Cancer Chemother Pharmacol (1993) 33: 191–196

Cancer Chemotherapy and Pharmacology

The role of reduced nicotinamide adenine dinucleotide phosphate in glucose- and temperature-dependent doxorubicin cytotoxicity

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Received 18 January 1993/Accepted 17 August 1993

Abstract. The mechanism of doxorubicin resistance induced by glucose deprivation was examined using an L929 cell system. Resistance developed even when the synthesis of glucose-regulated proteins was suppressed by supplementing glucose-deprived cultures with uridine. Resistance was also not correlated with pyruvate availability, with DNA strand breaks, or with intracellular drug or nucleotide levels. However, intracellular concentrations of reduced nicotinamide adenine dinucleotide phosphate (NADPH) decreased to undetectable levels in glucose-deprived cells with or without uridine supplementation. NADPH depletion induced by treating glucose-fed cells with low concentrations of methylene blue afforded the same degree of protection as glucose deprivation, and normal sensitivity could be restored to glucose-deprived cells by adding NADPH to the culture medium. These results suggest that decreased NADPH availability is responsible for the doxorubicin resistance induced by glucose deprivation. Although drug uptake and NADPH production increased with temperature, these effects could not fully account for the >1000-fold decrease in clonogenic survival observed over the 25°–37° C temperature range. Similarly, manipulation of NADPH levels confirmed a role for drug bioreduction in the cytotoxic mechanism but did not suggest that NADPH availability was rate-limiting for this process at any temperature employed.

Introduction

The widespread use of doxorubicin in cancer chemotherapy has stimulated intensive investigation of the mechanism by which this drug and other anthracyclines exert their cytotoxic effects. The chemical events elucidated thus far include DNA binding leading to blocked transcription [2], topoisomerase II-mediated DNA strand breaks [29], and redox cycling leading to free-radical generation [3, 8, 11, 26]. The rate of drug extrusion [13] and other less well-defined processes [7, 22] have also been shown to modulate anthracycline cytotoxicity. Doxorubicin resistance induced by glucose deprivation has been correlated with decreased glycoprotein synthesis [24] and with decreased topoisomerase II levels [25]. However, considering the large number of processes known to affect cytotoxicity, it is conceivable that additional mechanisms contribute to glucose-dependent resistance.

Doxorubicin cytotoxicity is also highly temperature-dependent. Clonogenic survival is increased, i.e., cytotoxicity is decreased, if cultured cells are exposed to the drug at temperatures below 30° C [28], and survival is dramatically decreased if drug exposure is performed at temperatures above 37° C [5, 10]. Understanding this phenomenon has been difficult because different mechanisms have been proposed to account for the changes in the drug's cytotoxicity at low versus high temperatures. Its increased cytotoxicity for CHO cells at high temperatures has been attributed entirely to increased uptake of the drug [6, 23]. Other workers have concluded that changes in uptake and distribution cannot account for its decreased cytotoxicity for L1210 cells at low temperatures and have proposed a mechanism that prevents DNA damage under such conditions [28].

Therefore, we examined the mechanism of doxorubicin cytotoxicity over a wide temperature range, taking advantage of our observation [17] that nucleotide supplementation reverses all of the known effects of glucose deprivation on L929-cell protein synthesis. We present evidence that decreased availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH) can account for resistance induced by glucose deprivation. Although the contributions of uptake and metabolism vary with temperature, these mechanisms do not appear to account fully for the temperature-dependent changes in cytotoxicity.

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Materials and methods

Reagents and chemicals. Doxorubicin was obtained from Farmitalia Carlo Erba (Milan, Italy); uridine was supplied by Sigma Chemical Co. (St. Louis, Mo.); methanol and acetonitrile were HPLC/Spectro-grade products of Alltech Associates, Inc. (Deerfield, Ill.); phosphoric acid (HPLC-grade) and methylene blue were obtained from Fisher Scientific (Fair Lawn, N.J.); and [1-^{14}C]-glucose and [6-^{14}C]-glucose were supplied by Amersham (1.0 μCi/ml).

Cells and culture conditions. Murine L929 cells (a permanent fibroblast-related line) were obtained from MA Bioproducts (Bethesda, Md.) and were routinely maintained in high-glucose Dulbecco's modified Eagle's minimum essential medium (DMEM) and 10% newborn calf serum as previously described [18]. This unsupplemented DMEM contained 25 m*M* glucose, 4 m*M* glutamine, and 1 m*M* pyruvate. Cells from the stock cultures were plated in 35-mm plastic tissue- culture dishes at a density of 1×10^5/cm^2. After 1–2 days, the monolayers had reached a final density of $2–3 \times 10^5$/cm^2. After cultures had been washed twice with Dulbecco's phosphate-buffered saline, 2 ml of the medium of interest was added and changed daily. Experiments designed to examine the effects of glucose deprivation utilized serum-free DMEM lacking glucose and pyruvate but containing 12 m*M* glutamine (GF-DMEM). This medium was supplemented with uridine from a neutralized concentrated stock solution to yield GFUS-DMEM. After maintenance for 3 days in these media with daily medium changes, cultures were exposed to the agent of interest for 2 h (unless indicated otherwise) by dilution directly into the maintenance medium. The desired temperature was maintained by placing cultures on an aluminum plate in an incubator as previously described [18]. Cell monolayers were rinsed with phosphate-buffered saline to remove extracellular agents, trypsinized, and plated for determination of clonogenic survival as previously described [18]. Although the figures show representative data, all experiments were performed two to four times with similar results.

Intracellular doxorubicin concentration. Following incubation with the drug (5 μg/ml for 1 h), cultures were washed several times with Dulbecco's phosphate-buffered saline and lysed in 0.3 *N* HCl-50% methanol, and the fluorescence intensity was measured in a fluorimeter using an excitation wavelength of 480 nm and an emission wavelength of 560 nm as described by Bachur et al. [4].

DNA strand breaks. Cellular DNA damage was assessed using an assay based on the enhancement of fluorescence after partial alkaline unwinding and binding of bisbenzamide [14]. F (the fraction of double-stranded DNA remaining after alkaline denaturation for 30 min) is inversely related to the extent of strand breakage. In these experiments, cells were exposed to doxorubicin for 4 h so as to obtain readily detectable extents of strand breakage.

Intracellular nucleotide concentrations. Monolayer cultures were maintained in 150-cm^2 glass petri dishes containing the media described above. After aspiration of the medium, cultures were quickly washed with saline, scraped into a small volume of 0.6 *N* $HClO_4$, and processed as described by Wynants and Van Belle [30]. Extracts were analyzed on a 250-mm × 4.6-mm reversed-phase column of Lichrosorb RP18 (Alltech) eluted as described by Wynants and Van Belle [30] using a Beckman model 165 high-performance liquid chromatography (HPLC) system. Reduced nucleotides [reduced nicotinamide adenine dinucleotide (NADH) and NADPH] were extracted and analyzed as described by Stocchi et al. [27]. Nicotinamide adenine dinucleotide phosphate (NADP) levels were considered to be too low for accurate determination. Nucleotides and related compounds were identified on the basis of the retention time and A_{254}/A_{280} ratio of standard compounds (all from Sigma).

Glucose metabolism. The glucose flux through the pentose shunt versus mitochondrial oxidative pathways was estimated as previously described [16]. Since the 6-carbon of glucose is removed only in the process of tricarboxylic acid cycle (TCA) oxidation, whereas the 1-carbon is removed in this step and in the pentose shunt as well, the difference (1-C)–(6-C), where each term denotes the rate of $^{14}CO_2$ production from the appropriately ^{14}C-labeled substrate, estimates the flux of glucose through the pentose shunt.

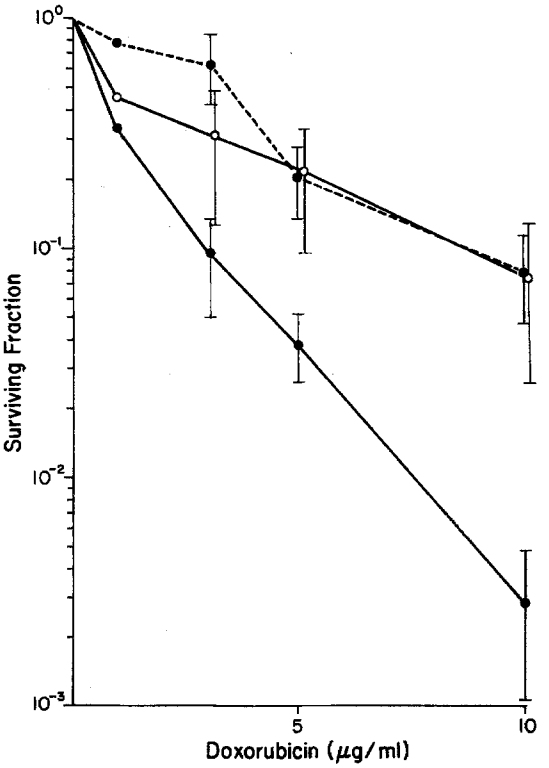

Fig. 1. Effect of medium supplementation on the survival of L929 cells following doxorubicin exposure. Cultures were maintained for 72 h in DMEM (●—●) or in GF-DMEM that was unsupplemented (●- - -●) or supplemented with 1 m*M* uridine (○—○). Data points represent the mean cloning efficiencies (±SD) of duplicate drug-treated cultures relative to the corresponding controls. Absolute values of duplicates differed by 20% or less

Results

Figure 1 shows that L929 cells were sensitive to doxorubicin over the dose range employed. Most of the cells rendered incapable of division by doxorubicin treatment persisted in the cultures as giant cells with a single large nucleus [19]. As described by Shen et al. [24] for Chinese hamster cells, glucose deprivation increased the clonogenic survival of L929 cells. Uridine supplementation did not restore doxorubicin sensitivity to the level characteristic of glucose-fed cells, i.e., nucleoside supplementation did not substitute for glucose in this regard. Supplementing cultures with 10 m*M* pyruvate or 12 m*M* glutamine had no effect on resistance to doxorubicin cytotoxicity (data not shown). Nevertheless, as previously reported, the protein-synthesis patterns of uridine-supplemented cultures were indistinguishable from those of the glucose-fed controls [17], whereas pyruvate did not suppress the induction of glucose-regulated protein synthesis [18].

Table 1. Effect of uridine supplementation on doxorubicin uptake by glucose-deprived L929 cells

Glucose deprivation (days)	Uridine supplement (1 m*M*)	Doxorubicin uptake (μg/mg protein)[a]
0	–	1.16±0.06
2	–	0.57±0.13
2	+	0.99±0.15

[a] Mean values ± SD of four determinations

Table 2. DNA damage in L929 cells

Medium	F[a]	
	Control	Doxorubicin (5 μg/ml)
DMEM	0.44±0.01	0.31±0.03
GF-DMEM	0.24±0.06	0.21±0.08
GFUS-DMEM	0.41±0.14	0.30±0.07

GF-DMEM, Glucose-free DMEM; GFUS-DMEM, glucose-free uridine supplemented

[a] Mean values ± SD of four determinations

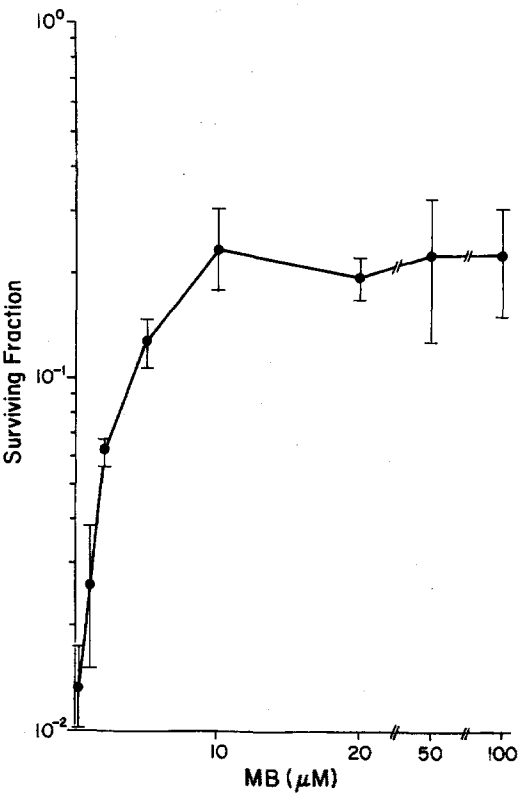

Fig. 2. Effect of methylene blue on doxorubicin cytotoxicity. Monolayer L929 cell cultures were maintained for 2 days in serum-free DMEM, simultaneously exposed to doxorubicin (5 μg/ml) and the indicated concentration of methylene blue for 1 h, washed, and trypsinized for determination of clonogenic survival

Intracellular levels of doxorubicin taken up under the various conditions of medium supplementation were determined directly. Table 1 shows that intracellular drug levels did not significantly differ in cultures maintained in DMEM versus GFUS-DMEM. Drug levels were lower in cultures maintained in GF-DMEM, but resistance was not increased beyond that observed for cultures maintained in GFUS-DMEM.

As found in many other studies, doxorubicin exposure resulted in extensive DNA damage as evidenced by increased susceptibility to alkaline denaturation (Table 2). However, the extent of strand breaks detected by this method was virtually identical when glucose-deprived, nucleoside-supplemented cultures were compared with controls maintained in DMEM. The extent of strand breakage was increased when cultures were maintained in GF-DMEM, probably reflecting cell death in the controls prior to drug exposure.

Analysis of intracellular nucleotides (Table 3) showed that levels fell markedly with glucose deprivation. Uridine supplementation maintained the intracellular triphosphates at levels slightly exceeding those in the glucose-fed controls, whereas dinucleotide levels were maintained to a lesser extent. Determination of reduced nucleotide levels (Table 4) showed that uridine supplementation partially maintained nicotinamide adenine dinucleotide (NAD) and NADH levels following glucose deprivation but that NADPH values fell below the limit of detection.

The hypothesis that glucose deprivation decreased doxorubicin cytotoxicity by abrogating an NADPH-dependent process was tested directly. The use of methylene blue treatment to deplete intracellular NADPH is expected to decrease doxorubicin cytotoxicity [1]. In our system, exceedingly low dye concentrations were effective in render-

Table 3. Effect of uridine supplementation on nucleotide levels in glucose-deprived L929 cells

Glucose deprivation (days)	Uridine supplement (1 m*M*)	Nucleotide level (nmol/mg protein)[a]				
		UTP	UDP	GTP	ATP	ADP
0	–	2.6±0.3	5.1±0.7	0.6±0.1	5.7±0.8	1.8±0.2
1	–	1.0±0.1	2.5±0.1	ND	4.0±0.4	1.3±0.3
2	–	0.2±0.3	0.6±0.2	ND	2.4±0.3	1.1±0.1
2	+	3.5±0.6	1.7±0.7	0.9±0.2	6.7±0.8	0.7±0.1
3	–	ND	0.4±0.1	ND	1.2±0.1	2.4±0.2

UTP, Uridine triphosphate; UDP, uridine diphosphate, GTP, guanosine triphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; ND, below the limit of detection (i.e., <0.5 nmol/mg protein)

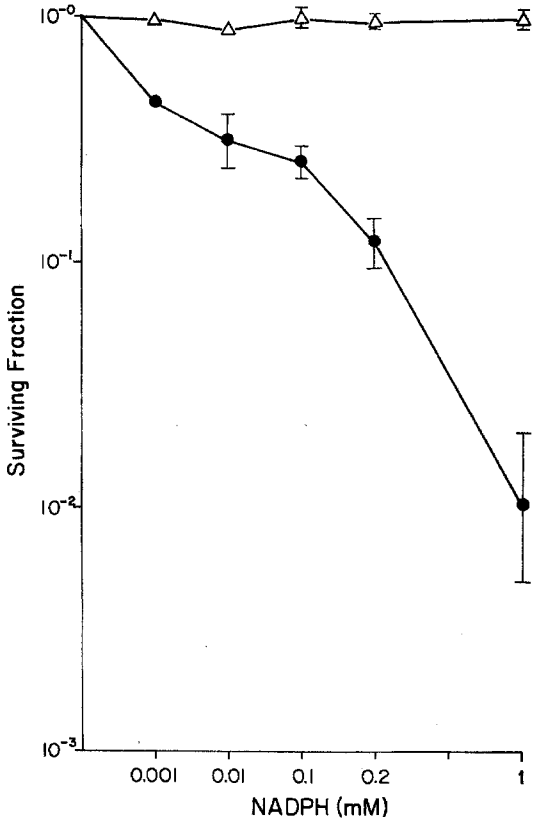

Fig. 3. Effect of NADPH on doxorubicin cytotoxicity. Cultures were maintained for 3 days in GFUS-DMEM, exposed simultaneously to doxorubicin (5 μg/ml) and the indicated concentration of NADPH for 3 h (●—●), washed, and trypsinized for determination of clonogenic survival. Control cultures (△—△) were exposed to NADPH in the absence of drug

Table 4. Effect of uridine supplementation on pyridine nucleotide levels in glucose-deprived L929 cells

Medium	Nucleotide level (nmol/mg protein)[a]			
	NAD	NADH	NADP	NADPH
DMEM	33.3±1.1	10.4±1.1	5.8±0.9	2.3±0.1
+doxorubicin (5 μg/ml)	25.9±7.5	6.1±1.8	4.7±0.5	1.2±0.3
GF-DMEM	1.7±0.5	1.2±0.7	ND	ND
GFUS-DMEM	13.1±5.7	4.7±1.7	1.4±0.5	ND

NADP, Nicotinamide adenine dinucleotide phosphate; ND, below the limit of detection (i.e., <0.5 nmol/g protein)

[a] Data represent mean values ± SD of four determinations

ing glucose-fed cells resistant to doxorubicin (Fig. 2). The maximal level of resistance was essentially the same as that produced by glucose deprivation. Adding NADPH to the medium of glucose-deprived cultures (Fig. 3) increased doxorubicin cytotoxicity in a dose-dependent manner, reaching a maximal level of sensitivity approximating that observed using glucose-fed cells.

Table 5. Temperature dependence of doxorubicin cytotoxicity and uptake

Temperature (°C)	Surviving fraction (%)[a]		Uptake in DMEM (μg mg protein^{-1} h^{-1})
	DMEM	GFUS-DMEM	
25	16 ±5	82 ±17	0.80±0.03
37	1.4±0.3	9.6± 1.5	1.2 ±0.1
42	4.3±1.4 × 10^{-3}	3.2± 2.2×10^{-2}	1.3 ±0.1

[a] Data represent mean values ± SE of 3–4 determinations following incubation for 2 h with doxorubicin (5 μg/ml)

Table 6. Temperature dependence of glucose oxidation by L929 cells

Temperature (°C)	Glucose oxidized (nmol mg protein^{-1} h^{-1})[a]	
	Pentose shunt	Mitochondrial
25	4.3± 0.8	1.4±0.7
+DOX	5.4± 1.4	1.7±0.4
37	42 ±13	13 ±0.5
+DOX	52 ±17	12 ±0.4
42	59 ±20	19 ±2
+DOX	59 ±11	19 ±2

DOX, Doxorubicin

[a] Mean values ± SE of four determinations

Table 5 shows that doxorubicin cytotoxicity was highly temperature-dependent in the L929 cell system, increasing by a factor of >10^3 over the span of 25°–42° C. In contrast, the increase in drug uptake over this temperature span was relatively small, i.e., about 60%. Since Fig. 1 shows that increasing the extracellular drug concentration by this amount would decrease survival by a factor of only 2–3, increased uptake cannot account for a large fraction of the temperature-dependent cytotoxicity observed in our system.

The role of reductive drug metabolism was evaluated by first asking whether NADPH production was temperature-dependent. Since NADPH is an obligatory product of pentose-shunt oxidation, NADPH production should be proportional to the flux through this pathway. Table 6 shows that glucose oxidation via the pentose shunt increased more than 10-fold over the 25°–42° C span, with the bulk of the increase occurring at between 25° and 37° C. This indicates that NADPH production is temperature-dependent.

The question as to whether NADPH availability limits drug reduction at low temperatures was approached by adding 1 m*M* NADPH to cultures maintained in GFUS-DMEM during drug exposure. This protocol enhanced the toxicity by a factor of only 2.4 and can be compared with the 100-fold increase observed when similar cultures were supplemented with NADPH at 37° C. The inference that NADPH availability is not responsible for temperature-dependent cytotoxicity is supported by the failure of doxorubicin to stimulate pentose-shunt oxidation (Table 6), as would be expected if NADPH availability were limiting the rate of drug reduction [31].

Discussion

In the course of examining the mechanism by which glucose deprivation protects cells against doxorubicin cytotoxicity, we found that intracellular drug levels, the extent of DNA damage, glucose-regulated protein induction, nucleoside triphosphate depletion, and pyruvate availability do not appear to play an important role. A number of other instances in which resistance is independent of intracellular drug levels have been described [5–7, 22], and in one thoroughly studied system [31], impaired NADPH-dependent free-radical formation has been implicated. Since cytoplasmic NADPH generation depends on the metabolism of glucose via the pentose shunt, glucose deprivation might decrease doxorubicin cytotoxicity by an NADPH-dependent mechanism.

Direct determination of intracellular NADPH concentrations showed that reduced nucleotide levels are, in fact, greatly decreased by glucose deprivation. This finding confirms our previous observation that glucose deprivation increases the pyruvate/malate ratio of L929 cells [20]. Because of coupling to NADP/NADPH by way of malic enzyme, this effect would be expected to depress NADPH levels. In addition, all of the cell lines that we have examined, including L929, synthesize large amounts of proline and release it into the culture medium [21]. Since this pathway proceeds via glutamate to pyrroline-5-carboxylic acid, which is reduced to proline in an NADPH-dependent reaction, NADPH is consumed in direct proportion to the amount of proline produced. Thus, the increased rate of glutamine metabolism observed in glucose-deprived cultures [15, 16] might be expected to deplete NADPH even further by this indirect mechanism.

NADPH depletion by treatment of glucose-fed cells with low concentrations of methylene blue afforded the same degree of protection as glucose deprivation, and NADPH supplementation markedly increased the cytotoxicity of doxorubicin in glucose-deprived cultures. These observations suggest that NADPH depletion is responsible for doxorubicin resistance in the glucose-deprived L929 system. Since NADPH-dependent drug reduction followed by oxidation and free-radical generation has long been postulated to be involved in cell killing by doxorubicin [3, 8, 11, 26], it is reasonable to suggest that glucose deprivation abrogates this mechanism by eliminating the major source of NADPH.

Thus, we conclude that an NADPH-dependent cytotoxic mechanism (probably free-radical generation) is important in the L929 system, accounting for essentially all of the increased killing caused by glucose feeding. Since NADPH could affect doxorubicin cytotoxicity as a reactant in both the formation and the detoxification of free radicals [31], the net effect of NADPH depletion depends on which process is rate-limited by physiological NADPH levels. The present study suggests that free-radical production is highly dependent on NADPH availability, whereas detoxification is less so.

Although drug uptake and metabolism do increase with temperature, these processes cannot account for the temperature-dependent cytotoxicity observed in the L929 cell system. Vichi et al. [28] also concluded that decreased uptake was not responsible for protection against DNA breaks at low temperatures, and our data suggest that mechanisms involving NADPH availability are not responsible for this phenomenon either. In the high temperature range, Rice and Hahn [23] attributed increased cytotoxicity entirely to increased uptake. More work is needed before a decision can be made as to whether different cell lines or different methodologies account for the relative importance of uptake vis à vis other mechanisms at temperatures of >37° C.

If our interpretation is correct, then the implications for cancer chemotherapy are significant. On one hand, the high rate of glucose metabolism characteristic of neoplastic cells may be partially responsible for their sensitivity to doxorubicin cytotoxicity. On the other hand, considering that extracellular glucose levels may be very low in some tumor regions [9], approaching zero at the boundary between viable and necrotic cells [12], resistance induced by NADPH depletion may be partially responsible for doxorubicin resistance observed clinically.

Acknowledgements. The technical assistance of V. Shah is greatly appreciated. This work was supported in part by grants from NIH (GM32725), the American Cancer Society (PDT-269), and the Research Foundation of SUNY.

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