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The role of lipid peroxidation in acute doxorubicin-induced cardiotoxicity as studied in rat isolated heart

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Doxorubicin induces an acute cardiotoxicity that becomes manifest in isolated hearts as a deterioration in mechanical function. The oxidative component in this myocardial damage has been investigated. The effects of doxorubicin on the activity of superoxide dismutase and the capacity of the glutathione system, factors of the cellular protective mechanism against free radicals, were examined in rat isolated heart. Doxorubicin was found to reduce the capacity of the protective mechanisms. Whether oxidative membrane damage due to excessive free radical formation plays a role in the pathogenesis of the acute cardiotoxic action of doxorubicin was also examined. Its acute effect on myocardial contraction amplitude, frequency of beating, coronary flow and on the above mentioned biochemical parameters was compared in rat hearts sufficient or deficient in vitamin E. Peroxidation of lipids was measured as the formation of malondialdehyde, one of the final products of this process. Vitamin E deficiency neither aggravated the decrease in the capacity of the cellular protective factors nor worsened the reduction in myocardial function. Nor did induction of lipid peroxidation by doxorubicin occur in vitamin E-deficient hearts. It was concluded that lipid peroxidative damage most probably is not decisive in the development of the acute cardiomyopathy in rats.

The underlying mechanism of the cardiomyopathy induced by the anti-tumour agent adriamycin, is still poorly understood, despite extensive investigation. The emphasis of most of the clinical and experimental studies has been on delayed, dose-limiting, degenerative cardiomyopathy. However, there is also an acute toxicity, manifested by ECG changes, left ventricular failure and arrhythmias in patients (Bristow et al 1978). The protective effect of the lipid-antioxidant vitamin E against the early toxicity of doxorubicin (Myers et al 1977; Ferrans 1978; Wang et al 1980), suggests that peroxidative damage of lipids could play a role. No such relation has been ascertained so far. We have investigated whether excessive free radical formation plays a role in the pathogenesis of the acute cardiotoxicity which becomes manifest in isolated hearts as a deterioration in mechanical function (Ohhara et al 1981; Höfling & Bolte 1981; Wikman-Coffelt et al 1983; Rabkin 1982). If doxorubicin-induced peroxidation of membrane lipids significantly contributes to this functional disturbance of cardiac tissue, vitamin E deficiency can be expected to exacerbate the loss of myocardial performance. For this reason we studied the acute effect of the drug on myocardial contraction amplitude, heart rate and coronary flow of spontaneously beating, isolated hearts of rats fed a normal or a vitamin E-deficient diet. In addition, the effect of the drug on the capacity of the cellular defence mechanisms against free radicals was established. The parameters studied were superoxide dismutase (SOD) and the factors of the glutathione system: glutathione-peroxidase (GSH-Px), the reduced and oxidized form of glutathione (GSH, GSSG). These factors are involved in the protection against the continuous formation of free radicals that normally occurs in tissues (Freeman & Crapo 1982).

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MATERIALS AND METHODS

Male Wistar rats (ca 250 g) were used. Those for vitamin E-deficient experiments were fed a diet from which the vitamin was omitted for 10 weeks; controls were fed the same diet with vitamin E (Muracon SSP-Tox standard; Trouw BV, Putten, The Netherlands). Doxorubicin (Adriamycin) was a gift from Dr F. Arcamone, Farmitalia Carlo Erba, Milan, Italy.

Perfusion method

Rats were anaesthetized with diethyl ether. After i.v. heparinization via the tail vein, the thorax was

opened, the aorta cannulated and the heart isolated and perfused according to Langendorff (1895). The standard salt solution contained (mM): NaCl 128·2; KCl 4·7; $CaCl_2$ 1·4; $MgCl_2$ 1·1; NaH_2PO_4 0·4; $NaHCO_3$ 20·2; glucose 11·1 and was continuously gassed with 95% O_2/5% CO_2 (pH 7·4) at 37 °C. For functional studies spontaneously beating hearts were perfused at a constant pressure of 8 kPa for 30 min. After this stabilization period, the hearts were perfused, non-recirculating, with the standard salt solution, with or without 35 μM doxorubicin, for 30 min, followed by a reperfusion with standard salt solution. Myocardial contraction amplitude was measured by recording apico-basal shortening of the heart muscle isotonically with a displacement transducer (HBM-W10). Frequency of contraction was derived directly from the heart via two electrodes. For biochemical studies hearts were perfused as described previously (Julicher et al 1984).

Assays

Superoxide dismutase (SOD) activity was assayed in the homogenate as described by Weisiger & Fridovich (1973), using the cytochrome c/xanthine oxidase-system. The procedure of Lawrence & Burk (1978) served as a method for the determination of Se-dependent GSH-peroxidase (GSH-Px). The amount of GSH and GSSG in homogenate and coronary effluent were determined as described by Griffith (1980). Lipid peroxidation was measured as thiobarbituric acid-reactive material, according to Buege & Aust (1978). Lipid peroxidation was expressed as nmoles malondialdehyde (MDA) per mg protein, using an extinction coefficient of $1{\cdot}56 \times 10^5\ M^{-1}\,cm^{-1}$ at 535–600 nm. Protein was determined according to Lowry et al (1951).

Microsomal and mitochondrial incubations

The microsomes were isolated by centrifugation as decribed previously (Sterrenberg et al 1984). The cardiac mitochondrial fraction was isolated as described by Idell-Wenger et al (1982). To obtain liver mitochondria, rat liver was minced, washed 4 times and homogenized (Potter-Elvehjem) in 3 volumes of a 10 mM Tris/HCl buffer pH 7·4, with 200 mM mannitol. The homogenate was centrifuged at 800*g* for 5 min at 0 °C and the resulting supernatant centrifuged at 13 000*g* and 22 000*g* both for 10 min at 0 °C. All treatments were on ice. Intactness of mitochondrial preparations was checked using a Clark type oxygen electrode. The mitochondria were used on the day of preparation and were resuspended before use in a 150 mM Tris buffer pH 7·4 with 50 mM KCl. Contamination of mitochondria with microsomes was low, as was established by measuring the activity of marker-enzymes. The microsomes were used in a final concentration of 0·3–0·7 mg protein ml^{-1}. Microsomal peroxidation was induced by an NADPH-regenerating system in a Tris/KCl buffer according to Mimnaugh et al (1981). Mitochondrial lipid peroxidation was determined in the same Tris/KCl buffer using 2 mM NADH and 0·5 mg protein ml^{-1} (Karash & Novak 1983). Both microsomes and mitochondria were made enzymatically inactive by heating for 2 min at 95 °C. All the water used during the preparations and incubations, had an exactly defined ion-content (Analar water; BDH Chemicals Ltd, Poole, UK).

RESULTS

The effect of doxorubicin on rat normal, isolated heart

Doxorubicin appeared to be able to change the capacity of the cellular defence mechanisms in whole heart (Fig. 1). A recirculating perfusion of rat isolated heart with 100 μM ADM led to a significant decrease in the activity of SOD ($P < 0{\cdot}01$). The activity of GSH-Px slightly increased, albeit not significantly ($P < 0{\cdot}1$). The amount of GSH, a cofactor of GSH-Px, was almost halved due to the treatment with the drug. The content of GSSG, which is indicative of increased free radical stress (Bartoli & Sies 1978), was not substantially raised in cardiac tissue. Both GSH and GSSG were, however, released into the coronary effluent during the recirculating perfusion with doxorubicin (Fig. 2). The total amount of glutathione (GSH + GSSG) in heart tissue and the coronary perfusate was reduced after perfusion with the drug compared with control treatment. These changes in the cardiac defence capacity against free radicals did not result in detectable lipid peroxidation, which means that MDA could not be found either in heart tissue or perfusate (results not shown).

It must not be inferred from this absence of detectable lipid peroxidation that doxorubicin is unable to generate reactive species in cardiac cell compartments, as was demonstrated with the following experiments. Fig. 3A shows that normal heart microsomes, which do not show NADPH-dependent lipid peroxidation in the presence of doxorubicin, are able to induce oxidative damage in heat-inactivated liver microsomes (which no longer possess the enzymatic activity to initiate lipid peroxidation). This was demonstrated by a substantial production of MDA. This NADPH-induced lipid

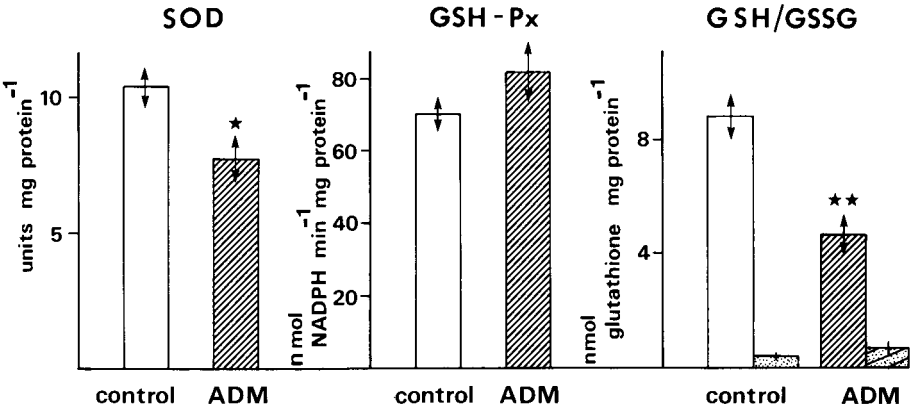

FIG. 1. The effect of a recirculating perfusion for 60 min on the activity of SOD, GSH-Px and the content of GSH and GSSG ((1) dotted column), with the standard salt solution ((2) open column) or with a 100 μM doxorubicin (ADM) solution ((3) hatched column). Each result represents the mean ± s.e.m. of six separate experiments. Significance of differences from the control value: $^{*}P < 0{\cdot}02$ and $^{**}P < 0{\cdot}01$.

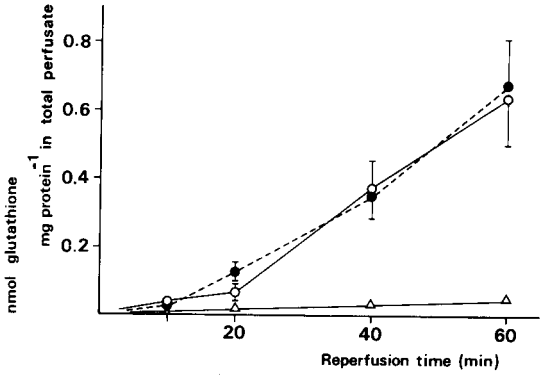

FIG. 2. The cumulative release of GSH (—○—) and GSSG (- -●- -) into the coronary perfusate during a recirculating perfusion with a 100 μM solution of doxorubicin. The total glutathione release (GSH + GSSG), as observed during a similar perfusion with standard salt solution, is depicted as (—△—). Samples were taken at the indicated times. Each value represents the mean ± s.e.m. of six separate experiments.

peroxidation process appeared to be stimulated by doxorubicin. The normal heart microsomes could not induce lipid peroxidation in heat-inactivated heart microsomes. These results imply that free radical generation can actually be enhanced by doxorubicin in cardiac microsomes. Heart mitochondria were not able to induce MDA production in heat-inactivated liver mitochondria, either in the absence or presence of doxorubicin (results not shown). In vitamin E-deficient heart mitochondria, in which a possible enhancement of free radical production can be made manifest more easily, a time-dependent production of MDA occurred (Fig. 3B). This NADH-induced production of MDA appeared to be enhanced in the presence of doxorubicin. In vitamin E-deficient heart microsomes a NADPH-induced formation of MDA was also observed in the presence of doxorubicin (Fig. 3C).

From these findings it may be concluded that doxorubicin is able to generate reactive species in cardiac membranes and also that vitamin E is necessary to protect microsomal and mitochondrial membranes against its peroxidative action.

The effect of doxorubicin on rat vitamin E-deficient, isolated heart

Fig. 4 shows that a non-recirculating perfusion with doxorubicin rapidly decreased the coronary flow in hearts from control rats. A partial restoration of this flow occurred upon normal reperfusion. Myocardial contraction amplitude and frequency of beating also diminished due to perfusion with doxorubicin, albeit less sharply. These effects were partially reversed upon normal reperfusion. Deficiency of vitamin E in rat isolated heart did not aggravate these effects of the drug. A smaller decrease in the frequency of contractions was observed in vitamin E-deficient hearts after a 30 min perfusion with doxorubicin. There was no difference found in the functional parameters between control and vitamin E-deficient rat hearts on perfusion with the standard salt solution.

The effect of perfusion with doxorubicin on the activity of SOD and GSH-Px as well as the content of GSH/GSSG in tissue and coronary effluent, was not affected by the concentration of vitamin E in the

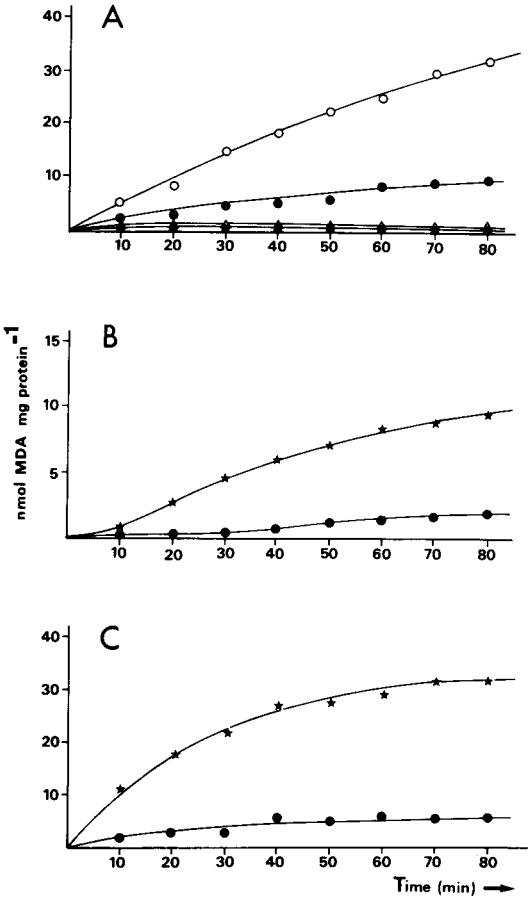

FIG. 3. The induction of lipid peroxidation by an NADPH-regenerating system in heat-inactivated heart (△, ▲) or liver (○, ●) microsomes by untreated heart microsomes, in the absence (●, ▲) or presence (○, △) of doxorubicin (A). The amount of MDA given was corrected for the MDA production in heat-inactivated liver or heart microsomes in the absence of untreated heart microsomes and for the MDA production in untreated heart microsomes themselves. The protein concentration was 0·5 mg ml^{-1} for the heart microsomes and 0·3 mg ml^{-1} for liver microsomes. Furthermore, the time course of lipid peroxidation, in the absence (●) or in the presence (★) of 100 μM doxorubicin is shown in heart mitochondria (B) and microsomes (C) of vitamin E-deficient rats. The protein concentration was 0·5 mg ml^{-1}. All data represent the mean of at least three experiments.

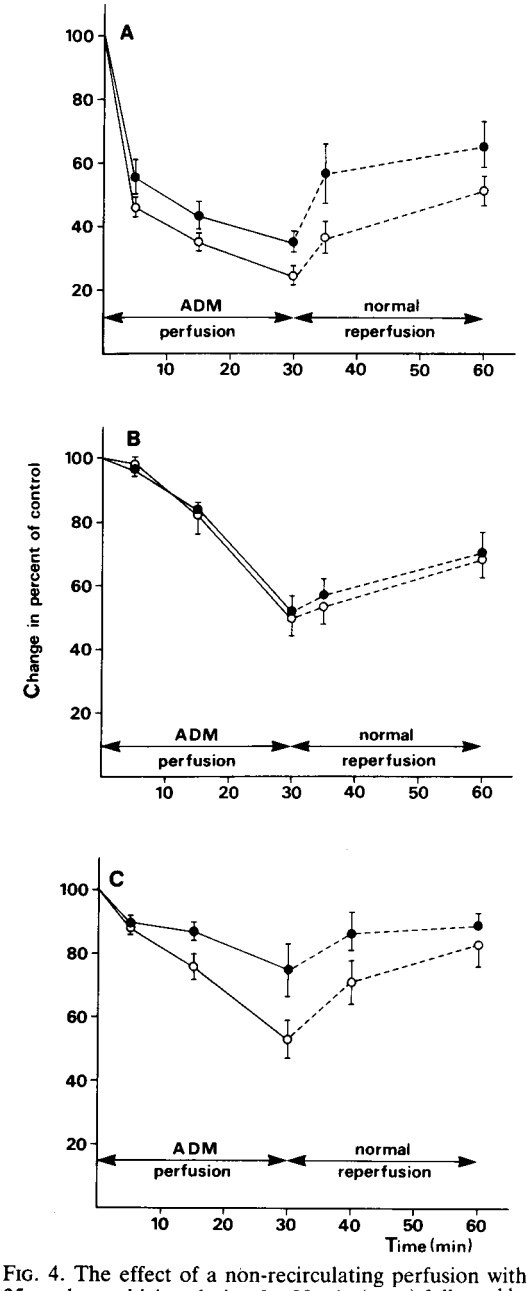

FIG. 4. The effect of a non-recirculating perfusion with a 35 μM doxorubicin solution for 30 min (——) followed by a normal reperfusion (– – –), on the (A) coronary flow, (B) contraction amplitude and (C) frequency of spontaneously beating hearts of rats fed a normal diet (○) or a diet from which vitamin E was omitted (●). The results represent the mean ± s.e.m. of 6 separate experiments.

cardiac tissue (Table 1). Also the ratio of GSH and GSSG content in tissue was not significantly different (results not shown). There was also an absence of peroxidation products of lipids, measured as MDA, not only in control hearts but even in vitamin E-deficient hearts after treatment with doxorubicin. There were no intrinsic differences present between rats fed a complete or a vitamin E-deficient diet, in the content or activity of those factors of importance in this study, i.e. SOD, the glutathione system, MDA and poly-unsaturated fatty acids (Julicher et al 1986).

Table 1. The activity of content of cellular protective factors and MDA in heart tissue of rats fed a complete or vitamin E-deficient diet after a recirculating perfusion for 60 min with a 100 μM doxorubicin solution. Each value represents the mean ± s.e.m. of 6 separate experiments. No statistically significant difference was found between the parameters studied in cardiac tissue of either group.

Doxorubicin treatment	Complete diet	Vitamin E-deficient diet
SOD (u mg protein^{-1})	9·4 ± 0·4	9·3 ± 0·6
GSH-Px (nmol NADPH min^{-1} mg protein^{-1})	86·4 ± 2·9	87·8 ± 3·7
GSH + GSSG in tissue (nmol GSH-eq. mg protein^{-1})	9·8 ± 0·6	9·3 ± 0·4
GSH in perfusate (nmol mg protein^{-1} in total perfusate)	0·23 ± 0·03	0·21 ± 0·02
GSSG in perfusate (nmol mg protein^{-1} in total perfusate)	0·36 ± 0·04	0·40 ± 0·08
MDA (nmol mg protein^{-1})	0·027 ± 0·004	0·024 ± 0·006

DISCUSSION

Doxorubicin induces a concentration-dependent reduction in myocardial contractility in a variety of isolated heart preparations (Ohhara et al 1981; Höfling & Bolte 1981; Wikman-Coffelt et al 1983; Rabkin 1982; Ditchey et al 1984), which is sometimes preceded by a transient positive inotropic effect (Höfling & Bolte 1981). Acute changes in subcellular structure (Taylor & Healy Bulkley 1982), a diminution in amplitude of ECG waves, which may be related to an increased perfusion pressure (Herman et al 1972), as well as a reduction in high energy phosphate content (Ohhara et al 1981), were also found as a result of exposure of isolated hearts to doxorubicin. The objective of this investigation was to determine whether excessive formation of reactive substances and subsequent peroxidation of lipids actually can be related to the doxorubicin-induced deterioriation in mechanical function in rat isolated heart. Our results show that the cellular defence against free radicals was affected by the drug in rat isolated heart. The observed rise in GSH-Px activity did not necessarily enhance the capacity to detoxify lipid hydroperoxides because the level of GSH was decreased significantly due to doxorubicin. The capacity of other GSH-dependent protective factors may have been reduced as well by the lowered GSH levels (Reddy et al 1982; Ursini et al 1982; Haenen & Bast 1983). The observed inactivation of cardiac SOD suggests a reduced protection within the cytosol and the mitochondrial compartment. Apparently, doxorubicin reduces the cellular defence capacity against one of its own toxic actions: the formation of free radicals. The reason for this decreased cellular defence is not clear, although some remarks can be made on it. Oxidative damage due to excessive free radical formation could account for both the increase in the activity of GSH-Px (Frank & Massaro 1980; Oei et al 1982) and decrease in the SOD-activity (Hodgson & Fridovich 1975). An increased formation of GSSG, which could account for the release of GSSG into the perfusate (Sies & Summer 1975), can occur due to exposure to oxidative stress. The reduction in the total amount of glutathione due to doxorubicin treatment is consistent with the formation of a reactive intermediate that can bind to GSH (Doroshow et al 1979; Wells et al 1980; Julicher et al 1985). Our findings with subcellular particles substantiated the idea that an increase in free radical formation occurs due to doxorubicin. The increase in MDA formation, which was caused by the drug in vitamin E-deficient microsomes and mitochondria, could not be detected, however, in the isolated, vitamin E-deficient heart. This points to an effective protection in whole heart, e.g. in the cytosol, against an increase in free radical formation. From this absence of detectable MDA formation following perfusion with doxorubicin of both control and vitamin E-deficient rat heart, it must be concluded that peroxidative damage of lipids is not a major component in the acute cardiotoxic action of the drug. This conclusion is strengthened by the finding that a lower content of vitamin E did not impose a higher stress on SOD or the factors of the glutathione system in rat isolated heart (Table 1). The finding that doxorubicin perfusion of vitamin E-deficient hearts did not aggravate the decrease in contraction amplitude, frequency of beating or coronary flow observed in control hearts, is in agreement with this. From the absence of detectable lipid peroxidative damage, even in the vitamin E-deficient rat heart, and the absence of aggravated functional and biochemical damage, it must be concluded that lipid peroxidation is not decisive in the development of the acute, doxorubicin-induced, cardiomyopathy in rat. The observed decrease in the water-soluble defence factors against reactive species might result from the presence of an excessive amount of free radicals in the water-soluble compartments of cardiac cells.

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