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Insulin Signaling in Regulation of Drug Metabolizing and Antioxidant Enzyme Expression

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Diabetes is a major cause of morbidity and mortality, and the incidence of diabetes has been increasing in the Korea. Complications resulting from diabetes have been attributed in part to increased oxidative stress. However, the reasons for oxidative stress observed in patients with diabetes are not clear. The signaling pathways involved in the regulation of drug metabolizing and antioxidant enzyme expression in response to insulin were examined using cDNA microarray, antibody microarray, real time RT-PCR, immunoblot analysis and enzyme assay in primary cultured rat hepatocytes. Alpha class glutathione S-transferases (GSTs), microsomal epoxide hydrolase (mEH), gamma-glutamylcysteine ligase catalytic subunit (GCLC), but not pi class GSTs, mu class GSTs and soluble epoxide hydrolase, were increased in response to insulin. Insulin resulted in a marked and prolonged activation of Akt and ribosomal p70S6 kinase (p70S6K), downstream effectors of the phosphatidylinositol 3-kinase (PI3K) and a time-dependent activation of mitogen activated protein kinase (MAPK) including, ERK, JNK and p38 MAPK. PI3K inhibitors, wortmannin and LY294002, dominant negative Akt, or rapamycin, an inhibitor of p70S6K phosphorylation, inhibited the insulin-mediated increase in drug metabolizing and antioxidant enzyme expression. PD98059, an inhibitor of MEK, SP600125, an inhibitor of JNK and SB203580, an inhibitor of p38MAPK failed to inhibit the insulin-mediated increase in drug metabolizing and antioxidant enzyme expression. This study shows that insulin signaling pathways involving PI3K/Akt/p70S6K are active in the insulin mediated regulation of drug metabolizing and antioxidant enzyme expression and implicates that oxidative stress observed in diabetics may be attributed to decreased expression of antioxidant enzyme in response to altered insulin level and/or insulin signaling.

It is generally recognized that the expression of drug metabolizing enzymes may be altered in response to development, aging, gender, genetic factors, nutrition, pregnancy and pathophysiological conditions such as diabetes, long-term alcohol consumption, inflammation, and protein-calorie malnutrition. The expression may also be altered by xenobiotics. Although the mechanisms by

which xenobiotics regulate drug metabolizing enzymes have been intensively studied, relatively less is known regarding the cellular mechanisms by which drug metabolizing enzymes are regulated in response to endogenous factors such as hormones and growth factors. Recent findings, however, have revealed that hormones and growth factors play an important role in the regulation of drug metabolizing enzyme expression. Furthermore, the cellular signaling pathways involved in hormone- and growth factor-mediated regulation of drug metabolizing enzymes are currently being studied.

Pathophysiological conditions such as diabetes, fasting, obesity and long-term alcohol consumption result in increased expression of several hepatic enzymes, including CYP1A1, 2B, 3A, 4A, 2E1 and bilirubin UDP-glucuronosyltransferase (UGT1A1), whereas decreased expression of CYP2C11, microsomal epoxide hydrolase (mEH) and sulfotransferases (SULTs), such as hydroxysteroid SULT-a (SULT2A1) and aryl SULT IV (SULT1A1), has been reported (Table 1).[1] In the other hand, studies of the expression and activity of glutathione S-transferase (GST) during diabetes are inconclusive, with both increased and decreased GST expression being reported *in vivo*. The reason for this discrepancy remains unknown. However, it may, in part, be associated with competing factors *in vivo* and with variations in 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.

Because these pathophysiological states all result in altered hormone (insulin, glucagon, growth hormone) secretion, these hormones may be etiologic factors affecting the expression of hepatic drug-metabolizing enzymes. It has been reported that insulin or growth hormone administration to chemically-induced or spontaneously diabetic rats restores drug-metabolizing enzyme activity and expression to control values (Table 1).[1] Our laboratory and others have demonstrated that the activity and/or expression of hepatic drug metabolizing enzymes such as CYP2B, CYP2E1, CYP2C11, CYP2A5, GST alpha class, GST pi class, UGT and mEH are regulated by insulin and glucagon.[2-12] These results indicate that changes in drug-metabolizing enzyme mRNA or protein levels observed in pathophysiological conditions may be attributed to alterations in these hormone levels. Thus, it is of interest to identify which cellular signaling pathways are involved in regulating the expression of these genes in response to hormones.

Our laboratory has demonstrated that the expression of CYP2E1 is suppressed by insulin and enhanced by glucagon in primary cultured rat hepatocytes.[7-8] In contrast, the expression of alpha-class GSTs, mEH and gamma-glutamylcysteine ligase catalytic subunit (GCLC) is enhanced by insulin and decreased by glucagon.[5,12-13] Treatment of cells with glucagon also inhibits the expression of pi-class GST.[5] Phosphatidylinositol 3-kinase (PI3K) and p70 ribosomal protein S6 kinase (p70 S6 kinase) appear to play a central role in mediating the suppression of CYP2E1 expression[14] as well as enhancement of mEH[12] and alpha-class GSTs (Kim et al., unpublished data). The regulation of CYP2E1 expression by insulin does not involved extracellular signal-regulated

kinases (ERK1/2) or p38 mitogen activated protein kinase (p38 MAPK).[14] This is in contrast to the findings for insulin-mediated expression of mEH, which appears to involve p38 MAPK.[12] Furthermore, our results implicate cAMP and protein kinase A (PKA) in mediating the effects of glucagon on CYP2E1, GSTs and mEH expression.[5,9,12]

In this presentation, an overview of the signaling pathways implicated in regulating drug metabolizing enzyme expression in response to insulin will be reported.

Table 1. Effect of diabetes, insulin and glucagon on drug-metabolizing enzyme expression and/or activity

	Diabetes	Restored by insulin	Insulin	Glucagon
CYP2B	Increased	Yes	Decreased	ND
CYP3A	Increased	Yes	Unchanged	ND
CYP4A	Increased	Yes	Marginally increased	ND
CYP2C11	Decreased	Yes	ND	Decreased
CYP2E1	Increased	Yes	Decreased	Increased
mEH	Decreased	Yes	Increased	Decreased
UGT1A1	Increased	Yes	ND	Increased
SULT2A1	Decreased	Yes	ND	ND
GSTs	Increase/ Decrease	ND	Increased	Decreased
GST alpha	ND	ND	Increased	Decreased
GST pi	ND	ND	Unchanged	Decreased
GST mu	ND	ND	Unchanged	Unchanged

ND is not determined.

Methods

Primary rat hepatocyte culture. Hepatocytes were isolated from the livers of male Sprague-Dawley rats (200-300 g) using collagenase perfusion as described previously.[7-8] Hepatocytes were plated onto dishes covalently coated with Vitrogen, and modified Chee's medium was fortified as described[7-8] and supplemented with 0.1 μM dexamethasone and 1 μM insulin. Cells were plated at a density of $3x10^6$ cells/60 mm dish or $1x10^7$ cells/100 mm dish. Four hours after plating, cells were washed with insulin-free medium several times and cultured for an additional 2 h in insulin-free medium prior to initiation of treatment. Cells were then treated with various concentrations of insulin (0-100 nM) or glucagon (0-100 nM). Kinase inhibitors were dissolved in DMSO and added 1.5 h prior to addition of insulin (10 nM). For adenovirus infection, 4 h after plating cells were washed with insulin-free medium several times and AdV-Akt (150 MOI; multiplicity of infection) or control virus (AdV-GFP, 15 MOI) was added to the cells in fresh medium. Following overnight infection, medium was changed, and hepatocytes were treated with insulin (10 nM) for 2 days. Hepatocyte viability was monitored by measuring released lactate dehydrogenase activity as described previously.[7-8]

Immunoblot analysis. Whole cell lysates were prepared as described.[5] For immunoblot analysis of drug metabolizing and antioxidant enzymes, lysates (5-20 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose (Bio-Rad, Hercules, CA), blocked in 5% milk powder in PBS-T (0.05% Tween 20 in PBS) and incubated with each specific antibody (1:10,000 in 5% milk powder in PBS-T) overnight at room temperature. To determine the phosphorylation state of Akt, p70S6K, ERK, p38 MAPK and JNK, cell lysates were prepared by scraping cells directly into 500 μL SDS-PAGE sample buffer. Lysates (10 μL) were separated by 10% SDS-PAGE, transferred to nitrocellulose, blocked in 5% milk powder in TBS-T (0.05% Tween 20 in Tris-HCl buffered saline) and probed with phospho-specific antibodies (1:250 in 5% bovine serum albumin in TBS-T) overnight at 4°C. Blots were stripped and re-probed with phosphorylation state-independent antibodies to Akt, p70S6K, ERK, p38 MAPK and JNK. Proteins were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma) and quantified by densitometry with a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences, Piscataway, NJ).

Kinase activity assay. Thirty minutes after 10 nM insulin treatment, whole cell lysates were prepared as described previously.[5] Akt immunoprecipitation and kinase assay were carried out following the procedures in the kit as described by the manufacture (Cell Signaling Technology).

Enzyme activity and GSH level determination. The concentration of GSH and the enzyme activities were measured as described previously.[15] None of the protein kinase inhibitors

resulted in interference of determination of GSH.

PCR determination of GCLC mRNA levels. Total hepatocyte RNA was isolated as previously described.[7-8] Reverse transcription was carried out using 2 μg of total RNA following the protocol for the Taqman Reverse Transcription Master Mix (Applied Biosystems, Foster City, CA). Ten nanograms of cDNA were subjected to PCR using the primer 5'-CATCAGGCTCTTTGCACGATAAC-3' and analyzed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Sybr Green was incorporated using the Sybr Green Master Mix from Applied Biosystems. Cycle threshold (Ct) values were obtained from the ABI Prism 7000 software and fold change was determined.

Microarray. Agilent cDNA microarrays were used. For each sample, Cy3- and Cy5-labeled cDNAs were generated from 20 μg of total RNA using the Agilent Direct Labeling Kit, product number G2557A. The labeling protocol uses Cy3-dCTP, Cy5-dCTP, MMLV-RT, and oligo-dT priming. On each microarray, labeled cDNA derived from the reference RNA was mixed with labeled cDNA from cultured cells and co-hybridized. Hybridization, blocking and washing of the microarrays was accomplished using Agilent's cDNA Microarray Kit Protocol. Microarrays were scanned using an Agilent dual laser DNA microarray scanner, model G2565AA, with 10 μm resolution.

Statistical analysis. Significant differences between groups were determined by ANOVA followed by the Newman-Keuls multiple comparison test ($p<0.05$). Statistical analysis was performed on triplicate cell lysates from a single hepatocyte preparation. Reproducibility of results was confirmed in two to four separate hepatocyte preparations.

Conclusion

1. Microarray, immunoblot analysis and enzyme assays show that insulin and glucagon regulate expression of drug metabolizing and antioxidant enzymes in opposing directions.
2. Reductions in GST, mEH and GCLC may be contributing factors to enhancement of oxidative stress observed in diabetes mellitus.
3. Insulin signaling pathways involving PI3K/Akt/p70S6K are active in the insulin-mediated regulation of drug metabolizing enzyme expression.
4. These results raise the possibility that insulin/glucagon-mediated changes in GST, mEH and GCLC expression may be related to a greater incidence of hepatic disease in diabetic patients.

References

1. Kim SK, Woodcroft KJ, Novak RF.: Insulin and growth factor signaling: Effects on drug-metabolizing enzymes. In Drug Metabolism and Transport (L.H. Lash, Ed.), pp 45-83. Humana Press Inc., Totowa (2004).
2. Constantopoulos A, Matsaniotis N.: Augmentation of uridine diphosphate glucuronyltransferase activity in rat liver by adenosine 3',5'-monophosphate. *Gastroenterology,* 75, 486-491 (1978).
3. Ricci GL, Fevery J.: Treatment of rats with glucagon, vasointestinal peptide or secretin has a different effect on bilirubin and p-nitrophenol UDP-glucuronyltransferase. *Biochem Pharmacol.*, 37, 3526-3528 (1988).
4. Carrillo MC, Monti JA, Favre C, Carnovale CE. Acute regulation of hepatic glutathione S-transferase by insulin and glucagon. *Toxicol Lett.,* 76, 105-111 (1995).
5. Kim SK, Woodcroft KJ, Novak RF. : Insulin and glucagon regulation of glutathione S-transferase expression in primary cultured rat hepatocytes. *J Pharmacol Exp Ther.,* 305, 353-361 (2003).
6. De Waziers I, Garlatti M, Bouguet J, Beaune PH, Barouki R. : Insulin down-regulates cytochrome P450 2B and 2E expression at the post-transcriptional level in the rat hepatoma cell line. *Mol Pharmacol.,* 47, 474-479 (1995).
7. Woodcroft KJ, Novak RF. : Insulin effects on CYP2E1, 2B, 3A, and 4A expression in primary cultured rat hepatocytes. *Chem Biol Interact.,* 107, 75-91 (1997).
8. Woodcroft KJ, Novak RF. : Insulin differentially affects xenobiotic-enhanced, cytochrome P-450 (CYP)2E1, CYP2B, CYP3A, and CYP4A expression in primary cultured rat hepatocytes. *J Pharmacol Exp Ther.,* 289, 1121-1127 (1999).
9. Woodcroft KJ, Novak RF. : The role of phosphatidylinositol 3-kinase, Src kinase, and protein kinase A signaling pathways in insulin and glucagon regulation of CYP2E1 expression. *Biochem Biophys Res Commun.,* 266, 304-307 (1999).
10. Iber H, Li-Masters T, Chen Q, Yu S, Morgan ET. : Regulation of hepatic cytochrome P450 2C11 via cAMP: implications for down-regulation in diabetes, fasting, and inflammation. *J Pharmacol Exp Ther.,* 297, 174-180 (2001).
11 Viitala P, Posti K, Lindfors A, Pelkonen O, Raunio H. : cAMP mediated upregulation of CYP2A5 in mouse hepatocytes. *Biochem Biophys Res Commun.,* 280, 761-767 (2001).
12. Kim SK, Woodcroft KJ, Kim SG, Novak RF. : Insulin and Glucagon Signaling in Regulation of Microsomal Epoxide Hydrolase Expression in Primary Cultured Rat Hepatocytes. *Drug Metab Dispos.,* 31, 1260-1268 (2003).
13. Kim SK, Woodcroft KJ, Khodadadeh SS, Novak RF. Insulin signaling regulates gamma-

glutamylcysteine ligase catalytic subunit expression in primary cultured rat hepatocytes. *J Pharmacol Exp Ther.*, 311, 99-108 (2004).

14. Woodcroft KJ, Hafner MS, Novak RF. : Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression. *Hepatology.*, 35, 263-273 (2002).
15. Kim SK, Choi KH and Kim YC (2003) Effect of acute betaine administration on hepatic metabolism of S-amino acids in rats and mice. *Biochem Pharmacol* 65:1565-1574.

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