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Origin of the gender differences of the natural resistance to antivitamin K anticoagulants in rats

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ABSTRACT

Vitamin K antagonists (VKA) are used either in human medicine to prevent thromboembolic disorders or as rodenticides for pest control management. In rodents, female rats are described to be more tolerant to the action of vitamin K antagonists than males. Nevertheless, the mechanism of this greatest tolerance is still unknown and this study aims to identify the origin of this greatest tolerance after VKA administration. Therefore, difethialone, one of the most powerful VKA was used in this study.

A possible difference in the pharmacokinetics of difethialone between males and females was first investigated. The determination of the pharmacokinetic parameters allowed to exclude a pharmacokinetic origin of the greatest tolerance of females to VKA. Thus, a natural resistance to difethialone of the liver VKOR activity, which is the target of VKA, was thus explored in females. The determination of Ki towards difethialone in liver microsomes allowed to also exclude this hypothesis. Therefore, equipment in vitamin K-dependent clotting factors and properties of vitamin K-dependent clotting factors were explored. Basal activity of clotting factors VII and X were found significantly higher in females of respectively 43% and 21%. Moreover, after VKA administration, half-lives of clotting factors II and X were found significantly longer in females of respectively 27% and 10% and a lag time of 4 h before the beginning of the decay of factor VII was observed only in females after difethialone administration. The greater tolerance of female rats to VKA is thus due a stronger basal pool of vitamin K-dependent clotting factors VII and X and to a slower decline of vitamin K-dependent clotting factors II, VII and X after VKA administration.

1. Introduction

In human medicine, vitamin K antagonists (VKA), derivatives of either 4-hydroxycoumarin or indane-1,3-dione, are widely prescribed in the prevention and the treatment of thromboembolic disorders. Warfarin, a derivative of 4-hydroxycoumarin, is the most used VKA worldwide and is prescribed to 2 million new patients in the USA each year. VKA are also used as rodenticides for pest control management. VKA exert their anticoagulant activity by stopping the recycling of vitamin K, which is the cofactor of oxydoreduction reactions, including the activation of vitamin-K dependent clotting factors. The activation of vitamin-K dependent clotting factors is performed by the gamma-glutamyl carboxylase (GGCX). In the endoplasmic reticulum, GGCX converts glutamate (Glu) of vitamin K-dependent proteins in carboxylated glutamate (Gla) (Furie et al., 1999). For each conversion, vitamin K hydroquinone has to be converted in vitamin K epoxide. Since the vitamin K dietary intake is not sufficient for the physiological needs (Ferland, 2012), vitamin K epoxide needs to be recycle in vitamin K hydroquinone by a vitamin K 2,3-epoxide reductase (VKOR) activity which involves mainly a vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) protein (Oldenburg et al., 2006). It is this enzyme which is the target of VKA.

Clotting factors II (*i.e.*, Prothrombin), VII, IX and X are vitamin K-dependent proteins involved in the coagulation. These clotting factors need to be gamma-carboxylated to be able to chelate calcium and have a biological activity (Bandyopadhyay, 2008). The uncarboxylated vitamin K-dependent clotting factors circulate in very low level in rat blood (Harauchi et al., 1986). Clotting factor VII is involved in the extrinsic pathway, clotting factor IX in the intrinsic pathway, prothrombin and factor X are involved in the common pathway. Consequently, active vitamin K-dependent clotting factors are necessary for coagulation. By inhibiting VKORC1 protein (Rost et al., 2004; Li et al., 2004), VKA block the

Abbreviations: Gla, carboxylated glutamate; Glu, glutamate; GGCX, gamma-glutamyl carboxylase; VKA, vitamin K antagonists.

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gamma-carboxylation of vitamin K-dependent clotting factors, impairing the coagulation function.

VKA are difficult to use because of a narrow therapeutic index and a wide variability of dosage necessary to achieve stable anticoagulation. This variability is partly due to basic physiological parameters such as age, body weight, but also to co-occurring disorders, drug and food interactions. Sex-differences in the response to VKA could exist. If these differences are difficult to demonstrate in human medicine, such differences have been demonstrated in rats (Gill et al., 1994; Kerins and MacNicoll, 1999). Female rats are more tolerant to VKA than males. The underlying mechanism of this resistance is still unknown. Differences of VKOR activity could be associated with the natural resistance of females. Indeed, mutations of VKORC1 enzyme can lead to resistance to VKA (Rost et al., 2004; Watzka et al., 2011; Hodroge et al., 2012; Pelz et al., 2005, 2012). If this VKORC1 mutation-induced mechanism can be excluded in the case of the natural resistance to VKA of females, differences in the expression level of genes encoding putative partner proteins of VKORC1, such as calumenin (Wallin et al., 2001), epoxide hydrolase (Guenthner et al., 1998) or glutathione transferase (Cain et al., 1998), could modify this VKOR activity. Other mechanisms could be suspected to explain the better tolerance of females to VKA, such the existence of metabolic differences between males and females (Huber et al., 1999; Markussen et al., 2007, 2008; Mugford and Kedderis, 1998; Waxman, 1988), or even a natural resistance of the coagulation cascade (Lemini et al., 2007) after VKA administration.

The aim of this study is to demonstrate first the greater tolerance of the female rats to VKA compared to males and to characterize this tolerance, and thus to identify the underlying mechanism of this greatest tolerance to VKA.

2. Materials and methods

2.1. Animals

OFA-Sprague–Dawley rats were obtained from a commercial breeder (Charle Rivers, l'Arbresles, France) and acclimated for a minimal period of 5 days. The rats were housed four per cage under a constant photoperiod and ambient temperature. In order to characterize the VKOR activity, 8-weeks old OFA-Sprague–Dawley rats (4 males and 4 females) were killed by decapitation. Liver was removed quickly and microsomes were immediately prepared. In order to study the decay kinetics of the vitamin K-dependent blood clotting factors after administration of anticoagulants, 8-weeks old OFA-Sprague–Dawley rats (44 males and 44 females) have been fed with vitamin K3-deficient food (Scientific Animal Food and Engineering, Augy, France), at least 48 h before the beginning and during the experience. To explore the basal levels of blood parameters, a first group of 8 males and 8 females were killed 48 h after the beginning of the vitamin K3-deficient feeding. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at −80 °C. The other animals received by force-feeding a *per os* administration of 10 mg/kg of difethialone 48 h after the beginning of the vitamin K3-deficient feeding, then a group of 3 males and 3 females was killed 1, 2, 4, 8, 10, 13, 16, 20, 24, 32, 48 and 72 h after the difethialone administration. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at −80 °C.

2.2. Microsome preparation

Liver microsomes were prepared by differential centrifugations as described by Moroni (Moroni et al., 1995). Briefly, liver cells were resuspended in 50 mM Phosphate Buffer (pH 7.4) containing 1.15% (w/v) of KCl. liver cells were broken by Potter and further submitted to differential centrifugation a continuously at 4 °C. The 100,000 × *g* pellet corresponding to the membrane fraction was resuspended by Potter homogenization in HEPES glycerol buffer (50 mM Hepes, 20% glycerol, pH 7.4). Microsomes were frozen at −80 °C and used for kinetic analysis. Protein concentrations were determined by the method of Bradford (Bradford, 1976) using serum albumin as a standard.

2.3. Vitamin K epoxide reductase activity

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed according to a modified protocol previously described by Thijssen (1987),Thijssen and Baars (1989) and Misenheimer and Suttie (1990). Briefly, standard reactions were performed in 200 mM Hepes buffer (pH 7.4) containing 150 mM KCl, 1 mM dithiothreitol, 1 mg of total proteins. The reaction was started by the addition of vitamin K > O solution in 1% Triton X–100. After incubation at 37 °C for 30 min, the reaction was stopped by adding 4 mL of iced 1:1 isopropanol/hexane solution. After centrifugation at 5000 × *g* for 10 min, the hexane layer was removed and dried under nitrogen. The dry residue was immediately dissolved in 0.2 mL of methanol and reaction product was analyzed by liquid chromatography–mass spectrometry.

2.4. Vitamin K1 measurement

The LC–APCI/MS/MS used was a 6120 Quadrupole LC/MS with an Atmospheric Pressure Chemical Ionisation (APCI) interface and a LCMS Chemstation software from Agilent Technologies (Palo Alto, CA, USA). Chromatographic separation was performed using a XTerra MS C18 column (2.1 mm × 50 mm, 2.5 μm, Waters, Milford, MA, USA) with a mobile phase of methanol, 0.1% acetic acid (96:4) in isochratic conditions. The column temperature was 48 °C. The flow rate in the LC column was 0.4 mL/min. The injection volume was 20 μL. The temperature of the autosampler tray was set to 5 °C and the samples were protected from the daylight. Detection was by MS with APCI source in positive mode. Nebulizer pressure was set to 60psi, dry gas temperature to 350 °C, dry gas flow to 5 L/min, and vaporizer temperature to 400 °C. Capillary voltage was set to 4000 V, corona needle to 10 μA. Identification criteria for vit K_1 are the retention time (tr = 3.9 min) and the selected ion 451.4. Identification criteria for vit K_1 > O are the retention time (tr = 2.9 min) and the selected ion 467.0. Linearity and accuracy were tested from 25 to 2000 ng/mL (n = 20). The response was linear throughout the concentration range tested with a coefficient of correlation (r^2) above 0.99. Accuracy was between 80 and 120% of the theoretical concentrations.

2.5. Kinetics

Kinetic parameters (K_m and V_{max}) were obtained from at least two separate experiments after the addition of increasing amounts of vitamin K > O (0.003–0.2 mM) to the standard reaction. The estimation of the kinetic parameters was achieved by the incubation of at least 8 different concentrations of vitamin K > O. Incubations were performed in duplicate. Data were fitted by nonlinear regression to the Michaelis–Menten model using R software.

In order to evaluate the inhibiting effect of warfarin on VKOR activity, inhibition parameters (Ki) were determined after addition of various concentrations of warfarin to the standard reaction. Inhibition parameters were first assessed with 4 different concentrations (0.01, 1, 10 and 30 μM) and further more precisely determined using concentrations from about 0.05 to 20 × Ki. Data