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RESEARCH ARTICLE

Carbonyl compounds methylglyoxal and glyoxal affect interleukin-8 secretion in intestinal cells by superoxide anion generation and activation of MAPK p38

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The carbonyl compounds methylglyoxal (MG) and glyoxal (GL) are reactive intermediates of glucose degradation pathways and capable of inducing cellular damage. Although immune-stimulating activity has been investigated in endothelial cells, little is known about the signaling pathways of cytokine induction of these compounds in the intestine. Hence, we investigated the impact of mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB) on IL-8 production by human intestinal cells (Caco-2 and HT-29) after stimulation by MG and GL. Both compounds induced a dose-dependent enhancement of IL-8 secretion in human intestinal cells. MAPK p38 and extracellular signal-regulated kinase (ERK) were phosphorylated in these cells after having been stimulated by MG and GL. Furthermore, inhibitors of MAPK p38 (SB 203580 and 239063), ERK1/2 (PD 98059) and NF-κB activation (SM-7368 and SC-514) reduced IL-8 secretion. The most important mechanism by which MG and GL induced IL-8 secretion was the generation of superoxide anions which was confirmed by the inhibition of the cytosolic NADPH oxidase with diphenyl iodonium (DPI) or by application of superoxide dismutase (SOD). Our data suggest that multiple pathways were simultaneously activated; however, superoxide dependent MAPK p38 activation seems to be the most dominant pathway for IL-8 secretion in intestinal cells.

Keywords:
Glyoxal / Interleukin-8 / Methylglyoxal / p38 MAPK / Superoxide anion

1 Introduction

In recent years, a variety of studies have demonstrated the role of ''carbonyl stress'' *in vivo* and *in vitro*. Until now, most of them have focused on effects of carbonyl compounds such as MG and GL as a consequence of hyperglycemia associated with endothelial dysfunction and inflammation [1–3].

In contrast, the effects of carbonyl compounds on intestinal cells are poorly documented. Several studies concerning the gastrointestinal tract dealt with their cytotoxic and mutagenic effects as microbial metabolic products in concentrations ranging between μM and mM [4–6]. Nonetheless, it was hypothesized that carbonyl compounds in the intestine can directly cause inflammatory processes similar to their inflammatory effects in endothelial cells. Although exact etiology and pathogenesis of inflammatory bowel diseases (IBD) remain unclear, there is substantial evidence that pro-inflammatory cytokines such as interleukin-8 (IL-8) play a key role in the inflammatory process [7]. Furthermore, increased secretion of IL-8 in the intestine of patients with IBD revealed the carbonyl compounds' relevance for intestinal inflammation [8–10]. Increased IL-8 levels support the role of the

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Abbreviations: Akt, v-akt murine thymoma viral oncogene homolog 1; **carboxy-H2-DCFDA,** 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; **CAT,** catalase; **DPI,** diphenyl iodonium; **ERK,** extracellular signal-regulated kinase; **FCS,** fetal calf serum; **GL,** Glyoxal; **HRP,** horse radish peroxidase; **IBD,** inflammatory bowel disease; **IKK,** inhibitor kappa B kinase; **IL-8,** interleukin-8; **JNK,** c-jun NH(2)-terminal kinase; **MAPK,** mitogen-activated protein kinases; **MG,** Methylglyoxal; **NF-κB,** nuclear factor kappa B; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase

epithelium in signaling between luminal factors and mucosal immune cells. We have recently shown that both carbonyl compounds, MG and GL, were able to influence cytokine release from intestinal epithelial cells, but the carbonyl compound mediated pathways involved in its gene and protein expression in the intestine have not been investigated [11]. The IL-8 expression and secretion is a tightly regulated process and numerous publications indicate that different signal cascades such as the mitogen-activated protein kinases (MAPK) and the NF-kappaB (NF-κB) pathway participate in this process. c-jun *N*-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAPK are representative members of the MAPK family and have been shown to be involved in IL-8 regulation in dependency of the stimulus [12]. Furthermore, it has been shown that oxidative stress activates both MAPK and NF-κB signaling pathways. Apart from that, MG and GL being able to generate reactive oxygen species (ROS) could influence MAPK and NF-κB signaling [13–16]. Therefore, we investigated the mechanisms by which MG and GL induce IL-8 secretion and the generation of ROS hypothesizing that MAPK signaling and NF-κB activation are involved.

2 Materials and methods

2.1 Cell culture

The human colon derived crypt like HT-29 and Caco-2 epithelial cell lines were obtained from LGC Promochem GmbH (Wesel, Germany) and maintained in Dulbecco's modified Eagle's medium with 4.5 g/L glucose (Caco-2) or Roswell RPMI-1640 (HT-29) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine (all materials supplied by Invitrogen; Karlsruhe, Germany). Cells were seeded at a density of 5×10^5 cells/75 cm^2 flask, incubated at 37°C with 5% CO_2 and passaged at 70% confluence. Passages between 10–25 (Caco-2) and 16–30 (HT-29) were used for exposure experiments.

2.2 Chemicals

MG and GL were obtained from Sigma Aldrich (Darmstadt, Germany). For inhibition studies, cells were pretreated for 1 hour with 5 μM PD98059 (ERK1/2 inhibitor), 5 μM SB239063 (p38α and -β inhibitor), 2.5 μM SB203580 (p38 inhibitor), 1 μM SC-514 (IKK-2 inhibitor that specifically blocks NF-κB dependent gene expression but not MAPK pathway), 5 μM SM-7368 (NF-κB inhibitor that targets downstream of MAPK p38 activation) [Calbiochem; Schwalbach, Germany], 250 U/mL superoxide dismutase (SOD), 250 U/mL catalase (CAT) and/or 1 mM diphenyl iodonium (DPI) [Sigma Aldrich; Darmstadt, Germany]. Cells were then exposed to MG and GL as indicated below.

2.3 IL-8 assay

To determine IL-8 secretion Caco-2 and HT-29 cells were seeded on 24-well culture plates at an initial density of 1×10^4 cells/well. While HT-29 cells were used as soon as they were confluent, Caco-2 cells were cultured for 14 days after reaching 100% confluence in order to acquire a differentiated phenotype. HT-29 or Caco-2 cells were finally incubated with components given in the figure legends. After 24 h, the supernatant was collected, cleared by centrifugation and immediately used for the cytokine assay. The amounts of cytokines secreted into the cell culture supernatant were determined using the commercially available IL-8 assay according to the manufacturer's instructions (BioCat; Heidelberg, Germany). Cytokine concentrations were determined in duplicate in three independent experiments on a 96-well plate reader (Asys; Eugendorf, Austria) at a wavelength of 405 nm. The sensitivity limit of the IL-8 assay was less than 10 pg/mL.

2.4 MAPK assay

After incubation with carbonyl compounds and/or inhibitors (see figure legends), cells were washed in phosphate buffered saline (PBS) and solubilized in lysis buffer. Protein concentration was determined using the Bradford protein assay; 300 μg of protein were used for the Human Phospho-MAP Array (R&D Systems; Heidelberg, Germany) according to the manufacturer's protocol. Briefly, arrays were incubated with whole cell lysates overnight at 4°C and then washed with the washing buffer supplied. Arrays were incubated with anti–-phosphotyrosine-HRP antibodies for 2 h at room temperature before incubation with a chemiluminescent reagent and film exposure. Spot intensity was calculated using the software Photo-Capt (Bio-Budget; Krefeld, Germany).

2.5 Determination of IL-8 and NF-κB mRNA expression

RNA extraction and cDNA synthesis. Total RNA was isolated using TRIzol reagent (Invitrogen; Karlsruhe, Germany), and cDNA synthesis was done as described previously [17].

Analysis of mRNA expression (Real-time quantitative RT-PCR). Messenger RNA expression of target genes was measured in intestinal cells using real-time RT-PCR. Real-time RT-PCR was performed using the 7500 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Gene-specific primers and probes used in this study (Table 1) were designed using the sequences accessible in the NCBI Reference Sequence and the software Primer Express (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences were chosen to avoid homologies with undesired genes and other coding sequences and checked

Table 1. Sequence of primers and probes used for real-time PCR

Gene	GenBank accession no	Primer and TaqMan probe sequence
IL-8	M28130	Forward primer:5′- AGCTGGCCGTGGCTCTCT -3′ Reverse primer: 5′- TTTAGCACTCCTTGGCAAAACTG -3′ Probe: 5′- Fam-CAGCCTTCCTGATTTCTGCAGCTCTGTG-Tamra-3′
NF-κB	M62399	Forward primer: 5′- AGCACAGATACCACCAAGACCC-3′ Reverse primer: 5′- CCAGGGAGATGCGCACTG-3′ Probe: 5′- Fam-CATCAAGATCAATGGCTACACAGGACCAGG-Tamra-3
GAPDH	NM_002046	Forward primer: 5′- CCACATCGCTCAGACACCAT-3′ Reverse primer: 5′- GTGACCAGGCGCCCAATA-3′ Probe: 5′-Fam-AGGTCGGAGTCAACGGATTTGG-Tamra-3′

with the BLAST™ software. All probes were labeled with the fluorescent dyes 5-FAM (6-carboxy-fluorescein) as reporter and 3-TAMRA (6-carboxy-tetramethyl-rhodamine) as quencher. Primers and probes were synthesized by Sigma (Deisenhofen, Germany). A total reaction volume of 25 μL contained 1 μL cDNA and Taqman PCR master mix (Eurogentec, Köln, Germany) including 250 nM of each primer and 150 nM probe. The PCR running conditions were: 10 min of initial denaturation at 95°C, followed by 45 cycles of 30 sec at 95°C, 30 sec at 58°C for annealing, 30 sec at 60°C and 15 sec at 75°C. Samples were run in triplicates. Real-time RT-PCR results are given as the relative expression level of normalized samples [Δcycle threshold (Ct)] in relation to the expression of the calibrator sample ($2^{-\Delta\Delta Ct}$), which was set at 100%. The Ct value refers to the cycle number at which the PCR plot crosses the threshold line, ΔCt is calculated by substracting the Ct value of the corresponding housekeeper gene GAPDH control (endogenous reference control) from the Ct value of the target gene, and ΔΔCt is obtained by substracting the ΔCt of each experimental sample from the ΔCt of the calibrator sample.

2.6 Determination of reactive oxygen species (ROS)

Cellular ROS generation was determined by 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2-DCFDA) (Invitrogen; Karlsruhe, Germany) by incubating cells in medium with 50 μM carboxy-H_2-DCFDA for 30 min at 37°C. An increase in carboxy-H_2-DCFDA fluorescence is primarily due to the reaction with intracellular superoxide, as noted by the manufacturer. Medium was replaced by Hank's balanced salt solution (HBSS) containing various concentrations of carbonyl compounds. Kinetic analyses were done up to 24 h of incubation at 37°C using the Ascent microplate fluorescence reader (Thermo; Karlsruhe, Germany). MitoSox Red mitochondrial superoxide indicator dye (Invitrogen; Karlsruhe, Germany) was used to determine ROS in mitochondria. Cells were treated with test compounds and then loaded with 5 μM MitoSox Red in HBSS for 10 min at 37°C. Cells were washed, and MitoSox Red fluorescence intensity was determined at 510 nm excitation and 580 nm emission.

2.7 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software 4.03 (San Diego, CA, U.S.A.). All results are given as mean ± SD of duplicate determinations representing three independent experiments. Statistical significance of the differences between various groups was evaluated by one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P<0.05$.

3 Results

3.1 Effect of glyoxal and methylglyoxal on constitutive expression of IL-8 mRNA and IL-8 protein by HT-29 and Caco-2 cells

Induction of IL-8 expression in response to the carbonyl compounds GL and MG was observed on mRNA as well as on protein levels. HT-29 (Fig. 1A) and Caco-2 cells (Fig. 1B) constitutively released IL-8 at a mean concentration of 2510 ± 450 pg/mL and 2250 ± 110 pg/mL, respectively. Caco-2 cells seemed to tolerate higher concentrations of GL and MG since concentrations above 500 μg/mL were accompanied by cytotoxic effects assessed by using the trypan blue vital dye exclusion technique (data not shown); however, in HT-29 cells, GL and MG concentrations above 250 μg/mL already were cytotoxic. Whereas the IL-8 inducing effects of MG were found to be at a similar level in both cell types, differences were observed for the treatment with GL. In HT-29 cells 100 μg/mL of GL significantly enhanced IL-8 secretion to 3340 ± 290 pg/mL reaching a maximum of 8170 ± 620 pg/mL at 250 μg/mL. In Caco-2 cells GL concentrations of 250 and 500 μg/mL revealed an IL-8 secretion of 3010 ± 140 and 3490 ± 260 pg/mL, respectively; lower concentrations had no effect. IL-8 secretion induced by MG was less pronounced. Incubation of HT-29 with 250 μg/mL of MG enhanced IL-8 secretion to 4940 ± 260 pg/mL (Fig. 1A). This substrate dependency was not observed in Caco-2 cells. Here, both carbonyl compounds at 250 and 500 μg/mL induced IL-8 secretion in a similar range (Fig. 1B).

Along with the IL-8 protein secretion, IL-8 mRNA levels in GL and MG treated cells increased dose-dependently. In HT-29, the highest concentration of carbonyl compounds (250 μg/mL) revealed a 7.5-fold and a 5.0-fold increase in IL-8 mRNA expression when cells were treated with GL and MG, respectively (Fig. 1C). The effect of GL and MG in Caco-2 cells

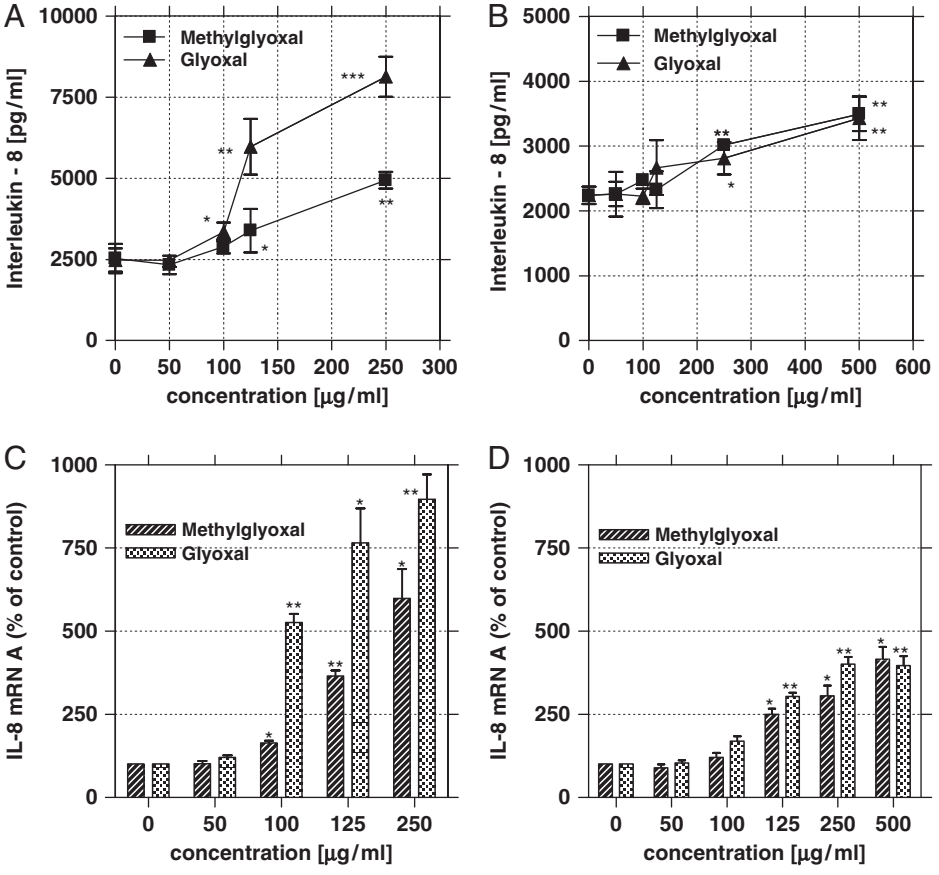

Figure 1. IL-8 protein and mRNA levels in intestinal cells. Secretion of IL-8 by HT-29 (A) and Caco-2 (B) cells incubated with increasing concentrations of GL and MG over 24 h. Basal secretion of IL-8 in control cells is shown at 0 mM. IL-8 mRNA levels in HT-29 (C), and Caco-2 (D) cells were determined using real-time RT-PCR. Results are expressed as mean ± SD of duplicate determinations in three independent experiments. Data of mRNA-levels were analyzed as means 2^{-ddCt} ± SD and are given as % of control ($^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$)

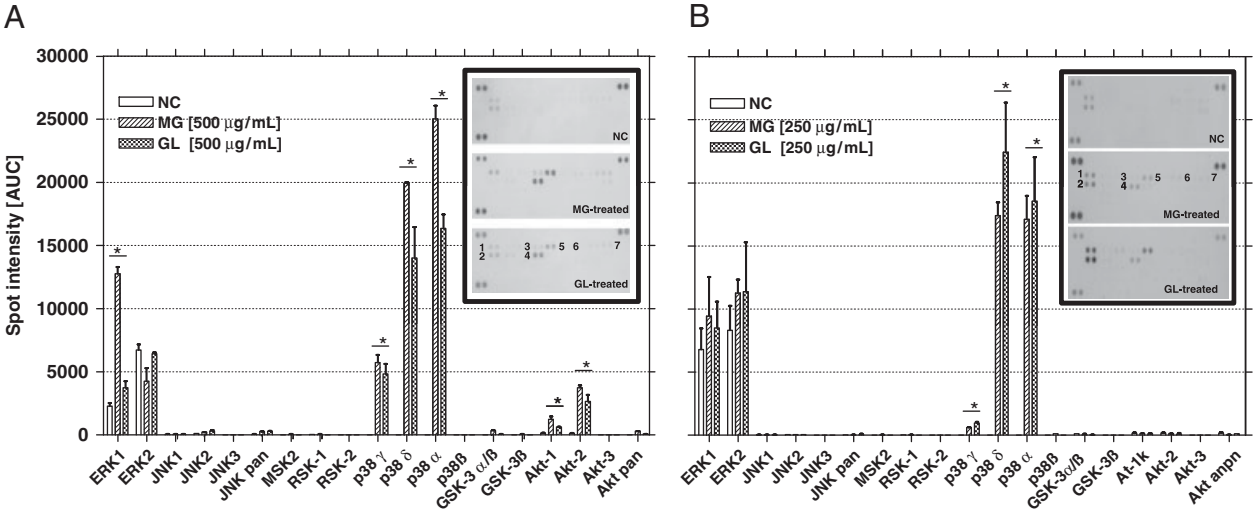

Figure 2. Detection of phosphorylated kinases in untreated (NC) and treated (MG and GL) Caco-2 (A) and HT-29 cells (B). Intestinal cells were either untreated or treated with indicated concentrations of MG or GL for 30 min, and finally protein was prepared. 300 μg of protein was then used for the MAPK assay. Array signals from scanned X-ray film images were analyzed and expressed as spot intensity [AUC]. Results are expressed as means ± SD of duplicate determinations ($^{*}P \leq 0.05$). Insert: Representative example of an array (1, ERK1; 2, ERK2; 3, p38γ; 4, p38δ; 5, p38α; 6, Akt-1; 7, Akt-2).

was slightly weaker revealing a 3-fold increase in IL-8 mRNA after incubation with 500 μg/mL GL or MG (Fig. 1D).

3.2 Effect of glyoxal and methylglyoxal on MAP kinases pathways in HT-29 and Caco-2 cells

IL-8 secretion is known to be activated by at least three different mechanisms including the MAPK pathway, receptor tyrosine kinases and the NF-κB pathway. We therefore investigated whether any of these mechanisms was involved in MG and GL mediated IL-8 secretion in the two cell lines. To determine the activation of specific MAPK in intestinal epithelial cells we used the Human Phospho-MAPK Antibody Proteome Profiler Array to screen 21 different tyrosine kinases.

As shown in Fig. 2, the analysis of MG and GL downstream signaling pathways revealed the role of MAPK p38 and partly the ERK1/2-pathway. Phosphorylation and activation of different MAPK p38 subtypes (α, δ and γ were induced by MG and GL. In Caco-2 cells the incubation with MG had an enhancing effect on ERK1 phosphorylation, but no significant effect on ERK2 was observed. In contrast, GL was unable to induce ERK1 and ERK2 phosphorylation. In HT-29 cells, neither MG nor GL induce ERK phosphorylation.

A slight Akt-1/2 activation induced by MG and GL was detected in Caco-2 cells, but not in HT-29 cells, which further indicates different mechanisms of MG and GL in the two cell lines.

3.3 Contribution of MAP kinase p38 to IL-8 secretion induced by carbonyl compounds

The role of MAPK on MG and GL induced IL-8 secretion in HT-29 and Caco-2 cells was investigated using inhibitors affecting different cellular targets which act upstream of transcription. We used the MAPK p38 inhibitor SB203580 and SB239063 because of their specificity for different isotypes of p38. These are SB203580, which is known to inhibit all isoforms of MAPK p38, but not MAPK p42/p44 (ERK1/2), and SB239063, which specifically inhibits the

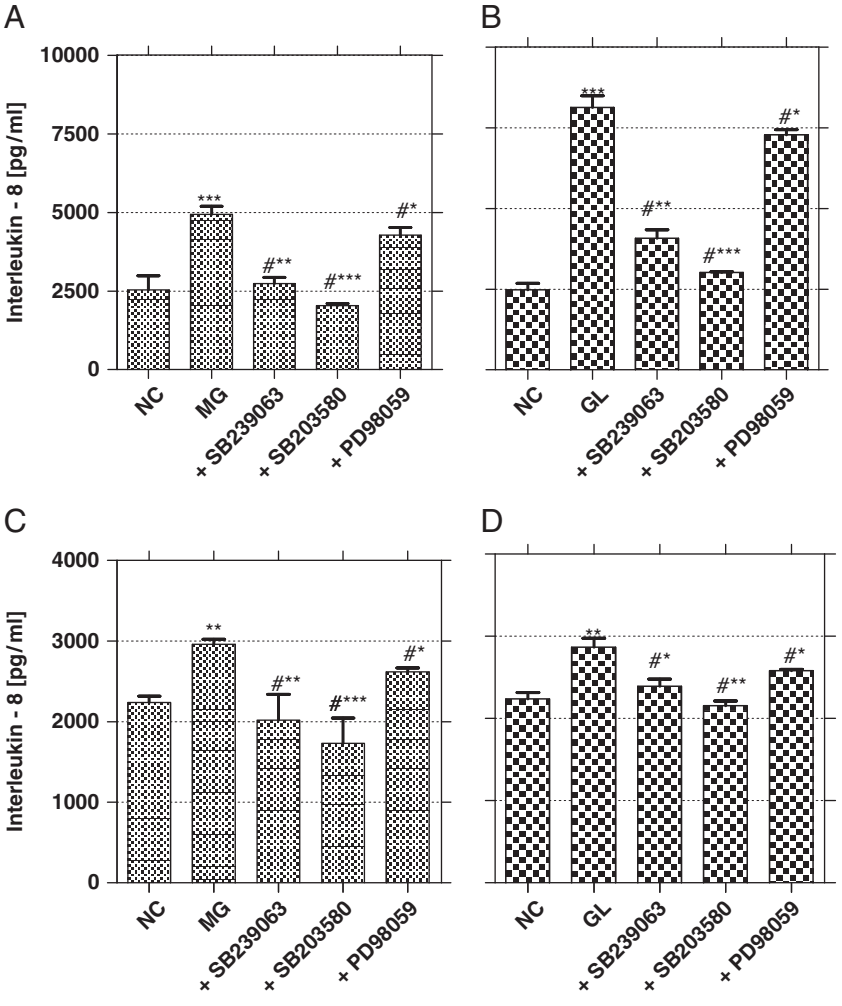

Figure 3. Effect of signal transduction inhibitors on MG and GL induced IL-8 secretion in HT-29 (A, B) and Caco-2 (C, D) cells. Intestinal cells were pretreated with SB203580 (2.5 μM), SB 239063 (5 μM) or PD 98059 (5 μM) for 1h and finally incubated for 24 h with MG and GL, whereas control cells remained untreated (NC). Supernatants were collected, and IL-8 was quantified using ELISA as described in the method section. Results are expressed as mean ± SD from triplicates. ($^{*}P \leq 0.05$ significantly different from control cells (NC) or $^{\#*}P \leq 0.01$ significantly different from MG or GL treated cells).

p38α and–β phosphorylation. As shown in Fig. 3, pretreatment of cells with these MAPK p38 inhibitors resulted in a virtually complete inhibition of MG and GL induced IL-8 secretion. In both cell lines, pre-incubation with SB 239063 and 203580 significantly inhibited MG and GL induced IL-8 secretion (Fig. 3). These results suggest a strong involvement of MAPK p38 in MG and GL induced IL-8 production in both intestinal cell lines.

Furthermore, we used the ERK1/2 inhibitor PD 98059 to evaluate the contribution of ERK1/2 to MG and GL induced IL-8 secretion. Pretreatment with the ERK1/2 signaling inhibitor slightly, but significantly reduced the MG and GL induced IL-8 secretion in HT-29 and Caco-2 cells.

3.4 Contribution of NF-κB signaling to IL-8 secretion induced by carbonyl compounds

Since NF-κB is a known regulator of IL-8 synthesis, we also investigated different NF-κB signaling inhibitors with regard to their effect on MG and GL induced IL-8 secretion (Fig. 4A and B) and determined NF-κB mRNA levels (Fig. 4C and D). To distinguish between the contribution of MAPK p38 and NF-κB signaling pathways, since both could be responsible for IL-8 transcription, we used the inhibitor SC-514 that specifically blocks NF-κB dependent gene expression at the level of IKK (inhibitor of kappa B kinase) without affecting MAPK pathway and SM-7368 that target downstream of MAPK p38 activation.

In both cell lines, MG-enhanced IL-8 secretion was slightly compensated by SC-514 but not by SM-7368, whereas both inhibitors reduced GL induced IL-8 secretion.

Based on mRNA levels, MG and GL regulated NF-κB in a different way. Neither MG nor GL treated HT-29 cells influenced NF-κB mRNA expression (Fig. 4C). As shown in Fig. 4D, only MG treatment led to a significant increase of NF-κB mRNA levels in Caco-2 cells, whereas GL treatment remained ineffective. The use of the inhibitors of NF-κB signaling did not change the expression level in MG-treated cells, indicating that MG affects both translocation and mRNA expression of NF-κB, whereas GL influenced only the translocation.

3.5 Contribution of reactive oxygen species to IL-8 secretion induced by carbonyl compounds

To investigate the role of ROS in MG and GL induced IL-8 secretion, cells were treated with different ROS-sensitive

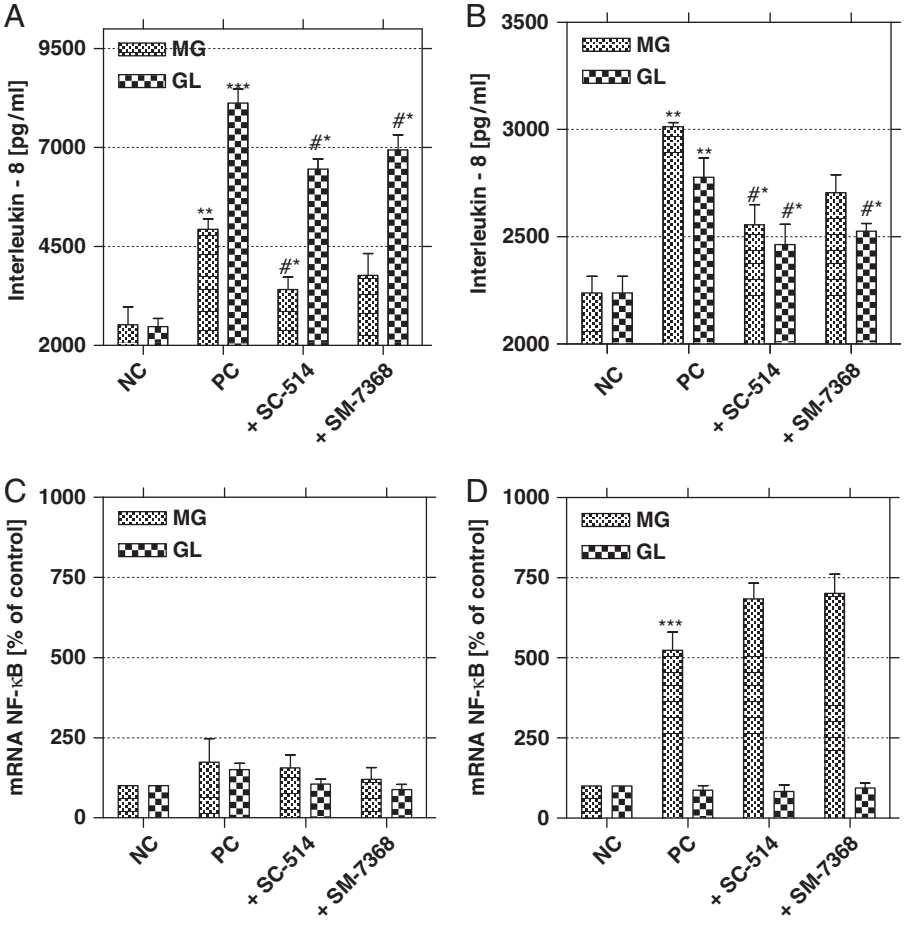

Figure 4. Effect of signal inhibitors on MG and GL induced IL-8 secretion and NF-κB mRNA expression in HT -29 (A, C) and Caco-2 cells (B, D). Intestinal cells were untreated (NC), pretreated with SC-514 (1 μM) or SM-7368 (5 μM) for 1h and then incubated with MG (Caco-2: 500 μg/mL and HT-29: 250 μg/mL) or GL (Caco-2: 500 μg/mL and HT-29: 250 μg/mL for 24 h. Thereafter, supernatant was collected, IL-8 was quantified using ELISA, and cells were prepared for real-time PCR as described in the method section. Results were expressed as mean ± SD from triplicates. (** $P \leq 0.01$ and *** $P \leq 0.005$ significantly different from control cells (NC) or #* $P \leq 0.01$ significantly different from control cells (PC, cells incubated with MG and GL without inhibitors).

fluorescence dyes. To differentiate which compartment was responsible for superoxide production, cells were treated with the cytosol-specific carboxy-H_2-DCFDA and the mitochondrial-specific MitoSOX. As shown in Table 2, both MG and GL caused an increase in carboxy-H_2-DCFDA fluorescence in HT-29 cells.

The generation of ROS induced by MG and GL seemed to be specific for superoxide anions since ROS decreased when the superoxide scavenger superoxide dismutase (SOD) was added, but no effect was observed using the hydrogen peroxide scavenger catalase (CAT). MG and GL treatment, however, caused no increase in MitoSOX fluorescence indicating that MG and GL induced cytosolic superoxides. Similar data were obtained in Caco-2 cells.

To verify that the observed MG and GL induced cytosolic superoxide generation was responsible for the enhanced IL-8 secretion, HT-29 cells were pretreated with different inhibitors. Pretreatment with SOD significantly reduced MG and GL induced superoxide generation and concomitant IL-8 secretion. Furthermore, pretreatment with the MAPK p38 inhibitor SB203580 reduced both superoxide generation and IL-8 secretion in GL-treated cells, whereas the use of the MAPK inhibitor only reduced IL-8 secretion without affecting superoxide generation in MG treated cells. The inhibition of the superoxide generation seemed to affect only MAPK p38 signaling; the ERK-inhibitor PD 98059 failed to influence superoxide induced IL-8 secretion in both, MG and GL treated cells.

We finally determined whether the impact of MG and GL on the superoxide generation was associated with a reduced oxidase activity. Therefore, HT-29 cells were pretreated with the NADPH oxidase inhibitor diphenyl iodonium (DPI) and superoxide generation and IL-8 secretion was assessed. Both MG and GL induced superoxide generation and IL-8 secretion were abrogated after pretreatment with DPI suggesting that NAPDH oxidase was responsible for MG and GL induced superoxide generation and subsequently IL-8 secretion.

4 Discussion

Carbonyl compounds such as MG und GL seem to play an important role in inflammatory response and oxidative stress [16, 18–20]. Their presence in the gastrointestinal tract due to the intake of processed food or bacterial metabolites may have a direct effect on the intestinal epithelium. Some previous data have shown that they were able to induce colonic injury, but the mechanism of action is not clear. We have recently shown that Caco-2 cells respond to MG and GL with an enhanced secretion of the pro-inflammatory cytokine IL-8 [11]. In this study we observed a dose-dependent enhancement of IL-8 secretion along with increased mRNA levels for IL-8 after MG and GL treatment of HT-29 and Caco-2 cells (Fig. 1). The simultaneous increase of IL-8 secretion and IL-8 mRNA suggested that IL-8 regulation is mediated by translational and transcriptional processes; however, the effects of MG and GL were weaker in Caco-2 cells than in HT-29 cells indicating that Caco-2 cells tolerate higher concentrations of carbonyl compounds. These cell type specific differences could be explained by different uptake kinetics for the carbonyl compounds in differentiated Caco-2 compared to undifferentiated HT-29 cells and/or by different activities of detoxification systems. The major dicarbonyl detoxifying system is the glyoxalase system. Physiological substrates of glyoxalase I are MG, GL and other acyclic α-oxoaldehydes. Although there are limited data on expression of glyoxalase and their isoforms in intestinal epithelial cells, different expression patterns of detoxification systems in the intestine may well occur in dependency of the phenotype [21, 22]. A recent study indicated that the anti-inflammatory and anti-cancerogenic action of the antioxidant curcumin may act via inhibition of glyoxylase I with concomitant enhanced levels of MG [23]. This effect seemed to be concentration-dependent: lower concentrations induced cell proliferation, whereas higher concentrations were associated with cytotoxicity. In addition, the effects of curcumin on cell growth were more effective in cancer cells than in non-cancer cells. It can thus be concluded that not only the concentration but also the cell-type specificity is important for the function of oxidants or antioxidants.

In order to elucidate the signaling pathways involved in IL-8 synthesis, we examined the ability of MG and GL to trigger the phosphorylation of different MAPK. In the present study we observed increased levels of phosphorylated p38 MAPK isoforms and ERK1/2 in MG and GL treated cells whereas other signaling molecules such as the JNK family remained unaffected (Fig. 2). It is a well known that p38 MAPK is activated in a variety of cell types and has participated in the control of epithelial IL-8 gene expression in intestinal cells [24, 25]. As shown recently by Liu *et al.* and Fukuda *et al.* MG was able to induce activation of p38 MAPK in mesangial or glial cells [26, 27]. This is in accordance with our results illustrating that p38 MAPK were involved in IL-8 secretion. Different isoforms seem to be responsible for these actions [28, 29]. After incubation with MG and GL, we observed a potent activation of the MAPK isoforms p38α and p38δ and to a lesser extent of p38γ. p38α is ubiquitously expressed in human tissue, and its relevance in inflammation has been shown by Kim *et al.* [30]. In contrast, the recently identified isoforms p38γ and δ have 60% identity, but less is known about their function. However, in our study both isoforms seem to be involved in IL-8 secretion.

In addition to the p38 MAPK pathway, the activation of ERK pathways was investigated. Whereas the treatment of HT-29 with MG and GL did not induce ERK1/2 activation, Caco-2 cells displayed a substrate specific regulation. Only MG treatment leads to a significant enhancement of ERK1, but not ERK2 phosphorylation (Fig. 2B). Reports about an activation of the ERK pathway by carbonyl compounds are contradictory. Portero-Otin *et al.* showed that MG and GL inhibit the ERK1/2 pathway in a variety of cell lines [31]; other authors demonstrated that activation of the ERK

pathway is essential for IL-8 secretion [25, 32]. However, the differences we observed between ERK1 and ERK2 activation in dependency of the cell line and the carbonyl compound suggest that the different phenotypes and concentrations of MG and GL influenced their action. This is in accordance with Akhand *et al.* who showed that MG and GL triggered distinct signal cascades [33].

To further confirm the participation of p38 and ERK1/2 MAPK in IL-8 expression and secretion we used different MAPK inhibitors. As shown in Fig. 3, MG and GL induction of epithelial IL-8 secretion was abrogated by blocking the p38 MAPK signaling pathway. However, the ability of MG and GL to enhance IL-8 secretion was slightly reduced by pretreatment with an inhibitor specific for the ERK1/2 pathway, i.e. carbonyl compounds induce IL-8 secretion primarily through p38 MAPK pathways.

In addition, ROS directly induce p38 MAPK signaling or conversely, p38 can be responsible for ROS generation and subsequent signal transduction [34, 35]. In several studies, MG was identified as an ROS generating molecule [36, 37]. In our experiments we demonstrated that both MG and GL generate superoxide anions and that the cytosol-associated NAPDH oxidase was the most important target for MG and GL since superoxide synthesis and IL-8 secretion were completely inhibited by the cytosol-specific NADPH oxidase inhibitor DPI (Table 2 and Fig 5). The influence of MG on the MAPK pathway has recently also been reported by others [38, 39]. Fan *et al.* used MG modified bovine serum albumin to identify the mechanism of TNF-α secretion. Their data indicate that MG modified bovine serum albumin causes cytokine release via ERK and p38 dependent pathways. These observations suggest that MG and GL trigger intracellular responses which regulate different members of the MAPK family and ROS formation.

Apart from p38 MAPK, NF-κB is an important redox sensitive factor which regulates IL-8 expression [40, 41]. NF-κB is mostly composed of an inactive homo- or heteromer consisting of different subunits (NF-κB1 (p50), NF-κB2 (p52) and c-REL, REL A (p65) and REL Bof). Activation of the heteromer p50:p65 is often linked with promoting inflammation. It is well documented that NF-κB activation takes place within minutes in a cell type and stimulus-specific manner independent of NF-κB protein expression. This activation leads to a site-specific phosphorylation of the inhibitor protein IκB by IκB kinases (IKK), consecutive rapid dissociation of the heteromer and subsequently transmigration into the nucleus. Here, they can induce gene transcription by binding to specific promoter elements in the IL-8 gene [39, 42]. Furthermore, enhanced transcription levels of NF-κB could also be responsible for the induced IL-8 expression and secretion.

Thus, we investigated if NF-κB mRNA expression and activation have any influence on IL-8 secretion which is induced by carbonyl compounds. Therefore, we used the NF-κB specific inhibitor SM-7368, a NF-κB inhibitor acting downstream of MAPK p38 and SC-514, a NF-κB inhibitor acting independently of the MAPK pathway including ERK1/2. As shown in Fig. 4, SM-7368 as well as SC-514 reduced GL induced IL-8 secretion in both cell lines, whereas MG induced IL-8 secretion was only moderately abrogated by SC-514. Hence, IL-8 secretion seems to be differently regulated dependent on the carbonyl compound that was used for its induction. Since NF-κB mRNA expression was only partly affected by MG treatment in Caco-2 cells, the carbonyl compound induced effects were mainly induced by p38 MAPK signaling. However, our experiments were limited to the NF-κB subunit p65 on a transcriptional level. Other monomers such as p50 might also have an effect on mRNA and protein levels of IL-8. Nevertheless, our data using the highly specific IKK-2 inhibitor of NF-κB activation, SC-514, are consistent with reports from other groups showing that NF-κB activation in MG treated cells is regulated by IKK-2 by mechanisms

Table 2. MG and GL induced ROS generation in HT-29 and Caco-2 cells[a)]

	HT-29		Caco-2	
	ROS generation (carboxy-H_2-DCFDA)	ROS generation (MitoSOX)	ROS generation (carboxy-H_2-DCFDA)	ROS generation (MitoSOX)
NC	100%	100%	100%	100%
MG	238±12%*	98±5%	156±3%*	114±13%
GL	378±10%*	103±10%	134±16%*	99±10%
MG+SOD	143±7%#*	99±3%	119±5%#*	106±6%
GL+SOD	138±5%#*	100±11%	109±3%#*	105±14%
MG+CAT	255±16%	104±6%	161±4%	108±20%
GL+CAT	388±13%	96±7%	148±12%	105±8%

a) ROS generation was determined fluorimetrically by using carboxy-H_2-DCFDA and MitoSOX. Cells were pretreated for 1 h with 250 U/mL SOD and 250 U/mL CAT and subjected to 250 μg/mL or 500 μg/mL MG and GL treatment. Fluorescence intensity was determined and negative control (NC) was set to 100%. (*$P \leq 0.05$ significantly different from control cells (NC) or #*$P \leq 0.05$ significantly different from treated cells; each experiment was run independently three times, $n = 3$).

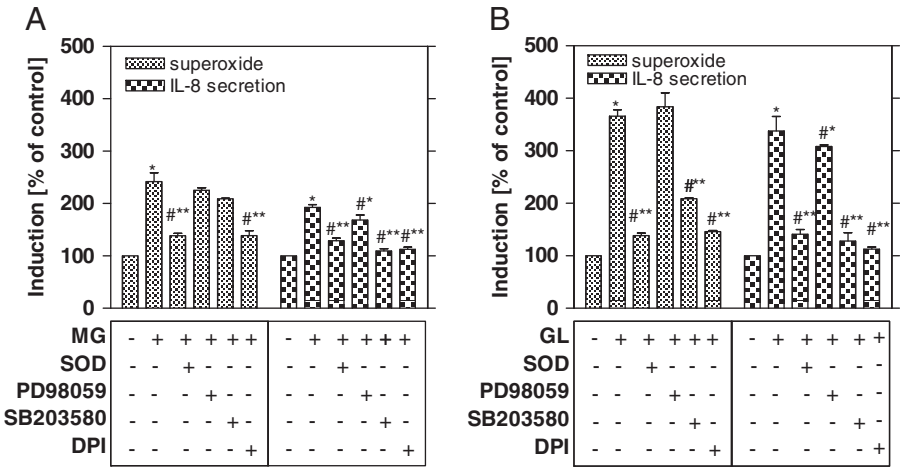

Figure 5. MG (A) and GL (B) induced ROS generation and influence on IL-8 secretion in HT-29 cells. Cells were pretreated with inhibitors for 1 h and then incubated with 250 μg/mL MG and GL for 60 min. Superoxide generation was determined by using carboxy-H_2-DCFDA and IL-8 secretion by ELISA ($^{*}P \leq 0.05$ significantly different from control cells (NC) or $^{\#}{}^{*}P \leq 0.05$ and $^{\#}{}^{**}P \leq 0.01$ significantly different from treated cells; each experiment was run independently three times, $n = 3$).

involving nuclear transmigration and/or p65 transactivation in addition to the degradation of IκBs [19, 38, 39].

In conclusion, we demonstrated that the exposure of intestinal cells to MG and GL resulting in increased IL-8 expression and secretion was mainly based on the induction of superoxide anions and the activation of p38 MAPK pathways. Unfortunately, until now it has been difficult to estimate the concentrations of MG and GL which were orally ingested. Thus, further studies are necessary to evaluate chronic exposure of the intestine to carbonyl compounds *in vivo*.

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5 References

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