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Use of the Comet Assay to Investigate the Role of Superoxide in Glutathione-Induced DNA Damage

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Although glutathione is an important scavenging molecule within the cell, it can also act as a pro-oxidant and at biological concentrations (1 mM) can induce DNA damage. We have used a sensitive cell-free Comet assay for DNA strand breakage to investigate this damage and to try to determine the active species involved. We show a substantial protection against glutathione-mediated DNA damage by superoxide dismutase (200 U/ml) and complete protection by combined superoxide dismutase and catalase. Damage is also prevented by EDTA but only at 100 mM and is not prevented by the chelating agent diethylenetriaminepentaacetic acid (100 μM). Although superoxide is known to potentiate DNA damage by other reactive species, none of these indirect mechanisms seem to account for our results and it is possible that superoxide may damage DNA directly. Under the same experimental conditions, S-nitrosoglutathione requires ultraviolet A photolysis to cause DNA strand breakage and superoxide dismutase increases the level of this damage. When intact human lymphocytes are incubated with glutathione (1 mM) in phosphate buffer, DNA damage is also observed, but in this case it is completely preventable by catalase, with no protective effect of superoxide dismutase. Since cellular scavenging systems are not completely protective against reactive species formed from autooxidation of extracellular glutathione and since glutathione and oxygen are ubiquitously present within cells, our results imply that cells may have a mechanism of preventing autooxidation, rather than simply relying on scavenging the reactive species formed. © 1998 Academic Press

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Abbreviations: DETAPAC, diethylenetriaminepentaacetic acid; GSH, glutathione, reduced form; GSNO, S-nitrosoglutathione; H_2O_2, hydrogen peroxide; PBSE, Dulbecco's phosphate buffered saline "A", + 1 mM EDTA; SOD, superoxide dismutase; UVA, ultraviolet light from a broad spectrum sunlamp with peak emission at 360 nm.

Key Words: **comet assay (single cell gel electrophoresis); DNA strand break; glutathione; superoxide; hydrogen peroxide; nitric oxide; S-nitrosoglutathione.**

The Comet assay (1-3) is a rapid and sensitive procedure for detecting DNA strand breakage in mammalian cells. We have used both a cell-free version (c.f. (4)) and a conventional version (1) of the assay to investigate the DNA-damaging action of glutathione (GSH) and of the naturally occurring nitrosothiol, S-nitrosoglutathione (GSNO). These compounds are likely to damage DNA through formation of reactive oxygen and nitrogen species, including superoxide, hydrogen peroxide (H_2O_2) and nitric oxide. Superoxide is scavenged by the enzyme superoxide dismutase (SOD) forming O_2 and H_2O_2 The H_2O_2 is in turn removed by catalase. Although both these enzymes can alter the response of cells to oxidative damage when added to the medium, they will not cross the cell membrane, so that failure to observe a protective effect may be because the reactive species is formed within the cell. The cell-free version of the Comet assay allows us to treat free DNA and measure strand breakage without the barrier of a lipid membrane or the presence of intracellular SOD or catalase, but with approximately 100-fold greater sensitivity than a plasmid nicking assay.

Using the cell-free assay with the naturally occurring nitric oxide donor S-nitrosoglutathione, we found DNA damage in the phosphate buffer and the glutathione controls. Glutathione in its reduced form (GSH) has an important protective role against a range of oxidative and other reactive species, and is present in mammalian cells at millimolar levels (5). Glutathione reacts directly with free radicals (6), protects against H_2O_2 via glutathione peroxidase, and detoxifies a range of potential mutagens and carcinogens via glutathione S-transferases (6, 7). However, like a number of other

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antioxidants, glutathione, especially in the form of the thiolate anion, has the capacity to act as a pro-oxidant and can cause the formation of superoxide anion from molecular oxygen (8, 9).

Superoxide is commonly thought not to be capable of reacting directly with DNA, but to cause DNA damage via dismutation to H_2O_2 and ultimate formation of hydroxyl radical. Thus SOD would not be expected to reduce the level of DNA damage observed in the presence of superoxide, and might even increase damage by increasing the level of H_2O_2. In certain cases, SOD has been shown to protect against oxidative DNA damage, but an indirect mechanism is involved. For instance, superoxide potentiates release of free transition metal from iron-sulfur proteins and can in this way increase the catalyzed formation of hydroxyl radical from H_2O_2 (10). Superoxide can also act as a reducing agent to recycle the transition metal (11) in a Haber-Weiss reaction, again allowing increased formation of hydroxyl radical from H_2O_2. Our present results show a substantial protective effect of SOD, which is not readily explained by either of these indirect mechanisms.

MATERIALS AND METHODS

Cells used. 50-80 ml blood samples from volunteer normal human subjects were taken with informed consent and the mononuclear fraction isolated by Ficoll separation using standard procedures (12). Aliquots of 3×10^6 cells were frozen in Heat-inactivated fetal calf serum +10% DMSO. For each series of experiments a sample was thawed and incubated in RPMI 1640 with 10% batch-tested human AB serum and glutamine (2 mM), penicillin (200 U/ml), streptomycin (200 μg/ml) and sodium pyruvate (200 μg/ml) at 37°C in a CO_2 incubator for at least 16 h and not more than 72 h (13).

Reagents. Materials for cell culture were obtained from Life Technologies Ltd., UK. Dulbecco's "A" Phosphate Buffered Saline, Oxoid, Basingstoke, UK; Laboratory biochemicals, BDH, Poole, UK or Sigma, Poole, UK. GSH was from Sigma (Cat. No. G4251, Lot No. 35H0010), was at least 99% pure, and contained no detectable cysteine by thin layer chromatography with an RF marker for cysteine (S Wells, Sigma, pers. comm.). Also from Sigma were SOD (Cat. no. S2515, Bovine erythrocyte); catalase, (Cat. no. C100, bovine liver,); diethylenetriaminepentaacetic acid (DETAPAC) (Cat no. D6518). GSNO was from Alexis, Nottingham UK. Solutions were prepared using water from an Elgastat Reverse osmosis/UHP system (Elga, High Wycombe, UK). In some experiments water was also passed through an Amberlite IR120plus (Sigma) cationic exchange column.

Comet assay. The standard Comet assay was performed as described previously (3). Briefly, mononuclear cells (2×10^4 per slide) were embedded on frosted slides, in duplicate, in 45μl of a 0.6% low melting agarose layer (made up with Dulbecco 'A' PBS + 1 mM EDTA (PBSE)), on top of an 85μl 0.6% base agar layer under a 22 mm $\times$ 22 mm coverslip. No third layer was applied (14). For treatment, 100μl of the test solution (containing damaging and protective agents) was applied to the surface of the agar and a coverslip added. Concentrations were calculated assuming that the test agents diffused uniformly through both agar layers. The slide was incubated at 37°C in a CO_2 incubator for 1 h in a closed light-proof container over moist tissue. After 5 min the coverslip was removed in order to facilitate perfusion of oxygen. Slides were transferred to lysis mixture (2.5 M NaCl, 200 mM NaOH, 100 mM EDTA-Na_2, 10 mM Tris base, 10% DMSO and 1% Triton X-100, pH 10) and incubated for at least 1 h at 4°C. Slides were transferred to alkaline buffer (300 mM NaOH, 1 mM EDTA-Na_2), incubated for 40 min and electrophoresis (20 V for 24 min) applied. Following electrophoresis, slides were rinsed with Tris buffer (400 mM Tris base, pH 7.5), 30μl ethidium bromide solution (20μg/ml) was applied and the slides viewed with a x10 objective under a fluorescence microscope. Comet length was determined using Casys software (Synoptics, Cambridge, UK).

For the cell-free Comet assay, a procedure similar to that of Collins *et al.* (14) was used. Cell suspensions were embedded on frosted slides, in duplicate as above. Slides were placed immediately in standard lysis mix and left for at least 1 h at 4°C. The levels of EDTA (100 mM) and Tris (10 mM) in the lysis mixture are strongly protective and must be rinsed away (see Results below). The slides were immersed three times for 5 min in a staining jar containing PBSE, then placed on a rack and a further rinse applied to the surface of the agar. (1 mM EDTA was added to the phosphate buffer to avoid variation in residual levels of EDTA). The test agent and protective enzymes were added in 100μl PBSE to the surface of the agar and the slides were incubated at 37°C for 1h as described above. Following treatment, slides were placed directly in alkaline buffer, incubated for 20 min and electrophoresis (20 V for 24 min) applied. The remaining steps were as above.

It should be noted that the cell-free system of Kasamatsu et al. (4) uses conditions designed to eliminate generation of the oxidative damage studied in the present paper.

For UVA irradiation of GSNO, a bank of two Thorn Atlas broad spectrum sunlamps (peak emission 360 nm) was used and 60 Jm^{-2} applied to the surface of the slide. This fluence causes no detectable DNA damage in the absence of GSNO.

Statistical analysis. A typical experiment consisted of 8 treatments or controls, with duplicate slides for each treatment and 25 comets scored for each slide. Mean comet length was determined for each treatment in each experiment. These values were in turn averaged and the standard error of the mean determined. Figures presented are mean comet length (μm) and its standard error for all experiments with that treatment. For statistical analysis, only those experiments where the comparison was made were considered and a paired t-test was performed. Numbers of experiments are indicated in each case. All results are based on at least three independent experiments.

RESULTS

In experiments using the cell free Comet assay to study activity of the naturally occurring nitric oxide donor GSNO, we found evidence of DNA damage in the GSH controls. Initially the level of damage was observed to be extremely variable between experiments. We eventually found that the inconsistency was linked to carryover of protective ingredients contained in the lysis mixture used to prepare the slides for treatment (see Methods). Figure 1 shows DNA damage following treatment with GSH (1 mM), but complete protection against GSH damage by the level of EDTA (100 mM) found in the lysis mixture. In all experiments rinses were performed with buffer containing the same level of EDTA as was used during the treatment with GSH. EDTA at 1 mM has only a small effect on GSH damage.

In contrast to GSH, GSNO causes little damage in a cell free system, and in order to detect significant activity, we have used photolysis by very low levels of UVA irradiation (60 J/m^{-2} from a broad spectrum sunlamp

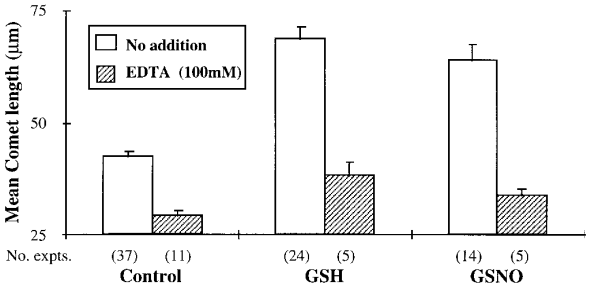

FIG. 1. DNA damage induced by GSH (1 mM) or GSNO (1 mM) + UVA (60 Jm^{-2}) in the cell-free Comet assay, in the presence or absence of EDTA (100 mM). Damage is determined as Comet length (μm). Shown are the mean and standard error of the mean for all experiments with the specific treatment. Figures in brackets represent the number of individual experiments. Statistical comparison (all by paired T-test for those experiments where the comparison was made) were as follows: Control vs. GSH, 24 experiments, $P<0.0001$; Control vs. GSNO + UVA, 5 experiments. $P<0.001$; Control vs. Control + EDTA, 7 experiments, $P<0.01$; GSH vs. GSH + EDTA, 3 experiments, $P<0.05$; GSNO vs. GSNO + EDTA, 4 experiments, $P<0.01$.

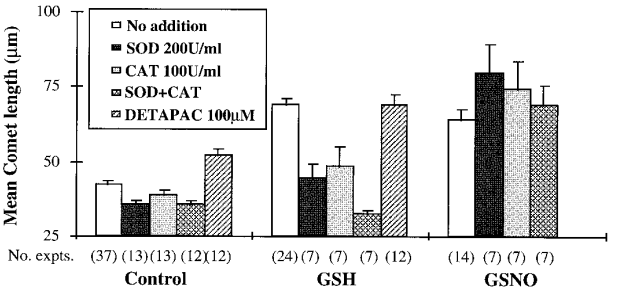

FIG. 2. DNA damage induced by GSH (1 mM) or GSNO (1 mM) + UVA (60 Jm^{-2}) in the cell-free Comet assay, in the presence or absence of (i) SOD (200 U/ml), (ii) catalase (100 U/ml), (iii) SOD (100 U/ml) + catalase (50 U/ml) or (iv) DETAPAC (100μM). Legend as for Fig. 1. Statistical comparisons: Control vs. Control + SOD 13 experiments, $P<0.002$; Control vs. Control + catalase 12 experiments, $P=0.2$; Control + DETAPAC vs. GSH + DETAPAC, 12 experiments $P<0.001$; Control vs. control + DETAPAC, 12 experiments $P<0.01$; GSH vs. GSH + SOD, 7 experiments, $P<0.03$; GSH vs. GSH + catalase, 6 experiments., $P<0.02$; GSH + SOD vs. GSH + SOD + catalase, 6 experiments, $P<0.05$; GSNO vs. GSNO + SOD, 7 experiments, $P<0.05$.

with peak emission at 360 nm) Figure 1 shows damage by GSNO + UVA and complete protection by 100 mM EDTA. It is clear that GSNO + UVA is a DNA-strand-breaking agent, presumably via photolytic release of nitric oxide or thiyl radical.

In all these experiments, a degree of DNA damage could also be observed in the controls, although this was moderated by the use of EDTA (1 mM) in the phosphate-buffered incubation mixture (data not shown). EDTA (100 mM) prevented this 'spontaneous' damage (Figure 1).

In order to characterize the mechanism of DNA damage further, we treated with GSH in the presence of SOD (200 U/ml), catalase (100 U/ml), both SOD and catalase (100 + 50 U/ml) or diethylenetriaminepentaacetic acid (DETAPAC, 100 μM). It can be seen from Fig. 2 that both SOD and catalase protected against GSH-mediated strand breakage. The combination of both agents (at half strength) was more effective than either agent alone and provided virtually complete protection. DETAPAC did not protect against GSH-induced strand breakage in the cell-free system.

Figure 2 also shows that SOD was effective in preventing 'spontaneous' damage but catalase was not protective and DETAPAC appeared to increase damage. In contrast to protecting against GSH and 'spontaneous' damage, SOD significantly increased the strand breakage induced by GSNO + UVA . Catalase did not affect GSNO + UVA damage, indicating that the enhancement of damage by SOD was not related to formation of H_2O_2.

Figure 3 shows that when intact lymphocytes were treated with 1 mM GSH DNA damage was still observed. However, in contrast to results with 'naked' DNA, SOD offered no protection to intact cells and catalase offered almost complete protection. DETAPAC appeared to provide protection but the effect did not achieve significance ($P=0.07$). With intact cells, negligible levels of damage were seen in the absence of GSH (Fig 3).

DISCUSSION

The cell-free Comet assay, as used in these experiments, enables us to measure very low levels of damage in high molecular weight DNA held in a simple phosphate buffer. Under these conditions, a significant level of 'spontaneous' DNA damage occurs, and a higher level of damage is observed in the presence of GSH. In both cases, damage is prevented by SOD rather than catalase. With intact cells, there is little spontaneous

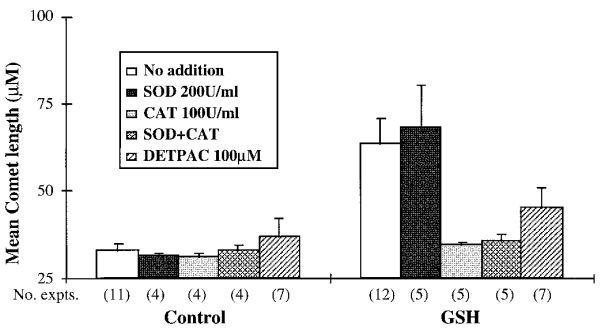

FIG. 3. DNA damage induced by GSH (1 mM) in intact mononuclear cells in the presence or absence of protective agents. Legend as for Fig 2. Statistical comparisons: Control vs. Control + GSH, 11 experiments, $P<0.01$; GSH vs. GSH + SOD, 5 experiments, NS; GSH vs. GSH + catalase, 5 experiments, $P<0.05$; GSH vs. GSH + DETAPAC, 7 experiments, $P=0.07$.

damage, SOD offers no protection, and catalase offers virtually complete protection against GSH-mediated damage.

The result with intact cells is entirely consistent with a conventional model of reactive oxygen species damage. Superoxide dismutates to H_2O_2, which can enter the cell and form hydroxyl radical via a Fenton-type mechanism. If H_2O_2 is removed by catalase, or transition metals are sequestered by DETAPAC, damage is likely to be reduced. In contrast, damage to free DNA appears to occur by a different mechanism, with SOD playing a significant role in protection. A protective effect of SOD does not constitute definitive evidence that superoxide can damage DNA directly. It is necessary to exclude indirect mechanisms whereby superoxide could increase the DNA damage caused by another reactive species.

We have considered four possible indirect mechanisms of superoxide-enhanced DNA damage but none of these appear to explain our results: (a) Brawn and Fridovich (11) have shown superoxide acting by recycling a transition metal and increasing formation of hydroxyl radical from H_2O_2. It is unlikely, however, that superoxide would be a rate-limiting reducing agent in the presence of glutathione. (b) Keyer and Imlay (10) have shown that superoxide can release intracellular bound iron which then catalyses the formation of hydroxyl radical from H_2O_2. The effect that we see occurs in phosphate buffer, following removal of other cellular constituents. (c) Winterbourn and Metodiewa (15) have shown that superoxide can catalyze the autoxidation of glutathione, which might lead to increased formation of H_2O_2. SOD, however, also protects against 'spontaneous' damage, where glutathione is not present. Moreover, if this were the mechanism of enhancement, one might expect SOD to protect against GSH damage in intact cells. Prevention of autoxidation outside the cell would reduce formation of H_2O_2. (d) Thiyl radical might be formed during autoxidation of GSH and is known to have DNA-damaging activity. Again, SOD protects against 'spontaneous' damage, where thiyl radical cannot be involved. Moreover, photolysis of GSNO may also yield thiyl radical and in this case DNA damage is enhanced by SOD.

We therefore suggest that the simplest explanation of our results is that superoxide has some ability to react directly with DNA and cause strand breakage. Sah et al. (9) have also shown protection against DNA damage by SOD, under conditions where an indirect mode of action of superoxide could not easily be envisaged. Since superoxide readily dismutates to H_2O_2, and transition metals are present in most assay systems, a direct damaging activity will normally be masked by formation of hydroxyl radical. Our particular assay system combines sensitive detection of strand breakage (better than 1 break per 10^7 base pairs) with very low levels of transition metal catalyzed formation of hydroxyl radical. Although our results suggest that superoxide can damage DNA directly, they are entirely consistent with the conventional view that H_2O_2 has no direct DNA-damaging activity, and acts via formation of hydroxyl radical.

In the cell-free Comet assay GSNO does not appear to be capable of GSH-like autoxidation but it readily causes DNA damage following a low fluence of UVA. Following photolysis, the oxidation product of nitric oxide is nitrite rather than nitrate. Damage is enhanced by SOD but is not affected by catalase, arguing against a mechanism involving formation of H_2O_2 by SOD. It is possible that damage is associated with formation of thiyl radical rather than nitric oxide, but it is not clear why SOD would increase such damage. Alternative explanations could be, (a) the enhancement of damage is through the direct reaction of nitric oxide with SOD to yield NO^- (16) or (b) SOD is removing spontaneously-formed superoxide which would otherwise react with NO to form peroxynitrite ($ONOO^-$) (17, 18). Although peroxynitrite is highly reactive, if a significant proportion isomerises directly to nitrate the net effect may be to reduce damage.

Although superoxide is normally considered a relatively unreactive radical, SOD is almost ubiquitous in living organisms. The capacity to convert superoxide to an apparently equally dangerous species appears essential. At least one familial neurodegenerative disorder, amyotrophic lateral sclerosis, is associated with a variant form of Cu/Zn SOD (19, 20). All this suggests that superoxide is not innocuous *in vivo*. The versatility and sensitivity of the Comet assay make it a useful tool in examining such issues.

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REFERENCES

1. Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988) *Expl. Cell Res.* **175,** 184–191.
2. Ostling, O., and Johanson, K. J. (1984) *Biochem. Biophys. Res. Commun.* **123,** 291–298.
3. Green, M. H. L., Lowe, J. E., Delaney, C. A., and Green, I. C. (1996) *Meth. Enzymol.* **269,** 243–266.
4. Kasamatsu, T., Kohda, K., and Kawazoe, Y. (1996) *Mutation Res.* **396,** 1–6.
5. Halliwell, B., and Gutteridge, J. M. C. (1989) Free Radicals in Biology and Medicine, Clarendon Press, Oxford.
6. Meister, A. (1995) *Meth. Enzymol.* **251,** 3–8.
7. Meister, A., and Anderson, M. E. (1983) *Ann. Rev. Biochem.* **52,** 711–760.
8. Winterbourn, C. C., and Metodiewa, D. (1995) *Meth. Enzymol.* **251,** 81–86.

9. Sah, N. K., Kumar, S., Subramanian, M., and Devasagayam, T. P. A. (1995) *Biochem. Molec. Biol. Intl.* **35,** 291–296.
10. Keyer, K., and Imlay, J. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13635–13640.
11. Brawn, K., and Fridovich, I. (1981) *Arch. Biochem. Biophys.* **206,** 414–419.
12. Cole, J., Green, M. H. L., James, S. E., Henderson, L., and Cole, H. (1988) *Mutation Res.* **204,** 493–507.
13. Green, M. H. L., Waugh, A. P. W., Lowe, J. E., Harcourt, S. A., Cole, J., and Arlett, C. F. (1994) *Mutation Res.* **315,** 25–32.
14. Collins, A. R., Duthie, S. J., and Dobson, V. L. (1993) *Carcinogenesis* **14,** 1733–1735.
15. Winterbourn, C. C., and Metodiewa, D. (1994) *Arch. Biochem. Biophys.* **314,** 284–290.
16. Murphy, M. E., and Sies, H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10860–10864.
17. Beckman, J., and Tsai, J.-H. (1994) *Biochemist* **16,** 8–10.
18. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.
19. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H.-X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeldt, B., Van den Bergh, R., Hung, W.-Y., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericack-Vance, M. A., Haines, J., Rouleau, G. A., Gusella, J. S., Horvitz, H. R., and Brown, R. H. (1993) *Nature* **362,** 59–62.
20. Beckman, J. S., Carson, M., Smith, C. D., and Koppenol, W. H. (1993) *Nature* **364,** 584.