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Differential AMPK phosphorylation by glucagon and metformin regulates insulin signaling in human hepatic cells

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ABSTRACT

Insulin and glucagon signaling in the liver are major contributors to glucose homeostasis. Patients with Type 1 and Type 2 diabetes have impaired glycemic control due, in part, to dysregulation of the opposing actions of these hormones. While hyperglucagonemia is a common feature in diabetes, its precise role in insulin resistance is not well understood. Recently, metformin, an AMPK activator, was shown to regulate hepatic glucose output via inhibition of glucagon-induced cAMP/PKA signaling; however, the mechanism for how PKA inhibition leads to AMPK activation in human hepatic cells is not known. Here we show that glucagon impairs insulin-mediated AKT phosphorylation in human hepatic cell line Huh7. This impairment of AKT activation by glucagon is due to PKA-mediated inhibition of AMPK via increased inhibitory phosphorylation of $AMPK^{Ser173}$ and reduced activating phosphorylation of $AMPK^{Thr172}$. In contrast, metformin decreases PKA activity, leading to decreased $pAMPK^{Ser173}$ and increased $pAMPK^{Thr172}$. These data support a novel mechanism involving PKA-dependent AMPK phosphorylation that provides new insight into how glucagon and metformin modulate hepatic insulin resistance.

1. Introduction

Glucagon is a hormone secreted by pancreatic alpha cells, and opposes many of the actions of insulin required for glucose homeostasis [1]. Since the discovery of insulin nine decades ago [2], the prevailing view has been that insulin is the most important metabolic regulator of glucose homeostasis and its dysregulation and peripheral resistance are the major determinants in diabetes. However, the notion that impaired insulin action is the primary cause for insulin resistance and hyperglycemia in diabetes has been challenged by emerging evidence that glucagon may play a significant role in the development of hyperglycemia in both Type 1 and Type 2 diabetes [3–6]. Several recent studies have demonstrated improvement in glycemic control when glucagon secretion is suppressed [5], glucagon receptor is knocked out in mice [7], or inhibited either by antibodies or small molecule antagonists [8]. Furthermore, hyperglucagonemia is a common feature of both Type I and Type II diabetes and patients with elevated circulating levels of glucagon also have impairment of insulin action in both types of diabetes [9,10]. Thus, it appears that glucagon is involved in the dysfunctional glucose regulation associated with insulin resistance. Binding of glucagon to its receptor on the hepatocyte plasma membrane leads to activation of adenylyl cyclase (AC), production of the second messenger cyclic AMP (cAMP), and stimulation of protein kinase A (PKA), which phosphorylates protein targets that work in concert to increase hepatic glucose output [11]. More recently AMPK-activating biguanides such as metformin has been shown to regulate hepatic glucose output via inhibition of glucagon-induced cAMP/PKA signaling [12]. However, the precise mechanism for PKA inhibition and AMPK activation is not known. Therefore, understanding the mechanism by which the glucagon inhibits and metformin enhances insulin signaling is a problem of considerable importance for diabetes research. In this study, we have identified a novel mechanism in which glucagon inhibits intracellular insulin signaling by decreasing AKT phosphorylation in hepatic cells via a PKA–AMPK pathway. In contrast, metformin, decreases PKA activity and stimulates AMPK phosphorylation. These findings show that glucagon impairment of insulin signaling may directly contribute to the development of hepatic insulin resistance in diabetes.

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; IRS, insulin receptor substrate; PKA, protein kinase A; pAKT, phosphorylated AKT.

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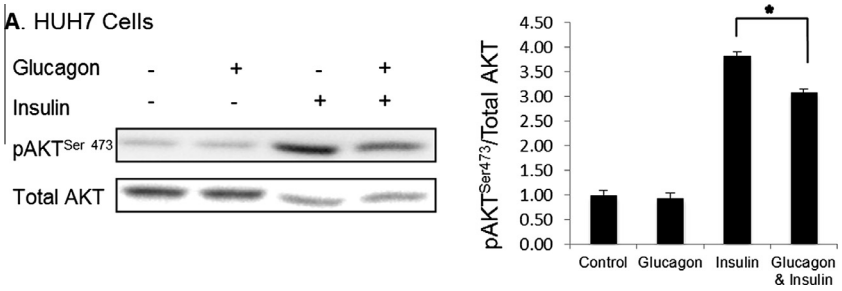

Fig. 1. Glucagon inhibits insulin signaling in hepatic cells. Effects of glucagon pretreatment on insulin-stimulated pAKT in Huh7 cells. Cells were cultured for 24 h in the presence or absence of 0.2 nM glucagon and with 10 nM insulin or no treatment for 15 min. Lysates then were analyzed by Western blotting for $pAKT^{Ser473}$ and AKT. Data represent the means of three experiments, $N = 3$; $^{*}P < 0.05$ for each experiment, error bars represent *s.e.m.*

2. Material and methods

2.1. Reagents

Synthetic glucagon peptides, human insulin solution, H89, cAMP analogous, metformin, AICAR were from Sigma Aldrich. Antibodies recognizing human AMPKα, AKT, IRS and PKA substrates were from Cell Signaling Technology. Actin antibody is from Santa Cruz Biotechnology. Anti-AMPK Ser 173 antibody was a kind gift from Prof. Wilhelm Krek (Institute of Cell Biology, ETH Zurich, Switzerland). AMPK siRNA (targeting the α subunit), culture media were from Invitrogen. FBS serum and BSA were from Sigma Aldrich.

2.2. Cell culture

Studies were performed in the human hepatoma cells Huh7 cells from American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) in a 5% CO_2 atmosphere.

2.3. Cell harvest and Western blotting

Huh7 cells grown to 80–90% confluence in 6 well plates were treated with the indicated reagents for the indicated timings. After treatment, the cells were stimulated with insulin (10 nM) for 15 min. The cells were then lysed using mammalian lysis buffer (Sigma Aldrich). Laemmli sample buffer (BioRad) was added and samples were heated to 100 °C for 10 min and stored at −80 °C until analysis. Equal amount of protein were separated by SDS gel electrophoresis and transferred immediately onto PVDF (Bio-Rad). Membranes were blocked in 5% milk and subsequently were incubated in 1% w/v BSA in PBST with specific antibodies overnight at 4 °C. Membranes were washed 3 times in PBST and subsequently incubated with species appropriate, peroxidase-conjugated secondary

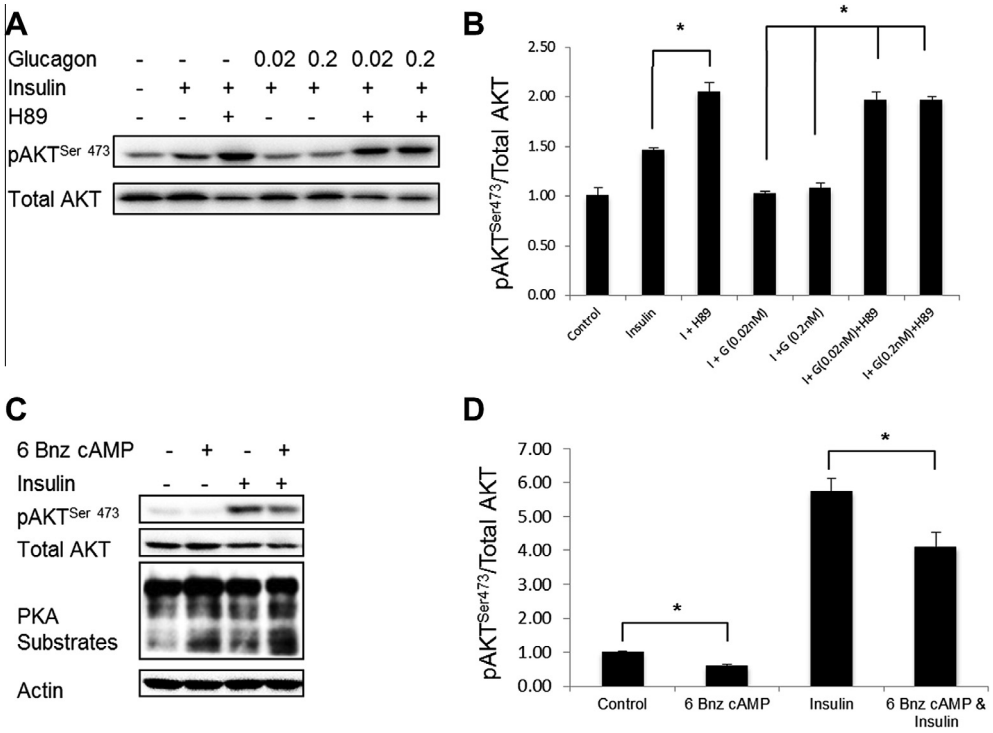

Fig. 2. Glucagon inhibition of pAKT is PKA-dependent in hepatic cells. (A, B) Effects of PKA inhibitor, H89 on pAKT in Huh7 cells treated with glucagon and insulin. Cells were initially treated with indicated doses of glucagon (nM) and 10 μM of H89 for 24 h; followed by 10 nM insulin for 15 min. They were then lysed and total cellular protein was analyzed by Western blot for total and phosphorylated AKT^{Ser473}. (C, D) Effects of cAMP analog on pAKT in the absence or presence of insulin. Huh7 cells were treated with 10 nM 6-Bnz cAMP for 24 h, cells were then treated with 10 nM insulin for 15 min lysed, and total cellular protein was analyzed by Western blot for total AKT and $pAKT^{Ser473}$ and PKA substrates. $N = 3$ for each experiment; $^{*}P < 0.05$ for each experiment, error bars represent *s.e.m.*

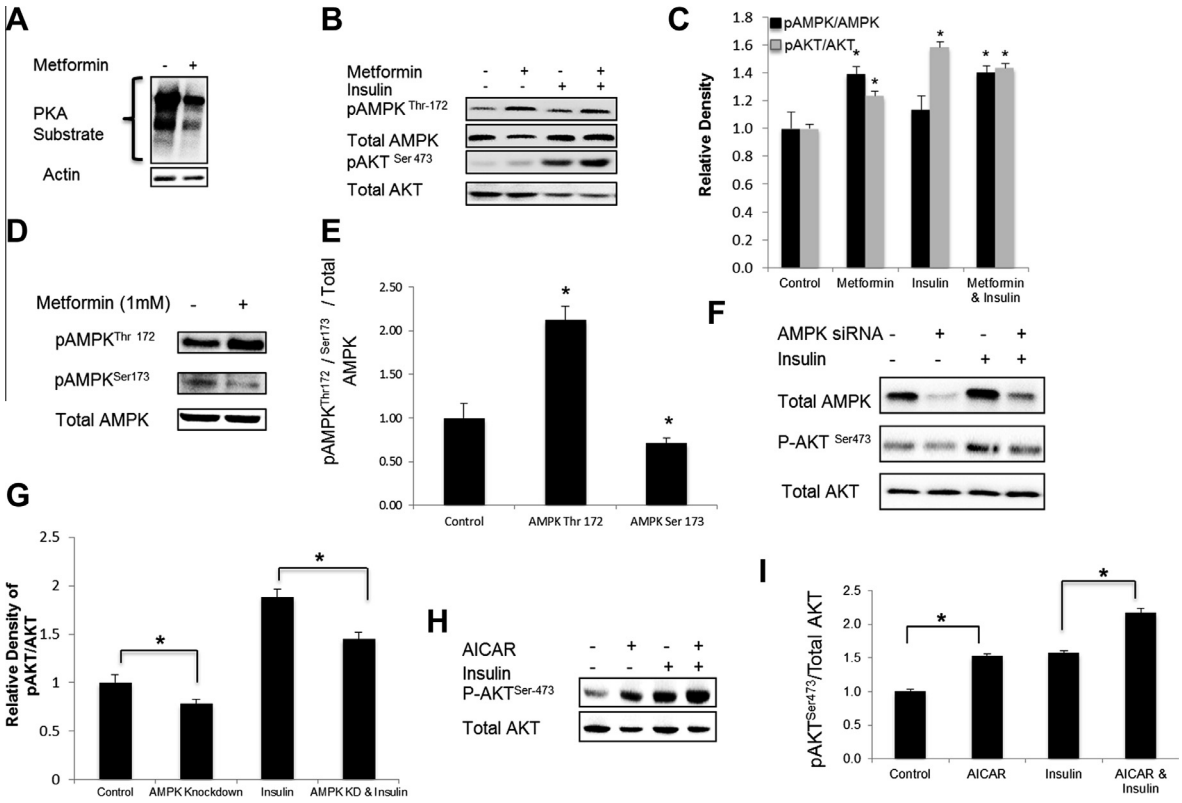

Fig. 3. Metformin stimulates AMPK^{Thr172} and decreases AMPK^{Ser173} phosphorylation. (A) Effects of metformin on PKA activity. (B, C) Effects of metformin on AMPK^{Thr172}, and AKT^{Ser473}. Huh7 cells were treated with 1 mM metformin for 24 h followed by 10 nM insulin or no treatment for 15 min. (D, E) Effects of metformin (1 mM/24 h) on differential AMPK phosphorylation in hepatic cells. Total cellular protein was analyzed by Western blot for total AMPK, AMPK^{Thr172} and AMPK$^{Ser-173}$. (F, G) Effects of AMPK knockdown on AKT phosphorylation. AMPK knockdown was achieved in Huh7 cells by transfecting 10 nM of AMPK siRNA for 48 h. Cells then were treated with 10 nM insulin for 15 min lysed, and total cellular protein was analyzed by Western blot for total and phosphorylated AMPK, AKT, mTOR. (H, I) Effects of AMPK activator, AICAR on insulin signaling. Cells were cultured for 24 h in the presence or absence of 1 mM AICAR and with 10 nM insulin or no treatment for 15 min. Lysates then were analyzed by Western blotting for total and phosphorylated AKT. ($N = 3$) *$P < 0.05$ for each experiment, error bars represent *s.e.m.* Although there was some variability in total AKT among samples, similar trends were observed when pAKT levels were normalized to total AKT controls. Moreover, most experiments were repeated at least 3 times with similar trends in pAKT/Total AKT ratios observed. The absolute magnitude of pAKT/Total AKT ratios may have differed slightly for each individual experiment but the trends remained similar.

antibodies (Santa Cruz) for 1 h. Blots were washed 3 times with PBST and once with PBS without tween. Densitometry analysis was performed using ImageJ software (NIH).

2.4. Knockdown of AMPK by siRNA

Stealth siRNA targeting AMPK (Invitrogen) were resuspended to make a 20 uM solution following the manufacturer's instructions. Transfections were carried out in Huh7 cells using 10 nM of AMPK siRNA and lipofectamine RNAiMAX (Invitrogen) following the reverse transfection protocol for Huh7 cells.

2.5. Calculations and statistics

Individual culture experiments were performed in triplicates and repeated 3 independent times using matched controls, and the data were pooled. Results were expressed as mean ± *s.e.m.*. The statistical significance of differences ($P < 0.05$) was assessed by 2-tailed *t* test.

3. Results

3.1. Glucagon impairs AKT^{Ser473} phosphorylation by insulin in hepatic cells

Huh7 cells, a relatively differentiated human hepatoma cell line that expresses the glucagon receptor and retains many of the metabolic functions found in normal hepatocytes and liver, were pre-treated with 0.2 nM glucagon for 24 h followed by insulin (10 nM) treatment for 15 min. The activation state of protein kinase B (AKT) was monitored using an antibody specific against AKT that is phosphorylated at Serine 473, a critical phosphorylation site regulating insulin action. Our results showed that glucagon significantly inhibited insulin induced AKT phosphorylation in Huh cells (Fig. 1A).

3.2. cAMP and protein kinase A are involved in glucagon's inhibition of AKT^{Ser473} phosphorylaton by insulin

We next examined inhibition of AKT^{Ser473} phosphorylation in hepatic cells co-treated with H-89 (PKA inhibitor) and glucagon. As shown earlier in Fig. 1, glucagon inhibited insulin-induced phosphorylation of AKT^{Ser473}. However, co-treatment with H89 prevented the inhibition of AKT^{Ser473} phosphorylation by glucagon in Huh7 cells (Fig. 2A, B). We next treated Huh7 cells with a PKA-specific cAMP analogue, 6-Bnz cAMP, for 24 h. 6-Bnz cAMP increased the activity of PKA as evident from the increased phosphorylation of PKA substrates (Fig. 2C) while it simultaneously suppressed the phosphorylation of AKT^{Ser473} (Fig. 2C, D). Taken together, these results show that glucagon inhibition of insulin-induced AKT phosphorylation likely involved activation of the classical PKA pathway.

3.3. Metformin-induced phosphorylation of AKT^{Ser473} is associated with reciprocal AMPK^{Thr172}/AMPK^{ser173} phosphorylation

To better understand the mechanism of PKA regulation of AKT phosphorylation, we treated Huh7 cells with metformin, a major

drug currently used for the treatment of insulin-resistant Type 2 diabetes. Metformin has been known to enhance insulin sensitivity by activating AMPK [13]. More recently, Birnbaum and associates showed that metformin can inhibit PKA activity by increasing AMP in mouse hepatocytes, leading to inhibition of adenylate cyclase. This, in turn, resulted in lower levels of cyclic AMP and protein kinase activity [12]. Therefore, we asked whether AMPK could serve as a vital link between PKA and AKT activities. Consistent with earlier findings, we observed that metformin decreased PKA activity in Huh7 cells (Fig. 3A). Furthermore, metformin increased phosphorylation of AMPK^{Thr172} along with increased basal AKT^{Ser473} phosphorylation (Fig. 3B, C). Since PKA previously was shown to decrease AMPK activity by stimulating an inhibitory phosphorylation at AMPK Ser173 (AMPK^{ser173}) [14], we next asked whether metformin might decrease phosphorylation at this site. Indeed, we observed that metformin's induction of activating phosphorylation pAMPK^{Thr172} was associated with a concomitant reduction in inhibitory phosphorylation at AMPK^{Ser173} (Fig. 3D, E). Taken together with previous findings [12,15], our results suggested that metformin decreased intracellular cAMP concentration leading to a subsequent decrease in PKA activity and AMPK^{Ser173} phosphorylation. These events, in turn, led to an increase in AMPK^{Thr172} phosphorylation and resulting in increased AKT phosphorylation. The role of AMPK in contributing to insulin-mediated AKT activation was further corroborated using genetic knockdown (Fig. 3F, G) or pharmacological activation using AICAR (Fig. 3H, I) to demonstrate that AMPK clearly was involved in enhancing AKT^{Ser473} phosphorylation by insulin.

3.4. PKA activation by glucagon suppresses phosphorylation of AMPK^{Thr172} by phosphorylating AMPK^{Ser173}

Based on the above results we next checked whether glucagon decreased AKT^{Ser473} phosphorylation through differential AMPK phosphorylation. Huh7 cells were treated with 0.2 nM glucagon for 24 h. Interestingly, AMPK^{Thr172} phosphorylation was inhibited by glucagon (Fig. 4A, B). Moreover, the PKA inhibitor, H89, increased AMPK^{Thr172} phosphorylation suggesting that PKA had similar action on AMPK activation in hepatic cells (Fig. 4A, B) as previously reported in adipocytes [14]. To further examine the inhibition of AMPK activity by PKA, we simultaneously measured the phosphorylation of AMPK^{Thr172} and AMPK^{Ser173} by glucagon using antibodies that were specific for each site. We observed increased phosphorylation of AMPK^{Ser173} and reciprocally decreased AMPK^{Thr172} consistent with a down-regulation of AMPK activity (Fig. 4C, D). These findings are consistent with the notion that glucagon activation of PKA increased phosphorylation of AMPK^{Ser173} which reciprocally decreased phosphorylation of AMPK^{Thr172}, and then led to decreased AKT phosphorylation by insulin (Fig. 4E).

4. Discussion

In this paper, we examined the mechanism for glucagon inhibition of insulin signaling in a relatively differentiated human hepatoma cell line that expresses the glucagon receptor and retains many of the metabolic functions found in the rodent and human

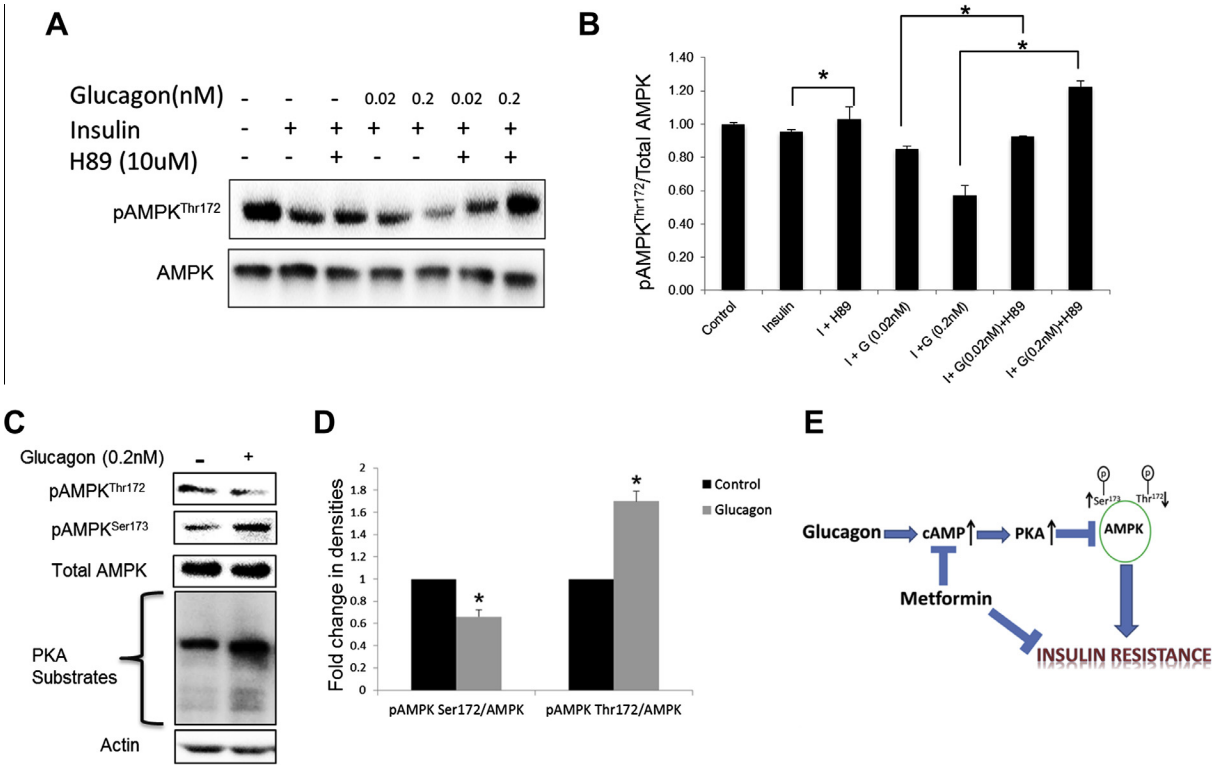

Fig. 4. Glucagon decreases pAMPK^{Thr172} in a PKA-dependent manner and increases pAMPK^{Ser173} in hepatic cells. (A, B) H89 effects on glucagon inhibition of pAMPK^{Thr172} in hepatic cells. Huh7 cells were pre-treated with 10 μM H89 for 24 h and then incubated with the indicated concentrations of glucagon for 24 h. Cells were then treated with 10 nM insulin for 15 min lysed, and total cellular protein was analyzed by Western blot for total AMPK and pAMPK^{Thr172}. (C, D) Reciprocal effects of glucagon on pAMPK^{Thr172} and pAMPK^{Ser173}. Huh7 cells were treated with 0.2 nM glucagon for the 1 h. Cells were lysed and subsequently analyzed by Western blotting for total AMPK, pAMPK^{Thr-72}, and pAMPK^{Ser173}. $N = 3$ for each experiment; $^{*}P < 0.05$ for each experiment; error bars represent *s.e.m.* (E) Proposed model for glucagon induced insulin resistance.

liver. Although, glucagon has been shown to inhibit insulin signaling, its mechanism in human hepatic cells is far from clear [16]. Here, we provide direct evidence for the existence of a PKA–AMPK–AKT signal transduction pathway that likely plays a significant role in insulin resistance in human hepatic cells. Our data showed that glucagon impairs insulin induced AKT activation via its classical cAMP–PKA pathway. PKA activation leads to $AMPK^{Ser173}$ phosphorylation and inhibition of $AMPK^{Thr172}$ phosphorylation. Our results are in agreement with earlier work that showed $AMPK^{Ser173}$ phosphorylation by PKA promoted lipolysis in adipocytes by inhibiting LKB1-mediated AMPK activation [14]. Also, it recently has been shown that the AMPK activator, metformin, inhibited glucagon signaling via increased intracellular cAMP although the precise mechanism was not known [12]. Of note, our results stand in contrast to an earlier report which showed that glucagon increased $AMPK^{Thr172}$ phosphorylation in rat liver [17]. Two possible explanations may account for this discrepancy: first, inter-species differences in the structural assembly of AMPK could explain its variable modulation in humans *vs.* rodents [18]. Second, PKA-independent cAMP signaling through Epac1 may induce activation of AMPK under certain conditions [19]. Therefore, at least in our model of human hepatic cells, we believe that phosphorylation of $AMPK^{Thr172}$ is critically important for mediating insulin sensitivity since it is decreased by glucagon and increased by metformin. A central feature in the proposed model of glucagon-induced insulin resistance involves phosphorylation of $AMPK^{Ser173}$ by PKA to inhibit $AMPK^{Thr172}$ phosphorylation. Moreover, it appears that this mechanism may be a general one since it affects insulin sensitivity in both adipocytes and hepatic cells.

AMPK activators have been shown to increase insulin sensitivity [20]. However, a recent paper presented evidence against an exclusive AMPK-mediated role for metformin's action and suggested that PKA signaling may be involved [12]. According to their findings metformin increases intracellular AMP, which in turn, inhibits adenyl-cyclase activity and reduces cAMP and decreases PKA activity in primary culture hepatocytes. Based on our results, metformin's enhancement of insulin signaling likely depends upon the phosphorylation of $AMPK^{Thr172}$ that occurs when PKA signaling is reduced and there is less inhibitory $AMPK^{Ser173}$ phosphorylation. Thus, our data present a novel unifying model in which PKA signaling regulates reciprocal activating and inhibitory AMPK phosphorylation sites contribute to the abilities of metformin or glucagon to modulate AKT.

In summary, we have provided direct evidence for a mechanism by which glucagon likely impairs insulin action. This mechanism also provides a unifying model to account for metformin's therapeutic effects utilizing both AMPK and PKA signaling (Fig. 4E). Our findings also strongly support the notion that glucagon may actually be a primary contributor to the progression of glucose and lipid deregulation in diabetes. Therefore, suppressing glucagon action through PKA–$pAMPK^{Ser173}$–AKT pathway in the liver presents the potential to improve deranged metabolic functions in diabetic patients.

Disclosure summary

No authors have any competing interest with this manuscript.

Acknowledgments

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