Influence of glutathione levels and heat-shock on the steady-state levels of oxidative DNA base modifications in mammalian cells

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The effects of thiols, ascorbic acid and thermal stress on the basal (steady-state) levels of oxidative DNA base modifications were studied. In various types of untreated cultured mammalian cells, the levels of total glutathione were found to be inversely correlated with the levels of DNA base modifications sensitive to the repair endonuclease Fpg protein, which include 8-hydroxyguanine (8-oxoG). A depletion of glutathione by treatment with buthionine sulfoximine increased the steady-state level in AS52 Chinese hamster cells by ~50%. However, additional thiols in the culture medium did not reduce the level of Fpg-sensitive base modifications: 0–10 mM N-acetylcysteine had no effect, whereas cysteine ethylster even increased the oxidative DNA damage at concentrations >0.1 mM. Similarly, ascorbic acid (0–20 mM) failed to reduce the steady-state levels. When AS52 cells were grown at elevated temperature (41°C), the steady-state level of the oxidative DNA modifications increased by 40%, in spite of a concomitant 1.6-fold increase of the cellular level of total glutathione. Depletion of glutathione at 41°C nearly doubled the already elevated level of oxidative damage. A constitutive expression of the heat-shock protein Hsp27 in L929 mouse fibrosarcoma cells at 37°C increased the glutathione level by 60%, but had little effect on the level of oxidative DNA damage.

Introduction

Basal (background) levels of oxidative DNA modifications such as 8-hydroxyguanine (8-oxoG) are detectable in apparently all types of cells by means of various techniques (1–5), although the correct absolute levels are controversial (6). The basal levels observed in untreated cells are assumed to reflect the balance (steady-state) between the generation of the lesions by endogenously produced reactive oxygen species (ROS) and their removal by excision repair. Since 8-oxoG and other DNA base modifications sensitive to the repair endonuclease Fpg protein (31) from Escherichia coli is directly or indirectly involved in many ROS-detoxifying reactions. Under cell-free conditions, it has been shown to inhibit the generation of 8-oxoG by ionizing radiation (17) and by H2O2 in the presence of Fe(II) (Fenton reaction) (18). In mitochondria of mouse and rat liver, the age-dependent oxidation of GSH was correlated with an increase of 8-oxoG in mtDNA (19). On the other hand, GSH can generate ROS via auto-oxidation and in the presence of transition metals (20–22), and the reaction of GSH with superoxide is slow, which may explain why GSH depletion had no effect on the cytotoxicity of the superoxide-generating agent paraquat (23).

Here, we report on a correlation of the intracellular levels of total glutathione with the steady-state levels of base modifications sensitive to the repair endonuclease, Fpg protein, which include 8-oxoG, for various types of mammalian cells. Depletion of GSH and thermal stress increased endogenous oxidative damage. In contrast, additional thiols in the medium did not reduce the level of oxidative damage.

Materials and methods

Cells and repair endonucleases

The types and sources of the mammalian cells used in this study are summarized in Table I. Human Hsp27-expressing L929-27-3-97 murine fibrosarcoma cell lines were derived from previously characterized L929-27-3 cells (30). L929-27-3-97 cells express ~0.2 ng Hsp27/mg total proteins, whereas original cells contained 0.9 ng Hsp27/mg total proteins (data not shown). These cells displayed a smaller increase in glutathione than that previously reported for L929-27-3 cells (1.6-fold instead of 3- to 5-fold). Cells were cultured as described (see refs in Table I).

Formamidopyrimidine-DNA glycosylase (Fpg protein) (31) from Escherichia coli was kindly provided by S.Boiteux (Fontenay aux Roses, France). Cysteine ethylster (CYSET) was a gift of G.Beijersbergen van Henegouwen (Leiden, The Netherlands) (32). N-acetylcysteine (NAC) and ascorbic acid were obtained from Sigma (Deisenhofen, Germany).

Treatment of cells with antioxidants and GSH depletion

To deplete GSH, cells were pre-incubated in culture medium with 1 mM buthionine[S,R]sulfoximine (BSO) for 24 h. When indicated, CYSET, NAC or ascorbic acid was added to the culture medium at 4 or 16 h before quantification of oxidative DNA damage.

Quantification of endonuclease-sensitive modifications by alkaline elution

Determination of modifications sensitive to Fpg protein was carried out by means of an alkaline elution assay, as described previously (33,34). Briefly, 105 cells were washed by centrifugation and resuspension in PBS–CMF buffer (140 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4, pH 7.4), collected on a polycarbonate filter (2 μm pore size) and lysed by pumping a lysis solution (100 mM glycine, 20 mM Na2EDTA, 2% SDS, 500 mg/l proteinase K, pH 10.0) through the filter for 60 min at 25°C. After extensive washing, the DNA remaining on the filter was incubated for 30 min at 37°C with Fpg protein (1 μg/ml). Under these assay conditions, the incision by the enzyme at its substrate modifications in the DNA was shown to be saturated.

Abbreviations: BSO, buthionine[S,R]sulfoximine; CYSET, cysteine ethylster; DTNB, 5,5'-dithio-(2-nitrobenzoic acid); GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); NAC, N-acetylcysteine; 8-oxoG, 8-hydroxyguanine; ROS, reactive oxygen species; s.s.b., single-strand breaks; TNFα, tumour necrosis factor α.

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III, which recognizes certain oxidized pyrimidines, were also quantified in several cell lines and found to be below or at the detection limit of ~0.05 modifications per 10^6 bp (data not shown). Intracellular levels of total glutathione varied between 2.3 nmol per 10^6 cells in primary human lymphocytes and 14.6 nmol per 10^6 cells in HeLa cells (Figure 1).

When the steady-state levels of Fpg-sensitive modifications in the various cell types are plotted against the cellular levels of total glutathione, an inverse correlation with a linear correlation coefficient of \( r = -0.751 \) and a significance of \( P = 0.008 \) is observed (Figure 1), i.e. high levels of total glutathione are associated with low basal levels of oxidative DNA damage. With respect to both parameters, there is no obvious general difference between human and rodent cells or between primary and transformed cells.

### Steady-state levels of oxidative DNA base modifications are not reduced by exogenous thiols and ascorbic acid

The observed inverse correlation of intracellular total glutathione with the steady-state levels of Fpg-sensitive modifications in the cells raised the question whether additional thiol in the culture medium could further reduce the oxidative DNA damage. The data shown in Figure 2 (upper panel) indicate that this is not the case. Incubation of AS52 cells for 16 h with 0.01–0.1 mM CYSET, a thiol that is well absorbed by cells (40), or with 1–10 mM \( N \)-acetylcysteine (NAC), a precursor of GSH, which raised the intracellular GSH levels in AS52 cells (data not shown) as well as in keratinocytes (40) by ~50%, had no effect on the steady-state level of Fpg-sensitive modifications. CYSET even increased the damage at higher concentrations (1 mM), i.e. it had a pro-oxidant effect.

The influence of ascorbic acid on the steady-state levels of Fpg-sensitive modifications in AS52 cells was measured for comparison. Addition of 0.01–1 mM to the culture medium had no significant effect on the levels of oxidative base modifications observed after 4 and 16 h. Again, unphysiologically high concentrations (10 mM, 20 mM) had a pro-oxidant effect after 16 h, i.e. they increased DNA damage (Figure 2, lower panel). The incubation time of 4 h is equal to the half-life of ascorbic acid in the culture medium (data not shown) and is between 2-fold and 4-fold longer than the repair times (\( t_{1/2} \)) reported previously for Fpg-sensitive modifications and 8-oxoG in various human and rodent cells (41–44).
Influence of glutathione levels and heat-shock

Fig. 2. Influence of various concentration of NAC and CYSET (upper panel) and ascorbic acid (lower panel) on the steady-state levels of oxidative DNA modifications sensitive to Fpg protein in AS52 cells. Cells were incubated with the antioxidants in full medium for the times indicated prior to DNA damage analysis. Data are means of three independent experiments ± SD.

Fig. 3. Influence of thermal stress and inhibition of GSH synthesis on the steady-state levels of oxidative DNA modifications sensitive to Fpg protein (left panel) and on the total levels of GSH (right panel) in AS52 cells. Cells were cultured at 37 and 41°C in the presence or absence of BSO (1 mM) in full medium for 24 h prior to analysis. Columns represent the means of three to five independent experiments ± SD.

decreased input of oxidative damage should become apparent as a reduced steady-state level after an incubation time that is longer than the repair time.)

Depletion of intracellular GSH and heat-shock increase the steady-state levels of oxidative DNA base modifications

When AS52 cells were depleted of GSH by incubation with BSO, an inhibitor of γ-glutamylcysteine synthetase (45,46), the steady-state level of Fpg-sensitive modifications increased by 50% (Figure 3, left panel). A rise in the cell culture temperature from 37 to 41°C in the presence of absence of BSO (1 mM) in full medium for 24 h prior to analysis. Columns represent the means of three to five independent experiments ± SD.

Table II. Influence of GSH depletion by BSO, thermal stress (41°C) and antioxidants on the plating efficiency of AS52 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative plating efficiency (%)a</th>
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<tbody>
<tr>
<td>1 mM BSO; 37°C; 24 h</td>
<td>93 ± 15 (3)</td>
</tr>
<tr>
<td>41°C; 24 h</td>
<td>51 ± 12 (3)</td>
</tr>
<tr>
<td>1 mM BSO; 41°C; 24 h</td>
<td>37 ± 20 (3)</td>
</tr>
<tr>
<td>1 mM CYSET; 37°C; 16 h</td>
<td>87 ± 21 (3)</td>
</tr>
<tr>
<td>10 mM NAC; 37°C; 16 h</td>
<td>110 ± 3 (2)</td>
</tr>
<tr>
<td>10 mM ascorbic acid; 37°C; 16 h</td>
<td>73 ± 11 (3)</td>
</tr>
</tbody>
</table>

aPlating efficiency of untreated control cells at 37°C defined as 100%. Number of experiments is given in parentheses.

these conditions, the plating efficiency of the AS52 cells was reduced to 37% of that of untreated control cells at 37°C (Table II).

The defence of cells against the deleterious effects of thermal stress involves the expression of heat-shock proteins such as Hsp27. The overexpression of Hsp27 in L929 mouse fibrosarcoma cells was shown to increase GSH, and the resistance to both heat and H2 O2 (30,47). To test the effect of Hsp27 on oxidative DNA damage, L929-27-3-97 cells, which were stably transfected with an hsp27 expression vector (see Materials and methods), were compared with the vector-only transformed L929-C2 cells. As shown in Figure 4, the overexpression of Hsp27 in the transformants was associated with a 1.6-fold increase in intracellular total glutathione, but the steady-state level of Fpg-sensitive modifications in the transformants was even slightly increased rather than decreased. Little or no effect of the Hsp27 overexpression on steady-state levels was observed in several other Hsp27-transformed clones that were analysed (data not shown).

Discussion

The results described here indicate a relatively high variation in the steady-state levels of oxidative DNA base modifications in different types of mammalian cells. In principle, differences in the metabolic generation of ROS, in the efficiency of
cellular antioxidants and in the efficiency of DNA repair, or a combination of these factors, could be responsible. The observed inverse correlation between the total cellular levels of glutathione and the basal levels of oxidative DNA base modifications (Figure 1) does not demonstrate a direct or linear dependence of the two parameters on each other. It is, however, an indication that GSH has an important role in the protection of cells from DNA damage by endogenously generated ROS, and that it is one of the factors that determines the steady-state level of oxidative DNA damage in cells. The high steady-state level of Fpg-sensitive modifications in the Fanconi’s anaemia cells H94-38T4 (Figure 1) was probably not caused by a repair deficiency of the cells, since the removal of additionally induced Fpg-sensitive modifications has been shown to be normal (48).

The protective effect of GSH is further indicated by the significant increase in the basal level of oxidative DNA base modifications in cells incubated for 24 h with BSO. This treatment reduces total glutathione concentration in the cells by ~90% (Figure 3). However, GSH depletion by BSO was shown to be less efficient in the nucleus than in the cytoplasm (49).

Hyperthermia is assumed to be associated with oxidative stress, possibly via mitochondrial uncoupling (50). Accordingly, the steady-state levels of Fpg-sensitive base modifications in AS52 cells were found to be increased by 40% under conditions of moderate hyperthermia (Figure 3). Heat-induced oxidative DNA damage is apparently alleviated by the concomitant 1.6-fold increase in cellular GSH levels, since a depletion of GSH by BSO at 41°C causes a much higher rise in the steady-state level of Fpg-sensitive base modifications, compared with that at 37°C (Figure 3).

Small heat-shock proteins induced by hyperthermia have been shown to provide protection against cytotoxicity and oxidative DNA damage induced by H2O2 and tumour necrosis factor α (TNFα) (30,47,51,52), although possibly not in all cell types (53). The data shown in Figure 4 indicate that Hsp27 overexpression in L929 cells does not reduce the steady-state level of Fpg-sensitive modifications. The overexpression might not only increase the glutathione levels but have more complex effects on the cellular factors relevant to the generation and removal of oxidative lesions.

The increase in oxidative DNA damage in AS52 cells after treatment with high concentrations of cysteine ethylester and ascorbic acid, but possibly also the failure of intermediate concentrations of these compounds to reduce the spontaneous level of oxidative DNA damage (Figure 2), may be explained by their potency to act as pro-oxidants, i.e. to generate ROS via auto-oxidation and/or the reduction of cellular transition metals (20–22,54). It is also possible that the protection by GSH and other thiols against endogenous ROS requires adequate concentrations of other factors in the cellular antioxidant defence system and is not, therefore, significantly improved if only the thiol concentrations are raised.

Acknowledgements

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References


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