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ScienceDirect
Toxicology in Vitro 21 (2007) 685–690
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Brief communication

Induction of cytochrome P450 1a1 by the food flavoring agent, maltol

Anwar Anwar-Mohamed, Ayman O.S. El-Kadi *

Faculty of Pharmacy and Pharmaceutical Sciences, 3126 Dentistry/Pharmacy Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2N8

Received 23 November 2006; accepted 5 January 2007
Available online 15 January 2007

Abstract

Maltol is used extensively as a flavor-enhancing agent, food preservative, antioxidant, and also in cosmetic and pharmaceutical formulations. However, a number of studies have shown that maltol may induce carcinogenicity and toxicity but the mechanisms involved remain unknown. Therefore, we examined the ability of maltol to induce the cytochrome P450 1a1 (Cyp1a1), an enzyme known to play an important role in the chemical activation of xenobiotics to carcinogenic derivatives. Our results showed that treatment of Hepa 1c1c7 cells with maltol significantly induced Cyp1a1 at mRNA, protein, and activity levels in a concentration-dependent manner. The RNA synthesis inhibitor, actinomycin D, completely blocked the Cyp1a1 mRNA induction by maltol, indicating a requirement of *de novo* RNA synthesis through transcriptional activation. In addition, maltol induced aryl hydrocarbon receptor (AhR)-dependent luciferase reporter gene expression in stably transfected H1L1.1c2 cells, suggesting an AhR-dependent mechanism. This is the first demonstration that the food flavoring agent, maltol, can directly induce *Cyp1a1* gene expression in an AhR-dependent manner and represents a novel mechanism by which maltol promotes carcinogenicity and toxicity.

Keywords: Aryl hydrocarbon receptor; Cytochrome P450 1a1; Maltol; Carcinogenesis

1. Introduction

The aryl hydrocarbon receptor (AhR) belongs to the helix-loop-helix protein family, it is found inactive in the cytoplasm attached to a complex of chaperone heat shock proteins 90 (HSP90), hepatitis B virus X-associated protein (XAP2), and p23 (Hankinson, 1995; Dogra et al., 1998; Mimura and Fujii-Kuriyama, 2003). Activation of the AhR upon binding with its ligand, causes translocation of the activated complex to the nucleus. In the nucleus, HSP90 dissociate from the activated AhR which subsequently heterodimerizes with a nuclear transcription factor protein, the aryl hydrocarbon receptor nuclear translocator (ARNT) (Whitelaw et al., 1994). The AhR/ARNT complex then binds to a specific DNA recognition sequence, GCGTG, within the xenobiotic responsive element (XRE), located in the promoter region of a number of receptor regulated genes, including the *CYP1A1* (Denison et al., 1989; Nebert et al., 2004). Among the AhR-regulated genes, *CYP1A1* is the most capable in producing polar, toxic, or even carcinogenic metabolites from various AhR ligands including aromatic and halogenated hydrocarbons (Schrenk, 1998). These metabolites have been shown to be involved in the mediation of a broad range of distinct toxic responses such as immune suppression, endocrine disruption, birth defects, and carcinogenesis (Poland and Knutson, 1982).

Maltol (2-methyl-3-hydroxy-1,4-pyrone) is a naturally occurring substance that is widely used as a flavouring agent. It is formed through thermal degradation of starch or sucrose pyrolysis, and is found in coffee, soybeans, baked cereals, and browned food (Bjeldanes and Chew, 1979). The usual amounts of maltol added to beverages, baked food, ice creams, and candy range from 80 to 110 mg/l (Bjeldanes and Chew, 1979). *In vivo* toxicity studies on male and female rats showed that treatment with maltol in the dose of 1000 mg/kg/day for 9 weeks causes significant weight loss, kidney lesions, and increases the incidence of albuminuria and mortality (Gralla et al., 1969). Similarly, treatment of dogs with maltol 500 mg/kg/day for 21–41 days caused

* Corresponding author. Tel.: +1 780 492 3071; fax: +1 780 492 1217.
E-mail address: aelkadi@pharmacy.ualberta.ca (A.O.S. El-Kadi).

0887-2333/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2007.01.003

significant increase in mortality with severe weight loss, increase in blood urea nitrogen, hepatorenal damage, and mid-zonal hepatic necrosis (Gralla et al., 1969). *In vitro* studies showed that maltol enhanced aluminium toxicity, and by itself is neurotoxic at micromolar concentrations (150 μM) to human and murine neuroblastoma cell lines, and primary murine fetal hippocampal neurons in a dose- and cell-type dependent manner (Hironishi et al., 1996; Johnson et al., 2005). In addition, maltol has been shown to cause mutagenesis in Salmonella typhimurium strains TA-98 and TA-100 using Ames plate assay system, but the mechanism remains unknown (Bjeldanes and Chew, 1979; Shibamoto et al., 1981).

The aim of this study was to investigate the mechanisms by which maltol induces toxicity or carcinogenicity. We therefore examined the effect of maltol on Cyp1a1 mRNA, protein, and enzyme activity in murine hepatoma Hepa 1c1c7 cells. Additionally the involvement of the AhR-dependent signaling pathway was also investigated by using luciferase reporter gene assay, using H1L1.1c2 cells. Here, we provide the first direct evidence for an AhR-dependent induction of *Cyp1a1* gene expression and enzyme activity by maltol.

2. Materials and methods

2.1. Cell culture and chemicals

Murine hepatoma Hepa 1c1c7 cells (generously provided by Dr. Oliver Hankinson, University of California, Los Angeles, CA), and recombinant mouse hepatoma H1L1.1c2 cells (generously provided by Dr. Michael S. Denison, University of California, Davis, CA), were maintained in standard DMEM supplemented with 10% fetal bovine serum (Sigma–Aldrich Chemical Co., St. Louis, MO). The cells were grown in 75 cm^2 tissue culture flasks at 37 °C under a 5% CO_2 humidified environment as described previously (Korashy and El-Kadi, 2005). 7-Ethoxyresorufin (7-ethoxy-3H-phenoxazin-3-one, CAS Number: 5725-91-7, purity >99.9%), maltol (2-methyl-3-hydroxy-1,4-pyrone, CAS Number: 118-71-8, purity >99.9%), protease inhibitor cocktail, and anti-goat IgG peroxidase secondary antibody were purchased from the Sigma–Aldrich Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was purchased from Cambridge Isotope Laboratories (Woburn, MA). CYP1A1 goat polyclonal primary antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Actinomycin D was purchased from Calbiochem (San Diego, CA). Luciferase assay reagents were obtained from Promega Co. (Madison, WI). All other chemicals were purchased from Fisher Scientific Co. (Toronto, Canada).

2.2. Effect of maltol on cell viability

The effect of maltol on cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Gharavi and El-Kadi, 2005).

2.3. RNA extraction and Northern blot analysis

After incubation with the test compounds for the indicated time periods, total RNA was isolated from the cells using TRIzol reagent, according to the manufacturer's instructions (Invitrogen Co., CA). Northern blot analysis was performed as described previously (Elbekai et al., 2004).

2.4. Protein extraction and Western blot analysis

Twenty-four hours after incubation with the test compound, cells were collected in lysis buffer and the total cellular proteins were obtained by incubating the cell lysates on ice for one hour, with intermittent vortex mixing every 10 min, followed by centrifugation at 12,000*g* for 10 min at 4 °C. Western blot analysis was performed using a previously described method (Gharavi and El-Kadi, 2005).

2.5. Determination of Cyp1a1 enzymatic activity

Cyp1a1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity was performed on intact, living cells using 7-ethoxyresorufin as a substrate, as previously described (Elbekai et al., 2004; Korashy and El-Kadi, 2004). Enzymatic activity was normalized for cellular protein content, which was determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993).

2.6. Measurement of luciferase activity

Recombinant mouse hepatoma H1L1.1c2 cells, stably transfected with integrated XRE-driven luciferase reporter gene plasmid, were grown for 48 h onto 6-well cell culture plates in DMEM culture media. Cells were then washed twice with PBS and incubated for 3 h with, 1 nM TCDD, or with 1 mM of maltol. Luciferase assay was performed according to manufacture's instructions (Promega) and as described previously (Jeuken et al., 2003). Briefly, after incubation with test compounds, cells were washed with PBS and a 200 μl of 1× lysis buffer was added into each well with continuous shaking for at least 20 min, then the content of each well was collected separately in 1.5 ml microcentrifuge tubes, followed by sudden freezing under liquid nitrogen and thawing of the tubes to ensure complete cell lysis. The tubes were then centrifuged to precipitate cellular waste, 100 μl cell lysate was then incubated with 100 μl of stabilized luciferase reagent and luciferase activity was quantified using TD-20/20 luminometer (Turner BioSystems, Sunnyvale CA).

2.7. Statistical analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, Inc, CA). A one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess

which treatment groups showed a significant difference from the control group. The differences were considered significant when $p < 0.05$.

3. Results

3.1. Effect of maltol on cell viability

To determine the optimal concentrations to use in our studies, maltol was tested for potential cytotoxicity using Hepa 1c1c7 cells. The MTT assay showed that the concentrations ranging from 50 to 1000 μM did not significantly affect cell viability, on the other hand the concentration of 10 mM decreased cell viability to 75% (Fig. 1). Based on these findings all subsequent studies were conducted at concentrations of maltol ranging from 10 to 5000 μM.

3.2. Induction of Cyp1a1 mRNA in Hepa 1c1c7 cells by maltol

In order to examine the effect of maltol on the Cyp1a1 mRNA, Hepa 1c1c7 cells were incubated for 6 h with increasing concentrations of maltol (10–5000 μM). These concentrations were chosen after determining the ability of a wide range of concentrations to modulate the *Cyp1a1* gene expression without significantly affecting Hepa 1c1c7 cell viability (Fig. 2). In the current study we demonstrated that maltol significantly induced the Cyp1a1 mRNA expression in a concentration-dependent manner (Fig. 2a). Furthermore the maximum induction of Cyp1a1 mRNA by maltol was observed at the concentration of 1 mM; however, there was slight induction at 500 μM concentration due to increased cytotoxicity at that concentration.

3.3. Induction of Cyp1a1 protein and catalytic activity in Hepa 1c1c7 cells by maltol

To further examine whether the induction of Cyp1a1 mRNA in Hepa 1c1c7 cells in response to maltol treatment is translated into functional protein and enzyme catalytic activity, Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of maltol, the same of those used in mRNA experiment, and the Cyp1a1 protein and catalytic activity levels were determined using Western blot analysis and EROD assay, respectively. Fig. 2b and c shows that maltol induces the Cyp1a1 protein and catalytic activity in a concentration-dependent manner with a maximum induction at 1 mM. These results are consistent with the mRNA data, and suggest that the mRNA-induced by maltol is translated into functional protein and enzyme catalytic activity.

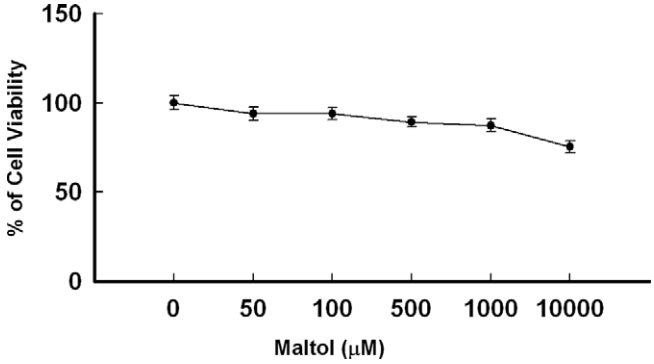

Fig. 1. Effect of maltol on cell viability. The cell viability was tested 24 h after treatment of Hepa 1c1c7 cells with maltol (0, 50, 100, 500, 1000, and 10,000 μM), by measuring the conversion of MTT to formazan crystals. Data are expressed as percentage of untreated control (which is set at 100%) ±SEM ($n = 8$).

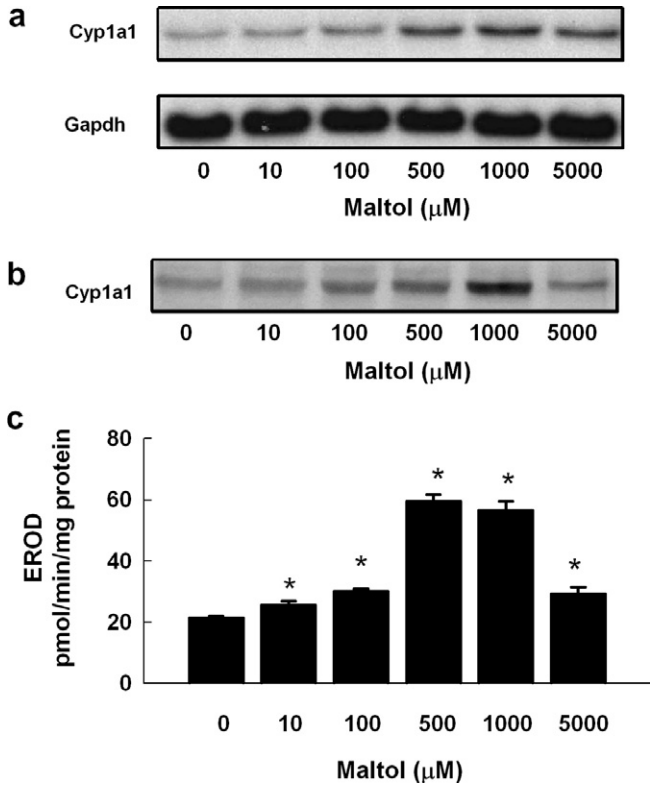

Fig. 2. Concentration-dependent induction of Cyp1a1 mRNA, induction of Cyp1a1 protein and catalytic activity by maltol. *a,* Hepa 1c1c7 cells were treated for 6 h with different concentrations of maltol. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [^{32}P]-labeled cDNA probe specific for mouse Cyp1a1. The blots were subsequently stripped and rehybridized with cDNA probe specific for Gapdh, which was selected as an endogenous RNA control. *b*, Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of maltol, protein (40 μg) was separated on a 10% SDS–PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C and then incubated with a primary Cyp1a1 antibody for 2 h at room temperature, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a1 protein was detected using the enhanced chemiluminescence method. *c*, Cyp1a1 activity was measured in intact living cells using a 96-well cell culture plates and 7-ethoxyresorufin as a substrate. Values are presented as mean ± SEM ($n = 8$). $^{*}p < 0.05$ compared to un-treated cells (concentration = 0).

3.4. Transcriptional induction of Cyp1a1 by maltol

In order to understand the mechanism by which maltol induces the Cyp1a1 mRNA, and to examine whether the

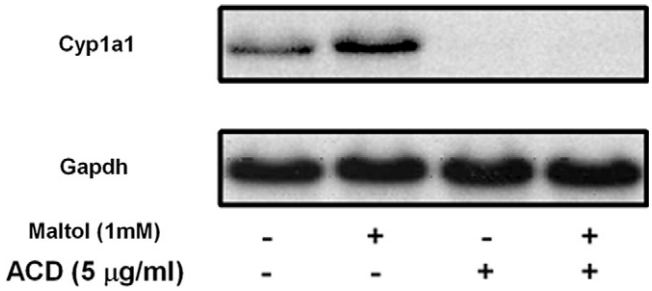

Fig. 3. Effects of RNA synthesis inhibitor on the induction of Cyp1a1 mRNA in response to maltol. Hepa 1c1c7 cells were pretreated with 5 μg/ml ACD, an RNA synthesis inhibitor, 30 min before exposure to 1 mM maltol for 6 h. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [^{32}P]-labeled cDNA probe specific for mouse Cyp1a1. The blots were subsequently stripped and rehybridized with cDNA probe specific for Gapdh, which was selected as an endogenous RNA control.

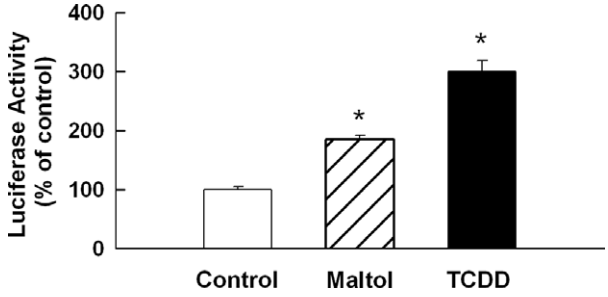

Fig. 4. Effect of maltol on luciferase activity. H1L1.1c2 cells, stably transfected with luciferase reporter gene, were grown into 6-well cell culture plates for 48 h. Thereafter, the cells were incubated with serum free medium, 1 mM maltol, or 2 nM TCDD for 3 h. Cells were lysed and luciferase activity was measured according to the manufacture's instructions. The graph represents the mean ± SEM ($n = 4$) luciferase activity expressed as relative to the activity obtained with control (which is set at 100%). *$p < 0.05$ compared to control.

increase in Cyp1a1 expression was a result of *de novo* RNA synthesis or a post-transcriptional effect, Hepa 1c1c7 cells were pretreated with the transcriptional inhibitor, actinomycin D (ACD) prior to the addition of maltol. Our results demonstrated that pretreatment of the cells with ACD completely abolished the induction of Cyp1a1 mRNA in response to maltol (Fig. 3), implying a requirement of *de novo* RNA synthesis. These results clearly show that maltol induction was through a transcriptional mechanism. To further evaluate the ability of maltol to induce the AhR-dependent gene expression, H1L1.1c2 cells, stably transfected with a luciferase gene whose expression is controlled only by XRE were used. H1L1.1c2 cells were incubated for 3 h with maltol or TCDD as positive control. Our results showed that treatment of the cells with maltol 1 mM significantly induced the reporter gene expression (Fig. 4).

4. Discussion

The present work provides the first demonstration that the food flavoring agent, maltol modulate *Cyp1a1* gene expression at the transcriptional level through an AhR-dependent mechanism(s).

Numerous chemicals have been identified as AhR ligands (Denison et al., 2002). Most of them, the "classical" ligands, including polycyclic aromatic hydrocarbons and halogenated aryl hydrocarbons, share the structural features of being planar, aromatic, and hydrophobic (Denison and Nagy, 2003). Recently, a relatively large number of AhR ligands whose structures and physiochemical characteristics differ from classical ligands have been identified (Denison and Nagy, 2003). The majority of these "nonclassical" AhR ligands has a low affinity to the AhR and is relatively weak inducers of Cyp1a1, compared with TCDD. A wide range of structural diversity in AhR ligands indicates that a greater spectrum of chemicals can interact with and activate this receptor than previously thought (Denison and Nagy, 2003).

CYP1A1 mRNA and protein are virtually undetectable in untreated animals or cells (Nebert, 1989). High levels of CYP1A1 mRNA, protein, and enzyme activity are detectable following induction by AhR ligands; in fact, many of the inducers are in turn metabolized by CYP1A1 (Nebert, 1989). Inducible CYP1A1 activity is ubiquitous, located in virtually every tissue of the body (Nebert et al., 2000). A direct role for CYP1A1 in toxicity has been well documented *in vitro* and *in vivo* (Nebert et al., 2000, 2004). It has been reported that CYP1A1 induction may lead to oxidative stress as a result of excessive generation of reactive oxygen species which can result in an imbalance in the cellular oxidative stress/antioxidant status and thus cause cell toxicity (Elbekai et al., 2004). In this context it has been reported that the cytotoxic effect of maltol maltol/transition metal complex is mediated by excessive generation of reactive oxygen species (Murakami et al., 2006).

Maltol is a white crystalline powder that has caramel smell, and is a γ-pyrone derivative that possesses metal chelating properties due to the presence of an α,β-unsaturated, keto–enol group (Kahn and Ben-Shalom, 1998). The WHO acceptable daily intake (ADI) of maltol is <1 mg/kg/day (WHO, 2006). Maltol is not only available in food like bread, milk, butter, uncured pork, beer, cocoa, coffee, and beans but also may be formed under baking and roasting conditions from simple sugars (WHO, 2006). It is not only used as a flavoring agent, but also may be used in medicine for example vanadyl maltolate for the treatment of diabetes, and ferric trimaltol for the treatment of iron deficiency anemia (Harvey et al., 1998; Thompson et al., 2003). It has been shown that maltol is absorbed rapidly through the gastro-intestinal tract and extensively metabolized and excreted as conjugates (Rennhard, 1971). However, several studies demonstrated its toxic effects on kidney and liver in rats and dogs specially after acute administration (Gralla et al., 1969) but the mechanisms are still a matter of debate.

To understand the mechanism by which maltol causes toxicity, we hypothesized that maltol acts as an AhR ligand and induces *Cyp1a1* gene expression. The reason is that the conversion of AhR ligands into electrophilic compounds by Cyp1a1 results in the formation of covalent adducts, which can react directly with intracellular nucleophiles, including DNA, and initiate the cancer process (Spink et al., 2002).

To test this hypothesis we examined the effect of maltol on Cyp1a1 mRNA, protein, and activity levels in Hepa 1c1c7 cells. Our data clearly show that treatment of Hepa 1c1c7 cells with maltol causes a concentration-dependent induction in Cyp1a1 mRNA, and activity levels. However, at the protein level, only the sub-maximal concentration of maltol used (1 mM) caused Cyp1a1 protein induction.

The regulation of *CYP1A1* gene expression involves activation of a cytosolic transcriptional factor, AhR, as the first step in a series of molecular events promoting *CYP1A1* transcription and translation processes (Denison et al., 1989; Korashy and El-Kadi, 2006). In the current study, the transcriptional regulation of *Cyp1a1* gene expression by maltol was demonstrated through different approaches. First, the inhibition of the RNA transcription, using ACD, completely abolished the induction of Cyp1a1 mRNA in response to maltol, implying that maltol increases the *de novo* Cyp1a1 RNA synthesis, in a manner similar to that obtained with TCDD. The second evidence for the transcriptional induction of *Cyp1a1* gene was demonstrated by the ability of maltol to increase the luciferase reporter gene that occurs only through the AhR activation, and that excludes the possibility of other mechanisms, such as mRNA stability (Sinal and Bend, 1997).

Taken all together, the results provided here present us with the first evidence that maltol can directly modulate the expression of Cyp1a1 through an AhR-dependent pathway by acting as an AhR ligand. This study provides a novel mechanism to explain the carcinogenicity and toxicity of maltol.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Council of Canada (NSERC) Grant RGPIN 250139 to A.O.S.E. A.A-M. is the recipient of Mike Wolowyk Graduate Scholarship award.

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