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Biochemical Pharmacology 63 (2002) 745–749

Biochemical Pharmacology

Investigations on the effects of oltipraz on the nucleotide excision repair in the liver

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Received 31 May 2001; accepted 6 November 2001

Abstract

Recent studies support the view that in addition to its effect on both phase I and phase II xenobiotic metabolizing enzymes, the synthetic chemopreventive agent oltipraz also increases the nucleotide excision repair (NER) which represents the major pathway of elimination of chemical carcinogen DNA adducts. Since most carcinogens are activated in the liver, we investigated the influence of oltipraz on NER activity of this target tissue by using two different approaches. First, we employed an assay based on the measurement of DNA repair in cisplatin-damaged plasmid DNA incubated in the presence of cell-free extracts prepared from either rat liver or human hepatoma HepG2 cells treated by oltipraz. Secondly, we analyzed the removal of aflatoxin B_1-derived DNA adducts formed in primary human hepatocytes exposed to oltipraz after treatment with this mycotoxin. Whatever the strategy used, NER activity was not altered in liver cells. These data demonstrated that liver cells actively repair bulky DNA adducts by NER and that oltipraz does not influence their NER activity neither *in vivo* nor *in vitro*, consequently strongly suggesting that the chemopreventive agent oltipraz is acting before the initiation step of cancer development.

Keywords: Nucleotide excision repair; Chemoprevention; Liver; Oltipraz; Cisplatin; Aflatoxin B_1

1. Introduction

Among the chemicals isolated from vegetables or their derivatives recognized to prevent cancer, oltipraz, a synthetic derivative of 1, 2-dithiole-3-thione, is receiving a growing interest. Its chemopreventive effects in animals have been well demonstrated [1], and the first clinical trials in humans are encouraging [2]. Oltipraz has been shown to be a potent inducer of detoxifying enzymes such as glutathione transferases, NADPH: quinone reductase, epoxide hydrolase and UDP-glucuronosyl transferases [3] and moreover, to be a powerful inhibitor of cytochromes P450 (CYP), especially, of those involved in the activation of major carcinogens such as aflatoxin B_1 (AFB_1) [4–6], indicating that it protects from the induction of DNA damage. In addition, this compound has been reported to protect against colon carcinogenesis when administered following azoxymethane, supporting the view that it is also effective after DNA damage induced by this carcinogen [7]. Moreover, an increased removal of platinum-derived DNA adducts has been observed in oltipraz-treated colon carcinoma cells [8]. These chemopreventive effects of oltipraz could be interpreted as resulting, at least partly, from an increase in DNA repair activity [7,8].

Among the different repair processes, NER represents the main pathway by which mammalian cells remove helix distorting lesions, bulky DNA damages [9]. To evaluate the direct effects of oltipraz on NER activity, we adapted to hepatocytes, the major cell type involved in the metabolism of chemicals, an assay initially developed by Wood *et al.* on Hela cells [10] and modified by Coudore *et al.* for tissue extracts [11]. It consists in the direct measurement of NER activity of damaged plasmid DNA incubated in the presence of protein extracts from cells or tissues. Our results show that oltipraz does not increase NER activity of cisplatin-modified plasmid DNA. It is well known that these cisplatin-derived DNA adducts are mainly removed

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Abbreviations: NER, nucleotide excision repair; AFB_1, aflatoxin B_1

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00906-6

by NER [9]. In addition, we also investigated the effect of oltipraz on the rate of AFB_1-derived DNA adducts in primary human hepatocyte cultures. Formation of AFB_1-derived DNA adducts was reduced by oltipraz, only when administered prior to or concomitantly with but not following carcinogen exposure, providing further evidence that this chemopreventive agent does not affect NER capacity.

2. Materials and methods

2.1. Chemicals

Williams'E medium was from Eurobio (Les Ulis, France) and fetal calf serum from Dominique Dutscher SA (Brumath, France). Oltipraz was kindly supplied by Rhone–Poulenc–Rorer (Alfortville, France). Cisplatin was purchased from Sigma and [^{3}H]AFB_1 from Moravek Biochemicals. All other compounds were readily available commercial products of the highest purity available.

2.2. Animal experiments

Male Wistar rats (weighing 180–200 g) were fed a diet containing oltipraz at a final concentration of 0.075% (w/w). All procedures were in accordance with the regulations laid down by the French Ministry of Agriculture and Forest, for the care and use of laboratory animals. At the end of the treatment, rats were killed and livers were immediately removed and kept at -80° until use.

2.3. Cell isolation and culture

Human hepatocytes were isolated by a two-step collagenase perfusion procedure [12]. All these experimental procedures complied with French laws and regulations, and were approved by the National Ethics Committee. Cell viability was 70–85%, as estimated by the trypan blue exclusion test. Hepatocytes were seeded at a density of 10^6 cells/35 cm^2 dish in 2 mL of a standard Williams'E medium containing 0.2% bovine insulin, 0.32% bovine serum albumin, 1% glutamine, 0.1% penicillin-streptomycin, 0.05% gentamycin and 10% fetal calf serum (all v/v). This medium, supplemented with 7×10^{-5} M hydrocortisone hemisuccinate but lacking fetal calf serum, was renewed after 18–24 hr and daily thereafter.

HepG2 cells, derived from a human hepatoblastoma [13], were cultured in the same standard Williams'E medium as before but supplemented with 7×10^{-7} M hydrocortisone hemisuccinate and without gentamycin.

Forty eight hours after hepatocyte seeding, 10 nM [^{3}H]AFB_1 (1.5 Ci/mmol) was added for 4 hr. Hepatocytes were treated with 50 μM oltipraz (dissolved in dimethylsulfoxide) 24 hr before, during, and 4, 24, 48 and 72 hr after [^{3}H]AFB_1 exposure. HepG2 cells were exposed to 50 μM starting 4 days after seeding for 48 or 96 hr. Control cultures received the vehicle alone at a concentration of 0.2% (v/v).

2.4. AFB_1-derived DNA adducts measurement

After three phosphate buffer saline washes, DNA was isolated as reported [14], ethanol precipitated, washed in 70% ethanol and dried. Amounts of [^{3}H]AFB_1 covalently bound to DNA were then determined by scintillation counting and normalized to amounts of DNA quantified by its absorbance at 260 nm.

2.5. Cell-free extracts

Frozen tissue specimens and HepG2 cell pellets (approximately 10^8 cells) were placed in a hypotonic lysis buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 5 mM dithiothreitol and subsequently extracted according to Manley *et al*. [15] with minor modifications. After elimination of DNA by a first precipitation with 10% ammonium sulphate, nuclear proteins were precipitated by addition of 0.45 g/mL ammonium sulphate and collected by centrifugation. The resulting pellet was then dialyzed for 12 hr in the cold against extract buffer containing 25 mM Hepes-KOH (pH 7.8), 0.1 M potassium glutamate, 2 mM EDTA, 2 mM dithiothreitol and 17% glycerol. The resulting extracts were immediately frozen and stored at -80°. Protein concentrations were determined using the Bradford's procedure [16].

2.6. In vitro repair synthesis assay

The assay was performed in a standard 50 μL reaction mixture with 200 μg protein extracts in the presence of the pBluescript KS$^+$ (pKS$^+$) from Stratagene (La Jolla, CA) containing platinum DNA adducts after reaction *in vitro* with cis-diamminedichloroplatinum (II) [17] confirmed by flameless atomic absorption spectrophotometry and pHM, a 3740 bp derivative plasmid, according to Wood *et al*. [10]. Optimal reaction conditions were obtained by addition of 150 mM and 100 mM glutamate potassium for tissue and cell extracts, respectively. After 3 hr at 30° with 2 μCi of [α-^{32}P]dCTP (Amersham, Buck, UK), the reaction was stopped. After extraction, the plasmids were linearized with 5 U EcoRV (Promega, Madison, WI) and electrophoresed overnight in a 1% agarose gel containing 0.03% ethidium bromide. The extents of incorporation of radiolabeled deoxynucleotides quantified by densitometry of the autoradiography were normalized to the amounts of recovered DNA.

2.7. Statistical analysis

Numerical values are expressed as the mean of arbitrary units ± SD, and significance, using the Student's *t* test, was set at a limit of $\leq$5%.

3. Results and discussion

The NER pathway represents the main mechanism for elimination of chemical carcinogen DNA adducts. Using an *in vitro* cell-free assay, we found a constantly detectable activity in normal liver tissue as well as in tumoral Hep G2 cells and both intra and inter-assay reproducible values were obtained.

The effects of oltipraz were first estimated on NER activity towards platinum DNA adducts following two different experimental approaches: whole extracts were prepared either from rat livers previously exposed to the chemopreventive agent oltipraz for various periods of time ranging between 24 and 120 hr, or from HepG2 cells treated by oltipraz at a concentration of 50 μM for 48 or 96 hr. As shown in Figs. 1 and 2, whatever the time of oltipraz treatment, NER activity was not increased significantly in either tissue or cell extracts. These results appear to contradict the conclusions of O'Dwyer *et al*. [8], which have suggested that oltipraz was capable of stimulating repair of damaged DNA. In fact, these authors showed that pre-treatment with oltipraz resulted in either decreased accumulation or enhanced removal of platinum DNA adducts in colon carcinoma HT29 cells. Using another colon carcinoma cell line, Caco-2 cells, we again did not observe any effect of oltipraz on NER (data not shown). Rao *et al*. [7] have also reported a protective effect of oltipraz in rat colon. However, the inhibition of colon tumor incidence, multiplicity and size by oltipraz administered following azoxymethane exposure could be related to the nature of DNA adducts formed. Indeed, azoxymethane metabolism results in the formation of alkylating species which are mainly repaired by the base excision repair or a reversion mechanism that involved the O^6-methylguanine methyl-transferase. An induction of alkyltransferases or glycosylases by oltipraz cannot be excluded. However, by measuring repair of methylene blue-damaged plasmid DNA, we came to the conclusion that oltipraz also did not affect DNA base excision repair of such an oxidative damage (data not shown).

To confirm the lack of NER modulation by oltipraz, we further investigated the influence of this chemopreventive agent on the removal of AFB_1-derived DNA adducts in primary human hepatocytes; this *in vitro* model has been shown to form the genotoxic AFB_1 *exo*-8,9-epoxide metabolite [4]. Human hepatocytes were treated with 50 μM oltipraz, a concentration known to strongly inhibit oxidative AFB_1 metabolism [4], either before, during or following a 4 hr exposure to the mycotoxin. As expected, a pre-treatment (Fig. 3) or a concomitant treatment (data not

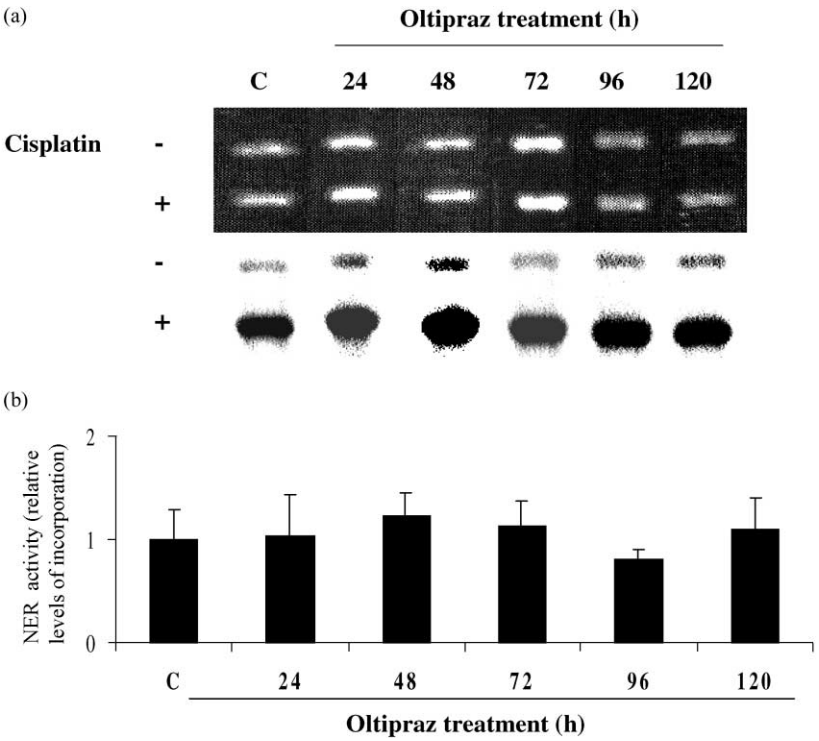

Fig. 1. Effect of oltipraz on the NER activity of protein extracts from rat livers. Cisplatin-damaged and undamaged plasmid DNAs were incubated for 3 hr at 30° in the presence of 150 mM potassium glutamate with 200 μg hepatic protein extracts from rats either untreated (C) or treated by oltipraz for 24, 48, 72, 96 and 120 hr. (a) Photograph of the ethidium bromide stained agarose gel and autoradiogram of the dried gel. (b) Quantification of NER activity into cisplatin-damaged and undamaged plasmid DNA. The results are the means of relative levels of [α-^{32}P]dCTP incorporation determined by densitometry of the autoradiograms normalized to the amounts of recovered DNA ± SD of rat liver extract preparations ($n = 4$). The ratios superior to 1.5 were considered as representative of an efficient NER activity. A statistical analysis (Student's t test) was performed by comparison of oltipraz-treated and control rats ($P < 0.05$).

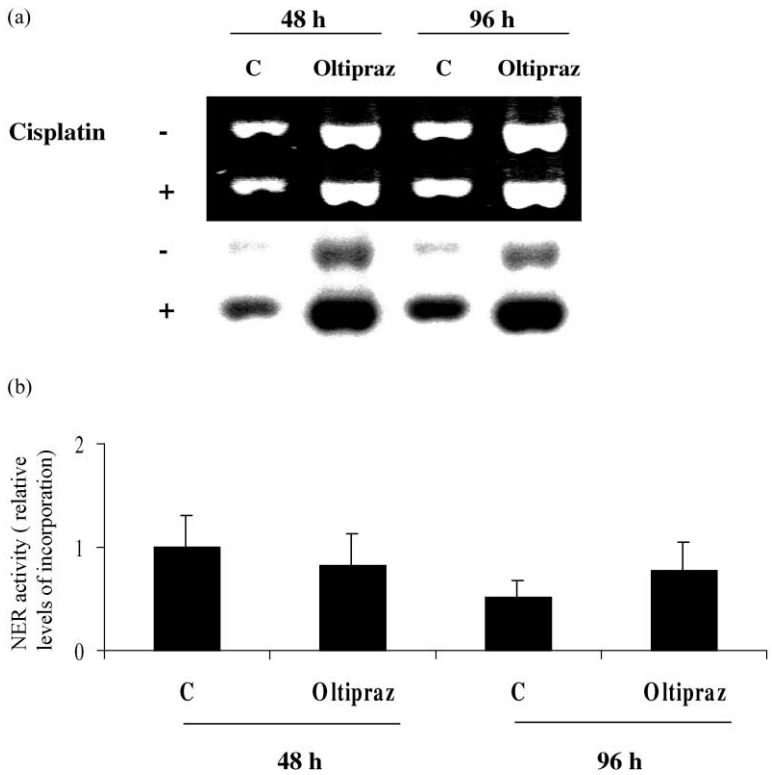

Fig. 2. NER activity of protein extracts from HepG2 cells treated or not with 50 μM oltipraz for 48 and 96 hr. Cisplatin-damaged and undamaged plasmid DNAs were incubated 3 hr at 30° in the presence of 100 mM potassium glutamate with 200 μg of protein extracts from HepG2 cells either untreated (C) or treated by 50 μM oltipraz for 48 or 96 hr. (a) Photograph of the ethidium bromide stained agarose gel and autoradiogram of the dried gel. (b) Quantification of NER activity into cisplatin-damaged and undamaged plasmid DNA. The results are the means of relative levels of [α-^{32}P]dCTP incorporation determined by densitometry of the autoradiogram normalized to the amounts of recovered DNA ± SD of 3 experiments for HepG2 cells. The ratios superior to 1.5 were considered as representative of an efficient NER activity.

shown) with oltipraz resulted in the formation of lower amounts of AFB_1-derived DNA adducts. These findings support the view that oltipraz is effective by protecting from hepatic AFB_1-derived DNA adducts formation, mainly through an inhibition of CYPs and an induction of glutathione transferases. By contrast, while the rate of removal of AFB_1-derived DNA adducts decreased to about 40% 72 hr after AFB_1 exposure, an oltipraz treatment

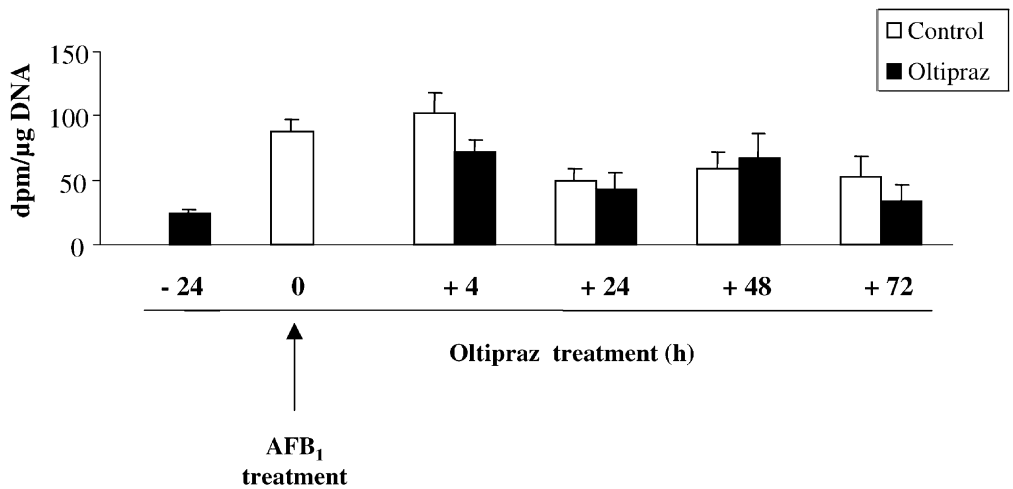

Fig. 3. Effect of oltipraz on the removal of AFB_1-derived DNA adducts in primary human hepatocytes. Primary human hepatocytes were treated in a serum-free medium with oltipraz at a concentration of 50 μM 24 hr before and 4, 24, 48 and 72 hr after a 4 hr exposure to 10 nM [^{3}H]AFB_1. Control cultures received the vehicle alone at a concentration of 0.2% (v/v). DNA was purified after different times of AFB_1 exposure and AFB_1-derived DNA adducts were measured by scintillation counting and normalized to amounts of recovered DNA. The results are expressed as dpm/μg DNA and are the means ± SD of a representative experiment performed in triplicate.

following AFB_1 exposure did not enhance significantly this decrease whatever the duration of treatment from 4 to 72 hr (Fig. 3). AFB_1-derived DNA adducts are known to be unstable and to lead to apurinic sites. However, since we measured radiolabeled AFB_1 bound to DNA at different time points and found no difference between control and oltipraz-treated cells, it may be concluded that if depurination occurred, it was similar in both conditions. In agreement with our results, Maxuitenko *et al*. [18] have also reported a lack of protective effect of oltipraz in the liver when administered to rats following exposure to this carcinogenic mycotoxin. Moreover, using similar experimental conditions, we also found that oltipraz did not stimulate NER activity of AFB_1-modified plasmid DNA in rat livers (data not shown). However, DNA repair processes are particularly complex. In the case of NER, more than 40 proteins are involved from the recognition, incision, excision up to the repair replication and ligation steps [19]. Therefore, it is not excluded that even if genes encoding some of them are sensitive to chemopreventive agents, this does not affect the overall repair activity in certain tissues. However, preliminary experiments in our laboratory using macroarrays (Clontech, Palo Alto, CA) did not show any change in several DNA repair proteins in primary human hepatocytes following exposure to oltipraz, including the endonuclease III homolog 1, a key enzyme in both NER and base excision repair (F. Morel, unpublished data).

Acknowledgments

This work was supported by the Institut National de la Santé et de la Recherche Médicale and the Association pour la Recherche sur le Cancer (to S. Langouët). L. Sparfel is a recipient of a fellowship from the Ministère de la Recherche et de l'Enseignement Supérieur.

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