Copper-Induced Stimulation of Extracellular Signal-Regulated Kinase in Trout Hepatocytes: The Role of Reactive Oxygen Species, Ca\(^{2+}\), and Cell Energetics and the Impact of Extracellular Signal-Regulated Kinase Signaling on Apoptosis and Necrosis

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Copper (Cu) is an essential transition metal, which, if present in excess, may exert significant toxicity both in vivo and in vitro. In trout hepatocytes, we recently showed that the main mechanisms underlying Cu toxicity include the disruption of Ca\(^{2+}\) homeostasis and the induction of the formation of reactive oxygen species (ROS) (Krumschnabel et al., 2005; Manzl et al., 2003, 2004). Ultimately, these effects result in necrotic and apoptotic cell death, with the former being critically dependent on cellular ROS formation, as it could be inhibited by radical scavengers, and the latter being closely linked to induction of the mitochondrial permeability transition (MPT), as it was prevented by the MPT inhibitor cyclosporine A. In addition to triggering apoptosis, which was presumably due to the release of proapoptotic factors from the intermembrane space during MPT, mitochondria were also identified as a prominent source of ROS, thereby further contributing to cell damage. The main site of ROS formation in the mitochondria seemed to be located at complex III, the inhibition of which by myxothiazol reduced cellular radical production by more than 50% during exposure to Cu (Krumschnabel et al., 2005). As another important origin of radicals, we identified the cellular chelatable iron pool, which is presumed to mainly reside in the lysosomes or may be released from lysosomes upon destabilization of these organelles (Krumschnabel et al., 2005). The critical role of this iron pool in mediating cell death has been previously demonstrated in rat hepatocytes, where, upon hypothermic exposure, an increase specifically of cytosolic chelatable iron was found to be the predominant cause of cold-induced apoptosis (Rauen et al., 2000, 2003). In the case of Cu, a similar condition could be created by direct or indirect interaction of the metal with the lysosomal iron pool to initiate Fenton-type reactions, producing radical intermediates (Pourahmad et al., 2001). ROS formation induced by Cu could thus be inhibited by the ferric ion chelator deferoxamine mesylate (DFO), a compound that is taken up only by endocytosis and thus mainly resides in the lysosomes (Antunes et al., 2001;
Lloyd et al., 1991). Interestingly, DFO not only prevented necrotic but also apoptotic cell death in trout hepatocytes (Krumschnabel et al., 2005), suggesting that lysosomal effects of Cu preceded those at the mitochondria. Similarly, it was shown that DFO, but not selective mitochondrial iron chelators, inhibited cold-induced cell injury in rat hepatocytes (Rauen et al., 2003). Together, these findings would be in line with the concept of a lysosomal-mitochondrial axis of cell death induced by oxidants, which has been put forward by Brunk and colleagues (Brunk et al., 2001; Persson et al., 2003). According to this concept, oxidative damage of lysosomes could lead to the release of lysosomal enzymes, e.g., cathepsins, the released enzymes would activate phospholipase A2 and Bid (among other proteins), and these would then act on the mitochondria, where enhanced ROS formation and/or the release of cytochrome c and other proapoptotic factors would ensue (Zhao et al., 2003). Alternatively, it would also appear possible that enhanced ROS production in the lysosomes could stimulate signaling pathways acting on mitochondria, thereby functionally linking both organelles. Among the most intensively studied signaling molecules are the mitogen-activated protein kinases (MAPKs), which are involved in fundamental processes such as cell growth, differentiation, and, importantly, apoptosis (Wada and Penninger, 2004). The best-characterized members of the MAPK family are the extracellular signal-regulated kinase (ERK), the p38-MAPK, and the c-Jun N-terminal kinase (JNK), all of which are known to be activated by radical stress (p38: Nusuetrong et al., 2005; JNK: Kim and Sharma, 2004; ERK: Park et al., 2005). Furthermore, there is increasing evidence that MAPKs are involved in stress signaling toward the mitochondria, although the exact mechanisms still need to be elucidated (Horbinski and Chu, 2005; Tamura et al., 2004; Wang et al., 2000). Previously, p38-MAPK and JNK have been mainly considered as proapoptotic, and ERK has been considered an important factor for cell survival (Wada and Penninger, 2004). However, in recent years it has become increasingly clear that the role of MAPKs as pro- or antiapoptotic factors may depend on cell type, the nature of the apoptotic stimulus, and the duration of MAPK activation evoked by the stimulus (Liu and Lin, 2005; Park et al., 2005; Song et al., 2005). Thus, several studies have shown that upon inducing radical stress, the concurrent activation of ERK promotes apoptotic cell death (Chu et al., 2004; Wang et al., 2000; Zhang et al., 2003), including investigations on the toxicity of zinc (Seo et al., 2001) or cadmium (Iryo et al., 2000). In fish cells the role of MAPKs in cell death signaling has not yet been addressed, but recent studies by Burlando and colleagues (Burlando et al., 2003; Marchi et al., 2005) have shown that in a trout hepatoma cell line, MAPKs are activated by radical stress induced either by directly adding H₂O₂ or by the addition of redox-active metals mercury or Cu. Interestingly, only ERK was activated by Cu alone, whereas p38-MAPK and JNK required the additional presence of H₂O₂ for significant Cu stimulation, suggesting a particular importance of ERK during Cu intoxication.

In view of these findings and our previous observations summarized above, the present study was designed to address the following questions. Firstly, we investigated if in trout hepatocytes, similar to the hepatoma cell line (Burlando et al., 2003), ERK is activated by Cu exposure. Secondly, we wanted to elucidate the mechanisms underlying the stimulation of ERK activity, specifically focusing on the hypothetical role of Ca²⁺ and ROS as stimuli. Regarding the latter, we also addressed the possible source of ROS stimulating the MAPK, i.e., lysosomes and mitochondria, which would allow us to get some insights into the hypothetical role of MAPKs as signal transmitters between both compartments. And thirdly, we studied the role of ERK, as well as that of p38-MAK and JNK, as mediators of cell death, in particular regarding their possible contribution to ROS-mediated necrotic cell death and their role in mitochondrial apoptotic cell death.

MATERIALS AND METHODS

Chemicals. Acridine orange (AO), benzylimidazolide, bovine serum albumin (BSA), chloroquine, cimetidine, collagenase (type VIII), DFO, digitonin, dimethyl sulfoxide, EGTA, ionomycin, leupeptin, myxothiazol, monensin, poly-l-lysine (PLL), rotenone (ROT), thienoyltrifluoracetonate (TTFA), tertiary-butyl hydroperoxide (tBH), trypan blue, and HRP-conjugated anti-rabbit secondary antibody were purchased from Sigma (Deisenhofen, Germany). Fura 2-AM and dichlorofluorescein diacetate (DCF-DA) were from Molecular Probes (Leiden, The Netherlands). Leibovitz L15 medium and fetal calf serum (FCS) were GIBCO products from Invitrogen (Vienna, Austria). The Apo-ONE Homogeneous Caspase-3/7 Assay kit and the luciferase/luciferin-ATP detection kit were purchased from Promega (Mannheim, Germany) and primary antibodies against total ERK and phosphorylated extracellular signal-regulated kinase (pERK) from Cell Signalling Technology (New England Biolabs, Beverly, MA, USA). Immuno-Blot polyvinylidene fluoride (PVDF) membrane was a product from Bio-Rad (Munich, Germany) and ECL Western blotting detection solution was from Amersham Biosciences (GE Healthcare, Vienna, Austria). U0126 and SB203580 were obtained from Calbiochem (Merck, Darmstadt, Germany), SP600125 from A.G. Scientific Inc. (Munich, Germany). Q-VD-OPH (N-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenyl)methyl ketone) was from Alexis Biochemicals (Lausen, Switzerland). All other chemicals were of analytical grade from local suppliers. Copper was applied from a stock solution of CuCl₂ prepared in distilled water.

Animals and preparation of hepatocytes. Rainbow trout (Oncorhynchus mykiss) were obtained from a local supplier and acclimated in a 200-l aquarium with running water at 15°C. The animals were fed daily with trout pellets (EWOS Aquaculture International, Södertälje, Sweden) ad libitum. Maintenance and use of experimental animals were in accordance with the Austrian federal law for care and use of laboratory animals. Hepatocytes were isolated as described previously (Krumschnabel et al., 1996). After isolation, cells were incubated for 1 h for recovery in a shaking water bath thermostated to 19°C. The standard incubation medium contained (in mM) 10 HEPES, 136.9 NaCl, 5.4 KCl, 1 MgSO₄, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 5 NaHCO₃, and 1.5 CaCl₂, 5 glucose, pH 7.6, including 1% BSA. Initial cell viability was determined from trypan blue exclusion and was > 95% for all preparations used in the experiments described.

Primary cultures of hepatocytes. For the measurement of cytosolic free calcium (Ca²⁺) and lysosomal membrane stability, hepatocytes (2 × 10⁶/ml) were suspended in sterile, modified Leibovitz L15 medium, containing 10mM
HEPES, 5mM NaHCO3, 50 µg/ml gentamicin, and 100 µg/ml kanamycin. pH titrated to 7.6, and supplemented with 5% FCS. The cells were then plated onto PLL-coated coverslips and left to attach overnight in an incubator with temperature set to 19°C and with 0.5% CO2. Before the use of these hepatocytes, cell cultures were washed several times with fresh standard saline so as to remove nonadherent cells and debris. Cells used for Western blot experiments were suspended in L15 medium containing 0.1% FCS, cell density being adjusted to a concentration of 10⁵/ml, and then plated onto culture dishes without coverslips.

Protein extraction and Western blot analysis. Following the experimental exposure of cells, total protein from 10³ cultured hepatocytes was extracted by a slightly modified standard lysis protocol. To this end, hepatocytes were removed from culture dishes by vigorous pipetting, transferred into precooled, 1.5-ml vials, and spun down by brief centrifugation at 13,000 × g. The resulting pellet was broken up in liquid nitrogen and then lysed on ice in a buffer containing 25% glycerol, 420mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 20mM HEPES in deionized H2O, with freshly added 0.5mM dithiothreitol, and 0.5mM phenylmethanesulfonyl fluoride. Finally, the cell lysate was centrifuged at 10,000 × g for 10 min at 4°C and the clear supernatant stored at –80°C. The protein concentration was determined using a colorimetric BioRad DC Protein Assay and measured on a Tecan Microplate photometer at 700 nm excitation wavelength.

Equal amounts of proteins (30 µg) were heated to 95°C for 5 min and then separated on a 10% sodium dodecyl sulfate–polyacrylamide gel using a NuPage system (Invitrogen) at 200 V for 50 min. Separated proteins were subsequently transferred onto a PVDF membrane at 25 V for 60 min. The membrane was washed with Tris-buffered saline (TBS) for 5 min and then blocked for 2 h in TBS containing 0.1% Tween-20 and 5% nonfat skimmed milk. In order to detect total ERK or pERK, the membrane was incubated at 4°C overnight with the polyclonal primary antibody used at a dilution of 1:1000 in TBST (TBS plus 0.1% Tween-20) containing 5% BSA. The membrane was washed several times with TBST and was subsequently incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000 in TBST) for 1 h at room temperature. After another wash of the membrane with TBST, the protein bands were visualized using an ECL Western blotting detection solution and exposing the membrane to a high-performance chemiluminescence film. The film was then scanned and protein abundance analyzed by densitometry using Quantity One (BioRad) software.

Cytosolic free calcium concentration (Ca²⁺). Alterations of intracellular free calcium (Ca²⁺) during metal exposure were determined in Fura 2–loaded single trout hepatocytes on a video-fluorescence microscope following the protocol described in detail elsewhere (Manzl et al., 2003).

Reactive oxygen species. The procedure for determination of the production of ROS has been previously described (Manzl et al., 2004). In brief, 100 µl of cell suspension (5 × 10⁵ cells) was transferred into the wells of a black, 96-well plate and mixed with 150 µl of standard saline containing Tris-HCl instead of HEPES and including 3µM DCF-DA. This nonfluorescent probe is oxidized in the presence of ROS to give a highly fluorescent product 2′,7′-dichlorofluorescein. In order to rule out possible extracellular interactions between Cu and the fluorescence dye, we also conducted experiments in the absence of DCF-DA. In these experiments, cell were washed in standard saline after exposure to control conditions or with Cu so as to remove the extracellular saline, resuspended in medium with DCF-DA, incubated for 10 min, and then measured. These studies produced similar results as those found with the other experimental procedures. In a separate set of experiments, hepatocytes were preloaded with the dye, subsequently washed, and then incubated with Cu. Results indicated that DCF is not well retained in the cells, and this procedure could, therefore, not be applied. However, since DCF-DA must be hydrolyzed by intracellular esterases to form the active probe and since various inhibitors of cellular metabolism or signaling pathways were able to reduce ROS formation, the method applied by us must clearly have detected radicals generated by the cells.

In order to obtain the dose dependence of ROS formation induced by Cu, hepatocytes were incubated with 1, 5, and 10µM of Cu and fluorescence measurements taken after 0, 5, 15, 30, 60, and 120 min using a Fluorescence Microplate Reader Molecular Devices (Munich, Germany) with 485 nm excitation and 538 nm emission. In addition, cells were incubated with or without Cu in the presence of the iron chelator DFO (200µM), with tBH (1mM) or with the inhibitors of mitochondrial electron transport chain complexes I, II, and III ROT (10µM), TTFA (25µM), and myxothiazol (10µM), respectively. To analyze the potential impact of MAPKs or caspase activity on Cu-induced ROS formation, cells were preincubated prior to measurements with U0126 (MEK inhibitor, 10µM for 60 min), SB203580 (p38-MAPK inhibitor, 20µM for 30 min), SP600125 (JNK inhibitor, 10µM for 60 min), or Q-V-D-OPH (caspase inhibitor, 20µM for 60 min). Finally, in order to test the importance of lysosomes in Cu-induced ROS production, hepatocytes were incubated with monensin (10µM), chloroquine (100µM), both blocking lysosomal acidification and endocytosis, and leupeptin, a protease inhibitor (10µM).

Cellular ATP contents. For the determination of cellular ATP contents, hepatocytes (15 × 10⁵/ml) were incubated under control conditions, with 10µM Cu, with the mitochondrial inhibitors myxothiazol, or ROT and TTFA for 30 min. Duplicate samples were taken at 0 and 30 min, extracted with perchloric acid, and then neutralized with KOH. Supernatants were stored at –80°C and ATP levels measured using a luciferase/luciferin-based ATP detection kit (Promega) according to the manufacturer’s protocol.

Lysosomal membrane stability. Stability of lysosomal membranes was estimated from the capacity of cells to retain AO. Hepatocytes, attached to PLL-coated coverslips, were loaded with AO (5 µg/ml) for 15 min at 19°C. Subsequently, cells were washed and mounted in a measuring chamber and fixed on the stage of an inverted fluorescence microscope. Fluorescence images were collected every 2.5 min at excitation/emission wavelengths set to 490/ >520 nm. Copper was added after obtaining a stable signal, and images were taken over 120 min of incubation. The fluorescence intensity at time zero was taken as the reference value, and subsequent measured values were expressed as % of this initial fluorescence.

Apoptotic and necrotic cell death. Caspases 3 and 7 are effector caspases, which, after their activation, cleave, degrade, or activate other cellular proteins. Generally, activation of these caspases is considered an early sign of apoptosis, which is not associated with other forms of cell death. Thus, in order to have a quantitative measure of apoptosis, we applied the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega) in the present study. Briefly, freshly isolated hepatocytes were diluted to a concentration of 10⁵ cells/ml and 100-µl aliquots of this suspension transferred and incubated in the wells of a black, 96-well plate for 2 h with or without Cu. Other experimental treatments were as those given for ROS measurements. Subsequently, the same volume of caspase substrate mix was added to each well, and the plates were briefly shaken and incubated at 19°C for another 30 min; finally, the fluorescence signal was detected with a Fluorescence Microplate Reader (Molecular Devices) at 485 nm excitation and 538 nm emission.

Necrotic cell death was determined using the trypan blue exclusion method by light microscopy. Incubations lasted for 2 h with experimental conditions as those for the detection of apoptosis.

Statistics. Data are presented as means ± SEs of n independent preparations or individual cells. At least three cell cultures from three different fish were used in the latter case. Statistical differences were evaluated by one-or two-factor ANOVA followed by the appropriate post hoc tests, with a p value < 0.05 being considered as significant.

RESULTS

ERK Is Activated during Cu Exposure

Exposure of trout hepatocytes to 10µM Cu caused a time-dependent increase of pERK levels, with an approximately 50% elevation detectable after 5 min of incubation and
a significant twofold increase after 30 min (Fig. 1). Levels of activated ERK remained elevated for at least 60 min of Cu exposure while no increase of pERK was seen in control hepatocytes over this period. As can be seen in the upper panels of Figure 1, this increase was due to enhanced phosphorylation of a protein migrating at around 44 kDa, presumably corresponding to ERK1, whereas a faint band corresponding to a 42-kDa protein/pERK2 could not be consistently detected by the antibody used. In contrast, an antibody directed against total ERK protein consistently detected two bands, one at around 42 kDa and one at 44 kDa, the abundance of which remained unaltered during Cu exposure. Since these experiments indicated maximal ERK activation after 30 min of incubation with Cu, all subsequent treatments were investigated after this incubation period.

**Dose-Dependent Effects of Cu**

Before examining the mechanisms suspected to underlie the stimulation of pERK by Cu, we first studied the dose dependence relationship between these parameters and Cu. In control hepatocytes, Ca\(^{2+}\) remained fairly constant throughout experimental incubation and amounted to 84 ± 7nM after 30 min. In contrast, the addition of 1, 5, and 10µM Cu caused a significant twofold increase of Ca\(^{2+}\), yielding final values of Ca\(^{2+}\) of 143 ± 9nM, 414 ± 25nM, and 737 ± 65nM, respectively (Fig. 2A). Similarly, Cu stimulated ROS formation in a dose-dependent manner, the relative increases amounting to 135 ± 9%, 228 ± 24%, and 277 ± 77% of the control rate at 1, 5, and 10µM Cu, respectively (Fig. 2B). Finally, we observed that Cu also induced an increase of pERK levels at all three concentrations applied (Fig. 2C), although in this case the dose dependence was less pronounced.

**Impact of Ca\(^{2+}\), ROS, and Cell Energetics on pERK-Stimulation by Cu**

Given the positive correlation between the dose of Cu applied and the increase of Ca\(^{2+}\), ROS, and pERK levels, we next investigated the importance of Ca\(^{2+}\) and ROS for the stimulation of the MAPK. When trout hepatocytes were incubated in Ca\(^{2+}\)-free saline in the absence and presence of Cu, pERK levels after 30 min amounted to 71 ± 9% and 127 ± 32% of the control value obtained in Ca\(^{2+}\)-containing medium, respectively (Fig. 3). Although these changes were not significant, the difference between cells exposed to Ca\(^{2+}\)-free saline with or without Cu was significant, the relative increase being 1.9 ± 0.5-fold (p < 0.05).

Upon incubating hepatocytes with the ferric ion chelator DFO, which totally abolished Cu-induced ROS formation during 30 min of exposure (Table 1), pERK levels remained completely unchanged, irrespective of the presence or absence of Cu (Fig. 3). Since DFO is presumed to mainly inhibit lysosomal ROS formation, we tested if tBH, which promotes oxidative stress also in other compartments, would also stimulate ERK phosphorylation. Indeed, when administered at 1mM concentration, which stimulated ROS formation by 12 ± 0.1-fold (n = 8), a significant elevation of pERK levels to 220 ± 28% of the controls was observed (Fig. 3). Together, this suggested that elevated ROSs are important stimuli of ERK activity, but the pronounced effect of DFO might indicate that lysosomal ROSs are particularly relevant in the presence of Cu. Thus, we next tested if inhibition of mitochondrial radical formation by use of the complex III inhibitor myxothiazol would also affect the stimulation of pERK. As shown in Table 1, myxothiazol completely suppressed Cu-induced ROS formation during 30 min of incubation. Surprisingly, this not only prevented the elevation of pERK induced by Cu but it also significantly reduced pERK levels to about 30% of the control level independently from the presence of the metal (Fig. 4A). At first sight, this might be taken to indicate an important role of mitochondrial ROS production in the control of ERK activity. However, when cells were incubated with ROT and TTFA, inhibitors of complexes I and II, respectively, Cu-induced ROS formation was not at all altered (Table 1), whereas ERK activity was reduced to a similar extent as seen with myxothiazol (Fig. 4A). Since both myxothiazol and ROT + TTFA inhibit mitochondrial ATP production and hence
the main source of ATP in these cells (Krumbschnabel et al., 2000), we measured ATP contents of the hepatocytes incubated with these compounds. As depicted in Figure 4B, cellular ATP remained constant in controls and, in agreement with previous findings (Manzl et al., 2003), was also not affected by Cu over 30 min of incubation. In contrast, a significant reduction of cellular ATP contents to 11 ± 2% and to 53 ± 10% of the initial value was observed following 30 min of exposure to myxothiazol and ROT \( \text{TTFA} \), respectively. This suggests that the lack of ATP but not mitochondrial ROS may have caused the reduction of pERK levels in the cells.

**Cu Effects on Lysosomes**

In order to further elucidate if Cu affects the lysosomes of the hepatocytes and to gain more insights into the importance of these organelles for Cu-induced ROS production, lysosomal stability and the action of various lysomotropic agents on ROS formation was studied. As shown in the upper panel of Figure 5, incubation of hepatocytes with the lysomotropic stain AO resulted in a granular staining pattern, reflecting the accumulation of the dye in lysosomes. During incubation of control hepatocytes over a period of 120 min, fluorescence emitted from the cells showed some fluctuations but was not diminished compared to the initial value. In contrast, upon addition of Cu, fluorescence significantly decreased to 47 ± 2% of the starting value. This was also reflected in a loss of granular staining, indicating that Cu did in fact induce a decrease of lysosomal stability. In line with this, addition of DFO to the cells before Cu exposure significantly diminished loss of the dye, resulting in a value of 80 ± 2% of the initial fluorescence after 120 min. Incubating cells with myxothiazol,
so as to compromise cell metabolism without directly stimulating lysosomal radical formation, caused a decrease of AO fluorescence to 76 ± 2%, and in this case, the decrease did not occur before 70 min of exposure to the inhibitor.

Since previous studies on rat hepatocytes have shown that besides DFO, which directly chelates the intralysosomal iron pool (Persson et al., 2003), other agents acting on lysosomes may as well interfere with ROS formation stimulated by redox-active metals (Pourahmad et al., 2001, 2003), we tested if this would also be the case in trout hepatocytes. As summarized in Table 1, we found that monensin and chloroquine, both of which inhibit lysosomal acidification and endocytosis (Stenseth and Thyberg, 1989), significantly diminished the production of ROS elicited by Cu. In contrast, no decrease of Cu-induced ROS formation was seen in the presence of the lysosomal protease inhibitor leupeptin.

Cu²⁺, ROS, Energetics, and Cell Death

In the next series of experiments we tested how the various treatments found to have an impact on the activation of ERK in Cu-exposed trout hepatocytes would affect cell viability and specifically if they could interfere with apoptotic or necrotic cell death induced by the metal. Since 30 min of incubation with 10μM Cu does not cause a significant decrease of viability (Manzl et al., 2003), these measurements were made after 120 min of exposure. Furthermore, as we previously found that the percentage of apoptotic cells detected by scoring alterations of nuclear morphology after 120 min incubation with Cu is rather low, we decided to measure activity of caspases 3 and 7. To our knowledge, enhanced activity of these executioner caspases is an early sign of apoptotic cell death exclusively associated with this mode of cell death. Thus, we considered caspase activity a more sensitive measure of apoptosis. Finally, given the important role of ROS formation in inducing cell death (present study and Krumschnabel et al., 2005; Manzl et al., 2004) and the fact that short-term effects of the tested compounds (Table 1) may not correspond to effect seen over 120 min, the extent of ROS formation was also studied for each condition in parallel to the evaluation of cell death.

As shown in Figure 6A, the effect of Cu on ROS formation measured over 120 min was indeed more pronounced as that determined over 30 min, reaching 756 ± 160% of the control rate in this experimental series. In cells incubated with Ca²⁺-free saline or with DFO, Cu elicited no significant elevation of radical formation. Cells treated with myxothiazol and Cu showed an elevated rate of ROS production of 382 ± 103%
of control rates, which corresponded to an approximately 50% reduction compared to the rate seen in the presence of Cu only.

The impact of these treatments on caspase 3/7 activity is depicted in Figure 6B, where it can be seen that Cu stimulated caspase activity 11.5-fold. All other treatments prevented a significant rise of caspase activity, an exception being the combined presence of myxothiazol and Cu. In this case, caspase activity was enhanced 5.9-fold, which again was half the activity triggered by Cu alone.

The percentage of necrotic cells, estimated from the uptake of trypan blue due to compromised membrane integrity, increased from 7% in controls to 24% in Cu-treated cells (Fig. 6C). The absence of extracellular Ca$^{2+}$, as well as the presence of DFO, prevented an increase of the number of necrotic cells, whereas myxothiazol both alone and in combination with Cu enhanced necrosis to levels indistinguishable from the effect of Cu.

**MAPKs, ROS, and Cell Death**

Finally, we aimed at directly addressing the role of ERK activity in Cu-induced cell death by pharmacologically interfering with its activation and studying the above-named parameters in this condition. Furthermore, although our present study focused on the role of ERK in this context, we also investigated the possible involvement of p38-MAPK and JNK in mediating Cu toxicity.

In these experiments, the stimulation of ROS formation by Cu amounted to 544 ± 62% of the control rate. Preincubating cells with an inhibitor of MEK, the upstream activator of ERK, or an inhibitor of p38-MAPK had no effect on radical production in the absence of Cu but, surprisingly, prevented a significant increase of ROS formation in the presence of Cu (Fig. 7A). In contrast, an inhibitor of JNK, which also had no significant effect on basal ROS production, did not inhibit, but stimulated, ROS formation upon addition of Cu, these cells reaching 1707 ± 556% of the radical production rate observed in controls. Finally, we also tested the impact of a pan-caspase inhibitor on radical formation, and we observed that it neither affected basal ROS production nor the Cu-stimulated rate.

The activity of caspases 3 and 7 determined after 120 min of incubation without Cu was not affected by any of the inhibitors of MAPK pathways applied (Fig. 7B). In contrast, although Cu

**FIG. 5.** Changes in lysosomal stability of hepatocytes, estimated from AO fluorescence, under control conditions (control) and in the presence of Cu (Cu), with Cu and the iron chelator DFO (Cu + DFO), or with the mitochondrial inhibitor myxothiazol (Myxo). Data are means ± SEs of ≥ 50 cells from greater than or equal to three independent experiments. Upper panel: fluorescence image of hepatocytes loaded with AO showing granular staining of the cells at the onset of the experiment and loss of granules, associated with decreased fluorescence, during Cu exposure.
FIG. 6. Changes in the rate of ROS formation (A), caspase 3/7 activity (B), and the percentage of necrotic cells (C) during 120 min of incubation in the absence or presence of Cu in control saline, in Ca\(^{2+}\)-free saline, with DFO, and with myxothiazol. Data are means ± SEs of (A) \(n \geq 8\) (three for Ca-free conditions) and (B, C) 5 independent experiments. *\(p < 0.05\) compared to controls.

FIG. 7. Changes in the rate of ROS formation (A), caspase 3/7 activity (B), and the percentage of necrotic cells (C) during 120 min of incubation in the absence or presence of Cu in control hepatocytes and in cells pretreated with the MEK inhibitor U0126, the p38-MAPK inhibitor SB203580, the JNK inhibitor SP600125, or the pan-caspase inhibitor Q-VD-OPH. Data are means ± SEs of four to five independent experiments. *\(p < 0.05\) compared to controls; *# \(p < 0.05\) compared to Cu-treated cells.
stimulated a three- to fivefold increase of caspase activity in hepatocytes pretreated with MAPK inhibitors, these elevations were far less pronounced than the stimulation of caspase activity seen with Cu only. Expressed relative to the increase triggered by Cu, the MAPK inhibitors reduced caspase activity by 73% (MEK inhibition), 54% (p38-MAPK), and 55% (JNK), each of these reductions being statistically significant. According to expectations, the pan-caspase inhibitor completely prevented any increase of caspase activity both with and without Cu.

In addition to reducing the induction of apoptotic cell death, the MEK inhibitor U0126 also completely prevented any increase of necrotic cell death in the presence of Cu (Fig. 7C). Similarly, inhibition of p38-MAPK prevented a significant increase of necrotic cells, although there was a clear tendency for an increase from 7% in controls to 18% in SB203580-treated, Cu-exposed cells. A comparable trend toward an increase of necrotic cells was also seen in hepatocytes pretreated with the JNK inhibitor, and in these cells, the increase of Cu-stimulated necrosis was not diminished, reaching 31% at the end of the 120-min incubation period. A similar elevation to 28% necrotic cells was observed in cells incubated with the pan-caspase inhibitor and Cu.

**DISCUSSION**

*Mechanisms Underlying Cu-Induced Stimulation of ERK Activity*

In agreement with a recent study on a trout hepatoma cell line (Burlando et al., 2003) and studies on oxidant-induced cell injury in other cells (Iryo et al., 2000; Park et al., 2005; Yu et al., 2000; Zhang et al., 2003), Cu induced a relatively rapid and sustained increase of ERK phosphorylation. Several lines of evidence suggest that the main mechanism responsible for the activation of ERK was enhanced formation of ROS triggered by Cu. Thus, we found that (1) the dose-dependent increase of ROS formation elicited by Cu was mirrored by a similar dose-dependent elevation of cellular pERK levels, (2) conditions diminishing radical production also prevented ERK stimulation, and that (3), conversely, stimulating ROS formation by means of the hydrogen peroxide analogue tBH enhanced cellular pERK levels. The dose-dependent increase of Ca2+i was not directly related to the stimulation of ERK, as the absence of Cu2+ only reduced the absolute, but not the relative, extent of ERK activation. Furthermore, in the present study the absence of Ca2+ almost completely suppressed the elevation of ROS formation by Cu, so this effect could as well have been an indirect one. On the other hand, a similar decrease of total pERK levels but no change in relative induction of the MAPK was also seen in trout hepatocytes exposed to hypoosmotic stress in Ca2+-free saline, where Ca2+i elevation is also exclusively dependent on influx from the extracellular space (Ebner et al., in press). Together, this indicates that Ca2+ may control the constitutive activity of ERK but not its relative increase upon stimulation.

Noteworthy, we previously found that at a higher concentration of Cu, i.e., 100μM, enhanced ROS formation appeared to be a cause of Ca2+i elevation, and not a consequence, since inhibiting radical formation prevented disruption of Ca2+ homeostasis, but not *vice versa* (Manzl et al., 2004). This underscores the notion that the damaging effects exerted by Cu, as well as by other toxicants, depend on its concentration and thus need to be taken into consideration before any generalizations can be made.

Regarding the origin of ROS triggering ERK activation, we specifically considered lysosomes and mitochondria as potential sources. In order to elucidate the importance of mitochondrial ROS formation, we exposed the hepatocytes to myxothiazol, an inhibitor of the electron transport chain complex III. Different from the effect of this compound over 120 min (Fig. 6), myxothiazol caused a complete inhibition of ROS formation during the first 30 min of Cu exposure. This treatment not only prevented the stimulation of ERK activity by Cu but also reduced pERK levels below basal control values. The same effect was noted upon exposing cells to myxothiazol without Cu, as well as after incubation with ROT and TTFA, which did not reduce ROS formation. Thus, the decrease of ERK activity was apparently not related to diminished radical production but rather due to a lack of ATP created by these treatments. In line with this, even though ERK activity may be enhanced during hypoxia in some cell models (Blaschke et al., 2002; Minet et al., 2000; Yung and Tolkovsky, 2003), complete inhibition of mitochondrial ATP production was found to reduce pERK levels (Abas et al., 2000; Yung and Tolkovsky, 2003). Similarly, a reduction of ATP to a comparable extent as that seen with myxothiazol (i.e., 10%) also diminished pERK levels in mammalian cells (Dlugosz et al., 2000). In contrast, a less pronounced decrease of ATP as seen with ROT and TTFA did not affect phosphorylation of protein kinase C in PC12 cells (Soltoff, 2001). This might either indicate different ATP dependence of different protein kinases or species- and cell type–specific differences. The role of mitochondria-derived ROS for ERK activation, therefore, remains unresolved at present, but our data lend support to the concept that mitochondrial ATP is an important requirement in this process (Abas et al., 2000).

*The Role of Lysosomes*

As outlined in the introduction, several studies indicate that lysosomes are critically involved in oxidant-induced cellular stress, both as a target and as a source of damaging effects (Brunk et al., 2001; Persson et al., 2003; Pourahmad et al., 2001; Rob erg et al., 1999). In keeping with these studies, we observed that exposure to Cu resulted in a rapid loss of granular AO fluorescence, indicating a decrease in lysosomal stability. As this could largely be inhibited by DFO, which also
prevented any elevation of ROS formation, it appears that the lysosomes or factors released from these organelles are involved in the increased radical production elicited by Cu. Furthermore, in agreement with other studies (Pourrahmad et al., 2001, 2003), we found that the lysosomotropic agents monensin and chloroquine prevented the enhancement of radical formation by Cu, although the exact mechanism of this effect is unknown. In contrast, the inhibitor of lysosomal proteases leupeptin did not diminish Cu-induced radical formation, arguing against a role of proteases released from damaged lysosomes, at least in the early phase of radical stress. Finally, we saw that cellular ATP depletion with myxothiazol promoted a significant increase of necrotic cell death (Fig. 6) but damaged lysosomes to a lesser extent and with considerable delay compared to Cu. This provides further support for the lysosomes being an early and important target during Cu toxicity.

The exact mechanism by which Cu may affect lysosomes and stimulate ROS formation in these organelles remains unclear at present, specifically in light of our previous finding that Cu is apparently not accumulated in trout hepatocytes during 2 h of incubation (Manzl et al., 2003). However, only little Cu is required to initiate redox cycling (see Fig. 2B), and a small, but still relevant, influx of Cu may have remained undetected previously. Furthermore, Cu is reduced to monovalent Cu at the cell membrane, e.g., via interaction with SH groups of membrane proteins (Bogdanova et al., 1999), which may subsequently promote lipid peroxidation and initiate a chain reaction (Chan et al., 1982). Once started, radicals can rapidly induce further radical formation in a feed forward cycle, e.g., by stimulating NAD(P)H oxidase (Li et al., 2001), by stimulating mitochondrial ROS production (see above; Tirmenstein et al., 2000), or particularly by interaction with redox-active transition metals, such as the lysosomal pool of iron (Persson et al., 2003). In addition, the cytosolic pool of iron has also been found to be important in inducing cell damage (Rauen et al., 2000, 2003). The pronounced protection afforded by DFO might thus indicate that DFO, in addition to chelating lysosomal iron, acts as a sponge, removing any labile iron from the cytosol and thereby reducing Fenton-generated ROS formation throughout the cell.

ERK and Its Role in Cu-Induced Cell Death

In addition to analyzing the mechanisms underlying Cu stimulation of ERK phosphorylation, this study also investigated the role of ERK as a factor contributing to either cell survival or cell death. In standard conditions, Cu caused an increase in the number of cells with a damaged membrane, interpreted as necrotic cells, as well as a huge increase in the activity of the executioner caspases 3 and 7, which is generally considered as an indicator for apoptosis. Regarding the role of ERK, our data suggest that, in Cu-exposed trout hepatocytes, activation of this MAPK is an important signal promoting cell death. Thus, we observed that conditions diminishing or preventing the activation of ERK, i.e., the absence of Ca\(^{2+}\) or the presence of DFO, concomitantly prevented an increase of necrotic and presumably apoptotic cell death. The fact that myxothiazol, despite suppressing pERK levels, only partially reduced apoptosis and not at all necrosis indicates that, under these conditions, pERK-independent cell death was triggered. Interestingly, myxothiazol alone killed the cells by necrosis but did not at all stimulate caspase activity, supporting the concept that a minimum level of cellular ATP is required to activate the apoptotic pathway (Eguchi et al., 1999; Leist et al., 1997). On the other hand, enhanced caspase activity seen in hepatocytes treated with myxothiazol and Cu could reflect the activation of caspases by lysosomal enzymes, e.g., cathepsins (Johansson et al., 2003; Zhao et al., 2003), released from lysosomes disrupted in the presence of Cu, which occurs to a much lesser degree in the presence of myxothiazol only.

More direct evidence for the involvement of ERK in cell death was obtained from our experiments using the specific inhibitor of the MEK/ERK pathway, U0126. Pretreating trout hepatocytes with U0126 reduced necrosis, diminished apoptosis, and, surprisingly, also prevented enhanced ROS formation in the presence of Cu. Given the importance of radical stress for Cu-induced cell death (present study and Manzl et al., 2004), the protective effect of ERK inhibition is most likely related to the latter effect. Specifically, this could be due to inhibition of ERK-induced activation of NAD(P)H oxidase, as ERK may stimulate the recruitment of components of this protein complex to the membrane (Dreiem et al., 2003; Hazan-Halevy et al., 2005; Matsunaga et al., 2005; Parinandi et al., 2003; Seru et al., 2004), although this remains speculative at present. In addition, inhibition of ERK may prevent apoptosis induced by ERK-dependent phosphorylation and thus inactivation of the antiapoptotic protein Bcl-2, which leads to mitochondrial translocation of proapoptotic Bax (Ishikawa et al., 2003; Tamura et al., 2004), causing the presumably MPT-mediated release of apoptotic factors from the mitochondria (Sharpe et al., 2004). In fact, it has been shown that Bax is upregulated by Cu and that Bcl-2 overexpression can partially inhibit Cu-induced apoptosis (Zhai et al., 2000). In addition, as apoptosis was not entirely blocked by inhibiting ERK phosphorylation, Cu seems to be capable of inducing apoptosis by another pathway, the nature of which remains to be elucidated.

The Role of p38-MAPK and JNK in Cu-Induced Cell Death

Inhibition of p38-MAPK resulted in a similar, but somewhat less pronounced, cytoprotection against Cu toxicity as seen upon inhibition of ERK activity. In line with this, the above-described effects of ERK on NAD(P)H oxidase (Hazan-Halevy et al., 2005; Parinandi et al., 2003), phosphorylation of Bcl-x(L) (Grethe et al., 2004), and mitochondrial Bax translocation (Ishikawa et al., 2003) have all been described for p38-MAPK as well, suggesting possibly similar mechanisms promoting
cell death. The fact that necrosis was only slightly reduced upon p38-MAPK inhibition may be related to ROS formation, which, although not quite significantly elevated by Cu, nevertheless more than doubled compared to controls.

In contrast, during inhibition of JNK, ROS formation was even stimulated, which could explain why it also slightly enhanced necrotic cell death. Inhibition of the JNK pathway nonetheless significantly reduced apoptosis, which may again be due to its well-known interaction with various anti- and proapoptotic proteins (Liu and Lin, 2005), as well as to direct interaction with the mitochondria (Aoki et al., 2002; Eminel et al., 2004). Noteworthy, JNK inhibition also doubled the number of necrotic cells in the absence of Cu, which could indicate that in trout hepatocytes it is generally cytoprotective under normal conditions. At the same time, however, it should be mentioned that the apparent proapoptotic function of all three MAPKs may merely be true for Cu intoxication, since a stimulus-dependent role as a factor promoting cell survival or death is known for each MAPK (Wada and Penninger, 2004).

Specifically, it has been shown for ERK that the duration of its activation is decisive in this regard (Apati et al., 2003; Chu et al., 2004), which is supported by experiments showing that in trout hepatocytes stimulated with epidermal growth factor, which does not kill the cells, pERK levels increase only transiently (Ebner and Krumschnabel, unpublished).

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