TNF- α /cycloheximide-induced oxidative stress and apoptosis in murine intestinal epithelial MODE-K cells

Short running title: TNF- α /CHX-induced oxidative stress and apoptosis

TNF- α /cycloheximide-induced oxidative stress and apoptosis in murine intestinal epithelial MODE-K cells

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Abstract

In the mouse postoperative ileus model, we have shown an increase in oxidative stress after intestinal manipulation occurring earlier in the mucosa than in the muscular layer, which might contribute to epithelial barrier dysfunction. To address these findings *in vitro*, we assessed TNF- α /cycloheximide (CHX)-induced oxidative stress and apoptosis in a mouse intestinal epithelial cell line, MODE-K. The influence of heme oxygenase (HO)-1-related products and agents known to reduce reactive oxygen species (ROS) production on TNF- α /CHX-induced oxidative stress and apoptosis were investigated. MODE-K cells were exposed to different concentrations of TNF- α /CHX in the absence/presence of the test agents. Cell viability, caspase-3/7 activity, apoptosis, reduced glutathione level (GSH) and intracellular ROS production were measured. TNF- α /CHX decreased cell viability, increased caspase-3/7 activity, induced apoptosis, reduced the GSH level and increased ROS production in a concentration-dependent manner in MODE-K cells. All these effects of TNF- α /CHX were partially prevented by pretreatment with a carbon monoxide-releasing agent (CORM-A1) and nitrite. The antioxidant resveratrol abolished TNF- α /CHX-induced increase in ROS production and caspase-3/7 activity, but apoptosis was only partially prevented. MODE-K cells are sensitive to TNF- α -induced apoptosis in the presence of CHX, which is associated with increased intracellular ROS production and caspase-3/7 activation. The effects were partially mitigated by CORM-A1, nitrite and resveratrol. Thus, these agents could be of potential use in protecting the epithelial barrier against oxidative stress during intestinal ischemia/reperfusion injury.

Key words

MODE-K cells, TNF- α /CHX, ROS, apoptosis, bilirubin, CORM-A1, nitrite, resveratrol

INTRODUCTION

Sepsis is an infection with a systemic inflammatory response syndrome, that can progress to multiple organ failure and septic shock. This complex disorder is triggered by an inflammatory cascade initiated by bacteria-derived molecules such as endotoxin, with subsequent formation of endogenous inflammatory cytokines such as tumor necrosis factor (TNF)- α , leading to ischemia/reperfusion, mitochondrial dysfunction and apoptosis [1]. The gut is a triggering factor in the progression of sepsis [2]. Severe stress caused by conditions such as major trauma, burns, hemorrhagia and major surgery leads to intestinal ischemia/reperfusion and local production of pro-inflammatory cytokines, that contribute to the systemic inflammation. In addition, ischemia/reperfusion causes disturbance of the intestinal epithelial barrier and bacterial translocation [3], which is facilitated by impairment of bowel motility (ileus) [4]. The mechanism of sepsis-induced ileus, assessed by injecting endotoxins in animal models, shows similarity to postoperative ileus occurring after abdominal surgery, as endotoxin exposure or intestinal manipulation results in activation of the resident macrophages in the intestinal muscle, leading to production of pro-inflammatory cytokines such as TNF- α , chemokines and adhesion molecules. The latter are essential for further recruitment of circulatory leukocytes into the intestinal muscularis [5]. The mucosal layer seems a victim rather than an instigator in the pathogenesis of ileus.

Notably, in a mouse model of postoperative ileus we recently reported an early and transient increase in oxidative stress in the mucosal layer, with a peak value at 1 h after intestinal manipulation, preceding the peak in the muscularis [6]. The combination of oxidative stress-induced epithelial cell damage, increased intestinal permeability and translocation of intraluminal endotoxins might trigger the muscular inflammation [7]; TNF- α may play a role

in this epithelial distress. When combined with the protein synthesis inhibitor cycloheximide (CHX), TNF- α was shown to initiate apoptosis in isolated gastrointestinal epithelial cells [8,9]. Moreover, TNF- α /CHX-induced oxidative stress has been implicated in intestinal epithelial cell apoptosis [10]. To our knowledge, no *in vitro* study has yet fully explored TNF/CHX- α -induced apoptosis in a murine epithelial cell line. The murine intestinal epithelial cell line MODE-K is an undifferentiated cell line, which has the advantage of not being derived from tumor tissue, but from the duodenum-jejunum from normal young mice. Hence, it is considered to be an important model exhibiting phenotypic and functional properties of normal mouse enterocytes [11,12]. The first aim of our present study was to investigate the relationship between oxidative stress and apoptosis by TNF- α /CHX in MODE-K cells.

Heme oxygenase (HO) is an ubiquitous rate limiting enzyme that catabolizes heme into ferrous iron, carbon monoxide (CO) and biliverdin, which is subsequently reduced to bilirubin. The stress inducible isoform HO-1, which is upregulated by numerous stimuli including oxidative stress and proinflammatory cytokines [13], serves as a cytoprotective gene by virtue of its potent antioxidant, anti-apoptotic and anti-inflammatory properties [14]. Biliverdin and its end product bilirubin are potent antioxidants and were shown to have cytoprotective effects [15,16]. Accumulating evidence indicates that also CO can mediate many of the biological functions of HO-1 [15]. Accordingly, CO-releasing molecules (CO-RMs) have been developed to deliver CO in a controllable manner under physiological conditions providing an advantage of reduced risk of systemic toxicity [17,18,19]. The water soluble CO-RMs (CORM-3 and CORM-A1) [20,21] have gained increasing interest recently due to their promising pharmacological properties as carriers of therapeutic doses of CO in models of disease including gastrointestinal disorders [22,23]. Indeed, in a murine model of postoperative ileus, we have shown that the “fast” CO releaser CORM-3 markedly reduced

the manipulation-induced inflammation of the muscularis and improved surgically induced intestinal dysmotility. This was accompanied by reduction of the early oxidative burst in the mucosa [6]. The second aim of our present study was therefore to investigate the influence of HO-1-related products (bilirubin, biliverdin and CO-RMs) on the oxidative/apoptotic response of the MODE-K cell line to TNF- α /CHX. For comparison, several agents that are known to reduce the excessive production of reactive oxygen species (ROS) were also tested: apocynin and diphenylene iodonium (DPI), which can inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity; nitrite, that can inhibit mitochondrial ROS production [24]; Mito-TEMPO, a mitochondria-targeted antioxidant; and dimethyloxallyl glycine (DMOG), a prolyl hydroxylase inhibitor, that attenuates hypoxia-induced intestinal mucosal inflammation [25] and decreases hypoxia-induced ROS production in a human synovial cell line [26]. Finally, resveratrol, a cytoprotective bioactive polyphenol present in red wine, was tested given its potential to attenuate intestinal ischemia/reperfusion injury [27] and its HO-1 related neuroprotective action against stroke [28].

MATERIALS AND METHODS

Chemicals and reagents

Bilirubin, biliverdin (biliverdin IX hydrochloride) and cobalt protoporphyrin IX (CoPP) were purchased from Frontier Scientific Europe Ltd. (Carnforth, UK). 2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl triphenylphosphonium chloride monohydrate (Mito-TEMPO) was purchased from Enzo Life Sciences. Apocynin, cycloheximide, dimethyloxallyl glycine (DMOG), dimethyl sulfoxide (DMSO), diphenylene iodonium (DPI), nitrite (as sodium nitrite), propidium iodide, resveratrol and ribonuclease-A were obtained from Sigma (St. Louis, MO, USA). Recombinant murine TNF- α was purchased from R&D system (Minneapolis, MN, USA). Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, and glutamax were obtained from Gibco BRL (Grand Island, NY, USA). All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

Cell culture

The mouse small intestinal epithelial cell (IEC) line, MODE-K (a generous gift from Dr. Ingo B. Autenrieth, University of Tübingen, Germany) was used in our study. These are small IECs from C3H/HeJ mice immortalized by simian virus (SV)-40 large T gene transfer, exhibiting morphological and phenotypic characteristics of normal enterocytes [29]. MODE-K cells (passage 10–35) were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 5% penicillin/streptomycin. Cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C and experiments were conducted on cells at approximately 80-90% confluence. MODE-K cells were seeded at either 3.5×10^5 cells per 2

mL of culture medium containing 10% serum in a six-well plate (for apoptosis assay) or at 1×10^4 cells per 200 μ L of culture medium in a 96-well microtiter plate (for all other assays), grown for 36 h and then serum starved overnight. On day 3, cells were treated with various concentrations of TNF- α /cycloheximide (CHX) for 0-6 h. Test drugs were pre-incubated for 1 h followed by co-incubation of drugs with various concentrations of TNF- α /CHX for 3-6 h, with the exception of CoPP that was incubated for 3 h from 24 h till 21 h before exposure to TNF- α /CHX. The influence of test drugs alone on cell viability was studied on day 3 by incubating MODE-K cells for 12 h.

Determination of cell viability

Cell viability was assessed by luminescent cell viability assay with CellTiter-Glo (Promega, Madison, WI, USA) according to the manufacturer's protocol. This assay determines the number of viable cells in culture based on quantitation of ATP present, an indicator of metabolically active cells. Briefly, MODE-K cells were plated into 96-well plates and treated as described under cell culture. At the end of the incubation period with agents on day 3, an equivolume of the luminescent substrate and lysis buffer mix from the assay kit was added. The mixture was transferred to an opaque 96-well plate and luminescence was recorded using a GloMax Microplate Luminometer (Promega). The index of cellular viability was calculated as percentage of luminescence with respect to untreated control cells.

Measurement of caspase-3/7 activity

Caspase-3/7 activity was determined using the chemiluminescent Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity, and cell lysis. Briefly,

MODE-K cells were plated into 96-well plates and treated as described under cell culture. After incubation, an equivolume of luminescent caspase-3/7 Glo substrate buffer was then added followed by gentle agitation for 30 min. The mixture was transferred to an opaque 96-well plate and luminescence was recorded using a GloMax Microplate Luminometer (Promega). Relative luminescence units were noted and the results are expressed as percentage of luminescence with respect to untreated control.

Flow cytometry analysis of apoptosis

Cellular DNA content and apoptosis were analyzed by flow cytometry as described previously [30]. Briefly, cells were plated into six-well plates and after treatment for the indicated time, the cells were rinsed with PBS, harvested and then fixed by the progressive addition of ice-cold 70% ethanol. The fixed cells were washed twice with PBS before staining with propidium iodide (PI) at $100\ \mu\text{g}\cdot\text{mL}^{-1}$ and RNase at $50\ \mu\text{g}\cdot\text{mL}^{-1}$ in PBS for 20 min. Cell viability was determined by flow cytometry (FACSCalibur, BD Bioscience, NJ) and CellQuest software (Becton Dickinson, San Jose, CA, USA). Cell debris was excluded from analysis by appropriate raising of the forward scatter threshold. The percentage of cells that had undergone apoptosis was assessed to be the ratio of the fluorescent area smaller than the G_0 - G_1 peak to the total area of fluorescence. The average of the results from at least three samples for each experimental condition is presented.

Measurement of intracellular reduced glutathione (GSH) levels

The intracellular content of reduced glutathione (GSH) was quantified using the bioluminescent GSH-Glo Glutathione Assay (Promega, Madison, WI, USA), essentially as recommended by the supplier. In brief, MODE-K cells were plated into 96-well plates and treated with $\text{TNF-}\alpha/\text{CHX} \pm$ test drugs as described above. After incubation, adherent cells

were directly lysed in 100 μ l GSH-Glo lysis and reaction buffer. After addition of 100 μ l GSH-Glo Luciferin detection reagent, the mixture was transferred to an opaque 96-well plate and luminescence was detected using a GloMax Microplate Luminometer (Promega). The GSH levels of treated cells are expressed as percentage of the value in untreated control cells.

Measurement of intracellular reactive oxygen species (ROS) generation

The intracellular ROS level was detected using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) (Molecular Probes, Eugene, OR, USA) as reported [31]. Carboxy-H₂DCFDA is a cell-permeable indicator for ROS that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. When oxidized by various active oxygen species, it is irreversibly converted to the fluorescent form, DCF. The fluorescence generated by DCF is proportional to the rate of carboxy-H₂DCFDA oxidation, which is in turn indicative of the cellular oxidizing activity and intracellular ROS levels. For ROS measurements, briefly, MODE-K cells were plated into black 96-well plates and treated as described. Cells were washed twice in Hanks' balanced salt solution (HBSS) and then incubated in HBSS containing carboxy-H₂DCFDA (10 μ M) for 40 min in the dark at 37°C. Fluorescence was measured using an excitation of 485 nm and emission of 530 nm, in a fluorescence plate reader (Wallac EnVision 2101 multilabel reader, Perkin Elmer, Zaventem, Belgium). Basal ROS generation in the cells treated without the stimulus was used as a control.

Western blotting

Total cell extracts were prepared using RIPA buffer and protein concentrations were measured with the BCA protein assay reagent (Pierce). Equal amounts of protein (20 μ g) were loaded onto NuPAGE-Novex 4 to 12% Bis-Tris electrophoresis gels (Invitrogen) and blotted

onto nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Membranes were blocked in Tris-buffered saline/0.1% Tween 20 containing 5% nonfat dry milk and incubated overnight with appropriate antibodies for the detection of HO-1 (cat. no. sc-10789, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and β -tubulin (cat. no. ab6046, 1:1000; Abcam, Cambridge, UK). Horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology Inc., Danvers, MA) were visualized by use of Amersham ECL Prime Substrate (GE Healthcare Life Sciences). Densitometry of the bands was performed using Image J software (National Institutes of Health, Bethesda, MD).

Statistical analysis

All data were expressed as mean $\pm$ SEM. Comparison of the means was performed using the Student's *t*-test for two groups of data and ANOVA followed by Bonferroni's multiple comparison test for comparison of more than two groups. Differences were considered to be significant at $P < 0.05$.

RESULTS

Influence of TNF- α /CHX on cell viability

The aim of the first set of experiments was to assess the sensitivity of MODE-K cells to the cytotoxic effect of TNF- α by testing its influence on cell viability. TNF- α is a cytokine inducing apoptosis but many cell lines only undergo apoptosis when TNF- α is combined with an agent such as CHX. Previous observations in MODE-K cells showed that TNF- α *per se* in concentrations up to 10 ng·mL⁻¹ and incubated for up to 24 h did not induce cell death [32]. We therefore first tested the apoptotic stimulus used in other intestinal epithelial cell lines (20 ng·mL⁻¹ TNF- α plus 25 μ g·mL⁻¹ CHX) [8,33,34]. Incubation of MODE K-cells for 6 h with

this combination decreased cell viability to 10% of untreated cells, while neither agent alone influenced cell viability, the latter corresponding to data with the same concentrations of both agents in rat IEC-6 cells [8]. As preliminary experiments showed that the very pronounced effect of TNF- α (20 ng·mL⁻¹)/CHX (25 μ g·mL⁻¹) on cell viability was not influenced by any of the HO-1 related products, lower concentrations of TNF- α were investigated, in combination with either 25 or 10 μ g·mL⁻¹ CHX, as 10 μ g·mL⁻¹ CHX is also often used in combination with TNF- α in cell lines [35,36,37]. This showed a high sensitivity of MODE-K cells to TNF- α /CHX as 0.1–1 ng·mL⁻¹ TNF- α together with 10 μ g·mL⁻¹ CHX induced a concentration-dependent decrease in cell viability (to $54 \pm 3\%$ at 0.1 ng·mL⁻¹ and $22 \pm 1\%$ at 1 ng·mL⁻¹ TNF- α , Fig. 1A), the effect with 1 ng·mL⁻¹ TNF- α /10 μ g·mL⁻¹ CHX being close to that of 20 ng·mL⁻¹ TNF- α /25 μ g·mL⁻¹ CHX. The concentration range 0.1–1 ng·mL⁻¹ TNF- α was therefore studied in the assays for caspase-3/7 activity, apoptosis and oxidative stress.

Influence of TNF- α /CHX on caspase-3/7 activity and apoptotic cell death

The combination of TNF- α /CHX for 6 h induced an increase in caspase-3/7 activity in a concentration-dependent manner (Fig. 1B). TNF- α (0.1 ng·mL⁻¹)/CHX (10 μ g·mL⁻¹) increased caspase-3/7 activity by 3.6 fold when compared to control while TNF- α (1 ng·mL⁻¹)/CHX (10 μ g·mL⁻¹) increased the caspase-3/7 activity by nearly five fold. Concomitantly, flow cytometric analysis of propidium iodide-stained cells for apoptosis showed that TNF- α induced a concentration-dependent apoptotic cell death (Fig. 1C). In both assays, the result with TNF- α (1 ng·mL⁻¹)/CHX (10 μ g·mL⁻¹) approached that obtained with TNF- α (20 ng·mL⁻¹)/CHX (25 μ g·mL⁻¹) studied in comparison. To evaluate the kinetics of TNF- α /CHX-induced apoptosis, MODE-K cells were incubated with TNF- α (1 ng·mL⁻¹)/CHX (10 μ g·mL⁻¹) for 0–6 h. Induction of apoptosis began after exposure to TNF- α /CHX for 2 h and increased further in a time-dependent manner (Fig. 1D).

Influence of TNF- α /CHX on reduced glutathione (GSH) and reactive oxygen species (ROS) levels

As a sequential pathway of “ROS production $\rightarrow$ caspase-3 activity $\rightarrow$ apoptosis” has been proposed for TNF- α /CHX-induced cell death in rat IEC-6 cells [10], TNF- α /CHX-induced oxidative stress in MODE-K cells was assessed by measurement of intracellular ROS generation and of the classic antioxidant GSH. Incubation of MODE-K cells with 0.1–1 ng·mL⁻¹ TNF- α /CHX (10 μ g·mL⁻¹) for 3 h did not influence GSH levels, while TNF- α (20 ng·mL⁻¹)/CHX (25 μ g·mL⁻¹) significantly decreased the GSH level to $62 \pm 2\%$ of untreated cells (Fig. 2A). However, after 6 h of exposure, TNF- α /CHX reduced cellular GSH levels in a concentration-dependent manner, with 0.1 and 1 ng·mL⁻¹ TNF- α decreasing GSH levels to $69 \pm 3\%$ and $44 \pm 2\%$, respectively. TNF- α (20 ng·mL⁻¹)/CHX (25 μ g·mL⁻¹) added for 6 h decreased the GSH level to $21 \pm 1\%$.

Incubation of cells with TNF- α /CHX for 3 h also induced a concentration-dependent increase in ROS production (Fig. 2B). When co-incubated with 10 μ g·mL⁻¹ CHX, 0.1 and 1 ng·mL⁻¹ TNF- α increased ROS production to 2.73 fold and 4.52 fold, respectively, compared to control. A time-course experiment with TNF- α (1 ng·mL⁻¹)/CHX (10 μ g·mL⁻¹) showed an increase in ROS production starting at 2 h (Fig. 2B) illustrating that the increase in ROS production paralleled the induction of apoptosis.

Effects of HO-1 and antioxidant-related products on TNF- α /CHX-induced decrease in cell viability

To determine the highest concentration of the HO-1 and antioxidant-related products that was not cytotoxic *per se*, MODE-K cells were incubated with increasing concentrations of each compound for 12 h ($n = 3$ for each compound, data not shown). This concentration was then

tested on the decrease in cell viability by $0.1\text{--}1\text{ ng}\cdot\text{mL}^{-1}$ TNF- α /10 $\mu\text{g}\cdot\text{mL}^{-1}$ CHX to screen for the protective effects versus the cytotoxic action of TNF- α /CHX.

As for the HO-1 related products, 100 μM of bilirubin and 3 μM of biliverdin did not influence TNF- α /CHX-induced decrease in cell viability (data not shown), but 1 μM of bilirubin partially protected against 0.1 and 0.25 $\text{ng}\cdot\text{mL}^{-1}$ TNF- α /CHX (Fig. 3A). CORM-A1 (100 μM) and CORM-3 (100 μM) induced significant partial protection from cell death at all concentrations of TNF- α /CHX tested (Fig. 3B-C). The most effective CORM (CORM-A1) and the low concentration of bilirubin were selected for further study.

With regard to the antioxidant-related products, apocynin (250 μM), MitoTEMPO (1 μM) and DMOG (500 μM) did not influence the decrease in cell viability by TNF- α /CHX (data not shown). However, DPI (100 μM) showed prevention from TNF- α -induced cytotoxicity at 0.1-0.5 $\text{ng}\cdot\text{mL}^{-1}$ TNF- α /CHX, while nitrite (10 mM) showed cytoprotection at all tested concentrations of TNF- α /CHX (Fig. 3D-E). The most effective agent was resveratrol (75 μM), which completely prevented cell death by 0.1 $\text{ng}\cdot\text{mL}^{-1}$ TNF- α /CHX and restored cell viability in the presence of 1 $\text{ng}\cdot\text{mL}^{-1}$ TNF- α /CHX from $22 \pm 1\%$ to $65 \pm 3\%$ (Fig. 3F). On the basis of these results, nitrite (10 mM) and resveratrol (75 μM) were selected to further examine the relation between their influence on TNF- α /CHX-induced oxidative stress and on TNF- α /CHX-induced apoptosis.

Effects of bilirubin, CORM-A1, nitrite and resveratrol on TNF- α /CHX-induced changes in caspase-3/7 activity and apoptosis

Treatment with 1 μM bilirubin, 100 μM CORM-A1, 10 mM nitrite or 75 μM resveratrol significantly attenuated the TNF- α /CHX-induced increase in caspase-3/7 activity at all tested concentrations of TNF- α (Fig. 4A-D). Among these agents, nitrite and resveratrol showed a more pronounced decrease of the induced caspase activities compared to bilirubin or CORM-

A1. The anti-apoptotic effect was tested versus 0.1 and 1 ng·mL⁻¹ TNF- α . Bilirubin was not effective in preventing the apoptotic cell death at both tested TNF- α concentrations, while CORM-A1, nitrite and resveratrol partially prevented the apoptotic cell death induced by the two tested concentrations of stimulus (Fig. **5A-D**). The results with bilirubin illustrate that inhibition of TNF- α /CHX-induced caspase-3/7 activity does not necessarily result in a reduction of TNF- α /CHX-induced apoptosis.

Effects of bilirubin, CORM-A1, nitrite and resveratrol on TNF- α /CHX-induced changes in GSH levels and ROS production

The effect of the various agents on cellular levels of GSH were measured in the presence of TNF- α /CHX for 6 h. TNF- α (0.1–1 ng·mL⁻¹)/CHX significantly reduced cellular GSH levels while pretreatment with 1 μ M bilirubin, 100 μ M CORM-A1, 10 mM nitrite or 75 μ M resveratrol partially prevented this effect (Fig. **6A-D**). We next investigated whether any of these agents could suppress TNF- α /CHX-induced ROS production by direct measurement of carboxy-H₂DCFDA fluorescence. TNF- α (1 ng·mL⁻¹)/CHX increased the ROS production by 4.63 fold compared to control, which was reduced to 3.51 fold by bilirubin, 2.93 fold by CORM-A1, and 3.57 fold by nitrite (Fig. **7**). On the other hand, resveratrol completely abolished the TNF- α /CHX-induced increase in ROS production. The results with CORM-A1, nitrite and resveratrol suggest that inhibition of TNF- α /CHX-induced ROS production can contribute to the anti-apoptotic effects of compounds in MODE-K cells.

Effects of bilirubin, CORM-A1, nitrite and resveratrol on TNF- α /CHX-induced changes in HO-1 protein expression

As induction of the expression of endogenous HO-1 has been suggested to contribute to the cytoprotective effects of CO-RMs and resveratrol [28,38], the expression of HO-1 was

assessed by Western blotting. In MODE-K cells, the baseline HO-1 protein level was significantly decreased by TNF- α (1 ng·mL⁻¹)/CHX (Fig. 8). The HO-1 inducer CoPP (10 μ M) [39], used as a positive control, increased HO-1 expression markedly both in the absence and presence of TNF- α /CHX. Treatment with 1 μ M bilirubin, 100 μ M CORM-A1 or 10 mM nitrite did not affect basal HO-1 expression; in co-presence with TNF- α /CHX, the HO-1 level was not significantly different from that with TNF- α /CHX alone. Notably, 75 μ M resveratrol *per se* induced HO-1 expression significantly to 1.63 fold as compared to the basal level. However, pretreatment of 75 μ M resveratrol did not prevent the TNF- α /CHX-induced decrease in HO-1 protein expression. The results illustrate that HO-1 expression does not contribute to the cytoprotective effect of CORM-A1, nitrite and resveratrol in MODE-K cells.

DISCUSSION

The aim of this study was 1) to investigate the oxidative and apoptotic effects of TNF- α /CHX in the murine MODE-K epithelial cell line and 2) to evaluate the influence of HO-1 and antioxidant-related products on the oxidative/apoptotic response to TNF- α /CHX in MODE-K cells.

Induction of oxidative stress and cell death in MODE-K cells by TNF- α

The study showed that the MODE-K IEC line is highly sensitive to the cytotoxic effects of TNF- α with CHX, 0.1 ng·mL⁻¹ TNF- α /10 μ g·mL⁻¹ CHX halving cell viability and 1 ng·mL⁻¹ TNF- α /10 μ g·mL⁻¹ CHX reducing cell viability to values close to that by 20 ng·mL⁻¹ TNF- α /25 μ g·mL⁻¹ CHX. The concentration range 0.1-1 ng·mL⁻¹ corresponds to TNF- α concentrations observed in mice under pathophysiological conditions of sepsis or postoperative ileus. After injection of endotoxin, circulatory peak levels of 0.78 and 2.11

ng·mL⁻¹ TNF- α have been reported [40,41]; in mesenteric lymph derived from the gastrointestinal tract, still higher and more sustained TNF- α levels of approximately 3 ng·mL⁻¹ were observed [41]. After intestinal manipulation, a TNF- α level of 0.08 ng·mL⁻¹ was obtained in peritoneal lavage fluid [42] and of 2 ng·mL⁻¹·mg⁻¹ in the supernatant of exteriorized and cultured muscularis layer [43].

TNF- α /CHX-induced apoptosis occurred in parallel with ROS production. TNF- α (0.1-1 ng·mL⁻¹)/CHX treatment for 6 h induced concentration-dependent apoptosis correlating with its influence on cell viability. When studying the time-dependency of 1 ng·mL⁻¹ TNF- α , apoptosis started from 2 h of TNF- α incubation on, and this corresponded with the occurrence of ROS production. The concentration-dependent induction of apoptosis by TNF- α at 6 h was accompanied by a corresponding increase in caspase-3/7 activity, so that the sequence “*ROS production* $\rightarrow$ *caspase-3/7 activity* $\rightarrow$ *apoptosis*” as reported in rat IEC-6 cells [10] might also occur in MODE-K cells. The concentration-dependent induction of ROS by TNF- α at 3 h was not paralleled by a decrease in GSH, which only occurred at 6 h. GSH is a potent intracellular anti-oxidant that attenuates oxidative stress, while cytochrome c is a protein located in the mitochondrial intermembrane space which becomes pro-apoptotic when triggered by increased and sustained ROS production [44]. Oxidative stress, due to ROS, can cause depletion of GSH levels in the mitochondria leading to the release and oxidation of cytochrome c to the cytosol and subsequent caspase activation. Since reduced GSH in mitochondria is the only defense provided to metabolize peroxides generated from the electron transport chain through the GSH redox cycle [45], from our observation, it seems that the production of ROS in the form of peroxides starts at a later time point (after 3 h) during TNF- α /CHX exposure, while other forms of ROS (hydroxyl radical and superoxide anion) might play a role in earlier apoptosis induction from 2 h of TNF- α /CHX exposure in MODE-K cells.

Influence of HO-1 related products on TNF- α /CHX-induced oxidative stress and apoptosis

At the highest non-cytotoxic concentration used, CORM-3 and more effectively CORM-A1 reduced the decrease of cell viability by TNF- α , while 100 μ M bilirubin or 3 μ M biliverdin had no effect. However, at much lower concentration (1 μ M), bilirubin has been shown to elicit antioxidant effects with significant reduction in ROS production and cell proliferation in human airway smooth muscle cells [46]. At this concentration, our studies revealed that bilirubin partially prevented cell death induced by the two lower concentrations of TNF- α tested; although partially preventing the TNF- α (1 ng·mL⁻¹)/CHX-induced increase in ROS production and caspase-3/7 activity, bilirubin failed to show any prevention of TNF- α /CHX-induced apoptosis. This is in agreement with a study by Basuroy *et al.* [36] showing that bilirubin had only a mild effect on TNF- α /CHX-induced apoptosis although it reduced caspase-3 expression in a pronounced way. The mild effect of bilirubin on TNF- α /CHX-induced ROS-production is thus not sufficient to reduce TNF- α /CHX-induced apoptosis; neither is its mild effect on caspase-3/7 activity. The latter can be related to caspase-independent pathways of TNF- α -induced apoptosis as TNF- α has been reported to also induce caspase-independent cell death in a variety of cells like macrophages [47], neutrophils [48] and, in particular, HT29 human adenocarcinoma cells [49].

The reduction of TNF- α /CHX-induced caspase-3/7 activity by CORM-A1 was also very mild, but in contrast to bilirubin, CORM-A1 was able to clearly reduce TNF- α /CHX-induced apoptosis. This dissociation can be attributed again to caspase-independent pathways of TNF- α -induced apoptosis. The exact mechanism of the anti-apoptotic effect of CORM-A1 in MODE-K cells remains to be clarified, but our study shows that its anti-apoptotic effect correlates with a reduction in ROS production, which was more pronounced than that

observed with bilirubin, as well as an increase in GSH levels. Indeed, caspase-independent induction of apoptosis by ROS has been reported in PC12 pheochromocytoma cells so that inhibition of ROS production *per se* can be expected to reduce apoptosis [50]. The finding that CORM-A1 exerts significant protection against both TNF- α /CHX-induced oxidative stress and apoptosis supports the notion that CO can mediate the well-known anti-apoptotic effect of HO-1.

CO-RMs were shown to induce HO-1 expression in different types of cells [51,52] and this was suggested to contribute to their anti-apoptotic effect [38]. In thyroid carcinoma cells, HO-1 induction by hemin was shown to protect against TNF- α /CHX-induced apoptosis [53]. In MODE-K cells in the actual study, basal HO-1 levels were decreased by 3 h exposure to TNF- α plus the protein synthesis inhibitor CHX, although TNF- α alone can reduce endogenous HO-1 expression in some cells [54]. CoPP, a well-known HO-1 inducer as also confirmed in control MODE-K cells, still clearly induced HO-1 when cells were also incubated with TNF- α /CHX. However, this was not the case with CORM-A1, excluding the possibility that endogenous HO-1 induction contributes to the anti-apoptotic effect of CORM-A1 in MODE-K cells. Correspondingly, exogenous CO protected pancreatic β -cells against TNF- α -induced apoptosis even when HO-1 activity was blocked [55].

Influence of antioxidant related products on TNF- α /CHX-induced oxidative stress and apoptosis

Apocynin is widely used as an NADPH oxidase inhibitor [56] and in Caco-2 cells, it was shown to inhibit polychlorinated biphenyls-induced NADPH oxidase activation and disruption of epithelial integrity [57]. However, in the present study we found that apocynin did not protect at all MODE-K cells from TNF- α /CHX-induced cytotoxicity. This does not discard the possibility that NADPH oxidase is participating to TNF- α /CHX-induced ROS

production in MODE-K cells since the inhibitory effect of apocynin on NADPH oxidase activity has been questioned [58]. Another NADPH oxidase inhibitor, DPI [59], partially protected MODE-K cells from TNF- α /CHX-induced cell death at lower concentrations of TNF- α , but not at 1 ng·mL⁻¹ TNF- α .

Mitochondrial ROS has also been implicated in TNF- α -induced cytotoxicity [60]. In our study, nitrite but not the mitochondrial-targeted antioxidant Mito-TEMPO influenced TNF- α /CHX-induced decrease in cell viability of MODE-K cells. The concentration of nitrite (10 mM) used was shown to provide complete rescue of UVA-induced apoptotic cell death in rat aortic endothelial cells [61]. We also found that nitrite reduced TNF- α /CHX-induced caspase-3/7 activation. It had a similar influence on TNF- α /CHX-induced ROS production as bilirubin, but in contrast to bilirubin, it also reduced TNF- α /CHX-induced apoptosis. In ischemia/reperfusion injury, nitrite has been shown to act by decreasing mitochondrial ROS generation through inhibition of complex I activity [24]. The fluorescent probe, carboxy-H₂DCFDA, used in this study as an indicator of generalized oxidative stress is not specific for a single type of ROS like H₂O₂, peroxynitrite and peroxides [62,63]. Consequently, this probe can detect different ROS types from mitochondria, lipid oxidation and other sources, and the similar degree of ROS reduction by nitrite and bilirubin does not necessarily mean that the same source of ROS is influenced by both agents. A more important role of mitochondrial ROS, rather than ROS from other sources, in the induction of apoptosis, might then explain why nitrite, preferentially reducing mitochondrial ROS production, is able to reduce apoptosis while bilirubin is not.

Despite the hydroxylase inhibitor DMOG has been reported to protect intestinal epithelial cells against TNF- α -induced apoptosis *in vitro* [64] and to decrease hypoxia-induced ROS production in human synovial cells [26], it failed to prevent TNF- α /CHX-induced cell death in MODE-K cells. Resveratrol, a bioactive polyphenol from red wine, has shown

cytoprotection in many models related to interaction with multiple targets, such as, for example, induction of silent information regulator-1 (SIRT-1) [65]. Its attenuating effect on dextran sulfate sodium-induced colitis was also related to SIRT-1 induction [66]. Recently, induction of HO-1 was implied in the neuroprotective effect of resveratrol against stroke [28]. In our study, resveratrol *per se* increased basal HO-1 protein expression in MODE-K cells, but it did not prevent the decrease by TNF- α /CHX excluding HO-1 induction as the mechanism of action for its protective effect versus TNF- α /CHX. In MODE-K cells, resveratrol was by far the most effective cytoprotective agent as it fully abolished TNF- α /CHX-induced ROS production and caspase-3/7 activation; surprisingly, it only reduced apoptosis by approximately 50%. This suggests that TNF- α /CHX-induced apoptosis of MODE-K cells can occur in a ROS- and caspase-3/7-independent way.

CONCLUSION

In summary, these data indicate that mouse intestinal epithelial MODE-K cells are highly sensitive to TNF- α -induced apoptosis in the presence of CHX. TNF- α /CHX-induced apoptosis was accompanied by increased intracellular ROS generation and caspase-3/7 activation. CORM-A1, nitrite and most importantly resveratrol attenuated TNF- α /CHX-induced ROS production, caspase-3/7 activation and apoptosis. These agents might therefore be useful to prevent epithelial damage during ischemia/reperfusion injury.

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LIST OF ABBREVIATIONS

TNF- α , tumor necrosis factor (TNF)- α ; CHX, cycloheximide; HO-1, heme oxygenase-1, CO-RMs, carbon monoxide-releasing molecules; CORM-A1, sodium boranocarbonate; ROS, reactive oxygen species; DPI, diphenylene iodonium; NADPH, nicotinamide adenine dinucleotide phosphate; DMOG, dimethyloxallyl glycine; IEC, intestinal epithelial cell; GSH, reduced glutathione

CONFLICT OF INTEREST

The authors report no conflict of interest.

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Figure Legends

Fig. (1). Influence of $0.1\text{--}1\text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $10\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX and of $20\text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $25\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX, incubated for 6 h, on cell viability (as % of control; A), caspase-3/7 activity (as % of control; B), and apoptosis (C). Control cells were incubated with serum free medium alone. Mean $\pm$ SEM of six independent experiments. $*P < 0.05$ versus control. D: Influence of $1\text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $10\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX, incubated for 0-6 h, on apoptosis. Mean $\pm$ SEM of three independent experiments. $*P < 0.05$ versus untreated (0 h) control group.

Fig. (2). Influence of $0.1\text{--}1\text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $10\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX and of $20\text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $25\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX, incubated for 3 h (A, B) or 6 h (A), on cellular glutathione (GSH; as % of control; A) and intracellular ROS levels (B). Control cells were incubated with serum free medium alone. Mean $\pm$ SEM of six (A) or two (B) independent experiments. $^{\#}P < 0.05$ versus control (for 3 h). $*P < 0.05$ versus control (for 6 h). C: Influence of $1\text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $10\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX, incubated for 0-3 h, on intracellular ROS levels. Mean $\pm$ SEM of two independent experiments. $*P < 0.05$ versus untreated (0 h) control group.

Fig. (3). Influence of $0.1\text{--}1\text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $10\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX, incubated for 6 h, on cell viability (as % of control) in the absence and presence of $1\text{ }\mu\text{M}$ bilirubin (BR, A), $100\text{ }\mu\text{M}$ CORM-A1 (B), $100\text{ }\mu\text{M}$ CORM-3 (C), $100\text{ }\mu\text{M}$ diphenylene iodonium (DPI, D), 10 mM nitrite (Nit, E) or $75\text{ }\mu\text{M}$ resveratrol (Res, F). Control cells were incubated with serum free medium alone. Mean $\pm$ SEM of six independent experiments. $*P < 0.05$ versus control. $^{\#}P < 0.05$ versus corresponding group with TNF- α /CHX alone.

Fig. (4). Influence of 0.1-1 ng·mL⁻¹ TNF- α with 10 μ g·mL⁻¹ CHX, incubated for 6 h, on caspase-3/7 activity (as % of control) in the absence and presence of 1 μ M bilirubin (BR, A), 100 μ M CORM-A1 (B), 10 mM nitrite (Nit, C) or 75 μ M resveratrol (Res, D). Control cells were incubated with serum free medium alone. Mean $\pm$ SEM of six independent experiments. * P < 0.05 versus control. # P < 0.05 versus corresponding group with TNF- α /CHX alone.

Fig. (5). Influence of 0.1 and 1 ng·mL⁻¹ TNF- α with 10 μ g·mL⁻¹ CHX, incubated for 6 h, on apoptosis in the absence and presence of 1 μ M bilirubin (BR, A), 100 μ M CORM-A1 (B), 10 mM nitrite (Nit, C) or 75 μ M resveratrol (Res, D). Control cells were incubated with serum free medium alone. Mean $\pm$ SEM of three independent experiments. * P < 0.05 versus control. # P < 0.05 versus corresponding group with TNF- α /CHX alone.

Fig. (6). Influence of 0.1-1 ng·mL⁻¹ TNF- α with 10 μ g·mL⁻¹ CHX, incubated for 6 h, on cellular glutathione (GSH; as % of control) in the absence and presence of 1 μ M bilirubin (BR, A), 100 μ M CORM-A1 (B), 10 mM nitrite (Nit, C) or 75 μ M resveratrol (Res, D). Control cells were incubated with serum free medium alone. Mean $\pm$ SEM of six independent experiments. * P < 0.05 versus control. # P < 0.05 versus corresponding group with TNF- α /CHX alone.

Fig. (7). Influence of 1 ng·mL⁻¹ TNF- α with 10 μ g·mL⁻¹ CHX, incubated for 3 h, on intracellular ROS levels in the absence and presence of 1 μ M bilirubin (BR), 100 μ M CORM-A1, 10 mM nitrite (Nit) or 75 μ M resveratrol (Res). Control cells were incubated with serum free medium alone. Mean $\pm$ SEM of four independent experiments. * P < 0.05 versus control. # P < 0.05 versus 1 ng·mL⁻¹ TNF- α /10 μ g·mL⁻¹ CHX alone.

Fig. (8). Influence of $1 \text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $10 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX, incubated for 3 h, on HO-1 protein expression (relative to β -tubulin) in the absence and presence of $10 \text{ }\mu\text{M}$ cobalt protoporphyrin (CoPP), $100 \text{ }\mu\text{M}$ CORM-A1, $1 \text{ }\mu\text{M}$ bilirubin (BR), $75 \text{ }\mu\text{M}$ resveratrol (Res) or 10 mM nitrite (Nit). Control cells were incubated with serum free medium alone. The influence of $20 \text{ ng}\cdot\text{mL}^{-1}$ TNF- α with $25 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX and DMSO, the solvent of Res was also studied. Mean $\pm$ SEM of three independent experiments. $*P < 0.05$ versus control. $^{\#}P < 0.05$ versus $1 \text{ ng}\cdot\text{mL}^{-1}$ TNF- α / $10 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ CHX alone. Representative immunoblots are shown at the top of the figure.

Fig. (1)

Fig. (2)

Fig. (3-i)

Fig. (3-ii)

Fig. (4)

Fig. (5)

Fig. (6)

Fig. (7)

Fig. (8)