Activation of Nrf2 Pathways using Dietary Supplements of Phytochemicals, PB123, to Mediate Effects of HIV-1

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BIOL 3520: Cell Physiology
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March 11th, 2022

Introduction

Human Immunodeficiency Virus (HIV)

Human immunodeficiency virus (HIV), the virus that attacks the body's immune system and ability to fight infection, is a global epidemic. Currently, there are 37.7 million people worldwide who are living with HIV, as of 2020, with 93% of infections recorded outside of sub-Saharan Africa (UNAIDS, 2021). 36.3 million people have died [from the beginning of the epidemic] from HIV-related illnesses, 680 000 of those deaths occurring in 2020 (Simon, et al., 2006; UNAIDS, 2021). First discovered in the 60's, it has since been classified as a Lentivirus, within the family of Retroviridae, and can be further classified into two sub-types, HIV-1, and HIV-2 (Hemother, 2016). Transmitted via bodily fluid, HIV-1 directly infects $CD4^+$ T cells, but almost every organ and tissue has been reported to be pathologically impacted (Karris et al., 2011). More specifically, pulmonary disorders have been reported co-morbidities among those with HIV, including issues relating to lung/airway epithelial tissue (Gingo & Morris, 2014). The mechanisms by which the lungs are affected by HIV are poorly understood; however, there has been a link between chronic-expression of HIV-related viral proteins within the lungs that cause oxidate stress and toxicity to airway epithelium and inhibition of Nrf2 (Kukoyi et al., 2019).

While there is no total cure for HIV, standard treatment involves antiretroviral therapy, which can increase quality and longevity of life (Sankaranantham, 2019). Nonetheless, since the successful integration and launch of mRNA vaccines to combat the SARS-CoV-2 virus, recent developments have been made after successful testing of novel vaccines for HIV-like infections in monkeys (Morris, 2021).

Nrf2 and PB123

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), regulates antioxidant defense and alveolar epithelial cell barrier function (Kukoyi et al., 2019). Nrf2 also counteracts oxidative stress, when working with its endogenous inhibitor, Keap1, and can typically mediate oxidative stress by translocating to the nucleus, heterodimerizing with transcription factor Maf proteins and binding to antioxidant responsive element(s) (ARE), to induce expression of antioxidants and metabolic genes (Bellezza et al., 2018). This is a critical for normal lung homeostasis and can easily be affected by HIV-1 infections. A study conducted by Staitieh et al., demonstrated that HIV-1 infections/exposure can inhibit Nrf2-ARE activity, effecting lung immunity and homeostasis. When Nrf2 activity is functional, it could reduce the number of pulmonary complications experienced by HIV-1 patients. Nrf2 activity is also shown to decrease with age, regardless of HIV-1 infections, and preliminary studies has been conducted for dietary supplements mediating the decreased Nrf2 activity (Hybertson et al., 2022).

PB123 is a dietary supplement composed of phytochemicals *Salvia rosmarinus, Zingiber officinale* and bioflavonoid luteolin. Rosemary (*Salvia rosmarinus)* is shown to have anti-inflammatory, antioxidant, and antimicrobial benefits, whereas Ginger (*Zingiber officinale)* shows anti-inflammatory, analgesic, and digestive benefits. Luteolin shows anti-inflammatory, antioxidant and neurological benefits, often used as a dietary supplement (Hybertson et al., 2022). PB123 has been effective at upregulating Nrf2 activity in HIV-1 transgenic rats (Hybertson et al., 2022; Kukoyi et al., 2019).

Objective

The objective of this research is to test the effectiveness of phytochemical dietary supplements, such as PB123, on the activation and upregulation of Nrf2 activity to enhance lung health and reduce pulmonary complications.

Materials and Methods

Permissions

Use of animals for this experiment will be pre-approved by the TRU Animal Care Committee and all animals will be treated in accordance with the TRU Animal Care policy.

Plant extract

Plant extracts will be required to create and standardized PB123 solution. Ginger root (from *Zingiber officinalis* – standardized to 20% gingerols) and rosemary (from *Salvia rosmarinus* – standardized to 6% carnosol; 15% carnosic acid) will be extracted from their respective plants, combined with luteolin (*Sophora japonica* – standardized to 98% luteolin) to create PB123 powder. Plant extracts will be obtained from Flavex (Rehlingen, Germany) and luteolin obtained form Jiaherb (Pine Brook, NJ, USA). PB123 powder will be prepared by mixing a 10:5:1 mass ratio of rosemary ginger and luteolin powders and will be extracted in ethanol to collect the supernatant (Hybertson et al., 2022).

Cell culture

Alveolar epithelial cells (AECs) will be cultured to determine the effects of Nrf2 inhibition. AECs were chosen as they are directly regulated by Nrf2 and are directly affected by HIV-1 infections. Primary AECs will be isolated, from six 9–12-month-old HIV-1 transgenic rats (HIV-1 Tg) and three littermate wild-type rats, both in Fischer 344 background and cultured

in DMEM/F12 (Cellgro, Manassas, VA) with 10% FBS (Atlanta Biological, Lawrenceville, GA) and the antibiotic-antimycotic agents: penicillin, streptomycin, and ampicillin (Sigma-Aldrich, St. Louis, MO) at 37ºC in 5% CO_2. The HIV-1 Tg rats will be obtained from Harlan Laboratories (Indianapolis, IN).

In both humans and rats, HIV-related proteins are found within the alveolar space. AECs monolayers will be cultured on a permeable transwell plates for 5-6 days. All procedures approved by the TRU Animal Care Committee. Western blotting will confirm HIV-1 infection (Kukoyi et al., 2019; Reid et al., 2001; Staitieh et al., 2017).

Cell viability assay

Cell viability will be tested to determine cell proliferation and the cytotoxicity of the compounds (PB123) being examined. If cells continue to proliferate with PB123 present, this will indicate that there is not a high cytotoxicity rate and will not be toxic to alveolar epithelial cells. Fan et al., suggested that Nrf2-Keap1 pathway promotes cell proliferation. Similar to this 2017 study, a MTT assay (Roth) (5 $mgml^{-1}$ at 37ºC, in 5% CO_2) will be used to measure cell proliferation rates. Cells will be plated in a 96-well plate and incubated at 37ºC, for 24 hours to adhere. After the 24-hour incubation period, the MTT solution will be removed, and cells will be treated with varying concentrations of PB123. The value will be measured with SLT Spectra (Crailsheim, Germany). The control group will be exposed to Isopropanol + 0.1N HCl, instead of PB123. For cell counts, the cells will be washed with PBS, 2 ml trypsin and incubated for 5 minutes. A Z2 cell counter (Beckmann-Coulter, Brea, CA, USA) will be used (Hybertson et al., 2022; Fan et al., 2017).

Enzyme fragment complementation assay

Enzyme fragment complementation (EFC) is a way to analyze the expression of an enzyme as two separate fragments, N- and C- terminus, that do not assemble or become functionally active. Using the N- and C- termini of firefly luciferase fragments, typically inactive in cells, they fuse to the termini of respective proteins, thus activating luciferase resulting in an active reporter enzyme which can be detected by luminescence imaging; therefore, the activity of fusing proteins is directly related to the signal generated (Ramankumar et al., 2013). Based on this previous research by Ramankumar et al., a highly specific and sensitive EFC can be created to monitor Nrf2-Keap1 activity. Cells will firstly be exposed to known Nrf2 activators, such as $CoCl_2$ and resveratrol, and tested at different concentrations for 24 hours. Cells will then be harvested and assayed for luciferase activity using a luminometer (Prometa, Madison, WI, USA). (Ramakumar et al., 2013).

Nrf2 Reporter gene assay

Reporter gene assays will be beneficial to this study as it will measure the regulatory ability of Nrf2, using previously mentioned luciferase. (ThermoFisher Scientific). Cells will be treated with the plant extract + luteolin. Synergy will be measured by comparing the Nrf2 activation signals with varying concentrations of PB123 and each plant extract (Hybertson et al., 2022; Hybertson et al., 2019; Rumankumar et al., 2013; ThermoFisher Scientific).

Epithelial monolayer permeability assay

A permeability assay will be employed to ensure PB123 is effectively absorbed to combat their prescribed issues. To ensure epithelial barrier function of AECs, the monolayers will be quantified using paracellular flux of FITC-labeled Dextran (Sigma-Aldrich, St. Louis, MO) over a two-hour period (Kukoyi et al., 2019; Volpe, 2010).

Dietary treatment with the Nrf2 activator PB123

Using a dietary treatment to activate Nrf2 is a feasible and practical option to ensure the public has access to the benefits of PB123. In a similar study, performed by Kukoyi et al., in 2019, three wild-type rats and six HIV-1 transgenic rats were segregated and treated with PBS123, after being fed a Leiber-DeCarli liquid diet. From this study, we will segregate control rats and diseased rats and treat with PB123, along with the Leiber-DeCarli liquid diet (Research Diets, New Brunswick, NJ), for two weeks. Rats AECs will then be harvested and assessed. The barrier function and monolayer formation of AECs will be compared to ensure Nrf2 function and barrier formation is improved in the HIV-1 rats. This will be done in vivo, and ex vivo to determine whether supplementing PB123 through diet or injecting it directly will produce better results (Kukoyi et al., 2019).

Statistical analyses

Experiments will be performed in triplicates, to ensure accurate results. Experimental comparisons between treatments will be analyzed using Students t-test. Image data will be analyzed using ImageJ software. Synergyfinder.org will be used for calculations relating to the synergy model.

Expected outcomes

This study will build on and support results found by other researchers in this field. Based on previous studies conducted by Hybertson et al., in 2022, they found using PB123 as a dietary supplement upregulates age-related inhibited Nrf2 activity and proves to be a potent Nrf2 activator. Similarly, in 2019, Kukoyi et al., used PB123 as an alternative method to mediate Nrf2 activity, using the dietary supplement for HIV-1 related Nrf2 inhibition, and provided

similar results to microRNA mediation. This research provides a proposed therapeutic cure for Nrf2 inhibition to lower pulmonary complications; these results are expected to be repeated in this study with improvement due to the proposed mediation of the mechanism, by focusing on PB123 as a treatment option.

Benefits of this research

Currently, there is no cure for HIV-1, and mortality rates are yet to drastically decline. Whilst antiretroviral therapy is an available and widely used option for treating individuals living with HIV-1, there are still adverse health effects, including pulmonary immunity impacts, which can lead to greater issues, complications and even death as the burden of HIV increases (Twigg & Knox, 2013). Antagonizing mRNAs using novel therapeutic agents [for oncogenic purposes] has been proposed by Nguyen & Chang, 2018, which demonstrated antagonizing mRNAs could provide therapeutic benefits to cancer. Based on previous studies by Kukoyi et al., antagonizing mRNAs associated with HIV-1 related proteins yields the same results as using PB123 as a dietary supplement. This proposed study could continue their theory and provide a novel therapeutic approach, by dietary supplements, to increase lung health for patients living with HIV-1, even when using antiretroviral therapies. If preventing the inhibition of Nrf2, HIV patients could increase their quality of life, and possibly prevent further lung complications. These finding could also roll over to age-related inhibited Nrf2 activity and protect the elderly from pulmonary complications that may arise

Dissemination

After results of the above methods has been determined, the findings will hopefully be published in the journal AJP Cell Physiology or OBM Integrative and Complementary Medicine. These two peer-reviewed journals are of interest as they are not only popular, but published

similar studies, including both articles this study is building on: miR-144 mediates Nrf2 inhibition and alveolar epithelial dysfunction in HIV-1 transgenic rats by Kukoyi et al., and Effects of the phytochemical combination PB123 on Nrf2 activation, gene expression, and the cholesterol pathway in HepG2 cells by Hybertson et al.

To relay this information to the general public, a blog post describing the study and its findings could be posted on HIV.gov, a website dedicated to providing up to date information, increasing knowledge and to increase the use of media tools to extend the reach of programs for people living with HIV. This post would include colloquial language and images, to encourage everyone, no matter their scientific knowledge, to understand the importance of this research. Additionally, a short video, including animations of pathways and processes, could be created by TED-Ed, allowing everyone to understand the findings in a relaxed and visual way.

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