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Effects of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) on histopathology, oxidative stress, and expression of c-fos, c-jun and p16 in rat stomachs

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ABSTRACT

2-Amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) is one of the most abundant heterocyclic amines (HCAs) generated from overcooking meat at high temperatures. To understand the possible mechanism of PhIP-associated stomach cancer, the effects of PhIP on morphology, oxidative stress, gene expression of c-fos, c-jun and p16 in rat stomachs were investigated. The results showed that (1) 15 mg/kg body weight PhIP induced obvious histopathological changes in gastric mucosa; (2) PhIP (10 and/or 15 mg/kg) significantly decreased superoxide dismutase (SOD) and glutathioneperoxidase (GPx) activities, while increased catalase (CAT) activity compared with the control. With the elevated doses of PhIP, malondialdehyde (MDA) contents, protein carbonyl (PCO) contents and DNA–protein crosslinks (DPC) coefficients were significantly raised in a dose-dependent manner; (3) PhIP at the doses of 10 mg/kg and/or 15 mg/kg significantly inhibited p16 mRNA and protein expression, whereas enhanced c-fos and c-jun expression relative to control. The data indicated that PhIP could cause stomach injury, oxidative stress in rat stomachs as well as the activation of c-fos and c-jun and inactivation of p16, which may play a role in the pathogenesis of PhIP-associated stomach cancer.

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1. Introduction

Heterocyclic amines (HCAs), a kind of low molecular organic amine compounds, are mainly formed in the pyrolysis process during cooking and frying of meat and fish at high temperatures (Sugimura et al., 2004). The high meat consumption is very typical of the Western diet, while recently the amount of meat production and consumption of the Eastern countries has grown rapidly by the globalization of food industry and rapid economic growth (Turesky, 2007; Nam et al., 2010). The literatures reported that the total HCA concentrations in cooked meat generally ranged from 1 to about 500 ng/g (from 0.001 to 0.5 ppm), and the dietary exposure to total HCAs was estimated to range from less than 1 to 17 ng/kg of body weight per day (Layton et al., 1995; National Toxicology Program, 2011). Additionally, HCAs have also been detected in processed food flavorings, cigarette smoke, wine, environmental particulates, surface water, etc. (Manabe et al., 1991, 1993; Dong et al., 2009; National Toxicology Program, 2011). Accordingly exposure to HCAs is considerable.

Mutagenic and/or carcinogenic HCAs were first found in meat and fish cooked at temperatures over 150 °C (Nagao et al., 1977). To date, more than 25 HCAs have been isolated and identified in cooked meat and meat products as potent mutagens in the Ames/Salmonella test (Kizil et al., 2011; Puangsombat et al., 2012). Many epidemiological studies showed that high intake of HCAs may increase the risk of stomach, colon, breast cancers and other cancer in humans (Ward et al., 1997; Pence et al., 1998; Kampman et al., 1999; Sinha and Snyderwine, 2001; Terry et al., 2003; Zheng and Lee, 2009). But some researchers say that the contribution ratio of HCAs to human cancer is very low (Delfino et al., 2000; Kobayashi et al., 2009).

2-Amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) is the most abundant HCA formed during the cooking of meat (Layton et al., 1995). Its concentration in cooked food can range from several parts per billion (ppb) up to 500 ppb (Ni et al., 2008). PhIP and

Abbreviations: HCAs, heterocyclic amines; PhIP, 2-Amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; PCO, protein carbonyl; DPC, DNA-protein crosslinks; HE, hematoxylin-eosin; IHC, immunohistochemistry; DAB, diaminobenzidine; AMV RT, avian myeloblostosis virus reverse transcriptase; dNTPs, deoxynucleoitide triphosphates; ROS, reactive oxygen species; CA, chromosomal aberrations; MN, micronuclei; SCE, sister chromatid exchanges.

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its metabolites were found in colon, blood, lymphocytes and urines of the individuals after intake of cooked meats (Boobis et al., 1994; Dingley et al., 1999; Magagnotti et al., 2003; Fede et al., 2009). More importantly, PhIP has been demonstrated to be a potential dietary risk factor related to some cancers such as breast, colon, stomach and prostate cancer (Ito et al., 1991; De Stefani et al., 1998; Tang et al., 2007; Choudhary et al., 2012), and the National Toxicology Program (2011) has concluded that PhIP is "reasonably anticipated to be a human carcinogen". Currently, the association between PhIP intake and stomach cancer was based on only three studies (De Stefani et al., 1998; National Toxicology Program, 2011; Cross et al., 2011). Therefore, the evidence from epidemiological and experimental animal studies is very inadequate to evaluate the relationship between human stomach cancer and exposure to PhIP.

Stomach cancer is the second leading cancer death in the world (Sun et al., 2004). Generally, the activation of proto-oncogenes (such as c-fos and c-jun) and the inactivation of tumor suppressor genes (such as p16) are considered to be very important in the carcinogenesis process. Activation of c-fos and c-jun always occurs at an early stage of tumor development induced by the chemical carcinogens (Vogt et al., 1992; Kogan et al., 1994), and it plays a key role in gastric carcinoma (Hou et al., 2006). Tumor suppressor genes including p16 exert a negative regulatory role in cellular proliferation and tumor formation, and inactivation of these genes may contribute to deregulation of cellular growth and malignant progression (Kim et al., 2003). Inactivation of p16 is a frequent event in stomach cancer (Fushida et al., 1996), and the p16 gene abnormality has been suggested to be one of the important molecular mechanisms in the carcinogenesis of gastric cancer and a useful biomarker for prediction of gastric cancer (Kanyama et al., 2003; Sun et al., 2004).

Most reports have also shown that oxidative stress induced by reactive oxygen species (ROS) is linked to carcinogenesis due to its ability to damage DNA (Oberley, 2002; Ishikawa et al., 2008; Klaunig et al., 2010). Batcioglu et al. (2006) pointed out that oxidative stress played an important role in gastric carcinogenesis. When oxidative stress is assessed, malondialdehyde (MDA) and protein carbonyl (PCO) are often used as biomarkers, for they are byproducts of oxidation of lipids and proteins (Imlay and Linn, 1988).

Taken together, PhIP is a health hazard related to stomach cancer. However, so far, the molecular basis of potential gastric carcinogenic effects of PhIP remains unclear. To understand the possible mechanism, the histopathology, oxidative stress, and expression of proto-oncogenes and tumor suppressor genes in stomachs from rats after oral administration of PhIP were studied using hematoxylin-eosin (HE) staining, real time quantitative RT-PCR, western blot, immunohistochemistry (IHC) and biochemical analysis methods, respectively. In the present study, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, MDA and PCO contents, DNA–protein crosslinks (DPC) coefficients, and gene expressions of c-fos, c-jun and p16 were determined. Elucidating the effects of PhIP on lipid peroxidation and protein carbonylation and the expression patterns of c-fos, c-jun and p16 is critical to our understanding of PhIP toxicity mechanisms and the relationships between human stomach cancer and exposure to PhIP.

2. Materials and methods

2.1. Preparation of animals

Healthy adult and clean grade male Wistar rats, weighing 180–200 g, were purchased from Animal Center of Hebei Medical University (Animal Certificate No: 1010031). Animals were housed in metallic cages under standard conditions (24 °C ± 2 °C and 50% ± 5% humidity) with a 12-h light–dark cycle. Rats were divided randomly into four equal groups of five animals each: (1) the control group (55% ethanol–saline, pH4.5), (2) 5 mg/kg body weight PhIP group (PhIP was dissolved in the solution of 55% ethanol–saline, pH4.5), (3) 10 mg/kg body weight PhIP group, and (4) 15 mg/kg body weight PhIP group. Different doses of PhIP and 55% ethanol–saline used in the present experiment were in accordance with the literatures by Lin et al. (1998) and Li et al. (2012), and they were respectively given to rats once by intragastric administration. PhIP ($C_{13}H_{12}N_4$, mol wt 224.11, CAS registry number: 105650-23-5) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada).

All animal procedures were approved by the Shanxi University Animal Investigational Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by Ministry of Health People's Republic of China and the Guiding Principles in the Use of Animals in Toxicology published by the Society of Toxicology in 1989.

2.2. Hematoxylin and eosin (HE) staining and immunohistochemistry

Rats were killed by anesthesia (sodium pentobarbital, 90 mg/kg, i.p.) 24 h after the intragastric gavage treatment. Then, the rat stomach was pulled up into the operative field, and cut open along the greater curvature. After washing the stomach contents with water, a piece of the fore-stomach was cut and fixed in 4% paraformaldehyde in PBS and paraffin-embedded for the HE staining and immunohistochemical analysis, while the rest sample was quickly frozen in liquid nitrogen, and stored at −80 °C until analysis.

Using the streptavidin biotin peroxidase method, immunohistochemistry for p16 protein expression in stomach tissues was performed as described previously (Bai and Meng, 2005). Briefly, 5 μm thick sections were immersed in citrate buffer solution (pH 6.0), then processed in a microwave oven twice for 5 min each time at high power, and incubated with normal goat serum for 20 min and left overnight at 4 °C with mouse anti-p16 monoclonal antibody (Santa Cruz, CA, USA) at 1:200 dilution. A horseradish peroxidase-conjugate polyclonal rabbit anti-mouse antibody was used as the secondary antibody reagent. The reaction product was visualized using diaminobenzidine (DAB) as a substrate. The negative control was accomplished by substituting p16 antibody with PBS. p16 positive staining was shown at 400× magnification. Positive immunohistochemistry expression of p16 was defined by nuclear and cytoplasmic staining pattern of gastric mucosa cells.

2.3. Measurement of antioxidant enzyme activities and MDA contents

Stomach samples were homogenized in 10% (w/v) in 0.1 mM PBS (pH 7.4) at 4 °C using a motor-driven homogenizer (Heidolph, Kehlheim, German) for determination of SOD, CAT and GPx activities and MDA contents. The homogenates were centrifuged at 1468*g* for 15 min at 4 °C, and the supernatants were collected and stored at −80 °C in polypropylene tubes until assay.

Measurements of enzymatic activities of SOD, CAT and GPx and the MDA contents in stomach tissues were performed spectrophotometrically using the corresponding kits from the Nanjing Jiancheng Biochemistry according to the manufacturer's protocols.

2.4. Measurement of PCO and DPC

Measurements of PCO and DPC were performed as described previously (Xie et al., 2007). Briefly, utilizing 2, 4-dinitrophenylhydrazine (DNPH) colorimetry, the carbonyl content was calculated from absorption at 370 nm (Hitachi U-3010, Tokyo, Japan) using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} and the results were expressed as nmol of carbonyl per mg protein. As for the measurement of DPC, the samples were treated with sodium dodecylsulfate (SDS)-KCl system. Since SDS binds tightly to protein but not DNA, the free protein and DNA protein crosslink complexes were formed in the SDS-K^+ precipitate while the unbound fraction of DNA (free DNA) existed in the supernatant. Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 460 nm (Hitachi F-4500, Tokyo, Japan). The results were expressed as percentage (DPC coefficient) of protein-bound DNA on total DNA (free DNA plus protein-bound DNA).

2.5. Real-time quantitative RT-PCR

The frozen stomach tissues were homogenized in TRIzol reagent using 1 ml TRIzol per 50 mg tissue. Total RNA was extracted from the tissue according to the manufacturer's suggested protocol. RNA pellets were dissolved in 100 μl DEPC (diethylpyrocarbonate)-treated water. Total RNA concentration was determined by spectrophotometric analysis at 260 nm. RNA quality was assessed by electrophoresis on a 1% agarose gel. First-strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (AMV RT) Transcriptor First Strand cDNA Synthesis Kit from the Sangon Biotech Co., Ltd (Shanghai, China) according to the manufacturer's protocols. The cDNA product was stored at −80 °C until use.

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