Metabolomics study of blood vessels-on-chip model

Kallakkudi Pandian, K.

Chapter 7

Conclusion and Future Perspectives

Conclusion

Endothelial dysfunction and microvascular diseases are the leading causes of cardiovascular diseases, which account for the highest number of fatalities globally. While studies on endothelial dysfunction have advanced significantly in recent decades, the lack of widely used human-specific in vitro models has been a limitation in conventional setups [1]. These setups lack the complexity, blood flow, cell–cell interactions, or proper extracellular matrix (ECM) environment that are typical of living tissues and are needed to assess the functionality of the microvascular system. Accurate micro vessel models are essential for replacing animal models, particularly in cardiovascular diseases (CVDs), as animal models often fail to accurately replicate human pathophysiology.

Microfabricated vessels, also known as "vessels-on-a-chip," are engineered to mimic physiological blood vessels. These hold promises for overcoming limitations of traditional in vitro and in vivo models. The rapid development of vessel-on-a-chip models in the past decade is likely due to the convergence of several key disciplines: blood vessel physiology, transport phenomena, cell and matrix biology, and tissue microfabrication [2]. As an advanced cell culture technology, microfabricated vessels-on-a-chip have successfully recapitulated the endothelial cells, 3D ECM, and structural components of blood vessels of the microcirculation under well-controlled conditions. Thus, as a collective, vessels-on-a-chip have proven to be a reliable and highly useful complement to in vivo studies of microvascular physiology and function [3]. This standing has been propelled by tremendous advances in microfabrication and microfluidic techniques combined with a deep understanding of vascular biology and physiology. Further strengthening the integration between microscale engineering technology and methods from cell and molecular biology is imperative for the continued advancement of vessel-on-a-chip models.

Microfluidic models incorporating a flow condition are particularly valuable for studying vasodilatory compounds like nitric oxide (NO) because physiological flow is a key

regulator of NO production [4]. NO plays a crucial role in various diseases and even possesses antiviral properties [5,6]. However, direct measurement of NO is challenging due to its short half-life, the lack of reserve storage in the cells, and limitations of existing techniques. Metabolomics offers a valuable alternative by providing a snapshot of NO metabolism through measurement of its metabolites. In place of direct measurement, NO biochemistry can be studied by analyzing the metabolites involved in NO mechanisms. Stable isotope labelling coupled with metabolomics offers an even more specific view of the NO pathways and can track a specific metabolic pathway without the interference of other complicated pathways. In addition, personalized medicine and targeted therapies will greatly benefit from considering the unique characteristics of individual cells. Single-cell technologies represent a rapidly evolving field poised to bridge the gap between traditional in vitro analysis and a more nuanced understanding of cellular heterogeneity.

Therefore, this thesis aimed to investigate endothelial dysfunction using in-vitro model systems, with the hypothesis that measuring eNOS activity is key to understanding this dysfunction. For this, we develop a mass spectrometry method for analyzing metabolites involved in NO production using a 3D microvessels-on-chip model. The ultimate goal was to leverage this method and the 3D model to investigate disease conditions and the impact of hypoxia on blood vessels. **Chapter 3** focused on facilitating the use of this method for studying disease by incorporating COVID-19 patient plasma samples into the microfluidic system. **Chapter 4** explored the effects of hypoxia on vessels using the established model. To further refine our understanding, **Chapter 5** delved into advancements in single-cell techniques to unravel cellular heterogeneity within the microvessels/tissues. Finally, **Chapter 6** explored the application of high-throughput screening for efficient analysis of potential therapeutic targets.

Tracer-based LC-MS method and 3D microvessels-on-chip model for the measurement of nitric oxide metabolites

The ultimate aim of tracer-based metabolomics is to precisely identify and track the marker metabolites involved in a specific pathway. **Chapter 2** investigated the development of a mass spectrometry method to measure the metabolites that are involved in NO production using a 3D microvessels-on-chip model. Therefore, we established a stable isotope Ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS)/MS method that measured NO substrate isotope labeled L-arginine and its downstream metabolites L-citrulline and L-ornithine. The exact isotopologue peaks were identified based on the m/z of each calculated transition state of these metabolites. Even more precisely, the level of metabolites was understood better by using an endothelial nitric oxide synthase (eNOS) enzyme stimulator and inhibitor and ornithine (arginase enzyme) production inhibitor. This assay helped us to track the NO metabolites during stimulation and inhibition conditions. To validate this method in 2D well plate cell culture, we confirmed eNOS expression using a reference staining technique (DAF-2DA). Additionally, we demonstrated the specificity of the assay by producing expected results with eNOS stimulator and inhibitors of eNOS and arginase. Next, the same method was employed to measure eNOS activity in two different culture conditions including 2D Culture, which is the standard cell culture format using 2D well plates for the initial studies, and 3D Microvessels model with Flow, which incorporates a microfluidic system to simulate physiological flow conditions. We explored two flow configurations: 1) Bidirectional Flow, which mimics the back-and-forth movement of blood observed in some vessels, and 2) Unidirectional Flow generated by microfluidic pumps producing a more constant flow pattern found in other vessels. This was confirmed through the mechanosensitive gene expressions measured in cells from all these platforms. Importantly, our study demonstrated two key advancements. Firstly, compared to traditional 2D cultures, the 3D microvessels model exhibits higher eNOS activity, potentially reflecting a more physiological environment for endothelial cells. Secondly, the optimized MS method offers a valuable tool for measuring nitric oxide (NO) marker metabolites. This method allowed us to quantify both extracellular and intracellular levels, providing a comprehensive picture of NO metabolism (metabolic flux). Furthermore, the MS assay offers distinct advantages: 1) Medium/Plasma Perfusion: By measuring the conversion of labelled arginine to citrulline in the perfused medium

(extracellular), we can assess the impact of the perfused solution (medium or plasma) on eNOS activity. This approach is significantly simpler than extracting the content from endothelial cells. 2) Reduced Background: Unlike fluorescent probes, which suffered from background interference from the extracellular matrix, the MS method offers greater specificity for NO metabolites. Future investigations could explore higher shear forces for the pump system to simulate diverse microvascular environments with different endothelial cell types. Furthermore, we could optimize the current model to different types of organ-specific endothelial cells with higher shear stress. Additionally, validating the system by comparing its NO metabolism data to in vivo data would strengthen its credibility. This comparison would highlight how well the platform recapitulates the complex environment of a living organism.

Investigation of microvascular diseases

Microvascular dysfunction and COVID-19

Emerging evidence suggests microvascular disease plays a critical role in driving complications and death in severe COVID-19 patients [7–9]. Therapeutic potential of NO for the treatment of deadly COVID-19 is emerging research, already many studies showed the significance of NO in mitigating COVID-19-assoicated symptomatic complications [10,11]. However, in vitro human micro vessel models were not explored much to understand the NO metabolism in COVID-19. To explore the relationship between COVID-19 and NO, in **Chapter 3**, we mimicked the COVID-19 conditions by perfusing the patient plasma samples into the 3D microvessels-on-chip model. This study aimed to investigate the impact of COVID-19 plasma components on the 3D endothelial system, focusing on metabolites involved in NO production. We employed the 3D microvessels cell model described in Chapter 2, incorporating a collagen-based extracellular matrix and a microfluidic channel mimicking a micro vessel lumen, which served as an optimal in vitro platform to study the complex effects of COVID-19 on the vascular system. An observational pilot case-control study was conducted with plasma samples from two

patients diagnosed with mild COVID-19 (low viral load) and severe COVID-19 (high viral load). We perfused these plasmas through the 3D microvessels with NO enzyme stimulators and inhibitors and arginase inhibitors, allowing us to analyze the intricate interactions between endothelial cells and patient plasma components. This approach provided valuable insights into the mechanisms underlying COVID-19 pathogenesis and NO metabolites levels. Using a tracer-based UPLC-MS/MS method, we observed significant effects on NO metabolites in response to patient plasma perfusion. Supplementation with eNOS stimulators, the eNOS cofactor compound biopterin, and arginase inhibition resulted in higher labelled citrulline levels, indicating increased eNOS activity. Additionally, the interplay between arginase activity and eNOS activity provided insights into the regulatory pathways governing citrulline and ornithine metabolism depending on disease severity. A positive correlation between arginase activity and eNOS activity was observed in the severe COVID-19 patient model but not in the mild COVID-19 patient. These findings underscore the importance of endothelial dysfunction in COVID-19 pathogenesis and highlight potential therapeutic targets for mitigating vascular complications associated with the disease. Notably, our study observed distinct NO metabolite responses based on the patients' clinical data and disease severity. This highlights the model's exceptional capacity to differentiate between disease states. This understanding holds promise for identifying pathological markers associated with vascular diseases. This 3D microvessels model offered a valuable tool for investigating the impact of plasma components on endothelial cells, particularly considering its ability to capture detailed eNOS mechanisms. Furthermore, such models facilitate the evaluation of potential therapeutics and interventions targeting endothelial dysfunction, inflammation, thrombosis, and other vascular complications associated with COVID-19. This preliminary study underscored the need for additional patient samples to establish a broader conclusion. Moreover, insights from patients' pathological history, medication practices, and clinical reports pertaining to endothelial dysfunction would be valuable. Future studies would benefit from measuring downstream metabolites of BH4, such as BH2 and neopterin [12,13] in patient plasma before and after treatment with the 3D micro vessel model. This could provide additional validation to our study and also provide

insights into the importance of BH4 in the eNOS mechanism and potentially serve as a more effective therapeutic target.

Metabolic alterations in microvessels under hypoxic condition

Cardiovascular injury results from ischemia, a condition characterized by insufficient blood flow to tissues. This ischemia is often caused by a blockage within the artery walls, typically due to atherosclerosis (plaque buildup). Additionally, inflammation and damage to the endothelium (the inner lining of the artery) can further contribute to impaired blood flow. Hypoxia, a condition characterized by insufficient oxygen supply to tissues, disrupts vascular homeostasis and could lead to endothelial dysfunction. To understand the role of oxygen on NO metabolism in endothelial cells, **Chapter 4** investigated the response of endothelial cells to transient and persistent hypoxia, focusing on metabolic changes in both 2D static cultures and our established 3D microvessels model. Using stable isotope-labeled L-arginine, we tracked the eNOS pathway by monitoring downstream metabolites L-citrulline and L-ornithine and performed additional measurements of inflammation as it is reflected in signaling lipids. Understanding how endothelial cells adapt to hypoxia is vital for elucidating their role in various physiological and pathological processes.

Oxygen-dependent pathways, particularly those involving NO and signaling lipids, are crucial for maintaining vascular homeostasis and resolving inflammation. Our research revealed significant effects of low oxygen levels on the metabolism of NO and signaling lipids, especially within the 3D microvessels model. This model offered a promising tool for analyzing metabolite levels under both normoxic and hypoxic conditions, including the combined effects of hypoxia and shear stress, an understudied area in in vitro culture setups. Our findings suggested a distinct response between the culture models. Exposure to short term hypoxia in the 2D static environment led to increased oxidative stress metabolites such as HDoHE, HEPE, TriHome, and HpETE potentially resulting from the peroxidation of their substrates. Conversely, the 3D platform, incorporating flow, demonstrated regulation of eNOS-derived NO metabolites (L-citrulline+ and L-

ornithine+7) and lower levels of bioactive lipids such as 2,3, dinor-11b-PGF2α and LPE:18.0, promoting an anti-inflammatory response. These observations were further supported by HIF1α immunostaining analysis. Overall, this study unveiled a novel mechanism governing HIF1α and flow-mediated metabolic regulation, potentially leading to new therapeutic strategies for addressing arterial endothelial dysfunction in prolonged hypoxic conditions. This study has certain limitations that warrant further investigation. Here, we focused on exploring the effects of hypoxia at two extremes: low oxygen and maximum hypoxia in short- and long-term exposure. Future studies could benefit from examining a wider range of oxygen concentrations and time of exposure to create a more comprehensive picture of how oxygen availability influences NO and signaling lipid metabolism. Additionally, this study focused on HIF-1α and NO and signaling lipid metabolites. Investigating a wider range of hypoxia-responsive genes and metabolite expressions would offer a more comprehensive view of the cellular response to oxygen deprivation. Including tissue-specific markers would be particularly informative, as these markers can provide insights into the specific adaptation processes occurring within different tissues. Finally, while this study explored some aspects of the hypoxic response, a more in-depth analysis of metabolic pathways, such as energy metabolism, could provide a clearer understanding of how hypoxia regulates cellular function. Future studies could delve deeper into these pathways to elucidate the full extent of the hypoxic regulatory mechanism.

Advanced technologies for single-cell metabolism

Advancements in single-cell metabolomics techniques over recent decades have revealed a significant heterogeneity within cell populations. This newfound awareness presents a crucial challenge for personalized medicine: understanding how these variations impact individual health. To address this challenge, researchers utilized microfluidic technologies, offering a diverse range of approaches with varying levels of throughput and resolution for studying single-cell activity. Especially challenges at single-cell level are real, requiring efficient ways to sample a single cell and match this with a sensitive mass spectrometry method. **Chapter 5** provided a concise overview of the latest

techniques in single-cell sampling and detection methods for metabolomics, particularly focusing on efficient analysis by mass spectrometry. It summarized various microfluidic chip models used for active single-cell separation or sorting based on size, biophysical, acoustic properties, and fluorescent markers. These techniques offer researchers a comprehensive toolbox for evaluating biological properties and responses at the individual cell level, addressing biological, clinical, and pharmacological questions. The chapter demonstrated the applicability of these technologies to diverse cell types. This chapter also highlighted the importance of single-cell metabolomics. Unlike bulk tissue analysis, which might dilute important metabolites present only in a few cell types, single-cell analysis reliably extracts these crucial molecules. Additionally, it serves as a powerful tool for predicting phenotypes and other information with high precision. The combination with imaging mass spectrometry offers the potential to extract spatial information. Advancements in technology and analytical methods have enabled researchers to comprehensively analyze cellular metabolism, leading to the discovery of novel metabolic pathways and diseases.

To advance needed technologies for single-cell metabolomics, in **Chapter 6**, we successfully developed and demonstrated the potential of an integrated electrical lysis setup, the single cell electrical lysis and nanospray (SCEL-nS) platform to the Orbitrap mass spectrometry. This platform seamlessly integrated live single-cell mass spectrometry (LSC-MS) and similar micro sampling techniques for single-cell metabolomics. The development process involved two key steps. First, we designed and manufactured specialized sampling capillaries that facilitated single-cell collection, lysis via electrical stimulation, and subsequent introduction into the mass spectrometer through nanospray ionization. Second, we employed multi-physics software to conduct detailed simulations of the electric field and potential distribution across a cell as it traversed the electrodes within the capillary. These simulation results guided the design of a novel, modified interface specifically tailored for electrical lysis and nanospray MS. To validate the efficacy of the SCEL-nS platform, we employed a two-pronged approach. Firstly, we utilized fluorescence imaging to confirm complete cell lysis. Secondly, we performed MS measurements on single cells spiked with tamoxifen. This allowed us to assess the

efficiency of the lysis process and the subsequent increase in MS signal intensity. Our research addressed the significant challenges associated with single-cell metabolomics, particularly sensitivity and dynamic range. Within a single cell, metabolite concentrations can vary dramatically, ranging from extremely low levels (nanomolar) to highly abundant metabolites (millimolar) alongside intermediates and signaling compounds. The SCEL-nS platform successfully addressed these challenges by delivering significantly higher peak area intensities compared to traditional LSC-MS methods. Notably, this platform achieved efficient cell lysis with minimal dilution, a crucial advantage for preserving precious cellular metabolites. Overall, the MS-compatible SCEL-nS platform emerges as a highly promising solution for future single-cell studies due to its automation potential and high-throughput capabilities. Future improvements should focus on developing more precise and automated methods for single-cell sampling which would improve the efficiency and accuracy of our platform. This could involve microfluidic technologies or robotic manipulation systems capable of isolating individual cells with minimal disruption. Seamless integration of our single-cell sampling methods with microfluidic platforms would enable streamlined analysis workflows. This could involve miniaturized, automated systems that handle cell isolation, lysis (if needed), and downstream analysis steps on a single chip. To broaden the applicability of our platform, testing it with a wider range of drugs and cell types would be crucial. This would involve systematically evaluating the platform's performance across different pharmacological agents and cell lines/tissues. By demonstrating its effectiveness with various drugs and cell types, we can establish the platform's versatility and robustness.

Future Perspectives

Metabolomics technologies play a crucial role in discovering metabolic biomarkers, which aid in clinical diagnosis, prognosis, and therapy evaluation. Notably, tracer-based metabolomics offers a powerful tool for dissecting precise metabolic pathways and identifying dysfunctional pathways in disease states.

Additionally, a complex in vitro model incorporating endothelial cells, smooth muscle cells, and pericytes would provide deeper insights into NO function by mimicking the physiological microvasculature. Future research should explore NO levels in specific disease contexts, correlating them with disease markers and patient history. This could reveal how NO levels change during inflammation (potentially higher) and vasoconstriction (potentially lower). Furthermore, an extensive molecular network integrating eNOS regulatory mechanism and other metabolites as well as associated proteins and genes are crucial for drug discovery and targeted therapy.

Future method development could focus on absolute metabolite quantification with chemically similar analog internal standards to allow to compare studies better. In addition, the method could be further extended in applicability by incorporating enzyme controls and profiling diverse biospecimens. With this methodology we would aim for having real impact in assisting drug development, clinical diagnosis and treatment. Close collaboration with clinical experts is crucial to ensure research relevance and facilitate follow-up studies for validating our preliminary findings. Collaboration with clinicians could facilitate the translation of our in vitro model to clinical practice. This would involve testing promising interventions, such as angiotensin-converting enzyme inhibitors [14], angiotensin AT1 receptors blockers [15], angiotensin-(1-7), antioxidants, sphingosine-1-phosphate and statins on the model and comparing the results with improved endothelial function/ NO production in patients undergoing these treatments. This approach would allow us to assess whether the model accurately reflects NO production changes observed in clinical settings. Moving forward, we should refine our strategy by integrating clinical and mechanistic studies, while combining metabolomics data with multi-omics data. This comprehensive approach using systems biology will pave the way for personalized medicine.

Additionally, this approach could be readily adapted to analyze various cell types and responses to treatments. Such advancements hold promise for identifying sensitive and low-abundant metabolic biomarkers within the complex landscape of heterogeneous cell populations. 3D microvessels-on-chip coupled with isotope-labeled tracers and single-

cell methods hold immense promise for future NO metabolite research. This powerful combination offers a physiologically relevant, controlled, and high-throughput platform. It allows us to delve deeper into the complexities of NO biology, paving the way for novel therapeutic strategies. The integration of single-cell metabolomics with microfluidic techniques further strengthens this approach. This enables high-throughput, precise analysis of cellular responses at the single-cell level, accelerating the development of targeted and personalized therapeutic strategies.

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