CYP1B1 mRNA inducibility due to benzo(a)pyrene is modified by the CYP1B1 L432V gene polymorphism

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Benzo(a)pyrene (BaP), a primary component of tobacco smoke, is activated by cytochrome P450 1B1 (CYP1B1). Smokers homozygous for the C-allele (*1/*1) at the CYP1B1 Leu432Val polymorphism have shown increased CYP1B1 expression, compared to smokers homozygous for the G-allele *3/*3. Since no difference has been shown in CYP1B1 expression between both genotypes in non-smokers, we assumed that the genetic impact is produced in combination with an exogenous induction (e.g., BaP). To confirm this theory and to quantify the effect, we induced human leucocytes with increasing BaP concentrations and determined CYP1B1 mRNA expression with real-time polymerase chain reaction (PCR). We incubated human leucocytes from 27 healthy donors with BaP concentrations ranging from 2.5 to 250 μM. We identified the CYP1B1 genotypes by melting curve analysis and assessed relative CYP1B1 mRNA expression using real-time PCR. Expression was related to β-2-microglobulin with the ΔΔCt method. Inducibility of CYP1B1 mRNA by BaP was higher in leucocytes carrying the CYP1B1*1/*1 genotype than in leucocytes carrying the CYP1B1*3/*3 genotype (P = 0.012). We revealed significant differences, with BaP concentrations of 2.5 μM (P = 0.0094), 5 μM (P = 0.027), 10 μM (P = 0.0006), 25 μM (P = 0.0007) and 50 μM (P = 0.017). Homozygous carriers of the C-allele (*1/*1) at the CYP1B1 Leu432Val polymorphism show a higher response to environmental factors, such as carcinogenic BaP, than homozygous carriers of the G-allele *3/*3.

Introduction

The majority of environmental compounds require metabolic activation by the cytochrome P450 mono-oxygenase system (CYP) in order to exert a carcinogenic effect (1). Cytochrome P450 1B1 (CYP1B1) is a Phase I enzyme belonging to a multi-gene super family of monomeric, mixed-function mono-oxygenases (2). It is involved in the activation of a broad spectrum of pro-carcinogens, e.g. components of tobacco smoke, such as polycyclic aromatic hydrocarbons (PAHs), of which benzo(a)pyrene (BaP) is typical. CYP1B1 gene expression is induced through the aromatic hydrocarbon receptor, which is activated by PAHs (3,4). CYP1B1 is expressed in many normal human tissues, including peripheral blood cells (5), and can easily be determined in peripheral blood leucocytes (6). When analysing the complexity of gene–environment interactions at the molecular level, gene polymorphisms should be considered. While gene mutations of high penetration often result in a high cancer risk (7), polymorphisms of genes with low penetration (such as CYP1B1 L432V) are thought to predispose an individual to risk if that person is exposed to carcinogenic chemicals (8). The CYP1B1 Leu432Val (rs1056836) polymorphism causes an amino acid exchange, and the C-allele codes for Leu432 (wild-type), while the G-allele codes for Val432 (variant). In this study, we used the designations CYP1B1*1 and CYP1B1*3 to denote the CYP1B1 wild-type and variant alleles, respectively, and CYP1B1*1/*1 and CYP1B1*3/*3 to denote the homozygous wild-type and variant genotypes, respectively. Epidemiological studies have described an influence of the CYP1B1 Leu432Val gene polymorphism (e.g. increased susceptibility to lung cancer), so we were interested in the reason for this effect. In addition, an association between the CYP1B1 Leu432Val gene polymorphism and a higher catalytic activity has also been described. The allele frequency of CYP1B1*3 is ~0.42, and although CYP1B1 expression has frequently been investigated as a biomarker, the CYP1B1 L432V polymorphism has rarely been considered. Our decision to examine this polymorphism in this study was primarily based on the results of epidemiological association studies, in which smokers homozygous for the C-allele (*1/*1) at the CYP1B1 Leu432Val polymorphism showed increased CYP1B1 expression, compared to smokers homozygous for the G-allele *3/*3 (9,10). Non-smokers did not show a difference in CYP1B1 expression between the genotypes. Therefore, we assumed that the genetic impact is produced in combination with an exogenous induction (e.g., PAHs). To confirm this theory and quantify the effect, we induced human leucocytes with increasing BaP concentrations and determined CYP1B1 mRNA expression with real-time polymerase chain reaction (PCR). We compared the CYP1B1 inducibility of both homozygote CYP1B1 Leu432Val genotypes in human leucocytes.

Materials and methods

Subjects

The study population consisted of a total of 27 healthy, non-smoking Caucasians, of whom 16 had homozygote wild-type genotypes (CYP1B1*1/*1) and 11 had homozygote variant genotypes (CYP1B1*3/*3). The participants were Universitätsklinikum Giessen und Marburg GmbH personnel and students of the Justus-Liebig-Universität Giessen, as well as patients recruited at the outpatient clinic of the Institute for Occupational and Social Medicine, Giessen, Germany. They had already taken part in a previous study and were again willing to be blood donors for this investigation. To quantify inducibility of CYP1B1 by BaP, we included only homozygote non-smoking individuals.

We interviewed all participants via a standardised questionnaire in order to obtain information regarding their lifestyle, including smoking habits, alcohol consumption, indoor air pollution (coal heating and cooking), eating habits, lifetime occupational history and disease history. The group consisted of 11 males and 16 females, and we excluded smokers.

The ethics committees of the university hospital, Giessen, Germany, approved the study (AZ 103/05).
Isolation of peripheral blood mononuclear cells

We diluted heparinised venous blood 1:1 in sterile Hanks’ balanced salt solution (Gibco®, layered on Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, UK) and centrifuged this for 30 min at 2250 xg, 20°C. We isolated peripheral blood mononuclear cells from the interface and washed them twice in Hanks’ balanced salt solution by centrifugation at 400 xg for 15 min. Subsequently, we performed erythrocyte lysis with PharmLyseTM erythrocyte lysis buffer (BD Biosciences®). We resuspended the pellet in 10 ml of RPMI 1640 with antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin; Gibco®), 5 μl β-mercaptoethanol (Mercck®) and 10 ml of human AB Serum.10 Mio cells were plated in 25-cm² culture flasks (Sarstedt®) with 10 ml of PPFF media for 24 h, after which we incubated them for 6 h with different concentrations of BaP: 2.5; 5; 10; 25; 75; 125 and 250 μM (Sigma-Aldrich®). We maintained cell cultures at 37°C in 5% CO₂ and 95% relative humidity until harvesting. We established controls using pure media alone and media plus dimethyl sulphoxide (DMSO).

Cell viability evaluation

We evaluated cell viability using PrestoBlue™ reagent. We incubated 100 000 cells in 90-μl media for 6 h with increasing concentrations of BaP. We added 10 μl of PrestoBlue™ to each sample and detected fluorescence at 570 and 600 nm after 20 min. Media alone (C), DMSO alone (DMSO) and cells plus media (Ccell) served as controls, and we measured all samples three times.

Real-time PCR and polymorphism detection

We isolated genomic DNA from whole blood (ca. 3 ml) using the Versagen® DNA Purification Kit (Genentra Systems, Minneapolis, MN, USA). The CYP1B1 Leu432Val (rs1056836) polymorphism causes an amino acid exchange. The C-allele codes for Leu432 and the G-allele codes for Val432. In this study, we evaluated all participants for the presence of the CYP1B1 Leu432Val (rs1056836 SNP) polymorphism only. We defined the C-allele as CYP1B1*1 and defined the G-allele as CYP1B1*3. For further CYP1B1 nomenclature, refer to the home page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (www.cypalleles.ki.se/). We detected the CYP1B1 Leu432Val polymorphism using a LightCycler® System, as described (6).

RNA extraction and quantitative PCR

We isolated the RNA of peripheral blood mononuclear cells with TRI-Reagent (Sigma-Aldrich®), according to the manufacturer’s protocol. We conducted all assays three times as independent PCR runs for each complementary DNA sample (6) and always related gene expression to expression of β-2-microglobulin as a housekeeping gene (11). We calculated expression using the 2-ΔΔCT method (12), and the primer sequences and PCR conditions were as published (6). We carried out all measurements without information regarding the origin of the samples.

Statistical analysis

We performed all statistical analyses using the statistical software package, SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The results are expressed as mean ± standard error of the mean. We analysed means in different subgroups using the Student’s t-test. In addition, we used analysis of variance (ANOVA) for analyses of statistical differences between groups. We regarded a value of P < 0.05 as significant.

Results

Viability

To determine the toxic BaP concentration, we evaluated cellular viability by analysis of cellular PrestoBlue™ reagent, and the results are shown in FIGURE 1. BaP concentrations of 100 μM and more revealed lower cell viability values, suggesting that BaP toxicity starts at concentrations higher than 100 μM.

CYP1B1 mRNA expression

To investigate the functional impact of the CYP1B1 allelic variants on the CYP1B1 expression, we induced leukocytes carrying the homozygote genotypes (CYP1B1*1/*1 and CYP1B1*3/*3) for the CYP1B1 Leu432Val polymorphism with increasing concentrations of BaP: 2.5; 5; 10; 25; 50; 75; 125 and 250 μM. Comparison of the means ± standard error of the relative CYP1B1 mRNA expression of the homozygote genotypes CYP1B1*1/*1 and CYP1B1*3/*3 revealed significant differences, with the exception of the BaP concentrations >75 μM (see Table 1).

In particular, CYP1B1 induction with BaP concentrations ranging from 2.5 to 50 μM revealed significant differences between the homozygote genotypes. The two highest BaP concentrations (125 and 250 μM) showed decreasing CYP1B1 mRNA expression, which is probably due to the toxic effects.
of BaP, as observed by decreasing viability at concentrations of >100 μM (see Figure 1).

The relative CYP1B1 expression of the CYP1B1*1/*1 genotype was generally higher than that of the CYP1B1*3/*3 genotype (see Figure 2). Testing the data with repeated measures ANOVAs also revealed significant differences between the two homozygote genotypes (P = 0.012).

Discussion

In this study, we demonstrated an influence of the CYP1B1 Leu432Val polymorphism on CYP1B1 mRNA expression induced by BaP in human leucocytes. In a previous study, we described significant higher CYP1B1 mRNA in non-smokers and smokers, carrying the wild-type C-allele (CYP1B1*1 allele) (9,10), and, in a larger study, in which we quantified the smoking habit by measuring the serum cotinine concentration, instead of questioning the participants, we found significantly higher CYP1B1 mRNA expression only in smokers carrying the CYP1B1*1/*1 genotype (10). Since there was no difference in CYP1B1 expression between the genotypes in non-smokers, we concluded that basic CYP1B1 expression is independent of genotype. Our results also implied that the genetic influence (the CYP1B1 polymorphism) is evident only via an induction of CYP1B1 expression (e.g. due to smoking) because we found significantly higher CYP1B1 expression in smokers, carrying the CYP1B1*1/*1 genotype. This was verified when leucocytes of non-smokers incubated with BaP, a key component of PAHs, showed a significantly higher response (CYP1B1 expression) when carrying the CYP1B1*1/*1 genotype than when carrying the CYP1B1*3/*3 genotype. This confirms our hypothesis that the CYP1B1 Leu432Val polymorphism modulates the effect of exogenous activators of the aromatic hydrocarbon receptor signalling cascade.

Evidence to support this theory can be found in a study by Hanaoka et al. (5), who described a positive correlation between CYP1B1 mRNA in blood cells and urinary 1-hydroxypyrene as an indicator of internal exposure to PAHs. However, this correlation was significant only in individuals with the *1/*1 genotype of the CYP1B1 Leu432Val polymorphism.

Shimada et al (13) examined the effects on catalytic properties of four polymorphic human CYP1B1 allelic variants. The two types of allelic variants with amino acid replacements of Ala by Ser at codon 119 and Leu by Val at codon 432 showed in a Salmonella typhimurium NM2009 umu response system a slightly higher (1.2- to 1.5-fold) catalytic activity to form reactive metabolites from pro-carcinogens (13). In contrast, Landi et al. (14) found that the CYP1B1*3 haplotype in particular was associated with a higher increase in CYP1B1 mRNA expression in vitro. When mitogen-treated lymphocytes were stimulated with 10 nM of 2,3,7,8-tetrachlorodibenzo-p-dioxin for 72h, CYP1B1 and CYP1A1 mRNA were strongly induced and were especially modified by CYP variant alleles. Mitogens lead to the proliferation of lymphocytes, and this occurs concomitantly with an activation of several cell signalling pathways and increases in gene transcription. Regardless, housekeeping genes are used for normalisation, and expression levels of CYP1B1 can be higher in mitogen-stimulated cells than in non-stimulated cells (15). Laroche-Clary et al. (16) showed that the L432V polymorphism was a major determinant of cell sensitivity to numerous drugs in a National Cancer Institute human tumour cell line panel. Cell lines homozygous for the V432 allele were more resistant (2-fold) to alkylating agents and camptothecins (4.5-fold), compared to cell lines homozygous for the L432 allele.

Ablation of the three members of the CYP1 family results in a profound phenotypic alteration, while the removal of (at least) the CYP2 and CYP3 subfamily clusters does not have the same effect (17). Therefore, a deletion of the CYP1B1 gene would probably lead to phenotypic changes. We checked the PCR products of all participants in our study by running them on agarose gel. The products were all the same size, and the melting curve showed the amplification of only one. The CYP1B1 gene and its promoter region have
been extensively investigated (18), but linkage disequilibrium with any polymorphism within the promoter of \textit{CYP1B1} has not been described to our knowledge. It has been suggested that post-transcriptional mechanisms are involved in \textit{CYP1B1} regulation; therefore, the genetic impact on the expression pattern of \textit{CYP1B1} is only one contributing factor (19–21). Regardless, further studies should consider the \textit{CYP1B1} Leu432Val polymorphism when investigating gene, as well as protein, expression of \textit{CYP1B1}.

Since \textit{CYP1B1-null} mice are protected against PAHs-induced tumours in most tissues, it has been suggested that CYP1B1 is required for metabolic activation and mediates the carcinogenicity of PAHs (22). Therefore, our findings support the theory that the \textit{CYP1B1}*3 allele has a modifying effect.

**Conclusion**

In conclusion, we demonstrated that the \textit{CYP1B1}*3/*3 genotype is associated with lower \textit{CYP1B1} mRNA expression than the \textit{CYP1B1}*1/*1 genotype after induction with BaP.

Conflict of interest statement: None declared.

**References**