

Oxidative Stress Activates Membrane Ion Channels in Human Biliary Epithelial Cancer Cells (Mz-Cha-1)

THORSTEN SCHLENKER¹, LUKAS SCHWAKE¹, AGNIESZKA VOSS²,
WOLFGANG STREMMEL¹ and CHRISTOPH ELSING³

¹Department of Gastroenterology, University Hospital Heidelberg, Heidelberg, Germany;

²Medical Biophysics, Institute of Physiology and Pathophysiology,
Ruprecht Karls University Heidelberg, Heidelberg, Germany;

³Department of Gastroenterology, St. Elisabeth Hospital Dorsten,
Katholisches Klinikum Ruhrgebiet Nord, Dorsten, Germany

Abstract. Oxidative stress is known to contribute to cell damage. In several cell types, one of the earliest effects of oxidative stress is a rapid and substantial decrease in cell volume, which in turn regulates a broad range of cell functions, including development of apoptosis. Since volume regulation is closely coupled to membrane ion permeability, activation of ion channels may play an important role in oxidative stress-related cell injury. Oxidative stress plays a major role in a variety of liver diseases and bile duct epithelia cells (BDE) represent an important site of injury. We, therefore, investigated the functional interactions of oxidative stress, cell volume and ion permeability in a BDE model. Whole-cell patch clamp studies were performed in the human Mz-Cha 1 cell line. Oxidative stress was produced by addition of H_2O_2 to the bath solution. Changes of intracellular Ca^{2+} concentration and of crosssectional area (for calculating cell volume) were monitored by laser scanning microscopy. Exposure of Mz-Cha 1 cells to H_2O_2 resulted in cell shrinkage and increase of the intracellular Ca^{2+} concentration. Patch-clamp studies revealed that exposure to H_2O_2 also resulted in the activation of ion currents with a threshold of 10^{-6} M H_2O_2 . Ion substitution studies and blocker experiments identified the currents as representing an increase in membrane K^+ and Cl^- permeability. Interestingly both ion channel activation and cell shrinkage had a close relationship to the applied H_2O_2 concentration and were significantly inhibited by

intracellular Ca^{2+} chelation. These data imply that in a BDE model, oxidative stress leads to cell shrinkage through activation of Ca^{2+} -dependent K^+ and Cl^- currents. Since cell shrinkage has been associated with increased cell damage, the opening of these ion channels might contribute to the high susceptibility of biliary epithelial cancer cells to oxidative stress.

Oxidative stress has an established role in cell damage in a variety of liver diseases, including ischemia reperfusion, primary biliary cirrhosis and non-alcoholic fatty liver disease (1-5). It results when the production of reactive oxygen species (ROS) surpasses the capacity of the cellular antioxidant systems and contributes to cell injury through effects on gene and protein expression (6, 7), DNA damage (8) and lipid peroxidation (9). The molecular mechanisms however, are yet not completely understood. Since cell volume has been shown to modulate a broad range of other cell functions, including gene and protein expression and the development of apoptosis, it is of interest that in a variety of cells, one of the earliest effects of oxidative stress is a rapid and substantial decrease in cell volume (10-12). In intact rat liver, cell shrinkage increased ischemia/reperfusion-related cell damage (13). In a number of cells, including hepatocytes, however, cell shrinkage is counteracted by Na^+ influx through ion channels or Na^+/H^+ exchange, providing a gradient favoring water influx and restoration of cell volume toward basal values (14, 15). This so-called regulatory volume increase (RVI) might provide a defense mechanism against oxidative injury and could explain the relative resistance of hepatocytes to oxidative stress (14).

Cholangiocytes, the epithelial cells which line the biliary tree, represent a focal site of injury in a number of liver diseases, including post-transplantation liver failure, cholangiocellular carcinoma, cholestatic liver diseases and ischemic-type biliary damage (3, 16-18). In cholangiocytes,

Correspondence to: Christoph Elsing, MD, Department of Gastroenterology, St. Elisabeth-Hospital, PO Box 580, 46225 Dorsten, Germany. Tel: +49 23622955101, Fax: +49 23629217044, e-mail: dr.c.elsing@kkrn.de

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oxidative stress reduces intracellular glutathione levels, which is followed by marked changes in genes associated with carcinogenesis (19). Palliative treatment of cholangiocellular carcinoma uses free radical production to destroy tumor cells, *e.g.* photodynamic therapies (20-22). Despite the fundamental role of oxidative stress in biliary cancer little is known of its influence on membrane ion channels. Consequently, the aim of the present study was to investigate the effects of oxidative stress on cell volume and membrane ion permeability in a biliary epithelial cancer cell model.

Materials and Methods

Cell isolation and culture. Studies were performed using the human cholangiocarcinoma cell line Mz-ChA-1 (23), which has been shown to express the biliary epithelia markers cytokeratin-19 and γ -glutamyl transpeptidase, and to possess phenotypic features of biliary origin, including Ca^{2+} - and cAMP-dependent Cl^- conductance (24). Cells were kindly provided by A. Knuth (Frankfurt/Main, Germany) and maintained in culture at 37°C in 5% CO_2 in HCO_3^- -containing Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$).

Patch clamp recordings. Whole-cell currents were measured using patch clamp recording techniques (25). Studies were performed at room temperature (22-25°C) 12 to 48 h after plating of cells. Cell-bearing coverslips were placed in a perfusion chamber and perfused at 2 ml/min with a NaCl-rich extracellular solution (see below). Cells were viewed through an Axiovert S100 inverted phase-contrast microscope (Carl Zeiss GmbH, Göttingen, Germany) using Hoffman optics at a magnification of $\times 400$. Patch pipettes were pulled from borosilicate glass (GB 150-8p; Science Products GmbH, Hofheim, Germany) and had resistances of 3-6 M Ω . Pulse protocols, data acquisition and data analysis were carried out using an EPC 9 amplifier (Heka, Lambrecht, Germany) and PulseFit software version 8.1 (Heka). Currents were sampled at 20 kHz and filtered by a 4-pole Bessel filter at 4 kHz. Pipette voltages refer to the bath, where V_p corresponds to the membrane potential and upward deflections of the current trace represent the outward membrane current.

Bath and pipette solutions. The standard extracellular bathing solution contained 140 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4 , 2 mM MgCl_2 , 1 mM CaCl_2 , 5 mM glucose, and 10 mM HEPES/NaOH (pH 7.3). The standard pipette (intracellular) solution contained, 130 mM KCl, 10 mM NaCl, 2 mM MgCl_2 , 10 mM HEPES/KOH, and 1 mM ATP (pH 7.3). Free Ca^{2+} was adjusted to ~ 100 nM (0.5 mM CaCl_2 , 1 mM EGTA) (26). Oxidative stress was induced by direct addition of H_2O_2 at defined concentrations (10-6 to 10^{-2} M). Currents were measured from a holding potential of -40 mV (between the equilibrium potentials for K^+ and Cl^-) at different test potentials. Ion channels were characterized in more detail using different inhibitors. The potassium channel was blocked by the use of the antagonist BaCl_2 (5 mM), the chloride channel was blocked by the use of 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB, 100 μM). In selected studies, NaCl was replaced by an equimolar amount of TrisCl to investigate the influence of sodium channels. To lower the intracellular Ca^{2+} concentration,

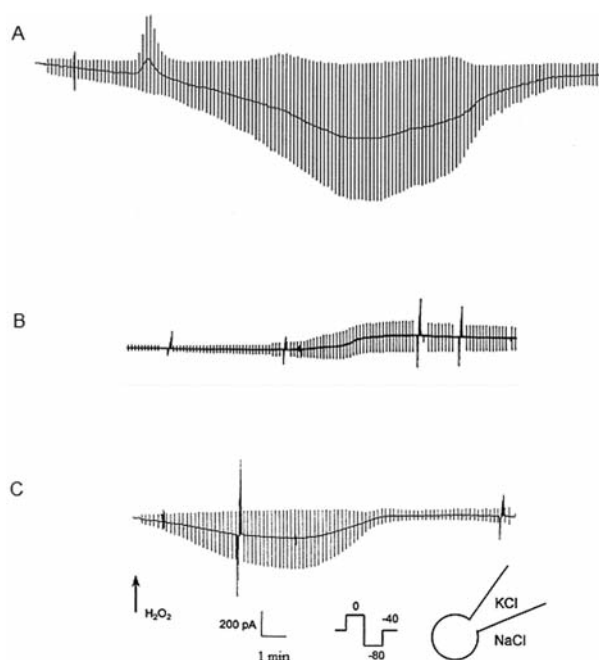


Figure 1. Effect of H_2O_2 on membrane ion permeability. Membrane currents were measured in the whole-cell configuration. From a holding potential of -40 mV, test potentials of 0 mV and of -80 mV were applied every 10 sec. Superfusion with H_2O_2 activated ion currents as indicated by an increase in current amplitude. Three different patterns were observed and representative recordings show initial outward current followed by a sustained inward current (at $V_p = -40$) (A), outward current only (B), and sustained inward current only (C).

intracellular Ca^{2+} ions were chelated by addition of 5 mM EGTA to the pipette or by 45 min pre-incubation of cells with 500 μM N,N'-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-2-[(acetyloxy)methoxy]-2-oxoethyl]-bis[(acetyloxy)methyl] ester.

All reagents were obtained from SIGMA-ALDRICH (Steinheim, Germany) unless indicated otherwise.

Estimation of cell volume changes. For determination of volume changes, cells were plated on coverslips. After 24 h (60-70% confluent monolayers), coverslips were placed on the stage of an LSM-310 confocal laser scanning microscope (Carl Zeiss GmbH, Göttingen, Germany) and superfused with extracellular solution. After an equilibration period of 3 min, the bath solution was replaced by H_2O_2 -containing buffer (0.01, 0.1, 1.0, 3.0 and 10.0 μM). The cell shape from confocal images using the transmission mode of the system were obtained every 15 s and digitized for storage on a computer. Only cells with a nearly round shape were used for these studies. Cell surface area (CSA) was analyzed using Zeiss LSM software (Carl Zeiss GmbH) and assuming a spherical shape, the cellular volume was calculated as previously described [27], using the equation:

$$V = (4/3) \times \pi^{1/2} \times (\text{CSA})^{3/2}$$

Determination of intracellular Ca^{2+} changes by confocal laser scanning microscopy.

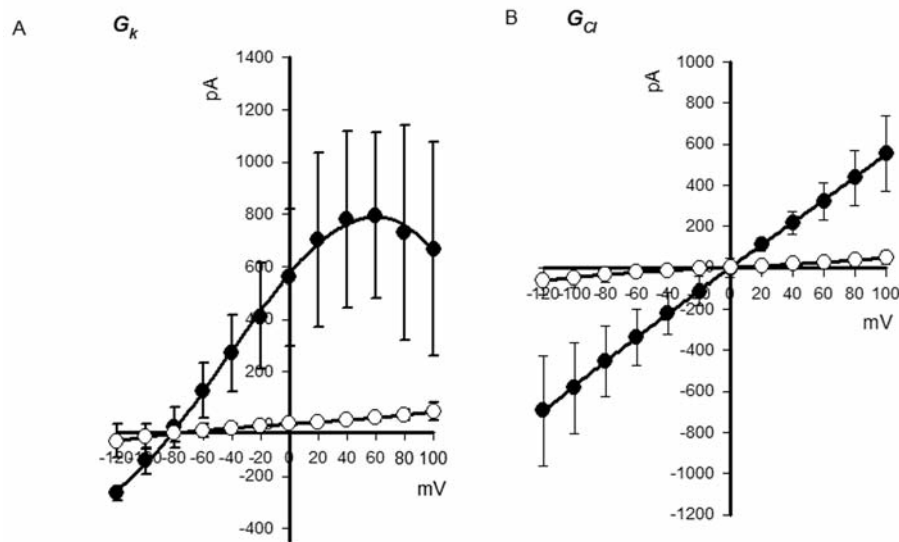


Figure 2. A: K^+ current: Average current-voltage relation from four cells prior to (open circles) and after (closed circles) H_2O_2 (10 mM) exposure. H_2O_2 caused a large increase in outward current at 0 mV and a negative shift in reversal potential towards K^+ equilibrium potential. B: Cl^- current: Average current-voltage relation from six cells prior to (open circles) and after (closed circles) H_2O_2 exposure. H_2O_2 caused a large increase in inward currents at -80 mV. Reversal potential remained at 0 mV.

Changes in intracellular Ca^{2+} concentration were measured using the Ca^{2+} -sensitive fluorescent dyes Fluo-3- and Fluo-4 acetoxymethyl ester (Fluo3/AM; Fluo4/AM) (Molecular Probes, Leiden, The Netherlands) as previously described [28].

Mz-Cha-1 cells were plated on coverslips 24 h (60-70% confluent monolayers), prior to studies. Cells were loaded with the membrane-permeant dye by incubation for 30 to 40 min at 37°C with 5 μ M Fluo3/AM in DMEM containing supplements as described above and 0.05% pluronic F-127 (Molecular Probes, Leiden, the Netherlands). Coverslips were placed in a superfusion chamber, allowing continuous laminar flow (1 ml/min). They were continuously superfused with the extracellular buffer (described above). In selected studies, the superfusion solution was switched to extracellular buffer containing H_2O_2 (10 mM). The chamber was specially manufactured and implemented in a fluoview-fv300-system containing of an omnichrome 643R-OLYM-AO3 argon/krypton ion laser (Melles Griot Inc., Helium Neon Lasers, Carlsbad, CA, USA), coupled to an IX70 inverted confocal microscope (Olympus Germany GmbH, Hamburg, Germany).

Cells were excited at 488 nm. Emission was recorded using a 525-565 nm band pass filter, and data were transferred to a computer. Images were digitized (12-bit). Data were analyzed using Olympus Fluoview FV300 software (Olympus Germany GmbH).

Cytotoxicity assay. For the assessment of H_2O_2 cytotoxicity, experiments were performed, by incubating the cells with H_2O_2 (3-10 mM for 10 min) or with staurosporine (1 μ M, 4 h) (Sigma-Aldrich, Munich, Germany); staurosporine induces cell death and apoptosis by protein kinase inhibition. Mz-Cha-1 cellular survival was assessed by means of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to the insoluble blue formazan catalyzed by mitochondrial and other cellular dehydrogenases (Roche Diagnostics, Mannheim, Germany). The

cellular absorbance which is indicative of the remaining cellular activity was read on a microplate photometer at 550 nm.

Presentation of data and statistical analysis. Pooled data are presented as the mean \pm SD. Statistical analysis was performed using the Student's *t*-test for unpaired (pclamp) or paired (volume) data and $p < 0.05$ was considered significant. Patch clamp data were normalized for cell size by presentation as current density picoamperes per picofarad (pA/pF). Fluorescence data are given as relative changes in fluorescence intensity over time.

Results

Effect of oxidative stress on membrane ion channels. To determine if oxidative stress mediates membrane ion permeability, whole-cell currents were investigated using patch clamp techniques. Basal currents in the standard buffer were small at both $V_p = 0$ mV and $V_p = -80$ mV with 0.1 ± 0.09 pA/pF and -1 ± 0.7 pA/pF respectively. Exposure to H_2O_2 activated currents independently in 40 out of 46 cells. Three basic patterns were apparent (Figure 1). In 77% of cells (Figure 1A), we observed both outward currents at 0 mV (I_0) and inward currents at -80 mV (I_{-80}), consistent with concurrent activation of K^+ and Cl^- conductance (G_K and G_{Cl}). Typically the increase in G_K occurred first and was transient, inactivating over 2-3 min, despite continued exposure to H_2O_2 buffer. The increase in G_{Cl} was more sustained and started later. In some cells, exposure to H_2O_2 was associated with activation of either G_K (5%) (Figure 1B) or G_{Cl} (18%) (Figure 1C) alone. Pre-incubation with the K^+ channel

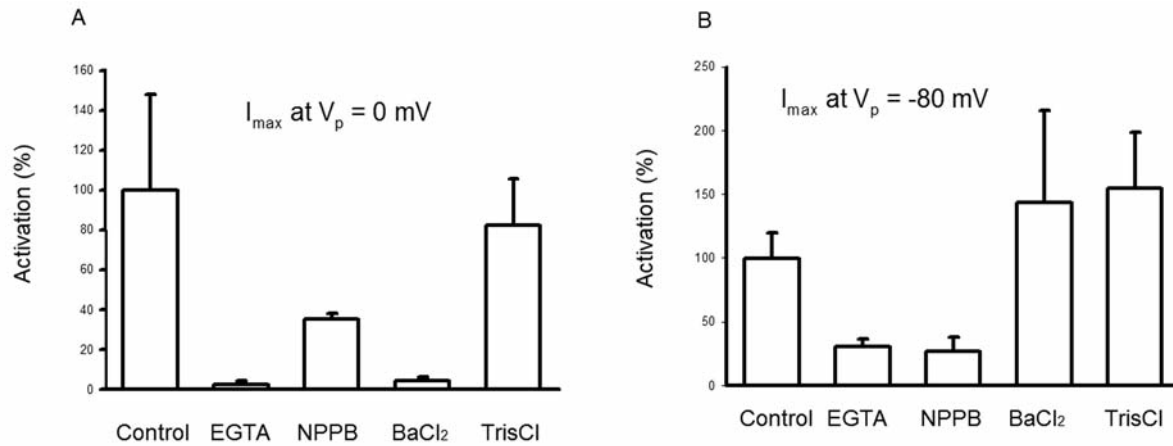


Figure 3. Effect of calcium chelation and ion channel blockers on H_2O_2 -related current activation. Data are presented as mean \pm SD percentage of the value for the controls (exposure to H_2O_2 (10 mM) only). A: Maximal current amplitude measured at $V_p=0$ mV. EGTA (n=6) and BaCl₂ (n=6) significantly reduced current activation, whereas replacement of bath Na⁺ with Tris⁺ (n=7) had no significant effect. B: Maximal current amplitude measured at $V_p=-80$ mV. EGTA (n=6) and NPPB (n=5) significantly reduced current activation, whereas replacement of bath Na⁺ with Tris⁺ (n=7) had no significant effect.

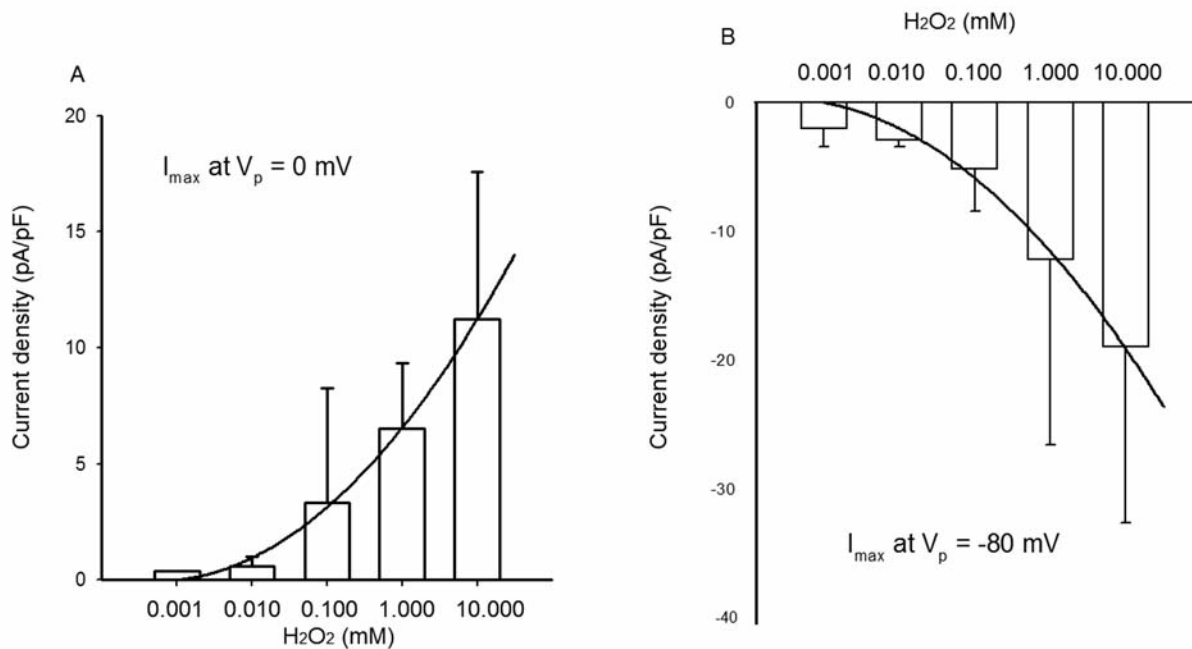


Figure 4. Concentration-dependent current activation. Maximal current density (mean \pm SD, n=3-14) was measured at different bath concentrations of H_2O_2 . A: Maximal outward current at $V_p=0$ mV; B: maximal inward current at $V_p=-80$ mV.

antagonist Ba²⁺ (5 mM) caused a significant decrease in the H_2O_2 -induced current density at $V_p=0$ mV (inhibition of 96%, $p<0.02$). No inhibition was found when Na⁺ in the bath was replaced by the membrane impermeant cation Tris⁺ (Figure 3A). K⁺ currents showed a linear I-V relation (Figure 2) over

physiological potentials but there was a tendency for time-dependent inactivation at depolarizing potentials. Consistent with a Cl⁻-selective anion channel, current density was markedly reduced when cells were pre-incubated with the Cl⁻ channel blocker NPPB ($p<0.014$). In contrast, no inhibition

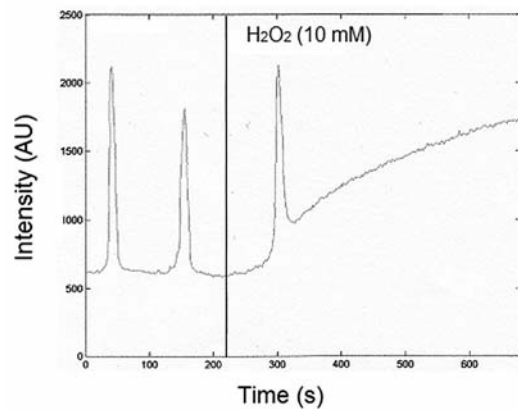


Figure 5. Intracellular Ca^{2+} concentration in Mz-Cha1 cells. Cells exhibited spontaneous oscillation of intracellular Ca^{2+} concentration. Exposure to H_2O_2 (10 mM) resulted in an increase of intracellular Ca^{2+} concentration and stopped the oscillatory pattern. Data from representative cells from 10 different preparations are shown.

was found when Na^+ in the bath was replaced by the membrane impermeant cation $Tris^+$, excluding opening of a non-selective cation channel (Figure 3B).

Overall, these findings indicate that exposure of biliary cancer cells to oxidative stress is associated with transient activation of GK, and sustained activation of GCl.

Concentration-dependent increases of K^+ and Cl^- conductance by H_2O_2 . To further quantify the sensitivity of current activation by oxidative stress, H_2O_2 was added to the bath at defined concentrations. The threshold for GK activation was 10^{-6} M H_2O_2 , and an increased current was detected up to 10^{-2} M ($p < 0.01$) (Figure 4A). The threshold for GCl activation was 10^{-5} M H_2O_2 , and an increased current was detected at 10^{-2} M ($p < 0.01$) (Figure 4B). Thus, maximal increases in oxidant stress cause a substantial increase in membrane K^+ and Cl^- permeability through opening of conductive pathways.

Channel activation is Ca^{2+} -dependent. Since oxidative stress has been shown to influence intracellular Ca^{2+} concentration in a variety of cells (25) and Ca^{2+} -dependent ion channels have been identified in the Mz-Cha 1 cell line (26), we performed studies with increased EGTA (5 mM) concentration in the pipette, to chelate intracellular Ca^{2+} . This resulted in inhibition of the maximal current amplitude of $72 \pm 13\%$ at $V_p = -80$ mV and $97 \pm 3.8\%$ at $V_p = 0$ mV, respectively, consistent with Ca^{2+} -dependent channel activation (Figure 3).

Characterization of intracellular Ca^{2+} changes. To further characterize the effects of H_2O_2 on intracellular Ca^{2+}

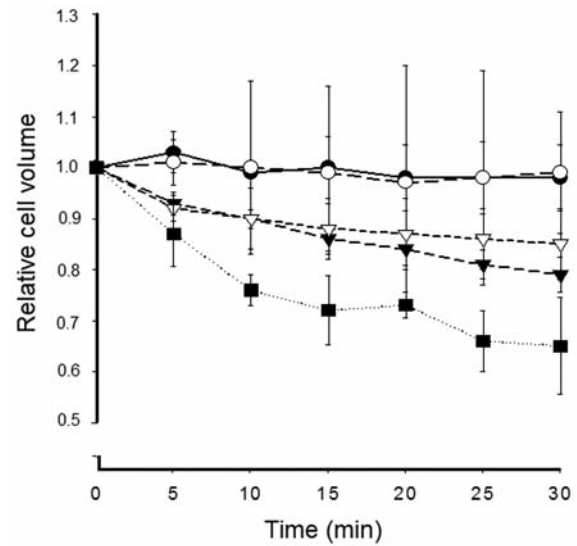


Figure 6. Effect of oxidative stress on cell volume. The effect of oxidative stress on cell volume was assessed in cells superfused with H_2O_2 -containing buffer. H_2O_2 was added at $t=0$ at a defined concentration (0.01 mM, closed circle; 0.1 mM, open circle; 1 mM, closed triangle; 3 mM, open triangle; and 10 mM, closed square). Changes in the relative cell size are shown, where the initial size was set as 1. Data are presented as the mean \pm SD ($n=4-12$).

concentration, cells were pre-loaded with a sensitive fluorescent dye and fluorescence was recorded by confocal LSM. When cells exhibited spontaneous oscillations of intracellular Ca^{2+} concentrations, H_2O_2 (10 mM) induced an increase of fluorescence intensity and oscillations were terminated (Figure 5).

Effect of oxidative stress on cell volume. To evaluate if the increase in ion membrane permeability after exposure to oxidants results in cell volume changes, CSA was measured under basal conditions and following exposure to H_2O_2 to calculate the cell volume, as described above.

In the absence of oxidative stress, the cell volume remained constant for more than 30 min. Figure 6 shows the H_2O_2 decrease of cell volume in a concentration range of 10 μ M to 10 mM. At 1 mM (closed triangle), cell volume decreased to $95 \pm 2\%$ of initial size after 3 min and $78 \pm 7\%$ after 30 min ($n=8$).

The putative correlation of K^+ and Cl^- efflux due to ion channel activation and cell shrinkage is supported by the following observations: With respect to the close dose response to H_2O_2 concentration and current density, we also assessed the sensitivity of volume changes by addition of H_2O_2 to the bath at defined concentrations. The threshold for detectable volume changes was 10^{-3} M with a dose-dependent increase of cell shrinkage for higher H_2O_2 concentrations (Figure 6) ($p < 0.01$).

Interestingly the co-incubation of cells with the Cl^- channel blocker NPPB (50 μM) significantly prevented cell shrinkage (Figure 7A) ($p < 0.04$). Incubation with BaCl_2 also tended to reduce shrinkage, although the reduction was statistically not significant (Figure 7B).

Chelation of intracellular Ca^{2+} , which significantly reduces ion current activation as indicated above, also inhibited H_2O_2 -induced cell shrinkage (Figure 7C) ($p < 0.01$).

We, thus, conclude that exposure of Mz-Cha 1 cells to oxidative stress leads to activation of Ca^{2+} -dependent K^+ and Cl^- channels resulting in cell shrinkage.

Cell toxicity. Cellular survival, as assessed by the MTT assay declined to about $76 \pm 3\%$ after H_2O_2 incubation (10^{-3} M for 10 minutes, $n=3$). In comparison, staurosporine reduced viability after 4 h incubation (1 μM) to about $67 \pm 4\%$ ($n=3$, n.s.).

Discussion

The present studies in a human biliary cholangiocarcinoma cell line demonstrate the functional interaction between oxidative stress, cell volume and ion channel opening.

The major findings are that oxidative stress leads to cell shrinkage and activates ion currents in biliary cancer cells. By analysis of the reversal potential and the use of specific ion channel blockers, currents were demonstrated to result from an increase in membrane K^+ and Cl^- permeability. In contrast to a renal cell line, where H_2O_2 activated Ca^{2+} -independent K^+ channels (29), current activation in our studies was shown to be closely coupled to the intracellular Ca^{2+} concentration, since chelation of intracellular Ca^{2+} inhibited current activation. This is of interest because prior studies in this cell line identified putative contributing ion channels (30) and described activation of K^+ and Cl^- currents by Ca^{2+} -dependent kinases under a variety of different conditions (31-34). In our study, however, we did not analyze in more detail the molecular machinery which is involved in the activation of the ion channels (35).

Previous studies of liver and other cell types demonstrated that an increasing local concentration of ROS causes an initial decrease in cell volume (10, 15, 29). In many cells, including the HTC hepatoma cell line, the adaptive response to cell shrinkage consists of an increase in membrane Na^+ permeability; the resulting influx of Na^+ and water contributes to partial recovery of volume toward basal values, a process generally referred to as RVI (14, 36). In contrast, exposure to oxidative stress leads to cell shrinkage without RVI in the Mz-Cha 1 cell line. This decrease in cell volume appears to be closely coupled to the activation of Ca^{2+} -dependent K^+ and Cl^- currents for three reasons: i) both ion current and cell volume decreases have a close dose-response relationship to the H_2O_2 concentration, ii) chelation of intracellular Ca^{2+} inhibits current

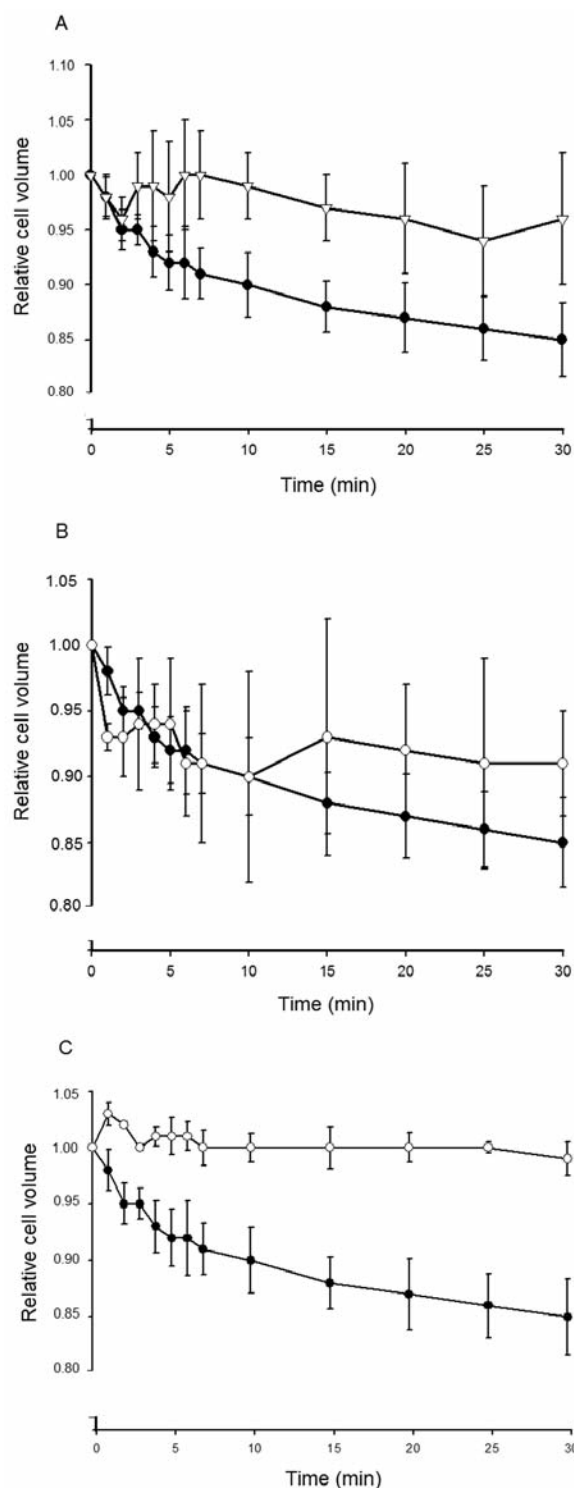


Figure 7. Influence of ion channel antagonists and Ca^{2+} chelation on cell volume during H_2O_2 perfusion. Cell volume was recorded during superfusion with buffer containing 3 mM H_2O_2 in the presence and absence (closed circle) of an ion channel antagonist or a Ca^{2+} chelator ($n=6$, mean \pm SD). A: Co-incubation with the Cl^- channel antagonist NPPB (open triangle); B: K^+ channel antagonist BaCl_2 (open circle); C: pre-incubation with BAPTA/AM (open circle).

activation and prevents cell shrinkage, and iii) the use of specific ion channel blockers prevents cell shrinkage. These data suggest that the decrease in cell size results from water loss consequent to K⁺ and Cl⁻ efflux through open channels. In our studies, no counteracting increase in membrane Na⁺-permeability, as reported for the HTC cell line (14), was observed. In addition, another important mechanism contributing to RVI, namely the activation of Na⁺/H⁺ exchange, has also been found to be inhibited during oxidative stress in the Mz-Cha 1 cell line (37).

The concentration of H₂O₂ used in our studies may appear relatively high. However, several reasons indicate that these studies are of relevance under *in vivo* conditions. Due to a very efficient detoxification capacity of most cells, high levels of intracellular ROS are actually needed to cause acute cell damage (38). On the other hand, pathological conditions often lead to severe deficiency of cellular antioxidants and can thus dramatically increase susceptibility to oxidative stress (38), which was demonstrated in the HTC cell line where the decrease in defense mechanisms resulted in a 105-fold decrease of the threshold for current activation by ROS (14). Another set of studies on the HTC cell line, which investigated the effect of co-incubation with activated polymorphonuclear leukocytes, suggested that local ROS concentrations may reach extremely high levels during inflammation (10⁻² M) (14) and cultured tumor cell lines usually are even more resistant to oxidative stress than cells *in vivo*.

In conclusion, these studies demonstrate that activation of Ca²⁺-dependent K⁺ and Cl⁻ channels by oxidative stress results in cell shrinkage in a human biliary epithelial cancer cell model. This could be of interest for further advancing treatment strategies, since recent research has focused on the development of oxidative stress-resistant cholangiocyte cell lines (39).

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