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INHIBITION OF FLAVIN METABOLISM BY ADRIAMYCIN IN SKELETAL MUSCLE*

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Abstract—Adriamycin (ADR), a potent antineoplastic agent, has been shown to interact with flavin derivatives and to compete with flavin coenzymes for their respective binding sites on flavin-containing enzymes. The present investigation determined that ADR administration inhibited flavin adenine dinucleotide (FAD) biosynthesis from riboflavin in rat skeletal muscle in a dose-related manner compared to results in pair-fed controls. Five groups of adult Holtzman rats of both sexes were given twice daily intraperitoneal injections of ADR for 3 days, representing cumulative doses of 6, 12, 18, 24, and 30 mg/kg body weight. At the cumulative dose of 6 mg/kg, there was no significant effect, but at 12, 18, 24, and 30 mg/kg levels significant increases in [^{14}C]FAD formation from [^{14}C]riboflavin occurred. ADR-induced myopathy may be due, at least in part, to inhibition of FAD formation, ultimately leading to changes in energy metabolism and oxidative capacity.

Adriamycin (ADR), also known as doxorubicin, is an anthracycline antibiotic extensively utilized for the treatment of a wide variety of human malignancies. These malignancies include leukemias, bone sarcomas, and carcinomas of the urinary and reproductive tracts [1]. The effectiveness of ADR as an antineoplastic agent is well established, but the most serious side effect of its use is an acute cardiotoxicity, characterized by abnormal electrocardiographic changes and arrhythmias. In later stages, a cumulative, dose-related chronic cardiotoxicity culminates in irreversible congestive heart failure [2]. This cardiotoxicity is well recognized biochemically, clinically, and histopathologically, and several pathogenetic mechanisms have been proposed as to the cause of ADR toxicity.

Biochemical studies have shown that ADR complexes with cardiolipin in the mitochondrial membrane, thereby inactivating cytochrome *c* oxidase [3]; ADR also inhibits oxidative phosphorylation [4], enhances lipid peroxidation [2], and prevents RNA transcription and DNA replication [5]. Clinical studies have demonstrated that the frequency of ADR-related heart failure increases markedly when the total dose administered exceeds approximately 550 mg/m^2 body surface area [6, 7]. Several studies suggest that the dosage schedule may also influence the amount of myocardial damage [8]. Electron microscopic examination of myocardial cells in patients treated with ADR reveals cytoplasmic vacuolization, central clumping of nuclear chromatin, contracted nucleoli, dilatation of sarcoplasmic reticulum cisternae, and a reduction of myofibrillar bundles [7, 9].

Evidence has been obtained that there are similarities in the responses of skeletal and cardiac muscle cell nucleoli to ADR treatment to the extent that both kinds of cells have similar responses to low doses of ADR but liver cell nucleoli respond differently. For example, as early as 1 hr after treatment with 5 mg/kg of ADR, segregation of nucleolar components was observed in both cardiac and skeletal muscle cells but it was not nearly as extensive in liver cells [7]. Limited microscopic investigations with skeletal muscle from animals treated with ADR also show vacuolar degeneration in the sarcoplasm similar to that in heart [10].

One factor that has not been considered in previous investigations of mechanisms of ADR toxicity is possible effects of this agent on riboflavin (vitamin B_2) metabolism. It is necessary to emphasize that the nucleotide derivatives of riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), serve as coenzymes for a wide variety of regulatory enzymes involved with the tricarboxylic acid cycle [11], the electron transport chain [12], fatty acid oxidation [13], the glutathione redox cycle [14], and drug-metabolizing enzyme systems [15]. Disturbances in the bioavailability of riboflavin due to either dietary deficiency or antagonism by various drugs adversely affect one or more of these important metabolic functions [16, 17]. Furthermore, it is not widely recognized that the four-membered anthracycline ring of ADR and the three-membered iso-

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alloxazine ring of riboflavin, including its physiological derivatives, FMN and FAD, form 1:1 stoichiometric complexes [18]. Adriamycin and several of its derivatives and metabolites can mimic flavins and compete successfully for their binding sites on a number of flavin-containing enzymes [19, 20].

These considerations prompted us to determine whether ADR directly inhibits the incorporation of riboflavin into FAD in skeletal muscle. Our initial studies showed that ADR does indeed inhibit FAD biosynthesis in rat cardiac muscle, but not in kidney or liver under similar conditions [21]. Flavin adenine dinucleotide is the predominant flavin nucleotide in tissues and is the coenzyme required by the majority of flavoenzymes [22]. Inhibition of FAD biosynthesis would be expected to have important implications for both the structure and function of metabolically active tissues, such as heart and skeletal muscle.

METHODS

Isotopes, chemicals, and diet

Adriamycin was purchased from Adria Laboratories, Columbus, OH, as a crystalline powder and reconstituted with saline prior to injection into animals. [^{14}C]Riboflavin, 28 mCi/mmol, was purchased from the Amersham/Searle Corp., Arlington Heights, IL, and the specific activity was determined in our laboratory prior to use. Non-radiolabeled flavins (riboflavin and FAD) were purchased from the Sigma Chemical Co., St. Louis, MO, and chromatographed in our laboratory before use. Absolute methanol (certified grade) was purchased from the Fisher Scientific Co., Pittsburgh, PA. Rat chow was purchased from the Ralston Purina Co., St. Louis, MO. Riboflavin content of the chow was assayed to be 8.0 μg/g of diet.

Animals

All experiments were conducted with adult male and female rats (Holtzman Rat Co., Madison, WI) weighing 140–400 g. Animals were housed individually in stainless steel metabolic cages, maintained on standard Purina Rat Chow, and allowed free access to tap water. Our animal care facility is equipped with controlled lighting (12 hr light/12 hr dark).

Analysis of [^{14}C]FAD formation in tissue

The formation of [^{14}C]FAD *in vivo* was measured in skeletal muscle using the method of reverse isotope dilution and anion exchange column chromatography with DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). This technique, developed by Fazekas in collaboration with our laboratory, entails extraction of flavins from tissues, separation of riboflavin from FAD by anion exchange chromatography, and determination of the radioactivity in the FAD fraction by scintillation counting [23, 24].

Aliquots of skeletal muscle (600 mg) were used for each assay. Tissues were homogenized in 3 ml of an aqueous solution containing equal volumes of nonradiolabeled riboflavin and FAD using a motor-driven, Potter–Elvehjem homogenizing apparatus. The glass homogenizing tubes were pre-chilled in an ice bath before use. The concentration of each nonradiolabeled flavin was determined earlier by spectrophotometry at 450 nm. Exactly 7.0 ml of absolute methanol, administered as two 3.5-ml portions, was added to the homogenized mixture. After further homogenizing, the homogenate was transferred to centrifuge tubes. The homogenizing vessels were rinsed with a small volume of 70% (v/v) methanol which was poured into the corresponding centrifuge tubes. After the tubes were balanced by additional aliquots of 70% (v/v) methanol, the homogenates were centrifuged at 100,000 *g* for 20 min in a refrigerated ultracentrifuge. The supernatant fractions were transferred to 50-ml round-bottom flasks, and the content of each flask was evaporated *in vacuo*. The radioactive residue was dissolved in 1 ml of 1% (w/v) ammonium sulfate, and the solution was chromatographed on a column of DEAE-Sephadex A-25. After separation of riboflavin and FAD, using 1% (w/v) ammonium sulfate as the eluent, the concentration of each extracted flavin was determined by spectrophotometry at 450 nm.

A 1-ml aliquot of each flavin sample was placed in a scintillation vial, and 10 ml of Hydrofluor Scintillation Counting Fluid (National Diagnostic, Somerville, NJ) was added to each vial. The total radioactivity incorporated into the FAD fraction of skeletal muscle is determined by multiplying the specific activity (counts per minute per milliliter) by the mass of nonradiolabeled FAD added. All results were initially calculated as picomoles of radiolabeled FAD formed per gram tissue per hour, and the results from the ADR-treated groups were expressed as percent of their corresponding control value. The percentage variation from the mean of duplicate determinations was approximately 4–5%. Student's *t*-test for paired samples was utilized for statistical analysis of the data.

Adriamycin treatment

Dose–response studies. After a 1-week acclimation period in metabolic cages, animals were divided into two groups designated as saline- and ADR-treated. The ADR-treated animals were subdivided into five treatment groups and were given twice daily intraperitoneal injections of ADR for 3 days, representing cumulative doses of 6, 12, 18, 24, and 30 mg/kg body weight. The age-matched, saline-treated animals were pair-fed to the ADR-treated animals and received isotonic saline identical in volume to their drug-treated counterpart.

Following an 18-hr fasting period and 1 hr prior to decapitation, all rats received a single subcutaneous injection of [^{14}C]riboflavin, 25 μCi/kg body weight. At the time of sacrifice, skeletal muscle surrounding the femur was excised from each animal and stored at −20° until the assay for incorporation of [^{14}C]riboflavin into [^{14}C]FAD could be performed. Tissues were analyzed within 1 week following sacrifice, but samples can be stored for up to 30 days without detectable loss of activity as FAD.

Time–course studies. A series of experiments was next conducted to ascertain the time–course of response of skeletal muscle to ADR at dose levels

of 10–30 mg/kg body weight. One group of animals was injected intraperitoneally with a single dose of ADR (10 mg/kg body wt) and was killed 6 hr later. Another group received a divided dose of ADR (5 mg/kg each at 10:00 a.m. and at 4:00 p.m.) and was killed 24 hr after the initial injection. A third group received divided doses of ADR (5 mg/kg each at 10:00 a.m. and at 4:00 p.m.) for 2 days and killed 48 hr after the initial injection of ADR. A fourth group of animals received ADR similarly for 3 days and was killed 72 hr after the initial dose of the drug.

This dose of 10 mg/kg of ADR in the rat was selected because it is equivalent to a dose of 60 mg per square meter (m^2) body surface area in humans. This dose, continued for a 3-day period, as in the initial study reported here, yields a cumulative dose of 180 mg/m^2, which represents the lowest dose shown to produce specific subclinical cardiac injury in patients, as demonstrated by electron microscopy [6].

RESULTS

As illustrated in Fig. 1, the biosynthesis of [^{14}C]FAD in skeletal muscle decreased with increasing cumulative dose of ADR administered during a 3-day period. The groups of controls, i.e. pair-fed, saline-treated animals matched to each of the drug-treated animals, exhibited no significant differences from one another in [^{14}C]FAD formation and, therefore, were pooled into one control group. The results in Fig. 1 are expressed as a percent of this control value. The mean ± SE of [^{14}C]FAD biosynthesis in these control animals was 239 ± 9 pmol·(g tissue)$^{-1}$·hr^{-1}. At a cumulative dose of 6 mg/kg of ADR, no significant effect was observed, but clearly at doses of 12, 18, 24, and 30 mg/kg significant and progressive decreases in [^{14}C]FAD formation occurred after 3 days of treatment.

Figure 2 represents data from the time–course studies and indicates that a significant decline from control values of [^{14}C]FAD formation was observed as early as 6 hr after one injection of ADR (10 mg/kg). Moreover, at 1, 2, and 3 days after the initial injection of ADR (10 mg/kg), a progressive and significant decline in the biosynthesis of [^{14}C]FAD occurred. At both 6 hr and 1 day after a cumulative dose of 10 mg/kg body weight of ADR, the formation of [^{14}C]FAD in rat skeletal muscle was reduced to 80% of the control value. A further reduction to 73% of control value of [^{14}C]FAD was observed after 2 days and to 53% at 3 days, representing cumulative doses of 20 and 30 mg/kg body weight of ADR respectively. Figures 1 and 2 suggest that, following acute administration of ADR, reduction of formation of [^{14}C]FAD in skeletal muscle appears to be dependent more upon the cumulative dose of ADR than upon the time–course of drug administration. This interpretation is corroborated by the observation that cumulative doses of ADR similar to those used in this study and given over different time intervals produce similar degrees of reduction in FAD biosynthesis.

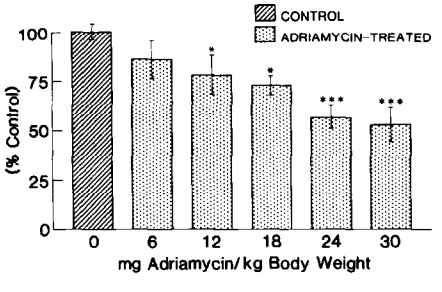

Fig. 1. Effects of graded doses of Adriamycin treatment on [^{14}C]FAD biosynthesis in skeletal muscle. All data are shown as mean ± SE, with five to eight rats per group. In control animals, the mean ± SE of [^{14}C]FAD biosynthesis was 239 ± 9 pmol·(g tissue)$^{-1}$·hr^{-1}. Key: (*) $P < 0.05$, and (***) $P < 0.001$.

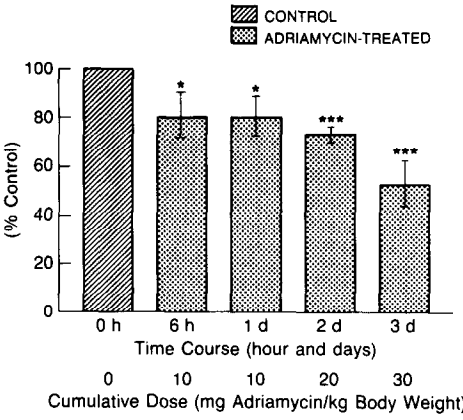

Fig. 2. Effect of Adriamycin treatment on [^{14}C]FAD biosynthesis in skeletal muscle with respect to cumulative dose and time–course of administration. Rats treated with ADR were killed at intervals of 6 hr, 1 day, 2 days, and 3 days, representing cumulative doses of 10, 10, 20, and 30 mg/kg body weight of ADR respectively. All data are shown as mean ± SE, with five to thirteen animals per group. See the legend of Fig. 1 for mean control value. Key: (*) $P < 0.05$, and (***) $P < 0.001$.

DISCUSSION

In this study, the formation of [^{14}C]FAD from [^{14}C]riboflavin in skeletal muscle was diminished by ADR administration in a dose-related manner. These results provide further evidence for direct interactions among flavins and anthracycline chemotherapeutic agents. Moreover, toxic aglycone metabolites of ADR have been shown to compete with flavins for their respective binding sites on certain flavoenzymes. The binding of ADR and its metabolites to flavoenzymes may be an important factor contributing to the toxicity observed in heart [19, 20].

Merski *et al.* [7] have presented ultrastructural evidence showing similarities in the responses of skeletal and cardiac muscle cell nucleoli to ADR treatment. These observations are of interest since a dose of ADR (3.5 mg/kg body weight) caused significant alterations of nucleolar structures of both cardiac and skeletal muscle cells, whereas a dose of 5.0 mg/kg was needed to produce similar changes in the nucleoli of hepatocytes. In terms of flavin metabolism, inhibition of FAD formation has been

demonstrated to occur in heart but not in liver and kidney at cumulative doses of 1–10 mg/kg body weight of ADR [21], providing biochemical evidence for what may be an organ-selectivity of the drug. Likewise, Lefrak *et al.* [25] demonstrated clinically that ADR cardiotoxicity is dose dependent.

These findings in their entirety suggest a possible correlation among the inhibition of flavin coenzyme biosynthesis, alterations in oxidative phosphorylation, and ADR-induced myopathy in metabolically active tissues. Hence, the inhibition of RNA synthesis by ADR in cardiac and skeletal muscle may be particularly critical in cardiac and skeletal muscle inasmuch as these tissues rely primarily on optimal activity of mitochondrial enzymes [7].

The glutathione redox cycle possibly may provide protection from ADR cytotoxicity [26]. This cycle operates through glutathione peroxidase and glutathione reductase to reduce cellular damage produced by ADR-generated H_2O_2, free radicals, and lipid peroxides. Glutathione reductase may offer cellular protection from ADR-induced peroxidation in a coordinated fashion with superoxide dismutase, alpha-tocopherol, and other related free radical scavengers [26]. Differing concentrations or activities of these enzymes in various tissues may account for the organ selectivity of ADR toxicity since cardiac tissue has low activities of superoxide dismutase, catalase, and glutathione peroxidase. Comparatively, liver possesses greater activities of these enzymes and exhibits less of a cytotoxic effect than does cardiac muscle [27]. In addition, since the reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH) is catalyzed by glutathione reductase, which requires flavin adenine dinucleotide for complete activation, the possible association of decreased FAD formation, impairment of intracellular reductive capacity, and ADR-induced toxicity becomes more apparent [28].

These considerations, taken with the findings of the present report, raise the possibility that ADR myopathy may be due, at least in part, to inhibition of FAD formation, ultimately leading to changes in energy metabolism and overall oxidative and reductive capacities of the cell. However, direct correlation between muscle weakness in patients receiving ADR and inhibition of FAD biosynthesis has yet to be established. The potential significance of this investigation to health and disease is that new information has been provided concerning the interrelationships among anthracycline drugs and inhibition of FAD biosynthesis. Since the clinical use of ADR as both a single agent and as a component of combination therapy for cancer is increasing [6], it is important to assess biochemical abnormalities produced by this class of chemotherapeutic compounds in skeletal and cardiac muscle as a means for predicting functional disturbances in these organs [7]. Moreover, further studies are required to elucidate the mechanism of inhibition of FAD formation by adriamycin and the role that this type of inhibition may play in the response to therapy of individual patients. It is tempting to speculate that there may be a possible role for the application of riboflavin in prevention or treatment of ADR-induced skeletal and cardiac muscle toxicity; however, the time–course and dose of administration have yet to be determined completely. The sensitivity of FAD biosynthesis in skeletal muscle to inhibition by ADR may serve as a possible marker for subsequent development of cardiotoxicity due to this drug. Further research is required before possible clinical applications of these findings can be assessed.

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