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Identifying proteins and metabolic pathways associated to the neuroprotective response mediated by tibolone in astrocytes under an induced inflammatory model.

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Bogotá, Colombia
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Resumen

En este trabajo, se identifican las proteínas y rutas metabólicas asociadas a la respuesta neuroprotectora mediada por el neuroesteroide sintético tibolona bajo un modelo inflamatorio inducido por palmitato usando análisis de balance de flujo (FBA). Para tal fin, se modelaron tres diferentes escenarios metabólicos ('saludable', 'inflamado' y 'medicado') bajo una reconstrucción metabólica tejido-específica de astrocitos maduros construida a partir de datos de expresión génica. La reconstrucción metabólica se construyó, validó y limitó usando tres paquetes de software ('minval', 'g2f' y 'exp2flux') liberados a través de los repositorios R CRAN durante el desarrollo de este trabajo. A partir de nuestros análisis, predecimos que la tibolona ejecuta sus acciones neuroprotectoras a través de la reducción de la neurotoxicidad mediada por el L-glutamato en astrocitos, induciendo la activación de varias rutas metabólicas con funciones neuroprotectoras asociadas como: Metabolismo de taurina, síntesis de ácidos biliares, gluconeogénesis y rutas de señalización PPAR y mediadas por calcio. Adicional a esto, encontramos un aumento en la tasa de crecimiento asociado a la tibolona que podría estar relacionado con efectos secundarios reportados para los compuestos esteroideos en otros tipos celulares humanos.

Palabras clave: Astrocitos, Tibolona, Neuroprotección, Inflamación, Análisis de Balance de Flujo.

Abstract

In this work, proteins and metabolic pathways associated to the neuroprotective response mediated by the synthetic neuro-steroid tibolone under a palmitate induced inflammatory model were identified by flux balance analysis (FBA). Three different metabolic scenarios ('healthy', 'inflamed' and 'medicated') were modelled over a gene expression data driven constructed tissue-specific metabolic reconstruction of mature astrocytes. Astrocyte reconstruction was built, validated and constrained using three open source software packages ('minval', 'g2f' and 'exp2flux') released through CRAN R repositories during the development of this work. From our analysis, we predict that tibolone execute their neuroprotective effects through a reduction of neurotoxicity mediated by L-glutamate in astrocytes, inducing the activation several metabolic pathways with neuroprotective actions associated such as taurine metabolism, bile acid biosynthesis, gluconeogenesis, calcium and PPAR signaling pathways. Also we found a tibolone associated increase in growth rate probably in concordance to previously reported side effects of steroid compounds in other human cell types.

Keywords: Astrocytes, Tibolone, Neuroprotection, Inflammation, Flux Balance Analysis.

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Objectives

Main:

Identify proteins and metabolic pathways involved in the neuroprotective effects of tibolone in human astrocytes based in metabolic scenarios comparison.

Specific

- Build a tissue specific computational model of astrocytes metabolism using gene expression data integration.
- Evaluate the effects caused by the increase of free fatty acids and tibolone presence in astrocytes metabolism.
- Determine metabolic pathways and relevant functional products in response to steroid tibolone through systems biology approximations.
- Evaluate the importance of proteins and metabolic pathways previously identified on the dynamics of the metabolic model.

1 Exploring the neuroprotective effects of tibolone during astrocytic metabolic inflammation: a flux balance analysis approach

Written by: *Daniel Osorio, Janneth Gonzalez and Andrés Pinzón-Velasco*

Abstract:

Inflammation is a complex biological response to injuries, metabolic disorders or infections and its dysregulation induce many complex diseases through astrocytic dysfunction. The increase of free saturated fatty acid produce a metabolic inflammation response, generally associated with the induction of diverse intracellular stresses, such as mitochondrial oxidative stress, endoplasmic reticulum stress, and autophagy defects. Astrocytes respond to inflammation through a complex reaction called astrogliosis. During astrogliosis, glial cells generally associated to several beneficial activities in the CNS, also act as a source of inflammatory mediators and as generators of ROS that have the potential to damage neurons. In search of compounds with neuroprotective effects that imitate the neuroprotective actions of estereoids without their perjudicial side effects; the syntetic neurosteroid Tibolone was identified. Tibolone acts as a estrogen receptor modulator (SERM), a selective tissue estrogenic activity regulator (STEAR) and has been shown neuroprotective effects in cultured and under ischemia injury rat neurons. Nevertheless, actually is not well know the effects of tibolone over glial cells that allows its neuroprotective action. In this work, we model and simulate the metabolic inflammation response in mature astrocytes through Flux Balance Analysis (FBA), and explore the neuroprotective effects of tibolone under the inflamed state. We focused in identification of changes in metabolic pathways activation, functional products, gliotransmitter release and the neuroprotective effects mediated by tibolone over inflamed scenario. The generated network, consisted of 1262 genes encoding for enzymes performing 2747 reactions distributed across eight compartments, which was studied using constrained-based modeling approach to recreate three different scenarios in mature astrocytes (healthy, inflamed and medicated), and validated with available experimental

evidences. From our analysis, we predict that tibolone execute their neuroprotective effects through a reduction of neurotoxicity mediated by L-glutamate in astrocytes. We also found a tibolone associated increase in growth rate probably in concordance to previously reported side effects of steroids in other human cell types.

1.1. Introduction

Astrocyte-Neuron Metabolic Relationships

Astrocytes are the most abundant cells in the human brain and play important roles in the central nervous system (CNS) [1]. They are highly associated to several homeostatic functions such as glutamate, ion, and water homeostasis, energy storage in the form of glycogen, synapse formation and remodeling, defense against oxidative stress, scar formation, tissue repair and modulation of synaptic activity via the release of gliotransmitters [2]. Astrocytes metabolize glucose in anaerobic way to produce lactate, which is released to neurons through monocarboxylate transporters [3]. Lactate is used in neurons as an energy substrate after its conversion to pyruvate and subsequently to ATP via oxidative phosphorylation [4]. Astrocytes play an important role in glutamate mediated synaptic activity [5]; according to the astrocyte–neuron lactate shuttle model, astrocytes respond to glutamate induced activation by increasing their rate of glucose uptake and the release of lactate into the extracellular space, increasing the lactate available to be used by neurons to supply their energetic needs [6]. Glutamate is uptaked by astrocytes through the glutamate aspartate transporter and glial glutamate transporter-1, inducing events that involves the activation of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ and maintaining extracellular glutamate at homeostatic levels [7]. Part of incorporated glutamate is converted to glutamine through glutamine synthetase, which is only associated to glial cells and released to neurons using electroneutral systems-N transporters coupled to Na^+ and H^+ [8]. In neurons glutaminase enzyme converts glutamine back into glutamate which can be used again for neurotransmission or metabolized into the neuronal Krebs cycle [9]. Astrocytes uptake and release many other substances related to synaptic transmission [10]. However D-serine, a neurotransmitter that act as a coagonist with glutamate at NMDA receptors is one of the most important [5]. Due in brain only glial cells can synthesize serine, all available D-serine at synapsis is associated to be primarily produced and secreted by astrocytes [8]. D-serine is synthesized in astrocytes by serine racemase from L-serine [11]. Serine and glycine are involved in a cycle between astrocytes and neurons similar to the glutamate-glutamine cycle [12]. Additionally to these energy and synaptic support associated functions, astrocytes also play an important role in the reduced glutathione (GSH) metabolism of the brain [13]. GSH is the major cellular antioxidant and plays an important neuroprotective role [14]. Cellular GSH levels are closely correlated with cell survival under adverse conditions [15]; it is synthesized from glutamate, cysteine, and glycine and release directly from astrocytes through GSH transporters ion-independent in a concentration-gradient dependent transport

[16]. This strong metabolic cooperation between astrocytes and neurons allows to predict that even an small astrocytic dysfunction might cause and/or contribute neurodegenerative processes [17]. Homeostatic astrocyte function is required for neuronal survival after different brain insults, such as inflammation, glucose deprivation, traumatic brain injury and ischemia [14, 18]. Astrocytes protect neurons of the most important factors that contribute to neuronal cell death such as glutamate-mediated excitotoxicity leading to disturbances in calcium and sodium intracellular metabolism, mitochondrial dysfunction, oxidative stress, cytokines and toxins [1, 2, 7, 19].

Astrocytes response to Inflammation

Inflammation is a complex biological response to injuries, metabolic disorders or infections and its dysregulation induce many complex diseases through astrocytic dysfunction [14, 20, 21]. In brain, inflammatory response acts as a defense mechanism against any threat to homeostatic state inducing changes in glucose metabolism and release of pro-inflammatory factors [15]. Inflammation responses in CNS are mediated by glial cells that acquire reactive phenotypes to participate in repair mechanisms [1, 14, 22]. Astrocytes, as glial cells are highly sensitive cells to inflammatory mediators, they respond to inflammation through a complex reaction named astrogliosis [23]. During astrogliosis, glial cells generally associated to several beneficial activities in the CNS, also act as a source of inflammatory mediators and as generators of reactive oxidant species (ROS) that have the potential to damage neurons [24]. Astrogliosis is characterized by a low regulation of mitochondrial dynamics that result in mitochondrial failure [25]. Mitochondrial failure induces the deregulation of Ca^{2+} homeostasis and increased ROS generation, both of which are linked to neurotoxicity [2]. At metabolic level, inflammatory process has been associated to an increase of free saturated fatty acid in comparison with healthy conditions in some brain tissues [26]. The increase of free saturated fatty acid induce metabolic inflammation, a response associated with the induction of diverse intracellular stresses, such as mitochondrial oxidative stress, endoplasmic reticulum stress, and autophagy defects [14]. Lipid excess in metabolic inflammation activates $\text{IKK}\beta$ and $\text{NF-}\kappa\beta$ signaling pathways, which ultimately impairs leptin and insulin hormonal signaling and further triggers the synthesis and release of increased amounts of ROS and pro-inflammatory cytokines ($\text{TNF-}\alpha$ and IL-6) from glial cells to sustain the neuroinflammatory state [27]. Enhanced ROS generation by reactive glial cells trigger mitochondria dysfunction in neuron, which induces neuronal apoptosis, the prerequisite for a diverse number of neurodegenerative conditions [28].

Systems Biology and Inflammation

Inflammatory pathways are evolutionarily conserved, complex, redundant and interconnected [29]. These characteristics difficult each attempt to understand any disease having inflam-

mation at its core using the traditional reductionism-based scientific method and the current regulatory framework [30]. Traditional methods generally focus on single molecules and genes as the targets of study and potential therapy development, nevertheless mechanistic simulation through a translational systems biology methods allows lead to an understanding of the origin of patterns based in omic data integration in order to facilitate the design of novel therapies [31]. Inflammation is a complex system, which is characterized by sensitivity to initial conditions, positive and negative feedback loops, combined robustness and fragility, and emergence of nonintuitive behaviors [32]. Translational Systems Biology to inflammation is focused on simulated clinical trials, trying to progress toward personalized diagnostics, personalized medicine, and the rational design of drugs [29].

Tibolone

Drugs as steroids compounds are the most potent and effective agents in controlling chronic inflammatory diseases [33]. However, steroids prescription is limited due their adverse side effects [34]. Some steroids synthesized in the nervous system, called ‘neurosteroids’, display beneficial neuroprotective properties, which may be of particular importance in the treatment of diseases where inflammation and neurodegeneration is predominant including age-dependent dementia, stroke, epilepsy, spinal cord injury, Alzheimer’s disease (AD) and Parkinson’s disease (PD) [35]. Neuroprotective actions of molecules that may imitate the neuroprotective actions of estereoids without the perjudicial side effects, such as selective estrogen receptor modulators (SERMs) and selective tissue estrogenic activity regulators (STEARs) have been tested in previous studies [36, 37]. Tibolone is one of these compounds with SERMs and STEARs activities, traditionally used as hormone replacement therapy in post-menopausal women [38]. Tibolone has been shown neuroprotective effects in cultured and under ischemia injury rat neurons [39]. Tibolone is a synthetic steroid drug with estrogenic, progestogenic, and weak androgenic actions; is metabolized in three compounds, two major active metabolites, 3α -hydroxytibolone and 3β -hydroxytibolone acting as potent agonists of the estrogen receptor (ER) and its metabolite $\Delta 4$ tibolone acting as agonists of the progesterone and androgen receptors [40]. Tibolone and their metabolites have tissue selective action mechanisms (progestogenic, androgenic and estrogenic) reported in liver, bone, breast and brain according to receptor interaction and activation [36]. Nevertheless, actually is not well know the effects of tibolone over glial cells that allows its neuroprotective effects [18]. Previous studies have shown that 3-hydroxy-metabolites of tibolone exert agonistic actions on human astrocytes through the activation of estrogen receptors, indicating that astrocytes are a target for tibolone [39].

In this work we simulate the metabolic inflammatory response in mature astrocytes caused by the increase uptake of palmitate, the most common free saturated fatty acid. We model and simulate the metabolic response using a translational system biology approach called Flux Balance Analysis (FBA) described in methods. We focused in identification of chan-

ges in metabolic pathways activation, functional products, gliotransmitter release and the neuroprotective effects mediated by tibolone in the inflamed scenario.

1.2. Material and Methods

Tissue Specific Model Construction

The tissue specific model construction process started with the identification of all enzyme-coding genes expressed over the mean in at least 50 % of samples for healthy human astrocytes indexed in the GEO database [41] as GSE73721 [42]. Gene identifiers conversion from GeneCards[43] to ENTREZ [44] was performed through ‘UniProt.ws’ R Package [45]. Reactions associated with the identified genes were mapped from the Human Genome Scale Metabolic Reconstruction RECON 2.04 downloaded from the VMH Lab (<https://vmh.uni.lu>) [46]. The R package ‘g2f’ [47] was used to identify and fill the gaps using all no gene associated reactions included in RECON 2.04, as well as to identify and remove all blocked reactions from the reconstruction. All reactions involved in the conversion of extracellular glutamate, glycine, cysteine and glucose to extracellular glutamine, glycine, serine-D, reduced glutathione, lactate and ATP respectively were added. Exchange reactions were limited to components of the Dulbecco’s Modified Eagle Medium (DMEM) as input and gliotransmitters (glutamine, D-serine, ATP, glutamate), reduced glutathione, lactate, glucose, nitric oxide, prostaglandins and leukotrienes as output. Finally, syntax, mass-charge validation and creation of SBML files were carried out through the ‘minval’ R Package [48]. Reaction limits (upper and lower bounds) were constrained proportional to the mean gene expression reported for genes included in Gene-Protein-Reaction (GPR) [49] associated to each reaction in samples of 47 to 63 years old using the ‘exp2flux’ R package [50]. All Flux Balance Analysis (FBA) were performed using the ‘sybil’ [51] R Package running under R 3.3.1 [52].

Flux Balance Analysis

FBA is a linear optimization method for simulating metabolism that allows to identify the set of reactions involved in the production of a biological response within a metabolic model [53]. The metabolic reactions are represented internally as a stoichiometric matrix (S), of size $m * n$, where m represents the compounds and n the reactions; the entries in the matrix are the stoichiometric coefficients of the metabolites participating in a reaction [54]. The flux through all of the reactions in a network is represented by the vector v , which has a length of n . The concentrations of all metabolites are represented by the vector x , with length m . The systems of mass balance equations at steady state, $\frac{d_x}{d_t} = 0$ or $S * v = 0$. FBA seeks to maximize or minimize an objective function which can be any linear combination fluxes, to obtain a flux for each reaction, indicating how much each reaction contributes to the objective function [53]. FBA for healthy, inflamed and medicated scenarios was resolved

using GLPK 4.60, setting the generic human biomass reaction included in RECON 2.04 and each one of reactions described in table **1-1** as objective functions. Models were analyzed by comparing fluxes between scenarios, metabolites production rate and a sensitivity analysis.

Table 1-1: Main metabolic capabilities associated to astrocytes represented as the set of objective functions used to evaluate neuroprotective effects of Tibolone under inflamed scenarios

ID	FORMULA REACTION	DESCRIPTION
Glu2Gln	1 glu_L[e] \Rightarrow 1 gln_L[e]	Glutamate - Glutamine Cycle
Gly2SerD	1 gly[e] \Rightarrow 1 ser_D[e]	Glycine to D-serine conversion
Glc2Lac	1 glc_D[e] \Rightarrow 2 lac_L[e]	Lactate production from Glucose
Glc2ATP	1 glc_D[e] \Rightarrow 36 atp[e]	ATP production from Glucose
Cys2GTHRD	1 cys_L[e] + 1 glu_L[c] + 1 gly[c] \Rightarrow 1 gthrd[e]	Catch of Cysteine to produce reduced Glutathione

Metabolic Scenarios

To test neuroprotective effects of tibolone during astrocytic metabolic inflammation we define three different metabolic scenarios. A ‘healthy’ scenario, where palmitate uptake rate was freely set by optimizer; an ‘inflamed’ scenario, where uptake rate of palmitate was forced to be stable in the mean of the half maximal inhibitory concentration (IC50) value for all objective functions included in table **1-1**. IC50 values were calculated through a robustness analysis performed using uptake of palmitate (‘EX_hdca(e)’ in RECON 2.04) as control reaction and a 1000 points in the range from 0 to 1 mMgDW⁻¹h⁻¹ for each objective function. Uptake value where each objective function reached IC50 was selected and subsequently averaged. Finally, a medicated scenario, defined as an inflamed scenario that include 279 reactions associated with estradiol-derived compounds and ten specific reactions associated to Tibolone action mechanism non included in RECON 2.04 described in table **1-2**.

Metabolic Changes

Metabolic changes across metabolic scenarios were measured through two different approximations. Flux differences for each reaction between optimized scenarios were measured using the fold change calculated as described in equation 1-1.

$$foldChange = \frac{valueModel2 - valueModel1}{|valueModel1|} \quad (1-1)$$

Table 1-2: Set of reactions added to recreate the medicated scenario model over the astrocyte tissue-specific model. Reactions are the representation of tibolone metabolism in brain following the reported by Kloosterboer (2004).

ID	FORMULA REACTION	DESCRIPTION
T1	tibolone[e] \Leftrightarrow	Tibolone exchange reaction
T2	tibolone[e] \Leftrightarrow a3OHTibolone[e]	3 α hydroxytibolone interconversion
T3	tibolone[e] \Leftrightarrow b3OHTibolone[e]	3 β hydroxytibolone interconversion
T4	tibolone[e] \Rightarrow d4tibolone[e]	Δ^4 tibolone isomer formation
T5	b3OHTibolone[e] \Rightarrow d4tibolone[e]	Δ^4 tibolone isomer formation from 3 β -hydroxytibolone
T6	a3OHTibolone[e] \Rightarrow estradiol[c]	Estradiol receptor agonist action mechanism of 3 α -hydroxytibolone
T7	b3OHTibolone[e] \Rightarrow estradiol[c]	Estradiol receptor agonist action mechanism of 3 β -hydroxytibolone
T8	d4tibolone[e] \Rightarrow prgstn[c] + tststerone[c]	Progesterone and androgen receptor activation by tibolone Δ^4 isomer
T9	a3OHTibolone[e] \Leftrightarrow a3SOTibolone[e]	3 α hydroxytibolone interconversion to sulfated inactive compounds
T10	a3SOTibolone[e] \Rightarrow	Tibolone inactive form in blood

Pro-inflammatory, Anti-inflammatory and Tibolone Action Mechanism Associated Enzymes

Identification of enzymes involved in pro-inflammatory and anti-inflammatory responses as well as in the tibolone action mechanism were identified through several sensitivity analysis as follows: Pro-inflammatory enzymes, are those that catalyze reactions that being knocked out allows an increase of objective function value. Antiinflammatory enzymes, are those associated to reactions that have a fold-change greatest equal to 2, and at being knocked out reduce even more the objective function value. Tibolone action mechanism associated enzymes are those that catalyze reactions that being knocked out inhibit entirely the metabolic effect of tibolone.

1.3. Results

All data, code, software and output files used in the develop of this work, are available to be downloaded from github URL: <https://github.com/dosorio/masterThesis> as a free repository.

Tissue Specific Metabolic Model

Generated astrocyte tissue-specific model describe the metabolism of 1956 compounds in a total of 2747 biochemical reactions associated to 1262 unique genes. Biochemical reactions include 60 exchange and 1080 transport reactions (79 % gene associated, facilitated or active transport) as is shown in figure 1-1A.

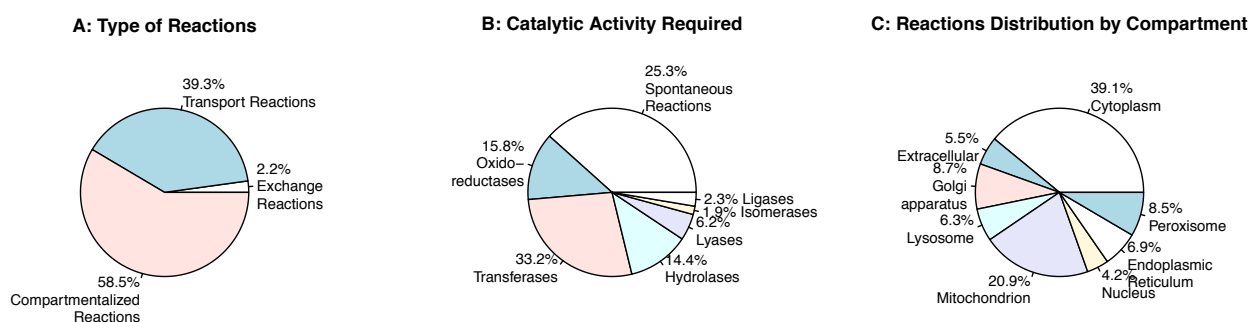


Figure 1-1: Distribution of biochemical reactions included in the astrocyte tissue-specific metabolic model, classification based in **A:** Type of reaction, **B:** Catalytic activity required and **C:** Associated compartment.

To describe the astrocyte tissue specific metabolic model, reactions were classified on the basis of required enzymatic activity to be catalyzed according to their Enzyme Commission (E.C) numbers (Fig. 1-1B), sub-cellular locations according to metabolites compartment (Fig. 1-1C), and metabolic pathways assigned in the KEGG database (Fig. 1-2). Based in the associated enzyme to each biochemical reaction 33.2 % of them are catalyzed by a transferase enzyme, 15.8 % by an oxidoreductase, 14.4 % by a hydrolase, 6.2 % by a lyase, 2.3 % by a ligase, 1.9 % by an isomerase enzyme and 25.3 % of them are spontaneous reactions without enzyme or gene associated. In the classification shown in figure 1-1C, the cytosolic and mitochondrial reactions contributed to 60 % of the total reactions in the model. The other 40 % of reactions are distributed in six other compartments as follows: 8.7 % occurs in golgi apparatus, 8.5 % in peroxisome, 6.9 % in the endoplasmic reticulum, 6.3 % in the lysosome, 4.2 % in nucleus, finally 5.5 % of them occurs outside the cell, in the extracellular space. Reactions included in astrocyte model are associated to 113 metabolic pathways reported in the KEGG database [55]. Almost 50 % reactions are associated to 10 main metabolic pathways, highly related to astrocytes metabolism and neuron support metabolic functions [6, 12, 56–59]. Entirely distribution of reactions in metabolic pathways is shown in figure 1-2.

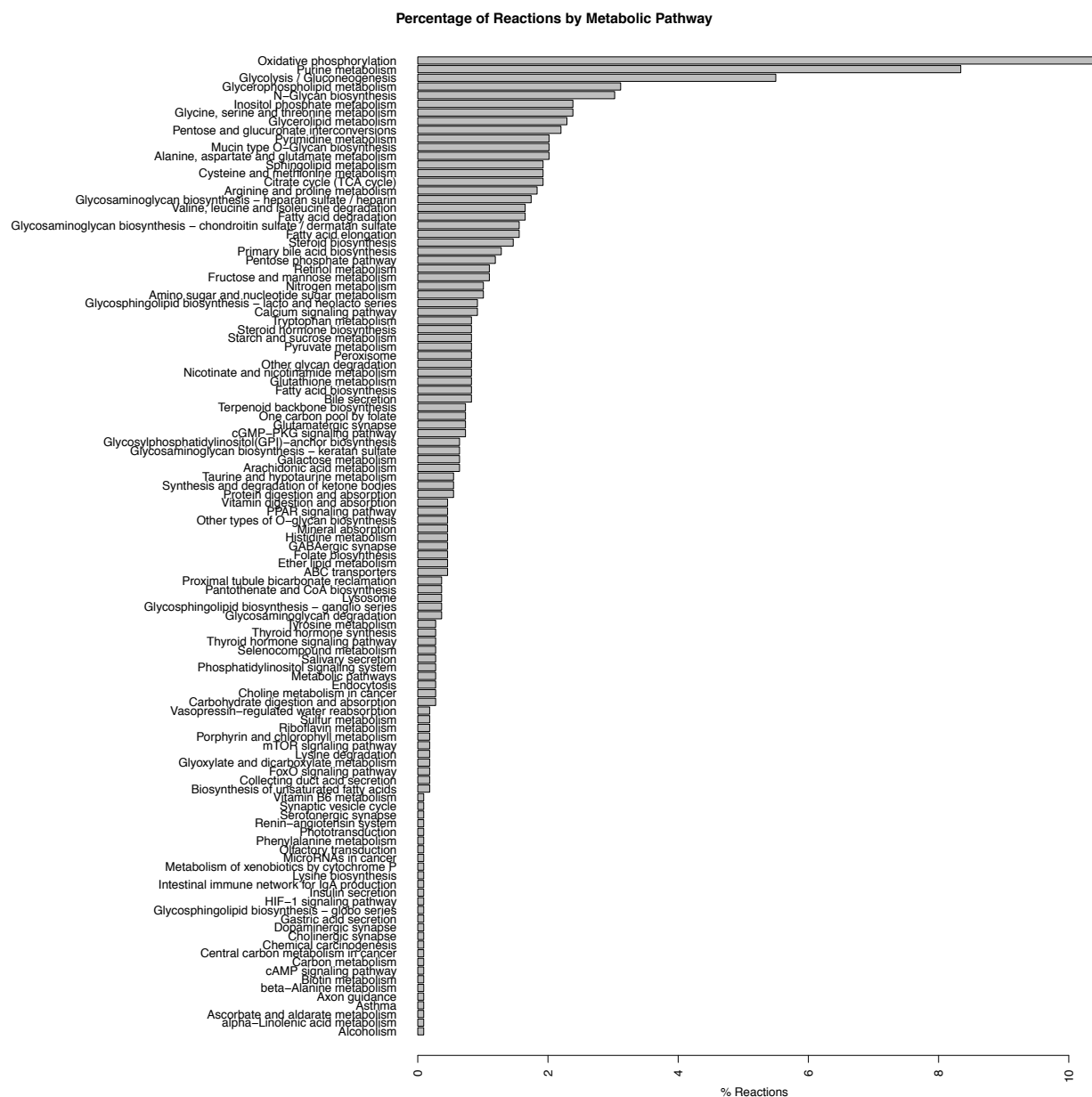


Figure 1-2: Pathways associated to biochemical reactions included in the astrocyte tissue-specific metabolic model. Pathway association was assigned based in the categorization of the KEGG database.

Healthy Scenario

As previously reported by the Das *et al.* wet lab, healthy human astrocytes grow up in DMEM culture medium [60]. Our metabolic simulation allow to predict an astrocytes slow grow rate ($0.37 \text{ mMgWD}^{-1}\text{h}^{-1}$) under DMEM medium. In our healthy scenario (Fig. 1-3), astrocytes activates the 52% of model reactions and prefer a glucose based metabolism, equal than found by Çakir *et al.* and Bhowmick *et al.* in resting conditions [12, 61]. Glucose is catabolized and constitutively released by astrocytes as lactate without any stimuli [62]. This observation is highly expected due astrocytes release large amounts of lactate in the extracellular space which can be used by neurons to supply their energy needs [15]. In our simulations, other gliotransmitters are synthetized and released by astrocytes only under specific stimulus (objective functions) and their release rate was used as reference to comparison between scenarios (Fig. 1-6).

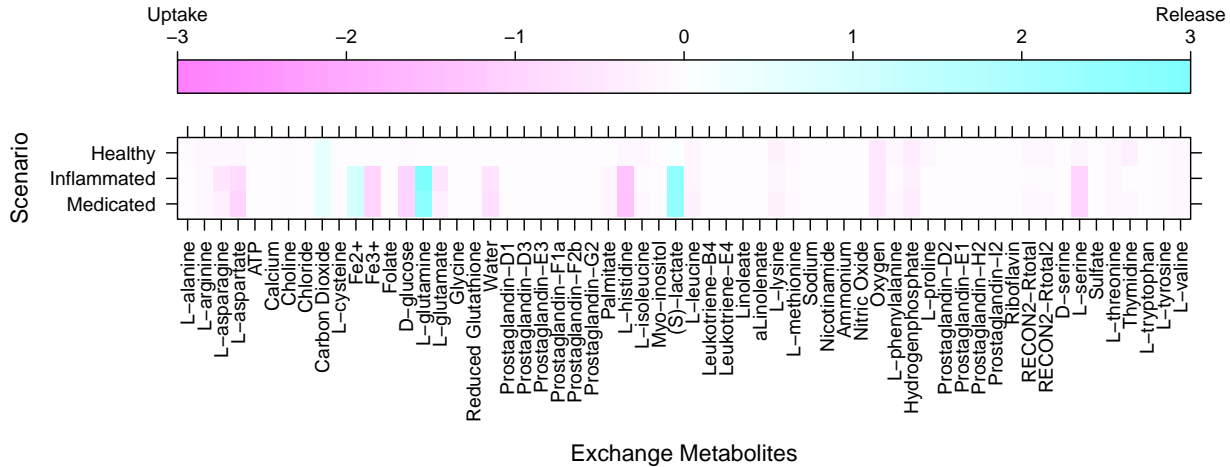


Figure 1-3: Exchange rate of metabolites between metabolic scenarios using the generic biomass reaction included in RECON 2.04 as objective function.

Inflamed Scenario

In the model, the healthy scenario was perturbed to generate the inflamed scenario. The palmitate induced IC50 value calculated for a set of metabolic functions (Table 1-1) was $0.208 \pm 0.024 \text{ mMgDW}^{-1}\text{h}^{-1}$. Calculated IC50 value is the same (0.2 mM) used by Liu *et al.* in wet lab to induce an astrogliosis reaction [63]. Inflamed scenario increases the demand of L-asparagine, L-aspartate, iron, D-glucose, L-glutamate, histidine, L-serine and the release of L-glutamine and lactate (Fig. 1-3). These response is typical of astrocytes in astrogliosis where neuroinflammation lead homeostatic disturbances [64], such as iron

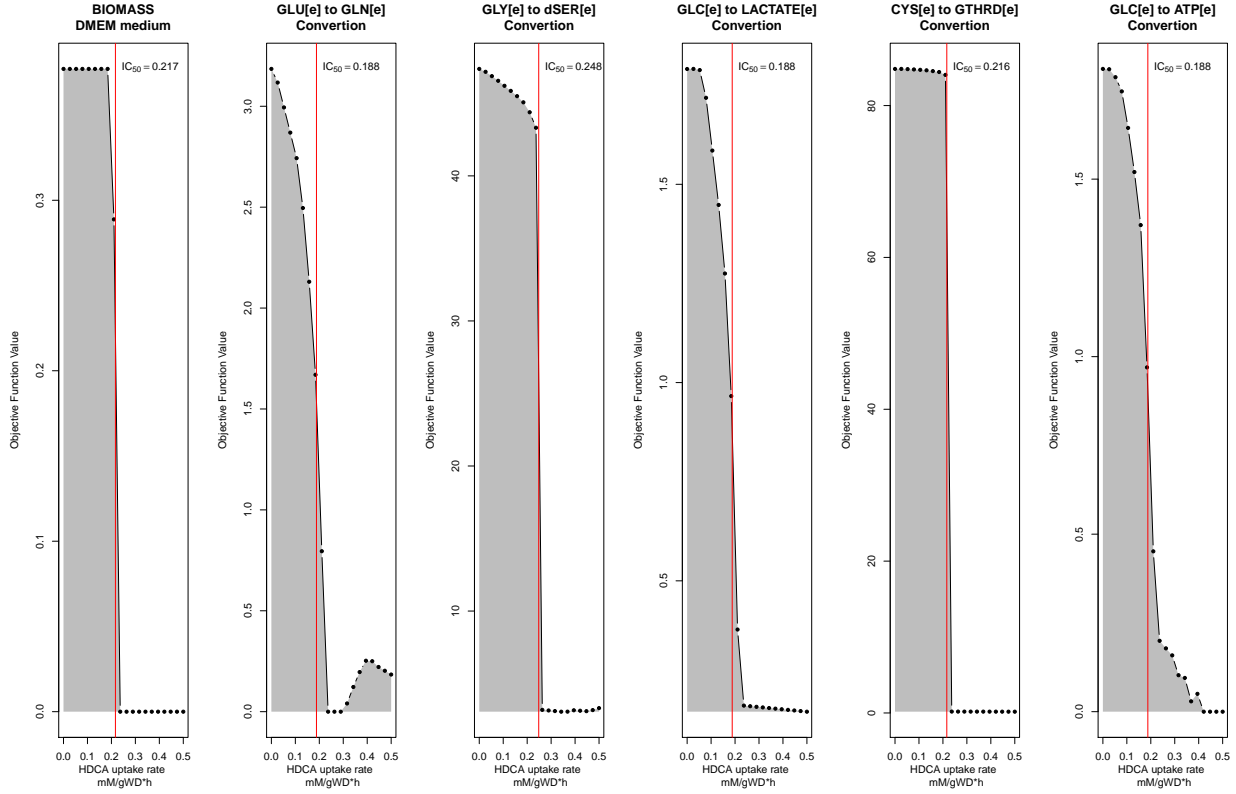


Figure 1-4: Robutness analysis to calculate palmitate induced IC50 value for each objective function described in table 1-1. Red line represents the calculated IC50 value.

accumulation, within CNS cells [14]. Iron accumulation has been demonstrated in several neurodegenerative diseases as AD, and PD, where it has been postulated to promote disease by augmenting microglial pro-inflammatory activity, altering mitochondrial function, and inducing ROS production [65]. Inflammation although induce neuronal release of glutamate that may result in the recruitment of neurons in the neuroinflammatory process [66]. Glutamate uptake into astrocytes disinhibits glycolytic enzymes that results in glucose uptake; this glucose is generally processed glycolytically, leading to the synthesis and release of lactate [14]. As neurons cannot generate glutamine from glutamate owing to the lack of the glutamine synthetase enzyme, uptaken glutamate is returned to neurons via synaptic clefts in the form of glutamine [67]. Histidine uptake increase was previously reported and suggested as a biomarker of metabolic inflammation [68]; it acts as a free-radicals scavenger and could reduce the levels of IL-6, TNF- α , CRP and inhibit the H₂O₂⁻ and TNF- α induced by IL-8 secretion [69, 70]. Aspartate, present in brain as N-Acetyl-L-aspartate (NAA) is synthesized and stored in the neurons but is hydrolyzed in glial cells [71]. NAA act as an anti-proliferation, anti-angiogenic, and anti-inflammatory molecule through the decrease of the amount of prostaglandin E2 (PGE2) in astroglial cells [72]. L-Asparagine in turn acts as a

regulator of ammonia toxicity through the increase of Na^+ intracellular concentration when is cotransported inside astrocytes [73]; asparagine induce a Ca^{2+} response comparable to GABA-induced Ca^{2+} transients in a dose-dependent manner [74]. L-serine and L-asparagine uptake increase may be related with a cell survival process that switch cellular metabolism to be highly dependent of nonessential amino acids available in extracellular space such as glutamine, serine, glycine, arginine, and asparagine [75]. Moreover, under inflamed scenario, our astrocyte model release a very small amount of prostaglandin D2. The release of this prostaglandin was previously associated to induce the depolarisation and potentiate the actions of simultaneously applied transmitters such as GABA, taurine, glutamate, and aspartate in astrocytes [76].

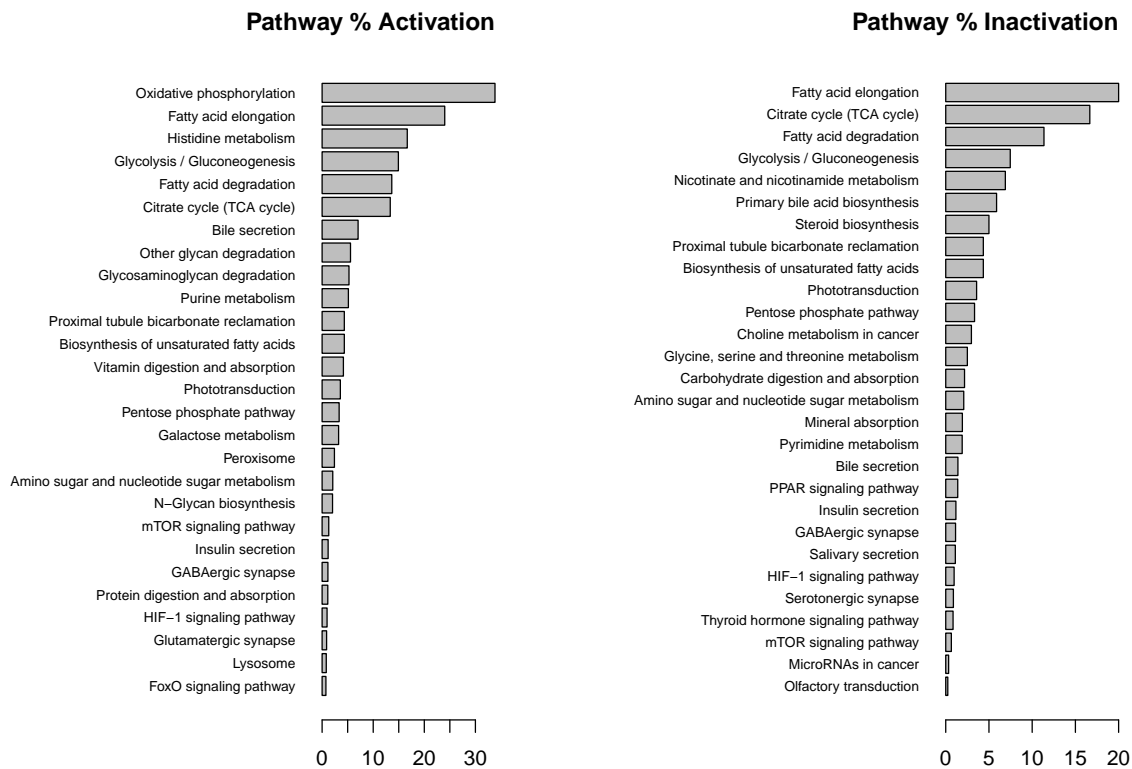


Figure 1-5: Metabolic pathways affected by metabolic inflammation. Percentage of activation and inactivation was calculated in comparison with genes associated to each pathway in the KEGG database.

In our inflamed scenario, astrocytes activates the 46.6 % of model (5.6 % less than healthy scenario) and affect the biomass flux rate of 586 reactions in comparison with healthy scenario. Main metabolic changes occurs in the activation of oxidative phosphorylation, histidine metabolism and fatty acid degradation pathways; as well as an inactivation of TCA and glycolysis pathways (Fig. 1-5). Inflammation affects in a negative way all metabolic objective

functions evaluated except the release of D-serine. In comparison to the healthy scenario, growth rate over DMEM medium decrease in a 15.6 %, the catch of cysteine to produce reduced glutathione in a 59.3 %, conversion of glucose to ATP in a 72 %, and to lactate in a 74.4 %; finally, conversion of extracellular glutamate in glutamine reduces in a 67.7 % (Fig. 1-6).

Table 1-3: Set of reactions with pro-inflammatory potential identified through a sensibility analysis over inflamed scenario.

ID	REACTION DESCRIPTION	H. FLUX	I. FLUX	FOLD CHANGE
FTCD	Formimidoyltransferase cyclodeaminase	0.39	1.28	2.28
H2Otm	H2O transport mitochondrial	-0.26	2.44	10.44

Based in performed sensibility analysis, we identify two pro-inflammatory reactions candidate to be knocked out (Table 1-3), that when being blocked increases the value of the objective function above the maximum value set (in a 11.45 % and 5.14 % respectively) for inflamed scenario. Reactions are associated to the formimidoyltransferase cyclodeaminase (FTCD) enzyme and the Aquaporin-8, a water transport protein.

Table 1-4: Set of reactions with anti-inflammatory potential identified through a sensibility analysis over inflamed scenario.

ID	REACTION DESCRIPTION	H. FLUX	I. FLUX	FOLD CHANGE
AKGMALtm	α -ketoglutarate/malate transporter	-0.17	-1.3	-6.85
NADH2_u10m	NADH dehydrogenase mitochondrial	0.12	0.37	2.17
r0639	Lauroyl-CoA: acetyl-CoA C-acyltransferase.	0.02	0.09	4.04
r0653	cMyristoyl-CoA: acetyl-CoA C-myristoyl transferase	0.02	0.09	4.04
r0714	(S)-3-Hydroxyhexadecanoyl-CoA: NAD ⁺ oxidoreductase	0.02	0.09	4.04
r0716	(S)-3-Hydroxyhexadecanoyl-CoA hydrolyase	0.02	0.09	4.04
r0718	(S)-3-Hydroxytetradecanoyl-CoA: NAD ⁺ oxidoreductase	0.02	0.09	4.04
r0720	(S)-3-Hydroxytetradecanoyl-CoA hydrolyase	0.02	0.09	4.04

FTCD enzyme, previously reported as over-expressed in high fat diets [77], contribute with one-carbon units from histidine degradation to the folate pool [78]. In turn, the Aquaporin 8,

generally associated to ammonia and water transport [79], has been proposed as a biomarker for inflammation processes where was in contrary way to our observations, highly correlated to cellular defence against severe oxidative stress [80].

As well as pro-inflammatory reactions, eight anti-inflammatory reactions were identified. Identified reactions (Table 1-4) have a change between scenarios greatest equal to 2-fold and when being blocked decreased even more the value of objective function in comparison with healthy scenario. Majority of identified reactions (r0639, r0653, r0714, r0716, r0718 and r0720) are involved to the fatty acid elongation in mitochondria through acyl-CoA association [81]. The elongation system, is responsible for the addition of two carbon units to the carboxyl end of a fatty acid chain, and play an important role in the maintenance of membrane lipid composition as well as in the generation of precursors for cell signaling molecules (such as eicosanoids and sphingosine-1 phosphate), energy production, and other unknown pathways involving with cancer growth. [82].

Medicated Scenario

Inflammatory scenario with the 279 reactions associated with tibolone and estradiol-derived compounds metabolism, defined as our medicated scenario displays several neuroprotective effects. Medicated scenario increase the demand of L-aspartate and in turn decreases the demand of L-asparagine, L-glutamate and the release of L-glutamine in comparison to the inflamed scenario (Fig. 1-3).

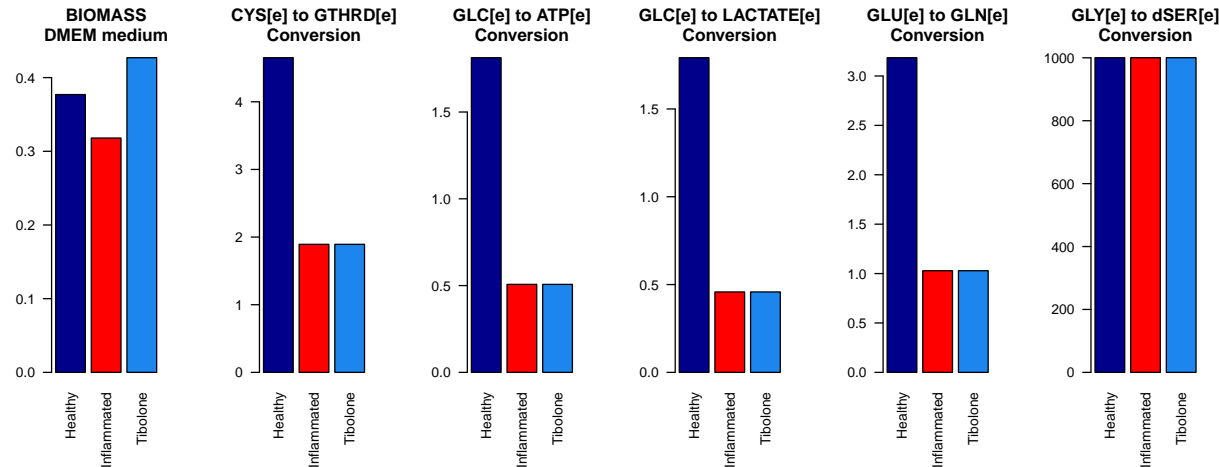


Figure 1-6: Response of the main astrocytes metabolic capabilities to different modelled scenarios.

The reduction of L-glutamate and L-glutamine uptake/release rate mediated by Tibolone effects could be associated to a neuroprotective effect through a reduction of neurotoxicity

mediated by L-glutamate in astrocytes [10]. L-glutamate is a contributing factor in neuronal damage induced by inflammation, traumatic brain injury, stroke, and in most of the chronic neurodegenerative diseases, such as PD and AD [83].

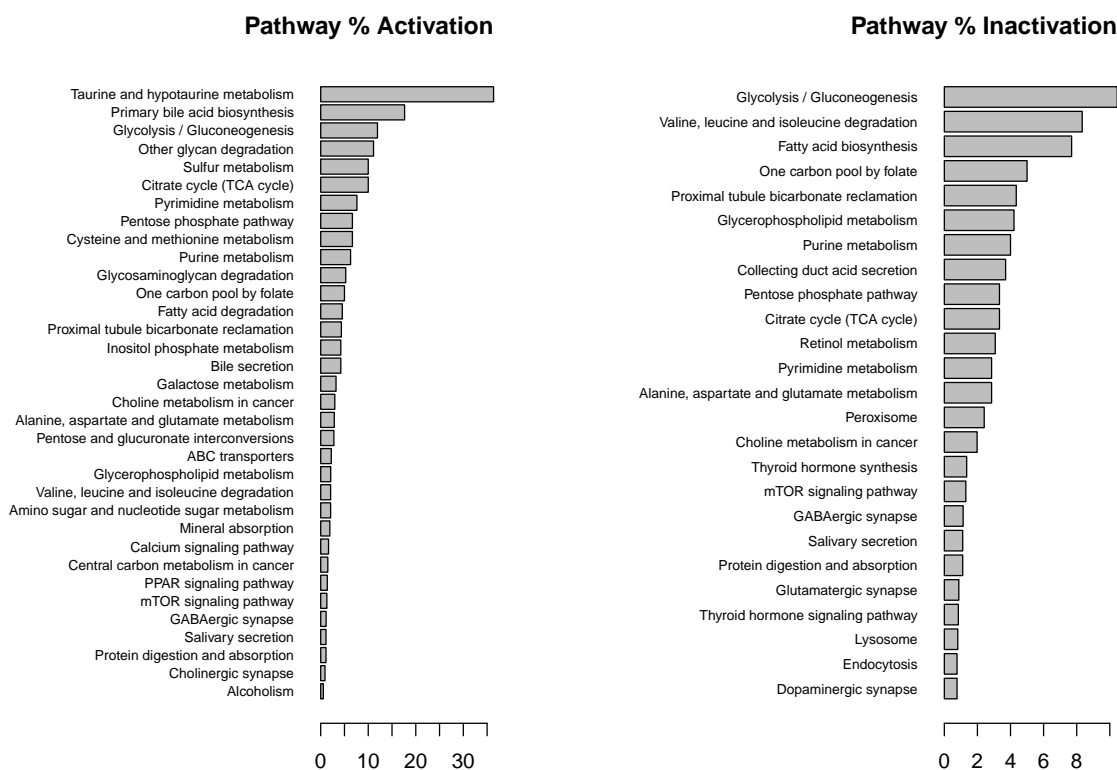


Figure 1-7: Metabolic pathways affected by tibolone effects over inflamed scenario. Activation and inactivation percentage was measured in comparison with genes associated to each pathway in the KEGG database.

In our medicated scenario, astrocytes activate 46.6 % of model, equal than in inflamed scenario. Nevertheless, tibolone effects affect the biomass flux rate of 948 reactions in comparison with the inflamed scenario; main metabolic changes occurs through the activation of several metabolic pathways with neuroprotective actions associated such as taurine metabolism, which has been shown to be tissue-protective in many models of oxidant-induced injury [84], bile acid biosynthesis, which traditionally has been associated to fatty acid metabolism in bile [85] but associated genes are expressed in astrocytes [42]; gluconeogenesis which is accelerated and facilitate the conversion of fatty acids into ketone bodies under steroid mediated effects [86], calcium and PPAR signaling pathways (Fig. 1-7). Tibolone do not show directly effect over the main associated neuron-supportive capabilities (Table 1-1) by affected by inflammation, however it shows a high related activity as potentiator of growth rate (Fig. 1-6). Increase of growth rate (13.26 % higher than in healthy scenario) could be

associated to an increase of cell viability or as an increase in the proliferative potential in cells [87]. Due that proliferative potential was not evidenced in inflamed scenario, this proliferative potential must not be associated to astrogliosis mediated proliferation in astrocytes [1]. However, it could be associated to proliferative side effect of steroid compounds as was previously reported in other cell types where tibolone was tested in wet lab [88, 89].

Table 1-5: Set of reactions associated to tibolone required to execute their neuroprotective effects. Reactions were identified through a sensibility analysis over the medicated scenario.

ID	REACTION DESCRIPTION	GENES IN ASTROCYTE DATA
r0739	Alcohol Dehydrogenase	ADH4, ADH5, ADH7
r2518	ATP-binding Cassette (ABC)	ABCD3
RE1804M	Cholestanetriol 26-monooxygenase	CYP27A1
RE1807M	Cholestanetriol 26-monooxygenase	CYP27A1

Based in performed sensibility analysis over the 289 reactions associated with tibolone and estradiol-derived compounds metabolism, we identify a set of four reactions that when being individually knocked out, block entirely the tibolone effects. Identified reactions are catalyzed by an alcohol dehydrogenase (E.C. 1.1.1.1) and a Cytochrome P450 associated to the PPAR signaling pathway. Both enzymes were previously reported with ROS reduction through redox reactions mediated by alcohol dehydrogenase (ADH) and posterior release associated to a cytochrome P450 [90, 91].

1.4. Conclusion

In this work, a tissue-specific metabolic network for mature astrocyte has been developed, and three different scenarios were modelled. Modelled scenarios allowed identify the metabolic changes between a healthy and a inflamed scenario as well as from an inflamed to a tibolone medicated scenario. Model was capable of yielding results which were in correspondence to the experimentally proved metabolic processes [60, 63]. From our study, adverse effects associated to the increase of palmitate uptake were described based in exchange, metabolite production and metabolic pathways perturbed under inflammatory response. Sensibility analysis performed through constrained-based modeling approach and FBA methods permitted recognize two possible reactions and their associated enzymes susceptible to be knocked out to reduce inflammatory processes. Based on literature reports we modelled a tibolone medicated scenario used to identify and describe the neuroprotective effects of this synthetic neurosteroid under an inflamed scenario in astrocytes; our main results suggest

that tibolone execute their neuroprotective effects through a reduction of neurotoxicity mediated by L-glutamate in astrocytes. L-glutamate [10]. We also found a tibolone associated increase in growth rate probably in concordance to previously reported side effects of steroids in other human cell types [88, 89]. Identified enzyme associated reactions with tibolone effects and their action mechanisms are highly consistent with reported previously by our associated wet lab [18, 92].

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