In Vitro Testing of Ellagic Acid Dihydrate (Naturalin®) for Cytotoxicity, Glucose Uptake, Glucose Transporter (GLUT4) Activity and Signal Transduction Events

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AIM: Glucose uptake using a fluorescent glucose analog is an established technique to provide information on the impact of small molecules with respect to glucose uptake. The glucose analog 2-NBDG is a fluorescently-labeled deoxyglucose analog that is used to directly monitor glucose uptake by living cells and tissues. Specifically, we performed the present study to assess the uptake of glucose (2-NBDG) induced by EADH (Naturalin) in skin (MEFs) and muscle (C2C12) cells. Additionally, we determined the impact of EADH (Naturalin) on the glucose transporter (GLUT4) using live-cell imaging and fluorescently-labeled transporter (GLUT4-GFP). Cytotoxicity was determined by cell counting and propidium iodide staining after exposure. Activity comparisons are made relative to insulin or metformin treatments. All analyses were performed on the Operetta (Perkin Elmer) or BD Accuri C6 (BD Biosciences).

Abbreviations:

GLUT4: Glucose Transporter 4; MEFs: Mouse Embryonic Fibroblasts; C2C12: Muscle Cells; 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2 Deoxyglucose: 2-NBDG; DMSO: Dimethyl sulfoxide; Phosphate Buffer Saline: PBS; EADH: Ellagic Acid Dihydrate (Naturalin)

Introduction

According to the Center for Disease Control, American Diabetes Association and the World Health Organization (WHO), more than two-thirds of US citizens are currently overweight or obese and the worldwide obesity rate is currently estimated at 30% (2.1 billion) and expected to reach 50% among adults by 2030. Obesity in US children has doubled and quadrupled among adolescents in the past 30 years. Strong links between obesity and chronic diseases, such as diabetes, cardiovascular disease, cerebrovascular disease and many cancers, are well established, and the economic and quality of life costs of obesity are a staggering $2+ trillion (USD) according to the McKinsey Global Institute obesity report published on November 2014. Diabetes presents as persistent hyperglycemia and agents that reduce glucose levels by decreasing insulin resistance or stimulating insulin secretion are being investigated. Additionally, genetic and behavioral (particularly diet and physical activity) factors are clearly important to the development of obesity and diabetes. Thus, there is an urgent need to identify new targets and strategies for preventing obesity, including identifying compounds that minimize related health consequences in life.

In this present study, we investigated the toxicity and molecular and cellular cascades associated with the anti-diabetic effect of EADH (Naturalin). Specifically, we focused on cytotoxicity, glucose uptake, GLUT4 translocation, and AMPK and MAPK-signal pathways for mediating glucose uptake in cells.

Material and Methods

MATERIALS, CHEMICALS, and REAGENTS

Tissue culture media, insulin, PBS, and Hank's Buffer were purchased from Life Science Technologies. C2C12 myoblasts were purchased from American Type Culture Collection. SNL 76/7 mouse embryonic fibroblasts (MEFs) were obtained from Texas Institute for Genomic Medicine. The primary antibodies against AKT, Phospho-Akt (SER^{473}), p44/42 MAPK, and phospho-p44/42 MAPK (Erk1/2) (Thr^{202}/Tyr^{204}) were purchased from Cell Signaling Technologies (Danvers, MA). Alexa conjugated secondary antibody (anti-rabbit Alexa-647) was purchased from Life Science Technologies. Metformin was purchased

from Sigma Aldrich. Green Fluorescent Protein-tagged SLC2A4 (GLUT4-GFP) was obtained commercially from Origene. Ellagic acid dihydrate (EADH; Naturalin) compounds were provided by the Halo Life Science (TX).

Cytotoxicity
SUMMARY PROTOCOL

Biological Material: Live Cells
Cellular background: MEF cells
Fluorescent Ligand: 2-PI

Glucose Uptake
SUMMARY PROTOCOL

Biological Material: Live Cells
Cellular background: C2C12 cells, MEF cells
Fluorescent Ligand: 2-NBDG

Glucose Transporter Activity
SUMMARY PROTOCOL

Biological Material: Live Cells
Cellular background: C2C12 cells
Fluorescent Tracer: GLUT4-GFP

METHODS - *In vitro* assays

Cellular assays were performed using mouse embryonic fibroblasts (MEFs) and myoblasts (ATCC). Cell proliferation rates were determined using automated cell counting of cells after 48hr exposure to EADH (Naturalin) in complete medium (DMEM w/ 10% FBS). Cell are then washed thrice with 1X PBS and incubated with Accutase (Invitrogen) for 10min at 37°C, neutralized with 10% FBS in 1XPBS, and analyzed by flow cytometry. Cell toxicity was determined by adding propidium iodide (PI) to suspended cells prior to flow cytometry. The PI is a red-fluorescent DNA stain that is excluded by live-healthy cells but will enter dead cells in a population.

Glucose uptake assays were carried out in a final incubation volume of 50 µl consisting of 2-NBDG (100µM) in 1XPBS w/ Ca+, Mg+ (PBS++). The same buffer is used for washing cells. Insulin was used as a positive control. Cells are culture to 70-90% confluence and then washed twice with PBS++. Cells are incubated for 30 minutes at 37°C in PBS++. Nuclear stain is added; HCS NuclearMask stain (blue, Invitrogen) and testing compounds are added EADH (Naturalin), insulin, or vehicle). All testing compounds are prepared in 10% DMSO and diluted 1:100 for a final concentration of 0.1% DMSO (vehicle). Cells are incubated for 30min at 37°C. Cells are washed twice with PBS++ and incubated with 2-NBDG for 10 minutes. Cells are washed thrice with PBS++ and then imaged for microscopy in PBS++. Alternatively, the final PBS wash is removed and cells are incubated with Accutase (Invitrogen) for 10min at 37°C, neutralized with 10% FBS in 1XPBS, and analyzed by flow cytometry.

The localization of glucose transporter (GLUT4-GFP) was performed in final incubation volume of 0.505 µL, consisting of 500 µl of Hank's Buffer (Invitrogen) and 5 µl of tested compounds. Insulin was used as positive controls and vehicle (0.1% DMSO) as the negative control.

Cells were rinsed in Hank's Buffer thrice and then incubated in Hank's at 37°C for one hour. Test compounds are added (EADH (Naturalin), insulin, or vehicle). All test compounds are prepared in 10% DMSO and diluted 1:100 for a final concentration of 0.1% DMSO (vehicle), and the cells were imaged using laser-confocal microscopy (Leica TCS SP5 X) with FITC fluorescent filters for changes in GLUT4-GFP localization. Time-lapse images (TIF) were collected and post-processed using Image-J (NIH) for video construction.

All test compounds of interest were initially dissolved in 100% DMSO. Estimated saturation occurred at approximately 4mM for EADH (Naturalin). The saturated solution was diluted 1:10 in 1XPBS; this 100X stock solution is 400µM. The working concentrations (1X) were 4µM, followed by 1:10 dilutions into 0.1% DMSO. The resulting six dilutions ranged from 4µM to 0.04nM in triplicate. Dose curves were analyzed using either Harmony software on the Operetta (Perkin Elmer) or Excel (Microsoft) for flow cytometry, which was performed on the BD Accuri (BD Biosciences).

RESULTS

Effect of EADH (Naturalin) on MEFs
Figure 1 depicts cell proliferation of MEFs after 48hr exposure to EADH (Naturalin). There is an increase in cell proliferation observed that appears dose related. The changes in cell proliferation produced were not statistically significant ($P > .05$).

Figure 2 shows cell membrane integrity of MEFs after 48hr exposure to EADH (Naturalin). Loss of cellular membrane integrity is an indication of cell death. Membrane integrity (% cell negative for PI) is increased relative to the vehicle or untreated cells for treatments 400nM-0.4nM. A decrease in integrity is observed relative to vehicle at 4000nM (95.48%, 95.40%, respectively). The changes in cell death/membrane integrity were not statistically significant ($P > .05$).

Effect of EADH (Naturalin) on Glucose Uptake in MEFs and C2C12 Cells

Figs. 3a, 3b and 4a, 4b show the uptake curves for test compounds. Figure 3a and 4a indicate the fluorescent

signal of glucose (2-NBDG) taken up by the cell. Figures 3b and 4b indicate the relative glucose uptake as a percent change relative to vehicle treated (0.1% DMSO) cells. As expected, insulin produced a significant increase in glucose uptake relative to the vehicle. At all concentrations tested, EADH (Naturalin) induced significantly more glucose uptake relative to insulin in both fibroblasts and myoblasts. The increase in glucose uptake produced by EADH (Naturalin) and insulin was ~4X greater in myoblasts (4a, 4b) relative to fibroblasts (3a, 3b). Figure 4(c) shows a comparison of glucose uptake in C2C12 cells exposed to metformin, insulin and EADH (Naturalin) (batch 2).

Figure 5 shows images taken of MEFs after incubation with EADH (Naturalin) (400nM). Cells were stained with Nuclear Cell Mask (blue, FITC) and glucose (green, FITC, 2-NBDG) uptake was imaged on the Operetta HCS (Perkin Elmer).

Effect of Insulin and EADH (Naturalin) on GLUT4 in C2C12 Cells

Figure 6 and 7 show changes in cells due to insulin or EADH exposure, respectively. Figure 6 indicates that the fluorescent signal (GLUT4-GFP) is predominately located around the nucleus prior to the insulin exposure. Exposure to insulin (4000nM) produced a decrease in nuclear GLUT4-GFP and rapid ruffling of filopodia at the surface of the cell (Figure 6, right panel). Exposure to EADH (Naturalin) (4000nM) also produced a decrease in nuclear GLUT4-GFP and rapid ruffling of filopodia at the surface of the cell (Figure 7, right panel). This process appeared slightly delayed in the EADH (Naturalin) treated cells. There was no indication of changes in nuclear localization of GLUT4-GFP or ruffling of filopodia in any time-lapse imaging of untreated or vehicle treated cells (supplemental data available).

Effect of Insulin, EADH (Naturalin) and Metformin on C2C12 Cells

Figs. 8 and 9 show the relative protein levels of AKT:phos AKT and p44/42 MAPK:phos p44/42 MAPK, respectively. The bars indicate the fluorescent signal (anti-rabbit Alexa 647) due to the detection using antibodies (rabbit) to the native and phosphorylated forms of the proteins. Serum starved cells (1hr) were treated for 30 minutes with each of the tested compounds. An increase in phos-AKT signaling was observed upon insulin treatment. Insulin stimulates glucose uptake into cells by increasing GLUT4 activation, and AKT, specifically phos-AKT signaling alters GLUT4 translocation. EADH (Naturalin) showed an increase in AKT signaling relative to GLUT4. EADH (Naturalin) and insulin both stimulated p44/42 MAPK relative to phos: p44/42 MAPK signaling. Metformin also showed stimulated p44/42
MAPK relative to phos: p44/42 MAPK signaling at 0.04nM treatment.

COMMENTS

At the onset of experimentation, EADH (Naturalin) was analyzed for direct interaction with glucose. Using a glucometer, glucose oxidase based senor, to measure serum glucose, no inhibition of the glucose oxidase reaction after incubation of EADH with serum at room temperature for five minutes or overnight at 37C was observed. To the best of our knowledge, EADH (Naturalin) does not directly interact with glucose and inhibit metabolism.

The increased uptake of glucose in multiple cell types by EADH (Naturalin) confirms that this compound is capable of stimulating glucose uptake in cultured myoblasts and fibroblast cells. At equal concentrations, the uptake induced by EADH (Naturalin) exceeded the uptake induced by insulin in both cell lines tested. This effect was significant at 4nM-4μM.

Myoblast cells transfected with GLUT4-GFP also demonstrated decreased nuclear localization of GLUT4-GFP after simulation of insulin (4000nM). A similar change in nuclear localization of GLUT4-GFP was observed after exposure to EADH (Naturalin) (4000nM). Exposure to insulin or EADH (Naturalin) also induced rapid ruffling of the cell membrane and numerous projections (filopodia) began billowing out of the cell. The projections appear to be directly related to an increase in cycling of GLUT4 to and from the cellular surface and cellular uptake of glucose by GLUT4.

The relevance of increased glucose uptake, changes in GLUT4 localization, altered intra-cellular signaling, and changes in cellular behavior consistent with insulin stimulation indicated that EADH (Naturalin) has the potential to supplement, mimic, or replace the function of insulin at a cellular level of organization. Weight loss has been consistently observed in exposed animals, but further studies in experimental models developed to assess the effects of EADH (Naturalin) *ex vivo* or *in vivo* will be needed to clarifying the mechanism by which weight loss is occurring.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper or the Study conducted.

Figures

Figure 1. Cell Proliferation of MEFs Exposed to Naturalin

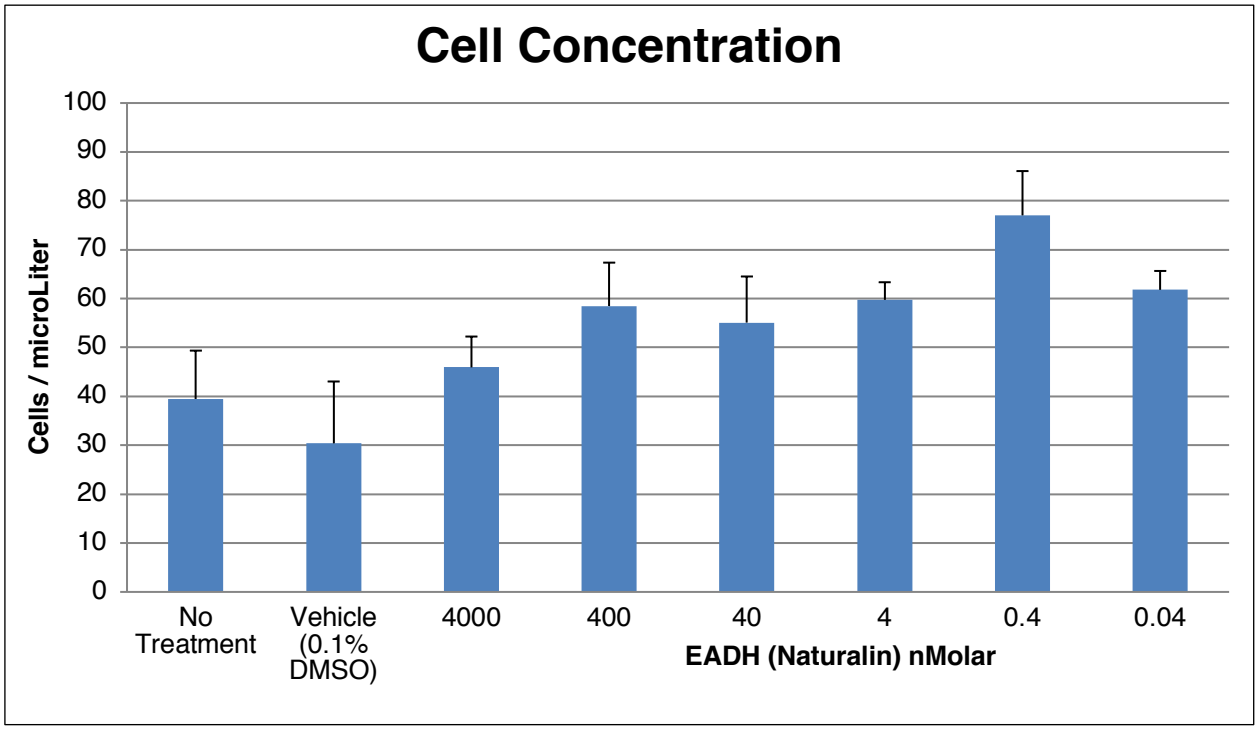


Figure 1. Cell concentrations were determined by cell counting (500-1000) on a flow cytometer (Accuri C6, BD Biosciences). Mouse embryonic fibroblasts (MEFs) were unexposed (No Treatment) or exposed to varying concentrations of ellagic acid dihydrate (EADH; Naturalin) (4μM-0.04nM) or vehicle (0.1% DMSO) for 48hrs. All assays were run in triplicate. The concentration of cells reflects the impact of EADH (Naturalin) on the cell population numbers (cell death and cell proliferation). An overall, yet non-significant, increase in cell concentration is observed with increasing concentration of Naturalin. These data indicate no negative impacts on cell population numbers upon exposure to Naturalin.

Figure 2. Cell Proliferation of MEFs Exposed to EADH (Naturalin)

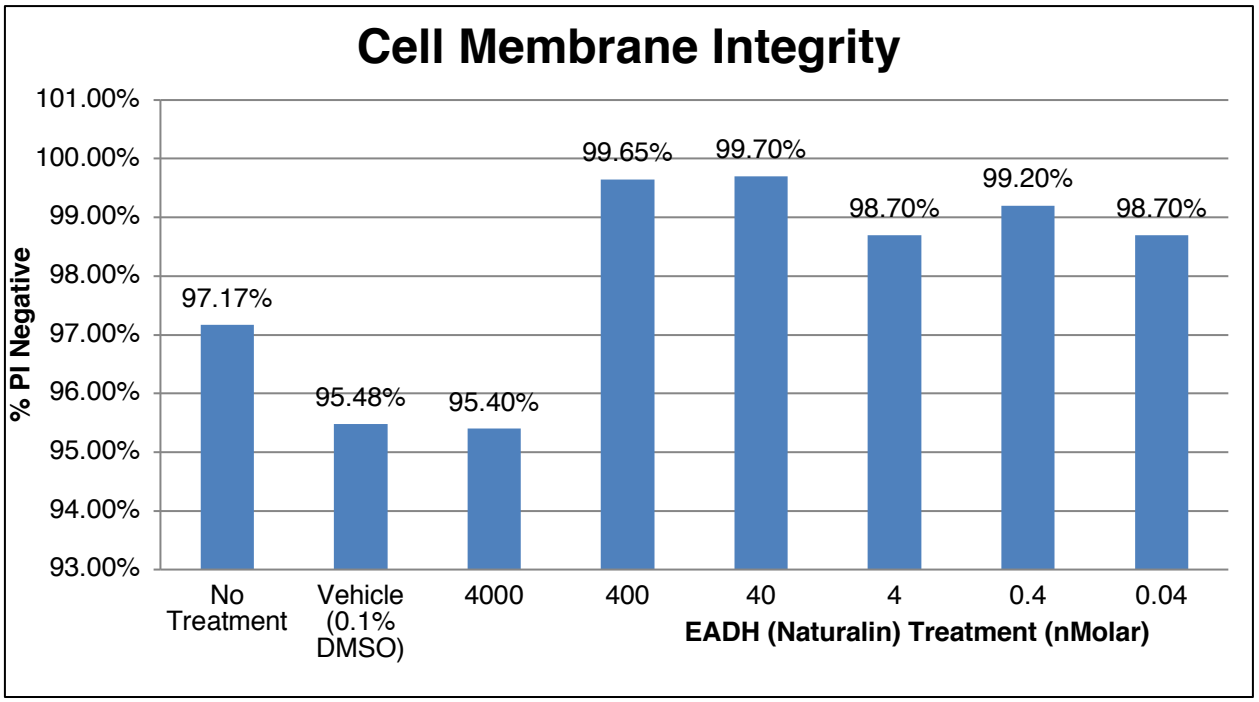


Figure 2. Cell membrane integrity was determined using propidium iodide (PI) labeling of cells (500-1000) on a flow cytometer (Accuri C6, BD Biosciences). All assays were run in triplicate. Cell with disrupted membranes, associated with cell death, are expected to be positively labeled with PI. The overall membrane integrity of cells was increased, non-significant) with EADH/Naturalin at 400nM-0.04nM. At the highest concentration tested (4μM) a non-significant decrease in membrane integrity is observed relative to vehicle treated cells (95.48% Vs 95.40%). These data indicate no negative impacts on cell membrane integrity upon exposure to EADH/Naturalin.

Figure 3. Glucose Uptake in MEFs Exposed to Insulin or EADH (Naturalin)

A.

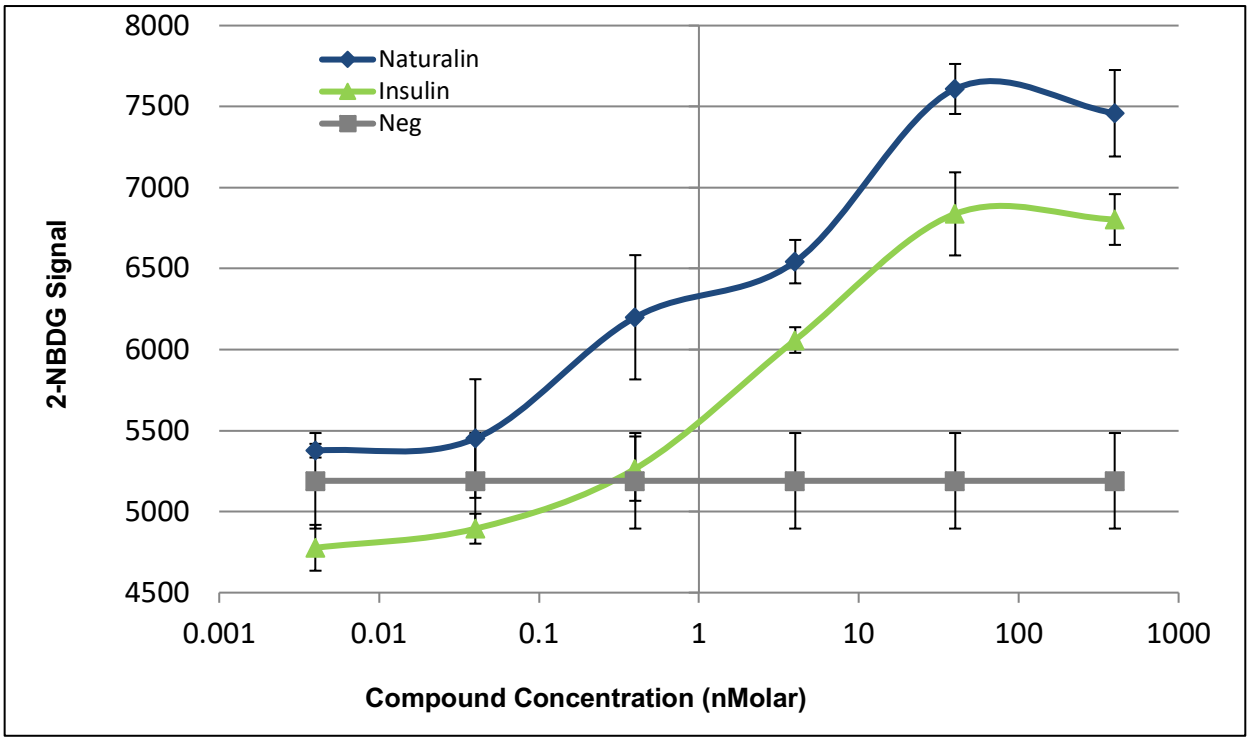


Figure 3. A. Glucose uptake was determined in MEFs using 2-NBDG labeling of cells (500-1000) on a flow cytometer (Accuri C6, BD Biosciences). Increased glucose uptake is indicated as increasing signal of 2-NBDG (488nM) in cells. Insulin shows a dose effect for increasing glucose uptake in MEFs. EADH (Naturalin) shows a dose effect for increasing glucose uptake in MEFs. The impact of EADH (Naturalin) on glucose uptake (2-NBDG signal) was significantly greater than vehicle treated (Neg, 0.1% DMSO) or Insulin treated cells. **Figure 3. B**. The percent increase in glucose uptake was calculated relative to untreated cells. EADH (Naturalin) and insulin increased glucose uptake by 47% and 32%, respectively, at 400nM exposures.

B.

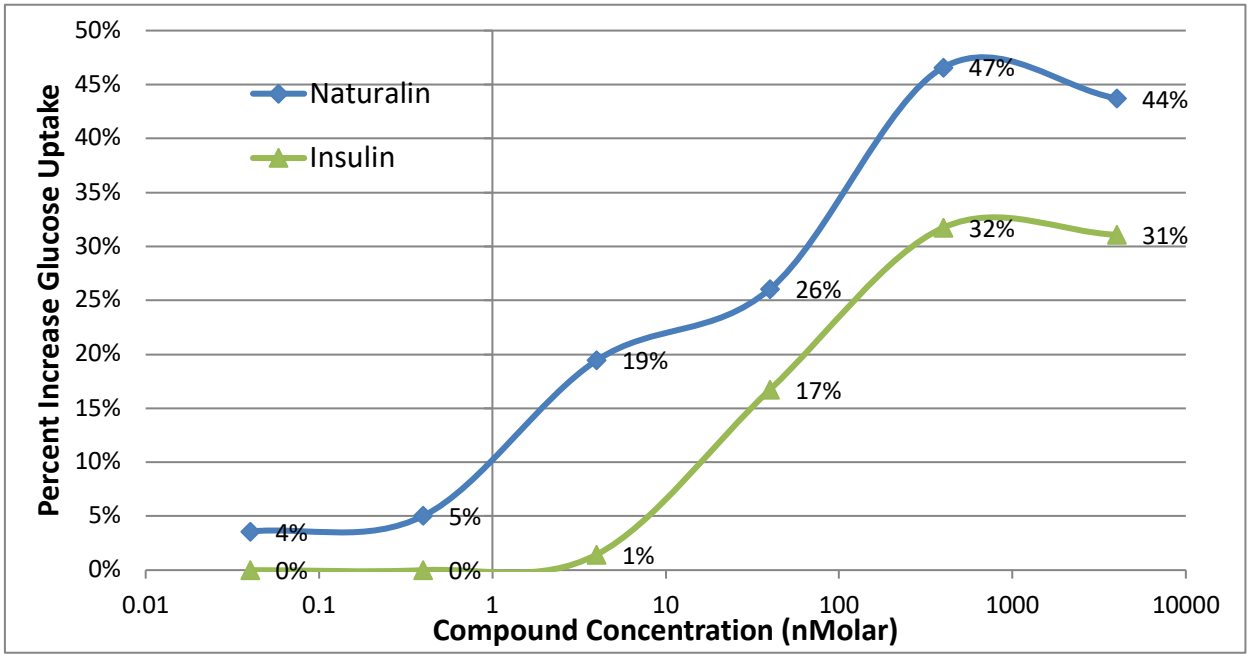
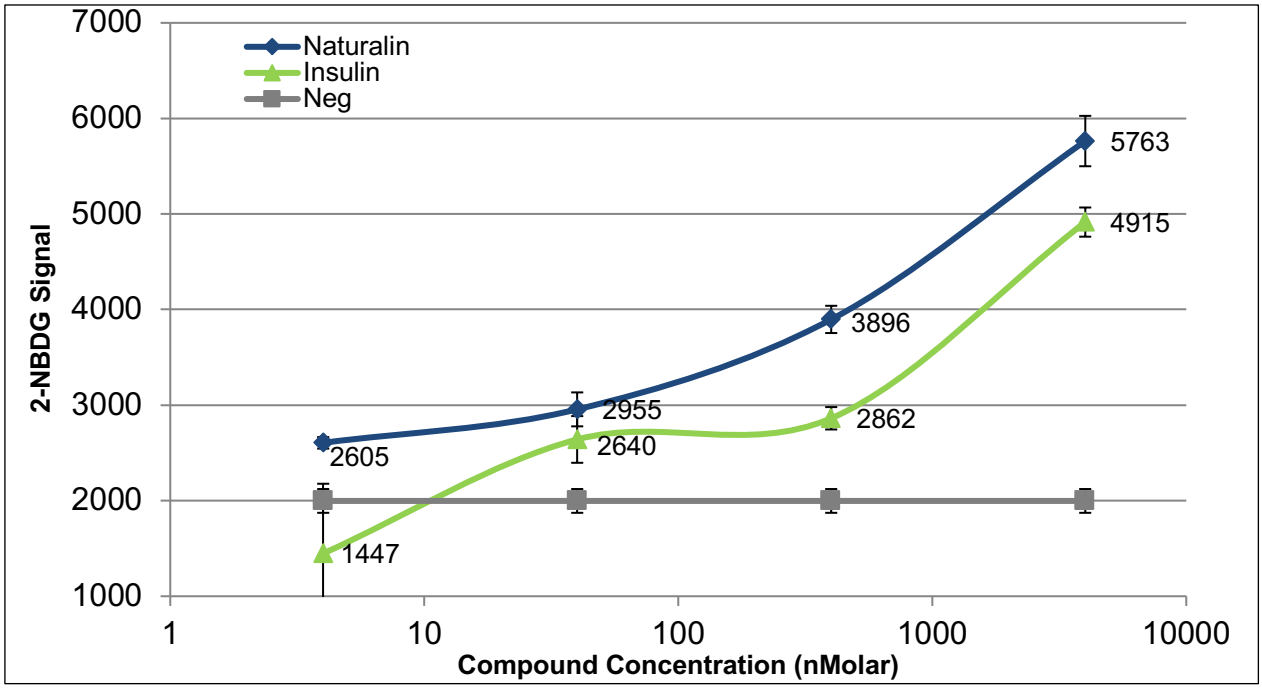


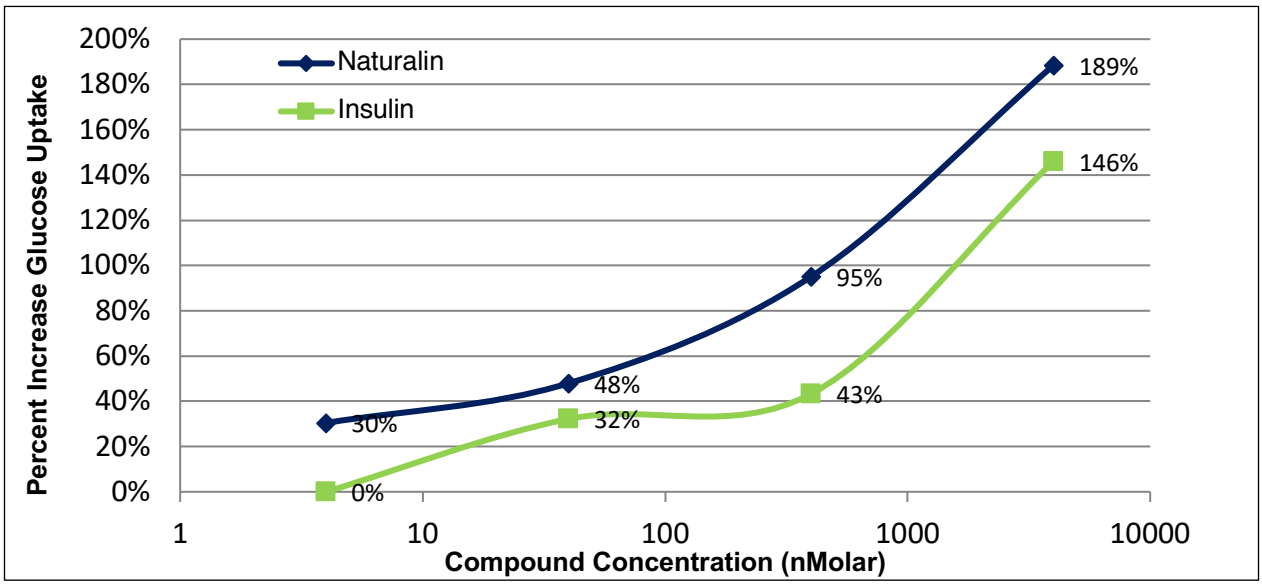
Figure 4. Glucose Uptake in C2C12 Cells Exposed to Insulin or EADH (Naturalin)

Figure 4. A. Glucose uptake was determined in muscle cells (C2C12) using 2-NBDG labeling of cells (500-1000) on a flow cytometer (Accuri C6, BD Biosciences). Increased glucose uptake is indicated as increasing signal of 2-NBDG (488nM) in cells. Insulin shows a dose effect for increasing glucose uptake in MEFs. EADH/Naturalin shows a dose effect for increasing glucose uptake in C2C12. The impact of EADH/Naturalin on glucose uptake (2-NBDG signal) was significantly greater than vehicle treated (Neg, 0.1% DMSO) or Insulin treated cells. **Figure 4. B.** The percent increase in glucose uptake was calculated relative to untreated cells. EADH/Naturalin and insulin increased glucose uptake by 189% and 146%, respectively, at 4μM exposures.

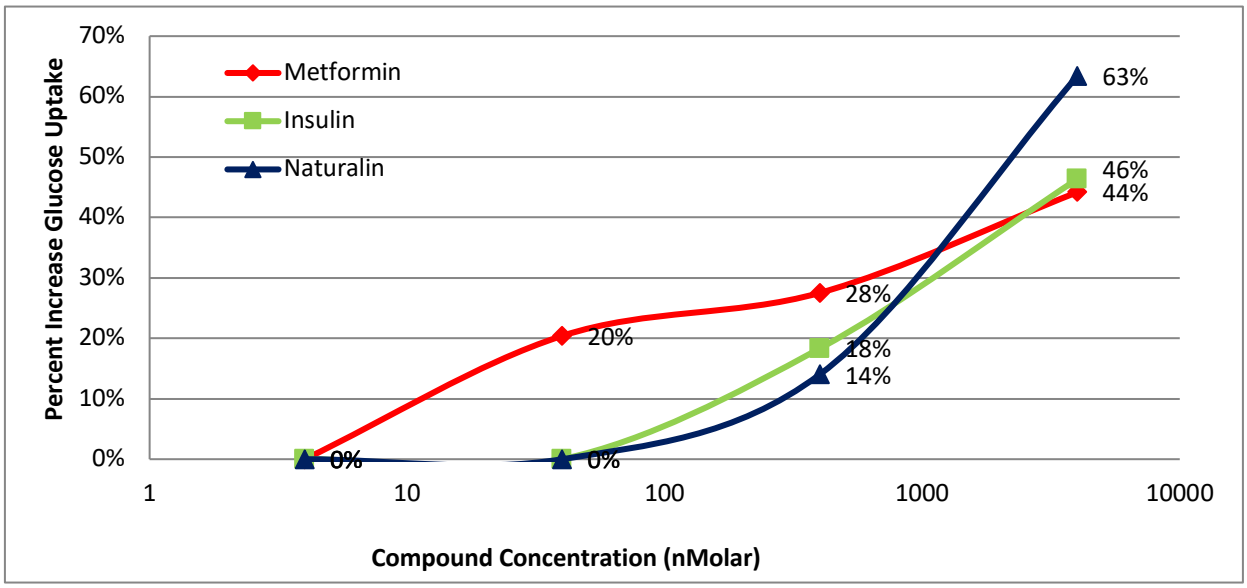
A.



B.



Glucose Uptake in C2C12 Cells Exposed to Metformin, Insulin and EADH 2 (Naturalin) C.



4.C. An additional experiment was performed to measure glucose uptake in C2C12 cells exposed to Metformin, insulin or EADH 2 (Naturalin) (batch 2).

Figure 5. EADH (Naturalin) Induced Glucose (2-NBDG) Uptake in MEFs

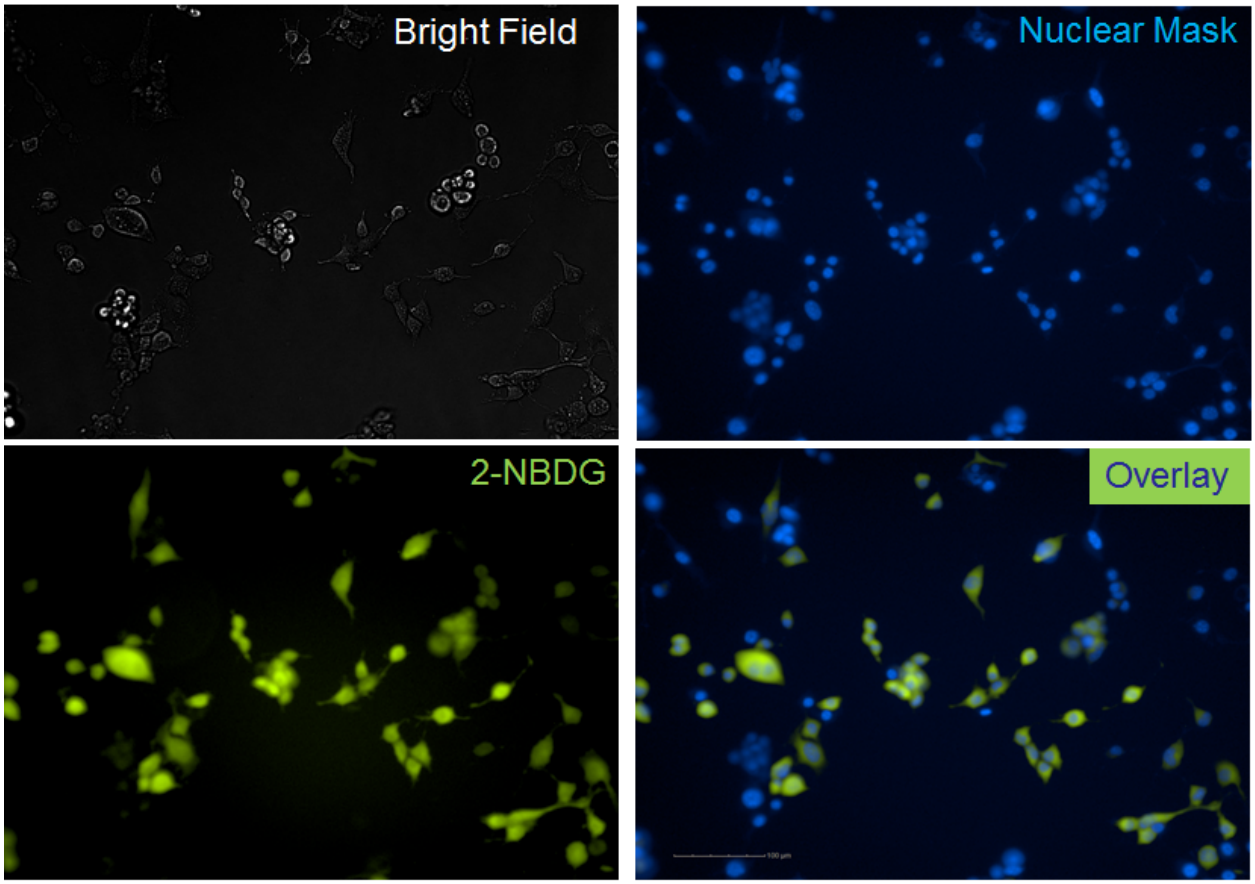
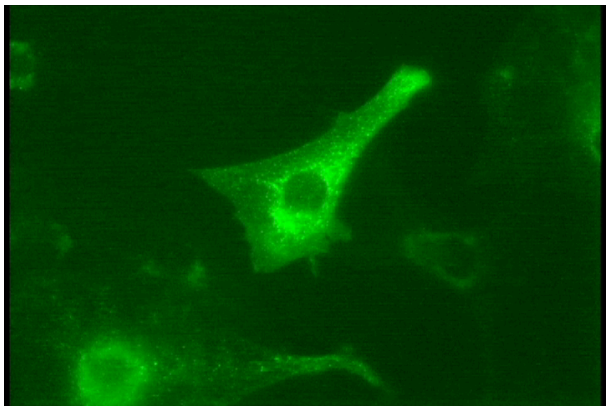
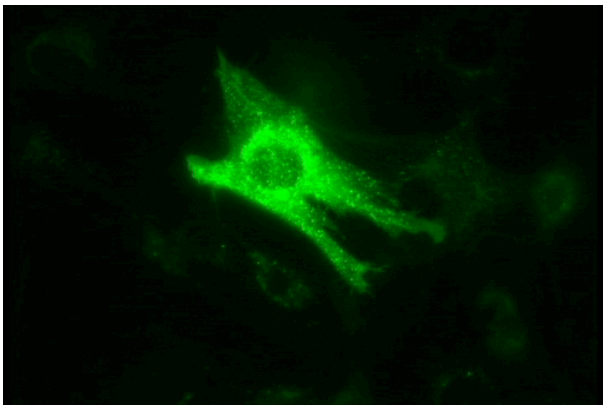


Figure 5. Glucose uptake was determined in MEFs using 2-NBDG labeling of cells on a high-content imaging system (Operetta, Perkin Elmer). Increased glucose uptake is indicated as increasing signal of 2-NBDG (488nM, green) in cells. Insulin shows a dose effect for increasing glucose uptake in MEFs. EADH shows a dose effect for increasing glucose uptake in C2C12. The impact of EADH on glucose uptake (2-NBDG signal) was significantly greater than vehicle treated (Neg, 0.1% DMSO) or Insulin treated cells (data not shown). Data parallel results shown by flow cytometry.

GLUT4 Translocation Videos: (Double Click Images Below to Play Video)



A. MEF cells treated with Insulin
Click here to play: Insulin Video
https://vimeo.com/257990872



B. MEF cells treated with EADH (Naturalin)
Click here to play: EADH Video
https://vimeo.com/257990756

Figure 6. Insulin Induced Changes in C2C12 (GLUT4-GFP) Cells

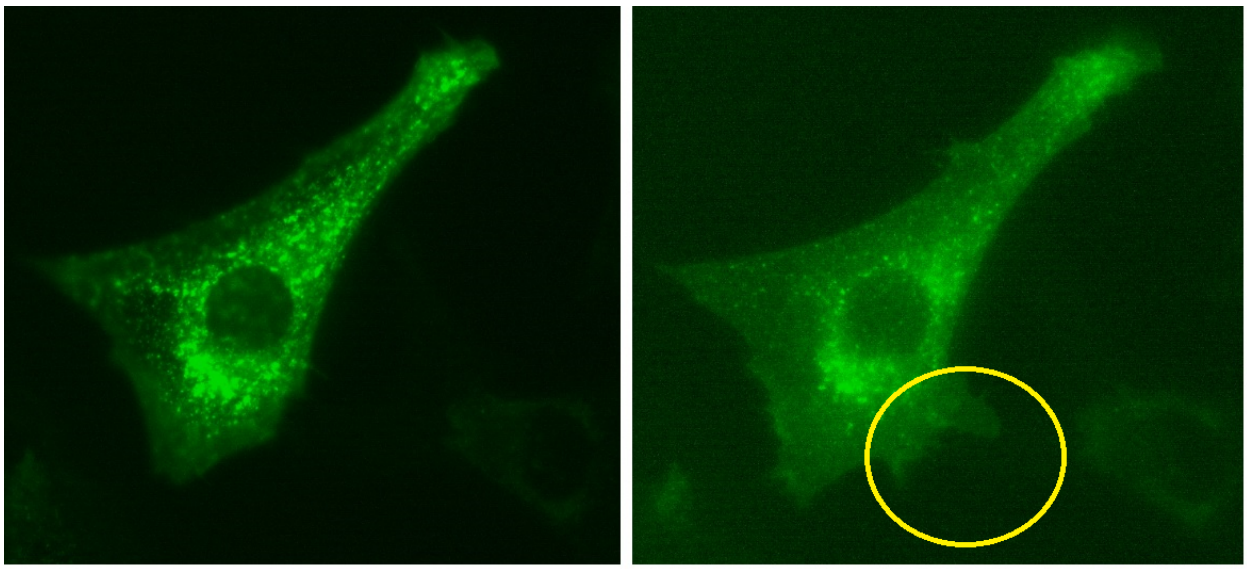


Figure 6. Muscle cells (C2C12) were infected with a fusion glucose transporter 4 green fluorescent protein (GLUT4-GFP). This protein allows the visualization of GLUT4 by imaging the GFP under fluorescent time-lapse microscopy. Frames are compiled to produce time-lapse images of cellular changes with no treatment or treatment with insulin. Untreated and vehicle treatment showed no net effect on cell behavior. Insulin produced a decrease in GLUT4-GFP around the cell nucleus and a parallel increase of movement at the membrane. Specifically, cellular filopodia (spikes) projections (shown circled) were found rapidly appearing around the cell with insulin treatment (4μM). The increase in membrane and GLUT4-GFP activity was directly associated with an increase in glucose uptake by insulin.

Figure 7. EADH (Naturalin) Induced Changes in C2C12 (GLUT4-GFP) Cells

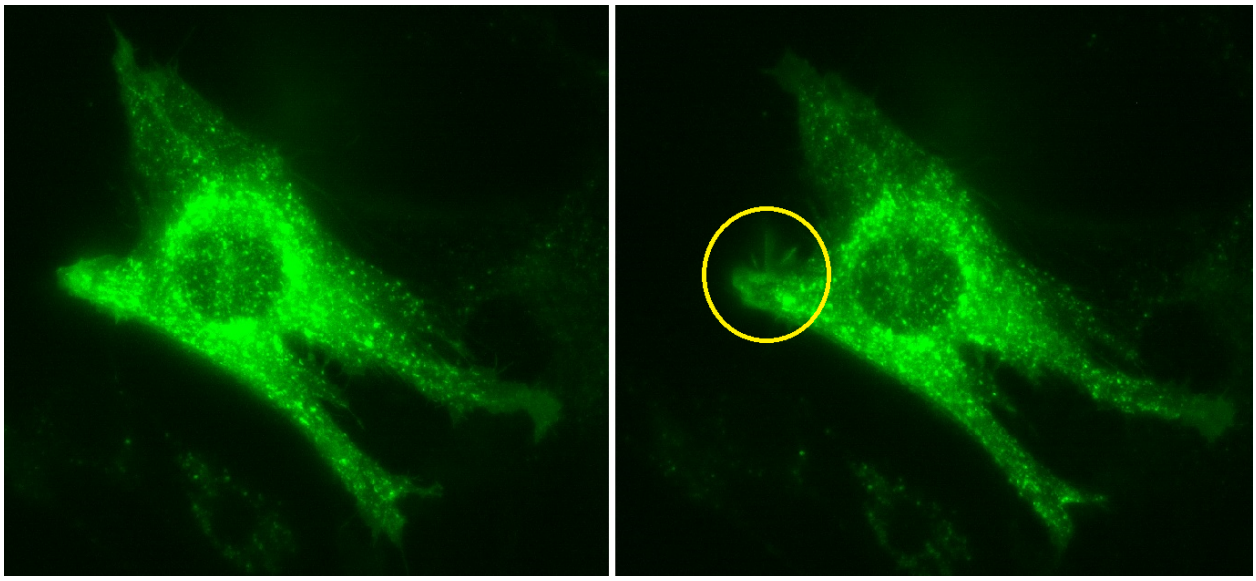


Figure 7. Muscle cells (C2C12) were infected with a fusion glucose transporter 4 green fluorescent protein (GLUT4-GFP). This protein allows the visualization of GLUT4 by imaging the GFP under fluorescent time-lapse microscopy. Frames are compiled to produce time-lapse images of cellular changes with no treatment or treatment with EADH (Naturalin). Untreated and vehicle treatment showed no net effect on cell behavior. EADH (Naturalin) produced a decrease in GLUT4-GFP around the cell nucleus and a parallel increase of movement at the membrane. Specifically, cellular filopodia (spikes) projections (shown circled) were found rapidly appearing around the cell with EADH treatment (4μM). These increase in membrane and GLUT4-GFP activity was directly associated with an increase in glucose uptake by EADH (Naturalin). These observations paralleled those seen with insulin (Figure 6).

Figure 8. EADH (Naturalin) Induced Changes in AKT Signal Transduction in C2C12

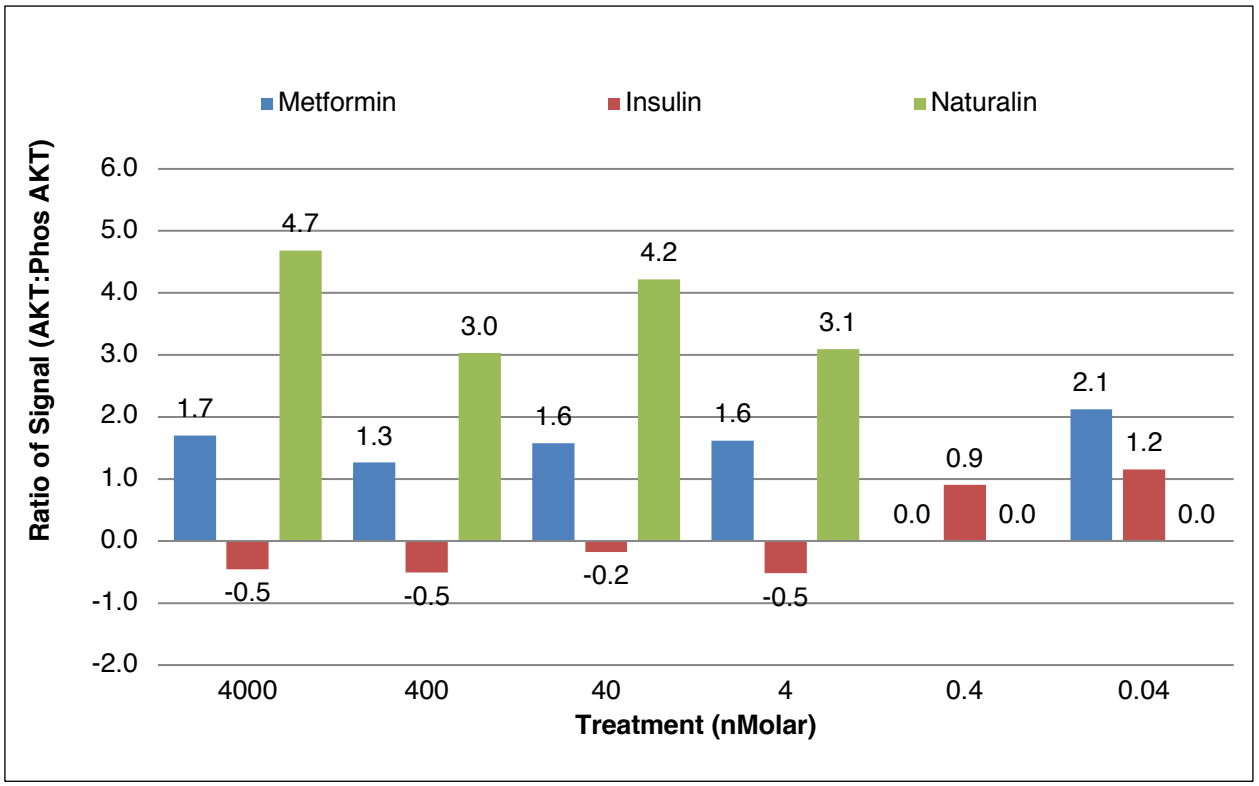


Figure 8. Altered AKT signaling was determined upon exposure to Metformin, insulin, or EADH (Naturalin). AKT and phosphorylated-AKT was determined in C2C12 using immunocytochemistry labeling (anti-AKT, anti-Phos AKT) on a high-content imaging system (Operetta, Perkin Elmer). Increased phosphorylation is indicated as decrease in signal of AKT:Phos AKT ratio (log_2 scale) relative to vehicle treated cells. Insulin shows an increase in Phos-AKT (-0.5 ratio) with treatment (4nM-4μM). EADH (Naturalin) shows an increase in AKT relative to Phos-AKT signal (4nM-4μM). Insulin increased phosphorylation of AKT. EADH (Naturalin) increased AKT relative to phosphorylation of AKT.

Figure 9. EADH Induced Changes in p44/42 MAPK Signal Transduction in C2C12

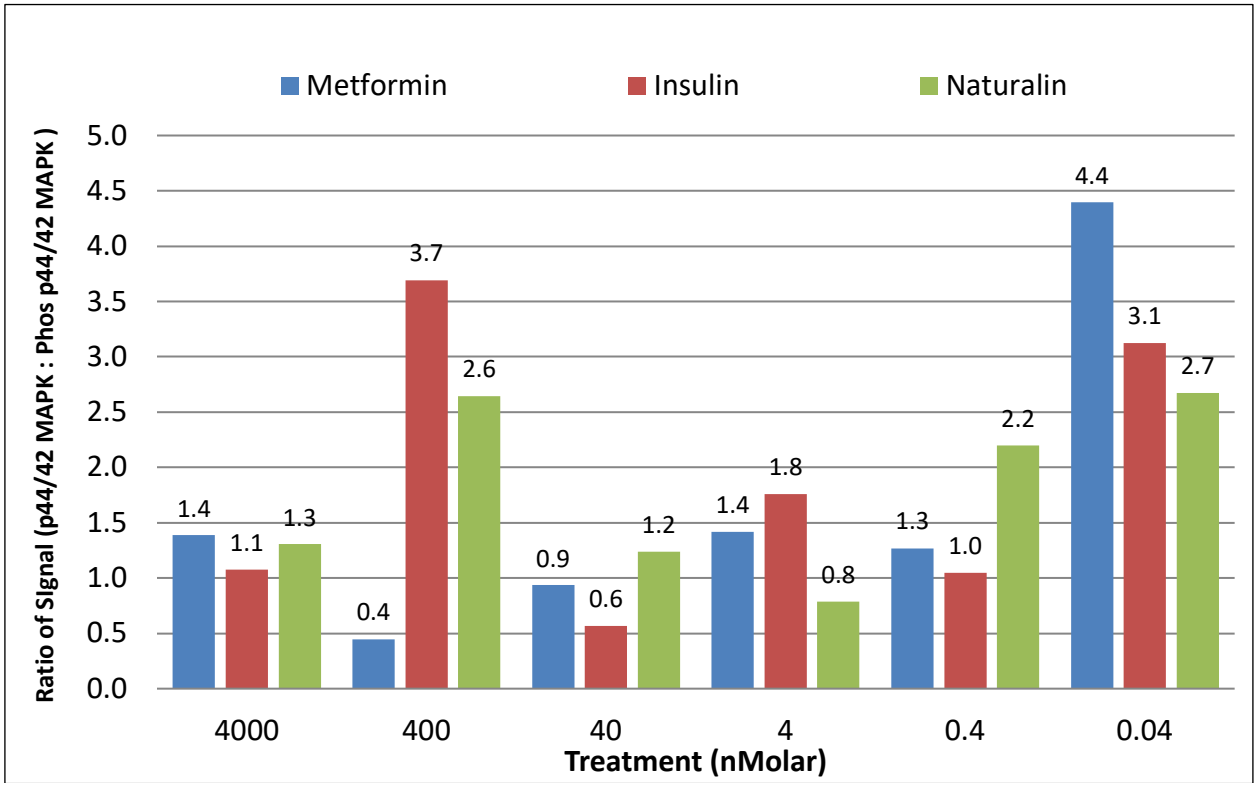


Figure 9. Altered p44/42 MAPK Signaling was determined upon exposure to Metformin, insulin, or EADH (Naturalin). The p44/42 MAPK and phosphorylated- p44/42 MAPK signals were determined in C2C12 using immunocytochemistry labeling (anti-p44/42 MAPK, anti-Phos p44/42 MAPK) on a high-content imaging system (Operetta, Perkin Elmer). Increased phosphorylation is indicated as decrease in signal of p44/42 MAPK:Phos p44/42 MAPK ratio (log_2 scale) relative to vehicle treated cells. Insulin and EADH (Naturalin) show an increase in p44/42 MAPK with treatment (400nM, 0.04nM). Metformin increased the ratio of p44/42 MAPK at 0.04nM treatment. No treatments were observed to increase the ratio of phosphorylated p44/42 MAPK relative to p44/42 MAPK signal.