Insulin and Glucagon Regulation of Glutathione S-Transferase Expression in Primary Cultured Rat Hepatocytes

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ABSTRACT
Diabetes is a major cause of morbidity and mortality, and complications resulting from diabetes have been attributed in part to increased oxidative stress. Glutathione S-transferases (GSTs) constitute a major protective mechanism against oxidative stress. Studies of the expression and activity of GSTs during diabetes are inconclusive, with both increased and decreased GST expression being reported in vivo. Insulin and glucagon effects on GST expression and the signaling pathway involved in the glucagon regulation of GST expression were examined in primary cultured rat hepatocytes. The addition of insulin resulted in the elevation of alpha-class GST protein levels, whereas glucagon concentrations resulted in increased GST activities and alpha-class GST protein levels, whereas glucagon concentrations resulted in decreased alpha- and pi-class protein levels and activity. Treatment of cells with 8-bromo-cAMP or dibutyryl-cAMP also resulted in decreased alpha- and pi-class GST protein levels. Pretreatment with N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89), a selective inhibitor of protein kinase A, before glucagon addition markedly attenuated the glucagon effect. This study demonstrates that insulin and glucagon regulate, in an opposing manner, the expression of alpha-class GSTs and that glucagon completely inhibits pi-class GST expression in vitro, suggesting that hepatic GST expression may be decreased during diabetes. Furthermore, the present study implicates cAMP and protein kinase A in mediating the inhibitory effect of glucagon on GST expression.
that insulin, in the absence of other hormonal or metabolic factors, dramatically decreases CYP2E1 mRNA and protein levels, while having little effect on CYP2B, 3A, or 4A mRNA levels (Woodcroft and Novak, 1997, 1999). A phosphatidylinositol 3-kinase (PI3-kinase) signaling pathway has been implicated in mediating the negative effect of insulin on CYP2E1 (Woodcroft et al., 2002).

The effects of diabetes or insulin on hepatic GST activities and expression, however, are controversial. An increase in hepatic GST activity was reported in streptozotocin-induced diabetic mice, but not in spontaneously (db/db and ob/ob) or alloxan-induced diabetic mice (Rouer et al., 1981; Agius and Gidari, 1985). In contrast, Thomas et al. (1989) reported that hepatic GST activity was decreased in chemical-induced diabetic rats and restored by insulin administration. The reason for this discrepancy remains unknown but may be due, in part, to the use of primarily nonselective GST enzymatic activities as indirect indicators of GST expression. However, it may also be associated with the opposing metabolic effects of insulin and glucagon and, hence, be related to glucagon levels in hepatic tissue. Another important condition affecting GST expression is oxidative stress, usually observed in diabetes. It has been reported that transcriptional activation of some GST genes may be associated with the change in the redox state in conjunction with oxidative stress (Wasserman and Fahl, 1997; Kang et al., 2001). Thus, the relative influence of hormones versus oxidative stress during diabetes may be the determining factor for regulation of GST levels and activity.

The objectives of the present study were to characterize, using primary cultured rat hepatocytes, the effects of insulin or glucagon on GST expression and activity and the signaling pathways involved in glucagon regulation of GST expression. We have previously reported a primary rat hepatocyte culture system that is responsive to xenobiotic-mediated increase in alpha-, mu-, and pi-class GST expression in a manner that parallels that monitored in vivo ( Dwivedi et al., 1993). We have used this primary rat hepatocyte culture system to demonstrate that culturing hepatocytes in the presence of insulin results in increased levels of GSTA1/2 and GSTA3/5 protein and GST activities. In contrast, treatment of cells with glucagon or cAMP analogs decreased GSTA1/2, GSTA3/5, and most notably GSTP1, protein expression and GST activities, and inhibition of protein kinase A (PKA) attenuated the glucagon effect on GST expression. These results provide novel data on insulin and glucagon regulation of GST expression in primary cultured rat hepatocytes and suggest that the relative levels of these hormones contribute to the modulation of the in vivo expression of the alpha- and pi-class GSTs.

**Materials and Methods**

**Chemicals.** Modified Chee’s medium and 1-glutamine were obtained from In VitroGen (Carlsbad, CA). Insulin (Novolin R) was purchased from Novo-Nordisk (Princeton, NJ). Collagenase (type I) was purchased from Worthington Biochemicals (Freehold, NJ). Vitrogen (95–98% type I collagen, 2–5% type III collagen) was obtained from Cohesion Technologies (Santa Clara, CA). Class-specific GST antibodies were prepared and characterized previously by our laboratory (Primiano et al., 1992; Gandy et al., 1996). Horseradish peroxidase-conjugated rabbit anti-goat antibody was obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Enhanced chemiluminescence reagents were purchased from Amersham Biosciences, Inc. (Piscataway, NJ). H89, Br-cAMP, and DB-cAMP were obtained from Calbiochem (La Jolla, CA). Glucagon, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD), etharycnic acid (EA) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Primary Rat Hepatocyte Cultures.** Hepatocytes were isolated from the livers of male Sprague-Dawley rats (250–350 g) using collagenase perfusion, as described previously (Woodcroft and Novak, 1997, 1999). Hepatocytes were plated onto dishes covalently coated with Vitrogen, and modified Chee’s medium was fortified as described (Woodcroft and Novak, 1997, 1999) and supplemented with 0.1 μM dexamethasone and 1 μM insulin. Cells were plated at a density of 3 × 10⁶ cells/60-mm dish or 10 × 10⁶ cells/100-mm dish. Four hours after plating, the medium was replaced with medium containing various concentrations of insulin (0–100 nM), glucagon (0–100 nM), Br-cAMP (0–100 μM), or DB-cAMP (0–100 μM). Control hepatocytes were cultured in the absence of insulin or glucagon. The PKA inhibitor H89 (0–25 μM) was added 1.5 h before the addition of glucagon (100 nM). The medium was changed every 24 h thereafter.

Toxicity was monitored by measuring released lactate dehydrogenase (LDH) activity, as described previously (Woodcroft and Novak, 1997). None of the treatments resulted in increased cell toxicity compared with control.

**Immunoblot Analysis.** Immunoblot analysis of whole cell lysates was used to examine the protein levels of GSTs. Cells were washed with 3 ml of 4°C phosphate-buffered saline (PBS; pH 7.4) and then lysed in 50 mM Hepes (pH 7.2), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM MnCl₂, 1 mM sodium orthovanadate, leupeptin (10 μg/ml), aprotonin (10 μg/ml), and 2 mM phenylmethylsulfonyl fluoride. Cells were scraped into lysis buffer, and the lysates were transferred into Eppendorf tubes and passed through a 25-gauge needle. Samples were incubated on ice for 30 min, and the lysates were centrifuged at 16,000g for 20 min at 4°C. The supernatant is termed the whole-cell lysate. Protein concentrations were determined using the bicinchoninic acid protein assay (Sigma-Aldrich).

For immunoblot analysis, protein samples (5–50 μg of protein per lane) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% gel, transferred to a nitrocellulose membrane (Bio-Rad, Inc., Hercules, CA), and blocked for 2 h in 5% milk powder in PBS-T (0.05% Tween 20 in PBS). For immunodetection, blots were incubated overnight with goat anti-rat GSTA1/2, GSTA3/5, GSTM1/2, or GSTP1 antibody (diluted in 5% milk powder in PBS-T) at room temperature, followed by incubation with secondary antibody conjugated to horseradish peroxidase (diluted 1:10,000 in 5% milk powder in PBS-T) for 3 h at room temperature. Proteins were detected by enhanced chemiluminescence on Kodak X-Omat film (Sigma-Aldrich) and quantified by densitometry with a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences, Inc.).

**Metabolic Assays.** The activities of GSTs in whole cell lysates were measured using CDNB, DCNB, NBD, and EA as substrates. The GST activity toward CDNB, DCNB, or EA was measured by the method of Habig et al. (1974), and the GST activity toward NBD was determined using the method of Ricci et al. (1994).

**Statistical Analysis.** Significant differences between groups were determined by ANOVA followed by the Newman-Keuls comparison test (P < 0.05).

**Results**

**Time-Dependence of Insulin or Glucagon Effects on GST Protein Levels and Activities.** GST protein levels were monitored in primary rat hepatocytes cultured in the
absence of insulin or glucagon for 4 days after plating (Fig. 1). The protein levels of alpha-class GSTs were maintained for 1 day after plating, compared with freshly isolated hepatocytes (Fig. 1, A and B), with a slight decline noticed at day 2. GSTA1/2 protein levels decreased further to ~40% of the level in freshly isolated hepatocytes by days 3 and 4 (Fig. 1A), whereas GSTA3/5 protein levels declined to ~50% at day 3 (Fig. 1B) and then increased to ~80% on day 4. The protein levels of mu-class GSTs, however, were unchanged during the culture period (data not shown). GSTP1 was not expressed in freshly isolated hepatocytes or in hepatocytes cultured for one or two days, but was expressed at detectable levels at day 3 and at 4-fold higher levels by day 4 (Fig. 1C).

The effects of insulin (100 nM) or glucagon (100 nM) on GST protein levels were monitored in hepatocytes over 4 days of treatment (Fig. 1). GSTA1/2 protein levels were increased 1.2- to 3.3-fold by insulin treatment, relative to control cells, over 4 days of culture (Fig. 1A). The presence of insulin elevated GSTA3/5 protein levels 1.6- to 2.8-fold, relative to control hepatocytes, over the 4-day culture period (Fig. 1B). In contrast, the addition of glucagon for 1, 2, or 3 days resulted in a 30% decrease in GSTA1/2 protein levels relative to control cells at identical time points, although the data are not statistically significant (Fig. 1A). GSTA3/5 subunit levels were significantly decreased, by 70 to 80%, in hepatocytes cultured for 1 to 4 days in the presence of glucagon compared with control hepatocytes at identical time points (Fig. 1B). Neither insulin nor glucagon altered GSTM1/2 protein levels, with the exception of 2-day glucagon treatment, which decreased GSTM1/2 levels slightly to ~80% of the level monitored in control hepatocytes at this time point (data not shown). The protein levels of GSTP1 were not significantly altered in response to insulin (Fig. 1C). In contrast, GSTP1 protein was decreased near the limit of detectibility in response to glucagon treatment (Fig. 1C).

GST activities were measured in primary cultured rat hepatocytes for 4 days after plating (Table 1). The GST activities toward CDNB and DCNB, standard nonselective substrates for nearly all GSTs, were decreased by ~20 to 40% from 1 day after plating, compared with freshly isolated hepatocytes, and remained relatively unchanged throughout the remainder of the culture period (Table 1). The activity toward NBD, a selective substrate for the alpha-class GSTs (Ricci et al., 1994), progressively declined to 44% of the level observed in freshly isolated hepatocytes after 4 days in culture in the absence of hormones (Table 1), paralleling changes in alpha-class GST protein expression (Fig. 1). The activity toward EA, a traditional substrate for pi-class GSTs (Awasthi et al., 1993), appeared to be relatively constant throughout this time period (Table 1). This result was unexpected, as expression of GSTP1 was not detected in freshly isolated hepatocytes or at day 1 or 2, but was markedly increased at days 3 and 4 after plating. These data suggest that EA may not be metabolized exclusively by pi-class GST.

The effect of insulin (100 nM) or glucagon (100 nM) on GST activity was also examined in hepatocytes over 4 days of culture. The presence of insulin resulted in elevation of GST activity toward EA, a traditional substrate for pi-class GSTs expected, as expression of GSTP1 was not detected in freshly isolated hepatocytes or in hepatocytes cultured for one or two days, but was expressed at detectable levels at day 3 and at 4-fold higher levels by day 4 (Fig. 1C).

In contrast, GST activity decreased toward CDNB or DCNB in hepatocytes cultured with glucagon (100 nM) from 2 to 4 days (Table 1). Treatment of cells with insulin (100 nM) for 3 or 4 days resulted in an ~2-fold increase in GST activity toward NBD (Table 1). The presence of glucagon (100 nM) progressively decreased this activity to 34% of the level monitored in corresponding control hepatocytes after 4 days in culture (Table 1). GST activity toward EA was increased 1.4-fold in hepatocytes treated with insulin for 3 or 4 days and decreased by 35% in hepa-

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**Fig. 1.** Immunoblot analysis of effects of insulin (100 nM) or glucagon (100 nM) on protein levels of GSTA1/2 (A), GSTA3/5 (B), and GSTP1 (C) in primary cultured rat hepatocytes. After a 4-h plating period in the presence of 1 µM insulin, hepatocytes were maintained in the absence of hormone (control) or in the presence of 100 nM insulin or 100 nM glucagon for 1, 2, 3, or 4 days. Day 0 refers to freshly isolated hepatocytes, and n.d. is not detectable. GSTA1/2 and GSTA3/5 levels are plotted as a percentage of the GST levels monitored in fresh hepatocytes (100%) and GSTP1 levels are plotted as a percentage of the GST levels monitored in 4-day control hepatocytes. Data are means ± S.D. of immunoblot band densities of three preparations of cell lysates. *, **, ***, significantly different than levels monitored in corresponding control hepatocytes, *P* < 0.05, **P** < 0.01, or ***P*** < 0.001, respectively.
TABLE 1
Effects of insulin (100 nM) or glucagon (100 nM) on GST activities toward CDNB, DCNB, NBD, and EA

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>CDNB</th>
<th>DCNB</th>
<th>NBD</th>
<th>EA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% 0 day</td>
<td>% 0 day</td>
<td>% 0 day</td>
<td>% 0 day</td>
</tr>
<tr>
<td>0 day</td>
<td>Control</td>
<td>100 ± 4</td>
<td>100 ± 6</td>
<td>100 ± 11</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>1 day</td>
<td>Control</td>
<td>78 ± 8</td>
<td>57 ± 9</td>
<td>90 ± 12</td>
<td>91 ± 5</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>86 ± 5</td>
<td>67 ± 4</td>
<td>110 ± 12*</td>
<td>94 ± 9</td>
</tr>
<tr>
<td></td>
<td>Glucagon</td>
<td>79 ± 3</td>
<td>81 ± 13</td>
<td>64 ± 11*</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>2 day</td>
<td>Control</td>
<td>80 ± 4</td>
<td>76 ± 6</td>
<td>67 ± 4</td>
<td>89 ± 11</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>86 ± 3*</td>
<td>70 ± 4</td>
<td>119 ± 25*</td>
<td>97 ± 9</td>
</tr>
<tr>
<td></td>
<td>Glucagon</td>
<td>71 ± 3*</td>
<td>52 ± 21*</td>
<td>55 ± 2</td>
<td>88 ± 16</td>
</tr>
<tr>
<td>3 day</td>
<td>Control</td>
<td>72 ± 7</td>
<td>78 ± 13</td>
<td>50 ± 5</td>
<td>76 ± 14</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>88 ± 4*</td>
<td>92 ± 5</td>
<td>100 ± 2*</td>
<td>112 ± 6*</td>
</tr>
<tr>
<td></td>
<td>Glucagon</td>
<td>65 ± 2</td>
<td>56 ± 7*</td>
<td>23 ± 3*</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>4 day</td>
<td>Control</td>
<td>71 ± 2</td>
<td>68 ± 5</td>
<td>44 ± 5</td>
<td>112 ± 16</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>87 ± 5*</td>
<td>83 ± 4*</td>
<td>93 ± 2*</td>
<td>153 ± 17*</td>
</tr>
<tr>
<td></td>
<td>Glucagon</td>
<td>56 ± 3*</td>
<td>44 ± 7*</td>
<td>15 ± 5*</td>
<td>73 ± 12*</td>
</tr>
</tbody>
</table>

* Significantly different than levels monitored in corresponding control hepatocytes, P < 0.05.

TABLE 2
Changes in GST activities toward NBD and EA in primary cultured rat hepatocytes maintained in culture in the presence of various concentrations of insulin for 3 days

<table>
<thead>
<tr>
<th>NBD</th>
<th>EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>% control</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>0.1 nM Insulin</td>
<td>121 ± 22</td>
</tr>
<tr>
<td>1 nM Insulin</td>
<td>142 ± 21*</td>
</tr>
<tr>
<td>10 nM Insulin</td>
<td>175 ± 10*</td>
</tr>
<tr>
<td>100 nM Insulin</td>
<td>183 ± 7*</td>
</tr>
</tbody>
</table>

* Significantly different than levels monitored in control hepatocytes, P < 0.05.

tocytes treated with glucagon for 4 days, relative to corresponding control hepatocytes (Table 1). These changes in metabolic activity, with the exception of EA, generally paralleled the changes in protein levels monitored by immunoblot analysis.

Concentration-Dependence of Insulin or Glucagon Effects on GST Protein Levels and Activities. To examine the concentration-dependence of insulin on levels of GST proteins and GST activities, hepatocytes were cultured for 3 days in medium supplemented with 0 to 100 nM insulin (Fig. 2, Table 2). GST alpha-class protein levels were increased significantly in a concentration-dependent manner (Fig. 2, A and B). GSTA1/2 protein levels were increased 134, 135, 171, or 201% in the presence of 0.1, 1, or supraphysiologic levels of 10 or 100 nM insulin, respectively, compared with hepatocytes maintained in culture in the absence of insulin (Fig. 2A). Treatment of hepatocytes with 0.1, 1, 10, or 100 nM insulin increased GSTA3/5 protein levels to 179, 146, 263, or 201% in the presence of 0.1, 1, or supraphysiologic levels of insulin (Table 2). The maximum elevated activity toward NBD or EA was ~183 or 194%, respectively, of the level in hepatocytes cultured without insulin (Table 2). The activity toward the nonelective substrates CDNB or DCNB was also increased in a concentration-dependent manner in response to insulin (data not shown).

The concentration-dependence of glucagon effects on GST protein levels and GST activities was determined in hepatocytes cultured for 3 days in the presence of 0 to 100 nM glucagon (Fig. 3, Table 3). The decrease in protein levels of GST alpha- and pi-class was monitored at 20 nM glucagon, with the most striking effect monitored for GST pi class, and no further decrease for either class of GST was observed up to 100 nM glucagon (Fig. 3). GSTA1/2 and 3/5 protein levels decreased to ~50% and 30% of control levels, respectively. In
The physiological effects of glucagon are mediated by elevation of cellular cAMP levels and activation of cAMP-dependent PKA. To examine whether glucagon effects on GST expression are associated with elevated cAMP levels, changes in GST protein levels were measured in hepatocytes cultured for 3 days in the presence of membrane-permeable cAMP analogs (Figs. 4 and 5). The addition of Br-cAMP resulted in a 40% decrease in GSTA1/2 protein levels at 100 μM. In contrast, GSTA3/5 protein levels were decreased ~50% by 10 μM Br-cAMP and were decreased maximally by 75 to 80% at 25 μM Br-cAMP. Most notably, virtually complete suppression of GSTP1 protein expression occurred at 25 μM Br-cAMP (Fig. 4).

DB-cAMP, another cAMP analog, significantly decreased the protein levels of GSTA1/2 and GSTA3/5 at concentrations ≥10 μM and decreased GSTP1 protein levels at concentrations as low as 1 μM (Fig. 5). The elevation of GSTP1 protein, which was observed in primary hepatocytes maintained in culture in the absence of hormones for greater than 3 days (Fig. 1C), was completely suppressed by the presence of 25 μM DB-cAMP. However, GST mu-class proteins remained relatively unchanged by each cAMP analog up to 100 μM (data not shown). Thus, treatment of hepatocytes with cAMP analogs (Br-cAMP and DB-cAMP) resulted in the same effect produced by glucagon treatment, including most notably that of decreased GSTP1 expression. These data implicate glucagon and the cAMP-dependent PKA signaling pathway in the regulation of GSTP1 expression.

Effect of the PKA Inhibitor H89 on the Glucagon-Mediated Decrease in GST Protein Levels. To investigate further the role of PKA in mediating the effect of glucagon on GST proteins, primary cultured rat hepatocytes were treated with the PKA inhibitor H89 (1–25 μM) 1.5 h before the addition of 100 nM glucagon. Under these conditions, H89 alone, at a concentration of 25 μM, did not affect GSTA1/2, GSTA3/5, or GSTP1 protein levels relative to hepatocytes treated with vehicle only (data not shown). H89 (1 μM) partially reversed the decline in GSTA1/2 levels resulting from 100 nM glucagon treatment (Fig. 6A). Elevated concentrations of H89 (10 and 25 μM) completely reversed the glucagon-mediated suppression of GSTA1/2 protein levels. The levels of GSTA3/5 protein were also restored to levels observed in control hepatocytes at H89 concentrations ≥10 μM (Fig. 6B). Thus, the glucagon-mediated suppressive effect on alpha-class GST proteins was completely reversed by H89 at 10 μM. H89 (25 μM) also partially prevented the glucagon-mediated decrease in GSTP1 protein levels, resulting in GSTP1 protein levels of ~40% of the level monitored in control hepatocytes, P < 0.05.

### Table 3

Changes in GST activities toward NBD and EA in primary cultured rat hepatocytes maintained in culture in the presence of various concentrations of glucagon for 3 days.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>NBD Activity</th>
<th>EA Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 11</td>
<td>100 ± 23</td>
</tr>
<tr>
<td>1 nM Glucagon</td>
<td>90 ± 13</td>
<td>86 ± 13</td>
</tr>
<tr>
<td>20 nM Glucagon</td>
<td>66 ± 7*</td>
<td>85 ± 13</td>
</tr>
<tr>
<td>100 nM Glucagon</td>
<td>45 ± 16*</td>
<td>86 ± 13</td>
</tr>
</tbody>
</table>

*Significantly different than levels monitored in control hepatocytes, P < 0.05.

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**Fig. 3.** Immunoblot analysis of GSTA1/2 (A), GSTA3/5 (B), and GSTP1 (C) protein levels in primary cultured rat hepatocytes maintained in culture in the presence of various concentrations of glucagon for 3 days. Values are shown as a percentage of the GST levels monitored in hepatocytes cultured in the absence of glucagon (100%). Data are means ± S.D. of immunoblot band densities of three preparations of cell lysates. *, **, significantly different than levels monitored in hepatocytes cultured in the absence of glucagon, P < 0.05 or P < 0.01, respectively.

Effect of the PKA Inhibitor H89 on the Glucagon-Mediated Decrease in GST Protein Levels. To investigate further the role of PKA in mediating the effect of glucagon on GST proteins, primary cultured rat hepatocytes were treated with the PKA inhibitor H89 (1–25 μM) 1.5 h before the addition of 100 nM glucagon. Under these conditions, H89 alone, at a concentration of 25 μM, did not affect GSTA1/2, GSTA3/5, or GSTP1 protein levels relative to hepatocytes treated with vehicle only (data not shown). H89 (1 μM) partially reversed the decline in GSTA1/2 levels resulting from 100 nM glucagon treatment (Fig. 6A). Elevated concentrations of H89 (10 and 25 μM) completely reversed the glucagon-mediated suppression of GSTA1/2 protein levels. The levels of GSTA3/5 protein were also restored to levels observed in control hepatocytes at H89 concentrations ≥10 μM (Fig. 6B). Thus, the glucagon-mediated suppressive effect on alpha-class GST proteins was completely reversed by H89 at 10 μM. H89 (25 μM) also partially prevented the glucagon-mediated decrease in GSTP1 protein levels, resulting in GSTP1 protein levels of ~40% of the level monitored in control hepatocytes, P < 0.05.
control cells, compared with undetectable levels in the presence of 100 nM glucagon (Fig. 6C).

**Discussion**

A number of observations illustrate that spontaneous and alloxan or streptozotocin-induced insulin-dependent diabetes in animals results in increased levels of CYP 2B, 2E1, 3A, and 4A protein and activity (Bellward et al., 1988; Barnett et al., 1990). In contrast, the data on the effects of diabetes on GST expression are variable with reports indicating both decreases and increases (Rouer et al., 1981; Agius and Gi-

dari, 1985; Thomas et al., 1989; Mukherjee et al., 1994). Considering that oxidative stress is usually observed in diabetes and plays an important role in regulating GST expression, both oxidative stress and hormonal conditions may affect GST expression in diabetes. To examine the singular effects of insulin or glucagon on hepatic GST expression, we employed primary cultured rat hepatocytes. We have reported that this system is responsive to xenobiotic-mediated increases in alpha-, mu-, and pi-class GST expression in a manner that parallels that monitored in vivo (Dwivedi et al., 1993).

The results of the present study demonstrate that alpha-class GST levels are fairly constant for 2 days after plating, but decline after 3 to 4 days in control hepatocytes. mu-Class
The activities toward CDNB or DCNB in cultured hepatocytes decreased to ~60 to 75% of the initial level observed in freshly isolated hepatocytes. These compounds have long served as standard substrates for nearly all GSTs, although the specific activities can vary greatly for the different GSTs. Therefore, these activities are not representative of individual GST expression. The decrease in activity toward NBD, a selective substrate for the alpha-class GSTs, was observed from 2 days after plating. The diuretic EA has been used as a selective substrate of pi-class GST (Awasthi et al., 1993). The activity toward EA was detected in freshly isolated hepatocytes and did not increase with time and elevated GSTP1 expression, suggesting that other GST family members may also catalyze this reaction. Other reports have also suggested that alpha-(Stenberg et al., 1992), mu- (Barycki and Colman, 1993), and theta- (Hiratsuka et al., 1990) class GSTs contribute to EA conjugation in rat. However, Henderson et al. (1998) reported that EA metabolism is lost in the liver of the GSTP knockout mouse. Moreover, GSTP is expressed in normal adult mouse liver, and hepatic GST activity toward EA is severalfold greater in adult mouse relative to rat. Thus, hepatic expression of pi-class GST, and GST specificity toward EA, appears to be species-specific.

It was reported that the intravenous injection of insulin plus glucose resulted in an increase in GST activity toward CDNB within 30 min, and the injection of glucagon resulted in a decline in this activity in an acute animal study (Carrillo et al., 1995). However, it has been reported that insulin or glucagon addition to cultured rat hepatocytes did not affect GST activity (Gebhardt et al., 1990). The discrepancy in the literature regarding changes in GST in response to hormones or diabetes in both rats and mice may stem from the use of enzymatic activities, especially using nonselective substrates, as an indicator of GST expression. Our immunoblot results showed that addition of insulin to hepatocytes resulted in increased alpha-class GST protein levels. And glucagon, a physiological antagonist of insulin, regulated this effect of insulin. In addition, the changes in GST activity toward NBD were correlated with the results of immunoblot analysis for alpha-class GSTs. These results demonstrate that insulin and glucagon can serve as physiological regulators of the expression of alpha-class GSTs. Alpha-Class GSTs are one of the major classes of GSTs expressed in adult liver and exhibit peroxidase activity toward lipid peroxides (Mannervik et al., 1985; Stenberg et al., 1992), mu- (Barycki and Colman, 1993), and theta- (Hiratsuka et al., 1990) class GSTs contribute to EA conjugation in rat. However, Henderson et al. (1998) reported that EA metabolism is lost in the liver of the GSTP knockout mouse. Moreover, GSTP is expressed in normal adult mouse liver, and hepatic GST activity toward EA is severalfold greater in adult mouse relative to rat. Thus, hepatic expression of pi-class GST, and GST specificity toward EA, appears to be species-specific.

The physiologic range of normal rat liver insulin concentrations has been reported as 0.4 to 2 nM (average, 1.2 nM) (Balks and Jungermann, 1984). Previously, we reported that tyrosine phosphorylation of insulin receptor was detected at an insulin concentration of 1 nM and was markedly increased at 10 nM insulin (Woodcroft et al., 2002). In the present study, significant changes in alpha-class GST activities or
protein levels were observed at 1 or 10 nM insulin, respectively. These data implicate a correspondence between insulin receptor-mediated activation of insulin signaling and the increase in alpha-class GST protein levels and activity in primary cultured rat hepatocytes. These results also suggest that the titration region for these events occurs in the physiological range of insulin concentration.

The regulation of GSTs is subject to a complex set of endogenous and exogenous parameters. These include developmental-, gender-, and tissue-specific factors, as well as a large number of xenobiotic-inducing agents such as polycyclic aromatic hydrocarbons, phenolic antioxidants, Michael acceptors, reactive oxygen species, isothiocyanates, trivalent arsenicals, barbiturates, and synthetic glucocorticoids (Hayes and Pulford, 1995). Reactive oxygen species and electrophiles induce some GSTs through activation of antioxidant response element (ARE), which involves Nrf proteins and Maf family members (Wasserman and Fahl, 1997; Kang et al., 2001). Recently, it has been reported that PI3 kinase is responsible for ARE-mediated antioxidant gene induction in H4IIE rat hepatoma and IMR-32 human neuroblastoma cells treated with tert-butylhydroquinone, which produces reactive oxygen species by redox cycling (Kang et al., 2001; Lee et al., 2001). However, it has been shown that the mitogen-activated protein (MAP) kinases are activated by oxidative stress and are involved in antioxidant gene induction via ARE activation (Kong et al., 2001). The cellular effects of insulin are mediated through activation of both PI3-kinase and MAP kinase signaling pathways. These observations raise the possibility that the insulin effects on GST expression may be mediated through activation of insulin-dependent signaling pathways, and preliminary data suggest that activation of PI3-kinase is involved in the insulin-mediated increase in alpha-class GST protein (S. K. Kim, K. J. Woodcroft, and R. F. Novak, unpublished observations).

The regulation of pi-class GST is of interest because its expression is significantly increased in many human tumors, in human cell lines made resistant to chemotherapeutic agents, and during hepatocarcinogenesis in rats (Tee et al., 1992; Tsuda and Sato, 1992). Thus, the presence of immunodetectable GSTP1 in rat liver is frequently used as an early marker of hepatic neoplasia (Tahir et al., 1989). Adler et al. (1999) showed that GSTP1 protein could act as an inhibitor of the Jun N-terminal kinase pathway, a stress-activated signaling pathway, indicating that GSTP1 protein can serve as a regulator of cell signaling pathways. In NIH 3T3 cells, GSTP protein protected against cell death induced by reactive oxygen species by controlling the balance of kinase activity elicited by Jun N-terminal kinase versus other cellular kinases, such as extracellular-signal-regulated kinase, nuclear factor κB, and p38 MAP kinase (Yin et al., 2000). The role of GSTP protein in controlling activity of protein kinases in hepatocytes, however, has not been determined.

The results of our investigation showed that the presence of 100 nM glucagon completely inhibited the increased expression of pi-class GST in cultured hepatocytes. Glucagon plays a significant physiological role in the regulation of metabolism by regulating the expression of a number of enzymes. The actions of glucagon are mediated by the glucagon receptor linked to a heterotrimeric G-protein complex, leading to increased cellular levels of cAMP and activation of PKA. In the present study treatment of hepatocytes with Br-cAMP or DB-cAMP, membrane-permeable cAMP analogs resulted in reduction of alpha- and pi-class GST protein levels identical to that resulting from glucagon treatment. Moreover, the PKA inhibitor H89 markedly attenuated the glucagon-mediated suppressive effect on alpha- and pi-class GST protein expression. These data support the involvement of cAMP and PKA as mediators of the decreased GST protein levels in response to glucagon and suggest that a glucagon signaling pathway is likely to be an inhibitory mechanism of alpha- and pi-class GST expression in primary cultured rat hepatocytes. This result also now enables investigators to suppress GST pi expression in primary cultured hepatocytes for additional mechanistic research.

In summary, the results of the present study indicate that insulin and glucagon regulate the expression of GSTs, particularly alpha-class GST, in an opposing manner in primary cultured rat hepatocytes and suggest that the development of oxidative stress observed in diabetes may be partially attributed to the reduction of alpha-class GST protein levels. This study also demonstrates that the glucagon signaling pathway is likely to be an inhibitory mechanism of pi-class GST expression in primary cultured rat hepatocytes. Neither glucagon nor insulin affected the expression of mu-class GST protein, indicating that each class of GSTs is differentially regulated by insulin or glucagon. This study also implicates cAMP and PKA involvement in mediating the inhibitory effect of glucagon on GST expression.

References
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