Heterocyclic aromatic amines and colorectal cancer risk

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Heterocyclic aromatic amines and colorectal cancer risk

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Heterocyclic aromatic amines and colorectal cancer risk

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"The difficulty in life is the choice"
- George Moore

Contents

CHAPTER I	9
General introduction and outline of the thesis	
CHAPTER II	35
Methodologies for heterocyclic aromatic amine-DNA adduct analysis and determination of urinary 2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine (PhIP) excretion levels	
CHAPTER III	47
Generation of free radicals and induction of DNA adducts by activation of heterocyclic aromatic amines via different metabolic pathways <i>in vitro</i>	
CHAPTER IV	61
Cyclooxygenase-mediated DNA adduct formation by heterocyclic aromatic amines in human adenocarcinoma colon cells	
CHAPTER V	79
CYP1A2 and NAT2 genotype/phenotype relations and urinary excretion of 2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine (PhIP) in a human dietary intervention study	
CHAPTER VI	93
Relationship between the CYP1A2-164A→C polymorphism (CYP1A2*1F) and risk for colorectal neoplasia in humans	
CHAPTER VII	105
Summary & general discussion	
SAMENVATTING	111
DANKWOORD	117
CURRICULUM VITAE	119
LIST OF PUBLICATIONS	119

Introduction

1.1 Colorectal cancer: etiological aspects

Facts and figures

Of all common diseases in industrialized Western countries, colorectal cancer (CRC) remains one of the most prevalent. In The Netherlands, approximately 8,600 new cases of CRC are diagnosed every year (1). In 2001, 3349 people died of colon cancer, which is about 9 % of all cancer deaths in that year (2). For women, CRC is second only to breast cancer, while lung and prostate cancer are the only cancer types that exceed CRC rates in men.

Many factors can influence the development of CRC in humans. Genetic predisposition is an important risk factor, underlying inherited syndromes like familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). FAP is characterized by the development of multiple colorectal adenomas (> 100) at a very young age, caused by an inherited mutation in the APC gene, mostly leading to truncated APC proteins (3,4). Patients with HNPCC show the tendency to early onset of CRC, without extensive polyposis. This syndrome is believed to be due to mutational loss of DNA mismatch repair genes.

1.1.1 Colorectal carcinogenesis: a genetic model

The development of cancer is a highly complex process, to which many factors may contribute, including environmental and dietary factors as well as inherited and somatic mutations. However, the precise mechanism behind the development of colon cancer has not been completely clarified yet. Although the genetic model presented by Vogelstein has undergone some modifications and elaborations, it is still believed to provide a molecular basis for CRC development, describing the complex multi-stage process in which cells undergo subsequent alterations in genes that control cell growth and differentiation (Figure 1) (5-8). In the initiation phase of tumor development, the Adenomatous Polyposis Coli (APC) gene, located on chromosome 5q, is believed to play a major role. In the majority of colorectal cancers, one or two alleles of the APC gene may be inactivated by somatic mutation at an early stage of tumor development (5,9,10), thus triggering a chain of other genetic alterations. As stated earlier, mutations in the APC gene are also responsible for the autosomal dominantly inherited syndrome called FAP.

The progression of tumors is believed to result from mutations in both oncogenes (K-ras) and tumor suppressor genes (on chromosomes 5q, 17p and 18q) (11-13). In patients without polyposis, allelic losses involving chromosome 5q21 are the most frequent genetic alteration found in early adenomas (5,9,14), while occurring with nearly the same frequency in more advanced tumors. Unchanged oncogenes or proto-oncogenes generally contribute to the normal growth regulation of cells in a positive manner, while mutated oncogenes can dominantly promote tumor development. The K-ras oncogene is mutated in approximately 50% of colorectal carcinomas and large adenomas, and these adenomas may have an increased risk of malignant progression (15). As the percentage of mutated K-ras genes is much lower in small adenomas (5), K-ras mutations are likely to occur in an early stage of colorectal carcinogenesis, during the conversion of small to large adenomas. Allelic losses in chromosomes 18q and 17p occur more frequently at later stages of tumorigenesis (5,16). On chromosome 18q, a common region of allelic loss was mapped, from which a candidate tumor suppressor gene was identified (17). Expression of this gene, termed DCC (deleted in colorectal cancer), is greatly reduced or absent in the majority of colorectal carcinomas. The common region of loss on chromosome 17p contains the gene encoding for p53, a protein that is believed to function as a tumor suppressor (18).

A GENETIC MODEL FOR HUMAN COLORECTAL TUMORIGENESIS

Fig.1. Accumulation of multiple genetic alterations in colorectal tumorigenesis, according to Fearon and Jones (8). APC = adenomatous polyposis coli; DCC = deleted in colon cancer; LOH = loss of heterozygosity (i.e. allelic loss). The three stages of adenomas refer to differences in size, dysplasia and villous content.

Without any other genetic alterations, mutations in the p53 gene are not common in carcinomas or adenomas. However, p53 point mutation in one allele coupled with the loss of the remaining wild-type allele, occurs frequently in later stages of the adenoma-carcinoma cascade (19).

Although a preferred order in the timing of the specific events appears to exist, this order is not invariant. Moreover, accumulation of multiple genetic alterations seems more important than the order of the successive events. It is believed that at least four to five alterations in oncogenes or tumor suppressor genes are required for a malignant tumor to develop (7).

1.1.2 Environmental factors and CRC risk

CRC incidence rates vary approximately 20-fold worldwide, with high colon cancer rates found in North America, Australia and Europe, and incidence being much lower in South America, Asia and Africa (20,21). Interestingly, these rates were found to increase among groups that migrate from low-incidence to high-incidence areas (22,23). Furthermore, CRC incidence increased over time within stable populations (24). These findings indicate that, besides the well-established genetic component (25), environmental factors are important modulating factors in CRC risk. Moreover, nowadays environmental influences are believed to account for most of the variation in incidence rates. In 1981, Doll and Peto already estimated that approximately 90 % of all colon cancer cases are due to environmental, and especially dietary, practices (26). In 1997, the World Cancer Research Fund (WCRF) together with the American Institute for Cancer Research (AICR), estimated that one-third of all cancer deaths are caused by the diet (27). In this perspective, the probable increased CRC risk associated with the consumption of red and processed meat seems very important and has gained much attention over the years.

1.2 Relationship between meat consumption and CRC risk

Although numerous studies have investigated the association between meat consumption and the risk for CRC (reviewed in 28), results are divergent and therefore, the relation remains controversial (e.g. 27,29-36). However, both red meat and processed meat are believed to be possible risk factors in the development of CRC. In 1989, the National Academy of Sciences in the USA issued a report which implicated red meat as a causative factor in the etiology of CRC (37). Subsequently, a WCRF report concluded that "red meat probably increases risk and processed meat possibly increases risk of CRC", while a report from the Committee on Medical Aspects of Food and Nutrition Policy stated that "there is moderately consistent evidence from cohort studies of a positive association between the consumption of red or processed meat and risk of CRC" (27,32). As a result, these groups of experts recommended a decrease in red meat consumption for Western populations in order to reduce CRC risk. Several hypothetical mechanisms have been proposed to link the intake of red meat to an increased risk of colon cancer. An interesting mechanism was described by Babbs (38), suggesting that high intake of red meat may increase fecal iron concentrations, thus leading to higher CRC risk via generation of hydroxyl radicals inducing oxidative DNA damage, which is a mutagenic lesion. Alternatively, transformation of meat constituents by reactive nitrogen species in the colon, resulting in DNA-binding reactive metabolites, may increase cancer risk (39).

Most of the hypotheses, however, are related to the fatty acid content of red meat. Reddy suggested that dietary fat induces the excretion of bile acids, which can be converted to carcinogens or promoters by normal colonic bacteria (40). Indeed, high

consumption of beef has been shown to increase concentrations of fecal bile acids (41). However, other sources of fat have not been found to increase CRC risk, which may be explained in different ways (42). For example, red meat may contain specific fatty acids that are particularly harmful. Furthermore, more of the red meat fat may reach the large bowel, because it is less well digested or absorbed in the small intestine. Finally, non-lipid components of meat may be responsible for an elevated CRC risk. In this perspective, the formation of carcinogens, such as polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines (HCA), during the cooking of meat may be of significant importance.

1.3 Discovery and characterization of heterocyclic amines

In 1939, Widmark firstly suggested the possible occurrence of "cancer-producing substances in fried meat", and demonstrated this by inducing carcinogenic effects in mouse skin by applying roasted horse meat extracts (43). In 1976, Sugimura wondered what might be in the smoke produced in the kitchen during cooking of fish and meat. He designed an apparatus to collect smoke particles, produced by broiling fish over a gas burner, on a glass-fiber filter. After being dissolved with dimethyl sulfoxide, the mutagenicity of the collected matter was tested in the Ames test, a sensitive and reliable assay using *Salmonella typhimurium* (44). Mutagenicity of the smoke condensate was demonstrated after metabolic activation with S9-mix (45,46). Furthermore, mutagenic activity was demonstrated in cooked fish and meat, and especially the surface of the cooked foods was found to be very mutagenic (46-48). As a result of these findings, research was initiated to characterize the substances in meat and fish responsible for its mutagenic activity.

A series of mutagenic compounds could be isolated from pyrolysates of amino acids and proteins and directly from cooked meat and fish by monitoring the mutagenic activity in *Salmonella typhimurium* strain TA98 with S9 mix during purification. These compounds were found to be heterocycles with an amine functional group, and this class of mutagens has since then been known as heterocyclic amines. The first HCA to be characterized were two imidazo-pyridine compounds, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), which were isolated from a pyrolysate of tryptophan (49). From a pyrolysate of glutamic acid, two dipyrdo-imidazole compounds were isolated, 2-amino-6-methyldipyrdo-[1,2-a:3',2'-d]-imidazole (Glu-P-1) and 2-aminodipyrdo[1,2-a:3',2'-d]imidazole (Glu-P-2) (50). Two derivatives of amino- α -carboline, 2-amino-9H-pyrido[2,3-b]indole (A α C) and 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeA α C), were found in a pyrolysate of soybean globulin (51). A few years later other mutagens were isolated directly from cooked fish and meat. Two imidazoquinoline compounds, 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), were purified from broiled sun-dried sardines (52,53), while 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) was found to be present in fried beef (54). In 1986, another major mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was isolated from fried beef (55). Eventually, all these HCA have been classified as non-IQ- and IQ-types. Their structures are depicted in Figures 2 and 3, and their names, abbreviations, source of isolation and year of characterization are given in Table 1. Formation of non-IQ-type HCA occurs at temperatures above 300 °C and these HCA are classified as 'pyrolytic mutagens'. Because these temperatures are far higher than normal domestic cooking temperatures, these particular HCA are not commonly found in the Western diet. The IQ-type HCA are formed at temperatures below 300 °C and are called 'thermic mutagens'. They possess a 2-amino-imidazo group fused to a quinoline (IQ and MeIQ), quinoxaline (MeIQx) or pyridine (PhIP) ring and are referred to as amino-imidazo-azarenes (AIA).

Fig.2. Structures of non-IQ-type heterocyclic amines.

Fig.3. Structures of IQ-type heterocyclic amines.

Table 1. Chemical names, abbreviations, source and year of isolation for heterocyclic amines.

<i>Chemical name</i>	<i>Abbreviation</i>	<i>Source</i>	<i>Year</i>
3-amino-1,4-dimethyl-5 <i>H</i> -pyrido[4,3- <i>b</i>]-indole	Trp-P-1	Tryptophan pyrolysate	1977
3-amino-1-methyl-5 <i>H</i> -pyrido[4,3- <i>b</i>]indole	Trp-P-2	Tryptophan pyrolysate	1977
2-amino-6-methyldipyrdo-[1,2- <i>a</i> : 3',2'- <i>d</i>]-imidazole	Glu-P-1	Glutamic acid pyrolysate	1978
2-aminodipyrdo[1,2- <i>a</i> : 3',2'- <i>d</i>]-imidazole	Glu-P-2	Glutamic acid pyrolysate	1978
2-amino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole	A α C	Soybean globulin pyrolysate	1978
2-amino-3-methyl-9 <i>H</i> -pyrido-[2,3- <i>b</i>]indole	MeA α C	Soybean globulin pyrolysate	1978
2-amino-3-methylimidazo-[4,5- <i>f</i>]quinoline	IQ	Broiled sun-dried sardine	1980
2-amino-3,4-dimethylimidazo-[4,5- <i>f</i>]-quinoline	MeIQ	Broiled sun-dried sardine	1980
2-amino-3,8-dimethylimidazo-[4,5- <i>f</i>]-quinoxaline	MeIQx	Fried beef	1981
2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]-pyridine	PhIP	Fried beef	1986

The synthetic pathway of the class of HCA seems to involve Maillard reactions in the presence of creatine (56-58). This is in agreement with the fact that the formation of HCA only occurs in foods containing creatine (*i.e.* mostly meat and fish), and not in other types of foods. Figure 4 shows a possible pathway for the formation of IQ by the Maillard reaction, as proposed by Jägerstad *et al.* (56). Other IQ-type HCA are formed in a similar way.

In this pathway, creatinine is formed from creatine by cyclization and elimination of water, eventually forming the amino-imidazo part of the HCA molecule. Other parts of the molecule are possibly made up of Strecker degradation products like pyridine, formed in the Maillard reaction between hexose and an amino acid. Finally, linkage of the two parts of the molecule is believed to result from an aldol condensation involving a Strecker aldehyde.

Fig. 4. Suggested pathway for the formation of IQ.

The important role of creatine has been confirmed by investigators adding creatine to meat products before frying, thereby strongly increasing the total mutagenic activity (59-62). The results of those studies suggest that the creatine content is a rate-limiting factor in food mutagen formation. Moreover, as suggested by the Jägerstad pathway in Figure 4, creatine has indeed been demonstrated to form a part of the PhIP molecule by using radiolabelled creatine (63). Many other studies have supported the hypothesis on the precursors and intermediates as suggested by Jägerstad (see 58).

With respect to other factors that influence the formation of HCA, temperature and time of preparation are both very important. Several studies have described an increase in HCA formation due to higher temperatures (64-66) or prolonged preparation times (67,68). When using a model system of creatinine, glucose and a mixture of amino acids, Arvidsson *et al.* found the amount of formed HCA to level out after reaching a maximum after some time (69).

Alternatively, several factors have been found to inhibit the formation of HCA. The presence of antioxidants (70-72), marinating before cooking (73,74), and pre-treatment by microwaves (75) have all been found to reduce HCA formation in meat.

In general, the formation of HCA in meat and fish cooked by ordinary household methods results in concentrations in the ppb (= ng/g) range, with PhIP being most abundantly present in cooked meat (76-78).

1.4 Metabolic fate of HCA

In humans, HCA are efficiently absorbed into the systemic circulation after ingestion, thus being almost completely bioavailable. With only 1-4 % to be excreted unchanged in the urine, the majority of ingested HCA are subject to metabolism in the body. Like many other xenobiotics, HCA have to be metabolically activated in order to exert their mutagenic and carcinogenic properties. This is believed to occur via *N*-oxidation to *N*-hydroxy metabolites followed by *O*-acetylation to form *N*-acetoxy arylamines, the reactive metabolites that may form DNA adducts. These reactions are respectively catalyzed by hepatic cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferase-2 (NAT-2), which are both polymorphic in humans. Figure 5 shows the proposed metabolic activation of HCA in relation to colon carcinogenesis (79).

Fig. 5. Metabolic activation of PhIP and other HCA in colorectal carcinogenesis, as proposed by Kadlubar *et al.* (79).

HCA have proven to be poor substrates for direct *N*-acetylation by NAT-2 (80). The *N*-hydroxy metabolites, however, are good substrates for the NAT-2 enzyme to undergo *O*-acetylation, either in the liver or in the colon after transportation via the blood stream. Alternatively, *N*-hydroxy metabolites may be subjected to glucuronidation and can thus be excreted in the urine, a detoxification step that is catalyzed by UDP glucuronosyltransferase (UDPGT). Another possibility is the formation of the sulfate ester from *N*-hydroxy-HCA by cytosolic sulfotransferase, a pathway that has also been found to occur in the human colon, rendering an ultimate carcinogenic species (81). Both the *N*-hydroxy form and the *N*-acetoxy form can be transported via the bloodstream to extrahepatic tissues like the colon. This circulation of highly reactive intermediates through the bloodstream has been confirmed by Schut and Herzog, who found high PhIP-DNA adduct levels in rat lymphocytes after an oral dose of PhIP (82).

Biliary transport after glucuronidation, with subsequent deconjugation of the *N*-glucuronides, appears to be a detoxification route (83). For PhIP, detoxification in the liver is believed to be caused by reaction of glutathione with the *N*-acetoxy derivative, thus explaining the carcinogenic properties of PhIP in rat colon rather than in the liver (84). This detoxification step is further enhanced by glutathione-*S*-transferases, which may explain organ-specificity in HCA carcinogenicity (85). The esters that result from either acetylation or sulfatation, undergo spontaneous hydrolyzation to form an arylnitrenium ion, the ultimate reactive intermediate that can form covalently bound DNA adducts.

In contrast, another detoxification pathway involves direct C-hydroxylation of HCA, resulting in non-mutagenic phenolic compounds like 4'-hydroxy-PhIP. These can subsequently undergo conjugation by phase II enzymes such as sulfotransferase, glutathione transferase or glucuronosyl transferase, that can be induced by vegetable extracts and green or black tea, which have been found to increase the detoxification reactions (86-88). The proposed pathway has been confirmed by several other investigators. For instance, Sawada and Kamataki found high reactivities for HCA in genetically engineered cells, stably expressing cytochrome P450 and *N*-acetyltransferase (89). Furthermore, Boobis *et al.* demonstrated that the CYP1A2-catalyzed primary conversion of HCA is the major metabolic route in humans (90). Next to CYP1A2, the isozymes CYP1A1 and, to a lesser extent, CYP1B1 could also play a role in extrahepatic PhIP metabolism in humans, with respect to the formation of 2-hydroxyamino-PhIP (*N*²-OH-PhIP). Although the rate of formation of this mutagenic metabolite is clearly higher for CYP1A2 than for CYP1A1 and CYP1B1, the catalytic efficiency of *N*²-OH-PhIP production was found to be higher for CYP1A1 than for CYP1A2 (91).

Specific intestinal bacterial systems can also participate in the metabolic activation of HCA. Both HCA that reach the gut directly, and especially HCA and their metabolites that reach the gut via biliary transport, can be converted in this way (92,93). IQ can be converted into the mutagenic 7-hydroxy derivative 7-OH-IQ by normal microflora (94), while *N*-hydroxy-IQ is formed by anaerobic microorganisms in the human small intestine (95). A major biliary metabolite of IQ was identified as the non-mutagenic sulfamate derivative, *N*-(3-methylimidazo[4,5-*f*]quinolin-2-yl)sulfamic acid, the formation of which thus being an important detoxification pathway in the rat (96).

Another pathway, that may be of particular interest with respect to the carcinogenicity of HCA in extrahepatic tissues such as colon and rectum, involves oxidation of HCA catalyzed by the enzyme prostaglandin H synthase (PHS) (97). PHS is found in most mammalian tissues and has two distinct activities, cyclooxygenase (COX) and peroxidase.

For COX, two isoforms are known; COX-1 is constitutively expressed in nearly all cells, while COX-2 can be induced in many cell types by a number of intracellular and extracellular stimuli (98,99). Cyclooxygenase catalyzes the formation of prostaglandin G_2 (PGG_2) from the polyunsaturated fatty acid arachidonic acid (AA), which is released from membrane phospholipids by phospholipases (100). The peroxidase moiety of PHS catalyzes the subsequent reduction of PGG_2 to prostaglandin H_2 (PGH_2), which is a precursor for several types of prostanoids, like prostaglandin E_2 (PGE_2) (97,101-103). During the conversion of PGG_2 to PGH_2 , electron-donating cosubstrates are necessary to allow the peroxidase catalytic cycle to proceed. In turn, these cosubstrates are then converted to chemically reactive, electron-deficient metabolites (97). It is believed that HCA can serve as cosubstrates for PHS peroxidase, thus undergoing one-electron oxidation, resulting in the formation of HCA free-radical metabolites that can bind covalently to DNA (104) (Figure 6).

A considerable amount of evidence indicates the involvement of COX-2 in colorectal carcinogenesis. Many studies have demonstrated that colorectal adenocarcinomas show higher levels of COX-2 expression than the adjacent normal tissue (e.g. 105-107). Furthermore, inhibitors of cyclooxygenases have been found to substantially decrease colorectal cancer risk. For instance, continuous use of non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin, which are known to inhibit both COX-1 and COX-2, has been correlated to a decreased CRC risk (108-112). This antineoplastic action is believed to result from the inhibition of isoform COX-2 (113).

Fig. 6. Cooxidation of HCA during the PHS-catalyzed conversion of arachidonic acid to prostanooids.

1.5 DNA adduct formation by HCA

Both the hepatic and the extrahepatic pathway lead to the formation of reactive HCA metabolites that may bind covalently to DNA, thus generating DNA adducts. It is generally believed that the formation of DNA adducts is an essential step in the carcinogenic process, although the exact relationship is not fully understood (114,115). Measurement of DNA adducts provides biologically relevant information on the ultimate result of exposure, absorption, distribution, metabolism, genetic susceptibility and DNA repair capacity. Because DNA adducts occur at very low levels, very sensitive assays are necessary to detect and quantify these lesions. Of the different methods developed over the years, the ^{32}P -postlabeling assay has been used most extensively. This technique involves the introduction of a radioactive label into the adduct, after it has been formed. Thus, DNA adducts can be regarded as biomarkers of potential mutagenicity and of cancer risk (114,116). Using the ^{32}P -postlabeling assay, adducts formed by HCA have been identified in rats (117-119), monkeys (120-122), dogs (79) and humans (79,123,124). In humans, HCA-DNA adducts were predominantly found in colon and rectum tissues, and not in pancreas, liver or urinary bladder, indicating that the colorectal mucosa is the target tissue for HCA.

After oral administration of the corresponding HCA to rats, the highest levels of IQ- and MeIQx-DNA adducts have been found in the liver, followed by kidney and heart; for PhIP, adduct levels were highest in colon, heart, lung and pancreas, and lowest in the liver (117-119,125). So far, various HCA-DNA adduct structures have been identified. All form a major adduct with the C8 atom of guanine, while IQ and MeIQx also form a minor adduct with the N^2 atom of guanine (120,122,126-129). Figure 7 shows the structures of the identified DNA adducts formed by IQ and PhIP.

Fig.7. Structures of DNA adducts formed by IQ and PhIP. dG-C8-IQ, *N*-(deoxyguanosin-8-yl)-IQ; dG-N²-IQ, 5'-(deoxyguanosin-N²-yl)-IQ; dG-C8-PhIP, *N*-(deoxyguanosin-8-yl)-PhIP.

Other assays used to determine HCA-DNA adduct levels comprise GC-MS (79,123), LC-MS (130) and accelerated mass spectrometry (AMS) (131-133). The latter technique, which has shown to be very sensitive, has been used to detect PhIP-DNA adducts in normal human colorectal tissue after exposure to a dietary-relevant dose of the food mutagen (134). However, this assay is limited to the use of ^{14}C -labeled substrates, and is therefore not applicable in standard human biomonitoring studies. Furthermore, by using the isolated compound, the food matrix effect in normal dietary exposure is not taken into account in these studies. As stated earlier, the ^{32}P -postlabeling assay, although less sensitive than AMS, has been used to detect HCA-DNA adducts in colon tissue, and may therefore be used in biomonitoring studies. Because the target tissues are usually not easily accessible, alternative tissues are considered for biomonitoring purposes. In this perspective, peripheral blood lymphocytes may be used for biomonitoring HCA exposure and investigating the peripheral bioavailability of HCA in humans. Although, for HCA-DNA adducts, such studies have not been described yet, the use of DNA adducts as biomarkers of exposure in peripheral blood lymphocytes was earlier described for another food carcinogen, the PAH benzo[a]pyrene (135).

Turteltaub *et al.* investigated covalent DNA-binding for MeIQx in the human colon using AMS, suggesting that humans may produce more DNA adducts per dose as compared to the rat (136). Furthermore, Lin *et al.* demonstrated that the human liver is more active with respect to *N*-hydroxylation of aromatic amines by CYP4501A2 as compared to the rodent liver (137), thus leading to higher levels of reactive HCA intermediates per ingested dose. These findings indicate that humans seem more sensitive to genotoxic damage by HCA than rodents (138,139), and suggest that extrapolation from rodent cancer data could lead to an underestimation of the risk for humans. In conclusion, it can be said that consumption of well-done meat results in HCA-DNA adduct formation in the colon, which may cause genetic alterations. These alterations can eventually lead to colorectal tumorigenesis, as described by the Vogelstein model in paragraph 1.1.1.

1.6 Mutagenicity and carcinogenicity of HCA

The AIA, with the exception of PhIP, are among the most potent mutagenic compounds known in bacteria, as tested in the Ames assay (140,141). Mutagenicities of the AIAs and some typical carcinogens in *Salmonella Typhimurium* strains TA98 and TA100 are summarized in Table 2.

Many studies in bacterial assays support the role of AIA-DNA adduct formation in mutagenesis (140-144). Malfatti *et al.* studied the efficiency of HCA-DNA adducts to form mutations in *Salmonella Typhimurium* TA98, showing that less than 25 adducts are required for one mutational event (*i.e.* frameshift mutation) (145). Furthermore, Kerdar *et al.* demonstrated for several AIAs that mutagenic potency was linearly related to higher adduct levels in bacterial DNA (143), thus indicating that quantitative rather than qualitative differences in DNA adduct levels determine mutagenicity differences among these AIAs.

Alternatively, in mammalian cell mutagenicity assays, AIA-DNA adducts have been found to primarily induce base substitution mutations in guanine, with G→T transversion mutations occurring most frequently (146,147). In general, in *in vitro* studies, mutations induced by AIA-DNA adducts appear to be site-specific in mammalian genes, as demonstrated for PhIP by Shibutani *et al.* (148).

Table 2. Mutagenic activities of IQ-type HCA in comparison with typical carcinogens in *Salmonella Typhimurium* TA98 and TA100 (149).

Mutagen	Mutagenic activity (revertants/ μ g)	
	TA98	TA100
IQ	433 000	7 000
MelQ	661 000	30 000
IQx	75 000	1 500
MelQx	145 000	14 000
4,8-DiMelQx	183 000	8 000
7,8-DiMelQx	163 000	9 900
PhIP	1 800	120
2-Furyl-3-nitrofuryl-acrylamide	6 500	42 000
Aflatoxin B ₁	6 000	28 000
4-Nitroquinoline 1-oxide	970	9 900
Benzo[a]pyrene	320	660
N-Nitrosodiethylamine	0.02	0.15

In vivo, AIAs have demonstrated to induce base substitution and/or deletion mutations in the *lacI* or *lacZ* genes of transgenic mice (150-152), in colonic cells of mice (153,154), and in mouse small intestine (155). In the transgenic mice, mutant frequency appears to depend on both the dose and the duration of exposure to AIAs (156,157). It is not certain whether *lacZ* or *lacI* genes can predict the sites for chemical-induced carcinogenesis (156), although PhIP has been shown to induce a single-base deletion in the *lacI* gene in transgenic mice colon, which was characteristic of the single-base deletions found in the APC gene of rat colon tumors induced by PhIP (151). Other studies have also suggested that PhIP leaves a characteristic mutational fingerprint in the APC gene (158,159). In general, HCA are less carcinogenic as they are mutagenic, when compared with other compounds (160). Still, HCA have proven to be potent carcinogens in numerous animal studies. For rodents, carcinogenicity of HCA has been well documented (160,161), showing that rats are generally more sensitive than mice. All of the AIAs that were found to be carcinogenic in rats and/or mice, form DNA adducts in these species (e.g. 83,84,129,162,163). IQ and MelQx are hepatocarcinogens in rats and mice, both producing relatively high levels of DNA adducts in the liver (118,125,164,165). Alternatively, PhIP, which is not a hepatocarcinogen in adult rodents, has shown to form relatively low levels of DNA adducts in the liver as compared to extrahepatic tissues such as mammary gland, prostate and colon (83,164,166,167). Similar findings were described in nonhuman primates, where hepatic adduct levels were found to be much higher for IQ than for MelQx, with IQ being a potent hepatocarcinogen, contrary to

MeIQx (121,168-170). Although these findings support the role of AIA-DNA adducts in carcinogenesis, the possible occurrence of relatively high adduct levels in non-target tissues, as well as the possible absence of elevated adduct levels in target tissues, contradict a simple relation between DNA-adducts and cancer (123,163,164,171). The capacity of human tissues to activate AIAs varies widely between individuals (168,172,173). The resulting variation in DNA adduct levels is suggested to be dependent on the balance between metabolic activation and detoxification (83-85,174,175). Polymorphisms and differences in expression of genes involved in activation or detoxification, may thus influence genotoxicity (176-178). For instance, high enzymatic activities for both CYP1A2 and NAT-2, which are key enzymes in HCA metabolism, have been associated with an elevated risk for colorectal cancer in humans (79,179,180). Such important determinants of HCA metabolism have not been taken into account in the numerous epidemiological studies where HCA exposure is directly linked to CRC (see 28), leaving uncertainties about the internal HCA exposure and the biological fate of HCA in the human body. Moreover, accurate assessment of human HCA exposure appears to be difficult, mainly due to the complexity of the food matrix, the low amounts of HCA present and the multi-step isolation procedure (181). Food questionnaires have often been used to estimate HCA exposure (182), but there are several flaws including bias, inconsistent reporting and day-to-day variation in diet (183). Therefore, it is necessary to monitor HCA and their metabolic activation in the human body, using biomarkers of exposure and incorporating the role of relevant genetic polymorphisms and enzyme activities.

In general, it is believed that individual genetic susceptibility may play a major role in human cancer risk. There is substantial evidence that certain individuals are at a higher risk for cancer when exposed to heterocyclic amines, the magnitude of risk most likely ranging from 1 per 10,000 for the average person to greater than 1 in 50 for those ingesting large amounts of well-done muscle meats (184).

1.7 Outline of the thesis

Many aspects concerning dietary HCA, their metabolism and the putative relation to human colorectal cancer risk, have been studied by other researchers who have amply provided insights into the features of these food-borne carcinogens. However, still many issues remain incompletely understood. With respect to the *in vitro* metabolic activation of HCA via different pathways, the extrahepatic pathway for HCA has been studied far less extensively as compared to the hepatic route. However, it is important to know the relative importance of either pathway, since both routes of activation, with their corresponding key enzymes and resulting reactive metabolites, may significantly contribute to the ultimate carcinogenic risk resulting from HCA exposure. Furthermore, interaction with other dietary factors, such as PUFA, may influence HCA metabolism.

As explained in paragraph 1.7, it is important to monitor the biological fate of HCA in humans. Although metabolic activation of HCA in relation to genetic polymorphisms and enzymatic activities for CYP1A2 and NAT-2 has been investigated in numerous studies, data on the relationship between the specific CYP1A2-164A→C polymorphism (CYP1A2*1F) and either biomarkers of exposure, such as urinary excretion of unmetabolized PhIP, or relative colorectal cancer risk are not available.

The aim of the present thesis is to further complete our understanding of the complex relationship between HCA and colorectal cancer risk in humans.

Therefore, the research topics of the present thesis are:

- To study the relative importance of the hepatic and extrahepatic metabolic pathways for HCA by investigating the formation of intermediate and ultimate reactive metabolites resulting from the activation of HCA via these different routes *in vitro*.
- To investigate the cyclooxygenase-catalyzed formation of DNA adducts by HCA in COX-2 expressing human colon cells, and to study the effect of polyunsaturated fatty acids and specific enzyme inhibitors on this DNA adduct forming capacity.
- To study the urinary excretion of PhIP as a biomarker of exposure, in relation to the dietary exposure and genetic polymorphisms and enzymatic activities for both CYP1A2 and NAT-2 in healthy volunteers.
- To investigate the influence of genetic polymorphisms and enzymatic activities for both CYP1A2 and NAT-2 on colorectal neoplasia risk in different risk groups of patients, as compared to healthy control subjects.

Chapter 2: This chapter describes the development and optimization of methods to determine different biomarkers of HCA intake used in this thesis. The ^{32}P -postlabeling assay was used for detection and quantification of DNA adduct levels formed by PhIP and IQ. In order to assess urinary PhIP excretion levels, gas chromatography-mass spectrometry (GC-MS) was used.

Chapter 3: As described in paragraph 1.5, the metabolic activation of HCA to genotoxic species can proceed via two different pathways. In this chapter, the formation of reactive metabolites via either of both pathways was investigated *in vitro*. The ^{32}P -postlabeling assay, as described in chapter 2, was used to assess HCA-DNA adducts levels resulting from either pathway, while the generation of free radical metabolites in the extrahepatic route was demonstrated using electron spin resonance (ESR) spectroscopy.

Chapter 4: The extrahepatic activation of HCA comprises cooxidation during the conversion of polyunsaturated fatty acids (PUFA) by the cyclooxygenase (COX) enzyme. DNA adduct formation by HCA was investigated in COX-2 expressing human colon cancer cells. Furthermore, the influence of PUFA and the effect of several specific enzyme inhibitors on the DNA adduct forming capacity by HCA was studied in these cells.

Chapter 5: To investigate the influence of genetic polymorphisms and enzyme activities on the metabolic process of HCA in humans, volunteers were exposed to a PhIP containing diet. Subjects were genotyped and phenotyped for the enzymes CYP1A2 and NAT-2. Urinary PhIP excretion levels were determined using GC-MS, as described in chapter 2.

Chapter 6: Patients undergoing endoscopic examination were divided into groups at different risk for colorectal cancer, based on occurrence and size of colorectal adenomas. For these subjects, the influence of genotypic and phenotypic variations in CYP1A2 and NAT-2 on colorectal cancer risk was investigated, as compared to control subjects.

Chapter 7: The findings of all studies described in this thesis are discussed and summarized.

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Methodologies for heterocyclic aromatic amine-DNA adduct analysis and determination of urinary 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) excretion levels

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Introduction

In our daily life we are continuously exposed to genotoxic chemicals that may interact with cellular DNA to form DNA adducts. Unless these adducts are removed by DNA repair systems, they may lead to mutations as a consequence of misreplication. There is convincing evidence that mutations in relevant target sequences, such as oncogenes or tumor suppressor genes, are associated with the carcinogenic process (1). From this, it stands to reason that accurate methods are necessary to measure human exposure to these environmental mutagens with high specificity and sensitivity. The development of extremely sensitive analytical methods for detecting DNA adduct levels in the range of 1 per 10^8 to 10^{10} normal nucleotides, offered the opportunity to use DNA adduct measurements as a quantitative tool in exposure and risk assessment. The advantage of using DNA adduct measurements in mutagen-exposed humans compared to environmental monitoring data in the context of risk assessment, is that these biomarkers integrate several aspects that determine the actual risk, including differences in exposure levels, absorption, distribution, metabolic (in)activation, genetic susceptibility and DNA repair capacity. Although there is still some debate on the biological significance of DNA adducts and the interpretation of adduct levels in terms of health risk (2,3), Tang *et al.* demonstrated that smoking-related DNA adducts in white blood cells significantly predict the risk for lung cancer in humans (4). There is however consensus that the use of adduct data in risk extrapolation has the greatest value when adduct structures have been characterized and the role of adduct removal and biological relevance of specific adducts are understood (2,5). As described in Chapter 1 of this thesis, for heterocyclic aromatic amines (HCA), the role of DNA adducts in carcinogenesis is supported by many studies. The most widely applied method for DNA adduct measurements is the ^{32}P -postlabeling assay, originally developed and described over 20 years ago (6,7). Since then, the number of studies using postlabeling techniques has been continuously growing, and the technique was greatly improved. The main improvement comprised the insertion of enrichment steps, allowing the selection of modified nucleotides among normal nucleotides (8,9). The general outline of the assay is as follows: [1] enzymatic digestion of DNA into 3'-mononucleotides; [2] adduct enrichment to enhance the sensitivity of the assay; [3] kinase-mediated phosphorylation of the adduct nucleotides with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of high specific radioactivity to form $[5'\text{-}^{32}\text{P}]\text{-3'}$ -biphosphates, and [4] chromatographic resolution of the labeled adducts (which are modified nucleoside 3'-5'-biphosphates) and their quantification.

Different modifications of the basic procedure have been applied, depending on the specific adduct under analysis. More specifically, for HCA-DNA adducts, several different methods have been described. With respect to the labeling conditions, next to the standard method (7), the intensification (*i.e.* ATP-deficient) method (10) has been used in many studies. The standard method comprises the use of a molar excess of AT^{32}P , thus labeling adducted and normal nucleotides to the same extent. With this method, an enrichment step is performed prior to the labeling, to increase the relative amount of adducted nucleotides in the labeling mixture. The intensification method is performed under ATP-deficient conditions and is based on the principle of preferential labeling, meaning that adducted nucleotides are ^{32}P -labeled rather than normal nucleotides. Furthermore, different methods have been described with respect to enrichment procedures and extra digestion steps after the labeling. The occurrence of large interlaboratory variations in HCA-DNA adduct analysis was demonstrated in an extensive interlaboratory trial (11). Therefore, it was important to determine the most optimal protocol in our laboratory, before the ^{32}P -postlabeling technique could be used in our studies.

Another way of investigating bioactivation of HCA in humans is by determination of parent compounds and/or metabolites that are excreted in the urine. These compounds may also be used as biomarkers of exposure. However, since the majority of the dose has been found to be excreted within the first 18 hours, it is only the recent exposure that is reflected in urine excretion levels (12). In order to determine the potential health risk associated with the HCA 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), several studies have been conducted on the metabolism and disposition of this compound in humans. Urinary excretion of unmetabolized PhIP was related to the dose received in well-done meat meals (13,14). Bioavailability of PhIP in humans was further demonstrated by the presence of PhIP conjugates in urine, although no information about specific metabolic pathways was obtained in this way (15,16). Also, in these studies, significant differences in excretion levels were observed between individuals, which may be due to variations in activities of enzymes responsible for the bioactivation of HCA. These enzymes, cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2), display polymorphic distribution in humans, and variabilities in their metabolic activities may influence cancer risk susceptibility (17,18). Boobis *et al.* found a 4-fold increase of urinary PhIP excretion levels in human subjects after administration of the CYP1A2 inhibitor furafylline (19). This study also showed that CYP1A2-mediated metabolism accounted for approximately 70% of the ingested dose. Recently, Stillwell *et al.* found an association between urinary excretion of the *N*²-glucuronide conjugate of PhIP and CYP1A2 activity, but not NAT2 activity in humans (20). These findings indicate that the metabolic fate of PhIP in humans strongly depends on CYP1A2 enzymatic activity, which shows interindividual differences.

Thus, monitoring HCA and their metabolites in urine can provide an overall view in assessing bioactivation and detoxification pathways (21,22). For this purpose, gas chromatography-mass spectrometry (GC-MS) is often used because of its high sensitivity and specificity, which are necessary to measure low levels in complex matrices. This assay generally comprises four steps: [1] urine hydrolysis, in which the HCA metabolites are quantitatively cleaved to liberate the parent compound; [2] extraction and possible clean-up; [3] derivatization of the polar HCA in order to make them volatile for GC; [4] GC-MS analysis. For each of these steps, several different procedures and modifications have been described (e.g. 13,14,23-25).

To be able to incorporate the use of DNA adducts and urinary HCA excretion as biomarkers of activation and exposure risk in our studies, we had to introduce and optimize the ³²P-postlabeling assay for HCA-DNA adducts and the GC-MS assay for urinary HCA in our laboratory.

Materials and methods

Chemicals and enzymes

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) and [²H₃]PhIP were obtained from Toronto Research Chemicals (Toronto, Canada). The DNA adduct standards for both IQ and PhIP were kindly provided by Dr. Herman Schut, Department of Pathology, Medical College of Ohio, Toledo, Ohio, USA. Spleen phosphodiesterases I and II were obtained from Worthington Biochemical Co. (Lakewood, NJ). T4 polynucleotide kinase was from Amersham (Braunschweig, Germany). PEI-cellulose plates were from Macherey-Nagel (Düren, Germany) and ³²P-ATP with a specific activity of ~7000 Ci/mmol was obtained from ICN (Meckenheim, Germany). Heptafluorobutyric acid anhydride (HFBA) was from Supelco (Bellefonte, PA, USA); 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), 3,5-bis(trifluoromethyl)benzyl

bromide (TFMB-Br), pentafluorobenzyl bromide (PFB-Br) and diisopropylethylamine from Aldrich (Steinheim, Germany). β -glucuronidase/arylsulfatase was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). All other chemicals were from Sigma Chemical Company (St. Louis, MO).

³²P-POSTLABELING METHODS

DNA extraction

DNA was extracted with equal volumes of phenol, phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol. Subsequently, DNA in the aqueous phase was precipitated by addition of sodium acetate (pH 6.0) and 96 % ethanol (4 °C). After drying, DNA was dissolved in 2 mM Tris and the final DNA concentration was determined spectrophotometrically at 260 nm.

DNA hydrolysis

DNA hydrolyzation was investigated by using different methods, as described by Gupta *et al.* (7), Wohlin *et al.* (26) and Phillips *et al.* (11). An alternative procedure was based on the protocol provided by Dr. Schut (Medical College of Ohio, Toledo, OH, USA), which was upscaled in terms of DNA and enzyme quantity. 15 μ g of DNA was hydrolyzed in air at 38 °C during 3.5 hours by 2.5 U micrococcal nuclease and 15 μ g of spleen phosphodiesterase, in a reaction mixture containing 10 mM sodium succinate (pH 6.0) and 5 mM CaCl₂.

Adduct enrichment

The butanol enrichment procedure we used was described by Wohlin *et al.* (26). To the DNA digest, 1 mM ammonium formate (pH 3.5) and 10 mM tetrabutyl ammonium chloride (TBA) were added. The mixture was extracted twice with water-saturated butanol, and the combined butanol phases were back-extracted twice with 0.1 mM butanol-saturated ammonium formate.

³²P-postlabeling analysis

Based on the methods described by Gupta *et al.* (7) and Randerath *et al.* (10), the labeling reaction was performed under standard or adduct-intensification conditions, respectively. For the intensification method, the protocol from Dr. Schut was used. Briefly, hydrolysed DNA (15 μ g) was incubated with [γ -³²P]ATP (225 μ Ci, 32 pmol), kinase buffer (180 mM bicine, 90 mM MgCl₂, 90 mM dithiothreitol, 4.5 mM spermidine) and polynucleotide kinase (0.45 U) for 40 min at 37 °C.

Nuclease P₁ hydrolysis

After the labeling reaction, zinc chloride, sodium acetate and hydrochloric acid were added, followed by NP₁, according to the method described by Pfau *et al.* (27). The mixture was incubated for 45 minutes.

Nuclease P₁ + phosphodiesterase I hydrolysis

After the labeling reaction, the mixture was incubated with HCl and sodium citrate buffer containing NP₁ for 10 min at 37 °C, as described by Fukutome *et al.* (28). Subsequently, the mixture was incubated with Tris and an aqueous solution of PDE I for 30 min at 37 °C.

Separation of adducts by thin layer chromatography (TLC) and adduct analysis

The reaction mixture was spotted on a PEI-cellulose thin layer plate (10 $\times$ 10 cm) with a wick of Whatman no. 1 paper (10 $\times$ 23 cm) stapled to the top. After overnight elution of

the plates in the D1 direction with 1 M sodium phosphate buffer (pH 6.5), the wicks were cut off and discarded. Elution in the D2 direction was performed with 5.3 M lithium formate, 8.5 M urea (pH 3.5) and elution in the D3 direction with 1.2 M lithium chloride, 0.5 M Tris, 8.5 M urea (pH 8.0). Finally, the plates were eluted with 1.7 M sodium phosphate (pH 6.0) overnight onto a wick of Whatman paper no. 1 (D4); again the wicks were cut off and discarded. Immediately before elutions D2-D4, the plates were predeveloped with water to ~1 cm from the bottom edge. Following each elution, the plates were washed twice with water and dried. Adduct spots on the chromatograms were located by a storage phosphor imaging technique (Molecular Dynamics, Sunnyvale, CA) (29), using a screen exposure time of 4-24 hours.

To evaluate the hydrolysis process and to determine the exact amount of DNA analyzed, a diluted aliquot of normal nucleotides was derived from the sample under analysis, and labeled with ^{32}P -ATP (50 μCi) using PNK. Individual nucleotides were separated from unused ^{32}P and unspecifically labeled material by monodirectional TLC in 0.12 M sodium phosphate (pH 6.8).

GC-MS METHODS

Internal standard

[$^2\text{H}_3$]PhIP, dissolved in methanol, was used as the internal standard, which was added to the urine sample before hydrolysis.

Urine hydrolysis

For chemical hydrolysis, either acid or alkaline conditions are applied. The alkaline hydrolysis used NaOH concentrations of 0.5 M (30) or 1 M (25), while 1 M (14,31) or 6 M (32-34) HCl was used for acid hydrolysis. Alternatively, urine samples were enzymatically hydrolyzed by β -glucuronidase/arylsulphatase (20,35).

Extraction and clean-up

Hydrolyzed urine samples were made alkaline by addition of sodium carbonate and then extracted twice with ethyl acetate. The organic extract was evaporated to dryness, resolved in HCl and washed twice with hexane. The acidic solution was made alkaline by adding sodium carbonate, and was subsequently extracted twice with ethyl acetate. The extract was evaporated to dryness before derivatization.

Derivatization procedure

Different methods describing the derivatization of HCA were explored. Sample extracts were incubated with either 3,5-bis(trifluoromethyl) benzyl bromide and diisopropylethylamine (e.g. 13,20,30,36), pentafluorobenzyl bromide and diisopropylethylamine (23,25,37,38), or heptafluorobutyric acid anhydride and diazomethane (14,24).

Clean-up after derivatization

Several investigators have applied the clean-up procedure after the derivatization reaction (13,25,36). Based on these methods, the sample was taken to dryness and resolved in HCl. The solution was washed twice with hexane and made alkaline by addition of sodium carbonate. Finally, the sample was extracted twice with ethyl acetate and evaporated to dryness before GC-MS analysis.

GC-MS analysis

The dried samples were dissolved in isooctane and the volume was reduced to 10 μl by evaporation under a stream of N_2 . For the analysis, 1 μl of the isooctane solutions were splitlessly injected into a HP5890 Series II gas chromatograph (Hewlett-Packard,

Portland, Oregon, USA), injector temperature 250 °C, using a 30 m, i.d. 0.25 mm, film thickness 0.25 µm CP-Sil8CB-MS column (Varian, Harbor City, CA, USA). Helium was used as the carrier gas at a column head pressure of 20 p.s.i. The temperature of the GC oven was 200 °C for 0.5 min, then rising to 300 °C at 20 °C/min, where it was held for 2 min. The GC column ended inside the ion source of the double focusing mass spectrometer of reversed geometry (Jeol-SX102A, JEOL Ltd., Tokyo, Japan), which was operated in either the negative ion chemical ionization (NICI) or the electron ionization (EI) mode, and mass resolution was varied between 1,000 and 10,000. Using EI, the base peaks $(M-C_3F_7)^+$ of PhIP ($m/z = 265.1089$) and $[^2H_3]PhIP$ ($m/z = 268.1277$) were recorded by selective ion monitoring (SIM).

Results

^{32}P -POSTLABELING ANALYSIS

All results, obtained by the various assays, were compared in terms of sensitivity and clarity of adduct patterns. For DNA hydrolysis, the methods by Gupta, Wohlin and Phillips were equally effective, as evaluated by monodirectional TLC of individual nucleotides. The best result, however, was obtained by the method of Schut. The butanol enrichment procedure was applied in the standard postlabeling assay, leading to better results as compared to the assay in which an enrichment step was omitted. This was not the case for NP₁ and PDE I hydrolysis after the labeling reaction, where no improvement was observed. However, application of the slightly modified adduct intensification assay resulted in the most optimal adduct sensitivity. Thus, after exploring all the different methods and modifications described, the ^{32}P -postlabeling method most suitable for application in our studies, was found to be the adduct intensification method by Dr. Schut. This means that no enrichment procedure or extra hydrolysis step was used in the eventual protocol. Figure 1 shows adduct standard patterns for both PhIP and IQ, obtained by this method. The detection limit for this assay was 1 adduct/ 10^8 normal nucleotides.

Fig. 1. DNA adduct standard profiles for (A) PhIP and (B) IQ, with modification levels of 1 adduct per 10^6 nucleotides and 1 adduct per 10^7 nucleotides, respectively. Adduct profiles were obtained using the ^{32}P -postlabeling method described by Dr. Schut.

GC-MS ANALYSIS

Enzymatic hydrolysis appeared to be effective and most useful, avoiding salt precipitations that were experienced when performing a chemical hydrolysis. The clean-up procedure with HCl was best applied in combination with the organic solvent extraction and thus before the derivatization procedure, also avoiding salt precipitations that were formed when the clean-up was performed after the derivatization. For the derivatization, the two-step procedure described by Reistad *et al.* (14), using HFBA and diazomethane for subsequent acetylation and methylation, was found to be most effective. In the eventual GC-MS analysis, the EI mode was preferred to NICI, rendering the best signal-noise ratio when the mass spectrometer was set at a mass resolution of 10,000. Thus, the most optimal assay in our laboratory setting was determined after comparing all different procedures and modifications.

Measured under these circumstances, the limit of detection for PhIP in urine was approximately 5 pg/ml. Figure 2 shows both a chromatogram and a mass spectrum for urinary PhIP, as obtained by the ultimate assay used in our studies.

Fig. 2. (A) Chromatogram of a urine sample containing PhIP, which is indicated by the arrow; (B) Spectrum and structure of the PhIP ion in the HREI mode, using selected ion monitoring.

Next to the parent compound, similar conditions were used to investigate the detection of N-OH-PhIP in urine, using the same assay that was described for 4'-OH-PhIP (14). Although the standard compound could be readily derivatized and measured using this assay, the metabolite could not be detected in human urine.

Discussion

In order to monitor the biological fate of heterocyclic aromatic amines in the human body, the use of suitable biomarkers of exposure, activation and risk is important. Covalent binding of reactive HCA metabolites to DNA, as well as the urinary excretion of HCA, can be used for this purpose. In our laboratory, we explored several modifications of ^{32}P -postlabeling methods, described for DNA adducts formed by PhIP and IQ, using TLC to separate labeled adducts. Variations in DNA hydrolysis protocols mainly comprised differences in enzyme concentrations and incubation time, except for the Schut protocol. Contrary to the other methods, where DNA was digested in a waterbath of 37 °C, in this method the incubation was performed in air at 38 °C. Hydrolysis was evaluated by monodirectional TLC analysis of individual nucleotides, demonstrating that the Schut method was most effective, showing better nucleotide patterns and less background disturbance as compared to the other assays.

If the labeling reaction is performed under standard conditions, usually an enrichment step is introduced. NP_1 selective digestion (*i.e.* dephosphorylation) of normal 3'-nucleotides enriches the sample in adducts, and is the most commonly used enrichment procedure. The dephosphorylated normal nucleotides are no longer substrates for kinase and will not be labeled. However, since HCA-DNA adducts are sensitive to NP_1 digestion (39), butanol extraction was used for enrichment of these adducts. This method mainly recovers hydrophobic adducts, which are transferred into the organic phase, while the normal nucleotides remain in the aqueous phase. Compared to postlabeling without an enrichment step, butanol enrichment prior to labeling resulted in higher recovery of adducts and less background radiation, due to a relatively higher concentration of adducts and less unspecifically labeled material. The adduct intensification (ATP-deficient) method has been employed in numerous studies to recover DNA adducts induced by HCA (*e.g.* 23,26,28,40-43). Some investigators found patterns of multiple spots after postlabeling, and suggested that some of these spots were those of undigested adducted di- or oligonucleotides. Therefore, additional digestion steps were introduced after the labeling reaction, leaving only adducted mononucleotides to be separated by TLC (27,28,44,45). Nuclease P_1 or a combination of nuclease P_1 and phosphodiesterase I were used for this purpose. In our study, incubation with these enzymes after postlabeling did not result in any noticeable improvement, indicating that DNA was already digested completely, not leaving di- or oligonucleotides to be labeled. Eventually, the adduct intensification method proved to be the most sensitive assay, and thus the most suitable method for application in our studies. Using this method, no enrichment steps or additional digestive steps were applied. Although lower detection limits have been reported for HCA-DNA adduct analysis using TLC- ^{32}P -postlabeling in some studies (*e.g.* 28; detection limit $1/10^{10}$), we were able to detect these adducts at a level of 1 per 10^8 nucleotides after optimization of the assay in our laboratory. Based on DNA-adduct levels formed by polycyclic aromatic hydrocarbons (PAH) in peripheral blood lymphocytes of humans after ingestion of PAH-containing hamburgers (46), we expect our detection limit to be adequate for DNA adduct measurements in our human studies. Especially in the colon, adduct levels are expected to be relatively high due to high metabolic activity. Techniques such as accelerated mass spectrometry (AMS), although much more sensitive with detection limits as low as 1 adduct per 10^{12} nucleotides, are not applicable in human biomonitoring studies due to the use of ^{14}C -labeled substrates (47,48). On the other hand, one might raise the question whether such low adduct levels can still be regarded as biologically relevant.

With respect to the GC-MS method to detect urinary PhIP levels, also different modifications were investigated. Although acidic conditions are most often used for hydrolyzation purposes, this method resulted in salt precipitations when reducing the

volume by evaporation, thus disturbing the assay. Therefore, enzymatic hydrolysis, not leading to any such side effects, became our method of choice. For the same reason, the HCl clean-up was performed in combination with the organic solvent extraction, as described by several other investigators (23,37). Applying the clean-up step after derivatization led to salt precipitation, possibly sodium carbonate. Alternatively, omission of the clean-up procedure was investigated as described by Friesen *et al.* (38), leading to many disturbing peaks and high noise signals in the chromatograms.

The derivatization procedure was tested in three different ways, using derivatization chemicals with either five (PFB-Br), six (TFMB-Br) or seven (HFBA) fluor atoms. Although the two-step procedure using HFBA and the carcinogenic and explosive diazomethane has not been used by many other investigators, this method was found to be the most optimal because of various chromatographic difficulties that were experienced when applying either of the other two methods. For the GC-MS analysis, the high resolution EI-mode (HREI) was adapted from Reistad *et al.* (14), who also applied the HFBA/diazomethane derivatization for determination of urinary HCA. With this method, higher selectivity and sensitivity were obtained than with the use of chemical ionization. The detection limit for PhIP measurement under these optimal conditions was 5 pg PhIP per ml urine, which is in agreement with the detection limit that was described in the original study (14), from which our protocol was adapted. Next to the parent compound, similar conditions were used to investigate the detection of N-OH-PhIP, one of the major metabolites of PhIP in the rat, in human urine. Although the standard compound could be readily derivatized, the metabolite was not detectable in urine. This was thought to be due to the clean-up procedure, during which the hydroxy-group might be lost. Therefore, our attention was focused on the analysis of unmetabolized PhIP in urine.

In conclusion, we succeeded in developing suitable methods for the detection of HCA-DNA adducts and urinary excretion of PhIP in our laboratory, which were to be used in our studies to monitor the biological fate of these food carcinogens in the human body.

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**Generation of free radicals and
induction of DNA adducts by
activation of heterocyclic aromatic
amines via different metabolic
pathways *in vitro***

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Abstract

Food-derived heterocyclic aromatic amines (HCA) have proven to be carcinogenic in both rodents and nonhuman primates. For the metabolic activation of HCA, two different metabolic pathways are suggested. The hepatic pathway proceeds via a 2-step process involving *N*-hydroxylation by CYP1A2 and subsequent *O*-acetylation by NAT2. An alternative pathway may be of particular interest in extrahepatic tissues and proceeds via one-electron oxidation catalyzed by prostaglandin H synthase (PHS), rendering free radical metabolites. In this study, we investigated the metabolic activation of two HCA, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), by two different enzyme systems *in vitro*, generating different primary and secondary reactive metabolites. Rat liver S9-mix or PHS was used as the activating system, representing the hepatic and extrahepatic pathway, respectively. Electron spin resonance (ESR) spectroscopy demonstrated that both IQ and PhIP exert inhibiting effects on the PHS-mediated formation of hydroxyl radicals during the conversion of arachidonic acid to prostaglandins. Evidence for the formation of HCA free radicals is presented in an indirect way by the formation of glutathione-derived thiyl radicals, using purified PHS as the activating system. Activation by S9-mix did not result in the formation of detectable radical metabolites, showing that the two metabolic routes primarily lead to the formation of different metabolites. In all ESR experiments, IQ appears more effective as compared to PhIP.

On the contrary, DNA adduct analysis by means of ^{32}P -postlabeling showed similar adduct patterns for S9 and PHS in single and double stranded salmon testes DNA after incubation with PhIP, indicating the ultimate formation of a common reactive intermediate. For IQ, activation by PHS led to an additional adduct spot that was not present after S9-activation. Furthermore, activation of IQ resulted in higher adduct levels as compared to PhIP for both activation pathways. Overall, adduct levels were higher in single stranded DNA as compared to double stranded DNA. Our results show that the hepatic and extrahepatic pathways result in different primary metabolites, while the ultimate formation of a similar reactive intermediate for PhIP, possibly an arylnitrenium ion, suggests that both pathways could play an important role in the onset of carcinogenesis.

Introduction

Heterocyclic aromatic amines (HCA) form a class of compounds that was first isolated and characterized by Sugimura *et al.* in 1977 (1). This class of compounds appeared highly mutagenic in the Ames mutagenicity assay after activation by both rat liver S9 (1-3) and prostaglandin H synthase (4,5). Furthermore, HCA have proven to be carcinogenic in both rodents (6-8) and nonhuman primates (9).

The bioactivation of HCA is hypothesized to occur via *N*-oxidation to *N*-hydroxy metabolites followed by *O*-acetylation to form *N*-acetoxy arylamines. These steps are catalyzed by hepatic cytochrome P4501A2 (CYP1A2) and colonic *N*-acetyltransferase 2 (NAT2), respectively. *N*-acetoxy arylamines are then further converted to chemically reactive nitrenium ions, ultimately leading to the formation of DNA adducts.

An alternative pathway, which may be of particular interest concerning the carcinogenicity of HCA in extrahepatic tissues such as colon and rectum, proceeds via one-electron oxidation catalyzed by prostaglandin H synthase (PHS) (10). PHS occurs in most mammalian tissues and features two distinct enzyme activities, namely cyclooxygenase (COX) and peroxidase. COX catalyzes the incorporation of molecular oxygen into arachidonic acid rendering a hydroperoxy endoperoxide, prostaglandin G₂ (PGG₂). Subsequent reduction of PGG₂ by the peroxidase activity of PHS yields the corresponding alcohol, prostaglandin H₂ (PGH₂) (Figure 1), which is further converted to other prostanoid metabolites (10-12).

Fig. 1. Generation of HCA free radical metabolites during peroxidase-catalyzed reduction of prostaglandin G₂ (PGG₂) to prostaglandin H₂ (PGH₂). Cyclooxygenase (COX) and peroxidase are the two distinct activities of the enzyme prostaglandin H synthase (PHS).

For the reduction of PGG₂ to PGH₂, reducing cofactors are required by PHS peroxidase in order to catalyze this reaction. Such cosubstrates donate single electrons to the peroxidase, while in turn being converted to chemically reactive, electron deficient metabolites (10).

The peroxidase catalytic cycle is also shown in Figure 1, starting with the resting PHS enzyme containing heme iron in the +3 oxidation state. By reducing the hydroperoxide

(PGG₂) to the corresponding alcohol (PGH₂) an iron-oxo complex (Compound I) is formed, in which the iron is in the formal oxidation state of +5. Compound I can be reduced by a single electron from an appropriate cosubstrate, thereby generating another iron-oxo complex (Compound II) in which the iron is in the +4 oxidation state. Finally, the ground state of the enzyme is regenerated by a second single electron reduction, while in turn another cosubstrate molecule is oxidized (10, 13).

It is generally assumed that HCA can be suitable cosubstrates for PHS peroxidase (Figure 1), thereby undergoing one-electron oxidation leading to the formation of HCA free radical metabolites producing DNA adducts (14). Indirect evidence for the formation of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) free radical metabolites by ram seminal vesicles was earlier described by Petry *et al.* (15). More recently, Wolz *et al.* (16) described the formation of identical IQ-DNA adduct patterns in hepatocytes, ovine seminal vesicle cells and *Salmonella typhimurium* after metabolic activation by either PHS or monooxygenases. However, in this study no adducts were found in isolated DNA after incubation with IQ activated by either of these enzyme systems.

In this study, we investigated the relative capacity of CYP- vs. PHS-mediated pathways to generate reactive metabolites from both IQ and 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP), and present evidence that both compounds are capable of forming adducts in isolated DNA after activation via either hepatic or extrahepatic pathways, represented by S9 mix and pure PHS, respectively. Furthermore, to our knowledge the combination of ESR spectroscopy and ³²P-postlabeling was never used before to investigate the mechanisms behind the metabolic activation of both IQ and PhIP by two different enzyme systems.

Materials and methods

Chemicals and enzymes

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) were obtained from Toronto Research Chemicals (Toronto, Canada). The DNA adduct standards for both IQ and PhIP were kindly provided by Dr. Herman Schut, Department of Pathology, Medical College of Ohio, Toledo, Ohio, USA. Purified prostaglandin H synthase (PHS) was purchased from Oxford Biomedical Research (Oxford, MI). Spleen phosphodiesterase II was obtained from Worthington Biochemical Co. (Lakewood, NJ). T4 polynucleotide kinase was from Amersham (Braunschweig, Germany). PEI-cellulose plates were from Macherey-Nagel (Düren, Germany) and ³²P-ATP with a specific activity of ~7000 Ci/mmol was obtained from ICN (Meckenheim, Germany). Glutathione, charcoal and ethanol were purchased from Merck (Darmstadt, Germany). All other chemicals were from Sigma Chemical Company (St. Louis, MO).

Electron spin resonance spectroscopy

Electron spin resonance (ESR) studies were performed at room temperature using a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12 kW power supply operating at X band frequencies. The following instrument conditions were used: scan range, 60 G; center magnetic field, 3490 G; modulation amplitude, 1.0 G; microwave power, 50 mW; receiver gain, 10⁵; modulation frequency, 100 KHz; microwave frequency, 9.85 GHz; time constant, 40.96 ms; scan time, 20.97 s; number of scans, 50. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was used for trapping hydroxyl radicals and was purified in nitrogen flushed milli Q water by mixing with 30 mg/ml charcoal during 20 min at 35 °C. This procedure was used three times to remove background ESR signals. Finally, the concentration of DMPO was determined

spectrophotometrically at 234 nm. Incubation mixtures (total volume 200 μ l) consisted of HCA (0.25, 0.50 or 1.0 mM) and DMPO (100 mM) in the absence or presence of glutathione (1 mM) and one of the following enzyme systems: (1) PHS (1 U/ μ l), hematin (5 μ M) and arachidonic acid (0.2 mM) or (2) phenobarbital induced rat liver S9 mix (equivalent to 0.3 mg protein/incubate). After 15 min of incubation at 37 °C, ESR spectra were recorded using 100 μ l capillary glass tubes. Quantitation of the spectra (in arbitrary units) was performed by peak integration using the WIN-EPR spectrum manipulation program. Simulation spectra were generated using the WIN-EPR SimFonia spectral simulation program.

Induction of HCA-DNA adducts

Stock solutions of salmon testes DNA in 2 mM Tris were used for all experiments. Single stranded DNA was obtained by denaturation of double stranded DNA at 100 °C for 5 minutes, immediately followed by chilling in ice-water. Both single stranded and double stranded DNA (100 μ g) were incubated in duplo during 1 hour at 37 °C with either 1 mM PhIP or 0.5 mM IQ and one of the following enzyme systems: (1) PHS (200 U), hematin (5 μ M) and arachidonic acid (80 μ M) or (2) phenobarbital induced rat liver S9 mix (equivalent to 0.3 mg protein/incubate). The total volume of the incubation mixtures was 0.5 mL.

Extraction and hydrolysis of DNA

The incubation mixture was extracted with equal volumes of phenol, phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol. DNA in the aqueous phase was precipitated by addition of 0.1 volumes of 1 M sodium acetate (pH 6.0) and 2 vol of 96 % ethanol (4 °C). After drying, DNA was dissolved in 2 mM Tris and the final DNA concentration was determined spectrophotometrically at 260 nm.

DNA was hydrolyzed at 38 °C during 3.5 hours in a total volume of 42 μ l containing 15 μ g DNA, 10 mM sodium succinate (pH 6.0), 5 mM CaCl_2 , 2.5 U micrococcal nuclease and 15 μ g of spleen phosphodiesterase.

³²P-Postlabeling and TLC analyses of HCA-DNA adducts

Based on the method described by Randerath *et al.* (17), the labeling reaction was performed under adduct-intensification conditions, thus preferentially ³²P-labeling the HCA-DNA adducts (18). Hydrolysed DNA (15 μ g) was incubated with [γ -³²P]ATP (225 μ Ci, 32 pmol), kinase buffer (180 mM bicine, 90 mM MgCl_2 , 90 mM dithiothreitol, 4.5 mM spermidine) and polynucleotide kinase (0.45 U) in a total volume of 62 μ l for 40 min at 37 °C. The reaction mixture was spotted on a PEI-cellulose thin layer plate (10 $\times$ 10 cm) with a wick of Whatman no. 1 paper (10 $\times$ 23 cm) stapled to the top. After overnight elution of the plates in the D1 direction with 1 M sodium phosphate buffer (pH 6.5) the wicks were cut off and discarded. Elution in the D2 direction was performed with 5.3 M lithium formate, 8.5 M urea (pH 3.5) and elution in the D3 direction using 1.2 M lithium chloride, 0.5 M Tris, 8.5 M urea (pH 8.0). Finally, the plates were eluted with 1.7 M sodium phosphate (pH 6.0) overnight onto a wick of Whatman paper no. 1 (D4); again the wicks were cut off and discarded. Immediately before elutions D2-D4, the plates were predeveloped with water to ~1 cm from the bottom edge. Following each elution, the plates were washed twice with water and dried. Adduct spots on the chromatograms were located by a storage phosphor imaging technique (Molecular Dynamics, Sunnyvale, CA) (19), using a screen exposure time of 4 hours.

Statistical methods

Differences between ESR signal peak areas were analyzed by the Mann-Whitney non-parametric two-sample test. Spearman rank correlation coefficients were determined between HCA concentrations and ESR signal peak areas.

Results

Generation of reactive oxygen species during HCA metabolism

HCA are believed to be oxidized by PHS via a one-electron mechanism, in which free radical metabolites are formed. Therefore, we attempted to detect HCA free radicals using ESR spectroscopy, both in a direct manner (i.e. without any spin trap) and by forming a spin adduct with the spin trap DMPO. Neither of these approaches led to an additional signal or an increase in the ESR control signal (data not shown). The control signal (in absence of any HCA) is a typical hydroxyl radical spectrum (DMPO-OH, Figure 2A), probably due to reactive oxygen species (ROS) that are formed in the PHS-catalyzed metabolism of arachidonic acid (20-23).

Fig. 2. Formation of spin adducts from oxygen radicals and thiyl radicals, measured by means of electron spin resonance spectroscopy. A, control incubation without IQ and glutathione. B, with 250 μM IQ. C, same as in A but with 1mM glutathione. D, same as in C but with 250 μM IQ. E, computer simulation of the spectrum for the thiyl radical spin adduct (DMPO-GS) with hyperfine splitting constants $\alpha^N = 15.4$ G, $\alpha_{\beta}^H = 16.2$ G and $\alpha^H = 0.64$ G. F, computer simulation of the spectrum for the hydroxyl radical spin adduct (DMPO-OH) with hyperfine splitting constants $\alpha^N = \alpha_{\beta}^H = 14.9$ G. G, computer simulation of the combination of spectra E + F.

Addition of IQ led to a decrease in the control signal (Figure 2B), indicating a decrease in the amount of ROS formed. This declining effect was found to be concentration

dependent (Figure 3, Spearman rank correlation coefficient $p = 0.65$, $p = 0.021$). For IQ, the difference between the decreased signal and its corresponding control was statistically significant for all concentrations ($P < 0.05$). This effect was not clearly demonstrated for PhIP, although the ESR signal was slightly inhibited at a concentration of 0.5 mM. Thus, inhibition of the DMPO-OH signal appeared to be much weaker for PhIP as compared to IQ (Figure 3), the difference between PhIP and IQ being nearly statistically significant ($P = 0.05$) for both 0.25 mM and 0.5 mM. When S9-mix was used as the activating system, no ESR signal could be detected in the absence or presence of a spin trap (data not shown).

Fig. 3. Decrease of oxygen radical formation during the PHS-catalyzed conversion of arachidonic acid by addition of IQ and PhIP, measured by means of electron spin resonance spectroscopy. Control experiments were performed without HCA. Values shown are averages ($n = 3$) with standard deviation; # indicates a statistically significant difference as compared to control ($P < 0.05$, Mann-Whitney).

Formation of HCA radical metabolites

Since no direct evidence for the formation of HCA free radicals was observed, an alternative method was used to obtain indirect evidence. This method, described by Petry *et al.* (15), is based on the fact that HCA free radicals are reduced by glutathione (24), while in turn glutathione is oxidized to form a thiyl radical. Because glutathione itself is relatively unaffected by PHS (21), the formation of thiyl radicals in presence of HCA indicates the formation of HCA free radical metabolites. These thiyl radicals form detectable spin adducts with the spin trap DMPO, leading to an ESR signal that is superimposed on the ESR spectrum of DMPO-OH, thus giving a combined spectrum of thiyl radicals (DMPO-GS) and hydroxyl radicals (DMPO-OH).

As can be seen in Figure 2C, the addition of glutathione does not affect the control signal (Figure 2A), confirming that glutathione itself is a poor substrate for PHS. However, after incubation with 0.25 mM IQ an increased ESR signal is obtained (Figure 2D). Since this spectrum matches the computer simulation of the combined spectrum of DMPO-GS and DMPO-OH (Figure 2G), it indicates the formation of thiyl radicals and thus the oxidation

of IQ, leading to IQ free radical metabolites. The formation of thiyl radicals after incubation with IQ proceeds in a concentration-dependent manner (Figure 4, Spearman rank correlation coefficient $\rho = 0.87$ at level of $P = 0.01$), showing statistically significant differences for all three concentrations as compared to their corresponding controls ($P < 0.05$).

Fig. 4. Thiyl radical formation during oxidation of IQ and PhIP. Control experiments were performed without HCA. Values shown are averages ($n = 3$) with standard deviation; # indicates a statistically significant difference as compared to control ($P < 0.05$, Mann-Whitney). #¹ indicates a significant difference between 0.25 mM PhIP and 0.25 mM IQ and #² indicates a significant difference between 0.5 mM PhIP and 0.5 mM IQ ($P < 0.05$, Mann-Whitney).

For PhIP, the same effect could be demonstrated (ESR spectra not shown), although there was no clear concentration-dependent relation and the signals showed no statistically significant differences as compared to the corresponding controls (Figure 4). In view of the poor solubility of PhIP in any organic solvent, incubation concentrations could not be further increased. Finally, the effect of PhIP on thiyl formation was significantly smaller as compared to IQ ($P < 0.05$). When S9-mix was used as the activating system, no ESR signal could be detected in the absence or presence of a spin trap (data not shown).

Formation of HCA-DNA adducts

Using the ³²P-postlabeling technique, DNA adducts were observed after incubation of both IQ and PhIP with single stranded or double stranded salmon testes DNA. HCA were activated by either S9-mix or PHS, representing the hepatic and extrahepatic metabolic pathway, respectively. For IQ, S9-activation leads to a multiple-adduct pattern for both single stranded and double stranded DNA (Figures 5A and 5B, respectively), matching the pattern of the IQ-DNA adduct standard (Figure 5E). However, for double stranded DNA, adduct spots 1 and 2 are both less intense than those in the single stranded DNA sample. In the case of PHS-activation, the profile of IQ adducts shows a pattern that is

slightly different from that after S9-activation. Besides the fact that adduct spots 1 and 2 are both less intense as compared to S9-activation, PHS-activation leads to an additional adduct spot (nr 3, Figures 5C and 5D). Also, single stranded DNA adducts are formed to a greater extent than double stranded DNA adducts after PHS-activation.

Fig. 5. Autoradiographic profiles of IQ-DNA adducts, obtained by ^{32}P -postlabeling after activation by rat liver S9-mix or prostaglandin H synthase (PHS). IQ was incubated in the presence of (A) single stranded salmon testes DNA and S9-mix or (B) double stranded salmon testes DNA and S9-mix or (C) single stranded salmon testes DNA and PHS or (D) double stranded salmon testes DNA and PHS. Panel (E) shows the profile of the IQ-DNA adduct standard with a modification level of 1 adduct per 10^6 normal nucleotides.

For PhIP, activation by two different enzyme systems leads to similar adduct profiles in single stranded DNA. When activated by S9-mix, the profile for single stranded DNA consists of three distinct adduct spots (Figure 6A), as is also the case for the PhIP-DNA adduct standard (Figure 6E). Incubation with double stranded DNA leads to only one adduct spot (Figure 6B), which is less intense than the matching spot in Figure 6A. For both single and double stranded DNA, the adduct profiles obtained after PhIP-activation by PHS (Figures 6C and 6D) are similar to the profiles in Figure 6A and 6E, again showing more intense spots for the single stranded DNA adducts.

Although the results from the ESR measurements show that different metabolic pathways initially lead to the formation of different reactive metabolites, the formation of similar DNA adduct profiles for PhIP indicates the ultimate formation of a common reactive metabolite. Furthermore, DNA adducts are formed more efficiently in single stranded DNA as compared to double stranded DNA for both enzyme systems. Comparison of the two heterocyclic amines shows that the activation of IQ leads to higher adduct levels as compared to PhIP at lower incubation concentrations for both activating systems.

Fig. 6. Autoradiographic profiles of PhIP-DNA adducts, obtained by ^{32}P -postlabeling after activation by rat liver S9-mix or prostaglandin H synthase (PHS). PhIP was incubated in the presence of (A) single stranded salmon testes DNA and S9-mix or (B) double stranded salmon testes DNA and S9-mix or (C) single stranded salmon testes DNA and PHS or (D) double stranded salmon testes DNA and PHS. Panel (E) shows the profile of the PhIP-DNA adduct standard with a modification level of 1 adduct per 10^7 normal nucleotides.

Discussion

The activation of dietary HCA can proceed via different metabolic pathways. Apart from the extensively studied hepatic pathway, there is also an extrahepatic pathway in which free radicals are believed to play a crucial role. Formation of IQ free radicals was demonstrated indirectly by Petry *et al.* (15), proving that this metabolic pathway proceeds via a one-electron mechanism. Furthermore, this study showed an inhibiting effect of IQ on both the cyclooxygenase and hydroperoxidase activity of ram seminal vesicles, measured as the inhibition of molecular oxygen incorporation into arachidonic acid and phenylbutazone, respectively. So far, no study has been described in which both hepatic and extrahepatic pathways are investigated with regard to the activation of IQ and PhIP *in vitro*, measured by both ESR spectroscopy and ^{32}P -postlabeling.

In our study, an inhibiting effect on the PHS-mediated formation of oxygen radicals during the conversion of arachidonic acid to PGH_2 was demonstrated for both IQ and PhIP, using ESR spectroscopy and purified PHS rather than ram seminal vesicles. Addition of IQ to the control incubation mixture caused a decrease of the ESR control signal (Figure 2B) in a concentration-dependent way, whereas the effect of PhIP was much weaker and not concentration-dependent (Figure 3). This difference between IQ and PhIP was nearly statistically significant ($P = 0.05$, Figure 3) and is probably due to structure-related properties. Although both IQ and PhIP belong to the HCA subclass of aminoimidazoazarenes (AIAs), for IQ the 2-aminoimidazo moiety is fused to a quinoline ring, while for PhIP this group is fused to a pyridine ring. A structure-activity relationship for HCA was also suggested by Felton *et al.* (25), with respect to mutagenic potency in the Ames test, in which IQ turned out to be far more mutagenic as compared to PhIP (26).

The ESR control signal, consisting of a typical DMPO-OH spectrum (Figure 2A), can be explained by the generation of reactive oxygen species during the PHS-catalyzed conversion of arachidonic acid to PGH_2 (20-23). These species could be both hydroxyl ($\cdot\text{OH}$) and superoxide ($\text{O}_2^{\cdot-}$) radicals. Both radical species form detectable spin adducts with the spin trap DMPO, but since the superoxide radical forms an unstable spin adduct that decomposes to a hydroxyl radical spin adduct within several minutes (27), the final ESR spectrum (i.e. after 15 minutes of incubation) is identical with the DMPO-OH signal. Inhibition of this signal means that less oxygen radical spin adducts are formed. This may be due to either a competition between the generation of reactive oxygen species and the oxidation of HCA, or reaction of HCA with the generated oxygen radicals. Either of these mechanisms would lead to the formation of HCA free radicals. However, we were not able to detect a spin adduct directly formed by any HCA free radicals, probably due to their extreme instability or reactivity (12). The exact mechanism behind these findings, however, remains unclear.

In order to obtain evidence for the formation of PHS-catalyzed oxidation products of HCA, an indirect approach was necessary. As explained earlier, glutathione can be used to reduce the obtained HCA free radicals, and in turn be oxidized to its thiyl radical ($\text{GS}\cdot$) (24). This thiyl radical can then form a stable spin adduct with DMPO, which is detectable by means of ESR spectroscopy. Indeed, after addition of glutathione to the incubation mixture, IQ caused an increase in the ESR signal (Figure 2D). This increase was concentration-dependent and was statistically significant ($P < 0.05$) for all three concentrations tested (Figure 4). The obtained ESR signal is a combination of 2 spectra, namely those of DMPO-OH (control signal) and DMPO-GS overlapping each other, as can be seen in the computer simulation of the combined spectrum (Figure 2G). For PhIP, the effect was significantly weaker as compared to IQ, although the increase was still statistically significant as compared to the corresponding control (Figure 4). Overall, for the ESR experiments it can be concluded that IQ shows a much stronger effect as

compared to PhIP, both for the decrease in oxygen radical generation and the formation of thiyl radicals. As mentioned earlier, this is most likely due to molecular structure differences. Since no ESR signal could be generated after activation of HCA by S9-mix with or without addition of glutathione, these results provide evidence for the formation of different primary metabolites by both IQ and PhIP after activation by different metabolic pathways.

The formation of DNA adducts by IQ after bioactivation by monooxygenases (MFO) or PHS was earlier described by Wolz *et al.* (16), suggesting that both activation pathways ultimately result in the same DNA-binding species (arylnitrenium ion), leading to identical DNA adduct profiles in the ^{32}P -postlabeling assay. The primary metabolites, however, were found to be different for the two pathways (28,29). The ultimate formation of a nitrenium ion as a common reactive intermediate for both metabolic routes was also suggested in other studies (30,31). So far, no adducts were found after incubation of PHS-activated HCA with isolated DNA. This was explained by the fact that the primary metabolites had to undergo a secondary metabolic step before ultimate DNA-binding species would be obtained (29). In our study, we compared the two different metabolic activation pathways in order to investigate whether different metabolites would be generated, rendering different adduct patterns in salmon testes DNA. In contrast to Wolz *et al.* (16), we found that HCA-DNA adducts can be generated in isolated DNA after both PHS- and S9-activation, demonstrating that no additional metabolic step is necessary for the formation of ultimate genotoxic species. Although only one of three adduct spots was detected in double stranded DNA after S9-activation (Figure 6B), for PhIP similar adduct profiles are obtained for both PHS- and S9-activation (Figures 6A, 6C and 6D). This indicates the formation of a common reactive metabolite, as suggested in other studies (29,30). For IQ, adduct patterns are different for PHS as compared to S9. While the pattern resulting from S9-activation is identical to the standard pattern (Figure 5E) for both single stranded and double stranded DNA (Figures 5A and 5B), the profile obtained after PHS-activation shows an additional adduct spot (nr. 3 in Figures 5C and 5D). Although the influence of species differences for the different enzyme systems cannot be ruled out, this spot may be due to the formation of DNA adducts by IQ free radical intermediates that are not yet converted to the suggested common intermediates (nitrenium ions), which are known to form different adducts to DNA than do free radical intermediates (28). The formation of a higher amount of radicals by IQ as compared to PhIP (Figure 4) may explain the presence of IQ radicals after 1 hour of incubation, in contrast to the apparent absence of PhIP radicals. Future research will focus on identification of this spot.

Furthermore, for both IQ and PhIP and for both activating systems, higher adduct levels are found in single stranded DNA as compared to double stranded DNA. This can be expected, since the reactive HCA metabolites do not experience any sterical hinder from a complementary DNA strand, thus able to form covalent bonds at greater ease as compared to double stranded DNA. Finally, IQ shows to have a greater adduct forming capacity, since IQ-DNA adducts are formed at higher levels than PhIP-DNA adducts, at an IQ-concentration only half that of PhIP (0.5 mM vs. 1 mM). In confirmation of our ESR data, these findings once again suggest that the structural differences between IQ and PhIP have great impact on their ability to form reactive metabolites. This could also be the explanation for the much higher mutagenic potential of IQ as compared to PhIP (25). In summary, our study has shown that metabolic activation of HCA via different pathways primarily leads to the formation of different reactive metabolites *in vitro*, with IQ exerting greater overall effects as compared to PhIP. Furthermore, our results show that activation via different metabolic routes ultimately leads to the formation of a common reactive genotoxic intermediate for PhIP, while for IQ an additional adduct spot is generated after PHS-activation, thus proving that no additional metabolic conversion is

necessary in order to obtain genotoxic species from both PhIP and IQ. Therefore, PHS-catalyzed activation of HCA should be taken into account when investigating the activation of HCA to genotoxic metabolites *in vivo*, especially in extrahepatic tissues. Further studies on the formation of HCA-DNA adducts in both human colon cell lines and animals could provide more clarity with regard to the relative importance of both metabolic pathways in human carcinogenesis.

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Cyclooxygenase-mediated DNA adduct formation by heterocyclic aromatic amines in human adenocarcinoma colon cells

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Abstract

Dietary heterocyclic aromatic amines (HCA) and polyunsaturated fatty acids (PUFA) are both believed to play a role in colon carcinogenesis, and are both substrate for the enzyme cyclooxygenase (COX). In HCA-7 cells, highly expressing isoform COX-2, we investigated DNA adduct formation by the HCA 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). Furthermore, we studied the role of COX, COX-2 in particular, and cytochrome P4501A2 (CYP1A2) by using the specific enzyme inhibitors indomethacin (IM), NS-398 and phenethyl isothiocyanate (PEITC), respectively. Finally, the influence of the PUFA arachidonic acid (AA, 20:4n-6) and eicosapentaenoic acid (EPA, 20:5n-3) on HCA-DNA adduct formation was investigated.

COX-mediated formation of prostaglandin E_2 (PGE_2) from linoleic acid (LA, 18:2n-6) showed that HCA-7 cells can convert LA into AA. Alternatively, EPA was found to compete with AA for COX. Strongly decreased PGE_2 levels by addition of IM demonstrated involvement of COX in PUFA metabolism. Both IM and NS-398 inhibited adduct formation by HCA to nearly the same extent, indicating involvement of COX-2 rather than COX-1, while CYP1A2 activity in HCA-7 cells was demonstrated by addition of PEITC. Overall, inhibiting effects were stronger for PhIP than for IQ. Finally, HCA-DNA adduct formation was stimulated by addition of PUFA, although high PUFA concentrations partly reduced this stimulating effect. These results show that COX, and COX-2 in particular, can play a substantial role in HCA activation, especially in extrahepatic tissues like the colon. Furthermore, the obvious interactions between PUFA and HCA in COX-2 expressing cancer cells may be important in modulating colorectal cancer risk.

Introduction

Heterocyclic aromatic amines (HCA) form a class of compounds to which humans are exposed daily through their diet. HCA are present in the ng/g range in meat and fish cooked by ordinary household methods (1,2) and they are believed to play a role in the development of colon cancer. For the metabolic activation of HCA, two different pathways have been described, both leading to the formation of HCA-DNA adducts (3). Of these two pathways, the hepatic activation by cytochrome P450 1A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2) has been studied most extensively. The alternative, extrahepatic pathway involves the enzyme cyclooxygenase (COX) and comprises co-oxidation of HCA during the metabolic conversion of polyunsaturated fatty acids (PUFA) (Figure 1), leading to the formation of free radical HCA metabolites that can undergo secondary reactions and subsequently bind to DNA (4).

PUFA form essential ingredients of the human diet, since the essential PUFA linoleic acid (LA; 18:2n-6) and α -linolenic acid (ALA; 18:3n-3) cannot be synthesized endogenously (5,6). By contrast, PUFA are also believed to play a role in colorectal cancer (e.g. 7-9). Longer-chain, more unsaturated fatty acids like arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) can be derived from linoleic acid and α -linolenic acid, respectively, by desaturation and elongation enzymes (10). Thus, there are two families of PUFA, named n-6 and n-3 fatty acids based on the position of the first double bond counted from the methyl end of the carbon chain. Special interest in these different fatty acids emerged from observations that colon cancer incidence is generally low in Eskimos in Greenland and Alaska as compared to Western populations (11). Eskimos mainly consume fish, whales and seals, thus ingesting predominantly n-3 fatty acids (6). The influence of PUFA on the development of colorectal cancer was further substantiated by Japanese emigrants, showing a progressive increase in cancer development upon moving to the United States. This phenomenon is believed to be caused by a westernization of the diet, thus shifting from food containing mainly n-3 fatty acids (e.g. fish) to food containing mainly n-6 fatty acids (e.g. meat and fat) (12). Also, numerous animal experiments confirmed beneficial effects of n-3 fatty acids, contrary to n-6 fatty acids (13-15). Nowadays, with regard to the dietary intake of PUFA, it is believed to be important to determine the (n-6) to (n-3) ratio rather than only the absolute level of each fatty acid family (16). Studies investigating the mechanisms behind these findings demonstrated involvement of the enzyme cyclooxygenase (COX), which has two active sites. The cyclooxygenase moiety catalyzes the conversion of AA into prostaglandin G_2 (PGG₂) and EPA into prostaglandin G_3 (PGG₃), while the endoperoxidase moiety catalyzes the subsequent reduction of PGG₂ to prostaglandin H_2 (PGH₂) and PGG₃ to prostaglandin H_3 (PGH₃) (Figure 1), which are precursors for several prostaglandins, thromboxanes and prostacyclins (5,6,17).

Of these products, prostaglandin E_2 (PGE₂) has been found to exert pro-carcinogenic effects by reducing the apoptotic rate and by increasing cell proliferation and cell motility in colon cancer cell lines (18-20). There are two distinct isoforms of COX, the constitutively expressed COX-1 and the inducible COX-2. Numerous studies have demonstrated an increased COX-2 expression in colorectal adenocarcinomas as compared to normal adjacent colonic tissue (e.g. 21-23). Thus, next to the metabolic activation of HCA, catalyzing the conversion of PUFA to prostaglandins represents another way in which COX may contribute to the development of colorectal cancer. Various studies have described the interaction between fatty acids and HCA. For instance, n-3 fatty acids have been found to inhibit the formation of HCA-induced DNA adducts in mice and rats (24-27). Furthermore, fatty acids have demonstrated to counteract the mutagenic effects of HCA in bacterial assays (28-30).

Fig. 1. Schematic representation of the conversion of AA (n-6) and EPA (n-3) by the cyclooxygenase and peroxidase moieties of the COX enzyme and the concomitant oxidation of HCA, leading to the formation of HCA radical metabolites.

Similar to HCA, n-3 and n-6 fatty acids are not only substrate to COX, but also to CYP1A2 (31,32). One way of PUFA to exert their chemo-preventive effect could be through affecting the catalytic activity or synthesis of CYP1A2, suggested by Schut (24). Thus, several studies have described an inhibiting effect of PUFA on DNA-adduct formation from both 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in rodents. To our knowledge, similar experiments have not yet been performed in human colon adenocarcinoma cells *in vitro*. In this study, we investigated the metabolic activation and DNA adduct forming capacity of the HCA PhIP and IQ in the human colon adenocarcinoma cell line HCA-7, which shows high constitutive COX-2 expression. Furthermore, the role of COX-2 in adduct formation by PhIP and IQ in HCA-7 cells was confirmed by using indomethacin (IM), NS-398 and phenethyl isothiocyanate (PEITC) as selective inhibitors for COX, COX-2 and CYP1A2, respectively. Finally, we studied the influence of the PUFA AA (n-6) and EPA (n-3) on HCA-DNA adduct formation in these cancer cells.

Materials and methods

Chemicals and enzymes

Bovine serum albumin (BSA; essential fatty acid-free), arachidonic acid (AA), eicosapentaenoic acid (EPA), linoleic acid (LA), indomethacin (IM), NS-398, phenethyl isothiocyanate (PEITC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and micrococcal nuclease (MN) were obtained from Sigma Chemical Company (St Louis, MO, USA). Triton X-100 and dimethylsulfoxide (DMSO) were purchased from Merck AG (Darmstadt, Germany). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) were obtained from Toronto Research Chemicals (Downsview, Ontario, Canada). The DNA adduct standards for both IQ and PhIP were kindly provided by Dr. Herman Schut, Department of Pathology, Medical College of Ohio, Toledo, Ohio, USA. Cell culture medium, fetal calf serum (FCS), sodium-pyruvate, trypsin and penicillin/streptomycin were purchased from Life Technologies (Breda, the Netherlands). Cell culture flasks were from Costar (Cambridge, MA, USA). Spleen phospho-diesterase II (SPD) was obtained from Worthington Biochemical Co. (Lakewood, NJ, USA). T4 polynucleotide kinase (PNK) was from Amersham (Braunschweig, Germany). Polyethyleneimine (PEI) cellulose plates were from Macherey-Nagel (Düren, Germany) and 32 P-ATP with a specific activity of ~7000 Ci/mmol was obtained from ICN (Meckenheim, Germany).

Cell culture

Human colonic adenocarcinoma cells (HCA-7) were kindly provided by Dr. S. Kirkland (University of London, England). Cells were grown in 10% FCS-containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 0.11 g/L sodium pyruvate, Glutamax-I, 4500 mg/L glucose and pyridoxin. During the postlabel experiments, the medium was also supplemented with 2% penstrep (100 U/ml penicillin G and 100 µg/ml streptomycin sulphate). Cells were cultured in a 5% CO₂ / 95% air atmosphere at 37 °C. For subculture, cells were treated with 0.25% trypsin (w/v) in phosphate buffered saline (PBS) after they had reached 70-90% confluence. Cells were cultured in 75 cm² culture flasks (Costar, Cambridge, MA, USA). All experiments were carried out within 25 passages.

Prostaglandin production

A PGE₂ enzyme immunoassay kit (Amersham Pharmacia Biotech UK, Buckinghamshire, England) was used for quantification of PGE₂ levels. Cells were plated at a concentration of 1.25×10^5 cells/ml in 500 µl of medium into 24 well tissue culture plates and cultured at 37 °C with 5 % CO₂. The medium was removed after 24 hours and replaced by 500 µl fresh serum-free medium for one hour. This medium was supplemented with 1 mg/ml fatty acid-free BSA and the indicated (non-cytotoxic) concentrations of AA, EPA and IM, and pre-incubated for 30 minutes at 37 °C before addition to the cells. AA, EPA and IM were dissolved in ethanol with a maximum final solvent concentration of 0.5% in the medium. The medium was collected after one hour and PGE₂ levels were determined according to the standard EIA kit protocol. The detection limit of the assay was approximately 20 pg/ml. PGE₂ levels are expressed as nanograms PGE₂ per ml medium or as mean percentages of control values (set as 100%).

Cytotoxicity analysis by LDH leakage

Leakage of the enzyme lactate dehydrogenase (LDH) in culture medium was used as an indicator of cytotoxicity, since leakage of this enzyme occurs when cells burst (33). The enzyme catalyses the conversion of pyruvate and NADH into lactate and NAD⁺, respectively. The decline of NADH is proportional to the enzyme activity and can be

measured spectrophotometrically. Cells were plated at a concentration of 5×10^4 cells/ml in 200 μ l of medium into 96 wells tissue culture plates and cultured at 37 °C with 5 % CO₂. After 24 hours, the medium was replaced by 100 μ l fresh serum-free medium, supplemented with 1 mg/ml fatty acid-free BSA and the indicated concentrations PhIP or IQ, and pre-incubated for 30 minutes at 37 °C before addition to the cells. HCA were dissolved in DMSO with a final solvent concentration of 0.2% in the medium. After 24 hours of incubation, the medium was removed and transferred to another 96 wells tissue culture plate. 100 μ l of 0.5% Triton X-100 in 50 mM potassium phosphate buffer (pH 7.5) was added to the cells for lysis. Subsequently, NADH and sodium pyruvate were added and LDH activity was determined by monitoring the decrease in NADH concentration at 340 nm using a Spectramax spectrophotometer (Molecular Devices Co., Sunnyvale, CA, USA). Cytotoxicity is expressed as LDH activity in the medium as a percentage of the total LDH activity in both cells and medium, corrected for the background leakage from control cells.

Cytotoxicity analysis by MTT colorimetric assay

The MTT assay was performed as described by Mosmann (34). The assay is based on the activity of mitochondrial dehydrogenase in viable cells, converting MTT to a blue formazan dye. Cells were plated into 96 wells tissue culture plates at 1×10^4 cells/100 μ l and cultured at 37 °C with 5 % CO₂. After 2 days, fresh medium was added to each well. At confluence, test compounds were added and incubated for 24 h at 37 °C in 5 % CO₂. Both PEITC and NS-398, inhibitors of respectively CYP1A2 and COX-2, were dissolved in DMSO with a final solvent concentration of 0.5% in the medium. Subsequently, 25 μ l of MTT dissolved in sterile PBS was added. After an incubation period of 3 h (37 °C, 5 % CO₂), the medium was replaced by 200 μ l DMSO. Formazan crystals were dissolved by gently shaking the plate (1 min), and absorption was read at 540 nm using a microplate reader (Biorad, Hercules, CA, USA). Cytotoxicity is expressed as the percentage of viable cells after exposure to the test compound.

Induction of HCA-DNA adducts

Cells were plated at a concentration of 1.8×10^5 cells/ml in 5 ml of medium into 25 cm² culture flasks. After 24 hours, the medium was removed and replaced by 5 ml of fresh serum-free medium. This medium was supplemented with 1 mg/ml fatty acid-free BSA and varying concentrations of PhIP, IQ, AA, EPA, IM, NS-398 and PEITC, and pre-incubated for 30 minutes at 37 °C before addition to the cells. After 24 hours of incubation, the cells were harvested and DNA was isolated for ³²P-postlabeling purposes. For PhIP and IQ, used concentrations (50 and 25 μ M, respectively) were determined from the linear part of the corresponding dose-response curve (data not shown).

DNA extraction and hydrolysis

After proteinase K digestion of the cells, DNA was extracted with equal volumes of phenol, phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol. Subsequently, DNA in the aqueous phase was precipitated by addition of 0.1 volumes of 1 M sodium acetate (pH 6.0) and 2 vol of 96 % ethanol (4 °C). After drying, DNA was dissolved in 2 mM Tris and the final DNA concentration was determined spectrophotometrically at 260 nm.

15 μ g of DNA was hydrolyzed at 38 °C during 3.5 hours in a total volume of 42 μ l, containing 10 mM sodium succinate (pH 6.0), 5 mM CaCl₂, 2.5 U MN and 15 μ g of SPD.

³²P-Postlabeling and TLC analysis of HCA-DNA adducts

Based on the method described by Randerath *et al.* (35), the labeling reaction was performed under adduct-intensification conditions, thus preferentially ³²P-labeling the HCA-DNA adducts (36). Hydrolysed DNA (15 µg) was incubated with [γ -³²P]ATP (225 µCi, 32 pmol), kinase buffer (180 mM bicine, 90 mM MgCl₂, 90 mM dithiothreitol, 4.5 mM spermidine) and PNK (0.45 U) in a total volume of 62 µl for 40 min at 37 °C. The reaction mixture was spotted on a PEI-cellulose thin layer plate (10 × 10 cm) with a wick of Whatman no. 1 paper (10 × 23 cm) stapled to the top. After overnight elution of the plates in the D1 direction with 1 M sodium phosphate buffer (pH 6.5) the wicks were cut off and discarded. Elution in the D2 direction was performed with 5.3 M lithium formate, 8.5 M urea (pH 3.5) and elution in the D3 direction using 1.2 M lithium chloride, 0.5 M Tris, 8.5 M urea (pH 8.0). Finally, the plates were eluted with 1.7 M sodium phosphate (pH 6.0) overnight onto a wick of Whatman paper no. 1 (D4); again the wicks were cut off and discarded. Immediately before elutions D2-D4, the plates were predeveloped with water to ~1 cm from the bottom edge. Following each elution, the plates were washed twice with water and dried. Adduct spots on the chromatograms were located and quantified using a storage phosphor imaging technique (Molecular Dynamics, Sunnyvale, CA) (37), with a screen exposure time of 24 hours. The detection limit was approximately 5 adducts per 10⁹ normal nucleotides.

Statistical analysis

Correlations (R²-values) were determined using trend line analysis in Microsoft Excel. Differences in PGE₂ levels and differences in DNA adduct levels were calculated using 95% confidence intervals.

Results*Cytotoxicity of HCA and enzyme inhibitors*

In the LDH leakage assay, compounds are considered to be cytotoxic when the relative LDH activity in the medium exceeds 10 %. Both PhIP and IQ did not exert cytotoxic effects up to a concentration of 100 µM (data not shown). Using the MTT assay, compounds were assigned cytotoxic when the resulting cell viability was less than 90 %. For PEITC, concentrations higher than 2.5 µM were cytotoxic, while exposure to NS-398 did not result in cytotoxic effects up to a concentration of 100 µM (data not shown). In further experiments, only non-cytotoxic concentrations were used.

PGE₂ production

PGE₂ levels in the medium of HCA-7 cells strongly increased after exposure to 10 µM AA for 1 hour, and a clear dose response relationship was observed (Figure 2; R² = 0.93). Exposure to EPA resulted in a dose dependent decrease in PGE₂ production (Figure 2; R² = 0.96), with a maximum decrease of approximately 30% for the highest EPA concentration tested (80 µM). When the cells were exposed to both AA and EPA, PGE₂ levels also decreased in a concentration dependent manner for all concentrations tested (Figure 2; R² = 0.85, 0.97, 0.99 and 0.88 for 10, 20, 40 and 80 µM AA, respectively). Exposing the cells to IM resulted in a dose dependent decrease of PGE₂ production, with an inhibition of almost 60 % after 24 hours of incubation with 500 µM IM (Figure 3; R² = 0.99 for 1 hour; R² = 0.89 for 24 hours).

Fig. 2. Effect of AA and EPA (alone or in combination) on the production of PGE₂ in HCA-7 cells after 1 hour. Results are expressed as mean values ($n = 2$) $\pm$ standard error. * indicates a statistically significant difference as compared to control (0 μ M AA) ($P < 0.05$). # indicates a significant difference as compared to the corresponding control (0 μ M EPA) ($P < 0.05$).

Fig. 3. Effect of indomethacin on PGE₂ production in HCA-7 cells after 1 hour and after 24 hours. Results are expressed as mean values ($n = 2$) $\pm$ standard error. * indicates a statistically significant difference as compared to control ($P < 0.05$).

Figure 4 shows that addition of LA also led to an increase in PGE₂ production, although the obtained concentrations were significantly lower as compared to AA-induced PGE₂ production for all concentrations tested ($P < 0.05$, 95% confidence interval). Furthermore, all PGE₂ concentrations were significantly lower after 1 hour of incubation as compared to 24 hours of incubation ($P < 0.05$, 95% confidence interval), contrary to PGE₂ production after exposure to AA under the same conditions. These results indicate that HCA-7 cells are likely to convert LA into AA.

Fig. 4. Effect of LA vs. AA on PGE₂ production in HCA-7 cells after 1 hour and after 24 hours. Results are expressed as mean values ($n = 2$) $\pm$ standard error. * indicates a statistically significant difference as compared to corresponding control ($P < 0.05$).

Effect of fatty acids and enzyme inhibitors on DNA adduct formation by HCA

Figure 5 shows the effect of both fatty acids AA and EPA on the DNA adduct forming capacity of IQ and PhIP. For IQ, a clear dose response relationship was observed up to concentrations of 60 µM for both fatty acids (Figure 5A; $R^2 = 0.86$ for AA; $R^2 = 0.94$ for EPA). However, not all induced adduct levels were significantly different from the corresponding control level, due to relatively large standard deviations. This stimulating effect was reduced by exposure to 80 µM of either fatty acid.

For PhIP, EPA appeared to have a stronger effect on adduct formation as compared to AA (Figure 5B). High concentrations of PUFA also led to a decrease in PhIP adduct levels, although with PhIP this effect was already observed with 60 µM of either fatty acid. Overall, increase of DNA adduct levels by addition of PUFA was stronger for IQ as compared to PhIP.

Fig. 5. Effect of AA and EPA on the formation of DNA adducts by (A) IQ and (B) PhIP in HCA-7 cells after 24 hours. Results are expressed as mean values ($n = 2$) $\pm$ standard error, with the exception of the 80 μM levels (single measurements). * indicates a statistically significant difference as compared to the corresponding control level ($P < 0.05$).

IM strongly inhibited adduct formation by PhIP, while for IQ this effect was only significant at a high concentration (Figure 6). The enzyme inhibitors PEITC and NS-398 both showed an inhibiting effect on DNA adduct formation by IQ and PhIP (Figure 7). For both heterocyclic amines, the inhibiting effects of PEITC and NS-398 were dose dependent when the inhibitors were added separately, in general leading to statistically significant differences as compared to the control level. Simultaneous exposure to both inhibitors did not result in a further significant decrease of DNA adduct levels. Both PEITC and NS-398 exerted stronger inhibiting effects for PhIP than for IQ. Overall, for PhIP the inhibiting effect of both IM and NS-398 were stronger as compared to PEITC. For IQ, the effects of PEITC and NS-398 were both stronger as compared to IM.

Fig. 6. Effect of indomethacin on DNA adduct formation by PhIP and IQ in HCA-7 cells after 24 hours. Results are expressed as mean values ($n = 2$) $\pm$ standard error. * indicates a statistically significant difference as compared to control level ($P < 0.05$).

Fig. 7. Effect of enzyme inhibitors PEITC and NS-398 on DNA adduct formation by PhIP and IQ in HCA-7 cells after 24 hours. Results are expressed as mean values ($n = 2$) $\pm$ standard error. * indicates a statistically significant difference as compared to control level ($P < 0.05$).

Discussion

Heterocyclic aromatic amines and polyunsaturated fatty acids are commonly ingested daily in the human diet. Furthermore, they both form classes of compounds that are believed to play a role in the development of colorectal cancer in humans. Thus far, several investigators have studied the interaction between PUFA and HCA in rodents, in which both n-3 and n-6 fatty acids were found to inhibit DNA adduct formation by HCA (24-27). However, the molecular mechanisms behind the interactive effects of HCA and PUFA remain to be established. HCA are known to be metabolized via both a hepatic and an extrahepatic pathway (3). The latter involves the enzyme COX, which is also important in the conversion of PUFA (Figure 1). Because of this analogy, in our study we investigated the COX-mediated pathways for both classes of compounds in HCA-7 cells, which highly express COX-2. Furthermore, selective enzyme inhibitors were used to investigate the relative importance of specific enzyme systems.

Figure 2 shows that the addition of relatively small amounts of AA results in a very strong increase in PGE₂ production after 1 hour of incubation, demonstrating highly expressed levels of COX-2 in these cells. By addition of higher concentrations of AA, a clear dose response relationship was observed ($R^2 = 0.93$). When the cells were exposed to only EPA, a clear dose dependent decrease in PGE₂ production was found (Figure 2, $R^2 = 0.96$). This can be explained by the fact that EPA has been shown to act as a competitive inhibitor for prostaglandin synthesis by AA (17, 38-41), leading to the formation of PGE₃ rather than PGE₂ (Figure 1). Moreover, EPA is believed to inhibit the activity of COX (39) and the expression of COX-2 (42), thus leading to the formation of less prostaglandins overall. Figure 2 also shows that HCA-7 cells produce large amounts of PGE₂ endogenously, as described earlier by Coffey *et al.* (43). Simultaneous exposure of the cells to AA and EPA led to an EPA dose dependent decrease in PGE₂ production for each AA concentration tested, demonstrating the competition between AA and EPA. When the general COX inhibitor IM was added to the medium of the cells, the endogenously produced PGE₂ levels were significantly decreased (Figure 3) and inhibition was strongly dependent on incubation time. The maximum inhibition measured was approximately 60% and was induced by an IM concentration of 500 μ M after 24 hours, thus demonstrating the involvement of COX in the AA-derived production of PGE₂ in these cells. In contrast to our findings, COX-independent mechanisms for IM were suggested in some studies. Smith *et al.* (44) suggested a COX-2 independent mechanism of action for IM. Moreover, IM-induced apoptosis was even found to occur in cells lacking both COX isoforms (45), possibly by activation of a receptor that is associated with apoptosis in human colorectal cancer cell lines (46,47).

In 1983, Nemoto and Takayama suggested that the number of carbon double bonds in PUFA molecules might determine their capacity to inhibit the metabolic activation of carcinogens by mixed-function oxidases in rat hepatic S9 fractions (48). By comparing the influence of LA (2 C=C bonds) and AA (4 C=C bonds) on the metabolic activation of HCA, we wanted to investigate whether this effect could be observed in HCA-7 cells. However, although HCA-7 cells have been investigated and characterized extensively (49-51), it was unknown whether these cells have the capacity to convert LA into AA *in vitro*. Figure 4 shows the effect of LA vs. AA on the production of PGE₂ after 1 hour and after 24 hours. As can be seen, addition of LA led to an increase in PGE₂ production, with significantly higher levels after 24 hours as compared to after 1 hour, contrary to the equal levels resulting from AA exposure. Since LA is not a direct substrate for COX, these findings demonstrate that HCA-7 cells can convert LA into AA, eventually leading to the formation of PGE₂. Thus, addition of LA is likely to result in a similar response as the addition of AA, although PGE₂ levels induced by LA exposure were significantly lower as compared to AA exposure after both 1 hour and 24 hours. As a consequence,

LA cannot be used to investigate the influence of the number of carbon double bonds on the metabolic activation of carcinogens in these cells.

The influence of AA and EPA on DNA adduct formation by IQ and PhIP is shown in Figure 5. For IQ, the adduct level was dose-dependently increased by addition of both AA and EPA up to concentrations of 60 μ M (Figure 5A). Similar results were found for PhIP up to concentrations of 20 μ M, although the effect of EPA was clearly stronger as compared to AA (Figure 5B). These effects can be explained by the fact that the addition of fatty acids stimulates the expression of COX-2, thus generating more HCA metabolites in the co-oxidation process during the conversion of fatty acids to prostaglandins, eventually leading to an increase in adduct formation. However, when the cells were exposed to 80 μ M of AA or EPA, IQ-DNA adduct levels were found to decrease. Although these levels were determined in single experiments, the decline in adduct level was obvious for both fatty acids. For PhIP, this inverting effect was already found after exposure to 60 μ M of either fatty acid. These latter results are in agreement with those found in most animal studies, where fatty acids showed a decreasing effect on HCA adduct formation (24-27). Several possible explanations have been suggested by other investigators, that may also apply to the results reported here. Hayatsu *et al.* suggested a limitation in cellular HCA uptake due to the presence of high concentrations of fatty acids, leading to the formation of micelles entrapping the HCA molecules (29). Another interesting explanation was provided by Corey *et al.*, who described inhibition of COX activity by EPA (39). In our study, this would then also apply to AA. Alternatively, in the presence of such high concentrations of PUFA, it could be hypothesized that HCA radical metabolites are scavenged by PUFA molecules before binding covalently to DNA. Next to interference in COX-mediated pathways, decrease in HCA-DNA adduct formation may also be due to interactions between PUFA and cytochrome P450 enzymes. Josyula and Schut suggested that n-3 fatty acids may affect the synthesis or catalytic activity of cytochromes P450 1A1 and 1A2 (27). Furthermore, various studies described the possible modulation of several cytochromes P450 by dietary fish oil, although specific effects on isoforms 1A1 and 1A2 remain unknown (52,53). Finally, competition between the fatty acids and HCA for the same site of the cytochrome P450 enzyme can be ruled out, as demonstrated by Hô *et al.* (54).

In a previous study, we demonstrated that HCA can be metabolized *in vitro* via CYP1A2- and COX-mediated pathways, both resulting in the formation of DNA adducts (3). In the present study, exposure of HCA-7 cells to both PhIP and IQ was firstly found to result in the formation of adducts. Furthermore, different selective enzyme inhibitors were used to investigate specific metabolic routes in these cells. Figure 6 shows the inhibiting effect of IM on the formation of DNA adducts by both PhIP and IQ. For PhIP, adduct levels decreased to approximately 20% of the control level after incubation with 300 μ M IM, while addition of 600 μ M showed no further decreasing effect. This could indicate that the remaining adducts are formed by cytochrome P450 catalyzed pathways, or that the inhibiting effect of IM may not be strong enough to totally inhibit the COX activity in these cells. In contrast, 300 μ M of IM did not effect the IQ adduct level. Although the highest IM concentration significantly reduced the adduct level, the effect was clearly less pronounced as compared to the effect on PhIP adduct formation.

NS-398 was used to inhibit COX-2, as this non-steroidal anti-inflammatory drug (NSAID) is reported to be highly specific for this COX isoform (55). For CYP1A2 inhibition we used PEITC, an isothiocyanate naturally occurring in cruciferous vegetables (56). As shown in Figure 7, both NS-398 and PEITC inhibited DNA adduct formation by HCA in a dose dependent manner, although for IQ only 2.5 μ M PEITC led to a statistically significant decrease. When both inhibitors were used simultaneously, decreases in adduct levels were significantly different from control levels for all combinations tested ($P < 0.05$). Although the exact cytochrome P450 isozyme activities for HCA-7 cells are not

known, these results indicate that the cells contain both CYP1A2 and COX-2 enzyme activity. However, cytochrome P450 activity in these cells may not be limited to isoform CYP1A2, since PEITC is also known to inhibit CYP1A1 activity (57). Furthermore, PhIP is known to be converted to the *N*-hydroxy derivative by both CYP1A2 and CYP1A1 (58-61). Remarkably, inhibition of DNA adduct formation by IM (Figure 6) was scarcely stronger as compared to inhibition by NS-398. This indicates involvement of mainly COX-2 and not COX-1, although involvement of the latter isoform cannot be ruled out completely. Similar findings were described by Wiese *et al.*, who found that metabolic activation of PhIP by COX-2 was clearly stronger as compared to COX-1, indicating a difference in substrate specificity (62). Furthermore, in that study the activation of IQ by COX was found to be very minimal, while in our study a decrease in IQ activation by addition of a selective COX-2 inhibitor appeared to be measured, although not statistically significant. Interestingly, Zhang *et al.* found anti-proliferative effects of NS-398 in embryo fibroblasts without any expression of COX-1 and COX-2, demonstrating a COX-independent mechanism of action for NS-398 (45). In agreement with these findings, Smith *et al.* suggested that NS-398 can exert anti-proliferative effects via both COX-2-dependent and -independent pathways in human colorectal cancer cells (44), while Elder *et al.* demonstrated COX-2-independent induction of apoptosis by NS-398 in different human colorectal cancer cell lines (63). However, the exact COX-2-independent mechanism of action of NS-398 remains unknown. Overall, Figures 6 and 7 show that the effects of the inhibitors were stronger for PhIP than for IQ. This could be due to the fact that IQ has a greater DNA adduct forming capacity as compared to PhIP (3), thus being relatively less sensitive for partial inhibition of either enzymatic pathway.

In summary, our present findings in HCA-7 cells for the first time show that these cells are capable of converting the essential fatty acid LA into AA, a precursor for many biologically active compounds. Next to high concentrations of endogenously produced PGE₂, addition of AA led to a further increase in PGE₂ production, while inhibition of PGE₂ production by EPA showed that this n-3 fatty acid competes with AA for the COX enzyme.

Furthermore, we demonstrated that both IQ and PhIP can readily form DNA adducts in these cells, while inhibition of adduct formation by specific enzyme inhibitors demonstrated the involvement of both COX-2 and CYP450 in the metabolic activation of the two HCA. Finally, simultaneous exposure to PUFA and HCA led to an increase of HCA-DNA adduct formation, an effect that was reduced by high concentrations of PUFA. Remarkably, no opposite effects were found for n-3 versus n-6 fatty acids in this perspective. In general, COX-catalyzed pathways can play an important role in the metabolic activation of xenobiotics like HCA, especially in extrahepatic tissues such as the colon. This might explain the decreased CRC risk that has been demonstrated after use of the NSAID aspirin, which is a COX inhibitor (64). Furthermore, one might speculate that the interaction between PUFA and HCA also applies *in vivo* and may therefore influence the risk for development of colorectal cancer.

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**CYP1A2 and NAT2
genotype/phenotype relations and
urinary excretion of 2-amino-1-
methyl-6-phenyl-imidazo-
[4,5-*b*]pyridine (PhIP) in a
human dietary intervention study**

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Abstract

Heating of protein-rich food like meat results in the formation of a class of food mutagens called heterocyclic aromatic amines (HCA), which are metabolically activated by cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2). Respective genes encoding for these enzymes, display polymorphic distribution in the human population and are thus believed to cause interindividual differences in cancer risk susceptibility. The present study investigated genotype/phenotype correlations for both CYP1A2 and NAT2. Furthermore, the influence of dietary exposure and CYP1A2 and NAT2 genotypes and phenotypes on urinary PhIP excretion levels was examined in 71 human volunteers. The subjects were exposed to a PhIP containing diet, and urinary PhIP concentrations were measured by means of GC-MS. The CYP1A2-164A→C polymorphism (CYP1A2*1F) and the NAT2 genotype were determined using PCR-RFLP. For NAT2, genotypes were divided into two categories (fast or slow). Subjects were phenotyped for both CYP1A2 and NAT2 by HPLC analysis of urinary caffeine metabolites. Urinary PhIP excretion levels were found to reflect recent dietary exposure levels in human volunteers. Urinary caffeine metabolite ratios were significantly different between the two NAT2 genotypes, whereas for CYP1A2, the apparent difference in metabolic ratios between the genotypes was statistically non-significant. For the first time, significant correlations were found between the CYP1A2*1F polymorphism and urinary PhIP excretion levels. This indicates that slow oxidizers excrete relatively high amounts of urinary PhIP as compared to fast oxidizers, implying that the latter group may be at higher risk for genotoxic damage due to higher doses of reactive PhIP metabolites in the systemic circulation. These results indicate that the CYP1A2*1F polymorphism could play a role in human cancer risk susceptibility.

Introduction

Diet represents a substantial part of all environmental conditions to which humans are exposed daily. Moreover, dietary exposure to mutagenic agents is believed to play a major role in the initiation process of carcinogenesis at various target sites in the human body, including the colon (1,2). Part of this mutagenic exposure is due to the fact that humans prepare their food at high temperatures prior to consumption. During the cooking process, numerous compounds can be formed, some of which are genotoxic. In 1977, Sugimura and co-workers described the occurrence of strongly mutagenic compounds in cooked meat and fish, leading to the discovery of a class of compounds nowadays known as heterocyclic aromatic amines (HCA) (3). So far, nineteen different HCA have been identified, with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) being most abundantly present in cooked meat (4,5). To exert their ultimate mutagenic/carcinogenic properties, HCA have to be metabolically activated, which can proceed via different pathways (6). In relation to colon carcinogenesis, the main steps in the metabolic activation of HCA via the hepatic pathway are *N*-hydroxylation and *O*-acetylation, catalyzed by cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2), respectively (7). HCA have proven to be poor substrates for direct *N*-acetylation by NAT2 (8). Multiple genes encoding for biotransformation enzymes such as CYP1A2 and NAT2, display polymorphic distribution in the human population and these enzyme activities are therefore liable to interindividual variation, leading to a specific metabolizing capacity profile for each person. Also, urinary excretion of unmetabolized PhIP may reflect these interindividual differences in HCA metabolism, as described earlier in other studies (9-11). In general, it is assumed that, as a consequence of these interindividual differences in bioactivating carcinogens, genetic polymorphisms cause variations in cancer risk susceptibility between individuals. Furthermore, while NAT2 activity is expressed constitutively (12), the activity of CYP1A2 is inducible by environmental factors, including the diet (13,14). With respect to NAT2, subjects can be designated either 'slow' or 'fast' acetylators, depending on their ability to acetylate many different kinds of substrates, including HCA.

For CYP1A2, several polymorphisms in the encoding gene have been described (15-17). The -164A→C polymorphism (CYP1A2*1F) is one of the most common in Caucasians and can be used in the routine assessment of the CYP1A2 genotype (18). Moreover, this polymorphism appears to be associated with differences in the inducibility of CYP1A2 (17). However, it has to be noted that, in general, solely genotyping cannot be used to predict enzymatic activities (*i.e.* phenotypes). In this respect it is of relevance that both high CYP1A2 activity and high NAT2 activity have been associated with a higher risk for developing colon cancer in humans (12,19). Moreover, high CYP1A2/NAT2 activities were found to correlate with an elevated CRC risk in individuals preferring well-done meat (20,21).

Therefore, in this study we investigated the correspondence between genotypes and phenotypic activities for CYP1A2 and NAT2. For this, CYP1A2 and NAT2 phenotypes were determined by caffeine administration, which provides valid estimates of the enzyme activities (22). Next, we exposed human volunteers to a PhIP containing diet and assessed urinary PhIP excretion as a function of CYP1A2 and NAT2 genotypes and phenotypes and dietary exposure. Since HCA are far better substrates for CYP1A2 than for NAT2, we hypothesize that interindividual variations in HCA metabolism are dominated by the CYP1A2 genotype/phenotype rather than by the NAT2 genotype/phenotype.

Materials and methods

Chemicals and enzymes

2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and [$^2\text{H}_3$]PhIP were purchased from Toronto Research Chemicals (Downsview, Ontario, Canada). Heptafluorobutyric acid anhydride (HFBAA) was from Supelco (Bellefonte, PA, USA); 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) from Aldrich (Steinheim, Germany). β -glucuronidase/aryl-sulfatase was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). Extrelut 20 columns and Extrelut refill material were from Merck (Darmstadt, Germany). Bond-Elut propylsulfonic acid (PRS) cartridges (500 mg) and Bond-Elut C_{18} cartridges (100 mg) were obtained from Varian (Harbor City, CA, USA). Taq polymerase was from Amersham (Braunschweig, Germany). 1-Methyluric acid (1U), 1-methylxanthine (1X), 1,7-dimethyluric acid (17U) and *N*-acetaminophen were from Sigma (St. Louis, MO, USA). 5-Acetylamino-6-formylamino-3-methyluracil (AFMU) was a gift from Dr. R. Fumeaux, Nestlé Research Center, Lausanne, Switzerland. Other chemicals were of HPLC- or analytical grade.

Protocol

71 healthy, non-smoking, female volunteers participated in this study. Ages ranged from 17 to 34 years. Participants were asked to consume a daily portion of 140 g of chicken for 10 consecutive days instead of their habitual lunch, while further maintaining their normal dietary habits. Subjects were assigned at random to either the high dose group ($n = 35$) or low dose group ($n = 36$). 24 hr urine samples were collected 4 days before and 7 days after the intervention period, while during this period, 24 hr urine samples were collected at all 10 days; the volumes were recorded and samples were stored at -20°C . Blood samples were collected from each individual for genotyping purposes. All participants gave their informed consent after being informed on the study aim and instructed about the procedure. This study was approved by the Medical Ethical Committee of the Academic Hospital in Maastricht.

Preparation of meat

Before frying, the boneless and skinless chicken breasts, purchased in one batch from a local supermarket, were cut into small pieces. Since two different doses of PhIP were to be compared in the intervention, two different cooking procedures were applied. For the low dose samples, meat was panfried at 200°C , just until done. For the high dose group, after panfrying, the meat was oven-broiled at 250°C for 16 min.

Extraction of HCA from prepared meat samples

Prepared meat samples were extracted and analysed by HPLC using the procedure described by Gross and Grüter (23) with minor modifications (24). Briefly, 3.5-g samples were homogenized in 1 M NaOH and mixed with Extrelut diatomaceous earth to fill an Extrelut 20 column. The HCA were eluted with 40 ml 5 % toluene in dichloromethane onto coupled Bond-Elut PRS columns. The PRS cartridges were washed with 6 ml 0.1 N HCl, 15 ml 40 % methanol in 0.1 N HCl to remove apolar heterocyclic amines, and 2 ml water; then the PRS cartridges were coupled to Bond-Elut C_{18} cartridges. The HCA were transferred to the C_{18} cartridges with 20 ml ammonium acetate (0.5 M, pH 8.0), and the C_{18} columns were subsequently eluted with 10 % ammonia in methanol. The eluate was evaporated to dryness at 50°C under N_2 , dissolved in 200 μl methanol and transferred to autosampler vials with inserts.

HPLC

HPLC separation of the HCA was performed as described by Gross and Grüter (23), on a TSK gel ODS80-TM column (TosoHaas, Montgomeryville, PA, USA, 220 mm × 4.6 mm i.d.) with a mobile phase of 0.01 M triethylamine phosphate (TEAP, pH 3.6) (solvent A) and acetonitrile (solvent B). A linear gradient (5-15 % B from 0-10 min; 15-25 % B from 10-20 min; 25-55 % B from 20-30 min) was used at a flow rate of 1 ml/min. Samples were analysed on a Gynkotek P580A HPLC system (Supelco, Bellefonte, PA, USA) with a Spark SP830 autosampler (Spark Holland, Emmen, The Netherlands) and a Perkin Elmer LS-30 programmable fluorescence detector (Perkin Elmer, Foster City, CA, USA). Excitation/emission wavelengths were 315/390 nm for PhIP. For quantification, the standard addition method was used, taking incomplete analyte recovery into account. The different HCA were identified based on retention times.

Enzymatic hydrolysis of urine

Urine samples were allowed to thaw at room temperature. After mixing, a 5 ml aliquot was transferred to a glass tube and 200 pg of [^3H]PhIP internal standard, dissolved in 100 μl of methanol, was added. After addition of 3 ml of 1 M acetate buffer (pH 4.9), 20 μl of enzyme mix, consisting of β -glucuronidase (2000 U) and arylsulfatase (16000 U), was added to the mixture. For hydrolysis, pH was adjusted to 4.9-5.1 with 1 M HCl and samples were incubated at 37 °C overnight.

Extraction of PhIP from urine

The extraction method was adapted from Lynch *et al.* (9). The hydrolysates were neutralized with 2.5 ml of 1 M sodium carbonate and subsequently extracted twice with 5 ml of ethyl acetate. The combined organic layers were evaporated to dryness at 40 °C under N_2 and the residues were dissolved in 200 μl of 0.1 M HCl. After extraction with 2 × 750 μl hexane, the aqueous phase was made alkaline with 100 μl of 1 M sodium carbonate and extracted with 2 × 750 μl ethyl acetate. The combined organic extract was again evaporated to dryness at 40 °C under a stream of N_2 .

Derivatization procedure

Derivatization procedures were adapted from Reistad *et al.* (10). The dried samples were dissolved in 100 μl ethyl acetate, which had been dried over calcium hydride. In order to derivatize the extracted HCA, 30 μl of freshly made trimethylamine-HCl (TMA) and 30 μl of HFBAA were added and after 1 hr at 40 °C the samples were dried at 40 °C under N_2 and the residue was dissolved in 100 μl ethyl acetate, which had been dried over calcium hydride. The acetylated HCA were subsequently *N*-methylated by addition of 200 μl of freshly made diazomethane, which was repeated after 30 min. Diazomethane was generated from MNNG by dissolving it in 1 ml of distilled water and 5 ml of hexane and adding 1 ml of 5 M NaOH dropwise. The methylated samples were left at room temperature overnight.

Analysis of urinary PhIP by GC/MS

The dried samples were dissolved in 300 μl isooctane and the solutions were transferred to a glass vial with insert. The volume was reduced to 10 μl by evaporation under a stream of N_2 . For the analysis, 1 μl of the isooctane solutions were splitlessly injected into a HP5890 Series II gas chromatograph (Hewlett-Packard, Portland, Oregon, USA), injector temperature 250 °C, using a 30 m, i.d. 0.25 mm, film thickness 0.25 μm CP-Sil8CB-MS column (Varian, Harbor City, CA, USA). Helium was used as the carrier gas at a column head pressure of 20 p.s.i. The temperature of the GC oven was 200 °C for 0.5 min, then rising to 300 °C at 20 °C/min, where it was held for 2 min. Under these conditions, the retention time for the HFBAA derivative of PhIP was 6.0 min.

The GC column ended inside the ion source of the double focussing mass spectrometer of reversed geometry (Jeol-SX102A, JEOL Ltd., Tokyo, Japan), which was operated in the electron ionization (EI) mode, with an ionizing energy of 70 eV and an ion source temperature of 220 °C, and set at a mass resolution of 10,000. The base peaks ($M-C_3F_7$)⁺ of PhIP (m/z = 265.1089) and [²H₃]PhIP (m/z = 268.1277) were recorded by selective ion monitoring (SIM). The limit of detection for PhIP was approximately 5 pg/ml.

Genotyping

The NAT2 genotyping procedure was adapted from the method described by Doll *et al.* (25). After proteinase K digestion, genomic DNA was extracted from peripheral blood lymphocytes with phenol-chloroform and precipitated with cold ethanol. For NAT2 genotyping, approximately 0.5 µg of DNA was amplified by polymerase chain reaction (PCR) in the presence of 0.5 µg of each of the NAT2 specific primers 5'-ATGGACATTGAAGCATATTTTGAAGAATT-3' and 5'-AAGGGTTTATTTTGTTCCTTA-TTCTAAAT-3', 0.2 mM of each dNTP, 1.1 U of Taq polymerase and PCR buffer composed of 50 mM KCl, 1.5 mM MgCl₂ and 10 mM Tris-HCl (pH 9.0) in a total volume of 50 µl. A PTC-200 PCR system (MJ Research, Watertown, MA, USA) was used to denature the mixture at 94 °C for 3 min and to subsequently subject it to 30 cycles of 94 °C for 0.5 min, 55 °C for 1 min and 72 °C for 0.5 min. Finally, incubation at 72 °C for 5 min was performed for complete extension. An aliquot of 10 µl of the 895-bp PCR product was then subjected to RFLP analysis. Nucleotide substitutions at G¹⁹¹A, A⁴³⁴C and C⁴⁸¹T were detected by digestion with the restriction enzymes *MspI* (10 U) and *KpnI* (10 U) at 37 °C for 3 h, nucleotide substitutions at G⁵⁹⁰A and G⁸⁵⁷A by digestion with *TaqI* (10 U) and *BamHI* (10 U) at 37 °C for 3h and then at 65 °C for 3h (restriction enzymes from Promega, Madison, WI, USA). DNA fragments were separated on a 5 % acrylamide (29:1) gel by electrophoresis at 30 V for 16 h. Following electrophoresis, the gel was stained with ethidium bromide and DNA fragments were visualized and photographed under UV light. Each individual was classified as a rapid acetylator when possessing 1 or 2 NAT2*4 alleles or as a slow acetylator when possessing 2 NAT2*5, NAT2*6 or NAT2*7 alleles (26,27).

For CYP1A2 genotyping, we used the method described by Christiansen *et al.* (28). Genomic DNA was amplified by PCR using the primers 5'-GGAAGGTATCAGCAGA-AAGCC-3' and 5'-GGCTCATCCTTGACAGTGCC-3' to give a 626-bp product. The total volume of the PCR reaction mixture was 50 µl and contained 100 ng of genomic DNA, 0.8 µM of both primers (Eurogentec, Seraing, Belgium), 1 U of Platinum Taq Polymerase (Invitrogen, Groningen, The Netherlands), 0.2 mM of each dNTP and PCR buffer consisting of 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂ and 50 mM KCl. The mixture was denatured at 95 °C for 5 min, followed by 35 cycles of 94 °C for 0.5 min, 62 °C for 0.5 min and 72 °C for 0.5 min, and a terminal extension at 72 °C for 10 min. Following PCR, the presence of the C/A polymorphism was detected by incubating a 20 µl aliquot of the PCR product with 1 U of the restriction enzyme *Apal* (Roche Diagnostics, Mannheim, Germany) at 30 °C for 1 h, cleaving the C alleles into two fragments of 181 and 445 bp. The digestion products were electrophoresed at 100 V for 2 h in a 2 % agarose gel containing ethidium bromide and visualised under UV light. Subjects were categorized into one of three possible genotypes: A/A, C/A or C/C (*i.e.*, fast, intermediate or slow oxidizers, respectively).

Phenotyping by caffeine administration

Urinary caffeine metabolites were measured by a procedure described by Krul and Hageman (29), with minor modifications. The subjects refrained from the consumption of products containing methylxanthine for 48 hours before and for the duration of the test. After consumption of one cup of coffee containing approximately 95 mg of caffeine, the

subjects collected a urine sample at $t=5$ h which was immediately stored at -20°C until analysis. For analysis, a 200 μl aliquot (pH adjusted to 3.5 with 1 M HCl) was saturated with 120 mg of ammonium sulphate and 200 μl of the internal standard, *N*-acetylaminophen (150 mg/l), was added. After extraction with 6 ml of chloroform:isopropanol (4:1), the organic phase was removed, gently evaporated to dryness under N_2 at 40–45 $^{\circ}\text{C}$ and resuspended in 1 ml of 0.05 % acetic acid. A 20 μl volume of the sample was injected onto a Hypersil 5 μm ODS HPLC column (15 cm $\times$ 4.6 mm i.d.) from Phase Separations (Deeside, UK), eluted with 33 % acetic acid:tetrahydrofuran:acetonitrile:milliQ-water (1:2.5:44:952.5, v/v) at a flow rate of 1 ml/min, and monitored by UV absorbance at 280 nm using an Axxiom 717-993 UV detector (Separations Analytical Instruments, Hendrik Ido Ambacht, The Netherlands). The identities of the caffeine metabolites AFMU, 1U, 1X and 17U were determined by retention time. Cytochrome P4501A2 (CYP1A2) activity was calculated from the molar ratio 5-acetylaminophen-6-formylamino-3-methyluracil (AFMU) + 1-methylxanthine (1X) + 1-methyluric acid (1U)/1,7-dimethyl uric acid (17U) and *N*-acetyltransferase 2 (NAT2) activity from the molar ratio AFMU/1X.

Statistical methods

Differences in urinary PhIP levels within and between groups were analyzed using the Student's *t*-test or Mann-Whitney nonparametric two-sample test. Differences in caffeine metabolite ratios between different genotypes were calculated using the Student's *t*-test for NAT2; in the case of CYP1A2, the Kruskal-Wallis test was used. Stepwise multiple linear regression models were used to examine the effect of CYP1A2 genotypes and phenotypes, NAT2 genotypes and phenotypes and dietary exposure levels on the urinary PhIP excretion levels in human volunteers on the various observed moments. In these models, NAT2 genotype and NAT2 phenotype were analyzed as dichotomous variables (slow = 0; fast = 1), as well as the dietary PhIP exposure level (low dose = 0; high dose = 1). The CYP1A2 polymorphism (CYP1A2*1F) was analyzed as a trimodal variable (C/C = 0; C/A = 1; A/A = 2) and the CYP1A2 phenotype as a continuous variable. Significance was accepted at $P < 0.05$. Finally, PhIP levels in chicken breast samples were compared using the Mann-Whitney test.

Results

NAT2 phenotypes were determined by the urinary molar ratio AFMU/1X, which ranged from 0.20 to 1.85. When the molar ratios were plotted as logarithmically modified values, the frequency distribution showed a bimodal distribution with a breakpoint at 0.6 for the ratio AFMU/1X (data not shown). This is in agreement with the findings in other studies (30,31). Hence, all subjects showing a ratio of 0.6 or higher were designated fast acetylators, all others were designated slow acetylators. In our study population, 35 individuals (49 %) were designated fast acetylators. CYP1A2 phenotypes were determined by the urinary molar ratio (AFMU + 1X + 1U)/17U, which ranged from 0.47 to 6.10 and showed a normal distribution when plotted as logarithmical values (data not shown).

NAT2 genotyping showed a population distribution of 37 slow vs. 34 fast acetylators (52 % vs. 48 %). For CYP1A2, 41 subjects had the A/A genotype (58 %), whereas 28 subjects had the C/A genotype (39 %) and only 2 were homozygous C/C (3 %).

When comparing individual genotyping results with caffeine-metabolite phenotyping, for NAT2 there was 79 % (56/71) overall concordance between genotype and phenotype. For the slow and fast acetylators, concordance was 80 % (29/36) and 77 % (27/35), respectively. The distribution of phenotype ratios within each genotype is shown in

Figures 1A and 1B. As can be seen, for NAT2 the bimodal phenotypic distribution can be clearly distinguished by the genotype, the difference between the phenotype distributions for the two different genotypes being highly statistically significant ($P < 0.001$, t-test). In the case of CYP1A2, the phenotypic caffeine ratios for the C/C genotype appear to be different from the ratios for the other genotypes (Figure 1B). However, due to the small number of subjects in that group ($n = 2$), the difference was not found to be statistically significant ($P = 0.104$, Kruskal-Wallis).

Fig. 1. Distribution of caffeine metabolite phenotypes categorized into genotypes for (A) NAT2 and (B) CYP1A2; • denotes outlying values.

The determined levels of PhIP in chicken breast for the low dose group and the high dose group are 1.7 ± 0.6 ng/g and 7.4 ± 2.0 ng/g, respectively (mean $\pm$ s.e.m.), showing a statistically significant difference between the doses ($P < 0.01$). Urinary excretion levels of PhIP were determined before the intervention period, on days 1, 5 and 9 during the intervention period, and 1 week after the intervention period. Figure 2 shows 24 hr urinary PhIP excretion levels of both the high dose group ($n = 35$) and the low dose group ($n = 36$). Before and after the intervention period, PhIP excretion levels are equal in both groups. During intervention, the amount of PhIP excreted in urine is higher in the high dose group as compared to the low dose group, with a statistically significant difference on day 5 ($P < 0.05$). For the low dose group, only the urinary PhIP excretion on day 9 was significantly different from the amount measured before the intervention, while for the high dose group, the difference was statistically significant at all observed moments during the intervention period ($P < 0.05$).

Fig. 2. 24 hr urinary excretion levels of PhIP; comparison between high and low dose groups.

* indicates a significant difference between the groups; # indicates a statistically significant difference as compared to corresponding pre-control value ($P < 0.05$, Student t-test).

The relationship between urinary PhIP excretion levels and the respective parameters CYP1A2 polymorphism (CYP1A2*1F), CYP1A2 phenotype, NAT2 genotype, NAT2 phenotype and PhIP exposure was tested in a stepwise multiple linear regression model. The parameters that were significantly correlated to the urinary PhIP excretion levels on the various observed moments, as well as their corresponding P-values and standardized regression coefficients, are presented in Table 1. For the pre-control values, only the NAT2 genotype showed a significant influence, indicating that fast acetylators excreted higher amounts of urinary PhIP as compared to slow acetylators. On both day 5 and day 9, the CYP1A2-164A→C polymorphism (CYP1A2*1F) was significantly correlated to the urinary PhIP excretion ($P = 0.025$ and $P = 0.028$, respectively), thus indicating that slow oxidizers (*i.e.* C/C genotype) excreted higher amounts of urinary PhIP as compared to fast oxidizers.

However, when the two C/C genotypes were excluded from the analysis, these correlations were no longer found to be significant. Furthermore, on day 5, the PhIP dose showed a significant effect on the urinary PhIP amount ($\beta = 0.258$). On day 1 and in the post-control values, no significant correlations were found. Overall, no relationship was found between urinary PhIP excretion levels and phenotypic differences between individuals.

Table 1. Parameters significantly correlated to urinary PhIP excretion, and their corresponding P-values and regression coefficients (β).

<i>Observed moment</i>	<i>Parameter significant in stepwise multiple regression</i>	<i>P-value</i>	<i>β</i>
Pre-control	NAT-2 genotype	0.025	0.266
Day 1	none	-	-
Day 5	CYP1A2*1F polymorphism	0.025*	0.260
	PhIP dose	0.026*	0.258
Day 9	CYP1A2*1F polymorphism	0.028	0.261
Post-control	none	-	-

*overall P-value = 0.009

Discussion

The metabolic activation of HCA is catalyzed by enzymes that are polymorphic in humans, leading to interindividual differences in metabolizing capacity. This may result in significant differences in cancer risk susceptibility between individuals. However, it is not only genetic variation in a human population that plays a role in the eventual cancer risk. Prior to the exposure to dietary HCA, food preparation methods determine the amount of HCA to be ingested. Due to individual dietary and cooking preferences, intake of HCA varies widely from day to day. In our study, we investigated the impact of the genotype/phenotype relationship for both CYP1A2 and NAT2 on PhIP metabolism in a dietary human intervention. In these subjects, the influence of the CYP1A2-164A→C polymorphism (CYP1A2*1F), the CYP1A2 phenotype, the NAT2 genotype and phenotype and the supplemented PhIP exposure on the urinary PhIP excretion was measured.

The distribution of NAT2 phenotypes was bimodal, with 49 % fast and 51 % slow acetylators. This ratio is representative for a Caucasian population, generally comprising 50-60 % slow acetylators (32). CYP1A2 phenotypes were determined using the urinary ratio (AFMU + 1X + 1U)/17U, showing a normal distribution. Although other molar ratios are also used for determining the CYP1A2 activity, this ratio has shown to give a reliable indication of caffeine clearance in humans (33-35).

Genotyping results for both NAT2 (48 % fast vs. 52 % slow acetylators) and CYP1A2 (58 % A/A, 39 % C/A and 3 % C/C) also showed representative distributions for a Caucasian population. For NAT2, the apparent relative discordance between genotype and phenotype (21 %) is somewhat higher as compared to findings in other studies (36-38) and may be explained by the fact that an observed phenotype cannot be simply linked to a well-defined genetic polymorphism. Figure 1A presents the distribution of NAT2 phenotype ratios within each of the NAT2 genotypes, showing that the bimodal nature of the phenotypic distribution can be clearly distinguished by the genotype. For the CYP1A2 genotypes (Figure 1B), an apparent difference in phenotype ratios was found between the C/C genotype and the other genotypes, although it was not statistically significant due to the small number of subjects.

Like for many other enzymes, the relation between genotype and phenotype is not straightforward, since the eventual metabolizing capacity (i.e. phenotype) may be influenced by many other factors. In the case of CYP1A2, the enzyme activity is not only determined genetically, but can also be induced by environmental factors such as smoking and diet (14) and even by polymorphisms in other genes such as CYP1A1 and glutathione S-transferase M1 (GSTM1) (39). Another complicating factor is that many other enzymes can be involved in the metabolic conversion of HCA (e.g., CYP1A1, CYP1B1, CYP2C9) (40,41), each with their own specific activities. Eventually, it is the phenotype that determines the metabolic outcome of the exposure to xenobiotics like HCA.

The two different cooking methods produced significantly different levels of PhIP in chicken, showing that the formation of heterocyclic aromatic amines in meat is strongly influenced by the preparation method, as also described in other studies (42-45). The difference in ingested PhIP levels was reflected in the urinary excretion when comparing the high and low dose group (Figure 2), although most differences were not statistically significant. For the high dose group, however, the PhIP levels found were significantly higher than the control value for all days measured, while for the low dose group, this was only the case for day 9 (Figure 2). Thus, urinary levels of unmetabolized PhIP clearly reflect differences in dietary PhIP exposure. The use of urinary HCA as biomarkers of exposure has been widely accepted, especially since it is believed that the use of dietary questionnaires may result in inaccurate estimates of HCA exposure (46,47). Because of the rapid excretion and short half-life of HCA in the body, only short-term exposure can be determined by measurement of urinary HCA levels (48).

Multiple linear regression showed that the urinary PhIP excretion was significantly correlated to the CYP1A2-164A→C polymorphism (CYP1A2*1F) on two observed moments during the intervention period. These observations are in agreement with the hypothesis that the primary HCA metabolism is dominated by CYP1A2 rather than by NAT2. This can be explained by the fact that the first step in the hepatic metabolic activation of PhIP in humans proceeds predominantly via *N*-hydroxylation, which is catalyzed almost entirely by CYP1A2 (49). Furthermore, although NAT2 may also metabolize PhIP directly by *N*-acetylation, HCA have proven to be poor substrates for NAT2. Therefore, the contribution of NAT2 to the primary conversion of PhIP is negligible (8). Thus, CYP1A2 mainly determines the amount of unmetabolized PhIP. Similar findings were described earlier for another HCA, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (27). Also, a significant influence of the PhIP dose on the urinary excretion was found on day 5 of the intervention period. Surprisingly, this correlation was non-significant on the other observed moments during intervention. It should be noted that the found correlations were highly influenced by the two C/C genotypes. To substantiate our findings, a larger number of C/C genotypes should be included in the study.

In contrast to the findings of Stillwell *et al.* (50), no correlations were found between the CYP1A2 caffeine ratios and the urinary PhIP excretion levels on any day. As stated earlier, this can be explained by the fact that the CYP1A2 enzymatic activity can be influenced by many other factors, resulting in a large range of metabolic ratios within a group of test subjects. Moreover, because of its inducibility, CYP1A2 enzyme activity is subject to intraindividual variation in course of time. Also, no correlation was found between NAT2 phenotypes and PhIP excretion levels. The latter was also found by Stillwell *et al.* (50), and is in agreement with the earlier described finding that HCA are poor substrates for direct *N*-acetylation by NAT2.

The apparent absence of CYP1A2 genotype influence on interindividual variability in CYP1A2 metabolic activity would be in agreement with findings in other studies (17,51,52).

In conclusion, our results show that the amount of unmetabolized PhIP in human urine clearly reflects the recent exposure to dietary HCA. Therefore, urinary PhIP levels can be used as biomarkers of short-term exposure in humans. Although the CYP1A2-164A→C polymorphism (CYP1A2*1F) does not appear to explain the variability in CYP1A2 metabolic activity in our study population, the significant correlations found between the studied CYP1A2 genotype and the urinary PhIP excretion indicate that this polymorphism could play a role in cancer risk susceptibility in humans.

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Relationship between the CYP1A2–164A→C polymorphism (CYP1A2*1F) and risk for colorectal neoplasia in humans

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Abstract

Colorectal cancer (CRC) is one of the most common malignancies in Western countries, and is believed to be strongly related to dietary factors. More specifically, the intake of processed meat and of the formed heterocyclic aromatic amines (HCA) herein, is believed to play an important role in the etiology of CRC. HCA are metabolically activated by the enzymes cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2). Respective genes encoding for these enzymes, show polymorphic distribution in the human population. These polymorphisms lead to variations in metabolic activities and may thus cause interindividual differences in cancer risk susceptibility. High activities for both CYP1A2 and NAT2 are hypothesized to be related to an elevated CRC risk. In the present study, we investigated the influence of genotypic and phenotypic variations for CYP1A2 and NAT2 on the risk for colorectal neoplasia.

The study population comprised 94 individuals at different risk of developing CRC as assessed by colonoscopy, who were phenotyped for both enzymes using urinary caffeine metabolite ratios. Furthermore, subjects were genotyped for polymorphisms of NAT2 and CYP1A2–164A→C (CYP1A2*1F) by means of PCR-RFLP.

Using logistic regression analysis, significant associations were found between the CYP1A2*1F polymorphism and the risk of colorectal neoplasia when comparing different risk groups or combinations of risk groups. Of all other variables, only age was found to be significantly associated to risk of colorectal neoplasia.

These results indicate that the CYP1A2*1F polymorphism could play a role in colorectal cancer risk susceptibility in humans. Further study in larger populations is necessary to substantiate and extend these findings.

Introduction

Colorectal cancer remains one of the most common malignancies in Western populations. In The Netherlands, more than 8000 persons are diagnosed with colon cancer each year. In a global perspective, colon cancer incidence varies widely between different regions (1). High rates are found in North America and northern Europe, while rates are lower in southern Europe and much lower in Asia and Africa. Furthermore, colon cancer rates have been found to increase in groups migrating from low- to high-incidence regions (2). Thus, although an important risk factor is formed by genetic predisposition, leading to hereditary forms of colon cancer (3), these findings indicate that environmental factors probably account for most of these distinct rate variations. Some 20 years ago, Doll and Peto already estimated that environmental, mostly dietary factors could account for 90 % of the human colorectal cancer risk (4). In numerous studies, an association was found between the intake of processed meat and risk of colon adenoma (e.g. 5,6) or colon cancer (e.g. 7,8). In this perspective, the formation of heterocyclic aromatic amines (HCA) during the processing of meat is considered to be of significant importance. Since their discovery in the 1970s, HCA have found to be highly mutagenic in bacteria, as well as carcinogenic in rodents (9) and non-human primates (10). More specifically, the HCA 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) has proved to be a colon carcinogen in rats (11). This might implicate that the mutagenic HCA may induce early mutations in the human colorectal tumorigenesis model as proposed by Vogelstein (12). In humans, HCA are efficiently absorbed into the systemic circulation after ingestion, thus being almost completely bioavailable. However, in order to interact with DNA, HCA are unactive until metabolized, which can proceed via different pathways (13). In the hepatic pathway, successive metabolic steps comprise *N*-oxidation and *O*-acetylation, which are catalyzed by cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2), respectively (14). These enzymes are encoded by genes that are polymorphic in humans, thus leading to interindividual differences in enzyme activities. Polymorphism in NAT2 results in a 'slow' or 'fast' acetylator phenotype, depending on the ability to *N*-acetylate HCA and other substrates. With respect to CYP1A2, several polymorphisms have been characterized in the past few years (15-17). Of these, the CYP1A2-164A→C polymorphism (CYP1A2*1F) is very common in Caucasians and is expected to result in a decreased capacity to activate HCA. Furthermore, the polymorphism appears to be related to variations in CYP1A2 inducibility (17). In this way, the CYP1A2*1F polymorphism may underlie to large interindividual differences in CYP1A2 phenotype.

It is assumable that these differences in bioactivating capacity may implicate variations in cancer risk susceptibility between individuals. However, besides genetic constitution, many other factors may affect enzyme activities. For instance, the CYP1A2 enzymatic activity can be influenced by environmental factors such as smoking and diet (18), but also by endogenous factors like gene-gene interactions (19). Analyzing both genotypes and phenotypes therefore provides complementary information on genetic impact. To obtain valid estimates of actual NAT2 and CYP activities, individuals are usually phenotyped using urinary caffeine metabolites (20).

In order to evaluate the role of CYP1A2 and NAT2 genotypes and phenotypes in CRC risk, we designed a case-control study with 3 risk groups based on occurrence and size of colorectal adenomas. In these subjects, we investigated CYP1A2 and NAT2 genotype/phenotype relations and their influence on the relative risk for developing colorectal neoplasia.

Materials and methods

Chemicals and enzymes

1-Methyluric acid (1U), 1-methylxanthine (1X), 1,7-dimethyluric acid (17U), *N*-acetaminophen and proteinase K were from Sigma (St. Louis, MO, USA). 5-Acetyl-amino-6-formylamino-3-methyluracil (AFMU) was a gift from Dr. R. Fumeaux, Nestlé Research Center, Lausanne, Switzerland. Taq polymerase was purchased from Amersham (Braunschweig, Germany). Other chemicals were of HPLC- or analytical grade.

Design and study population

A case-control study was designed to evaluate the roles of genotypes and metabolic phenotypes in the risk to develop colorectal neoplasia. The study population consisted of 94 individuals of Caucasian origin (58 men and 36 women, mean age 59 ± 12 years, range 29-82 years), who had to undergo endoscopic examination at the Maasland Hospital in Sittard (The Netherlands) between February 1999 and July 2002, and who agreed to participate in this study. Previous history of colorectal cancer or intraabdominal surgery were excluding criteria. During colonoscopy, rectal biopsies were taken from normal colon epithelium for DNA adduct analysis. Subjects were assigned to three different risk groups, based on the occurrence and size of adenomas (21). The low-risk control group comprised subjects showing no neoplastic or other abnormal bowel conditions. Subjects with small colorectal adenomas (diameter < 1 cm) were assigned to the medium-risk group, and those with large colorectal adenomas (diameter ≥ 1 cm) and/or colorectal carcinomas *in situ* were assigned to the high-risk group. All participants gave their informed consent after being informed on the study aim and instructed about the procedure. This study was approved by the Medical Ethical Committee of the Maasland Hospital in Sittard.

³²P-postlabeling

HCA-DNA adduct analysis in biopsies was performed by means of ³²P-postlabeling under adduct-intensification conditions, as described in detail elsewhere (13).

Genotyping

The NAT2 genotyping procedure was adapted from the method described by Doll et al. (22), leading to a classification of slow and fast acetylators (23). For CYP1A2 genotyping, we used the method described by Christiansen et al. (24), and subjects were categorized into one of three possible genotypes: A/A, C/A or C/C.

Phenotyping by caffeine administration

The urinary caffeine metabolites AFMU, 1U, 1X and 17U were determined by HPLC analysis as described by Krul and Hageman (20). Cytochrome P4501A2 (CYP1A2) activity was calculated from the molar ratio $(AFMU + 1X + 17U)/17U$ and *N*-acetyltransferase 2 (NAT2) activity from the molar ratio $AFMU/1X$.

Statistical analysis

Differences between phenotypic caffeine ratios for the different genotypes, age and smoking status were tested using the nonparametric Mann-Whitney two-sample test (NAT2) or the Kruskal-Wallis multi-sample test (CYP1A2). To assess the influence of age, smoking status, the CYP1A2*1F polymorphism, the CYP1A2 phenotype and the NAT2 genotype and phenotype on the CRC risk, logistic regression models were used. Because the CYP1A2 and NAT2 metabolic activity ratios appeared to be normally distributed, both phenotypes were introduced as continuous variables in the logistic regression models. Furthermore, the NAT2 genotype was analyzed as a dichotomous

variable (slow vs. fast) and the CYP1A2*1F polymorphism as a trimodal variable (C/C, C/A or A/A). Finally, different models were constructed to compare two risk groups or combinations of risk groups, thus enabling binary logistic regression.

Results

The total research population comprised 94 persons. Of these individuals, 19 (20%) were classified as low-risk control patients, showing no neoplastic bowel conditions upon endoscopic examination. Of the 75 patients, 37 (49%) were classified as medium-risk subjects, whereas the resulting 38 (51%) were assigned to the high-risk group (see Table 1 for demographic details). Of the latter group, 6 patients were diagnosed with a carcinoma *in situ*, however not showing any infiltration into colon mucosa.

³²P-postlabeling analysis of colon biopsies did not lead to any detectable levels of HCA-DNA adducts. The urinary AFMU/1X molar ratio (*i.e.* NAT2 phenotype) ranged from 0.08 to 3.29. CYP1A2 phenotypes were determined using the urinary caffeine metabolite ratio (AFMU + 1X + 1U)/(17U), which ranged from 1.44 to 14.24 within the study population. Plotted as logarithmic values, the frequency distributions of the phenotypic ratios over the whole study population showed normal distributions for both CYP1A2 and NAT2 (Figure 1). CYP1A2 metabolic activities did not differ significantly between smokers and non-smokers (P = 0.07; Student's t-test). Furthermore, neither CYP1A2 nor NAT2 metabolic activities were influenced by age. NAT2 genotyping resulted in a population distribution of 54 slow and 40 fast acetylators (57% and 43%, respectively). For CYP1A2, 49 subjects had the A/A genotype (fast oxidizers; 52%), 38 subjects had the C/A genotype (intermediate oxidizers; 40%) and 7 were homozygous C/C (slow oxidizers; 8%).

Table 1. Study population demographics.

	Group		
	Low-risk (n = 19)	Medium-risk (n = 37)	High-risk (n = 38)
Mean age (± SD)	48 (± 11)	61 (± 12)	62 (± 9)
Smoking subjects (%)	5 (26%)	10 (27%)	3 (8%)
Mean size of adenomas removed (cm ± SD)	-	0.5 (± 0.3)	1.1 (± 0.9)
Mean # of polypectomies per patient (± SD)	-	1.8 (± 1.0)	1.6 (± 0.8)

Fig. 1. Frequency distributions for the logarithmic values of the (A) CYP1A2 and (B) NAT2 urinary caffeine metabolite ratios.

The distributions of phenotypic values within the different genotype groups is presented in box-plot graphs (Figure 2). For CYP1A2 (Figure 2A), the difference between the phenotypic caffeine ratios was not significant ($P = 0.063$; Kruskal-Wallis), due to a small number of extreme values (*i.e.* cases with values more than 3 interquartile ranges from the upper edge of the box; cases not shown in Figure 2A). When these extremes were omitted from the analysis, the difference turned out highly significant ($P = 0.02$; Kruskal-Wallis). Figure 2B shows the distribution of NAT2 phenotypic caffeine ratios between the two different NAT2 genotypes, the difference being highly statistically significant ($P = 0.002$; Student's t-test). In general, the catalytic activities of both enzymes could be well related to the corresponding genotypes.

Fig. 2. Distribution of caffeine metabolite phenotypes categorized into genotypes for (A) CYP1A2 and (B) NAT2; • indicates outlying values.

The association between the risk for colorectal neoplasia and the respective parameters age, smoking status, CYP1A2*1F polymorphism, CYP1A2 phenotype, NAT2 genotype and NAT2 phenotype was tested in a logistic regression model. For the different analyses, most relevant parameters that appeared correlated to the CRC risk are presented in Table 2, with their corresponding P-values and odds ratios (OR). There was no statistically significant association between the risk for colorectal neoplasia and the smoking status, CYP1A2 phenotype, NAT2 phenotype or the NAT2 genotype in any of the various regression models. Furthermore, interaction terms including different phenotypes or genotypes appeared not to be significant variables in explaining CRC risk (data not shown). However, comparison of these different models showed that both age and the CYP1A2-164A→C polymorphism (CYP1A2*1F) were significant variables in the analysis (Table 2). The negative correlation coefficient for the CYP1A2*1F polymorphism indicates that the risk for developing colorectal neoplasia decreases when one or both of the CYP1A2 alleles possess the mutation. More specifically, comparison between the combined high-/medium-risk group and the low-risk group showed a significant association with age ($P < 0.001$, OR = 1.1 [95% CI, 1.0-1.2]) and a non-significant association with the CYP1A2*1F polymorphism ($P = 0.073$, OR = 2.0 [95% CI, 0.9-4.4]). For the low-risk group versus the medium-risk group, only age was found to be a significant variable ($P = 0.005$, OR = 1.1 [95% CI, 1.0-1.2]). When comparing the high-risk group with either the low-risk group or the combined medium-/low-risk group, age was only found to be a significant variable in the first analysis ($P = 0.002$ and 0.07, with ORs of 1.1 [95% CI, 1.0-1.2] and 1.0 [95% CI, 0.99-1.1], respectively), while the CYP1A2*1F polymorphism was of significant influence in both cases ($P = 0.017$ and 0.038, with ORs of 3.7 [95% CI, 1.3-10.7] and 2.2 [95% CI, 1.0-4.6], respectively).

Table 2. Results of logistic regression analysis, showing most relevant parameters that appear correlated to risk for colorectal neoplasia.

<i>Dependent variable</i>	<i>Independent variable</i>	β	OR (95% CI)	<i>P-value</i>
Low vs. (medium + high) risk	CYP1A2*1F	- 0.711	2.0 (0.9 – 4.4)	0.073
	age	0.089	1.1 (1.0 – 1.2)	< 0.001
Low vs. medium risk	age	0.074	1.1 (1.0 – 1.2)	0.005
Low vs. high risk	CYP1A2*1F	- 1.303	3.7 (1.3 – 10.7)	0.017
	age	0.123	1.1 (1.0 – 1.2)	0.002
(Low + medium) vs. high risk	CYP1A2*1F	- 0.783	2.2 (1.0 – 4.6)	0.038
	age	0.037	1.0 (0.99 – 1.1)	0.070

Discussion

The polymorphic nature of genes encoding for key enzymes in the metabolic activation of HCA in humans, is believed to result in interindividual differences in metabolizing potential. In this way, genetic polymorphisms may lead to differences in cancer risk susceptibility between individuals. Here, the influence of the CYP1A2-164A→C

polymorphism (CYP1A2*1F), the CYP1A2 phenotype and the NAT2 genotype and phenotype on the risk for development of colorectal adenomas was studied in a case-control setting, using logistic regression models.

Development of colorectal adenoma and cancer is known to occur more frequently at higher age. Therefore, it is not surprising that in this study, significant associations were found between age and CRC risk. However, to our knowledge, so far only one study investigated the relation between the CYP1A2*1F polymorphism and CRC risk, but no significant association was found (25). In contrast to these findings, our analysis is the first to clearly show a risk reducing influence of the CYP1A2*1F polymorphism on colorectal neoplasia risk, despite our relatively small study population. Because almost all colorectal cancers arise from preexisting adenomas (12), both adenoma patients and cancer patients were included in our study, and were divided into different risk groups. For the CYP1A2*1F polymorphism, statistically significant associations were found when comparing the high-risk group to the low-risk or to the combined low-/medium-risk group, with odds ratios of 3.7 (95% CI, 1.3-10.7) and 2.2 (95% CI, 1.0-4.6), respectively. Furthermore, there was an association of borderline significance when comparing the low-risk group to the combined medium-/high-risk group, the odds ratio being 2.0 (95% CI, 0.9-4.4). These findings indicate that individuals carrying the CYP1A2-164A/A constellation are at higher risk for producing mutagenic HCA metabolites, which may induce an early mutation in the sequence of the Vogelstein model for colorectal tumorigenesis (12). Thus, these subjects are at higher risk for developing colorectal neoplasia as compared to those with a C/A or C/C genotype.

The CYP1A2 phenotypic activity was not found to be correlated to the CRC risk, despite the significant association between CYP1A2 genotypes and phenotypes when omitting extreme values. High CYP1A2 activities have previously also not been found to correlate to CRC risk (26,27). In our study, CYP1A2 activities did not differ significantly between smokers and non-smokers and CRC risk was not influenced by smoking status.

With respect to NAT2 genotyping, a positive relationship between fast acetylators and CRC was found in only one study (28). Our results are therefore in agreement with most other studies (e.g. 29,30), showing no significant association between the NAT2 genotype and risk of colorectal neoplasia (data not shown). Several studies have found a significantly elevated CRC risk for individuals who possess both high NAT2 and CYP1A2 activities (31,32), whereas other studies have not (26). Furthermore, some studies found a significant association between high NAT2/CYP1A2 activities and CRC in individuals who preferred well-done red meat (27,33). Alternatively, patients with the hereditary familial adenomatous polyposis (FAP) syndrome showed lower NAT2 activities and higher CYP1A2 activities as compared to control subjects (34). In our study, we used logistic regression models to investigate the influence of genotypic and phenotypic variations in CYP1A2 and NAT2 on the risk of colorectal neoplasia. High CYP1A2 and NAT2 phenotypic expressions were neither independently nor interactively significantly correlated to CRC risk (data not shown).

The CYP1A2*1F polymorphism is a very common single nucleotide polymorphism (SNP) of the CYP1A2 gene, which has been associated with altered phenotypic activity in smokers (15,17). Recently, we found a significant association between the CYP1A2 SNP and urinary excretion levels of the HCA 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in a human dietary intervention study, demonstrating that high CYP1A2 activity leads to increased bioactivation of PhIP, thus leaving less of the unchanged HCA to be excreted in urine (submitted for publication). Although the CYP1A2*1F polymorphism is located in a non-coding region (intron 1) of the CYP1A2 gene, it may effect CYP1A2 phenotypic activity due to modification of a target sequence for the binding of regulatory proteins (15). Unfortunately, in the present study we were not able to identify HCA-DNA

adducts in polyps biopsies, apparently due to lack of sufficient sensitivity of the postlabeling assay.

The frequency distributions for both CYP1A2 and NAT2 enzymatic activities showed normal distributions when plotted as logarithmic values (Figure 1). For NAT2, this is contrary to most other studies using urinary caffeine metabolites to assess metabolic activity, where the AFMU/1X ratios showed a bimodal (e.g. 26,28) or even a trimodal distribution (35). This could be due to the relatively small study population in our study. Genotyping results showed population distributions that were comparable to those in Caucasian populations in other studies (17,36). Although NAT2 phenotypic ratios were found to be significantly different for the two genotypes, the NAT2 phenotype was introduced as a continuous variable in the logistic regression analysis, in view of the apparently normal distribution when plotted as logarithmic values (Figure 1B). In several studies, associations were found between high NAT2 enzymatic activities and susceptibility to colorectal neoplasia (e.g. 37), but other studies failed to demonstrate such a relationship (26,38).

For CYP1A2, the nearly significant difference in phenotypic ratios for the different genotypes reached a high degree of significance when the extreme values were omitted from the analysis. Although the expression of CYP1A2 enzymatic activity can be influenced by many other factors, such as smoking, diet (18) and polymorphisms in other genes (19), we confirm a borderline significant association between CYP1A2 genotypes and phenotypes. These findings are contrary to those of Sachse et al., who found no association between the CYP1A2*1F polymorphism and the CYP1A2 phenotype, using a slightly different urinary caffeine metabolite ratio to assess the phenotype (39). The caffeine metabolite ratio used in our study, [(AFMU + 1X + 1U)/17U], has proved to give a reliable indication of caffeine clearance in humans (40).

In conclusion, for the first time a significant association between the CYP1A2-164A→C polymorphism and the risk of colorectal neoplasia in a human study population is described.

These results are in agreement with the hypothesis that genetically susceptible subgroups are particularly at risk with respect to the development of colorectal adenomas, possibly due to increased capacity of wild types of bioactivating HCA into their ultimate carcinogenic metabolites. Unfortunately, we could not quantify levels of HCA binding to DNA of colon biopsies, in order to evaluate this test hypothesis. As our results demonstrate that the CYP1A2-164A/A genotype is quite common occurring at 52 % in the general population, in order to reduce overall colon cancer risk, it makes sense to avoid exposure to food ingredients such as HCA which require bioactivation for becoming mutagenic.

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Summary & general discussion

It is undisputed that humans are daily exposed to a large variety of genotoxic compounds. A substantial part of this exposure can be attributed to the diet, and there is convincing evidence that the intake of certain food compounds may contribute to cancer risks. More specifically, the intake of red and processed meat is believed to be related to colon cancer risk.

According to the Vogelstein model, the development and progression of colorectal tumors is resulting from an accumulation of multiple genetic alterations in both oncogenes and tumor suppressor genes. In the majority of colorectal cancers, the APC gene is inactivated in an early stage of tumorigenesis, which triggers a cascade of other genetic alterations. It is hypothesized that exposure to dietary mutagens, as present in foods, may be of significant importance in these early mutations. In this perspective, the occurrence of heterocyclic aromatic amines (HCA) in processed meat is suspected to be relevant. HCA, firstly discovered and characterized some 25 years ago, are formed during the cooking of meat and are pyrolytic products from reactions involving different meat constituents like sugars, amino acids and creatine. HCA have proved to be highly mutagenic in bacteria, and carcinogenic in rodents and non-human primates. In humans, numerous epidemiological studies have demonstrated a relationship between HCA intake and colorectal cancer risk (CRC), but these studies give no information about the metabolic and bioactivation pathways these HCA pass through in the human body. To obtain more insight in these processes, biomarkers have been developed that integrate several aspects that influence the ultimate risk. **Chapter 2** describes the development and optimization of two methods that are widely used to determine biomarkers for HCA exposure and activation. The formation of covalently bound DNA adducts is regarded as an essential step in the carcinogenic process and these adducts can be seen as markers of internal dose and therefore be used as biomarkers of risk at target sites, e.g. DNA. Adduct levels can vary widely between individuals, due to differences in exposure levels, absorption, distribution, metabolic (in)activation, genetic susceptibility and DNA repair capacity. The ^{32}P -postlabeling assay is a very sensitive method and is widely used for detection of DNA adducts. However, many different methods and modifications of basic methods have been developed since this technique was described for the first time, more than 20 years ago. As a result, large interlaboratory variations in DNA adduct analysis have arisen. Alternatively, the measurement of HCA compounds in urine reflects recent HCA exposure and bioactivation. GC-MS analysis has been widely applied for this purpose, because of its selectivity and sensitivity. Again, many different methods have been developed and described for this technique. For both ^{32}P -postlabeling and GC-MS, selection and optimization of the most appropriate assay was necessary to achieve the research goals of our particular studies. Eventually, we successfully developed suitable methods for application in our studies. The detection limits under the most optimal conditions were 1 adduct per 10^8 nucleotides for postlabeling and 5 pg PhIP per ml urine for GC-MS analysis, making it possible to study HCA metabolism *in vitro* and *in vivo*.

Metabolic activation of HCA may proceed via two different pathways. The hepatic pathway involves *N*-oxidation to *N*-hydroxy metabolites, catalyzed by cytochrome P4501A2 (CYP1A2), and subsequent *O*-acetylation to *N*-acetoxy arylamines, catalyzed by *N*-acetyltransferase 2 (NAT2). The extrahepatic pathway is dependent on the metabolic conversion of arachidonic acid (AA), during which HCA can serve as cosubstrates, thus being oxidized to free radical metabolites. This reaction is catalyzed by the enzyme prostaglandin H synthase (PHS), which is often termed cyclooxygenase (COX), featuring two distinct activities. In **Chapter 3**, the different routes for PhIP and IQ bioactivation were studied *in vitro*, using both electron spin resonance (ESR) spectroscopy and ^{32}P -postlabeling to monitor the formation of primary and secondary HCA metabolites. Using ESR spectroscopy, both PhIP and IQ were found to inhibit the

COX-mediated formation of oxygen radicals during the conversion of arachidonic acid to prostaglandins, possibly due to competition between the generation of reactive oxygen species (ROS) and HCA oxidation, or reaction of HCA with these ROS. The hepatic and extrahepatic pathway were represented by rat liver S9-mix and PHS, respectively, and led to different primary metabolites for both HCA. Formation of HCA radicals was indicated by the detection of glutathione-derived thiyl radicals. In this way, the generation of free radical metabolites was indirectly demonstrated for PhIP and IQ in the PHS-catalyzed pathway, but not in the S9-catalyzed route. HCA bioactivation can eventually lead to metabolites that can covalently bind to DNA, thus forming DNA adducts that are mutagenic. DNA adducts that are formed in the colon may induce early mutations in crucial genes, which can eventually result in colorectal adenomas, as described in the Vogelstein model. ³²P-postlabeling analysis showed identical DNA adduct profiles after S9- and PHS-activation of PhIP in the presence of single and double stranded salmon testes DNA, indicating the ultimate formation of a common reactive metabolite, presumably an aryl nitrenium ion. For IQ, an additional spot was found after PHS-activation, which may result from direct DNA adduct formation by IQ free radicals. Overall, in both the ESR and postlabeling assays, IQ showed stronger effects as compared to PhIP. These results indicate that the PHS-catalyzed activation of HCA should be taken into account when the formation of genotoxic HCA metabolites which may induce mutagenic events, is investigated *in vivo*, especially in extrahepatic tissues like the colon.

The difference in DNA adduct forming capacity of PhIP and IQ was also observed in human adenocarcinoma colon cells (HCA-7), as described in **Chapter 4**. These cells highly express enzyme isoform COX-2, and can therefore be used to investigate HCA activation via the extrahepatic pathway, comprising one-electron oxidation during the conversion of the polyunsaturated fatty acid (PUFA) AA to prostaglandin E₂ (PGE₂). Next to AA, addition of linoleic acid (LA) also resulted in PGE₂ formation, demonstrating that HCA-7 cells are capable of converting LA into AA. DNA adducts were readily formed by both PhIP and IQ, and their formation was stimulated by addition of the n-6 PUFA AA and the n-3 PUFA EPA, clearly demonstrating involvement of COX in this process. The promoting effect of both PUFA on DNA adduct formation by HCA is not in agreement with epidemiological findings, where n-6 PUFA exert beneficial effects, whereas n-3 PUFA show protective effects with respect to colorectal cancer risk. The stimulating effect was inverted by adding high amounts of PUFA, which might be explained by either fatty acid micelles entrapping the HCA, inhibition of COX activity by PUFA, or scavenging of HCA radicals by PUFA molecules. The role of COX, COX-2 in particular, and CYP1A2 was further investigated by using the specific enzyme inhibitors indomethacin (IM), NS-398 and phenethyl isothiocyanate (PEITC), respectively. Inhibition of PhIP- and IQ-DNA adduct formation by IM and NS-398 to nearly the same extent demonstrated involvement of mainly COX-2, not COX-1. PEITC also inhibited adduct formation, thus indicating that HCA-7 cells possess CYP450 activity, that may not be limited to CYP1A2. These study results confirm that COX, and especially COX-2, could play an important role in HCA bioactivation in extrahepatic tissues, causing the formation of DNA adducts and subsequent mutagenic events. This might be a reason for the decrease in CRC risk that has been observed after long-term use of aspirin, which is known to inhibit COX. Furthermore, interaction with PUFA in COX-expressing cancer cells imply that other (food) compounds could be important in modulating colorectal cancer risk *in vivo*.

In humans, efficient absorption of HCA into the systemic circulation takes place after ingestion, leading to a high degree of bioavailability. The enzymes that are responsible for the hepatic activation of HCA, CYP1A2 and NAT2, show interindividual differences in activities, due to polymorphic distribution of the corresponding genes in the

human population. However, since genotyping cannot be simply used to predict phenotypes, combined analysis of both geno- and phenotypes is considered necessary. In **Chapter 5**, the influence of dietary exposure and variations in enzymatic activities (phenotypes) and genetic constitutions (genotypes) for CYP1A2 and NAT2 on PhIP metabolism was studied. For 10 consecutive days, 71 healthy volunteers consumed daily portions of chicken, containing either a low (1.7 ± 0.6 ng/g) or high (7.4 ± 2.0 ng/g) dose of PhIP. 24 hr urine samples were collected before, during and after the intervention period. Subjects were phenotyped for CYP1A2 and NAT2 by HPLC analysis of urinary caffeine metabolites, and genotyping for NAT2 and CYP1A2 was performed using PCR-RFLP. CYP1A2 genotyping comprised analysis of the $-164A \rightarrow C$ polymorphism (CYP1A2*1F), one of the most common in Caucasians and suitable for routine assessment of the CYP1A2 genotype in humans. Urinary excretion levels, measured by GC-MS, were found to reflect the recent dietary exposure. NAT2 genotypes and phenotypes showed 79 % concordance, and the bimodal phenotypic distribution could be clearly distinguished by the genotype. For CYP1A2, no significant correlation was found between the genotype and enzyme activities. From the different phenotypes and genotypes, the CYP1A2*1F polymorphism was significantly correlated to urinary PhIP excretion, and this correlation was strongly influenced by the genotype that occurred in only two individuals. These results demonstrate that slow oxidizers (possessing the C/C genotype) excreted higher amounts of urinary PhIP as compared to fast oxidizers, implying that these fast oxidizers are exposed to higher internal doses of genotoxic PhIP metabolites. Therefore, this polymorphism could play a role in colon cancer risk susceptibility in humans.

To assess the health risk associated with CYP1A2 and NAT2 genotypes and phenotypes, in **Chapter 6** these parameters were studied in 94 patients at different risk of developing CRC, based on the occurrence and size of colorectal adenomas that were found and removed upon endoscopic examination. All individuals were phenotyped and genotyped for CYP1A2 and NAT2, using the same methodology as applied in the human intervention study described in Chapter 5. Although high enzyme activities for CYP1A2 and NAT2 have been correlated to an elevated CRC risk in some other studies, no such associations were found in the patients that were studied here. In contrast, the CYP1A2*1F polymorphism was found to be correlated to CRC risk when comparing different risk groups or combination of risk groups. This implies that individuals possessing the mutated CYP1A2 gene are at lower risk for developing CRC as compared to individuals possessing the wildtype constitution. With respect to the Vogelstein model, this genetic polymorphism may be of significance in the early stage of colorectal tumorigenesis most probably with respect to the induction of mutagenic events due to the bioactivation of food borne mutagens, and may thus influence the eventual colorectal cancer risk.

HCA-DNA adduct measurement in human studies

The ^{32}P -postlabeling assay, as described in Chapter 2, was intended to be applied in both the human studies described in this thesis, thus evaluating DNA damaging effects of activated HCA molecules. As stated earlier, DNA adducts can be used as biomarkers of internal exposure and risk, giving an overall view of the metabolic fate of HCA in the human body.

In the intervention study with healthy volunteers (Chapter 5), attempts were made to detect HCA-DNA adducts in peripheral blood lymphocytes, serving as surrogate tissue for the colon. Also, in this way, it would be possible to study the peripheral availability of reactive HCA metabolites. However, no adducts were detected in DNA isolated from these cells. HCA-DNA adduct levels in a target tissue like the colon are expected to be higher than in lymphocytes, based on metabolic activity and as demonstrated in animal

experiments. Therefore, the rectal biopsies obtained from the patients described in Chapter 6 were also tested for HCA-DNA adducts. Unfortunately, DNA isolated from normal mucosa nor adenoma tissue showed detectable adduct levels at our detection limit of 1 adduct per 10^8 normal nucleotides. Although more sensitive methods for DNA adduct measurement have been developed, they are not applicable in standard biomonitoring studies due to the use of ^{14}C -labeled substrates.

Thus, it can be stated that HCA-DNA adducts in both the colon tissues of the patients and the lymphocytes of the volunteers, if present, occur at levels lower than 1 adduct/ 10^8 nucleotides; the actual presence of these adducts can therefore not be excluded.

Future studies should focus on lowering the detection limit of the present postlabeling assay, or development and application of an alternative technique, enabling even more sensitive detection of HCA-DNA adducts.

In conclusion, the data presented in this thesis, show that HCA can be readily activated by two different pathways. Although the hepatic pathway has been studied and emphasized far more extensively, the free radical generating pathway may also be of significant influence in the onset of colorectal carcinogenesis. Therefore, metabolic activation of HCA via this route should be taken into account when investigating cancer risk induced by HCA exposure, especially in extrahepatic tissues such as the colon. Furthermore, it may be important to consider interactions with other compounds like PUFA, as these interactions can possibly modulate the eventual CRC risk. In humans, the CYP1A2*1F polymorphism influences HCA metabolism, and can thus play an important role in the ultimate health risk related to HCA exposure.

Overall, although our study results presented in this thesis provide much information on the metabolic activation of HCA and the influence of variations in genes and corresponding enzymes involved herein, no final conclusion can be drawn with respect to HCA exposure and human colorectal cancer risk. Nevertheless, our HCA metabolism studies did demonstrate that the extrahepatic pathway should always be taken into account when investigating metabolic activation of HCA in humans. Furthermore, our human studies show that certain individuals are at higher risk for developing genotoxic damage and colorectal neoplasia as compared to others, and these risk groups should thus be careful with frequent consumption of well-done meat.

Future research should further investigate the importance of the CYP1A2 genotype in HCA risk assessment in humans. Finally, studies in larger populations are necessary to substantiate and extend our findings, and the development of very sensitive methods should make it possible to measure relevant biomarkers of internal exposure and risk in such populations.

Samenvatting

Het staat vast dat de mens dagelijks wordt blootgesteld aan een grote verscheidenheid aan genotoxische stoffen. Een aanzienlijk gedeelte van deze blootstelling kan worden toegeschreven aan voeding, en er is overtuigend bewijs dat de inname van bepaalde voedingscomponenten een bijdrage zou kunnen leveren aan kanker risico's. Zo wordt de inname van rood en bereid vlees in verband gebracht met dikke darmkanker. Volgens het Vogelstein model is de ontwikkeling en progressie van dikke darmtumoren het resultaat van een opeenstapeling van meervoudige genetische veranderingen in zowel oncogenen als tumor suppressor genen. Bij de meerderheid van de dikke darmkanker gevallen is het APC gen geïnactiveerd in een vroeg stadium van tumorvorming, waardoor een cascade van andere genetische veranderingen wordt veroorzaakt. Het wordt verondersteld dat blootstelling aan mutagene stoffen, zoals die voorkomen in voeding, van groot belang kan zijn bij deze vroege mutaties. In dit verband is het voorkomen van heterocyclische aromatische amines (HCA) in bereid vlees waarschijnlijk van belang. HCA werden ca. 25 jaar geleden ontdekt en gekarakteriseerd, en worden gevormd tijdens de verhitting van vlees. Het zijn verbrandingsproducten van reacties waarbij verschillende vleesbestanddelen zoals suikers, aminozuren en creatine zijn betrokken. HCA zijn zeer mutageen in bacteriën en carcinogeen in knaagdieren en apen. Vele epidemiologische studies hebben een relatie aangetoond tussen inname van HCA en het risico op dikke darmkanker in de mens. Deze studies geven echter geen informatie over het metabolisme en de bioactivatie-routes van HCA in het lichaam. Om meer inzicht in deze processen te krijgen, zijn biomarkers ontwikkeld waarin verscheidene aspecten, die het uiteindelijke risico beïnvloeden, zijn geïntegreerd.

Hoofdstuk 2 beschrijft de ontwikkeling en optimalisatie van twee methoden die veelvuldig worden gebruikt om biomarkers voor HCA-blootstelling en -activatie te meten. De vorming van covalent gebonden DNA adducten wordt beschouwd als een essentiële stap in het carcinogene proces, en deze adducten kunnen gezien worden als markers van de interne dosis en kunnen dus gebruikt worden als biomarkers voor het risico op bepaalde doellocaties (b.v. DNA). Adductniveaus kunnen sterk variëren tussen mensen, als gevolg van verschillen in blootstelling, absorptie, verdeling, metabole (in)activatie, genetische gevoeligheid en DNA herstel capaciteit. De ^{32}P -postlabeling methode is een zeer gevoelige en wordt veel gebruikt voor detectie van DNA adducten. Ongeveer 20 jaar geleden werd deze techniek voor het eerst beschreven, en sindsdien zijn vele verschillende methoden en aanpassingen van basismethoden ontwikkeld. Het gevolg hiervan is dat er tussen verschillende laboratoria grote verschillen zijn ontstaan ten aanzien van de DNA adduct analyse.

Het meten van HCA verbindingen in urine weerspiegelt de recente HCA blootstelling en bioactivatie. GC-MS analyse is hiervoor veelvuldig gebruikt, vanwege de hoge selectiviteit en gevoeligheid. Ook hiervoor geldt dat vele verschillende methodes en aanpassingen zijn ontwikkeld en beschreven. Voor zowel ^{32}P -postlabeling als GC-MS was het noodzakelijk om de meest geschikte methode te selecteren en optimaliseren, teneinde de onderzoeksdoelen in onze studies te bereiken. Uiteindelijk zijn we er in geslaagd om geschikte methoden te ontwikkelen voor toepassing in onze studies. De detectiegrenzen onder de meest optimale condities waren 1 adduct per 10^8 nucleotiden voor de postlabeling en 5 pg PhIP per ml urine voor de GC-MS analyse, wat het mogelijk maakte om het HCA metabolisme zowel *in vitro* als *in vivo* te bestuderen.

Metabole activatie van HCA kan verlopen via twee verschillende routes. De hepatische route betreft *N*-oxidatie tot *N*-hydroxy metabolieten en daarop volgend *O*-acetylering tot *N*-acetoxy arylamines, gekatalyseerd door respectievelijk cytochroom P4501A2 (CYP1A2) en *N*-acetyltransferase 2 (NAT-2). De extrahepatische route is afhankelijk van de metabole omzetting van arachidonzuur, waarbij HCA kunnen dienen als co-substraten en zo kunnen worden geoxideerd tot vrije radicaal metabolieten. Deze reactie wordt gekatalyseerd door het enzym prostaglandine H synthase (PHS), ook wel cyclooxygenase (COX) genoemd, dat wordt gekenmerkt door twee verschillende activiteiten. In **Hoofdstuk 3** werden de twee verschillende routes voor de bioactivatie van PhIP en IQ *in vitro* bestudeerd, door gebruik te maken van zowel electron spin resonantie (ESR) spectroscopie en ^{32}P -postlabeling om de vorming van primaire en secundaire HCA metabolieten te volgen. Met behulp van ESR spectroscopie kon worden aangetoond dat zowel PhIP als IQ de vorming van zuurstof radicalen kunnen remmen, die ontstaan tijdens de COX-gemedieerde omzetting van arachidonzuur. Dit kan mogelijk verklaard worden door competitie tussen de vorming van reactieve zuurstof species en HCA oxidatie, of door reactie van HCA met de gevormde reactieve vormen van zuurstof. De hepatische en extrahepatische routes werden voorgesteld door respectievelijk S9-mix uit rattenlever en PHS, en leidden tot verschillende primaire metabolieten voor beide HCA. De vorming van HCA radicalen werd aangetoond door de detectie van thiyl radicalen, gevormd uit glutathion. Op deze manier werd de vorming van vrije radicaal metabolieten uit PhIP en IQ op een indirecte manier aangetoond in de PHS-gekatalyseerde route, maar niet in de S9-gekatalyseerde route. HCA bioactivatie kan uiteindelijk leiden tot metabolieten die covalent kunnen binden aan DNA, resulterend in mutagene DNA adducten. In de dikke darm gevormde DNA adducten zouden vroege mutaties kunnen veroorzaken in cruciale genen, die uiteindelijk kunnen resulteren in colorectale adenomen, zoals beschreven in het Vogelstein model. Met behulp van ^{32}P -postlabeling analyse kon worden aangetoond dat S9- en PHS-activatie van PhIP in aanwezigheid van enkelstrengs en dubbelstrengs DNA tot identieke DNA adduct profielen leidt. Dit duidt op de uiteindelijke vorming van een gemeenschappelijke reactieve metaboliet, vermoedelijk een arylnitrenium ion. Voor IQ werd een additionele spot gevonden na PHS-activatie, die mogelijk afkomstig is van directe DNA adduct vorming door IQ radicalen. Over het algemeen vertoonde IQ sterkere effecten dan PhIP in zowel de ESR als de postlabeling analyses. De resultaten laten zien dat bij de bestudering van de vorming van genotoxische HCA metabolieten *in vivo*, rekening gehouden moet worden met de PHS-gekatalyseerde activatie van HCA. Deze metabolieten kunnen mogelijk mutagene effecten induceren, met name in extrahepatische weefsels zoals de dikke darm.

Het verschil in adduct vormende capaciteit tussen PhIP en IQ werd ook gevonden in humane darmkanker cellen (HCA-7), zoals wordt beschreven in **Hoofdstuk 4**. In deze cellen komt de enzymatische isovorm COX-2 sterk tot expressie, en dit model kan daardoor gebruikt worden om de extrahepatische activatie van HCA te onderzoeken. Deze bestaat uit 1-electron oxidatie tijdens de omzetting van het meervoudig onverzadigde vetzuur (Engels: polyunsaturated fatty acid, PUFA) arachidonzuur naar prostaglandine E_2 (PGE_2). De toevoeging van zowel arachidonzuur als linolzuur leidde tot de vorming van PGE_2 , wat aantoonde dat HCA-7 cellen in staat zijn om linolzuur om te zetten in arachidonzuur. Blootstelling van de cellen aan zowel PhIP als IQ leidde tot de vorming van DNA adducten, en dit werd gestimuleerd door de toevoeging van het n-6 PUFA arachidonzuur en het n-3 PUFA EPA, wat duidelijk aangeeft dat COX betrokken is bij dit proces. Het stimulerende effect van beide PUFA op de DNA adduct vorming door HCA is niet in overeenstemming met de bevindingen in epidemiologische studies, waar n-6 PUFA bevorderende effecten en n-3 PUFA beschermende effecten vertonen met betrekking tot het risico op dikke darmkanker. Door toevoeging van hoge concentraties

PUFA nam het stimulerende effect op de adductvorming weer af, wat mogelijk verklaard zou kunnen worden door insluiting van HCA moleculen door PUFA micellen, remming van COX activiteit door PUFA, of reactie van HCA radicalen met PUFA. De rol van COX, COX-2 in het bijzonder, en CYP1A2 werd verder onderzocht door respectievelijk gebruik van de specifieke enzymremmers indomethacine (IM), NS-398 en fenethyl isothiocyanaat (PEITC). DNA adductvorming door PhIP en IQ werd na toevoeging van IM of NS-398 in nagenoeg dezelfde mate geremd, wat aangeeft dat voornamelijk COX-2, en niet COX-1, is betrokken bij dit proces. PEITC remde ook de adductvorming, wat dus betekent dat HCA-7 cellen ook CYP450 activiteit bezitten, die niet beperkt hoeft te zijn tot CYP1A2. Deze resultaten bevestigen dat COX, en met name COX-2, een belangrijke rol zouden kunnen spelen bij de bioactivatie van HCA in extrahepatische weefsels, resulterend in DNA adducten en de daaropvolgende mutagene effecten. Dit zou een verklaring kunnen zijn voor de waarneming dat het risico op dikke darmkanker daalt bij langdurig gebruik van aspirine, een bekende COX-remmer. Verder impliceert de interactie met PUFA in deze cellen met hoge COX-activiteit dat andere (voedings-) stoffen van invloed kunnen zijn op het dikke darmkanker risico *in vivo*.

In mensen vindt, na inname, efficiënte absorptie van HCA in de systeemcirculatie plaats, wat leidt tot een hoge mate van biologische beschikbaarheid. De enzymen die verantwoordelijk zijn voor de hepatische activatie van HCA, CYP1A2 en NAT2, vertonen interindividuele verschillen in activiteit als gevolg van een polymorfe verdeling van de bijbehorende coderende genen in de humane populatie. Omdat genotypering echter niet eenvoudigweg gebruikt kan worden om het fenotype te voorspellen, wordt een gecombineerde analyse van zowel geno- als fenotypes noodzakelijk geacht. In **Hoofdstuk 5** werd de invloed van blootstelling via voeding, alsook de invloed van variaties in enzymatische activiteit (fenotype) en genetische samenstelling (genotype) op het metabolisme van PhIP bestudeerd. Gedurende 10 opeenvolgende dagen consumeerden 71 vrijwilligers dagelijkse porties kip met een lage (1.7 ± 0.6 ng/g) of een hoge (7.4 ± 2.0 ng/g) dosis PhIP. 24-uurs urine samples werden vóór, tijdens en na de interventieperiode verzameld. Voor alle deelnemers werden de fenotypes bepaald met behulp van een HPLC analyse van urinaire cafeïne metabolieten, en de genotypes met behulp van PCR-RFLP. Genotypering voor CYP1A2 werd uitgevoerd door analyse van het -164A→C polymorfisme (CYP1A2*1F), een van de meest voorkomende in West-Europeanen en daardoor geschikt voor routinebepaling van het CYP1A2 genotype in mensen. Urinaire excretiegehalten, gemeten met behulp van GC-MS, bleken de recente blootstelling weer te geven. NAT2 genotypes en fenotypes vertoonden 79 % overeenstemming, en de bimodale verdeling van fenotypes kon duidelijk worden onderscheiden door de genotypes. Voor CYP1A2 was er geen significante correlatie tussen het genotype en de enzymatische activiteit. Van de verschillende fenotypes en genotypes was alleen het CYP1A2*1F polymorfisme significant gecorreleerd aan de urinaire PhIP excretie, en dit verband werd sterk beïnvloed door het genotype dat slechts in twee personen voorkwam. Deze resultaten geven aan dat langzame oxideerders (mensen met het C/C genotype) grotere hoeveelheden urinair PhIP uitscheiden dan snelle oxideerders, wat impliceert dat deze laatste groep wordt blootgesteld aan een hogere interne dosis genotoxische PhIP metabolieten. Dit polymorfisme zou dus een rol kunnen spelen in de gevoeligheid voor dikke darmkanker in de mens.

Om het gezondheidsrisico te bepalen dat samenhangt met CYP1A2 en NAT2 genotypes en fenotypes, werden deze parameters in **Hoofdstuk 6** onderzocht in 94 patiënten. Deze patiënten werden ingedeeld in verschillende risico-klassen, op basis van aanwezigheid en afmeting van darmadenomen die waren gevonden en verwijderd bij endoscopisch onderzoek. Alle personen werden gefenotypeerd en gegenotypeerd voor CYP1A2 en NAT2 met behulp van dezelfde methode als beschreven in Hoofdstuk 5.

Hoewel hoge enzymactiviteiten voor CYP1A2 en NAT2 in sommige andere studies in verband zijn gebracht met een verhoogd risico op dikke darmkanker, werd een dergelijk verband niet gevonden in de hier bestudeerde patiënten. Tegengesteld hieraan werd wel een correlatie gevonden tussen het CYP1A2*1F polymorfisme en kankerrisico in de dikke darm wanneer verschillende risicogroepen of combinaties van risicogroepen werden vergeleken. Dit impliceert dat mensen met een mutatie in het CYP1A2 gen een lager risico hebben op dikke darmkanker dan mensen met het wildtype. Ten aanzien van het Vogelstein model zou dit polymorfisme belangrijk kunnen zijn in een vroeg stadium van de tumorontwikkeling in de dikke darm, waarschijnlijk met betrekking tot de inductie van mutagene effecten als gevolg van de bioactivatie van voedselmutagenen, en zo het uiteindelijke dikke darmkankerrisico kunnen beïnvloeden.

HCA-DNA adduct metingen in humane studies

De ^{32}P -postlabeling methode, zoals beschreven in Hoofdstuk 2, was bedoeld om te worden toegepast in beide humane studies die beschreven zijn in dit proefschrift, om zo de schadelijke effecten van geactiveerde HCA moleculen op DNA te bestuderen. Zoals eerder gezegd kunnen DNA adducten gebruikt worden als biomarkers van interne blootstelling en risico, en geven ze een algemeen beeld van het metabole lot van HCA in het menselijk lichaam. In de interventiestudie met humane vrijwilligers (Hoofdstuk 5) werden pogingen ondernomen om HCA-DNA adducten te meten in lymfocyten in perifere bloed, als surrogaatweefsel voor de dikke darm. Op deze manier zou het ook mogelijk zijn om de perifere beschikbaarheid van reactieve HCA metabolieten te onderzoeken. Detectie van adducten in het DNA van deze cellen bleek echter niet mogelijk. Op basis van metabole activiteit en data uit proefdierstudies werd verwacht dat adductniveaus in doelwitweefsel zoals de dikke darm hoger zijn dan in lymfocyten. Daarom werden de rectumbiopsies van de patiënten beschreven in Hoofdstuk 6 ook onderzocht op HCA-DNA adducten. Helaas werden in het DNA van zowel normaal weefsel als adenoomweefsel geen meetbare adductniveaus aangetroffen bij een detectiegrens van 1 adduct per 10^8 nucleotiden. Hoewel gevoeliger methoden voor DNA adduct metingen wel zijn ontwikkeld, zijn deze niet bruikbaar in normale biomonitoringsstudies vanwege het gebruik van radioactief gelabelde substraten.

Het kan dus gesteld worden dat HCA-DNA adducten in zowel het dikke darmweefsel van de patiënten als in de lymfocyten van de vrijwilligers, indien aanwezig, voorkomen op niveaus lager dan 1 adduct/ 10^8 nucleotiden; de feitelijke aanwezigheid van deze adducten kan derhalve niet worden uitgesloten.

Toekomstige studies zouden zich moeten toelagen op de verlaging van de detectiegrens van de huidige postlabeling assay, of op de ontwikkeling en toepassing van een alternatieve techniek die een nog gevoeliger detectie van HCA-DNA adducten mogelijk zou maken.

Concluderend kan gesteld worden dat de data die in dit proefschrift zijn gepresenteerd, aantonen dat HCA via twee verschillende routes geactiveerd kunnen worden. Hoewel de hepatische route tot nu toe veel uitgebreider bestudeerd en benadrukt is, kan de vrije radicalen genererende route ook van significant belang zijn bij het begin van het carcinogene proces in de dikke darm. Daarom zou ook rekening gehouden moeten worden met de metabole activatie van HCA via deze route wanneer het aan HCA blootstelling gerelateerde kankerrisico wordt onderzocht, met name in extrahepatische weefsels zoals de dikke darm. Verder zou het van belang kunnen zijn om interacties met andere verbindingen zoals PUFA in aanmerking te nemen, omdat deze interacties van invloed zouden kunnen zijn op het uiteindelijke risico op dikke darmkanker. In de mens is het CYP1A2*1F polymorfisme van invloed op het HCA metabolisme, en dit zou dus een

belangrijke rol kunnen spelen in het uiteindelijke gezondheidsrisico dat gerelateerd is aan HCA blootstelling.

In het algemeen kan geen definitieve conclusie worden getrokken over de relatie tussen blootstelling aan HCA en het risico op dikke darmkankerrisico in de mens, hoewel de studies die in dit proefschrift gepresenteerd worden veel informatie verschaffen over de metabole activatie van HCA en de invloed van variaties in genen en bijbehorende enzymen die hierbij betrokken zijn. Niettemin laten de studies met betrekking tot HCA metabolisme zien dat de extrahepatische route altijd in beschouwing moet worden genomen als de metabole activatie van HCA in de mens wordt bestudeerd. Verder laten de humane studies zien dat bepaalde mensen een hoger risico hebben op het ontwikkelen van genotoxische schade en adenomen in de dikke darm dan anderen, en deze risicogroepen zouden derhalve voorzichtig moeten zijn met het frequent consumeren van doorbakken vlees.

Toekomstig onderzoek zou het belang van het CYP1A2 genotype in de risicobepaling van HCA verder moeten onderzoeken. Tenslotte zijn studies in grotere groepen belangrijk om onze bevindingen te onderbouwen en uit te breiden, en de ontwikkeling van zeer gevoelige methoden zou het mogelijk moeten maken om belangrijke biomarkers van interne blootstelling en risico te meten in dergelijke populaties.

Dankwoord

"It is never too late to be what you might have been" --- George Eliot

Aan het eind van het verhaal rest mij nog een aantal personen te bedanken, zonder wie dit proefschrift er niet in de huidige vorm (of helemaal niet) zou liggen.

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Harald.

Curriculum vitae

Harald Johan Joseph Moonen werd geboren op 27 april 1974 te Weert. Na het behalen van het VWO diploma aan de Philips van Horne Scholengemeenschap te Weert in 1992, begon hij zijn studie Chemische Technologie aan de Technische Universiteit in Eindhoven. Tijdens deze studie werden stage-onderzoeken verricht in Brazilië en bij de capaciteitsgroep Gezondheidsrisico Analyse en Toxicologie (GRAT) aan de Universiteit Maastricht. In augustus 1998 behaalde hij het ingenieursdiploma.

In november 1998 begon hij als assistent in opleiding bij de capaciteitsgroep GRAT, alwaar het onderzoek beschreven in dit proefschrift werd uitgevoerd. Tijdens deze periode werd tevens het certificaat 'Stralingshygiëne, deskundigheidsniveau 5b' behaald. Vanaf oktober 2003 is Harald werkzaam als post-doc bij de capaciteitsgroep GRAT aan de Universiteit Maastricht.

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