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


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-
re of rat liver mitochondria to 4-hydroxy-2-trans-
nonenal [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 esta-
blished.

There is also some experimental evidence from
animal studies suggesting increased expres-
sion and/or activity of UCP-2 in neurodegenerative
e (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 ciga-
rette smoke enhances free radicals production in
man [12–14], and recent studies have shown ele-

vations in several UCPs in mice exposed to ciga-
rette smoke [15, 16]. These findings have inspired
us (i) to develop a method for evaluation of UCP-2
expression in human peripheral blood lymphocy-
tes and (ii) to pilot-test the utility of the newly es-

tablished method by comparing UCP-2 levels between
volunteers of differing tobacco-smoking status.

**Material and methods**

Seven non-smokers and 10 smokers (for demo-

graphic data see Table 1), who smoked their last
cigarette at least two hours before donating their
blood samples, volunteered for the study after gi-
ving 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 respirato-
ry 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 ≥ 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 iso-

tated 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
MgCl2, 0.5 mM phenylmethylsulfonyl fluoride and
1 mM dithiothreitol, and were homogenized in a
Dounce homogenizer using 10 strokes. The homo-
genates were then centrifuged at 10000 × g at 4°C
for 10 min. The pellets were discarded and the su-

pernatants were centrifuged again at 17 000 × g and
4°C for 20 min to obtain mitochondria. The
crude mitochondrial fraction obtained was suspen-
ded in the homogenization buffer (250 µl) by vor-
texting, then mixed with 4 volumes of 12% Ficoll
in Krebs-Ringer buffer prior to the final centrifu-
gation (100 000 × g, 4°C, 30 min). The final mito-
chondrial 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, Wood-
stock, CT, USA) set at 0.4 W output power and
amplitude 80. The sonicated mitochondrial homo-
genate was snap-frozen and kept at −80°C until as-

essed for UCP-2. The protein content of the homo-
genate was determined by the method of Bradford
using bovine serum albumin as a standard.

Mitochondrial homogenates were mixed with
Laemmlı 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 rab-
bit polyclonal antibody against human UCP-2 (Cal-
biochem; dil. 1:500). Finally, the membranes were
incubated for 20 min with goat polyclonal anti-rab-
bit antibody conjugated with horseradish peroxi-
dase (Sigma; dil. 1:5000). After the incubation, the
blots were developed using ECL detection system
(Amersham). UCP-2 content was quantified den-
sitometrically using a model GelExpert 4 densito-

meter (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 be-
tween 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’
genomes are coded by genomic DNA, they are quick-
ly transferred to the mitochondrial inner mem-
brane posttranslationally and considerable amounts
of UCP-2 are found only in mitochondria [3]. The-
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

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Age (years)</th>
<th>Habitual smoking duration (years)</th>
<th>Pack-years</th>
<th>Gender</th>
<th>Weight (kg)</th>
<th>Height (m)</th>
<th>BMI (kg/m²)</th>
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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).

**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...
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.

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**References**


