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Nitric oxide and thioredoxin type 1 modulate the activity of caspase 8 in HepG2 cells

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ABSTRACT

Herein, we report that nitric oxide (NO) and the thioredoxin/thioredoxin reductase system affect the activity of caspase 8 in HepG2 cells. Exposure of cells to NO resulted in inhibition of caspase 8, while a subsequent incubation of the cells in NO-free medium resulted in spontaneous reactivation of the protease. The latter process was inhibited in thioredoxin reductase-deficient HepG2 cells, in which, however, lipoic acid markedly reactivated caspase 8. The data obtained suggest that extrinsic apoptosis can be subjected to redox regulation before induction of proteolytic damage by caspase 3.

Introduction

Tumor necrosis factor alpha (TNF-α) is a cytokine involved in regulation of immune cells, tumorigenesis, inflammation, and apoptotic/necrotic cell death (reviewed in [1]). Exposure of mammalian cells to TNF-α and inhibitors of transcription or translation, such as actinomycin D and cycloheximide (CX), leads to induction of apoptosis in a cell-specific manner by sequential activation of either caspases 8 and 3 or of 8, 9 and 3 [1–3]. Conversely, nitric oxide (NO) produced by low molecular mass compounds or intracellularly by nitric oxide synthases inactivates caspases 9, 8 and 3 via reactions of *S*-nitrosation [1,4–6]. The inhibitory effect of NO on caspase 3 is counteracted by thioredoxin type 1 (HTrxn), which catalyzes the denitrosation of *S*-nitrosocaspase 3 back to its proteolytically active SH state [7–9]. Hence, shifts in the rates of *S*-nitrosation and denitrosation following changes in either NO production or activity of HTrxn can modulate apoptotic pathways. Herein, we present experimental evidence that in human hepatocellular liver carcinoma cells (HepG2 cells) exposed to TNF-α and CX, both NO and HTrxn affect the caspase cascade at its initial stage of activation of caspase 8.

Materials and methods

Reagents. All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). The solutions used in the experiments were prepared in deionized and Chelex-100-treated water or potassium phosphate buffer.

Cell culture and treatments. HepG2 cells were cultured in the Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine in a humidified atmosphere in 5% CO_2 at 37 °C.

Activation of caspases in HepG2 cells. Cells (2×10^6 cells/flask) were grown in T75 flasks for 24 h and then incubated for 6 h with medium (control) or with medium containing TNF-α (1–50 ng/mL; Fig. 1)) and cycloheximide (40 μM). Whole-cell lysates for analysis of caspases 8 and 3 were harvested by repeated freeze and thaw cycles followed by centrifugation at 15,000*g* for 3 min at 4 °C. The activities of caspases 3 and 8 were measured fluorometrically on a LS50B Perkin Elmer spectrofluorimeter using either 0.1 mM Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin or 0.1 mM *N*-Acetyl-Ala-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin as a substrate (λ_{ex} = 380 nm; λ_{em} = 420 nm; excitation/emission slit, 5 nm). Activity calculations for caspases were corrected with the effect of specific inhibitors.

siRNA transfection. The *si*RNA directed against TrxnR was 5′-AGACCACGUUACUUGG GCAdTdT-3′ and the control was a scrambled sequence (5′-AGGCAAAUCACGGUGUCCUdTdT-3′) that does not match any sequence in the GenBank human database for >16 nt ([10]; Dharmacon RNA Technologies; Chicago, IL). Approximately 2×10^5 HepG2 cells were plated per well in a six-well plate.

Abbreviations: GSNO, *S*-nitrosoglutathione; HTrxn, human thioredoxin type 1; LA, lipoic acid; DHLA, dihydrolipoic acid; TrxnR, human thioredoxin reductase; CX, cycloheximide; TNF-α, tumor necrosis factor alpha.

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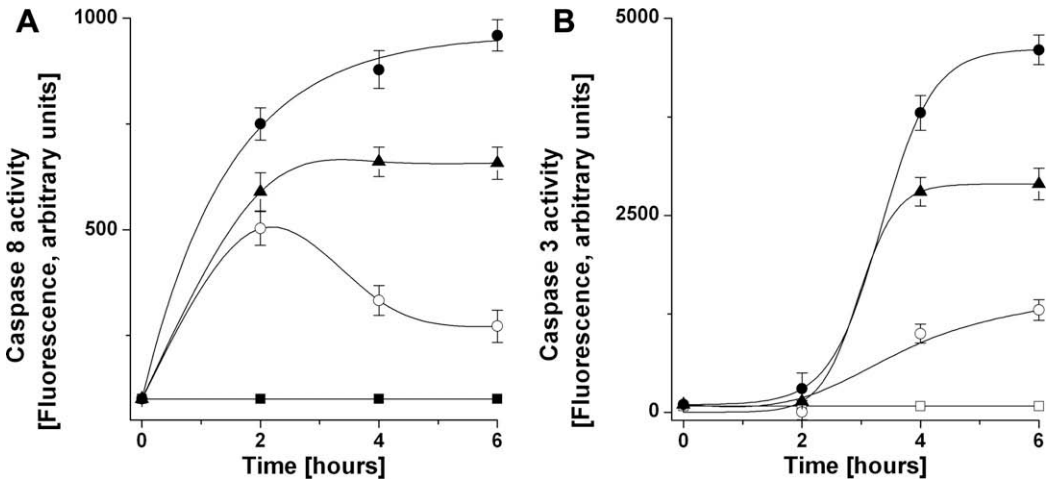

Fig. 1. TNF-α/CX-induced activation of caspases 3 and 8 in HepG2 cells. Incubations of cells and assessment of caspases were carried out as described in Methods. TNF-α was used at concentrations of 1 (open circles), 10 (triangles) and 50 (closed circles) ng/mL; CX, 40 μM. Control experiments for inhibition of caspases 3/7 and 8 were carried out with 5-[(*S*)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin (50 μM; A, closed rectangles) and acetyl-Ile-Glu-Thr-Asp-al (50 μM; B, open rectangles), respectively. Data are presented as mean + SE ($n = 3$).

The following day, cells were transfected with *si*RNA (30 pmol per well [11]) in the presence of lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) by following the manufacturer's recommendations. Transfection with the same amount of nonspecific *si*RNA was performed as control. The cells were harvested and analyzed at 48 h after transfection for cell viability and activity.

TrxnR assay. TrxnR activity was determined in a coupled assay with *Escherichia coli* Trxn (10 μM) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described in Ref. [8]. One unit of TrxnR activity was defined as the formation of 74 μmol of 5-mercapto-2-nitrobenzoic acid (1 absorbance unit at 412 nm; $\varepsilon_{412} = 13500\ M^{-1}\ cm^{-1}$) per min per mL at pH 7.0 at 25 °C.

Results and discussion

NO suppresses proteolytic processing and activation of multiple (pro)caspases in intact cells, including caspases 3 and 8 [12,13]. In model experiments with recombinant caspase 8, it was shown that 1 mol of protein binds 1.7 mol of NO with concomitant loss of enzymatic activity, whereas reduction of *S*-nitrosocaspase 8 with DL-dithiothreitol (DTT) resulted in reactivation of the native protease [13]. While the reductions of *S*-nitrosothiols by low molecular mass compounds with vicinal dithiols and by HTrxn follow a common reaction mechanism [8,9,14,15], we were interested to verify whether HTrxn affects the activity of caspase 8 in cells exposed to NO. To this end, control and TrxnR-deficient HepG2 cells were treated with TNF-α, CX and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and then the activity of caspase 8 was assessed in cell lysates. In contrast to hepatocytes, HepG2 cells are deficient in some phase I enzymes [16–18], including alcohol dehydrogenase class III, also referenced as *S*-nitrosoglutathione (GSNO) reductase, which catalyses the denitrosation of GSNO with consumption of NADH [19].

Exposure of HepG2 cells to TNF-α and CX led to a sequential activation of caspases 8 and 3, whereby the activity of caspase 3 was insignificant during the first 2 h of incubation (Fig. 1). Under these experimental conditions, no activation of caspase 9 was observed (data not shown). To minimize the possibility for activation of caspases 8 and 9 by caspase 3 via feedback mechanisms [20], the effects of HTrxn and NO on caspase 8 activity were assessed in control and TrxnR-deficient cells exposed to 10 ng of TNF-α mL for 1 h. In cells, TrxnR was silenced using *si*RNA (Fig. 2A; [8,21]). In turn, the cells were incubated for 1 h with 0.5 mM SNAP, washed with PBS, and the activity of caspase 8 was assessed in cell lysates either immediately (time for preparation of reaction solutions for spectral analysis, 5 min) or after an additional incubation for 60 min at 37 °C in SNAP-free incubation medium. The rationale for this experimental design followed the hypothesis that in SNAP-free medium time-dependent increases in caspase 8 activity would reflect the rates of endogenous reactions of *S*-denitrosation.

Treatment of control HepG2 cells with SNAP led to ~50% (5 min) and 5% (post-incubation for 60 min in SNAP-free medium) inhibition of caspase 8 (Fig. 2B). The regeneration of caspase 8 activity was insignificant in TrxnR-deficient cells (Fig. 2C), which suggests the requirement for HTrxn catalysis in this process. In this set of experiments, qualitatively the same effects were observed when SNAP was replaced with 0.1 mM *S*-nitrosocysteine ethyl ester (incubation time, 15 min; data not shown), which readily crosses cell membranes and *trans*-*S*-nitrosates cellular proteins [9,22].

The possibility for reductive reactivation of caspase 8 was further supported by experiments with DTT and lipoic acid (LA; 5-[1,2]dithiolan-3-yl-pentanoic acid), a cyclic disulfide that is an essential prosthetic group in the dihydrolipoyl transacetylase component of the alpha-ketoacid dehydrogenase complex in mitochondria. In cells, LA is reduced to dihydrolipoic acid (DHLA; 6,8-dimercaptooctanoic acid) by lipoamide dehydrogenase (LD) with consumption of NADH and by NADPH-dependent reductases [23,24]. The LA/LD/NADH system was shown to catalyze the denitrosation of both GSNO [9] and *S*-nitroso caspase 3 [21]. Accordingly, post-SNAP incubation of TrxnR-deficient cells with LA led to a marked reactivation of caspase 8 activity (Fig. 2C).

The highly conserved catalytic domains of caspases 3 and 8 contain a cysteine thiyl anion that adds to peptide carbonyls in protein substrates to form sulfanylpropanolates and then is regenerated following a C–N scission and hydrolysis of the corresponding sulfanylpropanones [25,26]. Caspase 3 can be inhibited by poly-*S*-nitrosation, whereby all SNO functions in its p12 subunit are denitrosated by GSH, except for a single SNO group [27]. Since the latter was not observed in a mutant form of caspase 3 lacking the active site cysteine, Zach et al. proposed that, in cells, NO nitrosates this cysteine to form *S*-nitrosocaspase 3, which is inert to reduction by GSH [27]. Recently, we reported that two enzymatic systems, LA/LD/NADH and HTrxn/TrxnR/NADPH, reduce *S*-nitrosocaspase 3 to caspase 3 [8,9,21]. In HepG2 cells, the effects of NO, HTrxn, and LA

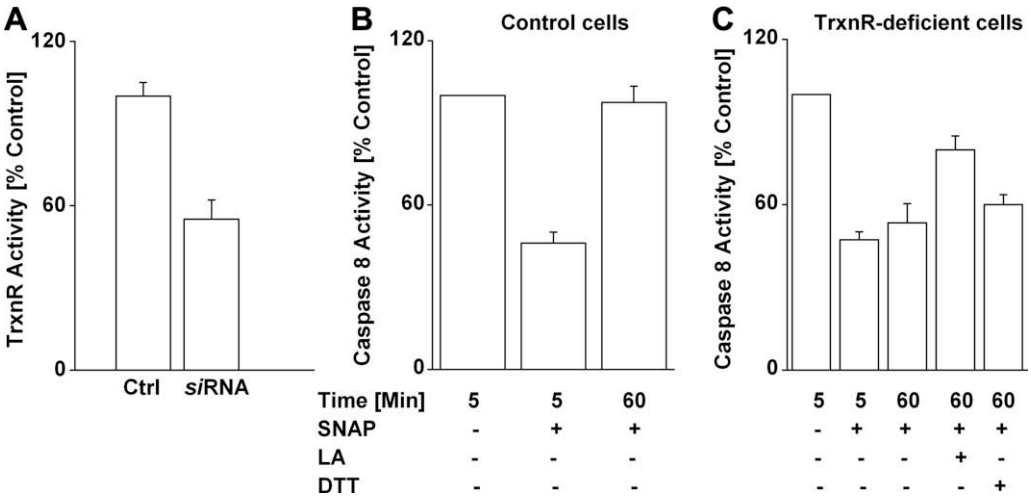

Fig. 2. Inhibition of TrxnR and activation of caspase 8 in HepG2 cells. Experiments aimed to silence TrxnR in HepG2 cells with *si*RNA (A,C) and to activate caspase 8 with TNF-α and CX (B,C) were carried out as described in Methods. SNAP, 0.5 mM; LA, 50 μM; DTT, 5 mM. The results represent the mean ± SE ($n = 3$).

on the activities of caspases 3 and 8 were comparable ([8,21]; Fig. 2), which is consistent with the similarity of the active sites of these proteases. However, because of its initiating role in Fas- or TNF-induced apoptosis, caspase 8 is a preferential target for drug therapy [28].

Conclusions

The data presented herein suggest that, prior to proteolytic damage induced by caspase 3, extrinsic apoptosis can be subjected to enzymatic redox regulation via *S*-(de)nitrosation of caspase 8. Studies are underway to verify whether in this model the reactivation of caspase 8 reflects direct reduction of SNO or SS functions in the nitrosated protein, or a shift in the equilibrium between caspase 8, GSNO, *S*-nitrosocaspase 8 and GSH in favor of caspase 8 due to reduction of GSNO by HTrxn [9,29].

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