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Original Article

The dose-dependent influence of antioxidant vitamins on electrochemically-driven cytochrome P450 3A4 catalysis

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Abstract

Electrochemical analysis of the catalytic activity of cytochrome P450 3A4 has shown that vitamins C, A and E exert influence on the Fe^{3+}/Fe^{2+} reduction process of this enzyme. Direct electron transfer of cytochrome P450 3A4 was investigated by use of cyclic voltammetry and square wave voltammetry. The increase of the reduction peak current in the presence of vitamins C, A and E appears to be associated with antioxidant properties of these vitamins. Vitamin C (in the range of 0.03-1 mM), vitamins A and E (in the range of 10-100 μM) stimulated the dose-dependent growth of the cathodic peak current of cytochrome P450 3A4. The electrochemical analysis of the catalytic activity of cytochrome P450 3A4 offers an unorthodox and efficient approach to pre-clinical registration of drug-drug and drug-vitamin interactions via the respective electrochemical responses.

INTRODUCTION

The cytochromes P450 (CYPs) are an ubiquitous superfamily of mixed function oxidases found in all kingdoms of life. They are one of the most extensively studied heme-thiolate proteins, their activity are crucial for the metabolic activation or inactivation of xenobiotics [1]. Cytochrome P450 3A4 (CYP3A4) is the most abundant CYP protein in human liver and is the most functionally significant among CYPs as it is responsible for biotransformation of 637 xenobiotics and participates in a metabolism of approximately 50% of currently available medical preparations and clinical drugs [2, 3]. CYP reactions are of high interest to the pharmaceutical industry, where lead compounds in drug development are screened as potential substrates or inhibitors of CYPs. Many clinically relevant drug-drug interactions are associated with inhibition and/or induction of this enzyme [4].

Investigation of the catalytic activity of isolated cytochromes from the P450 superfamily requires obligatory presence of redox partners and electron donors (NADPH) [4-9]. At the same time, redox partners are not obligatory in electrochemical reduction of these hemoproteins, so the catalytic system is essentially simplified [10-13]. Electrochemical systems can be applied to studying the catalytic activity of CYPs [14, 15] and their interactions with substrates and inhibitors [16, 17]. Electrocatalytic properties of CYP3A4, exhibited in the course of direct electrochemical reduction of this hemoprotein immobilized on didodecyldimethylammonium bromide/gold (DDAB/Au) nanostructured screen-printed electrodes (SPE), were used earlier for the investigation of the influence of vitamins B group on diclofenac metabolism. Electrochemical methods confirmed the inhibitory effects of vitamins of B group

(thiamine or vitamin B1, riboflavin or vitamin B2, pyridoxine or vitamin B6), on the monooxygenase activity of CYP3A4 enzyme [18].

According to catalytic cycle of CYP one of oxygen atoms is involved in oxidation of organic substance and the other is reduced to water (see below in equation 1). However, in some cases a reaction does not correspond to stoichiometry of the above equation. Part of electrons is spent on reduction of oxygen without substrate conversion (see equations 1.1-1.3). This phenomenon has come to be known as uncoupling. Part of redox equivalents participates in side oxidative reactions. In the course of the reaction there occurs formation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anion-radical with water formation (Scheme 1) [2, 4, 19-21].

Scheme 1.

$RH + O_2 \rightarrow ROH + H_2O$ (1)

$O_2 + 2H^+ + 2e \rightarrow H_2O_2$ (1.1) → ↘

$O_2 + H^+ e \rightarrow HO_2 \rightarrow O_2\bullet^- H^+$ (1.2) → → AO → O_2

$HO_2 + H^+ + e \rightarrow H_2O_2 + H^+$ (1.3) → ↗

-**RH;** the substrate for CYP

-**ROH;** the product of CYP-catalyzed monooxygenase reaction

-**AO;** antioxidant

Reactive oxygen species interact with CYP P450, causing an inactivation of the enzyme [4, 19-22]. Antioxidants can diminish the level of ROS by radical-scavenging effect or modulating the activities of CYPs which are known to generate reactive intermediates (Scheme 1).

The aim of this study was to investigate the influence of antioxidants, such as vitamin C, vitamin A, and vitamin E on the catalytic activity of CYP3A4 by electrochemical methods.

MATERIALS AND METHODS

Electrochemical measurements were carried out using a PGSTAT12 Autolab potentiostat/galvanostat, (Eco Chemie, Utrecht, The Netherlands) with GPES software. All the measurements were taken at room temperature.

A typical three-pronged screen-printed electrode (SPE, Elcom, Moscow, Russia) involved the working graphite electrode (d = 2 mm), the auxiliary graphite electrode and the silver/silver chloride (Ag/AgCl) reference electrode. The potentials are all referred to the Ag/AgCl reference electrode.

Cyclic voltammograms (CV) were registered at the scan rate of 10-100 mV/s. Parameters used in square wave voltammetry (SWV, reduction, aerobic conditions) were as follows: initial potential, 100 mV; final potential, −600 mV; step potential, 5 mV; amplitude, 20 mV; frequency, 10-100 Hz. For presentation of all electrochemical data the average values of maximum cathodic peak height of SWV from five independent experiments were used. The relative standard deviation (RSD) not exceed 10% for n = 5.

The following reagents were used: DDAB, gold acid chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium boro-hydride, testosterone (Sigma-Aldrich), diclofenac (Novartis).

The chemicals used in electrochemical experiments: freshly prepared aqueous 10 mM diclofenac; vitamin C (0.3 M in distilled water), vitamin A (0.1 M) and vitamin E (0.1 M) (water-soluble form of vitamins A and E were produced by DSM Nutritional Products, Herleen, The Netherlands); 10 mM itraconazole in dimethylsulfoxide; 20 mM testosterone in ethanol.

Synthesis of DDAB-stabilized solution of gold nanoparticles was carried out as described earlier [23]. Colloidal gold solution stabilized by DDAB in chloroform was spectrally characterized: λ_{max} = 520 nm.

Recombinant human CYP3A4 (165 μM) and CYP51 (165 μM) were kindly presented by Prof. S.A. Usanov (Institute of Bioorganic Chemistry, Minsk, Belarus). Concentration of CYP3A4 was determined based on formation of a complex of the CYP (reduced form) with carbon monoxide using the extinction coefficient ε_{450} = 91 $мМ^{-1}см^{-1}$ [24].

Applied onto the surface of the working graphite electrode were 2 μl of 5 mM colloidal gold solution in 0.1 M DDAB in chloroform; after evaporation of chloroform (10 min), 2 μl of CYP3A4 were loaded onto the electrode surface [25]. Electrodes (SPE/DDAB/Au/CYP3A4) were allowed to stay for 12 h at +4ºC in a humid chamber to prevent their total drying.

RESULTS

The influence of vitamin C on cytochrome P450 3A4 electrochemical reduction

We have shown that vitamin C at 0.03-1 mM concentration range stimulates the cathodic reductive peak current of SWV of CYP3A4. Diclofenac is a substrate for CYP3A4. CYP3A4 mediates 5-hydroxy-diclofenac and other mono- or dihydroxylated, methoxylated or decarboxylated metabolites [26-29]. Diclofenac alone produced a 128 ± 10% growth of catalytic current. With diclofenac plus vitamin C we have also observed the growth of catalytic current, testifying to electrocatalysis and to the stimulating action of ascorbic acid (Figs.1&2); it amounts to 135 ± 10% (vitamin C only) and 155 ± 7% (vitamin C plus diclofenac). However, at concentrations above 1 mM, vitamin C attenuated the cathodic peak current

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corresponding to electrochemical reduction of CYP3A4. Catalytic behavior of CYP3A4 towards testosterone [14] and the stimulating influence of ascorbic acid (0.03-0.6 mM) on testosterone (2 mM) were registered as well (data not shown). It was also revealed that 0.03 mM vitamin C stimulates the electrochemical reduction of sterol 14α-demethylase from *Mycobacterium tuberculosis* (CYP51b1): reductive peak current corresponds to 140 ± 10%.

The influence of vitamin A on cytochrome P450 3A4 electrochemical reduction

Retinol is reversibly oxidized by retinol dehydrogenases to yield retinal. Subsequently, retinal is irreversibly oxidized to all-trans retinoic acid (all-trans RA) by retinal dehydrogenases and is further oxidized by CYP enzymes (mainly CYP26) in hepatic tissue [30, 31]. These data show that vitamin A (retinol acetate) does not exhibit substrate properties in relation towards CYP enzymes. By our data, vitamin A exerts the concentration-dependent influence on electrochemical reduction of CYP3A4. 10-100 μM vitamin A produces the stimulatory effect on CYP3A4 electrochemical reduction: the cathodic peak increases proportionally to vitamin A concentration and reaches 229 ± 10% (Fig.3).

Diclofenac in the presence of 100 μM vitamin A does not give rise to the "additional" growth of catalytic current; the growth value is comparable to that obtained in case of vitamin A: 230 ± 10%. The effect of vitamin A on testosterone's catalytic current is more noticeable, exceeding by 20 ± 5% the growth produced by vitamin A alone. Vitamin A in concentration above than 0.1 mM (to 1 mM) does not influence the cathodic current of CYP3A4 reduction.

The influence of vitamin E on cytochrome P450 3A4 electrochemical reduction

Vitamin E in the concentration range 10-100 μM also exhibits the stimulatory effect: the cathodic peak of CYP3A4 reduction increases with increasing vitamin E concentration and reaches 162 ± 10% upon addition of 100 μM vitamin E (Fig.2). Diclofenac (just as in the case with vitamin A) does not produce an additional increase in catalytic current: it remains at the level of the vitamin E-containing system: 169 ± 10%. Vitamin E in the concentration range of 0.1-1 mM stimulates the growth of the cathodic peak current of CYP3A4 (by 119 ± 10%), while diclofenac in the presence of high concentration of vitamin E demonstrates the insignificant growth of catalytic current (by 121 ± 5%; just as with vitamin E alone).

Vitamin C, vitamin A and vitamin E were not electrochemically active in the investigated range of potentials (as were shown for vitamin C on Fig.2).

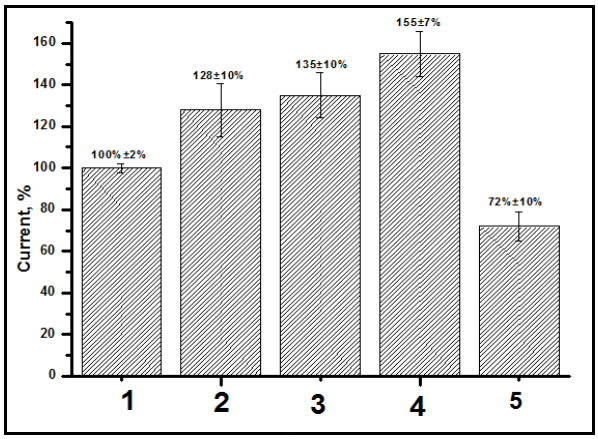

Figure 1. Peak intensity (%) of reductive square wave voltammograms of SPEs in aerobic buffer (with baseline correction): DDAB/Au/CYP3A4 *(1)*; DDAB/Au/CYP3A4 +100 μM diclofenac (DF) **(2)**; DDAB/Au/CYP3A4 + vitamin C (0.6 mM) **(3)**; DDAB/Au/CYP3A4 + vitamin C (0.6 mM), then 100 μM DF **(4)**; DDAB/Au/CYP3A4 +vitamin C (1.7 mM) **(5)**.

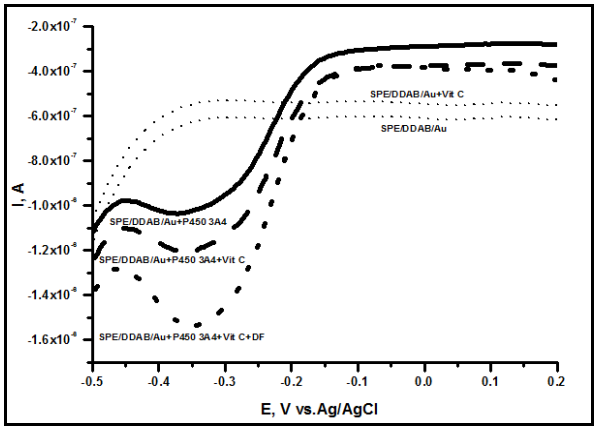

Figure 2. Reductive square wave voltammograms of screen-printed DDAB/Au/CYP3A4 electrode **(——)**; DDAB/Au/CYP3A4 +vitamin C (0.3 mM) **(– – –)**; DDAB/Au/CYP3A4 + vitamin C (0.3 mM), then 100 μM diclofenac (DF) **(- - -)**, DDAB/Au **(...)** and DDAB/Au + vitamin C (0.3 mM) **(…)**. Electrolyte volume is 1 ml of 100 mM potassium phosphate buffer plus 50 mM NaCl, pH 7.4.

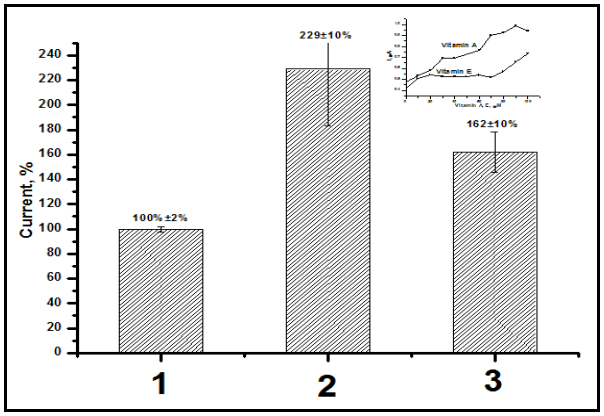

Figure 3. Peak intensity (%) of reductive square wave voltammograms of SPEs in aerobic buffer (with baseline correction): DDAB/Au/CYP3A4 **(1)**; DDAB/Au/CYP3A4 + 100 μM vitamin A **(2)**; DDAB/Au/CYP3A4 + 100 μM vitamin E **(3)**. Inset: concentration-dependent electrochemical response of DDAB/Au/CYP3A4 electrode on vitamin A or vitamin E addition.

DISCUSSION

Earlier, we have shown that electrochemical methods with nanostructured electrodes can be used for investigation of enzyme/substrate or enzyme/inhibitor interactions [14, 18, 23]. To obtain and analyze the electro-analytic characteristics of electrochemical systems with CYP immobilized on the electrode surface, we have resorted to cyclic voltammetry and square wave voltammetry. Substrates for CYP enzymes produce the enhancement of catalytic current under appropriate potentials, but inhibitors either do not change or reduce cathodic peak height. Itraconazole, an antifungal drug used in the therapy of different mycoses [32], acts as an inhibitor of CYP3A4 [33, 34]. The inhibitory action of itraconazole (10 μM) towards CYP3A4 activity was confirmed in electrochemical experiments with SPE/DDAB/Au/CYP3A4 electrodes. Itraconazole did not produce the growth of catalytic current, which is typical for electrochemical behavior of CYPs inhibitors [12-14, 18]. Diclofenac (electrochemical K_M for diclofenac corresponds to 40 ± 10 μM) in the presence of inhibitor itraconazole ($K_i = 0.45 \pm 0.15$ mM) had no effect on the cathodic peak of CYP3A4 [18].

In the course of catalytic reaction of CYP enzymes there occurs formation of ROS (Scheme 1). These reactive species can inactivate enzyme or destroy polypeptide chain of proteins [4, 19-22]. Scavenging substances are essential in the antioxidant defense against ROS, and can influence the catalytic functions of this hemoprotein.

In the present study, we investigated the role of antioxidants in CYP-mediated catalysis and have shown that vitamins C, A and E, possessing antioxidant properties, influence the catalytic activity of CYP3A4. Electrochemically-driven CYP 450 catalysis is also accompanied by ROS generation [25]; therefore the influence of free radical-scavenging substances (ROS "traps") on electrocatalysis may be reasonably expected.

Antioxidant compounds are routinely included in pharmaceutical formulations in order to minimize the oxidative degradation of the active pharmaceutical ingredient(s). To minimize drug-drug interactions it is necessary to choose safe drug-drug or drug-vitamin combination regimens and adjust drug dosage appropriately. Based on the analysis of electrochemical parameters of CYP, the algorithm, allowing elucidating the properties of antioxidants was developed.

Vitamin C (in the range of 0.03-1 mM), as well as vitamins A and E (in the ranges of 10-100 μM) stimulated the dose-dependent growth of the cathodic peak current of CYP3A4, corresponding to heme reduction according to the equation $Fe^{3+} + 1e \rightarrow Fe^{2+}$ (Figs.1&3).

To understand better the mechanism of stimulating effect of vitamins with antioxidant properties in CYP electrocatalysis, we have tested the influence of *tert*-butyl alcohol, a well-known ROS scavenger [35], on the cathodic reductive current, corresponding to heme reduction. It was found that t*ert*-butyl alcohol (0.05 M) stimulates the reductive current in the SPE/DDAB/Au/CYP3A4 electrode system ($138 \pm 10\%$) and neutralizes the antioxidant effect of vitamin C. These experiments confirm the participation of ROS in electrocatalysis of CYP3A4.

Our findings are in line with previously shown experimental data that vitamin C and cytochrome c could enhance electron transfer in reactions mediated redox processes by serving as nonspecific redox activity facilitators for heme peroxidases such as chloroperoxidase and horseradish peroxidase [36]. It is shown also that vitamin C, being a strong antioxidant, is capable of scavenging ROS in the low concentration ranges, and possesses pro-oxidant capacity in the high concentration [37].

In our experiments vitamins-antioxidants serve as modulating and/or stimulating additives with respect to CYPs electrochemical activity due to their free-radical scavenging, antihypoxant properties, or electron mediator features.

In conclusion, the electrochemically-driven CYP catalysis is an alternative model system for pharmacological research. The electrochemical experiments have elucidated the possible mechanism of dose-dependent interaction of vitamins exhibiting antioxidant properties with clinical drugs. These findings provide primary data for future clinical risk prediction studies, especially for those devoted to the interaction of drugs with antioxidants. Regulation and modulation of CYP3A4 activity through the action of vitamins-antioxidants, upon their appointment in a combination with clinical drugs metabolized by CYP, will probably become an essential requirement in clinical routine practice; antioxidants intake can lead to alteration in pharmacodynamic efficiency, which demands special attention from the physician since the prescribed medical product can bring about changes in an efficiency/safety profile.

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