Endosomal Acidification and Activation of NADPH Oxidase Isoforms Are Upstream Events in Hyperosmolarity-induced Hepatocyte Apoptosis*‡,§

Roland Reinehr‡, Stephan Becker†, Juliane Braun‡, Andrea Eberle‡, Susanne Grether-Beck†, and Dieter Häussinger‡,§

From the ‡Clinic for Gastroenterology, Hepatology, and Infectiology, Heinrich-Heine-University and §Institut für Umweltmedizinische Forschung, D-40225 Düsseldorf, Germany

Liver cell hydration is dynamic and changes within minutes under the influence of ambient osmolarity, nutrient supply, hormones, and oxidative stress (1–9). These hydration changes activate osmosensing and osmosignaling pathways, which modulate cell function, gene expression, and the stress response of hepatocytes (2–9). For example, hepatocyte swelling in response to hypo-osmolarity, insulin, or concentration of amino acid uptake involves volume sensing through the integrin system with downstream activation of focal adhesion kinase, c-Src, and the mitogen-activated protein kinases p38MAPK and ERKs (extracellular signal-regulated kinases) (8, 9). These osmosensing/signaling pathways then trigger functional consequences such as inhibition of autophagic proteolysis and stimulation of bile acid secretion (8, 9). Little is known about osmosensing/signaling in response to hepatocyte shrinkage; however, hyperosmotic exposure results in a rapid oxidative stress response (ROS)2 response (1), which triggers a proapoptotic state through activation of the CD95 system and sensitizes hepatocytes toward CD95 ligand-induced apoptosis (1, 10, 11).

Hyperosmotic exposure of rat hepatocytes induced a rapid oxidative stress (ROS) response as an upstream signal for proapoptotic CD95 activation. This study shows that hyperosmotic ROS formation involves a rapid ceramide- and protein kinase Cζ (PKCζ)-dependent serine phosphorylation of p47phox and subsequent activation of NADPH oxidase isoforms. Hyperosmotic p47phox phosphorylation and ROS formation were sensitive to inhibition of sphingomyelinas and were strongly blunted after knockdown of acidic sphingomyelinase (ASM) or of p47phox protein. Hyperosmolarity induced a rapid bafilomycin- and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid disodium salt (DIDS)-sensitive acidification of a vesicular compartment, which was accessible to endocytosed fluorescein isothiocyanate-dextran and colocalized with ASM, PKCζ, and the NADPH oxidase isoform Nox 2 (gp91phox). Bafilomycin and DIDS prevented the hyperosmolarity-induced increase in ceramide formation, p47phox phosphorylation, and ROS formation. As shown recently (Reinehr, R., Becker, S., Hönig, A., and Häussinger, D. (2004) J. Biol. Chem. 279, 23977–23987), hyperosmolarity induced a Yes-dependent activation of JNK and the epidermal growth factor receptor (EGFR), followed by EGFR-CD95 association, EGFR-catalyzed CD95-tyrosine phosphorylation, and translocation of the EGFR-CD95 complex to the plasma membrane, where formation of the death-inducing signaling complex occurs. These proapoptotic responses were not only sensitive to inhibitors of sphingomyelinase, PKCζ, or NADPH oxidases but also to ASM knockdown, bafilomycin, and DIDS, i.e. maneuvers largely preventing hyperosmolarity-induced endosomal acidification and or ceramide formation. In hepatocytes from p47phox knock-out mice, hyperosmolarity failed to activate the CD95 system. The data suggest that hyperosmolarity induces endosomal acidification as an important upstream event for CD95 activation through stimulation of ASM-dependent ceramide formation and activation of NADPH oxidase isoforms.

* This work was supported by Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 575 “Experimentelle Hepatologie” (Düsseldorf). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

‡ To whom correspondence should be addressed. Tel.: 49-211-811-7569; Fax: 49-211-811-8838; E-mail: haeussin@uni-duesseldorf.de.

1 The abbreviations used are: ROS, reactive oxygen species; ASM, acidic sphingomyelinase; CD95, CD95 receptor; CFP, cyan fluorescent protein; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid disodium salt; DISC, death inducing signaling complex; DPI, diphenyleneiodonium chloride; EGFR, epidermal growth factor receptor; JNK, c-Jun N-terminal kinase; FADD, Fas-associated death domain; FRET, fluorescence resonance energy transfer; Huh7, human hepatoma cell line 7; NAC, N-acetylcysteine; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PKCζ, protein kinase C; PKCζ inhibitor, cell-permeable myristoylated PKCζ pseudosubstrate; YFP, yellow fluorescent protein; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nox, NADPH oxidase.

2 The abbreviations used are: ROS, reactive oxygen species; ASM, acidic sphingomyelinase; CD95, CD95 receptor; CFP, cyan fluorescent protein; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid disodium salt; DISC, death inducing signaling complex; DPI, diphenyleneiodonium chloride; EGFR, epidermal growth factor receptor; JNK, c-Jun N-terminal kinase; FADD, Fas-associated death domain; FRET, fluorescence resonance energy transfer; Huh7, human hepatoma cell line 7; NAC, N-acetylcysteine; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PKCζ, protein kinase C; PKCζ inhibitor, cell-permeable myristoylated PKCζ pseudosubstrate; YFP, yellow fluorescent protein; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nox, NADPH oxidase.
duction (13), and these homologs of gp91phox are called Nox and Duox (12–14). Duox 1 and 2 reflect high molecular mass gp91phox homologs with an N-terminal peroxidase domain in addition to the C-terminal NADPH oxidase activity. NADPH oxidases are activated by assembly with regulatory proteins such as p47phox, p67phox, and Rac (15–18). Although being dispensable for NADPH oxidase activity under cell-free conditions, p47phox is critical for normal NADPH oxidase function, because p47phox acts as an adaptor protein, which facilitates stimulus-induced binding of p67phox to the enzyme complex. In neutrophils, p47phox is activated in response to inflammatory stimuli by multiple phosphorylations, which are thought to trigger intramolecular rearrangements thereby exposing Src homology 3 domains for binding to proline-rich regions of other NADPH oxidase components in order to form the active enzyme complex (19, 20). As shown recently (21), rat hepatocytes express mRNAs for Nox 1, 2, and 4 and Duox 1 and 2, and at the protein level the presence of Nox 2 and p47phox in hepatocytes was demonstrated (21, 22). Evidence has been given that such Nox isoforms are activated in hepatocytes in response to CD95 ligand (21) or hydrophobic bile acids (22). This study was undertaken in order to investigate the role of NADPH isoforms in hyperosmotic signaling toward CD95 activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The materials used were purchased as follows: collagenases from Roche Applied Science; William’s E medium, desipramine, A9944, d-(−)-neopterin, and FITC dextran (average molecular mass 70 kDa) from Sigma; penicillin/streptomycin from Biochrom (Berlin, Germany); fetal calf serum from Invitrogen; Dulbecco’s modified Eagle’s medium/NUT. Mix F12, pTOPO-TA vector and Lipofectamine 2000 from Invitrogen; diphenyleneiodonium chloride (DPI) from Biomol (Hamburg, Germany); myr-PKCζ-pseudosubstrate inhibitor (PKCζ inhibitor), chelerythrine, apocynin, bafilomycin A1, and DIDS from Calbiochem; and CM-H2DCFDA from Molecular Probes (Eugene, OR). The antibodies used were purchased as follows: rabbit anti-p47phox, rabbit anti-gp91phox (Nox 2), rabbit anti-acidic sphingomyelinase (ASM), mouse anti-PKCζ, rabbit anti-CD95, rabbit anti-FADD, and mouse anti-caspase 8 antibodies from Santa Cruz Biotechnology; rabbit anti-phospho-JNK-1/-2 antibodies from BioSource Int. (Camarillo, CA); monoclonal mouse anti-GAPDH and monoclonal mouse anti-annexin II antibodies from Biozol; goat anti-rabbit Cy3-linked to the C terminus. The EGFR-CFP-construct was grown to 70% confluency before transient transfection using expression vectors of the CD95-YFP and EGFR-CFP fusion proteins supplemented with Lipofectamine 2000 culture medium without antibiotics. About 54 ± 6% (n = 3) of the cells coexpressed the transfected EGFR-CFP and CD95-YFP constructs. Osmolarity changes were performed by appropriate addition or removal of NaCl from the medium.

**Reverse Transcription-PCR**—Total RNA was isolated from cultured wild type and p47phox knock-out mice hepatocytes, colon, kidney, and thyroid gland. PCR was conducted using standard protocols. For further details including primers used in this study see Supplemental Material.

**Plasmid Construction**—The nucleotide sequence encoding the CD95 receptor was amplified by reverse transcription-PCR using HepG2 mRNA and ligated into the pTOPO-TA vector (Invitrogen). After introduction of restriction sites and removal of the stop codon by PCR, the fragment was inserted into pEYFP-N1 (Clontech) to create a fusion protein with YFP linked to the C terminus. The EGFR-CFP-construct was obtained by replacing GFP of F7 erb B1-EGFP (kindly provided by Prof. Dr. Arndt-Jovin (25)) by CFP of pEYFP-N1 (Clontech). All constructs were confirmed by sequencing (MWG Biotech, Ebersberg, Germany).

**p47phox and ASM Protein Knockdown**—Antisense oligonucleotides directed to p47phox and the corresponding controls (i.e. nonsense oligonucleotides) were designed and manufactured by Biognostik (Göttingen, Germany). Immediately after hepatocytes were plated on collagen-coated culture plates (inner diameter, 6 cm; Falcon, Heidelberg, Germany) at a density of 4 × 10⁶ cells/plate, 4 μmol/liter of the respective oligonucleotides (nonsense, p47phox antisense, or ASM antisense) supplemented with Lipofectamine 2000 were instituted according to the manufacturer’s recommendations. Thereafter, cells were kept in culture for up to 4 days. In control experiments, uptake of FITC-labeled nonsense oligonucleotides (2 μmol/liter) was already visible after 1 h of incubation and lasted for up to 4 days.

**Western Blot Analysis**—At the end of the incubations, medium was removed, and cells were washed briefly with phosphate-buffered saline (PBS) and immediately lysed. Samples were transferred to SDS-PAGE, and proteins were then blotted to nitrocellulose membranes using a semi-dry transfer apparatus (Amersham Biosciences) as described recently (23). For further details see Supplemental Material.

**Immunoprecipitation**—Hepatocytes were cultured on collagen-coated culture plates (inner diameter, 10 cm; Falcon) at a density of 8 × 10⁶ cells/plate. They were harvested in lysis buffer as published recently (23). Equal protein amounts (200

**AUGUST 11, 2006• VOLUME 281 • NUMBER 32**
Hyperosmolarity and NADPH Oxidase

μg) of each sample were incubated for 2 h at 4 °C with a polyclonal rabbit anti-p47phox, rabbit anti-CD95, or rabbit anti-Yes antibody (dilution 1:100; Santa Cruz Biotechnology) to immunoprecipitate p47phox, CD95, or Yes. Then 10 μl of protein A- and 10 μl of protein G-agarose (Santa Cruz Biotechnology) were added and incubated at 4 °C overnight. Immunoprecipitates were washed three times as published recently (23) and then transferred to Western blot analysis as described above. Activation of p47phox by serine phosphorylation was detected using an anti-phosphoserine antibody (26).

Subcellular Fractionation—Hepatocytes were cultured on collagen-coated culture plates (inner diameter, 10 cm; Falcon) at a density of 8 × 10⁶ cells/plate. Cells were lysed in a buffer containing 10 mmol/liter Tris, 30 mmol/liter mannitol, and 10 mmol/liter CaCl₂ (pH 7.5). After centrifugation of the samples (5 min, 1200 × g), the supernatants were subjected to ultracentrifugation (35 min, 40,000 × g) in order to separate the plasma membrane fraction (pellet) from the cytosolic compartment, including intracellular endomembranes (supernatant). This supernatant was then again subjected to ultracentrifugation (2 h, 100,000 × g) in order to separate the intracellular endomembranes (pellet) from the cytosol (supernatant). The latter fractions underwent Western blotting as described above for p47phox, gp91phox, acidic sphingomyelinase, GAPDH, and annexin II. GAPDH and annexin II served as markers for the cytotoxic and the plasma membrane fraction, respectively. GAPDH was not detectable in the plasma membrane fraction, and annexin II was absent in the cytosolic fraction, indicating a high efficacy of separation.

Immunocytochemistry of ASM—To visualize ASP expression, 1.5 × 10⁶ hepatocytes were seeded on collagen-coated glass coverslips (inner diameter, 30 mm) in 6-well culture plates (Falcon) and cultured for 24 h. Cells were washed with PBS and then fixed using paraformaldehyde (4% v/v, 10 min). If indicated, cells were incubated for 1 h with 5 mg/ml FITC-dextran before fixation in order to visualize endocytotic vesicles. First antibodies against ASP (Santa Cruz Biotechnology; 1:100) were diluted in PBS and applied for 1 h. After intense washing with PBS, Cy3-conjugated secondary antibodies (1:500) were applied for another hour. Coverslips were mounted with diazabicyclo[2.2.2]octane coupled secondary antibodies (1:500) and washed with PBS and applied for 1 h. After intense washing with PBS, Cy3-conjugated secondary antibodies (1:500) were applied for another hour. Coverslips were mounted with diazabicyclo[2.2.2]octane coupled secondary antibodies (1:500) and washed with PBS and then incubated with FITC-dextran (5 mg/ml) for another 60 min at 37 °C. For fluorescence recording, the coverslips were mounted with PBS at 37 °C and equilibrated with room atmosphere resulting in a pH of 7.4. Measurements of apparent pHves in single cells were performed with an inverted fluorescence microscope (Zeiss, Axiovert) combined with the QuantiCell 2000-calcium imaging setup (VisiTech, Sunderland, UK). This apparatus allows FITC-dextran fluorescence measurements at the single-cell level at the excitation wavelengths of 488/440 nm with a time resolution of 20 Hz by a monochromator, and emission was measured at 515–565 nm using a CCD camera as provided by the QuantiCell 2000-calcium imaging setup. By use of regions of interest using the QuantiCell 2000 software, the field of measurement was chosen to be within one single cell. The raw fluorescence signals were corrected for autofluorescence as published previously (27). Apparent pHves were obtained from the corrected ratios of 488/440 nm after appropriate calibration according to Thomas et al. (28).

Fluorescence Resonance Energy Transfer (FRET) Experiments—FRET was used in order to visualize hyperosmotically induced interactions between EGFR and CD95. For this purpose HuH7 hepatoma cells were cotransfected with CD95-YFP and EGFR-CFP, as described in detail recently (29). Confocal pictures were taken using the LSM-510-META (Zeiss, Oberkochen, Germany). All cotransfections with YFP/CFP-coupled constructs were detected using the META scan avoiding bleed through of CFP in the YFP channel. CFP was excited with 405 nm and YFP with 514 nm (30). FRET efficiency was determined using the LSM-Image-Examiner version 3.1 software (Zeiss), and FRET pictures were normalized for the respective settings as indicated by the accompanying scale (FRET efficiency is given from blue/0 to red/255) (29).

Lipid Extraction and High Performance Thin Layer Chromatography—To measure intracellular ceramide concentrations, 500 μg of protein of the respective samples were transferred to quantification of lipids by Folch extraction (31). Thereafter, high performance thin layer chromatography and quantification of ceramide were performed as published previously (32–33). For details see Supplemental Material.

Statistics—Results from at least three independent experiments are expressed as means ± S.E. 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

Hyperosmolarity Induces Serine Phosphorylation of p47phox and Activates NADPH Oxidase Isoforms in Rat Hepatocytes—As shown recently (1, 11), hyperosmotic exposure (405 mosmol/liter) of 24-h cultured rat hepatocytes induced within 1 min an oxidative stress response, as detected by means of DCFDA fluorescence. This oxidative stress response was significantly inhibited by diphenyleneiodonium, apocynin, and neopterin, i.e., known inhibitors of NADPH oxidases (34–36) (Fig. 1). Rat hepatocytes were recently shown to express gp91phox (Nox 2) and the regulatory subunit p47phox, suggestive for the presence of at least one regulated NADPH oxidase (Nox 2) activity (21, 22). In addition, at the mRNA level also the Nox...
isoforms 1 and 4 and Duox 1 and 2 were detected in rat hepatocytes (21). Hyperosmotic exposure (405 mosmol/liter) of 24-h cultured rat hepatocytes led within 1 min to p47phox-Ser phosphorylation (Fig. 2), which persisted for more than 60 min. p47phox-Ser phosphorylation, which is known to be essential for Nox 2 activation in neutrophils and macrophages (19, 20), was osmolarity-dependent, and osmolarity increases by 20–40 mosmol/liter were sufficient to induce p47phox-Ser phosphorylation (supplemental Fig. 1).

Requirement of p47phox for the Hyperosmotic ROS Response—The role of p47phox in mediating the hyperosmotic oxidative stress response was substantiated in studies on 4-day cultured rat hepatocytes following transfection with nonsense or p47phox antisense oligonucleotides. As shown previously (see Fig. 3A in Ref. 21), 4 days after transfection with the antisense, but not the nonsense oligonucleotide, a substantial down-regulation of p47phox protein is achieved. Under these conditions, p47phox knockdown largely abolished the hyperosmolarity-induced increase of DCFDA fluorescence, which was preserved in

Hyperosmolarity and NADPH Oxidase

FIGURE 1. Generation of ROS in rat hepatocytes by hyperosmolarity is sensitive to inhibitors of NADPH oxidase. Hepatocytes were cultured for 24 h and then loaded with 5 μmol/liter CM-H2DCFDA as described under “Experimental Procedures.” When indicated, DPI (10 μmol/liter), apocynin (300 μmol/liter), or d-(-)-neopterin (100 μmol/liter) were preincubated for 30 min prior to hyperosmotic (405 mosmol/liter) exposure. In all experiments, the hyperosmolarity-induced increase of DCFDA fluorescence was measured 1 min after addition of the hyperosmotic medium. Fluorescence obtained in cells that were exposed to normo-osmotic medium (305 mosmol/liter) was arbitrarily set to 1. Hyperosmosmality induces a rapid ROS response compared with normo-osmotic treated control cells, which was significantly inhibited by DPI, apocynin, or neopterin (p < 0.05; *, n = 3).

FIGURE 2. Hyperosmolarity-induced p47phox-serine phosphorylation. Hepatocytes were cultured for 24 h and then exposed to hyperosmotic medium (405 mosmol/liter) for the time periods indicated. p47phox was immunoprecipitated as described under “Experimental Procedures” and detected for serine phosphorylation by Western blotting. Total p47phox served as loading control. Hyperosmotic exposure (405 mosmol/liter) induced within 1 min p47phox-Ser phosphorylation. Densitometric analysis revealed a significant increase in p47phox-Ser phosphorylation within 1 min, which lasts at least for 60 min (p < 0.05; *, n = 3).

FIGURE 3. Hyperosmotic ROS generation is abolished after p47phox protein knockdown in cultured rat hepatocytes. p47phox protein knockdown was achieved as described under “Experimental Procedures” by use of antisense oligonucleotides. Rat hepatocytes were cultured for 96 h in normo-osmotic control medium, supplemented with nonsense or p47phox antisense oligonucleotides, respectively. Effective knockdown of the NADPH oxidase subunit protein p47phox under these conditions was shown recently (21). Cells were then loaded with 5 μmol/liter CM-H2DCFDA as described under “Experimental Procedures” before hyperosmotic exposure (405 mosmol/liter). In all experiments, the hyperosmolarity-induced increase of DCFDA fluorescence was measured 1 min after hyperosmotic exposure. Fluorescence obtained in normo-osmotic (305 mosmol/liter) control cells was arbitrarily set to 1. The hyperosmotic ROS response was significantly inhibited after p47phox protein knockdown (p < 0.05; *, n = 3).

FIGURE 4. Hyperosmolarity-induced ROS formation in hepatocytes from wild type or p47phox knock-out mice. Hepatocytes were isolated from either control or p47phox knock-out mice as described under “Experimental Procedures” and cultured for 24 h. Hepatocytes from either wild type or p47phox knock-out mice were loaded with 5 μmol/liter CM-H2DCFDA as described under “Experimental Procedures” before the hyperosmotic challenge (405 mosmol/liter). In all experiments, the hyperosmolarity-induced increase of DCFDA fluorescence was measured after 1 min. Fluorescence obtained in normo-osmotic (305 mosmol/liter) control cells was arbitrarily set to 1. Hyperosmosmality induces a rapid ROS response in hepatocytes from wild type but not from p47phox knock-out mice (p < 0.05; *, n = 3).
Hyperosmolarity and NADPH Oxidase

p47phox served as a loading control. Hyperosmolarity-induced p47phox-Ser phosphorylation by Western blotting (see “Experimental Procedures”). Total bated for 30 min. p47phox was immunoprecipitated and detected for serine phosphorylation was sensitive to inhibition of sphingomyelinases (AY9944, desipramine) and PKC (Fig. 5B). Hyperosmotic exposure induced PKC phosphorylation. 24-h cultured hepatocytes were exposed to normo-osmolarity (305 mosmol/liter) or hyperosmolarity (405 mosmol/liter) for 1 min. When indicated, AY9944 (5 mol/liter), desipramine (5 mol/liter), PKC inhibitor (100 μmol/liter), or chelerythrine (20 μmol/liter) were preincubated for 30 min. p47phox was immunoprecipitated and detected for serine phosphorylation by Western blotting (see “Experimental Procedures”). Total p47phox served as a loading control. Hyperosmolarity-induced p47phox-Ser phosphorylation was sensitive to inhibition of sphingomyelinases (AY9944, desipramine) and PKCζ (PKCζ-inhibitory pseudosubstrate, chelerythrine) (n = 3). B and C, hyperosmolarity-induced increase of ceramide levels. Rat hepatocytes were cultured for 24 h and then exposed to hyperosmolarity (405 mosmol/liter). Hyperosmolarity induced within 30 s a significant increase in intracellular ceramide levels (p < 0.05; #, n = 4) (B). Inhibitor profile (C). When indicated, AY9944 (5 mol/liter) or desipramine (5 mol/liter) was preincubated for 30 min prior to hyperosmotic exposure. Relative ceramide levels are given after 1 min of hyperosmotic exposure. Inhibition of sphingomyelinase by AY9944 and desipramine significantly inhibited hyperosmotically-induced increase of ceramide levels. Rat hepatocytes were exposed to either normo-osmotic control medium (305 mosmol/liter) or hyperosmotic medium (405 mosmol/liter) for 1 min. Cells were stained for PKCζ by immunocytochemistry (see “Experimental Procedures”). Hyperosmotic exposure induced PKCζ translocation to the plasma membrane, suggestive for PKCζ activation (n = 3). C, inhibitor sensitivity of hyperosmolarity-induced ROS generation. Hepatocytes were cultured for 24 h and loaded with 5 μmol/liter CM-H2DCFDA as described under “Experimental Procedures” and then exposed to hyperosmolarity (405 mosmol/liter). When indicated, AY9944 (5 mol/liter), desipramine (5 mol/liter), PKCζ inhibitor (100 μmol/liter), or chelerythrine (20 μmol/liter) were preincubated for 30 min. The increase of DCFDA fluorescence was measured 1 min after hyperosmotic exposure, and the signal found in untreated control cells was set to 1. Hyperosmolarity-induced ROS generation was significantly inhibited by inhibition of sphingomyelinase and PKCζ (p < 0.05; *, n = 3).

untransfected cells or cells transfected with the nonsense oligonucleotide (Fig. 3).

The role of p47phox in triggering the hyperosmotic ROS response was also demonstrated in p47phox knock-out mice. Both wild type and p47phox knock-out mice expressed at the mRNA level Nox 2 and 4 as well as Duox 2, whereas the mRNAs for Nox 1 and 3 and Duox 1 were not detectable (supplemental Fig. 2). Thus, the pattern of NADPH oxidase isoform mRNA expression in mouse liver differed from that reported previously in rat hepatocytes (21), in that mRNAs for Nox 1 and Duox 1 were not detectable in mouse hepatocytes. Although hyperosmotic exposure of hepatocytes from wild type mice induced a rapid ROS response, this was not observed in hepatocytes from p47phox knock-out mice (Fig. 4). These findings suggest that the rapid oxidative stress response following hyperosmotic hepatocyte shrinkage is mediated by p47phox-regulated NADPH oxidases.

Mechanism of Hyperosmotic p47phox Activation—The hyperosmolarity-induced p47phox-Ser phosphorylation was sensitive to AY9944 and desipramine, i.e. known inhibitors of sphingomyelinases (37, 38) (Fig. 5A). This finding suggests that ceramide formation in response to hyperosmolarity may play a role in triggering hyperosmotic p47phox-Ser phosphorylation. In line with this, hyperosmotic exposure of rat hepatocytes resulted within 1 min in a marked increase of hepatocellular ceramide levels (Fig. 5B), which was largely abolished after inhibition of sphingomyelinases by AY9944 and desipramine (Fig. 5C). Hyperosmotic p47phox phosphorylation was also sensitive to inhibition of PKCζ by a specific synthetic inhibitory substrate or by the less specific chelerythrine (Fig. 5A). As a surro-
gate marker of hyperosmotic PKCζ activation, translocation of this PKC isoform to the plasma membrane occurred within 1 min of hyperosmotic exposure (Fig. 5D). Because ceramide was recently shown to directly activate PKCζ (39, 40), these findings suggest that hyperosmolality activates Nox/Duox isoforms through a sphingomyelinase-, ceramide-, and PKCζ-dependent serine phosphorylation of the regulatory subunit p47phox. In line with this, AY9944, desipramine, and PKCζ inhibitors strongly blunted the hyperosmolality-induced ROS response (Fig. 5E).

Role of ASM for Hyperosmotic Ceramide Formation and NADPH Oxidase Activation—The role of ASM for the hyperosmotically induced ceramide formation was studied in 4-day cultured rat hepatocytes after knockdown of ASM using an antisense approach. As shown in Fig. 6A, a substantial knockdown of ASM was achieved in hepatocytes transfected with the ASM antisense oligonucleotide but not after transfection of the nonsense oligonucleotide. ASM knockdown largely abolished the hyperosmolarity-induced increase in p47phox phosphorylation (Fig. 6A), as well as hyperosmolarity-induced ceramide (Fig. 6B) and ROS formation (Fig. 6C). This points to an involvement of ASM in the activation of NADPH oxidase isoforms by hyperosmolality, whereas other sphingomyelinase isoenzymes apparently play a minor role.

Cell fractionation studies revealed the presence of ASM not only in the plasma membrane and the cytosolic fraction but also in a presumably endosomal compartment, which sediments at 100,000 x g and also contains gp91phox (Nox 2), but not p47phox (Fig. 7A). Localization of ASM in the plasma membrane and in a putative vesicular compartment in the perinuclear region was also shown by immunocytochemistry (Fig. 7B). Subfractionation studies performed 5 min after hyperosmotic exposure showed that p47phox was now also detectable in the 100,000 x g sedimentable fraction, suggesting that cytosolic p47phox was recruited to NADPH oxidase complexes contained in the endosomal membranes after hyperosmotic stimulation.

Endosomal Acidification Is Required for Hyperosmotic Ceramide and ROS Formation—The data in Fig. 7 suggest a localization of ASM not only in the plasma membrane but also in an intracellular endosomal/lysosomal compartment, in line with previous reports (for reviews see Refs. 41 and 42). In rat hepatocytes, which were allowed to endocytose FITC-coupled dextran for 60 min, ASM colocalized to some extent with FITC-dextran-containing endocytic vesicles (Fig. 7B). As shown previously (27, 43, 44), such vesicles exhibit an apparent pH of about 6 and reflect a prelysosomal endocytotic compartment,

which was abolished after ASM knockdown (n = 3). B, ASM knockdown prevents the increase of ceramide levels in response to hyperosmolality. Ceramide was measured as described under “Experimental Procedures.” Hyperosmolality (405 mosmol/liter) induced a significant increase in intracellular ceramide levels in cells treated with nonsense oligonucleotides (p < 0.05; #) but not after ASM protein knockdown (p < 0.05; *). C, ASM knockdown prevents hyperosmotic ROS formation. Cells were loaded with 5 μmol/liter CM-H2DCFDA as described under “Experimental Procedures” before initiation of hyperosmolality (405 mosmol/liter). Hyperosmolality-induced increase of DCFDA fluorescence was measured 1 min after hyperosmotic exposure. Fluorescence obtained in normo-osmotic (305 mosmol/liter) treated control cells was arbitrarily set to 1. Hyperosmolality induces a rapid ROS response, which is largely abolished after ASM protein knockdown (p < 0.05; *, n = 3).
whereas a more acidic (pH around 5) and presumably lysosomal compartment is reached by endocytosed FITC-dextran after 6 h (43).

As reported earlier in studies on FITC-dextran or acridine orange-loaded hepatocytes (27, 43–45), hypo-osmotic exposure of rat hepatocytes leads to an alkalinization of endocytotic vesicles, whereas hyperosmotic hepatocyte shrinkage induced acidification. The functional significance, however, remained unknown. In view of the known pH dependence of ASM activity, the effect of hyperosmolarity on the pH in endocytotic vesicles is assessed. The results presented in panels A and B are consistent with the hypothesized model where hyperosmolarity induces acidification of the endocytotic vesicles, leading to an increase in ASM activity.
icicles (apparent pH\textsubscript{ves}) was studied in relation to hyperosmotic ceramide formation. Hepatocytes were allowed to endocytose FITC-dextran for 60 min, and apparent pH\textsubscript{ves} was monitored by means of FITC fluorescence, as described previously (27, 43, 44). Under these conditions FITC-dextran enters an endosomal compartment upstream of lysosomes (43), which is characterized by an apparent pH\textsubscript{ves} of around 6 (27, 43, 44). In line with this, in normo-osmotically exposed rat hepatocytes apparent pH\textsubscript{ves} was 5.99 ± 0.03 (n = 13; Table 1). Hyperosmotic exposure of rat hepatocytes decreased within less than 5 s of apparent pH\textsubscript{ves} by 0.36 ± 0.03 (n = 13). The hyperosmolarity-induced vesicular acidification was strongly blunted by bafilomycin, an inhibitor of vacuolar-type H\textsuperscript{+}-ATPase (Table 1). Likewise DIDS, an anion channel blocker, largely prevented the hyperosmosis-induced decrease of apparent pH\textsubscript{ves}, whereas AY9944 and apocynin, i.e. inhibitors of ASM and NADPH oxidases, respectively, were ineffective (Table 1). The finding that AY9944 and apocynin, which potently inhibit the hyperosmotic ROS response (Figs. 1 and 5E), had no effect on the hyperosmolarity-induced acidification of FITC-dextran-accessible vesicles suggests that hyperosmotic endosomal acidification is not a phenomenon secondary to hyperosmotic NADPH oxidase activation or ROS formation.

Both bafilomycin and DIDS inhibited not only the hyperosmotic decrease of apparent pH\textsubscript{ves} (Table 1) but also the hyperosmolarity-induced increase of ceramide levels (Fig. 8A) and p47\textsuperscript{phox}-Ser phosphorylation (Fig. 8B). In line with this, DIDS and bafilomycin also blunted the hyperosmotic ROS response, indicating translocation of p47\textsuperscript{phox} to the endosomal compartment. GAPDH and annexin II served as markers for the cytosolic and the plasma membrane fractions, respectively. GAPDH was not detectable in the cytosolic fraction, and annexin II was not detectable in the membrane fraction, indicating a high efficacy of separation (n = 3). B, colocalization of ASM with endocytosed FITC-dextran. Hepatocytes were allowed to endocytose FITC-dextran (5 mg/ml) for 1 h as described under “Experimental Procedures.” Thereafter cells were immunostained for ASM, which revealed some colocalization of ASM with endocytosed FITC-dextran in a presumably endosomal compartment, as studied by confocal microscopy.

### Table 1

| Condition | Apparent pH\textsubscript{ves} (305 mosmol/liter) | Apparent pH\textsubscript{ves} (405 mosmol/liter) | ∆pH\textsubscript{ves} induced by hyperosmolarity |
|-----------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Control   | 5.99 ± 0.03                                   | 5.63 ± 0.03                                   | −0.36 ± 0.03                                   |
| DIDS      | 5.86 ± 0.03                                   | 5.78 ± 0.02 (NS)                              | −0.08 ± 0.02 (NS)                              |
| Bafilomycin A1 | 6.11 ± 0.02                                   | 5.98 ± 0.03 (NS)                              | −0.14 ± 0.02 (NS)                              |
| AY9944    | 6.01 ± 0.02                                   | 5.63 ± 0.04 (NS)                              | −0.38 ± 0.05 (NS)                              |
| Apocynin  | 5.98 ± 0.04                                   | 5.61 ± 0.04 (NS)                              | −0.37 ± 0.04 (NS)                              |

* Statistically significant difference between hyperosmolarity and the corresponding normo-osmotic condition is shown (p < 0.05).

* Statistically significant inhibition of hyperosmosis-induced acidification is shown (p < 0.05).

### Figure 8

A, Bafilomycin and DIDS sensitivity of hyperosmotic acidification of ceramide formation (A), p47\textsuperscript{phox}-Serphorylation (B), and generation of ROS (C) in rat hepatocytes. Hepatocytes were cultured for 24 h and then exposed for 1 min to normo-osmotic (305 mosmol/liter) or hyperosmotic medium (405 mosmol/liter). When indicated, DIDS (500 μmol/liter) or bafilomycin A1 (100 nmol/liter) was preincubated for 30 min prior to hyperosmotic exposure. The hyperosmolarity-induced increase in ceramide levels (p < 0.05; #, n = 10) (A), p47\textsuperscript{phox}-Ser phosphorylation (n = 3) (B), and ROS formation (n = 3) (C) was largely abolished in presence of DIDS or bafilomycin (p < 0.05; *, n = 5). For further experimental details see “Experimental Procedures” and Fig. 5 legend.
Hyperosmolarity and NADPH Oxidase

as detected by DCFDA fluorescence (Fig. 8C). These data suggest that a hyperosmolarity-induced endosomal acidification may largely account for the ASM-dependent stimulation of ceramide formation.

Role of NADPH Oxidase for Hyperosmotic CD95 Activation—As shown recently (1, 10, 11), hyperosmotic hepatocyte shrinkage activates the CD95 system through a complex sequence of events, which is triggered by the upstream ROS signal (1, 11). Therefore, the role of hyperosmotic NADPH oxidase activation in triggering CD95 activation was studied. As shown recently, hyperosmotic CD95 activation involves downstream of the ROS response a Yes-dependent JNK and EGFR activation, subsequent EGFR-CD95 association, and EGFR-catalyzed CD95-tyrosine phosphorylation, which is the signal for membrane translocation of the EGFR-CD95 complex and formation of the death-inducing signaling complex (DISC) (1, 11). Although all these events are triggered already by mild or moderate hyperosmolarity (405 mosmol/liter), hepatocytes are only sensitized toward CD95 ligand-induced apoptosis under these conditions, but apoptosis is not executed by mild hyperosmolarity (405 mosmol/liter) itself (10). This is also reflected by the effect of moderate hyperosmolarity (405 mosmol/liter) on PARP cleavage as apoptotic readout (Fig. 9); however, more severe hyperosmotic stress (i.e., >505 mosmol/liter) induces PARP cleavage, suggestive for execution of hepatocyte apoptosis (Fig. 9).

To assess the role of NADPH oxidases in triggering upstream events of hyperosmotic CD95 activation, inhibitor studies were performed. As shown in Fig. 10A, hyperosmotic Yes activation was strongly blunted in the presence of inhibitors of NADPH oxidases (i.e., DPI, apocynin, and neopterin) and the antioxidant N-acetylcysteine (NAC), as reported recently (11). These inhibitors also prevented the hyperosmolarity-induced EGFR and JNK activation, Yes/EGFR association, EGFR activation, EGFR-CD95 association, CD95-tyrosine phosphorylation, and the recruitment of FADD and caspase 8 to CD95 (DISC formation) (Fig. 10, A and B). These findings suggest that a Nox-derived ROS signal triggers hyperosmotic Yes activation and the subsequent steps leading to CD95 activation.

Requirement of p47phox Serine Phosphorylation for Hyperosmotic CD95 Activation—As above, hyperosmotic activation of NADPH oxidases requires an ASM-dependent serine phosphorylation of p47phox. Inhibition of ASM by AY9944 or desipramine or of PKCζ by specific inhibitory substrate or chelerythrine inhibited the hyperosmolarity-induced ROS response (Fig. 5E). These inhibitors also strongly blunted EGFR (Fig. 10B) and JNK activation (supplemental Fig. 3) and largely prevented

FIGURE 9. Hyperosmolarity-induced PARP cleavage. Hepatocytes were cultured for 24 h and then exposed for 12 h to hyperosmotic media with osmolarities as indicated. Total PARP (116 kDa) and its cleavage product (cleaved PARP 85 kDa) were detected by Western blotting. PARP cleavage occurs at osmolarities above 505 mosmol/liter, suggestive for apoptotic cell death (n = 3).

FIGURE 10. Hyperosmolarity-induced activation of the CD95 system is sensitive to inhibitors of NADPH oxidase, sphingomyelinase, and PKCζ. Hepatocytes were cultured for 24 h and then exposed to hyperosmolarity (405 mosmol/liter). When indicated, DPI (10 μmol/liter), apocynin (300 μmol/liter), NAC (30 mmol/liter), AY9944 (5 μmol/liter), desipramine (5 μmol/liter), PKCζ inhibitor (100 μmol/liter), or chelerythrine (20 μmol/liter) were preincubated for 30 min. Yes, EGFR, and CD95 were immunoprecipitated as described under “Experimental Procedures” and analyzed by Western blotting. Activating Yes-Y418 phosphorylation, Yes/EGFR association, and EGFR tyrosine phosphorylation (EGFR-Tyr-P) was detected 1 min after hyperosmotic exposure, EGFR-CD95 association and CD95-tyrosine phosphorylation (CD95-Tyr-P) after 60 min, and DISC formation, i.e. caspase 8/CD95 and FADD/CD95 association, after 3 h of hyperosmotic exposure. Total Yes, EGFR, and CD95 served as respective loading controls. Hyperosmolarity induced within 1 min a Yes activation, Yes/EGFR association followed by EGFR-tyrosine phosphorylation, within 60 min EGFR-CD95 association and CD95-tyrosine phosphorylation, and within 3 h DISC formation. These events were sensitive to inhibitors of NADPH oxidase (DPI, apocynin, and neopterin) and antioxidants (NAC) (A) and of sphingomyelinase (AY9944, desipramine) and PKCζ (PKCζ inhibitory pseudosubstrate, chelerythrine) (B).
Hyperosmolarity induced within 30 min an intracellular EGFR-CFP/CD95-YFP association as indicated by the FRET signal that was followed by an enrichment of the EGFR-CFP/CD95-YFP protein complex to the plasma membrane. Inhibition of sphingomyelinase, PKC, or NADPH oxidase by AY9944, PKC inhibitor, or apocynin, respectively, prevented both the intracellular EGFR-CFP/CD95-YFP association and translocation to the plasma membrane, which were otherwise observed after 30 and 120 min, respectively. For statistical analysis see Table 2.

### TABLE 2

**Inhibitor profile of the hyperosmolarity-induced EGFR-CFP/CD95-YFP association and subsequent membrane translocation of the CD95-EGFR protein complex in Huh7 hepatoma cells**

Huh7 hepatoma cells were cotransfected with EGFR-CFP and CD95-YFP and then exposed to hyperosmolarity (405 mosmol/liter) for 0, 30, or 120 min, respectively. When indicated, DIDS (500 μmol/liter), bafilomycin A1 (100 nmol/liter), AY9944 (5 μmol/liter), PKC inhibitor (100 μmol/liter), or apocynin (300 μmol/liter) were added 30 min prior to hyperosmotic exposure. About 54 ± 6% (n = 3) of the cells expressed the transfected EGFR-CFP and CD95-YFP constructs. FRET pictures were taken as described under “Experimental Procedures” and then normalized with respect to FRET efficiencies (scale indicates FRET efficiency from blue to red).

| Huh7 cells condition | No FRET signal (%) | Intracellular FRET signal (%) | Plasma membrane FRET signal (%) |
|----------------------|--------------------|-------------------------------|--------------------------------|
|                      | 0 min              | 30 min | 120 min | 0 min | 30 min | 120 min | 0 min | 30 min | 120 min |
| 405 mosmol/liter     |                    |        |         |       |        |         |       |        |         |
| control              | 95.9 ± 1.6         | 77.7 ± 0.7 | 62.2 ± 2.2 | 2.7 ± 0.7 | 19.3 ± 0.4 | 16.9 ± 1.2 | 1.3 ± 0.9 | 3.0 ± 1.1 | 20.9 ± 1.0 |
| + DIDS               | 94.9 ± 0.5         | 90.7 ± 1.1* | 89.9 ± 2.8* | 2.7 ± 0.4 | 5.6 ± 0.7* | 3.7 ± 1.5* | 2.3 ± 0.9 | 3.7 ± 1.1 | 6.4 ± 1.2* |
|                      | 92.4 ± 1.3         | 91.0 ± 1.8* | 89.3 ± 0.2* | 4.8 ± 1.2 | 5.2 ± 1.6* | 2.6 ± 0.6* | 2.7 ± 0.2 | 3.8 ± 0.4 | 8.1 ± 0.7* |
| + Bafilomycin        | 95.0 ± 0.6         | 86.9 ± 1.1* | 88.0 ± 2.3* | 2.3 ± 0.3 | 9.4 ± 0.9* | 3.3 ± 0.7* | 2.7 ± 0.9 | 3.7 ± 0.8 | 8.7 ± 1.7* |
|                      | 94.3 ± 0.7         | 89.7 ± 1.1* | 87.7 ± 2.8* | 3.7 ± 0.5 | 7.1 ± 1.2* | 3.5 ± 0.4* | 2.0 ± 0.6 | 3.2 ± 1.2 | 8.8 ± 2.5* |
| + PKC inhibitor      | 96.3 ± 0.4         | 92.5 ± 1.6* | 89.2 ± 2.0* | 1.7 ± 0.7 | 5.1 ± 1.0* | 3.1 ± 1.1* | 2.0 ± 0.6 | 2.3 ± 0.8 | 7.8 ± 1.0* |
| + Apocynin           |                    |        |         |       |        |         |       |        |         |

Hyperosmotic EGFR-CD95 association, EGFR-catalyzed CD95-Tyr phosphorylation, and recruitment of FADD and caspase 8 to CD95, i.e. formation of the death-inducing signaling complex (Fig. 10B). In line with a requirement of p47phox phosphorylation for hyperosmotic CD95 activation, the osmdependence of p47phox. Ser phosphorylation...
Supplemental Fig. 1 paralleled that of Yes activation (supplemental Fig. 4).

Inhibition of hyperosmotic EGFR-CD95 association and subsequent translocation of this protein complex to the plasma membrane was also shown by FRET in Huh7 hepatoma cells, which were cotransfected with EGFR-CFP and CD95-YFP constructs, as described recently (21, 22, 29). Hyperosmotic exposure of these cells resulted within 30 min in an intracellular FRET signal, indicating EGFR-CD95 association in the cytosol (Fig. 11A; Table 2), as described recently (21, 22, 29). After 120 min, the FRET signal was found predominantly in the plasma membrane, suggestive of a translocation of the EGFR-CD95 protein complex from the cellular interior to the plasma membrane (Fig. 11B; Table 2). AY9944, the PKCζ inhibitory pseudosubstrate, or inhibition of NADPH oxidases by apocynin prevented both the intracellular EGFR-CD95 association and the subsequent translocation to the plasma membrane (Fig. 11; Table 2).

Also knockdown of the p47phox protein in rat hepatocytes using an antisense strategy blunted the hyperosmotic activation of Yes, JNK, and EGFR as well as EGFR-CD95 association, EGFR-catalyzed CD95-Tyr phosphorylation, membrane trafficking of CD95, and DISC formation, whereas treatment of hepatocytes with a nonsense oligonucleotide was ineffective (Fig. 12).

The requirement of p47phox for hyperosmotic CD95 activation was also investigated in experiments with hepatocytes from wild type or p47phox knock-out mice. In hepatocytes from wild type mice, hyperosmotic exposure induced Yes and EGFR activation, CD95-Tyr phosphorylation, and DISC formation, whereas these responses were largely abolished in hepatocytes from p47phox knock-out mice (Fig. 13). The lack of hyperosmotic CD95 activation in hepatocytes from p47phox knock-out mice was not explained by differences in the mRNA expression of Nox isoforms compared with wild type mice (supplemental Fig. 2).

**Requirement of ASM and Endosomal Acidification for Hyperosmotic CD95 Activation**—As shown above (Fig. 6), knockdown of ASM prevented the hyperosmotic increase in ceramide levels, p47phox phosphorylation, and the ROS response. In line with an important role of ASM-dependent NADPH oxidase activation for hyperosmotic CD95 activation, ASM knockdown also largely prevented hyperosmotic Yes, EGFR, and JNK activation, as well as CD95-Tyr phosphorylation and DISC formation (Fig. 14).

Also inhibition of vacuolar type H⁺-ATPase by bafilomycin or inhibition of anion channels by DIDS, which strongly
blunt the hyperosmolarity-induced acidification of ASM-containing, FITC-dextran-accessible vesicles (Table 1), ceramide formation, p47<sub>phox</sub> phosphorylation, and ROS formation (Fig. 8), prevented the hyperosmotic activation of Yes, JNK, EGFR, and the CD95 system including DISC formation (Fig. 15). Both inhibitors also prevented the hyperosmolarity-induced CD95/EGFR association and translocation of this protein complex to the plasma membrane in EGFR-CPF/CD95-YFP cotransfected Huh7 hepatoma cells, as shown by FRET analysis (Fig. 16; Table 2).

### DISCUSSION

**Hyperosmolarity Activates NADPH Oxidase Isoforms in Hepatocytes**—Hyperosmotic exposure is known to induce oxidative stress in rat hepatocytes (1, 11); however, the underlying mechanisms remained obscure. As shown in this study, the almost immediate hyperosmolarity-induced ROS response is apparently triggered by NADPH oxidase isoforms, which are found in many cell types, including rat hepatocytes (21), and which are increasingly recognized as an important site of cell signaling (12–16). In line with this, hyperosmolarity induced a rapid serine phosphorylation of the regulatory subunit p47<sub>phox</sub>, and the hyperosmolarity-induced ROS response was not only abolished by inhibitors of NADPH oxidases but also after knockdown of p47<sub>phox</sub> and was not observed in hepatocytes from p47<sub>phox</sub> knock-out mice. Ceramide formation, probably because of a hyperosmotic activation of ASM, and subsequent activation of PKC<sub>ζ/H9256</sub> are upstream events of hyperosmotic p47<sub>phox</sub> phosphorylation and Nox/Duox activation. In line with this, p47<sub>phox</sub> was recently shown to be a direct phosphorylation target of PKC<sub>ζ/H9256</sub> (46), and this PKC isoform participates in neutrophil respiratory burst (46, 47). In addition, evidence has been given that ceramide can bind to and activate PKC<sub>ζ/H9256</sub> directly (for review see Ref. 48). Also CD95 ligand and proapoptotic bile acids increase ceramide formation and trigger a PKC<sub>ζ/H9256</sub> inhibitor-sensitive phosphorylation of p47<sub>phox</sub> and ROS production in rat hepatocytes, as shown recently (21, 22).

Which Nox/Duox isoforms bring about the hyperosmotic ROS response are unclear. p47<sub>phox</sub> is an established essential activating component of the Nox 2 NADPH oxidase complex, whereas the role of p47<sub>phox</sub> for the in vivo activation of other Nox/Duox isoforms is not yet settled. Thus, the presence of Nox 2 at the mRNA and protein level in rat hepatocytes suggests that this isoform is a likely candidate for the induction of oxidative stress in response to hyperosmotic hepatocyte shrinkage. An involvement of other Nox/Duox isoforms, however, is not ruled out, because in Huh7 cells, which express Nox 1, 3, and 4 and Duox 1 and 2, but not Nox 2, the bile salt-induced ROS response was shown to be p47<sub>phox</sub>-dependent (22).

**Mechanism of Hyperosmotic Ceramide Formation**—Ceramide is formed by the action of sphingomyelinases, and at least five major classes of sphingomyelinases have been identified (for reviews see Refs. 41 and 49–52). Among them, ASM is probably the major enzyme, which triggers ceramide formation in response to hyperosmolarity in rat hepatocytes, because ASM knockdown prevented hyperosmotic ceramide formation, p47<sub>phox</sub> phosphorylation, and the hyperosmotic ROS response. However, a secondary and later involvement of neutral sphingomyelinase cannot be excluded. This is because hyperosmolarity induces a sustained elevation of ceramide levels in hepatocytes, to which the initial rapid ROS response could contribute through a secondary activation of neutral sphingomyelinase (50). ASM is activated by different kinds of stress by yet unknown pathways; potential mechanisms of ASM activation have been discussed recently (48, 53). ASM has a pH
optimum around 5 (37, 50), and acidity is thought to increase the affinity of ASM to its substrate (54). ASM was shown localize in the endosomal/lysosomal compartment and to some extent at the plasma membrane (49, 55, 56), and this was confirmed also in the present study. Apart from pH, further activators of ASM include activating proteins and lipids, phosphatidylinositol 3-kinase, and oxidants (for review see Refs. 50 and 57). Membrane rafts are specific sites for ceramide formation, because they are enriched in sphingolipids and ASM (for reviews see Refs. 58 and 59). They represent platforms for signaling components, including PKC isoforms, which may be involved in hyperosmotic NADPH oxidase activation.

The present data suggest that hyperosmotic ASM activation results from acidification of an ASM-containing endosomal compartment, which is accessible to endocytosed FITC-dextran. In view of the pH optimum of ASM at around 5, a shift of apparent pHves from 6.0 to 5.6, as it is observed upon hyperosmotic exposure, is expected to substantially increase ASM-catalyzed ceramide formation. However, one has to keep in mind that the apparent pHves determined in our study adds up the pH of FITC-dextran-containing compartments, which may be heterogeneous with regard to acidity in the individual vesicle, and this may also hold for the hyperosmotic acidification. Nonetheless, hyperosmotic acidification of this compartment appears to be an important trigger for hyperosmotic ceramide formation as deduced from the DIDS and bafilomycin sensitivity of hyperosmotic vesicular acidification, the hyperosmosis-induced increase in ceramide levels, and further downstream events leading to hyperosmotic NADPH oxidase activation, such as p47phox phosphorylation. In line with this, no significant increase of hepatocellular ceramide levels was found upon hypo-osmotic (205 mosmol/liter) hepatocyte exposure (not shown), which was recently shown to induce endosomal alkalinization (43, 44).

Endosomes as a Potential Osmosensor—Acidification of endosomes is brought about by vacuolar-type H\textsuperscript{+}/H\textsubscript{1001}-ATPase and requires an anion conductance to maintain electroneutrality during electrogenic proton pumping into the vesicular interior. Apart from Cl\textsuperscript{−} channels and the H\textsuperscript{+}/H\textsubscript{1001}-ATPase, further determinants of vesicular acidification are proton leaks, K\textsuperscript{+}/H\textsubscript{1001} channel activity, the Na\textsuperscript{+}/H\textsubscript{1001}/K\textsuperscript{+}/H\textsubscript{1001}-ATPase, and an Na\textsuperscript{+}/H\textsubscript{1001} exchanger in the vesicle membrane (for review see Ref. 60). Studies with acridine orange-loaded hepatocytes indicated vesicular alkalinization in response to hepatocyte swelling (45, 61, 62). Studies with endocytosed FITC-dextran showed that osmosensitivity of acidification occurs in the endosomal (pH around 6) but not the lysosomal (pH around 5) compartment (43). Only in these presumably endosomal vesicles hypo-osmotic hepatocyte swelling increased, whereas hyperosmotic hepatocyte shrinkage lowered the apparent pHves (27, 43, 44). The mechanisms, how
changes in cell hydration affect apparent $pH_{ves}$ are unclear and a matter of speculation. However, the finding that DIDS and bafilomycin largely abolished the hyperosmotically induced vesicular acidification supports the view that hyperosmolality may interfere with the activity of $H^+\text{-ATPase}$ and/or the chloride transport.

Hyperosmotic hepatocyte shrinkage increases the cytosolic Cl\(^-\) concentration (63–65) not only because of the almost immediate osmotic water efflux in response to hyperosmolality, which has a concentrative effect on cytosolic chloride concentration, but also because of the ionic mechanisms of volume regulatory increase. The latter involves a hyperosmotic activation of $Na^+\text{/}H^+$ exchange and $Na^+\text{/}K^+$-ATPase together with $HCO_3^-\text{/}Cl^-$ exchange, resulting in net accumulation of $Na^+$, $K^+$, and $Cl^-$ in rat hepatocytes (63–65). Conversely, hypo-osmotic exposure activates $K^+$ and $Cl^-$ channels, thereby decreasing intracellular $Cl^-$ (63–65). Evidence has been presented for a direct activation of the vesicular proton pump by $Cl^-$ (66–68), independent of a role of chloride channels for shunting the membrane potential, which is generated by the proton pump. Several types of intracellular chloride channels have been identified in hepatocytes at a functional, pharmacological, and molecular level (for review see Ref. 69). At least five members of the CIC-type chloride channel family (CIC3–7) are expressed in intracellular vesicles, including endosomes (for review see Ref. 70), and are also present in hepatocytes (69). Direct evidence for an involvement of CIC3 in endosomal acidification by $Cl^-$ shunting of the interior-positive membrane potential created by the vacuolar proton pump was recently presented in an excellent study using control versus CIC3-deficient hepatocytes (71). Thus, CIC3 may also be involved in the osmosensitivity of vesicular acidification. A hyperosmolarity-induced increase of the cytosolic chloride concentration may thus indirectly augment vesicular acidification, whereas a decrease in cytosolic chloride could explain the vesicular alkalization, which is observed in response to cell swelling (27, 63–65). However, it should be noted that the inhibitory effect of DIDS on the hyperosmolarity-induced vesicular acidification must not necessarily imply inhibition of DIDS-sensitive $Cl^-$ channels in the endosomal membrane. DIDS inhibits $HCO_3^-\text{/}Cl^-$ exchange at the plasma membrane (72–74) and may therefore impair not only regulatory volume increase but also the hyperosmotic increase in cytosolic $Cl^-$ concentration. If such interpretations were correct, endosomes would (via the cytosolic chloride concentration) act as intracellular osmosensors in addition to the osmosensing (mechanosensing) at the plasma membrane via integrins (8, 9).

On the other hand, the possibility is not excluded that osmosensitivity of apparent $pH_{ves}$ is a downstream consequence of another not yet identified osmosensing/signaling mechanism. Endosomal chloride channels exhibit consensus phosphorylation sites for a variety of protein kinases, which can enhance channel activity (75). Regardless of the mechanisms underlying the osmosensitivity of $pH_{ves}$, modulation of endosomal pH must be seen as one site of hepatocellular osmosensing through modulation of ASM activity and ceramide formation.
Role of NADPH Oxidase in Hyperosmotic Signaling toward Apoptosis—Several protein kinase systems are activated by hyperosmolarity as upstream events in CD95 activation and DISC formation. These include JNK and Yes (1, 11, 22, 76). Activated Yes triggers EGFR activation, which subsequently associates with CD95 and triggers CD95 activation (1, 11). The JNK signal occurs independent of EGFR activation (1) and is required for association of the activated EGFR with CD95 (11), which allows for subsequent CD95-Tyr phosphorylation by EGFR-tyrosine kinase activity and is a prerequisite for CD95 translocation to the plasma membrane and DISC formation (Fig. 17). These complex events leading to CD95 activation result in the recently reported sensitization of hepatocytes toward CD95 ligand-induced apoptosis by moderate hyperosmotic stress (10). However, as shown in this study, hyperosmotic stress can induce apoptosis, when the osmotic challenge becomes excessive. Hyperosmolarity also activates Fyn; however, in contrast to hyperosmotic Yes activation, this response was not sensitive to inhibition by antioxidants (1), indicating that hyperosmotic signaling toward Src family kinases occurs by ROS-dependent and -independent pathways. Clearly, NADPH oxidase-derived ROS triggers hyperosmotic Yes activation and the subsequent events leading to CD95 activation and DISC formation, because these events were not only sensitive to antioxidants, as described recently (23, 77), but also to inhibitors of NADPH oxidases, inhibitors of sphingomyelinase and PKCζ, as well as to ASM or p47phox protein knockdown. Interestingly, also bafilomycin and DIDS, which prevent the endosomal acidification in response to hyperosmolarity, thereby preventing hyperosmotic ceramide formation, largely abolished the hyperosmotic activation of the CD95 system. These findings suggest a very tight control of CD95 activation by hyperosmolarity through the pathways schematically depicted in Fig. 17. It should be noted that NADPH oxidases are not only activated in response to hyperosmolarity but also in response to CD95 ligand (21) and proapoptotic bile acids (22), and all these stimuli induce an activation of the CD95 system (1, 10, 11, 21–23). This points to a crucial role of NADPH oxidase activation in triggering hepatocyte apoptosis via CD95.

A role of ceramide and sphingolipids in apoptosis regulation is well established (for review see Ref. 48) and CD95-mediated apoptosis in vivo involves acidic sphingomyelinase (78). ASM−/− hepatocytes are resistant to anti-Fas antibody-induced apoptosis, and this was reversed by exogenous ceramide (79). With regard to tumor necrosis factor-α-induced apoptosis, ceramide formation depends on upstream caspases, and exogenous ceramide can activate downstream caspases (for review see Ref. 56). However, as shown in this study, ceramide also acts upstream of the ligand-independent, hyperosmotic activation of CD95. This, however, does not rule out further effects of ceramide in the downstream apoptotic program, for example at the mitochondrial level. Lung endothelial cells from ASM knock-out mice and lymphoblasts, but not thymocytes from patients with Niemann-Pick disease, are resistant to ion-
Hyperosmolarity and NADPH Oxidase

FIGURE 17. Hyperosmotic activation of the CD95 system. This figure summarizes our current view on hyperosmotic induced activation of the CD95 system. Hyperosmolarity rapidly induces endosomal acidification, which activates acidic sphingomyelinase and triggers ceramide formation. Ceramide in turn activates PKC\textsubscript{zeta}, which triggers NADPH oxidase activation and generation of ROS through a PKC\textsubscript{zeta}-dependent serine phosphorylation of p47\textsuperscript{phox}. ROS signaling toward the Src family kinase Yes allows for Yes association with and activation of the EGFR. Activated EGFR then dissociates from Yes and associates with CD95 in a JNK-dependent way, which leads to EGFR-tyrosine kinase-mediated CD95-tyrosine phosphorylation. The latter is a prerequisite for a microtubule-dependent translocation of CD95 to the plasma membrane and subsequent FADD and caspase 8 recruitment, i.e. DISC formation (1, 10, 11, 29).

Acknowledgments—The excellent technical assistance by Daniela Brammertz and Elisabeth Winands is gratefully acknowledged.
