OXIDATIVE DNA DAMAGE AND HOMEOSTASIS OF INTRACELLULAR IRON AND COPPER IONS

R. Meneghini*, E.A.L. Martins and M. Calderaro
Institute of Chemistry - Department of Biochemistry - CP 20780 - CEP 01498 - University of Sao Paulo - Sao Paulo - Brazil

The structure of DNA bases modified by hydroxyl radical attack have recently been identified. It is important to notice that these damages are detected in DNA of cells submitted to oxidative stress, showing that hydroxyl radicals are formed close to DNA, by metal-induced Fenton reactions. Some of these lesions have already been shown to be mutagenic. The presence of Fe and Cu in the nucleus, probably bound to DNA, is unexpected if we consider the potential hazard imposed to a critical target as DNA. One possibility is that the intracellular homeostasis of Fe and Cu is altered by the pro-oxidative status of the cell. Evidence on that was obtained in cells exposed to menadione. The levels of "free" Fe and Cu seem to increase substantially during menadione metabolism, and it is possible that part of the released Fe and Cu migrates to the nucleus.

Keywords: DNA damage; DNA strand breaks; oxyradicals; hydroxyl radical; copper; iron.

INTRODUCTION

In recent years it was established that genomic alterations are important triggers of cell malignant transformation[1]. It was also established that reactive oxygen species (ROS) are normally produced in the cell and that numerous chemical species present in the environment, including in the normal diet, are capable of increasing the cellular concentrations of ROS above the basal level[2,3]. Because some ROS are sufficiently reactive to produce DNA chemical modifications they must constitute a major source of malignant transformation[4-6] and of gene mutation[7-9]. Therefore it has become of great interest to know which ROS are capable of altering the DNA structure and to understand the mechanisms involved.

OXIDATIVE BASE MODIFICATIONS

It is well established that $O_2^{\cdot}$, lipid peroxides and H_2O_2 are not sufficiently reactive **per se** to attack DNA[10-12]. However OH radical, alkoxy radicals and singlet oxygen are capable of attacking DNA. From radiobiological studies it has long been known that OH radical produces several types of DNA damage[13]. The molecular mechanisms involved in the generation of these altered structures have been reviewed[14]. More recently, with two improved analytical techniques, namely, HPLC associated to electrochemical detection and GC-MS, the possibility of identifying DNA lesions at biologically significant levels became feasible. Over 10 base lesions have been identified upon DNA exposure to sources of OH radical generation. The most relevant were 8-hydroxyguanine, 4,6-diamino-5-formamido pyrimidine (Fapy Ade), 2,6-diamino,4-hydroxy-5-formamido pyrimidine (Fapy Gua), thymine glycol, cytosine glycol, 8-hydroxy adenine and dihydroxy cytidine[15]. Another significant base lesion seems to be 5-hydroxymethyluracil, which is formed in the DNA of activated polymorphonuclear leukocytes[16]. When chromatin is exposed to a source of OH radical a thymine-tyrosine cross-link is detected, in addition to base damages[17].

The sugar moiety is also a target for OH radical attack in DNA, although to a lesser extent than the bases. The preferential center of attack is at C-4, and the carbon-centered radical produced by hydrogen abstraction undergoes rearrangements which eventually leads to base loss and strand-break with two types of termini, phosphoryl and phosphoglycolate[13, 18, 19].

Singlet oxygen is also reactive towards DNA. However the spectrum of lesions produced is much shorter than that originated from the OH radical attack; the products so far identified were 8-hydroxyguanine, Fapy purines and strand breaks[20-22]. However the biological significance of this fact relies on obtaining evidence that singlet oxygen may be generated in the cell nucleus.

MUTAGENICITY OF OXIDATIVE DNA LESIONS

The mutagenicity of some DNA lesions produced by oxyradicals have been investigated. Recently it was shown that 8-hydroxyguanine led to the insertion of dA, dT, dG and dC in the opposite strand, with almost equal frequency[23]. Interestingly, the 3' vicinal base to 8-hydroxyguanine was also frequently misread. Other investigations have shown that G-T transversion is the predominant point mutation induced by 8-hydroxyguanine[8, 24, 25]; 5-hydroxymethyluracil has also been shown to be mutagenic, whereas thymine glycol seems to operate more as a block for DNA replication[26]. Several other oxyradical-induced DNA damages are likely to be mutagenic and it has been shown that phage DNA exposed to Fe^{2+} and transfected into bacteria induced a ratio of mutational event to lethal event higher than for any other agent tested[7]. Therefore, it is not surprising that the cells operate with distinct mechanisms to prevent the effects of these lesions; first, preventing its formation by means of antioxidant defenses. For instance, ascorbate in the seminal fluid has been shown to prevent 8-hydroxyguanine formation in sperm DNA[27]. In E.coli lacking FeSOD and MnSOD the mutation rate under aerobic growth is greatly enhanced in relation to the parental strain[28].

REPAIR OF OXIDATIVE DNA LESIONS

Once the lesion is formed DNA repair mechanisms are called into action. DNA strand breaks formed by attack of ROS usually have a phosphoryl or a phosphoglycolate group at the 3' terminus[19], both constituting a block for DNA replication. In **E.coli**, exonuclease III or endonuclease IV removes these blocks generating a 3'OH primer for DNA polymerase[18]. In mammalian cells an enzyme with the same properties of exonunuclease III has been identified[29]. Both 5-hydroxymethyluracil and thymine glycol are removed from DNA by specific glycosylases[30, 31]. More recently, an enzyme that removes 8-

hydroxyguanine and Fapy purine lesions from DNA was identified in **E. coli**; it has associated to it an endonuclease activity that cleaves DNA at the resulting abasic site, thus creating the appropriate substrate for further DNA repair process[22]. It is likely that several more enzymes involved in the repair of DNA damaged by ROS will be discovered in the near future. After all, there are many different DNA lesions produced by ROS and the repair enzymes have to be relatively specific. It is interesting that, even with such a elaborated machinery devoted to the prevention and repair of ROS-induced DNA lesions, normal oxidative damage to nuclear DNA is extensive, reaching one 8-hydroxyguanine per 130,000 bases[32].

ROLE OF METALS IN DNA DAMAGE BY ROS

It seems clear now that Fe is involved in the production of DNA damage by ROS. This was first proposed when it was shown that both 1,10-phenanthroline and 2,2'-dipyridyl prevented production of DNA strand breaks in mammalian fibroblasts exposed to H_2O_2[33, 34]. These two compounds are strong Fe chelators that prevent the metal from participating in the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^-$$

Because they have lipophilic structure, they can easily enter the cell and bind Fe(II), which is not the case of desferrioxamine, DTPA and bathophenanthroline[35]. Scavengers of OH radical also block the strand break formation and kinetic studies indicate that the OH radical travels, in average, 15 angstroms from the site of generation to the DNA target[36]. Collectively, these results suggest that H_2O_2 can reach the nucleus and react with Fe(II), which is close to or, more likely, bound to DNA. This would generate OH radicals that react practically at the site of formation[33]. Other genotoxic effects of H_2O_2 were prevented by these chelators, like sister chromatid exchanges[37], mutation at the HGPRT locus and cell transformation[9] and most of these results were reproduced in other laboratories[38-40]. The recent identification of several typical OH radical-induced base damages in cells exposed to a source of H_2O_2 clearly demonstrates that OH radicals are participating in site-specific Fenton reactions on DNA[41]. It also seems clear that, if OH radicals attack DNA bases, they should be able to attack the sugar moiety as well, giving origin to strand breaks. However, evidence has been produced that the increase in cytosolic Ca, caused by oxidative stress, activated nucleases, which would be responsible for the DNA breaks[42-44]. It is quite possible that both types of DNA strand breaks are formed upon oxidative stress, i.e., those produced by OH radical attack and those produced by Ca-activated nucleases, and that, depending on the cell, one or another will prevail. However, it is unlikely that the use of Ca and Fe chelators, neither exhibiting absolute specificity, will clarify this issue. To gain knowledge on this topic the better approach is to look for the structure of DNA at the site of the break, since this is expected to be different, depending on the causative agent (see above).

OXIDATIVE STRESS AND THE HOMEOSTASIS OF Fe AND Cu IN THE CELL

One intriguing aspect of the model proposed above is the assumption of the existence of Fe bound to DNA. In fact, Fe-DNA is a Fenton reagent[15, 45, 46]. Therefore one would not expect Fe being so closely associated with such a critical biological target. How much Fe or Cu is closely associated to DNA is still an open question, although some investigations have indicated the presence of these ions in the nucleus[47]. Another possibility is that under oxidative stress Fe and/or Cu intracellular homeostasis is altered, as in the case of Ca. For instance, Fe and Cu could be released from their storing sites under pro-oxidative conditions and part of these ions could leak into the nucleus. We have recently addressed the possibility of changes in the homeostasis of Fe and Cu in cells treated with the quinone menadione. In the cell menadione is enzymatically reduced, either partially to the semiquinone form, or fully to the diphenolic form. Both forms can react with dioxygen to generate superoxide, which by dismutation gives rise to H_2O_2. In fact, menadione has long been known to generate a strong pro-oxidative condition in the cell[48].

We and others have previously shown that menadione induces DNA strand breaks in the cell[49-52]. One interesting aspect is that menadione-induced DNA strand breaks are much less susceptible to inhibition by 1,10-phenanthroline than H_2O_2-induced DNA strand breaks. In fact, the more conspicuous effect of this chelator is to enhance the production of strand breaks, and this is a response that is dependent on menadione concentration (figure 1). At low menadione concentrations protection by 1,10-phenanthroline can be observed, similar to what has been determined in the case of H_2O_2-induced DNA strand breaks[34]. As the menadione concentration is increased, a dramatic increase in breaks caused by 1 mM 1,10-phenanthroline is observed. We reasoned that menadione could be inducing a release of copper from some storing source. Copper is known to form a clastogenic compound with 1,10-phenanthroline, which binds DNA and causes extensive degradation; this action is known to be prevented by neocuproine, a strong Cu chelator[53]. In fact, neocuproine presented a strong inhibitory effect on the production of strand breaks by menadione plus 1,10-phenanthroline (figure 2). It is interesting that neocuproine shows no inhibitory effect against the strand breaks caused by menadione alone; therefore these breaks are not mediated by Cu. It seems plausible to consider the following situation: the breaks induced by menadione alone are caused by H_2O_2, generated by menadione metabolism. Therefore they should be produced by a Fe-induced Fenton reaction in the nucleus[54]. The reason why 1,10-

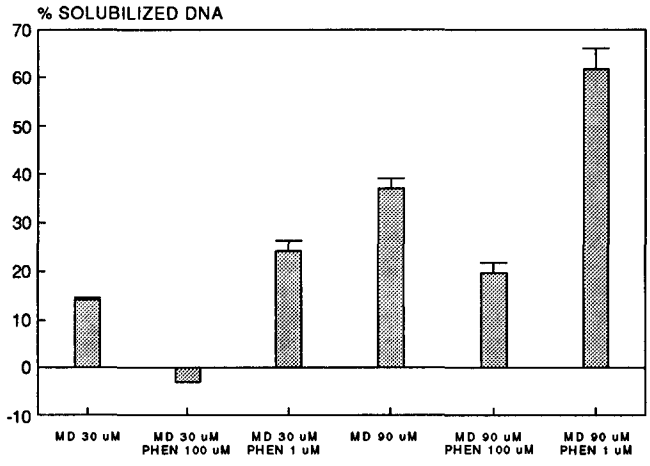

Figure 1. Inhibition and enhancement of menadione-induced DNA strand breaks by phenanthroline. Solubilized DNA is related to the production of DNA strand breaks. V-79 Chinese hamster cells were treated for 30 min with the indicated concentrations of the compounds. The production of DNA strand breaks was assessed by the assay previously described[58, 59]. MD, menadione; PHEN, 1,10-phenanthroline. Bars represent the standard deviations for 3 independent determinations.

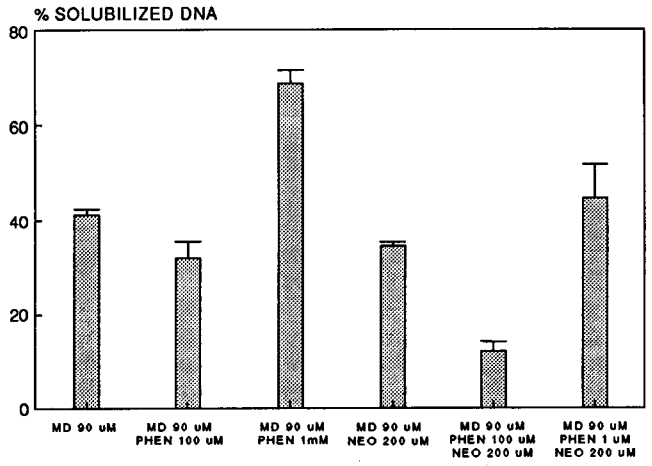

Figure 2. Effect of neocuproine on menadione-induced DNA strand breaks. Legend as in Figure 1. NEO, neocuproine.

phenanthroline is not inhibitory in this case is because Cu ions are released by menadione metabolism and the predominant effect becomes the clastogenic action of the Cu-phenanthroline complex. As possible sources of Cu donors, under the effect of menadione metabolites, we might consider glutathione-Cu and metallothionein-Cu complexes[55].

If this model is correct than it should be possible to prevent the Fe-mediated, menadione-induced strand breaks by an iron chelator that does not form clastogenic complexes with Cu. We have already shown that 2,2'-dipyridyl, which forms a Fe complex analogous to that of 1,10-phenanthroline, prevents Fe-mediated Fenton reactions[33, 34]. However, contrarily to the 1,10-phenanthroline molecule, the 2,2'-dipyridyl molecule is not planar; therefore, its Cu complex should not be capable of intercalating in DNA, a pre-requisite for the clastogenic action[53]. In fact, figure 3 shows the inhibitory action of two different concentrations of 2,2'-dipyridyl on strand breaks induced by menadione. Clearly, a strong protection is observed. Because neocuproine, a strong inhibitor of Cu-mediated (but not Fe-mediated) Fenton reaction[54], has no effect on the menadione-induced breaks, it is clear that 2,2'-dipyridyl is inhibiting Fe-mediated strand breaks. Also clear is that, as menadione concentration is increased, the inhibitory capacity of 2,2'-dipyridyl is proportionally decreased. This favors the idea that

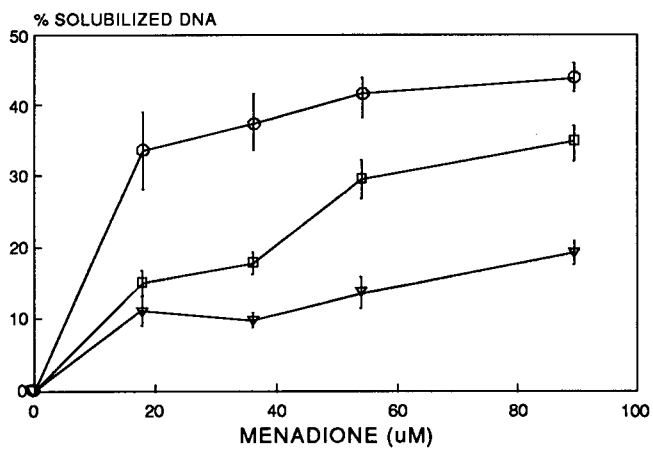

Figure 3. Effect of 2,2'-dipyridyl on the menadione-induced DNA sb. (○) menadione only; (□) menadione + 100 uM 2,2'-dipyridyl; (▽) menadione + 1 mM 2,2'-dipyridyl.

Fe ions are also being released from storing sources by menadione metabolites. In fact, it has been shown that two products of the menadione metabolism, superoxide anion[56] and menadione semiquinone[57], do promote iron release from ferritin. In conclusion, it seems clear that during menadione metabolism conspicuous alterations in Fe and Cu homeostasis can occur in the cell and that this may have important consequences in the genotoxic effects of oxygen radicals.

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