Pergamon

Biochemical Pharmacology, Vol. 50, No. 5, pp. 697–703, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00182-4

ENZYME KINETIC PROPERTIES OF HUMAN RECOMBINANT ARYLAMINE *N*-ACETYLTRANSFERASE 2 ALLOTYPIC VARIANTS EXPRESSED IN *ESCHERICHIA COLI*

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(*Received* 24 *October* 1994; *accepted* 6 *March* 1995)

Abstract—Arylamine *N*-acetyltransferase (NAT2) catalyses the *N*-acetylation of primary arylamine and hydrazine drugs and chemicals. *N*-Acetylation is subject to polymorphism, and humans can be categorized as either fast or slow acetylators according to their ability to *N*-acetylate certain arylamine substrates *in vivo*. Genetic variants at the polymorphic *NAT2* locus have been described. We expressed five of the most common NAT2 variants (NAT2 4, NAT2 5A, NAT2 5B, NAT2 6A and NAT2 7B) in *Escherichia coli* as a convenient source of the human variants. The apparent K_m values (at 100 μM acetyl CoA as co-substrate) of the different NAT2 variants for sulphamethazine, dapsone, *p*-anisidine, 2-aminofluorene, procainamide and isoniazid were determined. Data show that the apparent K_m of the slow variant NAT2 7B for the arylamine sulphamethazine was 10-fold lower than all the other allotypes. The apparent K_m for the structurally related sulphone antibiotic dapsone was 5-fold lower for the slow variant NAT2 7B when compared with the wild-type NAT2 4. These results indicate that the NAT2 7B specific amino acid substitution, Gly286-Glu, is important in promoting the binding of sulphamethazine and dapsone to the active site.

Key words: human; arylamine; acetyltransferase; enzyme kinetics; pharmacogenetics; recombinant

Human arylamine NAT‡ is encoded at two different loci. One locus encodes an enzyme that has a wide tissue distribution, is responsible for the acetylation of the model substrates *p*-aminobenzoic acid and *p*-aminosalicylic acid, and is termed *NAT1*. The second locus encodes an enzyme that has a distinct tissue distribution, is responsible for the inter-individual difference in acetylation of certain arylamine (e.g. SMZ) and hydrazine (e.g. isoniazid) drugs in humans, and is termed *NAT2*. Genetic variants have been described at both the *NAT1* locus [1, §] and the *NAT2* locus [2–6]. Recently, two variants at the *NAT1* locus have been shown to affect NAT1 functionality by causing a slow NAT1 phenotype in one compound heterozygous individual in a population of over 130 subjects.§ On the other hand, variants at the *NAT2* locus have long been shown to affect NAT2 activity *in vivo* with mutant alleles being correlated with slow acetylator status [2–7]. It is variation at the *NAT2* locus that is responsible for what is known classically as the human NAT polymorphism.

Human NAT polymorphism has been investigated for over 30 years by the use of probe drugs to measure the *in vivo* activity of NAT2 [8]. The population can be divided, in various proportions, depending on ethnic background, into slow and fast acetylators based on the *in vivo* metabolic clearance of NAT2 substrates such as SMZ. Phenotype determined in this way has been related to many disease states [see Ref. 8 for a review], most notably bladder cancer (slow), colon cancer (fast) and drug-induced systemic lupus erythematosus (slow). *NAT2* genotyping studies have been contradictory despite the availability of simple PCR-based genotyping assays [2, 7]. For example, bladder cancer was not linked to the slow *NAT2* genotype in a population of 229 smokers when compared with 211 frequency matched controls.‖ However, a recent study in a Caucasian population suggests a relationship between the slow acetylator genotype and bladder cancer in individuals with a known history of industrial

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‡ Abbreviations: CV, coefficient of variation; IPTG, isopropyl-β-D-thiogalactopyranoside; MTP, 3-methyl-*s*-triazolo[3,4-α]phthalazine; NAT, *N*-acetyltransferase; PCR, polymerase chain reaction; and SMZ, sulphamethazine.

§ Hughes NC and Grant DM, Cloning and expression of new mutant forms of human acetyltransferase NAT1 with defective function. In: *Proceedings of the 10th International Symposium on Microsomes and Drug Oxidations*, Toronto, Canada 18–21 July, 1994, p. 278.

‖ Taylor JA, Umbach D, Stephens E, Paulson D, Robertson C, Mohler J and Bell DA, Slow acetylation (NAT2) is not a risk factor in smoking-induced bladder cancer. In: *Proceedings of the 10th International Symposium on Microsomes and Drug Oxidations*, Toronto, Canada 18–21 July, 1994, p. 550.

exposure to arylamines and in cigarette smokers [9]. The reason for these apparent contradictions in proving a relationship between the *NAT2* genotype and disease probably resides in the multifactorial nature of the etiology of these diseases, which include both environmental and genetic elements. The clearest environmental element is exposure to hazardous chemicals. Genetic elements include certain heritable predispositions toward cancer and the particular composition of drug-metabolizing gene variants, of which many are polymorphic, including *NAT2*, and involved in activation, detoxification and clearance of chemicals from the body [10, 11]. One further confounding factor that has not been investigated is the potential role of inter-individual variation in the K_m of drug-metabolizing enzyme variants in determining the relationship between the level of exposure to hazardous chemicals (which can be at low levels [12]) and predisposition toward diseases such as bladder cancer. To investigate the K_m of individual NAT2 variants for different substrates, we expressed five of the known allelic variants at the *NAT2* locus in *Escherichia coli* and determined kinetic parameters for several NAT2 substrates.

MATERIALS AND METHODS

Cloning strategy for expression of NAT using the pET-5a expression vector

Insert DNA was amplified using Nat-Hu 23 $^{nt-10}$TTAGGGGCATATGGACATTGAA^{nt12} and Nat-Hu 21 ^{nt881}TCCGAATTCTAAATAGTAA-GGG^{nt860} from an individual with the desired *NAT2* allelle. The conditions for the PCR reaction were identical to those described previously [13] except that the *Pfu* DNA polymerase was substituted for *Taq* since the former possesses a 3′-exonuclease ability and, therefore, has a much higher fidelity during the amplification process [14]. The template for the PCR reaction was genomic DNA (100 ng) extracted from the white blood cells of human volunteers. The amplified product using Nat-Hu 23 and Nat-Hu 21 contained modified ends (modified bases underlined above) that allowed digestion with the *Nde*I and *Eco*RI restriction enzymes, respectively. The PCR product and the pET-5a plasmid (Novagen, UK) were digested with *Nde*I and *Eco*RI. The insert could only ligate into the plasmid in the correct orientation. The plasmid was established in a strain of *E. coli* that does not express the T7 RNA polymerase (NM554) and was sequenced across the coding region by a double-stranded plasmid method of Sanger *et al.* [15] using the Sequenase II kit (US Biochemical) to confirm that no errors were introduced as a result of the PCR process. Plasmid produced in NM554 was used to transform *E. coli* that carry a genomic copy of the T7 RNA polymerase gene under *lac* control [BL21 (DE3), Novagen, U.K.]. When induced with IPTG, the *E. coli* were then able to express the recombinant *NAT* gene [16].

Expression of NAT2 activity in E. coli

Cells containing a chromosomal copy of T7 RNA polymerase [BL21 (DE3)] and the pET-5a expression vector with the *NAT2*4* type *NAT2* insert were grown in the presence of ampicillin (50 μg/mL) at 37° to an absorbance at 550 nm of 0.6. The culture was induced to express NAT2 with IPTG (0.4 mM) for 4 hr to produce soluble enzyme for enzyme kinetic characterization. Cells from each 10 mL of culture were lysed by sonication on ice in 500 μL of 20 mM Tris–HCl, 1 mM EDTA and 5 mM dithiothreitol. The NAT activity was determined in the supernatant after centrifugation (10,000 *g*, 5 min, 4°).

Determination of NAT activity

Enzyme velocity experiments for Eadie–Hofstee transformation of NAT activity of the different variants of the NAT2 were conducted over the following range of concentrations: sulphamethazine (1–2000 μM), anisidine (1–200 μM), 2-aminofluorene (0.5–10 μM), procainamide (0.5–20 mM), isoniazid (0.1–5.6 mM), and dapsone (15–600 μM). Soluble NAT enzymes in bacterial supernatants from clones expressing each variant from the pET-5a plasmid were prepared as described above. NAT activity was assayed according to the method of Andres *et al.* [17]. The acetyl CoA concentration used for all apparent K_m determinations was 100 μM. NAT assays were incubated at 37° for 5 min such that no more than 20% of the substrate arylamine was converted to the acetylated product. Accumulation of the acetylated product was measured by reversed-phase HPLC with UV detection.

Analysis of acetylated metabolites by HPLC

A Waters 600E solvent delivery system was used with a Waters 484 UV/vis absorbance detector. Data were recorded using a BBC chart recorder and a Waters data module integrator or the Maxima HPLC analysis software (Waters). A reversed-phase C18 HPLC column (Dynamax 4.6 × 250 mm, 12 μm particle size, 30 nm pore size; Rainin Instrument Co., Emeryville, CA) was used with or without a guard column. Table 1 specifies the solvent conditions used to separate the arylamines from their acetylated metabolites without interference resulting from the other components in the assay.

Quantitation of *N*-acetylated metabolites in unknown samples was possible by comparison of peak height or peak area with injections of known standards. In all cases (except *p*-anisidine), there was a linear relationship between concentration of *N*-acetylated metabolite and peak height/area over the concentration range studied. The *N*-acetylanisidine standard was unavailable. Therefore, the concentration of acetylanisidine was estimated by following the progress of *N*-acetylation of *p*-anisidine (200 μM) over a 10-min time–course experiment using recombinant polymorphic NAT2. A linear increase in peak height over the time–course of the experiment was noted at a retention time of 7.6 min using 10% acetonitrile and 90% 50 mM acetic acid at a flow rate of 2 mL/min. A concomitant linear decrease in the peak height corresponding to *p*-anisidine was also noted. Therefore, approximate concentrations of *N*-acetylanisidine were deduced from peak heights by comparing the rate of increase of the peak eluting

Table 1. HPLC conditions for separation of arylamines from their acetylated derivatives

Arylamine/ arylhydrazine	Retention time (min)	Product	Retention time (min)	Solvent system	Flow rate (mL/min)	Detection wavelength (nm)
Sulphamethazine (SMZ)	6	Acetyl-SMZ	8	10% acetonitrile: 90% 50 mM acetic acid	2	266
p-Anisidine (AN)	1.8	Acetyl-AN	7.6	10% acetonitrile: 90% 50 mM acetic acid	2	266
2-Aminofluorene (AF)	3	Acetyl-AF	4.6	44% acetonitrile: 56% 50 mM acetic acid	2	270
Procainamide (PRO)	3.3	Acetyl-PRO	7	13.5% methanol: 86.5% 21 mM NaH_2PO_4, pH 4.4	1.5	266
Isoniazid (ISN)	5.4	Acetyl-ISN	4.1	100% NaH_2PO_4/Na_2HPO_4, pH 7.5	2	266
Hydralazine	1.6	MTP*	3.9	20% acetonitrile: 80% 50 mM acetic acid	2	241

* MTP (3-methyl-*s*-triazolo[3,4-*α*]phthalazine) is a stable urinary metabolite of hydralazine thought to be a ring stabilized spontaneous product of *N*-acetylhydrazine [18].

at 7.6 min with the decrease in peak height due to the *p*-anisidine substrate whose concentration was known.

The *N*-acetylation of all substrates (except hydralazine) was linear over the 5-min time period of the assay with up to 20% conversion of substrate to *N*-acetylated product. Enzyme preparations were diluted prior to assay if necessary such that no more than 20% of the substrate was converted in each case. Specific enzyme activity was calculated using protein concentration, estimated by the Bradford dye binding method [19]. The analysis of dapsone and its acetylated metabolite has been described separately [20].

Analysis of data

The ENZPAC program (Biosoft, Cambridge, U.K.) was used to calculate apparent K_m and apparent V_{max} values from initial velocity measurements at various concentrations of arylamine substrate or the cofactor acetyl CoA. The values of apparent K_m and apparent V_{max} were calculated using the direct linear plot method [21].

To estimate the "true" K_m of the NAT2 variants NAT2 4 and NAT2 7B for the arylamine sulphamethazine and acetyl CoA duplicate determinations of activity were made for each enzyme over a range of ten concentrations of SMZ (1–1000 μM) at each of six fixed concentrations of acetyl CoA (50–2000 μM). True K_m was estimated by fitting the Michaelis–Menten equation for Ping-Pong Bi-Bi enzyme kinetics to the velocity/concentration data (Equation 1 [22]) using non-linear regression.

RESULTS

Clones were constructed to express NAT2 in *E. coli* as the native polypeptide. Induction of the pET-5a expression vector at 37° gave a time-dependent increase in NAT activity (data not shown). Comparison of apparent Michaelis constants from our expressed NAT2 (NAT2 4 variant) gave values in close agreement with those reported previously for human liver cytosolic fractions using sulphamethazine, procainamide and aminofluorene [23], and from our own studies with dapsone [20], indicating that the expressed soluble enzyme was correctly folded. Other known NAT2 variants (NAT2 5A, NAT2 5B, NAT2 6A and NAT2 7B) were also expressed using the pET-5a expression system, and the apparent Michaelis constant for SMZ (Fig. 1) and for five other polymorphic substrates (Table 2) was determined.

Except for SMZ and dapsone, the apparent K_m of the substrates tested did not differ more than 2.5-fold between the different variants (Table 2). For SMZ and dapsone, the apparent K_m for NAT2 7B was, respectively, 10-fold and 5-fold lower than the other variants (Fig. 1 and Table 2), indicating a greater affinity of the NAT2 7B variant for these substrates. In contrast, the apparent K_m of NAT2 7B for the co-substrate acetyl CoA (at 100 μM SMZ) was 4-fold greater than for the other variants. These observations can be explained by the Ping-Pong Bi-Bi mechanism of the NAT reaction. A rearrangement of Equation 1, to a form resembling the single

$$v = \frac{([\text{AcCoA}] \cdot [\text{SMZ}])}{((K^{\text{true}}_{m_{\text{SMZ}}} \cdot [\text{AcCoA}]) + (K^{\text{true}}_{m_{\text{AcCoA}}} \cdot [\text{SMZ}]) + ([\text{AcCoA}] \cdot [\text{SMZ}]))} \cdot V_{\text{max}} \quad (1)$$

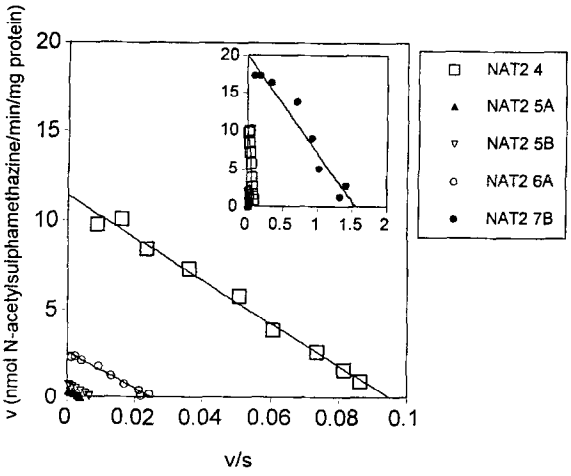


Fig. 1. Eadie–Hofstee transformation of *N*-acetyltransferase (NAT) activity of the different variants of the polymorphic arylamine NAT over a range of concentrations of sulphamethazine (SMZ). Soluble NAT enzymes from clones expressing each variant from the pET-5a plasmid were prepared as described in Materials and Methods. NAT activity was assayed using SMZ concentrations ranging from 5 to 2000 μM for the NAT2, 4, 5A, 5B, and 6A variants and from 1 to 500 μM for the NAT2 7B variant. The acetyl CoA concentration used was 100 μM. NAT assays were incubated at 37° for 5 min such that no more than 20% of the substrate SMZ was converted to acetyl-SMZ. Accumulation of the acetyl-SMZ product was measured by reversed-phase HPLC with UV detection. Each point represents the mean of two experiments. Eadie–Hofstee transformations of the data are shown [velocity divided by the substrate concentration (v/s) plotted against velocity (v)] with (inset) and without the data from the NAT2 7B variant.

substrate Michaelis–Menten equation, illustrates the relationship between "true" and apparent K_m values (Equation 2). It therefore becomes clear that any increase in saturation of the enzyme with one substrate (whether this be due to increased concentration or increased affinity of the enzyme for this substrate) causes an increase in the apparent K_m for the second substrate. Therefore, the increase in apparent K_m for the acetyl CoA is a likely consequence of a higher affinity of the NAT2 7B enzyme for the substrate SMZ.

$$v = \frac{V_{max}^{app} \cdot [S]}{K_m^{app} + [S]} = \frac{\dfrac{V_{max}}{1 + (K_{m_{SMZ}}^{true}/[SMZ])} \cdot [AcCoA]}{\dfrac{K_{m_{AcCoA}}^{true}}{1 + (K_{m_{SMZ}}^{true}/[SMZ])} + [AcCoA]} \quad (2)$$

To test this hypothesis we estimated the true K_m of the NAT2 4 and NAT2 7B isozymes for the co-substrates SMZ and acetyl CoA. Only a 2-fold difference in the true K_m for acetyl CoA [3489 μM (10.6%, CV) for NAT2 7B vs 5971 μM (16.6%, CV) for NAT2 4] was found, whereas there was a 14-fold difference in the true K_m for SMZ [260 μM (9.6%, CV) for NAT2 7B vs 3696 μM (16.5%, CV) for NAT2 4]. This demonstrates that the binding of SMZ, and not acetyl CoA, is changed by the Gly286-Glu amino acid substitution present in the NAT2 7B variant.

DISCUSSION

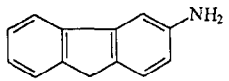
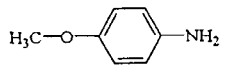
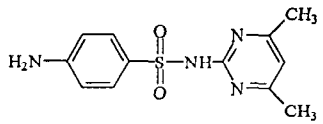
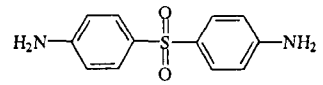
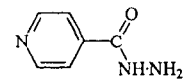
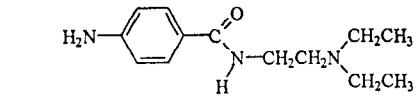
In this study, we report the expression of five variants of human polymorphic NAT2 in *E. coli*. All the variants gave rise to NAT2 activity in soluble extracts from cells grown and induced at 37°. The solubly expressed NAT2 possessed similar enzyme kinetic characteristics with four arylamine substrates compared with NAT2 activity extracted from human liver. This finding indicates that Michaelis constants derived from our expressed NAT2 provide information about the native molecule.

Apparent Michaelis constants were calculated for the NAT2 substrates sulphamethazine, *p*-anisidine, 2-aminofluorene, procainamide, isoniazid and the cofactor acetyl CoA. We also attempted to determine a K_m for the NAT2 substrate hydralazine; however, the enzymatic conversion of hydralazine to its stable *N*-acetylated and ring cyclized metabolite MTP was not linear over time so that no estimate of initial velocity was possible. We believe that this was due to a rate-limiting cyclization of the *N*-acetylhydralazine (the direct product of the acetylation of hydralazine by NAT2) to the stable product MTP [18].

Hein *et al.* [25] recently found essentially no change in the apparent K_m for 2-aminofluorene and the cofactor acetyl CoA determined with recombinant NAT2 4 and NAT2 7B in *E. coli*. We have shown that the single amino acid substitution Gly286-Glu causes an alteration in the apparent K_m of the NAT2 7B variant for the substrates sulphamethazine and dapsone, whilst the apparent K_m for other substrates remains relatively unaffected. Further investigation confirmed that the true K_m of the NAT2 7B variant for the arylamine sulphamethazine was decreased 14-fold relative to the wild-type NAT2 4, and that the decrease in the true K_m for acetyl CoA, at 2-fold, was relatively small. To determine if the K_m of the variant alleles for other substrates is affected, each variant/substrate combination will have to be tested individually. In addition, to determine if these K_m differences have functional significance *in vivo*, it will be important to determine if they can be detected in extracts from human liver of known genotype. Although the NAT2 7B allele is relatively rare in Caucasians (1% [4]), it is common in other ethnic populations such as aboriginal Australians (39% [26]), Central American Indians (23% [27]), Hispanics (10–17% [28]) and in Far Eastern populations (11–18% [28]).

A high-affinity enzyme alone is not enough to cause fast acetylator status. The *in vivo* phenotype of an individual will depend on the value of the drug concentration at the enzyme site relative to the value of the Michaelis parameters, V_{max} and K_m. For example, the lower K_m of NAT2 7B for the substrate SMZ does not make a carrier of NAT2 7B a fast acetylator since at clinical doses, its peak plasma concentration of 100 μM [29] far exceeds the estimated 10 μM K_m of NAT2 7B. At this concentration, assuming that the concentration at the enzymatic site equals plasma concentration,

Table 2. Apparent K_m (μM) and V_{max} (nmol·min^{-1}·mg protein^{-1}) of NAT2 variants for some arylamine and hydrazine substrates

Substrate	NAT2 4 (F1)		NAT2 5A (S1b)		NAT2 5B (S1a)		NAT2 6A (S2)		NAT2 7B (S3)	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
2-Aminofluorene 	1.85	16.8	1.79	1.3	0.97	1.3	1.35	4.5	2.29	37.5
p-Anisidine 	29.1	16.0	25	0.3	13.4	0.7	15.2	3.0	17.6	7.0
Sulphamethazine 	123	11.5	109	0.4	102	0.8	104	2.6	12.8	19.5
Dapsone 	117	0.84	—	—	—	—	—	—	22	1.3
Isoniazid 	374	16.3	ND		ND		547	1.5	366	22.3
Procainamide 	3220	11.7	ND		ND		3800	2.8	2060	8.8
Acetyl CoA	92	11.7	106	0.4	132	0.9	124	4.6	424	93.8

All experiments were done in the presence of 100 μM acetyl CoA with the exception of an additional experiment to determine the apparent K_m (μM) of NAT2 variants for acetyl CoA, which was done in the presence of 100 μM sulphamethazine. ND not detectable. Both the new nomenclature [24] and the old nomenclature, used previously by the authors [4], are shown.

the clearance of the drug will be determined predominantly by the V_{max}. In contrast, the *in vivo* clearance of a drug whose clinical concentration is substantially below the K_m for the enzyme will be determined predominantly by the ratio V_{max}/K_m. Since the *in vivo* plasma concentration of dapsone is approximately 10 μM [30], the change in K_m of dapsone for the different variants is likely to be significant. In either case, the magnitude of V_{max} will influence the clearance of the drug. For this reason it is important to determine the value of V_{max} for all the variants. Unfortunately, we are not able to draw conclusions regarding V_{max} from our expressed NAT2 variants since expression in artificial systems using unnatural promoters do not bear any relation to that found *in vivo*. However, an insight into the *in vivo* relevance of V_{max} of the different NAT2 variants could be obtained from extracts from carefully collected and stored liver samples of known genotype. V_{max} and K_m determinations from such sources would be of considerable interest in light of our finding that NAT2 variants can possess significantly different K_m values for at least one type of substrate (e.g. sulphamethazine). Since the range of liver NAT2 activities for fast acetylators extends from 2 to 20 times the liver activity of slow acetylator livers for the substrates sulphamethazine [31] and dapsone [20], it is likely that for some substrates, differences in K_m may be an important variable concerning drug and chemical metabolism by acetylation. At substrate concentrations substantially below the K_m of these enzymes, a 10-fold difference in V_{max} can be negated by a 10-fold change in the K_m of the low V_{max} enzyme (i.e. the V_{max}/K_m ratio is unchanged).

At present we can only speculate why the K_m for SMZ and dapsone are changed without affecting the other substrates tested. One common feature of these two substrates is the presence of the sulphone para to the N^1 amino nitrogen, which links a bulky aromatic side chain (Table 2). Presumably the Gly286→Glu mutation alters the enzyme structure in some way as to accommodate these substrates more readily. A battery of sulphonamides and sulphones is available with which to test which components (e.g. the sulphone or attached aromatic ring) are important. It would also be of great interest to test naturally occurring xenobiotic arylamines (such as the arylamine food pyrolysis products) as substrates for these common NAT2 variants, since it may have been the different environmental exposures of human subpopulations, including dietary exposure, that provided the evolutionary driving force behind the interethnic differences in the frequency of the different NAT2 variants [27].

Acknowledgements—This work was supported by The Wellcome Research Trust (E.S.), and Grants NIHAI27664 and NIHDK41978 (J.R.P., D.H. and J.D.U.). D.H. was also supported, in part, by a studentship from the Medical Research Council, U.K.

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