

Dichloroacetate Stimulates Glycogen Accumulation in Primary Hepatocytes through an Insulin-Independent Mechanism

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Dichloroacetate (DCA), a by-product of water chlorination, causes liver cancer in B6C3F1 mice. A hallmark response observed in mice exposed to carcinogenic doses of DCA is an accumulation of hepatic glycogen content. To distinguish whether the *in vivo* glycogenic effect of DCA was dependent on insulin and insulin signaling proteins, experiments were conducted in isolated hepatocytes where insulin concentrations could be controlled. In hepatocytes isolated from male B6C3F1 mice, DCA increased glycogen levels in a dose-related manner, independently of insulin. The accumulation of hepatocellular glycogen induced by DCA was not the result of decreased glycogenolysis, since DCA had no effect on the rate of glucagon-stimulated glycogen breakdown. Glycogen accumulation caused by DCA treatment was not hindered by inhibitors of extracellular-regulated protein kinase kinase (Erk1/2 kinase or MEK) or p70 kDa S6 protein kinase (p70^{S6K}), but was completely blocked by the phosphatidylinositol 3-kinase (PI3K) inhibitors, LY294002 and wortmannin. Similarly, insulin-stimulated glycogen deposition was not influenced by the Erk1/2 kinase inhibitor, PD098509, or the p70^{S6K} inhibitor, rapamycin. Unlike DCA-stimulated glycogen deposition, PI3K-inhibition only partially blocked the glycogenic effect of insulin. DCA did not cause phosphorylation of the downstream PI3K target protein, protein kinase B (PKB/Akt). The phosphorylation of PKB/Akt did not correlate to insulin-stimulated glycogenesis either. Similar to insulin, DCA in the medium decreased IR expression in isolated hepatocytes. The results indicate DCA increases hepatocellular glycogen accumulation through a PI3K-dependent mechanism that does not involve PKB/Akt and is, at least in part, different from the classical insulin-stimulated glycogenesis pathway. Somewhat surprisingly, insulin-stimulated glycogenesis also appears not to involve PKB/Akt in isolated murine hepatocytes.

Key Words: dichloroacetate; glycogen; insulin; insulin receptor; PKB/Akt; PI3K; hepatocyte.

Dichloroacetate (DCA) is a common by-product formed during drinking water chlorination and is hepatocarcinogenic in B6C3F1 mice and F344 rats (Bull *et al.*, 1990; DeAngelo *et al.*, 1991, 1996; Pereira, 1996; Stauber and Bull, 1997). A hallmark effect of DCA treatment in mice is a marked accu-

mulation of hepatocellular glycogen (Bull *et al.*, 1990; Kato-Weinstein *et al.*, 1998). The dose-response relationship for glycogen accumulation in mice closely parallels the dose-response relationship for the carcinogenic effects of DCA (Bull *et al.*, 1990; Kato-Weinstein *et al.*, 1998; Stauber and Bull, 1997). It is not known whether the mechanisms by which DCA causes hepatocellular carcinoma and stimulates hepatocellular glycogen content are related. However, a link between altered glycogen metabolism and liver cancer risk is suggested by the fact that patients with glycogen storage disease have a significantly increased incidence of liver cancer (Alshak *et al.*, 1994; Conti and Kemeny, 1992; Labrune *et al.*, 1997).

Glycogen synthase is the rate-limiting enzyme of glycogen biosynthesis, and its activation is regulated by a reversible dephosphorylation mechanism in which several insulin-controlled phosphatases and kinases can be involved (Pugazenthi and Khandelwal, 1995). The principal signaling pathway by which insulin stimulates glycogen synthase is via activation of the insulin receptor (IR), leading to phosphatidylinositol-3' kinase (PI3K)-dependent activation of protein kinase B (PKB/Akt), inactivation of glycogen synthase kinase-3 (GSK-3), and increased activity of glycogen synthase (GS) (Cohen, 1999; Cross *et al.*, 1995, 1997; Lawrence and Roach, 1997; Park *et al.*, 1999). PI3K-dependent activation of the p70 kDa S6 protein kinase (p70^{S6K}) as well as activation of the Ras/Raf/MEK/Erk1/2 signaling pathway have also been linked with GSK-3 inactivation and increased glycogen synthesis (Azpiazu *et al.*, 1996; Dent *et al.*, 1990; Park *et al.*, 1999; Shepherd *et al.*, 1995; Sutherland and Cohen, 1994; Sutherland *et al.*, 1993). In addition to their role in regulating metabolism, the activities of PI3K, PKB/Akt and Erk1/2 play important roles in regulating cell proliferation and apoptosis in hepatocytes (reviewed in Band *et al.*, 1999; Galetic *et al.*, 1999; Mounho and Thrall, 1999; Roberts *et al.*, 2000).

DCA has been shown to markedly increase liver glycogen levels in mice within one week, and the increase is sustained with continued treatment (Kato-Weinstein *et al.*, 1998). However, the glycogenic effect of DCA was associated with decreased glycogen synthase activity in the livers of these mice (Kato-Weinstein *et al.*, 1998). In more recent studies, serum insulin levels were found to be suppressed, but the effect

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required 2 weeks or more of DCA treatment to occur. At one week, insulin levels tended to be increased, but not significantly. Additionally, hepatic steady-state expression levels of the IR and PKB/Akt were significantly reduced during DCA treatment *in vivo* (Lingohr *et al.*, 2001). The decrease in expression of hepatic insulin signaling proteins was most dramatic after 10 weeks of DCA treatment, but readily apparent within 2 weeks (Lingohr *et al.*, 2001). These data suggested the accumulation of glycogen caused by DCA treatment might result in a compensatory downregulation of insulin signaling proteins and suppression of serum insulin levels. However, the lag observed in the decreased serum insulin concentrations also raised the question whether insulin and its signaling proteins were involved in the initial increase in liver glycogen caused by DCA treatment.

In the present study, the effect of DCA on glycogen levels in isolated B6C3F1 mouse hepatocytes has been examined to see if the effects of DCA could be distinguished from those of insulin. As found in livers of mice administered DCA in the drinking water, DCA increased glycogen accumulation in isolated hepatocytes. DCA-induced glycogen accumulation in hepatocytes was independent of insulin, but entirely dependent upon PI3K activity. Also, DCA downregulated IR expression in isolated hepatocytes, which could perhaps be the result of a regulatory loop involving glycogen content reminiscent of that occurring with insulin (Fleig *et al.*, 1985; Knutson, 1991).

MATERIALS AND METHODS

Chemicals and materials. Analytical grade dichloroacetic acid (Fluka Chemical Corp., Ronkonkoma, NY) was dissolved in double distilled water to a stock concentration of 1 M, neutralized with 1 N NaOH to a pH 6.8–7.2 and diluted in medium to the given concentrations. LY294002, PD98059, rapamycin, and wortmannin were purchased from Calbiochem (La Jolla, CA). Insulin, gentamicin and penicillin/streptomycin were from Life Technologies (Grand Island, NY). Dexamethasone, William's medium E, and all other reagents were purchased from Sigma (St. Louis, MO).

Animals and treatments. All animal care, use and experiment protocols were submitted and approved by the Institutional Animal Care and Use Committee (IACUC) of Washington State University or the IACUC of Battelle, Pacific Northwest Laboratories. Hepatocytes were isolated from male B6C3F1 mice 8–12 weeks of age as described below.

Primary hepatocyte isolation. Primary mouse hepatocytes were isolated by a retrograde perfusion method. Briefly, 4–10 week old mice were anesthetized by an ip injection of Nembutol (125 mg/kg), cannulated through the right ventricle, and livers were perfused with EGTA (0.5 mM) in HEPES-buffered Hank's Balanced Salt Solution (HBSS, pH 7.4) for 6–8 min (flow rate 5–7 ml/min). The livers were then perfused with HEPES-buffered HBSS solution containing collagenase (Worthington Type II–100 units/ml, Worthington Biochemical Corp., Freehold, NJ) for another 8–10 min (flow rate 7–10 ml/min). Hepatocytes were dispersed, washed, and purified on a Percoll density gradient (Sigma, St. Louis, MO). Cells were plated at 30,000 cells/cm² onto tissue culture dishes coated with Vitrogen (Collagen Biomaterials, Inc., Palo Alto, CA). Cell viability was >90% of cells in all experiments, as determined by trypan blue exclusion. Hepatocytes were allowed to attach 4 h in serum-free William's media E (Sigma, St. Louis, MO) containing 1% penicillin/streptomycin and 2 mM of L-glutamine. After attachment cells were washed with HBSS and given fresh medium, with or without test chemicals. Hepatocytes

were incubated in 95% air, 5% CO₂ until the end of treatment. Following treatment, hepatocytes were rinsed with ice-cold HBSS and lysed on the plate with 0.125 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1% nonidet P-40, 10 mM NaF, 1 mM pepstatin, 200 mg/ml leupeptin, 10 ug/ml aprotinin, 50 mg/ml PMSF, and 200 mM sodium orthovanadate). Protein content was determined by the method of Lowry *et al.* (1951), and lysates were frozen at –80°C until analysis.

Immunoblot analysis. Hepatocellular protein was diluted in gel loading buffer (final concentration of 50 mM Tris-HCl, pH 6.8, 2.5% glycerol, 2% SDS, 1% mercaptoethanol, 0.001% bromophenol blue, and 0.5 mM EDTA) and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to nitrocellulose membrane on a semi-dry blotting apparatus (Integrated Separation Systems, Natick, MA). Membranes were blocked for 1 h in 5% nonfat milk in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) and incubated overnight with primary antibodies directed against IR- β (Santa Cruz, Santa Cruz, CA) or phosphorylated PKB/Akt (New England Biolabs, Beverly, MA). A separate gel was run for each individual antibody tested and equal protein loading was confirmed either by gel staining or by blotting with a primary antibody directed against a protein (i.e., epidermal growth factor receptor, Santa Cruz). Blots were washed in 5 changes of TBS-T and incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Zymed Laboratories, San Francisco, CA). Detection was by horseradish peroxidase and chemiluminescence (Amersham Life Science, Arlington Heights, IL). Immunoblots were quantified using NIH Image 1.61 Software.

Glycogen analysis. Glycogen levels in hepatocyte monolayers were measured by a spectrophotometric enzymatic assay as described by Gomez-Lechon *et al.* (1995). Briefly, hepatocyte monolayers were washed twice with ice-cold PBS (20 mM, pH 7.4). Plates were frozen immediately in liquid nitrogen. Cells were scraped from the plates in 100 ml PBS and frozen and thawed twice. Lysates were added to 800 ml reaction buffer, containing 200 mM sodium acetate (pH 4.8). Amyloglucosidase (10 Units; Sigma, St. Louis, MO) was added to initiate enzymatic glycogen hydrolysis. Reactions were incubated for 2 h at 40°C with constant agitation. Glucose was determined by a colorimetric glucose oxidase method with reagents purchased from Sigma (St. Louis, MO). Phosphate buffer (100 mM, pH 7) containing 1 mg/ml ABTS, 0.0008U/ml peroxidase, and 0.5U/ml glucose oxidase were added to 200 ml of sample that were then incubated in the dark for 45 min. The intensity of the color reaction was measured at 405 nm. Glycogen content was normalized to protein concentration within each individual sample and expressed as glucose equivalents (nmol)/mg protein.

In experiments measuring glycogen degradation, hepatocytes were treated 16 h with DCA, followed by a 30-min treatment with 10 nM glucagon. Loss of intracellular glycogen levels was measured at the indicated time periods.

Other procedures. Data are presented as a mean \pm SEM. Statistically significant differences between samples and groups were analyzed using one-way ANOVA or Student's *t* test as indicated in the figure legends. Statistical significance was set at $p < 0.05$.

RESULTS

DCA increases hepatocellular glycogen levels in isolated hepatocytes. Primary hepatocytes isolated from B6C3F1 mice were treated 16 h with increasing concentrations of DCA in the media ($n = 3$ plates/treatment). Increases in glycogen were observed even at low DCA concentrations (10 mM) and were nearly 2-fold after treatment with 100 μ M DCA (Fig. 1). The dose-related effect of DCA on hepatocellular glycogen content was not influenced by the presence or absence of insulin (100 nM) (Fig. 1).

In the same experiments, treatment of hepatocyte monolay-

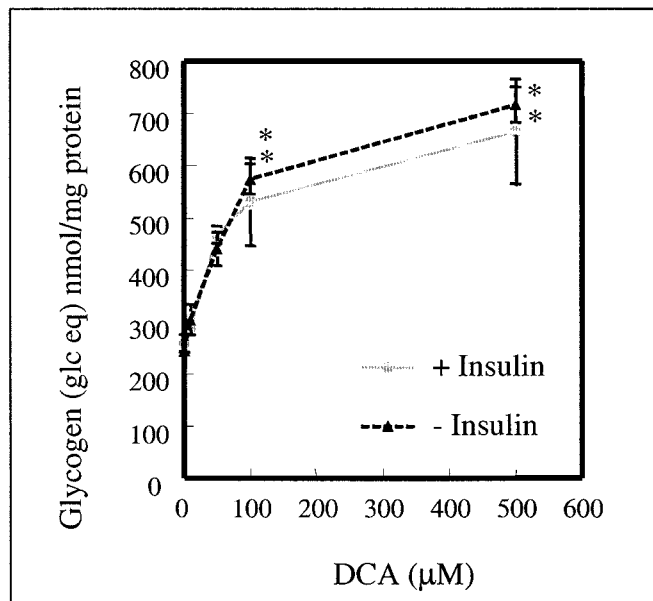


FIG. 1. DCA stimulates glycogen accumulation in isolated mouse hepatocytes independently of insulin. Hepatocytes plated as monolayers on 60-mm plates were treated with increasing concentrations of DCA (10–500 μ M) for 16 h in the presence or absence of 100 nM insulin. In each experiment glycogen levels were measured in 3 separate culture plates and the average content was determined. The data is taken from ≥ 3 experiments and is expressed as the mean nmol glucose equivalents/mg protein \pm SEM. *Significant difference as defined by one-way ANOVA occurring in DCA-treated hepatocytes, $p \leq 0.05$. The differences +/- insulin were not statistically significant.

ers with 100 nM insulin did not increase glycogen content (Fig. 1). In experiments in which 100 nM dexamethasone was added to the media, insulin (100 nM) significantly increased (>2 -fold) glycogen content after 16 h (see Figs. 3–5). Co-incubation of DCA with dexamethasone did not enhance DCA-induced glycogen synthesis (data not shown).

Glycogenolysis is not inhibited by DCA in isolated hepatocytes. Liver glycogen accumulation may result from either an increase in synthesis or a decrease in degradation. Primary hepatocyte monolayers were incubated 16 h in the presence or absence of 100 μ M DCA. In the last 30 min of treatment, 10 nM glucagon (GGN) was added to the media of all hepatocyte monolayers. DCA did not inhibit glucagon-stimulated glycogenolysis in hepatocytes (Fig. 2). Since the levels of glycogen at time zero were higher with DCA treatment, the initial rate of glycogenolysis induced by GGN was actually more rapid in hepatocytes treated 16 hours with DCA than untreated hepatocytes (Fig. 2). A similar result was obtained when glycogenolysis was induced by incubation of treated cells in glucose-free medium (data not shown). In each experiment, glycogen levels were reduced $>65\%$ in DCA-treated cells.

PI3K inhibitors completely abolish DCA-induced glycogen deposition, but only partially block insulin-induced glycogen

deposition in isolated hepatocytes. To determine whether DCA increased hepatocellular glycogen content by affecting glycogen synthase, we inhibited the insulin-controlled signaling pathways that regulate this enzyme (Cohen, 1999). The involvement of PI3K in mediating the glycogenic response of hepatocytes to DCA was assessed by using LY294002 and wortmannin, two structurally distinct PI3K inhibitors with different mechanisms of inhibition (Ui *et al.*, 1995; Vlahos *et al.*, 1994). Hepatocytes were pretreated with 25 μ M LY294002 or 100 nM wortmannin prior to the addition of DCA or insulin to the media. Others have demonstrated these doses of inhibitors do block insulin-stimulated PI3K activity (Cross *et al.*, 1995; Ui *et al.*, 1995; Vlahos *et al.*, 1994). Inhibitors remained in the media for the entire period of treatment. As in prior experiments, hepatocytes were incubated 16 h in the presence and absence of DCA (100 μ M) or insulin (100 nM). Whereas incubation with LY294002 or wortmannin alone had a minimal effect on basal levels of hepatocellular glycogen, both inhibitors completely blocked ($>85\%$, $p < 0.05$) the increase in glycogen induced by DCA (Fig. 5A).

As expected, insulin (100 nM) in the presence of dexamethasone (100 nM) significantly (>2 -fold, $p < 0.05$) increased hepatocellular glycogen content after 16 h (Figs. 3A–3C). Unlike DCA-stimulated glycogen synthesis, insulin-stimulated glycogen synthesis in hepatocyte monolayers was only partially inhibited ($\sim 50\%$) by the PI3K inhibitors (Fig. 3A).

Alternative signaling pathways, which may lead to stimula-

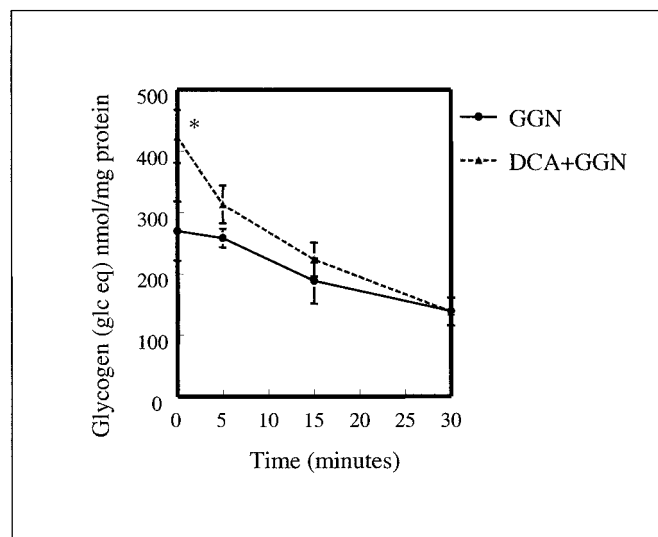


FIG. 2. Glucagon-induced glycogenolysis is not inhibited by DCA in isolated hepatocytes. Hepatocyte monolayers were treated for 16 h in the absence or presence of 100 μ M DCA and then were subjected to 10 nM glucagon (GGN), added at time = 0. In each experiment, glycogen levels were measured in 3 separate culture plates, and an average content was determined at each indicated time point. The data are from 3 experiments and are expressed as mean nmol glucose equivalents/mg protein \pm SEM. Experiments were analyzed using the Student's *t*-test at each time point. As expected from previous experiments, DCA significantly affected initial glycogen levels. *Significant difference between GGN and DCA+GGN-treated samples, $p \leq 0.05$.

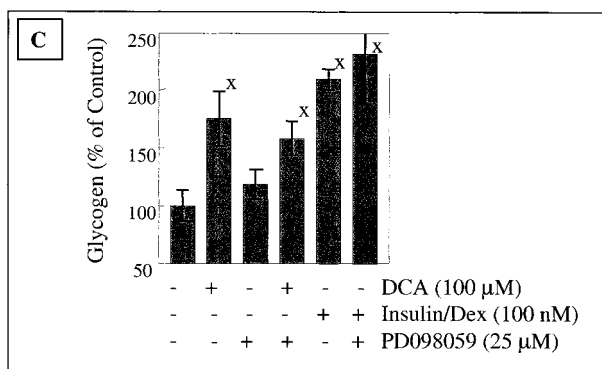
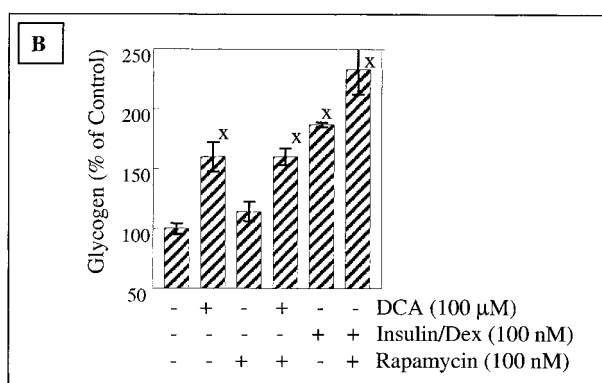
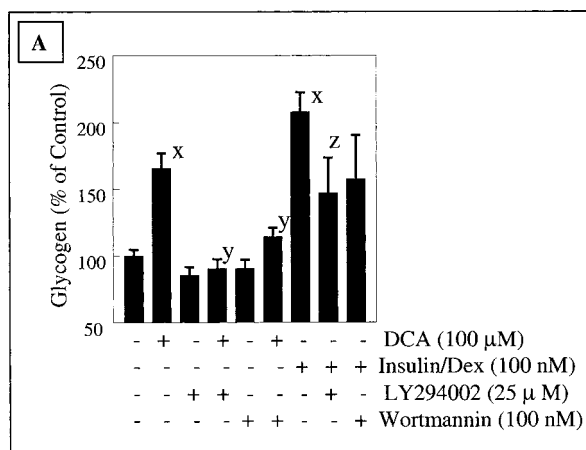


FIG. 3. Inhibitors of PI3K, but not $p70^{\text{S6K}}$ or Erk1/2, block the glycogenic response to DCA in isolated hepatocytes. Hepatocyte monolayers were pretreated with inhibitors 1 h prior to addition of DCA (100 μM) or insulin (100 nM) + 100 nM dexamethasone for 16 h. Inhibitors remained in the media during the entire treatment period. In each experiment, glycogen levels were measured in 3 separate culture plates, and an average content was determined for each treatment condition. (A) Hepatocyte monolayers were treated with PI3K inhibitor, LY294002 (25 μM) or wortmannin (100 nM) in the presence of DCA, insulin, or the absence of both. (B) Hepatocyte monolayers were treated with $p70^{\text{S6K}}$ inhibitor, rapamycin (100 nM) in the presence of DCA, insulin, or the absence of both. (C) Hepatocyte monolayers were treated with Erk1/2 kinase (MEK) inhibitor, PD098059 (25 μM) in the presence of DCA, insulin or, the absence of both. The data are taken from ≥ 3 experiments and are expressed as glycogen (% of control \pm SEM) in which the control is untreated hepatocyte monolayers. Significant differences, as detected by one-way ANOVA, between untreated and treated (DCA or insulin/dex) hepatocyte

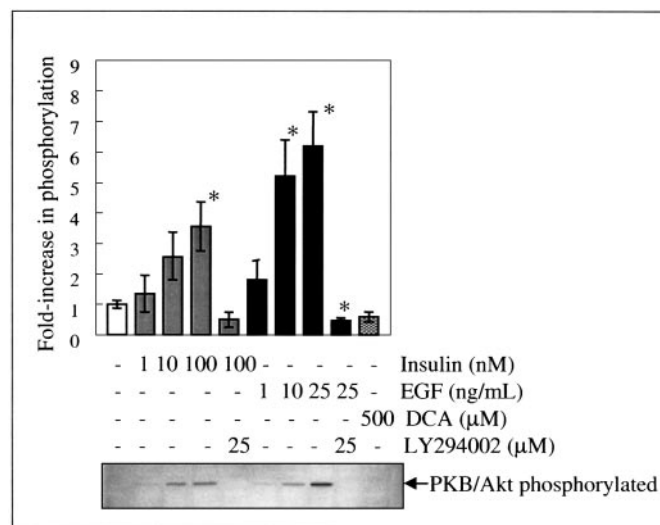


FIG. 4. Effect of insulin, EGF, and DCA on phosphorylation of PKB/Akt in isolated hepatocytes. Hepatocyte monolayers cultured on 60-mm plates were treated for 5 min with the indicated concentrations of insulin (+100 nM dexamethasone), EGF, and DCA. Cell lysates were prepared and subjected to immunoblotting analysis as described in the experimental section. Relative levels of phosphorylated PKB/Akt were quantified via NIH Image Software and expressed as the fold-increase above control \pm S.E.M from 4 independent experiments. *Significant differences as detected by one-way ANOVA between untreated and insulin, EGF or DCA-treated hepatocyte monolayers, $p \leq 0.05$. Below the graph is a representative immunoblot from which the quantitative results were obtained.

tion of glycogen synthase activity, include PI3K-dependent activation of the p70 ribosomal protein S6-kinase ($p70^{\text{S6K}}$) or activation of the Ras/Raf/MEK/Erk1/2 pathway (Azziazu *et al.*, 1996; Dent *et al.*, 1990; Shepherd *et al.*, 1995; Sutherland and Cohen, 1994). In primary mouse hepatocytes treated 16 h in the presence and absence of DCA and/or insulin, neither DCA- nor insulin-induced glycogen deposition was affected by rapamycin (10–100 nM) (Fig. 3B, data not shown).

Inhibition of the Erk1/2 kinase-signaling pathway via the MEK inhibitor, PD098059, also did not affect the increase in glycogen caused by DCA or insulin/dexamethasone in hepatocyte monolayers (Fig. 3C). Based on our previous work (Mounho and Thrall 1999), the concentration of PD098059 used in these experiments was sufficient to completely block Erk1/2 activation. Immunoblot analysis demonstrated that treatment of hepatocytes with DCA does not stimulate phosphorylation of Erk1/2 (data not shown).

DCA and insulin-induced glycogen deposition is PKB/Akt-independent. One of the most common downstream targets of PI3K reported to be involved in regulating glycogen synthesis is PKB/Akt (Cross *et al.*, 1995, 1997; Lawrence and

monolayers are indicated by X, $p \leq 0.05$; significant differences between the response to DCA in the presence and absence of inhibitors are indicated by Y, $p \leq 0.05$; and significant differences between the insulin + dexamethasone response in the presence and absence of inhibitors by Z, $p \leq 0.05$.

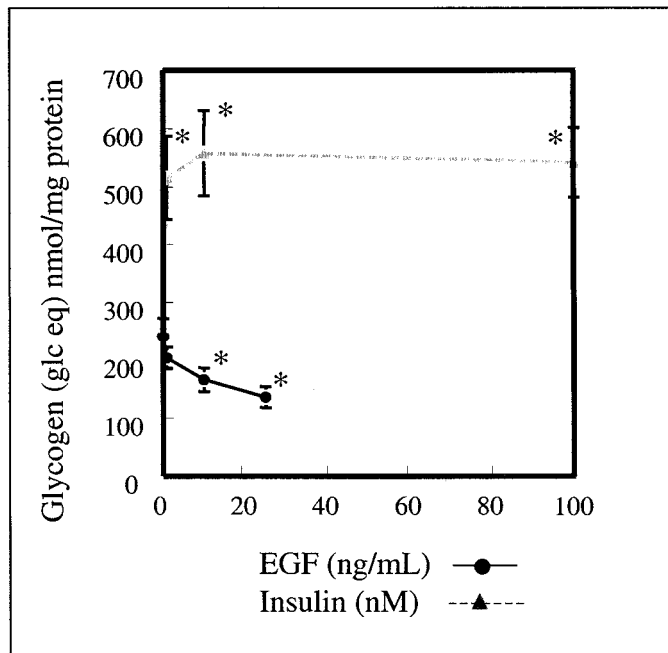


FIG. 5. Insulin, but not EGF, increases glycogen content in isolated hepatocytes. Hepatocyte monolayers were treated for 16 h with either increasing concentrations of insulin (1–100 nM) in the presence of dexamethasone or with increasing concentrations of EGF (1–25 ng/ml). In each experiment, glycogen levels were measured in 3 separate culture plates, and an average content was determined at each indicated concentration. The data are taken from 3 experiments and are expressed as the mean nmol glucose equivalents/mg protein \pm SEM. Experiments were analyzed using one-way ANOVA. *Significant difference between untreated and insulin/dex- or EGF-treated samples, $p \leq 0.05$.

Roach, 1997). Hepatocyte monolayers were treated for 5 min with increasing concentrations of insulin in the presence of 100 nM dexamethasone, increasing concentrations of epidermal growth factor (EGF, 1–25 ng/ml), or 500 μ M DCA. Lysates were immunoblotted with antibody that selectively recognized Ser⁴⁷³ phosphorylated PKB/Akt. Preliminary time-course experiments had delineated 5 min as the peak level of phosphorylation of PKB/Akt (data not shown). Insulin significantly increased PKB/Akt phosphorylation at doses of 100 nM (Fig. 4). The relative level of phosphorylation of PKB/Akt caused by 100 nM insulin was nearly 4-fold ($p < 0.05$) and was completely inhibited in the presence of 25 μ M LY294002 (Fig. 4). In contrast to insulin, DCA did not cause a measurable increase in PKB/Akt phosphorylation after 5-min treatment (Fig. 4), nor after 15, 30 or 60 min of treatment with DCA (10–500 μ M, data not shown). Therefore, the concentrations of DCA that stimulate hepatocellular glycogen accumulation do not cause PKB/Akt phosphorylation.

PKB/Akt is not an indicator of glycogen synthesis in isolated hepatocytes. As with insulin, EGF (1–25 ng/ml) treatment of hepatocytes results in an increase in PKB/Akt phosphorylation (>6-fold, $p < 0.05$) that is dependent on activation of PI3K

(Fig. 4). As observed in prior experiments by others (Chowdhury and Agius, 1987; Grau et al., 1996; Peak and Agius, 1994), EGF significantly decreased glycogen content of hepatocyte monolayers (Fig. 6). As before, insulin in the presence of dexamethasone increased glycogen content greatly ($p < 0.5$; Fig. 6).

DCA treatment downregulates IR expression in isolated hepatocytes. Hepatocyte monolayers treated 16 h with increasing concentrations of DCA, displayed a decrease in steady-state IR protein levels (Fig. 6). The decrease in IR expression was concentration-dependent, reaching a maximum reduction of approximately 50% ($p < 0.05$) at 100 μ M DCA (Fig. 6). As expected, IR protein expression levels were significantly reduced in hepatocytes treated with 100 nM insulin (Fig. 6).

DISCUSSION

DCA-induced glycogen deposition and effects on insulin signaling proteins seen *in vivo* (Kato-Weinstein et al., 1998; Lingohr et al., 2001; Stauber and Bull, 1997) were reproduced in isolated hepatocytes. The concentrations of DCA that increased glycogen accumulation in primary hepatocytes are in the same range of blood concentrations of DCA that increase glycogen in the liver of mice (Kato-Weinstein et al., 1998).

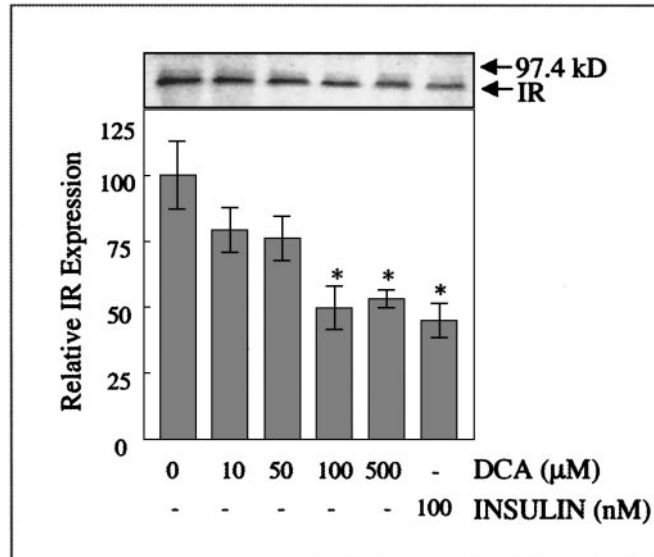


FIG. 6. DCA suppresses hepatocellular IR expression in isolated hepatocytes. Hepatocyte monolayers were treated for 16 h with increasing concentrations of DCA (0–500 μ M) or 100 nM insulin plus 100 nM dexamethasone. Cell lysates were prepared and subjected to immunoblotting analysis as described in the experimental section. Relative levels of IR protein were quantified via NIH Image Software and expressed as % of control \pm SEM. The data are taken from 3 experiments and were analyzed using one-way ANOVA. *Significant difference between untreated and DCA-treated samples, $p \leq 0.05$. Above the graph is a representative immunoblot from which the quantitative results were obtained.

The glycogenic effect of DCA in isolated hepatocytes was not related to a decrease in its degradation, as DCA was not effective in inhibiting glucagon-induced glycogen degradation. Also, we may infer from our data that glycogen accumulated with DCA treatment was susceptible to degradation. The increase in glycogen accumulation caused by DCA was similar to that of insulin, although it was independent of dexamethasone. Dexamethasone, which increases IR number in hepatocytes, is essential for insulin-induced glycogen synthase activation *in vitro* (Fleig *et al.*, 1985; Pugazenthi and Khandelwal, 1995).

In vivo, the temporal relationship of DCA's effects on liver glycogen, serum insulin, and hepatic expression of the IR suggest that the accumulation of glycogen induced by DCA treatment may lead to a compensatory downregulation of the IR and suppression of insulin. In isolated hepatocytes, the DCA-induced downregulation of the IR occurred in the absence of insulin. Earlier studies (Fleig *et al.*, 1985, 1987) have shown that pre-incubation of hepatocyte monolayers overnight with insulin downregulates IR binding via a decrease in IR on the cell surface, which then reduces the acute glycogenic response to insulin. When this occurs, there is an inverse correlation between glycogen content and insulin-induced glycogen synthase activity. These results support the hypothesis that the increased glycogen content induced by DCA treatment may trigger a regulatory pathway that results in downregulation of the IR, but through actions subsequent to the IR, because it does not require dexamethasone.

Selective inhibitors have proven to be useful probes for characterizing target signaling pathways involved in mediating particular insulin-induced biological responses (Cohen, 1999; Ui *et al.*, 1995). Based upon the inhibitor studies in isolated hepatocytes, the DCA-induced increase in glycogen is dependent on activation of PI3K. The concentrations of inhibitors that completely blocked the glycogenic response to DCA also significantly inhibited the accumulation of glycogen caused by insulin (in the presence of dexamethasone) in primary hepatocytes. However, the effects of the PI3K inhibitors on insulin-induced glycogen synthesis were only partial (<40%), suggesting that PI3K-independent signaling pathways also contribute to the glycogenic response to insulin in hepatocyte monolayers. This is not surprising since insulin stimulated GS activation is also dependent on the inactivation of protein kinase A and the activation of protein phosphatase-1 (PP1) (reviewed in Saltiel, 2001).

The serine/threonine kinase, PKB/Akt, is a downstream target of PI3K that has been shown to be involved in mediating the glycogenic response to insulin (Cheatham *et al.*, 1994; Osawa *et al.*, 1996; Shepherd *et al.*, 1995). Upon its activation by insulin, PKB/Akt phosphorylates GSK-3, leading to its inactivation and the enhancement of GS activity (Cross *et al.*, 1995, 1997; Ueki *et al.*, 1998). Although insulin stimulated PKB/Akt phosphorylation in hepatocyte monolayers, DCA did not affect PKB/Akt phosphorylation, even at concentrations

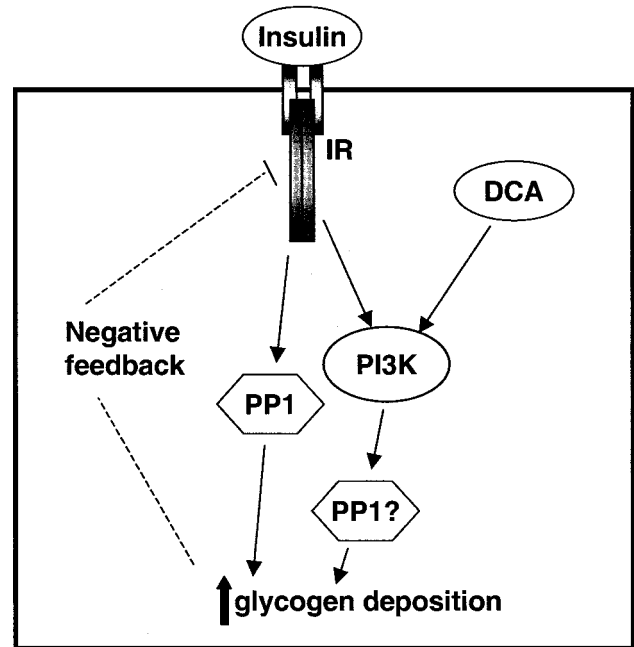


FIG. 7. DCA-induced glycogen accumulation is similar to insulin-induced glycogen deposition in hepatocytes. DCA increases glycogen deposition in hepatocytes by a PI3K-dependent mechanism. However, its effects appear to be downstream of the insulin receptor (IR). The increase in hepatocellular glycogen caused by DCA may negatively influence insulin-controlled signaling pathways by downregulating the IR. PI3K, phosphatidylinositol-3'-kinase; PP1, protein phosphatase 1.

that resulted in maximal increases in glycogen content. It is notable that the concentration of insulin required to produce a significant increase in PKB/Akt phosphorylation was 10-fold higher than that necessary to increase glycogen accumulation, suggesting that other PI3K-dependent signaling pathways (which could include PP1 activation) are more important in mediating the glycogenic response to insulin in hepatocytes.

The conflict between GSK-3 inhibition (via PKB/Akt) and increased glycogen synthesis has been described in adipocytes (Brady *et al.*, 1998) and more recently in hepatocytes (Lavoie *et al.*, 1999). Activation of PI3K is required for stimulation of GS in hepatocytes, but PKB/Akt activation and subsequent GSK3 inactivation is not sufficient to induce GS activation. This was reinforced in the present study by the finding that EGF activated PKB/Akt but did not increase glycogen synthesis. While our results strongly support a role for PI3K in DCA- and insulin-stimulated glycogen synthesis, it appears that PKB/Akt phosphorylation is neither a required nor a sufficient stimulus for glycogen synthesis in primary mouse hepatocytes. Our results also do not support a role for a rapamycin-sensitive signaling pathway or one involving the Ras/Raf/MEK/Erk1/2 signaling pathway in DCA- and insulin-induced glycogen synthesis.

The most important aspect of our results is the observation that DCA stimulates glycogen accumulation in hepatic paren-

chymal cells in the absence of insulin. In Figure 7, a diagram outlining the pathways implicated in DCA-induced glycogen deposition is shown. In turn, we hypothesize that the marked increase in hepatocellular glycogen caused by DCA may negatively influence insulin-controlled signaling pathways by downregulating the IR. The signaling molecules involved in DCA-stimulated glycogen accumulation downstream of PI3K are unknown. However one of the most likely modifiers is PP1, whose activity is highly correlative with changes in glycogen synthase activity (Brady et al., 1998). Insulin-stimulated PP1 activity is dependent on PI3K activation at least, in cardiomyocytes (De Luca et al., 1999). Therefore, it is a prospective candidate for mediating DCA-stimulated glycogen deposition in isolated hepatocytes.

PI3K is not only associated with glycogen synthesis but is also known to regulate cell proliferation and apoptosis in hepatocytes (Band et al., 1999; Mounho and Thrall, 1999). DCA-treatment suppresses replication of normal hepatocytes (Stauber and Bull, 1997), which could be a result of PI3K downregulation. Interestingly, cells within liver tumors induced by DCA are consistently glycogen-poor and have elevated expression of the IR in contrast to normal hepatocytes (Bull et al., 1990; Lingohr et al., 2001). It appears then that the signaling pathways by which DCA stimulates glycogen accumulation in normal mouse hepatocytes may be differentially regulated in precancerous cells, which are promoted to proliferate and form tumors by chronic DCA exposure (Stauber and Bull, 1997). Further identification of the signaling molecules affected by DCA should not only provide insight into the mechanisms by which DCA stimulates glycogen accumulation, but may also provide insights into mechanisms involved in DCA-induced tumor promotion.

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