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Biochemical Pharmacology, Vol. 30, pp. 676–678.

0006-2952/81/060676-03 $02.00/0

Lack of inhibition of glutathione reductase by anthracycline antibiotics*

(*Received* 3 *July* 1980; *accepted* 3 *October* 1980)

Although the mechanism by which the anthracycline antibiotics produce their cumulative dose-dependent cardiomyopathy remains to be fully elucidated, recent evidence suggests that reactive oxygen species may play an etiological role. Doxorubicin and daunorubicin undergo conversion to free radical semiquinones via the acceptance of one electron from NADPH–cytochrome P-450 reductase (EC 1.6.2.4), and the free radical thus formed may subsequently transfer an electron to molecular oxygen thereby generating superoxide [1, 2]. Superoxide and its decomposition product hydrogen peroxide have also been generated in an NADH-dependent system containing submitochondrial particles isolated from bovine heart [3], and hydrogen peroxide was identified as a product formed during incubations of doxorubicin with human erythrocytes [4].

Enzymes for the detoxification of superoxide and hydrogen peroxide are ubiquitous in mammalian tissues. Superoxide dismutase (EC 1.15.1.1) catalyses the conversion of superoxide to hydrogen peroxide. The latter may be converted to water either by glutathione peroxidase (glutathione : hydrogen peroxide oxidoreductase, EC 1.11.1.9) using reduced glutathione (GSH)† as a source of reducing equivalents, or by catalase (EC 1.11.1.6). The reaction of glutathione peroxidase with hydrogen peroxide results in the oxidation of GSH to GSSG which is normally reduced back to GSH by glutathione reductase (NADPH : oxidized-glutathione oxidoreductase, EC 1.6.4.2) and NADPH.

Cardiac tissue is ill-equipped to metabolize reactive oxygen species. It contains much less catalase than the liver—about 2–4 per cent of the hepatic activity in rabbits and rats [3] and less than 1 per cent in mice [5]. Additionally, superoxide dismutase activity in mouse heart was shown to be about one-fourth that found in mouse liver [5]. Although glutathione peroxidase activity in heart is similar

* Supported, in part, by the American Cancer Society, Illinois Division Grant 80-46 to R. F. N. and by NIH Training Grant GM07263 to the Department of Pharmacology.

† Abbreviations: GSH, reduced glutathione; and GSSG, oxidized glutathione.

to that in liver, doxorubicin administration depletes the heart of this enzyme, apparently by decreasing the concentration of selenium in the heart which is required for enzymic activity [5, 6]. Thus, doxorubicin may play a dual role in causing cardiotoxicity by generating superoxide and hydrogen peroxide and diminishing the already limited ability of the heart to metabolize hydrogen peroxide.

In addition to its participation in the metabolism of hydrogen peroxide, GSH is also a free radical scavenger. Doxorubicin has been shown to cause a moderate depletion of cardiac GSH levels [7, 8] with no concurrent increase in GSSG concentrations [8]. *N*-Acetylcysteine, a precursor substrate in glutathione biosynthesis and also a free radical scavenger, did, however, prevent doxorubicin cardiotoxicity [9].

α-Tocopherol is also capable of protecting against toxicity mediated by activated oxygen species. Presently a controversy exists regarding the role of α-tocopherol in protecting against doxorubicin-induced cardiomyopathy as manifested by morphological and biochemical changes. In particular, α-tocopherol has been shown to be both without activity [8], and effective [10], in protecting against doxorubicin-mediated decreases in cardiac GSH. Furthermore, α-tocopherol, in one study, prevented doxorubicin-induced cardiac ultrastructural changes [10], whereas in another, it was without effect [11]. Thus, the interrelationship between α-tocopherol and cardiac damage caused by doxorubicin remains unclear.

Doxorubicin and daunorubicin also inhibit important mitochondrial enzyme activities. The biosynthesis of coenzyme Q was impaired in beef heart mitochondria by these drugs [12], as were the activities of two coenzyme Q-dependent enzymes, succinoxidase and NADH-oxidase [13]. Isocitrate dehydrogenase (EC 1.1.1.42) was also inhibited by doxorubicin [14].

In view of the effects of the anthracycline antibiotics on the activities of various oxidoreductases and on their abilities to generate activated oxygen species, we undertook a study of the effects of doxorubicin and daunorubicin on the activity of glutathione reductase, an enzyme of great significance in the metabolism of hydrogen peroxide. The data presented in this paper demonstrate that, even in very high concentrations, the anthracyclines have no direct effect on glutathione reductase activity in human erythrocyte hemolysates.

Blood was obtained from normal adult human volunteers, using citrate as the anticoagulant. The cells were washed twice with, and then suspended in, isotonic saline to give a hematocrit of 20%. The cell suspensions were twice frozen in a dry ice/isopropanol bath and thawed to hemolyze the cells, and the solution was centrifuged to remove debris. The supernatant fraction was used as the source of glutathione reductase.

Doxorubicin hydrochloride was the gift of Adria Laboratories, Columbus, Ohio. Daunorubicin was obtained from the Drug Development Branch of the National Cancer Institute, Bethesda, MD. All other reagents were from the Sigma Chemical Co., St. Louis, MO.

Enzyme assays were carried out at 37° for 30 min. The incubation mixture contained, in a total volume of 1.0 ml, 0.3 mM NADPH, 1.0 mM GSSG, 5.0 mM EDTA, 20.0 mM potassium phosphate buffer (pH 7.5), and 0.1 ml of hemolysate. Glutathione reductase activity was assayed by measuring GSH formed from GSSG according to the method of Beutler *et al.* [15].

The effects of doxorubicin and daunorubicin, in the concentration range of 4–400 μM, on glutathione reductase activity in human red cell hemolysates is shown in Table 1. Neither doxorubicin nor daunorubicin, even at concentrations of 400 μM, produced inhibition of the enzyme activity. Nitrofurantoin, a known inhibitor of glutathione reductase, was able to inhibit glutathione reductase in this system. Nitrofurantoin in a concentration of

Table 1. Effects of doxorubicin and daunorubicin on human red cell hemolysate glutathione reductase activity

Drug	Activity*	% Inhibition† (% stimulation)
Control	0.845 ± 0.007	0
Doxorubicin (4 μM)	0.838 ± 0.007	0.8
Doxorubicin (40 μM)	0.850 ± 0.004	(0.6)
Doxorubicin (400 μM)	0.828 ± 0.008	2.0
Daunorubicin (4 μM)	0.836 ± 0.005	1.1
Daunorubicin (40 μM)	0.844 ± 0.006	0.1
Daunorubicin (400 μM)	0.832 ± 0.003	1.5

* Activity is expressed as μmoles GSH formed per min per ml erythrocytes. Values are means ± S.E.M. and represent the averages of triplicate determinations.

† None of the values is significantly different from control, using Student's *t*-test for unpaired data.

840 μM produced 92 per cent inhibition of glutathione reductase [16], while 40 μM nitrofurantoin caused 52 per cent inhibition [17]. Comparable effects of nitrofurantoin on glutathione reductase from rat blood hemolysate and from yeast were observed. In comparison, 50 μM doxorubicin produced half-maximal stimulation of superoxide formation in bovine heart submitochondrial particles [3], 176 μM doxorubicin caused an 8-fold increase in hydrogen peroxide generation in human erythrocytes [4], 5 and 12 μM doxorubicin and daunorubicin, respectively, produced 82 and 73 per cent inhibition of beef heart mitochondrial succinoxidase [13], and 200 μM doxorubicin inhibited isocitrate dehydrogenase by 90 per cent [14]. Thus, over the concentration range utilized which includes those previously shown to produce other biochemical alterations, the anthracycline antibiotics do not appear to be inhibitors of glutathione reductase.

Doxorubicin and daunorubicin generate superoxide and hydrogen peroxide in cardiac cells. Because these cells possess only very small amounts of catalase, the glutathione peroxidase/glutathione reductase system appears to play a major role in the detoxification of hydrogen peroxide. Since the anthracycline antibiotics cause cellular depletion of glutathione peroxidase, the concurrent inhibition of glutathione reductase is an attractive hypothesis that would partially explain the depletion of GSH. This inhibitory effect of the drugs would permit high levels of hydrogen peroxide to accumulate causing lipid peroxidation and cellular damage. The data presented herein do not support this hypothesis; hydrogen peroxide accumulation probably results from the loss of glutathione peroxidase activity, while the drop in GSH, without a concomitant increase in GSSG, supports the mechanism of GSH acting as a free radical scavenger of anthracycline semiquinone free radicals.

In summary, the anthracycline antibiotics doxorubicin and daunorubicin do not inhibit glutathione reductase. Their toxic effects may result from generation of activated oxygen species and impairment of hydrogen peroxide metabolism by drug-mediated depletion of glutathione peroxidase.

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Biochemical Pharmacology, Vol. 30, pp. 678–681.
Pergamon Press Ltd. 1981. Printed in Great Britain.

0006-2952/81/060678-04 $02.00/0

Hepatic microsomal oxidative drug metabolism in the spontaneously hypertensive rat

(*Received* 9 *April* 1980; *accepted* 10 *September* 1980)

One of the most extensively studied animal models of human essential hypertension is the spontaneously hypertensive rat (SHR) developed by Okamoto and Aoki [1] in 1962–1963 by means of selective inbreeding in a colony of Wistar rats. In 1974, Vainionpaa *et al.* [2] reported that the *in vivo* metabolism of hexobarbital, the *in vitro* oxidative metabolism of aminopyrine or of 3,4-benzpyrene, or the content of cytochrome P-450 in hepatic microsomes was not altered in SHR in comparison with normotensive Wistar rats. Willis and Queener [3], however, subsequently reported that pentobarbital sleeping times were significantly shorter in 10- to 14-week-old SHR than in Wistar–Kyoto rats (WKY), the normotensive control for SHR [1], and Hall *et al.* [4] have reported similar observations. More recently, Yates *et al.* [5] observed that the NADPH–cytochrome *c* reductase activity was slightly greater in hepatic microsomes of saline-pretreated SHR in comparison with the activity catalyzed by hepatic microsomes prepared from saline-pretreated normotensive Wistar rats. In an attempt to gain further insight into the effects of hypertension on the hepatic microsomal oxidative metabolism of xenobiotics, the present investigation was conducted using male SHR that were either developing hypertension or in a period of sustained hypertension. A preliminary account of this work has been published [6].

Male SHR were obtained from an inbred colony maintained at The University of Iowa and were of the forty-third through the forty-fifth generations when tracked back to the original pairing that resulted in the derivation of the strain. Male WKY were from a colony maintained in these facilities under the same conditions as were SHR. WKY are descended directly from a colony of Wistar–Kyoto rats from which SHR were originally isolated and are presently considered to be the most appropriate control for SHR [7]. Systolic arterial blood pressures of 10- to 13-week-old and 20- to 22-week-old unanesthetized animals were determined by a modification of the tail plethysmographic method described by Friedman and Freed [8]. Prior to pressure measurements, the rats were warmed to 35° for several minutes, and systolic arterial blood pressures were determined using an automated cuff-inflator-pulse detection system manufactured by Technilab Instruments (Pequannock, NJ). For each determination, three to five consecutive measurements were made on each animal that had been previously conditioned to the apparatus.

For the determination of hexobarbital sleeping times, hexobarbital was dissolved in saline made alkaline by the dropwise addition of 1 N NaOH and was administered intraperitoneally to 10- to 13-week-old animals at a dose of 200 mg/kg. The duration of sleep was determined from the time at which the righting reflex was lost to the time at which the rats regained the righting reflex. Upon regaining the righting reflex, rats were decapitated and trunk blood was collected. The plasma levels of hexobarbital were then determined as described by Cooper and Brodie [9].

Rats were fasted for 24 hr and then were decapitated. Livers were excised after perfusion *in situ* with ice-cold 0.154 M NaCl, and 10% (w/v) homogenates were prepared in ice-cold 0.25 M sucrose. The hepatic microsomal fraction was then isolated as described by Master *et al.* [10]. Protein was determined by the biuret method using bovine serum albumin as the standard [11].

Hepatic microsomal NADPH–cytochrome *c* reductase activity was determined at 25° as described by Masters *et al.* [10]. The *N*-demethylation of ethylmorphine catalyzed by hepatic microsomes was determined at 25° by measuring the rate of formation of formaldehyde employing the method of Nash [12] as modified by Cochin and Axelrod [13]. Each 7-ml reaction mixture contained 14 mg of microsomal protein, 8 mM ethylmorphine, 150 mM KCl, 10 mM $MgCl_2$, and 50 mM Tris–HCl buffer, pH 7.4. After the addition of 200 μM NADPH, 1-ml aliquots were removed every 30 sec for the determination of formaldehyde. Hepatic microsomal aniline hydroxylase activity was determined at 25° by measuring the rate of formation of *p*-aminophenol according to the method of Schenkman *et al.* [14]. The *O*-demethylation of *p*-nitroanisole catalyzed by