Exploring New Modalities of Restoring Insulin Sensitivity Using Lower Doses of C646 Combined with Polyphenolic Compounds

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Abstract

Over 500 million people worldwide, including 36 million Americans, are affected by type II diabetes, a metabolic disease characterized by insulin resistance and hyperglycemia. Complications associated with this disease include but are not limited to retinopathy, cardiovascular disease, neuropathy, and nephropathy. One of the major causes of insulin resistance stems from the protein IRS-1's insensitivity to activate downstream events when insulin binds to its receptor. Although there are numerous proteins that downregulate IRS-1 through serine phosphorylation, p300, a broad histone acetyltransferase responsible for the regulation of hundreds of transcriptions factors, can also inhibit IRS-1 through acetylation. Recent studies revealed that p300 inhibition using 20 μM of C646 promotes insulin sensitivity. Such high concentrations of C646 may significantly interfere with other cellular processes. Our study aims to investigate the use of lower concentrations of C646 combined with other natural compounds known to reverse insulin resistance. For example, cinnamon and curcumin are known to improve insulin resistance, but their effect of IRS-1 has not been directly investigated. Treatment with these compounds in conjunction with lower concentrations of C646 will potentially result in a more favorable outcome. For this investigation, we will culture lines of L6 rat cells, human HepG2 cells, and 3T3-L1 mouse cells and render them insulin resistant using various concentration of palmitic acid, insulin, and glucose. Each line will then be treated with cinnamon, curcumin, and common anti-diabetic medications in combination with C646. The results of this experiment have promising potential and significance in understanding insulin resistance in the IRS-1 pathway. Future research will involve treatment in an insulin resistant mouse model. These treatments may provide better understanding in novel ways to restore insulin sensitivity.

Introduction

With type II diabetes mellitus being a major health concern worldwide, new modalities, remedies, and solutions are becoming increasingly sought after in the scientific community. Type II diabetes is a disease in which the body becomes resistant to insulin, thereby allowing prolonged elevated blood glucose levels. A consequence of insulin resistance is an imbalance between metabolic effects and mitogenic effects. Some results of this imbalance includes increased inflammation, hypertrophy, and atherosclerosis. In the cellular response pathway to insulin, the insulin receptor substrate protein (IRS1) is targeted by the insulin receptor and is a key mediator that leads to glucose uptake into cells. Serine/threonine phosphorylation of IRS-1 leads to negative regulation of IRS1 and is one of the characteristics of insulin resistance. Acetylation by p300 is also know to inhibit the IRS1 protein. Recently, C646 has been used to traget and inhibit p300, but due to p300's involvement acetylation of histones, as well as many other proteins, treatment with high doses of C646 alone is not viable. Other natural polyphenolic compounds supplemented in addition to lower doses of C646 may produce more physiologically positive and beneficial results. For example, cinnamon is known to increase the expression of GLUT4 through its inhibition of GSK3 and PTP1, thus improving insulin signaling.

Materials and Methods

Cell Culture

Human hepatocellular carcinoma (HepG2) cells were grown in EMEM (Eagle's minimun essential medium) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen Strep) at 5% CO2 and 37°C until reaching ~80% confluency. The media was changed every two or three days. Once the total cell count reaches around 8 million, the cells will be transferred to two six-well plates, with each well containing around 300,000 cells. They will be supplied with 10% FBS and 1% Pen Strep. The cells will be grown for 48 hours until the confluency reaches 80%, and then then starved of their full serum media for 24 hours via aspiration. Cells will then be washed with sterile PBS. Serum free EMEM with only Pen Strep will then be supplied to the cells.

Induction of Insulin Resistance

After the 24 hours of being serum starved, to induce insulin resistance (IR), three wells on plate one will be treated with either 5nM insulin, 10nM insulin, or 55 mM glucose diluted in phosphate buffered saline (PBS) for 24 hours. The negative control will receive treatment of PBS only, and two wells will remain empty. To induce IR on the second plate, two wells will be treated with either 0.75mM or 0.25mM palmitic acid diluted in NaOH for 24 hours. The negative control will receive only treatment of NaOH, and the treatment will be performed in duplicate.

Combined Treatment with C646 and Polyphenolic Compounds

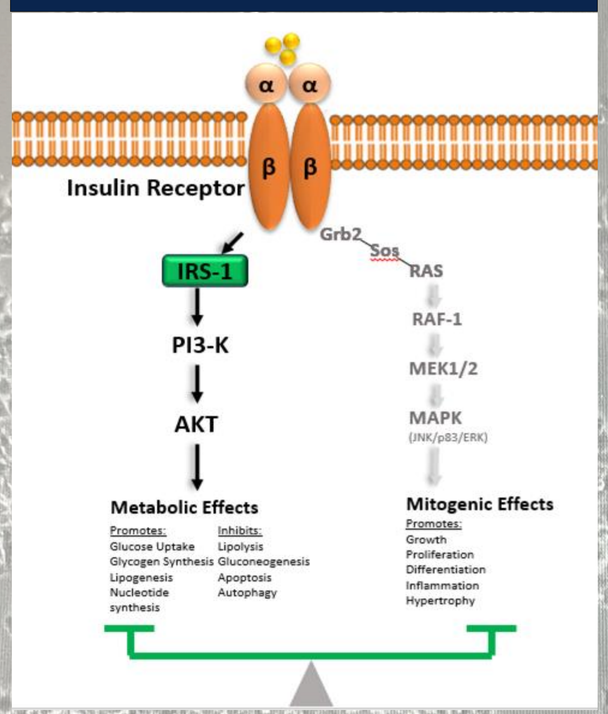
IR cells will be washed twice with sterile PBS and starved, then treated with 10μM of C646, either alone or in combination with 0.5mM cinnamon extract or 100ug/mL of curcumin for 24 hours. The control group will receive treatment with EMEM and no FBS. The exact concentrations of compounds will be determined after running a dose response.

Western Blot Analysis

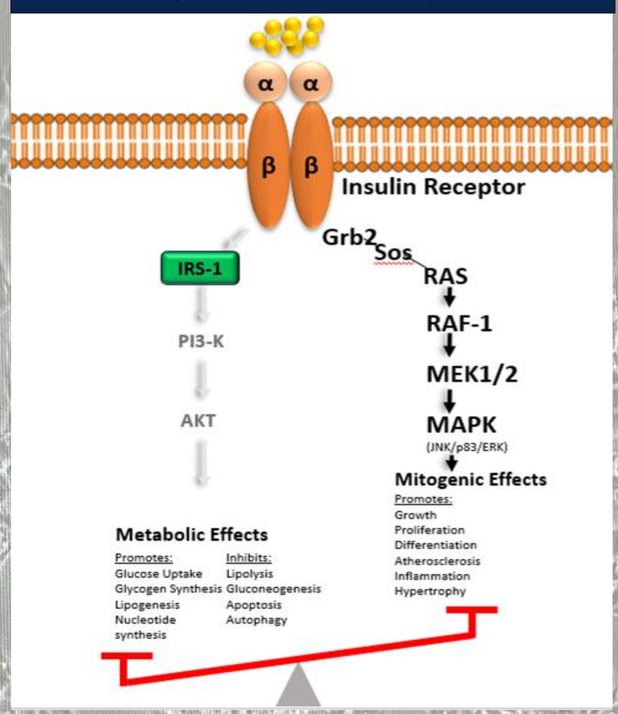
The HepG2 cells will be washed with sterile PBS after treatment with C646 or the polyphenolic compounds and collected with 2X Laemmli buffer. The collected samples will be boiled at 95°C for 5 min and centrifuged briefly. Protein samples will undergo gel electrophoresis using 4-20% precast polyacrylamide gels and then be transferred to a PVDF membrane according to the Bio-Rad Turbo Transfer protocol. The PVDF membrane will be wet in methanol and equilibrated with TBS-T (tris buffered saline tween-20) and blocked with 5% milk, either overnight at 4°C or for one hour at room temperature on a rocker. The membrane will be washed with TBST then incubated with the appropriate antibody overnight at 4°C with rocking . The antibody will be selected based on the following list of proteins we are investigating: IRS1, p70, S6K1, RPS6, PRAS, and p300. The membrane will be washed in TBS-T and incubated for one hour in LI-COR goat anti rabbit secondary antibody with TBS-T in 5% milk. The blots will be washed again with TBS-T and scanned and quantified using the LI-COR CLX imager and LI-COR software Image Studio.

Loss of Balance in the Insulin Signaling Pathway

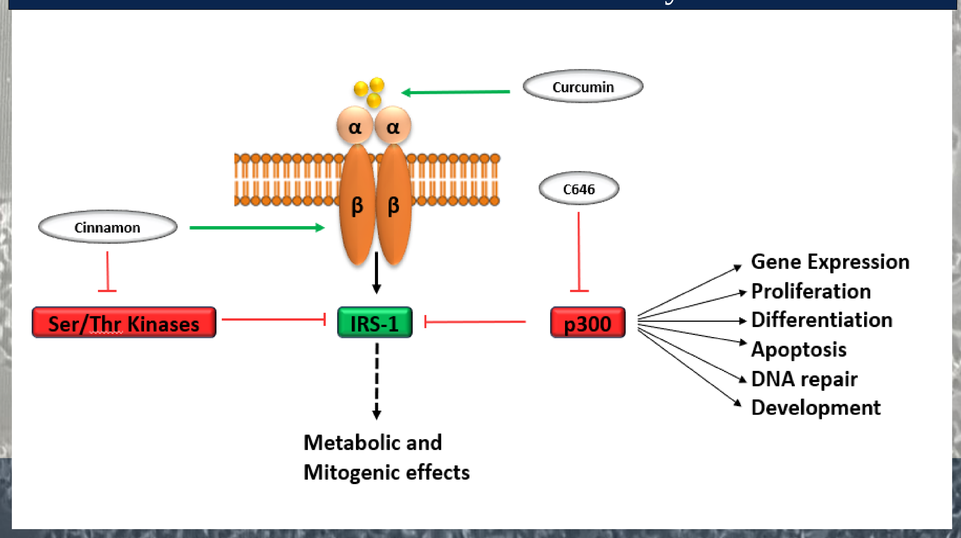
Insulin Sensitive Model



Insulin Resistant Model



Potential Treatment Pathways



Expected Results

Our expectation is that a lower dose of C646 in combination with commonly consumed polyphenolic compounds, such as cinnamon and curcumin, will lead to restored insulin sensitivity. Not only will insulin sensitivity be restored, but since a lower dosage of C646 will be used, p300 should remain functional enough to still regulate some mitogenic effects. The mechanism of action of curcumin is to increase the interaction between insulin and insulin receptor. On the other hand, cinnamon inhibits proteins that negatively regulate IRS-1, specifically GSK3 and PTP1. Since each cell line chosen in this experiment is a representation of muscle, adipose, and liver tissue, we expect to see restored insulin signaling in all three tissue types. These tissues are the major targets for insulin. Since p300 is indeed capable of acetylating over a hundred different proteins, some of these proteins will be used as markers to assess partial p300 acetylatranferase activity. Interactions between insulin receptor and IRS-1, as well as their tyrosine phosphorylation statuses as obtained from western blot analysis will allow us to assess whether insulin sensitivity was restored. To ensure that the balance was restored, the phosphorylation status of both the metabolic and mitogenic arms of the insulin signaling pathway will be assessed. The expectation is to see decreased MAPK activity and increased AKT activity.

Discussion

One of the many targets of p300 acetylation is IRS1, a major player in the insulin signaling pathway. When acetylated, IRS1 is prevented from associating with insulin receptor, resulting in the cessation of the insulin signaling pathway, thus contributing to insulin resistance and hyperinsulinemia. It is known that treatment with C646 will positively regulate the function of IRS1 and help reverse insulin resistance through inhibition of p300. However, due to the action of p300 in acetylating numerous and varied proteins involved in cell signaling, proliferation, and gene expression, treatment with high doses of C646 are not viable. For example, p300 acetylates Histone H3, directly controlling gene expression. Our aim is to combine a low concentration of C646 that is viable in physiological conditions with other known anti-diabetic compounds. Through this combination, our goal is to find a way that insulin sensitivity might be recovered without the disruption of other cellular pathways

There have been notable associations between type 2 diabetes and a number of other diseases, one of which is cardiac disease. Both type 2 diabetes and cardiac disease have been correlated with inappropriate quantities of p300 in the nucleus. In normal and healthy adults, p300 acetylates a regulatory protein involved in inhibiting transcription of important growth, anti-apoptosis, and pro-proliferation genes. An increase in p300 activity in cardiomyocytes has been noted to lead to increased growth and inhibited apoptosis, resulting in hypertrophy and cardiac necrosis. Likewise, increased p300 activity has been seen in mice with hyperinsulinemia. These data indicate that decreasing p300 activity not only holds the potential benefit of restoring insulin sensitivity, but it may also restore other pathways, particularly in cases where p300 activity is increased above normal levels.

References

Dubois-Deruy, E., Y. El Masri, A. Turkieh, P. Amouyel, F. Pinet, and J. Annicotte. 2022. Cardiac Acetylation in Metabolic Diseases. *Biomedicines.* 10:1834. doi: 10.3390/biomedicines10081834. doi: 10.3390/biomedicines10081834.

Geng, S., S. Wang, W. Zhu, C. Xie, X. Li, J. Wu, J. Zhu, Y. Jiang, X. Yang, Y. Li, Y. Chen, X. Wang, Y. Meng, M. Zhu, R. Wu, C. Huang, and C. Zhong. 2017. Curcumin attenuates BPA-induced insulin resistance in HepG2 cells through suppression of JNK/p38 pathways. *Toxicol.Lett.* 272:75-83. doi: 10.1016/j.toxlet.2017.03.011.

Hata, S., J. Hirayama, H. Kajiho, K. Nakagawa, Y. Hata, T. Katada, M. Furutani-Seiki, and H. Nishina. 2012. A novel acetylation cycle of transcription co-activator Yes-associated protein that is downstream of Hippo pathway is triggered in response to SN2 alkylating agents. *J.Biol.Chem.* 287:22089-22098. doi: 10.1074/jbc.M111.334714.

Jiang, N., W. Li, S. Jiang, M. Xie, and R. Liu. 2023. Acetylation in pathogenesis: Revealing emerging mechanisms and therapeutic prospects. *Biomedicine & Pharmacotherapy.* 167:115519. doi: 10.1016/j.biopha.2023.115519.

Li, P., L. Ding, S. Cao, X. Feng, Q. Zhang, Y. Chen, N. Zhang, and F. Qiu. 2020. Curcumin metabolites contribute to the effect of curcumin on ameliorating insulin sensitivity in high-glucose-induced insulin-resistant HepG2 cells. *J.Ethnopharmacol.* 259:113015. doi: 10.1016/j.jep.2020.113015.

Peng, J., B. Ramatchandirin, Y. Wang, A. Pearah, K. Namachivayam, R.M. Wolf, K. Steele, K. MohanKumar, L. Yu, S. Guo, M.F. White, A. Maheshwari, and L. He. 2022. The P300 acetyltransferase inhibitor C646 promotes membrane translocation of insulin receptor protein substrate and interaction with the insulin receptor. *J.Biol.Chem.* 298. doi: 10.1016/j.jbc.2022.101621.

Sheng, X., Y. Zhang, Z. Gong, C. Huang, and Y.Q. Zang. 2008. Improved Insulin Resistance and Lipid Metabolism by Cinnamon Extract through Activation of Peroxisome Proliferator-Activated Receptors. *PPAR Research.* 2008:581348. doi: 10.1155/2008/581348.

Lin, Z.; Guo, H.; Cao, Y.; Zohrabian, S.; Zhou, P.; Ma, Q.; VanDusen, N.; Guo, Y.; Zhang, J.; Stevens, S.M.; et al. 2016. Acetylation of VGLL4 Regulates Hippo-YAP Signaling and Postnatal Cardiac Growth. *Dev. Cell.* 39, 466–479.

Cao, J., J. Peng, H. An, Q. He, T. Boronina, S. Guo, M.F. White, P.A. Cole, and L. He. 2017. Endotoxemia-mediated activation of acetyltransferase P300 impairs insulin signaling in obesity. *Nat Commun.* 8. doi: 10.1038/s41467-017-00163-w.

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