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Alexandra Cok
Calvin University

Christina Plaisier
Calvin University

Matthew J. Salie
Calvin University

Daniel S. Oram
Calvin University

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Berberine acutely activates the glucose transport activity of GLUT1

Alexandra Cok, **Christina Plaisier**, **Matthew J. Salie**, **Daniel S. Oram**, **Jude Chenge**, and **Larry L. Louters***
Department of Chemistry and Biochemistry, Calvin College, Grand Rapids, MI, USA 49546

Abstract

Berberine, which has a long history of use in Chinese medicine, has recently been shown to have efficacy in the treatment of diabetes. While the hypoglycemic effect of berberine has been clearly documented in animal and cell line models, such as 3T3-L1 adipocytes and L6 myotube cells, the mechanism of action appears complex with data implicating activation of the insulin signaling pathway as well as activation of the exercise or AMP kinase-mediated pathway. There have been no reports of the acute affects of berberine on the transport activity of the insulin-insensitive glucose transporter, GLUT1. Therefore, we examined the acute effects of berberine on glucose uptake in L929 fibroblast cells, a cell line that express only GLUT1. Berberine activated glucose uptake reaching maximum stimulation of five fold at >40 μM. Significant activation ($P<0.05$) was measured within five minutes reaching a maximum by 30 minutes. The berberine effect was not additive to the maximal stimulation by other known stimulants, azide, methylene blue or glucose deprivation, suggesting shared steps between berberine and these stimulants. Berberine significantly reduced the K_m of glucose uptake from 6.7 ± 1.9 mM to 0.55 ± 0.08 mM, but had no effect on the V_{max} of uptake. Compound C, an inhibitor of AMP kinase, did not affect berberine-stimulated glucose uptake, but inhibitors of downstream kinases partially blocked berberine stimulation. SB203580 (inhibitor of p38 MAP kinase) did not affect submaximal berberine activation, but did lower maximal berberine stimulation by 26%, while PD98059 (inhibitor of ERK kinase) completely blocked submaximal berberine activation and decreased the maximal stimulation by 55%. It appears from this study that a portion of the hypoglycemic effects of berberine can be attributed to its acute activation of the transport activity of GLUT1.

1. Introduction

Berberine, an isoquinoline alkaloid isolated from several herbs including *Rhizoma Coptidis*, has a long history of use in Chinese medicine for the treatment of gastrointestinal infections, diarrhea, cardiovascular diseases, inflammation and hypercholesterolemia [1, 2]. More recently, berberine has been also proven to be efficacious for the treatment of type 2 diabetes [1, 3-5]. Studies using human patients [3-6], animal or cell models of insulin resistance [6-12], and insulin sensitive cell lines [13-16] have all established a clear hypoglycemic effect of berberine.

*Corresponding author. Larry L. Louters, Fax: 616-526-8551, Phone: 616-526-6493, lout@calvin.edu.

In general, there are two distinct pathways to activate glucose uptake in peripheral tissues; one stimulated by insulin through the IRS-1/PI 3-kinase and the other by exercise or hypoxia via activation of AMP activated protein kinase (AMPK). In muscle, which is the major tissue responsible for whole body glucose disposal, both pathways stimulate the translocation of GLUT4 to the cell membrane which accounts for the enhanced glucose uptake[17]. Current data suggest that the effects of berberine are complex and may activate portions of both the insulin and the exercise-induced glucose uptake pathways [13, 15]. In addition, berberine inhibits intestinal absorption of glucose which also contributes to berberine's hypoglycemic effect [18].

The effects of berberine on the insulin-stimulated glucose uptake pathway are varied and sometimes conflicting [1, 13], which can be, in part, attributed to the variety of cell types and treatment times utilized in these studies. However, there appears to be general agreement from multiple studies that berberine activates AMPK [6, 7, 13-16, 19]. These studies have been done in insulin sensitive cells where a change in glucose transport is typically attributed to a change in GLUT4 activity. Some studies have specifically implicated GLUT1 as the primary transporter responsible for the enhanced glucose uptake, but these studies investigated the chronic effects of berberine (6 -12 hour treatments) [15, 16]. The acute effects of berberine on GLUT1 activity uptake have not been studied.

There is increasing evidence that the more widely expressed GLUT1, initially thought to be responsible only for basal glucose uptake, can be acutely activated by cell stressors such as azide [20, 21], osmotic stress [22, 23], methylene blue [24], and glucose deprivation [25, 26]. In particular, the acute activation of GLUT1 by hypoxia or azide has been attributed to activation of AMPK [22, 27, 28]. In addition, it has been recently shown that peptide C activates GLUT1 transport activity in erthrocytes, establishing a potential link between GLUT1 activity and diabetes [29]. The specific purpose of this study was to systematically investigate the acute effects of berberine on glucose uptake in L929 fibroblast cells, a cell line that expresses only GLUT1 [30] and has been shown to respond to acute cell stress by increasing glucose uptake [24, 26].

2. Materials and Methods

2.1 Chemicals

Berberine, Compound C, Wortmannin, SB203580, PD98059, cyclohexamide, 2-deoxy-D-glucose-[1,2-^{3}H] (2DG) and D-mannitol-1-^{14}C were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.2 Cell culture

L929 mouse fibroblast cells were obtained from the American Type Culture Collection. To initiate each experiment, approximately 1.0×10^{5} L929 fibroblast cells were plated into each well of a 24-well culture-treated plate in 1.0 mL of low glucose (5.5 mM) DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were grown overnight at 37 °C in an incubator supplied with humidified room air with 5% CO_2. L6 myoblast cells were grown in 24-well plates in DMEM (25.0 mM glucose) supplemented with 10% FBS. The cells were then differentiated for six days by reducing FBS to 2%.

2.3 General experimental design

Cells grown overnight in 24-well plates were rinsed with 0.2 mL of treatment media and then incubated at 37 °C in 0.8 mL of fresh treatment media consisting of either low-glucose DMEM (0% FBS) (basal), or glucose-free DMEM (0% FBS) (activation by glucose

deprivation) plus the stimulant (berberine, azide, or methylene blue) at the concentrations indicated in the figure and table legends. The cells were maintained in this treatment media for varying times as indicated in the figure legends. Studies of inhibitors (Compound C, Wortmannin, SB203580, PD98059, cyclohexamide) included a prior 30-minute pretreatment incubation with the inhibitor at the concentration indicated. Following the treatment period that included both the stimulant and inhibitor, media were removed and glucose uptake was measured during a 10-minute glucose uptake phase as described below.

In experiments designed to measure recovery from the effects of berberine, the treatment phase was followed by a recovery period in which cells were washed and returned to low-glucose media (0.8 mL) without berberine for varying times as indicated in figure legend. Glucose uptake was measured as described below.

2.4 Glucose uptake assay

Glucose uptake was measured using the radiolabeled glucose analog 2-deoxyglucose (2DG) as previously described [23]. Briefly, the media was replaced with 0.3 mL of glucose-free HEPES buffer (140 mM NaCl, 5 mM KCl, 20 mM HEPES/Na pH=7.4, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 2 mM NaPyruvate, 1 mM mannitol) supplemented with 1.0 mM (0.3 μCi/mL) 2-DG (1, 2-^{3}H) and 1.0 mM (0.02 μCi/mL) mannitol (1-^{14}C). For the kinetics experiments the concentration of 2-DG in the uptake solution was varied as indicated in the figure legend. After a 10-minute incubation, cells were washed twice with cold glucose-free HEPES. The cells were lysed in 0.5 mL lysis buffer (10 mM Tris pH=7.4, 150 mM NaCl, 5 mM EDTA, 1.0% triton X-100, 0.4% SDS) and the ^{3}H-2 DG uptake with ^{14}C-mannitol as the extracellular marker was measured using scintillation spectrometry. Uptake of ^{14}C-mannitol only occurs if the cell membrane is compromised. Therefore, the use of a double-labeled uptake solution allows us to both measure surface binding and monitor potential toxic effects of the experimental treatments that would compromise the cell membrane.

2.5 Statistical analysis

Experimental conditions were repeated in triplicate or quadruplicate and glucose uptake was measured and reported as nmol/10 min/well ±standard error. Statistical significance was determined by either ANOVA followed by a post-hoc Dunnett test (dose and time dependent effects) or a two-tailed t-test. Statistical significance is reported at $P< 0.01$ or $P<0.05$. Experiments were repeated several times and results from representative experiments are reported. However, since the concentration dependent experiments (Figure 1) required multiple plates of cells, those data are normalized to control for the variation of cell density from plate to plate.

3. Results

3.1 Berberine activates glucose uptake in a dose dependent manner

Previous work showed that a six hour berberine treatment increases GLUT1 expression and subsequent glucose uptake in 3T3-L1 cells [15]. However, the acute effects of berberine on the activity of this transporter have not been measured. To investigate the acute effects of berberine on basal glucose uptake by GLUT1, L929 fibroblast cells were exposed to DMEM media containing 5.5 mM glucose and increasing concentrations of berberine for 30 minutes. The results, shown in Figure 1, show a dose dependent stimulation of glucose uptake that reached a maximum 5-fold stimulation at 100 μM berberine. Consistent with previous studies with L929 fibroblast cells, both basal and activated glucose uptake were blocked by cytochalasin B indicating the involvement of GLUTs in the glucose transport process [24, 26] (data not shown). There was a significant activation of uptake ($P<0.01$) at 10 μM (1.55×) and uptakes at concentrations greater than 40 μM were not significantly

different from each other. Therefore in subsequent experiments, concentrations >40 μM berberine were considered to be maximally effective. Berberine did not produce any visible toxic effects, as assessed by cell morphology and cell attachment, at any concentration tested.

3.2 Time course of activation and recovery of berberine-activated glucose uptake

To measure the effect of time on berberine activation of glucose uptake, L929 cells were incubated in DMEM media (5.5 mM glucose) containing berberine for times ranging from 5-60 minutes. The results shown in Figure 2 indicate that significant activation ($P<0.01$) was achieved within 5 minutes (from 1.79±0.09 to 2.97±0.18 nmol/10 min/well) and maximized by 30 minutes (4.97±0.17 nmol/10 min/well). The 0, 30, and 60-minute time points were also done in the presence of the eukaroyote translational inhibitor, cyclohexamide, which had no effect on glucose uptake (data not shown).

In a separate experiment, recovery from the effects of berberine was measured by exposing cells to berberine for one hour, followed by a wash and resuspension in media lacking berberine for various times up to 60 minutes. As shown in Figure 3, the effects of berberine were largely maintained for 30 minutes post exposure (86.9%). After 60 minutes of recovery, 50.7% of the activation remained.

3.3 Kinetic and additive effects of berberine activation

In order to gain some insight into to the activation pathway of berberine, we conducted a kinetic analysis and a series of additivity experiments. We had previously shown that glucose uptake in L929 fibroblast cells can be acutely activated by multiple agents including nitric oxide, troglitazone, azide, methylene blue and glucose deprivation [24, 26, 31]. In particular, acute activations by methylene blue and glucose deprivation were shown to be additive with different kinetic effects, suggesting that there are a least two distinct activation mechanisms for glucose uptake in L929 cells [24, 26].

The kinetics of 2DG uptake were measured in the presence and absence of berberine and the results are shown in Figure 4. A best fit analysis of the data using Michaelis-Menten kinetics indicates that berberine does not affect the V_{max} (13.0 ± 0.4 compared to 15.6 ± 1.7 nmol/10 min/well for control), but dramatically lowers the K_m of glucose uptake from 6.7 ± 1.9 mM in control cells to 0.55 ± 0.08 mM.

L929 cells were exposed to maximally effective concentrations of sodium azide (5 mM), methylene blue (50 μM), berberine (50 μM), or media lacking glucose for 30 minutes. Glucose uptake measurements are shown in Table 1 and as can be seen, each stimulant significantly activated glucose uptake. In a separate experiment, cells were exposed to a combination of maximally effective concentrations of berberine plus azide, or methylene blue, or glucose deprivation. As seen in Table 1, the effects of berberine were not further enhanced by any of the other stimulants.

3.4 Effects of inhibitors on berberine activated glucose uptake

Multiple studies have suggested that berberine activates AMPK [6, 7, 13-16, 19]. To explore this possibility, we pretreated cells with 0, 10, 20, or 50 μM Compound C, an inhibitor of AMPK, for 45 minutes, followed by a 30 minute exposure to 50 μM berberine in the continued presence of Compound C. The results, shown in Figure 5, again demonstrate robust activation of glucose uptake from 2.39±0.17 to 7.74±0.36 nmol/10min/well, which was not altered by pretreatment with Compound C.

We also measured the effects of pretreatment of wortmannin (inhibitor of PI3K), SB203580 (inhibitor of p38 MAP kinase), and PD98059 (inhibitor of ERK kinase (or MEK)). As expected and previously shown [15], wortmannin had no effect on either basal or berberine stimulated glucose uptake (data not shown). However, both SB203580 and PD98059 partially inhibited berberine stimulated glucose uptake. We pretreated cells with effective concentrations of either the p38 MAP kinase inhibitor (10 μM SB203580) or the ERK kinase inhibitor (50 μM PD98059) [15] followed by either a submaximal (10 μM) or maximal (50 μM) berberine activation in the continued presence of the inhibitor. As shown in Table 2, neither inhibitor alone significantly altered basal glucose uptake. SB203580 did not affect submaximal berberine activation but did lower maximal berberine stimulation by 26% (decrease from 4.13 ± 0.17 to 3.31 ± 0.09 nmol/10min/well). In contrast PD98059 was a much more effective inhibitor. It completely blocked submaximal berberine activation and decreased the maximal stimulation by 55% (from 4.13 ± 0.17 to 2.41 ± 0.05 nmol/10min/well).

4. Discussion

Berberine, an isoquinoline alkaloid isolated from a number of plants, has a rich history of use in Chinese medicine. It seems clear, considering the wide spectrum of therapeutic applications such as treatments for parasitic infection, bacterial diarrhea, inflammation, cardiovascular disease, hypercholesteremia, and diabetes, that berberine must trigger a number of physiological responses in biological systems.

Recent studies have documented berberine's effectiveness in the treatment of both human diabetes patients [3-5]and animal models of diabetes [6, 10-12]. This has lead to numerous studies designed to understand berberine's mechanism of action regarding its hypoglycemic effect. The results of these studies are somewhat confusing and sometimes conflicting. Some studies indicate that berberine works through the insulin pathway by enhancing either insulin secretion [11] or the signaling cascade [8, 9, 32-35]. Others suggest berberine stimulates AMPK in an insulin pathway-independent fashion [6, 14-16, 36] while others suggest that berberine has effects on both pathways [7, 13]. In most studies the berberine-enhanced glucose uptake has been attributed to increased GLUT4 activity. Some studies indicated that berberine increases GLUT4 production [37] or translocation [6, 34, 37], however other studies were unable to detect changes in GLUT4 [15, 38].

The acute effects of berberine on the transport activity of GLUT1 remain largely unexplored. A study in 3T3-L1 adipocytes showed that 6 hour exposure to berberine increases GLUT1 synthesis with no change in GLUT4 [15]. However, a second study reported that 24 hour exposure to berberine did not change either GLUT1 or GLUT4 content and suggested that berberine increases GLUT1 activity [16]. To explore the effects of berberine on GLUT1 activity, we measured glucose uptake in the presence of berberine in L929 fibroblast cells, a cell line that only expresses GLUT1 [30]. We report that berberine significantly activates transport at 10 μM and reaches a maximum stimulation of about 5-fold at concentrations above 40 μM (see Figure 1). Significant activation occurs within 5 minutes and plateaus at about 30 minutes. This berberine activation of glucose uptake is faster than previously reported in L6 or 3T3-L1 cells, which did not see an effect until after 2-6 hours of exposure [7, 14, 15]. Activation was not sensitive to cyclohexamide, indicating that the berberine effect does not depend on new protein synthesis. Recovery from berberine activation is slower than the activation with about 90% of the activation remaining after 30 minutes and 50% after 60 minutes.

Previous studies have suggested two distinct mechanisms for the activation of glucose uptake in L929 cells, one mechanism illustrated by the effects of methylene blue or sodium

azide and the other by glucose deprivation [24, 26]. The kinetic analysis of the berberine activation indicates that the K_m of glucose transport is decreased, but the V_{max} is unaffected, suggesting that sensitivity of the system is enhanced, but not the responsiveness. This kinetic behavior is similar to that observed upon glucose deprivation in this cell line [26]. To further explore the mechanism of berberine activated glucose uptake we explored the additivity of berberine's effects with maximally effective concentrations of methylene blue and azide or glucose deprivation. The results (Table 1) indicate that among these stimulants, berberine induces the most robust activation of glucose uptake and that its effects are not enhanced by the other stimulants. This suggests that the mechanism of berberine activation shares common steps with both pathways.

Key studies in other cell lines have strongly suggested that berberine activation of glucose uptake is mediated through activation of AMPK. To explore this possibility we measured the dose dependent effects of Compound C, an inhibitor of AMPK, on both basal and berberine-stimulated glucose uptake. Compound C had no effect on glucose uptake, suggesting AMPK is not involve in the stimulation of glucose uptake in L929 cells. This is consistent with previous work in L929 cells that reported that AICAR, an activator of AMPK, inhibited rather than activated glucose uptake [24]. This result was somewhat surprising given that previous work in L6 myotube cells had shown that the activation of glucose uptake by berberine could be completely blocked by compound C [14]. We were able to repeat those results with L6 myotube cells in our laboratory as well (data not shown) suggesting that AMPK is not involved in the activation of glucose uptake in L929 cells. Also, since L6 myotube cells express both GLUT1 and GLUT4, this may suggest that AMPK activation is required for the activation of GLUT4, but not for GLUT1. The lack of involvement of AMPK in regulating glucose uptake in L929 fibroblast cells indicate that additional studies need to be done investigate the expression levels and role of AMPK in this cell line.

Studies that support AMPK activation as a key step in berberine's effects are not all in agreement regarding which downstream kinases are involved. Some studies implicate activation of ERK kinase [15, 16], while others provide evidence of berberine's activation of p38 MAPK [6, 14]. To investigate the involvement of these two kinases we measured the effects of SB203580 (inhibitor of p38 MAPK), and PD98059 (inhibitor of ERK kinase) on berberine stimulated glucose uptake. SB203580 had no effect on submaximally effective concentrations of berberine, but reduced maximum stimulation by 26%, while PD98059 completely blocked submaximal berberine stimulation and reduced maximum stimulation by 55%. The combination of the two inhibitors did not further inhibit berberine stimulation of glucose uptake (data not shown). These data suggest that ERK kinase pathway may be more involved than p38 MAPK, but neither is able to account for the full stimulatory effect of berberine. It is important to note that these inhibitory studies should be considered to be just the initial steps in discerning the mechanism of berberine activation of GLUT1. One concern is a potential cross reactivity of the inhibitors. For example, recent work in striated muscle has demonstrated that SB203580 also significantly inhibits the activation of Akt, a serine/threonine protein kinase, activated in the insulin signaling system [39]. While this may not be important in L929 fibroblast cells, since this cell line is not insulin sensitive, it does point to potential complications in interpreting the results. Thus, future studies should look for a berberine stimulation of the phosphorylation of these kinases as well as identify other factors to account for the full activation of berberine.

5. Conclusions

This study demonstrates for the first time that berberine acutely activates the transport activity of GLUT1. In addition, the mechanism of activation is complex, but likely involves,

in part, the activation of ERK kinase and to a lesser degree p38 MAPK. The activation of these kinases in L929 cells appears to occur without the activation of AMPK. Thus it is likely, that a portion of the hypoglycemic activity of berberine, that has been observed by others, can be attributed to its acute activation of the transport activity of GLUT1. GLUT1 is widely expressed and is the unique transporter responsible for glucose uptake into the central nervous system. Thus, the ability of berberine to acutely up regulate the activity this transporter may suggest a useful strategy to help counter the damaging effects of strokes caused by arterial blockage as well as damage to cardiomycytes that occurs during a cardiac infarction.

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Research Highlights

Berberine acutely activates GLUT1 activity under basal conditions

Berberine decrease the Km of transport with no change in Vmax

Berberine activation does not require AMPK activation in L929 cells, but likely partially involves MEK and p38 MAP kinase

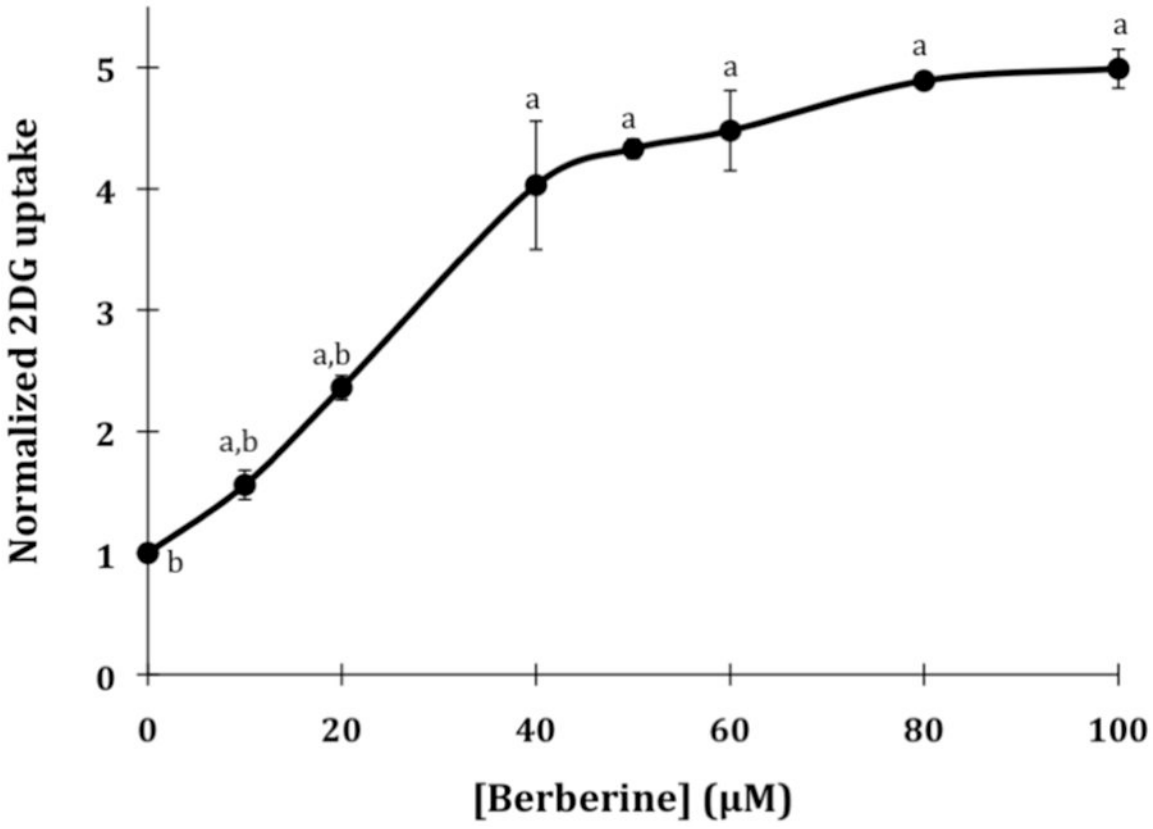

Figure 1.

Effects of berberine on 2DG uptake. L929 fibroblast cells were incubated at 37 °C for 30 minutes in DMEM media (5.5 mM glucose) supplemented with 0-100 μM berberine as indicated. Ten-minute 2DG uptakes were then measured as described in Materials and Methods. Data are means ± S.E. of normalized values from multiple experiments (samples size varied from 3-20). [a]Significantly different than 0 μM berberine and [b]significantly different than 100 μM berberine at $P<0.01$.

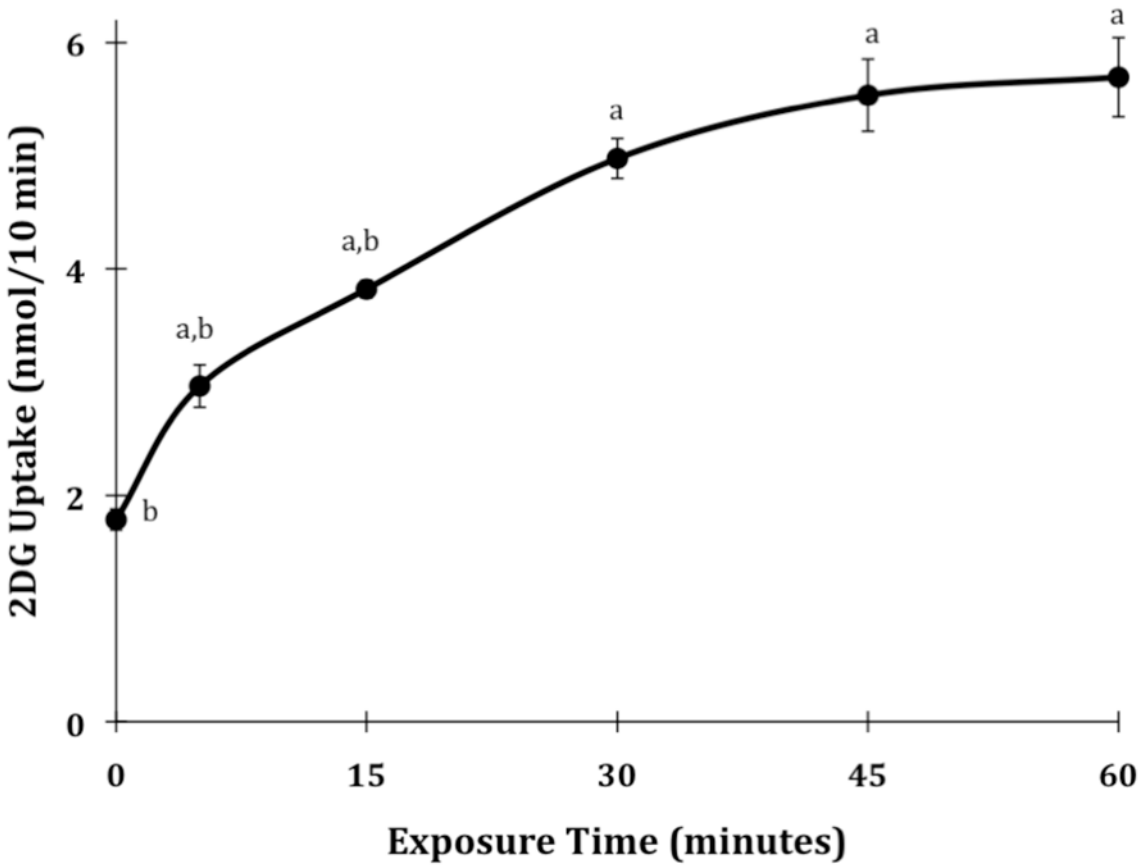

Figure 2.

Effect of time of berberine exposure on 2DG uptake. L929 fibroblast cells were incubated at 37 °C for 30 minutes in DMEM media (5.5 mM glucose) supplemented with 40 μM berberine for 0, 5, 10, 30, 45, and 60 min. Ten-minute 2DG uptakes were then measured as described in Materials and Methods. Data are means ± S.E. of a representative experiment. [a]Significantly different than 0 minute exposure to berberine and [b]significantly different than one hour at $P<0.01$.

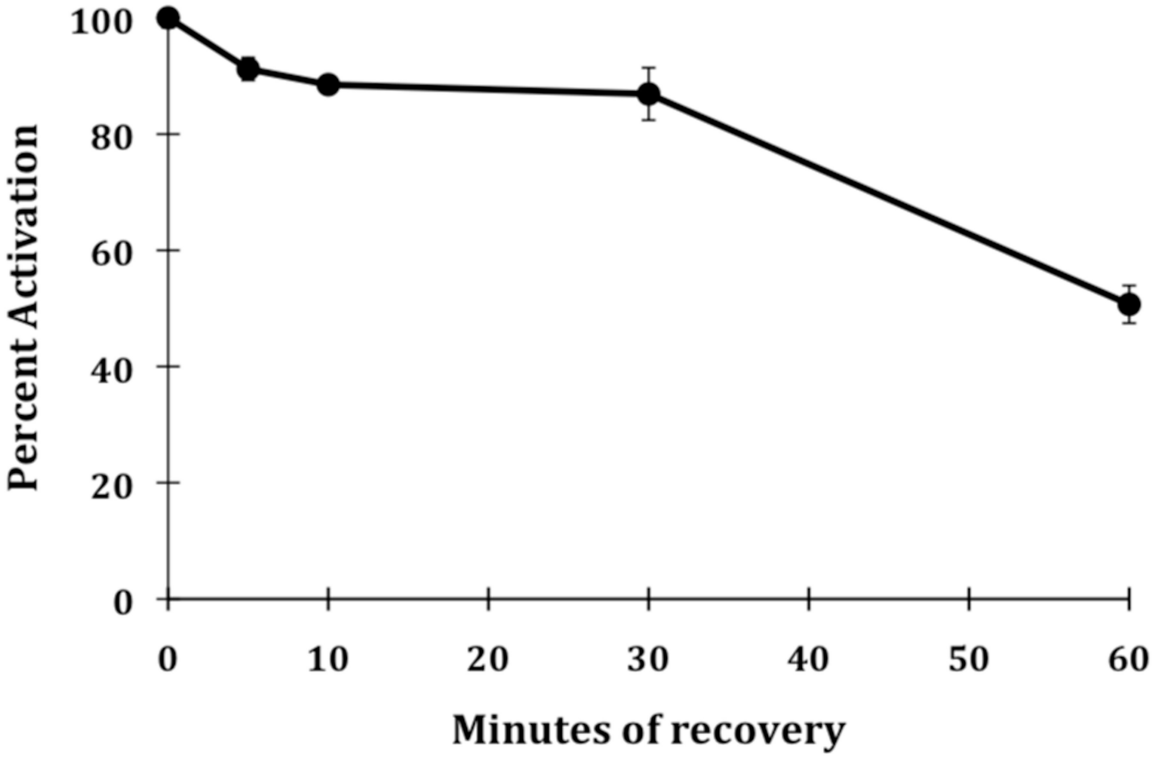

Figure 3.

Effect of recovery time on berberine-activated 2DG uptake. L929 fibroblast cells were incubated at 37 °C for 30 min in 5.5 mM glucose DMEM media supplemented with 40 μM berberine. Samples were washed and resuspended in media without berberine for 0-60 min. Ten-minute 2DG uptakes were then measured as described in Materials and Methods. Data are means ± S.E. of a representative experiment. 2DG uptake rates at all recovery times are statistically different from the control (0 min) at $P<0.05$.

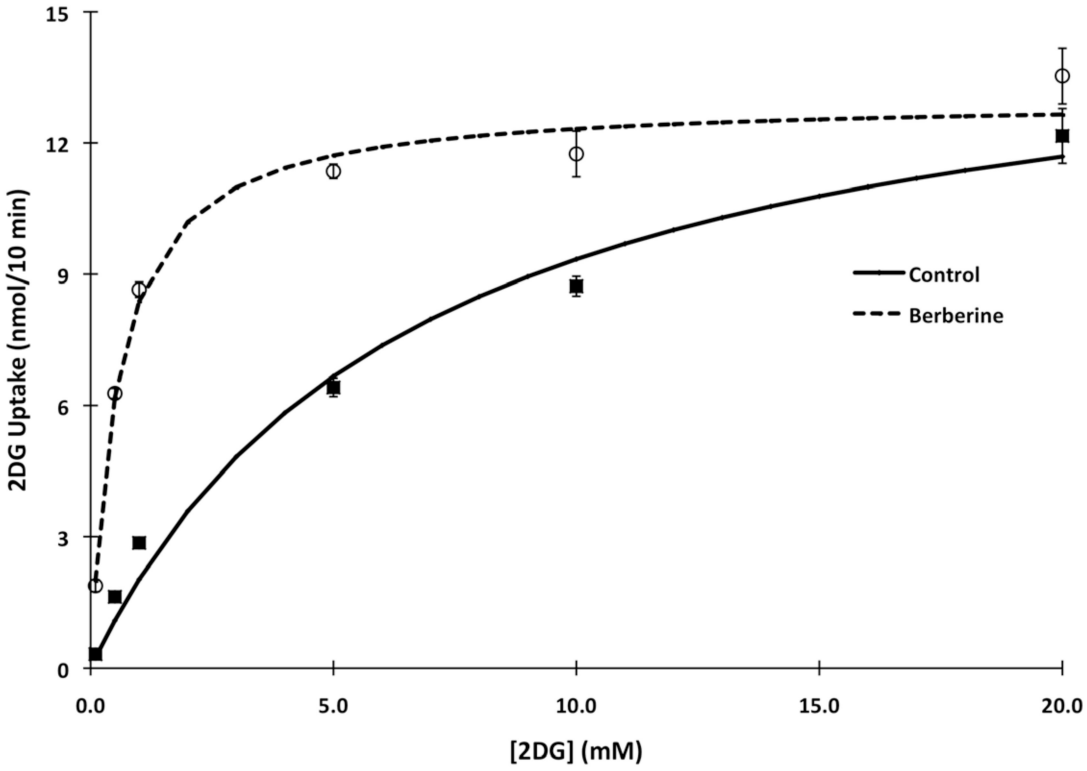

Figure 4.
Kinetics of 2DG uptake in control and berberine-activated cells. L929 fibroblast cells were incubated at 37 °C for 30 minutes in DMEM media (5.5 mM glucose) supplemented with either 0 or 80 μM berberine. Ten-minute 2DG uptakes were then measured at varying concentrations of 2DG (0.1, 0.5, 1, 5, 10, or 20 mM). Data are means ± S.E. of a representative experiment.

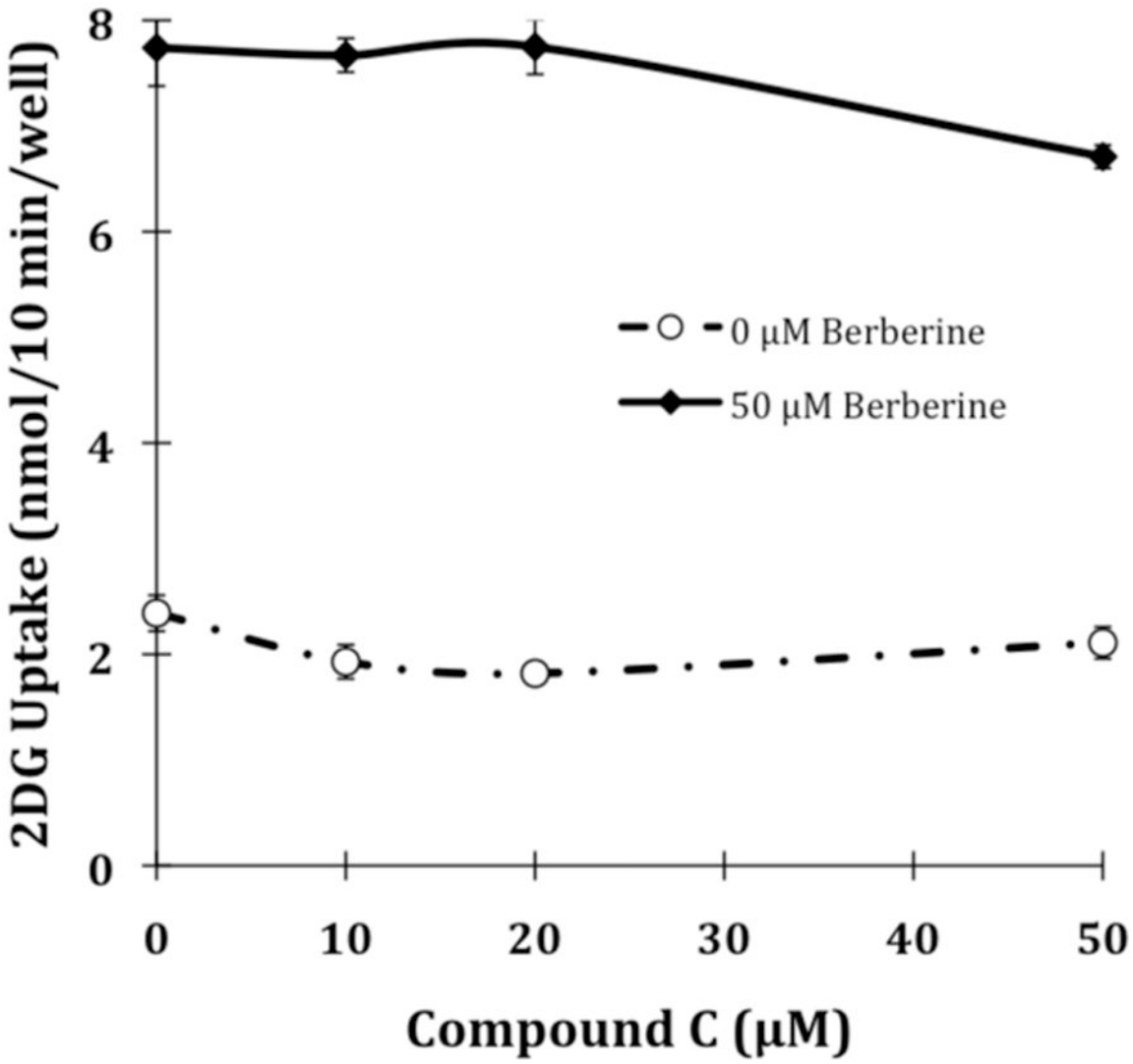

Figure 5.
Effect of Compound C on berberine activated 2DG uptake. L929 fibroblast cells were pretreated for 45 minutes at 37 °C in DMEM media (5.5 mM glucose) supplemented with 0, 10, 20, or 50 μM compound C, then incubated for 30 min in 5.5 mM glucose DMEM media still supplemented with compound C and either 0 or 50 μM berberine. Ten-minute 2DG uptakes were then measured as described in Materials and Methods. Data are means ± S.E. of a representative experiment.

Table 1
Combined effects of various stimulants of glucose uptake

[Berberine]	Controls	+ Azide	+ MB	+No glucose
0 μM	1.00±0.04	1.79±0.09	2.54±0.10	2.87±0.36
50 μM	3.17±0.10	3.23±0.18	3.37±0.18	3.04±0.40

L929 fibroblast cells were incubated for 30 minutes at 37 °in DMEM media (5.5 mM glucose or 0 mM glucose) containing either 0 or 50 μM berberine alone or in combination with either 5.0 mM sodium azide, or 50 μM methylene blue. Glucose uptake was measured as described in Materials and Methods and normalized to control with no berberine. All treatment uptakes were statistically higher than the untreated control cells (0 mM berberine) at P<.01, but all uptakes with 50 μM berberine were not statistically different from each other.

Table 2
Effects of inhibitors on berberine activated glucose uptake

[Inhibitor]		[Berberine]		
SB203580	***PD98059***	**0 μM**	**10 μM**	**50 μM**
0 μM	0 μM	1.00 ± 0.03	1.44 ± 0.08	4.13 ± 0.17
10 μM	0 μM	0.91 ± 0.02	1.46 ± 0.03	3.31 ± 0.09*
0 μM	50 μM	0.91 ± 0.10	1.06 ± 0.08*	2.41 ± 0.05#

As controls, cells were incubated for 30 minutes at 37 °in DMEM media containing either 0 (basal), 10 μM (submaximal stimulation), or 50 μM (maximal stimulation). The inhibitors, SB203580 (at 10 μM) and PD98059 (at 50 μM) were incubated with cells for 45 minutes prior to a 30-minute incubation with 0, 10, or 50 μM berberine plus the inhibitor. Glucose uptake was measured as described in Materials and Methods and normalized to control with no berberine and no inhibitor. Significant effects of the inhibitor compare to its berberine control (no inhibitor) were at P<.05 (*) and P<.01 (#).