Research Article

Impact of copper on the induction and repair of oxidative DNA damage, poly(ADP-ribosyl)ation and PARP-1 activity

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Copper is an essential trace element involved, among other functions, in enzymatic antioxidative defense systems. However, nonprotein bound copper ions have been shown to generate reactive oxygen species. To gain insight into the discrepancy between the protective properties of copper on the one hand and its toxicity on the other hand, we examined the genotoxic effects of $CuSO_4$ in cultured human cells. Here we report that copper, at cytotoxic concentrations, induces oxidative DNA base modifications and DNA strand breaks. However, at lower noncytotoxic concentrations, copper inhibits the repair of oxidative DNA damage induced by visible light. As a first mechanistic hint, inhibition of H_2O_2-induced poly(ADP-ribosyl)ation was identified in cultured cells and further experiments demonstrated a strong inhibition of the activity of isolated poly(ADP-ribose)polymerase-1 (PARP-1) by copper. Bioavailability studies of copper showed a dose-dependent uptake in cells and pointed out the relevance of the applied concentrations. Taken together, the results indicate that copper, under conditions of either disturbed homeostasis or overload due to high exposure, exerts defined genotoxic effects. Hence, a balance needs to be maintained to ensure sufficient uptake and to prevent overload.

Keywords: Base excision repair / Copper / Oxidative DNA damage / PARP-1, poly(ADP-ribosyl)ation

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1 Introduction

The essential trace element copper is a catalytic cofactor for enzymes that play critical roles, *e.g.,* in iron homeostasis (ceruloplasmin), catecholamine biosynthesis (tyrosinase, dopamine-β-hydroxylase), oxidative phosphorylation (cytochrome *c* oxidase) and oxidative stress protection (superoxide dismutase) [1]. Therefore, copper deficiency can result in multiple pathologies, which can in part be linked to alterations in the oxidant defense system and ultimately can lead to excessive oxidative stress [2]. In the general population the primary source of copper intake is diet, with an estimated intake of approximately 2 mg/day from food and 0.15 mg/day from drinking water. Potatoes and other vegetables make the largest contribution (approximately 30%), followed by meat, poultry, fish and bread (approximately 20%). The estimated average requirement for copper is 0.7 mg/day and the recommended dietary allowance (RDA) is 0.9 mg/day for adults [3]. This indicates that the dietary intake of copper in developed countries is well over the estimated average requirement for copper. Similar to copper deficiency, copper overload may also show adverse effects as copper toxicity can result in oxidative stress and subsequent tissue damage. Free copper ions or low molecular copper complexes catalyse Fenton-type reactions, generate reactive oxygen species (ROS) and induce protein and nucleic acid oxidation [2, 4, 5]. In addition, its ability to bind to proteins and nucleic acids enables copper to specifically promote oxidative modification reactions [6–8]. Living cells ensure essential copper functions and prevent copper toxicity by a tight control of the free copper level by highly efficient metal chelating proteins, resulting in almost undetectable amounts of free copper in intact cells [9–11]. However, when the concentration of free

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Abbreviations: Fpg, formamidopyrimidine-DNA-glycosylase; **ICC,** Indian Childhood Cirrhosis; **PARP-1,** poly(ADP-ribose)polymerase-1

copper increases, either by environmental or pathological causes, cell damage may occur [12–14].

Interestingly several population subgroups are more susceptible to copper toxicity. For example, the susceptibility of infants and children up to the age of 10 is evidenced by the incidence of Indian Childhood Cirrhosis (ICC) and there have been reports of adverse effects in children consuming drinking water containing elevated levels of copper [3, 11, 15]. It appears that children are not able to cope with excess copper, probably since their homeostatic mechanisms are not fully developed at birth and additionally newborns have elevated hepatic copper levels [16]. There is also an indication of a genetic susceptibility in the ICC data, because the incidence seems to be familial [3]. Additionally individuals with Wilson disease are highly susceptible to copper toxicity. Wilson disease, an autosomal recessive disorder, is associated with mutations in a copper-transporting protein, which initially leads to high copper accumulation in the liver due to an excess retention of hepatic copper and impaired biliary copper excretion. Copper overload causes death of hepatocytes and subsequent accumulation of copper in extrahepatic tissues, including the central nervous system. Observed symptoms in these tissues are thought to result from copper catalysed induction of ROS, oxidative DNA damage and lipid peroxidation [17–21]. Additionally, a study on Long-Evans Cinnamon (LEC) rats, an animal model for Wilson disease, provided evidence that acute hepatitis impairs the expression and function of two DNA glycosylases, responsible for the repair of oxidative DNA damage (base excision repair, BER) and strongly suggests that the repair of endogenous DNA adducts plays a critical role in the development of spontaneous hepatocellular carcinoma in LEC rats [22].

In the present study, we investigated the impact of copper on the induction and repair of oxidative DNA damage in cultured human cells. We found that at concentrations of copper that showed no elevation of oxidative DNA damage directly in HeLa S3 cells, copper inhibited the repair of oxidative DNA damage induced by visible light. As first mechanistic hint, the inhibition of H_2O_2-induced poly(ADP-ribosyl)ation was identified in cultured cells. Further experiments demonstrated a strong inhibition of the activity of the isolated repair protein poly(ADP-ribose)polymerase-1 (PARP-1) by copper, indicating that the observed decrease in cellular poly(ADP-ribosyl)ation is due to changes in the activity of PARP-1.

2 Materials and methods

2.1 Materials

Ham's F12 nutrient mixture, foetal bovine serum, trypsin and penicillin–streptomycin solutions were obtained from Sigma (Deisenhofen, Germany). The culture dishes were supplied by Biochrom (Berlin, Germany). mAb 10H directed against poly(ADP-ribose) [28] was prepared as described previously (Alvarez-Gonzalez, 1999) and kindly provided by Professor A. Bürkle (Konstanz, Germany) or purchased from Trevigen (Gaithersburg, MD, USA). FITC-conjugated goat antimouse IgG (Fab specific) antibody was provided BY Sigma and Vectashield mounting medium containing DAPI (1 μg/mL) from Vector Laboratories (Burlingame, CA, USA). Alexa Fluor 680 goat antimouse IgG antibody was purchased from Invitrogen (Paisley, UK). His-tagged PARP-1 was purified on a nickel chelating resin (Novagen, Madison, WI, USA) as recommended by the manufacturer. Triton X-100 was bought from Pierce (Oud-Beijerland, The Netherlands), hydroxyapatite (high resolution) from Calbiochem (Bad Soden, Germany), β-NAD^+ from MP Biochemicals (Irvine, CA, USA), *EcoR1* from New England Biolabs (Beverly, MA, USA). $CuSO_4$ (≥99.995% purity) was obtained from Aldrich (Buchs, Germany). H_2O_2 as well as all other chemicals were of p.a. grade and were procured from Merck (Darmstadt, Germany).

2.2 Cell culture and incubation with $CuSO_4$

HeLa S3 cells were grown in tissue culture dishes as monolayers in Ham's F12 nutrient mixture containing 10% foetal bovine serum, 100 U penicillin/mL and 100 mg streptomycin/mL. The cells were incubated at 37°C with 5% CO_2 in air and 100% humidity. Logarithmically growing cells were treated with $CuSO_4$ as described for the respective experiments. $CuSO_4$ stock solutions were prepared in sterile distilled water shortly before each experiment.

2.3 Uptake of $CuSO_4$

The uptake of copper in HeLa S3 cells was measured after ashing of cells by atomic absorption spectroscopy. Briefly, $1–2 \times 10^6$ logarithmically growing cells were trypsinized after 24 h incubation with $CuSO_4$, collected by centrifugation, and washed two times with ice-cold phosphate buffered saline (PBS). Cell number and cell volume were measured by an automatic cell counter (Casy-1, Schaerfe System). After incubation with the ashing mixture, 65% HNO_3/30% H_2O_2 (1/1) at 95°C for at least 12 h and dilution with water, copper was measured by atomic absorption spectroscopy (Perkin Elmer 4110 ZL, AS-72).

2.4 Irradiation with visible light in the presence of the photosensibilizator RO 19-8022

For treatment with visible light, cells were irradiated as monolayers in cell culture dishes covered with PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) containing 50 nM RO 19-8022 on ice, applying a special 1000 W halogen lamp (Hedler, Runkel/Lahn, Germany) for 10 min at a dis-

tance of 33 cm, which corresponded to 134 kJ/m^2. To avoid overheating, the dishes were gently shaken during the entire procedure.

2.5 Cell number and colony forming ability

Logarithmically growing cells were incubated for 2 or 24 h with $CuSO_4$, washed with PBS, trypsinized, counted and 300 cells/dish were seeded for determination of colony forming ability. After 7 days of incubation, colonies were fixed with ethanol, stained with Giemsa (25% in ethanol), counted and calculated as percent of control. Untreated controls exhibited colony forming abilities of about 85%.

2.6 Induction of oxidative DNA damage in cultured human cells

DNA strand breaks and formamidopyrimidine-DNA-glycosylase (Fpg)-sensitive sites were determined by the alkaline unwinding technique in combination with the bacterial Fpg as described elsewhere [23]. Briefly, 1×10^5 cells were seeded, allowed to attach for at least 24 h before treatment with $CuSO_4$ and/or visible light. At the end of the treatment medium was removed, cells were washed with cold PBS and a lysis buffer was added containing 0.006 M Na_2HPO_4, 0.001 M KH_2PO_4, 0.137 M NaCl, 0.003 M KCl and 0.1% Triton X-100. After 5 min on ice the solution was removed by aspiration and the cells were treated with a high salt solution containing 2 M NaCl, 0.01 M EDTA and 0.002 M Tris (pH 8) for 2 min on ice, where after the cells were left on ice for an additional 8 min. The nucleoids were then incubated with Fpg (1 μg/mL) in enzyme buffer (0.05 M sodium phosphate, 0.01 M EDTA, 0.1 M NaCl, pH 7.5) for 30 min at 37°C. For the detection of DNA strand breaks in the same experiment as Fpg-sensitive sites Fpg was omitted. At the end of incubation an alkaline solution was added yielding a final concentration of 0.07 M NaOH, 0.013 M EDTA, 0.37 M NaCl, pH 12.3 and the DNA was allowed to unwind for 30 min in the dark.

With respect to experiments investigating only DNA strand breaks, DNA strand breaks were quantified by a modified alkaline unwinding technique as described previously [24]. Briefly, 1×10^5 cells were seeded, allowed to attach for 24 h, preincubated with $CuSO_4$ for 24 h and coincubated with 50 μM H_2O_2 for 5 min. Thereafter the medium was removed, cells were washed with PBS and an alkaline solution containing 0.03 M NaOH, 0.02 M Na_2HPO_4, and 0.9 M NaCl (pH 12.3) was added and the DNA was allowed to unwind for 30 min in the dark.

After neutralization and sonication, separation of ssDNA and dsDNA was performed on 0.5 mL hydroxyapatite columns at 60°C. ssDNA and dsDNA were eluted with 1.5 mL of 0.15 M and 0.35 M potassium phosphate buffer, respectively. The DNA content of both fractions was determined by adding Hoechst 33258 dye to a final concentration of 7.5×10^{-7} M to 1 mL of each sample and measuring the fluorescence with a microtiter fluorescence reader (Spectra Fluor, Tecan) at an excitation wavelength of 360 nm and an emission wavelength of 455 nm. The fraction of dsDNA and lesion frequencies were calculated as described before [23, 24].

2.7 Determination of cellular levels of poly(ADP-ribosyl)ation

The extent of poly(ADP-ribosyl)ation was assessed in HeLa S3 cells as described previously [25–27]. The procedure is based on *in situ* immunofluorescence detection of poly(ADP-ribose) using a mouse mAb (10H) raised against poly(ADP-ribose) [28] in conjunction with an FITC-conjugated secondary antibody. Cells were grown as monolayers on coverslips (15 mm diameter) placed in cell culture dishes for 30 h and then incubated with $CuSO_4$ for 24 h. To induce poly(ADP-ribosyl)ation, the cells were treated with 100 μM H_2O_2 for 5 min. Subsequently, coverslips were removed, rinsed with PBS and fixed in ice-cold 10% trichloroacetic acid (TCA) for at least 10 min followed by successive 5 min washings in 70, 90% and absolute ethanol (−20°C). Coverslips were air-dried, rehydrated in PBS and incubated with 5 μg/mL purified mAb 10H directed against poly(ADP-ribose) in blocking reagent (PBS, pH 7.4, 5% skim milk powder and 0.05% Tween-20). This step was carried out in a humid chamber at 37°C for 30 min, followed by repeated washing of the coverslips in PBS. The secondary, FITC-conjugated antimouse antibody (dilution 1:50 in blocking reagent) was applied accordingly. Finally, the cover glasses were mounted on microslides in Vectashield mounting medium containing 1 μg/mL DAPI. Fluorescence intensity was evaluated using a Nikon Eclipse E400 microscope. Pictures of nuclear DAPI staining and poly(ADP-ribose) fluorescence signals were captured with a SPOT RT (realtime) camera (Visitron Systems, Puchheim, Germany). At least 50 cells *per* slide were selected using the DAPI stain. The selected areas were copied to the FITC fluorescence channel and FITC fluorescence intensity was quantified using MetaView imaging systems software, Version 4.1.5. For statistical analysis, a Student's *t*-test was performed by comparing the mean fluorescence intensities of at least five determinations measured after the respective treatments as compared to H_2O_2-treated cells serving as positive controls.

2.8 Activity of isolated PARP-1

To investigate the impact of $CuSO_4$ on the activity of isolated PARP-1, poly(ADP-ribosyl)ation was measured after activation of PARP-1 by a nicked plasmid. PARP-1 substrate was generated by nicking a pUC13-based circular plasmid, containing a single *EcoR1* restriction site, with *EcoR1* for 1 h at 37°C, followed by heat inactivation of the

enzyme at 65°C for 20 min. After 10 min preincubation of PARP-1 (100 ng) with $CuSO_4$ (10–250 μM) in preincubation buffer (83 mM HEPES, 10 mM $MgCl_2$, 11 mM KCl, 0.2 mM EDTA, 0.11 mM DTT, 5.6% v/v Glycerol, pH 7.9) at room temperature, the PARP-1 reaction was carried out for 5 min at 37°C in reaction buffer (50 μM β-NAD^+, 2 ng/μL PARP-1, 0.2 ng/μL nicked plasmid, 41 mM HEPES, 5 mM $MgCl_2$, 4 mM KCl, 0.1 mM EDTA, 0.04 mM DTT, 2% v/v glycerol, pH 7.9). Poly(ADP-ribosyl)ation was stopped by adding SDS/PAGE sample buffer (25 mM Tris/HCl, pH 6.8, 2.5% v/v 2-mercaptoethanol, 1% w/v SDS, 5% v/v glycerol, 1 mM EDTA, 0.15 mg/mL Bromophenol blue) and heating for 5 min to 95°C. The mixtures were separated on an SDS/10% polyacrylamide gel followed by transfer to a PVDF membrane. Finally poly(ADP-ribose) polymers were quantified by immunoblot analysis with a monoclonal PAR-antibody (10H), and a secondary Alexa Fluor 680-labelled antibody, and an infrared fluorescence imaging system (Odyssey, LI-COR Biosciences) was used for quantification.

3 Results

3.1 Uptake of copper in HeLa S3 cells

To assess bioavailability of copper, the uptake of copper was measured by atomic absorption spectroscopy. After 24 h incubation, a dose-dependent uptake and strong up to 8.5-fold accumulation of copper was observed in HeLa S3 cells (Fig. 1).

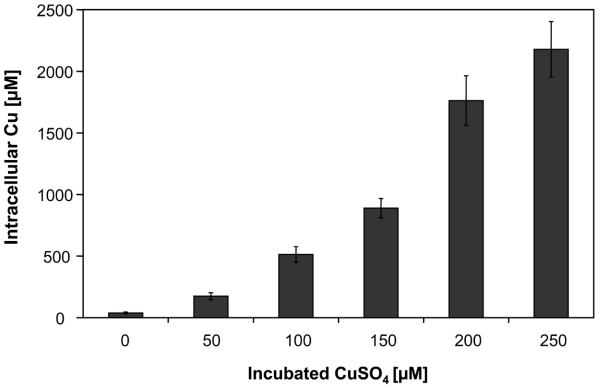

Figure 1. Uptake of copper after incubation of HeLa S3 cells with $CuSO_4$. Logarithmically growing cells were treated for 24 h with $CuSO_4$, trypsinized and counted, and after the cell volume was measured, copper was determined by atomic absorption spectroscopy. Shown are mean values of at least six determinations ± SD.

3.2 Cytotoxicity and induction of oxidative DNA damage

The cytotoxicity was determined by investigating the effect of $CuSO_4$ on cell number and colony forming ability after 2 (Fig. 2A) and 24 h (Fig. 2B) incubation. Cytotoxicity of $CuSO_4$ was more pronounced after 24 h incubation and stronger effects were seen on colony forming ability as compared to cell number.

To investigate the induction of oxidative DNA damage, HeLa S3 cells were incubated with $CuSO_4$ for short (2 h) and long term (24 h) incubation and lesion frequencies were quantified by the alkaline unwinding technique in combination with Fpg. Fpg recognizes 7,8-dihydro-8-oxoguanine (8-oxoguanine), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-Gua), 4,6-diamino-5-formamidopyrimidine (Fapy-Ade) and to a smaller extent 7,8-dihydro-8-oxoadenine (8-oxoadenine) as well as apurinic/apyrimidinic sites (AP sites) and converts them into DNA strand breaks by its DNA incision activity [29, 30]. After 2 and 24 h incubation, noncytotoxic concentrations of <1000 and <300 μM $CuSO_4$, respectively, did not induce any significant amount of oxidative DNA damage. At cytotoxic concentrations a strong induction of mainly DNA strand breaks was observed, reaching up to 5.1 lesions/10^6 bp at 2 mM (2 h incubation) and 2.3 lesions/10^6 bp at 400 μM $CuSO_4$ (24 h incubation), respectively.

3.3 Effects on the repair of oxidative DNA damage induced by visible light

To examine the impact of copper on the repair of oxidative DNA damage, HeLa S3 cells were irradiated with visible light in the presence of a synthetic photosensitizer. This treatment has been shown to cause the formation of singlet oxygen and excited intracellular photosensitizer molecules, generating predominantly Fpg-sensitive sites and to a lesser extent DNA strand breaks [31]. Irradiation with visible light (134 kJ/m^2) in the presence of the synthetic photosensitizer induced about 0.2 DNA strand breaks (Fig. 3A) and 1.2 Fpg-sensitive sites/10^6 bp (Fig. 3B). A 24 h preincubation with $CuSO_4$ increased visible light-induced amounts of DNA strand breaks starting at 250 μM $CuSO_4$, whereas no significant increase of Fpg-sensitive sites was observed. To investigate the repair of the respective lesions, the cells were postincubated for 4 or 8 h and the remaining DNA damage was quantified. Within 4 and 8 h about 65 and 85% of the induced Fpg-sensitive sites were repaired in control cells, respectively. In contrast, no significant change in the number of DNA strand breaks was observed within these repair times. Preincubation with $CuSO_4$ caused a significant and concentration-dependent repair inhibition of Fpg-sensitive sites and a slight accumulation of DNA strand breaks starting at 200 and 250 μM, respectively (Figs. 3A and B). At 300 μM $CuSO_4$ about 95% repair inhibition of Fpg-sensitive sites was observed after 8 h of repair. The total levels of DNA damage measured (DNA strand breaks and Fpg-sensitive sites) demonstrate the concentration-dependent inhibition of repair by $CuSO_4$ (Fig. 3C).

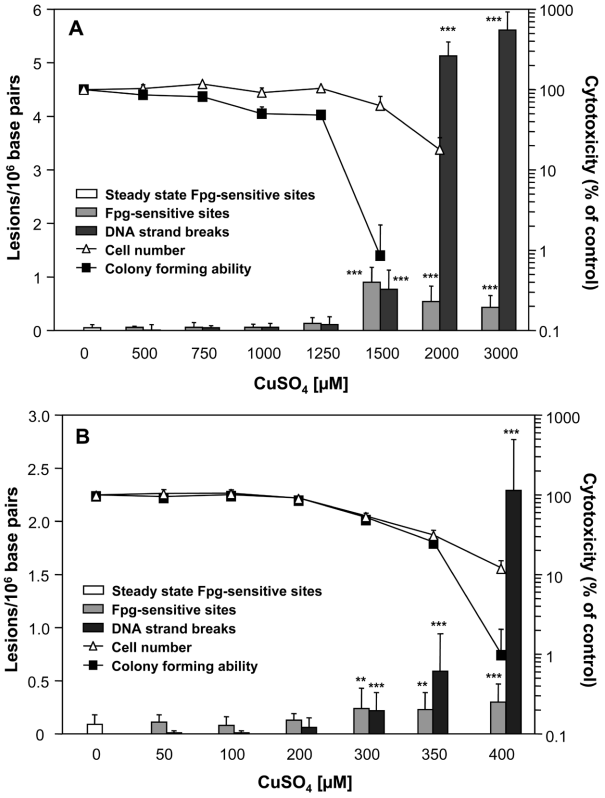

Figure 2. Cytotoxicity and induction of oxidative DNA damage after 2 h (A) and 24 h (B) incubation with $CuSO_4$. Cytotoxicity was determined by a decrease in cell number and colony forming ability. The data represent mean values of at least three (cell number) or nine (colony forming ability) determinations + SD. For assessment of oxidative DNA damage, logarithmically growing HeLa S3 cells were treated with $CuSO_4$ for 2 or 24 h and the frequencies of DNA strand breaks and Fpg-sensitive sites were determined by alkaline unwinding as described in Section 2. Steady state levels of Fpg-sensitive sites were derived from control cells. Shown are mean values of at least three determinations + SD. Statistically significant different from control: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by Student's *t*-test.

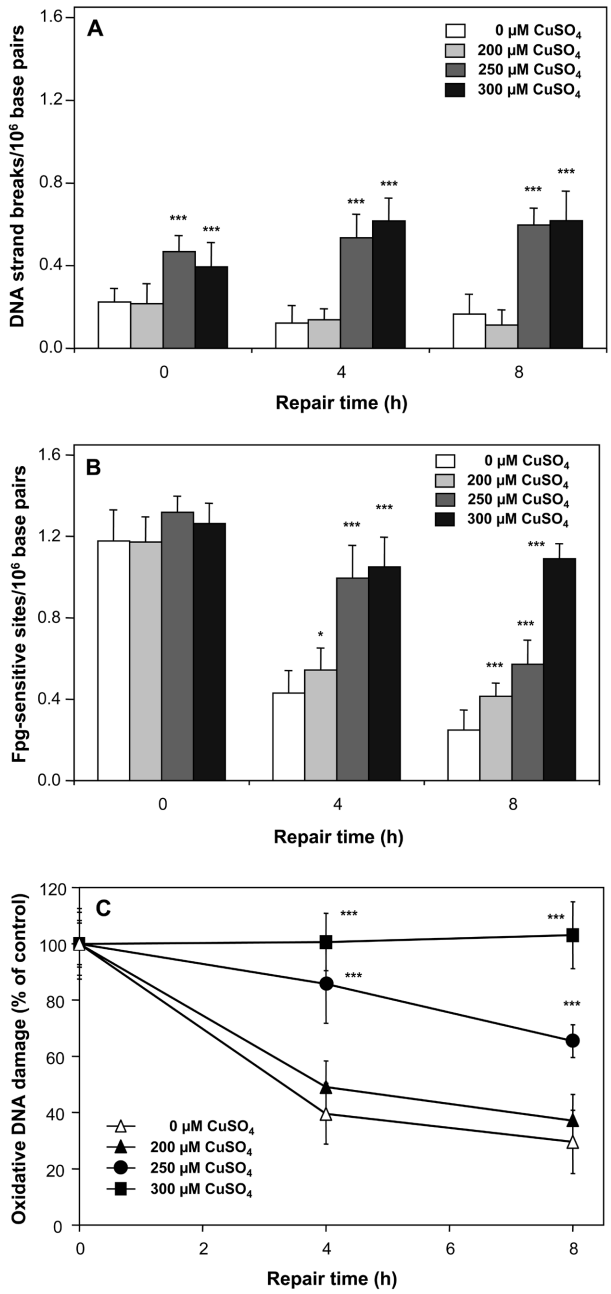

Figure 3. Effect of $CuSO_4$ on the induction and repair of oxidative DNA damage induced by visible light. For assessment of oxidative DNA damage, logarithmically growing HeLa S3 cells were preincubated with $CuSO_4$ for 24 h, irradiated with 134 kJ/m² visible light in PBS containing 50 nM RO 19-8022 on ice, and postincubated in the presence of $CuSO_4$ for 0, 4 or 8 h. Frequencies of DNA strand breaks (A) and Fpg-sensitive sites (B) were determined by alkaline unwinding as described in Section 2. For better clarity the steady state levels of Fpg-sensitive sites (derived from control cells) were subtracted. Data points in (C) represent the sum of DNA strand breaks and Fpg-sensitive sites for each time point. Shown are mean values of at least six determinations + SD. Statistically significant different from control in the absence of copper treatment: $^*p < 0.05$, $^{***}p < 0.001$ as determined by Student's *t*-test.

3.4 Effects on poly(ADP-ribosyl)ation

The extent of poly(ADP-ribosyl)ation in intact cells was determined by immunological detection of poly(ADP-ribose) *in situ* by applying a highly specific mAb and a secondary FITC-conjugated antibody. As a first approach the impact of 24 h incubation with $CuSO_4$ on poly(ADP-ribosyl)ation was investigated and copper showed no effect on poly(ADP-ribosyl)ation in nonstimulated cells (data not shown). Previous studies demonstrated that poly(ADP-ribosyl)ation is induced within 5 min after the induction of DNA strand breaks by 100 μM H_2O_2 [27], followed by a subsequent degradation of the poly(ADP-ribose) branches by poly(ADP-ribose) glycohydrolase (PARG) [32, 33]. In the present study after 5 min treatment with 100 μM H_2O_2, $CuSO_4$ significantly decreased the extent of poly(ADP-

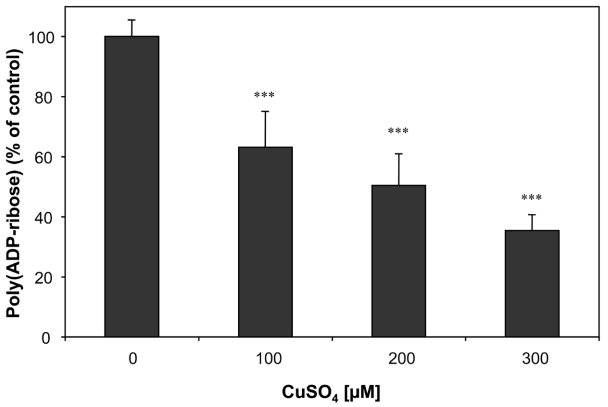

Figure 4. Effect of $CuSO_4$ on H_2O_2-induced poly(ADP-ribosyl)ation. Logarithmically growing HeLa S3 cells were preincubated with $CuSO_4$ for 24 h and treated with 100 μM H_2O_2 for 5 min in the continued presence of copper. Subsequently, cells were washed, fixed and further processed as described in Section 2. Fluorescence signals were quantified by microscopy and image analysis as described. Shown are mean values of at least four determinations based on evaluation of at least 50 cells each + SD. Statistically significant different from H_2O_2-treated cells: ***p <0.001 as determined by Student's *t*-test.

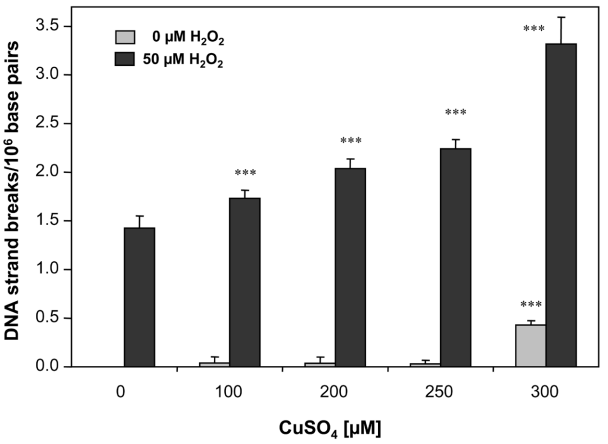

Figure 5. Dose-dependent increase of H_2O_2-induced DNA strand breaks in the presence of $CuSO_4$. Logarithmically growing HeLa S3 cells were preincubated with $CuSO_4$ for 24 h and treated with 50 μM H_2O_2 for 5 min in the continued presence of copper. DNA strand breaks were quantified by alkaline unwinding as described in Section 2. Shown are mean values of at least three determinations + SD. Statistically significant different from H_2O_2-treated cells in the absence of copper treatment (control): ***p <0.001 as determined by Student's *t*-test.

ribosyl)ation concentration-dependently at noncytotoxic concentrations, starting at 100 μM, reaching 40% residual activity at 300 μM (Fig. 4).

3.5 Effects on H_2O_2-induced DNA strand breaks

To investigate whether the observed reduction of poly-(ADP-ribosyl)ation by $CuSO_4$ was due to a lower number of

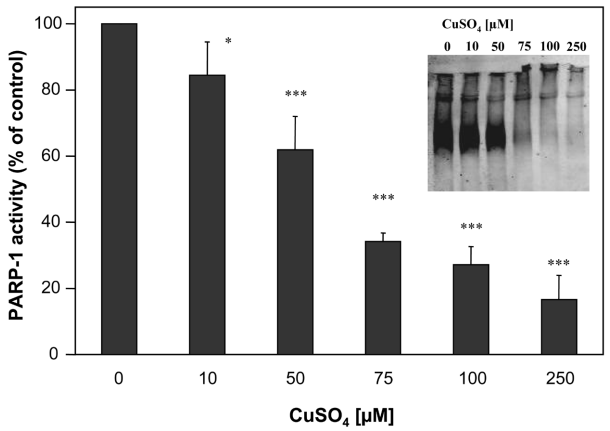

Figure 6. Effect of $CuSO_4$ on the activity of isolated PARP-1. After 10 min preincubation of PARP-1 with $CuSO_4$ at room temperature, PARP-1 reaction with a nicked plasmid as substrate was carried out for 5 min at 37°C and stopped by adding SDS/PAGE sample buffer. The mixtures were separated by SDS/PAGE, transferred to PVDF membranes, and analysed by immunoblotting with PAR antibody (10H) as described in Section 2. Shown is one representative immunoblot and mean values of at least four determinations + SD. Statistically significantly different from H_2O_2-treated cells in the absence of copper (control): ***p <0.001 as determined by Student's *t*-test.

DNA strand breaks induced after combined treatment of $CuSO_4$ and H_2O_2 as compared to H_2O_2 alone, the amounts of DNA strand breaks after incubation with $CuSO_4$, 50 μM H_2O_2 or a combination of both were measured by the alkaline unwinding technique. $CuSO_4$ alone only significantly induced strand breaks at 300 μM, as previously observed, however, in combination experiments with H_2O_2, $CuSO_4$ increased DNA strand breaks in a concentration dependent manner as compared to H_2O_2 alone (Fig. 5).

3.6 Effects on the activity of isolated PARP-1

To investigate whether the observed inhibition of cellular H_2O_2-induced poly(ADP-ribosyl)ation by copper might be due to an inhibition of PARP-1 activity, we analysed its effect on isolated recombinant PARP-1. After 10 min preincubation of isolated PARP-1, $CuSO_4$ decreased poly(ADP-ribosyl)ation concentration-dependently starting at 10 μM and reaching 80% inhibition at 250 μM (Fig. 6).

4 Discussion

The data presented in this study demonstrate that in cultured HeLa S3 cells copper induces a significant amount of oxidative DNA damage only at high cytotoxic concentrations, but interferes at lower noncytotoxic concentrations with the repair of oxidative DNA damage induced by visible light. It is generally known that damage to cells and organs can occur, if the ability to store copper is exceeded

[17] or if a sudden release of copper from metallothionein by oxidative stress is induced [34]. One of the mechanisms responsible for the observed damage is assumed to be an increase of radical formation leading to oxidative DNA damage and alterations in protein function. Thus in V79 cells copper nitrate has been reported to induce DNA strand breaks [35] and for copper sulphate an induction of DNA strand breaks was observed in rat hepatocytes [36] and leucocytes of mice [37]. In the present study, cytotoxic concentrations of copper induced mainly DNA strand breaks and only a small increase in the amount of oxidative DNA base modifications (Fpg-sensitive sites). A stronger induction of DNA strand breaks as compared to base lesions was also reported for isolated DNA by copper(II)/H_2O_2 [38]. Furthermore, another study on isolated DNA applying copper(II)/ascorbate/H_2O_2 assumed that DNA-bound copper(I) primarily mediates DNA base modifications and nonbound copper(I) primarily DNA strand breaks [39]. Oikawa *et al.* [40] demonstrated that Cu_{12}-MT caused cleavage of isolated DNA fragments, which was increased by piperidine treatment, suggesting both a breakage of the deoxyribose phosphate backbone as well as the induction of base damage and/or abasic sites. Additionally the observed accumulation of DNA strand breaks in the present study could also be linked to copper induced apoptosis [17, 41–43], especially as the strong increase in DNA strand breaks was only observed at cytotoxic concentrations.

Concerning Wilson disease animal models, in the liver of LEC rats an increased amount of DNA strand breaks and 8-oxoguanine was observed as compared to control rats [44, 45]. A recent study proposed that acute hepatitis in LEC rats impairs the expression and function of two DNA glycosylases, responsible for the repair of oxidative DNA damage [22]. This indicates that the observed higher amount of oxidative DNA damage in the liver of LEC rats could also result from repair inhibition and subsequent accumulation of endogenous oxidative DNA damage. The present study provides further evidence for this assumption; thus our data show for the first time a repair inhibition of oxidative DNA base lesions in intact cultured human cells at noncytotoxic copper concentrations.

Looking for potential molecular targets, $CuSO_4$ inhibited H_2O_2-induced poly(ADP-ribosyl)ation in HeLa S3 cells. Poly(ADP-ribosyl)ation is one of the first nuclear events following DNA strand break induction, where ADP-ribosyl residues from NAD^+ are covalently attached to proteins, and is mediated by members of the so-called 'PARP-family'. PARP-1, the founding member of this family is believed to mediate the main part of poly(ADP-ribosyl)-ation [46] and although its role is not fully understood up to now, there is strong evidence that PARP-1 contributes to BER [47–51]. The results of DNA strand break experiments exclude that the observed reduction of poly(ADP-ribosyl)-ation by $CuSO_4$ was due to a lower number of DNA strand breaks induced after combined treatment of $CuSO_4$ and H_2O_2 as compared to H_2O_2 alone. Moreover copper significantly increased the amount of H_2O_2-induced DNA strand breaks consistent with diminished DNA strand break protection by PARP-1. Further experiments demonstrated a strong inhibition of the activity of isolated PARP-1 by copper, indicating that the observed decrease in cellular poly-(ADP-ribosyl)ation might be due to changes in the activity of PARP-1. Concerning potential mechanisms, it is known that copper shows high affinity to thiols [52]. Binding of PARP-1 to DNA lesions is mediated *via* two zinc finger motifs, where zinc is coordinated by three cysteine and one histidine residue, forming a finger-like structure. Binding of $CuSO_4$ to the zinc finger domain would be expected to lead to displacement of zinc and/or oxidation of the cysteines complexing zinc in the zinc finger structure and consequently to a loss of DNA binding affinity of PARP-1 and a decrease of poly(ADP-ribosyl)ation. In this context, our group observed that the zinc finger repair proteins Fpg and XPA (Xeroderma Pigmentosum group A) could be damaged by copper. Thus, copper inhibited the activity of the isolated *Escherichia coli* glycosylase Fpg and decreased the binding ability of the isolated XPA to an UVC-irradiated oligonucleotide [53]. Interestingly, the addition of copper to a peptide representing the zinc finger domain of the human XPA resulted in the formation of a mixed Cu(I)/Zn(II) complex [52]. The tumour suppressor protein p53 also contains a zinc binding domain, which is responsible for DNA binding and its function as transcription factor. Tassabehji *et al.* [54] provided evidence that copper alters the conformation and transcriptional activity of p53 in human Hep G2 cells and just recently p53 has been assumed to be the major regulator of the expression and activity of the human 8-oxoguanin-DNA glycosylase (hOgg1) [55]. hOgg1 is responsible for the repair of the oxidative DNA base modification 8-oxoguanine. Thus, beside inhibition of PARP-1 the observed copper-induced inhibition of oxidative DNA base modifications in the present study could also result from copper-mediated inactivation of p53 and subsequently hOgg1 or also other DNA glycosylases responsible for the removal of oxidative base lesions. Additionally copper has been shown to inhibit isolated 8-oxo-GTPase [56].

Nevertheless, even not knowing the exact mechanism, the observed repair inhibition by copper in cultured human cells may also occur in tissues or organs after copper overload, *e.g.*, in the liver of Wilson disease patients or children with ICC. The hepatic copper content of healthy individuals rarely exceeds 50 μg/g dry weight (~220 μM as calculated in wet weight), a hepatic copper content of ≥250 μg/g dry weight (≥1090 μM as calculated in wet weight) is characteristic for Wilson disease patients and in heterozygotes hepatic copper concentrations are above normal, however do not exceed 250 μg/g dry weight (~1035 μM as calculated in wet weight) [57]. In children diagnosed with ICC liver copper levels ranging from 790 to 6654 μg/g dry

weight (3.5–29 mM as calculated in wet weight) were found, as compared to levels of 8–118 μg/g (35–500 μM as calculated in wet weight) in healthy controls [3]. The bioavailability studies of copper in the present study showed a dose dependent uptake and strong up to 8.5-fold accumulation in human HeLa S3 cells and pointed up the relevance of the applied concentrations. Thus, inhibition of poly(ADP-ribosyl)ation was observed after 24 h incubation with 100 μM $CuSO_4$, resulting in 500 μM intracellular copper and repair inhibition of oxidative base lesions was observed starting at 200 μM, resulting in 1.7 mM intracellular copper.

Taken together, the results indicate that copper, under conditions of either disturbed homeostasis or overload due to high exposure, can exert defined genotoxic effects. The carcinogenicity of copper has not been adequately studied. The EPA has classified copper in group D, not classifiable as to human carcinogenicity and the IARC has classified the pesticide copper-8-hydroxyquinoline in group 3, unclassifiable as to carcinogenicity in humans [3]. Concurrently patients with Wilson disease appear to be vulnerable to the formation of aggressive malignant intra-abdominal tumours, including hepatomas [58]. Interestingly, copper overload may also be relevant for healthy humans. Thus, two recent studies provided evidence that, in healthy humans after a high intake of copper for several months the homeostatic mechanisms controlling copper retention seem not to be sufficient to prevent accumulation of copper. Long-term high copper intake resulted in changes in several functional markers, suggesting that the high amount of dietary copper may adversely affect immune function and antioxidant defense [59, 60]. Hence, with respect to dietary copper intake or supplementation, a balance needs to be maintained to prevent overload on the one hand and to ensure sufficient uptake for essential copper functions on the other hand.

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