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NAD DEPLETION AFTER *IN VITRO* EXPOSURE OF MURINE LUNG SLICES TO BLEOMYCIN

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Abstract—Bleomycin (BLM), a DNA-cleaving, antitumor antibiotic, causes pulmonary fibrosis. It also causes cell injury and activates the nuclear enzyme poly(ADP-ribose) polymerase (PAP; EC 2.4.2.30) in lung slices exposed to the drug *in vitro*. 3-Aminobenzamide (3-AB), a PAP inhibitor, prevents enzyme activation and cell injury. We have examined the potential role of ATP and NAD depletion in injury of BLM-sensitive C57Bl/6N and -resistant BALB/cN murine lung slices treated with BLM or deprived of glucose, the major metabolic substrate of lung. Lung slices either were treated for 45 min with injurious concentrations of BLM (10–500 μg/mL) or were incubated without glucose, in the presence or absence of 2.5 mM 3-AB. Only the highest concentration of BLM, 500 μg/mL, caused any ATP depletion, and this 35% decrease was transient, occurring at 220 min in C57Bl/6N slices. In contrast, glucose deprivation caused 50–70% ATP depletion in slices from both strains. BLM alone at 100 and 500 μg/mL caused a sustained 30–70% NAD depletion from 75 min through 400 min in C57Bl/6N mouse lung slices. In the resistant BALB/cN lung slices, NAD depletion by BLM was only seen at 400 min. 3-AB almost completely antagonized NAD depletion in slices from both strains. In contrast to BLM, glucose deprivation did not decrease NAD levels unless 3-AB was present in C57Bl/6N slices. Thus, ATP depletion may play a role in the injurious effects of glucose deprivation, but does not appear to be a major factor in pneumocyte injury caused by BLM. NAD depletion or other effects of PAP activation appear to account for the strain-selective, injurious effect of BLM on lung tissue.

Pulmonary fibrosis is caused by many environmental contaminants, therapeutic substances and ionizing radiation [1–3]. Pulmonary fibrosis can be life-threatening and limits the clinical application of the anticancer drug bleomycin (BLM†). BLM-induced pulmonary fibrosis develops after acute cell injury and cellular repair responses [4]. We have examined acute cell injury by direct exposure of isolated lung slices to fibrogenic agents. In this model system, murine sensitivity and resistance to direct cytotoxic effects of BLM correlate with the *in vivo* sensitivity to pulmonary fibrosis [5]. This finding suggests that critical factors governing strain variation *in vivo* are retained in lung slices, and emphasizes the role of acute cell injury in initiating BLM-induced fibrosis. The initial site of cellular injury with BLM and a number of other fibrogenic agents may be DNA [6]. Previously, we investigated BLM-induced DNA damage in mice and found that BLM causes an equivalent level of initial pulmonary DNA damage in both BLM-sensitive C57Bl/6N and -resistant BALB/cN mice, but that the rate of repair differs [7]. Thus, murine strain sensitivity to BLM may be influenced by the acute response of the lung to DNA damage.

One common response to eukaryotic DNA damage is the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PAP; EC 2.4.2.30) [8, 9]. PAP polymerizes ADP-ribose from NAD on nuclear proteins at free carboxyl groups [9]. We found that BLM activated PAP acutely in slices of lung from C57Bl/6N mice, but activation in BALB/cN lung slices was delayed by several hours [5]. An antagonist of PAP, 3-aminobenzamide (3-AB), prevented the activation of PAP and the cytotoxicity in lung tissue. These results suggest that strain differences in the activation of pulmonary PAP may contribute to the murine strain differences in direct toxicity of BLM to lungs.

In the present study, we examined the biochemical consequences of PAP activation. Extreme activation of PAP is usually associated with substantial depletion of NAD and cell death [10–12]. The importance of PAP activation and/or NAD depletion in lung injury is suggested by the observations that BLM activates PAP and depletes NAD *in vivo* and that niacin, an inhibitor of PAP and a precursor of NAD, can increase lung NAD and mitigate BLM-induced pulmonary fibrosis [13–15].

Using various treatments with cultured cells, other investigators have observed ATP loss coincident with or subsequent to NAD depletion caused by PAP activation [10–12, 16–19]. It was suggested that depletion of NAD led to depletion of ATP and cell death. Two groups, however, have found that inhibition of PAP prevents NAD depletion and cell injury caused by enzymatically generated oxygen radicals, but not the fall in ATP levels [20, 21]. In addition, some cytotoxic agents such as methyl methanesulfonate and menadione appear to induce NAD depletion without affecting ATP [21, 22]. A specific role for NAD in the maintenance of cell viability, independent of energy status, could explain

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† Abbreviations: BLM, bleomycin; PAP, poly(ADP-ribose) polymerase; 3-AB, 3-aminobenzamide; and LDH, lactate dehydrogenase.

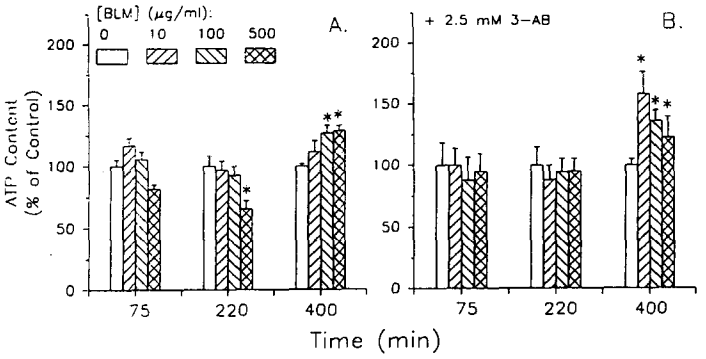

Fig. 1. Effect of BLM on the ATP content of C57Bl/6N mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Data are presented as mean per cent of control ± SEM; N = 3 for each treatment and time point. Key: (*) $P < 0.05$ when compared with control at each time point. Control values (mean ± SEM in nmol/mg protein) without 3-AB: 6.5 ± 0.29 at 75 min; 7.5 ± 0.52 at 220 min; 5.4 ± 0.08 at 400 min. Control values with 3-AB: 6.3 ± 0.33 at 75 min; 6.9 ± 0.85 at 220 min; 6.9 ± 0.29 at 400 min.

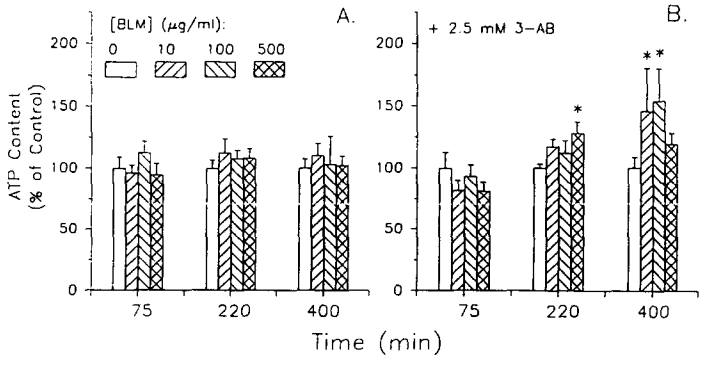

Fig. 2. Effect of BLM on the ATP content of BALB/cN mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Key: (*) $P < 0.05$ when compared with control at each time point. Control values (mean ± SEM in nmol/mg protein) without 3-AB: 6.5 ± 0.50 at 75 min; 7.6 ± 0,44 at 220 min; 6.4 ± 0.40 at 400 min. Control values with 3-AB: 6.7 ± 0.61 at 75 min; 7.0 ± 0.18 at 220 min; 6.3 ± 0.44 at 400 min.

these observations. These results indicate the importance of measuring NAD and ATP in BLM-treated lung slices.

Here we report the effects of BLM or glucose deprivation, in the presence and absence of 3-AB, on NAD and ATP content of lung slices from BLM-sensitive C57Bl/6N mice and BLM-resistant BALB/cN mice. Glucose deprivation was chosen as a positive control to deplete ATP because of the known dependence of the lung on glucose for intermediary metabolism and oxidative phosphorylation [23]. Furthermore, and in contrast to BLM, our previous results indicated that glucose deprivation causes extensive LDH release from lung slices of both strains of mice, which is not prevented by 3-AB [5].

MATERIALS AND METHODS

Lung slices and treatments. Three female mice (C57Bl/6N or BALB/cN; Charles River Laboratories, Kingston, NY) in each experimental group were anesthetized with sodium pentobarbital (100 mg/kg, i.p.). The lungs were perfused, removed, and sliced as described previously [5]. Slices were equilibrated (three washes × 5 min/wash) at room temperature in Krebs–Henseleit buffer (pH 7.2) with or without 4.5 g glucose (Sigma, St. Louis, MO) per liter (Krebs/glucose buffer). After the equilibration period, 6–8 slices (15–20 mg tissue) were transferred to a well in a 24-place culture plate containing 1.0 mL Krebs with or without glucose and shaken. The samples were preincubated at 37° for 15 min in 1.0 mL buffer with or without 2.5 mM 3-AB (Sigma), a concentration of 3-AB that is selective for PAP inhibition [24, 25].

Triplicate samples of lung slices were treated as described previously with 0–500 μg BLM (Blenoxane; Bristol Myers-Squibb, Wallingford, CT) per mL Krebs/glucose buffer (0.5 mL) with or without

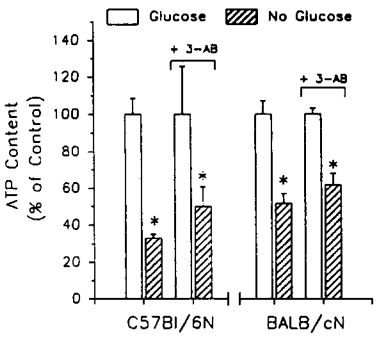

Fig. 3. Effect of glucose deprivation for 220 min on the ATP content of C57Bl/6N and BALB/cN mouse lung slices in the absence and presence of 2.5 mM 3-AB. Key: (*) $P < 0.05$ when compared with control at each time point. Control values in the presence of glucose (mean ± SEM in nmol/mg protein) in C57Bl/6N lung slices without 3-AB: 7.5 ± 0.52; with 3-AB: 6.9 ± 0.85. In BALB/cN lung slices without 3-AB: 7.6 ± 0.44; with 3-AB: 7.0 ± 0.18.

2.5 mM 3-AB for 45 min, a period equivalent to 4 half-lives of a fibrogenic i.v. dose of BLM *in vivo* in mice [26]. The slices were then washed three times and incubated in 1.0 mL BLM-free, Krebs/glucose buffer with or without 3-AB for the remainder of the experiment. A parallel experiment was performed using Krebs buffer without glucose as well. All slices were washed three times with Krebs buffer, with or without glucose at the end of incubation.

HPLC analysis of nucleotides. At appropriate times, slices were homogenized in 220 μl of phosphate-buffered saline at 4°. An aliquot (20 μL) was taken for protein assay [27]. Two hundred microliters of 20% trichloroacetic acid was added to the remaining sample, which was left on ice for 15 min and then centrifuged at 12,000 *g* for 5 min. The supernatant was extracted with 500 μL of 1,1,2-trichlorotrifluoroethane containing 0.5 M tri-*n*-octylamine (both from Sigma) [28]. The mixture was centrifuged at 12,000 *g* to separate the phases and the organic layer was discarded. Samples were further neutralized by the addition of 400 μL of 0.1 M K_2HPO_4 (pH = 9) and stored at −70° until used. Prior to injection, samples were extracted three times with 1,1,2-trichlorotrifluoroethane. Ten microliters of 500 μM nicotinamide (Sigma) was added to 100 μL of sample as an internal standard, and 100 μL was subjected to HPLC.

Sample and standard nucleotides (Sigma) were measured by reverse-phase HPLC by the method of Jones [29], employing a Waters U6K injector, two Waters 501 HPLC Pumps, a Whatman Partisil PXS C8 column (25 cm × 4.6 mm) and a Waters 994 Programmable Photodiode Array Detector. Waters Baseline 810 Software was used to control the gradient elution and for chromatogram analysis. Buffer A was 0.1 M potassium phosphate (pH 5.93) and Buffer B was Buffer A with 15% methanol. The flow rate was 3.0 mL/min. The gradient was 3 min Buffer A, switching to 66% Buffer B at 4 min.

Statistics. ANOVA was employed to determine the effects of treatments on nucleotide levels, normalized to protein content [30]. Differences were assumed significant at $P < 0.05$.

RESULTS

ATP. BLM did not cause ATP depletion in lung slices, except transiently at 220 min in C57Bl/6N lung slices treated with 500 μg/mL BLM (Figs. 1 and 2). ATP increased later at 400 min with 100 and 500 μg BLM/mL. In the presence of 3-AB, ATP was increased in BALB/cN slices at 220 min after 500 μg BLM/mL and at 400 min with exposure to 10 and 100 μg BLM/mL. In C57Bl/6 lung slices ATP was elevated at 400 min with 10, 100 and 500 μg BLM/mL in the presence of 3-AB.

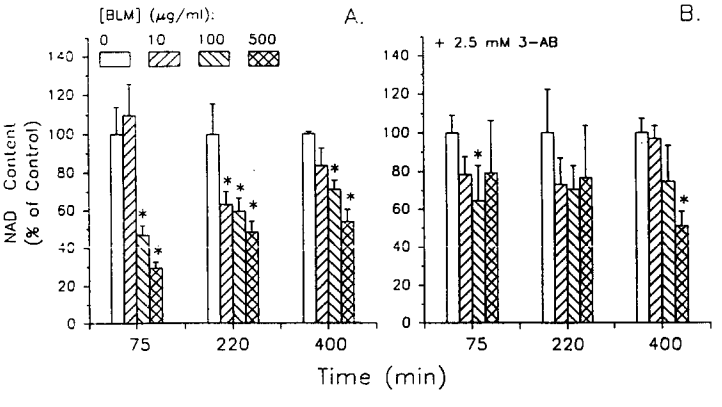

Fig. 4. Effect of BLM on the NAD content of C57Bl/6N mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Key: (*) $P < 0.05$ when compared with control at each time point. Control values (mean ± SEM in nmol/mg protein) without 3-AB: 2.8 ± 0.33 at 75 min; 2.8 ± 0.35 at 220 min; 5.4 ± 0.05 at 400 min. Control values with 3-AB: 3.6 ± 0.33 at 75 min; 3.2 ± 0.58 at 220 min; 3.6 ± 0.22 at 400 min.

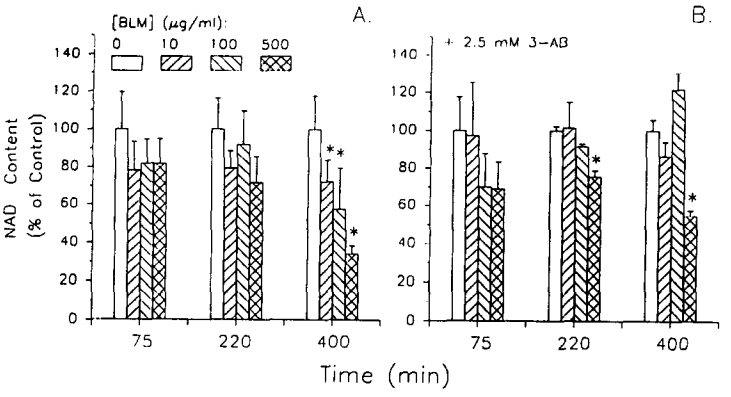

Fig. 5. Effect of BLM on the NAD content of BALB/cN mouse lung slices in the absence (A) and presence (B) of 2.5 mM 3-AB. Key: (*) $P < 0.05$ when compared with control at each time point. Control values (mean ± SEM in nmol/mg protein) without 3-AB: 3.4 ± 0.55 at 75 min; 3.4 ± 0.45 at 220 min; 5.6 ± 0.81 at 400 min. Control values with 3-AB: 2.8 ± 0.39 at 75 min; 1.8 ± 0.03 at 220 min; 3.8 ± 0.18 at 400 min.

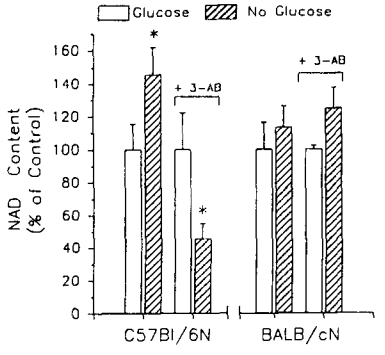

Fig. 6. Effect of glucose deprivation for 220 min on the NAD content of C57Bl/6N and BALB/cN mouse lung slices in the absence and presence of 2.5 mM 3-AB. Key: (*) $P < 0.05$ when compared with control at each time point. Control values in the presence of glucose (mean ± SEM in nmol/mg protein) in C57Bl/6N lung slices without 3-AB: 2.8 ± 0.35; with 3-AB: 3.2 ± 0.58. In BALB/cN lung slices without 3-AB: 3.4 ± 0.45; with 3-AB: 1.8 ± 0.03.

In contrast, glucose deprivation caused marked depletion of ATP in slices from both strains of mice at 220 min (33% of control for C57Bl/6N and 52% of control for BALB/cN; Fig. 3), and 3-AB did not prevent it. The effects of glucose deprivation are consistent with previous observations made by other investigators using cultured cells [31].

NAD. BLM caused marked NAD depletion in slices from C57Bl/6N mice (Fig. 4). The two highest concentrations of BLM, 100 and 500 μg/mL, depressed NAD at all time points, while the low concentration, 10 μg/mL, caused a transient effect at 220 min. The maximum decrease (70%) was seen with 500 μg BLM/mL at 75 min. Almost all of these decreases were blocked by 3-AB. BLM did not affect NAD in slices from BALB/cN mice until 400 min (Fig. 5). 3-AB antagonized the NAD depression at 400 min produced by 10 and 100 μg BLM/mL in BALB/cN lung slices, although a statistically significant reduction was seen with 500 μg BLM/ml at 220 and 400 min.

The effects of glucose deprivation were completely different from those of BLM (Fig. 6). Glucose deprivation enhanced NAD levels in C57Bl/6N lung slices at 220 min but had no effect in BALB/cN lung slices. 3-AB plus glucose deprivation resulted in loss of NAD in C57Bl/6N lung slices.

DISCUSSION

Early death of selected pulmonary cell types is a common morphological feature of drug-induced fibrosis [3]. *In vitro* lung slices provide a useful model to study acute pneumocyte injury associated with this disease. The viability of slices incubated with glucose is high, ranging from 95% at 75 min, to 90% at 220 min, and finally approaching 80% only after 500 min of incubation [5]. Effects of BLM occur within 75 min, both *in vivo* and in lung slices [5, 7] (Fig. 4).

We demonstrated previously that murine strain sensitivity to acute cell injury in isolated lung slices, measured by the release of lactate dehydrogenase (LDH), correlated with the sensitivity of the mice to pulmonary fibrosis. When C57Bl/6N, but not BALB/cN, mouse lung slices were exposed to 10–1000 μg BLM/mL, LDH release was enhanced in a concentration-related manner beginning at 360 min of incubation [5]. The maximum LDH release after 500 min incubation ranged from 35 to 45% of total LDH. Incubation in 3-AB blocked BLM-induced LDH release. These results contrast with the rapidity and effectiveness of glucose deprivation in causing LDH release from both C57Bl/6N and BALB/cN lung slices, where more than 70% was released at 300 min. 3-AB had no effect on the cytotoxicity of glucose deprivation. The efficacy of glucose

deprivation in causing LDH release relative to BLM may reflect a subpopulation of pneumocytes that are resistant to the cytotoxic action of BLM. The differences in LDH release may also reflect distinct mechanisms of injury caused by BLM exposure and glucose deprivation. For example, it seems likely that injury by BLM is mediated by DNA strand breakage [6, 7] whereas glucose deprivation may alter intermediary metabolism directly [23, 31].

Our previous results indicate that acute cell injury by BLM in sensitive lung slices involves rapid PAP activation [5]. Others have demonstrated the ability of activated PAP to consume NAD [9] and to deplete ATP in cultured cells [11, 12, 16–19]. Other studies, however, indicate that changes in NAD and ATP can be dissociated from each other after various injurious treatments in various cultured or isolated cells [20–22]. It was, therefore, important to determine whether BLM caused depletion of NAD or ATP in sensitive or resistant mouse lung slices and the temporal relationship between any changes and the onset of PAP activation and LDH release.

BLM caused marked NAD depletion in the BLM-sensitive C57Bl/6N lung slices throughout the 400-min incubation, which is prior to the loss of viability seen previously [5]. The lowest concentration of BLM caused a delayed and transient depletion of NAD. NAD loss was also delayed in the resistant BALB/cN lung slices even at high concentrations of BLM. This pattern is probably a function of the acute activation of PAP at 75 min in C57Bl/6N lung slices and delayed PAP activation at 400 min in BALB/cN lung slices [5]. 3-AB also partially antagonized the loss of NAD in slices from both strains. Prolonged suppression of NAD levels after the typically rapid, transient activation of PAP due to DNA damage in C57Bl/6N slices may be explained by conversion of PAP to non-polymerizing, NAD glycohydrolase [32–36]. The delayed loss of NAD seen in the low concentration group may also be related to this enzymatic conversion. We are currently assessing this possibility. BLM may activate other 3-AB-sensitive, NAD-metabolizing processes as well [33]. NAD depletion may occur by enhanced synthesis of NADP from NAD, as in hepatocytes treated with *t*-butyl hydroperoxide. This pathway, however, is completely insensitive to 3-AB [37]. Our results demonstrate that marked NAD depletion, sensitive to 3-AB, precedes BLM-induced injury to lung slices. NAD depletion may mediate the demise of BLM-treated lung cells. Alternatively, ADP-ribosylation of cellular components by PAP may alter cell function [9, 33] and contribute to injury.

ATP depletion was detected after treatment with the highest concentration of BLM in C57Bl/6N slices. This decrease could contribute to toxicity seen with high concentrations of the drug. The decrease was transient, however, and followed by an increase. Also, lower concentrations of BLM, which cause cell injury in lung slices, did not cause ATP depletion. Elevated ATP levels were seen late in 3-AB-treated slices from both strains and could contribute to protection by 3-AB.

In contrast to BLM, glucose deprivation caused substantial ATP depletion in slices from both BALB/cN and C57Bl/6N mouse lung. This result agrees with observations made in other systems where the supply of glucose was limited [31]. 3-AB did not block this effect, nor does 3-AB prevent LDH release in the absence of glucose [5]. Furthermore, glucose deprivation alone did not cause NAD depletion. One explanation of the results is that the injurious effect of glucose deprivation is due to loss of energy supply and ATP. Glucose deprivation may also stimulate oxidation of NADH, blunting any loss of NAD or actually increasing NAD levels (Fig. 6). The role of ATP depletion or potential NADH oxidation in the toxicity of glucose deprivation requires further investigation.

In conclusion, significant NAD depletion was caused by direct exposure of lung slices to BLM. The onset of these effects in sensitive lung slices was similar to the onset of PAP activation caused by 100 and 500 μg BLM/mL, but was delayed and transient after 10 μg/mL. Depletion of NAD occurred much earlier in slices from BLM-sensitive C57Bl/6N mice compared with those from BLM-resistant BALB/cN mice. NAD depletion was mitigated by 3-AB. In contrast, ATP depletion was detected only transiently in C57Bl/6N lung slices treated with 500 μg BLM/mL, whereas substantial ATP depletion was caused by glucose deprivation in slices from both murine strains. NAD depletion in response to BLM appeared to affect cell viability independently of energy status. PAP activation by BLM may influence cell viability by NAD depletion or ADP-ribosylation of critical substrates.

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