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Biological Response of Hepatomas to an Extract of *Fagopyrum esculentum* M. (Buckwheat) Is Not Mediated by Inositols or Rutin

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Buckwheat contains D-*chiro*-inositol (D-CI) and *myo*-inositol (MI), possible insulin-mimetic compounds; thus, this study investigated the insulin-mimetic activities of a buckwheat concentrate (BWC), D-CI, and MI on insulin signal transduction pathways and glucose uptake with H4IIE rat hepatoma cells. BWC stimulated phosphorylation of p42/44 extracellular-related kinase (p42/44 ERK) and its downstream target, p70^{S6K}, on Thr^{421}. In contrast, D-CI, MI, rutin, or its agylcone form, quercetin, did not activate these signal transduction proteins. Phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), another target of insulin, was also up-regulated upon BWC treatment. The effects of BWC on glucose uptake were subsequently investigated using H4IIE cells. Insulin and D-CI stimulated glucose uptake, whereas BWC inhibited basal and insulin-stimulated glucose uptake. Although results from this work suggest that BWC has insulin-mimetic effects on select protein phosphorylation events in H4IIE cells, D-CI and MI were not the active components responsible for the observed effects. The inhibition of glucose uptake by BWC suggests that buckwheat may affect hepatic glucose metabolism, possibly by inhibiting glucose flux. Furthermore, the fact that D-CI and MI stimulated glucose uptake in H4IIE cells suggests that other compounds are responsible for inhibition of glucose uptake by BWC.

KEYWORDS: Buckwheat; diabetes; p42/44 ERK; glucose uptake; inositols; rutin; *Fagopyrum esculentum* M.

INTRODUCTION

Insulin directly affects glucose uptake in skeletal muscle and adipose tissue through its regulation of the GLUT4 glucose transporter (*1*), whereas in the liver the effect is indirect. Although glucose entry into the hepatocyte is facilitated through the insulin-independent GLUT2 glucose transporter, binding of insulin to its receptor activates signal transduction pathways and enzymes involved in hepatic glucose metabolism, leading to increased glucose utilization through glycolysis, glycogenesis, and suppressed gluconeogenesis (*2*). Thus, initial increases in hepatocyte intracellular glucose concentrations are counteracted by activation of these pathways, eventually leading to a decrease in intracellular glucose concentrations. As a result, glucose enters the hepatocyte by facilitative diffusion through GLUT2, resulting in clearance of glucose from the portal vein and, consequently, a reduction in glycemia.

Buckwheat contains relatively high amounts of free D-*chiro*-inositol (D-CI), *myo*-inositol (MI), and galactosyl derivatives of D-CI known as fagopyritols (*3*). Both D-CI and MI have been identified as components of inositol phosphoglycan (IPG) molecules, which are released from cell membranes in response to insulin, and have demonstrated insulin-mimetic effects (*4*). The insulin-like activities of isolated IPGs and their chemically synthesized analogues have been widely investigated and are summarized elsewhere (*5*−*7*).

Like insulin, IPGs such as D-CI have been shown to lower serum glucose levels in rats (*8*, *9*). Among the reported insulin-mimetic effects of IPG are in vitro activation of enzymes and transcription of genes related to glucose metabolism, stimulation of glucose transport, GLUT4 translocation, glycogen synthesis, lipogenesis, and protein synthesis (*5*−*7*). An IPG containing MI has been reported to modulate key intermediates in the insulin-signaling pathway, such as phosphatidylinositol 3-kinase (PI3K), extracellular related kinase (ERK), and glycogen synthase kinase-3 (GSK-3) (*5*−*7*). Although it has been less studied, the D-CI-containing IPG also exerts insulin mimetic effects in vitro through the activation of key protein phosphatases known to be stimulated by insulin (*5*, *7*).

It has been previously demonstrated that intragastric administration of a buckwheat concentrate (BWC), containing D-CI, MI, and fagopyritols, effectively lowered serum glucose concentrations in streptozotocin (STZ) rats in the fed state (*10*).

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Table 1. Inhibitors of Signal Transduction Proteins in the Insulin Signaling Pathway[a]

compound	inhibits phosphorylation of	working concentration	reference
PD98059	P42/44 ERK	10^{-5} M	Yau et al., 1999 (*18*)
brefeldin A	phospholipases	180 μM	Li et al., 1998 (*29*)
1-butanol	PLD1	0.3%	Morton et al., 1995 (*30*)
PP1	Src	10^{-5} M	Zahradka et al., 2004 (*31*)
AG1024	insulin receptor	0.5×10^{-5} M	Zahradka et al., 2004 (*31*)
LY294002	PI3K	10^{-5} M	Saward and Zahradka, 1997 (*32*)
SB203580	P38 MAPK targets	10^{-5} M	Cuenda and Rousseau, 2007 (*22*)

[a] Inhibitors were prepared in DMSO unless otherwise indicated.

In humans, consumption of buckwheat is associated with a lower prevalence of hyperglycemia (*11*) and improved glucose tolerance in people with diabetes (*12*, *13*). Although the antihyperglycemic effects of free D-CI and BWC (containing D-CI) were presumed to be mediated via the insulin-mimetic activities of IPG, this was not directly investigated. Therefore, the purpose of the present study was to assess the insulin-mimetic activities of BWC, D-CI, and MI on activation of proteins in the insulin signaling pathway. Given the central role of the liver in glucose metabolism, the effects of BWC versus insulin on phosphorylation of signal transduction proteins and hepatic glucose uptake were investigated in H4IIE cells, a hepatoma cell line that has been previously used to characterize hepatic insulin signaling and glucose metabolism (*14*, *15*).

MATERIALS AND METHODS

Tissue culture media, antibiotics, fetal bovine serum, and Nunc tissue culture plates were purchased from Invitrogen. Insulin and MI were purchased from Sigma. D-CI was purchased from Industrial Research Limited. Polyclonal antibodies against phospho-Akt (Ser[473]), phospho-GSK3 (Ser[21/9]), phospho-insulin receptor (Tyr[1146]), p42/44 ERK, phospho-p42/44 ERK (Thr[202]/Tyr[204]), $p70^{S6K}$, phospho-$p70^{S6K}$ (Thr[389]), phospho-$p70^{S6K}$ (Thr[421]), p38 MAPK, phospho-38 MAPK (Thr[180]/Tyr[182]), phospho-STAT3 (Tyr[705]), and phospho-S6 ribosomal protein (Ser[235/236]) were purchased from Cell Signaling. The polyclonal antibody against phospho-Src (Tyr[529]) was obtained from Biosource, and phospho-insulin receptor substrate-I (Tyr[941]) (IRS-1) was obtained from Santa Cruz. HRP-coupled anti-rabbit IgG was purchased from Bio-Rad.

Compounds used as selective inhibitors of signal transduction were obtained from Calbiochem (AG1024 and SB203580), New England Biolabs (PD98059), Biomol Inc. (LY294002 and PP1), and Sigma (brefeldin A; 1-butanol). Inhibitor details are provided in **Table 1**. ^{3}H-Deoxyglucose was purchased from Perkin-Elmer. General laboratory chemicals were purchased from Sigma and Fisher. Ultrapure chemicals (Tris, glycine, SDS, acrylamide, glycerol, Tween 20) were obtained from Invitrogen, Bio-Rad, or Roche.

Preparation of the BWC. The BWC was prepared from the Koto variety of buckwheat (*Fagopyrum esculentum* Moench), which was provided by Kade Research (Morden, MB, Canada). The preparation and analysis of the BWC have been previously described (*10*).

Cell Culture. Rat H4IIE hepatoma cells (American Type Culture Collection, CRL 1548) were cultured as previously described (*16*). Briefly, cells were maintained in α-modified Eagle's media containing 10% fetal bovine serum, 2 mM glutamine, 25 $\mu g \cdot mL^{-1}$ streptomycin, and 25 units mL^{-1} penicillin. Cells were plated and grown to 70% confluence. All cells were placed into serum-free medium for 72 h before the addition of stimulating agents to ensure entry into a quiescent state.

Western Blotting. Cultures of quiescent H4IIE cells, in 12-well culture dishes containing 2 mL of serum-free medium, were stimulated by direct addition of the indicated compounds (volumes of additions were $\leq 10\ \mu$L) without replacement of the medium. Insulin was dissolved in water and added directly to cells at a concentration of 250 nM unless otherwise indicated. Two microliters (0.1% v/v final concentration) of BWC was added directly to cells unless otherwise indicated. This amount was chosen on the basis of equivalence to insulin with respect to p42/44 ERK phosphorylation. MI and D-CI were dissolved in water at a concentration equal to the amounts present in BWC (*10*), and 2 μL of each solution was added directly to cells. The final concentrations of D-CI and MI were 5.72×10^{-10} and 5.56×10^{-10} M, respectively. On the basis of initial time course experiments (data not shown), it was determined that exposure to treatments for 6 min was sufficient to elicit a response for phosphorylation of p42/44 ERK; therefore, 6 min was chosen for all phosphorylation experiments, in agreement with previous observations (*17*).

Inhibitors were added 10 min before the stimulating agents. The cells in 12-well culture dishes were incubated with stimulating agents for 6 min and rinsed with PBS. Cellular protein extract preparation and Western blotting were carried out as previously described (*18*), with the exception that all incubations were carried out at room temperature.

Assay of ^{3}H-Deoxyglucose Uptake in H4IIE Cells. ^{3}H-Deoxyglucose uptake was assayed as previously described (*19*). Inhibitors were added for 10 min prior to the 20 min stimulation with treatments (insulin, 10^{-6} M; BWC, D-CI, and MI, 0.4% v/v, unless otherwise indicated).

Statistical Analyses. All analyses were performed using SAS statistical software (SAS v. 9.1, SAS Institute Inc., Cary, NC). Statistical significance between treatment groups for Western blot data was determined using a mixed-model analysis with a random intercept for the repetitions and by estimate statements for individual comparisons of treatments versus the control. Statistical significance between treatments for glucose uptake data was determined by one-way ANOVA and by Duncan's multiple-range test for post hoc means testing. Differences were accepted as significant at $p < 0.05$. Data are presented as means ± SEM ($n = 3$) with the exception of **Figure 4**, which represents one-time experiments.

RESULTS

Effects of Buckwheat Concentrate on Protein Phosphorylation. To investigate the mechanism by which BWC lowers serum glucose (*10*), we monitored the phosphorylation status of key intracellular modulators of insulin signaling after treatment with BWC, insulin, D-CI, and MI. As shown in **Figure 1**, neither BWC, D-CI, nor MI stimulated changes in insulin receptor, IRS-1, Akt, Src, STAT3, and GSK3 phosphorylation, although insulin elicited the expected responses. In contrast, the phosphorylation of p42/44 ERK and p38 MAPK was increased by both insulin and BWC, whereas neither D-CI nor MI had any effect (**Figure 2A**,**B**). These results were confirmed in FAO hepatoma cells (data not shown). Finally, to establish if p42/44 ERK activation by insulin and BWC is mediated by a common signal transduction pathway, we compared the effect of combining these agents versus the agents alone. Our results indicate that the actions of insulin and BWC on p42/44 ERK, but not p38 MAPK, are additive (**Figure 2C**,**D**).

Cell Signaling Pathways Activated by BWC. The intracellular signaling pathways that contribute to the actions of BWC were evaluated through the use of selective inhibitors of phospholipase D (PLD), Src kinase (Src), and Arf3 in conjunction with Western blot analysis of protein phosphorylation. Panels **A** and **B** of **Figure 3** show representative results for insulin receptor and p42/44 ERK phosphorylation by insulin and BWC when PLD, Src, and Arf3 were inhibited. Phosphorylation of the insulin

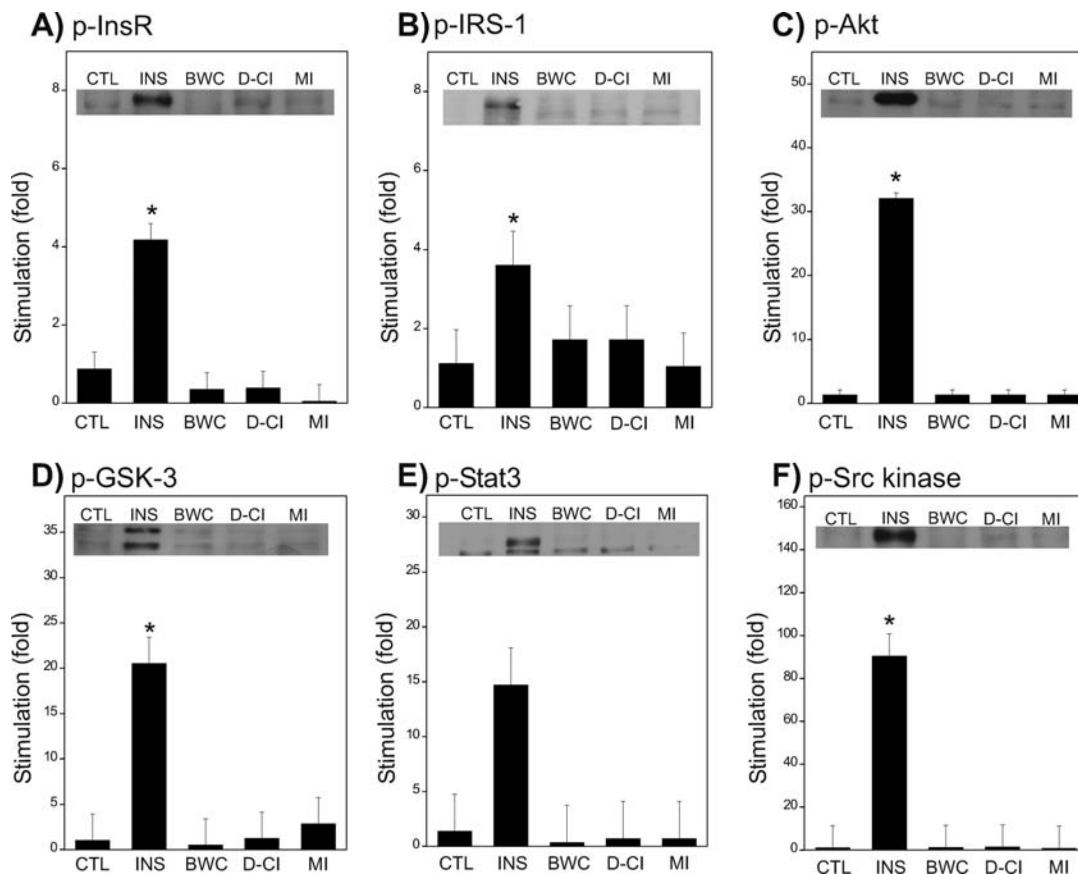

Figure 1. Comparative actions of insulin, buckwheat concentrate, D-CI, and MI on protein phosphorylation in H4IIE cells. Cells were treated with insulin (INS; 250 nM), BWC (0.1% v/v), D-CI (5.72×10^{-10} M), and MI (5.56×10^{-10} M) individually for 6 min, with untreated cells serving as the control (CTL). Protein phosphorylation was monitored by Western blotting with phospho-specific antibodies for (**A**) the insulin receptor, (**B**) IRS-1, (**C**) Akt, (**D**) GSK-3, (**E**) Stat3, and (**F**) Src kinase. Representative blots are shown in the insets. Band intensities were quantified by scanning densitometry and are plotted as means ± SE ($n = 3$) relative to control (set to 1). Significant differences ($p < 0.05$) between treatments versus the control (∗) are indicated. The *p* value for the comparison of insulin-stimulated phosphorylation of Stat3 versus the control was 0.06.

receptor was stimulated by insulin only, and PLD, Src, and Arf3 were not required. As seen previously (**Figure 1A**), BWC did not stimulate phosphorylation of the insulin receptor. However, as indicated in **Figure 3B**, the insulin and BWC stimulation of p42/44 ERK phosphorylation was blocked by 1-butanol, a PLD inhibitor. In contrast, the Src kinase inhibitor PP1 affected only the stimulation of p42/44 ERK by BWC. Arf3, which is sensitive to brefeldin, is not involved in this process.

We also compared the effects of BWC versus insulin on phosphorylation of proteins downstream from p42/44 ERK. As shown in **Figure 4A**, BWC stimulated the phosphorylation of $p70^{S6K}$ (Thr^{421}), but not $p70^{S6K}$ (Thr^{389}). In contrast, insulin stimulates the phosphorylation of $p70^{S6K}$ on both residues. Phosphorylation of ribosomal protein S6, which is immediately downstream from $p70^{S6K}$, occurred in the presence of insulin but not in the presence of BWC. The latter result is not surprising because S6 phosphorylation requires fully active $p70^{S6K}$, which is achieved when it is phosphorylated on both Thr^{421} and Thr^{389} (*20*). Panels **B** and **C** of **Figure 4** show that stimulation of $p70^{S6K}$ (Thr^{421}) by both insulin and BWC is p42/44 ERK dependent (PD98059 sensitive), but is independent of PI3-kinase (LY294002 sensitive). In contrast, insulin-dependent stimulation of $p70^{S6K}$ (Thr^{389}) phosphorylation requires activation of PI3K but is independent of p42/44 ERK (**Figure 4C**). These results illustrate that BWC is not capable of activating PI3-kinase, which is a critical mediator of insulin signaling (*21*).

Interactions between p42/44 ERK and p38 MAPK. To establish whether BWC operates through a common pathway to activate p42/44 ERK and p38 MAPK, selective inhibitors of these signaling enzymes were employed. As expected, PD98059, a potent inhibitor of p42/44 ERK phosphorylation (*18*), blocked activation of p42/44 ERK in response to BWC (**Figure 5A**). Likewise, SB203580, a p38 MAPK inhibitor (*22*), blocked the increase in phosphorylation of p38 MAPK caused by BWC (**Figure 5B**). Interestingly, PD98059 also prevented the stimulation of p38 MAPK (**Figure 5B**), whereas SB203580 inhibited p42/44 ERK phosphorylation (**Figure 5A**), suggesting crosstalk between the pathways.

In addition, we observed that SB203580 did not interfere with BWC- and insulin-dependent phosphorylation of $p70^{S6K}$ on Thr^{421}, but rather increased it (**Figure 6A**). This contrasts with the previous result wherein PD98059 clearly prevented this event (**Figure 4B,C**), thus implying p38 MAPK operates as a repressor whereas p42/44 ERK enhances phosphorylation of $p70^{S6K}$ on Thr^{421} by BWC and insulin. Furthermore, inhibition of p38 MAPK promoted insulin-stimulated phosphorylation of $p70^{S6K}$ on Thr^{389} (**Figure 4D**). These results suggest p42/44 ERK and p38 MAPK mediate distinct signal transduction pathways activated by BWC and insulin.

Glucose Uptake in H4IIE Cells Is Stimulated by Insulin, But Inhibited by BWC. In addition to our examination of signal transduction pathways, we compared the ability of BWC, insulin, D-CI, and MI to stimulate glucose uptake by H4IIE hepatoma

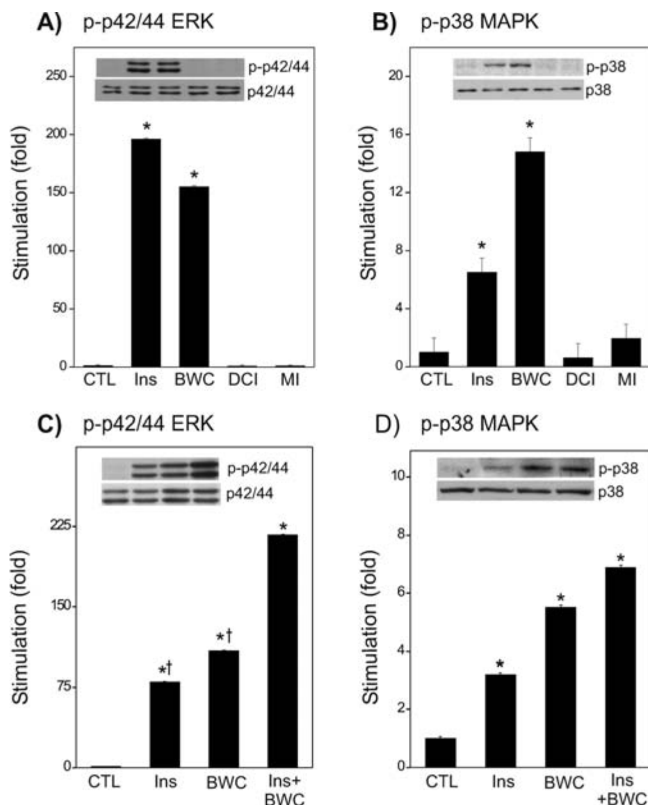

Figure 2. Activation of p42/44 ERK and p38 MAPK by insulin and BWC in H4IIE cells. Cells were treated with insulin (Ins; 250 nM), BWC (0.1% v/v), D-CI (5.72×10^{-10} M), or MI (5.56×10^{-10} M) for 6 min, with untreated cells serving as the control (CTL). Phosphorylation was monitored by Western blotting with phospho-specific antibodies for (**A**, **C**) p42/44 ERK and (**B**, **D**) p38 MAPK. Representative blots are shown in the insets. Band intensities on each blot were quantified by scanning densitometry and plotted as means $\pm$ SE ($n = 3$). Significant differences ($p < 0.05$) between treatments versus the control ($*$) are indicated. † denotes significant difference compared to Ins + BWC ($p < 0.05$).

cells. As shown in **Figure 7A**, insulin and D-CI stimulate glucose uptake above basal levels. Although MI also appears to slightly increase glucose uptake, the data were not statistically different from untreated cells. In contrast, BWC inhibited basal glucose uptake. We investigated the dose-dependent effects of BWC on inhibition of basal glucose uptake and found that 0.5 μL of BWC (0.1% v/v final concentration) had no effect on basal glucose uptake, whereas 2 and 10 μL of BWC (0.4 and 2%, respectively) apparently blocked basal glucose uptake

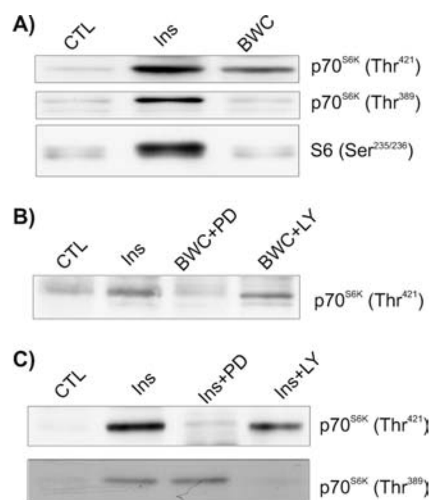

Figure 4. Contrasting effects of insulin and BWC on activation of $p70^{S6K}$ and ribosomal protein S6 in H4IIE cells. (**A**) Cells were treated with insulin (Ins; 250 nM) or BWC (0.1% v/v) for 6 min, with untreated cells serving as the control (CTL). (**B**) Cells were pretreated with PD98059 (PD; 10^{-5} M; inhibits p42/44 ERK) or LY294002 (LY; 10^{-5} M; inhibits PI3K) for 15 min prior to treatment with the BWC. (**C**) Cells were pretreated PD (10^{-5} M) or LY (10^{-5} M) for 15 min prior to treatment with insulin (Ins; 250 nM). Phosphorylation of $p70^{S6K}$ (Thr^{421}), $p70^{S6K}$ (Thr^{389}), and ribosomal protein S6 ($Ser^{235/236}$) was assessed using Western blotting. Representative blots ($n = 1$) are shown.

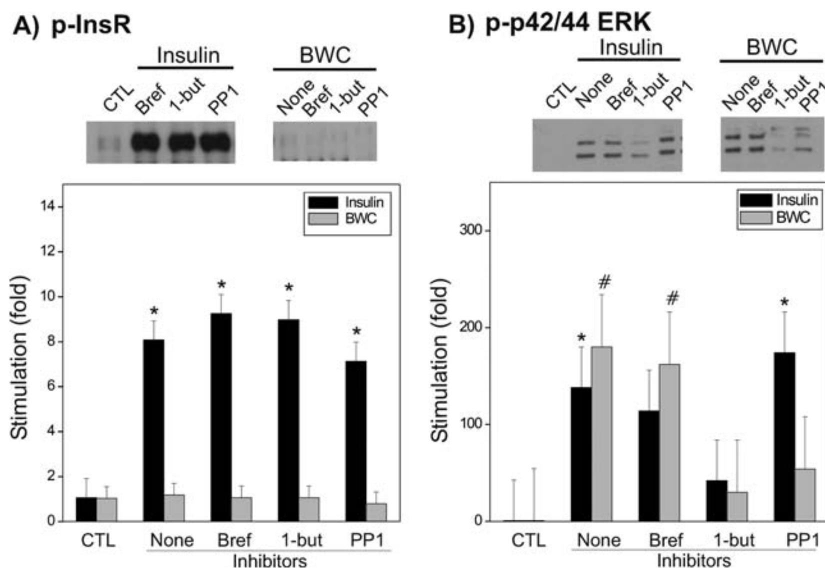

Figure 3. Insulin- and BWC-stimulated phosphorylation of the insulin receptor and p42/44 ERK after inhibition of phospholipase D, Src kinase, and Arf3 in H4IIE cells. Following a 15 min preincubation with butanol (0.3% v/v; inhibits phospholipase D), PP1 (10^{-5} M; inhibits Src kinase), or brefeldin (1.8×10^{-4} M; inhibits Arf), cells were treated with either insulin (250 nM) or BWC (0.1% v/v) for 6 min. The effects of inhibitors on BWC and insulin-stimulated phosphorylation of (**A**) insulin receptor (InsR) and (**B**) p42/44 ERK are shown. Representative blots are presented above each graph. Band intensities on each blot were quantified by scanning densitometry and plotted as means $\pm$ SE ($n = 3$). $*$ indicates statistical significance ($p < 0.05$) for insulin $\pm$ inhibitors versus the control, and # indicates statistical significance ($p < 0.05$) for BWC $\pm$ inhibitors versus the control.

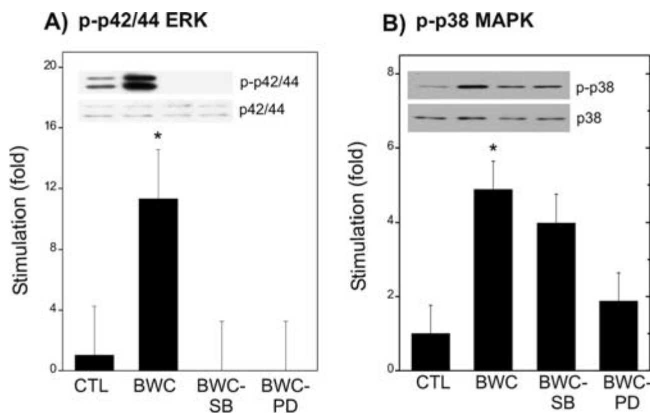

Figure 5. Mutual regulation of BWC-stimulated p42/44 ERK and p38 MAPK phosphorylation in H4IIE cells. Following a 15 min preincubation with 10^{-5} M of the indicated inhibitors, H4IIE cells were treated with BWC (0.1% v/v) for 6 min, with untreated cells serving as the control (CTL). Band intensities of (**A**) p42/44 ERK and (**B**) p38 MAPK on each blot were quantified by scanning densitometry and plotted as means ± SE (n = 3). Significant differences (p < 0.05) between treatments versus the control (∗) are indicated.

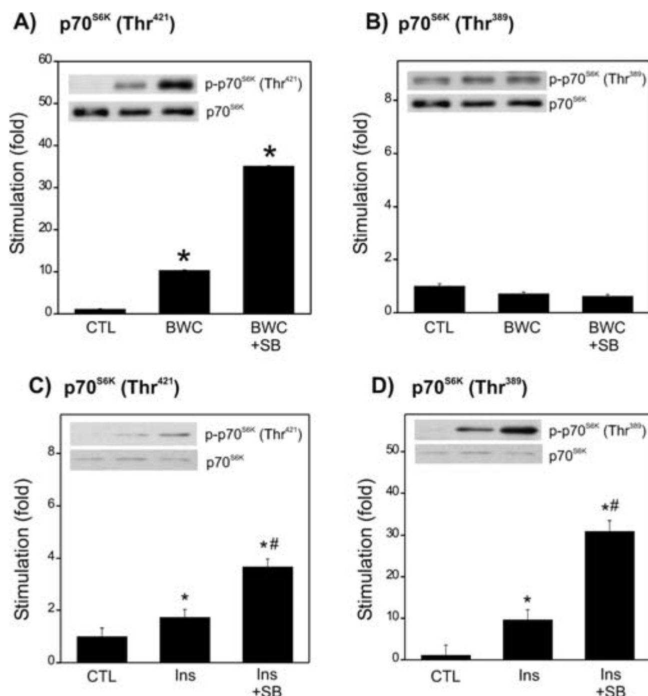

Figure 6. Insulin- and buckwheat-stimulated phosphorylation of $p70^{S6K}$ in response to p38 MAPK inhibition in H4IIE cells. Following a 15 min preincubation with the p38 MAPK inhibitor SB203580 (SB; 10^{-5} M), H4IIE cells were treated with Ins (250 nM) or BWC (0.1% v/v) for 10 min, with untreated cells serving as the control (CTL), and blotted for phospho-$p70^{S6K}$ (Thr^{421}) (**A**, **C**) or $p70^{S6K}$ (Thr^{389}) (**B**, **D**). Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SE (n = 3). Significant differences (p < 0.05) between treatments and the control are indicated (∗). Significant differences (p < 0.05) between Ins + SB203580 and the control are indicated (#).

(**Figure 7B**). Interestingly, addition of BWC to cells 10 min prior to treatment with insulin also prevented glucose uptake (**Figure 7C**).

In addition to D-CI and MI, buckwheat contains an abundant amount of rutin. Both rutin and the aglycone form of rutin, quercetin, have been previously shown to inhibit glucose uptake (*23*). We therefore examined the effect of rutin and quercetin relative to BWC on glucose uptake by H4IIE cells. Rutin had no effect on glucose uptake, but 50 μM quercetin was equipotent with BWC (**Figure 7D**). Neither rutin nor quercetin stimulated phosphorylation of 42/44 ERK (**Figure 7E**,**F**). Because ethanol/water, which was used to prepare BWC, would not be expected to remove rutin and quercetin from the buckwheat on the basis of incompatible polarities, we analyzed BWC for rutin and quercetin. Neither compound was detected (data not shown). We therefore conclude that neither rutin nor quercetin is responsible for the glucose uptake inhibitory actions of the BWC.

This study also examined the contribution of various signal transduction mediators to inhibition of glucose uptake by BWC. Unfortunately, identifying the contribution of p42/44 ERK to this process was not possible, as PD98059 was a potent inhibitor of insulin-mediated uptake (**Figure 8A**). As expected, insulin operated through the insulin receptor, as indicated by the decrease in glucose uptake seen with the insulin receptor tyrosine kinase inhibitor AG1024. On the other hand, addition of 1-butanol, a PLD inhibitor, had a partial effect, because uptake was reduced only to basal levels. When the same inhibitors were tested with BWC, no changes were observed (**Figure 8B**). These results suggest the actions of BWC on glucose uptake are independent of p42/44 ERK. We performed a separate series of experiments with the p38 MAPK inhibitor SB203580. This compound was unable to affect the actions of either insulin or BWC on glucose uptake (**Figure 8C**). These results confirm that p42/44 ERK and p38 MAPK operate via distinct pathways for both of these agents, with p42/44 ERK being linked to glucose uptake but not p38 MAPK.

DISCUSSION

Oral administration of BWC significantly lowers blood glucose levels in STZ rats (*10*). Because BWC contains the insulin-mimetic compounds D-CI and MI (*3*), it was hypothesized that BWC would have insulin-mimetic effects on cell signal transduction proteins and would promote glucose uptake in H4IIE rat hepatoma cells. The results of the present study indicate that BWC does not function as an insulin mimetic because it does not activate the same pathways as insulin. Likewise, neither D-CI nor MI stimulated phosphorylation of the proteins examined in this study. In contrast, both insulin and BWC activated p42/44 ERK and p38 MAPK. Furthermore, it was expected that the presence of D-CI and MI in BWC would enhance glucose uptake; however, BWC inhibited basal glucose uptake in the H4IIE cells, contrary to the effects of insulin, D-CI, and MI. This suggests that D-CI and MI may not be responsible for the previously observed antihyperglycemic effects of BWC.

Compounds having insulin-mimetic properties show promise in the treatment of diabetes (*24*). In addition to pharmacological insulin mimetics (*25*), naturally derived insulin mimetics also exist, including D-CI and MI-containing IPG (*5*−*7*). The insulin-mimetic effects of free D-CI and MI have not been evaluated previously at the cell signal transduction level; however, free D-CI has demonstrated blood glucose lowering effects in animal models of diabetes, presumably through a mechanism related to insulin-like activities of IPG (*8*, *9*). At the same time, in the current study, isolated D-CI and MI did not activate intermediates in the insulin signaling pathway, suggesting that these compounds do not act as insulin mimetics. Furthermore, BWC, which contained D-CI and MI, did not activate insulin signaling intermediates, suggesting that the presence of these compounds is not responsible for previously observed antihyperglycemic effects of BWC (*10*). Buckwheat contains a number of polyphenolic

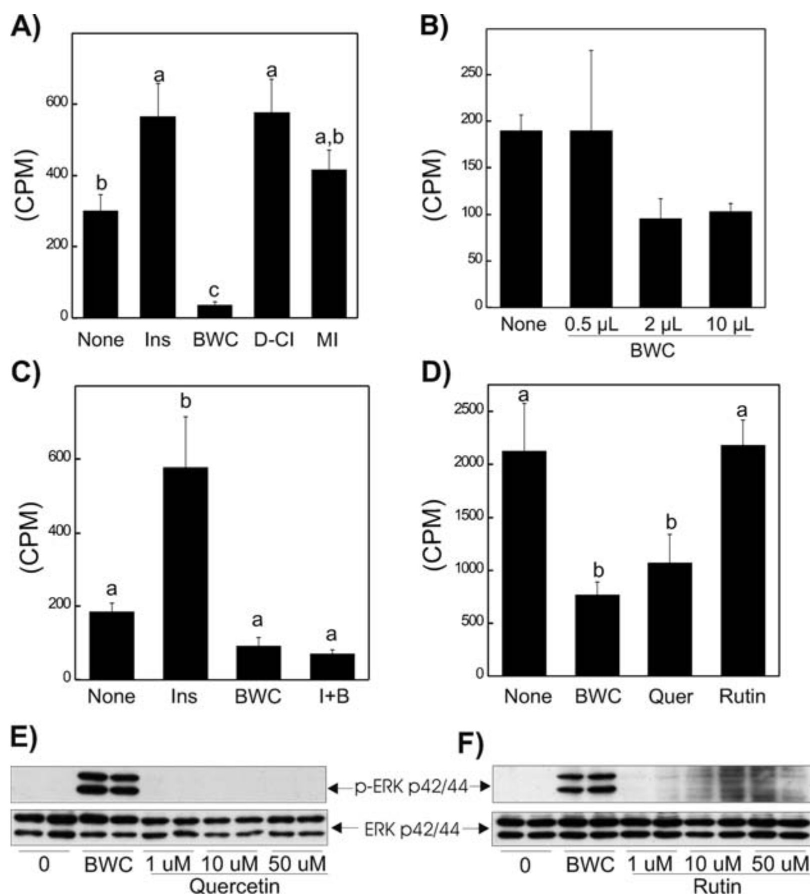

Figure 7. BWC inhibits ^{3}H deoxyglucose uptake in H4IIE cells: (**A**) effects of insulin (Ins; 10^{-6} M), BWC (0.4% v/v), D-CI (0.4% v/v), and MI (0.4% v/v) on glucose uptake; (**B**) dose-dependent effects of BWC on inhibition of glucose uptake; (**C**) effect of BWC (0.4% v/v) on basal and insulin (250 nM)-stimulated glucose uptake; (**D**) effects of quercetin (Quer; 50 μM) and rutin (50 μM) on glucose uptake; (**E**) dose-dependent effects of quercetin on phosphorylation of p42/44 ERK; (**F**) dose-dependent effects of rutin on phosphorylation of p42/44 ERK. The data are presented as means $\pm$ SEM ($n = 3$). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Duncan's multiple-range test. Bars with different letters are significantly different.

compounds including fagopyritols, flavonoids (rutin, quercetin, catechins, epicatechin, hyperoside, and proanthocyanidins), and lignans, and although we have ruled out rutin and quercetin, it is possible that one or more of these compounds is responsible for the insulin-mimetic effects of BWC as several bioflavonoid and phenol compounds have been reported to improve hyperglycemia in diabetes by affecting glucose transport and insulin-receptor function (*26*). It should be noted, however, that many polyphenolic compounds are metabolized in the intestine and do not circulate in their native forms in the bloodstream, but rather as metabolites. For example, quercetin can be rapidly conjugated to glucuronic acid or sulfate during first-pass metabolism and can also be subject to methylation (*27*). Therefore, to provide a more complete representation of in vivo conditions, future in vitro studies should test the effects of such polyphenol metabolites. Nonetheless, unconjugated quercetin (but not rutin) has been detected in plasma from humans who have consumed 100 mg of rutin, suggesting that free quercetin, derived from deconjugation of rutin, does circulate in plasma and may elicit biological effects (*28*).

Treatment of H4IIE cells with insulin and BWC stimulated phosphorylation of p42/44 ERK and p38 MAPK (**Figure 2A**,**B**). As a result, treatment with BWC also stimulated phosphorylation of p42/44 ERK-dependent p70S6K$^{(Thr421)}$, but not PI3K-dependent p70S6K$^{(Thr389)}$; consequently, BWC did not activate ribosomal protein S6, which requires phosphorylation of both Thr^{241} and Thr^{389} of p70S6K. Interestingly, both insulin and BWC require PLD activation for phosphorylation of p42/44 ERK; however, BWC also requires phosphorylation of Src kinase, suggesting BWC phosphorylates p42/44 ERK by activating a pathway that is separate from insulin. This is further supported by the observed additive effects of insulin and BWC on p42/44 ERK phosphorylation (**Figure 2C**).

Perhaps the most surprising result from the current study is the inhibition of both basal and insulin-stimulated glucose uptake by BWC (**Figure 8A**). Although both insulin and BWC stimulate phosphorylation of p42/44 ERK, their respective effects on glucose uptake are opposite and, furthermore, glucose uptake by insulin is dependent on p42/44 ERK, whereas there appears to be no role of p42/44 ERK in inhibition of glucose uptake by BWC (**Figure 8E**,**F**). This latter point reveals that activation of p42/44 ERK and inhibition of glucose uptake by BWC are two unrelated, independent processes and highlights a divergent pathway of p42/44 ERK signaling. Inhibition of PLD had no effect on the inhibition of glucose uptake by BWC.

Overall, the antihyperglycemic effects of buckwheat are not due to the insulin-mimetic activities of naturally present inositol phosphoglycans or rutin and its aglycone directly on the liver, although the effect of BWC in hepatocytes could somehow be related to its antihyperglycemic actions elsewhere in the body. A similar phenomenon of inhibited glucose uptake in the small intestine could potentially explain reduced hyperglycemia observed in rats; if so, buckwheat may represent a useful dietary agent for management of hyperglycemia. The mechanism of inhibited basal and insulin-stimulated glucose uptake by BWC in H4IIE cells can be addressed in future studies. Further research

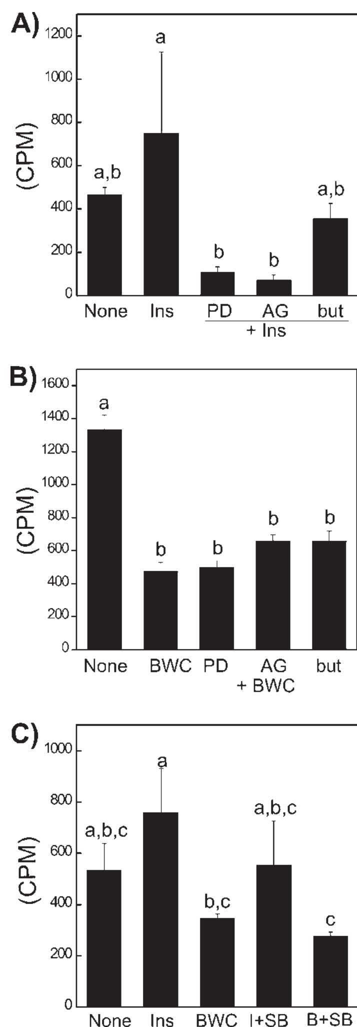

Figure 8. Inhibiting p42/44 ERK, the insulin receptor, PLD, and p38 MAPK ablates insulin-stimulated glucose uptake but has no effect on the inhibition of glucose uptake by BWC in H4IIE cells. H4IIE cells were treated with PD98059 (PD; 10^{-5} M), AG1024 (AG; 10^{-5} M), or 1-butanol (but; 0.3% v/v) 10 min before addition of (**A**) insulin (Ins; 250 nM) or (**B**) BWC (0.4% v/v). (**C**) H4IIE cells were treated with SB203580 (10^{-5} M) 10 min before addition of insulin (Ins) or BWC (0.4% v/v). The data are presented as means ± SEM (n = 3). Statistical significance ($p < 0.05$) was determined by one-way ANOVA and Duncan's multiple-range test. Bars with different letters are significantly different.

can clarify the significance of these findings in terms of glucose metabolism and can also elucidate the active component in BWC responsible for the observed effects.

ABBREVIATIONS USED

BWC, buckwheat concentrate; D-CI, D-*chiro* inositol; ERK, extracellular regulated kinase; GSK-3, glycogen synthase kinase-3; IPG, inositol phosphoglycan; MI, *myo*-inositol; p38 MAPK, p38 mitogen activated protein kinase; p42/44 ERK, p42/44 extracellular regulated kinase; PI3K, phosphatidylinositol 3-kinase; PLD, phospholipase D; Src, Src kinase; STZ, streptozotocin.

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