Effects of Dichloroacetate (DCA) on Serum Insulin Levels and Insulin-Controlled Signaling Proteins in Livers of Male B6C3F1 Mice

Melissa K. Lingohr,* † Brian D. Thrall,* † and Richard J. Bull* † 1

*Washington State University, Pullman, Washington 99164-6510; and † Pacific Northwest National Laboratory, Richland, Washington 99352

Received July 20, 2000; accepted October 5, 2000

DCA is hepatocarcinogenic in rodents. At carcinogenic doses, DCA causes a large accumulation of liver glycogen. Thus, we studied the effects of DCA treatment on insulin levels and expression of insulin-controlled signaling proteins in the liver. DCA treatment (0.2–2.0 g/l in drinking water for 2 weeks) reduced serum insulin levels. The decrease persisted for at least 8 weeks. In livers of mice treated with DCA for 2-, 10-, and 52-week periods, insulin receptor (IR) protein levels were significantly depressed. Additionally, protein kinase B (PKBα) expression decreased significantly with DCA treatment. In normal liver, glycogen levels were increased as early as at 1 week, and this effect preceded changes in insulin and IR and PKBα. In contrast to normal liver, IR protein was elevated in DCA-induced liver tumors relative to that in liver tissue of untreated animals and to an even greater extent when compared to adjacent normal liver in the treated animal. Mitogen-activated protein kinase MAP kinase phosphorylation was also increased in tumor tissue relative to normal liver tissue and tissue from untreated controls. These data suggest that normal hepatocytes down-regulate insulin-signaling proteins in response to the accumulation of liver glycogen caused by DCA. Furthermore, these results suggest that the initiated cell population, which does not accumulate glycogen and is promoted by DCA treatment, responds differently from normal hepatocytes to the insulin-like effects of this chemical. The differential sensitivity of the 2 cell populations may contribute to the tumorigenic effects of DCA in the liver.

Key Words: dichloroacetate; glycogen; insulin; signaling; insulin receptor PKB; hepatocarcinogen.

Dichloroacetate (DCA) is a contaminant of chlorinated drinking water (Singer and Chang, 1989) and is carcinogenic in the liver of male and female B6C3F1 mice (Bull et al., 1990; Daniel et al., 1992; DeAngelo et al., 1991; Pereira et al., 1997; Pereira and Phelps, 1996) and male F344 rats (DeAngelo et al., 1996). The weight of evidence suggests DCA is not genotoxic in in vitro tests at concentrations ≤1 mM or in in vivo tests at doses <3.5 g/l of drinking water (DeMarini et al., 1994; Fox et al., 1996; Fuscoe et al., 1996; Giller et al., 1997; Harrington-
strate that DCA treatment results in decreases in serum insulin levels and hepatocyte expression of the insulin receptor (IR) and protein kinase B (PKBα). We also report that liver tumor cells are refractory to the DCA-induced changes that occur in normal hepatocytes. The differential sensitivity of the 2 cell populations to DCA’s insulin-like activity may contribute to the tumorigenic effects of DCA in the liver.

MATERIALS AND METHODS

Chemicals and materials. Analytical grade dichloroacetic acid (Fluka Chemical Corp., Ronkonkoma, NY) was dissolved in double distilled water to concentrations of 0.1–2.0 g/l. This solution was neutralized with 1 N NaOH to a pH 6.8–7.2, and supplied to mice as their drinking water. In all experiments, a concurrent control group was included in which mice received double distilled water, pH 6.8–7.2, as their drinking water.

Animals and treatments. Male B6C3F1 mice were purchased from Simonsen Laboratories (Gilroy, CA) or Charles River (Raleigh, NC), allowed to acclimate for 1 week to a 12-h light/dark cycle, and placed on treatment. All animal care, use, and experiment protocols were submitted to and approved by the Institutional Animal and Use Committee (IACUC) of Washington State University or the IACUC of Battelle, Pacific Northwest Laboratories. Mice were treated with DCA (0.1–2.0 g/l) in their drinking water for 2–10 weeks. Water consumption was monitored and animals weighed weekly for the duration of the studies. Consistent with previous studies (Bull et al., 1990), DCA treatment does not significantly alter body weight or drinking water/food consumption for the duration of the studies at concentrations of DCA up to and including 2 g/l. At termination of treatment animals were sacrificed by CO2 asphyxiation. Blood was collected by cardiac puncture. Livers were excised and immediately frozen in liquid nitrogen. Most of these animals were sacrificed at 3:00 a.m. after discovering that blood concentrations of DCA at low doses rapidly decrease when lights are turned on and animals are no longer drinking water (Kato-Weinstein et al., 1998). Livers and tumors were available from prior experiments where mice were treated with 0.5 and 2.0 g/l DCA for 87 and 52 weeks, respectively, and age-matched controls (Orner et al., 1998). These animals were sacrificed during normal daylight hours.

Immunoblot analysis. Livers, previously frozen at −80°C, were weighed, minced, and homogenized in 1.0 ml ice-cold homogenization buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 50 units/ml bacitracin, 1 μM peptatin, 200 μg/ml leupeptin, 10 μg/ml aprotonin, 50 μg/ml PMSF, and 200 μg/ml orthovanadate) per 100 mg tissue (10% homogenate). Protein content was determined by the method of Lowry et al. (1951). Homogenate protein was diluted in 0.1 mM Tris-HCl (pH 7.4, 4°C) and in 2× SDS loading-gel buffer (100 mM Tris-HCl, pH 6.8, 5% glycerol, 3% SDS, 2% mercaptoethanol, 0.002% bromophenol blue, 1 mM EDTA) and 30 μg was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to either nitrocellulose or PVDF membrane on a semi-dry blotting apparatus (Integrated Separation Systems, Natick, MA). Membranes were blocked for either nitrocellulose or PVDF membrane on a semi-dry blotting apparatus polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to

RESULTS

Administration of DCA in the Drinking Water Decreases Serum Insulin Levels

In the first experiment (conducted at WSU), mice were 7-weeks old at the start of treatment and were sacrificed at ages 9, 11, and 15 weeks, corresponding to 2-, 4-, and 8-week treatment periods. Blood glucose concentrations tended to decrease with age, independent of DCA treatment, which is characteristic of the developing animal (Fig. 1c). Note that the age-related increase in serum insulin levels (Fig. 1a) in control mice corresponds with the decrease in blood glucose (Fig. 1c). In animals administered 2.0 g/l DCA for 2, 4, and 8 weeks serum insulin levels were decreased significantly below that of age-matched controls (Fig. 1a). The effect of DCA-treatment on serum insulin levels was greater than 50% at 4 and 8 weeks. All subsequent experiments were conducted at Battelle, Pacific Northwest Laboratories with mice purchased from...
TABLE 1
Circulating Blood Levels of IGF-I following a 2-week Administration of DCA

<table>
<thead>
<tr>
<th>Treatment (g/l)</th>
<th>Serum levels of IGF-I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
</tr>
<tr>
<td>Control</td>
<td>336.7 ± 27.1</td>
</tr>
<tr>
<td>0.5 DCA</td>
<td>ND</td>
</tr>
<tr>
<td>2.0 DCA</td>
<td>382.3 ± 47.8</td>
</tr>
</tbody>
</table>

Note. Serum IGF-1 levels are unaffected by DCA treatment. Serum was collected from mice administered DCA, and IGF-1 levels were measured by radioimmunoassay as described in Materials and Methods. The number of animals used in all treatment groups was = 5. Values are mean ± SEM and expressed as ng/mL. Data were analyzed using 2-way analysis of variance and the Tukey test; *indicates p < 0.05; n = 5.

Administration of DCA in the Drinking Water Decreases
Insulin Receptor Protein Levels

In a sub-group of randomly selected mice (n = 3), in which serum insulin levels were measured at 2 weeks, IR expression levels in livers were also measured. After 2 weeks of treatment, a trend towards decreased expression of IR protein was observed in livers of mice treated with DCA (Figs. 2a and 2c). The decrease in IR was significant at 0.5 and 2.0 g/l DCA. IR levels were also measured after 10 weeks of treatment. In the 10-week experiment, the high dose of DCA was decreased to 1.0 g/l because doses ≥ 2.0 g/l led to blood levels of DCA that approached the apparent Kᵢ of pyruvate dehydrogenase kinase (Kato-Weinstein et al., 1998; Pratt et al., 1979). After 10 weeks of treatment, a substantial decrease in IR was observed at both 0.5 and 1 g/l DCA (Figs. 2b and 2c).

Additionally, PKBα expression was measured in livers of animals treated for 10 weeks with DCA. Similar to the effect on IR expression, PKBα expression was significantly decreased in the livers of DCA-treated mice (Figs. 3a and 3b).

Insulin Receptor Expression in DCA-Induced Liver Tumors

IR levels were also examined in livers of mice from a previously conducted carcinogenesis study (Orner et al., 1998). In this experiment, DCA was administered at 0.5 or 2.0 g/l for 87 and 52 weeks, respectively. Animals on chronic DCA-treatment were from a prior study (conducted at WSU). In this case animals had been sacrificed between 9:00 and 11:00 A.M. Analysis of the livers from mice treated at the high dose of DCA revealed a decrease in IR protein expression in normal portions of the liver (approximately 30% relative to the concurrent control; p < 0.05 at 2.0 g/l; Fig. 4a). At 0.5 g/l, IR expression was also decreased, but the decrease was not significantly below that observed in livers from untreated animals (data not shown). The lack of significance of this change could be partially due to the fact that the sacrifice time occurred at a time when DCA concentrations in blood were reduced (Kato-Weinstein et al., 1998).
In contrast to findings in normal liver, liver tumors induced by 2.0 g/l DCA in the drinking water expressed higher amounts of IR than normal liver tissue taken from the same animal (Figs. 4a and 4b). The same was observed for tumors induced by 0.5 g/l DCA treatment (data not shown). The amount of IR protein present in tumors was nearly 2-fold greater than that of non-tumor-bearing portions of the same liver ($p$, 0.05; Figs. 4a and 4b). The amount of IR protein in tumors also tended to be higher (35%) than that in livers of mice that had no DCA treatment ($p$, 0.06; Fig. 4a).

**Phosphorylation of MAP Kinase Is Elevated in DCA-Induced Tumors**

Since increased expression of the insulin receptor can indicate increased activation of its signaling pathway, the relative phosphorylation level of MAP kinase, a downstream target protein, was measured. Phosphorylation of MAP kinase was increased nearly 4-fold within liver tumors when compared to adjacent normal liver tissue of the same animal ($p$, 0.05) and also to that of normal liver from untreated animals ($p$, 0.05; Figs. 5a and 5b).

**DISCUSSION**

In this study, DCA consistently suppressed levels of circulating insulin. The decrease in serum insulin levels required a minimum of a 2-week treatment with DCA. No significant decrease occurred after 1 week of DCA administration. However, hepatic glycogen levels are increased within 1 week (Kato-Weinstein *et al*., 1998). Therefore the accumulation in liver glycogen precedes the decrease of serum insulin concentrations. Previous work has shown that DCA treatment does not inhibit the release of insulin in response to glucose (Kato-Weinstein *et al*., 1998). Thus, serum insulin changes due to DCA-treatment are consistent with a feedback inhibition of insulin secretion that develops over time. Such feedback could be resulting from the accumulation of glycogen in the liver. However, no specific mechanism for such negative feedback could be identified in the literature.
PKB phosphatidylinositol-3 kinase (PI3-K)-dependent activation of suggested that insulin-stimulated glycogen synthesis occurs via 1995; Lawrence and Roach, 1997). Most recent work has been analyzed using analysis of variance and Students t-test; *indicates p < 0.05. In all analyses, n ≥ 5.

In addition to decreasing serum insulin levels, DCA-treatment resulted in decreased hepatic expression of the IR. The decrease in IR expression was significant at 2 weeks of DCA-treatment but progressed to an even lower level at 10 weeks of DCA-treatment. IR protein levels would normally be expected to increase with a fall in serum insulin levels (Kahn and White, 1995). However, it may be that the accumulation of glycogen in normal hepatocytes caused by DCA down-regulates the receptor responsible for initiating the activation of the signaling pathway leading to glycogen synthesis.

In support of this hypothesis, DCA decreased hepatic expression of PKBα, a downstream signaling component of the insulin-stimulated kinase cascade involved in mediating the glycogenic response to insulin (Cohen et al., 1997; Cross et al., 1995; Lawrence and Roach, 1997). Most recent work has suggested that insulin-stimulated glycogen synthesis occurs via phosphatidylinositol-3 kinase (PI3-K)-dependent activation of PKBα, which inactivates glycogen synthase kinase-3 (GSK-3), thereby reducing the level of phosphorylation of glycogen synthase and increasing glycogen synthesis (Cross et al., 1995; Lawrence and Roach, 1997; Park et al., 1999).

The decrease in IR expression was still apparent at the high dose of DCA (2.0 g/l) after 52 weeks of DCA-treatment. The decrease in IR expression was not significant after 87 weeks of treatment with 0.5 g/l DCA. However, this is most likely explained by the fact that the concentration of DCA in the blood is highest at night (Kato-Weinstein et al., 1998). At the 9:00 A.M. time of sacrifice, DCA had essentially disappeared from the blood of animals treated with 0.5 g/l DCA (Kato-Weinstein et al., 1998). The changes in IR protein are still evident at the higher dose of DCA, where the concentrations of DCA in blood are sharply increased and remain significantly elevated into the daylight hours as a result of auto-inhibition of metabolism (Gonzalez-Leon et al., 1997; Kato-Weinstein et al., 1998).

In contrast to normal hepatocytes of treated mice, insulin receptor protein was somewhat increased in liver tumors relative to livers from untreated animals and even increased significantly when compared to adjacent normal liver of the treated mice. As mentioned, DCA-induced tumors are uniformly glycogen-poor (Bull et al., 1990). In other studies, lesions expressing the glycogen-poor phenotype have been shown to have a significant reduction in activity of glycogen synthase and several other enzymes involved in glycogen metabolism (Bannasch et al., 1984, 1997). If the down-regulation of IR in normal hepatocytes is the result of increased glycogen content, then the elevated expression in DCA-induced tumors could be related to their glycogen-poor character.

Insulin is mitogenic in normal liver (Kahn and White, 1995) and even more so in malignant liver cells (Koontz and Iwahashi, 1981; Massague et al., 1982). The suppression of cell division in normal liver associated with extended DCA-treatment described by Stauber and Bull (1997) might be attributable to the decreases in serum insulin and IR expression in hepatocytes. In contrast, the increase in cell division in tumors induced by DCA (Stauber and Bull, 1997) may be explained by the greater sensitivity of these tumors to the mitogenic effects of insulin as a result of the higher expression of IR relative to the surrounding tissue.

Increased binding of insulin to its receptor has been observed in hepatocarcinomas in prior studies (Kurtaran et al., 1995). Also, increased IR expression is characteristic of hepatoma cells in culture (Taouis et al., 1994; Khamzina and Borgeat, 1998) and insulin-induced DNA synthesis in these cells is blocked by inhibitors of insulin-stimulated signaling pathways. Finally, the data shows that MAP kinase phosphorylation is significantly higher in DCA-induced tumors than in adjacent normal liver and also higher than that from liver of control animals. MAP kinase is a downstream target of the IR and its level of activity has been positively correlated to that of insulin-induced increases in cell proliferation (Porras et al., 1998).

In summary, the results of this study indicate that glycogen accumulation induced by DCA precedes decreases in serum concentration of free insulin.
insulin levels and expression of insulin-signaling proteins in normal liver. These data strongly suggest DCA-induced alterations in insulin, IR, and perhaps PKBα are a result of a compensatory down-regulation of the insulin pathway triggered by high glycogen levels in the liver. The mechanisms for compensatory down-regulation of the insulin-signaling pathway as a result of DCA-induced glycogen accumulation are not clear. Such mechanisms may be important, since cells promoted by DCA to form tumors are refractory to both glycogen accumulation and decreased IR expression.

ACKNOWLEDGMENT

We are grateful for the support of the Environmental Management Science Program, project 26748, under D.O.E. contract DE-AC06-76RLO 1830.

REFERENCES


