Indonesian Journal on Health Science and Medicine

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

Transcriptomic and Metabolomic Differential Responses in Hypoxia on RBL-2H3 Cell Through Sodium Cromoglycate Mediates

Rana Hussein Naser
Department of Science, College of Basic Education, University of Diyala, Iraq
Email: ranaalqaysi@gmail.com

Abstract. Hypoxia is a state in which sufficient oxygen is not available at the tissue level, a condition that leads to hypoxemia. Mast cells (MCs) are tissue resident cells that are widely distributed in vascularized tissues such as airways and gastrointestinal tract. Previously, studies have established the effect of pre-treatment of MCs with Sodium Cromoglicate (SCG) inhibitor in lung tissues of SD rats. Thus, due to the complexity and less understanding of the cell structure of lung tissues, and in order to understand the changes of MCs under hypoxia, RBL-2H3 were utilized in an experimental in-vitro study that aimed to evaluate the transcriptomic and metabolomic variations of these cells under hypoxic conditions and after pre-treatment with SCG. Differential expression analysis identified 25 genes as differentially expressed between HSCG vs HC group. On KEGG pathway enrichment analysis, Arachidonic acid metabolism, glutathione metabolism and AMPK pathway were among the enriched pathways in the HSCG vs HC group. A total of 1,513 metabolites were identified. These metabolites were amino acids, fatty acids, organic acids, lipids, carbohydrates, and others. Metabolic pathway enrichment analysis revealed that in the HSCG_vs_HC group, Glutathione, Val-Val, Arg-Glu, Pro-Glu and 1-Stearoyl-rac-glycerol were upregulated whereas, Cytidine, Hypoxanthine, Histamine and Pro-Asp were decreased. In conclusion, our study findings reveal that SCG impacts on cell mechanisms under hypoxic conditions.

Highlights:

1. **Hypoxia Impact on MCs: Hypoxic conditions lead to significant transcriptomic and metabolomic changes in mast cell-like RBL-2H3 cells.**
2. **SCG Effects: Pre-treatment with Sodium Cromoglicate modulates pathways such as arachidonic acid metabolism and glutathione metabolism.**
3. **Metabolite Shifts: Key metabolic changes include upregulation of Glutathione and Val-Val, and downregulation of Cytidine and Hypoxanthine.**

Keywords: Hypoxia, Transcriptome, Metabolomics, RBL-2H3 cells

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

Introduction

Vasoconstriction and remodelling of small pulmonary arteries and arterioles is the primary basis of hypoxic pulmonary hypertension that may result from prolonged exposure to low levels of oxygen [1]. Sudden change in hypoxic pulmonary hypertension (AHPH) is caused by immunological reactions due to oxygen deficit, aseptic inflammation and a sudden constriction of Mast cells (MCs) act as a modulator of asthma, infection responses, tumour growth, and ischaemia - induced inflammation [2]. Virtually all types of MCs are present in vascularised tissues which enable them to respond rapidly to allergens, pollutants and infections, especially those in the skin, thee digestive tract and the lungs [3]. In the lungs, MCs are located in strategic places because they exert their immune regulatory functions where they are closest to the pulmonary blood arteries, bronchi, and glandular tissues [4]. Chronic hypoxia restructures the blood vessels and leads to PAH in MCs [5]. Sodium cromoglycate (SCG) has been found effective to be used as a medicinal inhalant for allergic asthma, and was introduced in the year 1965. Due to its capacity to protect individuals from bronchial allergen challenges it was selected for this study [6] suggest that SCG may inhibit the ingression of calcium indirectly: they stipulate that chloride channels that function in MC activation are blocked. After performing a study of SD rats, it was observed that acute hypobaric hypoxia raised the level of MC, histamine, 5-HT and tryptase and chymase, which are specific to MC. In particular these markers were significantly suppressed by SCG pre-treatment [7], indicating that MCs are stimulated by hypoxia. Additionally, the published data indicate that, when exposed to hypoxic conditions, pulmonary arterial pressure (PAP) is increased, and SCG pre-treatment might alleviate this effect [8] potentiality implied that MC activity in hypoxic environments is involved in acute changes in PAP. For the MC biology studies, there is a great option to choose the RBL-2H3 cell line because of its genetically malleable nature and high culture growth [9]. Fortunately, cellular investigation has become achievable due to recent advances of omics technologies, namely multi-omics strategies. Metabolomics aims for identification of significant metabolites, which shed light on meaningful functions in cell metabolism; transcriptomics quantify genes in single cells [10]. While applying these technologies at once the understanding of cell functioning is developed significantly. This manuscript aims to investigate MC function and molecular changes during hypoxia and SCG treatment by analysing the transcriptome and non-targeted metabolome of RBL-2H3 cells. To understand MC control in hypoxic situations, this technique has tried to show cellular response to hypoxic condition and intervention to SCG.

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

Method

Sample Preparation and Cell Culture

RBL-2H3 cells at a density of 2X10^5 /ml were maintained in Minimum Essential Medium (MEM) containing 15% fetal bovine serum (FBS), 1% penicillin and were placed in incubator under 5% CO_2, 37°C, and humidity. Cells grown to 80% confluence after 24 hours of incubation were washed two times with 5 ml of 1× PBS and will be divided to the treatment groups mentioned on the table 1.

Table 1. Summary of Cell Culture Treatment Groups

Group Name	Type of Group	Cell Culture Conditions
NC	Control Group	5% CO_2
HC	Hypoxic Control Group	1% O_2, 5% CO_2
HSCG	SCG-Treated Group	1% O_2, 5% CO_2

In the NC and HC control groups, each of the four replicates was given 10 ml of MEM supplemented with 15% FBS, 1% penicillin and streptomycin. For the HSCG treatment group, 10 ml of the SCG medium was suspended in MEM supplemented with 10% FBS and antibiotics to give a final concentration of SCG 1 × 10^-6 g/ml. The NC cultures were then incubated at 37°C, 5% CO2 and 70% humidity and the HC and HSCG cultures were incubated at hypoxic conditions of 1%O2 5%CO2 at 37°C. 2, Cells were then washed three times at 48 hours after treatment with 5ml 1× PBS. To eliminate cell to cell contact, 1.5 ml of 0.25% protease was added and the cells were incubated for 5 min at 37°C. The enzyme activity was stopped by adding 5ml MEM. The cell suspension was then transferred to 15 ml sterile Centurk tube and was then centrifuged at 1000 rpm for 5 minutes. Cells were then washed with 1ml 1×PBS and resuspended and aliquotted in 2ml cryovials and stored at -80°C.

RNA Extraction and Transcriptome Sequencing

Consequently, the isolation of Total RNA from the tissue samples was done using Zymo Direct-zol kit (Zymo Research Corp, USA) according the producer's guideline. The quality of the RNAs was evaluated using the RNA Nano 6000 Assay Kit on the Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA) and a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). Types of samples with RIN > 8.0 were considered acceptable. RNA yield was determined using Qubit® RNA Assay Kit and Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). The library preparation procedure

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

was carried out using 3 µg of total RNA and Epicentre Ribo-zero™ rRNA removal kit (Epicentre, USA); further ethanol precipitation was done. To construct the cDNA library, NEBNext® Ultra™ RNA Library Prep Kit for Illumina® was used (NEB, USA). The RNA was treated with divalent cations at high temperatures for RNA fragmentation; reverse transcription used random hexamers and M-MuLV reverse transcriptase. The secondary cDNA synthesis was done by using DNA Polymerase I and RNase H, with dUTP instead of dTTP. The 3 'end of the DNA was adenylated and then ligated with NEBNext adaptors. Electrophoretic recovery of DNA ranged from 250 to 300 bp DNA fragments were purified using AMPure XP system (Beckman Coulter, USA) and further amplified through PCR. Essential quality of the libraries was further checked using the Agilent Bioanalyzer 2100 and sequencing was done in an Illumina Hiseq4000 Platform.

Non-Target Metabolomic Analysis by UHPLC-QTOF/MS

Cells were brought, pelleted at 1500 × g for 15 minutes, and then lysed before being stored at −80°C. Samples were then thawed at 4°C, and 100-µL aliquots were mixed with 400 µL of cold methanol/acetonitrile (1:The cell lysate was subjected to the conversion of proteins by the PCI method (Phenol: Chloroform: Isoamyl alcohol = 25:24:1, v/v). The mixture was centrifuged at 14.000 × g rpm for 20 min at 4°C; supernatants were then dried by Vacuum Centrifuge. Metabolic profiling was performed with ultra-high-performance liquid chromatography UHPLC Shimadzu Nexera XR LC-30AD equipped with QToF mass spectrometer AB SCIEX TripleTOF 6600. All mass spectrometry data were converted to MzXML format along using ProteoWizard tool MSConvert and then subjected to analysis in XCMS. Covariates with greater than or equal to 50% of values observed as nonzero in at least one group were included. These identified metabolites were referenced to an in-house database of known metabolite standards. All data, obtained for calibration and validation of the model, were normalized to the total peak intensity; processed data were analyzed in SIMCA-P (v14.1, Umetrics) to perform PCA and OPLS-DA. The generalisation abilities of the models were determined using seven fold cross validation and response permutation tests. Those variables which met the VIP value of more than 1 were tested for univariate Student's t-tests with $p < .05$ level of significance.

Bioinformatics Analyses

The sequencing data were trimmed of adapter sequences, excluded reads with N

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

content greater than 1% and low quality base >50% with Phred score ≤18. Clean reads were aligned to the reference genome using STAR v2.5.1b, and the HTSeq v0.6.0 was used to counts the mapped reads per gene, by FPKM. Data outputs were normalized and compared for relative gene expression with DESeq2 v3.8. Statistical differences were determined when the underlying q values were less than 0.05. We compared these DEGs to KEGG pathways using clusterProfiler (v 3.8) and analyzed significance thresholds using the BH method to control FDR.

Results

Raw Sequencing Data and Transcriptome Assembly

Sequencing process provided a huge 1,044.1 million of paired-end raw reads (2 × 125 bp) in twelve libraries. The quality control, and trimming yielded 1,034.4 million high quality reads, which served as a strong platform for subsequent analyses.. Read count of samples per study population for Illumina was 54,641,039(7.56 Gb) to 71,346,682(9.87 Gb), hence obtaining a correlation coefficient of 0.9 across samples, meaning more agreement within study groups (Fig 1).

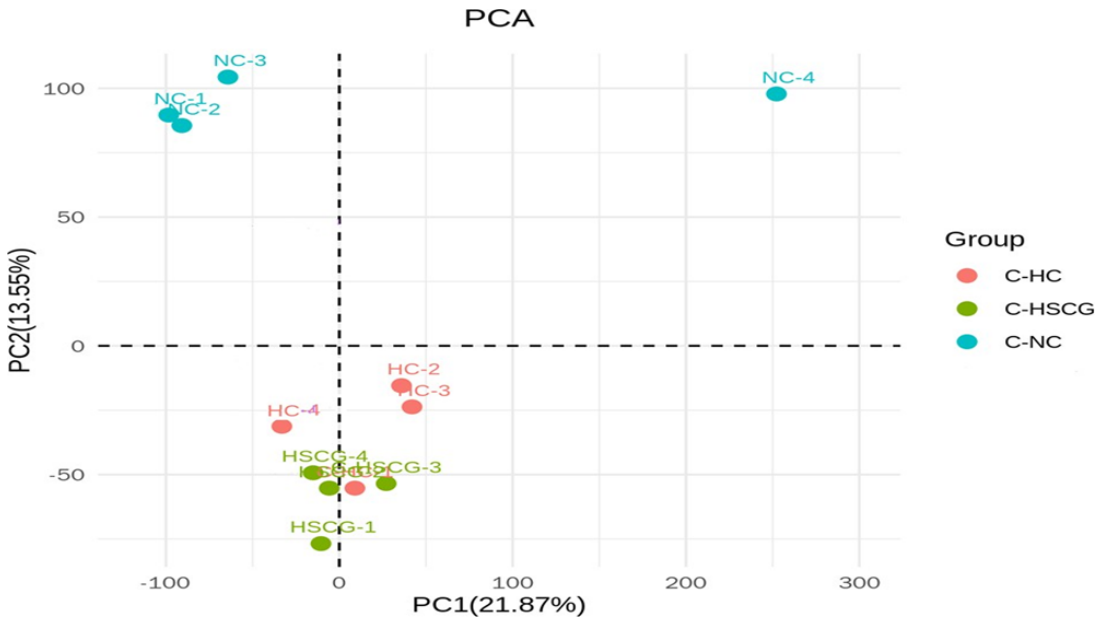


Figure 1. PCA Analysis of the treatment groups (HC, HSCG and NC groups).

Principal Component Analysis (PCA)

PCA further helped differentiate the groups from one another, and show that the expression profiles of these groups were vastly different. Distinctions between the control group (NC) and the hypoxia treated groups (HC and HSCG) were apparent to indicate that gene expression alterations are tightly linked with hypoxic treatment (Figure 2).

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

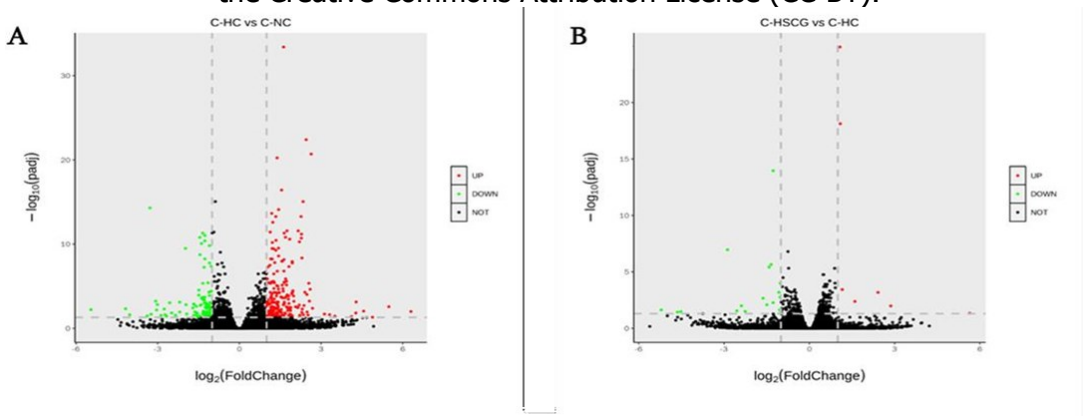


Figure 2. Overview of differentially expressed genes in (A) HC_vs_NC and (B) HSCG_vs_HC. Red spots indicate upregulated genes, green spots indicate downregulated genes, and black dots represent genes with no differential expression.

Gene Functional Annotation

Gene fragments were annotated by alignment against six public databases (Nr protein, Nt, Swiss-Prot, KEGG, GO, and COG) and when applying rather strict criteria, such as E-value <10^-5, their functions varied greatly. Average hit similarity varied from 18 to 100% with the E value of between 0 and 1.0E-15 indicating that the whole spectrum of the BLAST database was captured and variation within the dataset was well represented (Figure 3).

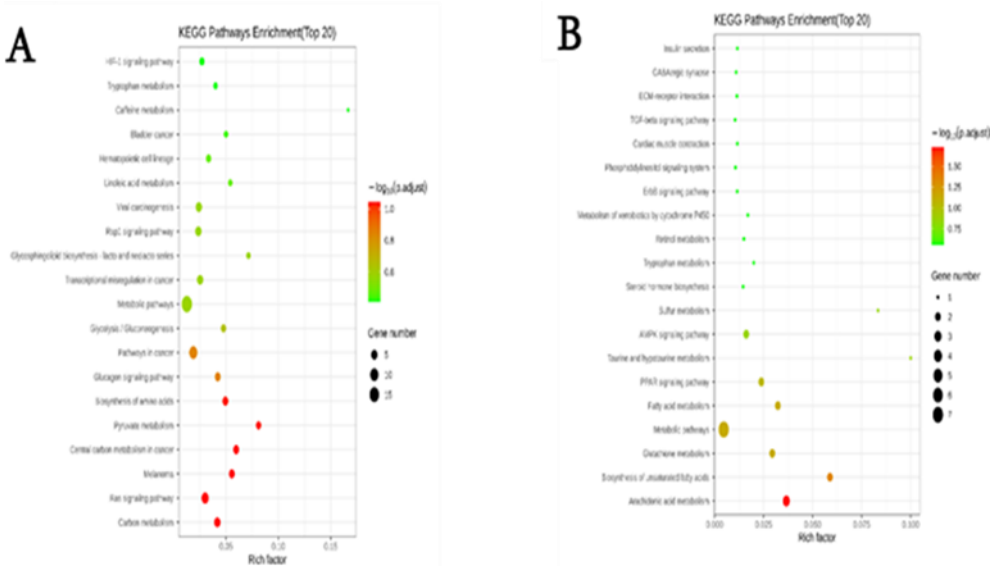


Figure 3. An outline of the significantly enriched KEGG pathways in differentially expressed genes in (A) HC_vs_NC, (B) HSCG_vs_HC.

Differentially Expressed Genes (DEGs)

Global RNA-Seq analysis identified the variation of gene expression levels

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

between the groups of the treated samples. A total of 344 genes were tested in the HC vs. NC comparison and 230 of these genes were upregulated while 114 downregulated. The comparison of HSCG and HC was determined to have 25 DEGs and including seven genes that were upregulated and eighteen genes that were downregulated. qRT-PCR validation of 11 DEGs in the four groups restored the reliability of the expression patterns shown by the sequencing data (Figure 4.).

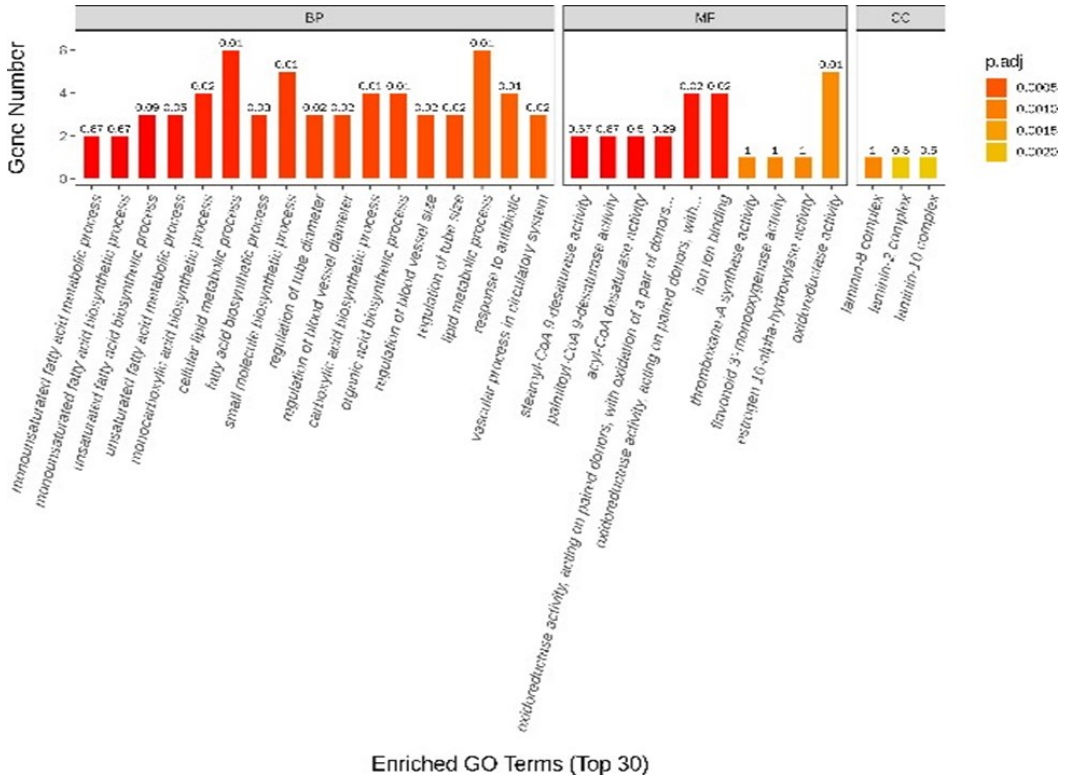


Figure 4. An overview of the GO enriched terms in differentially expressed genes in the groups, A: HSCG_vs_HC,

KEGG Pathway Enrichment Analysis

A KEGG pathway analysis revealed that metabolic reprogramming is significantly different between cells under hypoxia condition. Using the HC vs. NC group, we found numerous pathways, including carbon metabolism, Ras signaling, melanoma, and pyruvate metabolism (Figure 5). Conversely, based on DEGs in the HSCG vs. HC group, the metabolic pathways most significantly affected by SCG included arachidonic acid metabolism, unsaturated fatty acid biosynthesis, glutathione metabolism and AMPK signal pathway.

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

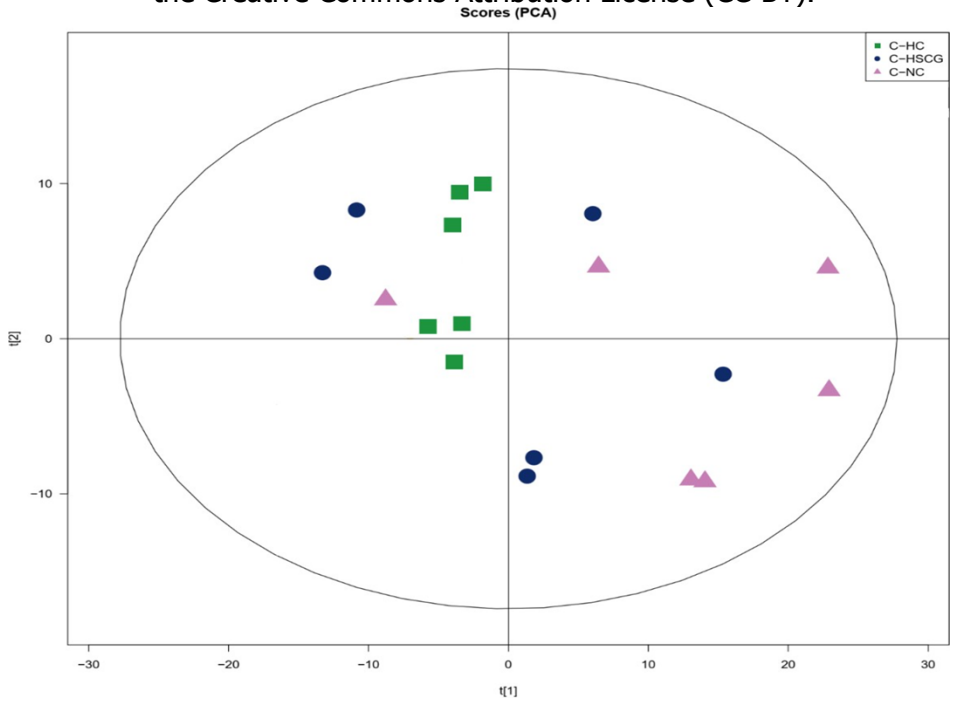
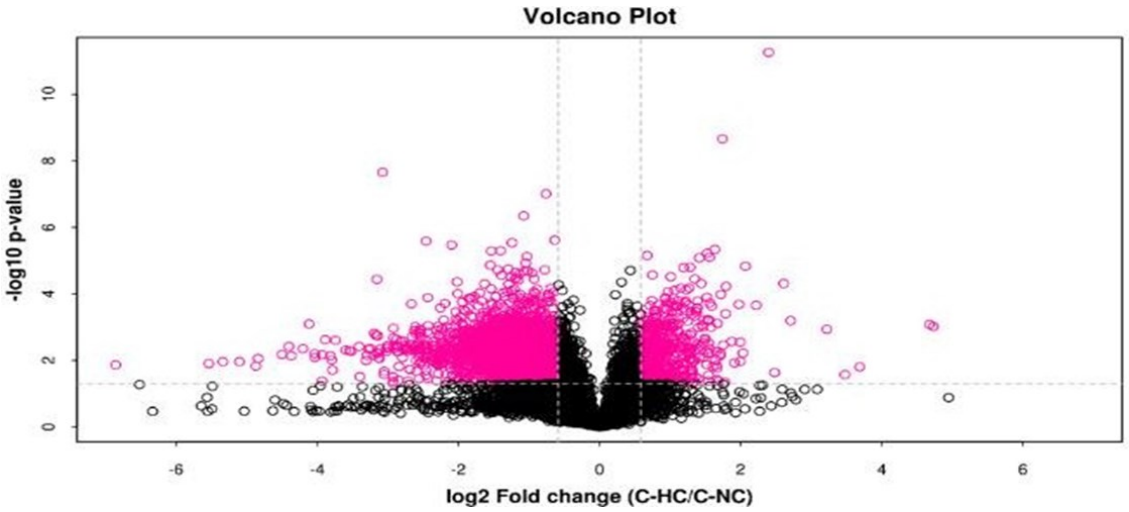


Figure 5. The PCA score plot of differentially expressed metabolites in the treatment groups (HC, HSCG and NC groups).

Gene Ontology (GO) Analysis

GO analysis of DEGs in the HSCG_vs_HC group highlighted specific biological processes, molecular functions, and cellular components. Lipid metabolism, small molecule biosynthesis, and monocarboxylic acid biosynthesis emerged as dominant biological processes, while oxidoreductase activity and iron binding were enriched in molecular functions. Cellular components were characterized by the presence of laminin complexes, pointing to structural and metabolic adaptations induced by SCG under hypoxic conditions (Figure 6).



ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

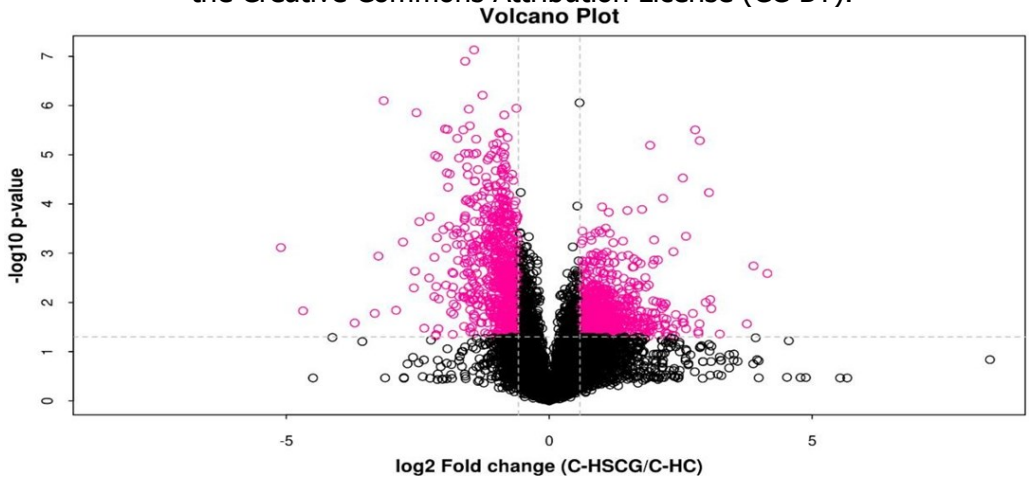


Figure 6. Volcano plot of differentially expressed metabolites in A: HC vs NC, and B: HSCG vs HC.

Untargeted Metabolomics

Using the QTOF/MS-based metabolomic profiling, 1,513 metabolites were detected across groups among which there were changes in amino acids metabolisms, fatty acids, lipids, organic acids and carbohydrates. The findings of metabolomics reflected the profound physiological changes to hypoxia and SCG treatment by showing separate clusters of the hypoxic and control groups in the PCA plot (Figure 7). This extensive characterization suggests a qualitative change in gene expression and metabolic profile under hypoxic condition and a probable impact or modulative role of SCG on hypoxia regulated processes.

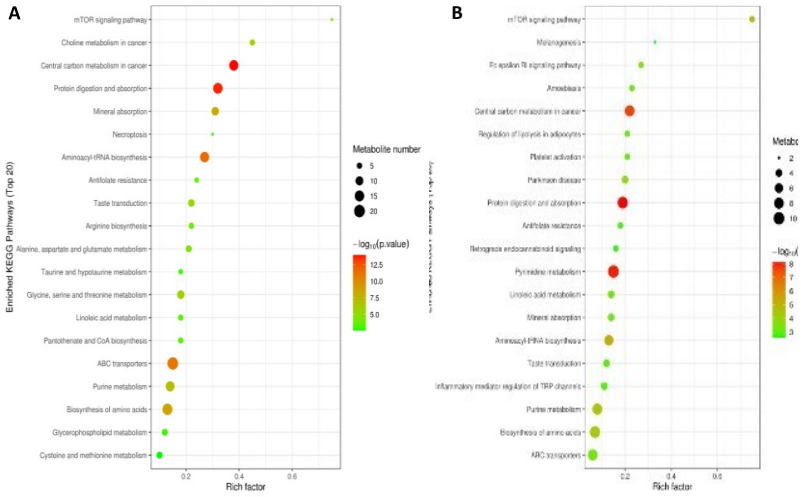


Figure 7. An outline of the significantly enriched KEGG pathways of differentially expressed metabolites A: HC vs NC and, B: HSCG vs HC.

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

Discussion

Examining immune processes at the cellular level has traditionally been difficult because of complexity inherent in immunologic derived culture techniques of primary degranulating cells such as the mast cells and basophils. The RBL-2H3 cell line has become highly essential in putting into light of pristine mast technology type functions and can also be relied on as an experimental system for immunity [11]. Environmental stress including hypoxia impacts essentially all aspects of cells' survival, growth and adaptation strategies in all the organisms. Hence, our work presents a transcriptomic and metabolomic investigation of the RBL-2H3 cells subjected to hypoxia with or without SCG treatment, reporting the protective mechanisms developed to help the cells change their metabolic and immune status to adapt to low oxygen conditions. The results provide important information on the effects of SCG with respect to cellular adaptation to hypoxia. Whenever the oxygen is scarce, cells are forced to down regulate energy consumption, which can either be in direct propinon to the available energy or indirectly through anaerobic respiration, which commonly occurs through glycolysis [12]. In this study, we found for the first time that aerobic metabolism-related genes were significantly downregulated in the hypoxic control group compared with the normoxic group, suggesting an alteration in metabolic demands when oxygen is scarce. However, the expression levels for these metabolic pathways were higher in SCG groups; consequently, we found some evidence to support that SCG may affect hypoxic stress by promoting energy metabolism. Monounsaturated fatty acid biosynthesis enzyme stearoyl-CoA desaturase (SCD1 and SCD2) was also upregulated in hypoxic cells treated with SCG. These enzymes have critical functions in lipid metabolism to change the SFA to MUFA a step within the biosynthesis of triglycerides within the fatty acid metabolic network [13][14]. This conversion assist with the preservation of cellular energy homeostasis, and it is interesting to note that SCD1 also offers cell defense against lipotoxicity which may be particularly advantageous under hypoxic circumstances where lipid metabolism is known to keep pace [15]. This adaptive response is in concord with previous studies indicating that elevated level of SCD1 is involved in hypoxia-mediated regulation of energy metabolism and cellular stress response in hepatoma cells. The isoform SCD2, similarly necessary for MUFA generation, sustains the epidermal barrier and lipid metabolism, key for cell proliferation under hypoxic stress, most significantly at the very early stages of development [16]. Because histamine has been described as an active principle in immediate hypersensitivity, in defense, immune reactions were suppressed by SCG treatment, which reduced histamine levels in the SCG-treated group.

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

The results of this study are in disagreement with prior research wherein histamine concentrations are increased under hypoxic conditions [17][18]. Some of the functions attributed to SCG are possibly involved in the decrease in mast cell stability and histamine release culminating from this substance since SCG act as an inhibitor of histamine and cytokine like tumor necrosis factor α from mast cells in the process of activation [19]. This decrease in histamine under hypoxia with SCG treatment proposes immunomodulatory role of the peptide which seems to reduce inflamma¬tory and hypersensitivity reactions under hypoxic conditions. The upregulated ErbB signaling pathway in the HSCG_vs_HC group is essential for controlling hypoxia-inducible factor -1α (HIF-1α). HIF-1α controls adaptational genes to hypoxia, such as angiogenesis, glycolysis, pH and metastatic changes in the cell, and its activity increases in pathologies like cancer and ischemia [20]. ErbB signaling pathway is also increased in hypoxic cells treated with SCG, indicating that SCG might affect HIF 1α activity and provide the direction that SCG might regulate HIF 1α activity to manage hypoxic stress through controlling HIF 1α related genes. Likewise, the PI3K/Akt signaling pathway, which was also activated by SCG in hypoxic cells, has many important roles in cell survival, division and metabolism. This pathway has been the mechanism of hypoxic stress survival where the pro-survival proteins are active while the apoptosis proteins are inactive this explains why this pathway is active in cellular adaptation to hypoxia [21]. These data suggest that ErbB and PI3K/Akt are important for every characteristic of hypoxic adaptation in SCG-treated cells, including increased survival and metabolic adaptability to hypoxic stress. Thus, The AMP-activated protein kinase (AMPK) works as a cellular energy regulatory molecule and brings energy balance in the critical condition by inhibiting the synthetic processes and activating the producers, when ATP levels in the cellular drop down [22]. The observed substantial upregulation of the AMPK in both SCG treated groups is consistent with other studies explaining hypoxia adaptation in several tissues such as the central nervous system, liver, skeletal muscle, cardiomyocytes and epithelial cells. When tissues are under hypoxic conditions, activation of AMPK is beneficial in minimizing energy consumption and is protective in hypoxic diseases including ischemic injury. Presenting similar data, our study contributes to this knowledge by showing that SCG also augments the activation of AMPK, which may reinforce cellular signaling against hypoxia. In the context of hypoxia, metabolomics has allowed to reveal 1,513 metabolites in study groups and evaluate the impact of SCG on cellular metabolism. Significant pathways related to hypoxia adaptation were determined as amino acid metabolism, ABC transporters, glutathione metabolism, starch and sucrose metabolism

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

and cysteine and methionine metabolism. Specifically, glutathione metabolism study was presented as crucial in hypoxic regulation since hypoxic adaptation involved glutamatergic signaling [19]. Moreover, starch and sucrose metabolism which is involved in energy metabolites exhibited adaptive function during hypoxia meaning that SCG may well control energy store and metabolism disparities in hypoxic situations.

Conclusion

Incorporating both transcriptomic and metabolomic data in this study, specific pathways and processes as a result of tissue hypoxia and the modulatory role of SCG in RBL-2H3 cells are uncovered. Overall, 789 differentially expressed genes and 1513 metabolites were identified and the hypoxic adaptation related pathway analysis showed that AMPK, ErbB and PI3K-Akt pathway were crucial for hypoxic adaptation. These pathways may seem fundamental to cellular response to SCG treatment possibly improving hypoxic stress tolerance. Other pathways including arginine biosynthesis, transporters and channels, N-acyl glutathamine, and starch & sucrose also contribute broadly to hypoxia responses complementing gene expression changes in modulating energy and immune responses. Interestingly, from the HSCG Vs HC metabolites comparison, metabolites like Glutathione, Val-Val, Arg-Glu, Pro-Glu and 1-Stearoyl-rac-glycerol were increased while Cytidine, Hypoxanthine Histamine and Pro-Asp were decreased again showing SCG's ability to change metabolism and immune response. Here, the data show that SCG modulates cellular perturbation under hypoxia which is an interesting premise for the development of hypoxia-linked pathological states treatment as SCG regulates cellular energetic status and metabolism, and affects immune response. Consequently, this work lays the groundwork for further investigations dealing with clinical hypoxic aspects of SCG and its hypoprotective potential.

References

[1] E. Y. Bissonnette, J. A. Enciso, and A. D. Befus, "Inhibition of tumour necrosis factor-alpha (TNF-α) release from mast cells by the anti-inflammatory drugs, sodium cromoglycate and nedocromil sodium," Clinical and Experimental Immunology, vol. 102, no. 1, pp. 78–84, 1995. doi: 10.1111/j.1365-2249.1995.tb06639.x.

[2] C. C. Cai et al., "Glycine protects against hypoxic-ischemic brain injury by regulating mitochondria-mediated autophagy via the AMPK pathway," Oxidative Medicine and Cellular Longevity, vol. 2019, pp. 1–12, 2019. doi: 10.1155/2019/4248529.

[3] R. Cavill, D. Jennen, J. Kleinjans, and J. J. Briedé, "Transcriptomic and metabolomic data integration," Briefings in Bioinformatics, vol. 17, no. 5, pp. 891–901, 2016. doi: 10.1093/bib/bbv090.

[4] W. C. Chang, "Store-operated calcium channels and pro-inflammatory signals," Acta Pharmacologica Sinica, vol. 27, no. 7, pp. 791–796, 2006. doi: 10.1111/j.1745-7254.2006.00395.x.

[5] A. Dalla Valle et al., "Induction of Stearoyl-CoA 9-Desaturase 1 protects human mesenchymal stromal cells against palmitic acid-induced lipotoxicity and inflammation," Frontiers in Endocrinology, vol. 10, p. 726, 2019. doi: 10.3389/fendo.2019.00726.

[6] F. Dengler, "Activation of AMPK under hypoxia: Many roads leading to Rome,"

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

International Journal of Molecular Sciences, vol. 21, no. 7, pp. 1–26, 2020. doi: 10.3390/ijms21072428.

[7] F. Dengler and G. Gäbel, "The fast lane of hypoxic adaptation: Glucose transport is modulated via a HIF-hydroxylase-AMPK-axis in jejunum epithelium," International Journal of Molecular Sciences, vol. 20, no. 20, pp. 1–19, 2019. doi: 10.3390/ijms20204993.

[8] F. Dengler, R. Rackwitz, H. Pfannkuche, and G. Gäbel, "Glucose transport across lagomorph jejunum epithelium is modulated by AMP-activated protein kinase under hypoxia," Journal of Applied Physiology, vol. 123, no. 6, pp. 1487–1500, 2017. doi: 10.1152/japplphysiol.00436.2017.

[9] K. J. Dunham-Snary et al., "Hypoxic pulmonary vasoconstriction: From molecular mechanisms to medicine," Chest, vol. 151, no. 4, pp. 940–951, 2017. doi: 10.1016/j.chest.2016.09.001.

[10] A. M. Edwards and J. B. L. Howell, "The chromones: History, chemistry, and clinical development," Clinical and Experimental Allergy, vol. 30, no. 5, pp. 756–764, 2000. doi: 10.1046/j.1365-2222.2000.00879.x.

[11] F. H. Falcone, D. Wan, N. Barwary, and R. Sagi-Eisenberg, "RBL cells as models for in vitro studies of mast cells and basophils," Immunological Reviews, vol. 282, no. 1, pp. 63–79, 2018. doi: 10.1111/imr.12628.

[12] M. T. Flowers and J. M. Ntambi, "Role of stearoyl-CoA desaturase in regulating lipid metabolism," Current Opinion in Lipidology, vol. 19, no. 3, pp. 248–256, 2008. doi: 10.1097/MOL.0b013e3282f9b54d.

[13] M. Gaczynska, P. A. Osmulski, Y. Gao, M. J. Post, and M. Simons, "Proline- and arginine-rich peptides constitute a novel class of allosteric inhibitors of proteasome activity," Biochemistry, vol. 42, no. 29, pp. 8663–8670, 2003. doi: 10.1021/bi034784f.

[14] S. J. Galli et al., "Mast cells as 'tunable' effector and immunoregulatory cells: Recent advances," Annual Review of Immunology, vol. 23, pp. 749–786, 2005. doi: 10.1146/annurev.immunol.21.120601.141025.

[15] C. Gu and J. C. Jun, "Does hypoxia decrease the metabolic rate?" Frontiers in Endocrinology, vol. 9, p. 668, 2018. doi: 10.3389/fendo.2018.00668.

[16] G. A. Gusarova et al., "Hypoxia leads to Na,K-ATPase downregulation via Ca2+ release-activated Ca2+ channels and AMPK activation," Molecular and Cellular Biology, vol. 31, no. 17, pp. 3546–3556, 2011. doi: 10.1128/mcb.05114-11.

[17] C. A. Hales and H. Kazemi, "Role of histamine in the hypoxic vascular response of the lung," Respiration Physiology, vol. 24, no. 1, pp. 81–88, 1975. doi: 10.1016/0034-5687(75)90123-1.

[18] J. Q. Han, K. Y. Yu, and M. He, "Effects of puerarin on the neurocyte apoptosis and p-Akt (Ser473) expressions in rats with cerebral ischemia/reperfusion injury," Chinese Journal of Integrated Traditional and Western Medicine, vol. 32, no. 8, pp. 1069–1072, 2012.

[19] T. Hashimoto et al., "Severe hypoxia increases expression of ATM and DNA-PKcs and it increases their activities through Src and AMPK signaling pathways," Biochemical and Biophysical Research Communications, vol. 505, no. 1, pp. 13–19, 2018. doi: 10.1016/j.bbrc.2018.09.068.

[20] H. Hu et al., "The cardioprotective effects of carvedilol on ischemia and reperfusion injury by AMPK signaling pathway," Biomedicine and Pharmacotherapy, vol. 117, p. 109106, 2019. doi: 10.1016/j.biopha.2019.109106.

[21] W. W. Hu and Z. Chen, "Role of histamine and its receptors in cerebral ischemia,"

ISSN 3063-8186. Published by Universitas Muhammadiyah Sidoarjo

ACS Chemical Neuroscience, vol. 3, no. 4, pp. 302–311, 2012. doi: 10.1021/cn200126p.

[22] Y. Hu and W. M. Kuebler, "The role of mast cells in pulmonary hypertension," Journal of Rare Diseases Research & Treatment, vol. 2, pp. 1–10, 2017.