0026-895X/88/050695-07$02.00/0

MOLECULAR PHARMACOLOGY, 134: 695–701

Oxygen-Dependent Hepatotoxicity Due to Doxorubicin: Role of Reducing Equivalent Supply in Perfused Rat Liver

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SUMMARY

Doxorubicin is an important anticancer drug that undergoes redox cycling leading to the production of oxygen radicals; however, its clinical use is limited by toxicity. Redox cycling due to doxorubicin was assessed in the perfused rat liver from increases in O_2 uptake by the organ, and toxicity was determined from lactate dehydrogenase release and trypan blue uptake. Doxorubicin increased O_2 uptake in a concentration-related manner with half-maximal increases at about 100 μM drug. Within 5 min after addition of 300 μM doxorubicin, lactate dehydrogenase was detected in the effluent perfusate. Enzyme release increased steadily and reached values of 600 units/liter after 60 min. Rates of O_2 uptake due to redox cycling of doxorubicin (300 μM) increased by 57 μmol/g/hr in oxygen-rich (mean $[O_2]$ = 473 μM) periportal regions of the liver lobule, but did not change in pericentral regions where O_2 tension was lower ($[O_2]$ = 247 μM). Concomitantly, fluorescence of NAD(P)H measured from the liver surface decreased in periportal but not pericentral regions. The zone-specific decrease in NADPH was attributed to redox cycling of doxorubicin. Trypan blue was taken up exclusively by cells in periportal regions of the liver lobule after perfusion with doxorubicin. When the average O_2 tension was lowered from 550 to 200 μM, O_2 uptake due to redox cycling of doxorubicin in periportal regions was reduced 3-fold and toxicity was abolished, indicating that toxicity due to doxorubicin is oxygen-dependent. Redox cycling of doxorubicin was minimal in regions of the perfused liver where the O_2 concentration was below 400 μM. In contrast, isolated microsomes displayed maximal changes in O_2 uptake due to redox cycling of doxorubicin at O_2 tensions of about 10 μM. Thus, oxygen per se is not rate-limiting for redox cycling of doxorubicin in the intact organ. Since NADPH is also required for redox cycling of doxorubicin, the effect of oxygen on the ability of mitochondria and the pentose cycle to supply reducing equivalents for redox cycling of doxorubicin was examined. NADPH supply from the pentose cycle was reduced by fasting while that from mitochondria was inhibited by cyanide. The increase in O_2 uptake due to redox cycling of doxorubicin was around 60 μmol/g/hr in livers from fed or fasted rats. In the presence of potassium cyanide, stimulation of O_2 uptake by doxorubicin was reduced by about one-half in livers from fed rats (29 μmol/g/hr) yet was abolished nearly completely in livers from fasted rats (7 μmol/g/hr). In livers from fasted rats perfused with potassium cyanide, fructose increased doxorubicin-stimulated O_2 uptake significantly indicating that NADPH supply was limiting for redox cycling. Neither fasting nor potassium cyanide diminished doxorubicin-stimulated O_2 uptake by microsomes incubated with excess NADPH. These data raise the interesting possibility that oxygen tension regulates the supply of NADPH which in turn determines the rate of redox cycling of doxorubicin in liver. Consequently, oxygen is a key determinant of hepatotoxicity of doxorubicin.

Doxorubicin (Adriamycin) is an anthracycline antibiotic used extensively in the treatment of human neoplasms including acute leukemia, Hodgkin's disease, and soft tissue sarcomas (1) and is the most efficient single drug used for the chemotherapy of breast cancer (2). The clinical usefulness of doxorubicin is limited, however, by its toxicity. The quinone nucleus of doxorubicin undergoes redox cycling where the reduction of doxorubicin to a semiquinone free radical is catalyzed by NADPH-dependent cytochrome P450 reductase (3), and reconversion to the quinone is accompanied by reduction of molecular oxygen to superoxide anion. Theoretically, 1 mol of oxygen and NADPH are utilized for the production of 1 mol of $NADP^+$ and superoxide anion. Generation of reactive oxygen species during redox cycling has been implicated in doxorubicin-induced toxicity (4–6).

A natural and well-defined oxygen gradient exists in the perfused liver where upstream, periportal regions of the liver lobule are oxygen-rich (average $[O_2]$ = 400–500 μM) and downstream, pericentral areas are relatively poor in oxygen (average $[O_2]$ = 200–300 μM) (7). Thus, the perfused liver presents a good model to study the influence of oxygen tension on biochemical processes. It has been demonstrated recently that oxygen is an important determinant of hepatotoxicity of allyl alcohol (8) and menadione, another quinone which undergoes redox cycling (9). Both allyl alcohol and menadione damage oxygen-rich periportal regions of the liver lobule, and toxicity due to allyl alcohol is diminished when oxygen tension is decreased. Since redox cycling of doxorubicin requires oxygen, the purpose of this study was to examine the effect of oxygen tension on doxorubicin-induced hepatotoxicity using the per-

This work was supported in part by Grant ES-02759 from the National Institute of Environmental Health Sciences. P. E. Ganey received a Postdoctoral Fellowship from the National Institute of Environmental Health Sciences (T32-ES01726) and from the American Liver Foundation.

fused rat liver as a model. Preliminary accounts of this work have appeared elsewhere (10).

Materials and Methods

Animals and liver perfusion. Female, Sprague-Dawley rats, 80–200 g, received sodium phenobarbital (1 mg/ml) in drinking water for at least 1 week prior to perfusion experiments to facilitate identification of periportal and pericentral regions of the liver lobule (11). Livers were perfused using a recirculating system as described previously (12). The perfusate was Krebs-Henseleit bicarbonate buffer containing 4% bovine serum albumin (pH 7.4, 37°C) saturated with 95% O_2/5% CO_2. In some experiments, inflow oxygen tension was lowered by saturating the perfusate with a mixture of 95% O_2/5% CO_2 and 95% N_2/5% CO_2. Doxorubicin (Adriamycin; a kind gift from Adria Laboratories, Inc., Columbus, OH) was added at concentrations indicated in the legends following a preperfusion period of approximately 20 min. Oxygen concentration in the effluent perfusate was monitored continuously with a Clark-type platinum electrode, and rates of oxygen uptake were calculated from inflow-outflow oxygen concentration differences, the flow rate, and the liver wet weight.

Measurement of regional rates of oxygen uptake. Oxygen tension in periportal and pericentral regions of the liver lobule was measured using a miniature oxygen electrode prepared by inserting a 50-μm diameter platinum wire into a glass capillary tube, pulling under heat, then coating the electrode tip with an oxygen-permeable acrylic ester polymer (Rhoplex) (11). To measure local rates of oxygen uptake, the electrode was placed on periportal and pericentral regions, and flow was stopped by simultaneously halting the inflow and clamping the outflow. Rates of oxygen uptake were then determined from the decrease in oxygen tension per unit time and the fluid content of the liver as described in detail previously (7).

Analytical measurements and histological procedures. Lactate dehydrogenase in the effluent perfusate was determined by standard enzymatic procedures (13). To detect toxicity in different regions of the liver lobule, trypan blue (0.2 mM) was infused into livers for 10 min followed by fixation of the liver by perfusion with paraformaldehyde (1% in Krebs-Henseleit buffer) for 5 min. Livers were processed for light microscopy and sections were stained with eosin only. This process allows easy identification of trypan blue in the nuclei of irreversibly damaged cells (14). With light microscopy five periportal and five pericentral regions were chosen randomly, and the percentage of cells stained with trypan blue in an area five cells wide radiating from the center of each region was identified (15).

Fluorescence of pyridine nucleotides and doxorubicin. Fluorescence was measured from the surface of the perfused liver using a large-tipped (2 mm) light guide or in periportal and pericentral regions using micro-light guides (diameter 70 μm). Construction and use of micro-light guides have been described in detail elsewhere (11). For measurement of pyridine nucleotide fluorescence, the liver was illuminated at 366 nm and fluorescence was measured at 450 nm. Doxorubicin fluorescence was measured by illuminating the liver at 485 nm and measuring fluorescence at 580 nm (16).

Preparation of microsomes. Microsomes were prepared by standard techniques of differential centrifugation (17) and were incubated at room temperature in 2 ml of Krebs-Henseleit buffer (pH = 7.4) in the presence of NADPH (5 mM) or an NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate, and 0.2 unit of isocitrate dehydrogenase (Sigma Chemical Co., St. Louis, MO). Microsomal protein was determined by the Biuret reaction (18). Decreases in microsomal oxygen concentration were monitored with a Clark-type platinum electrode in a 2-ml chamber, and rates of oxygen uptake were determined from tangents drawn to the oxygen tension versus time curve.

Statistical analyses. Results are represented as mean ± SE. The data were analyzed using Student's t test, paired or unpaired, or by analysis of variance. The criterion for significance was $p < 0.05$.

Results

Hepatotoxicity and redox cycling due to doxorubicin. Addition of 300 μM doxorubicin to the perfused liver caused a rapid increase in oxygen uptake due to redox cycling of about 60 μmol/g/hr which was sustained for up to 60 min (Fig. 1A). Lactate dehydrogenase was detectable within 5 min after addition of doxorubicin (Fig. 1B) and increased continuously reaching values of around 600 units/liter by 1 hr. The stimulation of oxygen uptake was concentration-dependent and was half-maximal with about 100 μM doxorubicin (Fig. 2). Cell death, assessed from uptake of trypan blue, occurred predominantly in oxygen-rich periportal regions of the lobule: 54% of cells in periportal regions were stained with trypan blue compared to 5% in pericentral areas (Table 1).

To determine whether redox cycling of doxorubicin occurred at different rates in sublobular zones, rates of oxygen uptake were measured in periportal and pericentral regions of the liver lobule using miniature oxygen electrodes. Prior to addition of doxorubicin, the initial oxygen concentration was approxi-

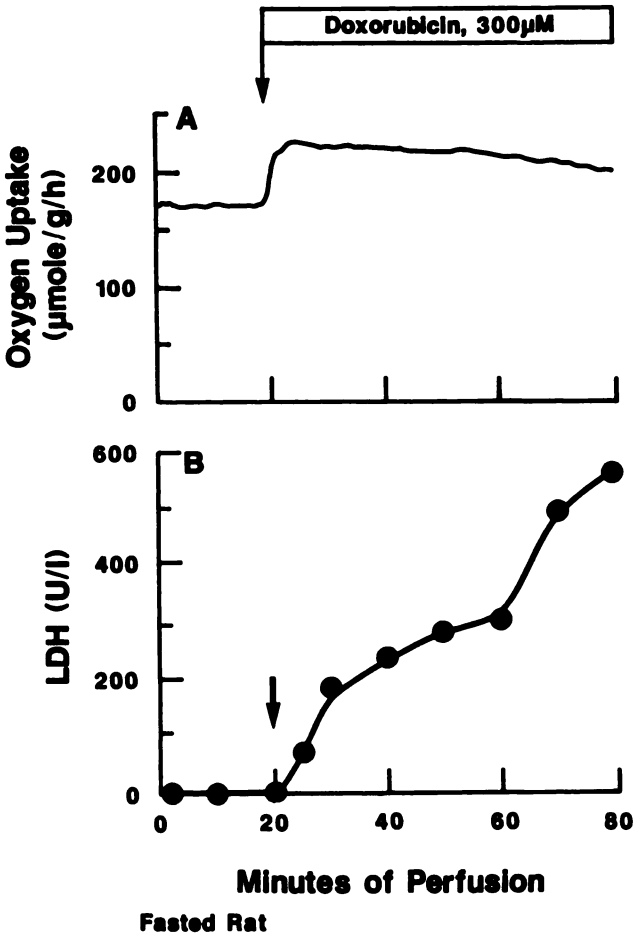

Fig. 1. Effect of doxorubicin on (A) oxygen uptake and (B) lactate dehydrogenase (*LDH*) release by perfused liver from a fasted, phenobarbital-treated rat. A liver was perfused in a recirculating system for 1 hr with Krebs-Henseleit buffer containing 4% bovine albumin saturated with 95% O_2/5% CO_2. Doxorubicin (300 μM) was added to perfusate over 1 min at the time indicated by the *arrow*. Oxygen uptake was monitored continuously using a Clark-type oxygen electrode as described under "Materials and Methods," and LDH was determined enzymatically in perfusate samples taken every 10 min. Typical experiment. In four livers of this design, LDH release reached maximal values of 1052 ± 370 units/liter.

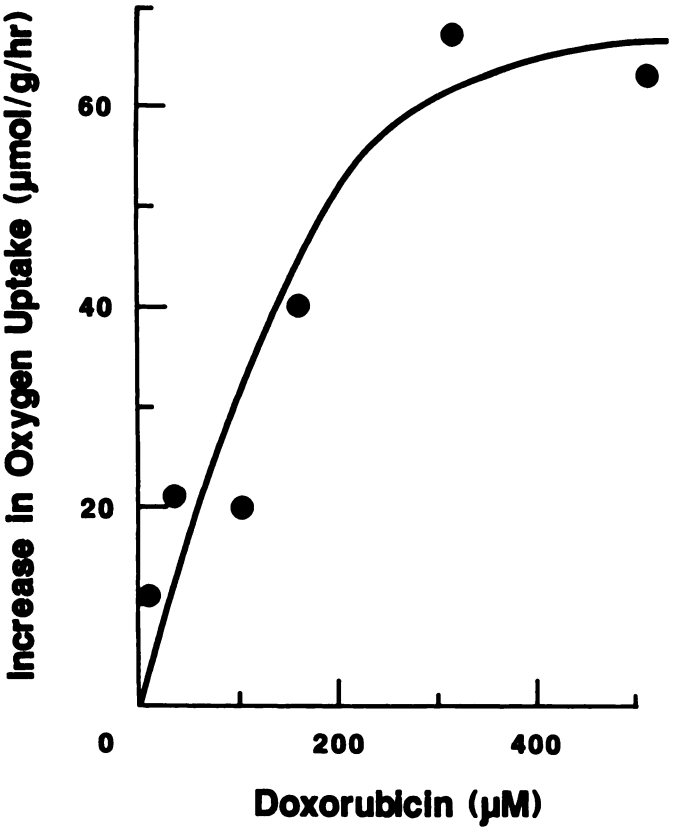

Fig. 2. Dependence of redox cycling in the perfused liver on doxorubicin concentration. Livers from fasted, phenobarbital-treated rats were perfused and oxygen uptake was measured as described under "Materials and Methods." Each point represents data from one liver perfused with one concentration of doxorubicin. Values represent maximal increase in oxygen uptake measured 5–20 min after addition of drug.

TABLE 1

Zone-specific uptake of trypan blue due to doxorubicin

Livers from fasted, phenobarbital-treated rats were perfused with buffer saturated with 95% O_2/5% CO_2 (average oxygen concentration in the liver = 550 μM) or with a mixture of 95% O_2/5% CO_2 and 95% N_2/5% CO_2 (average oxygen concentration = 200 μM). Doxorubicin (300 μM) was added over 1 min, and livers were perfused for 1 hr. Trypan blue (0.2 mM) was infused for 10 min, livers were fixed by perfusion with paraformaldehyde (1% in Krebs-Henseleit buffer) for 5 min and were processed for light microscopy. Percentage of cells stained in each region was determined as described under "Materials and Methods." Numbers of livers are given in parentheses.

Average Hepatic Oxygen Concentration	Sublobular Region	Local Oxygen Concentration	Cells Stained with Trypan Blue
		μM	%
550 μM (9)	Periportal	473 ± 16	54 ± 10
	Pericentral	182 ± 12	5 ± 2*
200 μM (5)	Periportal	247 ± 15	2 ± 1*

* Significantly different from the value for periportal regions of livers perfused at 550 μM oxygen ($p < 0.05$, Student's t test).

mately 580 μM in periportal regions and 300 μM in pericentral areas (Fig. 3). When inflow and outflow were halted simultaneously, oxygen concentration decreased linearly. Local rates of oxygen uptake were calculated from the rate of decline of oxygen concentration and the fluid content of the liver. Basal rates of oxygen uptake in periportal regions were 123 μmol/g/hr, approximately 3-fold greater than in pericentral regions (Table 2), confirming other studies (7, 19). Doxorubicin increased the rate of oxygen uptake in periportal regions by 57 μmol/g/hr, but had no effect in pericentral areas (Table 2). Because periportal and pericentral regions each occupy about 50% of the total liver mass (11), the sum of oxygen uptake in each region divided by 2 is approximately equal to oxygen uptake in the whole organ measured from steady state changes in oxygen concentration (7). In the present study, the increase in oxygen uptake caused by doxorubicin in periportal plus pericentral regions divided by 2 was approximately equal to the increase observed in the whole organ. Thus, oxygen uptake measured from the liver surface in distinct regions of the lobule by miniature oxygen electrodes represents metabolic activity of the organ as a whole.

Fluorescence of NAD(P)H and doxorubicin. In the basal state, approximately 90% of the $NADP^+$ is present in the reduced form, whereas only approximately 20% of the NAD^+ is reduced (20). Therefore, an increase in pyridine nucleotide fluorescence from the liver surface reflects predominantly an increase in NADH, whereas a decrease in fluorescence reflects mainly oxidation of NADPH. Basal fluorescence (366 → 450 nm) of the liver was lower in pericentral regions (Fig. 4) probably due to the higher content of cytochrome P450 which quenches fluorescence (11). Upon addition of doxorubicin, pyridine nucleotide fluorescence in periportal areas decreased rapidly to approximately 50% of the basal value, and maintained this new steady state level for 20 min most likely due to utilization of NADPH for redox cycling. Fluorescence in pericentral regions increased slightly in response to doxorubicin for reasons that remain unclear. Because fluorescence was not decreased in pericentral regions by doxorubicin, the possibility that the decrease in periportal areas was due to quenching of pyridine nucleotide fluorescence by doxorubicin can be ruled out.

Thus, the increase in oxygen uptake, toxicity, and oxidation of NADPH due to redox cycling of doxorubicin occurs nearly exclusively in upstream, periportal regions of the liver lobule (Tables 1 and 2; Figs. 3 and 4). Accordingly, the hypothesis that these effects were not observed in pericentral regions because doxorubicin did not reach downstream areas was evaluated by monitoring doxorubicin fluorescence in periportal and pericentral regions. Addition of doxorubicin caused a rapid increase in fluorescence of approximately 20–25% in both regions (Fig. 5) indicating that approximately equal amounts of doxorubicin reached both regions of the liver lobule.

Role of oxygen tension in hepatotoxicity and redox cycling of doxorubicin. To examine the relationship between oxygen tension, redox cycling, and toxicity due to doxorubicin, the inflow oxygen tension of the perfusion medium was decreased from an average oxygen concentration of 550 to 200 μM. At low oxygen tensions (200 μM), the increase in oxygen uptake due to doxorubicin was 20 μmol/g/hr, a value only about one-third as large as that observed in livers perfused at higher (550 μM) oxygen tensions (Table 2). Under these conditions, toxicity due to doxorubicin was prevented nearly completely: only 2% of cells in periportal regions were stained with trypan blue compared with 54% when the oxygen tension in periportal regions was high (Table 1).

The relationship between the increase in oxygen uptake due to doxorubicin and local oxygen concentration in the perfused liver is depicted in Fig. 6. Doxorubicin increased oxygen uptake only minimally at oxygen concentrations below 400 μM (half-maximal concentration = about 500 μM). In contrast to data

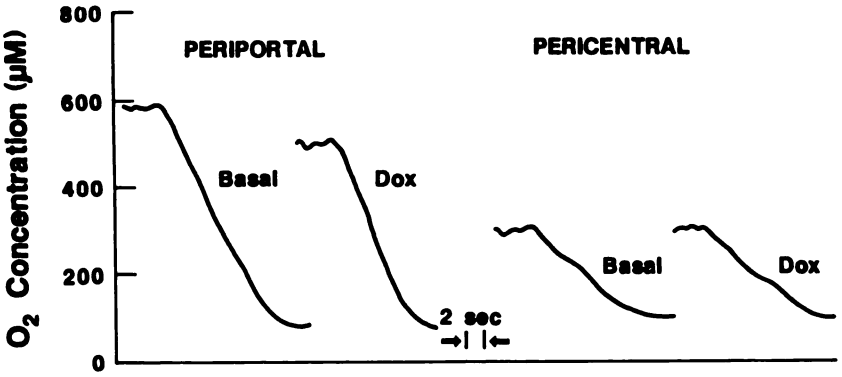

Fig. 3. Effect of doxorubicin on rates of oxygen uptake in periportal and pericentral regions of the liver lobule. Livers from fasted, phenobarbital-treated rats were perfused as described under "Materials and Methods." Periportal and pericentral regions were identified as light and dark spots, respectively, and local oxygen concentrations were measured using a miniature oxygen electrode placed on sublobular regions on the surface of the perfused liver. Inflow and outflow were halted simultaneously, and local oxygen concentration decreased linearly. Basal values were determined during the first 20 min of perfusion before addition of doxorubicin (*Dox*, 300 μM). Doxorubicin values were determined 5–20 min later. Typical experiment.

TABLE 2

Oxygen uptake due to doxorubicin in perfused liver

Livers from fasted, phenobarbital-treated rats were perfused with buffer saturated with 95% O_2/5% CO_2 (average hepatic oxygen concentration = 550 μM) or with a mixture of 95% O_2/5% CO_2 and 95% N_2/5% CO_2 (average hepatic oxygen concentration = 200 μM). Oxygen uptake was measured as described under Materials and Methods. Basal values were determined during the first 20 min of perfusion before addition of doxorubicin (300 μM). Doxorubicin values were determined 5–20 min after addition of the drug. Numbers of livers are given in parentheses.

	Oxygen Uptake		
	550 μM Oxygen (9)		200 μM Oxygen (5): Periportal
	Periportal	Pericentral	
	μmol/g/hr		
Basal	123 ± 5	42 ± 3	121 ± 2
Doxorubicin	180 ± 12[a]	43 ± 4	142 ± 9[a]
Increase	57 ± 11	1 ± 3	20 ± 7[b]

[a] Significantly different from basal value ($p < 0.05$, Student's t test).
[b] Significantly different from values in periportal regions for livers perfused with 550 μM O_2.

from the perfused liver, doxorubicin-stimulated oxygen uptake was half-maximal with less than 5 μM oxygen in isolated microsomes incubated with excess NADPH (Fig. 7). Maximal stimulation of oxygen uptake by doxorubicin in isolated microsomes was observed at concentrations around 10 μM, similar to values reported by deGroot *et al.* (21) for redox cycling of the quinone, menadione.

Role of NADPH supply in redox cycling of doxorubicin. The average local oxygen concentration of 182 μM in pericentral regions (Table 2) was more than 1 order of magnitude higher than the oxygen concentration (10 μM) required for maximal redox cycling of doxorubicin in microsomes incubated with excess NADPH (Fig. 7). Thus, regional differences in oxygen tension alone cannot explain the selective redox cycling and toxicity due to doxorubicin in periportal regions. Since NADPH is also necessary for redox cycling of doxorubicin, the hypothesis that oxygen was acting by influencing the supply of NADPH was examined. NADPH supply was decreased by perfusion with potassium cyanide to inhibit mitochondrial respiration, as well as by fasting for 24 hr to deplete glycogen. Glycogen is required for generation of NADPH via the pentose cycle when exogenous substrates are not supplied to the liver (22). Doxorubicin-stimulated oxygen uptake was around 60 μmol/g/hr in livers from fed and fasted rats (Table 3). Perfusion with KCN diminished oxygen uptake due to doxorubicin in livers from fed rats to 29 μmol/g/hr and in livers from fasted rats to 7 μmol/g/hr. Perfusion with fructose to generate NADPH in livers from fasted rats prior to infusion of

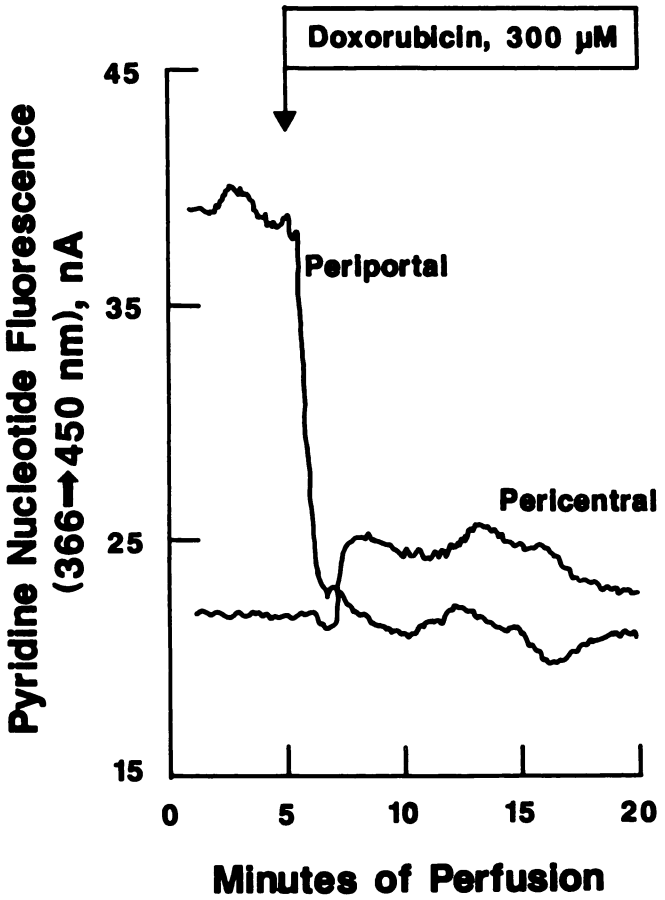

Fig. 4. Effect of doxorubicin on pyridine nucleotide fluorescence in perfused liver. Liver from a fasted, phenobarbital-treated rat was perfused as described under "Materials and Methods." Micro-light guides were placed on periportal (light) and pericentral (dark) regions of the lobule and surface fluorescence of pyridine nucleotides (366 → 450 nm), expressed as nanoamperes of photomultiplier current, was monitored continuously. Doxorubicin (300 μM) was added to the perfusate after approximately 5 min. Typical results from experiment repeated four times.

KCN resulted in significantly increased rates of oxygen uptake due to doxorubicin (17 μmol/g/hr; Table 3).

To test whether the differences in perfused livers from fed and fasted rats in the presence of KCN occurred at the level of the endoplasmic reticulum, redox cycling due to doxorubicin was examined in isolated microsomes. Basal rates of oxygen uptake measured in the presence of an NADPH-generating system were around 15 nmol/mg of protein/min (Table 4).

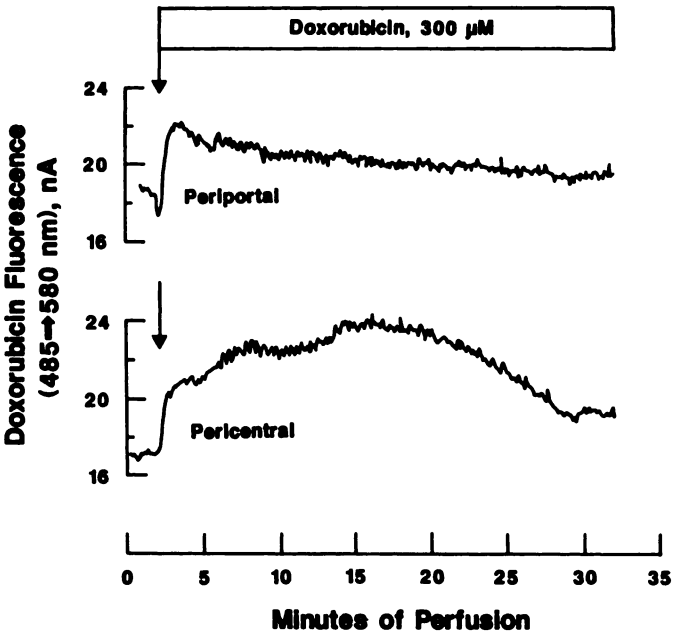

Fig. 5. Fluorescence of doxorubicin from the surface of perfused liver. Liver from fasted, phenobarbital-treated rat was perfused as described under "Materials and Methods." Micro-light guides were placed on periportal and pericentral regions of the lobule, and fluorescence of doxorubicin (485 → 580 nm) from the liver surface was monitored continuously. Doxorubicin (300 μM) was added to the perfusate after approximately 2 min. Typical experiment.

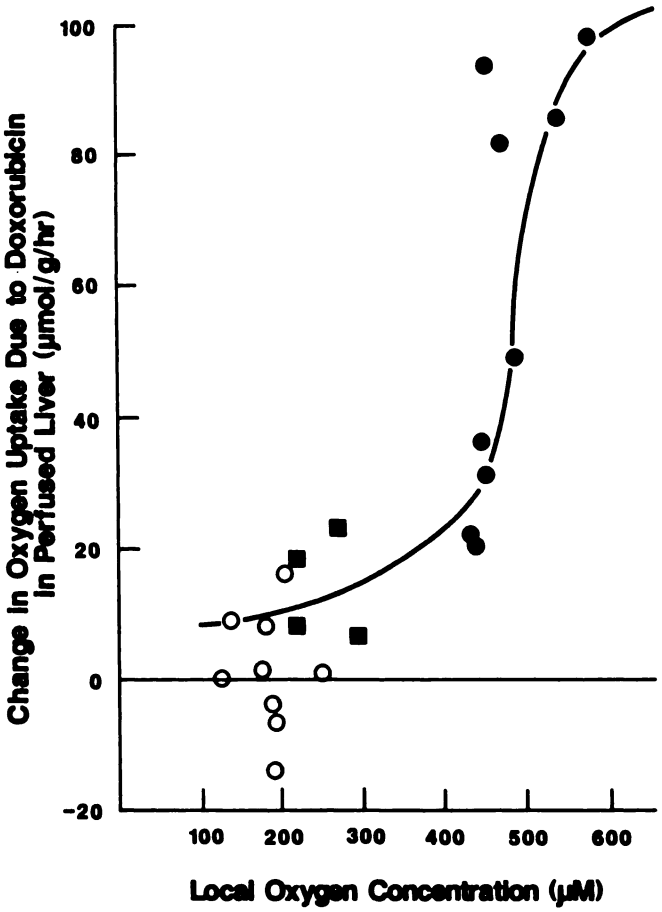

Fig. 6. Relationship between doxorubicin-stimulated oxygen uptake and oxygen tension in perfused liver. Data are from Tables 1 and 2. Local oxygen concentration represents values measured with miniature oxygen electrodes just prior to determination of the rate of oxygen uptake by the stopped-flow method. ○, pericentral, 182 ± 12 μM oxygen; ●, periportal, 473 ± 16 μM oxygen; ■, periportal, 247 ± 15 μM oxygen.

Doxorubicin increased oxygen uptake in a concentration-related manner: 0.1 mM doxorubicin increased oxygen uptake 2-fold and 1 mM doxorubicin increased values 3-fold. KCN augmented the increase due to doxorubicin by about 4-fold. Under all conditions studied, however, the stimulation due to doxorubicin was similar in microsomes from fed and fasted rats. The potentiation of doxorubicin-stimulated oxygen uptake in the presence of KCN was unexpected and may reflect inhibition of NADPH-dependent cyanide-sensitive fatty acid desaturase present in microsomes (23). Inhibition of this enzyme would increase the availability of NADPH for redox cycling.

Discussion

Role of oxygen in doxorubicin toxicity. Doxorubicin causes lipid peroxidation in isolated microsomes (5, 6, 24) and mitochondria (25) as well as *in vivo* (26, 27). Lipid peroxidation due to doxorubicin is reduced by antioxidants, suggesting the involvement of radical species (5, 6, 27). Since the generation of reactive oxygen species and peroxidation of membrane lipids requires oxygen, the role of oxygen in doxorubicin-induced hepatotoxicity was examined. Doxorubicin increased oxygen uptake markedly (e.g., by approximately 50%) in periportal regions of the liver lobule (Table 3). This increase was assumed to be due to redox cycling for two reasons. First, 1 mol each of doxorubicin, NADPH, and oxygen are theoretically required for redox cycling. Second, a substantial increase in oxygen uptake due to doxorubicin was observed in perfused liver in the presence of KCN (Table 3) indicating that this increase did not involve the mitochondrial electron transport chain.

Redox cycling and hepatocellular damage due to doxorubicin occurred in periportal regions of the liver lobule (Tables 1 and 2; Fig. 3) where the average oxygen concentration was more than twice as great as in pericentral areas (Table 1). Thus, redox cycling and toxicity due to doxorubicin occurred concomitantly in regions of the liver lobule with higher oxygen tension. Consistent with these results, NADPH fluorescence was decreased only in periportal regions by doxorubicin (Fig. 4). The hypothesis that doxorubicin toxicity is oxygen-dependent was tested by decreasing oxygen tension. Under conditions where the average oxygen concentration in periportal regions was reduced by approximately 50%, oxygen uptake due to redox cycling was decreased by 65% and hepatotoxicity due to doxorubicin was nearly completely abolished (Tables 1 and 2). Thus, it is concluded that hepatotoxicity due to doxorubicin is dependent on oxygen tension in the liver.

Redox cycling of doxorubicin in perfused liver was minimal at local oxygen concentrations below 400 μM (Fig. 6). The average oxygen concentration in periportal regions where redox cycling occurred was 473 μM, while values in pericentral areas were only 182 μM (Table 1). In isolated microsomes, however, doxorubicin-stimulated oxygen uptake was maximal at oxygen concentrations of about 10 μM (Fig. 7). Increasing oxygen concentrations did not increase rates of oxygen uptake (data not shown). Thus, redox cycling of doxorubicin and toxicity was minimal in pericentral regions of the liver lobule even though the average oxygen concentration was more than 1

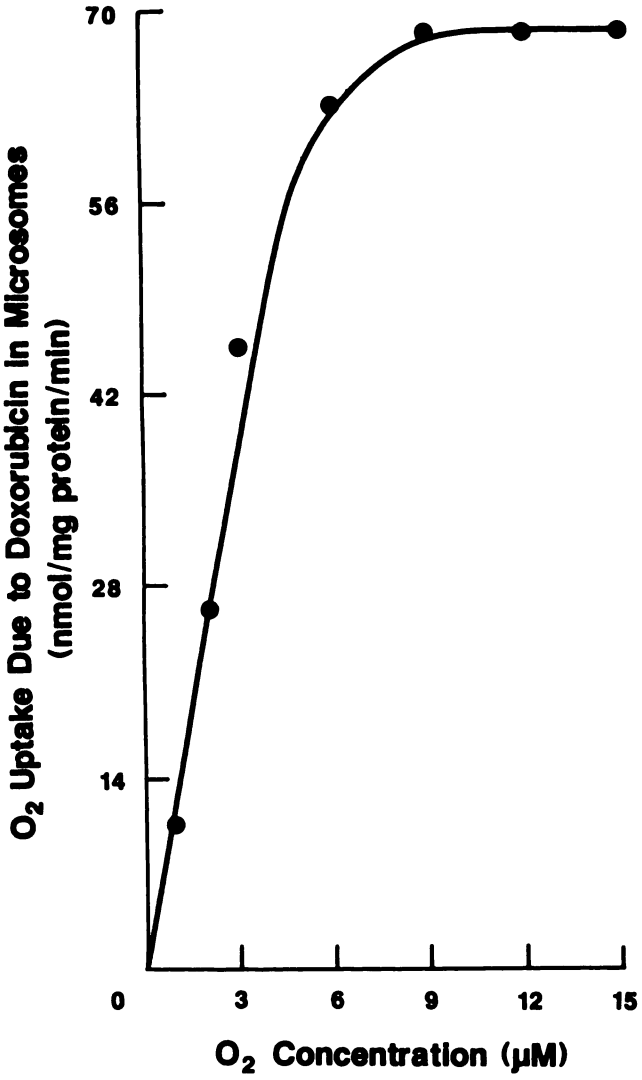

Fig. 7. Relationship between doxorubicin-stimulated oxygen uptake and oxygen concentration in isolated microsomes. Microsomes were isolated from livers of fed or fasted, phenobarbital-treated rats, and were incubated with doxorubicin (1 mM) and NADPH (5 mM) as described under "Materials and Methods." Oxygen concentration was monitored with a Clark-type oxygen electrode. Oxygen uptake was calculated from tangents drawn to the oxygen concentration versus time curve.

order of magnitude greater than values required for maximal redox cycling *in vitro*. Since the oxygen gradient from the sinusoid to the endoplasmic reticulum is quite shallow (28), differences in redox cycling of doxorubicin cannot be explained by an intracellular oxygen gradient. We suggest that oxygen affects redox cycling of doxorubicin in the perfused liver by an indirect mechanism (see below).

Role of NADPH in redox cycling due to doxorubicin. Redox cycling requires doxorubicin, oxygen, and NADPH. Neither insufficient doxorubicin delivery (Fig. 5) nor low oxygen tension could explain why redox cycling and hepatotoxicity did not occur in pericentral regions of the liver lobule. The possibility that oxygen acted indirectly by influencing NADPH supply was suggested by several findings. If NADPH supply is rate-limiting, then redox cycling should decrease when NADPH supply declines. In livers from fed rats there are two sources of NADPH: the mitochondria and the pentose cycle. Mitochondrial sources can be inhibited effectively by an inhibitor of

TABLE 3

Doxorubicin-stimulated oxygen uptake in perfused livers from fed and fasted rats

Livers from fed or fasted (24 hr), phenobarbital-treated rats were perfused in the presence or absence of KCN (2 mM; added 6 min prior to addition of doxorubicin) and the increase in oxygen uptake due to addition of doxorubicin (300 μM) to the perfusate was monitored as described under Materials and Methods. Fructose (8 mM) was added 5 min before addition of KCN. Number of livers are given in parentheses.

Addition	Increase in Oxygen Uptake Due to Doxorubicin	
	Fed	Fasted
	μmol/g/hr	
None	62 ± 8 (4)	57 ± 11 (5)
KCN	29 ± 4 (10)[a]	7 ± 2 (11)[a,b]
Fructose + KCN	ND[c]	17 ± 4 (5)[d]

[a] Significantly different ($p < 0.05$, analysis of variance) from no addition values.
[b] Significantly different from values for fed livers in the presence of KCN.
[c] ND, not determined.
[d] Significantly different from values for fasted livers in the presence of KCN.

TABLE 4

Effect of doxorubicin on oxygen uptake in isolated microsomes

Microsomes were isolated from livers of fed or fasted, phenobarbital-treated rats and were incubated with doxorubicin and an NADPH-generating system as described under Materials and Methods. Oxygen uptake was monitored with a Clark-type oxygen electrode. Means in each group were determined from four microsomal preparations from different livers.

	Oxygen Uptake	
	Fed	Fasted
	nmol/mg protein/min	
Basal	14 ± 1	15 ± 1
Doxorubicin, 0.1 mM	25 ± 5	22 ± 1
Doxorubicin, 1 mM	47 ± 5[a]	40 ± 10[a]
KCN, 2 mM	24 ± 6	20 ± 3
KCN, 2 mM + doxorubicin, 1 mM	135 ± 21[a]	117 ± 13[a]

[a] Significantly different from the respective control value ($p < 0.05$, analysis of variance).

cytochrome oxidase, KCN, and NADPH supplied by mitochondria should decline in the presence of KCN. Indeed, oxygen uptake due to redox cycling of doxorubicin declined by about 50% in livers from fed rats perfused with KCN (Table 3). In the fasted state, which is characterized by minimal glycogen stores, generation of reducing equivalents via the pentose cycle is nearly totally absent and the mitochondrial source predominates (29). Thus, inhibition of the mitochondrial source in the fasted state would be expected to abolish NADPH supply nearly totally. Again, this expectation was fulfilled by our observation that redox cycling was abolished (i.e., rates were only 7 μmol/g/hr) in fasted livers perfused with KCN (Table 3). These observations could not be explained by an action of KCN at the level of the endoplasmic reticulum since redox cycling of doxorubicin was identical in microsomes isolated from fed and fasted rats in the presence of KCN when NADPH was supplied in excess (Table 4).

Doxorubicin undergoes redox cycling at the level of NADH dehydrogenase in submitochondrial particles (30–32). KCN inhibits electron flux in the respiratory chain and would be expected to provide more reducing equivalents for doxorubicin metabolism at the level of NADH dehydrogenase in the mitochondria. However, since KCN decreased the stimulation of oxygen uptake due to doxorubicin in this study (Table 3), metabolism of doxorubicin via mitochondrial reduction appears unlikely in the perfused liver.

Another test of the hypothesis that NADPH is rate-limiting

for redox cycling comes from experiments where NADPH supply is increased. Fructose is metabolized to substrates for the pentose cycle even in the presence of KCN since it can produce ATP via glycolysis (33). Therefore, one would expect fructose to increase NADPH supply. In livers from fasted rats perfused with KCN, redox cycling due to doxorubicin was indeed greater in the presence than in the absence of fructose (Table 3).

The arguments made above allow us to conclude that redox cycling of doxorubicin is limited in the intact cell by the supply of NADPH. Since redox cycling and hepatotoxicity occur nearly exclusively in regions of high oxygen tension (Tables 1 and 2; Fig. 5), we also conclude that NADPH supply is regulated in the intact cell by oxygen tension. This conclusion is supported by the observation that redox cycling occurred at high rates in isolated microsomes supplied with excess NADPH at oxygen tensions above 10 μM (Fig. 6), yet was undetectable in pericentral regions of the liver lobule where the average oxygen tension was around 180 μM (Table 2). The possibility that NADPH supply is limited by oxygen tension is further supported by the occurrence of another NADPH-dependent process, namely urea synthesis, predominantly in upstream, oxygen-rich regions of the liver lobule (34).

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