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Bioelectrochemical profiling of two common polymorphic variants of human FMO3 in presence of graphene oxide

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ABSTRACT

Genetic variation of phase I drug metabolising enzymes has been shown to greatly influence inter-individual reaction to pharmacological treatments. Among these enzymes, human flavin-containing monooxygenase 3 (hFMO3) plays a crucial role and understanding its pharmacogenetics is fundamental for the prediction of individual drug response and the efficacy of therapy. In this work the altered drug metabolism of two common polymorphic variants of hFMO3 (E158K and E308G) are studied by using an electrochemical platform modified with graphene oxide (GO). Electrochemistry was used to characterise the properties of these two engineered and purified hFMO3 variants followed by electrocatalysis experiments in the presence of three different hFMO3 substrates benzydamine, tamoxifen and sulindac sulfide. HPLC quantification of the electrochemically produced metabolites showed that E158K mutation leads to an impairment of N-oxygenation activity while E308G mutation enhances the same activity.

Results demonstrate that electrocatalysis on GO modified glassy carbon electrodes provides a fast and reliable method for measuring kinetic parameters of hFMO3 polymorphic variants. This method can be considered suitable for deciphering metabolic implications of polymorphisms that might lead to adjustment of drug dosages depending on the individual's genetic makeup, a step closer to the development of personalised medicine.

1. Introduction

Drug metabolising enzymes are involved in the conversion of potentially dangerous foreign chemicals into less harmful and more readily excretable molecules in humans. Among these enzymes, human flavin-containing monooxygenase isoform 3 (hFMO3) is able to catalyse the oxygenation of a variety of xenobiotics with varied chemical structures, including many therapeutic drugs [1]. This enzyme is microsomal, FAD- and NADPH-dependent and, is able to activate molecular oxygen to catalyse substrate oxygenation [1–3,4]. Recently, in addition to xenobiotic metabolism, FMO3 has also been implicated in the development of atherosclerosis, cholesterol imbalance and to glucose and lipid metabolism [5–7]. Human FMO3 activity is widely recognized as being part of the first-pass metabolism of drugs together with the superfamily of cytochrome P450 enzymes [1]. Differently to cytochromes P450, hFMO3 metabolism is responsible for the polarization of drugs and xenobiotic molecules that are consequently converted into more excretable species so hFMO3 activity has clinical relevance in the detoxification and clearance processes. Therefore, the development of new chemical entities that can be metabolized by hFMO3 rather than cytochromes P450, can decrease the toxic side effects and be more tolerable by patients. In addition, while cytochromes P450 can be readily induced or inhibited with high probability of adverse drug-drug interactions, hFMO3 activity has not been shown to be influenced by foreign chemical species [1].

The natural polymorphism of FMO enzymes has been shown to strongly influence inter-individual drug response increasing the significance of these enzymes from a pharmacological as well as

Abbreviations: DDAB, didodecyldimethylammonium bromide; FAD, flavin adenine dinucleotide; FTIR, Fourier transform infrared spectroscopy; GO, graphene oxide; hFMO3, human flavin-containing monooxygenase isoform 3; HPLC, high performance liquid chromatography.

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toxicological point of view [7,8]. Human FMO3 is a highly polymorphic enzyme and many of its single nucleotide polymorphisms are present at reasonably high frequency within the population [9]. *In vivo* studies of the metabolism of drugs such as benzydamine, tamoxifen and sulindac sulfide, have demonstrated a strict relationship between some common hFMO3 nucleotide polymorphisms and their altered metabolism. It has also been demonstrated that simultaneous occurrence, like in the case of the two common polymorphic variants E158K and E308G, leads to a significant alteration in hFMO3 metabolism [10]. Furthermore, mutations leading to the total loss of hFMO3 function are the genetic basis for the trimethymaminuria disease [11,12]. People affected by this disease are not able to metabolize the odorous trimethylamine to its odourless N-oxide and the accumulated trimethylamine leads to the so-called fish-odour syndrome [13].

For these reasons, the understanding of hFMO3 pharmacogenetics would be fundamental in the prediction of individual drug response and therapy efficacy leading to a major contribution to drug design and development [9]. In light of this, much attention has been concentrated on the development of new methods useful for pharmacological research relevant to hFMO3 catalysis. One such technique which is applicable to these enzymes is electrochemistry where the direct electrochemical response of hFMO3 on gold and carbon surfaces has been previously demonstrated by our group [14–16]. The advantage of direct immobilization of hFMO3 on electrode surface is mostly related to the possibility of using reducing equivalents provided by the electrode surface instead of those of NADPH which is its natural redox partner, thus improving experimental conditions in terms of costs, reliability and efficiency. To further enhance the electrochemical response of this enzyme novel material such as graphene and/or graphene oxide (GO) can be employed [18]. GO has been found to have functionality in a variety of new applications such as electronic devices, sensors and energy-related techniques due to its interesting properties. The presence of oxygen functionalities causes a decrease of GO conductivity but, on the other hand, it improves GO solubility in aqueous solutions and allows the interaction with a number of chemical entities. Moreover, water solubility is consistent with the higher biological compatibility of GO compared to graphene that needs to be properly supported in order to be able to interact with biological substrates [19,20]. These peculiar characteristics have been considered promising for the development of a variety of biosensing strategies in which graphene material has been applied as a transducer of biological signals in electrochemical, fluorescence and impedance biosensors [21–24].

Here, we report the extension of the GO-based electrochemical approach [21] to two common polymorphic variants of hFMO3 enzyme, E158K and E308G. These two hFMO3 variants were expressed in a recombinant system and immobilised on didodecyldimethylammonium bromide (DDAB)/GO glassy carbon electrodes and their activity tested with three hFMO3 marker drugs: benzydamine, a non-steroidal anti-inflammatory drug that is metabolised to its N-oxide by hFMO3 [15–17,21,25], tamoxifen, a breast cancer drug with antiestrogenic effect, also N-oxygenated by hFMO3 [15–17,21,26,27],and sulindac sulfide, a nonsteroidal anti-inflammatory drug [11] that is selectively re-oxidised to sulindac by hFMO3 S-oxygenation [2,25].

2. Materials and methods

2.1. Chemicals

All the chemical products were obtained in analytical grade and dissolved in ultrapure deionized water immediately before use: GO (concentration of 4 mg/mL in water) from Graphenea (Spain); DDAB, benzydamine (hydrochloride), benzydamine N-oxide (hydrogen maleate), tamoxifen, sulindac sulfide and sulindac from Sigma-Aldrich (Italy); tamoxifen N-oxide from Biozol (Germany).

2.2. Site-directed mutants of hFMO3

WT hFMO3 cloning was performed using pJL2 expression vector and XbaI and HindIII restriction enzymes, as formerly reported [2]. E158K and E308G polymorphic variants were obtained using QuikChange Site-Directed Mutagenesis Kit (Stratagene, Italy). The primers used were constructed as follows with the mutation site highlighted in bold:

E158K

Forward primer: 5′ C AAC CTA CCA AAA **AAG** TCC TTT CCA GGA C 3′

Reverse primer: 5′ G TCC TGG AAA GGA **TTC** TTT TGG TAG GTT G 3′

E308G

Forward primer: 5′ C GTG AAG GAA TTC ACA **GGG** ACC TCG GCC ATT TTT G 3′

Reverse primer: 5′ C AAA AAT GGC CGA GGT **CCC** TGT GAA TTC CTT CAC G 3′

DNA sequencing of the clone confirmed the presence of the mutation in the correct position.

2.3. Expression and purification of human FMO3

WT, E158K and E308G hFMO3 proteins were obtained by expression in *Escherichia coli* (JM109) cells and purification by applying the protocol already optimized for the wild type enzyme [2]. In particular, the proteins were extracted from cells membrane fractions and purified using nickel affinity chromatography. After the purification, proteins purity was checked by visualization in a 10% SDS-polyacrylamide gel stained with Coomassie Blue. hFMO3 enzymes concentration was estimated considering an FAD equimolar content, an extinction coefficient of 11,300 $M^{-1}\,cm^{-1}$ at 450 nm and a molecular weight of 56 kDa [28].

2.4. FTIR spectroscopy

Infrared spectroscopy experiments on hFMO3 polymorphic variants were performed on gold-PET flat surface as protein support using the single reflection grazing angle attenuated total reflectance (GATR) tool (Harrick, USA) on a Bruker Model Tensor 27 FT-IR spectrometer (Bruker Instruments, USA) in nitrogen purged environment at room temperature. Protein samples were prepared by modifying gold-PET supports with 10 μL of 20 mM DDAB solution in chloroform plus 5 μL of GO water dispersion (1 mg/mL). After 10 minutes at 25 °C, to allow solvent evaporation, the gold-PET surfaces were further modified by adding 5 μL of protein solution. Substrates were then kept overnight at 4 °C before FTIR experiments. Wavenumber range of acquisition was from 4000 to 800 cm^{-1}; scan velocity and resolution were set at 10 kHz and 4 cm^{-1} respectively. Spectra were acquired in triplicates and averaged data, obtained using Opus software (Bruker Instruments, USA) were corrected by subtracting of the control spectra, obtained in the absence of protein. The obtained spectra were analysed by Fourier self-deconvolution (deconvolution factor 50, noise reduction factor 0.8) in order to investigate the composition of the amide I band in terms of number and location of single components. Afterwards, data analysis and curve fitting were carried out using PeakFit software (SPSS Inc., USA).

2.5. Electrode preparation

The preparation of glassy carbon electrodes was obtained as previously described [21]. In particular, 10 μL of 20 mM DDAB