ORIGINAL ARTICLE

Evaluation of genotoxic and cytotoxic effects of H_2O_2 and DMNQ on freshly isolated rat hepatocytes; protective effects of carboxymethyl chitin-glucan

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Abstract

OBJECTIVES: Utilizing primary rat hepatocytes we investigated the potential anti-mutagenic and anti-cytotoxic effects of carboxymethyl chitin-glucan (CM-CG) with respect to oxidative stress induced by the model free-radical-generating compounds hydrogen peroxide (H_2O_2) or 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). Different kinds of CM-CG action were studied by two different treatment protocols: a. pre-incubation of freshly isolated hepatocytes with the potential anti-mutagen followed by exposure to the oxidant or b. simultaneous treatment of hepatocytes with the potential anti-mutagen and the oxidant.

METHODS: As a measure of genotoxicity, the percentages of DNA in tails of comets by single cell gel electrophoresis were evaluated. The cytotoxicological endpoints analysed were the cell density (number of cells/cm^2), and the percentages of apoptotic and necrotic cells.

RESULTS: H_2O_2 and DMNQ, causing DNA single-strand breaks via the formation of •OH radicals, have been demostrated to induce both genotoxic and cytotoxic effects in primary rat hepatocytes resulting in increased percentages of DNA in tails of comets, and increased frequencies of apoptotic and necrotic cells accompanied by a decreased cell density. Further investigations were therefore focussed on possible modifications of these parameters by CM-CG. The results obtained clearly demonstrate that CM-CG (applied before and during treatment) protects primary rat hepatocytes against the genotoxic and cytotoxic effects of oxidative stress (H_2O_2 or DMNQ), whereas CM-CG itself has no effect on the endpoints of genotoxicity and cytotoxicity studied.

CONCLUSION: Our results indicate that carboxymethyl chitin-glucan represents a natural fungal polysaccharide that can inhibit the genotoxicity and cytotoxicity of experimentally induced oxidative stress in primary rat hepatocytes.

INTRODUCTION

The liver is the main organ for the metabolism of foreign compounds. Freshly isolated primary rat hepatocytes maintain xenobiotic metabolizing enzyme activities and represent the most reliable cell type not only for genotoxicity testing but also for the evaluation of different influences (detoxifying, anti-mutagenic, anti-oxidant) which potentially alter the response to mutagenic/pro-mutagenic compounds. Utilizing primary hepatocyte cultures we therefore assessed the potential anti-mutagenic and detoxifying effects of carboxymethyl chitin-glucan (CM-CG) with respect to oxidative stress induced by the model free-radical-generating compounds hydrogen peroxide (H_2O_2) or 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). Effects of H_2O_2 are mediated mainly by the highly reactive •OH radicals, generated by the reaction of reduced transition metals (e.g. Fe^{2+}) with H_2O_2 via the Fenton reaction (Halliwell & Auroma, 1991), resulting in a DNA damage profile consisting of approximately equal levels of oxidized DNA bases, abasic sites and strand breaks (Epe *et al*. 1993). DMNQ as a source of free radicals and capable of redox cycling (Bresgen *et al*. 2003; Kappus & Sies, 1981) has been demonstrated to induce genotoxic effects in primary hepatocytes (Bresgen *et al*. 2003). Our investigation therefore focused on possible modifications of the damaging effects of H_2O_2 and DMNQ by the polysaccharide carboxymethyl chitin-glucan (CM-CG) isolated from the waste material left upon the commercial production of citric acid by *Aspergillus niger*, an organism which represents a natural and inexpensive source of a natural non-toxic bioprotective and immunomodulatory drug (Kogan *et al*. 2003). The CM-CG used in this study represents a water-soluble derivative exerting DNA-protective effects *in vitro* (Križková *et al*. 2003; Slameňová *et al*. 2003), although the precise mechanism of action still remains unclear.

As a measure of genotoxicity the percentages of DNA in tails of comets by single cell gel electrophoresis were evaluated. Since the treatment regimens might also result in cytotoxicity, potential changes of the cell density (number of cells/cm^2), and the induction of apoptotic and necrotic cell death were analysed as well.

MATERIAL AND METHODS

Material

Water-soluble carboxymethyl chitin-glucan (CM-CG) was prepared according to the procedure described by Machová *et al*. (1999). Minimum essential medium (MEM) with Earle's salts and non-essential amino acids and antibiotics were obtained from Life Technologies, Vienna, Austria. Plastic culture dishes were from Sarstedt, Austria. Collagenase and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Company via Biotrade, Vienna; 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) from Eubio, Vienna.

Animals

Adult female Fisher 344 rats (8–14 weeks old) weighing ~100 g were obtained from Harlan-Winkelman, Germany. The animals were kept in a temperature- and humidity-controlled room with a 12 h light-dark cycle. Food and water was provided *ad libitum*.

Hepatocyte isolation and culture

Hepatocytes were isolated by the two-step *in situ* collagenase liver perfusion technique as described by Michalopoulos *et al*. (1982). According to Eckl *et al*. (1987) the isolated hepatocytes were plated at a density of 5×10^5 cells/dish on collagen-coated 60 mm diameter plastic culture dishes in 5 ml of MEM containing 1.8 mM Ca^{2+} supplemented with non-essential amino acids, pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM) and penicillin (100 U)/streptomycin (100 µg/ml). The cultures were incubated at standard culture conditions (37 °C, 5% CO_2 and 95% relative humidity). After an initial incubation period of 2 h, the medium was exchanged for fresh MEM and the cultures were returned to the incubator.

Treatment schedule

Approximately 18 h after the first exchange of medium hepatocyte cultures were treated: **1.** control (fresh medium for 6 h), **2.** CM-CG (50 µg/ml for 3 h), **3.** H_2O_2 (1 or 10 µM for 3 h), **4.** CM-CG (50 µg/ml for 3 h) as a pre-incubation before treatment with H_2O_2 (1 or 10 µM for 3 h), **5.** simultaneous treatment of CM-CG (50 µg/ml) + H_2O_2 (1 or 10 µM for 3 h), **6.** DMNQ (10 or 50 µM for 3 h), **7.** CM-CG (50 µg/ml for 3 h) as a pre-incubation before treatment with DMNQ (10 or 50 µM for 3 h), **8.** simultaneous treatment of CM-CG (50 µg/ml) + DMNQ (10 or 50 µM for 3 h). Thereafter, the plates were washed twice with fresh medium to completely remove the applied substances, and either DNA-damage analysis or cytotoxicity testing were carried out.

DNA-damage testing

The method of single cell gel electrophoresis (comet assay) of Singh *et al*. (1988) was followed with minor modifications made by Slameňová *et al*. (1997). We described the newest modification of the comet assay, used in this paper, recently (Horváthová *et al*. 2006).

Cytotoxicity assay

After removing of the applied substances, samples were either further cultivated in fresh MEM medium containing 1.8 mM Ca^{2+} for 24 or 48 h or just after the treatment (0 h) processed for cytotoxicity testing, i.e. cells were fixed in the dishes with methanol:glacial acetic acid (3:1) for 15 min, rinsed with distilled water and air dried. Thereafter, the fixed cells were stained with DAPI (4',6-diamidino-2-phenylindol) in McIlvaine buffer (0.2 M Na_2HPO_4 buffer adjusted to pH=7.0 with 0.1 M citric acid) for 30 min in the dark at room temperature. After washing with McIlvaine buffer, the dishes were rinsed with distilled water, air dried and mounted in

glycerol. Thousand cells per dish were analysed under the fluorescent microscope (Leitz Aristoplan) to determine the cell densities and the frequencies of apoptotic and necrotic cells. The morphology of DAPI-stained nuclei was used to discriminate between apoptotic and necrotic cells as described by Oberhammer *et al*. (1992).

Statistical analysis

Student's double-sided *t*-test for independent samples in both assays was used to calculate the levels of significance.

RESULTS

The most important results of this study are presented in Table 1 and Figure 1. Table 1 presents: 1. density of hepatocytes, 2. percentage of apoptotic cells and 3. percentage of necrotic cells during 48 h after treatment. As evident from this table, cytotoxic effects of H_2O_2 (1; 10 μM) and DMNQ (10; 50 μM) resulted in an increased percentages of apoptotic and necrotic cells accompanied by a decreased cell density of hepatocytes (Table 1), however, the cytotoxic effects of H_2O_2 and DMNQ were

Table 1. Cytotoxic effects of oxidative stress induced by H_2O_2 (1; 10 μM) or DMNQ (10; 50 μM) in primary rat hepatocyte cultures; protective properties of CM-CG

Samples	h	Density ×10³cells/cm²	Apoptotic cells (%)	Necrotic cells (%)
control	0	9.50 ± 1.41	0.15 ± 0.11	1.28 ± 0.11
	24	9.15 ± 1.35	0.48 ± 0.08	2.40 ± 0.12
	48	7.83 ± 0.86	0.40 ± 0.19	1.45 ± 0.34
CM-CG (50 μg/ml)	0	8.23 ± 0.48	0.28 ± 0.13	1.53 ± 0.31
	24	7.95 ± 0.36	0.38 ± 0.15	2.15 ± 0.26
	48	7.95 ± 0.72	0.43 ± 0.18	1.70 ± 0.22
H_2O_2 (1 μM)	0	4.95 ± 0.15$^{\#\#\#}$	0.40 ± 0.12$^{\#}$	4.38 ± 0.19$^{\#\#\#}$
	24	4.85 ± 0.18$^{\#\#\#}$	0.68 ± 0.08$^{\#}$	3.13 ± 0.47
	48	4.83 ± 0.15$^{\#\#\#}$	1.40 ± 0.07$^{\#\#}$	2.65 ± 1.18$^{\#\#}$
CM-CG → H_2O_2 (1 μM)	0	5.75 ± 0.36*	0.50 ± 0.14	3.30 ± 0.41*
	24	6.23 ± 0.26***	0.63 ± 0.31	2.48 ± 0.29
	48	5.43 ± 0.37*	0.68 ± 0.11***	1.40 ± 0.59*
CM-CG + H_2O_2 (1 μM)	0	6.70 ± 0.70**	0.33 ± 0.15	4.98 ± 0.53
	24	6.15 ± 0.43**	0.25 ± 0.11**	3.18 ± 0.51
	48	6.30 ± 0.58**	0.45 ± 0.11***	2.58 ± 0.83
H_2O_2 (10 μM)	0	4.63 ± 0.08$^{\#\#\#}$	0.30 ± 0.07	4.50 ± 0.48$^{\#\#\#}$
	24	4.65 ± 0.33$^{\#\#\#}$	0.93 ± 0.19$^{\#}$	3.05 ± 0.29$^{\#}$
	48	5.03 ± 0.46$^{\#\#}$	1.50 ± 0.49$^{\#}$	1.48 ± 0.24
CM-CG → H_2O_2 (10 μM)	0	5.40 ± 0.16***	0.43 ± 0.19	4.95 ± 0.32
	24	5.25 ± 0.29	0.50 ± 0.23*	3.90 ± 0.27*
	48	5.95 ± 0.40*	0.55 ± 0.22*	2.85 ± 0.48**
CM-CG + H_2O_2 (10 μM)	0	6.30 ± 0.67**	0.33 ± 0.22	4.78 ± 0.80
	24	5.10 ± 0.19	0.38 ± 0.15**	3.53 ± 0.58
	48	4.57 ± 0.21	0.53 ± 0.26*	2.53 ± 0.61
DMNQ (10 μM)	0	4.90 ± 0.75$^{\#\#}$	0.33 ± 0.18	4.93 ± 0.92$^{\#\#}$
	24	5.05 ± 0.18$^{\#\#\#}$	1.45 ± 0.18$^{\#\#\#}$	2.93 ± 0.28$^{\#}$
	48	5.00 ± 0.27$^{\#\#\#}$	1.65 ± 0.15$^{\#\#\#}$	2.03 ± 0.33
CM-CG → DMNQ (10 μM)	0	6.95 ± 0.47*	0.28 ± 0.04	2.50 ± 0.34*
	24	6.65 ± 0.26***	0.48 ± 0.29**	3.13 ± 0.16
	48	5.83 ± 0.23*	0.53 ± 0.23***	2.20 ± 0.68
CM-CG + DMNQ (10 μM)	0	7.25 ± 0.85*	0.18 ± 0.04	5.65 ± 0.47
	24	5.90 ± 0.24**	0.65 ± 0.15**	3.68 ± 0.54
	48	6.13 ± 0.11**	0.40 ± 0.22***	2.33 ± 0.65
DMNQ (50 μM)	0	4.50 ± 0.10$^{\#\#\#}$	0.55 ± 0.05$^{\#\#}$	6.50 ± 0.30$^{\#}$
	24	4.65 ± 0.05$^{\#\#\#}$	0.90 ± 0.10	2.30 ± 0.10
	48	4.10 ± 0.00$^{\#\#\#}$	1.30 ± 0.10$^{\#\#}$	2.35 ± 0.05$^{\#}$
CM-CG → DMNQ (50 μM)	0	7.10 ± 0.50*	0.50 ± 0.10	2.55 ± 0.05*
	24	6.75 ± 0.65	0.85 ± 0.45	1.65 ± 0.85
	48	6.30 ± 0.30*	0.75 ± 0.15	1.05 ± 0.05**
CM-CG + DMNQ (50 μM)	0	6.55 ± 0.35*	0.35 ± 0.15	4.50 ± 1.10
	24	5.95 ± 0.15*	0.80 ± 0.30	2.00 ± 0.40
	48	5.75 ± 0.45	0.55 ± 0.05*	0.80 ± 0.10*

Data represent the means ± SD of two independent experiments.

$^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared with the untreated control; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared with the values for H_2O_2 or DMNQ.

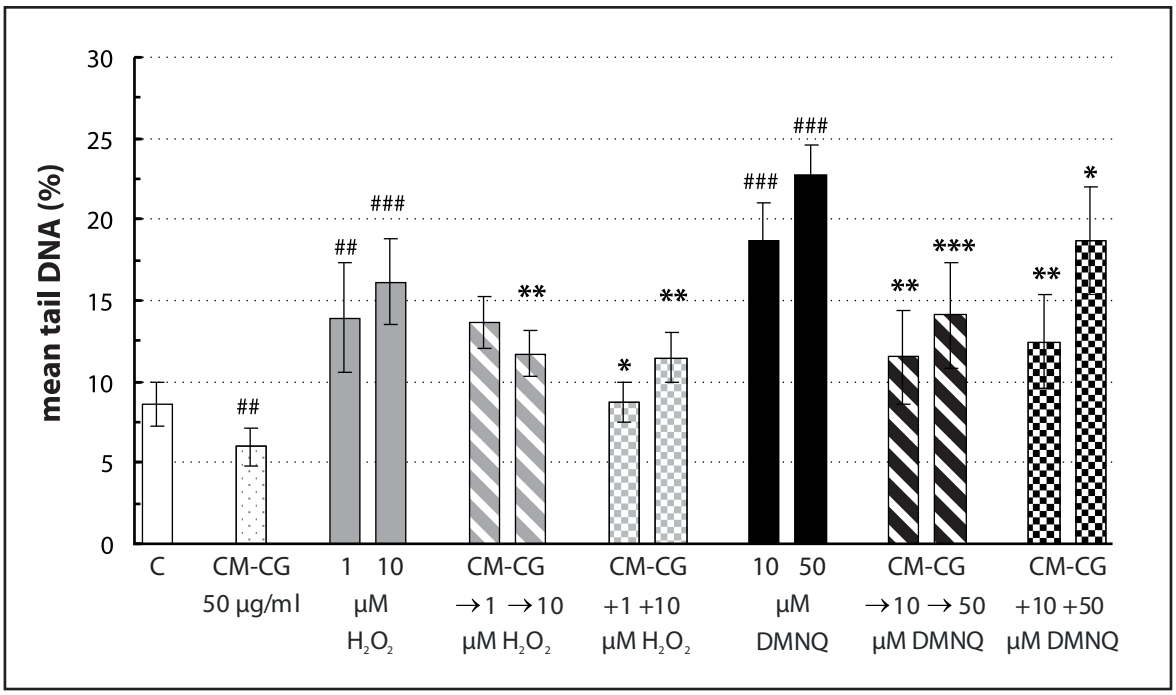

Figure 1. DNA-damaging effects of oxidative stress induced by H_2O_2 or DMNQ in primary rat hepatocyte cultures; influence of CM-CG. Levels of DNA damage were assessed in samples: control (open bar); CM-CG (50 µg/ml, dotted bar); H_2O_2 (1 or 10 µM, grey bars); CM-CG (50 µg/ml) as a pre-incubation → before treatment with H_2O_2 (1 or 10 µM, grey striped bars); simultaneous treatment of CM-CG (50 µg/ml) + H_2O_2 (1 or 10 µM, grey checked bars); DMNQ (10 or 50 µM, black bars); CM-CG (50 µg/ml) as a pre-incubation → before treatment with DMNQ (10 or 50 µM, black striped bars); simultaneous treatment of CM-CG (50 µg/ml) + DMNQ (10 or 50 µM, black checked bars). Data represent the means ± SD of two independent experiments.
##p<0.01 and ###p<0.001 compared with the untreated control; *p<0.05, **p<0.01 and ***p<0.001 compared with the values for H_2O_2 or DMNQ.

significantly reduced either by the pre-incubation or combined treatment with CM-CG. CM-CG alone has no effect on the cytotoxicity criteria studied.

Figure 1 shows genotoxic effects of H_2O_2, DMNQ and combinations of H_2O_2 or DMNQ with CM-CG applied either before or during the treatment with both oxidants. Genotoxicity was evaluated on the basis of DNA-damaging activity measured by the comet assay. Both tested oxidants manifested genotoxic effect, while CM-CG did not manifest any genotoxicity. CM-CG applied at non-toxic concentrations before or during treatment with H_2O_2 or DMNQ reduced genotoxicity induced by H_2O_2 or DMNQ as evidenced by decreased levels of DNA damage.

DISCUSSION

It has been demonstrated that carboxymethyl chitin-glucan, a natural fungal polysaccharide, is able to exert anti-mutagenic properties upon application in the diet as well as *in vitro*. These anti-mutagenic effects of CM-CG might be partly explained by its free radical scavenging activity (Babincová *et al*. 1999; Lazarová *et al*. 2006b; Patchen *et al*. 1987). In this study we demostrated that CM-CG significantly reduces the genotoxic and cytotoxic potential of H_2O_2 and DMNQ in freshly isolated rat hepatocytes. Oxidative stress induced by H_2O_2 and DMNQ, compounds that are considered to cause DNA damage via the formation of radicals (Halliwell & Aruoma, 1991; Morgan *et al*. 1992), exerted genotoxic effects in primary hepatocytes which corresponded with other reports on H_2O_2- and DMNQ-mediated genotoxicity (Lazarová *et al*. 2006a; Bresgen *et al*. 2003). Ability of CM-CG, applied either before or during treatment with oxidants, to decrease both cytotoxic and genotoxic effects of H_2O_2 and DMNQ are in accordance with the observed protective effects of either CM-CG enriched diet (*ex vivo* experiments) against oxidative damage induced in freshly isolated rat cells (Lazarová *et al*. 2006b) or CM-CG applied *in vitro* to different different cell types (Križková *et al*. 2003; Slameňová *et al*. 2003). Apart from this, several studies demonstrated that β-D-glucans can induce cytokines inhibiting the expression of cytochrome P450s (CYP450s), a class of drug- and xenobiotic-metabolizing enzymes highly expressed in the rat liver (Shon *et al*. 2003). Reduced expression of P450s could eventually also contribute to the protective mechanisms by modulating the metabolism of xenobiotics such as DMNQ. Cytokines, in addition, may also be responsible for the reduction of apoptosis by interfering with signal transduction pathways.

Though a precise mechanism of the observed DNA-protective and anti-cytotoxic effects of carboxymethyl

chitin-glucan applied before or during the treatment of hepatocytes with oxidants remains unclear, we can conclude that this β-D-glucan represents a natural polysaccharide contributing via the diet to the anti-oxidative defence mechanisms of organisms.

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