Doxorubicin Toxicity in Perfused Rat Heart
Decreased Cell Death at Low Oxygen Tension

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The purpose of these studies was to test whether O₂ tension influences cardiotoxicity due to doxorubicin. Isolated hearts were perfused by the method of Langendorff at constant pressure with Krebs-Henseleit buffer saturated with 5% CO₂ and either 95% or 20% O₂. Toxicity due to doxorubicin was evaluated from changes in heart rate and from uptake of trypan blue by nonviable nuclei. Heart rate was stable in control hearts perfused at 95% O₂ for 30 minutes but decreased in hearts perfused at 20% O₂. Doxorubicin (30 μM) increased O₂ uptake due to redox cycling by −75 μmol/g/hr at 95% O₂ but had no effect in hearts perfused at 20% O₂. Heart rate decreased during 30 minutes of perfusion with doxorubicin and 95% or 20% O₂, with greater decreases occurring with 95% than 20% O₂ compared with values for the respective untreated controls. Irreversible cell damage indexed from uptake of vital dye due to doxorubicin was threefold greater than control at 95% O₂ but was not different from control at 20% O₂. These data are consistent with the hypothesis that O₂ tension is an important determinant of toxicity due to doxorubicin. (Circulation Research 1991;68:1610–1613)

Doxorubicin is an important antineoplastic agent used widely in the treatment of soft tissue sarcomas, yet its usefulness is limited by cardiotoxicity. Symptoms of acute cardiotoxicity include decreases in ejection fraction and alterations in the electrocardiogram, whereas chronic toxicity is associated with congestive heart failure and cardiomyopathy. The mechanisms of toxicity are not clearly understood, although production of damaging free radicals due to redox cycling of the quinone nucleus has been suggested.

Redox cycling requires oxygen and a supply of reducing equivalents. Recently, we demonstrated that doxorubicin damages oxygen-rich perportal areas of the liver lobule without affecting oxygen-poor pericentral areas. In addition, reduction of inflow oxygen tension prevented hepatic damage, indicating that toxicity was dependent on oxygen tension. Since changes associated with acute doxorubicin toxicity may ultimately lead to cardiomyopathy, we have investigated the effect of oxygen tension on toxicity due to doxorubicin in perfused heart. In this study, hearts were perfused with doxorubicin at 95% and 20% O₂, and parameters of cardiac function, oxygen uptake, and cell death were examined.

Materials and Methods

Animals and Perfusion of Isolated Hearts

Sprague-Dawley rats (350–600 g, Charles River Breeding Co., Waltham, Mass.) were fasted overnight before use. Before perfusion, the rats received heparin (500 units i.p.) and were anesthetized lightly with ether. The heart and great vessels were excised rapidly and placed in ice-cold 0.9% NaCl solution. The aorta was attached to a Langendorff perfusion apparatus, and hearts were perfused at constant pressure (85 mm Hg) with Krebs-Henseleit bicarbonate buffer, pH 7.4, as described previously. In some experiments, hearts were arrested by perfusion with 16 mM KCl with a commensurate decrease in NaCl to maintain constant osmolarity. The perfusate was equilibrated with either 95% O₂–5% CO₂ or 20% O₂–75% N₂–5% CO₂ and was warmed to 37°C in a water-jacketed column. Doxorubicin was dissolved directly in Krebs-Henseleit buffer.

A small, compliant balloon-tipped catheter was placed in the left ventricle via the mitral valve and was filled with saline to produce a left ventricular end-diastolic pressure of 6 mm Hg. The peripheral end of the catheter was attached to a transducer (Gould, Cleveland, Ohio) and recorder (model 440,
Gould) to monitor heart rate, left ventricular systolic pressure, and dP/dt continuously.

Measurement of Oxygen Uptake

Oxygen tension was measured continuously in the influent and effluent perfusate with Clark-type oxygen electrodes. Flow rate (coronary flow) was measured using a graduated cylinder, and oxygen uptake was calculated from the difference in influent and effluent concentrations, the coronary flow rate, and heart weight.

Uptake of Trypan Blue

At the end of each perfusion, 20 ml trypan blue (0.2 mM in Krebs-Henseleit buffer) was infused through the aorta, followed by 100 ml Krebs-Henseleit buffer. Hearts were fixed in 1% paraformaldehyde in Krebs-Henseleit buffer and then processed for light microscopy. Two serial sections were cut from each tissue. One section was stained with eosin only so that irreversibly damaged cells could be identified from dark trypan blue–stained nuclei. The other section was stained with hematoxylin and eosin for determination of the total number of nuclei in a given field. For each heart, four areas of tissue were examined, and the percentage of trypan blue–stained nuclei was determined.

Statistical Analysis

Values are presented as mean±SEM. Data were analyzed by analysis of variance, and individual comparisons were made using the least significant difference test. The criterion for significance was \( p < 0.05 \).

Results

Oxygen Uptake Due to Doxorubicin in Beating Hearts

Doxorubicin (30 \( \mu \)M) increased oxygen uptake within 1 minute of addition from 140±16 to 215±42 \( \mu \)mol/g/hr in hearts perfused at 95% \( \text{O}_2 \) (Figure 1A). When the oxygen concentration was lowered from 95% to 20% \( \text{O}_2 \), oxygen uptake declined from 125 to 108±16 \( \mu \)mol/g/hr (Figure 1B). On perfusion with doxorubicin, oxygen uptake did not increase, as observed at 95% \( \text{O}_2 \) but continued to decrease, reaching values of ~35 \( \mu \)mol/g/hr by 30 minutes (Figure 1B). The increase in \( \text{O}_2 \) uptake due to doxorubicin observed in beating hearts perfused with 95% \( \text{O}_2 \) (+75±34 \( \mu \)mol/g/hr) was significantly different \( (p<0.05) \) from the change in \( \text{O}_2 \) uptake in hearts perfused with doxorubicin at 20% \( \text{O}_2 \) (-7±5 \( \mu \)mol/g/hr). In control hearts not exposed to doxorubicin, during perfusion at 95% \( \text{O}_2 \), basal rates of oxygen uptake were stable for 40 minutes at values of ~150 \( \mu \)mol/g/hr, but at 20% \( \text{O}_2 \), basal rates decreased steadily from ~150 to ~75 \( \mu \)mol/g/hr (data not shown).

The concentration–response curve for stimulation of oxygen uptake due to doxorubicin at 95% \( \text{O}_2 \) was very steep. Concentrations as low as 1 \( \mu \)M and as high as 30 \( \mu \)M produced similar increases in \( \text{O}_2 \) uptake (data not shown). On the other hand, cell death due to doxorubicin, determined as the percentage of cells with trypan blue–stained nuclei, was concentration dependent, with maximal cell death observed at 30 \( \mu \)M and half-maximal effects at ~10 \( \mu \)M doxorubicin (data not shown). Subsequent stud-
doxorubicin

squares) of 1

**Figure 2.** Graphs showing heart rate during perfusion with doxorubicin (Dox) at 95% (panel A) and 20% (panel B) oxygen. Hearts were perfused as described in the legend to Figure 1 in the absence (filled circles) or presence (filled squares) of 30 μM Dox. Heart rate was monitored as described in “Materials and Methods.” Values are mean±SEM (representative). For control hearts perfused with 95% or 20% O2, n=4 per group; for hearts perfused with Dox and 95% O2, n=5; for hearts perfused with Dox and 20% O2, n=6. *Significantly different from the control value at the same O2 tension (p<0.05).

Doxorubicin-Induced Alterations in Cardiac Function

During perfusion at 95% O2, control heart rate was constant at ~300 beats/min for 40 minutes (Figure 2A). As expected,9 heart rate decreased during perfusion at 20% O2 from 280 to ~180 beats/min over the first 20 minutes (Figure 2B). Perfusion with doxorubicin at 95% O2 caused heart rate to decline from ~290 to ~190 beats/min (Figure 2A). Values were significantly different from control after 20 minutes of perfusion with doxorubicin. At 20% O2, heart rate also decreased during perfusion with doxorubicin, from 230 to 120 beats/min, but values were not significantly different from those of control hearts (Figure 2B). After 30 minutes of perfusion with doxorubicin, heart rate was lower in hearts perfused at 20% (110 beats/min) than at 95% O2 (200 beats/min). A similar pattern of changes was observed in other parameters of cardiac function (left ventricular systolic pressure and dp/dt, data not shown). Coronary flow was not affected by perfusion with doxorubicin at 95% O2, whereas at 20% O2, coronary flow initially increased and then decreased at a similar rate in hearts perfused in the absence or presence of doxorubicin.

Irreversible Cell Injury Due to Doxorubicin

In hearts not exposed to doxorubicin, uptake of trypan blue was minimal: only 10–15% of cells were stained after 40 minutes of perfusion at 95% or 20% O2 (Table 1). The percentage of stained nuclei was increased threefold by doxorubicin at 95% O2 but was not affected at 20% O2.

**Table 1.** Trypan Blue Uptake Due to Doxorubicin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stained nuclei (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beating heart</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11±4</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>34±5</td>
</tr>
<tr>
<td>Arrested heart</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14±1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>35±8</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Hearts were perfused in the absence or presence of doxorubicin as described in the legend to Figure 1. After perfusion, 20 ml trypan blue (0.2 mM) was infused through the aorta, followed by 100 ml Krebs-Henseleit buffer. The hearts were fixed in 1% paraformaldehyde and processed for light microscopy as described in “Materials and Methods.” The percentage of stained nuclei was determined by comparison of the number of trypan blue–stained nuclei in a field on a section that was stained with eosin only with the total number of nuclei in the same field on a section that was stained with hematoxylin and eosin as described in “Materials and Methods.” Four fields were examined for each heart. For beating hearts, n=4 for controls at 95% or 20% O2, n=5 for doxorubicin at 95% O2, and n=6 for doxorubicin at 20% O2. For arrested hearts, n=4 for all groups.

*Significantly different (p<0.05) from control hearts perfused at 95% O2.

**Effect of Doxorubicin in KCl-Arrested Hearts**

To test whether oxygen uptake was not stimulated by doxorubicin at 20% O2 simply because the heart was already extracting all of the oxygen being delivered, oxygen uptake was examined in KCl-arrested hearts. Basal rates of oxygen uptake were reduced by approximately one order of magnitude in arrested hearts (values at 10 minutes in Figures 1C and 1D). Under these conditions, the increase in oxygen uptake due to doxorubicin was significantly greater (p<0.05) at 95% O2 (72±26 μmol/g/hr, Figure 1C) than at 20% O2 (3±4 μmol/g/hr, Figure 1B). As in beating hearts, uptake of trypan blue was significantly greater at 95% than at 20% O2 in arrested hearts: 35% and 13% of cells were stained, respectively (Table 1).

**Discussion**

In this study, basal values for cardiac function in hearts perfused at high O2 tension were in the normal range for all parameters evaluated.8 Basal rates of oxygen uptake (Figure 1) were also in the range of values reported previously for perfused heart.10 Lowering the oxygen tension from 95% to 20% suppressed the heart rate (Figure 2), as expected.9

Impaired contractility due to doxorubicin has been reported previously in perfused rat hearts.11 In the present study, doxorubicin decreased heart rate during perfusion with 95% O2 (Figure 2), and decreases
in left ventricular systolic pressure and dP/dt were also observed. In addition, coronary flow declined, a result consistent with increased coronary resistance observed in isolated rat hearts perfused with doxorubicin. Impairment of heart rate, left ventricular systolic pressure, dP/dt, and coronary flow was also observed in hearts perfused with doxorubicin at 20% \( \text{O}_2 \); however, these changes are difficult to interpret in view of the decrease in cardiac function observed in control hearts perfused at 20% \( \text{O}_2 \). When compared with the appropriate control, doxorubicin caused a greater decrease in heart rate at 95% than at 20% \( \text{O}_2 \). Despite the depression of cardiac function in hearts perfused at 20% \( \text{O}_2 \), cell death in these groups was not significantly different from control hearts perfused at 95% \( \text{O}_2 \) (Table 1). Thus, in the perfused heart, aberrations in cardiac function may not be good indicators of irreversible cell injury due to doxorubicin.

Doxorubicin stimulated oxygen uptake and caused cell death in hearts perfused at 95% \( \text{O}_2 \) (Figure 1 and Table 1). The increase in oxygen uptake was assumed to be due to redox cycling and subsequent reactions such as lipid peroxidation, because it is well known that doxorubicin undergoes redox cycling, producing superoxide anions and hydroxyl radicals, an oxygen-consuming process. In submitochondrial particles, production of superoxide anion due to doxorubicin occurred at the same rate as oxygen uptake. Further support for this idea is provided by the observation that antioxidants or radical scavengers inhibit oxygen uptake due to quinone-containing drugs in the presence of purified enzymes that catalyze redox cycling.

In summary, the stimulation of oxygen uptake and toxicity due to doxorubicin were greater at 95% than at 20% \( \text{O}_2 \). Thus, oxygen tension is an important determinant of cardiac toxicity due to doxorubicin.

References
7. Ganey PE, Kauffman FC, Thurman RG: Regulation of oxygen-dependent hepatotoxicity due to doxorubicin: Role of reducing equivalent supply in perfused rat liver. Mol Pharmacol 1988;34:695–701

Key Words: doxorubicin • oxygen tension • cardiac toxicity