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ADRIAMYCIN STIMULATES NADPH-DEPENDENT LIPID PEROXIDATION IN LIVER MICROSOMES NOT ONLY BY ENHANCING THE PRODUCTION OF $O_2^{\dot{-}}$ AND H_2O_2, BUT ALSO BY POTENTIATING THE CATALYTIC ACTIVITY OF FERROUS IONS

(Lipid peroxidation; adriamycin; ferrous ions; superoxide radicals; liver microsomes)

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SUMMARY

The antitumor drug, adriamycin, enhances NADPH-dependent lipid peroxidation in liver microsomes via the formation of superoxide anion radicals ($O_2^{\dot{-}}$) and hydrogen peroxide (H_2O_2). In the presence of metal ions additional reactive species are generated, causing stimulation of lipid peroxidation.

However, in this study it was found that the stimulation of NADPH-dependent lipid peroxidation by adriamycin was not only affected by the production of $O_2^{\dot{-}}$ and H_2O_2. Adriamycin also enhances the catalysis by metal ions of the formation of those reactive oxygen species which initiate peroxidation. This was inferred from the fact that adriamycin stimulated malondialdehyde production at low ferrous ion concentrations, whereas at high ferrous ion concentrations no stimulation was found. Additional evidence was found in experiments in which the enzymic redox cycle of adriamycin in microsomes was abolished by heat-inactivation of the microsomes, and $O_2^{\dot{-}}$ and H_2O_2 were only produced with xanthine and xanthine oxidase. In this case in the presence of ferrous ions, adriamycin stimulated lipid peroxidation.

INTRODUCTION

The use of the antitumor drug, ADM, is restricted by its cardiotoxicity. Based on experiments with liver microsomes, one of the speculations on the cause of this car-

Abbreviations: ADM, adriamycin; DMSO, dimethylsulfoxide; MDA, malondialdehyde.

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diotoxicity is that via ADM the production of free radicals is stimulated [1, 2]. This stimulation proceeds via a redox cycle in which ADM is enzymically reduced and reoxidized by oxygen [3–5]. From the oxygen species $O_2^{\dot{-}}$ and H_2O_2 which evolve from this cycle, in the presence of metal ions such as zinc, copper and ferrous ions, other oxygen species are formed [6–11]. The reactive oxygen species attack cell components and cause, for example, peroxidation of membrane lipids.

The role of ferrous ions in ADM-stimulated lipid peroxidation is unclear from the literature. Ferrous ions are reported to depress the stimulation of lipid peroxidation [2], but they are stated to be essential for stimulation [12].

We were confronted with a possible rôle of ferrous ions when we observed large variations in the stimulation of NADPH-dependent lipid peroxidation in Tris-buffered liver microsomes and found that these variations correlated with the conductivity of the deionised water used to prepare the buffer. Therefore, we decided to investigate further the rôle of ferrous ions in the stimulation of lipid peroxidation by ADM.

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Bakers Yeast), NADP, xanthine and xanthine oxidase (grade I from buttermilk) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Adriamycin was a gift from Dr. F. Arcamone (Farmitalia Carlo Erba, Milan, Italy). Water (Analar®) was from BDH Chemicals Ltd. (Poole, U.K.). All other chemicals used were of analytical grade purity.

Preparation of microsomes

Male Wistar rats (200–250 g, TNO, Zeist, The Netherlands) were killed by decapitation. The livers of 5 rats were removed and homogenized (1:2 w/v) in ice-cold 50 mM phosphate buffer, pH 7.4 containing 0.1 mM EDTA. To isolate microsomes, the homogenate was centrifuged at 10 000 × *g* for 20 min at 4°C. The supernatant was again centrifuged at 10 000 × *g* for 20 min. The resulting supernatant was centrifuged at 65 000 × g for 60 min. The microsomal pellet was resuspended in the phosphate buffer and stored at −80°C. To remove EDTA, before use the microsomes were diluted with ice-cold 50 mM Tris–HCl, 150 mM KCl, pH 7.4 and washed twice with this buffer by centrifugation at 115 000 × *g* for 40 min. The microsomal pellet was finally suspended in Tris-buffer, saturated with carbogen (5% CO_2/95% O_2).

Incubation conditions

Basic system: the microsomes were used in a final concentration of 0.5–1.0 mg protein/ml. Peroxidation was induced by an NADPH-regenerating system by the

method of Mimnaugh et al. [2]. The final concentrations of the ingredients were NADP 1.9 mM, glucose-6-phosphate 20 mM, $MgCl_2$ 9 mM. All solutions were prepared in 50 mM Tris-HCl, 150 mM KCl, pH 7.4, saturated with 5% CO_2/95% O_2. Peroxidation was started with the addition of glucose-6-phosphate dehydrogenase (0.85 U/ml final concentration). The incubation was performed at 37°C in a shaking water bath; air was freely admitted.

ADM, final concentration 100 μM, was added to the basic system in DMSO (final concentration 0.25%). Iron was added to the system as $FeSO_4$ (freshly prepared). When a xanthine/xanthine oxidase system was used to generate $O_2^{\bar{\cdot}}$ and H_2O_2, the final xanthine concentration was $5 \cdot 10^{-5}$ M.

Assays

Lipid peroxidation was assayed by the thiobarbituric acid test [13]. Zero-time samples were taken to correct for the extinction of ADM. Lipid peroxidation was expressed as nmol MDA/mg protein, using an extinction coefficient of $1.53 \cdot 10^5$ $M \cdot cm^{-1}$ at 533–600 nm. Protein was determined by the method of Lowry et al. [14].

RESULTS

Fig. 1 shows the lipid peroxidative process of liver microsomes, induced by an

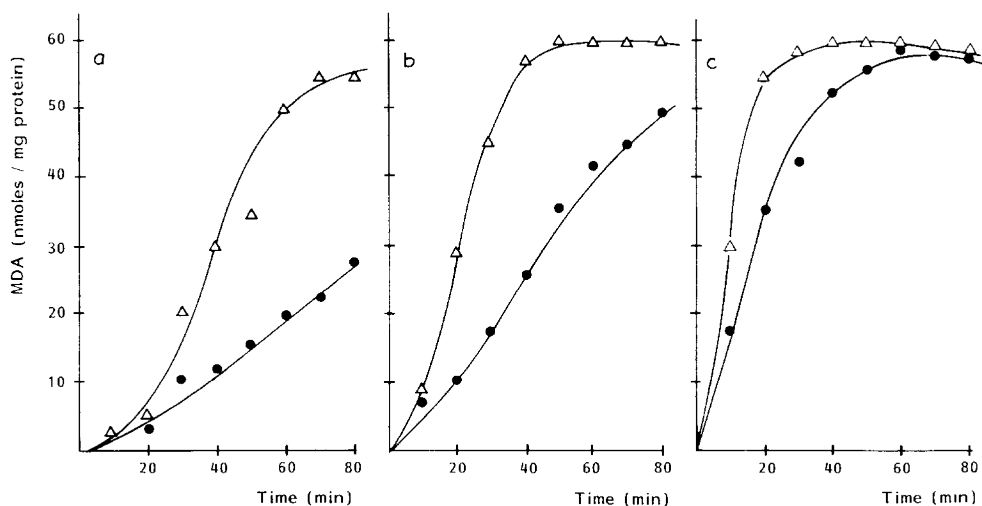

Fig. 1. Effect of ferrous ions on NADPH-induced and NADPH plus adriamycin-induced lipid peroxidation in liver microsomes. The reaction mixture was composed as described in MATERIALS AND METHODS (basic system). Protein concentration was 0.6 mg/ml. (a) No ferrous ions added. (b) Concentration added ferrous ions 0.5 μM. (c) 2.0 μM ferrous ions added. ●, MDA production in the absence of adriamycin; △ , MDA production in the presence of 100 μM adriamycin. Data represent one example of at least 4 duplicate experiments.

NADPH-regenerating system with different concentrations of added ferrous ions. Without added ferrous ions, MDA production increased with time and lipid peroxidation was stimulated by 100 μM ADM. When a small amount of ferrous ions was added (final concentration 0.5 μM), the NADPH-induced MDA production accelerated. ADM still stimulated the peroxidation, though the ratio stimulated/not stimulated MDA production after 60 min was decreased in comparison with the ratio obtained in the basic system. Enhancement of the concentration of added ferrous ions to 2 μM resulted in a further stimulation of MDA formation. The stimulation by ADM was restricted to the initial phase of MDA production; no stimulation was found after 60 min of incubation. Addition of 1 mM EDTA to the basic system to complex metal ions present in the buffer totally depressed MDA production. Also the stimulation of MDA production by ADM was abolished in that case (not shown).

Since ADM is thought to stimulate lipid peroxidation by enhancement of production of $O_2^{\dot{-}}$ and H_2O_2, it was decided to see if this extra production of the oxygen species in the microsomal system did increase MDA formation. Fig. 2 shows the results of experiments in which xanthine and xanthine oxidase were added to the

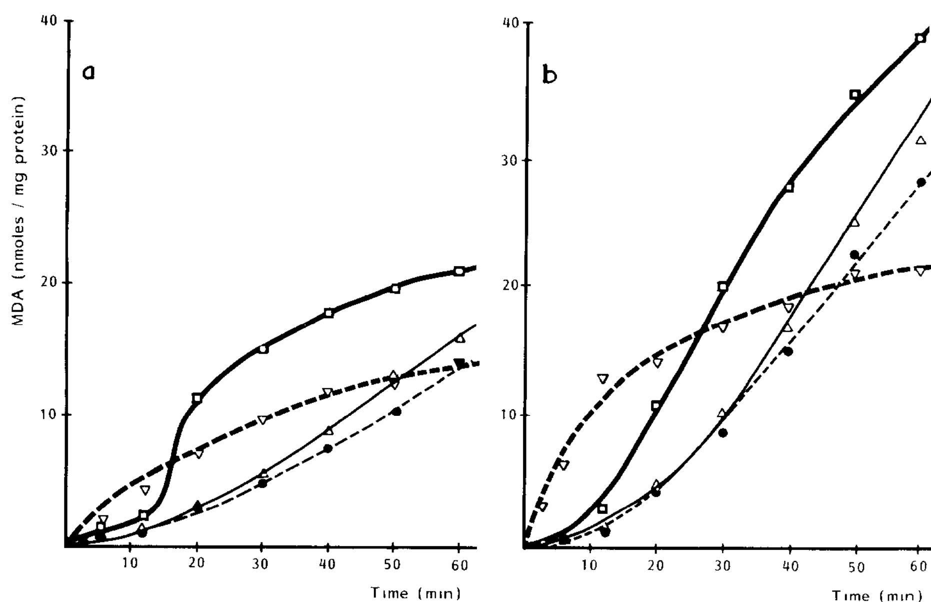

Fig. 2. Effect of xanthine and xanthine oxidase on the time course of lipid peroxidation in liver microsomes. Peroxidation mixture: see basic system as described in MATERIALS AND METHODS. To this basic system $5 \cdot 10^{-5}$ M xanthine and different amounts of xanthine oxidase were added. No ferrous ions were added. Protein concentration was 0.8 mg/ml. (a) NADPH-induced peroxidation. (b) NADPH plus 100 μM adriamycin-induced lipid peroxidation. ●, No xanthine oxidase added; △, xanthine oxidase concentration 5.5 mU/ml; □, xanthine oxidase concentration 55 mU/ml; ▽, xanthine oxidase concentration 550 mU/ml. Data represent mean of 5 experiments.

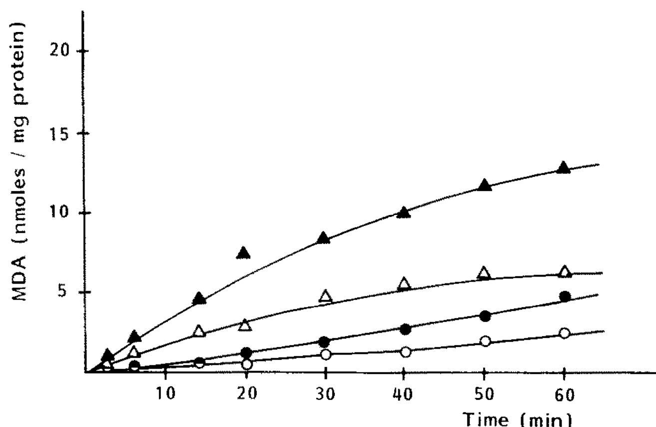

Fig. 3. Time course of lipid peroxidation in heated liver microsomes in the presence of xanthine oxidase. The incubation mixture was composed as described (basic system) in MATERIALS AND METHODS. Xanthine was added in a concentration of $5 \cdot 10^{-5}$ M, the xanthine oxidase concentration was 55 mU/ml. The microsomes were heated for 2 min at 95°C prior to the incubation to destroy enzymic activity. Protein concentration was 1.0 mg/ml. The data represent the mean of 3 experiments. ○, Basic system plus xanthine and xanthine oxidase (55 mU/ml); △, idem, plus 100 μM adriamycin; ●, basic system plus xanthine and xanthine oxidase (55 mU/ml) in the presence of 2 μM ferrous ions; ▲, idem, plus 100 μM adriamycin.

basic system to enhance the production of $O_2^{\overline{\cdot}}$ and H_2O_2. Both in the absence and in the presence of ADM, xanthine oxidase stimulated MDA production. A high production of $O_2^{\overline{\cdot}}$ and H_2O_2 (at a high xanthine oxidase concentration) led to a fall in the MDA level as compared with incubations with lower xanthine oxidase concentrations. Even in this case ADM increased the MDA production.

Finally, the effect of ADM on lipid peroxidation in heat-inactivated microsomes was measured. The heat treatment was done to abolish the enzymic activity of the microsomes, so that enzymic activation of ADM and the subsequent production of $O_2^{\overline{\cdot}}$ and H_2O_2 was absent. To facilitate lipid peroxidation, xanthine and xanthine oxidase were used to produce a certain amount of $O_2^{\overline{\cdot}}$ and H_2O_2. Fig. 3 shows the low MDA production in the absence of added ferrous ions in this system and somewhat higher production when the concentration of added ferrous ions was 2 μM. ADM stimulated the MDA production in both cases.

DISCUSSION

The main explanation for the stimulation by ADM of NADPH-dependent lipid peroxidation has been the increased production of $O_2^{\overline{\cdot}}$ and H_2O_2 via the enzymic reduction of ADM to its semiquinone radical. Subsequently the free electron of the semiquinone is transferred to oxygen [3–5] (mechanism *a*). From the literature [12, 15, 16] a dependence on ferrous ions for the stimulation of lipid peroxidation by

ADM is indicated. Also lipid peroxidation induced by NADPH alone depends on ferrous ions. This phenomenon is now being explained by the fact that not all reactive oxygen species (H_2O_2, $O_2^{\dot{-}}$, $HO_2^{\cdot}$, $OH^{\cdot}$) initiate lipid peroxidation, and that ferrous ions have a catalytic function in the generation of those species (whether OH radicals or iron–oxygen complexes [6–11]) that are able to attack the unsaturated bonds in polyunsaturated fatty acids. From these data, it would appear that ADM stimulates lipid peroxidation in the following way: ADM enhances the production of $O_2^{\dot{-}}$ and H_2O_2, and due to the presence of small amounts of metal ions lipid peroxidation-initiating oxygen species are formed which start the peroxidative process.

In the NADPH/microsomal system the ferrous ion concentration has a marked effect in the initiation of MDA production. As well as unstimulated MDA production, there is also stimulation by 100 μM ADM changes. For example, the ratio stimulated/not stimulated lipid peroxidation (measured as MDA production) at 60 min decreased from 2.5 when no ferrous ions were added to 1.0 in the presence of 2.0 μM iron. Although ferrous ions are necessary for the induction of lipid peroxidation (since EDTA abolishes MDA production) the stimulation of lipid peroxidation by ADM occurs only at low concentrations of metal ions. For ferrous ions, an optimal stimulation takes place in the nM/μM range. This dependence of ADM stimulation on ferrous ion concentration may be explained by the assumption that ADM is able to potentiate the catalytic function of ferrous ions in stimulating the generation of those oxygen species/complexes which initiate peroxidation (mechanism *b*).

In our system, extra production of H_2O_2 and $O_2^{\dot{-}}$ (mechanism *a*) can indeed contribute to the stimulation of MDA production by ADM: when in intact microsomes extra $O_2^{\dot{-}}$ and H_2O_2 were generated with xanthine and xanthine oxidase, MDA formation increased. Yet, even at the highest xanthine oxidase level when the MDA level fell (possibly due to an increased role of termination reactions) lipid peroxidation was enhanced by 100 μM ADM. This might mean an additional stimulatory effect of ADM due to its interaction with ferrous ions (mechanism *b*). More evidence for mechanism *b* was found in experiments using heat-inactivated microsomes. In this case enzymatic activation of ADM and subsequent redox cycling of ADM are eliminated, so that mechanism *a* cannot occur. However, our results show that ADM still stimulated MDA formation, thus confirming the notion that mechanism *a* is not exclusive and that mechanism *b* could be an additional way of the stimulation of lipid peroxidation.

In conclusion, our results suggest that ADM stimulates NADPH-dependent lipid peroxidation by a dual action, i.e. (mechanism *a*) increasing $O_2^{\dot{-}}$ and H_2O_2 production and (mechanism *b*) increasing the catalytic action of iron in the production of OH radicals or other reactive oxygen species that are able to initiate lipid peroxidation. The notion of an iron–ADM complex which can be reduced enzymically and at which formation of those oxygen species/complexes that initiate lipid peroxidation could directly and efficaciously take place [12, 15, 17], fits our data well.

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