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SHORT COMMUNICATIONS

Adriamycin-mediated inhibition of creatine phosphokinase binding to heart mitochondrial membrane

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Adriamycin (ADR), a clinically useful anticancer drug, causes a dose-limiting cardiotoxicity which is not observed with the administration of other anticancer drugs [1]. Although the precise etiology of adriamycin-mediated cardiomyopathy remains unclear, evidence has accumulated to suggest that myocardial membranes may be a specific target for this drug [2–5]. Current theories aimed at explaining the specificity of ADR-cardiomyopathy suggest that decreased levels of mitochondrial catalase and glutathione peroxidase [6, 7] may result in an impaired ability to adequately prevent ADR-mediated damage due to lipid peroxidation. Several investigators have demonstrated that adriamycin binds to the inner mitochondrial membrane in addition to DNA with a high degree of specificity. This may be due to direct interaction with membrane cardiolipin, a major phospholipid in mitochondrial membrane [4, 8, 9], or with enzyme–cardiolipin membrane complexes such as cytochrome *c* [10].

The broad spectrum of effects of ADR in inhibiting myocardial energy production and utilization has also been cited as a possible cause of a decline in cardiac pump efficiency. Few of these studies, however, have proposed specific drug-mediated alterations in mitochondrial morphology or function which can adequately explain the cardio-selective nature of ADR-mediated tissue damage. The existence of a distinct mitochondrial membrane bound isoenzyme of creatine phosphokinase (CPK) has been known for some time [11, 12]. Evidence suggests that it is located on the outer surface of the inner mitochondrial membrane [13] and that it serves a critical role in the transfer of high energy phosphate groups from heart mitochondria to myocardial muscle fibers [14]. Because of the demonstrated effect of anthracyclines on heart mitochondrial function and morphology, we examined the effect of adriamycin on the capacity of myocardial mitochondrial membranes to bind this specific isoenzyme of CPK.

Materials. Adriamycin, in the form of doxorubicin hydrochloride, was a gift from the Adria Laboratories (Columbus, OH). All other reagents used were of analytical grade and were obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ). CPK activity was determined by the coupled enzyme method [15] using CalBiochem CPK-single vial reagent (La Jolla, CA).

Preparation of mitochondria and crude CPK source. Heart mitochondria were isolated from 3 kg male New Zealand rabbits by differential centrifugation and were washed three times in 50 mM Tris–HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA and 1 mM β-mercaptoethanol. A crude mitochondrial CPK extract was obtained by treating the mitichondrial pellet with 3 vol. (w/v) of Buffer A (20 mM PO_4, pH 7.2, 1 mM β-mercaptoethanol) for 30 min at 2° as previously described [16]. The supernatant fraction resulting from a 10-min centrifugation at 10,000 *g*, was dialyzed for 12 hr at 2° against Buffer B (2 mM PO_4, pH 7.2, 1 mM β-mercaptoethanol) in preparation for the mitochondrial binding experiments. The extracted mitochondrial pellet was further extracted with Buffer A until less than 100 mU CPK activity/mg mitochondrial protein remained with the pellet. The final mitochondrial pellet was resuspended in 3 vol. (w/v) of Buffer B. Mitochondrial and enzyme protein contents were determined by the method of Lowry *et al.* [17].

Binding of CPK to mitochondrial membrane. The capacity of extracted mitochondria to rebind CPK was determined by incubating (2°, 30 min) 50 μl of mitochondria (0.2 to 0.3 mg protein) with various amounts of the dialyzed CPK preparation and Buffer B in a total assay volume of 0.15 ml [16]. The samples were then centrifuged (10,000 *g*, 10 min), the supernatant fraction containing unbound CPK was aspirated, and the mitochondrial pellet was resuspended in 0.5 ml of Buffer A. Aliquots of the resuspended mitochondria containing "released" CPK were then assayed directly.

ADR-mediated inhibition of CPK rebinding. Inhibition of the rebinding of CPK to heart mitochondrial membrane was studied using a final concentration of adriamycin that ranged from 0.1 μM to 1 mM. Adriamycin was added to 50 μl of mitochondria and Buffer B in a total volume of 0.15 ml. Tubes were incubated at 2°, 30° or 37° for 30 min after which they were centrifuged (10,000 *g*, 10 min) and the pellet was resuspended in 0.5 ml of Buffer B. CPK was then added to determine enzyme rebinding characteristics of the mitochondria as previously described.

ADR-mediated release of CPK from mitochondrial membrane. Release of bound mitochondrial CPK produced by incubation with adriamycin was also determined. Known, nonsaturating amounts of CPK were rebound to mitochondria as previously described. Bound enzyme was separated from unbound CPK by centrifugation and aspiration of unbound enzyme in the supernatant fraction. Samples of mitochondria with bound CPK were then resuspended in Buffer B and adriamycin (100 μM to 1 mM final concentration), incubated at 30° for 30 min, and recentrifuged at 10,000 *g* for 10 min. Following aspiration of the supernatant fraction, the mitochondrial pellet was resuspended in Buffer A for direct enzyme determination.

Results and discussion. Alteration of cardiac mitochondrial structure, commonly observed in adriamycin-treated animals [3, 18], implies a possible alteration in mitochondrial function. One of the most important changes in mitochondrial function described to date is ADR-mediated impairment of mitochondrial respiratory control. The inhibiton of flavin containing enzymes by adriamycin *in vitro* [19], for example, supports the role of a direct interaction of adriamycin with mitochondrial enzymes important in energy production. To examine the interaction of adriamycin with mitochondrial CPK, ADR (in concentrations up to 1 mM) was incubated directly with aliquots of the crude CPK preparation for up to 30 min (30°). However, no direct effect of ADR on CPK activity was observed (data not shown).

With the demonstration of the functional compartmentation of a specific isoenzyme of CPK on the mitochondrial membrane, it became clear that the actual form of energy transport in heart muscle fiber was phosphorylcreatine [13]. As recently pointed out by Bessman and Geiger [14], this established a molecular basis for a phosphorylcreatine–creatine shuttle for energy transport in heart muscle and provided an explanation for the inability to demonstrate experimentally a direct relation between muscle activity

and concentrations of adenine nucleotides.

The heart mitochondrial bound CPK isoenzyme has been shown recently to have different physical and kinetic parameters from previously characterized MM, BB or MB isoenzymes [20]. In addition, several groups have now demonstrated that the heart mitochondrial CPK isoenzyme is able to bind to partially purified inner mitochondrial membranes [13], that the binding is ionic in nature and that the degree of binding is dependent upon pH and P_i concentration [21, 22].

Release of bound CPK due to incubation with adriamycin occurred only when extremely high concentrations of drug were used (Fig. 1). Since the mechanism of ADR-mediated release of CPK from mitochondrial membranes is not known, optimal conditions for studying this phenomenon may not have been employed. It is unknown, for example, whether inclusion of a suitable electron donor, such as NADPH [23], in the incubation mixture would have augmented drug-mediated oxidative attack on the mitochondrial membrane and hence have augmented release of bound CPK. Since both cytochrome *c* and mitochondrial CPK are known to be bound to membrane phospholipids [9, 24], the extent of ADR-mediated lipid peroxidation may be a very important factor in determining the release and/or uncoupling of important membrane-bound enzymatic activities.

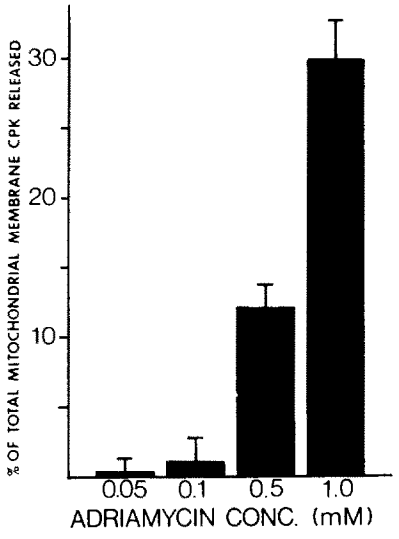

Fig. 1. Release of of mitochondrial bound CPK produced by incubation (30°, 30 min) with adriamycin. Each value is the mean ± S.E. from three experiments.

Membrane binding of adriamycin in the absence of significant levels of mitochondrial bound CPK did prevent rebinding of subsequently introduced enzyme in a dose-dependent manner (Fig. 2). This phenomenon was temperature dependent, with inhibition of CPK rebinding occurring to a much greater extent at 30° than it did at 2°. Preincubation of membranes with adriamycin at 37°, however, did not increase the inhibition of CPK rebinding beyond that observed at 30° (data not shown).

Lipskaya *et al.* [21] have suggested recently that under physiological conditions only part of the CPK normally remains bound to heart mitochondrial membranes, while the remainder of the CPK isoenzyme pool is in the free state contained within the intermembrane space. The ratio between bound and free forms of mitochondrial CPK may therefore vary under the influence of local changes in ATP or P_i concentrations [21, 22]. Such changes are known to readily occur, for example, under ischemic conditions [22] and may also occur following ADR treatment.

Our results suggest that the interaction of adriamycin with mitochondria can result in interference with the membrane binding of CPK. This may be an important factor in the etiology of ADR-induced cardiotoxicity, since it is the bound form of the enzyme that is responsible for the translocation of high energy phosphate groups from ATP to creatine. The recent observations of Burns and Dow [24] suggest, in fact, that anthracycline cardiotoxicity may involve a drug-induced dissociation between the ATP pool and myocardial muscle work. Drug-mediated interference with the rebinding of CPK to mitochondrial membranes may, therefore, significantly interfere with the required

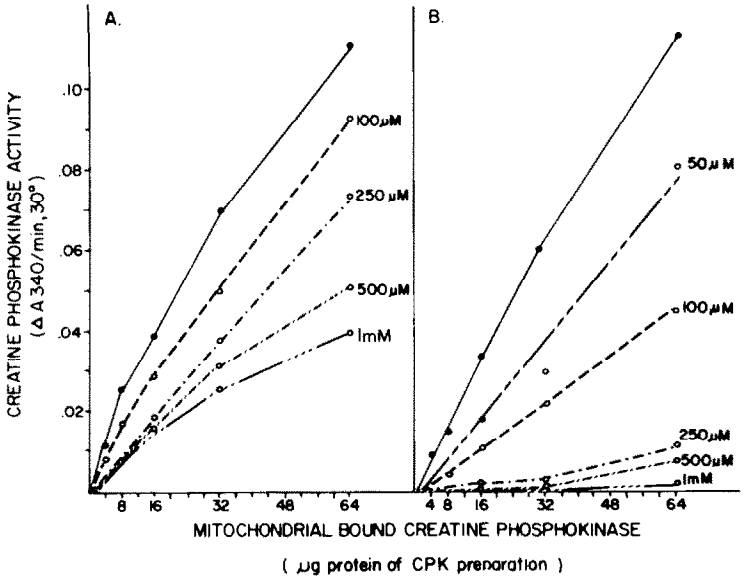

Fig. 2. Inhibition of rebinding of CPK to heart mitochondrial membranes. Each point represents the mean value from three experiments. Concentrations of adriamycin are as indicated; solid circles represent control value determinations. Experiments were performed at either 2° (A) or 30° (B).

ratio of bound/free CPK and consequently with optimal energy utilization in cardiac tissue.

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Decreases in the release of acetylcholine *in vitro* with low oxygen

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A decrease in the O_2 content of the inspired air (hypoxia) depresses higher integrative function in man and animals [1–3] but does not reduce brain energy reserves [4–8]. Thus, a decrease in the metabolism of neurotransmitters has been postulated as a possible molecular basis of hypoxic brain dysfunction [9]. Hypoxia impairs the *in vivo* synthesis of acetycholine (ACh)* [8, 10, 11], serotonin [12], the catecholamines [13] and the amino acids [11, 14, 15], while their concentrations remain unaffected. The decrease in synthesis during hypoxia without a corresponding reduction in levels implies that hypoxic insults may alter release mechanisms and that the non-released neurotransmitter may impair further synthesis. Indirect evidence suggests that low O_2 may impair dopamine release [16]. We tested this hypothesis directly with the cholinergic system, and found that a decrease in O_2 inhibits the Ca^{2+}-dependent release of ACh from brain slices and synaptosomes. Since the effects of hypoxia on release were dependent on the presence of Ca^{2+}, we tried to ameliorate the inhibitory effects of hypoxia with a pharmacological agent that interacts with Ca^{2+} homeostasis. 4-Aminopyridine increases neurotransmitter release in a Ca^{2+}-dependent manner [17, 18]. It increases the influx of Ca^{2+} into nerve terminals [19–21] although this increase may be secondary to alterations in K^+ channels [22, 23].

All reagents and procedures were as previously described [24]. Brain slices were prepared from male CD-1 mice (18–25 g). Slices (2–3 mg protein) were preincubated under 100% O_2 with 5 mM [U-^{14}C]glucose (1 μCi/μmole). After

* Abbreviations: ACh, acetylcholine; CDKS, Ca^{2+}-dependent-K^+-stimulated; EGTA, ethylene glycol-bis[β-aminoethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid; and 4-AP, 4-aminopyridine.