INFLUENCE OF DIABETES ON CYTOCHROME P450 ENZYME MEDIATED DRUG METABOLISM – CASE STUDIES ON DICLOFENAC AND K-48

Ph.D. Thesis

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SUMMARY

Insulin dependent diabetes mellitus (IDDM) is a complex metabolic disorder, which develops changes in the cytochrome P450 (CYP), mediated metabolism in the liver and in the small intestine and it may also produce altered bioavailability. The main goal of this study was to reveal these metabolic changes in experimental diabetic rats and to evaluate their significance in the drug metabolism.

Decreased intestinal CYP3A mediated metabolism in spite of the statistically unaltered total CYP content resulted, which suggests either posttranslational regulation of the enzyme via covalent down-regulation (e.g. phosphorylation) or a change in the intestinal isoenzyme composition. Insulin may be involved in the intestinal CYP3A regulation since inverse correlation was found between the blood glucose concentration (as a marker for insulin level) and the CYP3A function. The hepatic total CYP content and the hepatic CYP2E1 and FMO3 gene expression and function were seen to change remarkably in untreated long-term diabetes and following insulin treatment. Our study concentrated on rat hepatic CYP2C11, CYP2C13, CYP2C22 and CYP2C23 isoforms and reduced gene expressions with the exception of CYP2C23 were found in diabetes, which is explained, by its different physiological role and regulation. The mRNA level of CYP2C11 and CYP2C13 isoforms were sensitive to insulin showing the role of insulin in their regulation. The study resulted in unaltered CL_{int} of the CYP2C substrate; diclofenac in either insulin treated or untreated diabetic rats. Similarly, unchanged biotransformation of the cholinesterase reactivator oxime, K-48 was seen in diabetes. These results suggest no influence of diabetes and particularly compensated diabetes on the metabolism of the two drugs investigated. The *in vitro* and *in vivo* metabolism studies of K-48 resulted in a weak metabolism. None of the *in silico* predicted metabolites but the K-48 was found in serum, CSF and brain while an epoxide metabolite was detected in urine. The presence of K-48 in the brain shows a moderate penetration of K-48 to the CNS.

INTRODUCTION

Sex, age, nutrition, endogenous compounds (e.g., hormones), genetic polymorphism, other xenobiotics and pathological conditions have an effect on the drug metabolism. Any alteration in the metabolising capacity of cytochrome P450 enzymes in the liver and intestine may increase the incidence of altered biotransformation and bioavailability of the p.o. administered drugs. Insulin dependent diabetes mellitus (IDDM) produces changes in the cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) expression and function in the intestine and in the liver. Streptozotocin (STZ) induced experimental diabetes is a widely used model for the investigation of CYP and FMO mediated metabolism in IDDM. Its effects on the intestinal metabolism are much less represented in the literature than that of hepatic metabolism. It is generally accepted that the alterations usually up-regulations of cytochrome P450 enzyme system in both organs are due to metabolic and hormonal changes associated with diabetic state. Insulin may also play a direct or an indirect role -via insulin signalling pathway- in the regulation of these enzymes because most changes are either fully or partially restored following insulin treatment. CYP2C11 and CYP2C13 are down-regulated in experimental diabetes. The sex-dependent expression of these isoforms is known and their regulation by pituitary hormones are also suggested in diabetes. The changes of CYP2C22 and CYP2C23 in diabetes are much less reported. It is known that the regulation of CYP2C22 is similar to CYP2C11 in that it is also sensitive to gonadal hormones. The CYP2C23 has different regulation and it is rather involved in the metabolism of arachidonic acid than that of steroid hormones.

The effect of altered CYP mediated metabolism in diabetes on clinically used or newly developed drugs is underinvestigated. In the process of drug research and development the screening of drug safety is very important. Therefore, there is no need to emphasize how important to reveal the possibile drug metabolism changes due to e.g. pathological conditions such as diabetes.

The nonsteroidal anti-inflammatory drug, diclofenac was reported to have an increased AUC, decreased CL_{int} and V_{ss} following intravenous administration in diabetes which according to the suggestion of the authors is the result of the decreased CYP2C11 mediated metabolism. The other compound tested was K-48 which is a promising newly synthesized pyridinium aldoxime cholinesterase reactivator for organophosphate intoxication. The metabolism of diclofenac and K-48 was investigated in the diabetic state in this study.

RESEARCH OBJECTIVES

Our main goal was to study the small intestinal CYP function and regulation, and the influence of changed hepatic CYP mediated metabolism of the two selected compounds, diclofenac and K-48 in diabetic state. For this purpose we induced experimental diabetes on rats using streptozotocin a diabetogenic agent. Rats were divided into 3 groups in both short-term and long-term diabetic experiments: 1. control, 2. STZ-induced diabetic 3. insulin treated diabetic. Changes in CYP and FMO function and mRNA expression were determined using marker reactions for the isoenzymes and quantitative RT-PCR, respectively. The enzyme kinetic parameters (K_M, V_{max}) of diclofenac 4'hydroxylase were determined to reveal the influence of the diabetic state on the diclofenac metabolism. The biotransformation of K-48 was investigated by *in silico*, *in vitro* and *in vivo* methods.

The objectives were as follows:

1. How does the diabetic state influence the intestinal P450 mediated metabolism?

2. Is there any correlation between intestinal CYP3A catalytic activity and blood glucose concentration?

3. How does long-term diabetes affect the cytochrome P450 mRNA expression and catalytic activity?

4. Is the altered cytochrome P450 mediated metabolism sensitive for a nine day period insulin treatment?

5. Is the mRNA expression of CYP2C11 also reflected in the protein expression and catalytic activity in treated and untreated diabetes?

6. Have diabetes and insulin treatment any effect on the other CYP2C isoenzymes?

7. Does long term diabetes modify the enzyme kinetic parameters of diclofenac 4'-hydroxylase?

8. Does the metabolism of diclofenac change in diabetic state according to *in vitro* prediction?

9. Has diabetes any effect on the metabolism of K-48?

10. Do the *in silico* predicted metabolites of K-48 also appear in microsomal metabolism studies?

11. Can K-48 or its metabolite be found in rat serum, CSF and urine following i.m. injection?

12. Is it possible for K-48 or its metabolite to enter the brain?

METHODS

Animals and induction of diabetes

	Intestinal metabolism study (shor- term diabetes)	*Hepatic metabolism study (long-term diabetes)*
Animals	male Sprague-Dawley rats	male Sprague-Dawley rats
STZ administration (0th day)	i.p. 70 mg/kg in 0.1M citrate buffer, pH 6.0	i.v. 55 mg/kg in 0.1M citrate buffer, pH 6.0
Outset of insulin treatment	5th day	20th day
Duration of insulin treatment	14 days	9 days
Groups	control STZ treated diabetic (D70), insulin treated diabetic (ID70)	control STZ treated diabetic (D55), insulin treated diabetic (ID55)

Preparation of intestinal and hepatic microsomes
After isolating the liver and the small intestine they were homogenised in a Tris-HCl buffer (0.1 M, pH 7.4) containing 1.15 % KCl. Individual rat liver and intestinal microsomes were prepared by differential centrifugation. The microsomal pellets obtained were resuspended in the same buffer. The microsomal protein content was measured by the method described by Lowry.

Determination of cytochrome P450 content
CYP was essentially determined spectrophotometrically using the method described by Greim.

Enzymatic assays
CYP and FMO isoenzyme activities were determined using phenacetin O-deethylase (CYP1A), aminopyrine N-demethylase (CYP2B/3A), tolbutamide 4'-hydroxylase (CYP2C), mephenytoin 4'-hydroxylase (CYP2C), bufuralol 1'-hydroxylase (CYP2D), chlorzoxazone 6-hydroxylase (CYP2E1), testosterone 6β-hydroxylase (CYP3A) and benzydamine N-oxygenase (FMO) marker reactions and the metabolites were measured by HPLC-UV or spectrophotometer.

Determination of CYP and FMO mRNA expression

RNA isolation was carried out using the RNeasy Mini Kit. Full-length first strand cDNA was synthesized using "Superscript III First-Strand Synthesis SuperMix for qRT PCR" with a random primer and total RNA. Specific amplifications of cDNA were performed using TaqMan® Gene Expression Assays.

Western blot analysis

Tris-Glycine PAGEr GOLD Precast Gel and Immobilon-P PVDF membrane were used for the analysis of CYP2C11 protein level in rat liver microsomes. Primary labelling was carried out with rabbit polyclonal anti-Cytochrome P450 2C11 (1:1500) and goat Actin C-11 (1:200) antibody as a standard. Biotynilated anti-rabbit (1:2000) and anti-goat IgG (1:2000) were used as secondary antibodies. DAB Substrate kit was used for development.

Determination of enzyme kinetic parameters

Enzyme kinetic parameters for diclofenac 4'-hydroxylase were determined. The incubation time and microsomal protein content were in the linear range of metabolite formation and the substrate concentration was increased in 1.56-100 µM range. The kinetic constants (K_M, V_{max}) were calculated using Lineweaver-Burk plot by linear regression. The CL_{int} was calculated by using V_{max}/K_M formula.

K-48 metabolism studies

K-48 metabolism and lipophilicity were *in silico* predicted by the Pallas Program. In *in vitro* studies control and diabetic rat liver microsomes from long-term diabetic model were used. In the *in vivo* experiments 50 µM K-48 was i.m. administered to male rats. Serum, CSF, brain and urine samples were collected and K-48 and its metabolites were measured by HPLC-ECD or HPLC-MS.

Data analysis

Statistical analysis, mean, S.D. and correlation coefficient were calculated by GraphPad Prism 4.0.

RESULTS

Changes in physical and biochemical parameters in diabetic rats

Control rats consistently had blood glucose levels of approximately 100 mg/dL, while rats receiving STZ displayed about a five-fold increase in blood glucose concentration. Diabetic rats had an observably reduced body weight and wet liver weight compared to the control. Insulin treatment of diabetic animals only partially compensated for the rise of blood glucose in both short-term and long-term diabetes experiments and only the decrease in the long-term study was significant. The body weight restored only partially while wet liver weight was fully compensated following insulin administration which resulted in an enhanced relative wet liver weight.

Effect of diabetes and insulin treatment on intestinal cytochrome P450s

A significant (50%) decrease was seen in the intestinal testosterone 6ß-hydroxylase activity (CYP3A) in diabetic rats, while the total P450 content did not change statistically. The CYP3A activity was sensitive to insulin administration and increased by 40 % in comparison to 70 mg/kg STZ induced diabetic animals. The turnover number of CYP3A (calculated with total CYP content) mediated activity was reduced by 74 % and 54 % in diabetic and insulin treated diabetic rats, respectively. The regression analysis between blood glucose level and intestinal CYP3A isoenzyme activities resulted in inverse correlation (r= 0.6787; p= 0.0054; n=14).

Effect of diabetes and insulin treatment on hepatic cytochrome P450s and flavin-containing monooxygenases

Gene expression of CYP and FMO isoforms

The most remarkable changes in the hepatic CYP2E1 and FMO3 mRNA expression were seen. They resulted in a 2.2- and 3-fold increase, respectively. The tendency of both isoenzymes was to

reduce to the control level following insulin treatment, but only the decrease of the CYP2E1 expression was significant, however, neither differed statistically from control. The diabetes induced a decrease in the CYP2D2 gene expression (55 %) which did not increase greatly after insulin treatment. The other isoenzymes (CYP1A, CYP2B, CYP3A and FMO1) did not show considerable changes in diabetes. However, the CYP1A2 and CYP3A1 gene expressions were significantly reduced in insulin treated diabetes.

Gene expression of CYP2C isoenzymes
The mRNA expression of CYP2C11, CYP2C13 and CYP2C22 presented a significantly lower level in diabetic rats. The most evident decrease was seen at the gene expression of CYP2C11 (95 %). No substantial loss was noticed at the mRNA level of CYP2C23. Insulin treatment tended to compensate for the decrease of CYP2C11 and CYP2C13 mRNA expression. There was only a slight or no effect of insulin treatment on the gene expressions of CYP2C22 and CYP2C23, respectively.

Western blot analysis of CY2C11
CYP2C11 protein was highly expressed in the control animals, but protein was undetectable in the diabetic group. This result was in accord with the decreased CYP2C11 gene expression in diabetes. The insulin treatment seemed to slightly increase the protein level; however, it was considerably lower in comparison to the control group.

Total P450 content and hepatic CYP and FMO function
The hepatic total CYP content, the chlorzoxazone 6-hydroxylase (CYP2E1) and benzydamine N-oxygenase (FMO) activites elevated by 1.3-, 3.3- and 1.7-fold, respectively. In insulin treated diabetic animals, the total CYP content, CYP2E1 and FMO activities completely restored to control level, moreover, CYP content and FMO function resulted in a significantly lower level than that of control. The CYP2C activities measured by 4'-hydroxy tolbutamid and 4'-hydroxy mephenytoin formation showed no

statistically relevant changes in diabetes; however, the activity of mephenytoin 4'-hydroxylase reduced significantly by 42 % following insulin administration in comparison to control.

Changes in diclofenac metabolism in diabetes
The K_M and V_{max} values were determined at the formation of 4'-hydroxy diclofenac with rat liver microsomes prepared from control, diabetic and insulin treated diabetic animals. The K_M and V_{max} values increased in diabetic group in comparison to control and were not fully restored following insulin treatment, although they did not significantly differ from control. These changes did not appear in intrinsic clearance (CL_{int}) calculated since CL_{int} did not differ in the three investigated groups. Based on these *in vitro* results enzymatic changes in diabetes do not induce alteration in diclofenac biotransformation.

Result of K-48 metabolism study
The metabolism of K-48 was moderate, only approximately 15-20 % decrease was found with control and diabetic liver microsomes, suggesting no substantial alteration in K-48 biotransformation in diabetes. A hydroxy metabolite of K-48 was identified by HPLC-MS analysis. Various body compartments (serum, CSF, urine and brain) of rats were subjected to HPLC-MS analysis after i.m. administration of K-48. The parent compound and none of the *in silico* predicted metabolites were found in the serum, CSF and in the homogenate of the brain. At the same time, no unchanged K-48 could be detected, but presumably an epoxide metabolite was eliminated by urine. The low concentration of K-48 in the brain showed only a moderate, but some penetration of K-48 to the CNS.

CONCLUSION

1. It was revealed that diabetic state has an effect on the intestinal CYP mediated metabolism. Reduced CYP3A mediated metabolism in spite of the statistically unaltered total CYP content resulted in STZ induced diabetes, which can be explained either by posttranslational regulation of the enzyme via covalent down-regulation (e.g. phosphorylation) or by a change in the intestinal CYP enzyme composition. The intestinal CYP3A activity was sensitive to insulin administration.

2. Inverse correlation was found between blood glucose concentrations (regarded as a marker for insulin level) and CYP3A function suggesting the involvement of insulin in the intestinal CYP3A regulation.

3. Long-term, 28 days diabetes induced the most remarkable increase in hepatic total CYP content, and in hepatic CYP2E1 and FMO3 gene expression and function and a significant decrease in CYP2C11 and CYP2D2 mRNA expression. The CYP1A2, CYP2B, CYP3A1, CYP3A2 and FMO1 did not show statistical alteration either in expression or in function in diabetes.

4. Hepatic total CYP content and CYP1A2, CYP2E1, CYP3A1 gene expression significantly increased while CYP2C11 gene expression were decreased following a nine day period insulin administration. The catalytic activities of FMO and CYP2E1 were the most sensitive to insulin and their function resulted in a lower level in comparison to control after insulin treatment.

5. It was shown that the CYP2C11 gene expression is not in concert with the tolbutamide and mephenytoin 4'-hydroxylase activity, although the decreased gene expression in untreated

diabetes was also reflected in reduced CYP2C11 protein level. The insulin also restored the CYP2C11 mRNA and protein level.

6. It was further revealed that insulin treated and untreated diabetic state had an effect on the gene expression of CYP2C13 and CYP2C22, however, the CYP2C23 isoenzyme did not show any alteration. We suggested that the different attitude of CYP2C23 gene may be due to its different physiological function (arachidonic acid metabolism) and regulation.

7. In spite of altered (increased) enzyme kinetic parameters (K_M, V_{max}) of diclofenac 4'-hydroxylase in diabetic state the intrinsic clearance calculated remained unchanged. The changed *in vivo* pharmacokinetics of diclofenac in diabetes published might be explained by the differences between the *in vivo* and *in vitro* effective dose.

8. We found that changes in CYP2C enzymes in diabetes do not bring about alteration in the biotransformation of diclofenac which may suggest the redistribution of metabolic pathways.

9. K-48 showed only a moderate biotransformation (15-20 %) with both diabetic and control rat liver microsomes which suggests that diabetes does not affect the metabolism of K-48.

10. In *in vitro* microsomal metabolism studies only a hydroxyl metabolite of the pyridinium aldoxime cholinesterase reactivator, K-48 was identified. No other metabolites predicted *in silico* were seen *in vitro*.

11. It was demonstrated that only the parent compound, K-48 was found in serum, CSF and brain. Metabolites were absent in all three compartments. We did not find any unchanged K-48 in rat urine; presumably an epoxide metabolite could be identified by HPLC-MS.

12. Low concentration of K-48 in the brain homogenate shows a very poor penetration of K-48 to the CNS.

PUBLICATIONS

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1. <u>Benkő Bernadett</u>, Borbás Tímea, Dalmadi Balázs, Szeberényi Szabolcs, Leibinger János, Tihanyi Károly: Intesztinális gyógyszermetabolizmus jelentősége és stabil citokróm P450 tartalmú mikroszóma preparálása patkány vékonybélből. *Ph.D. TUDOMÁNYOS NAPOK 2003*, 2003. április 10-11., Budapest
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