Discussie

In obesitas en/of type-2-diabetes is een structurele onbalans tussen LCFA-opname, -opslag en/of -oxidatie hoogst waarschijnlijk verantwoordelijk voor het ontstaan van insulineresistentie in de skeletspier (1). LCFA-transporters hebben een belangrijke rol in het moduleren van de balans tussen plasma-LCFA-beschikbaarheid, -opname en/of -oxidatie.

Farmaceutische interventie van de LCFA-transporterhoeveelheid zou in eerste instantie een effectieve manier zijn om plasma-LCFA-opname in de spier te verminderen, echter onze data laten zien dat totale LCFA-transporterhoeveelheid niet betrokken is bij metabole stoornissen. Om de LCFA-transporters functioneel te laten zijn dienen ze gelokaliseerd te zijn op de plasmamembraan. Interessant genoeg blijken alle transporters ook intracellulair voor te komen in endosomale pools (7, 8). Alle drie transporters blijken te transloceren vanuit deze endosomale pools naar de plasmamembraan voor opregulatie van de LCFA-opname op een vergelijkbare manier als de translocatie van de glucosetransporter GLUT4. Als conclusie laat deze studie zien dat er geen verschillen zijn in mRNA- en totale-eiwithoeveelheid van de 3 LCFA-transporters FAT/CD36, FABPpm en FATP1 in skeletspier van type-2-diabetespatiënten ten opzichte van sedentaire controles. Tevens zien we, in contrast met FAT/CD36 en FATP1, een significante opregulatie van FABPpm-mRNA en totale-eiwithoeveelheid in skeletspier van getrainde mannen vergeleken met de sedentaire controles. Verder onderzoek naar de stoornissen in de bij de LCFA-transportertranslocatie betrokken signaleringscascades is daarom noodzakelijk.

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Detection of the CYP2D6*6 allele by LightCycler real-time PCR

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The enzyme debrisoquine 4-hydroxylase (CYP2D6) is involved in the oxidative metabolism and elimination of many commonly used drugs (1,2,3). The CYP2D6 gene (GenBank, M33388) is highly polymorph and the addition alleles result in deficient, reduced, normal or increased enzyme activity. Alleles with complete deficiency, such as CYP2D6*6, result in a poor metabolizer phenotype. CYP2D6*6 has an allele frequency of 1% (2).

The aim of this study was to develop a real-time PCR followed by melting curve analysis, using hybridization probes with a highly sensitive, rapid and efficient approach to mutation detection. The LightCycler instrument (LC) was used for the detection of the CYP2D6*6 allele. To evaluate the reliability of genotyping with the LightCycler the samples were also analyzed with a tetra-primer PCR.

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Material and methods

DNA was extracted from 400 µl EDTA blood and eluted in 200 µl elution buffer according to the manufacturer's protocol with a MagNaPure Compact (Roche Diagnostics). Anonymous DNA samples with a known CYP2D6*6 genotype were received from external laboratories. For the detection of the CYP2D6*6 polymorphism, PCR primers amplified a 563 bp fragment of the CYP2D6 gene. During PCR the amplicon was detected using two specific hybridization probes, one labeled with fluorescein and one with LightCycler Red 640 (LCRed640). The absence of a CYP2D6*6 allele introduces a destabilizing mismatch, which results in a decreased melting temperature. Primers and probes

Table 1. Oligonucleotides (5'-3') used for the detection of the CYP2D6*6 allele

	Position	length	GC (%)
TTggAgTgggTggTggATg	3156-3174	19	58
TATgCAAATCCTgCTCTTCCgA	3718-3697	22	45
probes			
TCggTCACCC_CTgCTCCAgC-FL	3336-3316	20	70
LC Red640-CTTCTTgCCCAggCCCAAgTTgC-ph	3313-3291	23	61

were designed and custom-made by TIB MOLBIOL, Berlin, Germany. The sequences are given in Table 1.
For mutation detection with the LightCycler, a 20 µl reaction was performed. The reaction mixture contained: 1x LightCycler DNA Master Hybridization Mix (Roche), 2.0 mM $MgCl_2$, 5 pmol of each primer, 4 pmol Sensor probe, 8 pmol Anchor probe, and 200 ng genomic DNA. PCR conditions were: 120 seconds at 95 °C for DNA denaturation, 40 cycles (0 seconds at 95 °C (denaturation), 10 seconds at 55 °C (annealing), 40 seconds at 72 °C (extension)). Next a melting curve analysis was performed by heating at 95 °C for 60 seconds, followed by cooling at 40 °C for 120 seconds and gradual heating (0.1 °C/s) up to 80 °C at. After the melting curve analysis a final cooling was performed at 40 °C for 30 seconds. To analyze the melting curves the corresponding melting peaks were calculated by plotting the first negative derivative of the fluorescence with respect to the temperature (-dF/dT vs. T).
DNA was amplified using the tetra-primer PCR in a T3 thermocycler (Biometra) and using primers as described by Hersberger et al. (3). PCR reaction mixture (25 µl) contained: 1x PCR buffer II (Perkin Elmer), 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol primer 1 new, 5 pmol primer 2 new, 3.75 pmol primer T mut, 7.5 pmol primer 11, 1U Taq polymerase (Perkin Elmer) and 250 ng genomic DNA. The PCR conditions were: 5 minutes at 95 °C, followed by 15 cycles of 94 °C for 30 seconds, 65 °C for 30 seconds, 72 °C for 60 seconds, and 32 cycles of of 94 °C for 30 seconds, 53 °C for 30 seconds, 72 °C for 60 seconds. The fragments were separated on a polyacrylamide gel (PAGE) and visualized with silver staining.

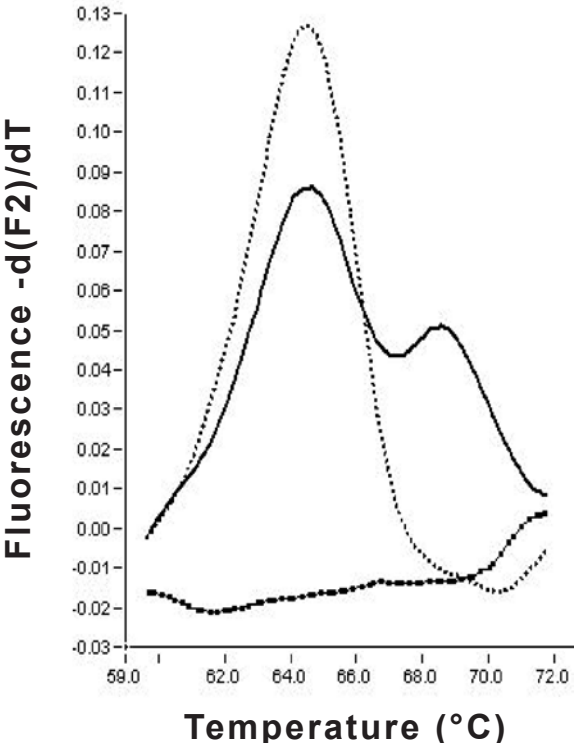

Figure 1. Melting peaks for CYP2D6*6 allele.
····· Homozygous wildtype T_m at 64.5°C, ——— heterozygous T_m at 64.5°C and 69.1°C, ····· H_2O.

Results

A new assay on the LightCycler was developed to analyze the CYP2D6*6 allel. The PCR products were tested on an agarose gel and the amplicons showed the expected size (563 bp). During the melting curve analysis the probes were dissociated from the target DNA. In case of a CYP2D6*6-allele (mutant genotype) the fluorescein-labeled probe has a fully homologous sequence with the target DNA and was dissociated from the target DNA at a melting temperature of 69.1 °C. In case of a wildtype-allele one mismatch occurs and the melting temperature was decreased to 64,5 °C (Fig. 1). In heterozygous genotypes the difference in melting temperature (Δ between melting peaks) was 4,53 °C.
In the tetra-primer PCR two gene-specific primers amplify the region of interest in the first set of cycles, 1106 bp. In the second set of cycles the allele specific primers amplify an amplicon of specific length if the allele is present. In case of a wildtype allele the allele specific primers amplify a 421 bp amplicon and in case of a CYP2D6*6 allele the allele specific primers amplify a 356 bp amplicon.
To evaluate the reliability of genotyping with the LightCycler, 8 human DNA samples (including two samples with a known genotype) were analyzed for CYP2D6*6 allele with the LightCycler and by tetra-primer PCR. Genotyping 8 DNA human samples resulted in 3 heterozygous CYP2D6*6 alleles and 5 wildtype alleles. In all samples tested a clear genotype was obtained with both methods and no discrepancies between the two methods were found.

Discussion and conclusion

With the described LC assay it is possible to detect the CYP2D6*6-allele. Comparison between LightCycler genotyping and tetra-primer PCR showed concordant results in all samples.
Performing a LightCycler assay in a closed system eliminates post-amplification processing. This considerable reduction in the number of manipulations reduces the risk of contamination. The use of the LightCycler clearly reduces analysis time (60 minutes vs. 1.5 days).

In conclusion real-time PCR followed by melting curve analysis is a rapid, simple, accurate method for genotyping the CYP2D6*6 allele.

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Monitoring of calcineurin activity under controlled systemic cyclosporine exposure after renal transplantation

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The calcineurin inhibitors (CNI) cyclosporine (CsA) and tacrolimus (TRL) are potent immunosuppressive drugs that are extensively used in organ transplantation. Unfortunately, severe adverse drug effects such as nephrotoxicity, diabetes mellitus, malignancies and cardiovascular toxicity are observed in CNI treated patients. Since large inter-individual variation in CNI pharmacokinetics is observed, therapeutic drug monitoring is required to control the therapeutic index. In practice, blood concentration of TRL and CsA is monitored, but since pharmacokinetic monitoring is a surrogate of effect, measurement of effect (pharmacodynamics) provides, at least in theory, a more accurate marker (1). We have therefore developed a calcineurin assay (2) and in this study we monitored calcineurin activity in renal transplant patients that were treated with CsA.

Methods

Forty-seven renal transplantation patients were monitored for leukocyte CN activity and CsA blood concentration. All patients received quadruple immune suppression including CsA, prednisolone, mycophenolate sodium and basiliximab prophylaxis. Samples were taken before transplantation and 2 weeks, 6 weeks, 6 months after graft implantation. After transplantation samples were taken before drug intake (12 hours after previous dose) and one patient was monitored for several hours after drug intake. CN leukocyte activity was measured as previously described (2) and CsA blood concentrations were measured using a fluorescence polarization immunoassay on an Abbott AxSYM system. CsA exposure was $AUC_{0\text{-}12}$ controlled and aimed at 5400 h*µg/L the first 6 weeks after transplantation and 3250 h*µg/L thereafter (3). Sudent's t-tests were performed to test significance and statistical significance was defined as $p < 0.05$.

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Results

An overview of the measured CN activities and CsA blood concentrations is found in table 1. When pre-transplantation (without CsA) leukocyte CN activities were compared to T0 CN activities, lower CN activities were found on week 2 (p=0.0003) and month 6 (p=0.02), but not on week 6 (p=0.2). A large spread in CN activities was observed between patients: coefficients of variations were 38%, 32%, 56% and 31% for Pre-Tx, week 2 (T0), week 6 (T0) and month 6 (T0) respectively. When a single patient is monitored in time for CN activity an inverse relation between CsA concentration and CN activity is found for the renal

Table 1. CN activity and CsA concentration in renal transplantation patients. Mean ± SD values of CN activities and CsA concentrations observed in renal transplantation patients just before drug intake (12 hours after previous dose). * CN activity is expressed as pmol · min^{-1} per 1 million leukocytes.

	CN activity*	[CsA] in µg/L
Pre-Tx	220 ± 84	
Week 2	153 ± 49	292 ± 108
Week 6	188 ± 106	228 ± 67
Month 6	149 ± 47	120 ± 38