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Human T lymphocytes Bioactivate Heterocyclic Aromatic Amines by Forming DNA adducts

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Abstract

Heterocyclic aromatic amines (HAA) are formed in cooked meat, poultry and fish but also arise in tobacco smoke and exhaust gases. HAA are potential human carcinogens, which require metabolic activation to exert their genotoxicity. Human tissues can bioactivate HAA to produce reactive intermediates that bind to DNA. HAA DNA adduct formation occurs in human hepatocytes; however, the potential of HAA to form DNA adducts has not been investigated in human T lymphocytes. In this study, we investigated the ability of human T lymphocytes activated with PMA/Ionomycin or CD3/CD28 to express functional CYP1 activity and bioactivate three major HAA: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) to form DNA adducts. Adducts were measured by ultraperformance liquid chromatography-electrospray ionization/multistage scan mass spectrometry. The highest level of DNA adducts occurred for AαC (16 adducts per 10⁹ nucleotides), followed by PhIP (9 adducts per 10⁹ nucleotides). In contrast, DNA adducts formed from MeIQx and the structurally related aromatic amine 4-aminobiphenyl, a known human carcinogen, were below the limit of detection (< 3 adducts per 10⁹ nucleotides). Moreover, we demonstrate that AαC is a potent inducer of CYP1A1 and CYP1B1 activity through a transcriptional mechanism involving the AhR pathway. Overall, our results highlight the capacity of activated human T lymphocytes to more efficiently bioactivate AαC to form DNA adducts than other prominent HAA or 4-ABP.

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Statement of Author Contribution

SL designed and conceptualized the study. MB performed most of the experiments with the help of LLH. RJT set-up, analyzed and interpreted all mass spectrometry data. MB prepared the first manuscript draft with the intellectual input of LV and GB. MB performed statistical analyses. RJT and SL finalized the manuscript. All authors read and approved the final manuscript.

Keywords

cytochrome P450; carcinogen metabolism; genotoxicity; 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC)

Introduction

Environmental exposure to hazardous chemicals is a major risk factor for cancer development [IARC 2010; IARC 2012]. Among the incriminated chemicals, heterocyclic aromatic amines (HAA) are one important class of potential human carcinogens. HAA are mainly formed in cooked meat, poultry and fish [Sugimura et al. 2004], but several HAA also arise in tobacco smoke and exhaust gases [Manabe et al. 1993; Zhang et al. 2011]. More than 25 HAA have been identified and are divided into two major classes: pyrolytic and aminoimidazoarenes. The pyrolytic HAA arise during the high-temperature (>250 °C) pyrolysis of proteins or amino acids such as tryptophan and glutamic acid [Sugimura et al. 2004]. 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) is by far the most abundant pyrolytic HAA formed in mainstream tobacco smoke, it occurs at amounts up to 258 ng/cigarette [Hoffmann 1998; Zhang et al. 2011]. These amounts are 25 to 100 times higher than those of 4-aminobiphenyl (4-ABP) and benzo(a)pyrene, two human carcinogens [Patianakos and Hoffmann 1979; Zha et al. 2002]. The aminoimidazoarene HAA are formed by the Maillard reaction at temperatures generally above 150 °C [Jagerstad et al. 1991]. Aminoimidazoarene formation involves the reaction of pyridine or pyrazine, which are derived from heat degradation products of amino acids and hexoses, followed by a condensation reaction with creatinine [Murkovic 2004]. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are two of the most abundant aminoimidazoarenes formed in cooked meat and fish; the amounts of MeIQx and PhIP formed are highly dependent upon the type of meat, the temperature, and method of cooking [Ni et al. 2008].

HAA require metabolic activation, which is carried out by *N*-oxidation of the exocyclic amine group by cytochrome P450 (CYPs) or other oxidases [Turesky and Le Marchand 2011]. The *N*-oxidation reaction of HAA is mainly catalyzed by CYP1A2 in the liver, and depending upon the HAA substrate, the *N*-oxidation reaction can be catalyzed by CYP1A1, CYP1B1, and several CYPs other than those in the Family 1 in extra-hepatic tissues [Joseph et al. 2001; Kim and Guengerich 2005; Turesky and Le Marchand 2011; Shimada et al. 2013]. CYP1A1 and CYP1B1 are the most active enzymes that carry out this reaction in extra-hepatic tissues [Kim and Guengerich 2005]. The *N*-hydroxylated HAA metabolites are substrates for *N*-acetyltransferases (NAT) or sulfotransferases (SULT), and form unstable esters [Turesky and Le Marchand 2011]. These intermediates undergo heterolytic cleavage to form the proposed reactive nitrenium/carbenium ions, which covalently bind to DNA [Turesky and Le Marchand 2011]. HAA form DNA adducts primarily at the C⁸ position of guanine (Figure 1) and also at lower amounts at the N² position of guanine and N⁶ position of adenine [Turesky and Vouros 2004; Bessette et al. 2009].

HAA are mutagenic in bacteria and mammalian cells, and all HAA assayed thus far are carcinogenic in animals [Sugimura et al. 2004]. DNA adducts are regarded as an important

factor in chemical carcinogenesis, and DNA adducts derived from HAA are believed to induce tumor formation by producing mutations in oncogenes and tumor suppressor genes such as *H-ras*, *p53*, *Apc* and β -*Catenin* [Turesky 2002; Sugimura et al. 2004]. Among the mutations induced by HAA in these tumor-related genes or *lacI* or *lacZ* genes of transgenic mice, GC to TA transversions and GC deletions are most frequent [Sugimura et al. 2004]. In addition to liver, colon and prostate, some HAA induce cancer in lymphoid tissue and the hematopoietic system in rodents [Sugimura et al. 2004]. Some epidemiological studies have reported that a high intake of red meat is associated with an increased risk in colon and prostate cancers, but also for the development of lymphoma cancer [Zheng and Lee 2009; John et al. 2011; Ferrucci et al. 2012; Yang et al. 2015].

Human lymphocytes express several CYPs enzymes including CYP1A1 and CYP1B1, mainly in response to their activation [Siest et al. 2008]. CYP1B1 mRNA expression is barely detected in quiescent cells; however, both CYP1A1 and CYP1B1 mRNA are expressed at high levels in activated cells [Crawford et al. 1997; van Duursen et al. 2005; Prigent et al. 2014]. Human T lymphocytes are physiologically activated by a double signal that involves T cell receptor (TCR) engagement and stimulation of the CD28 receptor [Smith-Garvin et al. 2009]. In vitro these cells can be activated by anti-CD3 and anti-CD28 antibodies, which mimic physiological activation by targeting TCR and CD28 receptors [Trickett and Kwan 2003]; or by the combination of Phorbol 12-Myristate 13-Acetate and Ionomycin (PMA/Iono), which target the same signal pathways but bypass the TCR [Truneh et al. 1985].

In this study, we examine the capacity of activated primary human T lymphocytes to express functional CYP1 activity and bioactivate HAA to form DNA adducts. The level of CYP1 activity was determined, and the DNA adducts derived from the N-oxidized metabolites of PhIP, MeIQx and A α C, three major HAA present in cooked meat or tobacco smoke, were measured by ultraperformance liquid chromatography-electrospray ionization/multistage scan mass spectrometry (UPLC-ESI/MS³). DNA adducts levels were compared to those formed with 4-aminobiphenyl (4-ABP), a structurally related aromatic amine present in tobacco smoke and a recognized human bladder carcinogen [IARC 2010]. Our results demonstrate that A α C formed the highest level of DNA adducts, followed by PhIP, whereas DNA adducts of MeIQx and 4-ABP were not detected. Moreover, our results demonstrate that A α C induced CYP1A1 and CYP1B1 activity through the AhR pathway. Overall, our findings highlight the ability of activated human T lymphocytes to efficiently form DNA adducts of HAA, particularly with A α C.

Materials and Methods

Caution

A α C, 4-ABP, MeIQx, and PhIP are potential human carcinogens, and they should be handled only in a well-ventilated fume hood while wearing appropriate protective clothing.

Chemicals and Reagents

Phorbol-12-Myristate-13-Acetate (PMA), 4-ABP, ionomycin, DMSO, ethoxyresorufin, 3-methylcholanthrene (3-MC), actinomycin D (Act D), cycloheximide (CHX), 2-methyl-2H-pyrazole-3-carboxylic acid (CH-223191) and α -Naphthoflavone (α -NF) were from Sigma Aldrich (St. Louis, MO, USA). MeIQx, A α C, and PhIP were purchased from Toronto Research Chemicals (Toronto, ON, Canada). FITC- and PE-conjugated antibodies were obtained from BD Biosciences (San Jose, CA, USA). *N*-(2'-deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-4-ABP), *N*-(2'-deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-A α C), *N*-(2'-deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-PhIP), *N*-(2'-deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (dG-C8-MeIQx) and labelled dG-C8-[²H₃C]-MeIQx, [¹³C₁₀]-dG-C8-4-ABP [¹³C₁₀]-dG-C8-A α C, and [¹³C₁₀]-dG-C8-PhIP were synthesized as previously described [Bessette et al. 2009].

Cells isolation, cultures and treatment with HAA or 4-ABP

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation from blood buffy coats of healthy donors, provided by the Etablissement Francais du Sang (EFS). The research protocol was conducted under French legal guidelines. After separation of monocytes by 1 h adhesion step, non-adherent PBMCs were activated by PMA (20 ng/ml) and Iono (1 μ M). Cells were cultured in RPMI medium (Gibco, Life technologies, Carlsbad, CA, USA) supplemented with 10% decompemented fetal calf serum (Life technologies), penicillin (100 IU/ml), and streptomycin (100 μ g/ml). After activation (72 h), two-thirds of the medium was replaced with fresh medium and the carcinogen (4- ABP (10 μ M), HAA (10 μ M) or 3-MC (1 μ M) was added to the medium.. All the treatment and control conditions contained DMSO (0.1% v/v) final concentration. For AhR inhibition experiments, after 72 h of activation, cells were pretreated with CH-223191 or α -NF for 1 h, followed by treatment with HAA 24 h with or without inhibitors. At the end of each experiment, the cells were centrifuged at 2000 rpm for 5 min at 4 °C and stored at -80 °C for further analysis. CD3⁺ T cells were purified from PBMCs. The cell purification was performed by negative selection using CD3 T cell Kit (Life Technologies, Carlsbad, CA, USA). The CD3⁺ T cells were activated with Dynabeads T-expander beads coated with anti-CD3 and anti-CD28 antibodies (Life Technologies) at a 1:1 bead/cell ratio.

Flow cytometry immunolabeling

Cells were incubated with PBS supplemented with 5% fetal calf serum for 30 min to avoid non-specific Ab binding. After, the cells were centrifuged at 2000 rpm for 5 min. The cells were washed with PBS, and the cell pellets were re-suspended in 50 μ l of PBS and incubated with specific FITC or PE conjugated mAb (CD25, CD69, CD40L, HLA-DR or CTLA-4) or the appropriate isotypic controls (PE or FITC). After 30 min, the cells were washed and analyzed by flow cytometry using a FC500 flow cytometer (Beckman Coulter, Villepinte, France).

Enzyme activity assays

Ethoxyresorufin O-deethylation (EROD) associated with CYP1 activity was measured in cultured T lymphocytes as described by Burke and Mayer [Burke et al. 1983]. Briefly, the cells were centrifuged at 2000 rpm for 2 min and washed twice with phosphate-buffered saline at 37 °C. Subsequently, salicylamide (1.5 mM) was added to block phase II-conjugation enzymes. 7-Ethoxyresorufin (3 µM) was added 1 minute later, and the fluorescence of the resorufin generated by the oxidation of ethoxyresorufin was measured every 2 min for 30 min at 37 °C. The reaction rates were linear with time and proportional to protein concentration. Cellular protein content was estimated with the Bradford procedure. The activities were expressed as pmol/min/mg protein.

Total RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the TRIzol method (Invitrogen, Life technologies, Carlsbad, CA, USA) using 3×10^6 cells. Total RNA (1 µg) was reverse-transcribed into cDNA using RT Applied Biosystems Kit (Foster City, CA). Real-time quantitative PCR was performed by fluorescent SYBR Green methodology using SYBR Green PCR Master Mix (Applied Biosystems, Life technologies, Carlsbad, CA, USA) and the ABI Prism 7000 detector (Applied Biosystems). The 18S ribosomal RNA subunit was used as the internal reference for all analyses. The sequences of human CYP1A1 and CYP1B1 primers were the following: CYP1A1 forward primer, 5'-AAAGATCCAGGAGGAGGAGTTAGACACA-3'; CYP1A1 reverse primer, 5'-GAGGTCTGTCAGAAAGCCGG-3'. CYP1B1 forward primer, 5'-TGGCTGCTCCTCCTCTTC-3'; CYP1B1 reverse primer, 5'-GGCTGGTCACCCATACAA-3'.

DNA isolation and enzymatic digestion for DNA adduct measurements

Cell pellets (3×10^6) were homogenized in 400 µl of TE buffer (50 mM Tris-HCl containing 10 mM EDTA and 10 mM β-mercaptoethanol, pH 8.0) and incubated with RNase T1 (318.75 U) and RNase A (20 µg) for 30 min at 37 °C. After, proteinase K (200 µg) and SDS (0.05% final concentration) were added and the homogenate was incubated for 1 h at 37 °C. The DNA was processed with the phenol/chloroform method and precipitated with ethanol. DNA was re-suspended in 200 µl of sterile water and quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Each DNA sample (3 µg) was spiked with isotopically labeled internal standards at a level of 1 adduct per 10^7 bases. DNA from the separately treated 4-ABP- and HAA-treated T lymphocytes samples were then pooled. DNA digestion was performed in 5 mM Bis-Tris-HCl buffer (pH 7.1) as previously described [Nauwelaers et al. 2011]. Briefly, DNA (3 µg) was incubated with DNase I and Nuclease P1 at 37 °C for 3.5 h, followed by incubation with alkaline phosphatase and phosphodiesterase at 37 °C for 18 h. Thereafter, samples were evaporated to dryness by vacuum centrifugation. The DNA digest was re-suspended in 30 µl of 1:1 water/DMSO and sonicated for 5 min. Samples were then centrifuged for 5 min at 22000 g, and the supernatants were transferred to LC vials.

Ultraperformance liquid chromatography-electrospray ionization multistage scan mass spectrometry (UPLC-ESI-MS³) measurement of DNA adducts

The measurement of DNA adducts was performed by UPLC-ESI/MS³ with a NanoAcquity UPLC system (Waters Corp., New Milford, MA) equipped with a Waters Symmetry trap column (180 µm×20 mm, 5 µm particle size), a Michrom C18 AQ column (0.3 mm×150 mm, 3 µm particle size), and a Michrom Captive Spray source interfaced with a linear quadrupole ion trap mass spectrometer LTQ Velos (Thermo Fisher, San Jose, CA). The chromatography conditions and MS parameters were described previously (Nauwelaers et al. 2011). The protonated ions of the DNA adducts were monitored at the MS³ scan stage employing the following transitions: dG-C8-MeIQx (m/z 479.1 > 363.1 > 239.2, 318.4, 346.4) and dG-C8-[²H₃C]-C8-MeIQx (m/z 482.1 > 366.1 > 242.2, 321.5, 349.5); dG-C8-AαC (m/z 449.1 > 333.1 > 209.2, 291.4, 316.4) and [¹³C₁₀]-dG-C8-AαC (m/z 459.1 > 338.1 > 210.2, 295.5, 321.5); dG-C8-PhIP (m/z 490.1 > 374.1 > 250.2, 329.2, 357.2) and [¹³C₁₀]-dG-C8-PhIP (m/z 400.1 > 379.1 > 251.2, 333.3, 362.2); and dG-C8-4-ABP (m/z 435.1 > 319.1 > 249.2, 277.2, 302.2) and [¹³C₁₀]-dG-C8-4-ABP (m/z 445.1 > 324.1 > 252.2, 281.2, 307.2). External calibration curves were constructed for quantification [Goodenough et al. 2007]. The limit of quantification was 3 adducts per 10⁹ DNA bases [Gu et al. 2012].

Results

Activation of human T lymphocytes induces CYP1 activity

We first verified that PMA/Iono mainly activated T lymphocytes. As shown in Figure 2A, PMA/Iono rapidly induced CD69 and CD40L, which are cell surface proteins known to be early biomarkers of human T lymphocyte activation [Castle et al. 1993; Simms and Ellis 1996]. The CD69 expression reached a maximum after 24 h, and then decreased between 24 and 72 h. CD40L was also rapidly increased within 6 h of activation and remained highly expressed for the 72 h. In addition to these early markers, we also analyzed the expression of CD25 (IL-2Rα), CTLA-4 and HLA-DR, which are recognized as late-activation markers [Rea et al. 1999]. The cell surface expression level of CD25 is highly expressed at 72 h. Cell surface expression of CTLA-4 is detected in unstimulated cells at a very low level and increased gradually at 72 h in the presence of PMA/Iono. Both the early and late activation markers' kinetics are similar to those observed with TCR/co-stimulation lymphocytes activation. Taken together, our data demonstrate that PMA/Iono, lead to an efficient activation of T lymphocytes. Figure 2B shows that after 72 h, more than 90% of cells are positive for the expression of CD25 and CD69, which demonstrates that the large majority of cells are activated human T lymphocytes.

CYP1 family plays a crucial role in the bioactivation of procarcinogens, as this family is responsible for the bioactivation of more than 40% of known procarcinogens [Rendic and Guengerich 2012]. We examined the enzymatic activity of CYP1 in activated human T lymphocytes by measuring EROD activity in unstimulated and PMA/Iono activated human T lymphocytes. In unstimulated cells, CYP1 activity was not detectable (limit of detection: 0.2 pmol/min/mg protein). After 72 h of activation with PMA/Iono, EROD activity increased and reached an average activity of 0.54 ± 0.32 pmol/min/mg protein; however, the activity varied substantially among the individuals and ranged from 0.23 to 1.28 pmol/min/mg

protein. Treatment of PMA/Iono activated lymphocytes with 3-MC, a well-known CYP1 inducer [Burke and Mayer 1983] resulted in statistically significant induction of EROD activity that was ~4 to 5 times higher from that of PMA/Iono activated lymphocytes (Figure 3A). This is the first report showing functional CYP1 activity in PMA/Iono activated lymphocytes.

HAA form DNA adducts in activated human T lymphocytes

Based on functional CYP1 activity data, we investigated the bioactivation of PhIP, MeIQx, AαC, and 4-ABP in PMA/Iono activated human T lymphocytes, by measurement of DNA adducts after 3, 8 and 24 h of cell treatment with procarcinogens (10 μM).. As illustrated in Figure 3B, DNA adducts were detected after treatment with AαC and PhIP, whereas DNA adducts were not detected with MeIQx or 4-ABP. The limit of quantification was 3 adducts per 10⁹ DNA bases, when 1 μg of DNA was assayed on-column. The amount of AαC adducts increased over the 24 hour time period and adduct levels exceeded those formed with PhIP. A representative UPLC-ESI/MS³ chromatogram and product ion spectra of DNA adduct are shown in supplementary data (Supplementary Figure S-1).

Interestingly, we show that treatment with anti-CD3/CD28 antibodies allowing human T lymphocytes activation through TCR leads to not only the expression of functional CYP1 activity, but also the formation of DNA adducts at similar levels to those observed in PMA/Iono activated T human lymphocytes (Figure 3C and 3D). Thus, bypassing TCR and CD28 receptors by PMA/Iono treatment did not influence ability of activated human T lymphocytes to biotransform HAA to reactive intermediates. Therefore, the activation of lymphocytes with PMA/Iono was performed for the remaining experiments of the present study.

AαC induces mRNA expression and CYP1A activity in human T lymphocytes

Several carcinogens are known to serve as both substrates and inducers of CYPs [Shimada et al. 2002]. As AαC formed high levels of DNA adducts, we investigated its potential to induce CYP1 activity in PMA/Iono activated human T lymphocytes. Following 24 h of treatment with AαC (10 μM), there was a significant induction of EROD activity, signifying that AαC significantly induces CYP1 activity. In contrast, PhIP and MeIQx had no effect on CYP1 activity. The level of CYP1 induced by AαC is comparable to that observed with the potent CYP1 inducer 3-MC (Figure 4A). Similar to 3-MC, AαC induced CYP1 within 8 h of treatment and the level of induction reached a maximum at 24 h, and then rapidly decreased within the ensuing 24 h of treatment (Figure 4B). AαC significantly induced CYP1 activity in a dose-dependent manner in lymphocytes treated with AαC at concentrations above 0.5 μM (Figure 4C).

To determine whether AαC induced CYP1 activity is linked to an increase of mRNA expression, the levels of expression of CYP1A1 and CYP1B1 mRNA were measured by qPCR. As shown in Figure 5A and 5B, AαC significantly induced CYP1A1 and CYP1B1 mRNA levels, whereas MeIQx and PhIP did not. Indeed, CYP1A1 and CYP1B1 mRNA levels were 20 to 30-times higher after treatment of PMA/Iono activated human T lymphocytes with AαC for 24 h than those levels measured in control cells. This level of

induction is comparable to that observed with 3-MC. In order to determine if AαC mediates the induction of CYP1A1 and CYP1B1 mRNA through a transcriptional mechanism, we blocked the transcription by Act D, a well-known transcription inhibitor [Perry and Kelley 1970]. Our results demonstrate that blockage of transcription by Act D completely suppressed the AαC-mediated induction of CYP1A1 and CYP1B1 mRNA levels. The treatment with CHX, a protein synthesis inhibitor [Obrig et al. 1971], leads to a super-induction of both CYP1A1 and CYP1B1 mRNA (Figure 5C and 5D). This finding is in agreement with previous reports showing that protein synthesis inhibition by CHX leads to a superinduction of CYP1A1 and CYP1B1 mRNA levels after TCDD treatment in various human tumor cell lines [Luska et al. 1992]. In total, our data demonstrate that AαC regulates CYP1A1 and CYP1B1 activities through a transcriptional mechanism, which requires an active protein synthesis.

HAA derived DNA adduct formation is AhR dependent

It is well established that the expression CYP1 family members is modulated through aryl hydrocarbon receptor (AhR) pathway [Nebert et al. 2004]. In order to determine whether AhR is involved in AαC-mediated CYP1 induction, we pre-treated cells with CH-223191 or α-Naphthoflavone (α-NF), two AhR inhibitors [Kim et al. 2006], prior to treatment with AαC, PhIP, MeIQx, or 3-MC. CH-223191 and α-NF are ligand-selective antagonists of AhR that exerts their antagonistic effect through direct competition for AhR binding [Merchant et al. 1993; Zhao et al. 2010]. Our results show that AαC-mediated increase in CYP1A1 and CYP1B1 mRNA expression was significantly reduced when cells were treated with either of these two AhR inhibitors (Figure 6A and 6B). We further evaluated the contribution of AhR pathway to the bioactivation of AαC and PhIP, by measuring DNA adduct formation in activated human T lymphocytes pre-treated or not with the two AhR inhibitors: CH-223191 and α-NF. As shown in Figure 6C, the inhibition of AhR leads to a significant decrease of the formation of DNA adducts derived from AαC and PhIP. These results are correlated with the significant inhibition of constitutive and induced EROD activity (Figure 6D). The findings reveal that AhR is involved in AαC-mediated increase of CYP1A1 and CYP1B1 mRNA expression and the ensuing HAA DNA adducts formed in activated human T lymphocytes.

Discussion

Certain HAA induce cancer in blood vessels, lymphoid tissue and the hematopoietic system, in addition to the liver, colon and prostate [Sugimura et al. 2004]. HAA require metabolic activation, mainly catalyzed by CYPs, to form DNA adducts which can induce mutations and lead to tumorigenesis. Thus, we proposed that lymphocytes that can express CYPs may be capable of bioactivating HAA. To investigate this hypothesis, we assessed the ability of activated human T lymphocytes in vitro to bioactivate HAA and form DNA adducts.

We have shown that the activation of human T lymphocytes results in the increase of both expression and activity of CYP1A1 and CYP1B1, two enzymes playing a critical role in the bioactivation of procarcinogens [Rendic and Guengerich 2012]. These results are in accordance with previous studies showing that CYP1A1 and CYP1B1 expression is

dependent on the activation state of the lymphocytes [Siest et al. 2008]. Indeed, while only CYP1B1 mRNA is barely detectable in unstimulated cells, both CYP1A1 and CYP1B1 mRNA are induced in PMA/Iono activated human leukocyte, CD3/CD28 activated human T lymphocytes and phytohemagglutinin (PHA) stimulated human lymphocytes [Crawford et al. 1997; van Duursen et al. 2005; Prigent et al. 2014]. PHA treatment is not regarded as a physiologically relevant activation mechanism since this lectin is a non-specific mitogen that does not trigger TCR engagement and absolutely requires an accessory signal to fully activate human T lymphocytes [Ceuppens et al. 1988; Jungo et al. 2001]. In our study, we demonstrate for the first time, that human T lymphocytes activated by PMA/Iono or CD3/CD28; two different and independent mechanisms that mimic physiological activation, express detectable CYP1 activity as measured using a specific substrate the ethoxyresorufin.

Furthermore, we have demonstrated that DNA adducts are formed by AαC and PhIP in activated human T lymphocytes at levels reaching 10 to 20 adducts per 10^9 DNA bases for AαC and 4 to 6 adducts per 10^9 DNA bases for PhIP. These levels of DNA adducts are about 100 times lower than those formed in human hepatocytes treated with the same concentration of HAA [Nauwelaers et al. 2011]. The lower levels of DNA adducts in lymphocytes can be explained by the ~hundred-fold lower mRNA levels of CYPs in human T lymphocytes compared to the liver [Siest et al. 2008], as well as by the absence of CYP1A2 activity in activated human T lymphocytes (data not shown). Indeed, CYP1A2 is the predominant enzyme involved in the bioactivation of HAA in human hepatocytes [Langouet et al. 2001; Langouet et al. 2002] and also in vivo [Boobis et al. 1994]. Previous reports on the expression of CYP1A2 mRNA in human lymphocytes T are variable and range from non-detectable [Spatzenegger et al. 2000] to barely detectable [Haas et al. 2005]. The lack of CYP1A2 activity observed in our study suggests that even if CYP1A2 mRNA is expressed in activated human T lymphocytes, the level is too low to detect enzyme activity. In addition to CYP1A2, AαC can be metabolized at lower rates by recombinant human CYP1A1 and CYP2C10 expressed in *E. coli*. [Raza et al. 1996], while PhIP can be bioactivated by CYP1A1 and CYP1B1 [Shimada et al. 1996; Hammons et al. 1997]. There is no data reported in the literature on the CYP2C10 expression in human lymphocytes. Therefore, our data suggests that the expression of CYP1A1 and CYP1B1 (and potentially CYP2C10) could lead to the bioactivation and formation of AαC and PhIP-derived DNA adducts in activated human T lymphocytes. In contrast to AαC and PhIP, DNA adducts of MeIQx and 4-ABP were not detected in activated human T lymphocytes. The absence of DNA adducts can be explained by the lack of expression of CYP1A2 in those cells and by the fact that both MeIQx and 4-ABP are poor substrates for CYP1A1 and CYP1B [Shimada et al. 1996].

It has been reported that human lymphocytes express SULT enzymes activities [Hoelzl et al. 2008], which play a crucial role in the formation of HAA derived DNA adducts [Turesky and Le Marchand 2011]. Further studies are needed to identify the specific human CYPs, NAT and SULT enzymes responsible for AαC and PhIP bioactivation in lymphocytes.

The highest level of DNA adducts formed in activated human T lymphocytes treated with HAA or 4-ABP occurred with AαC. It is noteworthy that adduct formation of AαC in human hepatocytes was also greater than that observed with other HAA but comparable to

adduct levels formed with 4-ABP [Nauwelaers et al. 2011; Nauwelaers et al. 2013]. The high level of DNA binding of AαC is in part attributed to the ability of AαC to induce CYP1 enzymes in human T lymphocytes. This is the first study to report an induction of CYP1 in human T lymphocytes by an HAA.

The induction of CYP1A1 and CYP1B1 by AαC occurs through a transcriptional mechanism that involves the AhR pathway. This regulation shows similarities to previous studies reported on the regulation of CYP1A1 and CYP1B1 by other carcinogens such as benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) [Harrigan et al. 2004]. However, the regulation of CYP1A and CYP1B by AαC seems to be tissue specific, as no significant CYP1 induction occurred in primary human hepatocytes treated with AαC (unpublished data. S. Langouët). The inhibition of AhR pathway, leads to a total inhibition of CYP activity and a complete inhibition of PhIP derived DNA adducts formation demonstrating the critical role of CYP1A1 and CYP1B1 enzymes in PhIP bioactivation in activated human T lymphocytes. In contrast, the total inhibition of CYP1 activity leads to a significant decrease but not to a total inhibition of AαC-derived DNA adduct formation. This result implicates other CYP enzymes or other non-CYP enzymes in the bioactivation of AαC in activated human T lymphocytes. Indeed, AαC is bioactivated by oxidase other than the CYPs since AαC derived DNA adducts were detected in the liver of P-450 reductase null mice [Turesky et al. 2015].

In conclusion, our data demonstrate that AαC undergoes efficient bioactivation in activated human T lymphocytes to form DNA adducts, and that AαC induces CYP1A1 and CYP1B1 expression and activity through AhR pathway. The up-regulation of CYP1A1 and CYP1B1 by AαC is of utmost interest and highlights the potential of AαC to increase the capacity of activated human T lymphocytes to bioactivate other HAA or other procarcinogens. Potential CYP1 induction caused by AαC or other constituents in tobacco must be taken into account when evaluating the health risk of AαC or other pro-carcinogens.

Supplementary Material

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Figure 1. Chemical structures of HAA and 4-ABP and their DNA adducts

Figure 2. Efficient activation of human T lymphocytes by PMA/Iono treatment

(A) Kinetics of human T lymphocytes activation markers in PMA/Iono activated human T lymphocytes. Cells were either unstimulated (UNS) or activated with PMA/Iono for 6, 24, and 72 h. Cell surface expression of CD25, CD40L, CD69, HLA-DR and CTLA-4 was measured by flow cytometry. Data are expressed as a ratio relative to the mean fluorescence intensity of the unstimulated cells, arbitrarily set at 1. (B) Cells were either unstimulated (UNS) or activated with PMA/Iono for 72 h and cell surface expression of CD25 and CD69 was measured by flow cytometry. Data are representative of five independent experiments and shown as mean $\pm$ SD. (ANOVA followed by Dunnett's multi-range test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus UNS)

Figure 3. Formation of HAA derived DNA adducts and CYP1 activity in activated human T lymphocytes

(A) CYP1 activity in PMA/Iono activated human T lymphocytes. After 72 h of activation with PMA/Iono, the cells were treated with 0.1% DMSO (Ctrl) or with 1 μ M of 3-MC for 24 h and CYP1 activity was measured. Data are expressed as the mean $\pm$ SD of eight independent experiments. (Student's t-test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$). ND: not detectable (B) Kinetics of 4-ABP and HAA DNA adduct formation in PMA/Iono activated human T lymphocytes. After 72 h of activation with PMA/Iono, the cells were treated with PhIP, MeIQx, A α C or 4-ABP (10 μ M) for 3, 8 or 24 h, and the DNA adducts were measured by UPLC/MS³. Data are representative of five donors; the DNA adduct level was measured in duplicate for each donor. Data are expressed as Mean $\pm$ SD. (C) CYP1 activity in CD3/CD28 activated human T lymphocytes. CD3⁺ T cells were either unstimulated (UNS) or activated with anti-CD3 and anti-CD28 Antibodies for 72 h and CYP1 activity was measured after 24 h of treatment with 0.1% DMSO (Ctrl) or 3-MC (1 μ M). Data are expressed as the mean $\pm$ SD of four independent experiments. (Student's t-test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$). (D) Levels of DNA adducts derived from 4-ABP, A α C and PhIP in CD3/CD28 activated human T lymphocytes. CD3⁺ T cells were activated with anti-CD3 and anti-CD28 antibody for 72 h, and the level of DNA adducts were measured after 24 h of treatment with PhIP, A α C or 4-ABP (10 μ M). DNA adducts levels were measured in duplicate for each donor and are expressed as the mean $\pm$ SD. The data are representative of four independent donors. ND: not detectable

Figure 4. Modulation of CYP1A activities in activated human T lymphocytes

(A) Effect of HAA on CYP1 activity in activated human lymphocytes. CYP1 activity was measured by EROD activity in PMA/Iono activated human T lymphocytes after 24 h of treatment with 0.1% DMSO (Ctrl), HAA (10 μ M) or 3-MC (1 μ M). Data are expressed as the mean $\pm$ SD of seven independent experiments. (Student's t-test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus Ctrl). (B) Time-dependent effect of AαC on CYP1 activity in activated human T lymphocytes. CYP1 activity was measured by EROD activity in PMA/Iono activated human T lymphocytes after 0, 8, 24 and 72 h of treatment with 0.1% DMSO (Ctrl), AαC (10 μ M) or 3-MC (1 μ M). Data are expressed as the mean $\pm$ SD of four independent experiments (ANOVA followed by Tukey's multiple comparison test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus Ctrl). (C) Effect of different concentrations of AαC on CYP1 activity in activated human T lymphocytes. CYP1 activity was measured by EROD activity in PMA/Iono activated human T lymphocytes after 24 h of treatment with different concentrations of AαC (0.1 – 10 μ M). Data are expressed as the mean $\pm$ SD of four independent experiments. (Student's t-test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus Ctrl).

Figure 5. Regulation of CYP1A1 and CYP1B1 mRNA expression in activated human T lymphocytes by HAA

Regulation of (A) CYP1A1 and (B) CYP1B1 mRNA expression by HAA in activated human T lymphocytes. PMA/Iono activated human T lymphocytes were treated for 24 h with 0.1% DMSO (Ctrl), HAA (10 μ M) or 3-MC (1 μ M) and CYP1A1 and CYP1B1 mRNA expression were analyzed by qRT-PCR. The levels of expression were normalized with 18s mRNA. Data are representative of five independent experiments and expressed as the mean $\pm$ SD. (ANOVA followed by Dunnett's multi-range test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus Ctrl). Effect of Actinomycin D (Act D) and Cycloheximide (CHX) on AαC induced (C) CYP1A1 and (D) CYP1B1 mRNA expression in activated human T lymphocytes. PMA/Iono activated human T lymphocytes were treated with PhiP, MeIQx and AαC (10 μ M) or 3-MC (1 μ M) for 16 h with or without Act D or CHX and the levels of CYP1A1 and CYP1B1 mRNA expression were analyzed by qRT-PCR. The data were normalized with 18s mRNA. Data are representative of three independent experiments and expressed as the mean $\pm$ SD. (ANOVA followed by Tukey's multi-range test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus Ctrl). Each treatment from Act D and CHX group has been compared to its corresponding treatment in the control group.

Figure 6. Effect of CH-223191 and α -Naphtoflavone (α -NF), two AhR antagonists on PhIP and A α C derived DNA adducts formation and CYP1 activity in activated human T lymphocytes

Effect of CH-223191 and α -NF on A α C induced (A) CYP1A1 and (B) CYP1B1 mRNA expression in activated human T lymphocytes. PMA/Iono activated human T lymphocytes were treated for 1 hour with 0.1% DMSO (Ctrl), CH-223191 (5 μ M) or α -NF (5 μ M), followed by 24 h of treatment with 0.1% DMSO (Ctrl), HAA (10 μ M) or 3-MC (1 μ M). The levels of CYP1A1 and CYP1B1 mRNA expression were analyzed by qRT-PCR and normalized with 18s mRNA. Data are representative of three independent experiments and expressed as the mean $\pm$ SD. (ANOVA followed by Dunnett's multirange test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$, versus Ctrl). Each treatment from CH-223191 and α -NF group has been compared to its corresponding treatment in the control group. (C) Effect of CH-223191 and α -NF on DNA adducts derived from A α C and PhIP formation in activated human T lymphocytes. PMA/Iono activated human T lymphocytes were pre-treated for 1 hour with 0.1 % DMSO (Ctrl), CH-223191 (5 μ M) or α -NF (5 μ M) followed by 24 h of treatment with 0.1% DMSO (Ctrl) or HAA (10 μ M). The DNA adducts were measured by UPLC/MS³. DNA adducts were measured in duplicate for each donor and expressed as the mean $\pm$ SD. (ANOVA followed by Tukey's multiple comparison test * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$, versus Ctrl). ND: not detectable. (D) Effect of CH-223191 and α -NF on CYP1 activity in activated human T lymphocytes. CYP1 activity was measured in PMA/Iono activated human T lymphocytes treated for 1 hour with 0.1 % DMSO (Ctrl), CH-223191 (5 μ M) or α -NF (5 μ M) followed by 24 h of treatment with 0.1% DMSO (Ctrl), PhIP (10 μ M), A α C (10 μ M) or 3-MC (1 μ M). Data are representative of three independent experiments and expressed as the mean $\pm$ SD. (ANOVA followed by Tukey's multiple comparison test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus Ctrl). ND: not detectable.