Free Rad. Res. Comms., Vol. 19, No. 2, pp. 125–134
Reprints available directly from the publisher
Photocopying permitted by license only

ENHANCED ENZYMATIC DEGRADATION OF RADICAL DAMAGED MITOCHONDRIAL MEMBRANE COMPONENTS

ADRIENNE J. GRANT, WENDY JESSUP and ROGER T. DEAN*

The Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, Australia, 2050

(Received May 4th 1993; in revised form June 10th 1993)

The location of a protein (soluble or membrane-bound) influences the extent of oxidative damage caused by free radicals. It has been established that after radical attack, soluble proteins can become more susceptible to hydrolysis by individual proteinases than native proteins.[1-4] We have now examined the hydrolytic susceptibility following radical attack of a protein that is located within a membrane environment, mitochondrial monoamine oxidase (MAO). After exposure to oxygen radicals generated by gamma irradiation, hydrolysis of sub-mitochondrial particles (SMP) containing MAO was increased in three respects. First, the generation of small fragments of MAO by the proteinases elastase and trypsin, was enhanced. Second, the generation by these enzymes and by phospholipase A2 of non-sedimentable membrane fragments containing MAO was also increased. Third, autolysis of SMP was enhanced. Hence, proteins located within membranes may become more susceptible to enzymatic degradation following oxidative damage.

KEY WORDS: Membrane proteins, oxidized, oxidised, protein damage, hydrolysis, proteolysis.

INTRODUCTION

Radical induced modifications to a protein depend amongst other factors upon its location.[5] Soluble proteins can be attacked directly by oxygen radicals. In membranes, phospholipids may provide alternative targets for radical attack thus sparing membrane bound proteins from direct attack. However, oxidation of membrane lipids may produce radical species and lipid peroxides and aldehydes which can in turn modify proteins indirectly.[4]

Monoamine oxidase (MAO) is an integral membrane enzyme which deaminates amines and is located in the outer mitochondrial membrane. It is extremely difficult to release from membranes, and can only be obtained lipid-free after extraction with organic solvents.[6,7] We have previously found that the extent of fragmentation of MAO induced by hydroxyl radicals is increased in lipid-depleted preparations confirming that lipids compete with proteins as targets for free radicals in these cellular membranes.[1,8] After radical fluxes caused by 1000 Gy of gamma irradiation in submitochondrial membrane particles, lipid peroxides were formed and the lipid phase antioxidant alpha tocopherol became significantly depleted.[9]

Previous studies by several groups[1-4] have shown that oxidized soluble proteins

*To whom correspondence should be addressed.

can become more susceptible to enzymatic hydrolysis *in vitro* than their native counterparts: this is believed to be due to loss of tertiary structure as a consequence of radical attack.[10,11] However, as unfolding of membrane proteins is less likely to occur, we did not foresee an increase in their hydrolytic susceptibility after radical attack. By studying MAO labelled with the active-site irreversible inhibitor [^{3}H] pargyline,[12] we have measured the effects of oxidative stress on its hydrolytic sensitivity. We have nevertheless found that MAO like several soluble proteins is more susceptible to hydrolysis by some proteinases after oxidation by gamma irradiation.

MATERIALS AND METHODS

Preparation of Labelled Monoamine Oxidase within Sub-Mitochondrial Particles

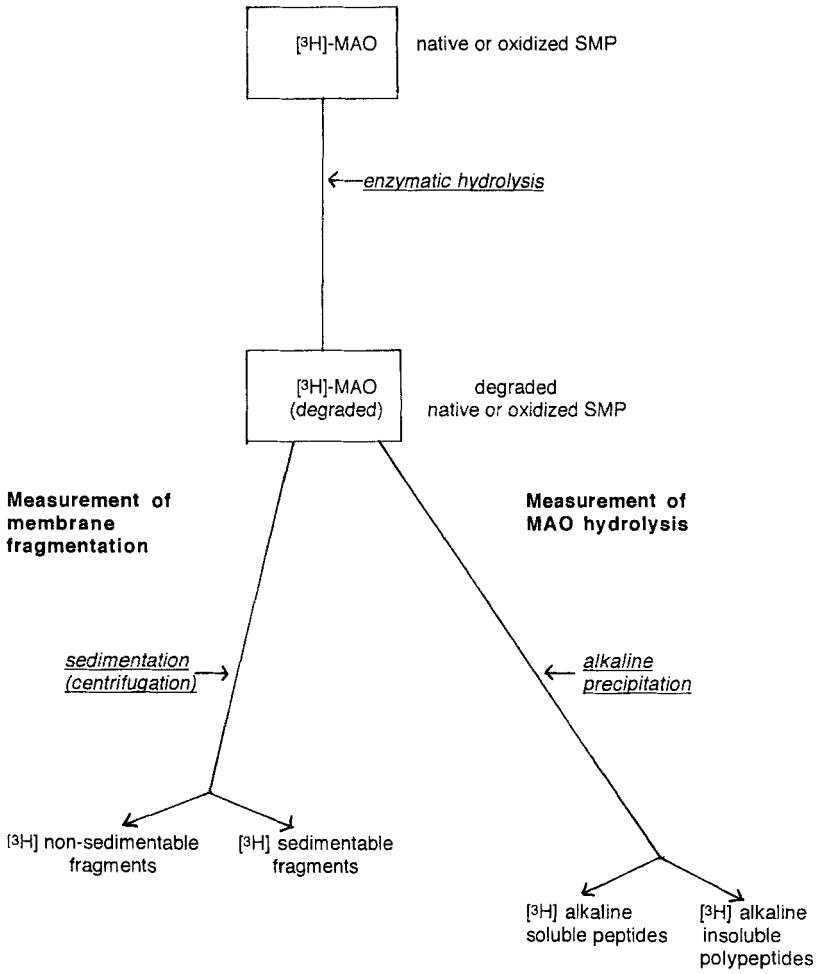
Mitochondria were prepared from rat liver and MAO in submitochondrial particles was labelled with [^{3}H] pargyline (NEN Research Products, Dupont, Australia) at 1 uCi/mg protein, as previously described.[9] [^{3}H] labelled MAO in SMP (~1 mg/ml) in 10 mM potassium phosphate buffer, (pH 7.2) was gamma irradiated with a [^{60}Co] source (at Macquarie University, Australia) under N_2O (80%), O_2 (20%) to generate predominantly hydroxyl radicals,[2,13] at a dose of 1400 Gy. These conditions were chosen on the basis of previous data as[8,9] to cause alpha-tocopherol depletion, lipid oxidation, and protein damage. Irradiated SMP were stored in 1 ml aliquots of ~2 mg protein/ml at −80°C and used only once after thawing. Protein was measured by the fluorescamine method.[14]

Hydrolysis of Monoamine Oxidase in Sub-Mitochondrial Particles

SMP (75 ug/ml) were incubated at 37°C in a gently shaking water bath with the following enzymes, in each case at 75 ug/ml: trypsin (Type 1), papain (P4762; 15 U/mg protein), elastase (Type IV) from Sigma, St. Louis, U.S.A. In addition, phospholipase A2 (PLA2, 120 ng/ml) from snake venom (400 U/mg Calbiochem, Australia) was used. SMP were incubated either: in buffer alone (autolysis); or with elastase, trypsin or PLA2 in 50 mM borate buffer (pH 8.3); or with papain (activated with 2 mM dithiothreitol (DTT) for 30 min at 37°C) in 50 mM phosphate buffer (pH 6.0).

Measurement of Monoamine Oxidase Hydrolysis and Release of Non-Sedimentable Components in Sub-Mitochondrial Particles

The hydrolysis of SMP by proteinases and phospholipases, causes two different types of degradation which influence MAO. The first is hydrolysis of the MAO polypeptide; some of these hydrolytic events release soluble MAO-derived peptides into the aqueous phase (detectable by the tritium label). The second is hydrolysis of other membrane components, such that small membrane fragments are generated from SMP. Some of these still contain MAO (again detectable by its tritium label), because it is an integral membrane protein. We wished to measure both processes, and thus used two methods of analysis of degradation, as shown in Scheme 1. The hydrolysis of the MAO polypeptide is represented by the 'alkaline soluble molecules'; while the generation of small membrane fragments is represented by 'non-



SCHEME 1 Diagram showing analysis of degradation.

sedimentable membrane fragments'. These parameters are expressed as the percentage of [^{3}H] respectively in (a) alkaline-soluble or (b) non-sedimentable forms.

a) MAO Polypeptide Hydrolysis: Alkaline soluble molecules

Alkaline soluble low molecular weight fragments ($\leq$10,000 M_r) containing [^{3}H] pargyline were measured in the supernatant solution following precipitation of macromolecules with $ZnSO_4/Ba(OH)_2$ as previously described.[8,9] In this system, the low pH of TCA may induce artefactual polypeptide breakage, and so should not be used for precipitation.[8]

b) Membrane Fragmentation: Non-sedimentable membrane fragments

Non-sedimentable components containing low density membrane fragments and any soluble molecules were measured as the release of ^{3}H labelled material from SMP. The non-sedimentable fraction was obtained following centrifugation at 16,000 g for 10 min (without precipitation with alkali).

SDS PAGE Identification of Label Distribution

To determine the site of radioactive labelling, samples of labelled SMP reduced with mercaptoethanol (2%) were examined in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) using pre-cast 4–20% gradient mini-gels (BioRad) in a Protean II system at 30 V constant for 4 h. Rainbow molecular weight markers (Amersham, Australia) 2.35 kDa-200 kDa were included. Gels were fixed in Entensify (NEN Research Products, Dupont, Australia) for 60 min then placed on Kodak X-OMat film and stored at −80°C for 28 days to produce autoradiographs. This SDS PAGE analysis showed that [^{3}H] pargyline treatment labelled a single band at ~60 kDa which corresponds with the molecular weight for monoamine oxidase in rat liver.[12]

RESULTS

Results described in all sections below were obtained from SMP that were irradiated under N_2O: O_2(4:1) which generates predominantly hydroxyl radicals (·OH).[2,13] However similar results were also obtained when SMP were irradiated under O_2 which generates approximately equal amounts of ·OH and superoxide radicals. Although there were variations in absolute values between batches of SMP, the trends were similar throughout the experiments. Data are expressed as the mean +/− S.D. of individual experiments performed in triplicate, which are representative of several experiments performed under identical conditions.

MAO POLYPEPTIDE HYDROLYSIS

Alkaline Soluble Fragments

(i) Autolysis

Autolysis, measured by hydrolysis of MAO in SMP incubated in buffer only was <5% for all preparations. After 24 h incubation in alkaline buffer (pH 8.3), autolysis of oxidized SMP to soluble fragments was higher than for native SMP as summarized in Table 1. In acidic buffer (pH 6.0) there was no significant difference.

(ii) Degradation by proteinases

Proteolysis of MAO in oxidized SMP by both trypsin (Figure 1A) and elastase (Figure 2A) was about double that in native SMP (Table 1). In contrast, during 24 h incubation, papain did not discriminate between MAO in oxidized and native SMP (Table 1). The results of short term kinetic experiments up to 2 h with all three proteinases (data not shown) were consistent with the long-term observations.

MEMBRANE FRAGMENTATION

Non-Sedimentable Fragments

(i) Autolysis

Autolysis, measured in the non-sedimentable fraction of MAO in SMP incubated in buffer only was <10% for all preparations. After 24 h incubation in alkaline

TABLE 1
Summary of the net degradation[1] of MAO after 24 h incubation

(A) Autolysis	Native SMP		Oxidized[2] SMP	
	pH 6.0	pH 8.3	pH 6.0	pH 8.3
Alkaline soluble	0.55 ± 0.4	1.12 ± 0.7	1.66 ± 1.7	3.46 ± 0.3
Non-sedimentable	0.7 ± 0.7	0	2.2 ± 1.8	6.13 ± 0.9

(B) Degradation by proteinases[3]

Proteinase	Native SMP	Oxidized[2] SMP
Alkaline soluble		
trypsin	4.71 ± 0.4	9.09 ± 1.3
elastase	8.24 ± 0.9	19.47 ± 1.1
papain	10.03 ± 3.0	11.30 ± 0.9
Non-sedimentable		
trypsin	31.89 ± 2.1	58.66 ± 1.2
elastase	64.11 ± 6.3	97.91 ± 4.3
papain	18.05 ± 6.4	22.53 ± 1.4

(1) Data are expressed as % of [^{3}H] present in either alkaline soluble molecules or non-sedimentable fractions as described in Methods. The small Oh values (shown in Figures 1 and 2) have been deducted.
(2) Oxidized SMP received a radical dose of 1400 Gy.
(3) SMP were incubated for 24 h at either pH 6.0 for papain or pH 8.3 for trypsin and elastase as described in Methods.

buffer (pH 8.3), or acidic buffer (pH 6.0) autolysis of oxidized SMP was higher than for native SMP as summarized in Table 1.

(ii) Degradation by proteinases

Degradation of oxidized SMP by trypsin (Figure 1B) and elastase (Figure 2B) was significantly higher than native SMP throughout the 24 h period of incubation.

After 24 h incubation there was no significant difference in the degradation of oxidized SMP compared with native SMP by papain (Table 1).

RELATIONSHIP BETWEEN MEMBRANE FRAGMENTATION AND PROTEOLYSIS

To determine whether release of non-sedimentable membrane fragments by enzymes permitted their increased access to sites for protein hydrolysis into soluble peptides, the kinetics of the two processes were studied in parallel up to 24 h (Figures 1 and 2). There was no indication that degradation to soluble peptides proceeded more rapidly following formation of non-sedimentable membrane fragments. From Figure 2, it remained possible that during the first six hours of incubation, membrane fragmentation by elastase, might accelerate concomitant MAO hydrolysis. However, separate detailed kinetic studies over shorter time courses ruled this out, in that hydrolysis of MAO was close to linear with time. The same was true in short time course studies for the more limited MAO hydrolysis achieved by trypsin. Other studies up to 50 h of incubation by both enzymes confirmed that membrane fragmentation did not accelerate MAO hydrolysis.

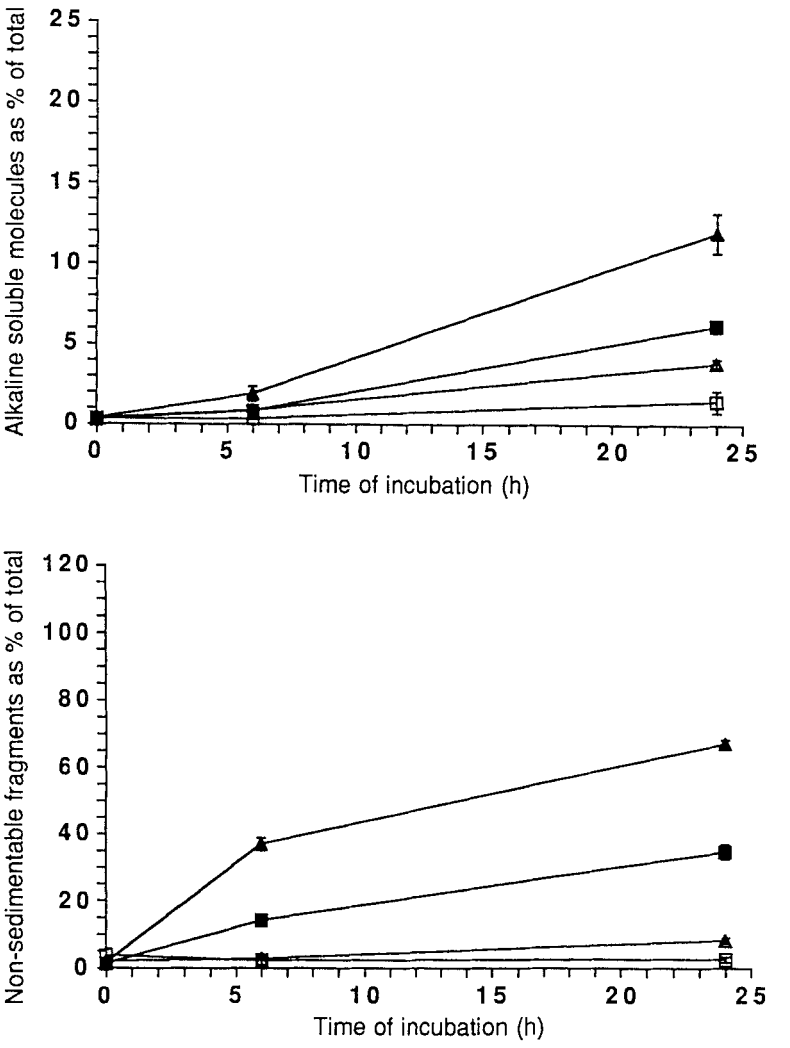


FIGURE 1 Degradation of [^{3}H] pargyline-labelled monoamine oxidase in SMP by trypsin. (A) Alkaline soluble molecules; (B) Non-sedimentable membrane fragments. Data are expressed as a percentage of the total dpm in the incubation. Experimental details are described in the text. Open squares = native SMP in buffer; closed squares = native SMP plus trypsin; open triangles = irradiated SMP in buffer; closed triangles = irradiated SMP plus trypsin.

DEGRADATION OF MEMBRANES BY PHOSPHOLIPASE A2

Because PLA2 has a preference for oxidized fatty acids,[17] we examined whether the sensitivity to hydrolysis of membrane lipids by PLA2 leading to the release of [^{3}H] labelled non-sedimentable material increased in SMP following irradiation. Figure 3 shows that PLA2 released nearly twice as much MAO from oxidized SMP in non-sedimentable fragments, compared with native MAO over 2 h, but it did not

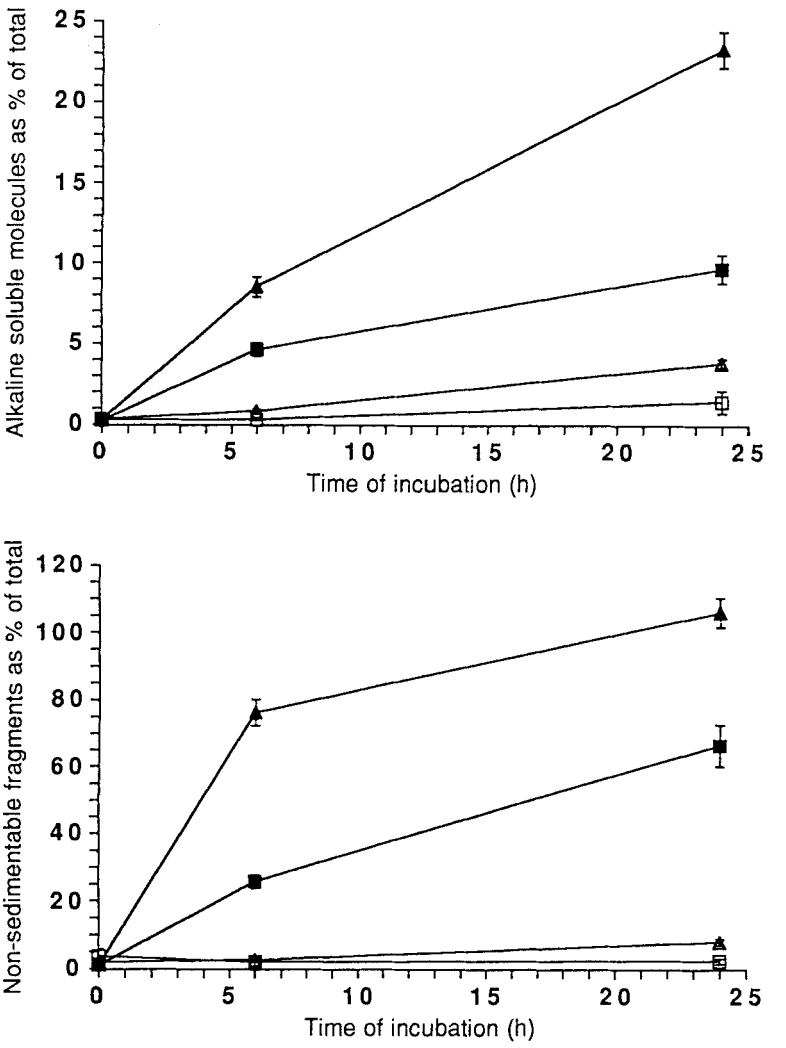


FIGURE 2 Degradation of [^{3}H] pargyline-labelled monoamine oxidase in SMP by elastase. (A) Alkaline soluble molecules; (B) Non-sedimentable membrane fragments. Data are expressed as a percentage of the total dpm in the incubation. Experimental details are described in the text. Open squares = native SMP in buffer; closed squares = native SMP plus elastase; open triangles = irradiated SMP in buffer; closed triangles = irradiated SMP plus elastase.

show signs of protein degradation consistent with its claimed lack of proteolytic contaminants (degradation to alkaline-soluble molecules was extremely straight). A lack of degradation of [^{14}C] labelled BSA incubated with PLA2 for 24 h also indicated that the hydrolysis of SMP by PLA2 was not due to a proteolytic contaminant.

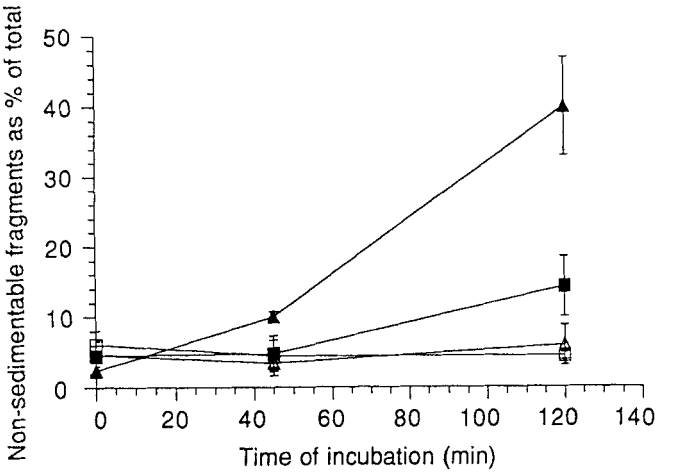


FIGURE 3 Degradation by phospholipase A2 of SMP containing [^{3}H] pargyline-labelled monoamine oxidase to non-sedimentable membrane fragments. Data are expressed as a percentage of the total dpm in the incubation. Experimental details are described in the text. Open squares = native SMP in buffer; closed squares = native SMP plus phospholipase A2; open triangles = irradiated SMP in buffer; closed triangles = irradiated SMP plus phospholipase A2.

DISCUSSION

Previous evidence has shown that oxygen-centred radicals can lead to degradation of MAO in SMP (even though lipids protect the protein in SMP). Furthermore radical treatment of SMP increases the sensitivity of its MAO to subsequent degradation induced by Fe^{2+}.[8]

We have now shown that radical attack also causes increased susceptibility to proteolysis of MAO embedded in the membrane of sub-mitochondrial particles. This result was unexpected, since enhanced proteolysis of *soluble* proteins after radical attack is thought to be due to unfolding, involving increased exposure of hydrophobic residues.[10,11] However, with a *membrane* protein, one would not predict such unfolding, nor increased exposure of hydrophobic residues, which would remain lipid-sequestered. Nevertheless, the protein modifications caused by radical attack even on an integral membrane protein such as MAO, may alter the degree of exposure of the active site region to proteinases. While MAO is anchored within the membrane,[6,7] its active site is available for reaction with aqueous phase substrates. Radical attack may make available to the aqueous phase additional parts of the polypeptide chain by fragmentation. Finally, the lipid oxidation which is concomitant with damage to SMP, may also contribute to changed susceptibility of MAO to external and endogenous mitochondrial proteinases, through a range of physicochemical effects, which 'open up' the membrane.[8,15] It is necessary to bear in mind that in both our assays, of MAO and SMP degradation, only molecules containing the MAO-active site label are followed, and changes in degradation of distal regions may not necessarily be detected.

Our other novel observation is that the known increased susceptibility of oxidized compared with unoxidized lipids to PLA2[15] also alters membrane structure in such a way as to permit PLA2 to release MAO in nonsedimentable but non-fragmented

(i.e. not alkaline soluble) forms (Figure 3). Indeed the increased autolysis of oxidized SMP to non-sedimentable fragments at alkaline pH observed in long incubations (Table 1) may incorporate the activity of an endogenous mitochondrial PLA2.[16] These were probably not proteolytic effects, since there was no change in the generation of low-molecular weight peptides. As PLA2 readily degrades oxidized membranes,[17] one might have predicted this enhanced formation of non-sedimentable membrane fragments.

There is some evidence that mitochondria contain a proteolytic system active at alkaline rather than acidic pH, which preferentially degrades oxidized proteins.[18] Our evidence of increased oxidized-MAO degradation at alkaline pH is consistent with this evidence.

The *in vivo* relevance of our observations remains to be established. Mitochondria are the site of the majority of radical flux in most cells,[19] as evidenced in part by their accumulation of much higher levels of oxidized DNA than accumulate in the nucleus.[20] Thus, they may be exposed to sufficient oxidative stress to overcome antioxidant defences and induce some of the types of damage we have investigated, particularly in conditions of cellular injury such as ischemia reperfusion.[21] Interrelated lipid and protein oxidation processes may contribute to changes in membrane permeability, ion transport and coupling which constitute mitochondrial swelling and the collapse of the protonmotive force.[22] These kinds of damage in turn may lead to enhanced overall catabolism of non-functional mitochondria.

References

1. R.T. Dean, S.M. Thomas, G. Vince and S.P. Wolff (1986) Oxidation-induced proteolysis and its possible restriction by some secondary protein modifications. *Biomedica Biochimica Acta*, **45**, 11-12, 1563-1573.
2. S. Wolff and R.T. Dean (1986) Fragmentation of polypeptides by free radicals and its effect on susceptibility to proteolysis. *Biochemical Journal*, **234**, 245-248.
3. K.J.A. Davies, S.W. Lin and R.E. Pacifici (1987) Protein damage and degradation by oxygen radicals IV. Degradation of denatured protein. *The Journal of Biological Chemistry*, **262**, 9914-9920.
4. J.V. Hunt, J.A. Simpson and R.T. Dean (1988) Hydroperoxide-mediated fragmentation of proteins. *Biochemical Journal*, **250**, 87-93.
5. R.T. Dean, J.V. Hunt, A.J. Grant, Y. Yamamoto and E. Niki (1991) Free radical damage to proteins: the influence of the relative localization of radical generation, antioxidants, and target proteins. *Free Radical Biology and Medicine*, **11**, 161-168.
6. A. Ito, T. Kuwaharra, S. Inadome and Y. Sagara (1988) Molecular cloning of a cDNA for rat liver monoamine oxidase B. *Biochemical and Biophysical Research Communications*, **157**, 970-976.
7. Z. Zhuang, B. Marks and R.B. McCauley (1992) The insertion of monoamine oxidase A into the outer membrane of rat liver mitochondria. *The Journal of Biological Chemistry*, **267**, 591-596.
8. R.T. Dean, S.M. Thomas and A. Garner (1986) Free radical-mediated fragmentation of monoamine oxidase in the mitochondrial membrane. *Biochemical Journal*, **240**, 489-494.
9. S.M. Thomas, J.M. Gebicki and R.T. Dean (1989) Radical initiated alpha-tocopherol depletion and lipid peroxidation in mitochondrial membranes. *Biochimica et Biophysica Acta*, **1002**, 189-197.
10. K.J. A. Davies (1986) Intracellular proteolytic systems may function as secondary antioxidant defenses: an hypothesis. *Free Radical Biology and Medicine*, **2**, 155-173.
11. S.P. Wolff, A. Garner and R.T. Dean (1986) Free radicals, lipids and protein breakdown. *Trends in Biochemical Sciences*, **11**, 27-31.
12. R.M. Cawthon and X.O. Breakefield (1983) Differences in the structures of monoamine oxidases A and B in rat clonal cell lines. *Biochemical Pharmacology*, **32**, 441-448.
13. C. von Sonntag (1987) The chemical basis of radiation biology, Taylor and Francis, London, pp 31-40.
14. S. Udenfriend, S. Stein, P. Bohlen and W. Dairman (1978) Fluorescamine: a reagent for assay of amino acids, peptides, proteins and primary amines in the picomole range. *Science*, **178**, 871-872.

15. F.J.G.M. van Kuijk, A. Sevanian, G.J. Handelman and E.A. Dratz (1987) A new role for phospholipase A 2: protection of membranes from lipid peroxidation damage. *Trends in Biochemical Sciences*, **12**, 31–33.
16 C. Levrat and P. Louisot (1992) Dual localization of the mitochondrial phospholipase A 2: outer membrane contact sites and inner membrane. *Biochemical and Biophysical Research Communications*, **183**, 719–724.
17. A. Sevanian and E. Kim (1985) Phospholipase A2 dependent release of fatty acids from peroxidized membranes. *Free Radical Biology and Medicine*, **1**, 263–271.
18. O. Marcillat, Y. Zhang, S.W. Lin and K.J. Davies (1988) Mitochondria contain a proteolytic system which can recognize and degrade oxidatively-denatured proteins. *Biochemical Journal*, **254**, 677–683.
19. B. Chance, H. Sies and A. Boveris (1979) Hydroperoxide metablism in mammalian organs. *Physiological Reviews*, **59**, 527–605.
20. C. Richter, J-W, Park, and B.N. Ames (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *National Academy of Science, Proceedings, US*, **85**, 6465–6467.
21. J.F. Turrens, M. Beconi, J. Barilla, U.B. Chavez and J.M. Mc Cord (1991) Mitochondrial generation of oxygen radicals during reoxygenation of ischemic tissues. *Free Radical Research Communications*, **12–13**, 681–689.
22. I. Rustenbeck, H. Eibl and S. Lenzen (1991) Structural requirements of lysophospholipid-regulated mitochondrial Ca^{2+} transport. *Biochimica et Biophysica Acta*, **1069**, 99–109.

Accepted by Professor B. Halliwell