Effect of Black Tea Intake on the Excretion of Mutagens in the Urine of Volunteers Taking a Beef Meal

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Abstract

The objective of this study was to investigate in a crossover study conducted in human volunteers whether black tea intake modulates the metabolism of heterocyclic amines, consumed in the form of well-cooked beefburgers, as exemplified by the excretion of mutagens in the urine. Mutagens were extracted from urine with blue rayon, and mutagenic activity was determined in the Ames test, in the presence of an activation system derived from Aroclor 1254–induced rats, and employing the *Salmonella typhimurium* O-acetylase over-expressing YG1024 bacterial strain. Volunteers consumed three well-cooked beefburgers, whereas a concurrently cooked fourth burger was analyzed for mutagenic activity. Following intake of the burgers, an increase in urinary mutagenic activity was observed, and mutagenic activity was completely excreted within 24 hours. A good correlation was obtained between the intake and excretion of mutagenic activity. The volunteers consumed the same burger meal on two different occasions, once following intake of 10 cups of strong black tea, and the second following intake of a corresponding volume of water. Urine was collected by each volunteer for 24 hours after the meal, and compliance was ascertained utilizing the excretion of p-aminobenzoic acid. The mutagenic ratio, defined as the ratio of urinary mutagenicity over the intake of mutagenicity, was not altered by the black tea, being 0.142 and 0.135 during the water and tea intake, respectively. It is inferred that short-term intake of high levels of black tea, as part of an otherwise normal diet, does not modulate the human metabolism of heterocyclic amines. (Cancer Epidemiol Biomarkers Prev 2004;13(12):2196–202)

Introduction

There is strong epidemiologic evidence that consumption of diets having a high content of fruits and vegetables is associated with reduced cancer incidence (1). The underlying mechanism(s) of action are far from being elucidated, and the current views are largely the result of conjecture based on limited experimental evidence emanating from animal studies. However, several phytochemical classes have been shown to display anticarcinogenic activity in animal models of the disease, and these include polyphenols (2), isothiocyanates (3), resveratrol (4), and many others.

Tea is one of the most promising chemopreventive agents consumed in the diet and has many beneficial attributes. It is a palatable beverage extensively consumed worldwide, it has been consumed for centuries with an impeccable record of safety, and in animal models it antagonized the carcinogenicity of chemicals to which humans are exposed including polycyclic aromatic hydrocarbons, heterocyclic amines, and nitrosocompounds (5, 6).

One of the mechanisms that is believed to contribute to the anticarcinogenic activity of tea, both green and black, is the decrease in the formation of DNA adducts (7, 8). Such an effect may be a consequence of impaired generation of reactive intermediates and/or enhanced detoxication once these have been produced. Indeed, in *in vitro* studies, both green and black tea suppressed the mutagenic activity of carcinogens through inhibition of their cytochrome P450–mediated bioactivation (9, 10). When given *in vivo* to rats, however, both types of tea elevated the levels of hepatic CYP1A2 (11), one of the most active cytochrome P450 proteins in the bioactivation of carcinogens such as aromatic and heterocyclic amines (12, 13). Caffeine, a major constituent of tea, is responsible for this effect (11, 14). To a lesser extent, tea also enhances the activity of phase II conjugation enzymes involved in carcinogen deactivation (15, 16). Indeed, exposure of rats to green tea for 6 weeks led to an increase in the metabolism of the heterocyclic amine 2-amino-3-methylimidazo-(4,5-*f*)quinoline (IQ), favoring metabolic pathways leading to deactivation (17, 18). As a result, in rats exposed to tea, excretion of mutagens in the urine, following treatment with a single dose of IQ, is decreased (19).

We have recently shown that even a short pretreatment with black tea, of only a single day, reduces the IQ-mediated urinary mutagenicity in rats (20). In the current study, we have extended the animal studies to human volunteers and investigated whether black tea intake, as part of an otherwise normal diet, influences the excretion of mutagens in urine, following ingestion of heterocyclic amines in the form of well-cooked beefburgers.

Materials and Methods

Blue rayon was purchased from ICN Biomedical, Inc. (Basingtoke, Surrey, United Kingdom), p-aminobenzoic acid, naphthylene ethylene diamine was from Sigma (Poole, Dorset, United Kingdom), and all cofactors were from Melford Laboratories (Ipswich, United Kingdom). Typhoo tea bags were obtained from the local supermarket and p-aminobenzoic acid tablets (80 mg) from Dunns Laboratory (Addenbrook Hospital, Cambridge, United Kingdom). Beefburgers were locally prepared from lean cut beefsteak, using a binding powder. Each burger weighed 100 g raw and was approximately 1 cm thick.

Human Study. The study was approved by the ethics committee of the University of Surrey. Twelve healthy, six male and six female, non-smoking volunteers were recruited by word of mouth. Initially, each volunteer completed a questionnaire providing personal information, including dietary habits, and nature of working environment. A diet diary was supplied, covering the experimental period, and volunteers were shown how to complete it.

Beefburger Preparation. All volunteers were supplied with eight beefburgers produced at the same time to ensure the same fat consistency. Four burgers were used in each of the two stages of the study (see below). Each volunteer was also provided with a large heavy-duty pan, cooking instructions, and a photograph of a cooked burger, indicating the desirable degree of cooking. The pan was heated on a hob, at high temperature, for 3 minutes. All four burgers were then put into the pan concurrently and cooked for a minimum of 4 minutes, taking care to flatten gently the burger against the pan intermittently. The burgers were then turned over and cooked in the same way. The burgers were then to be continuously turned for an equal period of time until the extent of cooking indicated in the photograph was attained. The volunteer consumed three of the burgers, whereas the fourth was retained for determination of the mutagenic activity.

Preparation of Tea. Volunteers were instructed to place a tea bag in a cup and to then add boiling water (~1.5% w/v concentration) and leave it to brew for 2 minutes. The tea bag was then stirred and squeezed against the side of the cup before being discarded. Volunteers could add milk, sugar, and lemon as they wished, and this was noted in the diet diary. It has previously been established that milk does not influence the anticarcinogenicity and antimutagenicity of tea nor the absorption of catechins (21, 22). In order to ensure palatability, brew time was investigated in preliminary studies employing a panel of four persons. The purpose of these initial studies was to maximize ingestion of tea without compromising the ''drop out'' rate as a result of nausea (22). A brewing time of 2 minutes was acceptable; tea is commonly brewed for < 2 minutes (23). It is relevant to note that a brew time of 1 to 2 minutes is sufficient to release the majority of the antimutagenic components of tea (24).

Study Design. This was a crossover study comprising two parts, one during which the volunteers consumed black tea and the second where tea consumption was replaced with the consumption of an equivalent volume of water. Day 1 served as a washout period during which the volunteers were asked to refrain from consuming grilled/fried meat, fish, fried eggs, beef stock flavorings or caffeine-containing products. Previous studies have established that heterocyclic amines and their metabolites are excreted in the urine within 16 hours (25). Fluid intake included 10 cups of tea/water, but the consumption of alcohol, coffee, drinking chocolate, and cola-containing drinks was not allowed. Volunteers were encouraged to consume a cup of tea (water) every 1 to 2 hours. During day 2, the volunteers continued to consume tea (water) and continued on the same diet specified for day 1. However, for their evening meal, they consumed three of the four cooked beefburgers. During day 3, the volunteers continued on the same dietary regime as on day 1.

For the collection of urine, volunteers were provided with labeled dark urine bottles and ice packs, which when transferred to the university were stored at $-20°C$ until analyzed. Baseline mutagenicity was determined in the urine sample collected between lunchtime until the evening beef meal. Urine was then collected for the subsequent 24-hour time period following the beef meal in three pools: urine was collected after the evening beef meal on day 2 until the first void on day 3, the second pool was collected from the second void of the morning until the lunchtime and, finally, the third pool was collected between lunchtime and the evening meal on day 3.

Compliance. In order to ensure that all volunteers fully complied with urine collection, p-aminobenzoic acid was used in preference to creatinine because the former would not be influenced by the high levels of creatinine present in the meat (26). Each volunteer was supplied with three tablets of p-aminobenzoic acid (80 mg). The first was taken with the beefburger meal on the evening of day 2, the second with breakfast on day 3, and the third with lunch on day 3. In order to allow sufficient time for the third p-aminobenzoic acid tablet to be excreted, the volunteers were asked to continue to collect urine overnight on day 3.

Analytical Procedures

Extraction of Mutagens From Urine. Heterocyclic amines from urine were extracted with a method recently developed in our laboratory, based on blue rayon (27). Briefly, the urine was extracted with blue rayon, and the adsorbed heterocyclic amines were eluted with methanol/ammonia (100:1, v/v). The solvent was evaporated to dryness and the residue redissolved in DMSO (1 mL). As the residue is contaminated with copper released from the blue rayon, the level was minimized by centrifugation at $9,000 \times g$ for 5 minutes, and the supernatant was carefully removed.

Extraction of Heterocyclic Amines From Beefburgers. Extraction was once again achieved using blue rayon as recently described (27). The burger was homogenized in boiling water, and a fraction was extracted with blue rayon, the adsorbed heterocyclic amines were eluted with methanol/ammonia (1000:1, v/v), and the solvent was evaporated to dryness, with the residue being redissolved in DMSO (1 mL). Copper salts were removed by centrifugation as described above.
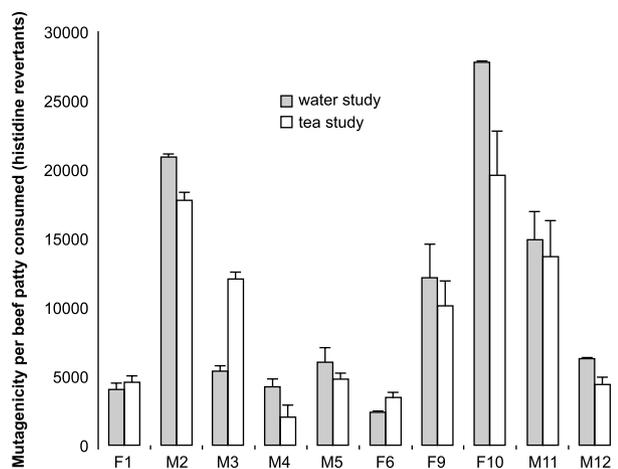
Figure 1. Mutagenic activity of beefburgers. The raw weight of each burger was 100 g. Mutagens were extracted using blue rayon, and the mutagenic activity of extracts was determined in the Ames test employing *S. typhimurium* strain YG1024, in the presence of an activation system (10%, v/v) derived from Aroclor 1254–induced rats. The mutagenic activity of both burgers supplied by each volunteer was determined on the same day to allow direct comparison. The spontaneous reversion rate, ranging from 21 to 54, has already been subtracted. Results, mean ± SD of triplicate plates. *M*, male volunteers; *F*, female volunteers.

Determination of Mutagenic Activity. Mutagenic activity in the urine and beef extracts was determined using the Ames test (28). Mutagenic activity of urinary extracts (70 μL) were determined using the *O*-acetylase over-expressing *Salmonella typhimurium* strain YG 1024, in the presence of an S9 activation system (10%, v/v) isolated from the liver of rats pretreated with Aroclor 1254–treated male Wistar albino rats. For the determination of direct-acting mutagens, urinary extracts (150 μL) were preincubated with the bacteria at 37C, in the absence of an activation system, for 90 minutes and then top agar was added. The mutagenic activity of the extracts (70 μL) from the beefburger were determined in the presence of the activation system as described above. The concentration of *p*-aminobenzoic acid excreted in the urine was determined as described by Bingham and Cummings (26). Data were analyzed using the Wilcoxon test within the Instat statistical package.

Results

Only ten of the volunteers (six males and four females) completed the study. Urinary analysis of *p*-aminobenzoic acid indicated that they all complied with urine collection. Several of the volunteers complained of headaches during the water study, i.e. during the period of abstinence from caffeine. Sudden withdrawal of caffeine intake is an established cause of headaches (29).

As each volunteer consumed only three of the four cooked beefburgers at each of the two stages of the study,

the fourth was returned to the laboratory. It was obvious from the degree of surface browning that not all of the volunteers cooked the beefburgers sufficiently, despite the provision of detailed instructions for cooking and a photo of a well-cooked burger. Indeed, surface browning is correlated to the generation of heterocyclic amines (30). It was apparent that each volunteer cooked the burgers to his/her personal taste. Consequently, it became essential for the mutagenic activity in each beefburger to be analyzed, so that urinary excretion of mutagenicity could be expressed in terms of the intake of mutagens. Figure 1 illustrates the mutagenic activity of the beefburgers prepared by the volunteers in each of the two parts of the study. A very marked variation exists in the mutagenic activity of the burgers prepared by each volunteer but, in contrast, the mutagenic activity of the beefburgers prepared by the same volunteers during the two stages of the study is relatively similar.

A marked, and statistically highly significant, increase in the excretion of promutagens in urine following intake of three well-cooked burgers was observed in all volunteers (Fig. 2). The highest mutagenic activity in urine was recovered in the first void following intake, i.e. the morning after the evening beef meal, and baseline mutagenicity was restored within 24 hours. A good correlation ($r = 0.772$) was evident between the mutagenic
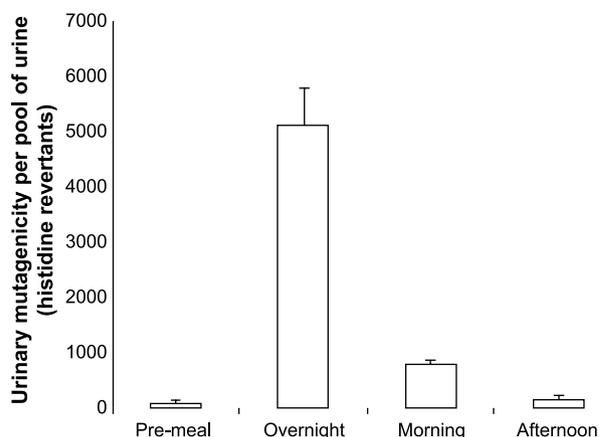
Figure 2. Typical urinary mutagenicity profile after consumption of a beefburger meal. An evening meal comprising three well-cooked beefburgers was consumed, and urine was collected thereafter until the first void the following day (overnight sample). Two further urine pools were collected, one between the second void of the morning until lunchtime (morning sample) and the last between lunch and dinner (afternoon sample). There was no intake of tea during the period. Mutagens were extracted with blue rayon and eluted with methanol/ammonia (100:1 v/v). Solvent was evaporated to dryness and the residue was redissolved in 1 mL DMSO. An aliquot (70 μL) was used in the Ames test using *S. typhimurium* strain YG1024, in the presence of an activation system (10%, v/v) derived from Aroclor 1254–induced rats. The spontaneous reversion rate of 39 ± 4 was subtracted. Results, mean ± SD of triplicates.
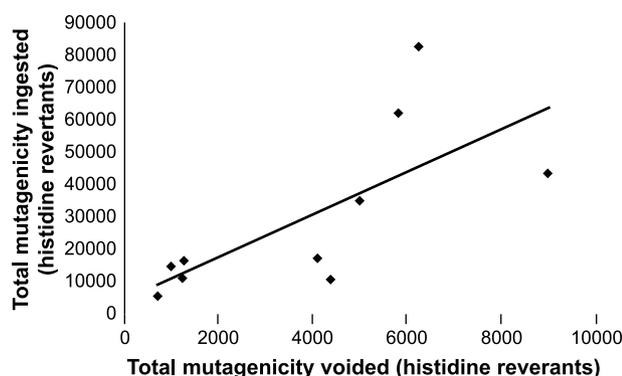
Figure 3. Correlation between mutagenicity ingested and mutagenicity excreted in urine of human volunteers. *Mutagenicity ingested*, mutagenic activity of the three beefburgers ingested by each of 10 volunteers. *Mutagenicity excreted*, mutagenic activity excreted by each volunteer during 24 hours after the meal consumption. The data are derived from the part of the study where volunteers drank water and were not exposed to tea. Mutagenic activity of beef extracts and urine was determined in the Ames test employing *S. typhimurium* strain YG1024, in the presence of an activation system (10%, v/v) derived from Aroclor 1254-induced rats.

activity consumed in the form of beefburgers and the urinary excretion of mutagenic activity determined in the presence of an activation system (Fig. 3).

The mutagenic activity in each urinary sample, determined in the presence of an activation system, during the two parts of the study, i.e. water and tea, is shown in Table 1. In all cases, the majority of the mutagenic activity was recovered in the overnight post-meal. Urinary mutagenic activity was expressed as total mutagenicity voided/total mutagenicity ingested, to account for variations in the intake of heterocyclic amines as a consequence of different extent of cooking. With one exception, this ratio remained within a reasonably narrow range (0.06-0.22). Statistical analysis revealed that

consumption of black tea did not significantly influence this ratio (Fig. 4; $P = 0.4316$).

When mutagenic potential was determined in the absence of an activation system, i.e. direct-acting mutagenicity, activity was detected only in the urine of one of the volunteers (M2), who had cooked the burgers extensively. Once again, excretion of direct-acting mutagens was highest in the first urine void after the beefburger consumption (Fig. 5), and intake of black tea significantly reduced the mutagenic response in the overnight sample.

Discussion

The principal aim of the current study was to investigate whether intake of black tea, as part of an otherwise normal diet, modulated the metabolism of heterocyclic amines in humans, as exemplified by the urinary excretion of mutagens. The design adopted has already been successfully tested in animal studies, where rats exposed to black tea for 24 hours prior to a low oral dose (5 mg/kg) of IQ excreted lower levels of mutagens in the urine, as assessed in the Ames test in the presence and absence of an activation system (20).

Heterocyclic amines were consumed in the form of well-cooked beefburgers. A major concern in designing these studies was that the ingested dose of heterocyclic amines might vary not only among the volunteers but also between the two parts of the study, where water or black tea is consumed with the beefburgers. Although each volunteer was given precise cooking instructions, as well as a photograph showing the desirable extent of cooking, it was felt that differences in the extent of cooking could compromise the study. In order to overcome this problem, volunteers cooked simultaneously an additional beefburger whose mutagenic activity was determined, so that the mutagenic activity excreted in the urine could be expressed in terms of mutagenic activity consumed. What emerged from these studies was that there was indeed a marked, more than 6-fold, difference in the extent of cooking of the beefburgers among the volunteers, which was evident both visually as

Table 1. Influence of tea on the urinary excretion of mutagens in human volunteers

Volunteer	Water					Black tea				
	Overnight post-meal	Morning post-meal	Afternoon post-meal	Total	Total ingested as beefburger	Overnight post-meal	Morning post-meal	Afternoon post-meal	Total	Total ingested as beefburger
F1	2,966 ± 305	1,055 ± 8	728 ± 220	4,389	12,233	5,920 ± 198	1,446 ± 222	662 ± 88	7,164	13,817
M2	5,129 ± 665	793 ± 91	153 ± 76	5,817	62,733	5,730 ± 574	587 ± 88	192 ± 68	6,218	53,300
M3	979 ± 237	256 ± 152	16 ± 0	1,015	16,217	4,704 ± 81	270 ± 167	326 ± 121	4,997	36,000
M4	1,186 ± 456	69 ± 10	0 ± 0	1,255	12,689	600 ± 30	0 ± 0	0 ± 0	600	6,222
M5	1,477 ± 30	110 ± 41	24 ± 20	1,282	18,033	746 ± 147	45 ± 48	27 ± 47	676	14,333
F6	678 ± 60	14 ± 25	29 ± 50	721	7,133	196 ± 92	43 ± 52	0 ± 0	211	10,306
F9	4,578 ± 283	608 ± 91	330 ± 63	5,000	36,200	3,024 ± 151	378 ± 46	226 ± 87	3,397	30,183
F10	5,680 ± 614	886 ± 36	479 ± 125	6,256	83,000	2,450 ± 114	413 ± 51	432 ± 51	2,425	58,567
M11	5,890 ± 194	2,962 ± 535	297 ± 131	8,990	44,367	5,080 ± 748	1,198 ± 210	413 ± 83	6,376	40,778
M12	3,405 ± 407	612 ± 81	403 ± 62	4,111	18,708	1,247 ± 41	182 ± 244	139 ± 41	1,343	13,000

NOTE: Mutagenic activity is expressed as histidine revertants (mean ± SD for triplicate plates) per urine pool from each volunteer. Total mutagenic activity is presented after subtraction of baseline mutagenicity, i.e. urinary mutagenicity prior to ingestion of the beefburger meal. Mutagenic activity in each sample was determined in the Ames test in the presence of an activation system, and utilizing *S. typhimurium* strain YG1024. Volunteers drank 10 cups of tea or the equivalent volume of water, and consumed three well-cooked beefburgers during the evening. Three urine voids were collected for each volunteer.
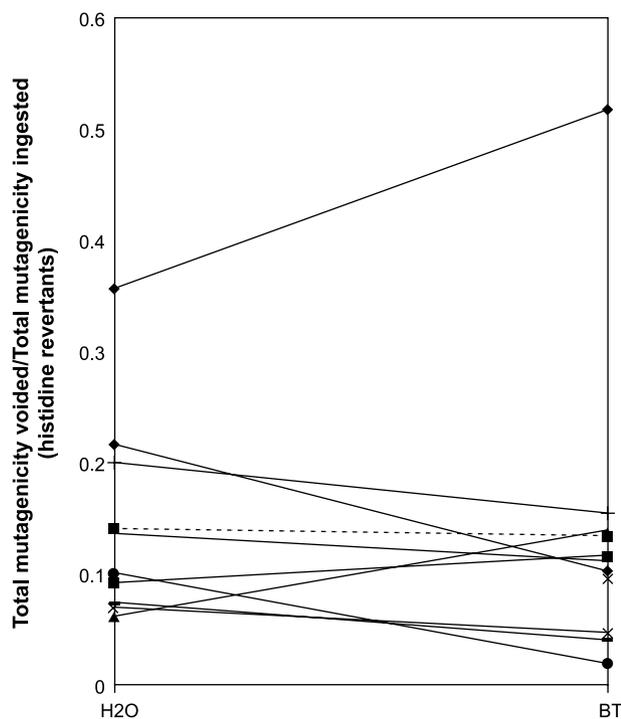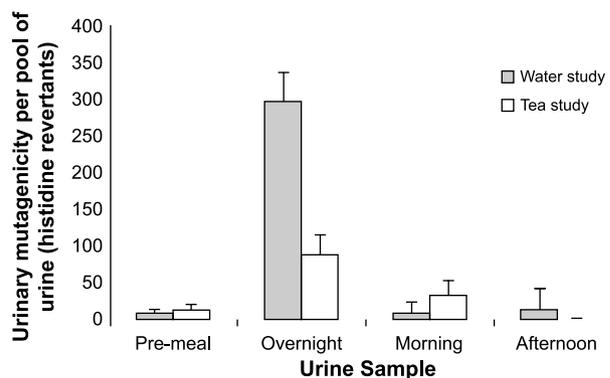
Figure 4. Effect of black tea intake on urinary mutagenicity in volunteers ingesting a beefburger meal. Total mutagenicity voided was calculated by adding the urinary mutagenicity voided during the 24 hours following the ingestion of the beefburger meal after baseline mutagenicity was subtracted. Total mutagenicity ingested was the mutagenic activity of the three beefburgers consumed by each volunteer. Mutagenic activity was determined in the presence of an activation system. *BT*, the part of the study where the burger meal was consumed following intake of black tea; *H₂O*, the part of the study where the burger meal was consumed following intake of the same volume of water. *Intermittent line*, mean values.

exemplified by the degree of surface browning, and also in terms of the mutagenic activity in the burger; the total intake of mutagenic activity ranged from 7,500 to 81,000 histidine revertants. Surprisingly, the mutagenic activity of the beefburger cooked by the same volunteer on the two different occasions was quite similar. Thus, it became apparent that some volunteers cooked the burgers to their own taste with little attention to the cooking instructions. However, all volunteers complied fully with the collection of urine, as evidenced by the excretion of *p*-aminobenzoic acid (26). Another factor that may influence the metabolism of this carcinogen is polymorphic expression of the enzymes involved in the metabolism of this carcinogen, such as CYP1A2 and acetylases (31).

Mutagenic activity in urine was very low but rose markedly after the ingestion of the burger meal, in agreement with previous studies (25). Excretion of mutagens in the urine of volunteers consuming the beefburger meal was, however, unaffected by the intake of black tea. As this was a crossover study, polymorphic

expression of the enzymes would not influence the outcome. In fact, it is interesting to note that with one exception, the ratio of mutagenicity intake to urinary mutagenicity did not differ markedly among the volunteers, and a good correlation was evident between mutagenicity intake and excreted urinary mutagenic activity. It has been reported that CYP1A2 expression in human liver can vary by more than 40-fold (32). In addition, it is unlikely that exposure to chemicals, e.g. through the diet, would modulate the levels of CYP1A2, because the two parts of the study were conducted in close proximity, in most cases on consecutive weeks. The lack of effect of tea is in marked contrast to the findings in animal studies, where a similar duration of black tea intake suppressed significantly the urinary excretion of mutagens in rats following oral to a single dose of IQ (20). This effect of black tea seemed to be associated with an elevation in CYP1A2 activity which stimulated the metabolism of IQ through 5-hydroxylation, a deactivation pathway (17, 18). A conceivable reason for the difference in the response of humans to the black tea intake may be the lack of induction of CYP1A2. In the rat studies, the animals were provided with 2.5% (w/v) of tea, brewed for 10 minutes, whereas in the human studies, the tea brew was weaker (1.5-2.0%, w/v) and was only brewed for 2 minutes. In general terms, the daily intake of

Figure 5. Effect of black tea intake on the direct-acting mutagenic activity in the urine of a single volunteer ingesting a beefburger meal. An evening meal comprising three well-cooked beefburgers was consumed, and urine was collected thereafter until the first void the following day (*Overnight*). Two further urine pools were collected, one between the second void of the morning until lunchtime (*Morning*), and the last between lunch and dinner (*Afternoon*). The beef meal was consumed either following intake of black tea or water as described in the text. Mutagens were extracted with blue rayon and eluted with methanol/ammonia (100:1, v/v). Solvent was evaporated to dryness and the residue was redissolved in 1 mL DMSO. An aliquot (150 µL) was used in the Ames test using *S. typhimurium* strain YG1024, in the absence of an activation system, following a 90-minute preincubation. The spontaneous reversion rate of 21 ± 5 was subtracted. Results, mean ± SD of triplicates.

caffeine by the volunteers was about 9 mg/kg, but was far higher in the rat studies, 56 mg/kg. It should be emphasized that it would not have been feasible to increase the daily intake of black tea. Almost invariably, the volunteers commented that they found it difficult to consume even the prescribed 10 cups per day. It is also worthwhile to note that the daily tea intake in the United Kingdom, which is one of the highest in the world, is only about five to six cups (22). Of more importance is the fact that a marked species variation in the metabolism of heterocyclic amines exists between rats and humans. In the rat, the principal metabolic pathway of heterocyclic amines is ring-hydroxylation, a deactivation step catalyzed by CYP1A2, the same cytochrome P450 protein that catalyzes its bioactivation through N-hydroxylation (12). However, in human liver ring-hydroxylation of heterocyclic amines is not catalyzed by CYP1A2 (33, 34), so that induction of this enzyme may not have impact on the deactivation of IQ. In fact, it may be argued that up-regulation of CYP1A2 may lead to elevated bioactivation through N-hydroxylation and increase in DNA adducts formation. However, at least in animals, induction of the CYP1 family leads to decreased bioactivation, presumably as a result of enhanced ring-hydroxylation (35, 36), which is not operative in human liver. It is important to point out that human CYP1A2 is far more efficient than the rat orthologues in catalyzing the N-hydroxylation of heterocyclic amines (37).

When mutagenic activity was assessed in the absence of an activation system, i.e. to determine the level of excretion of direct-acting mutagens, activity was only detectable in the urine of only one of the volunteers (M2), not surprisingly, a volunteer with a high mutagenicity intake. With the benefit of hindsight, it would have been desirable to instruct the volunteers to consume four beefburgers, rather than three, to make direct-acting mutagenicity detectable in the urine samples. It is worthwhile to note that in this single volunteer, the black tea intake reduced the excretion of direct-acting mutagens, as previously observed in animal studies (20). Further studies are required to ascertain whether this is a consistent effect in other human volunteers.

Very low levels of polycyclic aromatic hydrocarbons may also be generated during frying (38). Even if these are excreted in the urine unmetabolized, their contribution to the mutagenic activity, determined under the conditions employed here, are at best minimal. As mutagenicity in human urine after intake of a cooked beef meal can only be detected when O-acetylase over-expressing bacterial strains are utilized (39), it may be inferred that the urinary mutagenic activity is due to heterocyclic amines. Furthermore, the mutagenicity of unsubstituted polycyclic aromatic hydrocarbons is not enhanced by the use of O-acetylase over-expressing bacterial strains (40).

In conclusion, the present study has shown that short-term intake of black tea failed to influence the excretion of mutagens in the urine of volunteers ingesting heterocyclic amines in the form of well-cooked beefburgers. Under similar conditions, black tea suppressed excretion of mutagens in rats treated with a single oral dose of IQ. A limitation of this study is the small sample size of only 10 volunteers, and it would

be prudent for the current findings to be confirmed in larger groups. Finally, our observations advise caution in readily extrapolating chemoprevention data generated in animals to humans. In animal studies, the chemopreventive agents are frequently given to animals utilizing exposure patterns markedly different to those that are normally achievable or palatable to humans.

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