

# Inhibition of adriamycin-promoted microsomal lipid peroxidation by $\beta$ -carotene, $\alpha$ -tocopherol and retinol at high and low oxygen partial pressures

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Iron-dependent peroxidation of rat liver microsomes, enhanced by adriamycin, was measured in the presence of increasing concentrations of  $\alpha$ -tocopherol,  $\beta$ -carotene and retinol at low and high  $pO_2$ .  $\beta$ -Carotene and  $\alpha$ -tocopherol inhibited lipid peroxidation by more than 60% when present at concentrations greater than 50 nmol/mg microsomal protein at both high and low  $pO_2$ . Retinol inhibited peroxidation by 39% at concentrations greater than 100 nmol/mg microsomal protein. This maximal level of inhibition by retinol was unaltered by  $pO_2$ . However,  $\beta$ -carotene was more effective than  $\alpha$ -tocopherol or retinol at a  $pO_2$  of 4 mmHg, whereas  $\alpha$ -tocopherol was more effective under aerobic conditions. Since adriamycin-dependent lipid peroxidation is maximal at low  $pO_2$ ,  $\beta$ -carotene may play a role in protecting against this process.

Lipid peroxidation; Microsome; Adriamycin;  $\beta$ -Carotene;  $\alpha$ -Tocopherol

## 1. INTRODUCTION

The peroxidation of lipids has been proposed as a mechanism by which redox-active xenobiotics, including the anticancer drug adriamycin, cause cell damage [1–3]. Adriamycin is reduced by cellular enzymes and enhances lipid peroxidation in a variety of systems [3–5], suggesting that its dose-limiting cardiotoxicity could be a result of myocyte membrane peroxidation [4–6]. Recently, we have shown that adriamycin-dependent peroxidation of microsomes is 4–5-fold greater at low  $O_2$  partial pressures than in air [7]. Thus, lipid peroxidation in vivo should be favoured by low  $O_2$  partial pressures, such as exist in active muscle tissue [8].

Lipid peroxidation is inhibited by chain-breaking antioxidants.  $\alpha$ -Tocopherol, the best

known of these, is considered to be the major physiological inhibitor of lipid peroxidation [9]. Several in vitro studies of adriamycin-dependent peroxidation have shown inhibition by  $\alpha$ -tocopherol [10,11] although the effects of deficiency or supplementation have been equivocal [12–15]. The ability of  $\alpha$ -tocopherol to inhibit lipid peroxidation declines with decreasing  $pO_2$  [16]. In contrast, Burton and Ingold [16] have shown that  $\beta$ -carotene becomes more efficient at inhibiting peroxidation of linoleic acid as the  $pO_2$  decreases. This suggests that  $\beta$ -carotene and like compounds may provide better protection than  $\alpha$ -tocopherol against adriamycin-induced lipid peroxidation.

To test this proposal, we have compared the abilities of  $\alpha$ -tocopherol,  $\beta$ -carotene and retinol to inhibit adriamycin-dependent microsomal lipid peroxidation in air and at low  $pO_2$ .

## 2. MATERIALS AND METHODS

Biochemicals were from Sigma (St. Louis, MO) except for

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adriamycin (from Farmitalia, Carlo Erba, Italy). Liver microsomes were prepared from Wistar rats according to Ernster et al. [17], and stored in 10 mM phosphate buffer (pH 7.4), at  $-100^{\circ}\text{C}$ . Microsomal protein was measured by the method of Lowry et al. [18].  $\beta$ -Carotene and  $\alpha$ -tocopherol, at the required concentrations, were incorporated into the microsomal membrane by pelleting the microsomes at  $105\,000 \times g$  and gently homogenising the pellet with antioxidant solubilised in  $10\ \mu\text{l}$  chloroform, using a Potter-Elvehjem homogeniser. Once mixing was complete the microsomes were resuspended in buffer. When  $\beta$ -carotene-enriched microsomes were ultracentrifuged, all the  $\beta$ -carotene spun down with the microsomes, demonstrating that it was incorporated into the microsomal membrane. Cytochrome P450 reductase activity of the antioxidant-enriched microsomes was standardized such that  $50\ \mu\text{l}$  of each preparation, in 1 ml buffer with  $100\ \mu\text{M}$  NADPH, reduced cytochrome *c* at a rate of  $3\ \mu\text{M}/\text{min}$ . Procedures were carried out in acid-washed glassware and buffers were treated with chelex resin (Biorad Lab, CA) to remove adventitious iron.

### 2.1. Lipid peroxidation

Microsomes (0.5 mg protein/ml) were incubated with  $100\ \mu\text{M}$  NADPH,  $1.0\ \mu\text{M}$   $\text{FeCl}_3$  and  $30\ \mu\text{M}$  adriamycin for 30 min at  $22^{\circ}\text{C}$  in 10 mM phosphate buffer (pH 7.4). Reactions were performed in 10 ml rubber-stoppered glass tubes in which the  $p\text{O}_2$  was manipulated by bubbling solution with  $\text{O}_2$ -free  $\text{N}_2$  and replacing the requisite volume of  $\text{N}_2$  with  $\text{O}_2$  using a gas-tight syringe. Solutions were continually mixed by rotation. Lipid peroxidation was measured as  $A_{532}$  due to the formation of thiobarbituric acid-reactive products as described in [19]. Blanks contained adriamycin and microsomes but no NADPH or iron, and where appropriate, an equivalent amount of  $\beta$ -carotene.

### 2.2. $\beta$ -Carotene bleaching

Microsomes (0.5 mg), containing  $25\ \mu\text{M}$   $\beta$ -carotene, were incubated with  $1.0\ \mu\text{M}$   $\text{FeCl}_3$  and  $100\ \mu\text{M}$  NADPH in 1 ml phosphate buffer in the presence and absence of adriamycin.  $\beta$ -Carotene bleaching was continuously monitored at 466 nm.

## 3. RESULTS

In the absence of adriamycin, addition of NADPH and iron to rat liver microsomes caused a small amount of lipid peroxidation that was relatively independent of  $p\text{O}_2$  (table 1). As we have shown previously [8], adriamycin enhanced peroxidation less than 2-fold in air, but 4–5-fold at  $p\text{O}_2$  values of 4–8 mmHg (table 1).

Both  $\alpha$ -tocopherol and  $\beta$ -carotene gave a concentration-dependent inhibition of adriamycin-dependent peroxidation either at  $p\text{O}_2$  4 mmHg (fig.1A) or in air (fig.1B). Whereas in air  $\alpha$ -tocopherol gave more inhibition than an equivalent concentration of  $\beta$ -carotene, in low air  $\beta$ -carotene was a more efficient inhibitor. Max-

Table 1

Effect of  $p\text{O}_2$  on adriamycin-dependent peroxidation of microsomal lipids

$p\text{O}_2$ (mmHg)	$A_{532}$	
	–	+ adriamycin
0	0.09	0.11
4	0.14	0.73
8	0.15	0.70
80	0.14	0.28
160	0.14	0.27

Microsomes were incubated with or without  $30\ \mu\text{M}$  adriamycin, NADPH and  $1\ \mu\text{M}$   $\text{FeCl}_3$  as described in section 2. Each result is the mean of 4 experiments that agreed within 10%

imum inhibition was achieved with  $>50$  nmol of either antioxidant/mg microsomal protein, and the two together gave no greater inhibition than either one alone. The  $\text{O}_2$  dependence curves in fig.2 show

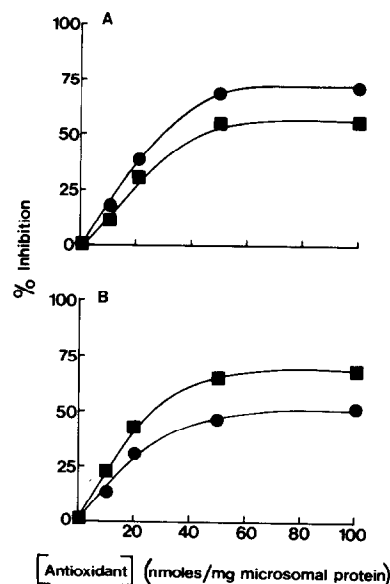


Fig.1. Effect of  $\beta$ -carotene and  $\alpha$ -tocopherol concentration on adriamycin-enhanced microsomal lipid peroxidation at (A)  $p\text{O}_2 = 4$  mmHg and (B)  $p\text{O}_2 = 162$  mmHg. Microsomes were incubated with adriamycin, NADPH,  $\text{FeCl}_3$  and  $\beta$ -carotene ( $\bullet$ ) or  $\alpha$ -tocopherol ( $\blacksquare$ ) at the concentrations shown. Blanks due to antioxidant, anthracycline and microsomes incubated without NADPH were subtracted and percent inhibition was calculated. Each point is the mean for two sets of duplicates and the SD falls within each symbol size.

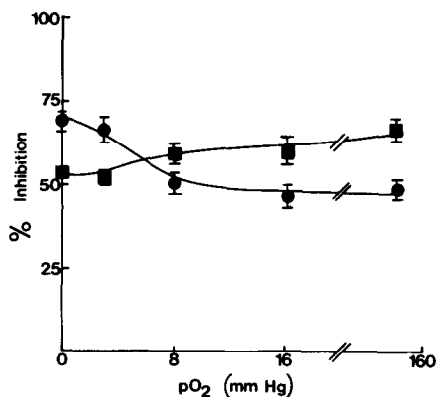


Fig. 2. Inhibition of adriamycin-dependent microsomal lipid peroxidation by (●) 50 nmol/mg  $\beta$ -carotene microsomal protein and (■) 50 nmol  $\alpha$ -tocopherol/mg microsomal protein under increasing  $pO_2$ . Microsomes were incubated with adriamycin,  $FeCl_3$  and NADPH. Blanks due to antioxidant, anthracycline and microsomes incubated without NADPH were subtracted and percent inhibition was calculated. Each point is the mean for two sets of duplicates.

that  $\beta$ -carotene inhibited more than  $\alpha$ -tocopherol only at very low  $pO_2$ .

Maximum inhibition by retinol required  $>100$  nmol/mg microsomal protein and was similar at the two  $O_2$  concentrations (table 2).

As evidence that  $\beta$ -carotene was consumed during lipid peroxidation, continuous monitoring of  $A_{466}$  showed bleaching of  $\beta$ -carotene-enriched microsomes (fig. 3). The rate of bleaching was enhanced 3-fold upon the addition of adriamycin.

Table 2

Effect of retinol on adriamycin-dependent peroxidation of microsomal lipids

[Retinol] (nmol/mg microsomal protein)	% inhibition of lipid peroxidation ( $A_{532}$ )
20	13
40	23
100	37
100 ( $pO_2 = 162$ mmHg)	39

Microsomes were incubated with NADPH, adriamycin,  $FeCl_3$  and retinol at  $pO_2 = 4$  mmHg, except where indicated.  $A_{532}$  values were read against a blank containing retinol, of the appropriate concentration, microsomes, and adriamycin, but no NADPH. Percent inhibition was calculated from  $A_{532}$  values of the complete system with and without retinol. Each result is the mean of 2 sets of duplicates which agreed within 15%

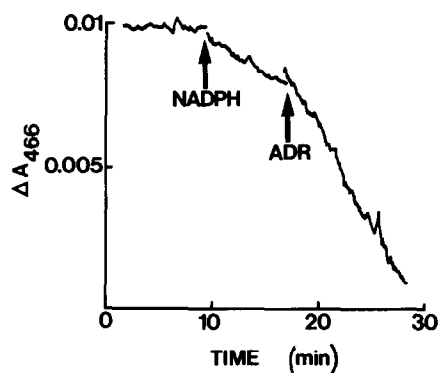


Fig. 3. Effect of adriamycin (ADR) on the bleaching of  $\beta$ -carotene incorporated into microsomal membranes. Incubations were with  $FeCl_3$  and 0.5 mg microsomes containing 25 nmol  $\beta$ -carotene, in 1 ml buffer, under aerobic conditions. The spectrophotometer trace shown is representative of 3 experiments.

#### 4. DISCUSSION

This work demonstrates that  $\beta$ -carotene significantly inhibits adriamycin-dependent lipid peroxidation of microsomes in air. It is slightly less efficient than  $\alpha$ -tocopherol, but as the  $pO_2$  declines and inhibition by  $\alpha$ -tocopherol decreases, there is an increase in inhibition by  $\beta$ -carotene. Retinol also inhibits adriamycin-dependent lipid peroxidation, although the degree of inhibition is similar at the different  $pO_2$  values.

$\alpha$ -Tocopherol inhibits lipid peroxidation by reacting with the lipid hydroperoxy radical [16], thus inhibiting propagation of the chain reaction (reaction 3):



The chain-breaking action of  $\alpha$ -tocopherol is most efficient at high  $pO_2$  and as the  $pO_2$  decreases this mechanism of inhibition becomes less efficient. However, as this happens, antioxidants such as  $\beta$ -carotene and retinol that can compete with  $O_2$  for the lipid radical ( $L'$ , reaction 2) become better scavengers. Thus, Burton and Ingold [16] have proposed that this class of compound should pro-

vide better protection than  $\alpha$ -tocopherol against peroxidation processes occurring at low  $pO_2$ .

Since adriamycin-dependent microsomal lipid peroxidation is optimal at low  $pO_2$ , their proposal implies that  $\beta$ -carotene should be better than  $\alpha$ -tocopherol at inhibiting this process. Our results show that this is indeed the case, although the differences between  $\beta$ -carotene and  $\alpha$ -tocopherol are not as great as might be expected from the conclusions of Burton and Ingold. However, in their studies on linoleic acid, the greatest difference in relative efficiencies of  $\beta$ -carotene and  $\alpha$ -tocopherol was between air and  $O_2$ , and their results at lower  $pO_2$  are comparable to ours.

The observed inhibition of lipid peroxidation by retinol, although not enhanced under low  $pO_2$ , was still significant. Retinol is frequently dismissed as an antioxidant, because, compared with  $\beta$ -carotene, it is ineffective at scavenging  $O_2$  [20]. However, this study has demonstrated that retinol does have antioxidant properties.

Normal levels of  $\beta$ -carotene,  $\alpha$ -tocopherol and retinol in chick liver are 0.17, 9.7 and 153 nmol/mg for liver tissue, respectively [21]. Supplementation is able to elevate levels of all three 4–7-fold [21]. However, the fact that levels of retinol are comparatively so much higher, and the demonstration of antioxidant activity in this study, suggest that the physiological significance of the antioxidant activity of retinol appears to have been underestimated. Our results provide supporting evidence for the proposed antioxidant role for  $\beta$ -carotene and retinol, and raise the possibility that they could protect against adriamycin-induced cardiotoxicity. Indeed, vitamin A in association with  $\alpha$ -tocopherol has been shown to decrease cardiotoxicity of adriamycin in rabbits [22].

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