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LIVER NUCLEAR NADPH-CYTOCHROME P-450 REDUCTASE MAY BE INVOLVED IN REDOX CYCLING OF BLEOMYCIN-Fe(III), OXY RADICAL FORMATION AND DNA DAMAGE

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When NADPH-cytochrome P-450 reductase isolated from rat liver microsomes was aerobically incubated with bleomycin, $FeCl_3$, NADPH and DNA parallel NADPH and oxygen were consumed and malondialdehyde was formed. A similar parallelism of NADPH- and oxygen-consumption and malondialdehyde formation was observed when cell nuclei isolated from rat liver were incubated under the same conditions. The formation of malondialdehyde which was identified by HPLC and which was most likely released from oxidative cleavage of deoxyribose of nuclear DNA required oxygen, bleomycin, $FeCl_3$ and NADPH. This indicates that a nuclear NADPH-enzyme, presumably NADPH-cytochrome P-450 reductase, is able to redox cycle a bleomycin-iron-complex which in the reduced form can activate oxygen to a DNA-damaging reactive species. The data suggest that the activity of this enzyme in the cell nucleus could play an important role in the cytotoxicity of bleomycin in tumor cells.

KEY WORDS: Bleomycin, liver cell nuclei, NADPH-cytochrome P-450 reductase, redox cycling, DNA damage, oxy radicals.

INTRODUCTION

The cytotoxic effects of bleomycin, a glycopeptide antibiotic drug used in tumor chemotherapy,[1] have been attributed to its ability to form an iron-complex which activates oxygen to DNA damaging reactive oxygen species.[2,3] However, it is still not known whether such a complex is formed intracellularly, by which mechanism oxygen is activated, what kind of reactive oxygen species is responsible for DNA damage and how the bleomycin-Fe(II)-complex is regenerated.

Most publications deal with oxygen activation by a bleomycin-Fe(II)-complex itself or by a complex of bleomycin-Fe(III) which has been reduced chemically (for review see[2]). Reduction of the bleomycin-Fe(III)-complex can also be performed by microsomes[4–6] as well as by isolated NADPH-cytochrome P-450 reductase.[7–10] The reductase catalyzes redox cycling of the bleomycin-iron-complex,[7] whereby cleavage of DNA when added can be observed,[7,8] most likely oxy radicals being responsible.[10]

Therefore, we wondered whether this enzyme which has been detected in the nucleus of liver cells[11] could be involved in the activation of the bleomycin-iron-complex associated with oxy radical formation and DNA damage. We determined

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bleomycin-iron-dependent NADPH- and oxygen-consumption and malondialdehyde formation in isolated liver cell nuclei. The latter is formed when DNA is incubated with bleomycin, Fe(II) and oxygen.[12–14] It originates from the oxidative cleavage of deoxyribose of DNA.[15,16] For comparison incubations with NADPH-cytochrome P-450 reductase isolated from liver microsomes were performed.

MATERIALS AND METHODS

Bleomycinum Mack® consisting of 55–70% bleomycin A_2 and 25–32% bleomycin B_2 was obtained from Mack, Illertissen, and the chemicals or biochemicals used either from Merck, Darmstadt, Sigma, München, or Boehringer, Mannheim, Germany.

NADPH-cytochrome P-450 reductase was isolated from liver microsomes as previously described.[7]

Cell nuclei were isolated from the livers of 200 g male Wistar rats by sucrose density gradient centrifugation as described elsewhere.[17] They were free of mitochondria as measured by the activity of succinate dehydrogenase and based on protein contained about 10% of glucose-6-phosphatase of microsomes, a constitutive activity normally present in microsome-free nuclear preparations.[18]

The isolated nuclei were incubated in 25 mM TES-buffer containing 2.5 mM $MgCl_2$ at 37°C. NADPH was added after preincubation of nuclei, bleomycin and $FeCl_3$ at 37°C. NADPH-consumption was followed fluorimetrically (λ_{ex} = 350 nm, λ_{em} = 465 nm). Oxygen-consumption was measured using a Clark-oxygen-electrode connected to an electrometer which was calibrated in ppm O_2. Malondialdehyde (MDA) was determined in the trichloroacetic acid supernatant by the thiobarbituric acid (TBA) reaction as previously described.[19]

In one experiment the MDA-TBA-adduct formed was separated by HPLC. After formation the reaction product was injected onto an HPLC column (Polyol RP-18, 5 μm) and eluted with H_2O, followed by an H_2O/methanol gradient similarly as described by Bird *et al.*[20] As standard tetramethoxypropane, a malondialdehyde precursor, was used. The optical density of the product formed was recorded at 531 nm, the absorption maximum of the authentic MDA-TBA-adduct.[21]

RESULTS

Figure 1 demonstrates that during incubation of DNA with a bleomycin-Fe(III)-complex and isolated liver microsomal NADPH-cytochrome P-450 reductase parallel to NADPH- and oxygen-consumption malondialdehyde is formed. Under these conditions NADPH- and oxygen-consumption and malondialdehyde formation were negligible in the absence of the enzyme as well as in the absence of the bleomycin-Fe(III)-complex. Without NADPH no malondialdehyde was formed (data not shown).

A similar experiment with isolated liver cell nuclei also shows a close relationship between NADPH- and oxygen-consumption as well as malondialdehyde formation in the presence of the bleomycin-Fe(III)-complex (Figure 2). However, a part of the nuclear NADPH- and oxygen-consumption was independent of bleomycin and $FeCl_3$ (Table I). Furthermore, some malondialdehyde was formed with $FeCl_3$ or with bleomycin alone (Table I). The latter effect could be due to traces of iron ions present

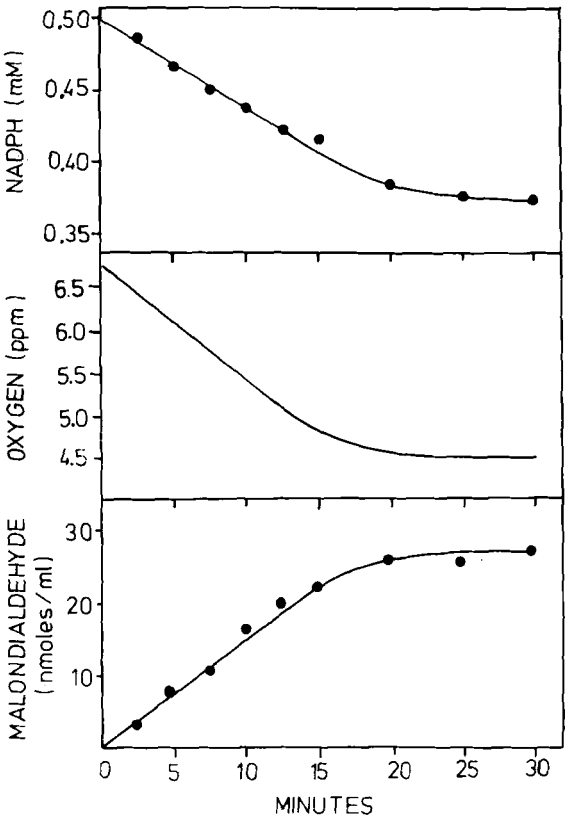

FIGURE 1 NADPH- and O_2-consumption and MDA formation when DNA (200 μg/ml) was incubated with bleomycin (500 μg/ml), $FeCl_3$ (0.15 mM), NADPH (0.5 mM) and isolated liver microsomal NADPH-cytochrome P-450 reductase at 37°C.

in the isolated nuclei. On the other hand, Table I clearly demonstrates that NADPH, oxygen and nuclei are required for malondialdehyde formation by bleomycin.

That indeed malondialdehyde (MDA) was formed can be seen from Figure 3. During incubation with the bleomycin-Fe(III)-complex and NADPH the nuclear suspension released a product which reacted with TBA. The resulting adduct coeluted on HPLC with the authentic MDA-TBA-adduct (Figure 3). No such peak occurred in the chromatogram when the nuclear suspension containing all ingredients was treated with TBA before incubation (data not shown).

DISCUSSION

Our results indicate that isolated liver microsomal NADPH-cytochrome P-450 reductase catalyzes redox cycling of a bleomycin-iron-complex whereby in the presence of DNA malondialdehyde is formed parallel to NADPH- and oxygen-consumption (Figure 1). Malondialdehyde most likely originates from the breakdown of deoxyribose of DNA. The underlying mechanism has not been completely worked out. But it has been shown that a bleomycin-Fe(II)-oxygen-complex cleaves DNA whereby base propenals are formed.[22–26] Under acidic conditions base propenals break down to malondialdehyde and free bases. The initiation of DNA breakage could either be

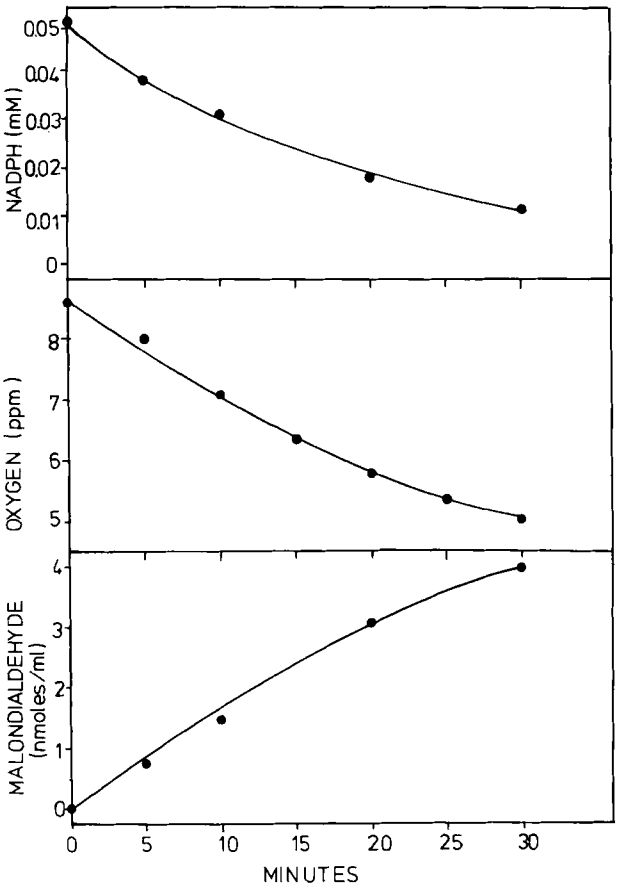

FIGURE 2 NADPH- and O_2-consumption and MDA formation when isolated liver cell nuclei (1 mg protein/ml) were incubated with bleomycin (100 μg/ml), $FeCl_3$ (0.1 mM) and NADPH (0.05 mM) at 37°C.

TABLE I

NADPH- and O_2-consumption and MDA formation in isolated liver cell nuclei (1 mg protein/ml) after 30 min incubation with bleomycin (100 μg/ml), $FeCl_3$ (0.1 mM) and NADPH (0.05 mM) at 37°C

	NADPH-consumption (nmol/ml)	O_2-consumption (ppm)*	MDA-formation (nmol/ml)
Complete system	39	3.5	4.9
– Nuclei	0	0	0
– NADPH	–	0	0
– Bleomycin, – $FeCl_3$	15	1.1	0
– Bleomycin	18	1.3	1.2
– $FeCl_3$	25	1.8	1.3
– O_2 (100% N_2)	15	–	0

*Difference of oxygen concentration between 0 min (air saturated) and 30 min is given.

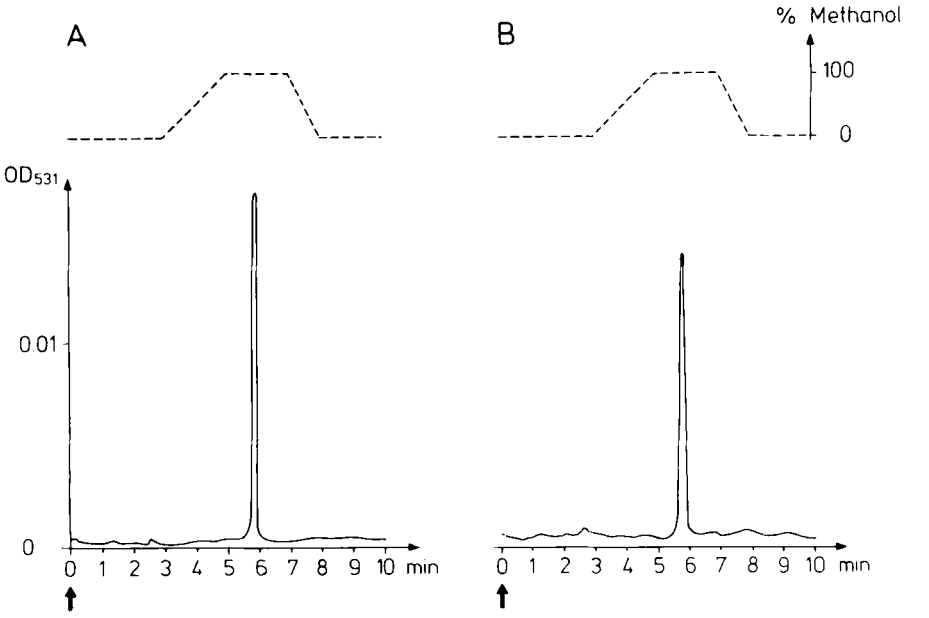

FIGURE 3 HPLC elution profile of the MDA-TBA-adduct chromatographed on a Polyol RP-18 (5 μm) column (250 × 4.6 mm) using a H_2O/methanol gradient (– – –). Injection volume 100 μl, flow rate 2 ml/min, pressure 100–150 bar, temperature 37°C. Arrows: injection. (A) Adduct formed with authentic malondialdehyde (1.2 nmol/ml); (B) Adduct formed in a sample which had been incubated for 30 min with isolated liver cell nuclei (1 mg protein/ml), bleomycin (100 μg/ml), $FeCl_3$ (0.1 mM) and NADPH (0.05 mM) at 37°C. Total dilution 1/6.

due to the bleomycin-Fe(II)-oxygen-complex itself[27,28] or due to hydroxyl radicals formed via the superoxide anion which may be released from this complex.[10,29-31]

Because only a reduced bleomycin-iron-complex is active cleaving DNA, the oxidized bleomycin-iron-complex must be continuously reduced after reaction with DNA. We and others have shown that isolated microsomal NADPH-cytochrome P-450 reductase, a one-electron-transferring flavoprotein, is able to reduce an oxidized bleomycin-iron-complex.[7-10] Therefore, this enzyme could also be responsible for the activation step of bleomycin in whole cells. On the other hand, it is mainly present in the endoplasmatic reticulum and therefore bleomycin activation and if involved also oxy radical formation would occur distant from cellular DNA. Therefore, we wondered whether nuclear NADPH-cytochrome P-450 reductase which is in close proximity to the bulk of cellular DNA might be able reduce an oxidized bleomycin-iron-complex.

Because our preparations of liver cell nuclei were essentially free of mitochondria and microsomes (Materials and Methods) our results indicate that in cell nuclei an NADPH-dependent enzyme catalyzes redox cycling of the bleomycin-iron-complex associated with the formation of malondialdehyde. This compound which we have identified here may be formed by cleavage of deoxyribose of nuclear DNA or by NADPH-dependent peroxidation of lipids of the nuclear envelope. NADPH-dependent lipid peroxidation is stimulated in microsomes by a bleomycin-iron-

complex.[32] However, when we incubated a comparable amount of liver microsomes – containing the same specific activity of glucose-6-phosphatase as our nuclei – only about 30% of the malondialdehyde formed in nuclei could be observed (data not shown). This suggests that at least 70% of the malondialdehyde formed in nuclei does not originate from lipid peroxidation. Malondialdehyde is most likely released from deoxyribose of nuclear DNA by a bleomycin-Fe(II)-oxygen-complex or by hydroxyl radicals formed.

The nuclear NADPH-enzyme involved in reduction of the bleomycin-iron-complex and in damage of nuclear DNA is probably identical to NADPH-cytochrome P-450 reductase which is a constitutive enzyme of isolated microsome-free liver nuclei.[33] When it regenerates the reduced bleomycin-iron-complex which can activate oxygen cytotoxicity of bleomycin could depend on the activity of this enzyme in the cell nucleus. Nuclear NADPH-cytochrome P-450 reductase of tumor cells might thus be an important factor in the efficacy of bleomycin as anticancer agent.

Acknowledgements

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