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SHORT COMMUNICATIONS

Stimulation by doxorubicin of adrenochrome formation by bovine heart sarcolemma

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There is accumulating evidence that relates the cardiotoxicity of the anthracyclines, including doxorubicin, to the generation of superoxide anions ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$) [1–3]. The anthracycline-related free radical production is a consequence of the cyclical oxidation and reduction of the anthracycline quinone catalyzed by certain flavin containing dehydrogenases [4, 5]. The recent study of Doroshow [6] clearly indicates that heart $O_2^{\cdot-}$ production was significantly increased by anthracycline in sarcoplasmic reticulum by NADPH-cytochrome P-450 reductase and in the mitochondrial membrane by the NADH dehydrogenase complex. Also in the cardiac cytosol depleted of superoxide dismutase, doxorubicin enhanced $O_2^{\cdot-}$ production probably by the xanthine oxidase enzyme [6]. On the other hand, the severity in heart muscle of the doxorubicin induced damages is the consequence of specific interactions of the drug with some myocardial compartments. In fact, several investigators have demonstrated that doxorubicin binds both to the inner mitochondrial membrane and to DNA with a high degree of specificity [7–9]. The interaction with the membranes is related to the high affinity of doxorubicin for acidic phospholipids [10] and although these components are scarcely present in the sarcolemmal membranes, doxorubicin can interact with the sarcolemma at the level of the ouabain inhibition site [11]. In addition, in this membrane Caroni *et al.* [12] demonstrated that doxorubicin inhibited the Ca^{2+} transport depending on the Na/Ca exchanger system. These effects could be relevant in inducing heart dysfunction both because doxorubicin affects the excitation–contraction coupling mechanism and because the sarcolemma is the first membrane which interacts with the drug. In order to investigate further the effects caused by the interaction between doxorubicin and the cardiac sarcolemma, we have performed some experiments to evaluate if the drug may favour the oxidation of adrenaline to adrenochrome considering that this reaction is initiated by $O_2^{\cdot-}$ and that the sarcolemma membrane is rich in adrenergic receptors.

Materials and methods

Bovine cardiac sarcolemma was prepared as described by Reeves and Sutko [13] except that phenyl methane sulfonyl fluoride, a protease inhibitor was added to the media [14]. After isolation, the vesicles were suspended in 160 mM KCl and 20 mM 3-(*N*-morpholino)-propane sulfonic acid, pH 7.4 at a protein concentration of about 1.5–2 mg/ml and stored frozen in small aliquots at −80°. The preparation was highly enriched in sarcolemmal membranes as was revealed by the high specific activity of the marker enzymes Na^+/K^+-ATPase-ouabain sensitive and 5′-nucleotidase.

The ATPase activity was estimated from samples which were preincubated for 30 min at 20° in the presence of 0.3 mg/mg protein sodium dodecylsulfate according to Lamers and Stinis [14]. The Na^+/K^+ ATPase activity was calculated as that activity which was inhibited by 2 mM ouabain and was 65.8 μmol/hr mg prot. The 5′-nucleotidase activity measured by the procedure of Edwards and Maguire [15] was 18.4 μmol/hr mg protein. The cross contamination of sarcoplasmic reticulum or of inner mitochondrial membranes was undetectable as demonstrated by the measure of the respective enzyme marker activities, NADPH-Cyt.c reductase [16] and succinate dehydrogenase [17]. The formation of adrenochrome was monitored at 480 nm at 25° in a double-beam Perkin Elmer spectrophotometer, model 559. The assay mixture consisted of 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 20–30 μg of sarcolemmal protein. Adrenaline was added at 1 mM final concentration. The concentration of adrenochrome was determined using $E_{480} = 4.02 \times 10^3$/M per cm. Protein concentration was evaluated using the procedure of Bradford [18].

Results

Figure 1 shows that the rate–time formation of adrenochrome was stimulated by sarcolemmal membranes when NADPH was added to the incubation mixture (trace 5).

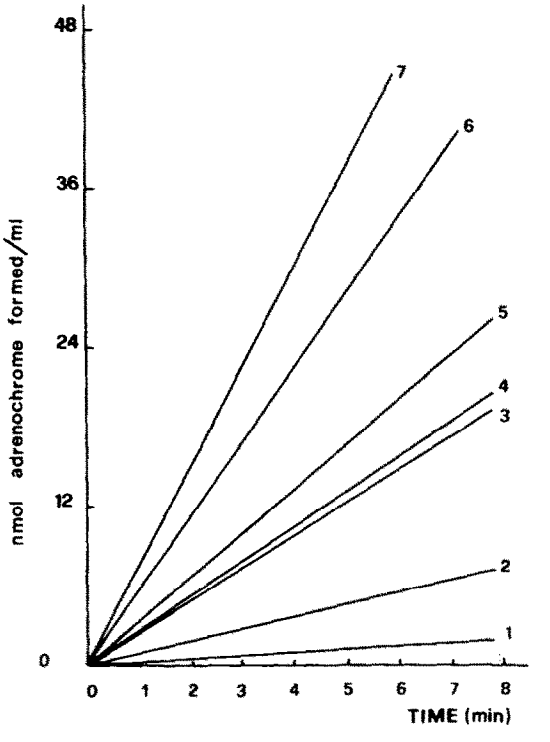

Fig. 1. Effect of doxorubicin on the formation of adrenochrome by bovine sarcolemmal membranes. The rate of production of adrenochrome was followed as described in Materials and Methods. Trace 1, incubation mixtures without sarcolemma; trace 2, incubation mixture plus sarcolemma; trace 3, as in trace 2 plus 60 μM doxorubicin; trace 4, as in trace 2 plus 120 μM doxorubicin; trace 5, as in trace 2 plus 0.4 mM NADPH; trace 6, as in trace 5 plus 60 μM doxorubicin; trace 7, as in trace 5 plus 120 μM doxorubicin.

NADH produced a stimulatory effect, but it was almost 50% less than NADPH (data not shown). Doxorubicin slightly increased the oxidation of adrenaline when the drug was added to the sarcolemmal membranes (traces 3–4). In the presence of NADPH this stimulatory effect was more progressively marked in a doxorubicin concentration ranging from 60 μM to 120 μM (traces 6–7). Adrenaline and doxorubicin incubated together in the presence or not of NADPH produced an oxidation of adrenaline that in all cases coincided with trace 1.

Table 1 shows that the formation of adrenochrome evaluated after 10 min of preincubation of the sarcolemmal membranes with doxorubicin and NADPH was strongly reduced by the addition of superoxide dismutase. Alpha-tocopherol phosphate also reduced significantly the rate of adrenaline oxidation, while the effect of catalase was less evident.

Discussion

In cardiac microsomes the oxidation of adrenaline to adrenochrome has been studied by Gudbjarnason and Doell [19] who demonstrated that this oxidation was stimulated by polyene fatty acid. More recently, Monfoort and Lamers [20] confirmed this reaction by using liposomes constituted of polyunsaturated fatty acids.

The present research shows that the cardiac sarcolemmal membranes, which are components of the microsomal membranes, are able to stimulate the oxidation of adrenaline to adrenochrome. The rate of this reaction was enhanced when the sarcolemma was incubated with NADPH. A further increase was caused by doxorubicin in a concentration dependent manner or after the preincubation of the membranes with the drug. It is significant that doxorubicin produced these effects at doses close to the plasma level which caused severe cardiac lesions in rabbit hearts [21]. Moreover, the sarcolemmal membranes denaturated by heating lost their ability to promote adrenochrome formation in each condition (data not shown). Therefore, all these results support the hypothesis that the formation of adrenochrome might result not only from an autoxidation reaction mediated by the polyunsaturated fatty acids, but it is likely that a dehydrogenase component of the sarcolemma, as in consequence of its autoxidation, produces $O_2^{\cdot}$ radicals that in turn stimulate the oxidation of adrenaline. Doxorubicin can accentuate this reaction probably through the production of superoxide by activation of the antracycline to a free radical state. It has been already described in heart and liver microsomes that the interaction of doxorubicin with these membranes and NADPH generates drug semiquinone radicals that may transfer electrons to molecular oxygen forming $O_2^{\cdot}$ [4, 5]. This mechanism can be taken in account also in our experimental conditions because the superoxide dismutase abolished almost completely the adrenaline oxidation induced by the drug. Alpha-tocopherol, which is a scavenger of $O_2^{\cdot}$ radicals, also reduced the rate of this reaction. On the contrary, since catalase had a partial inhibitory effect it is likely that H_2O_2 does not play an important role during adrenochrome formation.

The pathophysiology of the doxorubicin-induced cardiotoxicity is poorly understood despite the proposal of several mechanisms including the disturbance of the calcium homeostasis with an abnormal intracellular calcium overload [22], inhibition of transcription and protein synthesis [23] or augmented release of vasoactive substances which potentiates cardioactivity [24]. The fact that doxorubicin causes a larger release of catecholamines in heart muscle might be of interest considering the present results. In fact it is possible that the disturbances of cardiac function provoked by doxorubicin are due to the elevated heart catecholamine release followed by their oxidation in the sarcolemma. In fact, it is known that the oxidized products of catecholamines are toxic for heart muscle, producing in particular cellular calcium overload [25] and contractile failure [26].

In conclusion, the present data provide additional evidence on a possible mechanism by which doxorubicin can damage heart muscle by increasing the rate of formation of oxidized catecholamines at the sarcolemmal level where there are abundant adrenergic receptors.

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Table 1. Effect of incubation of doxorubicin with sarcolemmal membranes on the rate of adrenochrome formation

	nmol/mg prot. min
(a) Without sarcolemma	6.2 ± 0.2
NADPH	9.3 ± 0.2
(b) With sarcolemma	168.2 ± 4.2
NADPH	322.4 ± 4.8
NADPH + superoxide dismutase	48.1 ± 0.8
NADPH + α-tocopherol phosphate	82.6 ± 0.4
Catalase	199.5 ± 2.6

The incubation medium consisted of Tris–HCl buffer, pH 7.5, 1 mM adrenaline and 60 μM doxorubicin. When scheduled 20–30 μg of sarcolemmal proteins, 0.4 mM NADPH, 2 μM SOD, 600 μM α-tocopherol phosphate or 2 μM catalase were added.

The mixtures were incubated for 10 min at 25° before the measurement of adrenochrome formation.

The values are means ± S.D. of 3 samples from 3 hearts.

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A decrease in the capacity of hepatocytes isolated from aged male BN/BiRij rats to metabolize digitoxin

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Lipid soluble drugs are generally metabolized by the liver in two successive phases, the Phase I and Phase II reactions. Phase I reactions include oxidations, reductions, dealkylations and hydrolyses. Phase II metabolism involves those reactions in which the drug or its Phase I metabolites are transformed into less lipophilic metabolites by conjugation with small endogenous molecules such as glucuronic or sulphuric acid. Probably, the effect of age is different for the different metabolic pathways, which might explain the apparently contradictory literature data on the effect of age on the metabolism of lipid soluble drugs. To obtain insight into this problem, the effect of age on digitoxin (DT_3*) metabolism was investigated in the present study. DT_3 is metabolized via different metabolic pathways, viz. degraded by hydrolysis and hydroxylation reactions. The resulting active Phase I metabolites and DT_3 itself can be inactivated by conjugation with glucuronic or sulphuric acid. Therefore, by studying the effect of age on the metabolic pattern of DT_3, information on the influence of age on hydroxylation, hydrolysis and conjugation reactions can be obtained.

Hepatocytes isolated from rats represent a suitable system for studying the metabolism of DT_3 for the following reasons. It was previously observed that the pattern of active DT_3 metabolites formed by hepatocytes isolated from 3-month-old male Wistar rats was similar to that reported to be present in serum and urine of patients on DT_3 therapy, although the amounts of the inactive conjugated metabolites were smaller in the rat hepatocytes [1]. The amount of metabolized DT_3 was linear for at least 1 hr of incubation and was proportional to the number of hepatocytes present in the incubation medium [1]. In the present study, the effect of aging on the pattern of metabolites and on the kinetic characteristics (K_m, V_{max} and Cli ($=V_{max}/K_m$)) of DT_3 was determined with hepatocytes isolated from various age groups of male BN/BiRij rats.

A preliminary report incorporating some of these data has been previously published [2].

Materials and methods

Inbred 3-, 18-, 30- and 36-month-old male BN/BiRij rats were used. The 90%, 50% and 10% survival ages were 21, 32 and 39 months respectively. The rats were maintained under "clean conventional" conditions as described by Hollander [3].

Digitoxin (DT_3), digitoxigenin-bis-digitoxoside (DT_2), digitoxigenin-mono-digitoxoside (DT_1), digitoxigenin (DT_0), digoxigenin-bis-digitoxoside (DG_2), digoxigenin-mono-digitoxoside (DG_1) and digoxigenin (DG_0) were obtained from Roth (Karlsruhe, G.F.R.), digoxin (DG_3) from Merck (Darmstadt, G.F.R.). (^{3}H) Digitoxin (13.8 C/mmole) was purchased from New England Nuclear (Boston, MA, U.S.A.). Collagenase (type 1) was purchased from Sigma (St. Louis, MO, U.S.A.), Waymouth MB 752/1 medium from GIBCO (New York, NY, U.S.A.) and Seppak Cartridges from Water Associates Inc. (Etten-Leur, The Netherlands).

Thin layer chromatography (t.l.c.) was performed with D.C. Fertigplatten, Kieselgel 60 of 20 × 20 cm (Merck, Darmstadt, G.F.R.). A Dunnschicht-Scanner LB 2723 Berthold was used for locating the radioactive spots on t.l.c. plates.

* Abbreviations used: DT_3, digitoxin; DT_2, digitoxigenin-bis-digitoxoside; DT_1, digitoxigenin-mono-digitoxoside; DT_0, digitoxigenin; DG_3, digoxin; DG_2, digoxigenin-bis-digitoxoside; DG_1, digoxigenin-mono-digitoxoside; DG_0 digoxigenin; t.l.c., thin-layer chromatography.