Free Rad. Res., Vol. 21, No. 1, pp. 9-17
Reprints available directly from the publisher
Photocopying permitted by license only

THE AMBIVALENT ROLE OF GLUTATHIONE IN THE PROTECTION OF DNA AGAINST SINGLET OXYGEN

M. VINCENT M. LAFLEUR*, JASPER J. HOORWEG*, HANS JOENJE†, E. JOKE WESTMIJZE* and JAN RETÈL*

**Department of Medical Oncology and †Institute of Human Genetics, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT, Amsterdam, The Netherlands*

(Received October 12, 1993; in revised form January 13, 1994)

Glutathione (GSH) was examined with respect to its ability to protect DNA against $^1O_2$ damage. We have found that GSH protected, at least partly, the DNA against inactivation by $^1O_2$. Up to 10 mM the protection increased as a function of GSH concentration. Above 10 mM the protection remained constant and less than expected on the basis of scavenging/quenching of $^1O_2$, in contrast to the protection offered by sodium-azide. Especially at the higher concentrations of GSH the protection against the biological inactivation is accompanied by an increase in single-strand breaks and also probably lethal base damage. However, all together the data suggest that at least in the physiologically important range (0.1-10 mM) GSH is able to protect efficiently against $^1O_2$-induced inactivating DNA damage.

KEY WORDS: $^1O_2$, GSH, DNA, Inactivation, Protection.

INTRODUCTION

A major ever-existing threat to living cells is oxidative stress leading to the formation of reactive oxygen species. The most important oxidative damage is supposed to be inflicted into the cellular DNA and seems to be related to processes like aging, mutagenesis and carcinogenesis.[1-3]

An important oxidizing species, although less-well documented than the OH radical, is singlet oxygen, which is capable of damaging various cell constituents including DNA. In DNA singlet oxygen reacts exclusively with the guanine base resulting in the formation of several products (more than 14) like 7,8-dihydro-8-oxoguanine, alkali-labile sites and a few strand-breaks.[4-6]

The tripeptide glutathione (GSH) is the major non-protein sulphydrylic compound in mammalian cells known to play an important role in the cellular defence against various hazardous agents of endogenous as well exogenous origin. It has been well established that thiols can protect against the action of the OH radical.[7-9] Also for singlet oxygen it has been demonstrated that it reacts with thiols resulting in several oxidation products.[10,11] Recently some papers reported enhancement of DNA damage (single-strand breaks; 7,8-dihydro-8-oxoguanine) by thiols such as GSH in the presence of singlet oxygen.[12,13] However, it is unknown whether such a

Corresponding author: Dr. M.V.M. Lafleur, Dept. of Medical, Oncology, c/o Institute of Human Genetics, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.

thiol-induced increase of the DNA damage is of any relevance for the biological functioning of the DNA. To fill in this gap we decided to investigate the biological consequences of such reactions by using biologically active DNA. We have found that despite an increase in damage (both base damage and single-strand breaks) the DNA still will be protected against inactivation by singlet oxygen, especially at physiologically relevant concentrations of GSH (0.1–10 mM).

MATERIALS AND METHODS

Drugs and Chemicals

The singlet oxygen generator $NDPO_2$ was synthesized as described by Aubry.[14] Thermal dissociation of $NDPO_2$ yields 3,3'-(1,4-naphtylidene) dipropionate (NDP) and molecular O_2, half of which is in the triplet ground state, the other half being in the excited singlet state. The flux and cumulative concentration of $^1O_2$ was calculated from the increase in absorbance at 288 nm, at which NDP (but not $NDPO_2$) has an absorbance peak ($\epsilon = 7780\ M^{-1}\,cm^{-1}$).[14] Glutathione (GSH) was obtained from Sigma Chemical Company, U.S.A., and sodium-azide (NaN_3), *tert*-butanol and isopropanol from Merck A.G., Germany. Before use the chemicals were freshly dissolved and the solutions were deaerated by flushing nitrogen through the solution. All solutions were prepared in triple distilled water. All standard chemicals were of analytical grade and used as supplied.

Exposure Conditions

$NDPO_2$ was added to solutions containing single-stranded ΦX174 DNA (5–10 μg/ml) in the absence or presence of GSH, sodium-azide, *tert*-butanol or isopropanol. Incubation was at 37°C in the dark in 50 mM K/Na phosphate buffer pH 7.4. At various time intervals samples were taken and diluted at least 100-fold before transfection to *E. coli*. For analysis on sucrose gradients the samples were diluted 10-fold.

DNA and Transfection Assays

Single-stranded ΦX174 DNA was prepared as described by Blok *et al.*[15] The plaque-forming activity of the ΦX174 DNA was determined on *E. coli* (AB 1157) spheroplasts essentially as described by Lafleur *et al.*[16] Survival curves were obtained by plotting the logarithm of the fraction of surviving DNA (measured as the number of produced bacteriophages) against time or (cumulative) concentration of singlet oxygen.

Determination of Strand Breaks

Linear neutral sucrose gradients (5–40% (w/v) in 10 mM NaCl, 0.1 mM EDTA, 1 mM phosphate buffer pH 7.2) were prepared in cellulose nitrate tubes. Centrifugation was for 20 h at 20°C, and 36000 r.p.m. in a Beckman SW41 rotor. After centrifugation the gradients were analysed as described.[17]

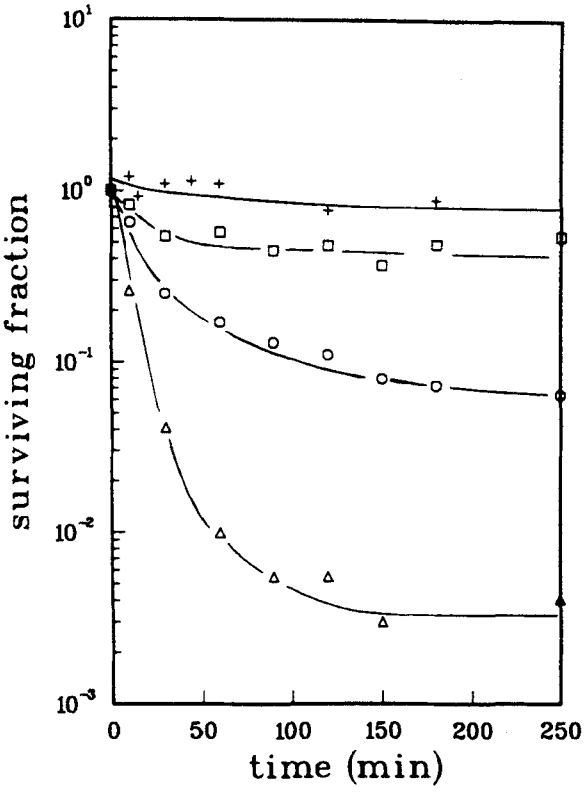

FIGURE 1 Loss of biological activity of single-stranded ΦX174 DNA (5 μg/ml) as a function of time in the presence of $^1O_2$-generating $NDPO_2$ with or without GSH. △-△, 1.1 mM $^1O_2$ (cumulative conc.); □-□, 0.26 mM $^1O_2$; ○-○, 1.1 mM $^1O_2$ +25 mM GSH; +-+, 0.26 mM $^1O_2$ + 25 mM GSH.

RESULTS

The Effect of Glutathione on the DNA Inactivation by $^1O_2$

To get an impression of the protective potential of thiols different concentrations of $NDPO_2$, releasing singlet oxygen, were allowed to react with single-stranded ΦX174 DNA for increasing periods of time in the presence or absence of glutathione (representative curves are given in Figure 1). As is demonstrated in the figure the inactivation can be described in both situations with kinetics that parallels the kinetics of $NDPO_2$ decomposition, which has been completed after about 180 min. and a plateau is reached. Prolonging of the incubation to 24 h did not induce more inactivation. Therefore the plateau value can be used as a measure for the degree of inactivation. In Figure 2a the surviving fraction obtained from plateau values as function of the cumulative $^1O_2$ concentration are presented. In Figure 2b the survival is shown after 15 min. of incubation. The latter experiments were performed to determine the effects at initially high concentrations of $^1O_2$ where no plateau values could be expected because of too extensive inactivation. In both situations the DNA is protected against $^1O_2$ by GSH to the same extent.

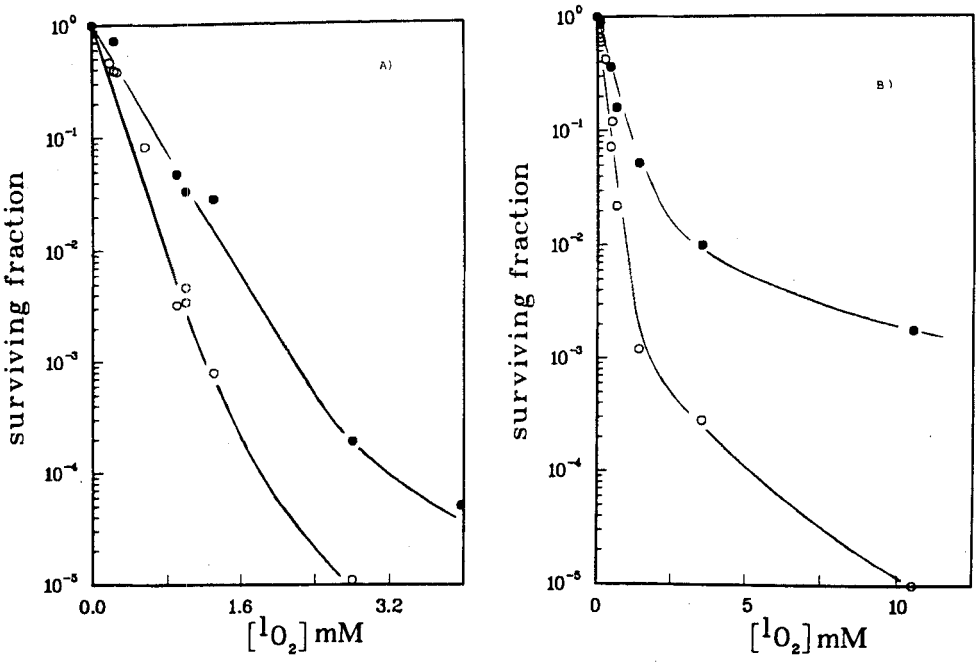

FIGURE 2 Representative survival curves for single-stranded ΦX174 DNA (5 μg/ml) after exposure to $^1O_2$ generated from $NDPO_2$ for (a) ≥ 180 min and (b) 15 min of incubation. ○-○, in the absence or, ●-●, in the presence of 25 mM GSH.

Modulation of $^1O_2$-induced Strand Breaks by GSH or NaN_3

As can be inferred from the work with plasmid DNAs[12,13] it can be expected that thiols will influence the amount of breaks in the reaction of singlet oxygen with single-stranded DNA. Indeed the induction of single-strand breaks has been altered by the presence of GSH as can be seen in Figure 3. The single-strand break curves are presented together with their corresponding survival curves showing that protection against inactivation is accompanied by a considerable increase in single-strand break formation. From the slopes of the curves the concentration of $^1O_2$ (dose D) at which on the average one lethal event, with respect to the survival, (D = 0.21 mM in the absence and 0.41 mM in the presence of GSH) or one single-strand break (D = ≥ 14 mM in the absence and ≥ 3.8 mM in the presence of GSH) per DNA molecule is introduced can be calculated. From these data the maximum relative contribution of the single-strand breaks, which are lethal in single-stranded DNA, to the biological inactivation can be determined, ≤ 2% for $^1O_2$ and ≤ 10% in the presence of GSH/$^1O_2$. In contrast to GSH, NaN_3, a wellknown scavenger/quencher for $^1O_2$, inhibited the induction of strand breaks as well as inactivation induced by $^1O_2$ (an example has been given in Figure 4), leaving the contribution of single-strands to the inactivation unchanged (≤ 2%). The specific OH radical scavengers *tert*-butanol (Figure 4) and isopropanol showed neither protection nor increase of damage, both in the absence and presence of GSH, ruling out the possible involvement of oxygen radicals.

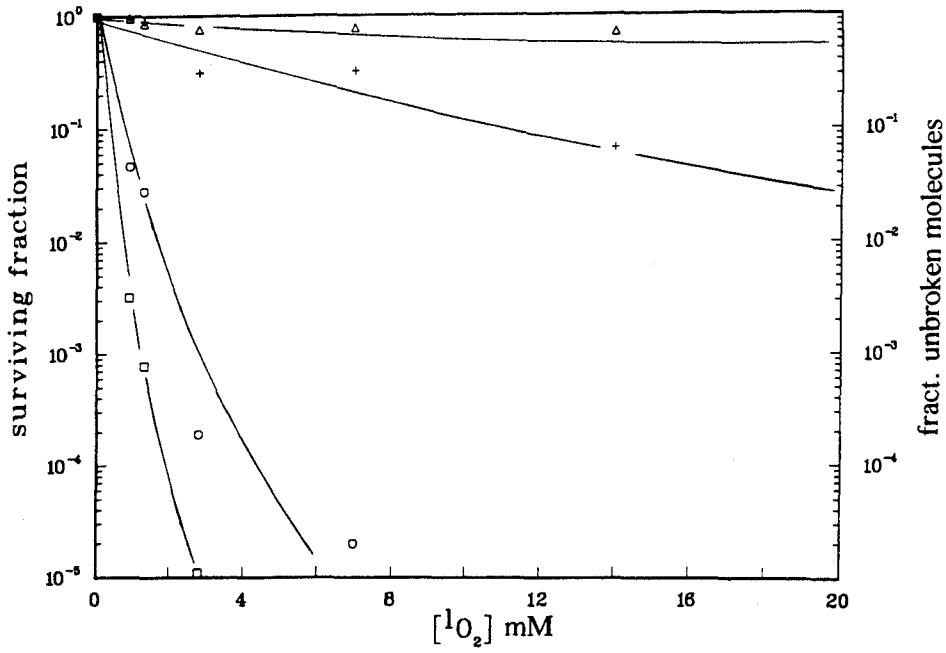

FIGURE 3 Representative survival curves (□-○) together with their respective single-strand break induction curves (+-△) for single-stranded ΦX174 DNA (10 μg/ml) after exposure to $^1O_2$ generated from $NDPO_2$ for ≥ 180 min. □-△, in the absence or, ○-+, in the presence of 25 mM GSH.

Comparison between GSH and NaN_3 as Protectors against DNA Inactivation

As is shown in Figures 1, 2 it appears that 25 mM GSH protects the DNA against singlet oxygen inactivation. To investigate to what extent GSH is able to protect, experiments were performed in which the survival at different concentrations of GSH was determined. From these data the protection factor (dose-modifying factor; DMF), defined as the ratio of doses required to give the same biological effect in the absence or presence of GSH (see e.g. Figure 2a), as a function of the GSH concentration could be calculated (Figure 5), showing a maximum protection factor of about 2.4. Again data obtained from experiments as presented in Figure 2b yielded similar results. We observed that at concentrations higher than about 40 mM GSH, GSH itself has a considerably lethal effect on the biological activity of the DNA. For that reason those concentrations of GSH were not employed. In order to detect possible sensitizing effects (extra lethal damage) of thiols we compared GSH with sodium-azide (NaN_3), which is known to function only as a scavenger/quencher for $^1O_2$, for its protecting efficiency. In Figure 5 the dependence of the protection factor (DMF) on the concentration ([S]) of GSH or NaN_3 expressed as the scavenging/quenching capacity, k_s[S], is shown. k_s is the rate constant for the reaction $^1O_2$ + S. It is evident that the data obtained with GSH do not fit the sodium-azide protection very well, especially in the higher region the protection offered by GSH is lower and does not longer increase with increasing concentration.

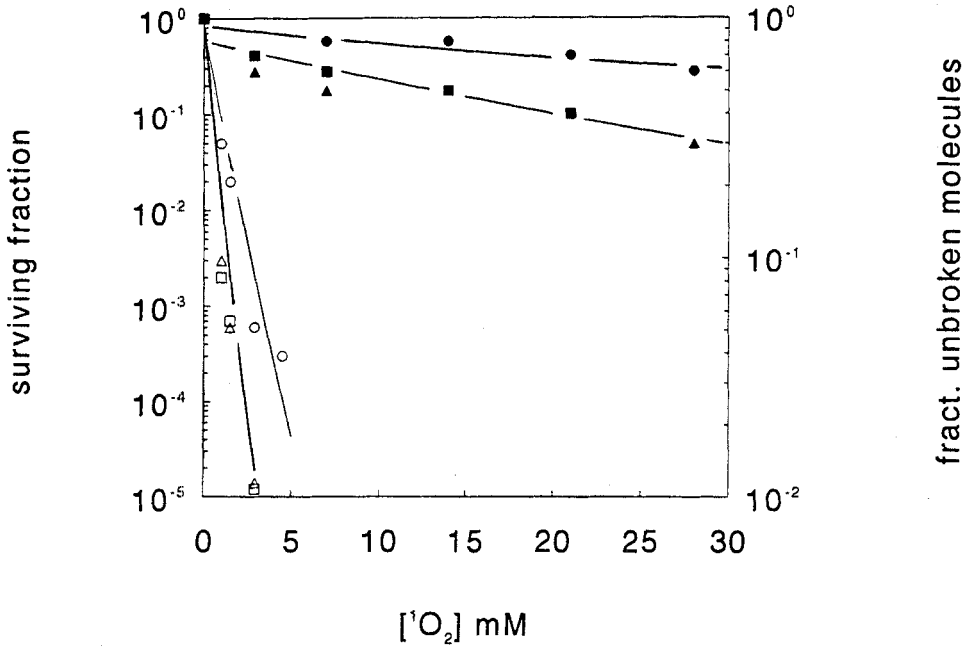

FIGURE 4 Representative survival curves (□-○-△) together with their respective single-strand break induction curves (■-●-▲) for single-stranded ΦX174 DNA (10 μg/ml) after exposure to $^1O_2$ generated from $NDPO_2$ for ≥180 min. □-■, in the absence; ○-●, in the presence of 0.1 mM NaN_3; △-▲, or in the presence of 100 mM *tert*-butanol.

DISCUSSION

Almost all of the experiments have been performed with single-stranded DNA, because of its higher reactivity for $^1O_2$ than double-stranded DNA. Moreover single-stranded DNA is more sensitive to lethal damage than the double-stranded form, because no enzymatic repair can take place. Therefore damage can be detected in a more distinct and easier way in single-stranded DNA. In the presence of a thiol like GSH there is a significant protection against the DNA inactivation by $^1O_2$ resulting in a maximum protection of a factor of about 2.4. At first sight these results seem to be in contrast with the reported damage-enhancing effects of thiols like more single-strand break.[12,13] Therefore the breaks formed by $^1O_2$ in the presence and absence of GSH were analysed. The results (Figure 3) show that the amount of breaks inflicted into the DNA by $^1O_2$ contributes less than 2% to the biological inactivation of single-stranded ΦX174 DNA, demonstrating that the majority of the lethal lesions is different in nature. This small yield of breaks is consistent with earlier data.[4,5,17,18] In the presence of 25 mM GSH the number of strand breaks increases with a factor of about 4. However, at the same time there is a 2-fold protection against inactivation. This can be explained as follows: As stated above less than 2% of the lethal damage by $^1O_2$ is due to single-strand breaks. This means that although in the presence of GSH the number of strand breaks increases considerably and every strand break is lethal in single-stranded DNA, these strand breaks still will represent

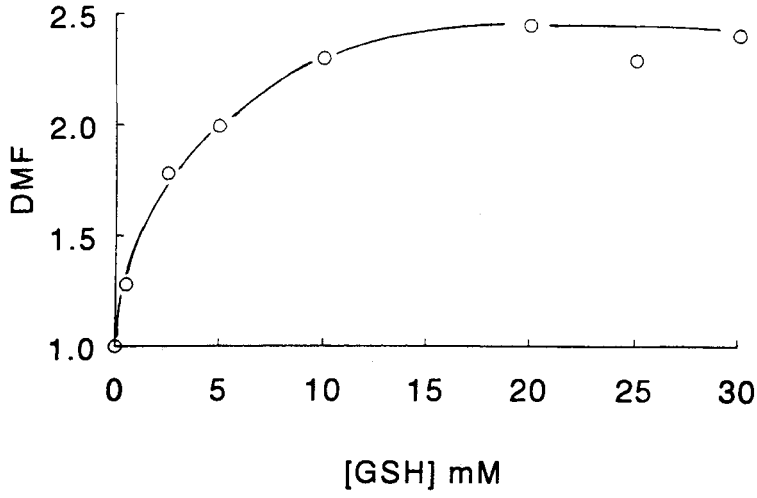

FIGURE 5 Dependence of the protection factor (DMF) of single-stranded ΦX174 DNA (5 μg/ml) exposed to $^1O_2$ on the concentration of GSH. Data, which are presented at the mean from the independent experiments, are derived from similar curves as presented in Figure 2a.

only a small part (at most 10%) of the total lethal damage. Furthermore the results demonstrate that protection against lethal damage due to quenching/scavenging of $^1O_2$ by glutathione is still the determining factor.

Moreover the lack of inhibition of the effects by *tert*-butanol and isopropanol, which are specific OH radical scavengers, in contrast to sodium-azide, which is a specific scavenger/quencher of $^1O_2$, demonstrates clearly that as might be expected $^1O_2$ but not OH radicals are involved.[13]

Preliminary results obtained with double-stranded ΦX174 DNA suggest that also here the amount of breaks increases dramatically in the presence of GSH, in line with the data of Devasagayam *et al.* with pBR322 plasmid DNA,[12] but that again substantial protection against the inactivation will be observed. This might imply that these extra single-strand breaks do not contribute to lethality, which is not surprising because single-strand breaks are usually enzymatically repaired in double-stranded ΦX174 DNA after transfection to its host.[4]

From the comparison with sodium-azide, which acts solely as a scavenger/quencher for $^1O_2$, it can be concluded that the protection against lethality offered by GSH at higher concentrations is lower than can be expected based on its capacity to scavenge/quench $^1O_2$. Moreover, the GSH-curve (see Figures 5,6) is rather similar to the curves obtained with DNA-inactivating secondary radicals, which are derived from reactions of radiation-produced ·OH with various organic compounds.[8,19] These results indicate that most probably a reactive species formed through the reaction of $^1O_2$ with GSH is capable of inducing, apart from single-strand breaks, lethal damage into DNA, which is not associated with single-strand breaks, but rather with base damage. Although this finding seems to be in line with the observation of Devasagayam *et al.*[13] who found an increase in the production of 8-hydroxy-(d)G in the presence of thiols it is rather unlikely that these products are responsible for the increased lethality, since it was shown that 7,8-dihydro-8-oxoguanine does not act as a blocking lesion and that cytosine is preferentially incorporated opposite this

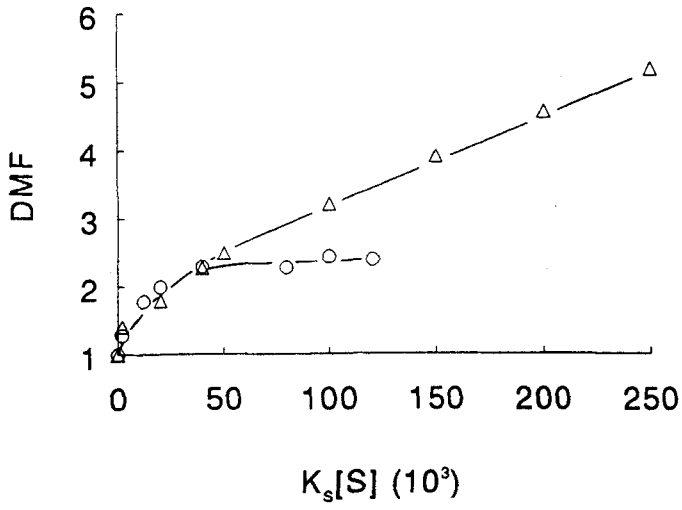

FIGURE 6 Dependence of the protection factor (DMF) of single-stranded ΦX174 DNA (5 μg/ml) on the scavenging/quenching capcity (k_s[S]) for $^1O_2$. ○-○, GSH; △-△, NaN_3. Data (mean; n = 3) are derived from comparable experiments as presented in Figure 2a. (k_s: GSH = 2.9×10^6 M^{-1} s^{-1}; NaN_3 = 2.2×10^8 M^{-1} s^{-1}).

particular base modification.[20,21] Furthermore Lutgerink *et al.*[5] showed that the contribution of 7,8-dihydro-8-oxoguanine to $^1O_2$ induced inactivation of single-stranded DNA is at most rather small. Finally the results demonstrate that at concentrations of GSH <10 mM, GSH protects as efficient as NaN_3 against DNA inactivation by $^1O_2$.

In conclusion, our results show that GSH plays an ambivalent role in the protection of DNA against inactivation by $^1O_2$. On the one hand it increases the production of single-strand breaks and base damage on the other hand it still protects the DNA against lethality. This means that in most physiological situations scavenging/quenching of $^1O_2$ by GSH will be an important cellular defence mechanism.

Acknowledgement

This work was supported by the Netherlands Cancer Foundation (Grant IKA 88-11).

References

1. D. Harman (1981) The aging process. *Proceedings National Academy of Science USA*, **78**, 7124-7128.
2. B.N. Ames, L.S. Gold (1991) Endogenous DNA damage as related to cancer and aging. *Mutation Research*, **250**, 3-16; 1991.
3. P.A. Cerutti (1991) Oxidant stress and carcinogenesis. *European Journal Clinical Investigations*, **21**, 1-5.
4. H. Joenje, M.V.M. Lafleur and J. Retèl (1991) Biological consequences of oxidative DNA damage. In: *Membrane lipid Oxidation*. Vol 3. (ed. C. Vigo-Pelfrey) Boca-Raton: CRC Press, pp. 87-113.
5. J.T. Lutgerink, E. Van den Akker, I. Smeets, D. Pachen, P. Van Dijk, J-M. Aubry, H. Joenje, M.V.M. Lafleur and J. Retél (1992) Interaction of singlet oxygen with DNA and biological consequences. *Mutation Research*, **275**, 377-386.

6. H. Sies and C.F.M. Menck (1992) Singlet oxygen induced DNA damage. *Mutation Research*, **275**, 365–375.
7. E.A. Bump and J.M. Brown (1990) Role of glutathion in the radiation response of mammalian cells in vitro and in vivo. *Pharmacology Therapy*, **47**, 117–136.
8. M.V.M. Lafleur and H. Loman (1986) Radiation damage to ΦX174 DNA and biological effects. *Radiation Environmental Biophysics*, **25**, 159–173.
9. M.V.M. Lafleur and J. Retèl (1993) Contrasting effects of SH-compounds on oxidative DNA damage: repair and increase of damage. *Mutation Research*, **295**, 1–10.
10. M. Rougee, R.V. Benasson, E.J. Land and R. Pariente (1988) Deactivation of singlet molecular oxygen by thiols and related compounds, possible protectors against skin photosensitivity. *Photochemistry Photobiology*, **47**, 485–489.
11. T.P.A. Devasagayam, A.R. Sundquist, P. Di Mascio, S. Kaiser and H. Sies (1991) Activity of thiols as singlet molecular oxygen quenchers. *Journal Photochemistry Photobiology*, **B9**, 105–116.
12. T.P.A. Devasagayam, P. Di Mascio, S. Kaiser, and H. Sies (1991) Singlet oxygen induced single-strand breaks in plasmid pBR322 DNA: the enhancing effect of thiols. *Biochimica et Biophysicsa Acta*, **1088**, 409–412.
13. T.P.A. Devasagayam, S. Steenken, M.S.W. Obendorf, W.A. Schulz and H. Sies (1991) Formation of 8-hydroxy(deoxy)guanosine and generation of strand breaks at guanine residues in DNA by singlet oxygen. *Biochemistry*, **30**, 6283–6289.
14. J.-M. Aubry (1991) New chemical sources of singlet oxygen. In: *Membrane Lipid Oxidation*. Vol 2. (ed. C. Vigo-Pelfrey) Boca-Raton: CRC Press; pp. 65–102.
15. Joh. Blok, L.H. Luthjens, and A.L.M. Roos (1967) The radiosensitivity of bacteriophage DNA in aqueous solution. *Radiation Research*, **30**, 468–482.
16. M.V.M. Lafleur, E.J. Pluijmackers-Westmijze and H. Loman (1984) Contrasting effects of cytochrome C on the radiosensitivity of single-stranded ΦX174 DNA in the presence of misonidazole or phenol under anoxia. *International Journal Radiation Oncology Biology and Physics*, **10**, 1195–1197.
17. M.V.M. Lafleur, A.W.M. Nieuwint, J.-M. Aubry, H. Kortbeek, F. Arwert and H. Joenje (1987) DNA damage by chemically generated singlet oxygen. *Free Radical Research Communications*, **2**, 343–350.
18. A.W.M. Nieuwint, J.-M. Aubry, F. Arwert, H. Kortbeek, S. Herzberg and H. Joenje (1985) Inability of chemically generated singlet oxygen to break the DNA backbone. *Free Radical Research Communications*, **1**, 1–9.
19. M.V.M Lafleur, J. Retèl and H. Loman (1988) Inactivation efficiencies of radical reactions with biologically active DNA. *Radiation Physics and Chemistry*, **32**, 329–333.
20. M. Moriya, C. Ou, V. Bodipudi, F. Johnson, M. Takeshita and A.P Grollman (1991) Site-specific mutagenesis using a gapped duplex vector; A study of translesion synthesis past 8-oxodeoxyguanosine in *E. coli*. *Mutation Research*, **254**, 281–288.
21. S. Shibutani, M. Takeshita, and A.P-Grollman A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation damaged base 8-oxo-G. *Nature*, **349**, 431–434.

Accepted by Prof. H. Sies