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HEME METABOLISM IN LIVER AND SPLEEN OF 1-(2-CHLOROETHYL)-3-CYCLOHEXYL-1-NITROSOUREA (CCNU)-TREATED RATS*

ROKEA A. EL-AZHARY and AHMED E. AHMED†

Departments of Pathology, Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

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Abstract—The effect of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), an anticancer alkylating agent of the nitrosourea group, on liver and spleen enzymes involved in the control of heme metabolism was studied. A single oral dose of 50 or 100 mg/kg CCNU caused a time-dependent loss in weight of both spleen and liver. Seven days after CCNU treatment (100 mg/kg) the weights were at 45 and 65% of controls respectively. The activity of δ-aminolevulinic acid synthetase (ALA-S), the rate-limiting enzyme in heme biosynthesis, declined in spleen and liver to 11 and 24% of control values, respectively, 7 days after CCNU treatment. Heme oxygenase activity, the rate-limiting enzyme of heme breakdown, was moderately increased in liver and spleen following CCNU administration. In liver, heme oxygenase activity was 142% of control values at 24 hr, and in spleen the activity was 180% of controls at 1 week. Pretreatment of the animals with phenobarbital (PB) (40 mg/kg/day, i.p.) for 4 days caused a reversal in the decline of liver weight with no effect on the decline in spleen weight following CCNU treatment. Similarly, PB pretreatment reversed the decline in hepatic ALA-S activity after CCNU administration but had no effect on the decline in splenic ALA-S activity. This study indicates that CCNU causes significant decreases in the activity of enzymes of heme biosynthesis in spleen and liver. The CCNU hemotoxicity in the liver was reversed by PB pretreatment whereas the splenic hemotoxicity was unchanged.

CCNU‡ [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] is an antineoplastic alkylating and carbomylating agent used in the treatment of a wide variety of tumors [1]. Major side effects limiting the use of CCNU include hematopoietic depression and hepatic injury reported in both animals and man [2–5]. Studies from our laboratory [6] as well as others [7] have lead us to believe that CCNU may cause perturbations of heme metabolism. Severe hyperbilirubinemia in rats was observed after a single oral dose of BCNU or CCNU [4, 6]. This effect was reversed by pretreatment of the animals with phenobarbital [6]. Litterst [7] recently described prolonged depression of hepatic microsomal cytochromes P-450 and b_5 and total heme contents of rat livers after a single dose of CCNU. This effect was also reversed in PB-pretreated animals.

The decrease in the major hepatic hemoproteins and heme content observed in CCNU-treated animals [6, 7] could be due to a decrease in heme biosynthesis or an increase in heme breakdown. Hepatic heme levels are mainly controlled by the activity of δ-aminolevulinic acid synthetase (ALA-S), the rate-limiting enzyme of heme biosynthesis [8, 9], and microsomal heme oxygenase, the key enzyme in heme breakdown [10]. Furthermore, heme oxygenase in spleen and liver is known to play a predominant role in the breakdown of heme, derived from hemoproteins or hemoglobin, to bilirubin [11, 12]. Conceivably, induction of this enzyme system may be a contributing factor in the CCNU-induced hyperbilirubinemia.

The objective of this study was to investigate the mechanisms of CCNU-induced heme depletion and the associated hyperbilirubinemia, by measuring the early changes following CCNU treatment in the key enzymes of heme metabolism in both the liver and spleen of normal and PB-pretreated rats.

MATERIALS AND METHODS

Materials. [2,3-^{14}C] Succinic acid was purchased from the New England Nuclear Corp., Boston MA. δ-Aminolevulinic acid, Dowex X-50 resin (200–400 mesh), pyridoxal phosphate, glycine, hemin (equine), $NADP^+$, $NADPH^+$, and glucose-6-phosphate were purchased from the Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase was purchased from the Boehringer Mannhein Corp., New York, NY. CCNU was a gift from the Bristol Laboratories, Syracuse, NY.

Treatment of animals. Male, Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200–250 g were fed Purina Rat Chow *ad lib.* Unless otherwise indicated, the number of

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† To whom reprint requests should be addressed at the Department of Pathology, 230 Keiller Bldg., University of Texas Medical Branch, Galveston, TX 77550.

‡ Abbreviations: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ALA, δ-aminolevulinic acid; ALA-S, δ-aminolevulinic acid synthetase; and PB, phenobarbital.

animals for each treatment was four. A group of four control rats was given 0.4 ml corn oil orally, and killed at each time point. CCNU, a suspension in corn oil, was given in a single oral dose of either 50 or 100 mg/kg. When phenobarbital (PB)-pretreated animals were used, the drug was injected i.p. in a dose of 40 mg/kg/day for four consecutive days and CCNU (100 mg/day) was given 48 hr prior to killing. Animals were killed by decapitation, and their livers were perfused *in situ* with ice-cold 1.15% KCl. Spleens and perfused livers were excised, blotted dry, and weighed.

Methods. Tissues were homogenized in NaCl–Tris buffer (0.9%–0.01 M, pH 7.4). ALA-S was determined in 1% liver or 5% spleen homogenates as described by Ebert *et al.* [13]. Microsomes were prepared from liver 9000*g* fractions as previously described [14]. Heme oxygenase activity in liver microsomes and in 18,000*g* supernatant fraction of spleen was determined as described by Tenhunen *et al.* [15] using 17 μM hemin (as methemalbumin) as substrate. The rate of increase in optical density at 468 nm was determined in a Gilford spectrophotometer (model 250, Gilford Instruments, Oberlin, OH) equipped with a constant temperature cuvette at 37°.

Statistical analysis. Data were subjected to statistical analysis using Student's t-test and expressed as mean ± S.E. The 0.05 level of probability was used as the criterion of significance.

RESULTS

Organ weight. A single dose of CCNU caused a time- and dose-dependent decline in weight of both liver and spleen (Fig. 1). In the liver, a gradual weight loss was observed, reaching a maximum loss of 20 and 40% within 7 days following treatment with 50 and 100 mg/kg CCNU respectively. The decline in weight was more pronounced in the spleen than in the liver. A maximum loss of 40 and 54% was observed within 7 days following treatment with 50 and 100 mg/kg CCNU respectively.

When rats were treated with PB for 4 consecutive days, liver weight was increased to 135% of control

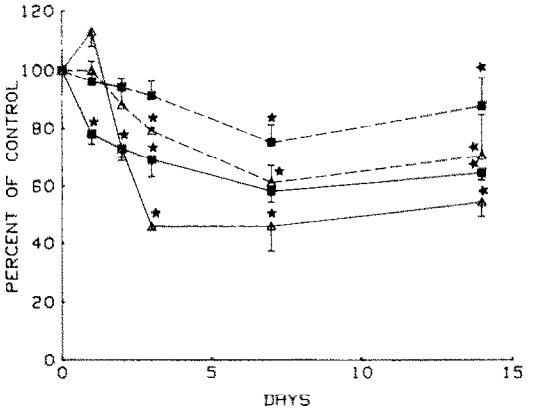

Fig. 1. Effects of a single oral dose of CCNU on the weight of rat spleen and liver. Animals received a single 50 mg/kg (■) or 100 mg/kg (△) dose of CCNU orally and were killed at various times thereafter. One hundred percent control values: spleen weight (——) = 0.79 ± 0.04; liver weight (– – –) = 12.6 ± 0.70. Key: (★) significantly different from control values ($P < 0.05$).

(Table 1). When calculated on an organ/body weight ratio, this increase was 139% of controls. This value was essentially unchanged (136% of controls) in animals treated with both PB and CCNU. In these rats, body weight was decreased to 89% of controls and liver weight was increased to 119% of controls. PB treatment did not alter spleen weight. Animals receiving both PB and CCNU showed the same decline in spleen weight as the rats treated with CCNU alone. Thus, CCNU caused a 28% loss of spleen weight and a 26% loss when given to PB-pretreated animals.

ALA-S activity in spleen and liver. CCNU, in a dose of 50 or 100 mg/kg (Fig. 2), caused early and dramatic losses in splenic ALA-S activity. By 24 hr, ALA-S activity was at 26 and 21% of control values with 50 and 100 mg/kg respectively. The decline in ALA-S activity was maximal at 7 days with only 11% of the activity remaining after 100 mg/kg CCNU.

Hepatic ALA-S activity declined gradually reaching 24 and 70% of control values 7 days after 100 and 50 mg/kg CCNU respectively. Thus, loss in hepatic

Table 1. Effects of CCNU on body, liver and spleen weights in normal or phenobarbital-pretreated rats*

Treatment	Body weight (g)	Liver weight (g)	Liver weight (g/g body weight)	Spleen weight (g)	Spleen weight (g/g body weight)
Control	245 ± 4 (100)	12.6 ± 0.70 (100)	0.05 (100)	0.79 ± 0.04 (100)	0.003 (100)
CCNU†	215 ± 7‡ (88)	12.09 ± 1.05 (96)	0.056 (112)	0.57 ± 0.05§ (72)	0.0027 (88)
PB¶	244 ± 2 (99.6)	17.7 ± 1.19§ (135)	0.07 (139)	0.73 ± 0.03 (92)	0.003 (100)
PB + CCNU#	219 ± 5§ (89)	14.93 ± 0.63§ (119)	0.068 (136)	0.59 ± 0.05§ (74)	0.0027 (88)

* Values are mean ± S.E. of four rats. Values in parentheses are percent of control.

† Rats received 100 mg/kg CCNU, 48 hr before killing.

‡ Significantly different from controls ($P < 0.05$).

¶ Rats were given 40 mg/kg/day phenobarbital (i.p.) for 4 consecutive days, before killing.

Rats were treated with phenobarbital (40 mg/kg/day) for 4 days. CCNU (100 mg/kg, p.o.) was given 48 hr prior to killing.

Table 2. Effect of CCNU on ALA-S activity in liver and spleen of normal or phenobarbital-pretreated rats*

Treatment	Hepatic ALA-S (nmoles ALA/g liver/hr)	Splenic ALA-S (nmoles ALA/g spleen/hr)
Control	44.39 ± 5.10 (100)	61.94 ± 5.65 (100)
CCNU†	27.87 ± 3.98‡ (63)	10.59 ± 3.80‡ (17)
PB§	73.56 ± 5.06‡ (166)	47.71 ± 7.66 (77)
PB + CCNU¶	36.18 ± 4.5 (82)	13.57 ± 2.90‡ (22)

* Values are mean ± S.E. of four rats. Values in parentheses are percent of control.
† Rats received 100 mg/kg CCNU, 48 hr before killing.
‡ Significantly different from controls ($P < 0.05$).
§ Rats were given 40 mg/kg/day phenobarbital (i.p.) for 4 consecutive days, before killing.
¶ Rats were treated with phenobarbital (40 mg/kg/day) for 4 days. CCNU (100 mg/kg, p.o.) was given 48 hr prior to killing.

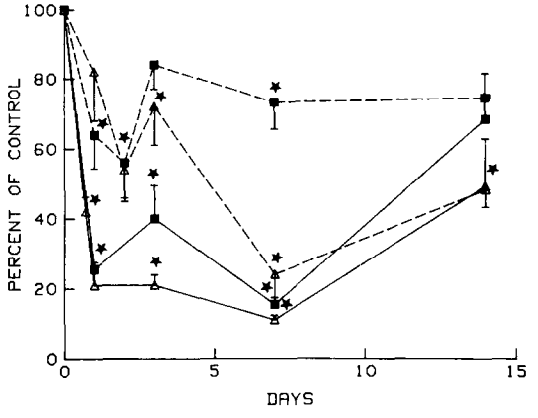

Fig. 2. Effects of a single oral dose of CCNU on the ALA-S activity of rat spleen and liver. Animals received a single 50 mg/kg (■) or 100 mg/kg (△) dose of CCNU orally and were killed at various times thereafter. One hundred percent control values: spleen ALA-S activity (——) = 61.94 ± 5.7; liver ALA-S activity (– – –) = 44.4 ± 5.1. Key: (★) significantly different from control values ($P < 0.05$).

ALA-S was more pronounced with the 100 mg/kg than the 50 mg/kg dose.

Phenobarbital treatment, 40 mg/kg for 4 days, had no effect on splenic ALA-S but caused an increase in hepatic ALA-S activity to 166% of control values (Table 2). CCNU, 100 mg/kg for 48 hr, caused decreases in ALA-S activity in liver and spleen to 63 and 17% of control values respectively. In rats receiving PB and CCNU, hepatic ALA-S activity was not significantly different from untreated controls; however, the activity was intermediate between the CCNU- and PB-treated controls. Splenic ALA-S activity was decreased to 22% of control values in animals receiving PB and CCNU. Thus, PB pretreatment partially reversed the decrease in hepatic ALA-S activity caused by CCNU treatment. The loss in splenic ALA-S activity following CCNU was not altered by pretreatment with PB.

Heme oxygenase activity. Table 3 shows the effects of a single oral dose (100 mg/kg) of CCNU on hepatic and splenic heme oxygenase activities. A transient increase (142% of controls) was observed in liver heme oxygenase activity 24 hr after 100 mg/kg CCNU. In the spleen, no significant early changes

Table 3. Effect of a single, oral (100 mg/kg) dose of CCNU on hepatic and splenic heme oxygenase*

Time after CCNU treatment	Liver heme oxygenase (nmoles bilirubin/mg protein/10 min)	Spleen heme oxygenase (nmoles bilirubin/mg protein/10 min)
0 hr	0.24 ± 0.03 (100)	0.18 ± 0.02 (100)
16 hr	0.17 ± 0.02 (71)	0.19 ± 0.03 (111)
24 hr	0.34 ± 0.03† (142)	0.23 ± 0.02 (130)
48 hr	0.2 ± 0.03 (85)	0.22 ± 0.03 (123)
1 week	0.28 ± 0.05 (117)	0.32 ± 0.05† (180)

* Values are means ± S.E. of four rats. Values in parentheses are percent of control.
† Significantly different from controls ($P < 0.05$).

were observed; however, the activity was increased to 180% of control values 1 week after CCNU treatment.

DISCUSSION

The results of this study show that a single oral dose of CCNU caused early losses in the rate-limiting enzyme of heme biosynthesis, namely ALA-S, in both liver and spleen. The decline of ALA-S activity in the spleen occurred prior to its decline in the liver. Furthermore, the decrease in ALA-S activity in the spleen was more pronounced, with only 11% of the activity remaining after a CCNU dose of 100 mg/kg. We have shown recently that, in rats treated with melphalan, a myelosuppressive alkylating nitrogen mustard and a non-hepatotoxin, spleen and bone marrow ALA-S are selectively decreased whereas the liver enzyme is not affected [16]. On the other hand CCNU, an alkylating nitrosourea and myelosuppressive agent, is a known hepatotoxin [17]. Organ selectivity was not observed with CCNU in this study, which is consistent with the CCNU-induced hepatic injury. In rodents, the spleen is an active site for hematopoiesis [18]. Suppression of ALA-S activity in spleen by CCNU may be related to its well known hematopoietic depressive action.

The decline in the hepatic ALA-S activity was quite significant as well as time and dose dependent after CCNU treatment. The early decline in hepatic heme biosynthesis, as indicated by the decrease of ALA-S activity, may result in the losses of hepatic heme and hemoprotein content observed in CCNU-treated animals [6, 7].

Heme oxygenase activity was increased slightly after CCNU treatment in both spleen and liver. This moderate increase in splenic heme oxygenase activity would not account for the massive hyperbilirubinemia observed following CCNU treatment. Furthermore, the increase in activity of splenic heme oxygenase occurred at 1 week, whereas the observed hyperbilirubinemia started as early as 24–48 hr after CCNU administration [6]. In the liver, the moderate and early rise in heme oxygenase may occur in conjunction with the decrease in hepatic cytochrome P-450, and heme content, previously reported [6, 7].

Phenobarbital treatment of rats prior to CCNU administration is known to raise the LD_{50} of CCNU and to decrease its antitumor activity [17]. We have also shown that PB is capable of reversing all the hepatic toxic manifestations of CCNU, including the hyperbilirubinemia [6]. In this study PB reversed the decreases in hepatic weight and ALA-S activity but had no effect on the decline in splenic weight or ALA-S activity in CCNU-treated animals. These effects show for the first time *in vivo* a dissociation of the individual toxic manifestations of CCNU.

May *et al* [19] have shown that microsomes from control rats are capable of hydroxylating CCNU on the cyclohexyl ring, leading to the formation of 4–6 isomers, all of which have antitumor and toxic potential. When PB microsomes were used *in vitro*, a preferential shift of the metabolic pattern was observed to the *cis*-4-hydroxy metabolite. Current work in our laboratory is in progress to attempt to dissociate the individual toxic and chemotherapeutic actions of each metabolite of CCNU. PB is also known to induce the synthesis of several hepatic enzymes, including cytochrome P-450. Induction of hepatic ALA-S by PB is directly related to the increased biosynthesis of cytochrome P-450 apoprotein [20]. However, such a role for PB has not been described in the spleen. Indeed, ALA-S in spleen was not induced by PB treatment as was the case for the hepatic enzyme. Thus, the PB reversal of the hepatic toxicity of CCNU may be attributed to the increased potential of the liver to detoxify CCNU to less reactive metabolites. Such a mechanism may not be operative in the spleen.

In conclusion, this work shows several important aspects of CCNU toxicity. (a) A decline in splenic and hepatic heme biosynthesis was evident. The decrease in hepatic heme biosynthesis may be the direct cause for the depletion of hepatic heme content. On the other hand, the decline in splenic heme biosynthesis may be involved in the hematopoietic suppressive actions of CCNU. (b) Moderate increases in splenic or hepatic heme oxygenase activity were observed, indicating that increased heme turnover may not be the major cause of the observed hyperbilirubinemia after CCNU treatment and that other mechanisms may be involved. (c) PB was capable of reversing the hepatic, but not the splenic losses in weight and in ALA-S activity after CCNU treatment. This may indicate that either the drug may be under different metabolic regulation in the two tissues or different metabolites may initiate different toxic manifestations in the liver and spleen.

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