Assessed Molecular Mechanism of Action of Milagro de la Selva

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Results

1. *Muscle - C2C12 cells.*

1.1. Glucose uptake, Glycolysis and Tricarboxylic acid cycle (TCA).

Glucose uptake by muscle and its metabolism is important for reducing the glucose level in the serum. Treatment of C2C12 cells with ***Milagro de la Selva*** does not enhance the expression of the main glucose transporter GLUT4. As the expression of most of the transcription factors of GLUT4, like MEF2D, KLF15 is slightly downregulated, the expression of GLUT4 also is reduced. GLUT4is endocytosed and is recycled, by vesicle-mediated transport, to the surface in face of glucose overload.In response to treatment with ***Milagro de la Selva***, expression of proteins associated with vesicle-mediated transport like VAMP2, VAPA and VAPB is reduced suggesting that GLUT4 recycling to the surface is not actively taking place. It therefore appears that glucose uptake is not specifically activated by ***Milagro de la Selva***. There is also no significant increase in theexpression of enzymes involved in Glycolysis and Tri-Carboxylic Acid (TCA) cycle.

1.2. Glycogen synthesis and Glycogenolysis

Glucose is converted to glycogen and stored in muscle. The two main enzymes involved in the process are Glycogen Synthase (GYS) and branching enzyme (GBE1). In response to ***Milagro de la Selva*** treatment the expression of both the enzymes is reduced and as such glycogen synthesis is not specifically enhanced in muscle cells. There appears to be no differential regulation of genes involved in the process of glycogenolysis.

1.3. Mitochondrial biogenesis and Oxidative-phosphorylation.

In mitochondrial biogenesis, a gene expression cascade that includes, among others, PPARGC-1 (peroxisome proliferator activated receptor gamma coactivator-1), its target genes NRF-1 (Nuclear Respiratory Factor 1) and their target genes-TFAM (mitochondrial transcription factor), cytochrome C oxidase etc. results in increase in number and size of mitochondria that enhances the oxidative capacity of the muscle fibres. But in response to ***Milagro de la Selva*** the expression of many of the transocription factors involved in mitochondrial biogenesis like CAMK4, PPARGC-1, ESSRA, TFAM, etc is reduced. Also many of the enzymes participating in oxidative phosphorylation like cytochrome C oxidase and NDUFs show a reduced expression. It appears that the important process of mitochondrial biogenesis and oxidative phosphorylation is not enhanced by ***Milagro de la Selva*** .

1.4. Fatty Acid Transport and Oxidation

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Fatty Acids in circulation are taken up by muscle cells and oxidized to obtain energy and this represents an important mechanism to counter obesity and triglyceridemia. The transcription of the different SLC27A transporters and of CD36 is not affected by ***Milagro de la Selva***. Similarly most of the enzymes involved in actual fatty acid oxidation process are not differentially regulated. Importantly the expression of 2 main transcription factors PPAR alpha and PPAR delta is reduced. It appears that the process of fatty acid transport and oxidation in muscle is not activated by treatment with ***Milagro de la Selva***.

1.5. Insulin Receptor signaling.

The growth hormone insulin signals through its receptor and associated proteins leading to the activation of Protein Kinase B (Akt) and controls numerous cellular processes. ***Milagro de la Selva*** increases transcription of Insulin Receptor

Substrates IRS1 and 2 and of the regulatory subunit of PI3 kinase while expression of Akt is reduced. It appears that insulin signaling is not affected by
Milagro de la Selva.

2 Adipose Tissue . 3T3L1 cells.

Adipose tissue is the body.s largest energy reservoir with the primary role to store triacylglycerol during periods of excess calorie and to mobilize the reserve when expenditure exceeds intake. The fat cells, more importantly, secrete many members of the cytokine family like TNFα (Tumor Necrosis Factor Alpha), IL-6 (Interleukin-6) and other adipokines that have endocrine, paracrine and autocrine activity that influence peripheral fuel storage, mobilization and combustion, energy homeostasis and peripheral insulin sensitivity in the organism. The targets of these mediators now seem to include immune and inflammatory systems.

2.1. Glucose uptake and Glycolysis

Treatment of 3T3L1 cells with ***Milagro de la Selva*** does not appear to enhance either the process of glucose uptake or of glycolysis. There is no increase in transcription of either the glucose transporter GLUT4 or of the many proteins involved in vesicle mediated GLUT4 translocation to the plasma membrane. In fact

the expression of some of these proteins like VAMP2, KIF3A, STX1A and TRIP10 is educed upon ***Milagro de la Selva*** treatment. The expression of PARD3, an inhibitor of protein kinase C isoforms Zeta (PRKCZ) and Eata (PRKCI), is increased suggesting that the kinases are not activated and so the process of
GLUT4 translocation to the plasma membrane and consequently glucose transport is inhibited. The transcription of hexosekinase, the first enzyme in glycolysis, is reduced and so is the process of glycolysis.

2.2. Fatty acid transport, synthesis and triacylglycerol formation.

Adipocytes take up fatty acids in circulation, transport and incorporate into triacylglycerol (TAG) and this function enhances insulin sensitivity in the periphery. Adipocytes can also perform de novo fatty acid synthesis. Treatment of 3T3L1 cells with ***Milagro de la Selva*** does not enhance transcription and expression of any of the fatty acid transporters but increases expression of SCD1, which increases fatty acid synthesis, and of DGAT1 which is involved in TAG formation. ***Milagro de la Selva*** also enhances expression of PLIN, a protein that coats the lipid droplets and prevents access to Lipase and thus prevents TAG breakdown. Further the expression of Lipase is also reduced. It therefore appears that ***Milagro de la Selva*** enhances TAG formation in adipose tissue and thus increases insulin sensitivity in the periphery as a result of a reduction in circulating fatty acid level.

2.3. Fatty acid Oxidation and thermogenesis.

Enhanced fatty acid oxidation and thermogenesis in adipose tissue can protect against obesity. While PPARGC-1 determines the level of fatty acid oxidation, the expression of UCP 1 (uncoupling protein 1) enhances thermogenesis.Fatty acid oxidation involves transport of fatty acids into mitochondria, through 2 transporters

CPT1(Carnitine Pamitoyl Transferase) and CPT2 present in the outer and inner mitochondrial membranes respectively. CPT1 is inhibited by malonyl CoA synthesized by Acetyl CoA Carboxylase B (ACACB). Treatment of 3T3L1 cells with ***Milagro de la Selva*** enhances expression of ACACB and reduces expression of CPT1 and CPT2. It appears that the process of fatty acid oxidation is not increased in adipocytes by treatment with ***Milagro de la Selva***. The expression of UCP1 increases release of energy as heat as against synthesis of ATP and thus protects against obesity. Treatment with ***Milagro de la Selva*** also does not increase expression of UCP1 and so there is no change in thermogenesis compared to wild type.

2.4. Proliferation of Adipocytes.

Adipocyte proliferation is an important step in the increase in adipose tissue mass and the adipocytes also produce factors that can influence neovascularization of adipose tissue. Proliferation is controlled by an interplay of factors that either help progression or arrest of cell cycle and factors that contribute to the process of apoptosis. The effect of ***Milagro de la Selva*** a on adipocytes is difficult to comprehend. On the one hand there is an increase in the expression of cell cycle inhibitor like CDKN1B and decrease in expression of proteins like CCND1, CDK4, E2F that allow cell cycle progression indicating that there is inhibition of cell division. On the other hand there is increase in expression of proteins like APAF1, CASP3 and CASP9 that enhance apoptosis. Treatment with ***Milagro de la Selva*** a also increases expression of VEGF, vascular endothelial growth factor, that helps vascular tissue growth which in turn is essential for adipose tissue growth.

3. Liver- HepG2 cells

Liver is a major target for insulin action, contributing to energy storage in the fed state by regulating catabolic and anabolic pathways. Insulin decreases gluconeogenesis enzyme mRNAs and increases lipogenic enzyme mRNA expression. Liver is the organ where metabolism of xenobiotic compounds at times leads to severe toxicity.

3.1. Insulin sensitivity

Treatment of HepG2 cells with ***Milagro de la Selva*** enhances expression of insulin receptor and of insulin receptor substrate 4 which can improve insulin signaling. At the same time there is a reduction in expression of MAPK8,9 and PRKCQ- kinases that serine phosphorylate insulin receptor and its substrates and cause insulin resistance. Expression of PTPN1, PTEN, INPPL1, SNF1K2 and TCEB1- phsophatases which can dephosphorylate tyrosine residues and terminate insulin signaling- is reduced thereby enhancing insulin sensitivity.

3.2. Glycogen metabolism

Treatment with ***Milagro de la Selva*** up regulates AGL, GBE1, GYS2 and UPG2- enzymes that are involved in synthesis of glycogen, indicating that glycogen synthesis as a process is enhanced in liver. Simultaneously expression of GSK3beta, PHKA2 and PHKG2- enzymes that promote glycogenolysis is reduced.

3.3. Fatty acid metabolism.

Treatment of HepG2 cells with ***Milagro de la Selva*** increases expression of proteins NR1H3, NR1H2 and SREBP1 that are transcription factors for FASN, ELOVL5 and FADS2-enzymes involved in fatty acid synthesis. But the effect on fatty acid oxidation is not very clear. Expression of some of the enzymes like ACADM, CPT1A and CROT is increased while that of CPT2 and ECHS1 is decreased. Also there is a reduction in expression of peroxisomal genes ECH1, HSD17B4 and SCP2, indicating that the process of peroxisomal fatty acid oxidation is down regulated.

3.4. Cholesterol metabolism.

Treatment with ***Milagro de la Selva*** reduces expression of HMGCR, MVD, FDFT1, LSS, IDI1 and SQLE- enzymes that are involved in cholesterol synthesis. The effect of ***Milagro de la Selva*** could be similar to that of statins. ***Milagro de la Selva*** also reduces expression of mitochondrial enzymes IDH2, IDH3A and NNT that are involved in generation of NADPH which is required for cholesterol synthesis. Further expression of genes involved in steroidogenesis like CYP11A1 and SRD5A1 is reduced. Not only synthesis but the degradation of cholesterol is also increased by treatment with ***Milagro de la Selva*** suggesting that it is a potent inhibitor of atherosclerosis. Genes involved in degradation of cholesterol- CYP7A1, CYP7B1 and AKR1C2 and CYP39A1 are up regulated.

3.5. Lipoprotein metabolism

High-density lipoprotein (HDL) and its major apolipoprotein A1 are inversely correlated with the incidence of atherosclerotic cardiovascular disease and low serum levels of HDL and ApoA1 is seen

in type 2 Diabetes Mellitus patients. Treatment of HepG2 cells with ***Milagro de la Selva*** enhances expression of ApoA1, A2, C1, C2 and ApoE- proteins that are required for HDL maturation. ApoC3 is an apolipoprotein that inhibits Lipoprotein lipase (LPL) and prevents uptake of fatty acids from VLDL/LDL particles by cells in the periphery. This results in increase in serum triglyceride concentration. ***Milagro de la Selva*** reduces expression of ApoC3 in liver and thus reduces its concentration in VLDL/LDL particles. The net effect is reduction of hypertriglyceridemia and the risk of atherosclerosis.

3.6. Gluconeogenesis

The blood glucose level is kept within tight limits owing to the absolute dependence of brain on glucose for its energy needs. The liver and to some extent kidney can convert three carboncompounds to glucose by the process of gluconeogenesis and release it into the blood stream. The expression of three genes viz Phosphoenolpyruvate kinase (PEPCK), Fructose 1.6 bisphosphatase (FBP) and Glucose-6-phosphatase (G6PC) are essential for the process of gluconeogenesis to occur. The catabolism of aminoacids generates the carbon skeleton that can be converted to glucose and the ammonia released in the process activates the urea cycle which again produces fumarate which can be converted to glucose. ***Milagro de la Selva*** reduces expression HAL, AGXT, TAT and GLUD1 which catabolize amino acids and ASL and ARG1 which are involved in the urea cycle. The activity of CEBPA, an important transcription factor of the urea cycle, is also reduced by enhanced expression of its inhibitors- DDIT3, TCERG1 and CALR. One of the important transcription factor for the gluconeogenic enzymes is 11HSDB1 which produces the glucocorticoid ligands to activate the glucocorticoid receptor.

Milagro de la Selva specifically reduces expresión of 11HSDB1 and thus inhibits gluconeogenesis. Another mechanism by which gluconeogenesis is inhibited is by reducing expression of pyruvate carboxylase that synthesizes oxaloacetate a substrate for gluconeogenesis. Glucose output from the liver is thus prevented by multiple mechanisms.

3.7. Energy metabolism.

Milagro de la Selva seems to enhance expression of genes that increase the cells. ATP and NADH level. Enzymes of the glycolysis and TCA cycle- ALDOA, ACO1, CS, GCK, PFKFB3, PFKL, PKLR and PGAM1 are upregulated. By increasing expression of ME1, G6PD and PGD increased NADPH is formed. Similarly increase in transcription of ATP5A1, COX6A1 and COX7A2 oxidative phosphorylation and ATP generation are enhanced. ***Milagro de la Selva*** reduces expression of ADK, enzyme that converts ATP to AMP. Surprisingly ***Milagro de la Selva*** decreases expression of FRAP1, EIF4E, RPS6AK3 etc and reduces protein synthesis in the cells.

4. Pancreas- Beta-TC6 cells

The β-cells of the pancreatic islets of Langerhans sense glucose directly via its metabolism and respond by secretion of insulin. Type 2 diabetes is characterized by reduced β-cell insulin stores.

4.1. Insulin gene transcription.

Insulin gene transcription is the first event necessary for insulin synthesis and secretion. ***Milagro de la Selva*** has no effect on insulin gene transcription probably because some of the transcription
factors like NEUROD1, FOXA2, CALM, and PAX4 are down regulated. C20orf67, a factor that inhibits insulin gene transcription, shows enhanced expression.

4.2. Insulin secretion.

Pancreatic β-cells sense glucose directly via its metabolism and secrete insulin from storage granules through the regulated secretory pathway. Metabolism of glucose and other nutrient secretogogues within β-cells results in increase in ATP levels and closure of the ATP-sensitive K_+ channel. This closure leads to depolarization of the cell and influx of $Ca2_+$ through voltage-sensitive channels and results in insulin secretion. ***Milagro de la Selva*** does not increase the transcription of the glucose transporter GLUT2 or of the enzymes involved in glycolysis and TCA cycle indicating that there is no enhancement of metabolism that generates ATP. Infact F0F1ATPases and cytochrome C oxidases are down regulated and expression of UCP2, which reduces cellular ATP level, is increased. The expression of CPT1 and CPT2, which control the entry of fatty acyl CoA into mitochondria, is reduced indicating the there may be no increase in the level of ATP inside the cell. The consequence of reduced metabolism will be a decrease in insulin secretion.

High intracellular level of Ca2+ is essential for the process of insulin secretion. Increase in intracellular Ca2+ can come from increased Ca2+ entry and release from endoplasmic reticulum. The G protein coupled receptor, GNAS, signals through

Adenylate Cyclase (ADCY) for synthesis of cAMP which actives protein kinase A (PRKA) and the subsequent processes lead to an increase in intracellular Ca2+. Treatment with ***Milagro de la Selva*** causes a small increase in expression of GNAS, ADCY and even 3 subunits of the calcium channels. It is the phosphorylation status of protein kinase A and of its target proteins that can confirm if the CAMP/PRKA signaling mechanism is activated in ***Milagro de la Selva*** treated pancreas.

Insulin exocytosis follows vesicle-mediated transport to the cell-membrane and the granules are then release into the blood. SNAP 25 and STX1A are membrane-associated vesicle proteins that fuse with VAMP2, present on the vesicle, and thus help in regulated transport of insulin granules. DNAJC5 is a chaperone which helps proper folding of proinsulin in the endoplasmic reticulum. A few other proteins as Osteopontin and Neuronatin enhance insulin secretion. In beta-TC6 cells, the transcription of STX1a, SNAP25, DNAJC5, VAMP2, Osteopontin and Neuronatin is decreased in response to treatment with ***Milagro de la Selva*** and insulin secretion is not enhanced.

4.3. Apoptosis.

Eukaryotic cells undergo .programmed cell death. or .apoptosis. in response to certain stimuli. From the increased expression of some of the proapoptotic proteins such as Caspase 1,2, 6 and 8, TRADD, TRAF6 and APAF1, it appears that ***Milagro de la Selva*** treatment could be preventing apoptosis in a small was in beta-cells. This aspect would need to be confirmed with additional biochemical tests.

4.5. Proliferation.

There is no transcriptional evidence for an increase in beta-cell replication being enhanced by treatment with ***Milagro de la Selva***.

5. Summary.

Following is the summary of the anti-diabetic action of ***Milagro de la Selva***;

a. ***Milagro de la Selva*** appears to enhance the process of triacylglycerol formation and inhibits lipolysis in adipose tissue. The combined effect is to reduce free fatty acids in circulation and thus enhance insulin sensitivity in the periphery.

b. ***Milagro de la Selva*** has the potential to reduce hyperglycemia by the following actions in liver; - by enhancing the process of glycogen synthesis. - by inhibiting the process of glycogenolysis and gluconeogenesis.

c. ***Milagro de la Selva*** is an excellent anti-atherosclerotic agent as it increases cholesterol degradation and HDL formation while at the same time inhibiting cholesterol synthesis. These actions are beneficial to the T2DM patients.