigenetic mechanisms that are linked with exercise firstly act in transcription factors related with glucose and lipid metabolism, such as histone deacetylase (HDAC), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), cAMP response element-binding protein (CREB), forkhead box protein O1 (FOXO1), and peroxisome proliferator-activated receptors (PPARs) [31].

Intriguingly, we observed that the *PRKAA2* gene methylation was positively correlated with triglycerides (CpG5), total cholesterol (CpG1, CpG3), and LDL-C (CpG1) in the LCF group. Although the AMPK enzymatic activation downregulates the anabolic pathways as lipogenesis and glycogen synthesis [1], the impact in the metabolic processes of epigenetic mechanisms involving AMPK is still unclear. Interestingly, a study with an animal model and physical exercise showed that AMPKα2 mRNA and protein expression were lower in the exercise group, but there was no difference in methylation percentages [30]. Nevertheless, our study provides important insights for future research concerning metabolic processes and physical exercise.

It should be emphasized that the use of peripheral blood mononuclear cells (PBMC) instead of tissue biopsies to identify gene methylation may not reflect the metabolic processes that occur in muscle cells. However, the ethical and operational difficulties of using tissue biopsies from healthy children justify our choice of PBMC. Owing to its ease of use and accessibility compared with biopsy, peripheral blood has indeed been considered a promising tool for molecular diagnostics, particularly for generating genetic methylation profiles [34, 35]. On the basis of DNA extracted from PBMC, several studies have shown that obesity, diet, and physical activity are associated with global DNA methylation [36]. In the recent years, there has been an increase in the number of studies employing body fluids, such as urine, bronchial lavage, breast milk, expectoration, plasma, and peripheral blood, as biomarkers [35].

Conclusions

In conclusion, the VO_2max was not able to cause changes in the *PRKAA2* gene methylation profile. How epigenetic mechanisms related with AMPKα2 can influence metabolic processes linked with physical exercise remains unclear. Although all subjects in the study were normoglycemic, the HCF group showed low fasting glucose and a correlation between glucose and insulin when compared to the LCF group. Therefore, our study provides important insights for future research on the mechanisms through which VO_2max influences glucose metabolism.

Disclosure statement

No author has any financial interest or received any financial benefit from this research.

Conflict of interest

The authors state no conflict of interest.

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