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Maternal intake of quercetin during gestation alters *ex vivo* benzo[*a*]pyrene metabolism and DNA adduct formation in adult offspring

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Variation in xenobiotic metabolism cannot entirely be explained by genetic diversity in metabolic enzymes. We suggest that maternal diet during gestation can contribute to variation in metabolism by creating an *in utero* environment that shapes the offspring's defence against chemical carcinogens. Therefore, pregnant mice were supplemented with the natural aryl hydrocarbon receptor (AhR) agonist quercetin (1 mmol quercetin/kg feed) until delivery. Next, it was investigated whether the adult offspring at the age of 12 weeks had altered biotransformation of the environmental pollutant benzo[*a*]pyrene (B[*a*]P). *In utero* quercetin exposure resulted in significantly enhanced gene expression of *Cyp1a1*, *Cyp1b1*, *Nqo1* and *Ugt1a6* in liver of foetuses at Day 14.5 of gestation. Despite cessation of supplementation after delivery, altered gene expression persisted into adulthood, but in a tissue- and gender-dependent manner. Expression of Phase I enzymes (*Cyp1a1* and *Cyp1b1*) was up-regulated in the liver of adult female mice *in utero* exposed to quercetin, whereas expression of Phase II enzymes (*Gstp1*, *Nqo1* and *Ugt1a6*) was predominantly enhanced in the lung tissue of female mice. Epigenetic mechanisms may contribute to this adapted gene expression, as the repetitive elements (SINEB1) were hypomethylated in liver of female mice prenatally exposed to quercetin. Studies on *ex vivo* metabolism of B[*a*]P by lung and liver microsomes showed that the amount of B[*a*]P-9,10-dehydrodiol, B[*a*]P-7,8-dihydrodiol and 3-hydroxy-B[*a*]P did not change, but the amount of unmetabolised B[*a*]P was significantly lower after incubation with lung microsomes from offspring that received quercetin during gestation. Moreover, *ex vivo* B[*a*]P-induced DNA adduct formation was significantly lower for liver microsomes of offspring that were exposed to quercetin during gestation. These results suggest that prenatal diet leads to persistent alterations in Phase I and II enzymes of adult mice and may affect cancer risk.

Introduction

A vast amount of research has been performed to determine the effects of genetic polymorphisms in enzymes involved in xenobiotic metabolism on interindividual variation in cancer susceptibility (1–4). However, analysis of genetic polymorphisms did not explain a large part of the variation and other factors, especially the diet, have been postulated as additional contributors to individual reactions after exposure to xenobiotics (5). For instance, intake of dietary flavonoids is common in the human diet as they are found in fruit, vegetables and beverages, such as wine, tea and coffee. They are mostly known for their anticancer properties, as they are potent antioxidants (6). Quercetin is the most widely consumed flavonoid in the diet. This polyphenolic compound is not only a potent free radical scavenger and iron chelator (7,8) but can also modulate the expression and activity of cytochrome P450 (CYP450) and Phase II detoxifying enzymes (9–11). The expression of Phase I and II enzymes is important for the metabolism of many xenobiotics, including benzo[*a*]pyrene (B[*a*]P). B[*a*]P is produced during the incomplete combustion of organic compounds like tobacco, fuels, wood and meat (12–14). On its own, B[*a*]P has no mutagenic or carcinogenic properties (10,15). However, it can diffuse into the cell due to its lipophilic character, bind to the aryl hydrocarbon receptor (AhR) and translocate into the nucleus. Here, AhR heterodimerizes with the aryl hydrocarbon receptor nuclear translocator and this complex binds to xenobiotic responsive elements, leading to the transcription of genes of Phase I and II enzymes (10,16). The metabolic activation of B[*a*]P is thought to be principally performed by cytochrome P450 (CYP450) enzymes (CYP1A1 and CYP1B1), resulting in the formation of the reactive B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE), leading to DNA adduct formation and subsequent mutations, thus increasing the risk of cancer. Although it is known that quercetin prevents B[*a*]P-induced DNA damage, there is a disagreement about the underlying mechanism (10,17–20). Similar to B[*a*]P, quercetin is a potent ligand for AhR (9) and could thus alter the expression of enzymes involved in both activation as well as detoxification of B[*a*]P.

A diet rich in fruit and vegetables and therefore containing high amounts of flavonoids is thought to protect against cancer development (6). However, the time point of exposure to these flavonoids can also contribute to the interindividual differences (21). Without any doubt, maternal diets can affect foetal programming by inducing epigenetic alterations that can lead to long-lasting changes in gene expression (21,22). Due to altered genetic imprinting, individuals may acquire distinct responses to carcinogenic exposures and may therefore have different risks for developing DNA damage and cancer. The foetal origin of adult cancer needs more attention.

Therefore, in this study, we aim to investigate whether gene expression of B[*a*]P metabolising enzymes can be modulated by prenatal exposure to quercetin [1 mmol (302 mg) quercetin/kg feed] via the maternal diet, starting 3 days before conception

until delivery. At the adult age of 12 weeks, male and female offspring mice were sacrificed and Phase I and II gene expression was determined. Direct effect of quercetin was investigated by determining gene expression levels of Phase I and II enzymes in the liver of foetuses at Day 14.5 of gestation. B[*a*]P metabolism and DNA adduct formation were determined *ex vivo* using S9 mix of liver and lung tissue of 12-week-old male and female offspring.

Material and methods

Mice and sample collection

Mice (129/SvJ:C57BL/6J background) ~8 weeks of age, received either normal chow (n = 8, low phytoestrogen content complete feed for mice breeding; ssniff®, Soest, Germany) or the same chow (ssniff®) supplemented with quercetin [n = 8, 1 mmol (302 mg)/kg feed] (Sigma, Zwijndrecht, The Netherlands) from 3 days before conception until the end of gestation. Male mice were placed in the cage only for the duration of copulation. After delivery, all mothers and pups received normal chow. Offspring mice were anaesthetised and sacrificed by cardiac puncture at 12 weeks of age, and liver and lung were removed and frozen at −80°C until analysis.

To study the direct effects of the maternal diet on foetal Phase I and II gene expression, a different group of mice were mated overnight. The presence of a vaginal plug the next morning was defined as 0.5 day post-conception. On Day 14.5 of gestation, mice were sacrificed to isolate the foetuses and their livers, as AhR mRNA and protein expression peaks between gestational Days 14 and 16 (23).

Quantitative real-time polymerase chain reaction

The liver of foetuses and one-half of the lung and liver of 12-week-old mice were homogenised, using the Ultra-Turrax homogeniser (IKA, Staufen, Germany). Next, RNA was isolated using TRIzol Reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions.

Using iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA), 1 μg RNA was transcribed into complementary DNA (cDNA). An aliquot of one-tenth of the resulting cDNA was applied for quantitative polymerase chain reaction (PCR) amplification together with 12.5 μl SYBR Green (Qiagen, Venlo, The Netherlands) and 7.5 pmol of each primer (see Table I; Eurogentec, Maastricht, The Netherlands) in a volume of 25 μl. The reactions were carried out using a MyiQ single color RT–PCR detection system (Bio-Rad) under the following conditions: 1 cycle at 95°C for 3 min and then 40 cycles at 95°C for 15 sec and 60°C for 1 min followed by 1 cycle at 95°C for 1 min and 1 cycle at 65°C. Data were analysed by MyiQ Software system (Bio-Rad), *C*t values were normalised for an endogenous reference gene (β-actin) compared with the calibrator (i.e. average *C*t value of control samples) and expressed as fold change ($2^{-\Delta\Delta Ct}$).

Methylation-sensitive McrBC real-time PCR assay of foetuses and adult mice

Analysis of methylation patterns of the short and long interspersed nucleotide elements (SINEB1, SINEB2 and LINE1) was performed using the methylation-sensitive McrBC real-time PCR assay (24). One microgram of genomic DNA was digested overnight at 37°C using 10 U of McrBC (New England Biolabs, Beverly, MA, USA), an endonuclease that cleaves DNA containing 5-methylcytosine but will not cleave unmethylated DNA. The DNA strand breaks prevent amplification of methylated DNA in the quantitative real-time PCR assay. Two-step quantitative real-time PCR was performed using IQ SYBR Green Supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands) with 4 ng of McrBC-digested DNA and 25 pmol of each primer (Table I; Eurogentec) in a reaction volume of 25 μl. The cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 45 sec, 58°C for 90 sec, using the iCycler (Bio-Rad Laboratories). Data were analysed by MyiQ Software system (Bio-Rad Laboratories), *C*t values were normalised for *C*t values of undigested DNA and subsequently compared to a calibrator (i.e. average *C*t value of five control samples for the adult mice and of four control samples for the foetuses). Results were expressed as relative expression ($2^{-\Delta\Delta Ct}$). An increase in PCR amplification is indicative of hypomethylation, whereas a decrease in PCR amplification products indicates hypermethylation. Alterations in DNA methylation were assessed in foetal liver and adult liver and lung.

High-performance liquid chromatography and fluorescence detection of B[a]P metabolites

To mimic lung and liver biotransformation, an aliquot of S9 mix (4 mg of proteins), obtained from homogenising the other half of the lung and liver of 12-week-old mice in lysis buffer (10 mM Tris, 150 mM KCl, pH 7.4) using the Ultra-Turrax homogenizer (IKA), was incubated with a mixture consisting of 1 μM B[*a*]P in DMSO and a NADPH-generating system [10 mM NADPH, 100 mM glucose-6-phosphate, 40 mM $MgCl_2$, 400 mM Tris (pH 8), 17.8 μg glucose-6-phoshate dehydrogenase and milli-Q in a final volume of 500 μl], without addition of cofactors for Phase II metabolising enzymes. The incubation was carried out at 37°C for 1 h. Afterwards, metabolites were extracted using ethyl acetate, evaporated and dissolved in methanol. Nextly, samples were applied to a high-performance liquid chromatography system equipped with a fluorescence detector (excitation 257 nm and emission 350 nm) and an analytical reversed-phase column (ODS Hypersil, length 2.5 cm, internal diameter 3 mm, particle size 5 μm; Supelco, Bellefonte, PA, USA). Separation was performed with Solvent A (100% methanol) and Solvent B (40% methanol, mobile phase) using the following elution gradient: 30% A and 70% B (0–29 min), 90% A and 10% B (30–35 min), 30% A and 70% B (36–40 min) while the flow rate was maintained at 0.5 ml/min.

^{32}P-postlabelling analysis

To determine the BPDE-induced DNA adduct formation, 10 μg calf thymus DNA (Sigma) was incubated with the S9 mix of lung and liver tissue of 12-week-old mice and 1 μM of B[*a*]P. The DNA was isolated using a phenol–chloroform extraction method and BPDE–DNA adduct levels were determined by the nuclease P1 enrichment technique as described by Reddy and Randerath (25) with the modifications described by Godschalk *et al*. (26). In all experiments, three BPDE–DNA standards with known BPDE–DNA adduct levels (one adduct per 10^6, 10^7 and 10^8 normal nucleotides) were analysed in parallel for quantification purposes. Adduct spots on the chromatograms were quantified using Phosphor-Imaging technology (Fujifilm FLA-3000, Rotterdam, The Netherlands) using the Aida Imager Analyser (Raytest, Milan, Italy) with a detection limit of approximately one adduct per 10^9 nucleotides per individual DNA adduct spot.

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS version 17 for Windows; SPSS Inc., Chicago, IL, USA). Nested analysis of variance (ANOVA) was used to compare the mRNA expression level of Phase I and II enzymes in the liver of foetuses. The non-parametric Mann–Whitney test was used to determine the mRNA expression level of Phase I and II enzymes in the liver and lung of adults, unconjugated B[*a*]P metabolites,

Table I. Forward and reverse primers used for quantitative real-time PCR

	Forward primer (5′–3′)	Reverse primer (5′–3′)
Cyp1a1	TGCCCTTCATTGGTCACATG	CGTCCCCATACTGCTGACTCA
Cyp1b1	AACGCAGCCGGTGATTGT	TGTACCGACAGCCGAAGCA
Comt	AGAGAAGGAGTGGGCCATGA	CCGAATCACTGCATCCATGA
Gstp1	AGAACCAGGGAGGCAAAGCT	CAGGTCCAGCAAGTTGTAATCG
Nqo1	CAGTTCCCATTGCAGTGGTTT	CCTGCTACGAGCACTCTCTCAA
Ugt1a6	GCTACCCCAAAATGATCTGCTT	AATACCATGGGAGCCAGAGTGT
AhR	TGTGCAGAATCCCACATCCG	AATCAAGCGTGCATTGGACTG
β-Actin	ACGGCCAGGTCATCACTATTG	CAAGAAGGAAGGCTGGAAAAGA
SINEB1	GTGGCGCACGCCTTTAATC	GACAGGGTTTCTCTGTGTAG
SINEB2	GAGATGGCTCAGTGGTTAAG	CTGTCTTCAGACACTCCAG
LINE1	TTTGGGACACAATGAAAGCA	CTGCCGTCTACTCCTCTTGG

unmetabolised B[*a*]P and BPDE–DNA adducts formed after incubation with liver or lung S9 mix of adult mice. To avoid litter effects, no more than two adult mice per litter were used for analysis. One-way ANOVA was used to test differences in methylation pattern of SINEB1, SINEB2 and LINE1 between control and quercetin exposed foetuses and adult mice.

Results

Quercetin exposure increased gene expression of Phase I and II enzymes in foetuses

To investigate the effects of quercetin exposure on the foetuses' gene expression of Phase I and II enzymes, mouse foetuses were exposed to quercetin (1 mmol/kg feed) via the maternal diet. Pregnant mice were sacrificed at Day 14.5 of gestation. Phase I and II gene expression levels were examined in the liver of the foetuses ($n = 5$ per diet group). In line with the AhR agonistic action of quercetin, maternal exposure resulted in a significant increase in expression of both Phase I (Cyp1a1: $F_{1,3}$: 22.5, $P = 0.004$ and Cyp1b1: $F_{1,3}$: 76.1, $P < 0.001$; Table II) and Phase II (Gstp1: $F_{1,3}$: 5.2, $P = 0.07$; Nqo1: $F_{1,3}$: 20.5, $P = 0.005$ and Ugt1a6: $F_{1,3}$: 1.8, $P = 0.004$) enzymes, without a statistically significant litter effect. *AhR* gene expression did not change by quercetin exposure.

Prenatal exposure to quercetin affects gene expression profile of B[a]P metabolising enzymes in adult liver and lung

As *in utero* exposure to quercetin up-regulated gene expression of Phase I and II enzymes, we investigated whether these changes persisted into adulthood. Therefore, mice were *in utero* exposed to quercetin (1 mmol/kg feed) and they were sacrificed at the age of 12 weeks. Basal gene expression of Phase I and II enzymes was determined in liver and lung tissue of five male and five female offspring mice. As shown in Table III, prenatal exposure to quercetin enhanced gene expression in the liver of female offspring mice of Phase I enzymes, Cyp1b1 ($P = 0.01$) and Cyp1a1 ($P = 0.05$), involved in B[*a*]P metabolism in the liver of female offspring mice. However, the gene expression of AhR was significantly induced in the liver of males prenatally exposed to quercetin ($P = 0.009$). On the contrary, the expression of Phase II enzymes was not altered in the liver.

In lungs of these mice, no significant changes in Phase I or AhR gene expression levels were observed (Table III). On the other hand, female mice prenatally exposed to quercetin showed an increase in expression of the following Phase II enzymes in their lung: Gstp1 ($P = 0.009$), Nqo1 ($P = 0.009$) and Ugt1a6 ($P < 0.05$) compared to control females.

Also interesting to see were the significant tissue specific differences in gene expression between male and female mice (Table IV). In case of the liver, Cyp1b1 ($P = 0.05$), Gstp1 ($P = 0.009$), Nqo1 ($P = 0.009$) and Ugt1a6 ($P = 0.02$) were differentially regulated in control males and females. In the lung, only the expression of Gstp1 ($P < 0.05$) and Nqo1 ($P = 0.009$) differed between male and female control mice. Moreover, prenatal exposure to quercetin seemed to counteract these gender differences for almost all genes, with an exception for Gstp1 in liver ($P = 0.009$) and Nqo1 ($P = 0.01$) in lung.

Prenatal exposure to quercetin results in long-lasting epigenetic modifications of repetitive elements in liver of female mice prenatally exposed to quercetin

As gene expression of Phase I and II enzymes was altered by prenatal exposure to quercetin and was maintained till adulthood, we also investigated whether exposure to quercetin could affect the methylation status of the repetitive elements, SINEB1, SINEB2 and LINE1. SINE and LINE repetitive elements are normally expressed during early embryogenesis, after which their expression rapidly decreases with development. Later in life, expression of SINEB1, SINEB2 and LINE1 is associated with cell stress, for instance by DNA-damaging agents (27,28). At gestational Day 14.5, no changes in methylation of repetitive elements were detected in foetuses exposed to quercetin (Figure 1A), suggesting that quercetin exposure did not influence methylation status at that point in gestation.

Although no differences in global methylation status could be detected in the liver at gestational Day 14.5, SINEB1 in the liver of adult female mice prenatally exposed to quercetin was hypomethylated compared to control female mice ($P = 0.01$, Figure 1B). DNA methylation of SINEB2 and LINE1 repetitive elements was also decreased in female mice prenatally exposed to quercetin, though not significantly when analysed separately. Combined analysis of all three repetitive sequences also indicated that these were hypomethylated in the liver of female offspring that received quercetin *in utero*, with borderline significance ($P = 0.06$). No alterations in methylation were observed for male liver or lung of both genders (Figure 1B and C). These data suggest that *in utero* exposure to quercetin results in the hypomethylation of the repetitive elements SINEB1, SINEB2 and LINE1 in the liver of adult female mice.

Prenatal exposure to quercetin diminished the B[a]P-induced DNA adduct formation ex vivo using liver microsomes of adult mice

Prenatal exposure to quercetin resulted in long-lasting alterations in the gene expression profile of enzymes involved in B[*a*]P metabolism in liver and lung of mainly female offspring. However, as mRNA expression levels do not necessarily reflect enzyme activity, S9 mix from liver or lung tissue of five male and five female mice prenatally exposed to quercetin was incubated *ex vivo* with B[*a*]P. S9 mix of liver of mice

Table II. Relative gene expression profile of Phase I and II enzymes in the liver of quercetin exposed foetuses at Day 14.5 of gestation

	Control ($n = 5$)	Quercetin ($n = 5$)	Diet	Litter effects
	Mean ± SE	Mean ± SE		
Cyp1a1	1.0 ± 0.4	16.5 ± 3.1	$F_{1,3}$: 22.5; $P = 0.004$	$F_{5,3}$: 0.9; $P = 0.6$
Cyp1b1	1.0 ± 0.4	7.9 ± 1.3	$F_{1,3}$: 76.1; $P < 0.001$	$F_{5,3}$: 0.1; $P = 1.0$
Comt	1.0 ± 0.4	2.5 ± 0.2	$F_{1,3}$: 3.8; $P = 0.1$	$F_{5,3}$: 3.5; $P = 0.2$
Gstp1	1.0 ± 0.4	2.5 ± 0.4	$F_{1,3}$: 5.2; $P = 0.07$	$F_{5,3}$: 3.7; $P = 0.2$
Nqo1	1.0 ± 0.2	2.5 ± 0.3	$F_{1,3}$: 20.5; $P = 0.005$	$F_{5,3}$: 0.5; $P = 0.7$
Ugt1a6	1.0 ± 0.5	3.9 ± 1.0	$F_{1,3}$: 1.8; $P = 0.004$	$F_{5,3}$: 4.3; $P = 0.6$
AhR	1.0 ± 0.4	1.2 ± 0.1	$F_{1,3}$: 0.1; $P = 0.7$	$F_{5,3}$: 9.0 $P = 0.05$

F-value with corresponding degrees of freedom for diet groups and number of litters, respectively.

Table III. Relative gene expression of Phase I and II enzymes in the liver and lung of 12-week-old male and female offspring mice prenatally exposed to quercetin

	Males[a] (n = 5)		Females[a] (n = 5)	
	Control	Quercetin	Control	Quercetin
Liver				
Cyp1a1	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.5 ± 0.2*
Cyp1b1	1.0 ± 0.2	1.9 ± 0.6	1.0 ± 0.3	2.9 ± 0.8**
Comt	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	1.2 ± 0.3
Gstp1	1.0 ± 0.4	0.9 ± 0.2	1.0 ± 0.2	1.4 ± 0.4
Nqo1	1.0 ± 0.1	4.5 ± 2.9	1.0 ± 0.5	2.4 ± 1.0
Ugt1a6	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.4
AhR	1.0 ± 0.1	2.6 ± 0.4**	1.0 ± 0.3	1.5 ± 0.4
Lung				
Cyp1a1	1.0 ± 0.5	0.9 ± 0.3	1.0 ± 0.2	0.9 ± 0.1
Cyp1b1	1.0 ± 0.2	1.2 ± 0.5	1.0 ± 0.4	1.4 ± 0.2
Comt	1.0 ± 0.3	1.5 ± 0.2	1.0 ± 0.1	1.2 ± 0.2
Gstp1	1.0 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	2.2 ± 0.3**
Nqo1	1.0 ± 0.7	1.6 ± 0.6	1.0 ± 0.2	11.3 ± 3.8**
Ugt1a6	1.0 ± 0.3	1.6 ± 0.6	1.0 ± 0.1	2.3 ± 0.4*
AhR	1.0 ± 0.4	1.9 ± 0.6	1.0 ± 0.6	1.0 ± 0.2

[a]Control male and female mice were used as reference sample to calculate $\Delta\Delta CT$ for males and females, respectively. Results represent mean ± SE.
Significant differences between diets: $*P < 0.05$, $**P \leq 0.01$.

Table IV. Gender effect on Phase I and II gene expression in the liver and lung of 12-week-old mice

	Control		Quercetin	
	Males[a] (n = 5)	Females[a] (n = 5)	Males[a] (n = 5)	Females[a] (n = 5)
Liver				
Cyp1a1	1.0 ± 0.3	0.9 ± 0.1	1.0 ± 0.1	1.4 ± 0.2
Cyp1b1	1.0 ± 0.2	0.5 ± 0.1*	1.9 ± 0.6	1.2 ± 0.3
Comt	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2
Gstp1	1.0 ± 0.4	0.1 ± 0.02**	0.9 ± 0.2	0.1 ± 0.04**
Nqo1	1.0 ± 0.1	3.3 ± 1.4**	4.5 ± 2.9	6.2 ± 2.6
Ugt1a6	1.0 ± 0.2	2.1 ± 0.3*	1.0 ± 0.3	2.1 ± 0.7
AhR	1.0 ± 0.01	1.2 ± 0.3	2.6 ± 0.4	1.7 ± 0.5
Lung				
Cyp1a1	1.0 ± 0.5	1.1 ± 0.2	0.9 ± 0.3	0.9 ± 0.1
Cyp1b1	1.0 ± 0.2	0.6 ± 0.2	1.2 ± 0.5	0.7 ± 0.1
Comt	1.0 ± 0.3	0.9 ± 0.1	1.5 ± 0.2	1.0 ± 0.3
Gstp1	1.0 ± 0.1	0.8 ± 0.1*	1.4 ± 0.2	1.8 ± 0.3
Nqo1	1.0 ± 0.7	0.3 ± 0.1**	1.6 ± 0.6	7.3 ± 1.8**
Ugt1a6	1.0 ± 0.3	0.8 ± 0.1	1.6 ± 0.7	2.0 ± 0.2
AhR	1.0 ± 0.4	3.4 ± 1.7	1.9 ± 0.6	2.2 ± 1.0

[a]Control male mice were used as reference sample to calculate $\Delta\Delta CT$ for males and females. Results represent mean ± SE.
Significant differences between gender: $*P < 0.05$, $**P \leq 0.01$.

prenatally exposed to quercetin did not show an altered B[*a*]P metabolism (Table V). On the other hand, lung S9 mix of mice prenatally exposed to quercetin resulted in decreased amounts of B[*a*]P-9,10-dihydrodiol (9,10-diOH-B[*a*]P, $P = 0.007$) and B[*a*]P-7,8-dihydrodiol (7,8-diOH-B[*a*]P, $P = 0.05$). The amount of 9,10-diOH-B[*a*]P was especially decreased in case of S9 mix derived from male mice prenatally exposed to quercetin compared to control males ($P = 0.03$). In addition, the total amount of unmetabolised B[*a*]P was significantly lower ($P = 0.01$) when using lung S9 mix of mice prenatally exposed to quercetin compared to the control group, especially in the males ($P < 0.05$).

Despite the clear differences in gene expression between male and female mice, no gender differences were observed for

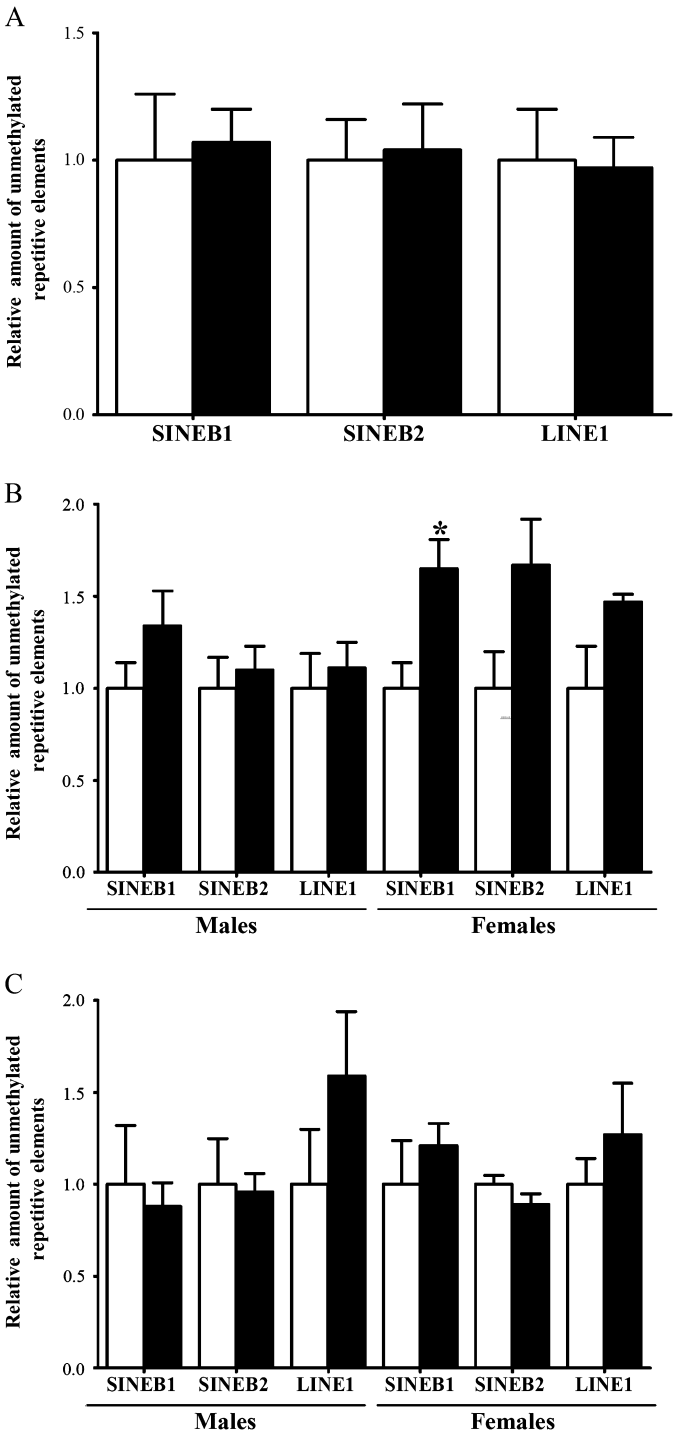

Fig. 1. Methylation status of SINEB1, SINEB2 and LINE1 repetitive elements in foetal and adult liver and lung after (prenatal) exposure to quercetin. Short-term effects of prenatal exposure to quercetin on DNA methylation of SINEB1, SINEB2 and LINE1 repetitive elements in foetal liver of quercetin exposed foetuses (n = 4, black bars) compared to control foetuses (n = 4, white bars) at Day 14.5 of gestation (**A**). Long-term effects of prenatal exposure to quercetin on DNA methylation of SINEB1, SINEB2 and LINE1 repetitive elements in liver (**B**) and lung (**C**) of male and female mice prenatally exposed to quercetin (n = 5 for each gender, black bars) compared with control mice (n = 5 for each gender, white bars). The degree of methylation of SINEB1, SINEB2 and LINE1 repetitive elements was determined by methylation-sensitive McrBC real-time PCR assay. Bars represent the normalised average expression level of the unmethylated repetitive elements. Error bars represent standard error. Statistical significance was determined by one-way ANOVA. $*P < 0.05$.

Table V. B[*a*]P metabolism by liver and lung S9 mix of 12-week-old male and female offspring mice prenatally exposed to quercetin and resulting BPDE adducts

Mean ± SE	Overall (n = 10)		Males (n = 5)		Females (n = 5)	
	Control	Quercetin	Control	Quercetin	Control	Quercetin
Liver						
9,10-diOH-B[*a*]P (ng/ml)	12.4 ± 3.0	8.6 ± 1.4	17.3 ± 3.5[a]	10.6 ± 2.2	6.2 ± 3.2[a]	6.7 ± 1.6
7,8-diOH-B[a]P (ng/ml)	1.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	0.8 ± 0.1	0.8 ± 0.4	0.8 ± 0.2
3-OH-B[a]P (ng/ml)	2.7 ± 0.4	2.6 ± 0.3	2.3 ± 0.7	2.4 ± 0.2	3.1 ± 0.6	2.7 ± 0.7
Unmetabolised B[*a*]P (AUC)	359.7 ± 35.0	375.3 ± 15.6	339.3 ± 64.1	389.5 ± 8.1	380.1 ± 34.5	361.2 ± 30.4
BPDE–DNA adducts/10^8 nucleotides	33.4 ± 7.4	17.0 ± 3.3*	28.6 ± 3.7	13.1 ± 3.7	36.2 ± 11.9	20.1 ± 5.1
Lung						
9,10-diOH-B[*a*]P (ng/ml)	0.5 ± 0.04	0.3 ± 0.03**	0.5 ± 0.03	0.3 ± 0.06*	0.5 ± 0.07	0.3 ± 0.04
7,8-diOH-B[*a*]P (ng/ml)	0.2 ± 0.04	0.1 ± 0.02*	0.3 ± 0.06	0.1 ± 0.03	0.2 ± 0.04	0.1 ± 0.02
3-OH-B[*a*]P (ng/ml)	3.1 ± 0.4	3.1 ± 0.8	3.0 ± 1.5	3.3 ± 1.5	3.4 ± 0.7	2.9 ± 0.9
Unmetabolised B[*a*]P (AUC)	148.9 ± 14.0	81.9 ± 14.8**	154.3 ± 26.3	65.9 ± 17.8*	143.4 ± 13.1	98.0 ± 23.3
BPDE–DNA adducts/10^8 nucleotides	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Results represent mean ± SE. AUC, area under the curve; n.d., not detected.
Significant differences between diets: *$P \leq 0.05$, **$P \leq 0.01$.
Significant differences between gender within diet: [a]$P = 0.09$.

B[*a*]P metabolism, except maybe for the liver S9 mix where 9,10-diOH-B[*a*]P levels were higher for control males compared to their female littermates, but this did not reach statistical significance ($P = 0.09$). The overall profile of metabolites strongly differed between lung and liver S9 mix; using S9 mix obtained from the lung, the major metabolite was 3-hydroxy-B[*a*]P (3-OH-B[*a*]P), which is considered to be a detoxification product of B[*a*]P. No B[*a*]P DNA adducts could be detected in calf thymus DNA that was added to the S9 mix obtained from lung tissue, regardless of the prenatal diet (Table V). When using liver-derived S9 mix, prenatal quercetin exposure resulted in a significant decrease in DNA adduct formation ($P = 0.04$), with no differences between genders.

Discussion

As genetic polymorphisms (1–4) do not entirely explain the interindividual differences in xenobiotic metabolism, we investigated whether prenatal exposure to the natural flavonoid quercetin, which is a potent AhR ligand, could permanently alter the expression of enzymes involved in both activation as well as detoxification of B[*a*]P.

We found that *in utero* exposure to quercetin affected the gene expression of Phase I and II enzymes of the foetal liver at gestational Day 14.5. Gene expression of Cyp1a1 and Cyp1b1 was significantly increased, indicating that quercetin or its metabolites functioned as an AhR ligand in the foetus. However, these alterations in gene expression could also have other reasons. For instance, Santini *et al.* (29) showed that exposure of swine granulose cells *in vitro* to a low dose of quercetin resulted in increased levels of 17β-oestradiol. As 17β-oestradiol is a substrate for CYP1B1 and 1A1 (30) and because foetuses, both males and females, are continuously exposed to 17β-oestradiol throughout gestation via the mother (31), this route of exposure could additionally increase the gene expression of Phase I enzymes. Moreover, as CYP's are haemeproteins that contain iron (32), the iron-scavenging properties of quercetin (7) could result in less CYP activity; therefore, enhanced gene expression is needed as compensation. Gene expression of the Phase II enzymes Ugt1a6 and Nqo1 was also significantly enhanced in the liver of quercetin-exposed foetuses. This increase could theoretically be the result of an increase in CYP activity, detoxifying the reactive compounds formed by the Phase I reactions. Regardless of the underlying mechanism, from our experiments, it is clear that supplementation with quercetin altered gene expression *in utero*.

According to the hypothesis of 'foetal programming', impaired maternal nutrition during pregnancy results in foetal adaptations that are meant to improve survival. However, when these adaptations are permanent, they could also alter the risk for diseases later in life. In this study, we investigated whether the changes in Phase I and II gene expression due to the *in utero* environment are maintained throughout life. Indeed, 12-week-old female mice prenatally exposed to quercetin had increased Phase I (Cyp1a1 and Cyp1b1) gene expression levels in their liver, while the Phase II enzymes (Gstp1, Nqo1 and Ugt1a6) were predominantly enhanced in the lung tissue of these female mice. Interestingly, AhR gene expression was only increased in the liver of male mice prenatally exposed to quercetin.

The assumed AhR-induced up-regulation of Cyp450s seen in the liver due to prenatal exposure to quercetin did not occur in the lung. Since the lung develops mainly in the final stages of pregnancy and even continues to develop after birth (33), while the liver is largely differentiated before birth (34), the different responses of both tissues to quercetin exposure could be due to the difference in maturation state of the organ during exposure. The enhanced expression of Phase II enzymes in mice prenatally exposed to quercetin could be the result of the pro-oxidant capacity of quercetin (35,36), resulting in the up-regulation of Phase II enzymes through the Nrf2 pathway (9), which could then be maintained throughout life.

In the control group, significant differences were observed between male and female offspring regarding the gene expression of certain Phase I and II enzymes both in liver as well as lung. This has already been reported for several species (37–40). However, prenatal exposure to quercetin seemed to reduce this gender difference. Only for Gstp1 (liver) and Nqo1 (lung), the gender effect was persistent. Makaji *et al.* (11) also reported that prenatal intake of flavonoids only resulted in long-term alterations of CYP activity in female rat offspring, by making the CYP profile more masculine, as was also observed in our study. They suggested that quercetin could influence Phase I

and II gene expression by regulating constitutive androstane receptor or Ah receptor. They also suggested that the gender differences in mRNA expression of Phase I enzymes seen in adult animals is imprinted by the presence (or absence) of increased androgen levels at puberty.

We suspected that epigenetic modulations underlie the persistent changes in Phase I and II gene expression. Therefore, the potential of quercetin to alter the epigenome was assessed and showed that prenatal quercetin exposure resulted in the hypomethylation of repetitive elements in liver of adult female mice. Interestingly, most pronounced changes in gene expression were observed in liver and lung of female mice prenatally exposed to quercetin. However, changes in DNA methylation of repetitive elements were only seen in liver. As we did not measure gene-specific DNA methylation levels, we can only assume that *in utero* exposure to quercetin resulted in gene-specific hypomethylation for the genes or their regulators that were up-regulated in liver and lung tissue of male and female mice prenatally exposed to quercetin. Since these epigenetic modifications could not be detected in foetuses at Day 14.5 of gestation, we suggest that they occurred at a later time point in gestation or even after birth. This is plausible as during the late gestational and early postnatal period, tissue maturation results in most epigenetic modifications. Moreover, it has been suggested that transcriptional inactivity attracts *de novo* methylation, while transcriptional activity can eventually override DNA methylation (41).

To study the effects of maternal quercetin intake during gestation on B[*a*]P metabolism by adult liver and lung, we used S9 mix of lungs and livers of animals that did not receive B[*a*]P at adult age. In that case, analysis of *ex vivo* B[*a*]P metabolism using S9 mix is more straightforward because there is no bias by, for instance, B[*a*]P uptake and kinetics, DNA repair and altered cell turnover. The identified metabolites formed using liver and lung S9 mix did not differ between control and quercetin exposed animals. However, due to the limited number of metabolites detected, false representation of the B[*a*]P metabolite profile cannot be excluded. Although there were no differences in the amount of unmetabolised B[*a*]P for liver S9 mix after the 1-h incubation, the BPDE–DNA adduct levels were lower for mice prenatally exposed to quercetin. This suggests that prenatal exposure to quercetin did not result in an accelerated metabolism of B[*a*]P (as seen in lung), but in B[*a*]P detoxification, e.g. less formation of DNA-reactive metabolites including BPDE.

No BPDE–DNA adducts could be detected for the lung S9 mix of both control and quercetin-exposed mice, indicating that the B[*a*]P adduct forming ability of the lung is lower compared to the liver. Moreover, for the lung, less unmetabolised B[*a*]P was detected after incubation, indicating that the conversion of B[*a*]P to water-soluble derivatives was significantly increased in offspring that received quercetin during gestation. This suggested that more detoxification of B[*a*]P can take place in the lung of mice prenatally exposed to quercetin as a result of the adapted gene expression of Phase II enzymes, which is an important finding as the lungs are the primary target organ for airborne B[*a*]P (42). This finding therefore indicates that prenatal exposure to quercetin could decrease the risk on B[*a*]P-induced DNA damage and therefore the susceptibility to lung cancer.

These results show that prenatal exposure to the natural AhR agonist quercetin leads to tissue- and gender-specific long-lasting alterations in the gene expression of Phase I and II enzymes of adult mice, probably by epigenetic modifications. This seems to result in increased protection against B[*a*]P-induced DNA adduct formation. However, as gene expression levels of Phase I and II enzymes do not reflect actual enzyme activities, and because B[*a*]P metabolism and BPDE–DNA adduct formation experiments occurred *ex vivo*, it is now warranted to continue with similar studies with *in vivo* exposure to B[a]P of adult mice prenatally exposed to quercetin to confirm our theory.

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