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EFFECTS OF BIS-CHLORONITROSOUREA (BCNU) ON PULMONARY AND SERUM ANGIOTENSIN CONVERTING ENZYME ACTIVITY IN RATS

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Abstract—The anticancer drug bis-chloronitrosourea (BCNU) causes potentially life-threatening lung injury in a high percentage of patients. Because changes in the activities of pulmonary and serum angiotensin converting enzyme (ACE) have been reported previously to reflect toxic pulmonary damage by certain agents, we have investigated the effects of BCNU on pulmonary and serum ACE of rats. *In vitro*, BCNU had a direct inhibitory effect on both serum ACE and pulmonary ACE. *In vivo*, a large single dose of BCNU (80 mg/kg, i.p.) did not alter pulmonary ACE nor cause histologically observable acute lung damage. However, serum ACE dropped by 25% within 1 hr after drug. A multi-dose regimen, consisting of 5 mg BCNU/kg once per week for 6 weeks, caused marked pulmonary injury, which continued to develop in severity over several weeks following the completion of dosing. Lung ACE after four doses (total of 20 mg BCNU/kg) was depressed by 40% and remained low until dosing was completed. Following the final dose, lung ACE returned to control within 2 weeks. However, after an additional 2 weeks both lung and serum ACE had decreased by 35 and 25% respectively. It appears that ACE may provide a useful biochemical monitor for BCNU-induced pulmonary toxicity, but careful attention must be given to the time-course of changes in the enzyme activity.

The antitumor activity of bis-chloronitrosourea (BCNU; Carmustine) has been found useful in combination with other chemotherapeutic agents in the treatment of a variety of malignancies and as the sole therapeutic agent in the treatment of brain tumors [1, 2]. In the past few years, there have been numerous reports linking BCNU administration with progressive pulmonary disease [3–6]. The pulmonary toxicity of BCNU is dependent upon the total cumulative dose of BCNU administered [7, 8] and it has been estimated that 10–30% of patients treated with high doses of BCNU (10–80 mg/kg) develop pulmonary toxicity [8]. Experimentally, BCNU has been found to cause pulmonary toxicity in F344 rats [9, 10].

Recently, it has been suggested that serum and pulmonary levels of angiotensin converting enzyme (ACE) may be useful for monitoring the pulmonary toxicity of certain xenobiotics [11–13]. ACE is believed to be located primarily on the outer membrane of capillary endothelial cells, and most ACE activity is found in pulmonary tissue [14]. The purpose of the present study was to examine the effects of BCNU, *in vitro* and *in vivo*, on pulmonary and serum ACE activities in the F344 rat.

MATERIALS AND METHODS

Animals. Male F344 rats (120–170 g) were used throughout these studies. BCNU was supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. For multiple dosing experiments, the appropriate amount of BCNU was dissolved in 1 vol. of ethanol and then diluted with 9 vol. of distilled water to give a final BCNU concentration of 0.5 mg/ml. For single-dose studies, in which larger doses of BCNU were used, it was necessary to administer BCNU in sesame oil vehicle (40 mg/ml) because of the limited solubility of the drug in other media. All animals were injected intraperitoneally with drug or the corresponding drug vehicle. Animals were killed by a pentobarbital overdose (60 mg/kg), and blood samples were taken by cardiac puncture. Lungs were perfused *in situ* with heparinized saline, removed, trimmed, weighed, and frozen at −70° until assayed.

Angiotensin converting enzyme assay. Serum ACE was measured by the method of Rohrbach [15] using [^{14}C-glycine]hippuryl-histidyl-leucine (New England Nuclear Corp., Boston, MA). Pulmonary ACE was determined in lung homogenates as described by Newman *et al.* [13]. The solubilization of the membrane-bound pulmonary ACE was accomplished by adding 0.05% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), a procedure that released 95% of the total particulate activity. The specificity of the substrate for ACE was determined by using the specific inhibitor, Captopril, in the assay. Virtually 99% of ACE activity was inhibited by 0.2 μM Captopril in both the lung and serum enzyme assays. Pulmonary ACE was normalized per mg protein as determined by the method described by Lowry *et al.* [16].

The *in vitro* effect of BCNU on ACE activity was

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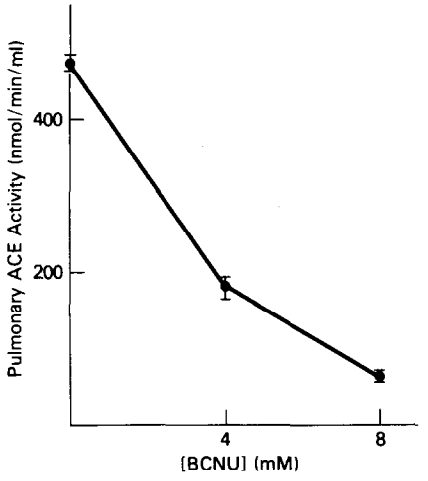

Fig. 1. Effect of BCNU on pulmonary ACE. Pulmonary homogenates (see Materials and Methods) were incubated with various concentrations of BCNU for 30 min before ACE activity was assayed. Values represent the mean ± S.E.M. of triplicate determinations.

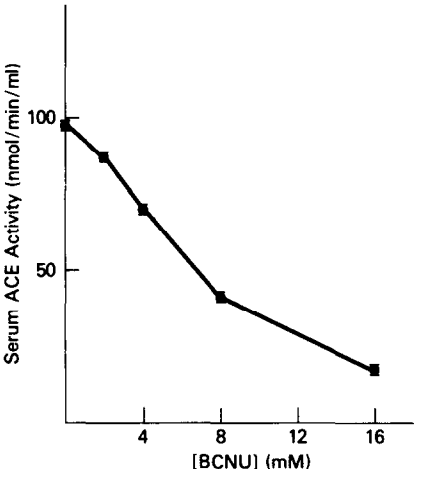

Fig. 3. Effect of BCNU concentration on serum ACE. Serum samples were incubated with various doses of BCNU for 30 min before ACE activity was determined. Values represent the mean ± S.E.M. of triplicate determinations.

determined by preincubating serum or solubilized lung homogenate with the drug at 37°. After the preincubation period, ACE activity was determined using the standard assay.

Statistics. A paired *t*-test was used to analyze the data presented.

RESULTS

BCNU inhibited both pulmonary and serum ACE *in vitro*. The pulmonary activity was decreased by 60% after incubation with 4 mM BCNU for 1 hr (Fig. 1). The serum enzyme activity was decreased by 57% after a 1-hr incubation with 4 mM BCNU. The degree of inhibition was shown to be dependent both upon the incubation time (Fig. 2) and the concentration of BCNU (Fig. 3).

Single doses of BCNU (up to 80 mg/kg) markedly decreased serum ACE activities (Fig. 4) but did not alter pulmonary ACE nor cause histologically observable acute pulmonary damage (detailed histopathological studies to be reported elsewhere). Serum ACE dropped to 75% of control within 1 hr after the administration of 80 mg BCNU/kg. The

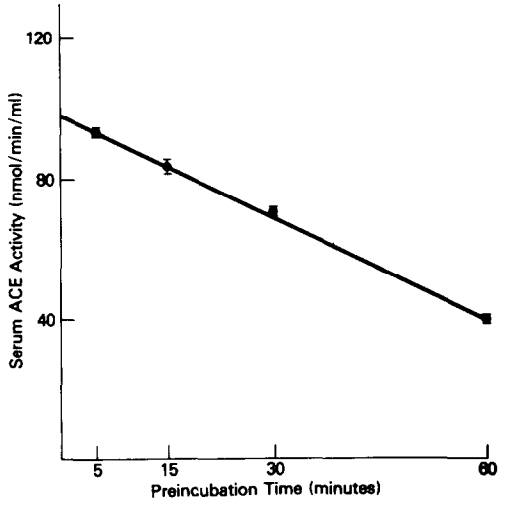

Fig. 2. Effect of incubation time on the inhibition of serum ACE by BCNU. Serum samples were incubated with 4 mM BCNU for the indicated time before ACE activity was determined. There was no change in ACE activity in samples incubated in the absence of BCNU over the 60 min incubation period. Values represent the mean ± S.E.M. of triplicate determinations.

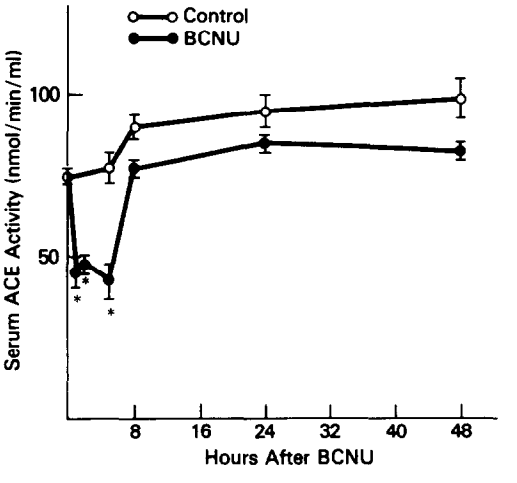

Fig. 4. Effect of a single dose of BCNU on serum ACE *in vivo*. Male F344 rats were treated with 80 mg/kg, i.p., BCNU or vehicle (sesame oil). Serum samples were collected at the indicated time after drug or vehicle administration. Values represent the mean ± S.E.M. of four to six animals. An asterisk indicates that the mean is statistically different from control values (paired *t*-test, $P < 0.05$).

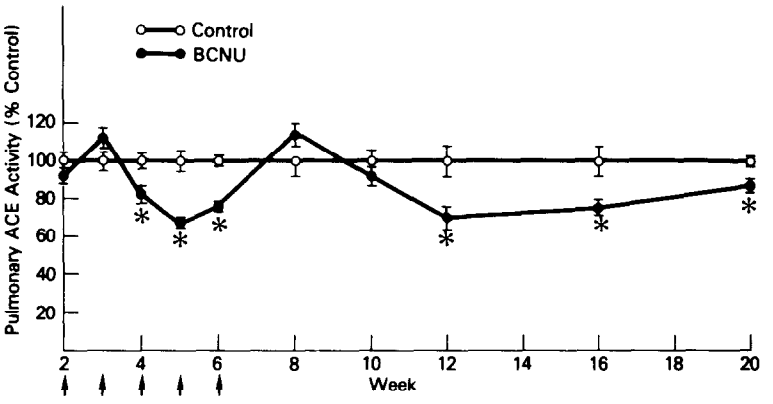

Fig. 5. Effect of multiple doses of BCNU on pulmonary ACE activity. Male F344 rats were treated with 5 mg BCNU per kg per week, i.p., for 6 weeks (dosing times indicated by ↑). During the dosing period, samples were taken 6 days after the last dose of BCNU. Pulmonary ACE was determined as described in Materials and Methods. Values represent the percent control ± S.E.M. of five to ten animals and represent data normalized per mg protein. An asterisk indicates that the mean is statistically different from control values (paired t-test, $P < 0.05$).

inhibition of serum ACE was relatively short lived with the activities returning to control by 24 hrs after the BCNU treatment.

In contrast to a single dose of BCNU, multiple doses of this drug caused marked pulmonary damage (to be described in detail elsewhere). A multi-dose regimen of 5 mg BCNU/kg once a week for 6 weeks resulted in massive pulmonary injury which developed over several weeks following the completion of dosing. There was no change in the lung weight of BCNU-treated rats; however, there was a slight (~70 g) decrease in net body weight gain in the BCNU group. Changes in serum and pulmonary ACE levels were followed during the dosing period (6 days after the last dose of BCNU) and for 14 weeks after the dosing was completed. Pulmonary ACE activity dropped to 60% of control values after a cumulative dose of 20 mg BCNU/kg (Fig. 5). This activity remained low until the administration of BCNU was completed, after which time the pulmonary ACE returned to control values within 2 weeks. A delayed drop in pulmonary ACE subsequently became apparent 6 weeks after the BCNU dosing period was completed; the activities then fell to 65% of control values and remained low through the end of the study.

Serum ACE, unlike pulmonary ACE, did not change during the BCNU dosing period (Fig. 6). However, within 4 weeks after BCNU dosing was completed, serum levels had dropped to 70% of control and remained low throughout the remainder of the study.

DISCUSSION

Angiotensin converting enzyme is presumed to be located on the outer membrane of endothelial cells, and most ACE activity is found in pulmonary tissue

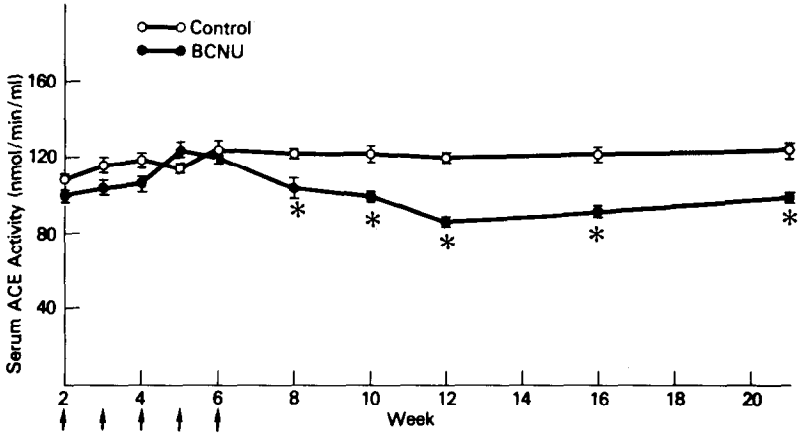

Fig. 6. Effect of multiple doses of BCNU on serum ACE activity. Male F344 rats were treated with 5 mg BCNU per kg per week, i.p., for 6 weeks (dosing times indicated by ↑). During the dosing period, samples were taken 6 days after the last BCNU injection. Serum samples were collected at the indicated times during the study. Values represent the mean ± S.E.M. of five to ten animals. An asterisk indicates that the mean is statistically different from control values (paired t-test, $P < 0.05$).

[14]. Recently, this enzyme has been used as a marker to study the lung damage caused by a number of pulmonary toxins, including thiourea [11], paraquat [12], bleomycin [13], and carbon tetrachloride [17]. Doses of these compounds, which cause acute pulmonary damage, typically cause a transient increase of ACE activity in lung lavage fluid [11, 13] and in serum [11–13, 17] levels while pulmonary tissue levels of the enzyme decrease [11–13]. The current view is that acute lung cell damage releases ACE into the serum, thereby presumably decreasing the overall pulmonary ACE activity while transiently increasing the serum ACE activity [11, 17].

Single doses of BCNU did not cause acute changes in pulmonary ACE *in vivo*, although the drug had a direct inhibitory effect on the lung enzyme *in vitro*. This was consistent with the apparent lack of any striking histological damage after single doses of BCNU. Interestingly, BCNU inhibited serum ACE activity both *in vitro* and *in vivo*. This inhibition seems relatively specific since other serum enzyme activities, lactate dehydrogenase and glutamate oxaloacetate transaminase, were unchanged after BCNU (unpublished observations). Others have reported that a large number of red blood cell enzymes are unaffected by large concentrations of BCNU [18]. However, red blood cell glutathione reductase is also markedly inhibited by small doses of BCNU [18–20]. It has been hypothesized that BCNU or one of its metabolites produces this inhibition of the reductase by binding to an essential sulfhydryl at the active site of the enzyme [20]. The direct inhibition of serum ACE by BCNU conceivably could occur by a similar mechanism.

Repetitive administration of low doses of certain pulmonary toxins are known also to affect pulmonary and serum ACE levels. For example, multiple doses of the anticancer drug, bleomycin, administered subcutaneously to rabbits, results in a 39% decrease in pulmonary ACE and 65% decrease in serum ACE [21]. A similar drop in lung ACE was described in rats treated with multiple doses of this agent [22]. On the other hand, one study reported that mice administered multiple doses of bleomycin had elevations of both serum and pulmonary ACE [23], while another study reported that both serum and pulmonary ACE levels in mice were decreased after multiple doses of the drug [24]. In contrast to the effects of multiple doses of bleomycin, single subcutaneous or intraperitoneal doses of bleomycin do not apparently result in any alteration of lung or serum ACE activity in rats or rabbits, nor does this treatment seem to cause any pulmonary damage [21, 22].

Thus, the effects of BCNU on lung and serum ACE were somewhat similar to those observed for other lung-toxic chemicals, especially bleomycin. Administration of multiple doses of BCNU to rats caused marked decreases of pulmonary and serum ACE. Pulmonary ACE activities were depressed during the BCNU treatment period, possibly reflecting an initial phase of pulmonary damage. After BCNU pretreatment was completed, lung ACE temporarily recovered but subsequently both the pulmonary and the serum ACE became depressed. At the time the late-occurring deficiencies in pulmonary and serum ACE were observed, the lungs showed severe histopathological changes. Thus, it appears that the effect of multiple doses of BCNU on pulmonary ACE is biphasic, the initial decrease possibly representing an early phase of reversible pulmonary injury and the delayed decrease possibly corresponding to a more chronic, irreversible form of lung damage. Further attempts at correlating these observed changes in ACE with different phases of lung injury must await a more detailed characterization of the histopathogenesis. At present, however, we can conclude from these studies that ACE may provide a useful way to biochemically monitor BCNU-induced pulmonary toxicity, but careful attention must be given to the time-course of changes in the enzyme activity.

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