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Induction of 8-oxo-dGTPase activity in human lymphoid cells and normal fibroblasts by oxidative stress

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Abstract

The pre-mutagen 8-Oxo-2′-deoxyguanosine-5′-triphosphate (8-oxo-dGTP) is formed during normal cellular metabolism and its incorporation into DNA leads to transversion mutations. Human cells possess the hMTH-1 gene encoding the enzyme 8-oxo-dGTPase, which catalyzes the hydrolysis of 8-oxo-dGTP to the corresponding 8-oxo-dGMP, preventing mutations. To elucidate the involvement of 8-oxo-dGTPase in carcinogenesis, we studied hMTH-1 gene expression and enzyme activity in response to oxidative stress to human skin fibroblasts and Jurkat cells. In fibroblasts, ranges from 0 to 100 μM H_2O_2 caused a 2-fold induction of hMTH-1-mRNA expression and a 3-fold induction of enzyme activity. A 1.7-fold induction of mRNA expression and a 3.5-fold induction of enzyme activity was obtained in Jurkat cells after treatment ranging from 0 to 300 μM H_2O_2. Cytotoxic concentrations of hydrogen peroxide lead to an almost complete loss of enzyme activity and an inhibition of hMTH-1 mRNA expression. Induction of hMTH-1 gene expression was prevented by addition of actinomycin D and cycloheximide. These data indicate the inducibility of the hMTH-1 gene expression and enzyme activity by prooxidative molecules, such as hydrogen peroxide. These parameters can thus be used as a marker of oxidative stress.

Keywords: 8-oxo-dGTPase; Oxidative stress; Gene expression

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, selenium-dependent glutathione peroxidase; GSH, glutathione; 8-oxo-dGTP, 8-oxo-2′-deoxyguanosine-5′-triphoshate; dGTP, 2′-deoxyguanosine-5′-triphoshate; 8-oxo-dGDP, 8-oxo-2′-deoxyguanosine-5′- diphosphate; dGDP, 2′-deoxyguanosine-5′- diphosphate; 8-oxo-dGMP, 8-oxo-2′-deoxyguanosine-5′- monophosphate; dGMP, 2′-deoxyguanosine-5′- monophosphate; 8-OhdG, 8-oxo-2′-deoxyguanosine; dG, 2′-deoxyguanosine; hMTH-1, human MutT homologue; 8-oxo-dGTPase, 8-oxo-2′-deoxyguanosine-triphosphate-pyrophosphohydrolyase; BSA, bovine serum albumin; LDH, lactate dehydrogenase; DTT, dithiothreitol; Tris, Tris(hydroxymethyl)aminoethan; TBA, Tetrabutylammoniumhydroxid; PMSF, phenylmethylsulfonylfluoride; HPLC, high performance liquid chromatography; RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glycerin-aldehyd-3-phosphate-dehydrogenase.

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1. Introduction

Reactive oxygen species (ROS) are continually generated during normal cellular metabolism. To minimize oxidative damage of important macromolecules, cells possess an antioxidant defense system, including enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP_x), as well as low molecular weight substrates, such as glutathione (GSH), ascorbic acid and tocopherols. Reaction of oxygen radicals with DNA results in the formation of a set of oxidized base products (Gajewski et al., 1990; Demple and Harrison, 1994). Among these an oxidized form of dG, 8-oxo-dG appears to be important in mutagenesis and carcinogenesis (Kasai et al., 1986; Ames and Gold, 1991). This oxidized base is formed either from the direct interaction of oxygen free radicals with the guanine base in cellular DNA or after reaction with the dGTP of the free nucleotide pool. The resulting 8-oxo-dGTP may then be incorporated into the DNA during DNA replication or DNA repair synthesis (Akiyama et al., 1989; Maki and Sekiguchi, 1992). 8-Oxo-dG can pair with cytosine as well as with adenine and, as a result, A:T to C:G as well as G:C to T:A transversion-mutations can occur (Shibutani et al., 1991; Cheng et al., 1992). Organisms are equipped with elaborate mechanisms to prevent mutations. In *Escherichia coli* two glycosylases have been identified. One, the MutM protein, removes 8-oxoguanine paired with cytosine (Cabrera et al., 1988; Chung et al., 1991; Michaels et al., 1991) and a second, the MutY protein, removes adenine paired with 8-oxoguanine from DNA (Nghiem et al., 1988; Au et al., 1989; Michaels et al., 1992). Similar repair processes have been identified in mammalian cells (Bessho et al., 1993; McGoldrick et al., 1995). In addition to these glycosylases a third enzyme, MutT, also involved in 8-oxo-dG lesion repair and preventing system, was identified in *E. coli* (Treffers et al., 1954). The MutT protein of *E. coli* and its human homologue the hMTH-1 protein were shown to be a specific pyrophosphohydrolase (8-oxo-dGTPase) (Furuichi et al., 1994), which hydrolyzes 8-oxo-dGTP to the corresponding monophosphate 8-oxo-dGMP (Maki and Sekiguchi, 1992; Mo et al., 1992). This substrate cannot be rephosphorylated to the triphosphate and thus cannot be incorporated into DNA (Hayakawa et al., 1995; Sekiguchi, 1996). The expression of the 8-oxo-dGTPase varies widely among cell types and tissues. High levels are found in Jurkat cells, a human T-cell leukemia cell line. The enzyme is inducible by growth stimulation (Sakumi et al., 1993; Wani and D'Ambrosio, 1995; Oda et al., 1997). To examine the role of the 8-oxo-dGTPase in prevention of mutagenesis and carcinogenesis, the expression of the 8-oxo-dGTPase in response to oxidative stress has to be assessed. We treated Jurkat cells and primary human skin fibroblasts with various concentrations of hydrogen peroxide for 24 h and studied the hMTH-1 mRNA expression and the 8-oxo-dGTPase enzyme activity. For this purpose we modified the method reported by Bialkowski and Kasprzak (1998).

2. Materials and methods

2.1. Chemicals

RPMI-1640 medium, fetal bovine albumin and SuperScript II H^- Reverse Transcriptase were purchased from Gibco BRL (Grand Island, NY). Ascorbic acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, 30% (w/w) hydrogen peroxide, disodium ethylenediaminetetraacetate (Na_2EDTA), sodium chloride, ammonium sulfate, Tris-(hydroxylmethyl)aminomethan (Tris), magnesium chloride, hydrochloric acid, ammonium acetate, acetonitrile (HPLC grade) and methanol (HPLC grade) were from Merck (Darmstadt, Germany). Tetrabutylammoniumhydroxide (TBA) was obtained from Aldrich (Milwaukee, WI). Bovine serum albumin (BSA) was from ICN Biochem. (Costea Mesa, CA). A deoxynucleotide set (dNTPs) and random Hexamers ($pd(N)_6$) were from Pharmacia Biotech (Uppsala, Sweden). Lactate dehydrogenase determination kit was purchased from Boehringer Mannheim (Mannheim, Germany). Protein determination kit was from Bio-Rad (Muenchen, Germany). Penicillin, streptomycin, glutamine, dithiothreitol (DTT), phenyl-

methylsulfonyl fluoride (PMSF), glycerol, cycloheximide, actinomycin D, potassium chloride (molecular biological grade), magnesium chloride, Tris, Tris-HCl (all of molecular biological grade), Triton X-100, collagenase (type II), 2′-deoxyguanosine (dG) and its 5′-mono-, di- and triphosphates: dGMP (free acid), dGDP (disodium salt) and dGTP (trisodium salt) were obtained from Sigma Chemical (St Louis, MO). Thermoprime Plus DNA-Polymerase was from Advanced Biotechnologies (Surrey, UK).

2.2. Oligonucleotides

The oligonucleotides for the RT-PCR for human GAPDH (5′-CCA TGG AGA AGG CTG GGG-3′ and 5′-CTA AGC AGT TGG TGG TGC A-3 ′) and for human 8-oxo-dGTPase (5′-CGG CTC TGC GCC ACT CAA-3′ and 5′-GAG CGG CGG TGC AGA ACC-3)′ were obtained from NAPS GmbH (Göttingen, Germany).

2.3. Preparation of 8-oxo-dGMP, 8-oxo-dGDP and 8-oxo-dGTP

All the 8-oxo-dG-5′-phosphates were prepared according to the method of Mo et al. (1992) and Kasai and Nishimura, (1984), with minor modifications. The reaction mixture containing 100 mM sodium phosphate (pH 6.8), 30 mM ascorbic acid, 100 mM hydrogen peroxide and 6 mM of dGMP, dGDP or dGTP was incubated for 2.5 h at 37°C in the dark. 100 μl of the reaction mixture was loaded onto a Hamilton PRP X-100 column (10 μm particle size, 4.1×100 mm) equilibrated with 25 mM citric acid, 7% acetonitrile (pH 5.4) and chromatographic separation was carried out with the same buffer at a flow rate of 1 ml/min. 8-Oxo-dG-5′phosphates were monitored with an UV detector (254 nm) and eluted after their corresponding unreacted dG-5′-phosphates. Fractions containing 8-oxo-dGMP, 8-oxo-dGDP or 8-oxo-dGTP were combined, concentrated and applied to a Beckman Ultrasphere C_{18} column (5 μm particle size, 4.6×250 mm). The 8-oxo-dG-5′-phosphates were eluted isocratically with 0.03% ammonium acetate, 5% methanol at a flow rate of 1 ml/min.The fractions containing individual 8-oxo-dG-5′-phosphates were pooled, evaporated to dryness under vacuum and dissolved in 2 ml 0.03% ammonium acetate. Purity and concentration were examined by electrochemical measurements and UV spectrum analyses (molar absorbance coefficient $\varepsilon_{293} = 10\,300$). The nucleotide solutions were stored at −20°C.

2.4. Cell culture and harvesting

Normal human neonatal foreskins were obtained after surgery from Hamburg University Hospital. The skin was cut into small pieces and treated with collagenase (type II) in RPMI 1640 medium at 37°C for 15 min with gentle stirring. After filtration through gaze the suspension was centrifuged. The pellet was resuspended in media and plated into 80 cm^2 flasks for growing.

The skin fibroblasts and the Jurkat cells were grown at 37°C, under air containing 5% CO_2, in 15 ml RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). The Jurkat cells were harvested at a density of 2×10^5 cells/ml by centrifugation and washed twice with ice-cold phosphate-buffered saline. When the skin fibroblast reached near confluence, the medium was removed from the flask. Cells were washed twice with PBS, scraped off the dish, suspended into 10 ml PBS and recovered by centrifugation.

2.5. Cell-free extracts

Cells from one culture flask were suspended into 500 μl of 20 mM Tris-HCl (pH 7.5) and lysed by passing four times through a 25-gauge needle and four cycles of freezing (dry ice) and thawing (37°C). The resulting cell lysates were ultracentrifuged for 2 h at $200\,000 \times g$ at 4°C. The supernatants, termed below as extracts, were dialyzed over night against 500 ml 20 mM Tris-HCl (pH 7.5) and stored at −70°C. Alternatively, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM DTT, 200 mM NaCl, 5% glycerol and 0.5 mM PMSF were used as lysis buffer. To the cell lysates ice-cold saturated ammonium sulfate solution (pH 8.0) was added to give a final saturation of 30%. The lysate was left on ice for 1 h and centrifuged

at 15 000 × *g* for 15 min at 4°C. To the supernatant 0.33 mg/μl solid ammonium sulfate was added and the mixture was left on ice for 30 min. After centrifugation at 15 000 × *g* for 15 min at 4°C the precipitate was resuspended in 400 μl 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM DTT, 20 mM NaCl, 5% glycerol and 0.5 mM PMSF and dialyzed over night against 500 ml of the same buffer. The fraction of low molecular weight proteins were isolated by filtering 200 μl of the extract through a 30 kDa cut-off, low protein-binding ultrafiltration membrane (Ultrafree-MC Filtration Unit, Millipore), with centrifugation at 3000 × *g* for 30 min. The resulting ultrafiltrate was stored at − 70°C.

2.6. Determination of 8-oxo-dGTPase activity

The assay was conducted at 37°C in a total volume of 50 μl containing 100 mM Tris-HCl (pH 4 – 12), 5 mM $MgCl_2$, 5–120 μM 8-oxo-dGTP, 5 – 20 μl extract or ultrafiltrate (protein concentration ranges from 0.015 to 1 mg/ml) and other additives as specified in the Results for 5 – 40 min. The reaction was terminated by adding 50 mM EDTA (molar ratio $EDTA/Mg^{2+} = 3.3$). The reaction mixture was diluted 1:50 fold with water and finally analyzed by HPLC.

2.7. High pressure liquid chromatography (HPLC) analysis

The HPLC system consisted of a Merck Hitachi intelligent L-6200 pump with an UV-VIS L-4200 detector, an ICI 1260 EC-detector and a Merck Hitachi 655A-40 autosampler. Separation of 100μl samples were performed on a Beckman Ultrasphere C18 column (5 μm particle size, 4.6 × 250 mm) by isocratic elution with 200 mM sodium phosphate buffer (pH 6.0), containing 2.5 mM TBA and 10% methanol, at a flow rate of 1 ml/min. The 8-oxo-dG-5′-phosphates in the samples were quantified electrochemically at 600 mV and by UV absorbance at 254 nm by integrating the chromatograms. Solutions of known concentrations of 8-oxo-dG-5′-phosphates were used for calibration.

2.8. Treatment with hydrogen peroxide

The skin fibroblasts and Jurkat cells were treated at ∼70% confluence with hydrogen peroxide. The final concentration of H_2O_2 varied between 0 and 1000 μM. Each group consisted of six separate cultures. After addition of H_2O_2 the cells were allowed to grow for additional 24 h. Three cultures at each hydrogen peroxide concentration were harvested, lysed, ultracentrifuged and ultrafiltered for measuring the 8-oxo-dGTPase activity. The other three cultures were harvested and the RNA was isolated for RT-PCR.

2.9. Inhibition of protein and RNA synthesis

Jurkat cells were exposed to nontoxic concentrations of inhibitors for 24 h. 50 μM cycloheximide was used to inhibit protein synthesis and 4 μM actinomycin D was used to inhibit RNA synthesis. ROS exposed cells received 100 μM hydrogen peroxide.

2.10. Protein determination

Protein concentration in the cell extracts was determined in triplicate according to the method of Bradford (1974). Crystalline BSA was used as a protein standard.

2.11. Cell viability

Cell viability was analyzed by Trypan blue exclusion. Extracellular lactate dehydrogenase (LDH) activity was determined using the LDH determination kit from Boehringer (Mannheim, Germany). Triton X-100 was used as positive control

2.12. Semiquantitative RT-PCR

Total RNA was isolated as described by Chomczynski and Sacchi (1987). 1 μg of total RNA was reverse-transcribed (Sambrook et al., 1989) with minor modifications. Briefly, a reaction mixture (50 μl) containing 1 μg total RNA, 50 mM KCl, 3 mM $MgCl_2$, 0.1 mg/ml BSA, 10 mM Tris-HCl (pH 9.3), 50 mM dNTPs, 250 pM $pd(N_6)$ and 0.5

U SuperScript II H$^-$ Reverse Transcriptase was incubated for 1 h at 39°C. The reaction was terminated at 94°C for 3 min. PCR was performed in a reaction mixture (50 μl) containing an appropriate amount of the first-strand cDNA, 10 mM Tris-HCl (pH 9.3), 50 mM KCl, 3 mM $MgCl_2$, 0.1 mg/ml BSA, 250 μM dNTPs, 10 pM of each primer and 1 U Taq-Polymerase. The amplification was performed by up to 32 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min, using a DNA thermal cycler (Stratagene, La Jolla, CA). 10 μl of the PCR products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide and quantified using the GelPrint 1000i documentation system (MWG Biotech, Ebersberg, Germany).

The mRNA levels in treated and untreated cells were estimated by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) using GAPDH as reference gene.

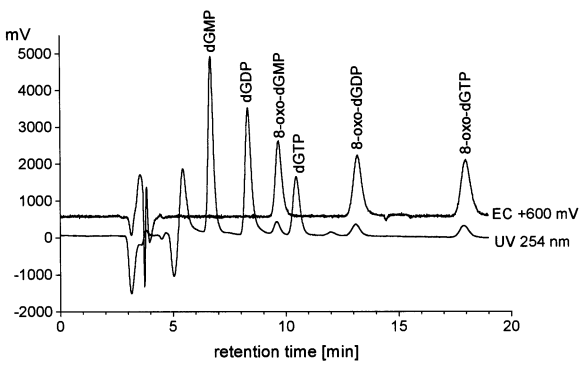

Fig. 1. HPLC separation of dGTP/8-oxo-dGTP and their hydrolytic dephosphorylation products dGDP/8-oxo-dGDP and dGMP/8-oxo-dGMP. A standard solution containing dGTP, dGDP, dGMP (each 80 μM) and 8-oxo-dGTP, 8-oxo-dGDP, 8-oxo-dGMP (each 8 μM) was chromatographed by HPLC as described in the experimental procedures. The dG-5′ phosphates were determined by UV absorbance at 254 nm and the 8-oxo-dG-5′-phosphates by electrochemical detection at + 600 mV.

3. Results

3.1. HPLC analysis of the 8-oxo-dGTPase assay

The determination of 8-oxo-dGTP/ dGTP and their hydrolytic dephosphorylation products 8-oxo-dGDP/ dGDP and 8-oxo-dGMP/ dGMP was achieved using ion-pairing chromatography on a Beckman Ultrasphere C18-column. The elution order of the compounds on this system depends (i) on the number of phosphate residues in the molecules (monophosphates < diphosphates < triphosphates) and (ii) on the hydroxylation on C8 position (dG-5′-phosphates < 8-oxo-dG-5′-phosphates) (Fig. 1). Using the HPLC conditions described, the dG-5′-phosphates and the 8-oxo-dG-5′-phosphates have subsequent retention times: dGMP: 6.5 min; dGDP: 8.3 min; dGTP: 10.5 min; 8-oxo-dGMP: 9.2 min; 8-oxo-dGDP: 13 min and 8-oxo-dGTP: 18 min.

3.2. Effects of dGTP, dGDP and 8-oxo-dGDP on the kinetic parameters, K_M and V_{max} of the 8-oxo-dGTPase

After removal of nonspecific phosphatases in the cell lysate by ultrafiltration, hydrolyzation of 8-oxo-dGTP to 8-oxo-dGMP was observed. This magnesium dependent reaction has a pH optimum at 8.3 and follows Michaelis–Menten kinetics. The K_M value and V_{max} for 8-oxo-dGTP hydrolysis at pH 8.0 in Jurkat cells is 14 μM and 36.2 pmol/min; in primary human skin fibroblasts these values were 8 μM and 34 pmol/min.

To investigate the influence of dGTP, dGDP and 8-oxo-dGDP on the kinetic parameters, K_M and V_{max}, of the 8-oxo-dGTPase activity in Jurkat cells, 5 μM dGTP, dGDP or 8-oxo-dGDP were added to the reaction mixture. A competitive inhibition of the 8-oxo-dGTPase activity was observed after dGTP and dGDP addition. Both compounds caused negligible changes of V_{max} from 36.2 to 36 and 35.8 pmol/min and increased the K_M value from 14 to 18 and 22 μM respectively. In contrast, 8-oxo-dGDP addition caused a uncompetitive inhibition of the 8-oxo-dGTPase activity, increasing the K_M value to 30 μM and decreasing V_{max} to 28.9 pmol/min (Fig. 2(A)). The

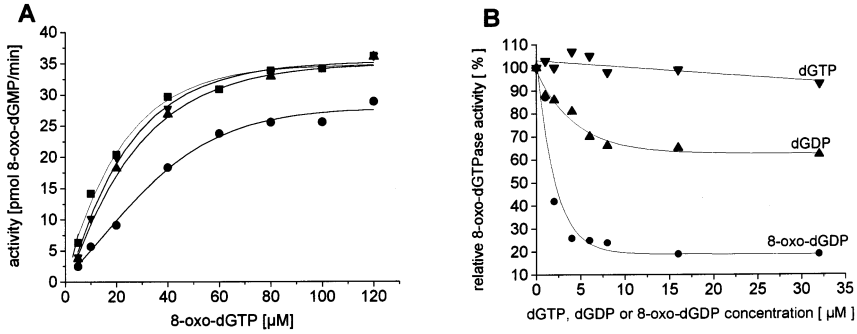

Fig. 2. Inhibitory effect of 8-oxo-dGDP, dGDP and dGTP on the activity of 8-oxo-dGTPase of Jurkat cells. The enzyme activity in the ultrafiltrate was determined under standard conditions described in the Experimental procedures except that, (A) the reactions contains 5–120 μM 8-oxo-dGTP (■) in the absence or presence of 5 μM 8-oxo-dGDP (●), dGDP (▲)or dGTP (▼) or, (B) that the reaction contained increasing concentrations of 8-oxo-dGDP (●), dGDP (▲) or dGTP (▼) ranging from 0 to 40 μM. Neither 8-oxo-dGDP, dGDP or dGTP decomposed under these conditions. Experiments were performed in duplicates.

presence of 32 μM of each of the different nucleotides caused the following inhibitory effects on the 8-oxo-dGTPase activity: dGTP, 3%, dGDP, 34% and 8-oxo-dGDP, 83%. (Fig. 2(B)). The results demonstrate the potent inhibitory effect of 8-oxo-dGDP on the 8-oxo-dGTPase activity.

3.3. Effect of hydrogen peroxide on cell viability

The cell viability was analyzed by Trypan blue exclusion and determination of extracellular lactate dehydrogenase activity. Treatment with hydrogen peroxide resulted in a dose-dependent increase of extracellular lactate dehydrogenase activity. In primary human skin fibroblasts 300 μM H_2O_2 caused a 9-fold and 1000 μM H_2O_2 a 14-fold increase in extracellular lactate dehydrogenase activity. In Jurkat cells 1000 μM H_2O_2 caused a 4-fold increase and 3000 μM H_2O_2 a 6-fold increase in enzyme activity (Fig. 3). In comparison, treatment with 0.5% Triton X-100 as positive control caused a 8.5-fold increase in extracellular lactate dehydrogenase activity in Jurkat cells and a 17-fold increase in primary human skin fibroblasts. Lactate dehydrogenase levels more than 50% of the positive control were defined as cytotoxic. These observations indicate that in Jurkat cells hydrogen peroxide concentrations more than 1 mM and in primary human skin fibroblasts more than 300 μM are cytotoxic.

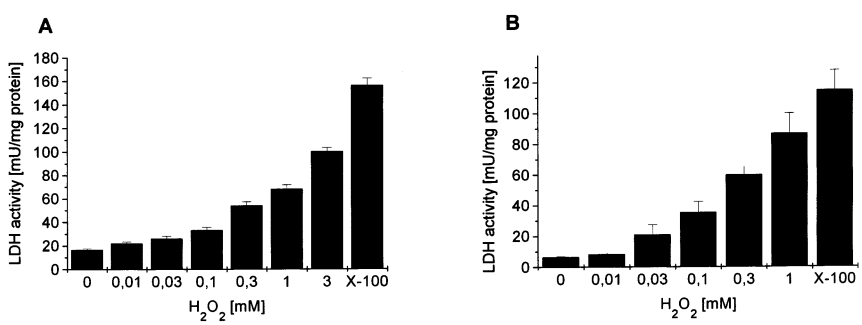

Fig. 3. Influence of hydrogen peroxide on the viability of Jurkat cells and primary human skin fibroblasts. Extracellular lactate dehydrogenase (LDH) activity was estimated after 24 h exposure of primary human skin fibroblasts (B) and Jurkat cells (A) with various concentrations of hydrogen peroxide. Triton X-100 (X-100) was used as a positive control. Lactate dehydrogenase levels $>50\%$ of the positive control were defined as cytotoxic. The error bars represent standard error values for three independent cultures treated in the same way.

3.4. Effect of hydrogen peroxide on 8-oxo-dGTPase mRNA levels and enzyme activity

The addition of hydrogen peroxide to Jurkat cells and primary human skin fibroblasts caused a dose-dependent increase in the levels of 8-oxo-dGTPase mRNA and enzyme activity. In Jurkat cells the greatest induction was observed with 300 μM H_2O_2 which resulted in an 1.7-fold increase of the levels of 8-oxo-dGTPase mRNA and a 3.5-fold increase in the levels of 8-oxo-dGTPase enzyme activity (Fig. 4). In human skin fibroblasts 100 μM H_2O_2 caused a 2-fold induction in 8-oxo-dGTpase mRNA expression, whereas a 3-fold induction in enzyme activity (Fig. 5) was obtained. These concentrations are non or only slightly cytotoxic. Cytotoxic concentrations of H_2O_2 caused a downregulation of 8-oxo-dGTPase mRNA expression and an almost complete loss of the enzyme activity.

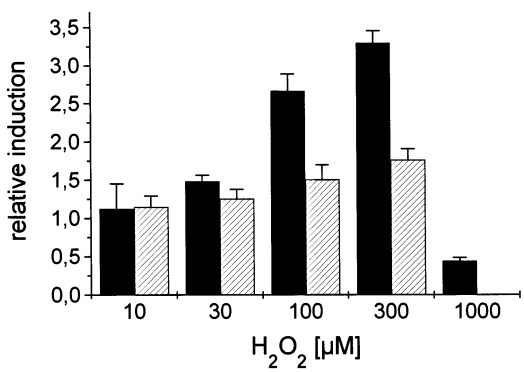

Fig. 4. 8-Oxo-dGTPase activity and hMTH-1-mRNA expression in Jurkat cells after treatment with 0–1000 μM hydrogen peroxide for 24h. 8-Oxo-dGTPase activity (■) in treated Jurkat cells were determined under standard conditions and compared with the activity of untreated Jurkat cells, which was set as 1. Total RNA was isolated from Jurkat cells treated with 0–1000 μM hydrogen peroxide for 24 h. The hMTH-1 mRNA levels (▨) were estimated by semiquantitative RT-PCR using GAPDH as reference gene. The data are expressed as a ratio of treated hMTH-1/untreated hMTH-1 mRNA level. The control (untreated cells) was set as 1. The error bars represent standard error values for three independent cultures treated in the same way.

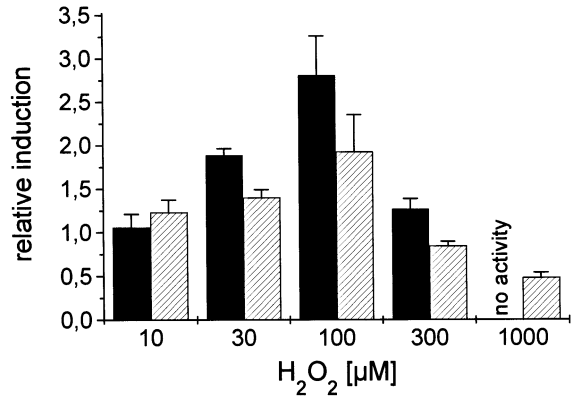

Fig. 5. 8-Oxo-dGTPase activity and hMTH-1 mRNA expression in primary human skin fibroblasts after 24 h treatment with 0 to 1000 μM hydrogen peroxide. 8-oxo-dGTPase activity (■) in treated fibroblasts were determined under standard conditions and compared with the activity of untreated fibroblasts, which was set as 1. Total RNA was isolated from primary human skin fibroblasts treated with 0–1000 μM hydrogen peroxide for 24 h. The hMTH-1 mRNA levels (▨) were estimated by semiquantitative RT-PCR using GAPDH as reference gene. The data are expressed as a ratio of treated hMTH-1/untreated hMTH-1 mRNA level. The control (untreated cells) was set as 1. The error bars represent standard error values for three independent cultures treated in the same way.

3.5. Effect of actinomycin D and cycloheximide on 8-oxo-dGTPase mRNA

In order to evaluate the contribution of transcription to the induction of 8-oxo-dGTPase mRNA, we used the RNA synthesis inhibitor, actinomycin D. Exposure of Jurkat cells to actinomycin D simultaneous with 100 μM hydrogen peroxide resulted in a 47% decrease in 8-oxo-dGTPase activity compared with Jurkat cells exposed to actinomycin D alone (Fig. 6). This suggests that the induction of 8-oxo-dGTPase mRNA observed with hydrogen peroxide requires active transcription of mRNA.

The involvement of protein synthesis was examined by addition of cycloheximide to Jurkat cells. Cells exposed to Cycloheximide and hydrogen peroxide contained 50% less 8-oxo-dGTPase message compared with control Jurkat cells (cycloheximide alone) (Fig. 6). This observation suggests the involvement of protein synthesis in the induction of 8-oxo-dGTPase mRNA.

4. Discussion

8-Oxo-dGTPase is an enzyme present in all organisms from bacteria to humans. It catalyzes the hydrolyzation of 8-oxo-dGTP, a promutagenic substrate derived from dGTP by reaction with oxygen radicals in the nucleotide pool of a cell, to 8-oxo-dGMP. During DNA synthesis 8-oxo-dGTP can pair with cytosine as well as with adenine at almost equal efficiency, leading to A:T to C:G and G:C to T:A transversions (Maki and Sekiguchi, 1992). This type of mutation was frequently observed in activated oncogenes or tumor suppressor genes during cancer development (Hollstein et al., 1991; Shibutani et al., 1991; Kamiya et al., 1992). Sufficient activity of 8-oxo-dGTPase is apparently necessary prerequisite for accurate DNA synthesis in replicating cells and for maintaining a low incidence of spontaneous mutations induced by oxidations. The expression of the 8-oxo-dGTPase gene in cancer cells and its possible use as a marker of oxidative stress have been recently characterized (Wani and D'Ambrosio, 1995; Kennedy et al., 1998; Wani et al., 1998). However, no current information about the regulation of this enzyme in human cells in response to oxidative stress is available.

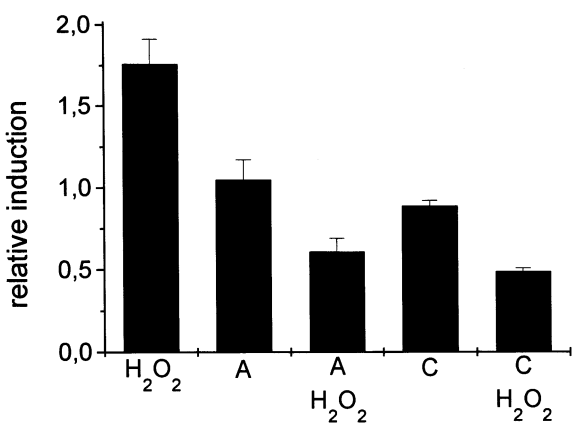

Fig. 6. Effect of actinomycin D and cycloheximide on H_2O_2 induction of hMTH-1 mRNA. The hMTH-1 mRNA level of Jurkat cells, exposed to 100 μM hydrogen peroxide (H_2O_2), 4 μM actinomycin D (A) alone, both actinomycin D and hydrogen peroxide (A, H_2O_2), 50 μM cycloheximide (C) alone and cycloheximide and hydrogen peroxide (C, H_2O_2) for 24 h was estimated by semiquantitative RT-PCR using GAPDH as reference gene. The data are expressed as ratio of treated hMTH-1/untreated hMTH-1 mRNA level. The control (untreated cells) was set as 1. The error bars represent standard error values for three independent cultures tested in the same way.

The 8-oxo-dGTPase enzyme activity in cultured cells was determined according to Bialkowski and Kasprzak (1998) with modifications. As shown, dGTP, dGDP and particularly 8-oxo-dGDP are inhibitors of the 8-oxo-dGTPase activity. These observations are in agreement with the results published by Bialkowski and Kasprzak (1998) and have subsequent practical implications: (i) the 8-oxo-dGTP preparations should be virtually free of dGTP, dGDP or 8-oxo-dGDP and (ii) unspecific phosphatases, hydrolyzing 8-oxo-dGTP to 8-oxo-dGDP, should be excluded. Therefore, we optimized the synthesis of all 8-oxo-dG-5′-phosphates described by Mo et al. (1992), based on the oxidation of dG-5′-phosphates by the oxygen radical generating system ascorbic acid/hydrogen peroxide. The 8-oxo-derivatives were separated from the parent compounds and byproducts from the reaction by a two step semipreparative HPLC purification, using anion-exchange and reversed-phase material. The 8-oxo-dGTP remains stable in 0.03% ammonium acetate at −20°C for months. The 8-oxo-dGTPase was separated from the interfering phosphatases by using a 30 kDa cut-off ultrafiltration membrane (Bialkowski and Kasprzak, 1998). All dG-5′-phosphates and their corresponding 8-oxo-derivates could be definitively separated by using ion-pairing chromatography on a Beckman Ultrasphere C18 column. These compounds were detected by a UV–VIS detector at 254 nm and a highly sensitive electrochemical detector at +600 mV (Fig. 1). This application allows further studies of the 8-oxo-dGTPase as well as other enzymes involved in the metabolic pathway of guanine nucleotides.

We also characterized the 8-oxo-dGTPase enzyme from Jurkat cells and primary human skin fibroblasts. In both cells types the molecular mass of the enzyme was below 30 kDa, the pH optimum was 8.3 and the activity dependent Mg^{2+}-concentration. In Jurkat cells, the K_M value and V_{max} were found to be 14 μM and 36.2 pmol/min. In primary human skin fibroblasts these values

were 8 μM and 34 pmol/min, respectively. In comparison, Mo et al. (1992) characterized the purified human 8-oxo-dGTPase as a 18 kDa protein. They also observed the dependence of enzyme activity on Mg^{2+}-concentration with a pH optimum of 8.0 and $K_M = 12.5$ μM. A pH-optimum of 8.5 and $K_M = 9.3$ μM were described for CHO cells, whereas a $K_M = 8.5$ μM was observed in human fibroblasts (Bialkowski and Kasprzak, 1998).

In the present study we investigated the response of the 8-oxo-dGTPase activity to oxidative stress caused by hydrogen peroxide in Jurkat cells, a human T-cell leukemia line, and in primary human skin fibroblasts. In Jurkat cells we observed a 3.5-fold increase and in human skin fibroblasts a 3-fold increase in enzyme activity after 24 h treatment low, nontoxic concentrations of hydrogen peroxide. The 8-oxo-dGTPase mRNA was induced 1.7-fold in Jurkat cells and 2-fold in human skin fibroblasts. Cytotoxic concentrations of hydrogen peroxide lead to a loss of enzyme activity and caused a down regulation of 8-oxo-dGTPase-mRNA expression (Figs. 4 and 5). Our investigation demonstrated that 8-oxo-dGTPase enzymatic activity correlates with its mRNA level. Whether differential translation or post-translational modifications may be involved in the regulation of this enzyme will be considered in further studies.

The induction of the 8-oxo-dGTPase-mRNA by hydrogen peroxide was inhibited by co-treatment with actinomycin D as an RNA synthesis inhibitor (Fig. 6). This observation suggests that transcription is required for the induction of 8-oxo-dGTPase mRNA. Previous studies determined a potential binding site for Ets family proteins near the putative initiation site of the 8-oxo-dGTPase gene. These transcription factors may be involved in the regulation of the 8-oxo-dGTPase gene expression (Oda et al., 1997). It has been pointed out that hydrogen peroxide can readily penetrate cells and modulate the oxidized/reduced state of transcription factors either directly by oxidation or indirectly through changes in the cellular levels of reduced or oxidized glutathione (Burdon, 1995). Co-treatment of cells with cycloheximide, a protein synthesis inhibitor, also caused an inhibition of 8-oxo-dGTPase-mRNA expression induced by hydrogen peroxide (Fig. 6). This observation indicates the requirement of protein synthesis for the induction of 8-oxo-dGTPase mRNA.

In conclusion, we have demonstrated that oxidative stress causes an induction of 8-oxo-dGTPase-mRNA expression and subsequently of the activity of this enzyme in human cells. Whether an adequate induction of this enzyme is critical for the prevention of mutations caused by the incorporation of 8-oxo-dG into genomic DNA, with the consequence of the development of cancer and degenerative diseases, remains to be elucidated.

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