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A Newly Adapted Pulsed-field Gel Electrophoresis Technique Allows to Detect Distinct Types of DNA Damage at Low Frequencies in Human Dermal Fibroblasts upon Exposure to Non-toxic H_2O_2 Concentrations

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Reactive oxygen species (ROS) comprise several oxygen containing compounds, among them hydrogen peroxide (H_2O_2), which are generated by internal and external sources and play pleiotropic roles in physiological and pathological states. Skin cells as well as cells from other tissues have developed antioxidant defense mechanisms to protect themselves from high concentrations of ROS. Although biological and pathological roles of ROS have previously been elucidated, so far only limited knowledge exists regarding ROS-mediated generation of DNA breaks and base lesions occurring at low frequency in intact skin cells. This study was therefore designed to probe a newly adapted pulsed-field gel electrophoresis technique for the adequate measurement of high molecular weight DNA fragments as well as to investigate the protective role of the antioxidant enzyme catalase against H_2O_2-mediated damage in human dermal fibroblasts. We stably transfected and overexpressed the full-length catalase cDNA in the human dermal fibroblast cell line 1306 in culture and found that these cells are significantly more protected from cytotoxicity, overall DNA strand breaks, and 8-oxodeoxyguanine base lesions resulting from H_2O_2-triggered oxidative stress compared to vector-transfected 1306 cells or secondary dermal fibroblasts. This work has outlined the importance of catalase in the protection from H_2O_2-mediated cytotoxicity and DNA damage which – if unbalanced – even when occurring at low frequency are known to lead to genomic instability, a hallmark in carcinogenesis and premature aging.

Keywords: Hydrogen peroxide, oxidative stress, DNA damage, catalase, overexpression, fibroblast

INTRODUCTION

Reactive oxygen species (ROS) are generated from molecular oxygen including the free radicals superoxide anion ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$) and nitric oxide ($NO^{\bullet}$), as well as non-radical intermediates such as hydrogen peroxide (H_2O_2),

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lipid peroxides (LOOH), and singlet oxygen ($^1O_2$).[1,2] ROS play multiple roles in physiological and pathological states.[2–4]

Hydrogen peroxide (H_2O_2) arises intracellularly directly by a variety of endogenous oxidases, such as xanthine oxidase,[5,6] and indirectly by spontaneous or catalyzed dismutation of the superoxide anion ($O_2^{\bullet-}$) during mitochondrial respiration.[7] Exogenous sources for the production of cellular H_2O_2, especially in the skin, are ultraviolet UVA[8] and UVB[9] irradiation. However, the observation that a wide variety of normal and malignant cell types can intracellularly generate and release H_2O_2 at low rates led to the hypothesis that H_2O_2 may function as a crucial second messenger.[10–13]

If produced at higher concentrations, H_2O_2, however, represents a highly dangerous molecule being causally involved in a variety of pathologies.[14,15] Its small size and lack of charge allow it to diffuse considerable distances, to easily cross cellular membranes, and to reach the nucleus. Subsequently, hydrogen peroxide can react with transition metal cations such as iron (Fe^{2+}) or copper (Cu^{1+}) resulting in the generation of the highly toxic and aggressive hydroxyl radical ($HO^{\bullet}$). Since these metal cations are bound to DNA, the hydroxyl radical will be generated adjacent to and preferentially reacts with these critical target[4] in a so-called site-specific Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + [H_2O_2^-] \rightarrow HO^{\bullet} + HO^-$)[16] to produce DNA single-strand breaks (SSBs),[17] double-strand breaks (DSBs)[18,19] and oxidized bases.[20–22] Both the formation of DNA breaks and oxidized bases arise *in vivo* and *in vitro* upon oxidative stress and analysis of the extent of base modifications has been widely used to investigate DNA damage.[19] Recently, 8-oxodeoxyguanine (8-oxodG) became accepted as a sensitive marker for oxidative damage upon $H_2O_2/HO^{\bullet}$ exposure.[20,23]

The antioxidant defense system against H_2O_2 in mammalian cells comprises two enzymes, the selenium-dependent glutathione peroxidase (GSHPx, EC 1.11.1.9) and the heme group containing catalase (EC 1.11.1.6) which, at the subcellular level, is mainly found in peroxisomes (80%) and cytosol (20%).[24] Both enzymes metabolize H_2O_2 in a catalytic reaction producing water and oxygen (O_2).[24,25]

However, only limited knowledge exists regarding (i) ROS-mediated generation of overall DNA breaks (SSBs and DSBs) and base lesions occurring at low frequencies in skin fibroblasts upon *non*-toxic H_2O_2 concentrations, and (ii) the potentially protective role of catalase in these types of DNA damage. We stably transfected and overexpressed the human catalase cDNA in the human dermal fibroblast cell line 1306 (LipoCat). Oxidative stress-mediated DNA damage of these cells was compared with the DNA damage profile of only vector-transfected cells (LipoV), non-transfected 1306 cells, and secondary human dermal fibroblasts (HDF) using a newly adapted pulsed-field gel electrophoresis (PFGE) technology. As compared to the vector-transfected and non-transfected fibroblasts, the catalase overexpressing cells showed a significant protection from cytotoxicity, DNA strand breaks, and an increase in DNA fragment sizes reflecting the reduction in 8-oxodG base lesions, the preferential cleavage site of the repair enzyme formamidopyrimidine–DNA glycosylase (Fpg protein).[26] By means of a herein adapted PFGE, we were able to detect DNA damage at low frequency and, furthermore, provide several lines of evidence for the importance of the antioxidant enzyme catalase in the protection from oxidative stress-mediated DNA damage triggered by non-toxic H_2O_2 concentrations in human dermal fibroblasts.

MATERIALS AND METHODS

Cell Culture

Primary HDF cultures were established by outgrowth from skin biopsies of healthy human donors at age between 3 and 10 years as described

earlier.[27] Cells were used for the experiments at cumulative population doublings (CPD) 12 to 30, now called secondary fibroblasts (HDF).[28] The human skin fibroblast cell line 1306[29] was obtained from ECACC, Salisbury, UK (ECACC No: 90011887). Both cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCOBRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), glutamine (2 mM), penicillin (400 U/ml) and streptomycin (50 mg/ml) at 37°C in a humidified atmosphere at 5% CO_2 and 95% air. Secondary dermal fibroblasts were passaged once a week and seeded at a density of 7×10^5 cells per 55-cm^2 tissue culture dish, 1306 cells were passaged at a 1 : 3 dilution every three days. Recombinant cells were selected in neomycin (G418) (GIBCOBRL) at 150 μg/ml, a concentration which completely inhibited the growth of non-transfected cells. For some experiments the catalase-transfected cells were pretreated for 4 h with 100 or 200 μM of the specific catalase inhibitor aminotriazole (ATZ) (Sigma, Deisenhofen, Germany).[11]

Expression Vector for Human Catalase cDNA and Cell Transfection

The 1.578 kb human catalase cDNA fragment flanked by the Pst I restriction site was blunt-end ligated into the EcoR V site of the expression vector pcDNA3 (Invitrogen, San Diego, USA). The expression vector contains the cytomegalovirus (CMV) major intermediate early promotor/enhancer region and a neomycin selection marker which is driven by the Simian Virus 40 (SV40) promotor. Subconfluent 1306 fibroblast cultures (~80% confluence) were transfected with the pcDNA3 vector 2 μg/ml serum-free medium) containing or not containing the catalase cDNA by using polycationic liposomes (LIPOFECTAMINE, GIBCOBRL).[30] For selection of stable transfectants G418 was added to the cells 24 h after transfection. Individual neomycin-resistant cell clones were screened by measurement of the catalase activity.

Assay for Determination of the Activity of the Antioxidant Enzyme Catalase

The assay was performed with cells in a logarithmic growth phase. The decomposition of hydrogen peroxide could be followed directly by the decrease in absorbance at 240 nm (A_{240} = 39.4 $mol^{-1} \times liter \times cm^{-1}$) within 2 min in a 1 ml reaction volume which contained up to 200 μl of the cell lysate in 50 mM phosphate buffer (pH 7.0) and 100 μl of 50 mM H_2O_2 in the same buffer. The difference in absorbance (A_{240}) per unit time is a measure of the catalase activity.[31]

Cytotoxicity (MTT) Assay, Clonal Growth Assay and Viability

The viability of the transfectants, non-transfected 1306 cells, and secondary dermal fibroblasts was measured 24 h after incubation with H_2O_2 for 1 h in DMEM plus 10% FCS,[32] with aphidicolin, a tetracyclic diterpene antibiotic and specific inhibitor of DNA polymerase alpha, delta and epsilon involved in excision repair[33,34] for 2 h, and with the mild detergent saponin, which reversibly can permeabilize cell membranes,[35] for 10 min on ice. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) was used for the quantification of living metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to purple formazan dye.[36] Cytotoxicity was calculated as percent of formazan formation in cells treated with the agents compared to mock-treated cells. Furthermore, long-term viability was tested by means of the clonal growth assay as previously described[37] with minor modifications. Briefly, mock-treated or H_2O_2-treated cells were trypsinized and seeded at a clonal density of 1.5×10^3 cells in DMEM plus 20% FCS on tissue culture dishes 10 cm in diameter.[25] Fourteen days

thereafter, the cells were fixed, stained with 0.05% Coomassie G 250 in 7.5% acetic acid/20% methanol, and photographed.

Trypan Blue Exclusion Test and Cell Membrane Permeability

The trypan blue test was performed as described[38] with minor modifications. Briefly, 500 µl of 0.4% trypan blue and 500 µl phosphate-buffered saline (PBS) were added to a ∅3.5-cm tissue culture dish containing cells with about 70% confluence which prior to the trypan blue test were incubated for 10 min on ice with non-toxic concentrations of saponin in Hepes-buffered saline (HBS; 100 mM KCl, 20 mM NaCl, 1 mM EGTA, 30 mM Hepes, pH 7.0) to get the cells permeable. The cell permeability was investigated by light microscopy using a magnification of 100×. Cells with permeable membranes remained blue, while white cells had intact membranes.

H_2O_2 Treatment and Pulsed-field Gel Electrophoresis

Subconfluent transfectants and non-transfected fibroblasts were treated with 10 µM aphidicolin for 15–20 min at 37°C in DMEM plus 10% FCS to prevent base excision repair[34] which begins already within minutes after damage. Thereafter, H_2O_2 concentrations of up to 1 mM were added to the complete medium for 1 h at 37°C in a 5% CO_2 humidified atmosphere to prevent unspecific cellular and DNA injury.[32] This experimental approach allowed to specifically produce and detect H_2O_2 mediated oxidative stress resulting in DNA damage. The medium was removed and the cells were either subjected to PFGE or incubated for 10 min on ice with different non-toxic concentrations of saponin in HBS to get the cells permeable. Thereafter, the cells were washed in ice-cold Fpg (Fapy glycosylase) working buffer (25 mM Tris-HCl, 2 mM Na_2EDTA, 100 mM KCl, pH 7.4–8.0) plus aphidicolin and incubated for 2 h at 37°C with 150 and 300 units (U) Fpg protein per ml buffer containing aphidicolin (specific activity 1×10^5 units per mg Fpg (kindly provided by S. Boiteux, Fontenay aux Roses, France).

The cells were harvested in PBS/10 µM aphidicolin by a plastic scraper, centrifuged (2000 rpm, 5 min, 4°C), resuspended in 0.9% low melting agarose (InCert, Biozym, Oldendorf, Germany) in PBS/10 µM aphidicolin pipetted into a plug mould (80 µl/well, 5×10^5 cells), and allowed to solidify at 4°C for 10 min. The plugs were pushed out and transferred into 50°C lysis solution for 48 h containing 0.5 M Na_2EDTA (pH 8.0), 1% sarkosyl and 1 mg/ml proteinase K, and with a buffer change after 24 h. Thereafter, the plugs were washed twice for 1–2 h each in 10× volume of 1× TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.8) containing 1 mM of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) at room temperature. After the PMSF treatment the plugs were washed three times for 2 h each in 10× volume of TE buffer without PMSF at room temperature. For restriction endonuclease digestion the agarose plugs (inserts) containing the lysed cells were incubated in a microcentrifuge tube overnight at 37°C in 300 µl enzyme buffer supplied by the manufacturer (AGS, Heidelberg, Germany), and 30 U of the rare-cutting Not I enzyme (AGS).[39,40] The digestion was stopped by the addition of 30 µl 0.5 M Na_2EDTA (pH 8.0) for 30 min at 4°C. For the detection of DNA SSB and alkali-labile sites, the double-stranded DNA in the agarose plugs was then denaturated in 10× volume of 20 mM NaOH, 2 mM Na_2EDTA (pH ≈ 12.0) for 90 min on ice in the dark, neutralized and subjected to neutral PFGE.[41]

We purposely run gels under neutral conditions. Under complete alkaline conditions and at the voltages used here, the resulting dramatic increase in current and in ohmic heating artificially damaged DNA during the run and, furthermore, destroyed the electrodes. (Brenneisen, unpublished results). These observations are in agreement with earlier findings that using conventional alkaline agarose gels the voltages are

recommended not to be over 0.25 V/cm[42] which, however, is not feasible for the optimal separation of the high molecular weight DNA fragments by means of PFGE.

The electrophoresis was performed with the ROTAPHOR apparatus (Biometra, Göttingen, Germany). The plug pieces were inserted into 0.9% gels made from Qualex PFGE agarose (AGS) in 0.25× TBE buffer (1× TBE is 89 mM Tris, 89 mM boric acid, and 2 mM Na_2EDTA, pH 8.0). The samples run in 0.25× TBE under standardized conditions (running time: 72 h; pulse time: 360–60 s, logarithmic ramping; reorientation angle: 110–100, linear ramping; voltage (V): 6.0–2.5 V/cm, linear ramping; temperature: 12°C) which were modified dependent on the experimental setting. Significant differences from the standard conditions are indicated in figure legends. Following electrophoresis the gels were stained in 10 µg/ml ethidium bromide containing 0.25× TBE buffer for 1 h, then destained in TBE for about 3 h to remove excess ethidium bromide. The following DNA size markers were used: Chromosomal DNA from yeast *Schizosaccharomyces pombe*, from *Saccharomyces cerevisiae YPH 149*, from *Hansenula wingei*, and DNA from phage *Lambda* (Hind III digested).

Data Evaluation and Statistics

The ethidium bromide-stained gels were excited at 312 nm with an UV transilluminator resulting in a fluorescence emission. The fluorescence signals were detected by a high-resolution CCD camera system (756 × 581 pixel with a pixel distance of 11.0 × 11.0 µm) (Biometra). The information from the gel pictures was then digitized (256 Gy values were attached to the different fluorescence intensities), transferred to a personal computer, and processed by means of a modified software (ScanPack 3.0. Biometra) which allows for correction of the camera's shading effect and background fluorescence. In order to measure DNA damage, the number average molecular length of a DNA sample (L_n) was used as an index for the number of DNA (single-strand) breaks and fragment sizes of the DNA, respectively.[40,43,44] The L_n value does not represent the molecular length of DNA at which the fluorescence shows the maximum, but rather represents median lengths, at least under circumstances that the distribution of DNA fragment sizes is sufficiently random.[43] A decrease in the L_n value would indicate an increase in the frequency of the studied strand breaks. The determination of the L_n needed knowledge about the size distribution of DNA. The size distributions of the DNA entering the gel were obtained from standard curves generated by measurements of the least-squares relation between mobility in neutral gels and molecular length of the PFGE size markers.[43,45] Additionally, the data were subjected to statistical calculations such as runs test,[46] Student's t-test,[47] and one-way analysis of variance (ANOVA).[48]

RESULTS

Generation of 1306 Fibroblasts Expressing Elevated Levels of Human Catalase

The HDF cell line 1306 was stably transfected with the eukaryotic expression vector pcDNA3 containing the full length cDNA of human catalase and a neomycin (G418) resistance cassette. Several G418 resistant cell clones were isolated with a catalase activity ranging from 3.3 ± 0.4 to 64.0 ± 4.0 U/mg total protein. Thus, the catalase activity of these clones was 2.5- to 49-fold increased compared to that of the non-transfected wild-type 1306 cells and 1306 cells transfected with the expression vector alone. Furthermore, the catalase activity in catalase overexpressing cells was 1.4- to 27-fold increased compared to secondary HDF (Figure 1). The catalase clone (LipoCat) with a 49-fold increase in catalase activity and the vector-containing clone (LipoV) with a catalase activity of 1.3 ± 0.1 U/mg total protein were used for subsequent experiments (Figure 1).

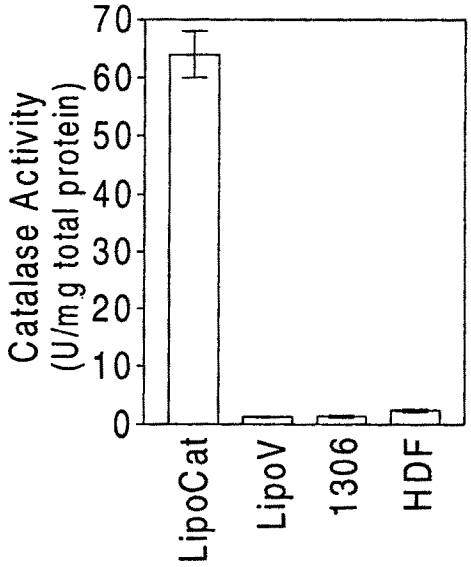

FIGURE 1 Catalase activity of transfected and non-transfected dermal fibroblasts. Fibroblasts of the cell line 1306 were stably transfected with the expression vector pcDNA3 containing full length catalase cDNA (LipoCat), the vector alone (LipoV) or were non-transfected (1306, HDF). The activity of the antioxidant enzyme catalase was measured as an indication for the expression of the enzyme. The mean ± SD, represents four independent measurements of activity. HDF, human dermal fibroblasts; SD, standard deviation.

Protection of Catalase Overexpressing Cells from H_2O_2-mediated but Not from Aphidicolin Cytotoxicity

The MTT assay was used to establish the conditions for the protection of catalase-transfected fibroblasts against H_2O_2-mediated oxidative cell injury. Furthermore, the assay was applied to determine the highest concentrations at which 75% of the four experimental groups of mitotic fibroblasts used in the subsequent experiments survived at least 24 h after incubation with the oxidant H_2O_2 and the repair inhibitor aphidicolin, repectively, with no change in morphology (Figure 2A and C).

The H_2O_2-treated and catalase overexpressing cells (LipoCat) did not show a reduced viability between 0.1 and 3.0 mM H_2O_2 compared to viability of the mock-treated cells which was set at 100%. A concentration of 5 mM H_2O_2 reduced viability of the LipoCat cells to 75%. In contrast, the vector-transfected (LipoV) and

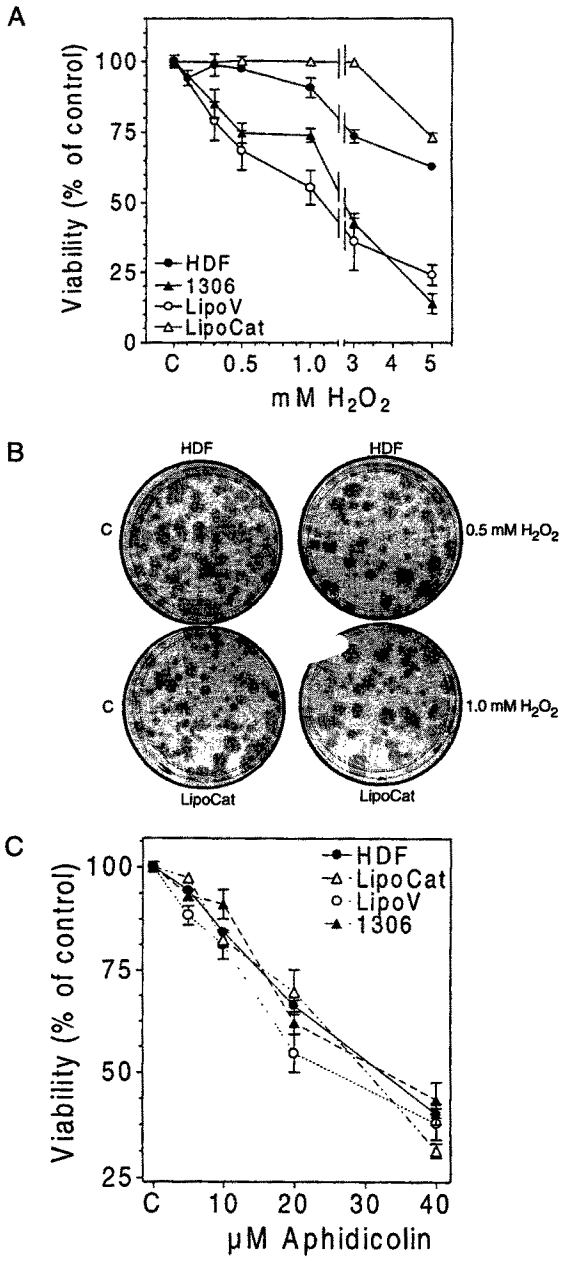

FIGURE 2 Cytotoxicity and clonogenicity upon treatment of cells with the oxidant H_2O_2 and DNA repair inhibitor aphidicolin. Confluent fibroblasts were incubated at the indicated H_2O_2 concentrations for 1 h (A) and with aphidicolin for 2 h (C) at 37°C, respectively. The percentage of living cells was measured 24 h after treatment. The experiments were performed in three independent experiments. To test long-term viability mock-treated or H_2O_2-treated cells were seeded at clonal density in DMEM containing 20% FCS (B). Fourteen days thereafter, the cells were fixed, stained and photographed. C, mock-treated controls; HDF, human dermal fibroblasts; LipoCat, catalase overexpressing 1306 fibroblasts.

non-transfected 1306 cells already showed a reduction in viability to 93% at a concentrations of 0.1 mM. Viability of LipoV cells was reduced to $69 \pm 7\%$ at a concentration of 0.5 mM H_2O_2, to $36 \pm 10\%$ at 3 mM H_2O_2, and to $24 \pm 4\%$ at 5 mM H_2O_2. At the same concentrations non-transfected 1306 fibroblasts showed a reduced viability to $75 \pm 3\%$, $43 \pm 25\%$, and $14 \pm 3\%$. Interestingly, HDF were more protected from H_2O_2-mediated cytotoxicity at all H_2O_2 concentrations studied compared to LipoV and non-transfected 1306 fibroblasts. However, HDF were significantly more sensitive against H_2O_2 than catalase overexpressing cells (LipoCat) (Figure 2A).

Furthermore, the clonal growth assay was performed for HDF and catalase overexpressing cells (LipoCat) to determine long-term effects concerning cytotoxicity and inhibition of autonomous proliferation by H_2O_2 (Figure 2B). Two weeks after H_2O_2 treatment, no significant loss of clonogenic potential was observed for both cells at concentrations of 0.5 (HDF) and 1.0 mM (LipoCat), respectively, compared to mock-treated controls (C) (Figure 2B).

In conclusion, to minimize cytotoxic effects a *non*-toxic though oxidative stress-inducing H_2O_2 concentration of 0.5 mM was used for subsequent experiments to minimize cytotoxic effects. At the H_2O_2 concentration of 0.5 mM applied for 1 h in DMEM containing 10% FCS at 37°C, cells of all four experimental groups showed a viability $\geq 75\%$ as tested in the clonal growth and/or MTT assay. These results are on perfect agreement to earlier findings.[32]

The cytotoxicity of aphidicolin was evaluated and shown in Figure 2C. The highest non-toxic concentration of aphidicolin was found to be 10 μM. All tested fibroblast cultures revealed a viability of $> 75\%$ at this concentration. Similar results were obtained for LipoCat, LipoV, non-transfected 1306, and HDF cultures. Incubation of the four experimental groups of fibroblasts for 1 h with a combination of both 0.5 mM H_2O_2 and 10 μM aphidicolin resulted in a viability of $93 \pm 3\%$ for catalase overexpressing cells (LipoCat), of $90 \pm 4\%$ for secondary HDF, of $77 \pm 4\%$ for vector-transfected cells (LipoV), and of $79 \pm 6\%$ for non-transfected 1306 fibroblasts.

Linearity of Increasing H_2O_2 Concentrations and Increase in DNA Damage of Secondary Dermal Fibroblasts

In order to determine the linearity of DNA damage[49] upon increasing H_2O_2 concentrations as a measure for the suitability of the PFGE technique for subsequent studies on oxidative stress, secondary HDF were incubated with increasing concentrations of H_2O_2. Figure 3A shows a representative fluorescence-stained neutral gel of these experiments. It could be demonstrated that DNA damage significantly increased upon increasing H_2O_2 concentrations ranging from 0.1 to 1.0 mM (lanes 6–9) compared to mock-treated controls (lane 5). This was confirmed by the number average molecular length (L_n) values of the DNA ranging from 4335 ± 255 kb for the control to 500 ± 70 kb for cells treated with 1 mM H_2O_2 (Figure 3B). A mathematical approach for the analysis of a linear relationship between H_2O_2 concentrations and DNA damage demonstrated a linearity between L_n values and concentrations up to 0.5 mM H_2O_2 reflecting the high regression coefficient (r^2) of 0.88. Furthermore, the runs test revealed a P value of 0.67. In case the P values of the runs test are small (< 0.5) it would argue in favor for the view that the data do not follow the model of linearity. In fact, this was the case, when linearity was tested up to 1 mM H_2O_2 (data not shown) suggesting that at a concentration of 1 mM H_2O_2 the DNA damage has reached saturation (Figure 3B, inset).

Furthermore, the evidence of linearity over a defined concentration range implied that the pulsed-field electrophoresis technique is a valuable tool for subsequent experiments.

Protection of Catalase Overexpressing Fibroblasts from H_2O_2-triggered Strand Breakage Compared to Control Fibroblasts

Transfected (LipoCat, LipoV) and non-transfected (1306, HDF) fibroblasts were treated with

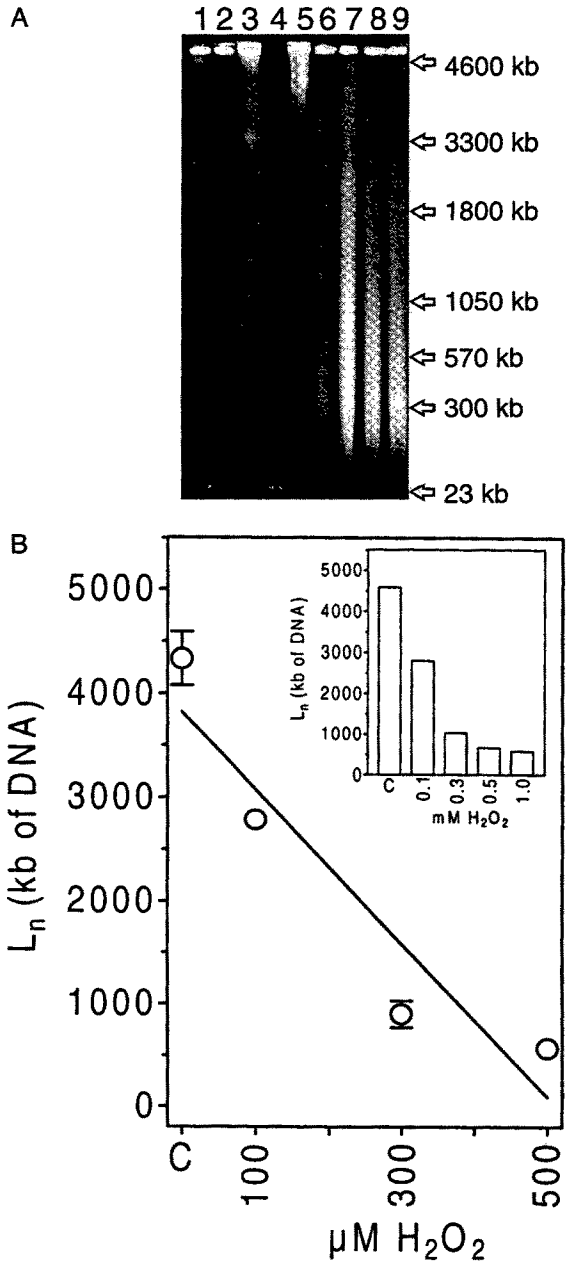

FIGURE 3 Linear correlation between DNA breakages and H_2O_2 concentrations. Confluent HDF were treated with different *non-toxic* concentrations of H_2O_2 for 1 h at 37°C. After lysis of the cells, Not I digestion, and subsequent alkali treatment of agarose plugs, samples were subjected to neutral PFGE. (A) Representative ethidium bromide-stained gel reveals H_2O_2 concentration-dependent overall DNA breaks. Lanes 6 to 9 represent H_2O_2 concentrations of 100, 300, 500 µM, and 1 mM. Lanes 1 to 4 represent DNA markers and lane 5 displays the mock-treated control. The gel run was performed for 48 h at a pulse time of 240–40 s and at 8.5–7.5 V/cm. (B) Densitometric evaluation of the number average molecular length (L_n) and linear least-squares analysis of L_n values with a regression coefficient (r^2) of 0.88 from gels as shown in (A). The runs test indicated a P value = 0.67. The bar chart (inset (B)) indicates L_n values of the gel shown in (A). Four independent experiments were performed.

0.5 mM H_2O_2 to evaluate the number average molecular length (L_n) of the DNA as an index for H_2O_2-dependent DNA breaks and size of the resulting DNA fragment in the high molecular weight range (> 100 kb). Figure 4A shows a representative fluorescence-stained gel of these experiments. No significant differences in the fragment sizes of mock-treated (lane 5) and H_2O_2-treated (lane 6) catalase overexpressing LipoCat cells were observed. These results were confirmed by the L_n values of 655 ± 50 kb of DNA and 580 ± 64 kb, respectively, and a P value of 0.185 (Student's t-test) (Figure 4B). However, catalase-overexpressing cells (LipoCat) pretreated with the catalase inhibitor ATZ (Figure 4A, lane 7) resulted in a significant decrease in the fragment sizes reflected in a L_n value of 380 ± 28 kb and $P = 0.0011$ (t-test) compared to the untreated LipoCat cells (Figure 4B). These findings indicate an increase in the frequencies of DNA strand breaks. Furthermore, mock-treated vector-transfected cells (LipoV) (Figure 4A, lane 8), non-transfected 1306 lane 10), and secondary HDF (lane 12) did not show differences in L_n values compared to H_2O_2-treated LipoCat fibroblasts (P value = 0.513, ANOVA) (Figure 4B), whereas the L_n values of the mock-treated compared to the H_2O_2-treated fibroblasts among the individual experimental groups revealed significant differences. This was also true for the L_n (Figure 4B) of ATZ/H_2O_2-treated LipoCat fibroblasts (Figure 4A, lane 7), H_2O_2-treated LipoV cells (lane 9), H_2O_2-treated non-transfected 1306 cells (lane 11), and H_2O_2-treated HDF (lane 13) compared to that values of the treated LipoCat fibroblasts (lane 6) ($P = 0.0045$, ANOVA).

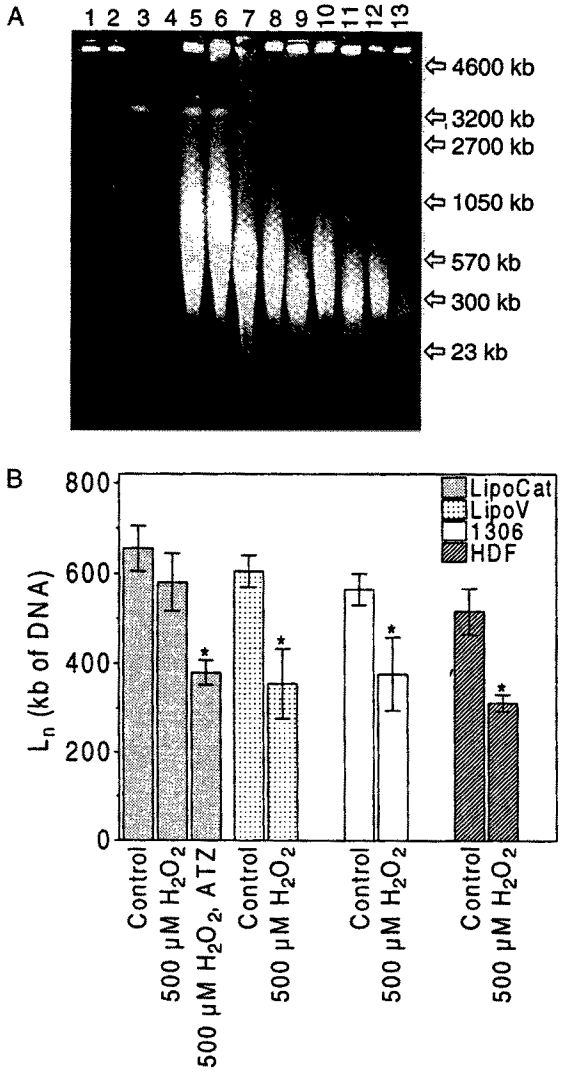

FIGURE 4 Overexpression of catalase and its protective effect against strand breaks. Confluent monolayer cultures of transfected (LipoCat LipoV) and non-transfected 1306 cells and HDF were treated with 500 µM H_2O_2 for 1 h at 37°C. After cell lysis, Not I digestion, and alkali treatment, the samples were subjected to neutral PFGE. (A) Representative ethidium bromide-stained gel showed significant differences in the DNA fragment sizes of the four different experimental groups. Lanes 1 to 4 represent DNA markers, lanes 5, 8, 10 and 12 show DNA samples of mock-treated catalase overexpressing (LipoCat), vector-transfected (LipoV), wild-type 1306 fibroblasts, and secondary HDF, lanes 6, 9, 11 and 13 represent DNA samples of H_2O_2-treated LipoCat, LipoV, 1306, and HDF cells, and lane 7 represents LipoCat cells which prior to exposure to H_2O_2 were treated with 200 µM aminotriazole for 4 h. (B) Densitometric evaluation of the number average molecular length (L_n) from gels as shown in (A). The ANOVA indicated a $^*P = 0.0045$ compared to the H_2O_2-treated LipoCat fibroblasts. The experiments were performed in triplicate.

Reversible Permeabilization of Transfected and Non-transfected Fibroblasts by Saponin

The reversible permeabilization of cells definitely is an important prerequisite for the introduction of enzymes detecting oxidative damage into cells, as we intended for the Fpg enzyme. The MTT assay was applied to determine the highest concentration at which 75% of cells of the four experimental groups survived at least 24 h after incubation following treatment with the detergent saponin. Also, morphology of cells were monitored (Figure 5A). Cells treated at these saponin concentrations were introduced into the trypan blue exclusion tests to determine the percentage of permeable cells and to study the reversibility of this permeability (Figure 5B).

Twenty-four hours after saponin treatment at different concentrations for 10 min on ice, all tested transfected and non-transfected fibroblasts displayed a viability $\geq 75\%$ at a concentration of 0.005% saponin, while a concentration of 0.015% already reduced viability below 65% (Figure 5A).

The trypan blue exclusion test applied at a concentration of 0.005% saponin clearly demonstrated that directly after the treatment between 70% and 90% of the cells were permeable. Interestingly, 24 h after treatment the percentage of permeable cells was similar to that of the mock-treated controls (Figure 5B). Accordingly, in subsequent experiments a concentration of 0.005% saponin was used.

Protection of Catalase Overexpressing Fibroblasts from H_2O_2-mediated 8-Oxodeoxyguanine Base Lesion

Apart from SSBs and DSBs, the 8-oxodG base lesion is a sensitive marker for oxidative damage upon $H_2O_2/HO^\bullet$ exposure. Catalase overexpressing 1306 fibroblasts (LipoCat) and secondary HDF were treated with 0.5 mM H_2O_2 to evaluate the occurrence of 8-oxodG base lesions

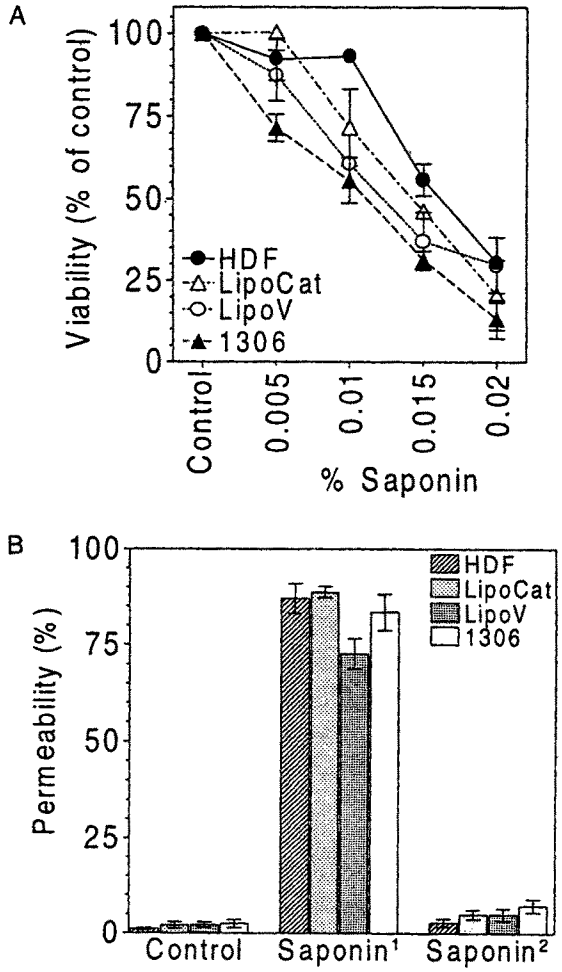

FIGURE 5 Cytotoxicity and membrane permeability of fibroblasts of the four experimental groups after saponin treatment. (A) Confluent dermal fibroblast monolayer cultures of transfected 1306 fibroblasts (LipoCat, LipoV), non-transfected 1306 cells, and secondary HDF were incubated with increasing concentrations of the detergent saponin on ice for 10 min. The percentage of living cells was measured 24 h after treatment. (B) Furthermore, subconfluent cell cultures were treated with a non-toxic concentration of 0.005% saponin, as shown in (A), and the percentage of permeable cells was measured directly after treatment (saponin[1]) and 24 h after treatment (saponin[2]). The experiments were performed in four independent experiments.

based on the analysis of changes in the number average molecular length (L_n) of the DNA. Figure 6A shows a representative fluorescence-stained gel of these experiments. LipoCat cells (lanes 7, 10 and 13) which have been treated with

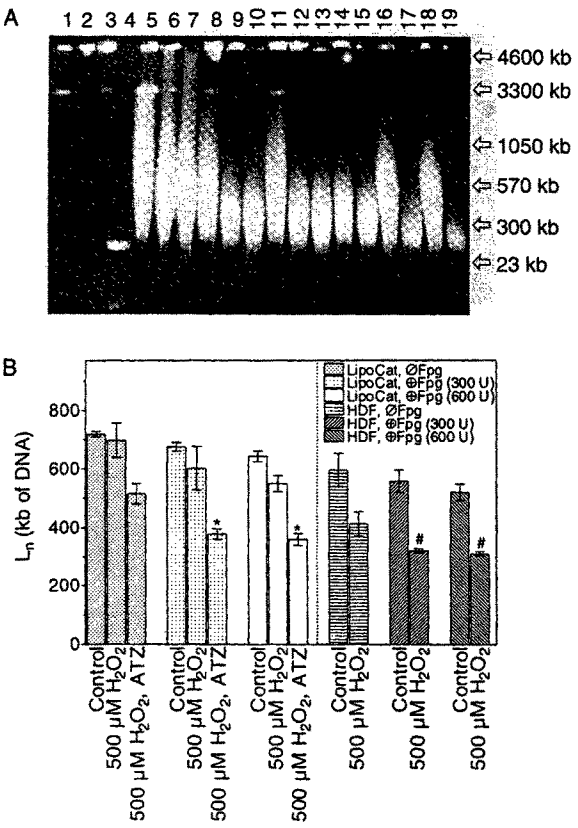

FIGURE 6 Overexpression of catalase and its protective effect against H_2O_2-dependent 8-oxodG formation. Confluent monolayer cultures of transfected LipoCat cells and secondary HDF were treated with 500 µM H_2O_2 at 37°C for 1 h and permeabilized with saponin. Thereafter cells were treated with different concentrations of Fpg enzyme prior to cell lysis in agarose plugs, Not I digested, and alkali-treated. DNA samples were then subjected to neutral PFGE. (A) Representative ethidium bromide-stained gel displayed significant differences in the size of the single-stranded DNA fragments. Lanes 1 to 4 represent DNA markers, lanes 5 to 13 reveal DNA samples of LipoCat cells, and lanes 14 to 19 DNA samples of HDF which were untreated (Ø) or treated (⊕). The fibroblasts were treated as follows: lane 5 (Ø), lane 6 (⊕ 500 µM H_2O_2), lane 7 (⊕ 100 µM ATZ, ⊕ H_2O_2), lane 8 (⊕ 300 U Fpg), lane 9 (⊕ H_2O_2, ⊕ Fpg), lane 10 (⊕ ATZ, ⊕ H_2O_2, ⊕ Fpg), lane 11 (⊕ 600 U Fpg), lane 12 (⊕ H_2O_2, ⊕ Fpg), lane 13 (⊕ ATZ, ⊕ H_2O_2, ⊕ Fpg), lane 14 (Ø), lane 15 (⊕ 500 µM H_2O_2), lane 16 (⊕ 300 U Fpg), lane 17 (⊕ H_2O_2, ⊕ Fpg), lane 18 (⊕ 600 U Fpg), lane 19 (⊕ H_2O_2, ⊕ Fpg). (B) Densitometric evaluation of the number average molecular length (L_n) from gels as shown in (A). The analysis of variance (ANOVA) indicated $^*P = 0.017$ and $^\#P = 0.043$ compared to ⊕ ATZ/⊕ H_2O_2-treated LipoCat cells and ⊕ H_2O_2-treated HDF, respectively. The mean ± SD values resulted from two independent experiments.

a combination of H_2O_2 and ATZ, a specific inhibitor of catalase,[11] revealed a significant decrease in the mean ± SD of the L_n values compared to only H_2O_2-treated (lanes 6, 9 and 12) and mock-treated fibroblasts (lanes 5, 8 and 11) (Figure 6B). However, the ATZ- and H_2O_2-treated LipoCat cells, which were treated with 300 U (Figure 6A, lane 10) or 600 U (lane 13) of the repair enzyme Fpg showed distinctly reduced L_n values (Figure 6B) of 378 ± 18 and 360 ± 21 kb of DNA, respectively, as compared to the L_n of 515 ± 35 kb of LipoCat cells not treated with Fpg (Figure 6A, lane 7) (P value = 0.017, ANOVA). Treatment of ATZ- plus H_2O_2-exposed catalase overexpressing cells (LipoCat) with 300 or 600 U Fpg did not result in any significant difference in Fpg enzyme-introduced strand breaks ($P = 0.45$, two-sided t-test).

Furthermore, H_2O_2-treated HDF (Figure 6A, lanes 15, 17 and 19) revealed a significant decrease in the mean ± SD of the L_n values (Figure 6B) compared to mock-treated fibroblasts (Figure 6A, lanes 14, 16 and 18). However, the H_2O_2-treated HDF which were treated either with 300 U (lane 17) or 600 U (lane 19) of Fpg showed reduced L_n values of 321 ± 8 and 310 ± 8 kb of DNA, respectively, compared to the L_n of 413 ± 41 kb of HDF not treated with Fpg (lane 15) (P value = 0.043, ANOVA). Comparison of H_2O_2-treated HDF either treated with 300 or 600 U Fpg did not result in a significant L_n difference ($P = 0.303$, two-sided t-test). The differences in the L_n values of ATZ-treated LipoCat cells (Figure 6) in comparison with the results of Figure 4 may be due to the different ATZ concentrations.

Taken together, these results suggest that apart from the occurrence of H_2O_2-mediated strand breaks, specific base lesions such as 8-oxodG were generated at relatively low frequencies as reflected by high L_n values and were reliably detected by means of the herein newly adapted technique of PFGE. The sensitivity to detect this specific type of DNA damage by this technique was determined to be in the range of five megabase pairs (Mb) of genomic DNA.

DISCUSSION

Intracellular accumulation of ROS to concentrations at which the cellular antioxidant system is overwhelmed leads to oxidative stress.[1] An interesting strategy for protection against oxidative stress is the support of the endogenous antioxidant system. In this study we have used a newly adapted technique of PFGE to study the protective effect of catalase on H_2O_2-triggered DNA damage. By means of this technique we were able to demonstrate that stable transfection and overexpression of human catalase protected the HDF cell line 1306 from oxidative stress-mediated cell killing and, furthermore, significantly reduced the number of overall DNA strand breaks (SSB, DSB) and 8-oxodG base lesions, distinct sensitive markers[15,17,21] for oxidative damage upon $H_2O_2/HO^{\bullet}$ exposure.

Our study was intended to better understand the effects of *non*-toxic H_2O_2 concentrations and the potentially protective role of enzymatic antioxidants in biological systems in terms of prevention of H_2O_2-induced specific DNA damage. In the literature, the significance of catalase in the overall prevention of H_2O_2-mediated oxidative damage is discussed controversially.[25,50–54] In this study, we found that stable overexpression of catalase, in fact, confers substantial protection of LipoCat cells even from H_2O_2 concentrations of 3.0 mM which normally reveal significant cytotoxicity. This protection was antagonized by the catalase inhibitor ATZ (data not shown). Interestingly, fibroblasts from catalase-deficient patients did not show decreased survival after a single UV-dose when compared to normal cells,[25] suggesting that under these conditions catalase is not necessarily required to maintain cell and tissue integrity.

By contrast to previous studies which have routinely used toxic H_2O_2 concentrations up to 60 mM,[18,48] the major finding of this study is that *non*-toxic H_2O_2 concentrations of 500 μM resulted in significant DNA strand breaks and 8-oxodG base lesions at low frequency which could

be reduced by overexpression of catalase and which we were able to detect by an adapted pulsed-field electrophoresis technique. Our data are in agreement with several reports published previously. First, an increase in catalase activity of transfected mouse epidermal cells have been reported to lead to a protection from UVB-induced DNA damage.[51] Secondly, human catalase overexpressing endothelial cells[50] as well as lens epithelial cells of glutathione peroxidase (GSHPx)-deficient mice[52] showed protection from H_2O_2-induced oxidative stress.

Furthermore, there is evidence that the crucial antioxidant role of catalase is amplified by preventing the destruction of other antioxidant enzymes.[53] In this regard catalase also reduces damage to antioxidant enzymes like GSHPx and superoxide dismutase (SOD) maintaining their activities during chronic UV exposure.[53] Furthermore, catalase seems to play the most important role in case H_2O_2 concentrations overwhelm the protective effect of the H_2O_2-detoxifying glutathione peroxidase[54] which is easily achieved at concentrations of 100 μM H_2O_2.[50] Although our data point to the crucial role of catalase in the protection from oxidative damage, it cannot completely excluded that in our system other antioxidant pathways, among them glutathione,[55] are involved at least in part in the protection from H_2O_2-induced damage.

To study the protective role of catalase on H_2O_2-induced DNA damage in terms of strand breaks and 8-oxodG base lesions, we have chosen transfection experiments using the HDF cell line 1306 to get stable transfectants containing catalase cDNA under the control of the CMV major intermediate promotor/enhancer region. A variety of other techniques have been used to study catalase effects including exogenous addition of catalase to the culture medium of murine fibroblasts,[17] transient transfection with SV40-transformed monkey kidney COS-1 cells,[56] or infection of human endothelial cells with human catalase cDNA by adenovirus-mediated transfer.[50] However, transient transfection leads to an increase in enzyme activity for only 2–3 days in the case of conventional expression vectors and up to 7 days in the case of adenovirus-mediated transfer.[50] In addition, repeated transfections and infections lead to differences in the catalase activity. Therefore, both approaches were not suitable for our investigations.

The adapted PFGE technique was considered to be a valuable tool and a new experimental approach in our studies to detect DNA damage (strand breaks, base modifications) triggered by low *non*-toxic concentrations of H_2O_2. The resulting high molecular weight DNA fragments have not been accessible to conventional electrophoresis. So far, the most commonly used techniques to investigate DNA break induction at high frequencies related to large doses or concentrations of various noxious agents are sucrose sedimentation and filter elution techniques[40,57,58] which have been shown to be sensitive assays. However, these methods suffer from a lack of sensitivity (sedimentation) and a possible lack of specificity (elution). Furthermore, quantitation of strand breaks at low concentrations is difficult because large mammalian DNA molecules may sediment anomalously in neutral gradients and the rate at which large molecules elute from filters at neutral pH can be affected by extrinsic factors. These problems, however, were reported not to be relevant in PFGE.[40,57,59] In addition, the here reported experiments have been performed with intact cells to maintain as long as possible the real state of the damaged DNA. By contrast, in case isolated genomic DNA[19] or plasmid DNA[58] is used for the experiments, artificial DNA damage can easily be introduced. Thus, the main advantage of the here reported PFGE is the special preparation of cells enclosed in agarose plugs guaranteeing to avoid mechanical shear forces, and the optimal preservation of genomic DNA.

In conclusion, our data strengthen the relevance of antioxidant enzyme activity to maintain genomic stability of cells upon oxidative challenge. These findings may allow to develop novel

strategies for protection from oxidative stress and thereby preventing oxidative stress-induced pathological disorders.

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