1 Neuroprotective effects of tibolone during astrocytic metabolic inflammation: a network based approach

Abstract:

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 convertion 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 Na^+–K^+-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 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 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]. Additionally to these energy and synaptic support associated functions, astrocytes also play an important role in the reduced glutathione (GSH) metabolism of the brain [12]. GSH is the major cellular antioxidant and plays an important neuroprotective role [13]. Cellular GSH levels are closely correlated with cell survival under adverse conditions [14]. GSH is synthesized from glutamate, cysteine, and glycine and release directly from Astrocytes through GSH transporters ion-independent, net transport is concentration-gradient dependent [15]. This strong metabolic cooperation between astrocytes and neurons allows to predict that even an small astrocytic dysfunction might cause and/or contribute neurodegenerative processes.

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 [13, 16, 17]. In brain, inflammatory response acts as a defense mechanism against any threat to homeostatic state inducing changes in glucose metabolism and release of proinflammatory factors [14]. Inflammation responses in CNS are mediated by glial cells that acquire reactive phenotypes to participate in repair mechanisms [1, 13, 18]. Astrocytes, as glial cells are highly sensitive cells to inflammatory mediators, they respond to inflammation through a complex reaction named astrogliosis [19]. During astroglyosis, 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 [20]. Astrogliosis is characterized by a low regulation of mitochondrial dynamics that result in mitochondrial failure [21]. 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 [22]. 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, ER stress, and autophagy defects [13]. Lipid excess in metabolic inflammation activates hypothalamic IKKβ and NF-$\kappa\beta$ signaling pathways, which ultimately impairs hypothalamic leptin and insulin signaling and further triggers the synthesis and release of increased amounts of ROS and proinflammatory cytokines (TNF-α and IL-6) from glial cells to sustain the neuroinflammatory state [23]. 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 [24].

Systems Biology and Inflammation

Inflammatory pathways are evolutionarily conserved, complex, redundant and interconnected. These characteristics difficult each attempt to understand any disease having inflammation at its core using the traditional reductionism-based scientific method and the current regulatory framework. 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 framework allows lead to an understanding of the origin of patterns in omic data in order to facilitate the design of novel therapies. 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. 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.

In this work we simulate the metabolic inflammatory response caused by the increase uptake of palmitate, the most common free saturated fatty acid in healthy mature astrocytes. We model and simulate the metabolic response using a translational system biology approach called Flux Balance Analysis (FBA) described in methods. This work focused in identification of changes in metabolic pathways activation, functional products, gliotransmitter release and the neuroprotective effects mediated by tibolone in an inflammated scenario.

Tibolone

Tibolone is a synthetic steroid drug with estrogenic, progestogenic, and weak androgenic actions. Tibolone 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 Δ4-tibolone acting as agonists of the progesterone and androgen receptors.

Estrogen neuroprotective effects are well known, but the use of estradiol for neuroprotective therapies is limited by its non desirable side effects [4,5]. Several laboratories have assessed the neuroprotective actions of molecules that may imitate the neuroprotective actions of estradiol without side effects of the hormone, such as selective estrogen receptor modulators (SERMs) and selective tissue estrogenic activity regulators (STEARs) [6,7]. Tibolone is used for the treatment of climacteric symptoms and osteoporosis in post-menopausal women [13–15]. Tibolone is considered a STEAR, since its estrogenic activity depends on its differential metabolism in each tissue, combined with the different affinity of its metabolites for hormone receptors and the different action of its metabolites on the inhibition of steroid sulphatase activity [10,11,16–18]. Brain tissue is a target for Tibolone, which exerts neuroprotective actions, reducing infarct volume, increasing Bcl-2 expression and acting as an anti-apoptotic agent in the brain of adult female Sprague-Dawley rats exposed to middle cerebral artery occlusion [19]. Several studies have explored the neuroprotective mechanisms

of Tibolone, showing that the drug increases antioxidant activity in primary neuronal cultures [21] and in the brain of adult female Wistar rats [22]. Little is known about the role of glial cells in the neuroprotective actions of Tibolone. However, previous studies have shown that 3-hydroxy-metabolites of Tibolone exert agonistic actions on human astrocytes through the activation of estrogen receptors [23], indicating that astrocytes are a target for Tibolone. Astrocytes play an important role in the maintenance of homeostasis in the central nervous system (CNS), regulating neuronal function and metabolism [24–27] and proper astrocyte function is fundamental for neuronal survival after different brain insults, such as glucose deprivation, traumatic brain injury and ischemia [25,27–33]. Astrocytes are known to participate in the neuroprotective mechanisms of estradiol [34] and the hormone protects primary astrocytes from metabolic insults, including oxygen glucose deprivation [1,35,36]. This protective action of estradiol on astrocytes may contribute to the protective mechanism of the hormone after stroke. Therefore, it is important to determine whether Tibolone, which is used for hormonal therapy in women, exerts similar protective actions on human astrocytes. In Tibolone possesses a complex pharmacology.[14] Its two major active metabolites, 3α-hydroxytibolone and 3β-hydroxytibolone, act as potent, fully activating agonists of the estrogen receptor (ER), with a high preference for ERα.[14][15][16] Tibolone ,[15] while 3α-hydroxytibolone and 3β-hydroxytibolone, conversely, act as antagonists of these receptors.[14] Lastly, tibolone, 3α-hydroxytibolone, and 3β-hydroxytibolone act as antagonists of the glucocorticoid and mineralocorticoid receptors, with preference for the mineralocorticoid receptor.[14]

Tibolone has tissue-selective estrogenic effects, with desirable effects in bone, the brain, and the vagina, and lack of undesirable action in the endometrium and breasts.[16] Its tissue selectivity is the result of metabolism, enzyme modulation (e.g., of estrogen sulfatase and estrogen sulfotransferase), and receptor modulation that vary in different target tissues, and differs mechanistically from that of selective estrogen receptor modulators (SERMs) such as tamoxifen, which produce their tissue-selectivity via means of modulation of the ER.[15][16] As such, to distinguish it from SERMs, tibolone has been described as a "selective tissue estrogenic activity regulator"(STEAR),[16] and also as a "selective estrogen enzyme modulator"(SEEM).[17]

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 [25] as GSE73721 [26]. Gene identificators convertion from GeneCards[27] to ENTREZ [28] was performed throught 'UniProt.ws' R Package [29]. Reactions associated with the identified genes were mapped from the Human Genome Scale Me-

tabolic Reconstruction RECON 2.04 downloaded from the VMH Lab (https://vmh.uni.lu) [30]. The R package 'g2f' [31] 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 [32]. Reaction limits (upper and lower bounds) were constrained proportional to the mean gene expression reported for genes included in Gene-Protein-Reaction (GPR) [33] associated to each reaction in samples of 47 to 63 years old using 'exp2flux' R package [34]. All analysis were done by the 'sybil' [35] R Package running under R 3.3.1 [36].

Flux Balance Analysis

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 [37]. 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 [38]. 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 [37]. FBA for healthy, inflammated 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 sensitivity analysis.

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 'inflammated' 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 robutness

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

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

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 $\mathrm{mMgDW^{-1}h^{-1}}$ for each objective function. Uptake value where each objective function reached IC50 was selected and subsequently averaged. Finally, a medicated scenario, defined as an inflammated scenario that include 279 reactions associated with tibolone and estradiol-derivated compounds metabolism. Ten specific reactions described in table **1-2** associated to specific Tibolone action mechanism non included in RECON 2.04 were added to medicated scenario.

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.

$$foldChange = \frac{valueModel2 - valueModel1}{|valueModel1|} \tag{1-1}$$

Additionally, to obtain a full perspective about inflammation effects in metabolites production, the production of each metabolite was set as objective function in each metabolic scenario and differences were evaluated as well as flux differences.

Proinflammatory, Antiinflammatory and Tibolone Action Mechanism Associated Enzymes

Identification of enzymes involved in proinflammatory and antiinflammatory responses as well as in the tibolone action mechanism were identified through several sensitivity analysis as follows: Proinflammatory enzymes, are those that catalyze reactions that being knocked out allows an increase of objective function value. Antiinflammatory enzymes, are those

Table 1-2: Set of reactions associated to tibolone specific action mechanism in brain reported by Kloosterboer, H. J. (2004) added to medicated scenario model.

ID	FORMULA REACTION	DESCRIPTION
T1	tibolone[e] $\Leftrightarrow$	Tibolone exchange reaction
T2	tibolone[e] $\Leftrightarrow$ a3OHtibolone[e]	3αhidroxytibolone interconvertion
T3	tibolone[e] $\Leftrightarrow$ b3OHtibolone[e]	3βhidroxytibolone interconvertion
T4	tibolone[e] $\Rightarrow$ d4tibolone[e]	Δ-4Tibolone isomer formation
T5	b3OHtibolone[e] $\Rightarrow$ d4tibolone[e]	Δ-4Tibolone isomer formation from 3β-hidroxytibolone
T6	a3OHtibolone[e] $\Rightarrow$ estradiol[c]	Estradiol receptor agonist action mechanism of 3α-hidroxytibolone
T7	b3OHtibolone[e] $\Rightarrow$ estradiol[c]	Estradiol receptor agonist action mechanism of 3β-hidroxytibolone
T8	d4tibolone[e] $\Rightarrow$ prgstrn[c] + tststerone[c]	Progesterone and androgen receptor activation by tibolone Δ^4 isomer
T9	a3OHtibolone[e] $\Leftrightarrow$ a3SOtibolone[e]	3αhidroxytibolone interconvertion to sulfated inactive compounds
T10	a3SOtibolone[e] $\Rightarrow$	Tibolone inactive form in blood

associated to reactions that 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

1.4. Conclusion

Bibliography

[1] Kazuhiro Takuma, Akemichi Baba, and Toshio Matsuda. Astrocyte apoptosis: Implications for neuroprotection. *Progress in Neurobiology*, 72:111–127, 2004.

[2] Sofie C. Lange, Lasse K. Bak, Helle S. Waagepetersen, Arne Schousboe, and Michael D. Norenberg. Primary cultures of astrocytes: Their value in understanding astrocytes in health and disease. *Neurochemical Research*, 37:2569–2588, 2012.

[3] Harold K Kimelberg. Functions of Mature Mammalian Astrocytes: A Current View. *The Neuroscientist*, 16(1):79–106, feb 2010.

[4] Nicola J Allen and Ben Barres. Neuroscience: Glia - more than just brain glue. *Nature*, 457(7230):675–677, 2009.

[5] Michael M Halassa and Philip G Haydon. Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. *Annual review of physiology*, 72(2):335–355, 2010.

[6] Christian Giaume, Annette Koulakoff, Lisa Roux, David Holcman, and Nathalie Rouach. Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nature reviews. Neuroscience*, 11(fEbRuARy):87–99, 2010.

[7] Cora H. Nijboer, Cobi J. Heijnen, Vincent Degos, Hanneke L M Willemen, Pierre Gressens, and Annemieke Kavelaars. Astrocyte GRK2 as a novel regulator of glutamate transport and brain damage. *Neurobiology of Disease*, 54:206–215, 2013.

[8] Ben Barres. The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron*, 60(3):430–40, nov 2008.

[9] Jun Shen. Modeling the glutamate-glutamine neurotransmitter cycle. *Frontiers in Neuroenergetics*, 5(JAN):1–13, 2013.

[10] Francesco Petrelli and Paola Bezzi. Novel insights into gliotransmitters. *Current Opinion in Pharmacology*, 26(Table 1):138–145, 2016.

[11] Andrea R Durrant and Uriel Heresco-levy. D-Serine in Neuropsychiatric Disorders : New Advances. 2014, 2014.

[12] Stephen P. Raps, James C K Lai, Leif Hertz, and Arthur J L Cooper. Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. *Brain Research*, 493(2):398–401, 1989.

[13] Mithilesh Kumar Jha, Dong Ho Park, Hyun Kook, In-Kyu Lee, Won-Ha Lee, and Kyoungho Suk. Metabolic Control of Glia-Mediated Neuroinflammation. *Current Alzheimer research*, 13(4):387–402, 2016.

[14] Igor Allaman, Mireille Bélanger, and Pierre J. Magistretti. Astrocyte–neuron metabolic relationships: for better and for worse. *Trends in Neurosciences*, 34(2):76–87, feb 2011.

[15] Xue Feng Wang and Max S. Cynader. Astrocytes provide cysteine to neurons by releasing glutathione. *Journal of Neurochemistry*, 74(4):1434–1442, 2000.

[16] Brent E Masel and Douglas S DeWitt. Traumatic brain injury: a disease process, not an event. *Journal of neurotrauma*, 27(8):1529–1540, 2010.

[17] Qing Yan. *Systems biology in drug discovery and development.* 2013.

[18] Michael T. Fitch and Jerry Silver. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Experimental Neurology*, 209(2):294–301, 2008.

[19] James a. Dowell, Jeffrey a. Johnson, and Lingjun Li. Identification of astrocyte secreted proteins with a combination of shotgun proteomics and bioinformatics. *Journal of Proteome Research*, 8(8):4135–4143, 2009.

[20] Anna V. Molofsk, Robert Krenick, Erik Ullian, Hui Hsin Tsai, Benjamin Deneen, William D. Richardson, Ben. Barres, and David H. Rowitch. Astrocytes and disease: A neurodevelopmental perspective. *Genes and Development*, 26:891–907, 2012.

[21] Marta Sidoryk-Wegrzynowicz and Michael Aschner. Role of astrocytes in manganese mediated neurotoxicity. *BMC pharmacology & toxicology*, 14:23, 2013.

[22] Sunita Gupta, Alecia G. Knight, Shruti Gupta, Jeffrey N. Keller, and Annadora J. Bruce-Keller. Saturated long-chain fatty acids activate inflammatory signaling in astrocytes. *Journal of Neurochemistry*, 120(6):1060–1071, 2012.

[23] Sudarshana Purkayastha and Dongsheng Cai. Neuroinflammatory basis of metabolic syndrome. *Molecular Metabolism*, 2(4):356–363, 2015.

[24] Suk K. Proteomics-based discovery of biomarkers and therapeutic targets in neurodegenerative diseases: Perspective of microglia and neuroinflammation. *Expert Opinion on Therapeutic Patents*, 16(3):237–247, 2006.

[25] Ron Edgar, Michael Domrachev, and Alex E Lash. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*, 30(1):207–210, 2002.

[26] Ye Zhang, Steven A. Sloan, Laura E. Clarke, Christine Caneda, Colton A. Plaza, Paul D. Blumenthal, Hannes Vogel, Gary K. Steinberg, Michael S B Edwards, Gordon Li, John A. Duncan, Samuel H. Cheshier, Lawrence M. Shuer, Edward F. Chang, Gerald A. Grant, Melanie G Hayden Gephart, and Ben A. Barres. Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron*, 89(1):37–53, 2016.

[27] Michael Rebhan, Vered Chalifa-Caspi, Jaime Prilusky, and Doron Lancet. GeneCards: integrating information about genes, proteins and diseases. *Trends in Genetics*, 13(4):163, 1997.

[28] Donna Maglott, Jim Ostell, Kim D Pruitt, and Tatiana Tatusova. Entrez Gene: gene-centered information at NCBI. *Nucleic acids research*, 33(suppl 1):D54—-D58, 2005.

[29] Marc Carlson. *UniProt.ws: R Interface to UniProt Web Services*, 2016.

[30] Ines Thiele, Neil Swainston, Ronan M T Fleming, Andreas Hoppe, Swagatika Sahoo, Maike K Aurich, Hulda Haraldsdottir, Monica L Mo, Ottar Rolfsson, Miranda D Stobbe, and Others. A community-driven global reconstruction of human metabolism. *Nature biotechnology*, 31(5):419–425, 2013.

[31] Kelly Botero, Daniel Osorio, Janneth Gonzalez, and Andres Pinzon. *g2f: Find and Fill Gaps in Metabolic Networks*, 2016.

[32] Daniel Osorio, Janneth Gonzalez, and Andres Pinzon. minval: MINimal VALidation for Stoichiometric Reactions, 2016.

[33] Ines Thiele and Bernhard Ø Palsson. A protocol for generating a high-quality genome-scale metabolic reconstruction. *Nature Protocols*, 5(1):93–121, jan 2010.

[34] Daniel Osorio, Kelly Botero, Janneth Gonzalez, and Andres Pinzon. *exp2flux: Convert Gene EXPression Data to FBA FLUXes*, 2016.

[35] Gabriel Gelius-dietrich, Abdelmoneim Amer Desouki, Claus Jonathan Fritzemeier, and Martin J Lercher. sybil – Efficient constraint-based modelling in R. 2013.

[36] R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2016.

[37] Jeffrey D Orth, Ines Thiele, and B O Palsson. What is flux balance analysis? *Nature Biotechnology*, 28(3):245–248, 2010.

290 [38] Karthik Raman and Nagasuma Chandra. Flux balance analysis of biological systems:
291 Applications and challenges. *Briefings in Bioinformatics*, 10(4):435–449, 2009.