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Osthole activates glucose uptake but blocks full activation in L929 fibroblast cells, and inhibits uptake in HCLE cells

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Abstract

Aims—Osthole, a coumarin derivative, has been used in Chinese medicine and studies have suggested a potential use in treatment of diabetes and cancers. Therefore, we investigated the effects of osthole and other coumarins on GLUT1 activity in two cell lines that exclusively express GLUT1.

Main Methods—We measured the magnitude and time frame of the effects of osthole and related coumarins on glucose uptake in two cells lines; L929 fibroblast cells which have low GLUT1 expression levels and low basal glucose uptake and HCLE cells which have high GLUT1 concentrations and high basal uptake. We also explored the effects of these coumarins in combination with other GLUT1 activators.

Key findings—Osthole activates glucose uptake in L929 cells with a modest maximum 1.7-fold activation achieved by 50 μM with both activation and recovery occurring within minutes. However, osthole blocks full acute activation of glucose uptake by other, more robust activators. This behavior mimics the effects of other thiol reactive compounds and suggests that osthole is interacting with cysteine residues, possibly within GLUT1 itself. Coumarin, 7-hydroxycoumarin, and 7-methoxycoumarin, do not affect glucose uptake, which is consistent with the notion that the isoprenoid structure in osthole may be important to gain membrane access to GLUT1. In contrast to its effects in L929 cells, osthole inhibits basal glucose uptake in the more active HCLE cells.

Significance—The differential effects of osthole in L929 and HCLE cells indicated that regulation of GLUT1 varies, likely depending on its membrane concentration.

Keywords

GLUT1; glucose uptake; osthole and coumarins; acute activation; membrane transport

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1. Introduction

Osthole (7-methoxy-8-(3-methy-2-butenyl) coumarin) is a bioactive coumarin derivative found in a variety of medicinal plants including Cniddium monnieri (L.) Cusson. Osthole exerts a wide range of biological effects including antiosteoporotic (Zhang, et al. 2007, Ming, et al. 2011, Tang, et al. 2010) anti-inflammatory (Zimecki, et al. 2009), antiseizure (Luszczki, et al. 2009), antiallergic (Matsuda, et al. 2002), and anti-fatty liver activities (Zhang, et al. 2007, Sun, et al. 2009). However, the bulk of studies have reported on osthole's effects on cell growth, its proapoptotic effects and its potential as an anticancer or antitumor agent (Lopez-Gonzalez, et al. 2004, Riviere, et al. 2006, Xu, et al. 2011, Yang, et al. 2010, Hung, et al. 2011, Zhang, et al. 2012).

More relevant to this study are recent studies demonstrating that osthole also stimulates glucose uptake, and may have efficacy as an antidiabetic agent (Lee, et al. 2011, Liang, et al. 2009). These studies suggest that osthole activates glucose uptake by the initial activation of AMP-kinase. Osthole enhances the phosphorylation of AS160 suggesting that the translocation of GLUT4 is the direct cause for the enhanced glucose uptake. However the effects of osthole on the transport activity of GLUT1 are unknown.

There is accumulating evidence that the ubiquitously expressed GLUT1 can be acutely activated by a host of reagents or stress conditions including glucose deprivation (Kumar, et al. 2004, Roelofs, et al. 2006), osmotic stress (Barnes, et al. 2002, Barros, et al. 2001), exposure to azide (Shetty, et al. 1993, Rubin and Ismail-Beigi 2003), methylene blue (Louters, et al. 2006), or peptide C (Meyer, et al. 2008). The mechanism of this acute activation is not known. However, in contrast to GLUT4 activation, the activation of GLUT 1 appears to occur without a change in the membrane concentration of the transporter (Shetty, et al. 1993, Mercado, et al. 1989). One model for the acute activation of GLUT1, based on work in erythrocytes (Graybill, et al. 2006, Carruthers, et al. 2009, Zottola, et al. 1995, Hebert and Carruthers 1992, Pessino, et al. 1991), suggests that GLUT1 is activated by the formation of an internal disulfide bond that stabilizes the oligomerization (tetramer) and activation of GLUT1. While this mechanism has not been confirmed in other cell types, several studies with L929 fibroblast cells, which express GLUT1 as the exclusive glucose transporter (Liong, et al. 1999), have suggested that acute activation of GLUT1 involves thiol chemistry of key cysteine residues. Thiol reactive compounds such as phenylarsine oxide (Scott, et al. 2009), cinnamaldehyde (Plaisier, et al. 2011) and nitroxyl (Salie, et al. 2012) all activate GLUT1 within minutes. The structure of osthole includes an α-β unsaturated carbonyl, a good Michael acceptor of thiols. Therefore, to better understand the effects of osthole on GLUT1 activity, we measured the acute effects of osthole on the glucose uptake in both L929 fibroblast cells and HCLE cells, two cell lines where glucose uptake is mediated exclusively by GLUT1, but have different basal uptake rates (Liong, et al. 1999, Kuipers, et al. 2013). In contrast to L929 cells, HCLE cells have about a 7-fold higher concentration of GLUT1, but have a 25-fold higher basal glucose uptake rate, suggesting GLUT1 is in an activated state in HCLE cells (Kuipers, et al. 2013).

2. Materials and Methods

2.1 Chemicals

Osthole (Os), coumarin (Co), 7-hydroxycoumarin (umbelliferone (Um)), 7-methoxycoumarin (7-MC), phenylarsine oxide (PAO), methylene blue (MB), hydroxylamine (HA), sodium azide (Az), cinnamaldehyde (CA), 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). Angeli's salt (AS) was a generous gift of Dr. John P. Toscano (Johns Hopkins University).

2.2 Cell culture

L929 mouse fibroblast cells were obtained from the American Type Culture Collection. The immortalized human corneal–limbal epithelial (HCLE) cell line was obtained from Dr. Ilene Gipson (Department of Opthalmology, Harvard Medical School) and maintained as monolayer cultures in Keratinocyte-Serum Free medium (K-SFM) (Invitrogen, Carlsbad, CA), as previously described (Gipson, et al. 2003). To initiate each experiment, a 24-well plate was seeded either 2 or 3 days (HCLE cells) or 1 day (L929 cells) prior to experimentation. Experiments were done with cells near confluency, which is about 1.0×10^5 cells per well for HCLE cells and 3.2×10^5 for L929 fibroblast cells. The cells were grown at 37 °C in an incubator supplied with humidified room air with 5% CO_2.

2.3 General experimental design

To initiate an experiment, the media from cells in 24-well plates were removed and then incubated in 0.4 mL of fresh treatment media consisting of either low-glucose DMEM alone (0% FBS) or low-glucose DMEM plus the chemical of interest (see figure legends) or glucose-free DMEM (activation by glucose deprivation). Cells were maintained at 37 °C for the times indicated. Reagents were added to the media or 2DG uptake solution from 100–200× aqueous (Az, HA), or ethanol (MB, CA) or DMSO (PAO, Os, Co, Um, 7-MC,) stock solutions. Ethanol and DMSO have no effect on glucose uptake at the concentrations added (Scott, et al. 2009, Plaisier, et al. 2011). In the experiment using AS, the solid compound was quickly dissolved in uptake media at room temperature immediately distributed to the cells in the 24-well plate (process took about 30 seconds).

2.4 Glucose uptake assay

As a reflection of glucose uptake we determined the uptake of the radiolabeled glucose analog, 2-deoxyglucose (2DG), as previously described (Van Dyke, et al. 2003). Briefly, the media was replaced with 0.2 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). Uptake media was supplemented with additional compounds, such as Os, AS, HA, as indicated in the figure legends. After a 10-minute incubation at 37 °C, 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 and ^{14}C-mannitol were measured using scintillation spectrometry.

Mannitol is not normally taken up by cells, therefore the inclusion of ^{14}C-mannitol in the uptake media allows us to account for any surface binding, to monitor potential toxic effects of the experimental treatments that would compromise the cell membrane, and to account for excess radioactivity that might remain after the washes. Uptake of radiolabeled 2DG in both L929 and HCLE cells is fully inhibited by both cytochalasin B and quercetin, which are known inhibitors of GLUT1 (Kuipers, et al. 2013).

2.5 Statistical analysis

Experimental conditions were repeated in quadruplicate and glucose uptake was measured and reported as nmol/10 min/well ±standard error. Statistical significance was determined by a two-tailed t-test or by an ANOVA with a Dunnett's post test. Statistical significance is reported at $P < 0.01$. Each experiment with quadruplicate samples was repeated a minimum of three times to insure that results could be replicated. Results from a representative experiments are reported. In some cases, multiple experiments were combined after normalizing of data to control conditions and reported as fold increase in 2DG uptake (Figure 1, Tables 1 and 2)..

3. Results

3.1 Osthole activates glucose uptake in a dose dependent manner

Osthole activates glucose uptake via GLUT4 in skeletal muscle (Lee, et al. 2011), but its effects on GLUT1 activity are not known. Therefore, we measured glucose uptake in the presence of increasing concentrations of osthole (0–200 μM) in L929 fibroblast cells, where glucose uptake is mediated exclusively by GLUT1 (Liong, et al. 1999). Results, shown in Figure 1, indicate that a small, but significant increase at 25 μM osthole and a maximum, 1.6-fold increase, is achieved at 50 μM. There were no toxic effects observed for osthole at any of the concentrations used as monitored by changes in cell morphology or growth rates (data not shown). Therefore, in subsequent experiments we utilized 100 μM concentrations of osthole to ensure maximum response.

3.2 Osthole inhibits full activation by other activators of glucose uptake

We were curious to understand the combined effects of osthole with other reagents that acutely activate glucose uptake in L929 cells. We have previously observed that a number of thiol reactive agents such as CA, and PAO have stimulatory activity under basal conditions, but block full activation by other agents (Scott, et al. 2009, Plaisier, et al. 2011). In our initial experiment we measured glucose uptake in L929 cells in the presence of maximum effective concentration of either osthole (100 μM), or HA (5.0 mM) which is a robust and fast activator (Louters, et al. 2013), or in the presence of both reagents. The results are shown in Figure 2. As expected, osthole alone has small significant activating effect (1.56-fold). HA stimulated glucose uptake 3.6-fold, which is significantly reduced in the presence of osthole. We explored this inhibitory effect further by investigating the combined maximally effective concentrations of osthole with other activators. We investigated both slower acting reagents (present during a 10–20 minute pretreatment) such as glucose deprivation, MB, or Az (Roelofs, et al. 2006, Rubin and Ismail-Beigi 2003, Louters, et al. 2006) as well as reagents that activate within minutes, such as AS and PAO, that are only present during the uptake

measurement (Scott, et al. 2009, Salie, et al. 2012). The results are shown on Table 1 and reveal that in all cases osthole significantly decreases the activation of transport by these reagents.

3.3 Recoveries from the activating and inhibitory effects of osthole are different

To investigate the recovery from the glucose uptake activating effects of osthole, L929 cells were incubated with 100 μM osthole for ten minutes followed by an immediate measure of glucose uptake without osthole, or a measurement of uptake after a 10 or 20-minute recovery in media. The results, shown in Figure 3, indicate maximum activation is maintained if glucose uptake is measured immediately after treatment with osthole (matches measured uptake in the presence of osthole), but uptake returns to basal levels within ten minutes. In contrast, the inhibitory effects of osthole on HA activation of uptake are longer lasting. Cells were incubated for 10-minutes with 100 μM osthole and then allowed to recover in media lacking osthole for 0, 5, 10, or 20 minutes before measurement of glucose uptake under activating conditions (5.0 mM HA). The data is expressed as a percent of maximum activation and are shown in Figure 4. Results indicate that HA-activation recovers from about 15% to 52% after 20 minutes, but essentially half of the inhibitory effect of the osthole remains even after 20 minutes.

3.4 Inhibitory effects are not seen during a long time exposure to osthole

The thiol reactive compounds, PAO and CA, like osthole, also activate glucose uptake under basal conditions, but inhibit the full activation of glucose uptake by HA. The inhibitory effects of these PAO and CA were evident under two conditions. First, at higher concentrations the magnitude of stimulation decreased significantly, and second, after longer exposure times to maximally effective concentrations these compounds inhibited rather than activated glucose uptake (Scott, et al. 2009, Plaisier, et al. 2011). For osthole, we did not observe a decrease in the magnitude of activation at higher concentrations (see Figure 1). However, we also wanted to measure the effects of exposure time. If inhibitory effects appear as a function of exposure time we would expect that the uptake would not remain linear over 45 minutes (slope would decrease). Likewise, if osthole activates a signaling pathway, we would predict an increase in the rate of uptake over the signal transduction period (eg 10–15 minutes). Therefore, glucose uptake was measured in the presence and absence of 100 μM Os for increasing times up to 45 minutes. The results shown in Figure 5 indicate that both control and osthole-activated uptake remains linear over the 45-minute exposure time. There is no evidence of either a secondary inhibitory action or a continuing activating effect for osthole within this time frame. The effects are immediate.

3.5 Other coumarin derivatives are not active

We tested other coumarin derivatives for their effects on GLUT1 activity. We measured glucose uptake in L929 cells in the presence of 100 μM concentrations of osthole, coumarin, 7-hydoxycoumarin (umbelliferone), or 7-methoxycoumarin. The results shown on Table 2 indicate that only osthole stimulates glucose uptake. When these compounds were co-incubated with the activator, HA, again, only osthole significantly blocked the activating effects of HA (see Table 2). None of these compounds, except osthole, were active at 200 μM concentrations either (data not shown).

3.6 Osthole inhibits glucose uptake in HCLE cells

Previous work in our lab demonstrated that glucose uptake in HCLE cells was 25 times greater than in L929 cells (Kuipers, et al. 2013). This enhancement was attributed to both a higher expression level of GLUT1 and to an activation of the transporter. We also demonstrated that thiol active compounds, such as CA, did not activate, but inhibited glucose uptake in HCLE cells. To measure the effects of osthole in HCLE cells, uptake was measure in the presence of varying concentrations of osthole (0–200 μM). The results, shown in Figure 6, demonstrate that osthole has a dose dependent inhibition of glucose uptake in HCLE cells. The data are fit by a simple exponential decay and the relatively poor fit suggests that the osthole may involve multiple inhibitory effects. Regardless, the inhibition if glucose uptake HCLE cells is in sharp contrast to the activation observed in L929 cells.

4. Discussion

This study adds to the list of physiological effects of osthole and reports for the first time the acute effects of osthole on the glucose uptake activity of GLUT1. Our data demonstrate that osthole activates glucose uptake in a dose dependent manner with maximum stimulation of uptake of 170% at 50 μM (Fig. 1). This effect is very fast and requires only exposure to osthole during the 10-minute glucose uptake measurement. This activating effect of osthole is also short lived and glucose uptake returns to baseline within 10 minutes after removal of osthole (Fig 3). A previous study showed that osthole stimulates glucose uptake in skeletal muscle by an activation of AMP kinase via a phosphorylation and an increase in the AMP/ATP ratio. They also showed an increase in the phosphorylation of AS160, a Rab-GTPase activation protein, and implicated a subsequent translocation of GLUT4 to the cell membrane as the direct mechanism for increased glucose uptake. However, there are several reasons that this mechanism is not a likely mechanism for the effects of osthole in L929 cells. First, full activation of glucose uptake by osthole in skeletal muscle, as monitored by phosphorylation of AMP kinase and ACC, required a 2–4 hour treatment, while the effects of osthole in L929 cells occur within minutes. Second, we have previously shown that neither AICAR, an activator of AMP kinase, nor compound C, an inhibitor of AMP kinase, alter glucose uptake in L929 cells (Louters, et al. 2006, Cok, et al. 2011). Given the speed and reversibility, it seems more likely that osthole is having a more direct effect on the transporter rather than an activation of a signaling system. Previous studies have indicated that the acute activation of GLUT1 is independent of protein synthesis and occurs without a change in the membrane concentration of GLUT1 (Shetty, et al. 1993, Rubin and Ismail-Beigi 2003, Louters, et al. 2006, Barnes, et al. 2004, Rubin and Ismail-Beigi 2004). However, a recent study in blood brain endothelial cells has shown that acute stress does increase the membrane concentration of GLUT1 (Cura and Carruthers 2012).

One insight to the mechanism is the potential involvement in cysteine residues in the activation of GLUT1. The chemical structure of osthole contains a α-β unsaturated carbonyl, which is a good Michael acceptor of thiols. We have previously shown that other thiol reactive compounds, such as cinnamaldehyde, PAO, and nitroxyl show a similar, fast activation of glucose uptake (Scott, et al. 2009, Plaisier, et al. 2011, Salie, et al. 2012).

In addition to activating glucose uptake, osthole also inhibits full activation of GLUT1 by other, more robust activators of glucose uptake. Treatment of osthole with activators such as HA, Az, PAO, MB, glucose deprivation, and nitroxyl (via AS) all result in a significant reduction in activation by these activators (see Fig 2 and Table 1). It is not likely that osthole is reacting directly with these activators. Az, MB, and glucose deprivation, are pretreatment conditions and not in contact with osthole during uptake. While HA and osthole are in direct contact is not likely that osthole reacts directly with HA since other coumarins have no effect on HA activation, and there is no chemical logic for a reaction between HA and the isoprenoid structure at position 8 in osthole.

This dual action of osthole is similar, but not identical, to the dual effects previously reported for PAO and CA (Scott, et al. 2009, Plaisier, et al. 2011). Similar to osthole, both PAO and CA activate basal glucose uptake, as well as inhibit full activation by other activators. However, the inhibitory features of PAO and CA and other thiol active compounds such as nitroxyl and HA (Salie, et al. 2012, Louters, et al. 2013), were evident at either at a higher concentration of the compound or after a longer treatment time with the maximally effective concentration of the activator. In contrast, osthole did not exhibit any inhibitory effects under basal conditions at either higher concentrations (eg 200 μM osthole, see Fig. 1), or after longer treatment times (Fig 5). The activation and inhibitory effects may result from different actions of the compound. Additionally, the recovery from this inhibitory effect is much slower than recovery from the activating effects of osthole. Even after 20 minutes, only 50% of the activating effects of the activator, HA, are restored (Fig. 4).

The consistency in the actions of osthole with other thiol reactive compounds suggests that the coumarin ring structure is essential to its activity. However, somewhat surprisingly, other coumarin derivatives did not exhibit either the activation or inhibitory effects (Table 2). This does not appear to be simply a matter of concentrations because even 200 μM concentrations of these other coumarin compounds did not exhibit either an activation of basal uptake or inhibition of full activation (data not shown). It may be that the isoprenoid structure at position 8 is essential to deliver the compound to a membrane protein, such as GLUT1 itself.

In sharp contrast to its activating effects in L929 cells, osthole has a dose dependent inhibition of glucose uptake in HCLE cells. HCLE cells are fast growing cells, with glucose uptake rates that are about 25-fold higher than L929 cells (uptake/cell) (Kuipers, et al. 2013). This enhance uptake rate can be accounted for by a 6–7 fold higher expression of GLUT1 coupled to a 3–4 fold activation of the transporter in HCLE cells. This inhibitory effect of osthole mimics the effects of other thiol reactive compounds in HCLE cells. CA, PAO and nitroxyl all reduced glucose uptake in HCLE cells while strongly active glucose uptake in L929 cells (Kuipers, et al. 2013). HCLE (human) and L929 cells (mouse) do express different GLUT1 alleles, however, the gene products are similar enough to bind to the same antibodies. Therefore, the differential effects of osthole in HCLE and L929 cells are more likely due to differences in the membrane concentrations and activation states of GLUT1 than to species differences between the transporters.

Previous work has shown that acute activation of glucose uptake in L929 cells occurs without a detectable change in the concentration of membrane GLUT1 (Louters, et al. 2006)

and with varied kinetic effects including an increase in Vmax (Louters, et al. 2006), a decrease the Km (Roelofs, et al. 2006, Cok, et al. 2011) or a change in both parameters (Gunnink, et al. 2013). Given this, it is not expected that the fast acting osthole would alter membrane concentrations of GLUT1. The data presented in this study are actually more consistent with a direct effect of osthole on GLUT1 than with a model that requires a signal transduction mechanism. There was no change in the rate of glucose uptake from 2 minutes to 45 minutes (Fig 5), and therefore, if osthole interacts with a signaling pathway it had to fully activate that pathway within 2 minutes.

However, these results are consistent with a model for GLUT1 activation, based on work in erythrocytes (Graybill, et al. 2006, Zottola, et al. 1995, Carruthers, et al. 2009), that suggests that GLUT 1 can exist in multiple states ranging from low activity monomers, containing reduced cysteine residues, to high activity oligomers, likely tetramers. The more active tetramer is a noncovalent complex that is stabilized by the conformation change that occurs when an internal disulfide bond forms within GLUT1. If the bulk of the GLUT1 transporters in HCLE cells are in a highly activated state, that is, tetramers with oxidized cysteine residues, the model predicts, as we observe, that reagents that react with thiols would stabilize a less active form of GLUT1 and shift the overall equilibrium away from the highly active state.

The high growth rate and dependence on glucose in HCLE cells is a feature shared by many cancers, which overexpress GLUT1 (Furuta, et al. 2010, Amann and Hellerbrand 2009, Ganapathy, et al. 2009, Young, et al. 2011, Macheda, et al. 2005). It is interesting to note that the majority of literature studies are on the effects of osthole on cancer cells. It has been shown to be effective in the treatment of a number of cancers including hepatocellular carcinoma (Zhang, et al. 2012), lung (Lopez-Gonzalez, et al. 2004, Xu, et al. 2011), leukemia (Yang, et al. 2003), and breast (Yang, et al. 2010, Hung, et al. 2011). These studies point to multiple mechanisms of action to account for the efficacy of osthole, but none of the studies cite the possible inhibition of glucose uptake as a potential mechanism to limit cancer cell growth. The data presented in this study suggest that the effects of osthole on glucose uptake in cancer cells should be systematically explored.

5. Conclusion

This study reveals that osthole has a dual effect on the glucose uptake activity of GLUT1. In L929 cells, where glucose uptake is relatively low, osthole activates basal glucose uptake but blocks full activation by other, more robust activators of glucose uptake. In contrast, osthole inhibits glucose uptake in HCLE cells, where glucose uptake rates are high under basal conditions. This differential effect of osthole appears to be related to the concentration and initial activation state of GLUT1.

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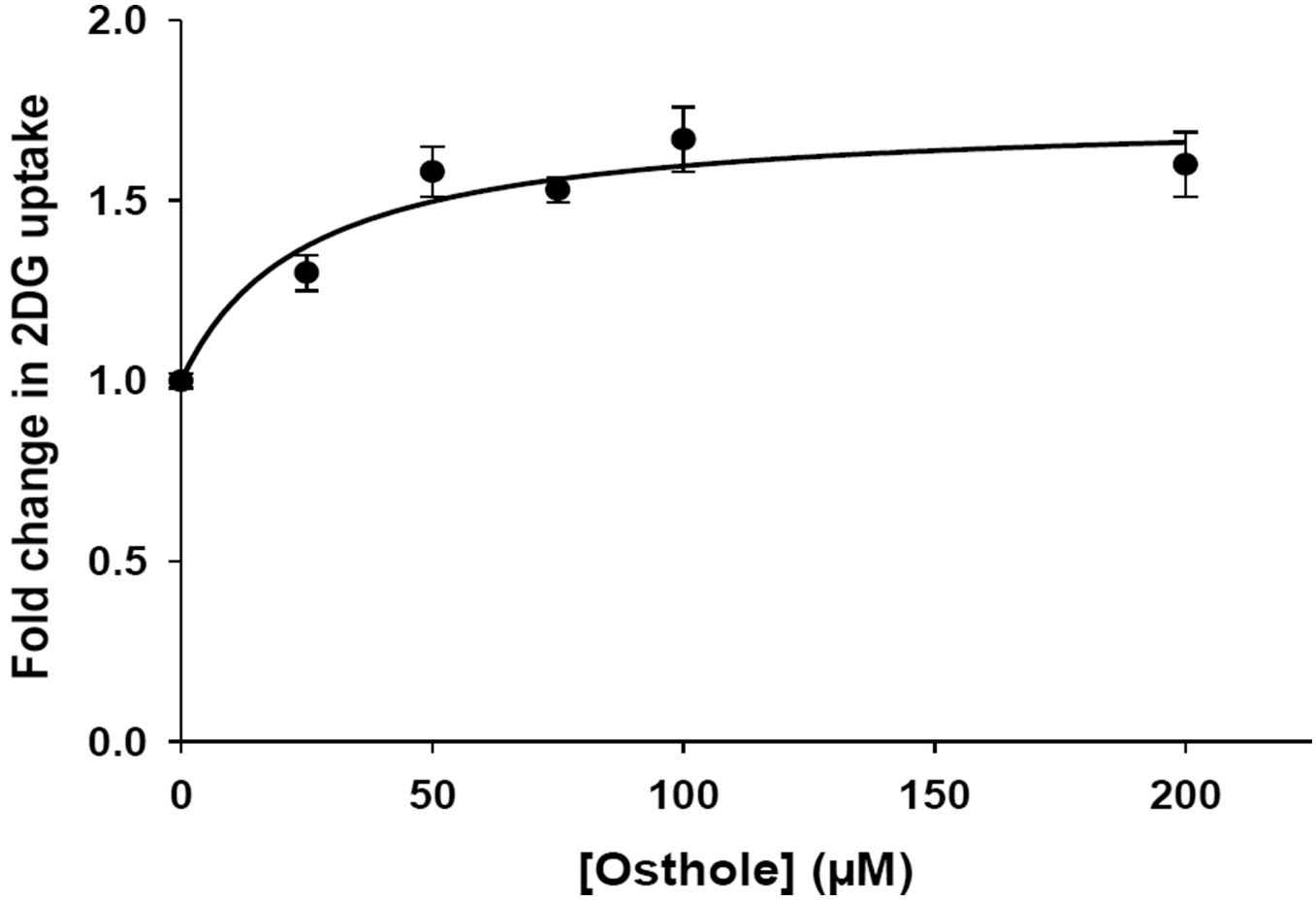


Figure 1.

Dose dependent effects of osthole on 2DG uptake in L929 fibroblast cells. Ten-minute 2DG uptakes were measured in the presence of osthole concentrations ranging from 0–200 μM. Data were normalized to basal uptake (no osthole) and are reported as means of fold change ± S.E. (n=12–16). Regression analysis indicates a maximum 1.74 ± 0.10-fold activation with $K_{0.5}$ of 24.7 ± 13 μM. All osthole concentrations are significantly different than basal 2DG uptake (0 μM osthole) at $P<0.01$.

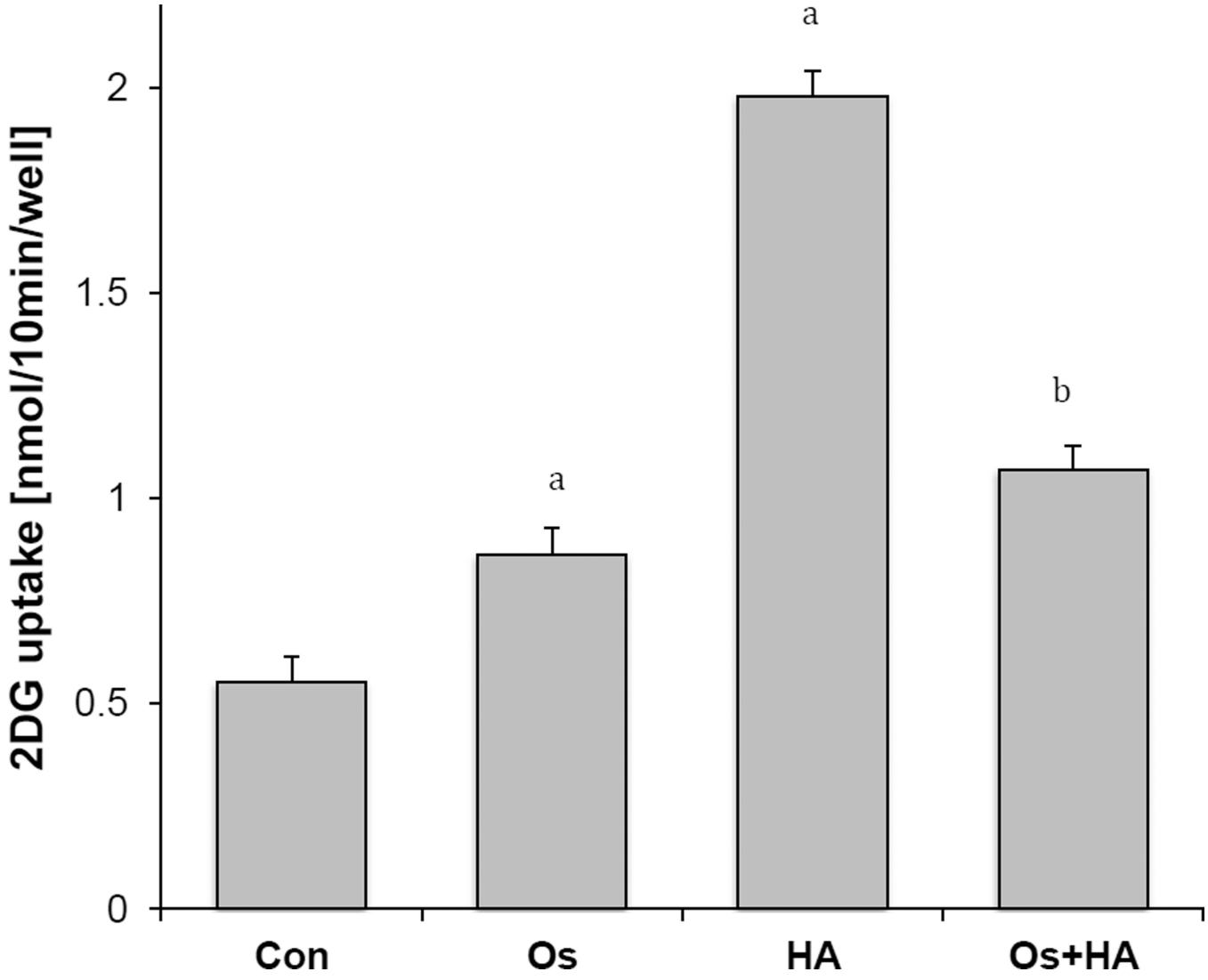


Figure 2.

Effects of osthole on hydroxylamine activated 2DG uptake. Ten-minute 2DG uptakes were measured in the absence of additives (Con) or in the presence of either 100 μM osthole (Os), or 5.0 mM hydroxylamine (HA), or both (Os+HA). A representative experiment is reported as means of nmol/10min/well ± S.E. (n=4) [a]Significantly different than control at P<0.01 and [b]significantly different than both control and HA treated cells at *P*<0.01.

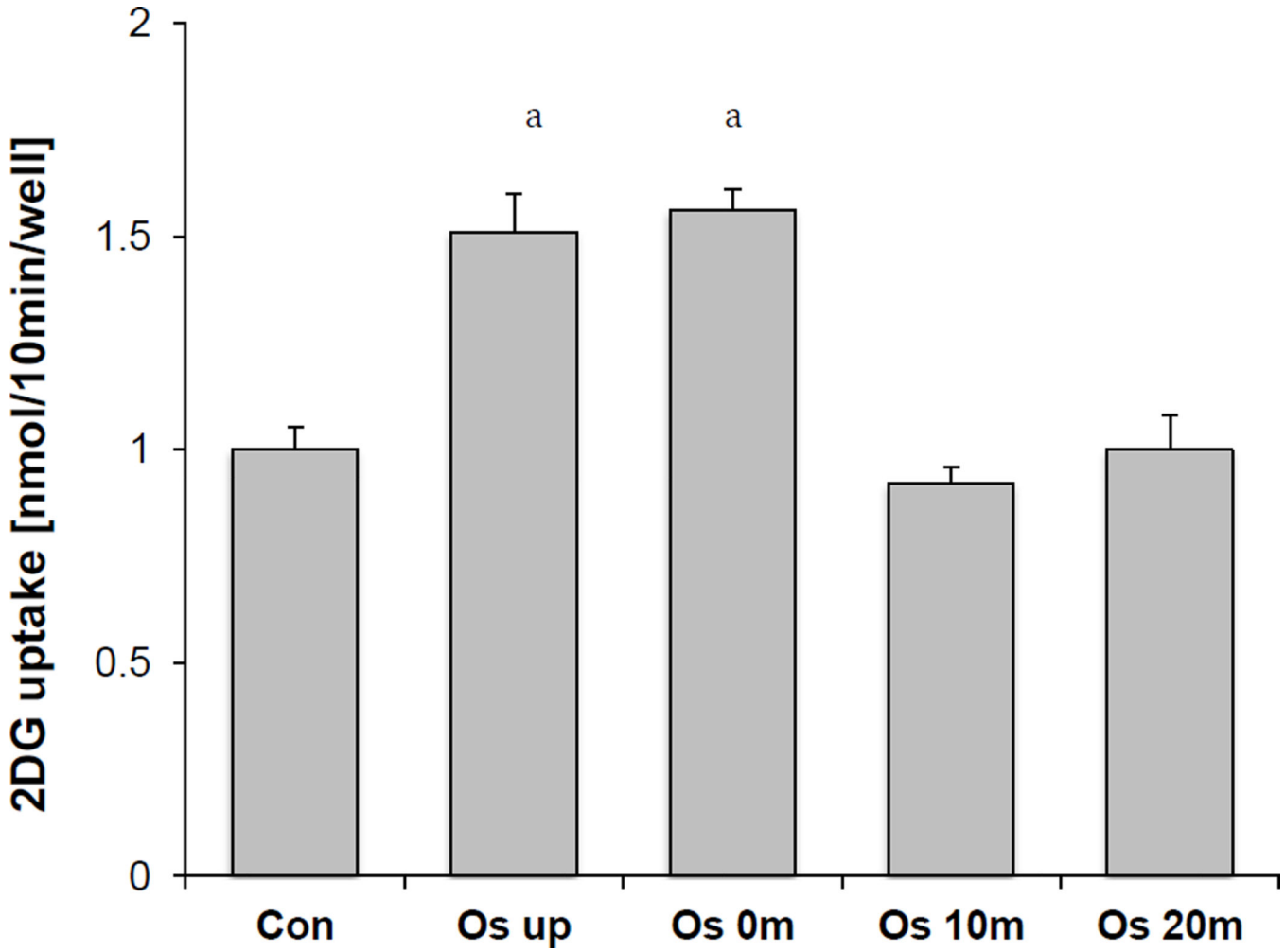


Figure 3.

Recovery from activating effects of osthole. Cells were exposed to 0 μM (Con) or 100 μM osthole during uptake (Os up) or exposed to media containing 100 μM osthole for ten minutes followed by a recovery of 0, 10 or 20 minutes in media without osthole (Os 0m, Os 10m, and Os 20m respectively). Osthole was not present during measurement of 2DG uptake. Data are reported as means of nmol/10min/well ± S.E. (n=4) of a representative experiment. [a]Significantly different than control at $P<0.01$.

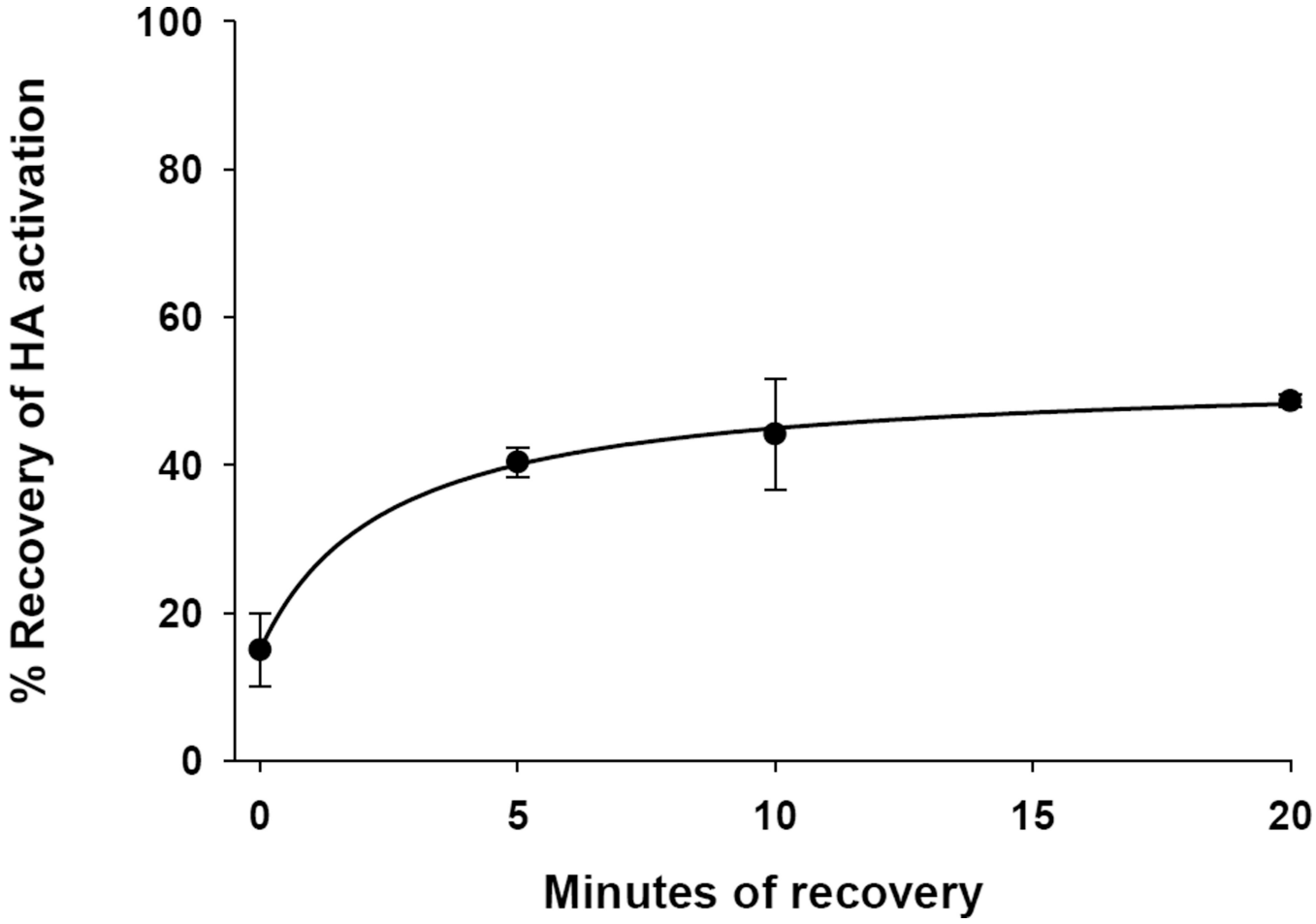


Figure 4.

Recovery from inhibitory effects of osthole. Cells were exposed 100 μM osthole for ten minutes followed by a recovery of 0, 5, 10 or 20 minutes in media without osthole. 2DG uptake was measured in the presence of 5.0 mM HA (activating conditions). The magnitude of activation was determined by the difference in 2DG uptake under basal conditions (no additives) and under activating conditions (5.0 mM HA in uptake only, which was a 2.56-fold activation (not shown)). Data are reported as percent of activation (means ± S.E., n=4) of a representative experiment. Regression analysis indicates that a 52.4 ± 1.2% maximum recovery within 20 minutes and $K_{0.5}$ of recovery of 2.46 ± 0.38 minutes. All recovery times are significantly different than zero minute recovery at $P<0.01$.

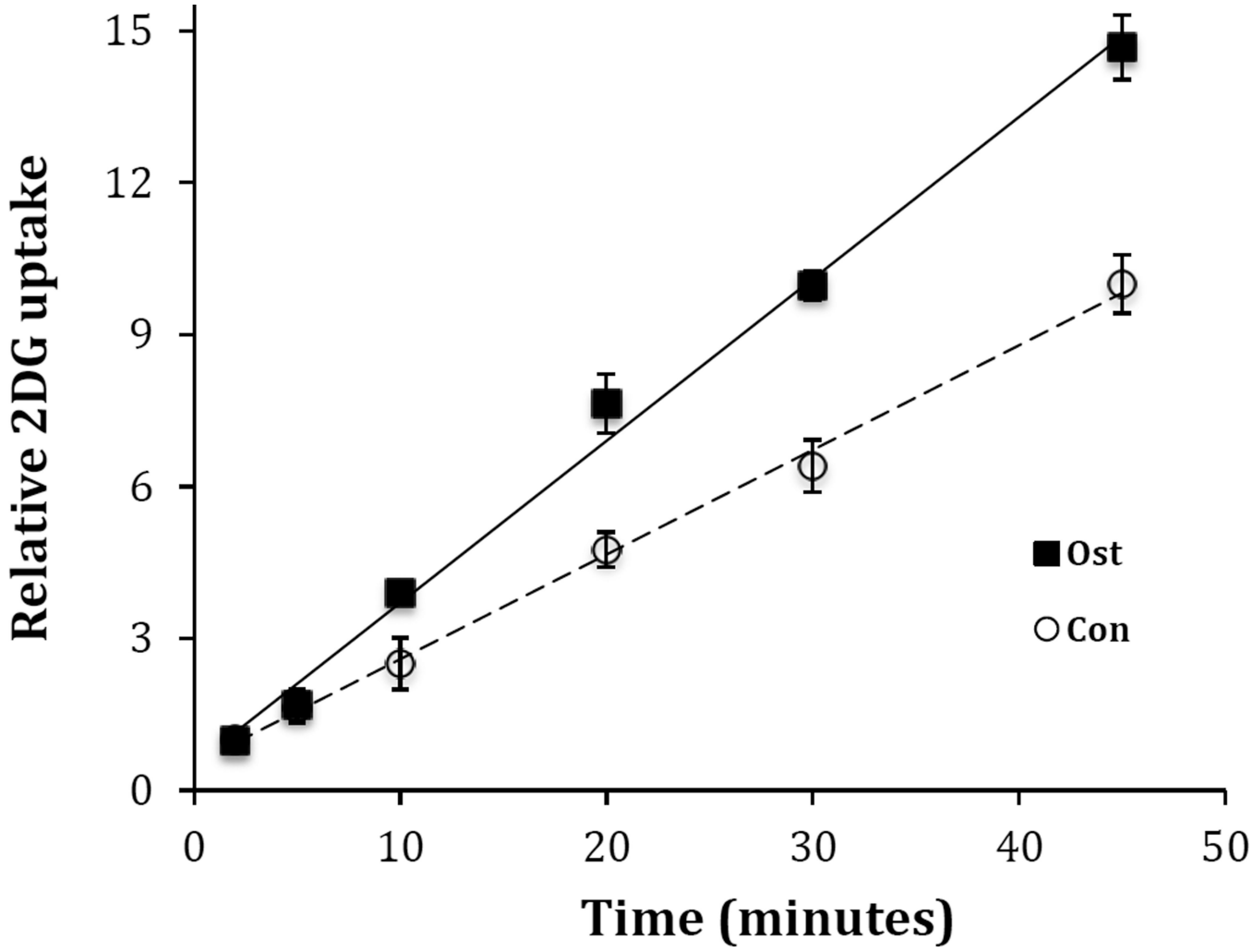


Figure 5.

2DG uptake in the presence and absence of osthole remains linear over 45-minutes. 2DG uptakes in the presence or absence of 100 μM osthole were measure for 2, 5, 10, 20, 30, or 45 minutes. Data are normalized to uptake at 2 minutes and reported as relative uptakes (means ± S.E., n=4).

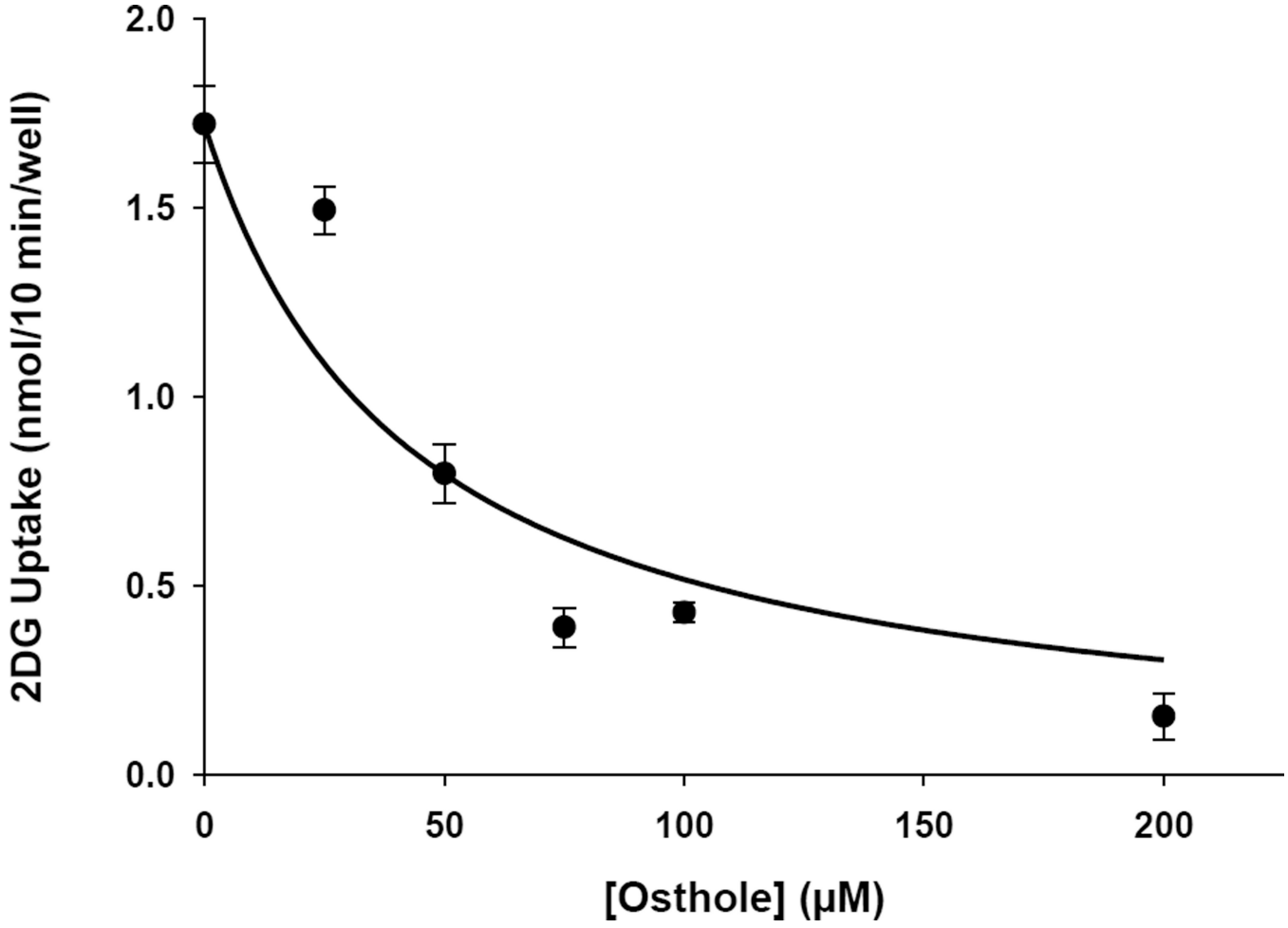


Figure 6.

Dose dependent effects of osthole on 2DG uptake in HCLE cells. Ten-minute 2DG uptakes in HCLE cells were measured in the presence of osthole concentrations ranging from 0–200 μM. Data are reported as means of nmole/10min/well ± S.E. (n=4) of a representative experiment. Line is a simple hyperbolic decay analysis indicating a K_I(app) of 43 ± 11 μM. Concentrations greater than 50 μM are significantly different than basal 2DG uptake (0 μM osthole) at $P<0.01$.

Table 1

Effects of osthole on various activators of glucose uptake.

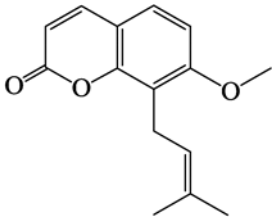
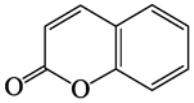
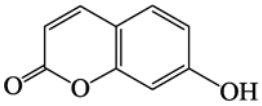
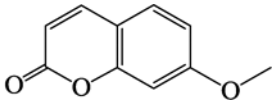
	Normalized 2DG uptake (nmole/10min/well)	
Stimulant	***Control***	***+Os (100 μM)***
None	1.00 ± 0.03	1.42 ± 0.05 *
Az (5 mM)	3.41 ± 0.51	1.59 ± 0.21 *
MB (50 μM)	2.98 ± 0.17	2.01 ± 0.18 *
NG (0 mM glucose)	2.92 ± 0.07	1.61 ± 0.04 *
PAO (10 μM)	2.53 ± 0.12	1.74 ± 0.09 *
AS (10 mM)	2.45 ± 0.10	1.27 ± 0.03 *

Glucose uptake was measured under glucose activating conditions alone or under activating conditions plus 100 μM osthole. Activation with methylene blue (MB), sodium azide (Az), or glucose deprivation (NG) required a 30-minute pretreatment, but Angelie's salt (AS), phenylarsine oxide (PAO) are fast activating and were only present during the measurement of glucose uptake. Uptakes were measure as nmol/10min/well and are normalized to control. Data are presented as means ± S.E.

* Significantly different than its respective control (no osthole) at P< 0.01.

Table 2

Effects of coumarin analogs on basal and activated glucose uptake.

Compound	Structure	Basal	HA (5.0mM)
Control	no coumarin analog	1.00 ± 0.02	3.33 ± 0.11
Osthole		1.67 ± 0.09 *	1.23 ± 0.03 *
Coumarin		1.08 ± 0.07	3.19 ± 0.04
Umbelliferone		1.12 ± 0.02	3.29 ± 0.04
7-methoxycoumarin		1.15 ± 0.06	3.08 ± 0.07

Glucose uptakes were measured in the presence of four coumarin derivatives each at 100 μM under basal conditions or under glucose activating conditions (plus 5.0 mM hydroxylamine (HA)). Uptakes were measure as nmole/10min/well are normalized to basal control. Data are means ± S.E.

* Significantly different than its respective control at P< 0.01.