Cell nuclei generate DNA-nicking superoxide radicals

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Rat liver nuclei generate superoxide radicals in the presence of NADPH. Active oxygen species induced nicks in nuclear DNA. This was prevented by superoxide dismutase and catalase as well as by anaerobiosis. EDTA-Fe^{3+} dramatically increased the active oxygen-dependent DNA nicking.

Superoxide generation *NADPH oxidation* *DNA damage* *Active oxygen species* *(Rat liver nucleus)*

1. INTRODUCTION

Superoxide radicals ($O_2^{\cdot -}$) are produced by many metabolic pathways in oxygen-metabolizing cells [1]. Cellular membranes are the principal source of $O_2^{\cdot -}$ in the cell. It also seems interesting to investigate the $O_2^{\cdot -}$ generation in nuclear membranes in view of their close proximity to the genetic apparatus of the cell.

Oxygen radicals have been shown to be able to cleave DNA [2] and to inactivate enzymes [3]. Moreover, $O_2^{\cdot -}$ acts as a promoter of transformation and as a weak complete carcinogen [4]. Also, isolated nuclei and nuclear membranes have been shown to generate $O_2^{\cdot -}$ in an NAD(P)H-dependent manner [5–9]. An intriguing aspect of $O_2^{\cdot -}$ generation by nuclei is the possibility that it generates breaks in the DNA molecules.

$O_2^{\cdot -}$ generation by nuclei as well as by other membranous structures is usually monitored by spectroscopic methods which measure SOD-sensitive reduction of cytochrome *c*, adrenaline, spin traps, etc. To detect $O_2^{\cdot -}$ generation by membranes, however, it is important to develop new approaches since conventional techniques have limitations and often do not provide unambiguous evidence of the intrinsic $O_2^{\cdot -}$ generation by the membrane. The most reliable way is to measure $O_2^{\cdot -}$ generation by 2 or more independent methods.

Here, we have investigated $O_2^{\cdot -}$ generation by isolated nuclei by monitoring the formation of nicks in DNA molecules and their sensitivity to SOD and catalase. We found that incubation of nuclei in the presence of NADPH leads to the appearance of nicks in the nuclear DNA. SOD plus catalase as well as anaerobiosis prevented DNA damage.

Abbreviation: SOD, superoxide dismutase

2. MATERIALS AND METHODS

2.1. *Isolation of nuclei*

Nuclei from Wistar rat liver were prepared by the modified method of Blobel and Potter [10]. Isolated nuclei were suspended in ice-cold sucrose buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$). The purity of the final preparation was monitored by electron microscopy.

2.2. *Detection of nicks in DNA by exogenous DNA polymerase*

Nuclei were incubated in 100 μl of medium containing 20 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 150 mM KCl, 0.01% albumin at 30°C for 30 min. In some incubations NADPH, SOD (Sigma, St. Louis, MO) and catalase (Serva, Heidelberg) were also included. To provide anaerobiosis 5 mM glucose, 0.5 U/ml glucose oxidase (Boehringer

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Mannheim, grade 1) and 25 μg/ml catalase (to scavenge H_2O_2 produced by glucose oxidase) were added. After incubation at 30°C for 30 min all samples were heated at 65°C for 10 min and cooled. Then 4 deoxyribonucleoside triphosphates (50 μM each), 1 μM [^{3}H]TTP (22 × 10^3 Ci/mol) and 1 U DNA polymerase from *Bacillus stearothermophilus* [11] (kindly supplied by Dr Kaboev) were added. The reaction mixture was incubated at 60°C for 30 min, stopped with 5 ml of ice-cold 5% trichloroacetic acid and placed on Whatman GF/B glass fibre paper. Radioactivity was measured as described [12] in an SL-4000 scintillation counter (Intertechnique, France).

2.3. *Detection of nicks in DNA by alkaline gel electrophoresis*

Nuclei were incubated in 100 μl of medium containing 150 mM KH_2PO_4, pH 7.4, 5 mM EDTA, 1 mM EGTA and EDTA-Fe^{3+} (concentration of iron, 25 μM) at 30°C for 30 min. Then 105 μl alkaline buffer (100 mM NaOH, 2 mM EDTA, 5% glycerine), followed by 5 μl of 20% SDS were added. Samples were analyzed by alkaline gel electrophoresis in 0.5% agarose as described [13,14].

3. RESULTS AND DISCUSSION

We expected that during NADPH-dependent $O_2^{\cdot-}$ generation by nuclei the amount of nicks in the nuclear DNA would be increased. Therefore, if a polymerase preferably utilizing nicked DNA and labelled dNTP is added to the test tube, incorporation of the acid-insoluble label would reflect $O_2^{\cdot-}$ generation. In our experiments we used the DNA polymerase from *B. stearothermophilus*, which preferably uses nicked DNA as a template [11].

Isolated rat liver nuclei were incubated with NADPH. The polymerase was added to the reaction system after stopping the $O_2^{\cdot-}$ generation and inactivation of endogenous polymerase activity by incubating the mixture at 65°C for 10 min. As shown in table 1, addition of NADPH did not result in an increase of DNA synthesis. Moreover, in the presence of 1 mM NADPH the labelling of DNA decreased. The probable explanation for this is that dismutation of $O_2^{\cdot-}$ that also occurs in the absence of specific catalysis [15] could yield H_2O_2 which could inactivate the polymerase. Indeed, when catalase was added after the stopping of $O_2^{\cdot-}$ generation, but before polymerase addition to destroy H_2O_2, increased labelling of DNA was observed (table 1). Anaerobiosis also protected DNA (table 2), providing evidence for involvement of active oxygen species in the process. Table 2 also shows that SOD and catalase prevented DNA nicking.

Table 1

Effect of NADPH on nick-dependent DNA labelling

Additions	cpm[b]
(1) None	406 (100%)
(2) + 0.1 mM NADPH	417 (103%)
(3) + 1.0 mM NADPH	318 (78%)
(4) + 0.1 mM NADPH + catalase[a]	502 (124%)
(5) + 1.0 mM NADPH + catalase[a]	611 (150%)

[a] Catalase was added before polymerase to the heated samples

[b] Nonspecific label of DNA in the absence of the added polymerase (127 cpm) was subtracted. 1.0 × 10^6 nuclei were in test tubes

In the presence of trace amounts of iron, $O_2^{\cdot-}$ and H_2O_2 co-operate in production of $^{\cdot}OH$ radicals which can attack DNA and produce breaks in the DNA chain [16]. In particular, $^{\cdot}OH$ formation is markedly augmented by catalytic amounts of EDTA-Fe^{3+} [17], which has been shown to stimulate $O_2^{\cdot-}$ and $^{\cdot}OH$ generation by microsomes [18,19]. The data in table 3 indicate that EDTA-Fe^{3+} greatly stimulates the appearance of NADPH-dependent nicks in DNA. The 7.5-fold increase in labelling of DNA is in good agreement with the data on EDTA-Fe^{3+} stimulation of

Table 2

Effect of active oxygen species formation on nuclear DNA nicking

(1)	Control	100%
(2)	+ NADPH + catalase[a]	132%
(3)	+ SOD + catalase + NADPH	102%
(4)	+ anaerobiosis + NADPH + catalase	102%

[a] Catalase was added before polymerase to the heated samples

Additions: NADPH, 1.0 mM; SOD, 50 μg/ml; catalase, 25 μg/ml

NADPH oxidation and $O_2^{\cdot-}$ and $^{\cdot}OH$ generation [18,19]. However, SOD plus catalase failed to reduce the high level of nicking of the DNA in the presence of EDTA-Fe^{3+} (table 3).

A possible explanation for the lack of enzymatic defence against EDTA-Fe^{3+}-catalyzed nicking could lie in the large size of the catalase molecule which could prevent its entrance into the nucleus. For example, microinjection studies have demonstrated that catalase can cross the nuclear envelope, but requires 4–6 h to acquire uniform distribution between the nucleus and cytoplasm [20]. Obviously, under our experimental conditions, catalase did not have enough time to permeate into nuclei. At a low rate of $O_2^{\cdot-}$ and H_2O_2 production, the small SOD molecules presumably effectively scavenge $O_2^{\cdot-}$ within the nucleus, and H_2O_2 is able to diffuse out of the nucleus, where catalase destroys it. At the high rate of formation of active oxygen species in the presence of EDTA-Fe^{3+}, the rates of H_2O_2 diffusion outside the nuclei, and of $O_2^{\cdot-}$ dismutation by SOD, may be insufficient to prevent accumulation of active oxygen species to concentrations critical for the onset of $^{\cdot}OH$ production.

To test the validity of the above explanation we carried out experiments in the presence of high concentrations of EDTA. It is known that EDTA treatment of nuclei results in an 8-fold increase in the nuclear volume [21]. Consequently, EDTA-dependent swelling of nuclei would: (i) decrease the concentration of $O_2^{\cdot-}$ and H_2O_2 inside the nucleus and consequently of $^{\cdot}OH$ inside the nucleus; (ii) break nuclear integrity and allow catalase to penetrate.

Alkaline gel electrophoresis was used to estimate single-strand breaks in DNA [13]. This method was used because the DNA polymerase reaction depends on Mg^{2+} [11] and the DNA polymerase assay employed above does not allow detection of nicks in DNA in the presence of EDTA. Fig.1 shows patterns of DNA analyzed by alkaline electrophoresis in agarose after incubation of nuclei in the presence of EDTA. Addition of EDTA-Fe^{3+} to the NADPH-oxidizing nuclei resulted in a decrease in the amount of high-M_r DNA (fig.1, lane 5), and SOD plus catalase provided some protection (fig.1, lane 6). The appearance of nicks in intermediate-

Table 3

Nick-dependent labelling during active oxygen species formation

Additions		cpm[b]
(1)	None	1901 (100%)
(2)	+ NADPH + catalase[a]	2596 (137%)
(3)	+ NADPH + EDTA-Fe^{3+} + catalase[a]	14478 (762%)
(4)	+ SOD + catalase + NADPH + EDTA-Fe^{3+}	13277 (698%)

[a] Catalase was added before polymerase to the heated samples

[b] Nonspecific label of DNA in the absence of the added polymerase (605 cpm) was subtracted. 3.2×10^6 nuclei were in test tubes

Additions: EDTA-Fe^{3+}, concentration of iron, 25 μM; other conditions as in table 2

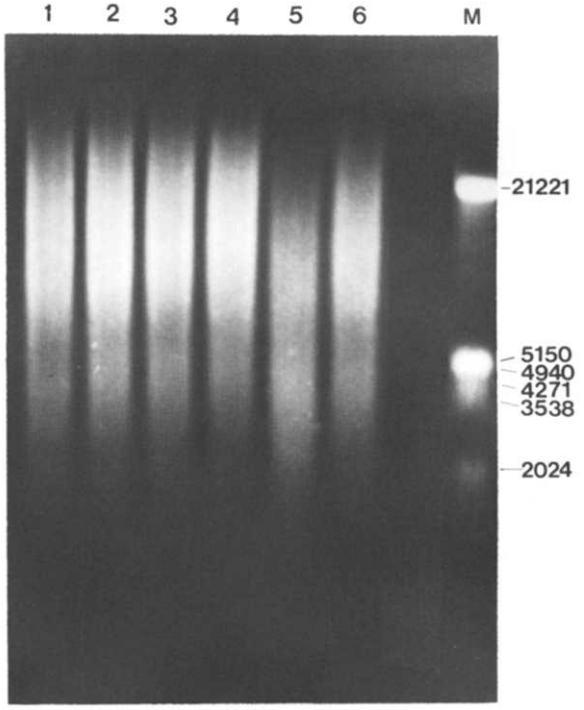

Fig.1. Alkaline gel electrophoresis of nuclear DNA. Nuclei were incubated as described in section 2, but EDTA-Fe^{3+} was omitted from lanes 1–3. Incubations – lanes: 1, control; 2, 1.0 mM NADPH; 3, 1.0 mM NADPH; 50 μg/ml SOD, 25 μg/ml catalase; 4, EDTA-Fe^{3+}; 5, 1.0 mM NADPH, EDTA-Fe^{3+}; 6, 1 mM NADPH, EDTA-Fe^{3+}, 50 μg/ml SOD, 25 μg/ml catalase; M, size marker, base pairs (DNA cleaved with a mixture of *Eco*RI and *Hin*dIII).

sized DNA cannot be visualized since the smear distributes rather uniformly due to the high amount of nicks in the DNA (the genomic DNA was already fragmented by endogenous nucleases during incubation), but extensive nicking of high-M_r DNA, leading to disappearance of material in the upper zone of the gel, is obvious.

EDTA-Fe^{3+} alone did not cause the appearance of nicks in DNA (cf. lanes 1 and 4 in fig.1). When nuclei were incubated with NADPH in the absence of EDTA-Fe^{3+} and nuclear DNA was subjected to electrophoresis in an alkaline gel, a pattern essentially indistinguishable from that obtained for the nuclei incubated without NADPH was observed (fig.1, lanes 1 and 2). This result is in agreement with data on the lack of NADPH-dependent nicking of DNA that were obtained by related method [22]. Presumably the number of nicks in nuclear DNA formed in the presence of NADPH alone is too small to be measured by most methods including alkaline treatment but can be revealed using the polymerase assay.

Our results are consistent with earlier observations that nuclei generate $O_2^{\cdot -}$ in the presence of NADPH [5–9]. It also shows that formation of nicks in DNA can be used as a probe for $O_2^{\cdot -}$ generation by membranes.

Another important conclusion of this work concerns the ability of DNA to accumulate $O_2^{\cdot -}$-dependent nicks during nucleus functioning. While this work was being performed, a report on isolated DNA strand scission by incubation with cytochrome P-450 reductase (nuclear membrane enzyme [23]), NADPH and aminoquinone containing anthracyclines was published [24]. We cannot exclude completely the possibility of involvement in the process in our experiments of other NADPH-dependent membranes that invariably contaminate the nuclear preparation [25]. However, in any case the appearance of active oxygen-dependent nicks in nuclear DNA during incubation with NADPH remains the main observation of this work.

There are many ways for $O_2^{\cdot -}$ formation in a cell [1], as well as strong enzymatic and non-enzymatic defence mechanisms against active oxygen species [26]. Formation of active oxygen species is increased by radiation [27], hypoxia [28], hyperoxia [29] and many drugs [1,7]. We feel that some active oxygen species can overcome the antioxidant defence permanently as proposed by Gerschman [27]. One can suggest that active oxygen species not only damage DNA but also inactivate the DNA repair enzymes. In our experiments nick-dependent DNA labelling was decreased after incubation in the presence of NADPH (table 1), which suggests partial inhibition of the polymerase by accumulated H_2O_2, inasmuch as catalase abolished the effect. One must take into account that $O_2^{\cdot -}$ and $^{\cdot}OH$ are more suicidal species than H_2O_2. The polymerase from *B. stearothermophilus* is insensitive to sulfhydryl blocking agents [11]. SH groups react with oxygen radicals [30]. Consequently SH-dependent polymerase should be much more sensitive to active oxygen species than the SH-independent ones. In accordance with this proposal, inhibition of solubilized nuclear DNA polymerases in the presence of microsomes or nuclear membranes, when NADPH was present, was observed, DNA polymerases α and γ were affected more by the treatment than was DNA polymerase β which is highly resistant to SH-reacting substance [31].

Changes in liver chromatin [32] and a significant decline in DNA repair capacity [33] have been shown to occur with increasing age. It has been calculated that 'spontaneous' cancer could arise from about one single strand break on average in the DNA of each cell of the body each day [34]. It is tempting to speculate that permanent leakage of active oxygen species over the antioxidant defence system contributes to these events.

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