INSULIN AND GLUCAGON SIGNALING IN REGULATION OF MICROSMAL EPOXIDE HYDROLASE EXPRESSION IN PRIMARY CULTURED RAT HEPATOCYTES

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ABSTRACT:
Microsomal epoxide hydrolase (mEH) plays an important role in the detoxification of a broad range of epoxide intermediates and has been reported to be decreased during diabetes and fasting. The signaling pathways involved in the regulation of mEH expression in response to insulin and glucagon were examined in primary cultured rat hepatocytes. mEH protein levels were increased 2- to 6-fold in hepatocytes cultured for 1 to 4 days, respectively, in the presence of insulin. Concentration-response studies revealed that insulin concentrations ≥1 nM resulted in increased mEH protein levels. The phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin or LY294002 [2-(4-morpholino)-8-phenyl-4H-1-benzoypuran-4-one], and rapamycin, an inhibitor of p70 S6 kinase phosphorylation, ameliorated the insulin-mediated increase in mEH protein levels. The p38 mitogen-activated protein (MAP) kinase inhibitors SB203580 and SB202190 also abrogated the insulin-mediated increase in mEH protein. Treatment of cells with glucagon, 8-bromo-cAMP, or dibutyryl-cAMP for 3 days resulted in decreased mEH protein levels. Pretreatment with the protein kinase A (PKA) inhibitor H89 (N-[2-(4-bromocinnamylamino)ethyl]-5-isooquinoline) prior to glucagon addition markedly attenuated the glucagon effect, implicating PKA signaling in the regulation of mEH expression. These data demonstrate that insulin and glucagon regulate, in an opposing manner, the expression of mEH in primary cultured rat hepatocytes. Furthermore, these data suggest that PI3K and p70 S6 kinase are active in the regulation of insulin-mediated mEH expression. We also provide data implicating p38 MAP kinase in the insulin-mediated increase in mEH levels. Moreover, cAMP and PKA are implicated in mediating the inhibitory effect of glucagon on mEH expression.

mEH catalyzes the \textit{trans}-addition of water to a broad range of epoxide substrates. There has been great interest in mEH because of its potential involvement in the bioactivation of carcinogenic polycyclic aromatic hydrocarbons (Shou et al., 1996). However, mEH also plays an important role in the detoxification of many reactive epoxide intermediates from xenobiotics. A number of investigations have indicated that an individual’s mEH genotype may be associated with altered susceptibility to various syndromes, such as hepatocellular carcinoma, colon cancer, and lung cancer (McGlynn et al., 1995; Harrison et al., 1999; To-Figuera et al., 2001).

It has been reported that pathophysiologic conditions such as diabetes, fasting, and long-term alcohol consumption result in decreased activity of hepatic mEH (Thomas et al., 1989; Van de Wiel et al., 1993). Because these pathophysiological states all result in altered hormone (insulin, glucagon, growth hormone) secretion, hormonal changes may be etiologic factors affecting the expression of hepatic mEH. Thomas et al. (1989) have reported that insulin administration to diabetic rats returned mEH activity to control values, and the age and gender-dependent expression of hepatic mEH has been largely attributed to alterations in hormone levels (Horsfield et al., 1992; Kim and Kim, 1992). The effects of individual hormone(s) on mEH expression, however, have not be determined.

Insulin serves as a metabolic regulator of enzymes involved in the metabolism of nutrients such as carbohydrates, lipids, and proteins, and enzymes involved in xenobiotic metabolism (Woodcroft and Novak, 1997, 1999; Woodcroft et al., 2002). The metabolic effects of insulin are mediated through activation of PI3K and a variety of downstream effectors, including Akt, p70 S6 kinase, and protein kinase C (PKC) (Shepherd et al., 1998; Farese, 2001). Insulin receptor signaling also leads to activation of mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-JUN NH2-terminal kinase (JNK) (Avruch 1998;
Chen et al., 1998; Haussinger et al., 1999). Recently, Kang et al. (2001b, 2002) showed that sulfur amino acid deprivation activated PI3K/Akt and JNK, which served as an essential pathway for mEH induction by oxidative stress in H4IIIE cells. Also, Kim et al. (1998) reported that acrilavine, a PKC inhibitor, suppressed both constitutive and inducible hepatic mEH gene expression in rats.

Glucacon, a physiological antagonist of insulin, regulates the expression of several gene products important in cellular metabolism. The actions of glucacon are mediated by the glucacon receptor linked to a heterotrimetric G-protein complex, leading to increased cellular levels of cAMP and activation of PKA. Although the effects of glucacon on mEH expression have not been examined in primary cultured rat hepatocytes, a previous report indicated that cAMP analogs effectively repressed its phenobarbital-mediated induction (Sidhu and Omiecinski, 1995).

These studies, using primary cultured rat hepatocytes, were conducted to test the hypothesis that insulin, through PI3K and/or MAP kinase signaling pathways, regulates expression of mEH protein, and that glucacon, via PKA-mediated signaling, regulates mEH in an opposing manner to insulin. We report that insulin and glucacon regulate, in an opposing manner, the expression of mEH protein. While these data implicate PI3K and p70 S6 kinase in the insulin-mediated induction of mEH protein levels, we also provide data implicating p38 MAP kinase involvement in this response. The inhibitory effect of glucacon on mEH expression appears to be mediated via cAMP and PKA.

Materials and Methods

Materials. Modified Chee’s medium and t-glutamine were obtained from Invitrogen (Carlsbad, CA). Insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals Inc. (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 Inc. (Palo Alto, CA). Anti-mEH was prepared previously by our laboratory. These studies, using primary cultured rat hepatocytes, were conducted to test the hypothesis that insulin, through PI3K and/or MAP kinase signaling pathways, regulates expression of mEH protein, and that glucacon, via PKA-mediated signaling, regulates mEH in an opposing manner to insulin. We report that insulin and glucacon regulate, in an opposing manner, the expression of mEH protein. While these data implicate PI3K and p70 S6 kinase in the insulin-mediated induction of mEH protein levels, we also provide data implicating p38 MAP kinase involvement in this response. The inhibitory effect of glucacon on mEH expression appears to be mediated via cAMP and PKA.

Primary Rat Hepatocyte Culture. Hepatocytes were isolated from the livers of male Sprague-Dawley rats (200–300 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). Hepatocytes were treated with various concentrations of insulin (0–100 nM), glucagon (0–100 nM), Br-cAMP (0–25 μM), or DB-cAMP (0–100 μM). Untreated hepatocytes were cultured in the absence of insulin and glucagon. Kinase inhibitors were added 1.5 h prior to addition of insulin (10 nM) or glucagon (100 nM). Medium was changed every 24 h and cultures were continued for 24 h to 4 days.

Hepatocyte viability was monitored by measuring released lactate dehydrogenase (LDH) activity as described previously (Woodcroft and Novak, 1997). None of the protein kinase inhibitors resulted in increased cell toxicity at the doses used in this study as compared with untreated cells.

Effects of Insulin on Levels of mEH Protein. mEH protein levels were monitored in primary rat hepatocytes cultured in the absence of insulin for 4 days after plating (Fig. 1A). The protein levels of mEH were maintained initially for 2 days after plating, relative to freshly isolated hepatocytes. However, mEH protein levels declined to 55 and 39% of the level measured in freshly isolated hepatocytes by day 3 and 4 of culture, respectively. In contrast, hepatocytes maintained in culture in the presence of insulin over 4 days exhibited mEH protein levels ~2- to 2.5-fold higher than that measured in freshly isolated hepatocytes, and 2- to 6-fold greater than mEH levels in untreated hepatocytes at identical time points (Fig. 1A).

To assess cell viability, total cellular LDH levels, as well as LDH release, were examined in hepatocytes maintained in the presence or absence of 100 nM insulin for 24, 48, 72, or 96 h. Cell viability was unaltered by the absence of insulin since LDH release into the medium was identical in cells cultured in the presence or absence of insulin. However, total cellular LDH activities were decreased to 99, 80, 73, or 61% in hepatocytes cultured in the presence of insulin for 24, 48, 72, or 96 h, respectively, relative to corresponding 100 nM insulin-treated hepatocytes. The results likely reflect a decrease in cell adhesion to the substratum in the absence of insulin rather than cell destruction.

To examine the concentration dependence of insulin on mEH protein levels, hepatocytes were cultured for 24 h in medium supplemented with 0.01 to 100 nM insulin (Fig. 1B). mEH protein levels were increased in a concentration-dependent manner and were increased maximally ~2.4-fold in response to 10 nM insulin, relative to untreated cells (Fig. 1B). Thus, insulin regulates the basal expression of mEH in cultured hepatocytes.

Effects of PI3K Inhibitors on the Insulin-Mediated Increase in mEH Protein Levels. To determine whether PI3K may play a role in
Insulin regulation of mEH expression, hepatocytes were pretreated with wortmannin (0.1–0.5 μM) or LY294002 (1–10 μM) prior to addition of 10 nM insulin for 24 h (Fig. 2). Both wortmannin (Fig. 2A) and LY294002 (Fig. 2B) pretreatment resulted in a concentration-dependent inhibition of the insulin-mediated increase in mEH protein, with complete inhibition of the insulin effect observed at 0.5 μM wortmannin or 10 μM LY294002. In the absence of insulin, wortmannin (Fig. 2A) or LY294002 (Fig. 2B) alone resulted in an ~30 to 40% decrease in mEH protein levels, relative to untreated hepatocytes.

The inhibitory action of wortmannin and LY294002 on PI3K was confirmed by monitoring the Ser-473 phosphorylation of Akt in response to 10 nM insulin (Fig. 3). Insulin treatment (3 h) resulted in a 20- or 26-fold increase in Akt phosphorylation. Wortmannin (Fig. 3A) and LY294002 (Fig. 3B) inhibited insulin-mediated Akt phosphorylation in a concentration-dependent manner, with complete inhibition occurring at 0.5 μM wortmannin or 10 μM LY294002. Thus,
with either wortmannin or LY294002, inhibition of the insulin-mediated elevation in mEH expression was accomplished in an inhibitor concentration-dependent manner consistent with inhibition of PI3K signaling. Because mEH protein levels were decreased ~30–40% by wortmannin and LY294002 in hepatocytes cultured in the absence of insulin, we examined whether the suppressive effects of wortmannin or LY294002 on mEH protein levels were associated with inhibition of basal PI3K activity (Fig. 4). In the absence of insulin, basal phosphorylation of Akt was decreased in a concentration-dependent manner by the addition of wortmannin (Fig. 4A) or LY294002 (Fig. 4B), suggesting that wortmannin- or LY294002-induced suppression of mEH protein levels in untreated hepatocytes is likely associated with inhibition of basal PI3K activity. These data show that PI3K plays an important role in the basal as well as insulin-induced expression of mEH protein in hepatocytes.

Sidhu et al. (2001) reported that wortmannin and LY294002 increased LDH leakage and insulin-mediated phosphorylation of JNK and p38 MAP kinase in a concentration-dependent manner. The authors suggested that some of the effects of wortmannin and LY294002 might involve cytotoxicity as opposed to effects on signal transduction. To assess the effect of wortmannin (500 nM) and LY294002 (10 μM) on cell viability, LDH release was examined in hepatocytes maintained in the presence or absence of 10 nM insulin for 24 h. Neither wortmannin nor LY294002 was toxic to hepatocytes as determined by this method, with LDH release varying less than

Fig. 3. The effects of the PI3K inhibitors wortmannin (A) and LY294002 (B) on the insulin-mediated phosphorylation of Akt in primary cultured rat hepatocytes.

Hepatocytes were treated with wortmannin or LY294002 for 1.5 h before addition of 10 nM insulin for 3 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Phospho-Akt levels were normalized to total Akt levels. Values are shown as a percentage of the level of phospho-Akt/total Akt in untreated hepatocytes [100%; 84 arbitrary densitometry units of phospho-Akt and 776 arbitrary densitometry units of Akt (A), 36 arbitrary densitometry units of phospho-Akt and 473 arbitrary densitometry units of Akt (B)]. Data are means ± S.D. of Western blot band densities of two preparations of cell lysates from a single hepatocyte preparation.

Fig. 4. The effects of the PI3K inhibitors wortmannin (A) and LY294002 (B) on phosphorylation of Akt in rat hepatocytes cultured in the absence of insulin.

Hepatocytes were treated with wortmannin or LY294002 for 4.5 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Phospho-Akt levels were normalized to total Akt levels. Values are shown as a percentage of the level of phospho-Akt/total Akt in untreated hepatocytes [100%; 1245 arbitrary densitometry units of phospho-Akt and 1522 arbitrary densitometry units of Akt (A), 705 arbitrary densitometry units of phospho-Akt and 1471 arbitrary densitometry units of Akt (B)]. Data are means ± S.D. of Western blot band densities of two preparations of cell lysates from a single hepatocyte preparation.
20% from that monitored in untreated or insulin-treated hepatocytes. Furthermore, wortmannin (Fig. 5) and LY294002 (data not shown) failed to augment the phosphorylation of p38 MAP kinase or JNK induced by 10 nM insulin treatment. To examine the effects of wortmannin and LY294002 on p38 MAP kinase and JNK phosphorylation in hepatocytes cultured in the absence of insulin, hepatocytes were treated with wortmannin for 1.5 h prior to addition of 10 nM insulin for 3 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Phospho-p38 MAP kinase and phospho-JNK levels were normalized to total p38 MAP kinase and total JNK levels, respectively. Values are shown as a percentage of the level of phospho-p38 MAP kinase/total p38 MAP kinase (A) or phospho-JNK/total JNK (B) in untreated hepatocytes [100%; 48 arbitrary densitometry units of phospho-p38 MAP kinase and 225 arbitrary densitometry units of p38 MAP kinase (A), 154 arbitrary densitometry units of phospho-JNK and 1494 arbitrary densitometry units of JNK (B)]. Data are means ± S.D. of Western blot band densities of two preparations of cell lysates from a single hepatocyte preparation.

Hepatocytes were treated with wortmannin for 1.5 h prior to addition of 10 nM insulin for 24 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and wortmannin. Values are shown as a percentage of the level monitored in untreated hepatocytes (100%; 909 arbitrary densitometry units). Data are means ± S.D. of Western blot band densities of three preparations of cell lysates from a single hepatocyte preparation. Values with different letters are significantly different from each other, \( P < 0.05 \).

which indicate that the basal phosphorylation of Akt is detectable in hepatocytes cultured in the absence of insulin and markedly inhibited by PI3K inhibitors (Fig. 4). This difference in the role of PI3K inhibitors in cytotoxicity and signal transduction may reflect differences associated with cell culture conditions.

**Effect of p70 S6 Kinase Inhibition on the Insulin-Mediated Increase in mEH Protein Levels.** We used rapamycin, which inhibits the phosphorylation and activation of p70 S6 kinase (Shepherd et al., 1998), to determine whether p70 S6 kinase plays a role in mediating the insulin effect on mEH protein levels (Fig. 6). Insulin increased mEH protein expression by ~175% over untreated cells. Rapamycin pretreatment produced a concentration-dependent decline in the insulin-mediated increase in mEH protein levels, resulting in a maximal 50% inhibition of the insulin effect. These results suggest that p70 S6 kinase may also play a role in the insulin-mediated increase in mEH protein levels.

**Effects of PKC or Src Kinase Inhibitors on the Insulin-Mediated Increase in mEH Protein Levels.** To examine the possible involvement of PKC in the insulin-mediated effect on mEH protein levels, the broad spectrum PKC inhibitor bisindolylmaleimide was used. Bisindolylmaleimide, up to 10 \( \mu \)M (sufficient to inhibit multiple PKC isoforms), failed to inhibit the insulin-mediated increase in mEH protein levels (data not shown), suggesting that PKC does not contribute to the insulin induction of mEH protein expression.

Src kinase is activated in response to insulin and can serve as a mediator of insulin signaling through activation of 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (Chiarugi et al., 1997; Park et al., 2001). To investigate whether Src kinase is involved in mediating the positive effects of insulin on mEH expression, primary cultured rat hepatocytes were pretreated with geldanamycin or SU6656. Geldanamycin is a benzoquinone antibiotic that disrupts...
Hsp90 complexes, resulting in increased degradation and decreased activity of Hsp90-chaperoned proteins, including Src kinase (Pratt, 1998). Unfortunately, geldanamycin is not a specific inhibitor of Src kinase. SU6656, however, has been reported to be a selective inhibitor of Src kinase (Blake et al., 2000). But neither geldanamycin (0.1 μM) nor SU6656 (1 μM) inhibited the insulin-mediated increase in mEH protein levels (data not shown). These data suggest that Src kinase is not involved in mediating the positive effect of insulin on mEH protein expression.

Effects of MAP Kinase Inhibition on the Insulin-Mediated Increase in mEH Protein Levels. To investigate whether MAP kinase signaling pathways might be involved in the insulin-mediated increase in mEH protein levels, the MEK inhibitor PD98059, the JNK inhibitor SP600125, and the p38 MAP kinase inhibitors SB203580 and SB202190 were used. Neither PD98059 (up to 100 μM) nor SP600125 (up to 20 μM) affected the insulin-mediated increase in mEH protein levels (data not shown), suggesting that neither ERK nor JNK signaling pathways are involved in the insulin-mediated increase in mEH expression. On the other hand, SB203580 (1–20 μM) and SB202190 (10 μM) effectively inhibited the 10 nM insulin-mediated increase in mEH protein levels (Fig. 7). These data suggest that p38 MAP kinase might also play a role in controlling the insulin-mediated increase in mEH protein expression.

It was reported that SB203580 abolished interleukin-1-induced Akt phosphorylation with an IC_{50} value of 3 to 5 μM and activation of p70 S6 kinase with an IC_{50} value above 10 μM by inhibiting PDK-1 (Lali et al., 2000). Also Wang et al. (2001) observed that the p38 MAP kinase inhibitors, SB203580 (10 μM) and SB202190 (10 μM), markedly inhibited the phosphorylation of Akt induced by 10 nM insulin in adult rat ventricular cardiomyocytes. These results suggest that the activities of Akt and p70 S6 kinase, downstream effectors of PDK-1, may be inhibited by SB203580 and SB202190 at concentrations above 1 μM. We examined the effect of these p38 MAP kinase inhibitors on 10 nM insulin-stimulated phosphorylation of Akt at Ser-473. SB203580 and SB202190, however, failed to inhibit the phosphorylation of Akt (data not shown), indicating that the inhibitory effect of SB203580 or SB202190 on the insulin-mediated increase in mEH protein expression is not due to inhibition of PDK-1 or its downstream effectors.

Effects of Glucagon on Levels of mEH Protein. The effects of glucagon (100 nM) on mEH protein levels were monitored in rat hepatocytes over 4 days of treatment. Hepatocytes maintained in culture with glucagon in the absence of insulin exhibited mEH protein levels of 105, 50, 28, or 13% of the level in freshly isolated hepatocytes at day 1, 2, 3, or 4, respectively (Fig. 8A). mEH protein levels were decreased by 50 to 70% relative to the levels monitored in the respective untreated cells, when hepatocytes were cultured in the presence of glucagon (100 nM) for more than 2 days.

The concentration dependence of the glucagon effect on mEH protein levels was determined in hepatocytes cultured for 3 days in the presence of 0 to 100 nM glucagon (Fig. 8B). The levels of mEH protein were decreased by 25, 53, or 66% in the presence of 1, 20, or 100 nM glucagon, respectively, compared with cells maintained in culture in the absence of glucagon. These results clearly show that glucagon is capable of significantly suppressing mEH levels in primary cultured rat hepatocytes.

Effects of cAMP Analogs and PKA Inhibitor on mEH Protein Levels. 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 mEH expression are associated with elevated cAMP levels, changes in mEH protein levels were monitored in hepatocytes cultured for 3 days in the presence of membrane-permeable cAMP analogs (Fig. 9). Addition of Br-cAMP or DB-cAMP to hepatocytes decreased mEH protein levels by as much as 90%, in a concentration-dependent manner (Fig. 9). Thus, treatment of hepatocytes with cAMP analogs resulted in the same effect as glucagon treatment, implicating PKA signaling in regulating the expression of mEH.

Hepatocytes were treated with SB203580 or SB202190 for 1.5 h prior to addition of 10 nM insulin for 24 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Values are shown as a percentage of the level monitored in untreated hepatocytes [100%; 506 arbitrary densitometry units (A), 445 arbitrary densitometry units (B)]. Data are means ± S.D. of Western blot band densities of three preparations of cell lysates from a single hepatocyte preparation. Values with different letters are significantly different from each other, P < 0.05.

**Fig. 7.** Immunoblot analysis of the effects of the p38 MAP kinase inhibitors SB203580 (A) and SB202190 (B) on the insulin-mediated increase in mEH protein levels in primary cultured rat hepatocytes.

Hepatocytes were treated with SB203580 or SB202190 for 1.5 h prior to addition of 10 nM insulin for 24 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Values are shown as a percentage of the level monitored in untreated hepatocytes [100%; 506 arbitrary densitometry units (A), 445 arbitrary densitometry units (B)]. Data are means ± S.D. of Western blot band densities of three preparations of cell lysates from a single hepatocyte preparation. Values with different letters are significantly different from each other, P < 0.05.
protein expression, whereas a concentration of 25 μM H89 completely inhibited the 100 nM glucagon-mediated decrease in mEH protein levels, resulting in mEH protein levels comparable to that monitored in cells cultured in the absence of glucagon (Fig. 10B).

Discussion

Expression of mEH is altered in response to pathophysiologic conditions such as diabetes, fasting, protein-calorie malnutrition, and long-term alcohol consumption as well as by a variety of xenobiotics (Thomas et al., 1989; Van de Wiel et al., 1993; Cho et al., 2001). In the present study we have demonstrated that insulin increases, and glucagon decreases, mEH protein expression in primary cultured rat hepatocytes. These results suggest that insulin and glucagon can serve as physiological regulators of the expression of mEH and further suggest that the suppression of hepatic mEH expression in diabetics or during fasting may be attributed to altered levels of insulin and glucagon.

Oxidative stress is increased in diabetic conditions and is a major factor contributing to the extent of chronic diabetes complications. A higher incidence of hepatic cancer has been reported to be associated with diabetes (Adami et al., 1996). Recently, it has been reported that when mEH genotypes were combined to express a metabolic phenotype, very slow metabolizers were highly prevalent among cirrhotic and hepatocellular carcinoma patients (Sonzogni et al., 2002). It has
also been reported that hepatic mEH activity toward styrene oxide was decreased in diabetes (Thomas et al., 1989). Our previous studies have demonstrated decreased expression of glutathione S-transferase, one of the major antioxidant enzymes, and increased expression of cytochrome P4502E1, an enzyme whose metabolic activity leads to increased oxidative stress, in response to glucagon and lower insulin concentrations (Woodcroft et al., 2002; Kim et al., 2003). Increased cytochrome P4502E1 levels during diabetes, especially in conjunction with decreased glutathione S-transferase levels, may be a contributing factor to the increased oxidative stress observed during diabetes.

These results raise the possibility that the insulin/glucagon-mediated changes in cytochrome P4502E1, glutathione S-transferase, and mEH expression may be related to a greater incidence of hepatic disease in diabetic patients.

The numerous and varied cellular effects of insulin are mediated by the insulin receptor. Previously, we reported that tyrosine phosphorylation of insulin receptor in primary cultured rat hepatocytes was detected at an insulin concentration of 1 nM and was markedly increased at 10 nM insulin (Woodcroft et al., 2002). 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). In the present study a significant increase in mEH protein levels was observed at 1 nM insulin, and a further increase was observed in hepatocytes cultured in the presence of 10 nM insulin. These data show a correspondence between insulin receptor phosphorylation and the increase in mEH protein levels in primary cultured rat hepatocytes. These results also suggest that the titration region for these events occurs in the physiologic range of insulin concentration.

In the present study, PI3K inhibitors, wortmannin and LY294002, inhibited the insulin-mediated increase in mEH protein expression. Our findings implicate PI3K as an obligatory component in the stimulation of mEH protein expression by insulin. Also, wortmannin or LY294002 addition to hepatocytes cultured in the absence of insulin produced an ~30 to 40% decrease in mEH protein levels and a marked inhibition of basal Akt phosphorylation. These results suggest that basal activity of PI3K in the absence of insulin plays an important role in regulating the expression of mEH protein in hepatocytes under these conditions.

Recently, it has been reported that PI3K is responsible for the activation of antioxidant-responsive element (ARE) binding proteins and subsequent induction of phase II detoxifying enzymes including alpha class glutathione S-transferase and NADPH/quinone oxidoreductase (Kang et al., 2001a; Lee et al., 2001). Moreover, Kang et al. (2001b) showed that PI3K was essential for the induction of mEH by oxidative stress following sulfur amino acid deprivation in H4IIE cells. These results raise the possibility that induction of mEH by insulin treatment may involve ARE activation mediated by a PI3K signaling pathway, although the ARE has not yet been identified for the mEH gene.

Our results suggest that p70 S6 kinase, a downstream effector of PI3K, may also play a role in the insulin-mediated increase in mEH protein expression. A consistent body of evidence indicates that p70 S6 kinase plays an important role in the regulation of protein synthesis in response to nutrients and hormones (Shah et al., 2000). These results raise the possibility that the insulin effects on mEH protein levels can be related with the increased translational capacity through activation of p70 S6 kinase.

Insulin receptor signaling also leads to activation of MAP kinase signaling pathways (Avruch 1998; Chen et al., 1998; Haussinger et al., 1999). In the present study, neither the ERK inhibitor PD98059 nor JNK inhibitor SP600125 affected the insulin-mediated increase in mEH protein levels, excluding ERK and JNK from a major role in regulating mEH protein expression. In contrast, the p38 MAP kinase inhibitors, SB203580 and SB202190, prevented the insulin-mediated enhancement of mEH expression. Recently, it was reported that sulfur amino acid deprivation-induced cell death in H4IE cells was prevented by SB203580 or by dominant-negative JNK1 stable transfection (Kang et al., 2002). The induction of mEH, which occurred in parallel with cell death, was partially decreased in H4IE cells only by dominant-negative JNK1 stable transfection, but not by SB203580. This difference in the role of p38 MAP kinase and JNK in regulating mEH protein expression may reflect differences associated with cell context.

**FIG. 10.** Immunoblot analysis of the effects of the protein kinase A inhibitor, H89, on the glucagon-mediated decrease in mEH protein levels in primary cultured rat hepatocytes.
A number of studies have shown that the phosphorylation of MAP kinases, including p38 MAP kinase, by activation of a variety of receptors was regulated by PI3K (Assefa et al., 1999; Smalley et al., 1999; Hirasawa et al., 2000). We raised a further question: whether PI3K might be involved in insulin activation of p38 MAP kinase. The PI3K inhibitors wortmannin and LY294002, however, failed to inhibit insulin-induced phosphorylation of p38 MAP kinase. Thus, p38 MAP kinase may represent a distinct pathway responsible for the induction of mEH in response to insulin treatment.

The p38 MAP kinase inhibitors used in the present study were reported to inhibit the activation of PDK-1 and its downstream effectors, including Akt and p70 S6 kinase, in T-lymphocytes and adult rat ventricular cardiomyocytes (Lali et al., 2000; Wang et al., 2001). But it was shown that PDK-1 activity remained unaffected by in vitro incubation with SB202190 or SB203580 (Davies et al., 2000). In L6 myotubes, activation of p70 S6 kinase by insulin was also not diminished by SB203580 (Somwar et al., 1998). Given the apparent cell-specific differences, we determined the effect of SB203580 or SB202190 on insulin-stimulated phosphorylation of Akt at Ser-473 in primary cultured rat hepatocytes. SB203580 and SB202190 had no significant effect on insulin-mediated Akt phosphorylation, indicating that the inhibitory effect of these p38 MAP kinase inhibitors on insulin-stimulated stimulation of mEH protein expression is likely due to a specific effect on p38 MAP kinase-dependent processes.

Glucagon, a physiological antagonist of insulin, regulates the expression of several gene products important in cellular metabolism in an opposing manner to insulin. The results of our investigation showed that glucagon markedly decreased mEH protein levels in primary cultured rat hepatocytes. SB203580 and SB202190 had no specific effect on p38 MAP kinase-dependent processes.

In summary, the results of the present study indicate that insulin and glucagon regulate the expression of mEH in an opposing manner in primary cultured rat hepatocytes and implicate the involvement of cAMP and PKA as mediators of the decreased mEH protein levels in response to glucagon.

References