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Succinate aggravates NAFLD progression to liver cancer on the onset of obesity: An *in silico* model

Indrani Ray, Abhijit Dasgupta and Rajat K. De*

Machine Intelligence Unit, Indian Statistical Institute
203, B. T. Road, Kolkata 700108, India
**rajat@isical.ac.in*

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The incidence and prevalence of nonalcoholic fatty liver disease (NAFLD) have been increasing to epidemic proportions around the world. NAFLD, a chronic liver disease that affects the nondrinkers, is mainly associated with steatohepatitis and cirrhosis. The progression of NAFLD associated with obesity increases the risk of liver cancer, a disease with poor outcomes and limited therapeutic options. In order to investigate the underlying cellular dynamics leading to NAFLD progression towards cancer on the onset of obesity, we have integrated human hepatocyte pathway with hypoxia-inducible factor1-α (HIF1-α) signaling pathway using state space model based on classical control theory. Modified Michaelis–Menten equation and mass action law have been used to define flux vectors of the proposed model. We have incorporated feedback inhibition/activation and allosteric effects into the simulink-based model. The values of kinetic constants have been taken from the literature. It is found that on the onset of obesity, HIF1-α-induced proteins stabilize approximately 62 times that in the case of a normal cell. Consequently, the HIF1-α-induced proteins enhance the enzymatic activities of hexokinase (HK), phosphofructo kinase (PFK), lactate dehydrogenase (LDH), and pyruvate dehydrogenase (PDH), which induce Warburg effect promoting an environment suitable for cancer cells.

Keywords: Obesity; succinate; NAFLD; liver cancer; state space; simulink.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), resulting from liver inflammation, has become a serious form of disease nowadays. It leads to scarring and irreversible damage. Similar type of damage can be found due to heavy alcohol consumption. It may start with chronic inflammation followed by replacing liver tissues with scar tissues. Consequently, NAFLD results in hepatic steatosis, steatohepatitis and cirrhosis,[2,26] and ultimately leads to liver cancer.[28] Being a prominent comorbid disease of obesity,[15] NAFLD is the most prevalent form of chronic liver disease nowadays. It arises when liver accumulates excess amount of fat due to genetic

reasons and/or habits except excessive alcohol consumption. The human liver consists of an interesting architectural organization that allows metabolic cells, i.e. hepatocytes, to be in close proximity to immune cells, i.e. Kupffer cells (stellate macrophages). Here, both the cells have an instant access to a huge network of blood vessels. Subsequently, hepatic tissues get privilege to a suitable environment for continuous and dynamic interactions between immune and metabolic responses.[13]

On the onset of obesity, the liver accumulates excess amount of fat. An evidence[6] suggests that free fatty acid (FFA) increases in the blood of obese individuals. Moreover, obesity alters the gut microbiome.[12,14,22] Besides, it replaces beneficiary bacteria with the harmful ones. This circumstance facilitates an enhancement in lipopolysaccharides (LPS) absorption through gut.[3,7] Here, the proportion of an LPS-containing microbiota in the gut increases on the onset of obesity.[3] In case of the mice, similar effects can be found during inducing metabolic endotoxemia for four weeks through continuous subcutaneous infusion of LPS and during feeding with high fat.[3] Specifically, the effect has been observed in the case of fasting glycemia, insulinemia, and weight gain of whole body as well as in the case of liver and adipose tissue.[3] Subsequently, LPS alters cellular pathways as well as activates macrophages to initiate inflammation. It blocks succinate dehydrogenase (SDH), an important enzyme of citric acid cycle (CAC). SDH is responsible to transform succinate into fumarate. As SDH is blocked on the onset of obesity, succinate accumulates in the cell. It is reported that succinate plays an important role in stabilizing hypoxia-inducible factor1-α (HIF1-α) transcription factor.[40] Besides, succinate stimulates dendritic cells via succinate receptor1 (SUCNR1). Furthermore, succinylation can perform post-translational modifications of proteins. Thus, succinate alters the immuno-metabolic homeostasis.[27]

In the context of the above discussion, we have considered two situations on the onset of obesity. First, the alteration of gut microbiome leads to the enhancement of LPS. As a result, LPS inhibits succinate to fumarate transformation in CAC. It causes accumulation of succinate in the cells. Second, succinate plays an important role in activating HIF1-α transcription factor. In this study, we aim to analyze how the dynamic behavior of a cell changes due to the two above-mentioned scenarios in an *in silico* model in integrated fashion.

Several mathematical models of human liver have already been developed with partially overlapping content. Among them, Recon 1 (a global human metabolic reconstruction)[37] including its final version Recon 2.2,[39] Edinburgh Human Metabolic Network (EHMN)[24] and Human Metabolic Atlas (HMA)[1] are considered to be well-known tools for analyzing human metabolism. Besides, manually curated cell-type specific reconstructions, including a part of the Virtual Liver Network (called HepatoNet1),[10] are also available. Moreover, Steatonet is another comprehensive reconstruction of human hepatocyte[31] to analyze the progression of NAFLD disease. The above-mentioned extensive models based on Flux Balance Analysis (FBA) have potentially contributed to investigate liver functions. However, in these processes, it is difficult to integrate metabolic pathways with both gene expression and signaling

networks.[11] Here, the robustness and genotype–phenotype correlation are notably compromised. Parameter estimation plays an important role in large computational models, since kinetic constants derived from *in vitro* experiments have been poorly documented till now. Dynamic models, based on numerical integration of ordinary differential equations (ODEs) for calculating reaction rates, enable one to predict the metabolite concentration and the reaction rates at a given time. There are several ODE-based hepatocyte metabolic models;[30] particularly, the model of gluconeogenesis and lipid metabolism in perfused liver.[5] Moreover, a previous investigation has shown the implications of glucose metabolism in type 2 diabetic individuals with rigorous insulin therapy.[20] However, the extensive use of higher order and complicated mathematical formulations makes the ODE-based models of biochemical pathways unsuitable for large-scale modeling.[9,33] Besides, we could not find a model that integrates human hepatocyte pathway with HIF1-α signaling pathway to analyze the effect of NAFLD progression under two above-mentioned situations.

In this study, we have developed a state space model to investigate how a normal hepatocyte cell becomes prone to cancer due to NAFLD progression on the onset of obesity and insulin resistance scenario associated with two phenomena mentioned earlier. We have integrated central carbon metabolic (CCM) pathways that include glycolysis, CAC and pentose phosphate pathways along with fatty acid β oxidation pathway, and HIF1-α, using state space model based on classical control theory. Here, each metabolite or signaling molecule has been considered as state components of the system. Subsequently, reaction rates (fluxes) form an output vector. The state vector is the product of stoichiometric matrix and flux vector. We have defined flux vectors using modified Michaelis–Menten equation[34] and mass action law. Here, we have included every possible perturbation, i.e. feedback inhibition/activation, and allosteric effects of different enzymes and hormones acting as catalyzer/inhibitor of the participating reactions in the integrated CCM and HIF1-α pathways. Following this process, the model has been simulated in the simulink environment of MATLAB. We have taken the values of kinetic constants from Refs. 9, 19 and 33.

As LPS inhibits the activity of SDH, we have blocked SDH during simulation. The result shows that succinate accumulates more compared to normal situation. Besides, the translation of HIF1-α-induced proteins enhances due to increased accumulation of succinate. HIF1-α is an important transcription factor that can determine the dynamics of both tumor and immune cells. The activation of the transcription factor, HIF1-α, contributes significantly to the alteration of cellular pathways. It leads to increase of the activities of hexokinase (HK), phosphofructo kinase (PFK), and lactate dehydrogenase (LDH).[25] On the other hand, HIF1-α inhibits the translation of pyruvate dehydrogenase (PDH).[18] As a result, oxidative phosphorylation slows down. The situation resembles "Warburg effect". Our result confirms the above-mentioned claims.

In summary, we have analyzed the altered dynamics of integrated CCM and HIF1-α signaling pathways based on the known knowledge, i.e. high LPS absorption along with succinate accumulation due to NAFLD progression on the onset of

obesity and alteration of HIF1-α-induced protein translation. Although previous investigations[28,38] suggest that NAFLD progression may lead to liver cancer, the underlying cellular dynamics is still not reported. Our simulation results show that on the onset of obesity, NAFLD progression may cause "Warburg effect", an ideal environment for tumorous cells to become malignant.

2. Method

In order to model the CCM pathway, fatty acid and glycogen synthesis pathways of human hepatocyte along with HIF1-α signaling pathway, we have considered Michaelis–Menten kinetics, Mass action law, Stoichiometric matrix, Nonlinear state space model, and Simulink (described in the supplementary material). The modified Michaelis–Menten equation, formulated in our previous investigation,[34] is used here to include feedback inhibition/activation in terms of the feedback constant F. In HIF1-α signaling pathway model, mass action law and modified equations[4,32] have been used. Thus, the steps of the methodology are described below.

(1) Feedback inhibition/activation is a natural phenomenon for any biochemical pathway. Thus, every possible feedback inhibition/activation as well as the allosteric effect of different enzymes and hormones play an important role to control the dynamics of integrated CCM and HIF1-α pathways. In this context, we have used a modified Michaelis–Menten equation, developed in our previous investigation,[34] to include every possible perturbation into our model. Here, feedback constant F has been used to determine the relative strength of feedback inhibition/activation. The value (in [0, 1]) of F can be chosen randomly. Higher the value of F, the stronger is the effect of inhibition or activation. Different choices of feedback constant values have not altered the dynamic behavior of the biological networks. However, the steady state values have been changed.
Here, we have considered a hypothetical pathway depicted in Fig. S1 of supplementary material. The hypothetical pathways show that the molecule D accelerates the reaction $A \to B$, whereas the molecule B inhibits the reaction $C \to D$. The modified–Michaelis–Menten equation for the reaction $C \to D$ is

$$V_{C\to D} = \frac{V_{\max_{C\to D}} \cdot [C]}{(K_{m_{C\to D}} + [C])(1 + F_{C\to D} \cdot [B])}. \tag{1}$$

On the other hand, the modified Michaelis–Menten equation for the reaction $A \to B$ is

$$V_{A\to B} = \frac{(V_{\max_{A\to B}} \cdot [A])(1 + F_{A\to B} \cdot [D])}{K_{m_{A\to B}} + [A]}. \tag{2}$$

Here, the terms $V_{\max_{C\to D}}$ and $V_{\max_{A\to B}}$ represent the maximum rates achieved by the reactions $C \to D$ and $A \to B$, respectively, at maximum (saturating) substrate concentrations. Michaelis constants $K_{m_{C\to D}}$ and $K_{m_{A\to B}}$ are the substrate

concentrations at which the reaction rates become half of $V_{\max_{C\to D}}$ and $V_{\max_{A\to B}}$, respectively. The terms $F_{C\to D}$ and $F_{A\to B}$ are feedback constants associated with the reactions $C \to D$ and $A \to B$, respectively. The rates of the reactions $C \to D$ and $A \to B$ are $V_{C\to D}$ and $V_{A\to B}$, respectively. For simplicity, the values of all feedback constants F have been considered as 0.9, i.e. we have considered strong inhibition/activation effect. Likewise, we have modified Michaelis–Menten kinetic equation as well as mass action equation for the signaling pathway in order to incorporate feedback.

(2) We have defined the rate expressions (Eqs. (6)–(60) in supplementary document) of each enzymatic reaction (Fig. 1) participating in CCM pathway along with fatty acid and glycogen synthesis of human hepatocyte using modified Michaelis–Menten kinetic equation as mentioned above. We have considered each reversible reaction as two irreversible reactions. Besides, we have included activation of HK, PFK, LDH and pyruvate kinase as well as inhibition of PDH by HIF1-α (Eqs. (6), (12), (26), (27) and (28) in supplementary document) using the ratio $\mathcal{R}$ which is defined as

$$\mathcal{R} = \frac{\text{Concentrations of HIF1-}\alpha\text{-induced proteins during perturbed condition}}{\text{Concentrations of HIF1-}\alpha\text{-induced proteins during normal condition}}. \tag{3}$$

In perturbed condition, $\mathcal{R}$ is calculated and applied to CCM pathway. To analyze the normal condition of a cell, no perturbation has been applied to the proposed model. As a result, the numerator of Eq. (3) becomes zero. Thus, the value of $\mathcal{R}$ has been considered as zero while simulating the normal scenario of cell.

(3) Similarly, the rate expressions (Eqs. (61)–(87) in supplementary document) of the reactions involved in HIF1-α signaling pathway[32] (Fig. 2) are defined using mass action law and Michaelis–Menten equation with a little modification (described earlier). During modification, we have included the activation of HIF1-α by inhibiting prolyl hydroxylases (PHD) using succinate[36] with the feedback constant $F_{\text{succinate}}$ in Eqs. (63), (67), (71), (72), (75), (79) and (85) in supplementary document.

(4) The stoichiometric matrix (described in supplementary document) of the pathway has been calculated. It helps us in defining all the state components of the pathway using nonlinear state space model as mentioned in supplementary document.

(5) We have constructed the simulink model (supplementary Fig. S2) of the integrated CCM pathway along with fatty acid and glycogen synthesis, and HIF1-α signaling pathway. The general description of simulink model can be found in supplementary document.

(6) In the case of CCM pathway (supplementary Tables S1 and S2), we have accumulated the values of kinetic parameters $V_{\max}$ and K_m from Refs. 9, 19 and 33. Besides, we have initialized the concentrations of different metabolites as well as hormones and calcium with very small random values near zero

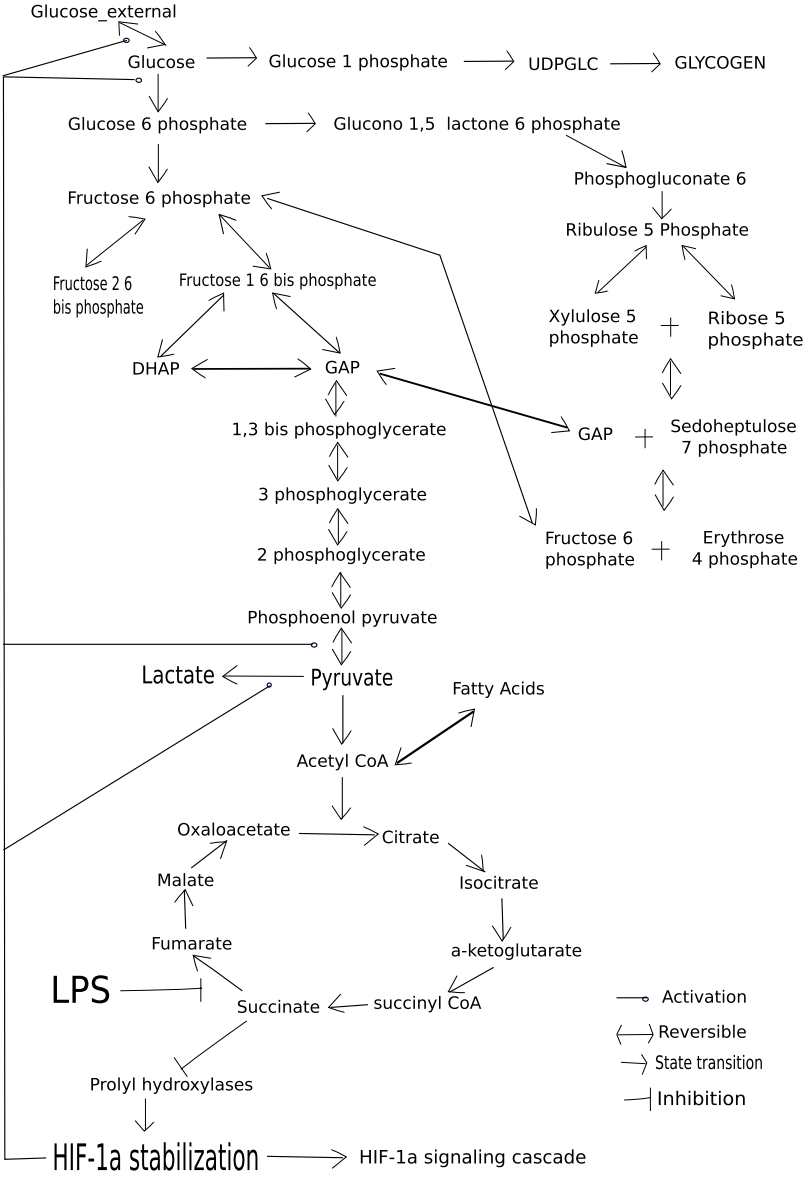

Fig. 1. Human hepatocyte pathway with LPS and HIF1-α.

(supplementary Table S4). The input of fatty acid level varies[16,35] in 0.1–1.5 mM during normal and perturbed conditions. Besides, the activation due to the ratio $\mathcal{R}$ (Eq. (3)) has been applied to the metabolic network. During normal condition, the value of $\mathcal{R}$ remains at zero. The value of $\mathcal{R}$ is calculated during perturbed condition and applied to CCM pathway. Here, only 4 nM HIF1-α-induced protein has been translated during normal condition, whereas 250 nM

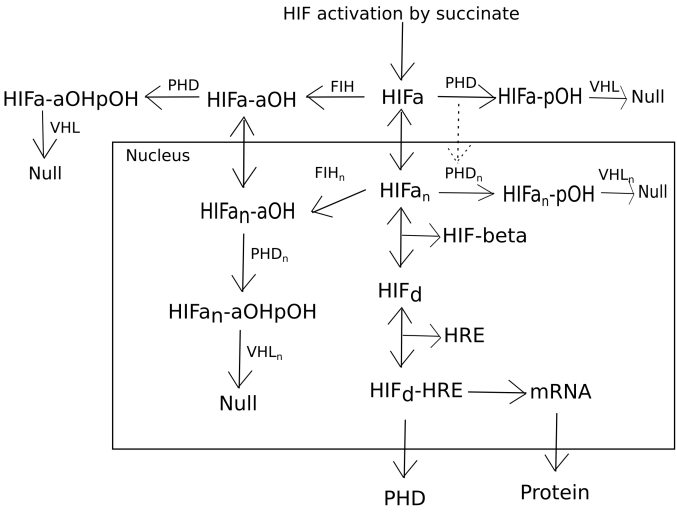

Fig. 2. HIF1-α signaling pathway.

HIF1-α-induced protein has been translated due to perturbations. As a result, the value of $\mathcal{R}$ is 62 (approximately) and has been used for CCM pathway during perturbations.

(7) According to Refs. 29, 36 and 41, the inhibition of PHD by succinate does not take place during normal condition. Thus, the input succinate, applied to HIF1-α signaling pathway, remains at zero. However, we have applied excess amount of succinate (converted from milli-Mole to nano-Mole) to this signaling pathway during perturbations. The initial concentration values of signaling molecules and kinetic parameters used in our model are provided in Tables S3 and S4 of supplementary document.[4,9,19,33]

(8) We have considered $V_{\max}$ of the reaction succinate $\rightarrow$ fumarate as zero to block SDH due to LPS. Subsequently, we have varied fatty acid level according to different perturbations.

(9) Finally, we have executed the model with "ode23tb", a differential equation solver on the matlab–simulink platform.

3. Results

Here, we have considered three cases to check the concentrations of succinate, lactate, and HIF1-α-induced proteins.

Case 1: In the first case, we have compared the concentrations of succinate, lactate, and HIF1-α-induced proteins during normal condition with the situation when SDH is blocked by LPS (Fig. 3). It has been found that when LPS blocks SDH, there is higher accumulation of succinate (Fig. 3). As succinate accumulates more, it translates higher amount of HIF1-α-induced proteins. The higher concentrations of

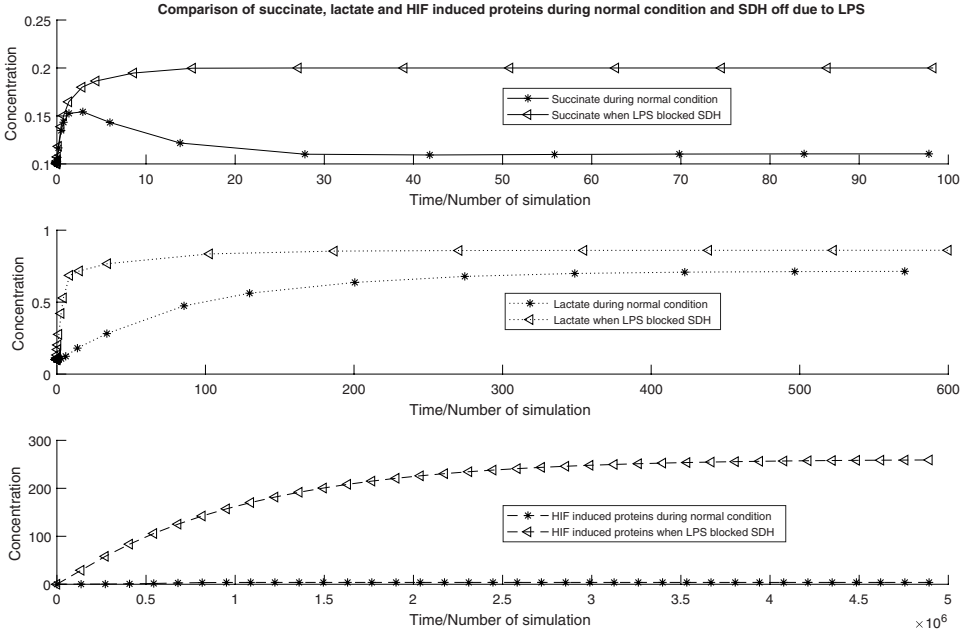

Fig. 3. Case 1: Concentrations of succinate, lactate, and HIF1-α-induced proteins during normal and blocked SDH by LPS condition, against time/number of simulations. It is clearly shown that the concentrations of succinate, lactate, and HIF1-α-induced proteins increase when LPS blocks SDH.

HIF1-α-induced proteins (Fig. 3) suggest that succinate plays an important role in translating HIF1-α-induced proteins. It leads to increased activity of LDH. Higher concentration of lactate than normal one (Fig. 3) confirms the enhanced activity of LDH.

Case 2: Obesity is associated with increased FFA. In order to determine how the increased FFA affects the normal functioning of metabolic pathways, particularly the concentrations of succinate (Fig. 4), lactate (Fig. 4), and HIF1-α-induced proteins (Fig. 4), we have stimulated the model with varying concentration of initial FFA (0.1–1.5 mM). In this case, SDH is not blocked by LPS. High level of fatty acid is present in blood on the onset of obesity.[35] It has been found that there is an increase in succinate concentration as well as increase in concentrations of lactate (Fig. 4) and HIF1-α-induced proteins (Fig. 4). This suggests that higher concentration of FFA can also induce more HIF1-α stabilization.

Case 3: In this case, we have considered the presence of increased FFA and LPS as both are associated with obesity. We have observed the concentrations of succinate, lactate, and HIF1-α-induced proteins under the above-mentioned scenario compared to the normal condition (Fig. 5). It has been found that there is an increase in the concentration of succinate (Fig. 5), lactate (Fig. 5), and HIF1-α-induced proteins (Fig. 5). Here, we have examined the rates of glucose uptake, ATP and NADH

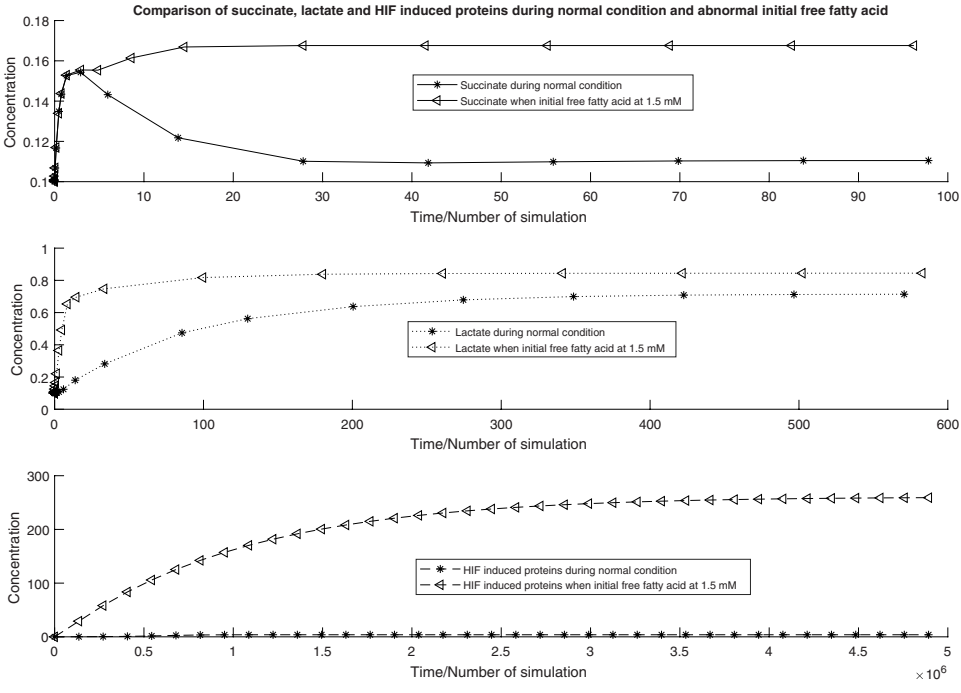

Fig. 4. Case 2: Concentrations of succinate, lactate, and HIF1-α-induced proteins during normal and when initial FFA concentration is high (abnormal), against time/number of simulations. It is clearly shown that the concentrations of succinate, lactate, and HIF1-α-induced proteins increase when initial FFA is high. There is no perturbation in SDH activity.

production to check whether oxidative phosphorylation slows down or not. Figure 6 shows less glucose uptake, ATP, and NADH production during perturbation compared to normal ones due to enhanced stabilization of HIF1-α-induced proteins. This result confirms PDH inhibition along with slowing down oxidative phosphorylation.

In order to monitor the effect of LPS as it blocks the SDH, the model has been perturbed by altering $V_{\max}$ of SDH to zero. Ceasing the conversion from succinate to fumarate, it has been found that the translation of HIF1-α-induced proteins stabilize 62 times (approximately) than normal scenario. The steady state value of succinate in normal case has been found to be 0.11 mM, while in SDH blocked condition (case 1), the steady state value of succinate has become 0.2 mM. The excess amount of succinate is calculated in nanomole as

$$(\text{abnormal succinate in millimole} - \text{Normal succinate in milimole}) \times 10^6. \quad (4)$$

The excess amount of succinate is applied as an input to HIF1-α signaling pathway during perturbed condition. Any change in concentration in nanomole scale affects signaling pathway drastically. This explains why the translation of HIF1-α-induced protein stabilizes by 62 times (approximately) higher rate during perturbed

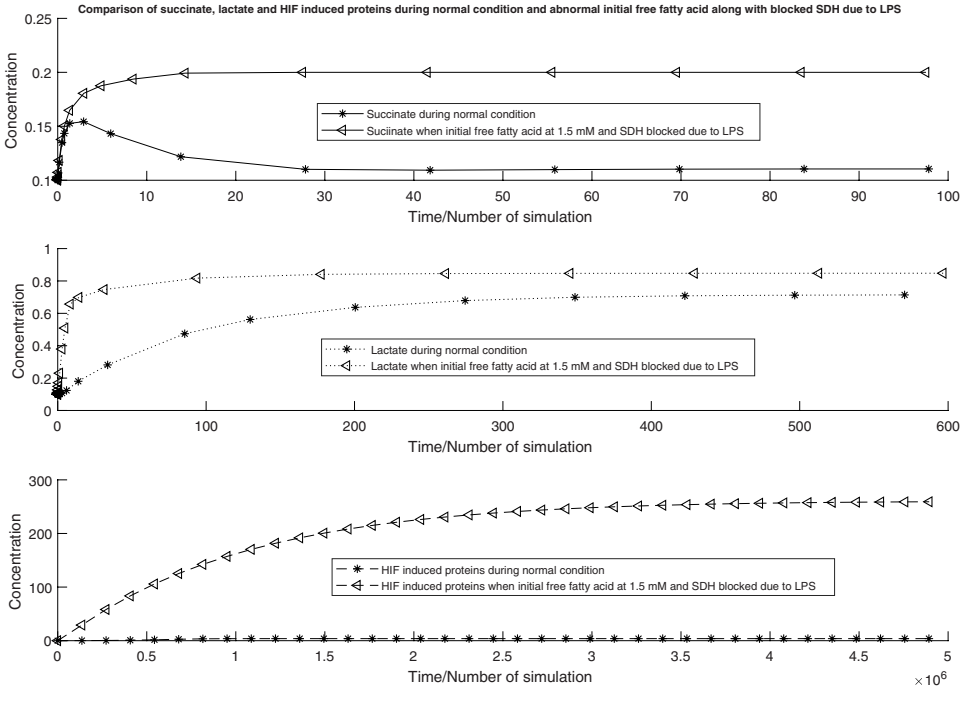

Fig. 5. Case 3: Concentrations of succinate, lactate, and HIF1-α-induced proteins during normal condition and abnormal initial FFA along with blocked SDH due to LPS. The concentrations of succinate, lactate, and HIF1-α-induced proteins are plotted against time/number of simulations.

condition compared to normal situation. Similar patterns in concentrations of succinate, lactate and in stabilization of HIF1-α-induced proteins are found in cases 2 and 3 (Figs. 4 and 5).

4. Discussion

Succinate is a part of many pathways including a macrophage-specific metabolic pathway generating itaconate as well as the metabolism of branched chain amino acids, heme synthesis, ketone body utilization, and γ-aminobutyric acid (GABA) shunt. Succinate takes part in paracrine and endocrine signaling pathways, mediated by G-protein-coupled receptor (GPR91)/SUCNR1.[8] Finally, SDH, an enzyme that converts succinate to fumarate in CAC, plays as an important controller of reactive oxygen species (ROS) homeostasis. Thus, the accumulation of succinate in hepatocyte leads to HIF1-α activation.[41] If we look deep into the pathological condition, i.e. obesity, it has been found that obesity is always associated with high FFA in blood.[17] Besides, it alters gut microbiome with LPS secreting bacteria.[3] Additionally, NAFLD is a prominent comorbid disease on the onset of obesity.[15] Depending on the above-mentioned condition associated with NAFLD on the onset of obesity, we have

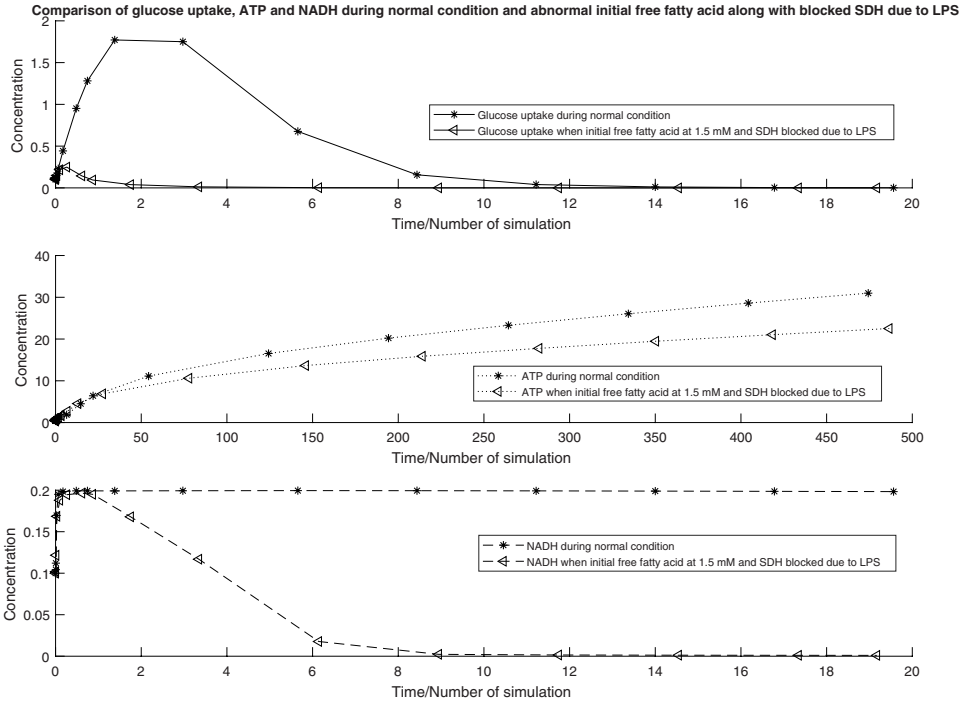

Fig. 6. Decrease in glucose uptake, ATP, and NADH production indicates the slow oxidative phosphorylation, i.e. a common phenomenon of "Warburg effect" leading to cancer.

simulated through the proposed model to examine the possible altered cellular dynamics responsible for liver cancer due to NAFLD progression.[28,21,42]

According to the simulation results, it is clear that accumulation of succinate leads to increased synthesis of HIF1-α-induced proteins and lactate. In the scenario of obesity, when FFA is high irrespective of SDH being blocked by LPS or not, the concentrations of lactate and HIF1-α-induced proteins have been increased. Besides, glucose uptake, ATP, and NADH production slow down. Thus, our results confirm the enhanced activity of LDH and the inhibition of PDH. It slows down the oxidative phosphorylation. The results also suggest glucose fermentation to form higher lactate than that in normal cells.

The activation of HIF1-α, a transcription factor, significantly contributes to the alteration of cellular pathways. HIF1-α stabilizes and increases the activities of HK, PFK, LDH, and pyruvate kinase.[25] Besides, the activity of PDH is inhibited. Thus, less oxidative phosphorylation and glucose fermentation may create a phenomenon similar to "Warburg effect".[17] This kind of scenario promotes tumorous cell to become malignant.[23]

In conclusion, we can suggest that NAFLD patients having obesity are at high risk of NAFLD progression towards liver cancer. Here, succinate plays an important role. In future, it is needed to analyze other inflammatory pathways[38] that are also

responsible for liver cancer in the scenario of obesity. Our simulation results suggest that the effect of succinate accumulation in obesity may have much implications on hepatocyte pathways *in vivo* and/or by *in vitro* experiments. Thus, the proposed *in silico* model is quite successful in capturing the aforesaid key features of the NAFLD progression on the onset of obesity.

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Indrani Ray has completed Master of Technology in Biotechnology from Indian Institute of Technology, Guwahati, India. She started her Ph.D. research work in 2014 at the Indian Statistical Institute, Kolkata, India. Her research work is focused on systems biology, mathematical modeling of biological pathways, database design and bioinformatics in the area of obesity and its co-morbid diseases.

Abhijit Dasgupta is currently working as a Senior Research Fellow, under Visvesvaraya Ph.D. Scheme of the Government of India, at the Indian Statistical Institute, Kolkata, India. After passing Master of Technology in Information Technology from University of Calcutta, Kolkata, India, he has joined Indian Statistical Institute, Kolkata, West Bengal, India for working in a research project. His research interest lies on the broad area of pattern recognition, machine learning, bioinformatics, system biology, computational biology, mathematical modeling and control engineering. Presently, he is doing research on integrative biochemical pathway modeling, brain networks, omics data integration and personalized medicine.

Rajat K. De is a Professor of the Indian Statistical Institute, Kolkata, India. He obtained his received his Ph.D. degree from the Indian Statistical Institute, India, in the year 2000. He was a Distinguished Postdoctoral Fellow of the Whitaker Biomedical Engineering Institute, the Johns Hopkins University, USA during 2002–2003. He visited the Department of Medicine, University of California, San Diego, under Fulbright-Nehru Academic and Professional Excellence Fellowship Program, during 2017–2018. Dr. De has authored about 100 papers published in international journals, edited books and in conference proceedings. His research interest includes modeling biochemical pathways, multi-omics data analysis, pattern recognition, machine learning, deep neural networks, and data sciences.