CD95-tyrosine nitration inhibits hyperosmotic and CD95 ligand-induced CD95 activation in rat hepatocytes

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CD95-Tyr-NO₂; CD95-tyrosine nitration
CD95-Tyr-P; CD95-tyrosine phosphorylation
DISC; death inducing signaling complex
FADD; fas associated death domain
EGFR; epidermal growth factor receptor
Huh7; human hepatoma cell line 7
JNK; c-Jun-N-terminal kinase
MPO; myeloperoxidase
NOS; nitric oxide synthase
PKC; protein kinase C
ONOO⁻; peroxynitrite
PSG; phosphate-saline-glucose buffer
TUNEL; terminal deoxynucleotidyl transferase-mediated X-dUTP nick-end labelling
YFP; yellow fluorescent protein
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Abstract

Epidermal growth factor receptor (EGFR)-dependent CD95-tyrosine phosphorylation was recently identified as an early step in apoptosis induction via the CD95 system (Reinehr et al., FASEB J 2003). The effect of peroxynitrite (ONOO$^-$) on modulation of the hyperosmotic and CD95L-induced CD95 activation process was studied. Pretreatment of hepatocytes with ONOO$^-$ inhibited CD95 ligand (CD95L)- and hyperosmolarity-induced CD95 membrane trafficking and formation of the death-inducing signaling complex (DISC), but not EGFR activation and its association with CD95. Under these conditions, however, no tyrosine phosphorylation of CD95 occurred; instead CD95 was tyrosine-nitrated. When ONOO$^-$ was added after induction of CD95-tyrosine phosphorylation by CD95L or hyperosmolarity, tyrosine nitration of CD95 was largely prevented and DISC formation occurred. CD95-tyrosine nitration abolished the hyperosmotic sensitization of hepatocytes towards CD95L-induced apoptosis. Also in CD95-YFP-transfected Huh7-hepatoma cells, ONOO$^-$ induced CD95 Tyr-nitration and prevented CD95L-induced Tyr-phosphorylation and apoptosis. Tyrosine-nitrated CD95 was also found in rat livers derived from an in vivo model of endotoxinemia. The data suggest that CD95-tyrosine nitration prevents CD95 activation by inhibiting CD95-tyrosine phosphorylation. Apparently, CD95-tyrosine phosphorylation and nitration are mutually exclusive. The data identify critical tyrosine residues of CD95 as another target of the antiapoptotic action of NO.
Introduction

Apoptosis plays an important role in the pathogenesis of liver injury with activation of the CD95 (also known as Fas/APO-1) system in response to hyperosmolarity, CD95 ligand, hydrophobic bile acids or ethanol.\(^1\)-\(^{11}\) In hepatocytes, CD95 activation is a complex process, which involves rapid activation of the EGFR, its JNK-dependent association with CD95 and subsequent tyrosine phosphorylation of CD95 by the EGFR-tyrosine kinase activity.\(^4\),\(^6\) CD95-tyrosine phosphorylation then triggers CD95 membrane trafficking, DISC formation and in the case of hydrophobic bile acids and CD95L execution of apoptosis.\(^4\),\(^6\) CD95 activation and DISC formation are also triggered by hyperosmotic cell shrinkage, however, despite this hepatocytes do not undergo spontaneous apoptosis, but are sensitized towards CD95L-induced apoptosis.\(^1\) The critical role of CD95-tyrosine phosphorylation for apoptosis induction/sensitization is underlined by the findings that inhibitors of EGFR activation or its tyrosine kinase activity abolish CD95 activation, membrane trafficking and apoptosis.\(^4\),\(^6\) Recent studies indicate that nitric oxide (NO) can protect various cell types from apoptotic cell death.\(^12\)-\(^{15}\) This protective role of NO involves cyclic guanine monophosphate formation,\(^13\) caspase 8 S-nitrosylation, prevention of loss of mitochondrial membrane potential, Bid cleavage and cytochrome c release.\(^16\)-\(^{18}\) Nitric oxide is also a precursor of peroxynitrite, a very potent nitrating agent which can induce nitration of critical protein tyrosine residues with important consequences for signal transduction pathways.\(^19\),\(^20\) The aim of present study therefore was to examine a possible interplay between phosphorylation and nitration of the CD95-system and its impact on hyperosmotic- and CD95L-induced apoptosis. The data identify CD95-nitration as a novel mechanism for apoptosis inhibition by NO, which competes with proapoptotic CD95-tyrosine phosphorylation.
Materials and Methods

Materials

The materials used were purchased as follows: collagenases from Boehringer (Mannheim, Germany); William's E medium and taurolithocholate-3-sulfate (TLCS) from Sigma-Aldrich (Deisenhofen, Germany); penicillin/streptomycin from Biochrom (Berlin, Germany); fetal calf serum from Gibco Life Technologies (Gaithersburg, MD, USA); DMEM/nut.mix F12, Lipofectamine 2000 and pTOPO-TA vector from Invitrogen (Karlsruhe, Germany); caspase-3 and -8 assays from R&D Systems (Wiesbaden, Germany); TUNEL assay from Roche Diagnostics (Mannheim, Germany). Soluble CD95-ligand (CD95L) was obtained from Alexis Corp. (Grünberg, Germany) and was always employed with 10-fold amount of enhancer protein (as provided by the supplier). The antibodies used were purchased as follows: rabbit anti-CD95, mouse anti-GFP (which crossreacts with YFP), rabbit anti-FADD and mouse anti-caspase 8 antibodies from Santa Cruz Biotechnology (CA, USA); goat anti-rabbit Cy3-conjugated antibody from Dianova (Hamburg, Germany); rabbit anti-phospho-JNK-1/-2 and rabbit anti-phospho-EGFR antibodies from BioSource Int. (Camarillo, CA, USA); mouse anti-phospho-serine and anti-phospho-threonine antibodies from Biomol (Hamburg, Germany); mouse anti-3'-nitrotyrosine from Calbiochem (Bad Soden, Germany); sheep anti-EGFR and mouse anti-phospho-tyrosine antibodies from Upstate Biotechnology (Lake Placid, NY); horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG from Bio-Rad (Hercules, CA, USA). All other chemicals were from Merck (Darmstadt, Germany) at the highest quality available.
Plasmid construction

The nucleotide sequence encoding the CD95 receptor was amplified by RT-PCR using HepG2 mRNA as template with the following primers: 5’-GTC GAC CAC TTC GGA TTG CTC AAC-3’ and 5’ – CTC TAG ACT AGA CCA AGC TTT GGA TTT C –3’. The resulting 1 kb fragment was ligated into the pTOPO-TA vector to proceed pTOPO-TA-CD95. For construction of CD95-YFP, PCR-directed mutagenesis was performed in the template pTOPO-TA-CD95R using the oligonucleotide primers 5’-TGC TCG AGA TGC TGG GCA TCT GGA CCC TCC TAC CT- 3’ and 5’- CGG TAC CGT CGA CAC CAA GCT TTG GAT TTC ATT TCT-3’ to remove the stop codon of CD95 and introduce a Xho1 site on the 5’ end and a Kpn1 on the 3’end. The Kpn1-Xho1 fragment of CD95 was subsequently inserted into pEYFP-N1 (Clontech, Palo Alto, CA, USA) to create a receptor fusion protein with YFP linked to the C-terminal cytoplasmatic tail. The construct was confirmed by sequencing (MWG Biotech, Ebersberg, Germany).

Cell preparation and culture

Hepatocytes were isolated from livers of male Wistar rats, fed ad libitum with a standard diet, by a collagenase perfusion technique as described previously. Aliquots of 1.5 x 10^6 cells were plated on collagen-coated 6-well culture plates (Falcon, Heidelberg, Germany) and cultured as published recently. The viability of the hepatocytes was more than 95 % as assessed by trypan blue exclusion.

Human hepatoma cell line 7 (Huh7) cells were cultured in a humidified 5% CO_2_ atmosphere at 37°C in DMEM/nut.mix F12 supplemented with 10% fetal calf serum and 1% penicillin/streptomycin resulting in a final osmolarity of 305 mosmol/L as measured using a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany). Cells were grown to 70%
confluency before transient transfection using expression vectors of the CD95-YFP fusion protein supplemented with Lipofectamine 2000 culture medium without antibiotics.

Osmolarity changes were performed by appropriate addition or removal of NaCl from the medium.

**Incubations with peroxynitrite (ONOO⁻)**

According to the manufacturer’s recommendations (Calbiochem, Bad Soden, Germany), the concentration of peroxynitrite (ONOO⁻) was determined spectrophotometrically using its absorbance at 302 nm with an extinction coefficient of 1670 M⁻¹ cm⁻¹ in 0.1 M NaOH prior to the experiments. ONOO⁻ was instituted for 15 min either before or after the experimental periods, i.e. exposure to normoosmolarity (305 mosmol/L), hyperosmolarity (405 mosmol/L) or CD95L for the time periods indicated. For this purpose, medium was removed from the respective culture dishes and cells were washed twice with phosphate buffered saline (PBS). Thereafter phosphate-saline-glucose buffer (PSG) containing 100 mmol/L KH₂PO₄, 10 mmol/L NaCl and 5 mmol/L glucose (pH 7.4) was installed for 15 min. If indicated, ONOO⁻ was added as a bolus at the respective concentration to the PSG buffer, followed instantly by tilting of the culture dish. The same procedure, including institution of PSG buffer for 15 min was used in the respective control experiments, i.e. without ONOO⁻ addition. At the end of ONOO⁻ exposure, PSG buffer was removed and when indicated, culture medium was reinstalled. The rationale for using PSG buffer during ONOO⁻ exposure was its high buffer capacity and to avoid reactions between ONOO⁻ and constituents of the culture medium.

**Western blot analysis**

At the end of the incubation periods, medium was removed, and cells were washed briefly with PBS and immediately lysed. Samples were transferred to sodium dodecyl sulfate
(SDS)/polyacrylamide gel electrophoresis (PAGE) and proteins were then blotted to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia Biotech, Freiburg, Germany) as recently described. Blots were blocked for 2 hours in 5 % (w/v) BSA-containing 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl and 0.1% Tween 20 (TBS-T), and then incubated at 4°C overnight with the first antibody [antibodies used: anti-phospho-JNK-1/-2 (1:1000), anti-phospho-EGFR (1:2500), anti-EGFR (1:5000), anti-CD95, anti-FADD, anticaspase 8, anti-phospho-tyrosine (all 1:10000)]. Following washing with TBS-T and incubation with horseradish peroxidase-coupled anti-mouse, anti-sheep or anti-rabbit IgG antibody (all diluted 1:10000) at room temperature for 2 hours respectively, the blots were washed extensively and developed using enhanced chemiluminescent detection (Amersham Pharmacia Biotech, Upsala, Sweden). Blots were exposed to Kodak X-OMAT AR-5 film (Eastman Kodak Co., Rochester, NY, USA).

**CD95-immunoprecipitation**

Hepatocytes or Huh7 cells, respectively, were cultured on collagen-coated culture plates (Ø 10 cm; Falcon, Heidelberg, Germany) at a density of 8×10⁶ cells/well. They were harvested in lysis buffer containing 136 mM NaCl, 20 mM Tris-HCl, 10 % (v/v) glycerol, 2 mM EDTA, 50 mM β-glycerophosphate, 20 mM Na-pyrophosphate, 0.2 mM pefablock, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 4 mM benzamidin, 1 mM Na-vanadate and 1 % (v/v) Triton X 100. The lysates were kept for 10 min on ice and then centrifuged at 10000 g for 25 min at 4°C, and aliquots were taken for protein determination using the Bio-Rad protein assay (Bio-Rad Labs., Hercules, CA, USA). The supernatants containing equal protein amounts (200 µg) were incubated for 2 h at 4°C with a polyclonal rabbit anti-rat CD95 antibody (dilution 1:100; Santa Cruz, CA, USA) in order to immunoprecipitate CD95 from rat hepatocytes or were incubated for 2 h at 4°C with a monoclonal mouse anti-GFP antibody (dilution 1:100; Santa Cruz, CA, USA) in order to immunoprecipitate the transfected CD95-
YFP-fusion protein in Huh7 cells, respectively. Then 10 µl of protein A- and 10 µl of protein-G-agarose (Santa Cruz, CA, USA) was added and incubated at 4°C overnight. Immunoprecipitates were washed 3 times with a buffer containing 136 mM NaCl, 20 mM Tris-HCl, 10 % (v/v) glycerol, 2 mM EDTA, 50 mM β-glycerophosphate, 20 mM Na-pyrophosphate, 0.2 mM pefablock, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 4 mM benzamidin, 1 mM Na-vanadate and 0.1 % (v/v) Triton X-100 and then transferred to Western blot analysis as described above.

**CD95-membrane trafficking**

For determination of membrane surface trafficking of CD95 in primary rat hepatocytes, cells were cultured for 24 hours on collagen-coated glass coverslips (Ø 30 mm) in 6-well culture plates (Falcon, Heidelberg, Germany) and subsequently exposed to hyperosmotic medium or CD95L at the given concentrations. Permeabilized and non-permeabilized cells were stained as published recently¹ using a polyclonal rabbit anti-CD95 antibody (dilution 1:500 in PBS) and a secondary anti-rabbit Cy3-conjugated antibody. Cells were visualized using an Axioskop (Zeiss, Oberkochen, Germany) and pictures were taken with a 3CCD-Camera (Intas, Göttingen, Germany). Receptor membrane trafficking was defined as the appearance of fluorescent spotting on the surface of the non-permeabilized cells compared to the non-permeabilized control cells.¹,4,6 For each condition, a blinded observer scored at least 100 cells per independent experiment from at least three different cell preparations for CD95-membrane trafficking.

For determination of membrane surface trafficking of CD95-YFP in Huh7, cells were transfected as described above and plated on glass bottom dishes (Mattek, Ashland, MA, USA). Confocal pictures were taken using the LSM 510 META (Zeiss, Oberkochen, Germany). YFP was excited with 514 nm. 24 h after transfection CD95-YFP was detected in
living cells. At least 100 cells from three independent experiments were counted in a humidified 5% CO₂-atmosphere at 37°C.

**Detection of apoptosis**

Caspase 3 and 8 activation was determined by in vitro caspase assays from cytosol of rat hepatocytes treated with hyperosmotic medium or CD95L for the indicated time periods. The colorimetric assay (R&D Systems, Wiesbaden, Germany) and the Bio-Rad Lowry-protein assay (Bio-Rad Labs., Hercules, CA, USA) were used as published recently. Data were corrected for total protein amount and are expressed as relative increase compared to the control level (normoosmotical medium without CD95L set to 1).

Terminal deoxynucleotidyl transferase-mediated X-dUTP nick-end labelling of FITC-conjugated deoxyuridine triphosphate (TUNEL) technique was performed as described recently. The number of apoptotic cells was determined by an independent examiner by counting the percentage of fluorescein-positive cells. At least 100 cells from three independent experiments from three different cell preparations were counted for each condition. Cells were visualised on an Axioskop (Zeiss, Oberkochen, Germany).

**Statistics**

Results from at least three independent experiments are expressed as means ± SEM (standard error of the mean). n refers to the number of independent experiments. Results were analyzed using the Student’s t-test: p<0.05 was considered statistically significant.
Results

Peroxynitrite (ONOO\(^-\))-induced protein-tyrosine nitration

24 h-cultured and normoosmotically exposed rat hepatocytes were exposed for 15 min to a bolus of peroxynitrite (ONOO\(^-\); initial concentration 1-500 µmol/L), which has a half-life at pH 7.4 of approximately 2 seconds.\(^{24}\) Thereafter, hepatocytes were lysed, proteins separated by gel electrophoresis and analyzed for protein-tyrosine nitration. As shown in Fig. 1A, ONOO\(^-\) induced a concentration-dependent increase of protein-tyrosine nitration of distinct protein bands with a prominent band with a molecular mass of approximately 48 kDa. In line with previous reports,\(^{20,25}\) these data suggest that ONOO\(^-\) induces protein-tyrosine nitration.

Peroxynitrite (ONOO\(^-\)) induces CD95-tyrosine nitration

CD95-tyrosine nitration was studied in 24 h cultured, normoosmotically (305 mosmol/L) exposed rat hepatocytes by CD95 immunoprecipitation and subsequent NO\(_2\)-tyrosine immunoblotting. As shown in Fig. 1B, neither hyperosmolarity (405 mosmol/L) nor CD95L (50 ng/mL) induced CD95-Tyr-nitration within 3 h, whereas this was observed when the cells were preincubated for 15 min with ONOO\(^-\) (500 µmol/L) prior to the experimental period, \(i.e.\) 3 h normo-, hyperosmotic or CD95L-exposure (Fig. 1B). When, however, ONOO\(^-\) was added for 15 min after the experimental period, \(i.e.\) after a 3 h incubation period with hyperosmolarity or CD95L, CD95-Tyr-nitration was strongly blunted (Fig. 1B), although an overall formation of Tyr-nitrated proteins was still observed. As expected, addition of ONOO\(^-\) after a 3h exposure to normoosmotic medium, however, increased CD95-Tyr-nitration. This suggests that CD95-Tyr-phosphorylation, which is induced within 60 min by
hyperosmolarity or CD95L, but not by normoosmotic exposure, protects CD95 from becoming Tyr-nitrated in response to ONOO$^-$. As shown in Fig. 1C, ONOO$^-$ at a concentration of 5-10 µmol/L was sufficient to induce CD95-Tyr-nitration. There was a reciprocal relationship between ONOO$^-$-induced CD95 Tyr-nitration and CD95-Tyr-phosphorylation induced by subsequent hyperosmotic exposure (Fig. 1C). On the other hand, when CD95-Tyr-phosphorylation was induced by hyperosmotic treatment prior to ONOO$^-$ addition, only very small amounts of CD95-nitrotyrosine were generated in response to ONOO$^-$ (Fig. 1D). However, the ONOO$^-$-induced formation of total tyrosine-nitrated proteins was not different between experiments, in which ONOO$^-$ was added either before or after the 3h-period of hyperosmotic exposure (Fig. 1C,D, bottom lanes). Significant protein-Tyr-nitration was found already at ONOO$^-$ concentrations of 10 µmol/L.

**Hyperosmotic- and CD95L-induced activation of the epidermal growth factor receptor (EGFR)- and of c-Jun-N-terminal kinase (JNK) is not affected by peroxynitrite (ONOO$^-$)**

In order to examine the mechanism underlying the ONOO$^-$-induced inhibition of CD95-tyrosine phosphorylation in response to hyperosmolarity or CD95L, the effects of ONOO$^-$ on the early steps of CD95 activation were studied. 24 h-cultured hepatocytes were exposed to CD95L (50 ng/mL), normo- (305 mosmol/L) or hyperosmotic medium (405 mosmol/L) for 30 min. As shown in Fig. 2 and in line with previous data, hyperosmolarity and CD95L induced an activation of JNK. Also ONOO$^-$ (500 µmol/L) instituted for 15 min induced JNK activation (Fig. 2A), however, the JNK-activating effects of ONOO$^-$ on the one hand and of hyperosmolarity or CD95L on the other, were roughly additive.
As further shown in Fig. 2, hyperosmolarity (405 mosmol/L) and CD95L (50 ng/mL), but not ONOO\(^-\) (500 µmol/L) induced within 5 min EGFR-tyrosine phosphorylation, which was not affected by ONOO\(^-\) pretreatment or ONOO\(^-\) exposure after the experimental period (Fig. 2B). These data suggest that ONOO\(^-\) does not affect hyperosmotic or CD95L-induced JNK- and EGFR-activation, which were recently shown to be important initial steps in the machinery leading to CD95 activation.\(^4\,6\)

CD95-Tyr-nitration inhibits CD95-tyrosine phosphorylation (Tyr-P), CD95-membrane trafficking and DISC-formation, but not EGFR/CD95 association

To answer the question, whether phosphorylation and nitration compete on critical CD95-tyrosine residues or whether ONOO\(^-\) inhibits the hyperosmotically- or CD95L-induced CD95/EGFR-interaction, CD95-immunoprecipitation with subsequent immunoblotting for EGFR and Tyr-P was performed in 24 h cultured rat hepatocytes. In line with previous data,\(^4\) hyperosmotic exposure (405 mosmol/L) and CD95L (50 ng/mL) led within 1 hour to an association of the CD95 with the EGFR, subsequent CD95-Tyr-phosphorylation and FADD/caspase 8 recruitment (DISC formation) (Fig. 3), whereas ONOO\(^-\) (500 µmol/L) by itself was ineffective. When ONOO\(^-\) was added 15 min prior to hyperosmotic or CD95L exposure, CD95/EGFR-association still occurred, however, CD95-Tyr-phosphorylation and DISC formation were prevented (Fig. 3). These findings suggest that ONOO\(^-\)-induced CD95-Tyr-nitration does not affect the physical association of CD95 with the hyperosmotically or CD95L-activated EGFR, but prevents EGFR-catalyzed CD95-Tyr-phosphorylation and DISC formation, probably as a consequence of ONOO\(^-\)-induced CD95-Tyr-nitration. In line with this, CD95-Tyr-phosphorylation and DISC formation were not affected, when ONOO\(^-\) was added for 15 min after hyperosmotic or CD95L-exposure (Fig. 3), i.e. at a time point when CD95-Tyr-phosphorylation and DISC formation had already occurred.\(^4\) These data further
suggest that phosphorylation and nitration of CD95-tyrosine residues are apparently mutually exclusive and that Tyr-nitrated CD95 can no longer be Tyr-phosphorylated by hyperosmolarity or CD95L. Likewise, after hyperosmotically- or CD95L-induced CD95 Tyr-phosphorylation, ONOO\(^{--}\) is no longer able to induce CD95-Tyr-nitration.

CD95-Tyr-nitration also abolished CD95 membrane trafficking in response to hyperosmolarity and CD95L (Table 1). The immunolocalization of CD95 was studied in 24 h cultured, normoosmotically (305 mosmol/L) exposed rat hepatocytes. Non-permeabilized cells were used in order to detect CD95 in the plasma membrane, whereas CD95 immunostaining of permeabilized cells reflects CD95 at the cell surface and the cellular interior. As shown in Fig. 4A, no significant CD95 immunostaining was found at the cell surface in non-permeabilized hepatocytes, whereas staining in the cellular interior was intense, when permeabilized hepatocytes were used (Fig. 4B). This indicates that CD95 is located inside the cell with little or no CD95 at the cell surface under normoosmotic conditions.\(^1\) When, however, hepatocytes were exposed to hyperosmotic medium (405 mosmol/L, Fig. 4C) or CD95L (50 ng/mL, Fig. 4G) for 3 h, a strong CD95 immunostaining was found in non-permeabilized cells, indicating hyperosmotic- and CD95L-induced targeting of CD95 from the cellular interior to the plasma membrane in line with previous data\(^1,4\).

ONOO\(^{--}\) (500 µmol/L) by itself did not induce CD95-membrane targeting in normoosmotic incubations within 3 h (Fig. 4D). However, when added for 15 min prior to hyperosmolarity or CD95L, ONOO\(^{--}\) fully prevented CD95 membrane trafficking (Fig. 4 F,H, Table 1). Again, addition of ONOO\(^{--}\) for 15 min after a 3 h-exposure to hyperosmolarity or CD95L had no effect on CD95 membrane surface staining. These data suggest, that inhibition of CD95-Tyr-phosphorylation due to ONOO\(^{--}\)-induced CD95-Tyr-nitration prevents CD95-membrane trafficking and, as shown in Fig. 3 DISC formation.
**ONOOCO\(^{-}\)** prevents CD95 activation in Huh7 hepatoma cells

As shown recently, in the human hepatoma cell line 7 (Huh7) cells CD95L fails to induce apoptosis probably due to a lack of CD95 expression.\(^27\) Therefore this cell line was chosen and transiently transfected with a CD95-YFP fusion protein. Transfected cells exhibited a strong expression of the CD95-YFP fusion protein (Fig. 5A). CD95-YFP was functionally active, because a 2 h-exposure to hyperosmolarity or CD95L induced Tyr-phosphorylation of the CD95-YFP fusion protein, while no Tyr-nitration was detectable (Fig. 5B). In addition, CD95L (50 ng/mL) induced a significant apoptosis only in Huh7 transfected with CD95-YFP, but not in cells, which were transfected with YFP only (Table 2).

Also in transfected Huh7 cells, a 15 min-incubation of ONOCOCO\(^{-}\) prior to the 2 h of normo-, hyperosmotic or CD95L-treatment resulted in CD95-YFP-Tyr-nitration. Under these conditions, however, hyperosmolarity and CD95L failed to induce CD95-YFP-Tyr-phosphorylation (Fig. 5B). When ONOCOCO\(^{-}\) was incubated after the experimental period (hyperosmotic or CD95L-exposure), \textit{i.e.} at a time point when CD95-Tyr-phosphorylation had already occurred, no CD95-YFP-Tyr-nitration was observed, although the overall formation of Tyr-nitrated proteins was not affected (Fig. 5C). These findings indicate that phosphorylation and nitration of CD95-YFP-tyrosine residues are mutually exclusive also in Huh7 cells.

In order to visualize CD95-YFP-membrane trafficking in living cells, CD95-YFP transfected Huh7 cells were examined by confocal laser scanning microscopy (Table 1, Fig. 6). Under control conditions, transfected Huh7 cells showed a homogenous CD95-YFP distribution, without significant membrane localization (Fig 6A). Whereas a 2 h-exposure to normoosmotic medium (305 mosmol/L) did not change the CD95-YFP distribution (Fig 6B), hyperosmotic medium (405 mosmol/L, Fig. 6C) and CD95L (25 ng/mL, Fig. 6D) led to a marked enrichment of CD95-YFP in the plasma membrane. ONOCOCO\(^{-}\) (500 µmol/L) by itself did not induce CD95-YFP-membrane targeting in normoosmotic incubations, regardless of
whether ONOO$^-$ was added before (Fig. 6E) or after (Fig. 6H) the 2 h of normoosmotic incubation. However, ONOO$^-$ pretreatment fully abolished CD95-YFP membrane targeting in response to hyperosmolarity or CD95L (Fig. 6 F,G). On the other hand, ONOO$^-$ addition after a 2 h-exposure to hyperosmolarity or CD95L had no effect on CD95-YFP-membrane translocation (Fig. 6 I,J).

**Peroxynitrite (ONOO$^-$) inhibits CD95L-induced apoptosis in CD95-YFP transfected Huh7 cells**

The effect of ONOO$^-$-induced CD95-Tyr-nitration on CD95L-induced apoptosis was studied in CD95-YFP transfected Huh7 cells, using the TUNEL reaction as apoptotic readout. Whereas YFP-transfected cells were fairly resistant towards CD95L-induced apoptosis (Table 2) due to the low endogenous CD95 expression level in these cells,\textsuperscript{27} CD95L (50 ng/mL) induced apoptosis within 12 h in 34.9 ± 1.2 % of CD95-YFP-transfected Huh7 cells. A 15 min preincubation with ONOO$^-$ (10 µmol/L), however, significantly lowered the number of TUNEL-positive cells to 12.2 ± 3.4 % (p<0.05) in the latter cells (Table 2). These data indicate that CD95-Tyr-nitration protects against CD95L-induced apoptosis.

**Peroxynitrite (ONOO$^-$) inhibits hyperosmolarity-induced caspase 8- and 3-activation and sensitization towards CD95L-induced apoptosis in rat hepatocytes**

Hyperosmolarity (405 mosmol/L) was recently shown to activate caspases 8 and 3 and to sensitize rat hepatocytes towards CD95L-induced apoptosis.\textsuperscript{1} This hyperosmotic caspase 3 and 8 activation was fully abolished by a 15 min preincubation of the hepatocytes with ONOO$^-$ (500 µmol/L) before the onset of a 12 h hyperosmotic exposure. However, ONOO$^-$ induced some caspase activation by itself, irrespective of the ambient osmolarity (Fig. 7). This may reside in the known proapoptotic potential of ONOO$^-$, which shown for the ONOO$^-$
concentration employed here and which interestingly depends upon glutathione peroxidase-1.\textsuperscript{28}

The significance of ONOO\textsuperscript{−}-induced CD95-Tyr-nitration for apoptosis induction by hyperosmolarity and CD95L was studied in 24 h-cultured hepatocytes. These cells were after a priming period exposed to normoosmotic medium with or without CD95L (50 ng/mL) for 12 h and the percentage of apoptotic cells was assessed using the TUNEL technique as apoptotic readout. The priming period consisted either of a 15 min preincubation with ONOO\textsuperscript{−}, being followed by 3 h exposure to normo- or hyperosmotic medium or a 3 h hyperosmotic exposure being followed by a 15 min ONOO\textsuperscript{−} exposure. As shown in Table 3, ONOO\textsuperscript{−} induced by itself a dose-dependent increase of apoptosis. Without ONOO\textsuperscript{−} pretreatment however, CD95L induced within 12 h 30.2 ± 1.4 % apoptotic (TUNEL-positive) cells and there was no significant increase in apoptosis under these conditions, when the cells had been preincubated with ONOO\textsuperscript{−} at concentrations up to 100 μmol/L. Because ONOO\textsuperscript{−} by itself induces apoptosis, this finding may suggest some inhibition of CD95L-induced apoptosis, as it was also shown above for Huh7 cells. As shown recently,\textsuperscript{1} hyperosmotic preconditioning had by itself had no effect on hepatocyte apoptosis and the number of TUNEL-positive cells (1.1 ± 0.2 %) did not significantly differ from normoosmotic controls (0.9 ± 0.2 %) (Table 3). However, a 3 h-hyperosmotic priming period sensitized the hepatocytes towards CD95L (50 ng/ml)-induced apoptosis, as reported previously.\textsuperscript{1} Upon exposure to CD95L about 96 % of the cells were TUNEL-positive after hyperosmotic priming, compared to only 30.2 ± 1.4 % with normoosmotic priming conditions. As further shown in Table 3, hyperosmolarity did not affect ONOO\textsuperscript{−}-induced apoptosis (CD95L absent). This is an expected finding, because ONOO\textsuperscript{−}-induced apoptosis is unlikely to proceed via the CD95 system. However, pretreatment with ONOO\textsuperscript{−} dose-dependently attenuated the hyperosmotic sensitization towards CD95L-induced apoptosis. On the other hand, when
ONOO$^-$ was added for 15 min after 3 h of exposure towards hyperosmolarity, no effect on the hyperosmotic sensitization towards CD95L-induced apoptosis was observed. These data suggest that CD95-Tyr-nitration abolishes the hyperosmotic sensitization towards CD95L-induced apoptosis.

**LPS treatment induces CD95-Tyr-nitration *in vivo***

*In vivo* treatment of rats with LPS (4 mg / kg body weight *i.p.*) is known to induce iNOS and results within 24 h in an increased myeloperoxidase (MPO) expression in the liver (Fig. 8B). MPO was recently suggested to mediate the production of nitrating oxidants *in vivo*. In *in vivo* LPS treatment also induced tyrosine nitration of distinct proteins with a molecular mass of 35-75 kDa (Fig. 8A,C), however, the pattern of Tyr-nitrated proteins in response to LPS was apparently more specific than that obtained after ONOO$^-$ treatment of hepatocytes (Fig. 1A). In CD95 immunoprecipitates from livers of LPS-treated animals strong Tyr-nitration of CD95 was detectable (Fig. 8D). These findings suggest that CD95 Tyr-nitration may also occur *in vivo*. 
Discussion

CD95-Tyr-nitration

Recent studies have identified CD95-Tyr-phosphorylation as a crucial step in the activation of CD95 in response to proapoptotic stimuli, such as hydrophobic bile acids, CD95L and hyperosmolarity.\textsuperscript{1,4,6} The present study describes a novel covalent CD95-Tyr-modification, \textit{i.e.} CD95-Tyr-nitration, which can be induced \textit{in vitro} by peroxynitrite and \textit{in vivo} following LPS treatment. The mechanisms underlying protein-tyrosine nitration are a matter of debate,\textsuperscript{31} however, recent studies point to the existence of multiple pathways including myeloperoxidase\textsuperscript{30} and superoxide dismutase-catalyzed reactions.\textsuperscript{32} Clearly, the appearance of distinct Tyr-nitrated protein bands upon ONOO\textsuperscript{-} treatment \textit{in vitro} or LPS-treatment \textit{in vivo} is suggestive for some selectivity of the nitration process, as it was recently shown for ammonia- or benzodiazepin-induced protein-Tyr-nitration in astrocytes.\textsuperscript{33,34} Peroxynitrite is a very potent nitrating species and is formed \textit{in vivo} from a near diffusion-limited reaction between O\textsubscript{2}\textsuperscript{-} und NO or from nitrite and H\textsubscript{2}O\textsubscript{2}.\textsuperscript{35} 3-Nitrotyrosines in proteins were shown to be poor substrates for tyrosine phosphorylation reactions\textsuperscript{36,37} and evidence for an impairment of signal transduction cascades that depend on reversible tyrosine phosphorylation events by 3-nitration has been given recently.\textsuperscript{19,20,38} In line with this and as shown in the present study, CD95-Tyr-nitration blocks CD95 signaling towards apoptosis, which may be relevant for the known antiapoptotic effect of NO.\textsuperscript{16-18}

CD95-Tyr-nitration and inhibition of apoptosis

Induction of hepatocyte apoptosis by hydrophobic bile acids, CD95L or hyperosmolarity involves a complex mechanism, which leads to an oxidative stress response, a subsequent antioxidant-sensitive JNK and EGFR activation, being followed by EGFR/CD95 association
and a EGFR-catalyzed CD95-Tyr-phosphorylation, which is required for CD95 membrane trafficking and DISC formation.\textsuperscript{4,6} ONOO\textsuperscript{−} has little effects on the initial steps of CD95 activation, \textit{i.e.} also after ONOO\textsuperscript{−} pretreatment proapoptotic stimuli still induce JNK and EGFR activation and EGFR/CD95 association. However, CD95-Tyr-nitration prevents the CD95 from becoming Tyr-phosphorylated by the EGFR-tyrosine kinase activity. Failure to become Tyr-phosphorylated then prevents CD95 membrane trafficking as well as recruitment of FADD and caspase 8, \textit{i.e.} DISC formation, underlining the importance of CD95-Tyr-phosphorylation for CD95 activation and apoptosis. When, however, CD95 had already undergone Tyr-phosphorylation, ONOO\textsuperscript{−} was no longer able to Tyr-nitrate CD95 and to inhibit apoptosis. An antiapoptotic effect of NO, as a precursor of ONOO\textsuperscript{−}, has been established in the past, and multiple effects of NO on the apoptosis cascade have been described,\textsuperscript{12,39,40} such as suppression of Bel-2 binding protein BNIP3 expression,\textsuperscript{41} caspase inhibition by S-nitrosylation,\textsuperscript{17,18} interruption of the mitochondrial pathway\textsuperscript{42-45} or induction of heatshock proteins.\textsuperscript{46} It is likely that some of these mechanisms are downstream consequences of NO-mediated CD95-Tyr-nitration, which blocks CD95 activation and the activation of upstream caspases, which are required for mitochondrial amplification of the apoptotic signal.

ONOO\textsuperscript{−} by itself induced JNK activation, but evidence has also been presented that the JNK pathway can be negatively regulated by NO, depending on the experimental conditions employed. For example, inactivating S-nitrosylation in response to interferon-γ-induced NO formation has been described,\textsuperscript{47} which may occur via NO\textsuperscript{+} but not ONOO\textsuperscript{−}.

**Physiological relevance**

NOS and MPO induction are hallmarks of inflammation,\textsuperscript{48} which are expected to be accompanied by an enhanced nitrating potential. Under these conditions CD95-Tyr-nitration...
may serve to limit the apoptotic cell death in response to inflammatory stimuli. It is also an interesting speculation whether inactivation of CD95 via tyrosine nitration provides a link between inflammation and carcinogenesis.\textsuperscript{49}
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Figure legends

**Figure 1: Peroxynitrite (ONOO⁻)-induced CD95-tyrosine nitration**

**A)** Hepatocytes were exposed to ONOO⁻ for 15 min as described in the Methods section and analyzed for protein tyrosine nitration. Proteins were separated by SDS-PAGE electrophoresis and protein-tyrosine nitration was detected by Western blotting technique. ONOO⁻ induces tyrosine nitration of distinct proteins in a dose dependent manner. GAPDH was used as loading control.

**B-D)** Hepatocytes were cultured for 24 h and then exposed for 3 h to normo- (305 mosmol/L), hyperosmotic medium (405 mosmol/L) or CD95L (50 ng/mL). When indicated, ONOO⁻ (500 µmol/L) was added for 15 min either before this incubation period (ONOO⁻ → 305 mosmol/L, 405 mosmol/L or CD95L) or ONOO⁻ was added for 15 min after the 3 h incubation period (305 mosmol/L, 405 mosmol/L or CD95L → ONOO⁻) as described in the Methods section. In control experiments, PSG without ONOO⁻ addition was instituted for 15 min prior to the normo-, hyperosmotic or CD95L-exposure.

**B)** CD95 was immunoprecipitated as described in the Methods section. The immunoprecipitated CD95-samples (~ 48 kDa) were detected for nitro-tyrosine by Western blotting. Total CD95-amount from each sample served as loading control. Total protein-Tyr-nitration was shown using dot blot analysis. Representative blots from 5 independent experiments are shown.

All samples treated with ONOO⁻ showed increased total protein-nitration levels. ONOO⁻ preincubation (500 µmol/L) leads to a marked CD95-Tyr-nitration. Hyperosmotic or CD95L-treatment before ONOO⁻ exposure strongly blunts CD95-Tyr-nitration.
C) CD95 was immunoprecipitated as described in the Methods section. ONOO\textsuperscript{−} concentrations above 10 µmol/L induced CD95-Tyr-NO\textsubscript{2} and a reciprocal decrease of subsequent hyperosmotic-induced CD95-Tyr-P. Increasing doses of ONOO\textsuperscript{−} induced increasing amounts of total protein-nitration as indicated by dot blot technique.

D) CD95 was immunoprecipitated as described in the Methods section. Hyperosmolarity-induced CD95-Tyr-phosphorylation largely abolished CD95-Tyr-NO\textsubscript{2} in response to ONOO\textsuperscript{−}. However, a dose-dependent increase of total protein-nitration as indicated by dot blot technique, was still found, indicating specific inhibition of CD95-Tyr-nitration under these conditions.

**Figure 2: Hyperosmotic and CD95L-induced activation of c-Jun-N-terminal kinase (JNK) and the epidermal growth factor receptor (EGFR) are not impaired by peroxynitrite**

Hepatocytes were cultured for 24 h and then exposed for 30 min to normo- (305 mosmol/L), hyperosmotic medium (405 mosmol/L) or CD95L (50 ng/mL). When indicated, ONOO\textsuperscript{−} (500 µmol/L) was incubated for 15 min either before or after the osmolarity was changed or CD95L added. In control experiments, PSG without ONOO\textsuperscript{−} addition was instituted for 15 min prior to the normo-, hyperosmotic or CD95L-exposure.

A) Hyperosmotic exposure, CD95L and ONOO\textsuperscript{−} itself led within 30 min to JNK-activation compared to normoosmotically exposed control cells. After preincubation with ONOO\textsuperscript{−} a hyperosmotic- or CD95L-induced JNK-activation was still detectable. Total JNK-amount (~ 46/54 kDa) from each sample served as loading control. Phospho-JNK-1/-2 (~ 46/54 kDa), as marker of JNK-activation, were detected by Western blotting. Representative blots from 3 independent experiments are shown.
B) Samples were examined after 30 min of hyper-/normoosmotic incubation or CD95L addition for EGFR-tyrosine phosphorylation (~ 170 kDa) as marker for EGFR-activation using Western blotting. ONOO\(^{-}\) treatment was introduced before or after the experimental period as described above. Total EGFR-amount (~ 170 kDa) from each sample served as loading control. Representative blots from 3 independent experiments are given.

Hyperosmolarity and CD95L, but not ONOO\(^{-}\) induce EGFR phosphorylation which is not affected by ONOO\(^{-}\) preincubation.

**Figure 3: CD95-Tyr-nitration inhibits hyperosmotic- and CD95L-induced CD95-tyrosine phosphorylation and DISC formation**

Hepatocytes were cultured for 24 h and then exposed to normo- (305 mosmol/L), hyperosmotic medium (405 mosmol/L) or CD95L (50 ng/mL) as described below. When indicated, ONOO\(^{-}\) (500 µM) was incubated for 15 min either before (“ONOO\(^{-}\) → 305/405/CD95L”) or after the osmolarity was changed or CD95L was added (“305/405/CD95L → ONOO\(^{-}\)”). In control experiments, PSG without ONOO\(^{-}\) addition was also instituted for 15 min prior to the normo-, hyperosmotic or CD95L-exposure. CD95 was immunoprecipitated as described in the Methods section after the time periods indicated below. The immunoprecipitated CD95-samples (~ 48 kDa) were detected for EGFR-association (~ 170 kDa), for tyrosine phosphorylation (P-Tyr, ~ 48 kDa), for Fas-associated death domain (FADD, ~ 28 kDa)- and caspase 8 (Casp8, ~ 54/55 kDa)-association by Western blotting. Total CD95 (~ 48 kDa) served as loading control. Representative blots from at least 3 independent experiments are shown.

A) **EGFR/CD95-association:** Hyperosmolarity and CD95L induce within 1 hour a CD95-EGFR-association, which was not affected by ONOO\(^{-}\).
B) CD95-tyrosine phosphorylation: Hyperosmolarity and CD95L induce within 90 min CD95-tyrosine phosphorylation, which was strongly blunted, when ONOO$^-$ was preincubated and CD95 thereby nitrated (see Fig. 1). On the other hand, CD95-tyrosine phosphorylation was not affected when ONOO$^-$ was given after CD95 had been tyrosine phosphorylated by a previous hyperosmotic- or CD95L-exposure.

C) DISC-formation: Hyperosmolarity and CD95L induce within 3 hours formation of the death inducing signaling complex (DISC), as shown by the association of CD95 with FADD and Caspase 8. DISC formation was inhibited, when ONOO$^-$ was preincubated in order to induce CD95-Tyr-nitration (see Fig. 1). On the other hand, DISC-formation was not affected when ONOO$^-$ was given after the hyperosmotic- or CD95L-exposure.

Figure 4: Effect of peroxynitrite (ONOO$^-$) on hyperosmolarity- and CD95L-induced CD95 membrane trafficking in primary rat hepatocytes

24 h-cultured hepatocytes were exposed for 3 h to either normo- (305 mosmol/L, A,B), hyperosmotic medium (405 mosmol/L, C, E, F), peroxynitrite (500 µmol/L, D) or CD95L (100 ng/mL, G,H) and then immunostained for CD95. ONOO$^-$ (500 µmol/L) was given for 15 min after the hyperosmotic exposure (E) or incubated for 15 min prior to the hyperosmotic- (F) or CD95L-exposure (H) as described in the Methods section. In control experiments, i.e. experiments without ONOO$^-$ addition, PSG was also instituted for 15 min prior to the normo-, hyperosmotic or CD95L-exposure. Representative samples from at least three independent experiments are shown.

A,B: While non-permeabilized normoosmotically exposed hepatocytes (A) virtually show no CD95-immunostaining, permeabilized cells (B) show strong staining for CD95, indicating the intracellular localization of CD95 in normoosmotically exposed hepatocytes.
C-F: Hyperosmotic exposure (C), but not ONOO⁻ (D), leads to the appearance of CD95-staining in non-permeabilized hepatocytes, indicating hyperosmotic CD95-membrane trafficking. Hyperosmotic CD95 membrane targeting is abolished after a 15 min preincubation with ONOO⁻ (500 µmol/L, F), but not, when ONOO⁻ was given for 15 min after the 3 h-hyperosmotic exposure period (E).

G+H: In normoosmotic incubations CD95L leads to a CD95-membrane staining in non-permeabilized cells (G), which was abolished after a 15 min preincubation with ONOO⁻ (500 µmol/L, H).

Inserts show phase contrast recordings, indicating the presence of cells in case of CD95 was not detectable by surface immunocytochemistry (A, D, F, H).

**Figure 5: CD95-Tyr-nitration inhibits hyperosmotic- and CD95L-induced CD95-YFP-tyrosine phosphorylation in Huh7 cells**

Huh7 cells were transfected with CD95-YFP as described in the Methods section and then exposed for 2 h to normo- (305 mosmol/L), hyperosmotic medium (405 mosmol/L) or CD95L (25 ng/mL). When indicated, ONOO⁻ (500 µmol/L) was incubated for 15 min either before or after the osmolarity was changed or CD95L added. In control experiments, PSG without ONOO⁻ addition was also instituted for 15 min prior to the normo-, hyperosmotic or CD95L-exposure. Representative blots from 3 independent experiments are shown.

A) CD95-YFP-transfection led to expression of the CD95-fusion protein as detected by anti-GFP immunoblotting (CD95-YFP, ~ 74 kDa). GAPDH served as loading control.

B) CD95-YFP was immunoprecipitated as described in the Methods section. The immunoprecipitated CD95-YFP-samples were detected for tyrosine nitration (NO₂-Tyr, ~ 74 kDa) and tyrosine phosphorylation (P-Tyr, ~ 74 kDa) by Western blotting. Total CD95-YFP (CD95-YFP, ~ 74 kDa) served as loading control.
C) Total protein-nitration was shown using dot blot analysis and served as a marker for total nitration. All samples treated with ONOO\(^-\) showed increased total protein-nitration levels.

**Figure 6: Effect of peroxynitrite (ONOO\(^-\)) on hyperosmolarity- and CD95L-induced CD95 membrane trafficking in CD95-YFP-transfected Huh7 cells**

Huh7 cells were transfected with CD95-YFP as described in the Methods section resulting in a homogeneous intracellular distribution of the fluorescent CD95-YFP-fusion protein (A).

Huh7 cells were subsequently exposed for 2 h to either normo- (305 mosmol/L, B), hyperosmotic medium (405 mosmol/L, C), or CD95L (25 ng/mL, D). ONOO\(^-\) (500 µmol/L) was given for 15 min before or after normoosmotic (E,H), hyperosmotic medium (F, I) or CD95L (G,J). In control experiments, i.e. experiments without ONOO\(^-\) addition, PSG was also instituted for 15 min prior to the normo-, hyperosmotic or CD95L-exposure (B,C,D).

Representative samples from three independent experiments are shown.

**A-D:** While normoosmotically exposed cells (B) show a homogeneous CD95-YFP-distribution, hyperosmotic exposure (C) or CD95L-addition (D) led to an enrichment of CD95-YFP in the plasma membrane compared to untreated control (A).

**E-H:** Hyperosmotic (F) or CD95L (G)-induced CD95-YFP membrane targeting is abolished by ONOO\(^-\) pretreatment, but not, when ONOO\(^-\) was given for 15 min after the 2 h-hyperosmotic (I) or CD95L (J)-exposure period. ONOO\(^-\) itself did not induce CD95-YFP membrane enrichment regardless of whether it was added before (E) or after (H) a 2 h normoosmotic exposure.

**Figure 7: Peroxynitrite-pretreatment inhibits hyperosmotic caspase 3- and 8-activation**

Data are given as means ± SEM from 3 independent experiments. Hepatocytes were cultured for 24 h and then exposed for 15 min to PSG buffer with (closed symbols) or without ONOO\(^-\)
(open symbols) before either normo- (305 mosmol/L; ○) or hyperosmotic medium (405 mosmol/L; □) was instituted for the time periods indicated. ONOO$^-$ (500 µmol/L) was preincubated for 15 min before the normo- (●) or hyperosmotic medium (■) was installed. (A) Caspase 3- and (B) caspase 8-activation is expressed relative to the activity found in normoosmotic controls.

Hyperosmotic exposure induces activation of caspases. ONOO$^-$ induced some caspase activation by itself, however, abolished the hyperosmotic caspase activation.

Figure 8: LPS-induced CD95-tyrosine nitration in vivo

Rats were treated with LPS (4 mg / kg body weight i.p.) for 24 h and then liver samples were taken and transferred to immunoprecipitation or immunoblotting. 3 animals for the control and LPS condition, each were studied (n1-n3).

A) Proteins were separated by SDS-PAGE electrophoresis and proteine-tyrosine nitration was detected by Western blotting. LPS induced an increase of tyrosine nitration of distinct protein bands. One of these bands is found at 48 kDa, i.e. the molecular weight of CD95. GAPDH was detected as a loading control.

B) Myeloperoxidase (MPO) protein expression, as detected by Western blot analysis, is increased as one marker of LPS action.

C) In vivo LPS treatment induces a marked increase in total protein-tyrosine nitration, as detected by dot blot analysis. CD95 served as a loading control.

D) In vivo LPS treatment induced marked CD95-Tyr-nitration. CD95 was immunoprecipitated as described in the methods section and then detected for tyrosine nitration by Western blot. Total CD95 served as a loading control.
Table 1: Inhibition of hyperosmotic and CD95L-induced CD95-membrane trafficking by peroxynitrite (ONOO\(^-\))-pretreatment in rat hepatocytes and CD95-YFP transfected Huh7 cells

Hepatocytes were kept in culture for 24 h and were then exposed for 3 h to hyperosmotic medium (405 mosmol/L) or CD95L (50 ng/mL). Thereafter the percentage of cells with positive CD95 membrane staining was determined. Hyperosmolarity and CD95L induce CD95 membrane trafficking, which was largely abolished after a 15 min-preincubation with ONOO\(^-\) (500 µmol/L; ONOO\(^-\) \rightarrow\) 405/CD95L), whereas addition of ONOO\(^-\) (500 µmol/L) for 15 min after the 3 hours of hyperosmotic or CD95L exposure (405/CD95L \rightarrow\) ONOO\(^-\)) was ineffective.

Similar data were obtained in Huh7 cells transfected with CD95-YFP in order to detect CD95-YFP-membrane trafficking. Here the experimental period (i.e. normo-, hyperosmotic or CD95L exposure) was 2 h.

Data are given as means ± SEM and are from 3-5 independent experiments for each condition. Statistically significant (p<0.05) inhibition of hyperosmotic or CD95L-induced CD95-membrane trafficking by ONOO\(^-\) is indicated by \#; “n.s.“ indicates no significant inhibition.

* indicates significant difference from normoosmotic control.
Table 1

| Condition                                      | % cells with positive CD95-membrane staining | rat hepatocytes | Huh7  |
|-----------------------------------------------|--------------------------------------------|----------------|-------|
| 305 mosmol/L                                  |                                            |                |       |
| 305 mosmol/L → ONOO^- (500 µmol/L)            | 0.2 ± 0.1 n.s.                             | 3.7 ± 0.6 n.s. |       |
| ONOO^- (500 µmol/L) → 305 mosmol/L            | 0.4 ± 0.1 n.s.                             | 3.3 ± 0.3 n.s. |       |
| 405 mosmol/L                                  |                                            |                |       |
| 405 mosmol/L → ONOO^- (500 µmol/L)            | 12.5 ± 0.9 n.s.                            | 17.3 ± 3.9 n.s.|       |
| ONOO^- (500 µmol/L) → 405 mosmol/L            | 2.2 ± 0.3 #                               | 4.6 ± 0.2 #    |       |
| CD95L (50 ng/mL)                               |                                            |                |       |
| CD95L (50 ng/mL) → ONOO^- (500 µmol/L)         | 8.2 ± 0.4 n.s.                             | 17.9 ± 3.5 n.s.|       |
| ONOO^- (500 µmol/L) → CD95L (50 ng/mL)         | 1.6 ± 0.3 #                               | 3.0 ± 0.6 #    |       |
Table 2: Inhibition of CD95L-induced apoptosis by peroxynitrite (ONOO\textsuperscript{−}) in Huh7 cells

Huh7 cells were either transfected with CD95-YFP (Huh7\textsuperscript{CD95-YFP}) or YFP (Huh7\textsuperscript{YFP}) as described in the Methods section and were then exposed for 12 h to either normoosmotic medium (305 mosmol/L) or CD95L (50 ng/mL). When indicated, ONOO\textsuperscript{−} (10 µmol/L) was incubated for 15 min prior to the 12 h normoosmotic or CD95L-exposure. In control experiments, normoosmotic PSG without ONOO\textsuperscript{−} addition was instituted for 15 min prior to the normoosmotic or CD95L-exposure. Thereafter the number of apoptotic cells was determined using the TUNEL-technique.

CD95L induces significant apoptosis in Huh\textsuperscript{CD95-YFP} cells, but not in Huh7\textsuperscript{YFP} cells. Preexposure of Huh\textsuperscript{CD95-YFP} cells for 15 min to ONOO\textsuperscript{−}, strongly blunted the apoptotic potency of CD95L.

Data are given as means ± SEM and are from 3 independent experiments for each condition. * indicates significant difference from normoosmotic control; “n.s.” not significantly different. # indicates significant difference from the corresponding experiment without ONOO\textsuperscript{−} treatment; “n.s.” not significantly different.

Table 2

| Condition                              | % TUNEL positive cells |
|----------------------------------------|------------------------|
|                                        | Huh7\textsuperscript{YFP} | Huh7\textsuperscript{CD95-YFP} |
| 305 mosmol/L (12 h)                    | 1.4 ± 0.3              | 2.5 ± 0.6               |
| CD95L (12 h)                           | 2.9 ± 0.6 \textsuperscript{n.s.} | 34.9 ± 1.2 \textsuperscript{*} |
| ONOO\textsuperscript{−} (15 min) → 305 mosmol/L (12 h) | 3.4 ± 1.2              | 4.0 ± 0.8               |
| ONOO\textsuperscript{−} (15 min) → CD95L (12 h) | 3.5 ± 0.9 \textsuperscript{n.s.} | 12.2 ± 3.4 \textsuperscript{#} |
Table 3: Inhibition of hyperosmolarity-induced sensitization towards CD95L-induced apoptosis by peroxynitrite (ONOO$^-$)

24 h-cultured rat hepatocytes were exposed for 15 min to ONOO$^-$ (0-500 µmol/L in PSG) and thereafter for 3 hours to normo- (305 mosmol/L) or hyperosmotic medium (405 mosmol/L). In another set of experiments ONOO$^-$ was added for 15 min after the 3 h hyperosmotic exposure period. After this 195 min lasting priming period, normoosmotic medium with or without CD95L (50 ng/mL) was instituted for another 12 h and the number of apoptotic cells was determined using the TUNEL-technique.

In the absence of CD95L, ONOO$^-$ induced dose-dependent apoptosis, which was not affected by hyperosmotic priming. Hyperosmolarity by itself did not induce apoptosis. CD95L increased significantly the number of apoptotic cells from $0.9 \pm 0.2$ to $30.2 \pm 1.4$ % under normoosmotic conditions and from $1.1 \pm 0.2$ to $95.6 \pm 3.1$ % in hyperosmotically primed hepatocytes, indicating the known hyperosmotic sensitization of the cells towards CD95L-induced apoptosis.1

In normoosmotically exposed hepatocytes, ONOO$^-$ pretreatment apparently attenuated CD95L-induced apoptosis, because the apoptotic effects of ONOO$^-$ alone and CD95L alone were not additive. No significant increase of CD95L-induced apoptosis was observed in normoosmotic experiments irrespective of whether concentrations of ONOO$^-$ were varied from 0-100µmol/L (which by themselves already induced apoptosis). ONOO$^-$ pretreatment however strongly and dose-dependently attenuated the hyperosmotic sensitization of hepatocytes towards CD95L-induced apoptosis, which was not observed when ONOO$^-$ was added after the hyperosmotic priming period.
Data are given as means ± SEM and are from 3-6 independent experiments for each condition. * indicates significant difference from the corresponding control without ONOO$^-$ treatment; “n.s.” not significantly different.
| Priming condition | % TUNEL-positive cells after 12h of normoosmotic exposure |
|-------------------|----------------------------------------------------------|
|                   | CD95L absent | CD95L present |
| ONOO<sup>-</sup> (0 µmol/L) → 305 mosmol/L | 0.9 ± 0.2 | 30.2 ± 1.4 |
| ONOO<sup>-</sup> (10 µmol/L) → 305 mosmol/L | 6.3 ± 1.2 * | 32.4 ± 2.2 n.s. |
| ONOO<sup>-</sup> (50 µmol/L) → 305 mosmol/L | 10.0 ± 1.4 * | 25.5 ± 1.2 * |
| ONOO<sup>-</sup> (100 µmol/L) → 305 mosmol/L | 15.4 ± 1.8 * | 33.6 ± 2.3 n.s. |
| ONOO<sup>-</sup> (500 µmol/L) → 305 mosmol/L | 38.1 ± 2.8 * | 50.1 ± 3.3 * |
| ONOO<sup>-</sup> (0 µmol/L) → 405 mosmol/L | 1.1 ± 0.2 | 95.6 ± 3.1 |
| ONOO<sup>-</sup> (10 µmol/L) → 405 mosmol/L | 6.9 ± 1.0 * | 77.7 ± 3.2 * |
| ONOO<sup>-</sup> (50 µmol/L) → 405 mosmol/L | 10.8 ± 1.1 * | 66.5 ± 4.0 * |
| ONOO<sup>-</sup> (100 µmol/L) → 405 mosmol/L | 13.7 ± 2.5 * | 56.9 ± 3.4 * |
| ONOO<sup>-</sup> (500 µmol/L) → 405 mosmol/L | 35.9 ± 3.5 * | 54.8 ± 3.8 * |
| 405 mosmol/L → ONOO<sup>-</sup> (0 µmol/L) | 0.8 ± 0.3 | 96.3 ± 3.2 |
| 405 mosmol/L → ONOO<sup>-</sup> (10 µmol/L) | 7.4 ± 1.1 * | 93.3 ± 3.4 n.s. |
| 405 mosmol/L → ONOO<sup>-</sup> (50 µmol/L) | 11.2 ± 1.0 * | 94.4 ± 2.2 n.s. |
| 405 mosmol/L → ONOO<sup>-</sup> (100 µmol/L) | 14.3 ± 2.7 * | 95.6 ± 1.8 n.s. |
| 405 mosmol/L → ONOO<sup>-</sup> (500 µmol/L) | 37.6 ± 2.7 * | 82.7 ± 4.2 n.s. |
Figure 1

A

| ONOO⁻ (µmom/L) |
|----------------|
| 0  | 1  | 5  | 10 | 50 | 100 | 500 |

---

NO₂-Tyr

---

48 kDa

---

GAPDH

---

B

NO₂-Tyr-CD95

---

total CD95

---

total NO₂-Tyr
Figure 1

405 → control
405 → 1 µM ONOO−
405 → 5 µM ONOO−
405 → 10 µM ONOO−
405 → 50 µM ONOO−
405 → 100 µM ONOO−
405 → 500 µM ONOO−

control → 405
1 µM ONOO− → 405
5 µM ONOO− → 405
10 µM ONOO− → 405
50 µM ONOO− → 405
100 µM ONOO− → 405
500 µM ONOO− → 405
Figure 2

B

A

305
405
CD95L
ONO - → 305
ONO - → 405
ONO - → CD95L
305 → ONOO -
405 → ONOO -
CD95L → ONOO -

total EGFR
P-EGFR
total JNK-1
P-JNK-1
Figure 3

A

EGFR/CD95
total CD95

B

P-Tyr-CD95
total CD95

C

Caspase 8/CD95
FADD/CD95
total CD95
Figure 4

A

B

C

D

E

F

G

H
### Figure 5

| 305 | 405 | CD95L | ONOO → 305 | ONOO → 405 | ONOO → CD95L | 305 → ONOO | 405 → ONOO | CD95L → ONOO |
|-----|-----|-------|------------|------------|--------------|------------|------------|--------------|

#### A

- **CD95-YFP**
- **GAPDH**

#### B

- **NO₂-Tyr-CD95-YFP**
- **P-Tyr-CD95-YFP**
- **total CD95-YFP**

#### C

- **total NO₂-Tyr**
Figure 7

A

B

relative caspase 3-activity

relative caspase 8-activity

| 0  | 3  | 6  | 9  | 12 |
|-----|----|----|----|----|
| 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| 3.5 | 4.0 |

| 0  | 3  | 6  | 9  | 12 |
|-----|----|----|----|----|
| 1.0 | 1.2 | 1.4 | 1.6 | 1.8 |
| 2.0 | 2.2 |

| 0  | 3  | 6  | 9  | 12 |
|-----|----|----|----|----|
| 1.0 | 1.2 | 1.4 | 1.6 | 1.8 |
| 2.0 | 2.2 |

time (h)

305 mOsm
405 mOsm
PN + 305
PN + 405 mOsm
Figure 8

A

| control | LPS |
|---------|-----|
| n1      | n2  |
| n1      | n2  |

105 kDa - 75 kDa - 50 kDa - 35 kDa - 30 kDa - 25 kDa -

NO$_2$-Tyr

48 kDa

GAPDH

B

| control | LPS |
|---------|-----|
| n1      | n2  |
| n1      | n2  |

MPO
Figure 8

C

|     | control (n1) | LPS (n1) | control (n2) | LPS (n2) | control (n3) | LPS (n3) |
|-----|--------------|----------|--------------|----------|--------------|----------|
|     | total NO2-Tyr |          | total CD95   |          |              |          |

D

|     | control (n1) | LPS (n1) | control (n2) | LPS (n2) | control (n3) | LPS (n3) |
|-----|--------------|----------|--------------|----------|--------------|----------|
|     | NO2-Tyr-CD95 |          | total CD95   |          |              |          |
CD95-tyrosine nitration inhibits hyperosmotic and CD95 ligand-induced CD95 activation in rat hepatocytes
Roland Reinehr, Boris Görg, Andrea Höngen and Dieter Häussinger

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