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Application of Western blotting for the detection of uncoupling protein-2 (UCP-2) in mitochondria from smokers and non-smokers

Abstract

Introduction: Uncoupling proteins (UCPs) are a family of transmembrane anion transporters present in the inner mitochondrial membrane. UCP-2, which exhibits the widest distribution in various tissues, plays an important role in many physiological processes. Human UCP-2 studies have been hampered by the lack of a method for measuring this protein in an easily accessible human tissue, e.g. blood. The aim of this study was to develop such a method and test its utility by comparing UCP-2 levels in smokers and non-smokers.

Material and methods: Venous blood samples from 10 smoking and seven non-smoking volunteers were used for the study; lymphocytes were isolated employing Lymphoprep. UCP-2 levels were measured by Western blotting combined with chemoluminescence detection.

Results: Total lymphocyte homogenates were found useless for measuring UCP-2 levels, but it was possible to measure UCP-2 in homogenates of purified lymphocyte mitochondria. There was a significant, though moderate, linear correlation between UCP-2 level and daily cigarette use. UCP-2 level in peripheral blood lymphocytes from smokers was higher than that in non-smokers.

Conclusion: The method for measuring UCP-2 in peripheral blood lymphocytes opens the possibility of UCP-2 screening studies in humans and thus may be useful for studying the role of the protein in human physiology and pathology.

Key words: uncoupling protein-2, lymphocytes, mitochondria, tobacco smoking, Western blotting

Pneumonol. Alergol. Pol. 2009; 77: 118–122

Introduction

Uncoupling proteins (UCPs) are transmembrane anion transporters present in the inner mitochondrial membrane [1]. Various UCPs have been found in the majority of human tissues including adipose tissue, spleen, skeletal muscle, lung and peripheral blood macrophages [2]. UCP expression varies depending on a given physiological or pathological state. For example, it is elevated by starvation and hyperthyroidism [1, 3].

UCP-2 is a member of the UCP protein family that shows the widest distribution in various tissues. The most likely principal function of this protein is uncoupling of mitochondrial oxidative phosphorylation [2, 4] which may subserve important physiological functions. UCP-2 has also been reported to be involved in lipid metabolism, glucose utilisation, thermoregulation, macrophage-mediated immunity, inhibition of inflammation, and inhibition of apoptosis [5].

There is some evidence that UCP-2 plays an important role in the regulation of reactive oxygen species (ROS) production in mitochondria, and in cellular defence against oxidants [1, 4–6]. Mitochondrial ROS production is very sensitive to the proton-motive force [7] and is strongly diminished by mild uncoupling. On the other hand, ROS-related oxidation products potently activate proton conductance through increase in the expression of UCPs including UCP-2. Increases in UCP-2 expression *in vitro* have been observed upon the exposu-

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Received: 26.05.2008

ISSN 0867–7077

re of rat liver mitochondria to 4-hydroxy-2-transnonenal [8], and of rat kidney mitochondria [9] and rat spleen and thymus mitochondria [10] to ROS. The mechanism of this activation is yet to be established.

There is also some experimental evidence from animal studies suggesting increased expression and/or activity of UCP-2 in neurodegenerative (e.g. Alzheimer's and Parkinson's Diseases) and other diseases (e.g. diabetes, obesity, atherosclerosis) [6, 11]. However, translation of these observations into human clinical studies has been hampered by the lack of a method for measuring this protein in an easily accessible human tissue, e.g. blood.

It has been known for many years that cigarette smoke enhances free radicals production in man [12–14], and recent studies have shown elevations in several UCPs in mice exposed to cigarette smoke [15, 16]. These findings have inspired us (i) to develop a method for evaluation of UCP-2 expression in human peripheral blood lymphocytes and (ii) to pilot-test the utility of the newly established method by comparing UCP-2 levels between volunteers of differing tobacco-smoking status.

Material and methods

Seven non-smokers and 10 smokers (for demographic data see Table 1), who smoked their last cigarette at least two hours before donating their blood samples, volunteered for the study after giving their informed consent. The volunteers were interviewed regarding their general health using a special questionnaire. They all claimed to be in good health, did not suffer from flu and/or other acute conditions that might affect their respiratory health and immune system status at the moment of blood collection, nor from any chronic thyroid, heart and/or respiratory system diseases. There were no differences in age, gender, weight, height, or BMI between the two groups ($p \geq 0.49$). From each participant a 20 ml venous blood sample was drawn using trisodium citrate as an anticoagulant. Lymphocytes were isolated using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). The isolated cells were resuspended in 500 μl of ice-cold 15 mM Tris-HCl buffer pH 7.6 supplemented with 0.25 M sucrose, 2 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol, and were homogenized in a Dounce homogenizer using 10 strokes. The homogenates were then centrifuged at 1000 × g at 4°C for 10 min. The pellets were discarded and the supernatants were centrifuged again at 17 000 × g and 4°C for 20 min to obtain mitochondria. The crude mitochondrial fraction obtained was suspended in the homogenization buffer (250 μl) by vortexing, then mixed with 4 volumes of 12% Ficoll in Krebs-Ringer buffer prior to the final centrifugation (100 000 × g, 4°C, 30 min). The final mitochondrial pellet was mixed with 250 μl of ice-cold homogenization buffer and sonicated for 1 min in an ice-water bath using a model CP-130 ultrasonic processor (Linemaster Switch Corporation, Woodstock, CT, USA) set at 0.4 W output power and amplitude 80. The sonicated mitochondrial homogenate was snap-frozen and kept at –80°C until assessed for UCP-2. The protein content of the homogenate was determined by the method of Bradford using bovine serum albumin as a standard.

Mitochondrial homogenates were mixed with Laemmli buffer [17], heated for 5 min at 100°C and equal amounts of protein (40 μg) were loaded onto a 12% polyacrylamide gels (SDS-PAGE) together with prestained broad-range molecular weight standards (Bio-Rad), electrophoresed and then electrotransferred to nitrocellulose membranes (Amersham). The membranes were blocked with skimmed milk and incubated overnight with rabbit polyclonal antibody against human UCP-2 (Calbiochem; dil. 1:500). Finally, the membranes were incubated for 20 min with goat polyclonal anti-rabbit antibody conjugated with horseradish peroxidase (Sigma; dil. 1:5000). After the incubation, the blots were developed using ECL detection system (Amersham). UCP-2 content was quantified densitometrically using a model GelExpert 4 densitometer (NucleoTech, San Mateo, CA, USA).

All statistical analyses were done using the Statistica v. 7.1 software (StatSoft, Tulsa, OK, USA). Spearman's correlation by ranks and linear regression, and the Mann-Whitney *U* test were used, respectively, for assessing correlations between cigarette use and UCP-2 expression, and the significance of between-group differences in UCP-2 level; $p < 0.05$ was considered significant.

Results

Our attempts to measure UCP-2 expression in total lymphocyte homogenates were unsuccessful. We believed this was because of too low UCP-2 level in total cell homogenates. Whereas UCPs' genes are coded by genomic DNA, they are quickly transferred to the mitochondrial inner membrane postranslationally and considerable amounts of UCP-2 are found only in mitochondria [3]. Therefore, we next attempted to measure UCP-2 using homogenates of mitochondrial fractions obtained from total lymphocyte homogenates. Using this

Table 1. Somatic and demographic characteristics of the study participants

Smoking status	Age (years)	Habitual smoking duration (years)	Pack-years	Gender	Weight (kg)	Height (m)	BMI (kg/m^2)
–	25	0	0	M	75	1.76	24.4
–	37	0	0	F	80	1.68	28.3
–	28	0	0	F	65	1.74	21.5
–	32	0	0	F	52	1.55	21.5
–	58	0	0	F	70	1.75	22.9
–	49	0	0	F	62	1.59	24.5
–	29	0	0	F	45	1.58	17.9
+	27	3	1.5	F	65	1.69	22.8
+	26	8	15	F	50	1.66	18.1
+	51	30	0.5	F	78	1.65	28.7
+	54	30	12.5	M	78	1.82	23.5
+	24	5	3	F	60	1.67	21.5
+	50	30	1.75	F	52	1.60	20.3
+	26	10	3.6	F	45	1.60	17.6
+	55	25	2	F	60	1.60	23.4
+	39	3	22.5	F	58	1.70	20.1
+	36	7	60	F	74	1.74	24.4

M — *male*; F— *female*

approach, we found detectable UCP-2 levels in all study participants. There was a significant, though moderate, positive linear correlation between UCP-2 level and daily cigarette use (Fig. 1), but not between pack-years of smoking and UCP-2 level (linear correlation coefficient r = 0.22, p = 0.53; Spearman's correlation by ranks coefficient R = 0.21, p = 0.56). The difference in UCP-2 levels between smokers and non-smokers did not reach statistical significance in our study (p = 0.079; Fig. 2), but the tendency for increased UCPs levels in smokers was in agreement with animal studies [15]. The highest UCP-2 level in the non-smokers' group, but not in the smokers' group, differed from its respective group mean by two standard deviations; after excluding this result from the analysis, the difference between the two groups reached significance (p = 0.030).

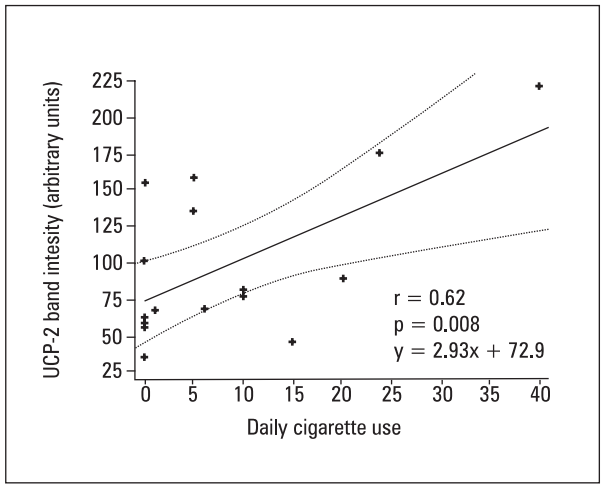

Figure 1. Correlation between UCP-2 level and cigarette use. Dashed curves denote 95% confidence limits

Discussion

It is well known that smokers have, on average, lower BMI than non-smokers of matching age and gender [16–18], and that cessation of smoking without nicotine replacement therapy is usually accompanied by weight gain [13]. This is because some component(s) of cigarette smoke disturb energy homeostasis, a phenomenon which may crucially contribute to the weight and adipose tissue loss observed in experimental animals [15]. However, the exact mechanism(s) by which smoking affects body weight remain unclear. A number of human and animal studies have suggested that nicotine suppresses appetite and decreases food intake [15, 16]; this would imply that cessation of smoking may be associated with increased food intake. Some researchers have suggested that nicotine

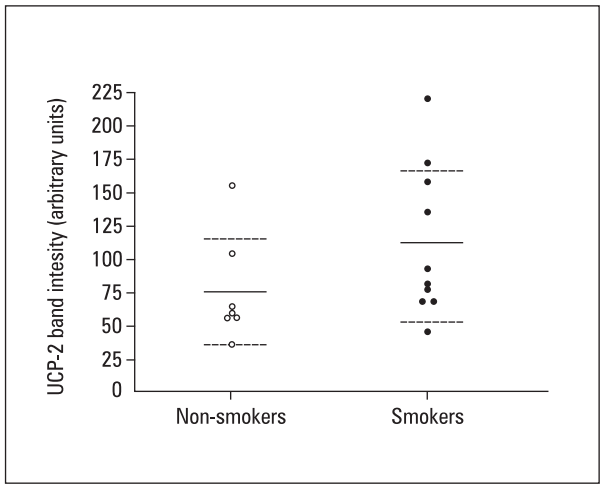

Figure 2. UCP-2 band intensity in non-smokers and smokers. Solid lines denote mean values, dashed lines denote standard deviation

from tobacco smoke may decrease plasma leptin concentration and subsequently reduce body weight [16]. Another report suggested that smokers with high nicotine uptake use more lipids to sustain fasting resting energy expenditure than non-smokers. Therefore, if subjects who stop smoking do not decrease their lipid intake, the imbalance in lipid intake and fat oxidation may induce an increase in body fat [18].

There are also suggestions that exposure to cigarette smoke enhances the production of inflammatory cytokines, such as TNF-α and IL-6 [16]. TNF-α can inhibit adipose cell differentiation *in vitro*, affect lipid metabolism by increasing lipolysis in adipose tissue, and inhibit appetite [15]. It would be highly interesting to see if TNF-α can up-regulate UCP genes' expression, which may also contribute to alterations in lipid metabolism in smokers.

As mentioned in the Introduction, UCP-2 expression can be enhanced by increases in oxidative stress. Cigarette smoking may increase ROS production and therefore alter the expression of UCP-2 in peripheral blood lymphocytes. UCP-2 is believed to protect cells by limiting mitochondrial free radicals' production [3]. There is a growing body of evidence that UCP-2 is involved in modulation of lipid metabolism and energy expenditure [2, 19–21]. For example, it has been reported that UCP-2 mRNA levels in adipose tissue were positively related to resting metabolic rate [19]. Moreover, administration of triiodothyronine to humans, which causes a pronounced increase in energy expenditure, has been shown to up-regulate UCP-2 mRNA expression in skeletal muscles [20]. This suggests that decreased UCP-2 expression may be involved in the development of obesity. We have found that the level of UCP-2 in non-smokers tends to be lower than that in smokers. Expression of UCP-2 may thus be involved in weight gain after smoking cessation.

Conclusion

The method presented in this study allows measuring UCP-2 in peripheral blood lymphocytes and thus may be convenient for studying the role of the protein in human physiology and pathology.

Acknowledgments

This study was supported by the Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland. The authors would also like to thank Dr. Stanisław J. Chrapusta of the Department of Experimental Pharmacology, Mossakowski Medical Research Institute, PAS, for his critical reading of the manuscript and helpful suggestions.

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