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Article

Association of the *calpain-10* gene with microvascular function

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Abstract

Aims/hypothesis. Genotype could influence vascular function. In some populations, *Calpain 10* gene polymorphisms increase susceptibility to diabetes or insulin resistance. Alterations in microvascular function could contribute to insulin resistance. This study investigated whether polymorphisms in the *Calpain-10* gene influence microvascular function.
Methods. Skin maximum microvascular hyperaemia to local heating on the dorsum of the foot (30 min at 43°C) was measured by Laser Doppler Fluximetry in 37 healthy volunteers. All were normoglycaemic according to World Health Organisation criteria, normotensive and not on any medication.

Four polymorphisms in the *calpain-10* gene were typed: SNP-44, SNP-43, SNP-19, SNP-63. The SNP common to all the described high risk haplotypes is the G-allele at SNP-43. This intron 3 polymorphism appears to influence gene expression. Microvascular function was examined in relation to polymorphisms at this site alone as well as the effects of the known extended high risk haplotypes using the SNP's above.
Results. Maximum microvascular hyperaemia was increased in the 21 subjects with *G/G* genotypes at SNP-43 compared to the combined group of subjects (*G/A* genotype at SNP-43 (n=12) + *A/A* genotype at SNP-43 (n=4)), and the minimum microvascular resistance was reduced 49.4 (39.6–94.2) vs 67.5 (39.1–107.3) mmHg/V, p=0.007). Haplotype analysis of the hyperaemic response revealed no significant differences between haplotypes. The two groups did not differ in terms of anthropometric measures, blood pressure, insulin resistance or glucose.
Conclusions/interpretation. The polymorphism that confers susceptibility to Type II (non-insulin-dependent) diabetes mellitus in some populations is associated in United Kingdom Caucasians with enhanced microvascular function in the presence of normoglycaemia. [Diabetologia (2002) 45:899–904]

Keywords Calpain 10, microvascular function, skin hyperaemia, genotype, normoglycaemia.

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Abbreviations: MODY3, Maturity onset Diabetes of the Young (hepatocyte nuclear factor-1α); ACE, angiotensin I converting enzyme; BP, Blood pressure; WHO, World Health Organisation; MMH, maximum microvascular hyperaemia; MVR, minimum microvascular resistance; HOMA, homeostasis model Assessment; I/D, insertion/deletion; G/G, homozygous for G allele at SNP-43; G/A, heterozygous for C/A alleles at SNP-43; A/A, homozygous for A allele at SNP-43; CAPN10, calpain-10 gene; SNP, sodium nitroprusside; D/D, deletion/deletion; eNOS, endothelial nitric oxide synthase

Normal microvascular function is vital for tissue health. The microvasculature is the site of nutrient and waste product exchange and it plays an important role in the maintenance of tissue fluid economy and immune responses.

In patients with both Type I (insulin-dependent) and Type II (non-insulin-dependent) diabetes mellitus, microvascular complications are a major cause of morbidity and are accompanied by marked microvascular dysfunction [1, 2, 3]. Even before clinical microvascular complications are evident, abnormalities of the responsiveness of the microvasculature have been described, for example the hyperaemic response is im-

paired in prepubertal children with Type I diabetes [4, 5]. Furthermore impaired microvascular function could even precede the development of diabetes [6, 7, 8] leading to the suggestion that there could be an intrinsic microvascular abnormality in those destined to develop diabetes [9].

Although many studies have examined relations between various genotypes and the risk of cardiovascular disease or macrovascular function, few have examined the effects of genotype on in vivo resistance vessel or microvascular function. In patients with the type 3 form of maturity-onset diabetes of the young (MODY3) which results from mutations in the beta-cell transcription factor hepatocyte nuclear factor-1α, we described an impaired skin microvascular hyperaemic response with characteristics similar to those previously seen in patients with Type I diabetes [10]. Several groups have investigated the vascular effects of insertion/deletion (I/D) polymorphisms of the angiotensin I converting enzyme (ACE) gene with conflicting results. In healthy individuals, forearm resistance vessel vasodilatory function might be unaffected or blunted in those with the D-allele [11, 12]. However, others report an impaired forearm vasodilatory response only in subjects with the D-allele in combination with the angiotensinogen T174M genotype [13]. Individuals with the ACE D/D genotype display an enhanced vasoconstrictor response to angiotension I [14] although the vasoconstrictor response to noradrenaline is unaltered [12]. In healthy individuals the beta 2 adrenoreceptor genotype is also associated with altered forearm vascular responses to catecholamines [15, 16]. A lack of biological effects of the endothelial nitric oxide synthase (eNOS) Glu298Asp polymorphism on forearm vasculature has been described [17].

Variation in the calpain-10 gene (*CAPN10*) has been associated with Type II diabetes in the Mexican American population of Starr County, Texas, and in some Northern Europeans including the Finnish population from the Botnia region, Germans and British [18, 19]. These studies suggest that risk is not conferred by a single DNA polymorphism but rather there are multiple risk alleles of *CAPN10*. The single nucleotide polymorphism (SNP)-43 (*CAPN10*-g.4852G/A) is associated with increased risk in Mexican Americans and Finns/Botnians with odds ratios (OR, 95%-CI) of 1.54 (0.88–2.14) and 1.84 (1.18–2.88), respectively. Inheritance of a haplotype combination defined by SNP-43, SNP-19 (*CAPN10*-g.7920indel32 bp) and SNP-63 (*CAPN10*-g.16378C/T) is associated with a threefold risk in Mexican Americans and in a combined Finnish and German group. In the UK, the association with SNP-43 is less clear; there was no association with Type II diabetes [19] but in a non-diabetic cohort *G/G* genotype at SNP-43 was associated with higher blood glucose concentrations. An adjacent SNP, SNP-44 (*CAPN10*-g.4841T/C) shows linkage and association with Type II diabetes in a group of UK parent-offspring trios (OR=1.59 (1.15–2.20) [19]. SNPs 43 and 44 have been shown to alter transcription of Calpain 10 in vitro [18]. In addition SNP-43 is associated with reduced skeletal muscle calpain-10 mRNA content and increased measures of insulin resistance in normal glucose tolerant Pima Indians although it is not associated with Type II diabetes in itself in this population [20].

The molecular mechanism(s) by which calpain-10 affects insulin action and/or insulin secretion and hence insulin resistance or risk of Type II diabetes are unknown. Calpain-10 mRNA is found in all fetal and adult tissues tested [18]. Calpains have been implicated in the regulation of adipocyte differentiation [21] and insulin-induced down regulation of insulin receptor substrate 1 [22] and could affect insulin action and diabetes risk through effects on these processes. An alternative explanation might be that Calpain-10 has an affect on microvascular function.

Microvascular function is abnormal not only in individuals with diabetes, but also in prediabetic hyperglycaemic subjects as well as in those with increased susceptibility to diabetes [7, 23, 24]. Indeed redistribution of blood flow from nutritive to non-nutritive capillaries contributes to insulin resistance in animals [25, 26] and skin capillary recruitment, endothelial responses and maximum hyperaemia correlate with insulin resistance in man suggesting a key role for the microcirculation in the determination of insulin resistance [27, 28, 23]. Calpains have been implicated in processes that could potentially affect vascular function including platelet activation, integrin-mediated cell adhesion, signalling and cytoskeletal associations, and the protein kinase C pathway [29, 30, 31, 32]. Calpain-10 could be involved in regulating these processes and thus provide a link between microvascular function, insulin resistance and diabetes risk. We tested this hypothesis by assessing the skin microvascular hyperaemic response in a group of normal subjects with different *CAPN10* genotypes.

Subjects and methods

Subjects. Thirty seven healthy Caucasian subjects were recruited by advertisement from the local population. Their age ranged from 21 to 55 years, mean 38.5 years, 27 were women. The phase of the menstrual cycle was not standardised. All were normotensive (blood pressure (BP) ≤138/95 mmHg) with BMI ranging from 16.6 to 38.2 Kg/m^{-2}. None were taking any medication. All subjects underwent a 2-h 75 g oral glucose tolerance test and were normoglycaemic according to WHO criteria. All subjects gave written informed consent to take part in this study which was approved by the Local Medical Research Ethics Committee.

Vascular measurements. Maximum microvascular hyperaemia was determined following a maximum heating stimulus as de-

Table 1. Clinical features of study groups based on SNP-43 genotype

	G/G (Group 1)	G/A + A/A (Group 2)	*p*
Age (years)	39.0 (25.1–54.7)	39.0 (21.0–49.0)	0.964
Smokers	5	2	
Sex	17F, 4 M	10F, 6 M	0.211
Weight (kg)	71.3 (48.2–109.3)	71.9 (46.2–98.2)	0.617
BMI (kg/m^2)	24.3 (18.5–38.0)	25.5 (16.6–37.8)	0.617
Waist to hip ratio	0.79 (0.66–1.27)	0.82 (0.70–1.08)	0.962
Systolic BP (mmHg)	112.0 (97.1–136.6)	119.5 (96.0–137.0)	0.165
Diastolic BP (mmHg)	73.0 (57.2–89.9)	74.0 (53.0–95.0)	0.774
Fasting plasma glucose (mmol/l)	4.4 (3.3–5.0)	4.6 (3.7–4.9)	0.055
2 h plasma glucose (mmol/l)	4.6 (3.8–7.2)	5.1 (3.6–7.4)	0.220
HOMA (% normal)	86.1 (23.8–943.4)	74.4 (50.2–495.7)	0.400
Insulinogenic index ($\times 10^9$)	139 (57.9–432.2)	124.3 (1.49–276.3)	0.622
Plasma Cholesterol (mmol/l)	4.95 (2.66–6.97)	5.05 (2.90–6.10)	0.671

scribed previously in detail [23]. Briefly a thermostatically controlled brass heating element set at 43°C is used to heat the skin on the dorsum of the foot for a minimum of 30 min which achieves maximum microvascular dilatation. The red cell flux, a measure of microvascular blood flow, through the microvessels underlying the heating element, is then measured using a laser Doppler flowmeter (model PF3B, Perimed, Upsala, Sweden). The probe is placed eccentrically through the heating element which can be rotated in its holder so that eight spaced measurements of flux values can be made and a mean value calculated. Employed in this way highly reproducible estimations of maximum microvascular hyperaemia (MMH) could be derived (coefficient of variation 5.6% for subjects measured on multiple occasions over a 6-month period).

Minimum microvascular resistance (MVR) was calculated to correct for differences in blood pressure by dividing mean blood pressure by the value for maximum microvascular hyperaemia. Brachial artery blood pressure was measured as the mean of three resting values using an automated device (Dinamap, Critikon, Tampa, Fla., USA).

Metabolic assessments. An index of insulin sensitivity was calculated by the HOMA model using two fasting insulin and glucose measurements [33, 34]. Subjects underwent a 75 g oral glucose tolerance test and the insulinogenic index, an index of beta-cell function, was calculated as the increment in insulin at 30 min (pmol/l) in relation to the increment in glucose at 30 min (pmol/l) [35].

Insulin was measured using an immunoenzymometric assay (Medgenix EASIA, Biosource, Nivelles, Belgium) calibrated against IRP 66/304 with no detectable cross-reactivity with intact proinsulin or 32,33 split proinsulin. Interassay coefficients of variation (CV) were less than 10% over the range 95–1038 pmol/l.

Genotyping. Four polymorphisms in the calpain-10 gene were typed as described by Evans and colleagues [19]. SNP-44 (*CAPN10*-g.4841T/C), SNP-43 (g.4852G/A), SNP-19 (g.7920indel32 bp) and SNP-63 (g.16378C/T). Alleles are numbered as previously described [18, 19].

Statistics. Data are presented as median (5 and 95 centiles). Comparisons between high and low risk genotypes (e.g. G/G v G/A + A/A) were made by Mann-Whitney U test. Comparisons between the three genotypes were made using Kruskal Wallis analysis of Variance.

Results

Homozygosity for SNP-43 allele 1 is associated with all haplotype combinations showing increased risk of diabetes in Mexican Americans as well as insulin resistance in the Pima Indians suggesting that this polymorphism has an important role in determining susceptibility to Type II diabetes. The subjects of this study were therefore first analysed for polymorphisms at this site. Twenty one subjects were homozygous for the G-allele (allele 1, genotype G/G) at SNP43, 12 where heterozygous (G/A) and four were homozygous for the A-allele (allele 2, genotype A/A). As the susceptibility is associated with homozygosity and CAPN10 mRNA is reduced in Pima Indians homozygous for the SNP-43G allele compared to individuals not homozygous for SNP-43 G-allele, we compared microvascular function in those with the G/G-genotype (Group 1) and a combined group of those with the G/A + A/A genotypes (Group 2). In addition the three genotypes were investigated separately.

No differences were observed between the G/G and G/A + A/A genotype groups with regard to age, sex, weight, BMI waist-to-hip ratio, systolic or diastolic blood pressure, fasting plasma glucose or plasma glucose concentration 2 h after the 75 g oral glucose load, HOMA or insulinogenic index (Table 1).

Subjects with SNP-43 G/G-genotypes exhibited an increased MMH compared to those with G/A + A/A genotypes (1.6 (0.95–2.28) v 1.4 (0.78–1.72) volts, p=0.011), Fig. 1; MVR was correspondingly lower 49.4 (39.6–94.2) v 67.5 (39.1–107.3) mmHg/V, p=0.007) Fig. 2. When the three genotypes were analysed separately there was still a significant difference in both MMH (p=0.017) and MVR (p=0.010).

Additional analysis of extended haplotypes (SNPs 43/44/16/63 including the at risk Caucasian haplotype described by Evans [19]) did not show significant results. The haplotype identified by Horikawa [18] as the highest risk combination for relative risk of diabe-

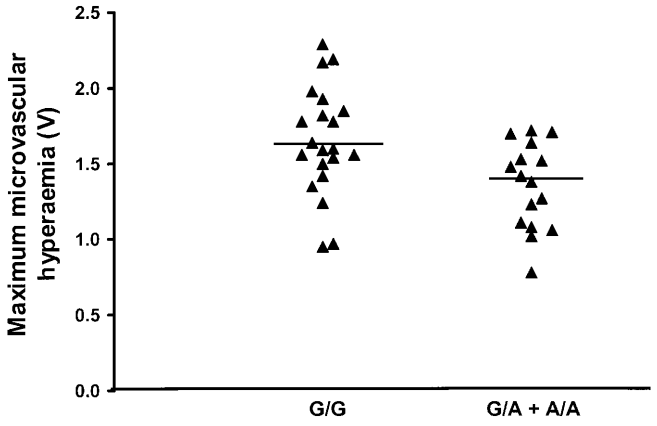

Fig. 1. Maximum microvascular hyperaemia (MVH) based on SNP-43 genotype: G/G v G/A + A/A

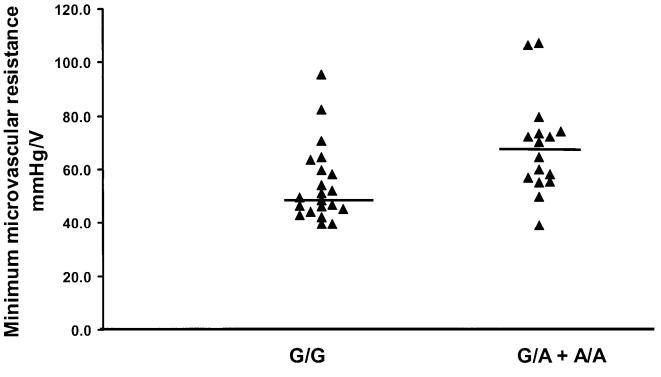

Fig. 2. Minimum microvascular resistance (mean systemic blood pressure/ maximum microvascular hyperaemia) (MMR) based on SNP-43 genotype: G/G v G/A+A/A

tes in the Mexican American 112/121 (SNPs 43/19/63) occurred in only one of the 30 subjects with full haplotype data thus the effect of this haplotype on microvascular function is still not unknown.

Discussion

This study is the first demonstration that variation in a gene that predisposes certain populations to the development of Type II diabetes or insulin resistance is associated with altered microvascular function in healthy normoglycaemic Caucasian subjects. Interestingly homozygosity for SNP-43 allele 1 in this group appeared to confer an increased microvascular vasodilatory response rather than the depressed response commonly observed in those at risk of Type II diabetes. The explanation for the enhanced vasodilatation in this group is not clear but suggests the presence of an early hyperaemia which progresses to an impaired response as subtle abnormalities of the insulin resistant syndrome develop. The enhanced hyperaemia itself could contribute to the subsequent impairment in function [36, 37] through a delterious effect on the endothelium. The magnitude of the difference in the hyperaemic response between the two groups is modest being about half that seen when comparing controls and either fasting hyperglycaemic individuals or normoglycaemic individuals who have previously had gestational diabetes [38, 39].

The G-allele (allele 1) at SNP-43 is the only allele common to all the high-risk haplotypes seen in Mexican Americans suggesting that it either determines susceptibility to Type II diabetes or is the best marker of the true susceptibility allese(s). The SNP43 G allele or an allele in strong linkage disequilibrium (LD) with it appears to play a role in regulation of calpain expression. For example in vivo studies in Pima Indians demonstrate that G/G homozygotes have lower skeletal muscle CAPN10 mRNA and increased insulin resistance [20]. However studies in UK subjects did not find any association between Type II diabetes and SNPs 43, 19 and 63, either individually or as part of the previously described risk haplotypes or genotypes. Similarly alterations in vascular function were not associated with these extended haplotypes. In UK Caucasian non-diabetic subjects enriched with first-degree relatives of patients with Type II diabetes, investigators reported hyperglycaemia and beta-cell dysfunction associated with SNP-43 genotype [40]. In the current smaller study of individuals homozygous for the G allele in SNP-43, there was no evidence of increases in plasma glucose, indeed there was a trend for lower fasting glucose. This study did not, however, set out to assess effects of genotype on glucose metabolism and differences are likely to be explained by the small number of subjects studied.

This study was not designed to examine the mechanism responsible for the enhanced microvascular vasodilatory response observed in those homozygous for SNP-43 1 allele. MMH is a complex response involving neurogenic, endothelial dependent as well as structural determinants. Given the range of actions of the cysteine proteases it is quite conceivable that one or more of such determinants might be influenced by altered expression of Calpain 10.

In conclusion this study provides proof of principle that allelic variation in the intron region of the *Calpain 10* gene could have consequences for microvascular function. The G allele of SNP43 that confers susceptibility to Type II diabetes in some populations is associated in UK Caucasians with enhanced microvascular hyperaemia in the presence of normoglycaemia.

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