olism in females is mostly independent of circulating sex hormones, in contrast to men. This might contribute to the observed age-dependent decrease in muscle carnitine in males. The down-regulation of carnitine transporters attributable to reduced serum testosterone may be crucial in males. The down-regulation of carnitine transporters attributable to reduced serum testosterone may be crucial in males. This might contribute to the physiologic process of sarcopenia, especially in men.

In summary, this study provides evidence that there is a sex-dependent decrease in carnitine concentrations in skeletal muscle, affecting only men. Men >60 years showed significantly lower free and total carnitine in skeletal muscle than did younger controls. In sera from females, there was an age-dependent increase of carnitine concentrations, but mean values were higher in men. Therefore, age- and sex-dependent reference values have to be considered. It is not clear whether the observed effects are only an indicator of age-dependent changes, or whether, in addition, they are a cause of functional impairments accompanying aging.

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References

Rapid Detection of the C–1496G Polymorphism in the CYP2D6 *2 Allele, Jeremy D. Claassen,1 Nina Pascoe,1,2 Alan F. Schatzberg,3 and Greer M. Murphy, Jr.1,2* (1 Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA 94305; 2 Department of Veterans Affairs Sierra-Pacific Mental Illness Research, Education, and Clinical Center, Palo Alto, CA 94304; * address correspondence to this author at: Neuroscience Research Laboratories, Department of Psychiatry and Behavioral Sciences, MSLS P-104, Stanford University School of Medicine, Stanford, CA 94304-5585; fax 650-725-5714; e-mail gmurphy@stanford.edu)

The CYP2D6 gene, encoding debrisoquine hydroxylase, is involved in the metabolism of a large number of medications (1). Recently, Raimundo et al. (2) described a C–1496G polymorphism in the promoter region of the CYP2D6 *2 allele that has a strong effect on debrisoquine metabolic phenotype when present in combination with a null allele. The *2 allele is the most common allele encoding intermediate debrisoquine hydroxylase activity in Caucasians (3), but to date, substantial variability among *2 carriers in metabolic activity has been reported. To accurately predict debrisoquine phenotype from...
CYP2D6 genotype in *2 carriers, determining C–1496G genotype will be necessary. We sought to develop a rapid, high-throughput genotyping assay for the CYP2D6 C–1496G promoter polymorphism described by Raimundo et al. (2). Because no available restriction enzyme differentially digests the polymorphic site (4), we introduced a BsrI restriction site (actggggt) into the amplicon using a forward primer with a 3’ single-nucleotide mismatch (underlined and in bold). The primers 2D6–1496F [gctgagacaattggaagaac] and 2D6–1496R3 (gtgccacagctcagttt) (5) amplify a 203-bp amplicon.

The following were mixed with 1 μL of genomic DNA at 0.23–1.1 μg/μL (extracted from whole blood using a Gentra reagent set) in a total volume of 25 μL: 2.5 μL of 10× PCR buffer (Applied Biosystems); 0.5 μL of 10 mM dNTP (Amersham Pharmacia Biotech); 0.1 μL of each primer at 100 pmol/μL; and 0.25 μL of Taq polymerase at 5 U/μL (Applied Biosystems). After an initial 5-min denaturing step at 94°C, the reaction mixture went through 40 cycles of PCR (94°C for 30 s, 64°C for 30 s, and 72°C for 30 s), followed by a final extension at 72°C for 10 min; 5 μL of this PCR product was digested with 0.5 μL of 5 U/μL BsrI (New England Biolabs), 1.5 μL of 10× NEBuffer 3, and 8 μL of water for at least 2 h at 65°C. The digested samples were then assayed for 1 h on a 2% agarose gel in a field of 4 V/cm (Fig. 1).

Allele C undigested, yielding the 203-bp amplicon, whereas allele G was cut, yielding 177- and 26-bp fragments. We selected the PCR products from four C/C, three G/G, and four C/G samples for DNA sequencing (Biotech Core) by primer extension with the forward primer. For all samples, direct sequencing confirmed the BsrI genotype.

Raimundo et al. (2) observed an exclusive association of the G allele with the CYP2D6 *2 allele. As an early confirmation of our genotyping method, we looked at this association, and indeed, found that all samples that were found to be G/G homozygous according to our assay were either homozygous for *2 (n = 10) or had one *2 allele and one deletion allele (CYP2D6 *5; n = 6). Furthermore, 10 of 10 samples lacking the *2 allele were also found to lack the G variant.

In a previous sampling of 221 Caucasians, we detected 109 heterozygotes for the *2 allele and 22 homozygotes, using the Affymetrix GeneChip CYP450 system (Affymetrix; unpublished observations). All procedures were approved by the Stanford Medical Human Subjects Committee, and all participants provided informed consent. The *2 allele frequency in this sample thus was 0.346, close to the 0.324 reported by Sachse et al. (3). According to the method described here, 111 of the 153 chromosomes harboring *2 alleles showed the G variant of the C–1496C polymorphism. The overall frequency of the G allele was thus 0.25 in this sample. Raimundo et al. (2) estimated a G allele frequency of 0.19 in 39 people studied (ethnicity not specified).

We were concerned that we might be amplifying the rare pseudogene CYP2D7BP because sequence comparisons indicated that our primers had essentially 100% identity with CYP2D7BP (6, 7) (GenBank Accession No. X58468). We undertook two courses of action to try to resolve this issue. First, we took advantage of the fact that published sequences of CYP2D6 (GenBank Accession No. M33388) and CYP2D7 differ at nucleotide –1338, which lies 6 nucleotides upstream from our reverse primer. CYP2D6 has a C at this position, whereas CYP2D7 has a T. The PCR product from six samples was selected to be sequenced (Biotech Core) by primer extension with the forward primer. In each instance, a “C” was determined to lie at position –1338.

We also knew that the reverse primer used by the Affymetrix GeneChip CYP450 system for amplifying exons 1 and 2 of CYP2D6 (which we called 2D6ex1–2 R) has no significant homology with CYP2D7BP (6). When used with the primer upf14 (2), 2D6ex1–2 R should amplify CYP2D6 exclusively. Thus, if we used the amplicon generated by upf14 and 2D6ex1–2 R as the starting material for our 2D6–1496F/R3 PCR, then any amplicon would have to be derived from CYP2D6. Eighteen samples were tested with both the 2D6–1496F/R3 method and the nested PCR method, and the two methods yielded identical genotypes.

The nested PCR was executed as follows: 7.5 μL of 3.3× L Buffer II PCR buffer (Applied Biosystems), 1.0 μL of 25 mM Mg(OAc)2 (Applied Biosystems), 0.5 μL of 10 mM dNTP (Amersham), 0.25 μL of primer upf14 (gccggga- caacctggaagaac) at 100 pmol/μL, 0.25 μL of primer 2D6ex1–2 R (ggctccacagtagctgctctg) at 100 pmol/μL; and 0.5 μL of rTth polymerase at 2 U/μL (Applied Biosystems) were mixed with 1 μL of genomic DNA at 0.23–1.1 μg/μL (extracted from whole blood using a Gentra reagent set) in a total volume of 25 μL. After an initial 1-min denaturation at 93°C, the reaction mixture went through 35 cycles of PCR (93°C for 1 min, 65°C for 30 s, and 72°C for 2.5 min), followed by a final extension at 72°C for 10 min. After the amplicon was confirmed to be 2701 bp by electrophoresis on a 1% agarose gel, 2 μL of the remaining amplicon was diluted to 1:11 in 1× PCR buffer (Applied Biosystems), and 1 μL of this diluted

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**Fig. 1.** Amplification products digested with BsrI.

Photograph of a 2% agarose gel stained with ethidium bromide and viewed via ultraviolet light. Lane M, molecular markers (200, 300, 400, and 500 bp); lane 1, heterozygous (203, 177, and 26 bp; 26-bp fragment not visible); lane 2, homozygous C (203 bp); lane 3, homozygous G (177 and 26 bp; 26-bp fragment not visible).
product was used as the starting material for the 2D6–1496F/R3 PCR described above.

In summary, our method provides a simple means for detecting the C–1496G polymorphism in the CYP2D6 *2 allele, which should be useful in genotype–phenotype correlation studies.

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References

New Enzymatic Colorimetric Assay for Total Homocysteine, Naoto Matsuyama, Masahiro Yamaguchi, Mitsuyoshi Toyosato, Masaharu Takayama, and Koji Mizuno (R & D Division, Azwell Inc., 2-24-3 Shō, Ibaraki, Osaka 567-0806, Japan; * author for correspondence: fax 81-726-22-4909, e-mail nskrd-06@mbd.sphere.ne.jp)

In 1969, McCully (1) observed that increased plasma homocysteine (Hcy) was linked with vascular disease. Subsequent studies demonstrated that even mild hyperhomocysteinemia is an independent risk factor for cardiovascular, cerebrovascular, and peripheral vascular disease (2–8). Increased plasma Hcy is associated with a labilizable variant of 5,10-methylenetetrahydrofolate reductase, smoking, lack of exercise, and excessive use of alcohol and coffee.

Plasma or serum total Hcy (tHcy) concentrations are most commonly measured by HPLC (9), which is time-consuming and expensive, and by immunochemical (10–12) or enzymatic (13) methods, which may not be applicable to all colorimetric-based clinical chemistry analyzers.

The thiol group of Hcy allows it to form a disulfide bond with other thiol-containing molecules, such as Hcy itself, cysteine, and the cysteine residue of plasma proteins. Biologic fluids may often contain both reduced and oxidized species of Hcy, and the sum of all the forms of Hcy is usually called total Hcy (tHcy) (14). Most clinical studies concerning Hcy have relied on the measurement of tHcy. An initial chemical reduction step of the sample is inevitable in the tHcy assay. Because reducing agents can interfere with the oxidation of redox indicators, such as Trinder’s reagents and derivatives of methylene blue generally used in diagnostic reagents, methods for tHcy that use these indicators have not been developed.

The present method is a new enzymatic colorimetric assay for tHcy in biologic samples. The principle is as follows. In the first step, samples are reduced by dithiothreitol to generate free reduced Hcy. Simultaneously, Hcy methyltransferase (EC 2.1.1.10) transfers the methyl group of d-methionine methylsulfonylum to Hcy, leading to the generation of t-methionine and d-methionine. In the second step, d-amino acid oxidase (EC 1.4.3.3) oxidizes d-methionine with the simultaneous production of hydrogen peroxide, followed by oxidation of 10-(carboxymethylaminocarbonyl)-3,7-bis(dimethylamino)phenothiazine (DA-67) to yield methylene blue with an absorbance maximum at 660 nm. This assay system contains N-ethylmaleimide in the second step to capture the thiol group of the remaining dithiothreitol, enabling the oxidation of the redox indicator and generation of the colored product.

1-L-Homocysteine and d-amino acid oxidase were from Sigma. d-Methionine methylsulfonylum bromide was obtained from Acros. DA-67 was obtained from Wako Pure Chemical Industries. Dithiothreitol and N-ethylmaleimide were obtained from Nacalai Tesque. Hcy methyltransferase was prepared from baker’s yeast (15). One unit of Hcy methyltransferase activity was defined as the amount of enzyme that catalyzed the synthesis of 1 µmol of d-methionine per minute. All other materials used were of analytical reagent grade.

The tHcy assay was performed as follows. One hundred microliters of saline (for reagent blank), calibrator, or sample was added to 50 µL of reagent A, containing 9.6 mmol/L Hcy methyltransferase, 15 mmol/L diithiothreitol, 1.5 mmol/L d-methionine methylsulfonylum bromide, 0.5 mmol/L ZnBr2, 35 mmol/L sodium phosphate (pH 7.0), and 0.1 g/L Triton X-100. To serve as the background, 100 µL of the same sample was also added to the reagent that did not contain Hcy methyltransferase. The mixtures were incubated for 90 min at 37 °C, and the reactions were terminated by the addition of 150 µL of reagent B, containing 18 mmol/L N-ethylmaleimide and 0.1 g/L Triton X-100. The generated d-methionine was measured with the Hitachi Model 7170 analyzer with a two-point assay. The reaction mixture (20 µL) was added to 160 µL of reagent 1, containing 0.24 mmol/L DA-67, 0.025 mmol/L diithiothreitol, 0.003 mmol/L potassium ferrocyanide, 97 mmol/L sodium phosphate (pH 7.0), and 0.1 g/L Triton X-100. The mixture was incubated for 5 min at 37 °C, followed by the addition of 100 µL of reagent 2, containing 1.4 kU/L d-amino acid oxidase, 4.4 kU/L peroxidase, 1 mmol/L FAD, 93 mmol/L sodium phosphate (pH 7.0), and 0.1 g/L Triton X-100. The mixture was again incubated for 5 min at 37 °C. Absorbance [660 nm...