Matairesinol and its metabolite enterolactone promote glucose uptake in 3T3-L1 adipocytes via a Glut4/Akt-dependent mechanism

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Purpose of Project

In this study, we aimed to determine the effects of enterolactone (ENL) and matairesinol (ML) on glucose uptake in differentiated 3T3-L1 adipocytes and begin to characterize the molecular mechanism(s) by which they exert their effect(s).

1. Determine whether these compounds promote glucose uptake in differentiated 3T3-L1 adipocytes.
2. Characterize the molecular mechanism(s) by which these compounds exert their effect(s).

Introduction

Type-2 Diabetes (T2D) consistently poses a mounting challenge in the realm of public health. As of 2021, 537 million people between the ages of 20-79 years old have been diagnosed with T2D. Domestically, 38.4 million diagnoses have been made in the United States alone [1]. Notably, it holds the unfortunate distinction of being the costliest chronic health condition in the United States, with an annual expenditure eclipsing $413 billion dedicated solely to diabetic medical care (2023) [2].

T2D manifests as chronically elevated blood glucose levels stemming from acquired insulin resistance, a phenomenon wherein cells crucial for glucose uptake become insensitive to insulin's regulatory cues [3]. Consequently, this insulin resistance precipitates chronic hyperglycemia, exacerbating the disease's progression and inflicting damage upon vital organs such as the kidneys and heart. Given the prevalence and economic toll of T2D, the exploration for novel, cost-effective agents for its management and prevention becomes imperative.

Reviews delving into habitual coffee consumption and its relationship to T2D development consistently suggest an inverse association between these variables [4, 5]. Through a review of the literature, six compounds, either naturally occurring in coffee or its metabolic derivatives, emerged as potential anti-diabetic agents.

Of particular interest were two compounds singled out for this study. One, enterolactone (ENL), exhibited the capacity to bolster basal glucose uptake in L6 myotubes, while also impeding triacylglycerol uptake in HEPA1-6 cells and adipogenesis in 3T3-L1 fat cells [6]. Meanwhile, the other compound, matairesinol (ML), the immediate precursor for ENL, demonstrated efficacy in restraining body weight gain and the accrual of fat mass—both common hallmarks associated with diet-induced T2D [6]. These findings hint at the potential of these compounds to mitigate T2D progression by safeguarding the health of β-cells and fostering glucose uptake in skeletal muscle (SkM) and adipose tissues.

A comprehensive review of literature suggests that these compounds may serve to enhance β-cell function and viability, promote glucose uptake in SkM and adipocytes, and possibly elicit anti-diabetic effects in vivo. Nonetheless, a gap in understanding persists regarding their impact across multiple tissue types and the intricate mechanisms underlying these effects.

Consequently, this study endeavors to shed light on their influence on differentiated 3T3-L1 adipocytes.

Methods

Cell Culture
3T3-L1 fibroblasts were grown to ~80% confluency in complete growth medium comprised of DMEM supplemented with 10% bovine calf serum. The fibroblasts were then differentiated (day 0) in complete growth medium supplemented with 0.5 mM IBMX, 1.0 µM dexamethasone, and 10 µg/mL human insulin. On day three of differentiation, differentiation medium was replaced with post-differentiation medium which consisted of complete growth medium and 10 µg/mL insulin. Cells were maintained in post-differentiation medium until day six at which point the experiments were conducted.

Fluorescence Glucose Uptake Assay with Ritonavir
Cells were seeded in black, clear-bottom, 96-well plates. Cells were treated with either 10 nM insulin (positive control), ethanol (ENL negative control/vehicle), DMSO (ML negative control), or a combination of 10 nM insulin or the predetermined optimal concentrations of ENL (1, 10 µM) or ML (50 µM) plus Ritonavir. To measure glucose uptake, fluorescently labeled glucose, 2-NBDG (100 µg/mL) was also added to each treatment. Cells that were to receive selective GLUT inhibitors as part of the treatment were pretreated with such compounds for 6 minutes prior to receiving the treatment including the test compound and inhibitor. After treatments were applied, cells were incubated for 15 minutes and washed with 1X PBS according to manufacturer protocol (Cayman, Ann Arbor, MI). A Tecan Infinite 200 Pro plate reader (Grödig, Austria) was used to analyze fluorescence at excitation/emission 485/535 nm.

Akt/IRS-1 Phosphorylation Assays
Cells were seeded in 6-well plates at a density of 3 x 10^5 cells/well. On day six, cells were treated with compounds previously shown to significantly increase 2-NBDG uptake (ENL: 1, 10 µM) for 15 minutes. Cell lysates were diluted in 1X lysis buffer to 45 µg/µL protein. 45 µg of cell lysates from cells treated with either insulin (10 nM), vehicle (ethanol), 1 µM ENL, or 10 µM ENL were transferred into white, clear-bottom, 96-well plates containing antibodies for either phospho-specific or total (Pan) Akt. Akt assays were conducted according to the manufacturer's protocol (RayBioTech, Peachtree Corners, GA). IRS-1 assays were also conducted according to the manufacturer's protocol (Cell Signaling Technologies, Danvers MA). Absorbance (450 nm) was measured in each assay by using a Tecan Infinite 200 Pro plate reader (Grödig, Austria). Data are displayed as a normalized ratio of pAkt:Akt and pIRS-1:IRS-1.

Protein Quantification
Cell lysate protein was quantified by using a BCA assay kit according to the manufacturer protocol (Prometheus, Raleigh, NC).

Statistical Analysis
Data were analyzed by one-way ANOVA. Duncan's multiple range test was performed for pairwise comparison of observed significant differences ($p < 0.05$). Values are expressed as mean ± the standard error of the mean.

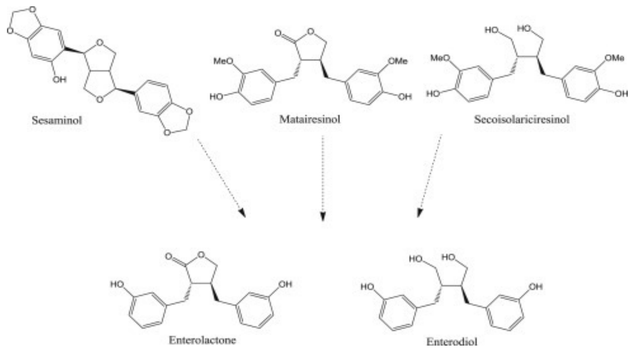

Figure 1. Metabolization of matairesinol (Molar mass : 358.14 g/mol) and other lignans into bioavailable substrates enterolactone (Molar mass : 298.338 g/mol) and enterodiol (Molar mass : 302.36 g/mol).

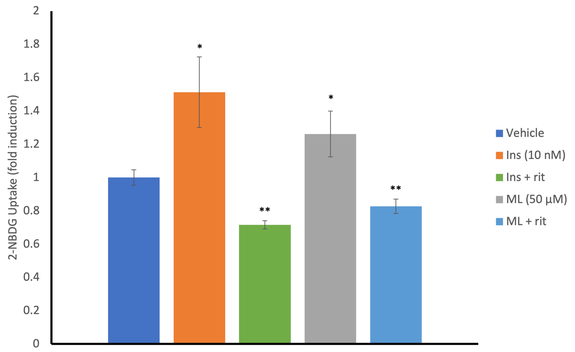

Figure 2. Matairesinol (ML)-stimulated glucose uptake is blunted by ritonavir. Cells were treated with either vehicle (DMSO), insulin (10 nM), ML (50 µM), or ML (50 µM) plus ritonavir (50 µM). ML significantly increased glucose uptake relative to the negative control (*$p < 0.05$). Further, ritonavir significantly blunted the effect of insulin and 50 µM ML on glucose uptake (**$p < 0.05$). The date represent three biological replicates.

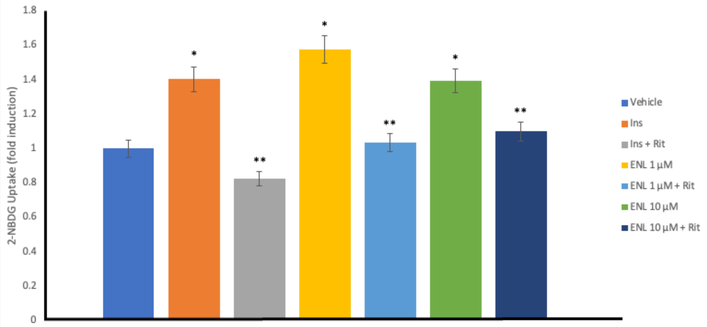

Figure 3. Enterolactone (ENL) significantly increased glucose uptake in 3T3-L1 differentiated adipocytes. Cells were treated with either vehicle (ethanol), ENL (1 or 10 µM), insulin (10 nM), insulin (10 nM) plus ritonavir (50 µM), or ENL (1 or 10 µM plus ritonavir (50 µM). The 1 and 10 µM concentrations of ENL significantly increased glucose uptake (*$p < 0.05$) relative to the negative control. Further, ritonavir significantly blunted the effect of the insulin, 1, and 10 µM ENL (** $p < 0.05$). The data represent three biological replicates.

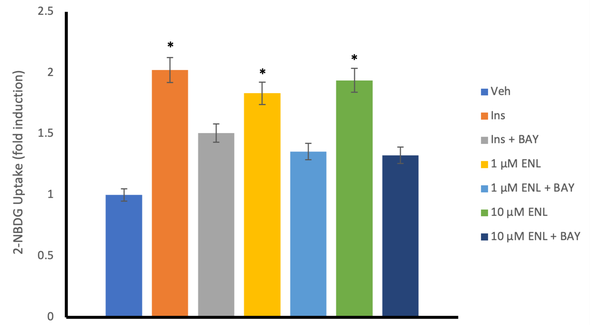

Figure 4. ENL-stimulated 2-NBDG uptake is suppressed by the selective GLUT4 inhibitor, BAY-876, in differentiated 3T3-L1 adipocytes. Cells were treated with either vehicle (ethanol), insulin (10 nM), insulin + BAY-876 (290 nM), ENL (1 or 10 µM), or ENL (1 or 10 µM) + BAY-876 (290 µM). The 1 and 10 µM concentrations of ENL along with insulin increased 2-NBDG uptake relative to the negative control. Further, BAY-876 (290 µM) blunted the glucose uptake stimulating effects of insulin, 1 µM ENL, and 10 µM ENL. Data represent three biological replicates.

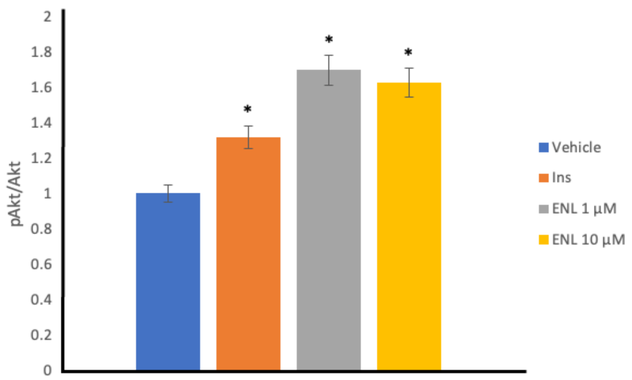

Figure 5. Enterolactone (ENL) significantly increased Akt phosphorylation in 3T3-L1 differentiated adipocytes. Cells were treated with either vehicle (ethanol), insulin (10 nM), or ENL (1 or 10 µM). pAkt and Akt were measured via ELISA and expressed as a normalized ratio of pAkt/Akt. The data represent three biological replicates (*$p < 0.05$).

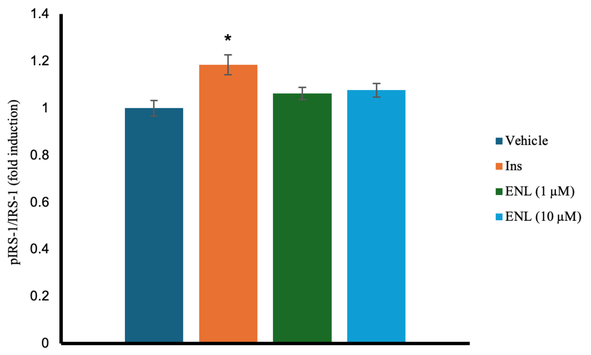

Figure 6. Enterolactone (ENL) increased IRS-1 phosphorylation in 3T3-L1 differentiated adipocytes. Cells were treated with either vehicle (ethanol), insulin (10 nM), or ENL (1 or 10 µM). pIRS-1 and IRS-1 were measured via ELISA and expressed as a ratio of normalized pIRS-1:IRS-1. The data represent three biological replicates (*$p < 0.05$).

Results and Conclusions

Results
Both ENL and ML significantly increased glucose uptake in 3T3-L1 adipocytes relative to the negative control (Fig 2, 3, 4).

Ritonavir, which has been shown to be a selective inhibitor of Glut4, significantly inhibited glucose uptake that was otherwise promoted by ENL (1, 10 µM) and ML (50 µM) (Fig 2, 3, 4).

Neither compound promoted insulin-stimulated glucose uptake or demonstrated an additive effect when combined with insulin (data not shown).

ENL (1, 10 µM) significantly increased Akt phosphorylation when compared to the negative control to the extent that it was statistically indistinguishable from the positive control (Fig 5).

ENL (10 µM) did not increase IRS-1 phosphorylation compared to the negative control (Fig 6).

Conclusions
Significant enhancements in glucose uptake were observed following treatment with both ENL and ML, indicating their potential utility as anti-diabetic agents. Moreover, the concurrent inhibition of glucose uptake in combination with ritonavir and BAY876 suggests that these compounds facilitate glucose uptake through a Glut4-dependent mechanism. While ritonavir has been shown to inhibit both Glut1- and Glut4- stimulated glucose uptake, the timing and concentrations utilized in this study align with a Glut4-mediated mechanism. Additionally, ritonavir attenuates the insulin effect to a similar degree as it inhibits the effects of the compounds.

Our data further reveal that ENL stimulates Akt phosphorylation, but not IRS-1 phosphorylation, both of which are pivotal in Glut4 translocation to the plasma membrane. The timing and degree of Akt phosphorylation induced by ENL are statistically comparable to that of insulin. Although the effect of ML on Akt and IRS-1 phosphorylation remains unanalyzed, the chemical similarity as well as similarities in glucose uptake promotion and Glut4 inhibitor sensitivity between ENL and ML suggests a shared mechanism of action. Furthermore, neither compound enhances insulin-stimulated glucose uptake, indicating potential pathway overlap in their mode of action.

Current evidence supports an Akt/Glut4-mediated pathway for stimulating glucose uptake in adipocytes that is activated downstream of IRS-1 phosphorylation and thus an intracellular mode of action. Future research directions for ENL involve further elucidation of its molecular mechanism, while interactions between ML and differentiated 3T3-L1 adipocytes will be continuously examined using established methodologies. Additionally, we plan to analyze other biomarkers of Glut signaling, including AMPK and PI3K phosphorylation, utilizing selective protein inhibitors such as compound C and wortmannin, respectively.

Future Work

ENL
1. Repeat glucose uptake assays using selective PI3K and AMPK inhibitors to determine whether the effects depend on these enzymes.
2. Determine the cellular transporter through which ENL enters the cell.
3. Confirm ELISA data via Western blot analysis
4. Examine ENL's translational potential by conducting an *in vivo* study.

ML
1. The mechanism(s) by which ML contributes to glucose uptake will be further characterized by employing a variety of proteomic assays and arrays.
2. Conduct Akt and IRS-1 Phosphorylation assays on ML.

References and Acknowledgments

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