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The presence of the −866A/55Val/Ins haplotype in the *uncoupling protein 2* (*UCP2*) gene is associated with decreased *UCP2* gene expression in human retina

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ABSTRACT

Uncoupling protein 2 (UCP2) is a mitochondrial transporter present in the inner membrane of mitochondria, and it uncouples substrate oxidation from ATP synthesis, thereby dissipating the membrane potential energy and consequently decreasing ATP production by mitochondrial respiratory chain. As a consequence of the uncoupling, UCP2 decreases the reactive oxygen species (ROS) formation by mitochondria. ROS overproduction is related to diabetic retinopathy (DR), a chronic complication of diabetes mellitus (DM). Recently, our group reported that the −866A/55Val/Ins haplotype (−866G/A, Ala55Val and Ins/Del polymorphisms) of the *UCP2* gene was associated with increased risk for DR in patients with DM. The purpose of this study was to analyze the effect of this haplotype on *UCP2* gene expression in human retina. In addition, *MnSOD2* gene expression was also investigated according to different *UCP2* haplotypes. This cross-sectional study included 188 cadaveric cornea donors. In a subset of 91 retinal samples differentiated according to the presence of the mutated *UCP2* haplotype and risk alleles of the −866G/A and Ins/Del polymorphisms, *UCP2* and *MnSOD2* gene expressions were measured by semi-quantitative RT-qPCR. Mutated *UCP2* haplotype carriers (homozygous + heterozygous) had a lower *UCP2* gene expression than reference haplotype carriers (8.4 ± 7.6 *vs.* 18.8 ± 23.7 arbitrary units; $P = 0.046$). Accordingly, *UCP2* gene expression was decreased in −866A carriers when compared with G/G carriers ($P = 0.010$). *UCP2* gene expression did not differ between Ins allele carriers and Del/Del carriers ($P = 0.556$). Interestingly, subjects carrying the heterozygous *UCP2* haplotype showed increased *MnSOD2* gene expression ($P = 0.025$). This is the first report suggesting that the presence of the −866A/55Val/Ins haplotype is associated with decreased *UCP2* gene expression in human retina. Possibly, *MnSOD2* expression might influence the UCP2 effect in the protection against oxidative stress.

1. Introduction

The uncoupling protein 2 (UCP2) belongs to the mitochondrial inner membrane carrier family and it is expressed in many tissues including white adipose tissue, pancreatic islets and retinal cells (Cui et al., 2006; Erlanson-Albertsson, 2002; Fisler and Warden, 2006; Fleury et al., 1997). UCP2 mildly uncouples substrate oxidation from ATP synthesis, thereby dissipating the membrane potential energy and consequently decreasing ATP production by mitochondrial respiratory chain (Cui et al., 2006; Erlanson-Albertsson, 2002; Fisler and Warden, 2006; Fleury et al., 1997). The uncoupling thus leads to tissue-specific functions such as regulation of free fatty acids metabolism, inhibition of insulin secretion from beta-cells and decreasing ROS formation by mitochondria (Erlanson-Albertsson, 2002).

Diabetic retinopathy (DR) is a common sight-threatening microvascular complication affecting patients with diabetes mellitus (DM) and it is a major cause of new cases of blindness in adults (Fong et al., 2004). Proliferative DR (PDR), which is the most severe form of DR, might affect 10–20% of patients with DM (Fong et al., 2004; Kohner, 1993). Overproduction of reactive oxygen species (ROS) or a failure in intracellular antioxidant defenses against ROS are considered to be causal links between elevated glucose and the other abnormalities important in the development of DR (Brownlee, 2001; Fong et al., 2004).

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Mitochondria are the major source of superoxide production and this makes them the target of direct attack of ROS (Liang and Godley, 2003). There is a positive correlation between mitochondrial inner membrane potential and ROS production. At high membrane potentials, even a small increase in membrane potential causes a large stimulation of hydrogen peroxide (H_2O_2) production. Therefore, "mild uncoupling", i.e., a small decrease in membrane potential, has been suggested to have a natural antioxidant effect (Papa and Skulachev, 1997). Since even a "mild uncoupling" has a large effect on reducing ROS production, the hypothesis that UCP2 protects against oxidative stress now has strong support (Brand et al., 2004; Fisler and Warden, 2006). Manganese superoxide dismutase (MnSOD) catalyzes the breakdown of superoxide into H_2O_2 scavenging superoxide, and, because of its mitochondrial localization, this enzyme is considered as the first line of defense against oxidative stress (Kowluru et al., 2006).

Taking into account the role of UCP2 in the protection against oxidative stress, we recently investigated whether three *UCP2* gene polymorphisms (−866G/A, Ala55Val and Ins/Del), previously described in association with type 2 DM and/or obesity (Jia et al., 2009), were also associated with DR in Brazilian subjects with DM (Crispim et al., 2010). Interestingly, our data showed that the −866A/55Val/Ins haplotype was associated with an increased risk for PDR in both type 2 and type 1 DM patients (Crispim et al., 2010). Therefore, in the present study, we evaluated the effect of the mutated −866A/55Val/Ins haplotype on *UCP2* gene expression in retina from cadaveric cornea donors. Additionally, *MnSOD2* gene expression in retina was also investigated according to different *UCP2* haplotypes.

2. Patients and methods

2.1. Samples

Three hundred and seventy-six eyes were obtained from 188 cadaveric cornea donors identified through the *Central de Transplantes do Rio Grande do Sul* (a Brazilian organization that regulates organ donations in Rio Grande do Sul [RS], Brazil), and collected at two Porto Alegre (RS) hospitals, namely Hospital de Clínicas de Porto Alegre and Hospital Santa Casa de Misericórdia. A standard questionnaire was used to collect information from medical records about age, gender, presence of arterial hypertension and DM, smoking, occurrence of other diseases and cause of death.

After enucleation and separation of corneas for donation, retinas were visually separated from the remaining intraocular structures, snap-frozen in liquid nitrogen and stored at −80 °C until analysis. The mean duration of time (±SD) from death of the donor to dissection and conservation of the retinal tissue was 5.54 ± 2.2 h. Blood samples were also collected from each subject for DNA extraction and genotyping of the −866G/A, Ala55Val e Ins/Del polymorphisms. Following genotyping, subjects were divided into groups according to the presence of the mutated haplotype (−866A/55Val/Ins) or different genotypes of the analyzed polymorphisms.

To estimate the allele frequencies of *UCP2* polymorphisms in the general population of RS, 458 healthy blood donors were also genotyped. The protocol of this study was approved by the Hospital Ethics committees, and relatives of all donors gave their written informed consent authorizing the use of the retinas.

2.2. Genotyping

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The −866G/A polymorphism (rs659366) in the promoter region of the *UCP2* gene was determined by digesting polymerase chain reaction (PCR) products with the enzyme *Mlu*I (Invitrogen Life Technologies, CA, USA), as previously described (Sasahara et al., 2004), and using primers depicted in Table 1. Evaluation of the 45 bp Ins/Del polymorphism in the 3′untranslated region (UTR) of exon 8 was done by PCR using primers depicted in Table 1. Primers amplified products of 457 bp (insertion allele) or 412 bp (deletion allele), which were resolved on 2% agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., CA, USA) and visualized under ultraviolet light. Genotyping of the Ala55Val (C/T) polymorphism (rs660339) in exon 4 was performed using primers and probes contained in the Human Custom TaqMan Genotyping Assay 40x (Applied Biosystems, CA, USA) and shown in Table 1. Reactions were conducted in 96-well plates, in a total 5 μl volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1× (Applied Biosystems) and Custom TaqMan Genotyping Assay 1×, as previously described (Crispim et al., 2010).

2.3. RNA isolation

Retinal tissues (250 mg) were homogenized in phenol–guanidine isothiocyanate (Invitrogen Life Technologies). RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 × g) at 4 °C. RNA pellet was washed twice with 75% ethanol and ressuspended in 10–50 μl of diethylpyrocarbonate treated water.

Concentration and quality of total RNA samples were assessed using a NANODROP 2000 spectrophotometer (Thermo Scientific Inc., DE, USA). Only total RNA samples which achieved adequate purity ratios (A260/A280 = 1.9–2.1) were used for subsequent analyses (Bustin et al., 2009). In addition, RNA integrity and purity was also checked on agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium Inc.). The mean RNA concentration (±SD) isolated was 14.9 ± 8.6 μg/250 mg retina.

2.4. Quantification of UCP2 and MnSOD gene expressions by RT-qPCR

Real-time reverse transcription-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by quantitative real-time PCR (RT-qPCR). Reverse transcription of 1 μg of RNA into cDNA was carried out using the SuperScript™ III First-Strand Synthesis System for RT-PCR

Table 1
Primer and probe sequences used for genotyping of *UCP2* gene polymorphisms or gene expression analyses.

	Sequences
−866G/Apolymorphism	F 5′-CAGGCTGCTTCTGCCAGGAG-3′
	R 5′-AGGCGTCAGGAGATGGACCG-3′
Ala55Val polymorphism	F 5′-GTCTTGGCCTTGCAGATCCA-3′
	R 5′-GTCAGAATGGTGCCCATCACA-3′
	FAM dye 5′-TGGGCGCTGGCTGTA-3′
	VIC dye 5′-TGGGCGCTGACTGTA-3′.
Ins/Del polymorphism	F 5′-CAGTGAGGGAAGTGGGAGG-3′
	R 5′-GGGGCAGGACGAAGATTC-3′
UCP2 gene[a]	F 5′-TTGGGTTCAAGGCCACAGAT-3′
	R 5′-CCAGCCCCAAGAAACTTCAC-3′
MnSOD2 gene[a]	F 5′-AAATTGCTGCTTGTCCAAATCAG-3′
	R 5′-ATCAATCCCCAGCAGTGGAAT-3′
B-actin gene[a]	F 5′-GCGCGGCTACAGCTTCA-3′
	R 5′-CTTAATGTCACGCACGATTTCC-3′

F = forward primers and R = reverse primer.
[a] Primers were designed using published human gene sequences and the Primer Express 3.0 Software (Applied Biosystems), and they were projected to target two consecutive exons of a gene in order to prevent the amplification of any contaminating genomic DNA.