Clinical research

Cardiovascular risk in healthy men and markers of oxidative stress in diabetic men are associated with common variation in the gene for uncoupling protein 2

Sukhbir S. Dhamrait\textsuperscript{a,*}, Jeffrey W. Stephens\textsuperscript{a,b,1}, Jacqueline A. Cooper\textsuperscript{a}, Jayshree Acharya\textsuperscript{a}, Ali R. Manic\textsuperscript{c}, Kevin Moore\textsuperscript{c}, George J. Miller\textsuperscript{d}, Steve E. Humphries\textsuperscript{a}, Steven J. Hurel\textsuperscript{b}, Hugh E. Montgomery\textsuperscript{a}

\textsuperscript{a} Centre for Cardiovascular Genetics, British Heart Foundation Laboratories, Royal Free and University College London Medical School, Rayne Building, 5 University Street, London WC1E 6JF, UK
\textsuperscript{b} Department of Diabetes and Endocrinology, UCL Hospitals, London W1T 3AA, UK
\textsuperscript{c} Centre for Hepatology, RFUCL Medical School, London NW3 2PF, UK
\textsuperscript{d} Medical Research Council, Cardiovascular Research Group, Wolfson Institute of Preventive Medicine, London EC1M 6BQ, UK

Received 13 January 2004; revised 21 January 2004; accepted 23 January 2004

Background Oxidative stress reduces total antioxidant status (TAOS) and is implicated in atherogenesis. Mitochondrial uncoupling protein 2 (UCP2) negatively regulates reactive oxygen species generation. The UCP2 gene demonstrates a common functional promoter variant (-866G>A).

Methods and results Amongst 465 diabetic men (age 61.7 ± 13.3 years), an association of the UCP2-866A allele with significantly lower TAOS in those without CHD was even more pronounced in those with CHD (TAOS 30.1 ± 16.1% vs. 41.6 ± 12.4% for AA vs. GG; \(P = 0.016\)). In a sample of 20 diabetic men selected for homozygosity for the UCP2-866G>A variant, matched for baseline characteristics, plasma markers of oxidative stress in those with CHD were significantly higher in AA genotype men (TAOS 31.7 ± 7.3% vs. 52.6 ± 6.3%; \(P = 0.001\) and F2-isoprostanes 220.6 ± 37.2 pg ml\(^{-1}\) vs. 109.9 ± 51.1 pg ml\(^{-1}\); \(P = 0.005\) for AA vs. GG). Amongst 2695 healthy men (age 56.1 ± 3.5 years) prospectively studied for a median 10.2 years, AA homozygotes had a highly significant doubling in CHD risk after adjustment for established risk factors (HR 1.99 [1.37–2.90]; \(P = 0.002\)). Risk associated with this genotype was substantially increased by the presence of other risk factors (obesity, hypertension and diabetes).

Conclusions This study provides the first in vivo evidence of a role for UCP2 in modifying oxidative stress and CHD risk in humans.

© 2004 The European Society of Cardiology. Published by Elsevier Ltd. All rights reserved.

KEYWORDS
UCP2; Genetics; Coronary heart disease; Diabetes mellitus; Inflammation; Oxidative stress
Introduction

The generation of reactive oxygen species (ROS) is implicated in coronary heart disease (CHD) pathogenesis, being increased in the presence of human atherogenesis or its classical risk factors.1–3 However, whilst numerous enzymes play a role in ROS production,3 the fundamental processes regulating ROS production remain obscure. As a component of aerobic metabolism, mitochondrial electron transport chain (ETC) activity is associated with substantial mandatory ROS generation.4,5 However, the contention that the ETC is the predominant ROS source in non-phagocytic cells,6 and its putative role in atherogenesis, both remain unproven.

Uncoupling proteins (UCPs 1–3)7 are thought to dissipate the inner mitochondrial membrane proton electrochemical gradient that drives ATP synthesis.8 UCP1 expression is restricted to brown adipose tissue (BAT),9 whilst UCP3 is predominantly expressed in skeletal muscle.10 UCP2 is ubiquitous11–13 and shares 59% and 73% sequence homology with UCP11,12 and UCP3,10 respectively.

The ubiquity and electrochemical actions of UCP2 make it a plausible negative regulator of ROS production.14,15 In support, decreased UCP2 expression (through endothelial anti-sense strategies13 or macrophage gene-deletion16) increases ROS generation and bone marrow transplant from UCP2 knockout donor mice increases markers of oxidative stress (OS) and lesion size in atherosclerotic-prone mice.17 Meanwhile, UCP2 expression is induced by OS,18 protecting against further ROS generation. Increased UCP2 activity may therefore limit ROS generation, decreasing atherosclerotic risk. We have examined these issues using a genetic strategy.

A common functional variant exists in the promoter of the human UCP2 gene (−866G>A),20 the UCP2-promoter A-allele construct being associated with impeded UCP2-expression.21 If UCP2 expression does regulate ROS generation, then UCP2 genotype should be associated with markers of OS amongst patients with type 1 (T1DM) or type 2 diabetes (T2DM), in whom OS is elevated.22 Secondly, if ROS generation does play a role in atherogenesis, UCP2 genotype should be similarly associated with the development of CHD. We have examined these hypotheses.

Methods

Institutional ethical committee approval was obtained and all subjects gave written informed consent. Both studies complied with the Declaration of Helsinki.

Plasma markers of OS in diabetic men

The University College Diabetes and Cardiovascular disease (UDAC) study is a cross-sectional sample designed to study the association between common variants in inflammatory/meta-bolic genes and biochemical risk factors implicated in CHD in patients with diabetes. It comprises 1020 subjects consecutively recruited from the diabetes clinic at UCL Hospitals in 2001–2. Analyses were confined to Caucasian men only (n = 465; mean age 61.1 ± 13.3 years; Table 1). CHD was defined at the time of...
recruitment as documented myocardial infarction (MI), percutaneous/surgical coronary revascularisation, symptomatic/treated angina, or positive exercise tolerance test, cardiac thallium scan or coronary angiography. Plasma total anti-oxidant status (TAOS), which is inversely related to ROS generation, was measured by Sampson’s modification of Laight’s photometric microassay, using 2.5 μl citrated plasma samples in 96-well ELISA plates. Inter- and intra-assay coefficients of variation were 14.1% and 4.3%, respectively.

In addition, plasma esterified F2-isoprostanes were measured using gas chromatography and mass spectroscopy as previously described23 in 20 UCP2-866G>A homozygous subjects (5 GG and 5 AA with CHD and 5 GG and 5 AA without CHD), with the mean for each group closely matched for baseline characteristics including drug treatment as shown in Table 2. Results were calculated by reference to deuterated 8-iso-PGF2α internal standards.

**Prospective CHD risk in healthy men**

Subjects were those of the Second Northwick Park Heart Study (NPHSII), detailed elsewhere.24 In brief, 3012 unrelated healthy Caucasian middle-aged male subjects (mean age 56.1 ± 3.5 years) recruited from nine UK general practices were prospectively followed for a median 10.2 years (interquartile range 8.1–11.4 years). Baseline exclusion criteria were a history of MI, cerebrovascular disease, life-threatening malignancy or regular medication with aspirin or anticoagulants. Time to first CHD event (defined as sudden cardiac death, symptomatic/silent MI (the appearance of a new major Q wave on the follow up ECG, with Minnesota codes 11,12,1 to 112,122 plus 51 or 52,27) or coronary revascularisation) was recorded, yielding only one event/subject. To date, 204 events have occurred in the 2775 subjects with DNA available for analysis: 148 (72.5%) acute MI, 38 (18.6%) coronary surgery and 18 (8.8%) silent MI. Obesity and systolic hypertension were defined as body mass index ≥30 kg m⁻² and systolic blood pressure ≥160 mmHg.

**UCP2-866G>A genotyping**

Genotypes were determined using leucocyte DNA polymerase chain reaction amplification (PCR) using published primers and conditions25 and products resolved by MADGE28 and confirmed by two independent technicians blind to subject outcome, with discrepancies resolved by repeat genotyping.

**Statistical analysis**

Analysis was performed using ‘Intercooled STATA’ (version 7.0, STATA Corporation, Texas) and two-sided tests were performed throughout. Data are represented as means ± SD unless otherwise stated. Genotype distributions in both studies were consistent with Hardy Weinberg equilibrium. To assess the differences between CHD and non-CHD patients in UDACS, two-sided T tests were performed on normally distributed data or after appropriate transformation (log or square root). Analysis of variance (ANOVA) was used to assess the association between genotypes and baseline characteristics. The relationships between baseline parameters and plasma TAOS were tested by Spearman rank correlation coefficient. An ANOVA was also performed to test the association between genotype and TAOS after adjustment for the potential confounders using multiple regression analysis to obtain a residual. In both studies, an overall comparison of the three genotype groups was made using ANOVA, followed by a comparison made after combining the GA and GG groups where appropriate recessive effects of the A allele were demonstrated. In NPHSII, survival analysis with respect to genotypes was carried out using Cox proportional hazards model, ‘failure’ being the first CHD event. Results are

---

**Table 2** Plasma TAOS and esterified F2-isoprostanes in relation to UCP2-866G>A genotype and CHD status in the UDACS substudy

<table>
<thead>
<tr>
<th></th>
<th>No CHD</th>
<th></th>
<th>CHD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA (n = 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA (n = 5)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.6 (4.4)</td>
<td>65.4 (5.3)</td>
<td>0.80</td>
</tr>
<tr>
<td>BMI (kg m⁻²)</td>
<td>32.7 (6.9)</td>
<td>29.6 (4.8)</td>
<td>0.43</td>
</tr>
<tr>
<td>Duration DM (years)</td>
<td>6 (0.5–20.5)</td>
<td>6 (4–12)</td>
<td>0.78</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.5 (0.7)</td>
<td>7.5 (1.3)</td>
<td>0.19</td>
</tr>
<tr>
<td>Glucose (mmol l⁻¹)</td>
<td>9.2 (5.0–15.7)</td>
<td>8.1 (7.2–10.1)</td>
<td>0.47</td>
</tr>
<tr>
<td>Cholesterol (mmol l⁻¹)</td>
<td>5.1 (0.8)</td>
<td>5.5 (1.0)</td>
<td>0.50</td>
</tr>
<tr>
<td>LDL (mmol l⁻¹)</td>
<td>2.8 (0.6)</td>
<td>3.4 (1.0)</td>
<td>0.28</td>
</tr>
<tr>
<td>HDL (mmol l⁻¹)</td>
<td>1.3 (0.4)</td>
<td>1.4 (0.3)</td>
<td>0.93</td>
</tr>
<tr>
<td>Tg (mmol l⁻¹)</td>
<td>2.1 (0.6)</td>
<td>2.3 (1.5)</td>
<td>0.81</td>
</tr>
<tr>
<td>TAOS (%)</td>
<td>42.06 (6.12)</td>
<td>54.68 (6.99)</td>
<td>0.02</td>
</tr>
<tr>
<td>F₂ isoprostanes (pg ml⁻¹)</td>
<td>119.1 (40.2)</td>
<td>105.9 (27.48)</td>
<td>0.58</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>141 (12)</td>
<td>145 (12)</td>
<td>0.63</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81.2 (8.4)</td>
<td>80.4 (8.1)</td>
<td>0.88</td>
</tr>
</tbody>
</table>

| % Proteinuria     | 30% (n = 3) | 40% (n = 4) | 0.64 | 60% (n = 3) | 60% (n = 3) | 1.00 |
| % Smokers         | 20% (n = 1) | 20% (n = 1) | 1.00 | 0% (n = 0)  | 20% (n = 1) | 0.29 |
| % on Aspirin      | 20% (n = 1) | 20% (n = 1) | 1.00 | 40% (n = 2) | 60% (n = 3) | 0.53 |
| % on Statin       | 20% (n = 1) | 20% (n = 1) | 0.49 | 60% (n = 3) | 80% (n = 4) | 0.49 |
| % on ACEI         | 40% (n = 2) | 20% (n = 1) | 0.49 | 60% (n = 3) | 80% (n = 4) | 0.49 |

Mean (SD) shown or Median (IQR); Compared with Student’s t test/Mann–Whitney/U² test.

Genotype groups were closely matched for baseline characteristics.

Comparing AA (No CHD vs. CHD).

*P < 0.05.

**P < 0.01.
presented as hazard ratios (HR) with their corresponding 95% confidence interval (CI) and CHD event rate per 1000 patient years calculated from survival analysis. To allow for differences in baseline data according to age and practice, age was included as a covariate in the model and data stratified by practice (using the strata option in STATA). The assumption of proportional hazards was checked by testing for a non-zero slope in a generalized linear regression of the scaled Schoenfeld residuals on time (using the stph test command in STATA). The relative excess risk due to interaction (RERI) was used as a measure of deviation from additive effects. A value of zero represents no deviation from additive effects, and 95% CI were calculated using bootstrapping. No adjustment was made for multiplicity of testing. Whilst making such an adjustment reduces the type I error, it leads to increases in the type II error, and fewer errors of interpretation occur when no adjustment is made. In all cases a P value of <0.05 was considered statistically significant.

Results

The UCP2-866G>A gene variant, plasma TAOS and CHD risk in diabetic men

We successfully genotyped 465 of the 485 (95.9%) Caucasian men with diabetes from the UDAC study (Table 1). The rare (A) allele frequency was 0.34 (0.31–0.37). CHD was present in 105 individuals (22.5%), whose mean age, characteristics (including treatment or duration of diabetes) by UCP2 genotype (data not shown). There was no genotype or allelic association with CHD (A allele frequency 0.34 in both those with and without CHD, P = 0.99).

To further corroborate these data, 20 men from UDACS were selected for homozygosity for the UCP2-866G>A variant and closely matched for baseline characteristics (Table 2). There were non-significant differences in plasma markers of OS between the presence/absence of CHD; with TAOS lower (CHD 42.2 ± 12.7% vs. no-CHD 48.4 ± 12.9%; P = 0.22) and F₂-isoprostanes higher (CHD 168.3 ± 74.2 pg ml⁻¹ vs. no-CHD 111.7 ± 79.1 pg ml⁻¹; P = 0.05). However, there were highly significant differences in plasma TAOS between AA and GG homozygotes overall (AA 36.9 ± 8.4% vs. GG 53.6 ± 10.5%; P < 0.0001), in those without CHD (AA 42.1 ± 6.1% vs. GG 54.7 ± 7.0; P = 0.016) and in those with CHD (AA 31.7 ± 7.3% vs. GG 52.6 ± 6.3%; P = 0.001). AA homozygotes with CHD had the lowest TAOS of all groups (Fig. 2(a)). In accordance with this, plasma F₂-isoprostane concentrations were highly significantly elevated in AA homozygotes both overall (AA 175.4 ± 64.5 pg ml⁻¹ vs. GG 104.6 ± 72.8 pg ml⁻¹; P = 0.011) and in those with CHD (AA 220.6 ± 37.2 pg ml⁻¹ vs. GG 109.9 ± 51.1 pg ml⁻¹; P = 0.005), but not significantly in AA homozygotes without CHD (AA 119.1 ± 40.2 pg ml⁻¹ vs. GG 105.9 ± 27.5 pg ml⁻¹; P = 0.58; Fig. 2(b)). There was evidence of significant interaction between genotype and CHD status in determining F₂-isoprostanes (P = 0.014) but not plasma TAOS (P = 0.19).

The UCP2-866G>A gene variant and CHD risk in NPHSII

Genotypes were obtained in 2695 (97.1%) of the 2775 subjects with available DNA (Table 3). Genotype distribution and rare (A) allele frequency of 0.37 (95% CI 0.35–0.38) were similar to that of healthy controls previously reported and to the UDAC sample (χ² = 2.54, P = 0.28).

Homozygosity for the A allele was more prevalent amongst obese subjects (64/375 obese vs. 297/2316 non-obese; P = 0.03), and was associated with a significant elevation in baseline diastolic blood pressure
GG, GA, AA, respectively, respectively, \( P < 0.0001 \) for AA vs. GA+GG; Table 3). The hazard ratios demonstrated a recessive effect of the A allele (HR 2.08 [1.49–2.86]; \( P < 0.0001 \) for AA vs. GA+GG). The doubling in risk remained highly statistically significant even after adjustment for accepted risk factors (excluding CRP) in the study group overall (HR 1.86 [1.33–2.59]; \( P < 0.0001 \) for AA vs. GG; Table 3) and in the 721 subjects in whom CRP was also measured (HR 2.05 [1.28–3.26]; \( P = 0.003 \) for AA vs. GA+GG; Table 3). Even in those AA homozygote men without accepted risk factors for CHD, risk was elevated, being 77% higher in non-obese subjects (Fig. 3(a)), 94% higher in non-hypertensives (Fig. 3(b)) and 98% higher in non-diabetics (Fig. 3(c)).

In general, CHD risk was substantially elevated amongst those with the UCP2-866AA genotype (HR 2.22 [1.53–3.22] for AA vs. GG genotype; \( P = 0.0002 \); Table 3). The hazard ratios demonstrated a recessive effect of the A allele (HR 2.08 [1.49–2.86]; \( P < 0.0001 \) for AA vs. GA+GG). The doubling in risk remained highly statistically significant even after adjustment for accepted risk factors (excluding CRP) in the study group overall (HR 1.86 [1.33–2.59]; \( P < 0.0001 \) for AA vs. GG; Table 3) and in the 721 subjects in whom CRP was also measured (HR 2.05 [1.28–3.26]; \( P = 0.003 \) for AA vs. GA+GG; Table 3).

Discussion

This is the first report to demonstrate that a common functional variant in the UCP2 gene is associated with both increased OS and with prospective CHD risk. Such data support a role for UCP2 (and hence the mitochondrial electron transport chain) in the regulation of ROS generation, and highlight its potential impact upon CHD risk. The oxidation of vulnerable cell membrane unsaturated lipids by ROS33 modulates diverse signal transduction pathways, leading to increased expression of cell adhesion molecules, induction of pro-inflammatory pathways, activation of matrix metalloproteinase, vascular smooth muscle cell proliferation and death, and endothelial dysfunction and lipid peroxidation — factors implicated in atherogenesis, to which the formation of
oxidized LDL (OxLDL) may contribute. Elevated OxLDL is independently associated with increased atherosclerotic burden and increased CHD risk.

In vitro, UCP2 is activated by ROS, whilst selective down-regulation of UCP2 increases endothelial cell ROS generation. Thus, under conditions of OS, increased UCP2 expression should prove vasculo-protective, and in support, UCP2 protects against atherosclerosis in LDL-receptor deficient mice.

Diabetes is associated with increased OS, and thus a fall in TAOS. In keeping with a causal role for ROS in atherogenesis, we found that plasma TAOS was significantly lower in diabetic men with CHD than those without. In diabetic men, the UCP2-866A allele was independently associated with lower TAOS, suggesting a modulating influence of UCP2 genotype on OS burden, although it was not clear whether this was a recessive or dominant effect. Patients with the UCP2-866AA genotype who also had CHD, demonstrated the lowest levels of plasma TAOS of all groups tested, with TAOS 30% lower than those non-CHD AA subjects and 33% lower than non-CHD G allele carriers. In a subset of diabetic men with CHD matched for baseline characteristics (including age and treatment), UCP2AA homozygosity was associated with 40% lower TAOS mirrored by a 100% increase in F2-isoprostane concentrations. UCP2 activity and expression is induced by oxidative stress (which is itself induced by CHD and its risk factors), thus protecting from further mitochondrial ROS generation. This may explain the dependence of the observed genotypic effect on the presence/absence of CHD. These data therefore suggest the UCP2-866A allele to be strongly associated with increased ROS burden. Given the putative role of ROS in atherogenesis, we predicted a similar genotype association with prospective CHD risk, which was confirmed. In prospectively studied middle-aged men, CHD risk was doubled amongst those homozygous for the UCP2-866AA allele, even amongst the normotensive, lean, non-smokers and non-diabetics. However, the risk associated with genotype was substantially increased by the presence of conventional CHD risk factors known to be associated with increased OS, such as hypertension,
been associated with insulin resistance21 and1 2 3 22 31 35 (see Table 4). Indeed, the risk of CHD was elevated nearly 8-fold amongst the small number of diabetic subjects of UCP2-866AA genotype, when compared to non-diabetic G allele carriers and almost 4-fold in obese AA compared to non-obese G allele carriers.

The association of the UCP2-866A allele with reduced TAOS, increased esterified F2-isoprostanes and with prospective CHD risk is mechanistically consistent, supporting the validity of our findings. The UCP2-866G>A gene variant is located within a multifunctional cis regulatory site, involving putative binding sites for pancreatic and hypoxia-induced transcription factors.20 The UCP2-866G>A polymorphism has been shown to be functional in vitro and in vivo. The UCP2-866A allele has been associated with insulin resistance21,42 and with type 2 diabetes,21 both being conditions associated with increased OS and CHD risk. Although promoter constructs of the -866A allele are associated with greater transcriptional activity in pancreatic β cells, they are associated with greater repression of transcription in somatic non-β cells.21 It is likely, therefore, that the UCP2-866A allele is related to lower inducible UCP2 expression within the vasculature or circulating immune cells. As such, one would anticipate the A allele to be associated with increased OS and higher risk of CHD as demonstrated in these studies. Furthermore, the -866G>A variant appears to be strongly associated with functionality across the gene cluster.20

At first sight, the lack of any significant difference in genotype distribution between those with and without CHD in the diabetic subjects would appear to conflict with the prospectively derived data. However, this is not the case. Firstly, prospective gene-association studies are more powerful than case-control studies.43 Secondly, increased obesity, increased OS, inflammation, and hyperglycaemia might all overwhelm the UCP genotype ‘strength of signal’ in diabetes. Thirdly, case-control cross-sectional studies are prone to intrinsic bias, for example due to possible altered rates of disease progression, subsequent progression of secondary phenotypes, or genotype associations with death or treatment changes. Indeed, the presence of the A allele might be associated with both earlier disease presentation and earlier death in some, subsequently balanced by more aggressive secondary prevention strategies. Such influences are well-recognised confounders.43 45

The number of diabetic men recruited to NPHSII is small, and confirmation of these findings should be sought in other diabetic groups. However, the substantially increased CHD risk amongst diabetic men of UCP2-866AA genotype in NPHSII is congruent with the finding of increased markers of OS found in the plasma of diabetic men from UDACS. The association of UCP2 genotype with altered markers of OS also requires examination in other ‘high ROS’ groups and these observations should also be extended to those of other races, and to women. In addition, further in vitro functional studies are required. However, the conclusions from these two independent studies are consistent and statistically robust, and, if confirmed, will have important implications. There is a global epidemic of diabetes in which CHD is the major cause of mortality,46 and diabetes is one of the major risk factors for CHD.47 However, no more than 25% of the excess CHD risk in diabetes can be accounted for by modulation of established risk factors,48 and a search for mechanistic understanding may thus have profound implications for the development of novel therapeutic options. These data suggest that modulation of UCP2 expression may be one such important mechanistic target.

### Acknowledgments

The British Heart Foundation supported SSD, SEH and HEM: FS/2001044, RG2000 015, SP98003; Diabetes UK – JWS (BDA: RD01/0001357); and the NPHSII study by the Medical Research Council, the US National Institutes of Health (Grant NHLBI 33014) and Du Pont Pharma.

### References

Cardiovascular risk in healthy men and markers of oxidative stress in diabetic men


