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In Vitro Antimutagenicity of Capsaicin Toward Heterocyclic Amines in *Salmonella Typhimurium* Strain TA98

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Abstract. *Capsicum fruits are widely consumed as a component of the human diet. Capsaicin is the principle substance responsible for their hot, pungent taste. Heterocyclic amines (HCAs) are formed during cooking of meats and are mutagenic/carcinogenic compounds. In this study, we looked at whether capsaicin showed anti-mutagenic effects toward HCA-induced mutagenesis in Salmonella typhimurium TA98 when incubated with 0.5 mg liver S9 protein from rat, hamster and human. The HCAs used were Trp-P-2, Glu-P-1 and PhIP. Capsaicin, at non-toxic amounts of 0.25 and 0.5 μmole/plate, expressed a dose-dependent inhibition of the mutagenicity of Glu-P-1 and PhIP when they are metabolically activated by rat, hamster and human liver S9 and of Trp-P-2 when activated by rat and hamster liver S9. In contrast, capsaicin enhanced the mutagenicity of Trp-P-2 in TA98 when incubated with human liver S9. The lack of consistency in the anti-mutagenic action of capsaicin toward HCAs is puzzling and currently unresolved.*

Considerable attention has been given in recent years to the role of diet in the carcinogenic process. Foods that comprise the human diet have been shown to contain mutagenic/carcinogenic substances, either naturally or as a consequence of the manner in which they are prepared (1,2). Heterocyclic amines (HCAs) are a class of compounds that exist as pyrolisates of protein formed by the heat cooking of beef, fish and chicken (2,3). HCAs are mutagenic in *Salmonella typhimurium* strain TA98 bacteria when activated by cytochrome P450 enzymes (4). HCAs cause tumors in the liver, colon and mammary gland of rats (5,6). The risk associated with mutagens/carcinogens in foods may be modulated by the presence of other naturally occurring substances in foods. These phytochemicals include an array of phenolic and polyphenolic compounds (7, 8). *Capsaicin* (trans-8-methyl-N-vanillyl-6-nonenamide) is the major component in *Capsicum* fruits that is responsible for their hot, pungent properties (9). *Capsicum* fruits are consumed throughout the world as a component of the human diet (10). Capsaicin has been shown to exhibit chemoprotective properties. It altered the metabolism and mutagenicity of aflatoxin B1 and of the tobacco-specific nitrosamine NKK (11, 12). It preferentially repressed growth of cancer cell lines (13) and has recently been shown to inhibit angiogenesis both *in vitro* and *in vivo* (14). Capsaicin is reported to possess antimutagenic and anticarcinogenic properties (15). The chemical structure of capsaicin is shown in Figure 1.

In the study presented here, we report the effects of non-toxic amounts of capsaicin on the mutagenicity of three HCAs in *Salmonella typhimurium* TA98 in the presence of liver S9 protein from the rat, hamster and human. The HCAs were 3-amino-1-methyl-5H-pyrido [4,3-b] indole acetate (Trp-P-2), 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (Ph1P) and 2-amino-6-methyl-dipyrido [1,2-a:3',2'-d] imidazole hydrochloride (Glu-P-1).

Materials and Methods

Chemicals. DMSO, NADP, D-glucose-6-phosphate, capsaicin, L-histidine and d-biotin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Nutrient agar was obtained from Fisher Scientific (Pittsburg, PA, USA) and nutrient broth was purchased from Unipath, Oxoid Division (Columbia, MD, USA). 3-Amino-1-methyl-5H-pyrido[4,3-b] indole (Trp-P-2) acetate, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) and 2-amino-6-methyldipyrido [1,2-a:3',2'-d] imidazole hydrochloride (Glu-P-1) were purchased from Toronto Research Chemicals, Inc. (Downsview, Ontario, Canada). *Salmonella typhimurium* TA98 was a gift from Dr. Bruce Ames (Univ. California, Berkeley, USA).

Liver S9. Liver S9 protein from male Sprague-Dawley rats, male Syrian hamsters and humans pooled from 5 donors was purchased and shipped on dry ice from Molecular Toxicology, Inc. (Boone, NC, USA).

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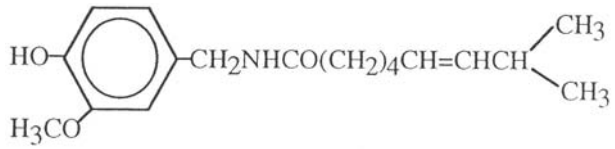

Figure 1. *Chemical structure of capsaicin.*

Toxicity tests. Salmonella typhimurium TA98 was cultured for 6 h in oxoid nutrient broth at 37˚C in a shaking waterbath. The bacteria were diluted 1:10,000 and 100 μl of the dilution were placed in sterile culture tubes to which different amounts of capsaicin dissolved in DMSO and 2 ml of molten agar containing 0.1 mM histidine and 0.4 mM d-biotin were added, mixed and poured onto minimal essential agar plates (16). The agar plates were incubated at 37˚C for 36 h. The mean number of surviving TA98 colonies was determined for each amount of capsaicin tested and compared with controls (0 capsaicin). Non-toxic amounts of capsaicin were determined to be those at which no statistically significant difference in the number or size of surviving colonies was observed compared to colonies grown in the absence of capsaicin.

Mutagenicity assays. Sterile culture tubes containing 0.5 ml S9 cofactor buffer (4 mM NADP, 5 mM glucose-6-phosphate, 33 mM KC1 and 8 mM $MgCl_2$ in 0.1M sodium phosphate buffer, pH 7.4), 0.1 ml overnight culture *Salmonella typhimurium* TA98, 0.5 mg S9 liver protein from rat, hamster or human, 5 μg Trp-P-2, 50 μg PhIP or 500 ng Glu-P-1 and 0, 0.25 or 0.5 μmole capsaicin were incubated for 25 min at 37˚C in a shaking waterbath. Two ml molten top agar was then added to each tube, mildly vortexed and poured onto minimal glucose agar plates and incubated at 37˚C for 48 h (16). The number of revertant colonies on each agar plate was scored. Plates without S9 protein, capsaicin and mutagen were part of each experiment in order to determine the number of spontaneous revertant colonies. The mean number of spontaneous revertant colonies was subtracted from experimental values.

Statistical analysis. Experiments were conducted in triplicate and data was analyzed using the Student's paired two-tailed *t*-test. Results were expressed as the mean±SEM and statistical significance was defined as $p \leq 0.05$ when compared to control values (0 capsaicin).

Results

Assays to determine the amount of capsaicin affecting the survival of *Salmonella typhimurium* TA98 indicated that toxicity occurred at between 1 and 2 μmoles/plate (data not shown). The subtoxic doses selected to study the effects of capsaicin on the mutagenicity of Trp-P-2, PhIP and Glu-P-1 in TA98 were 0.25 and 0.5 μmole/plate.

Figure 2 shows the effect of capsaicin on the *in vitro* mutagenicity of Trp-P-2, PhIP and Glu-P-1 when incubated in the presence of 0.5 mg S9 protein from rat, hamster and human liver. Capsaicin produced a statistically significant dose-dependent inhibition of the expressed mutagenicity of Glu-P-1 and PhIP in all three metabolic activation systems. Capsaicin also exhibited a dose-dependent inhibition of the mutagenicity of Trp-P-2 in TA98 when activated by rat and hamster liver S9. However, as Figure 2 illustrates, capsaicin enhanced the mutagenicity of Trp-P-2 in TA98 when metabolically activated by human liver S9. Although only the 0.5 μmole dose caused a statistically significant change, the 0.25 μmole dose also reflected the trend. The results expressed in Figure 2 were only observed when capsaicin was present during the 25-min incubation prior to the addition of top agar. When capsaicin was added to the top agar after the 25-min incubation, the colony counts were not statistically different from the control plates (data not shown).

Discussion

Numerous compounds that possess the potential to alter the development of cancer have been identified in foods consumed by humans. The scientific literature is replete with discussions of experimental studies that give evidence of this. Since the development of cancer is a multi-step process, chemoprevention of this process by phytochemicals may occur at different stages in the initiation, promotion and progression of the disease. The *Salmonella* mutagenicity assay (16) has provided an excellent correlation between mutagenicity in bacterial strains of *Salmonella* and carcinogenicity in animal models (17, 18). This well-established assay has been useful in screening for compounds with anticarcinogenic potential.

HCAs are promutagens requiring metabolic activation by microsomal associated cytochrome P450 isoenzyme 1A2 (19). They exist as imidazoazaarenes (*e.g.*, PhIP), pyridoindole and dipyridoimidazole derivatives of which Trp-P-2 and Glu-P-1 are examples, respectively. They are mutagenic in *Salmonella typhimurium* TA98 (4) and induce tumors in laboratory animals (5, 6). HCAs are produced when muscle protein is subjected to high temperatures and/or prolonged cooking. As meats are cooked combinations of creatine, creatinine, amino acids and sugars yield various HCAs. PhIP is the predominant HCA present in cooked fish and chicken, whereas other HCAs form in cooked beef (20). Various studies have described a chemopreventive action by citrus flavonoids, isothiocyanates, tea compounds and capsaicin toward HCA-induced mutagenesis and carcinogenesis (21-24).

In view of the widespread use of *Capsicum* fruits as a food additive and the presence of HCAs in cooked meats, we undertook the present study to examine the effects of capsaicin on the *in vitro* mutagenesis of HCAs using liver S9 from the rat, hamster and human for metabolic activation. Human S9 protein was pooled from five disease-free donor liver samples as purchased from Molecular Toxicology, Inc.. The results described herein were consistent with a dose-dependent inhibition of the formation of mutant colonies in *Salmonella typhimurium* TA98 by PhIP and Glu-P-1 in the

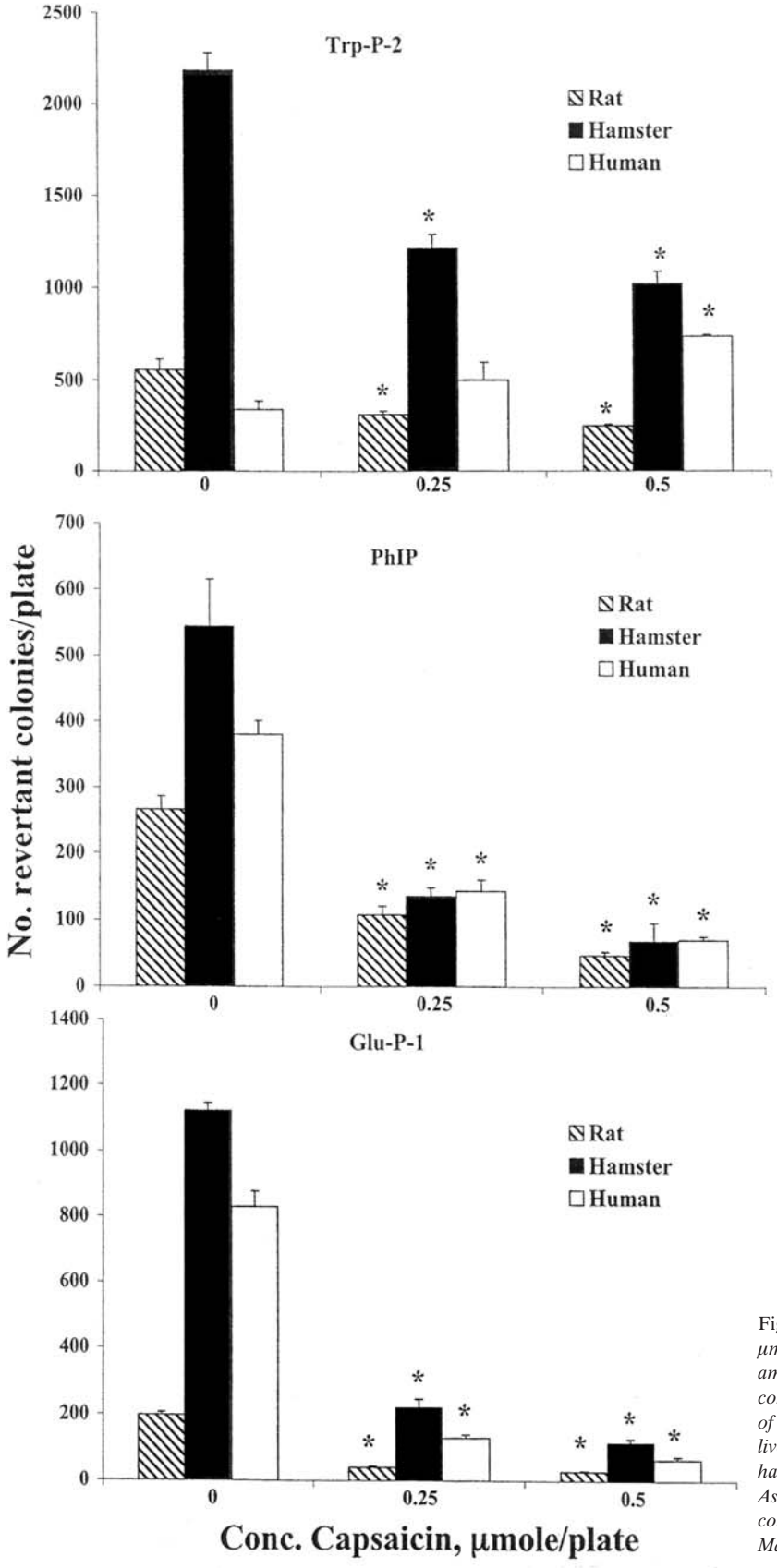

Figure 2. *Effects of capsaicin at 0.25 and 0.5 μmole/plate on the mutagenicity of the heterocyclic amines Trp-P-2, PhIP and Glu-P-1 compared to controls (0 μmole/plate). In vitro metabolic activation of Trp-P-2, PhIP and Glu-P-1 was by incubation with liver S9 protein from the Sprague-Dawley rat, Syrian hamster and human. Values are the means±S.E. Asterisks indicate a statistical significant difference from controls, $p \leq 0.05$. Procedural details are given in Materials and Methods.*

presence of rat, hamster and human liver S9 protein. There was also a dose-dependent inhibition of the mutagenic action of Trp-P-2 in TA98 by capsaicin when Trp-P-2 was activated by rat and hamster liver S9. The mutagenicity of PhIP, Glu-P-1 and Trp-P-2 was highest when activated by hamster liver S9, shown in Figure 2. This correlated with the product data supplied by Molecular Toxicology, Inc. which indicated that hamster liver S9 had several-fold greater methoxyresorufin demethylase (MROD) activity than either the rat or human S9. MROD activity is linked to the activity of P450 1A2. Capsaicin inhibited MROD activity in hamster liver microsomes (23).

Two inconsistencies are expressed in Figure 2. Both relate to results obtained with human liver S9. Firstly, the incubation with the mutagen Trp-P-2 generated fewer revertant colonies in TA98 than when the mutagen was incubated with rat liver S9. This is contrary to that seen with PhIP and Glu-P-1. Secondly, there was the absence of a dose-dependent inhibition of Trp-P-2 mutagenesis by capsaicin when compared to the effect observed with PhIP and Glu-P-1. These vagaries are puzzling. Liver S9 contains phase II enzymes which play a role in the detoxification of xenobiotics in addition to microsomal cytochrome P450 activity (25). Why capsaicin would selectively affect the metabolic activation and detoxification of Trp-P-2 differently than either PhIP or Glu-P-1 is unresolved at this time and complicates any conclusion regarding the chemoprotective action of capsaicin toward the mutagenesis and carcinogenesis of HCAs.

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