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Reducing Atherosclerotic Plaque Development and Endothelial Hemichannel Activity with WIN-55,212-2: A Research Protocol

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Abstract

Introduction: Endothelial cells (ECs) are critical regulators of vascular homeostasis, and their dysfunction leads to the development of atherosclerosis – the main underlying cause of cardiovascular diseases (CVDs). This dysfunction can be promoted by prolonged endothelial connexin43 hemichannel activity, which is caused by the combination of high glucose levels and cytokines IL-1β/TNF-α. WIN-55,212-2 (WIN) is a synthetic agonist of CB1/CB2 receptors and can counteract the proinflammatory effects of high glucose and IL-1β/TNF-α. We hypothesize that WIN treatment on ECs will reduce connexin43 hemichannel activity, thus preventing endothelial dysfunction and atherosclerotic progression.

Methods: We will use the Apolipoprotein E Knockout ($ApoE^{-/-}$) mouse model to assess the impact on atherosclerotic lesions. Hyperglycemia will be generated in these mice with Streptozotocin injections. The increased levels of glucose should induce IL-1β expression and stimulate prolonged hemichannel activity. ECs will be isolated from a subset of mice and cultured to test WIN-efficacy. ATP release will be assessed through an ATP viability assay. More *in vitro* assessments on subsets of $ApoE^{-/-}$ mice treated or not with WIN will be performed. Flow cytometry will evaluate monocyte-derived macrophage concentration and other pro and anti-inflammatory cytokines in tissue samples. Furthermore, atherosclerotic plaque volume in the aortic sinus will be quantified and characterized.

Results: We expect that WIN-treated ECs will reduce ATP synthesis compared to those from the control group. Moreover, we expect to see a reduction in the inflammatory response with a consequent decrease in atherosclerotic progression.

Discussion: This manuscript outlines the use of a novel compound that could prevent atherosclerosis progression. The results of this study could outline a potential mechanism that may be targeted to treat or forestall atherosclerosis progression.

Conclusion: Overall, we aim to determine if WIN may not only hinder this pervasive condition but inhibit CVDs through curtailing atherosclerotic plaque development. The following steps include performing the experiment, confirming results through repetition, and using other animal models.

Keywords: WIN-55,212-2; endothelial cells; endothelial connexin43 hemichannel; IL-1β/TNF-α cytokines; ATP assays; $ApoE^{-/-}$ mice; flow cytometry; aortic sinus

Introduction

Atherosclerosis is an inflammatory disease that constitutes a buildup of plaque in arterial walls, leading to the hardening and narrowing of arteries [1]. Diabetes, and other forms of chronic hyperglycemia, are strongly associated with an increased risk of developing atherosclerosis [1]. The significant immune reaction to this condition contributes to the formation and activation of atherosclerotic plaques [2]. These plaques are prone to rupture, which, in conjunction with constricted blood flow, may result in myocardial infarction and ischemic strokes [1]. Given that atherosclerosis is a primary cause of CVDs, interventions directed towards treating or slowing its progression are needed [1].

Endothelial cells (ECs) are particularly vital in regulating vascular homeostasis, and their dysfunction is a known cause of atherosclerotic development [1]. One 2018 study revealed that endothelial deterioration is precipitated by prolonged connexin43 hemichannel activity [3]. Connexins are molecules that form gap junctions and hemichannels on the plasma membrane [3]. The endothelial hemichannels (EHs) generally control the release of ligands to facilitate biological processes like the exchange of ions between intracellular and extracellular compartments [3]. A prolonged opening of hemichannels is involved in disease progression by inducing the increased release of paracrine substances like ATP, alterations in Ca^{2+} handling, and ionic and osmotic imbalance [3]. Together, hyperglycemia and pro-inflammatory cytokines IL-1β and TNF-α lead to an increased hemichannel activity and affect intracellular signalling pathways in ECs [3]. This reduces endothelial

barrier function while also impacting vascular tone regulation and insulin resistance [3].

WIN-55,212-2 (WIN) is a synthetic agonist of CB1/CB2 receptors, which are expressed by ECs. The endothelial CB1/CB2 receptors can produce an anti-inflammatory effect that was shown by the previous study to counteract the pro-inflammatory effects of hyperglycemia by modulating the pro-inflammatory cytokines, including IL-1β/TNF-α [3]. With WIN, both the mediated release of ATP and hemichannel function were reduced – as measured through ethidium uptake [3]. Furthermore, changes to ATP-dependent Ca^{2+} dynamics, NO production, and a reduction in endothelial cell-cell coupling were all prevented [3].

We hypothesize that the treatment of WIN on ECs will reduce endothelial connexin43 hemichannel activity and thereby decelerate endothelial deterioration and disease progression. WIN is illegal in the United Kingdom due to the Misuse of Drugs Act banning all compounds structurally derived from 3-(1-naphthoyl) [4]. However, targeted treatment with this compound might not cause severe side effects, and there is a history of it benefiting other parts of the body. For example, WIN decreases brain damage in fetal lambs after hypoxic-ischemic injury, as determined by reduced apoptotic cell numbers and an increased percentage of S-100 proteins in all investigated regions [5]. Furthermore, cannabidiol (CBD) has a positive history with cardiomyopathy – with an anti-inflammatory and anti-oxidant effect that benefits diseases like diabetes [6]. Therefore, we have designed that WIN will not be provided as a full-body fix due to its high potency as a CBD substance [7].

Methods

Spontaneous atherosclerotic lesions may be examined through the Apolipoprotein E Knockout ($ApoE^{-/-}$) mouse model. $ApoE^{-/-}$ mice naturally have an elevated expression of IL-1β in plasma, the brain, and the aorta [8]. Since both hyperglycemia and IL-1β/TNF-α are necessary to increase hemichannel activity, Streptozotocin (STZ) injections will also be used to induce high glucose levels [3]. STZ (40 mg/kg, intraperitoneally) will be injected once daily for five days starting when mice are eight weeks of age.

ECs will be isolated from the mouse model cultured in vitro to observe hemichannel activity. They will then be treated with WIN (0.1mg/kg) daily for one week. Given that prolonged hemichannel activity leads to an increase in ATP, an ATP assay will be adopted to ensure that WIN does reduce the EH activity. The CellTiter-Glo® 2.0 Cell Viability Assay – which can be used along with other assays – will be applied daily to assess the gradual effect of WIN [9]. Before use, the CellTiter-Glo® 2.0 Reagent must be set to room temperature [9]. Then the multiwell plates can be prepared with mammalian cells in a culture medium, equilibrated to room temperature, and mixed with the reagent on an orbital shaker [9]. The luminescent signal produced is correlated to the number of cells in culture [9].

Flow cytometry will be used to determine the impact of WIN on the immune response. Monocyte-derived macrophages accumulate on atherosclerotic plaques, so we can observe the pro and anti-inflammatory cytokines in tissue samples to examine the influence of WIN treatment on disease progression [2]. On day 7 of WIN treatment, blood plasma will be collected to assess the impact of WIN through differences in the immune cell population and inflammatory cytokines (T cells and macrophages) compared to the control group – which constitutes STZ injections sans WIN.

The heart will also be collected from the experimental mice to measure atherosclerosis in the aortic sinus, as this is where the condition begins formation in mice [10]. The aortic sinus slides will be stained by monoclonal antibodies to MAC3 with immunofluorescence. The immune response with WIN treatment will be compared to the control group (untreated). This will serve as a secondary verification of WIN efficacy in reducing atherosclerotic lesions. Figure 1 depicts the methods used to assess the impact of WIN on EH activity.

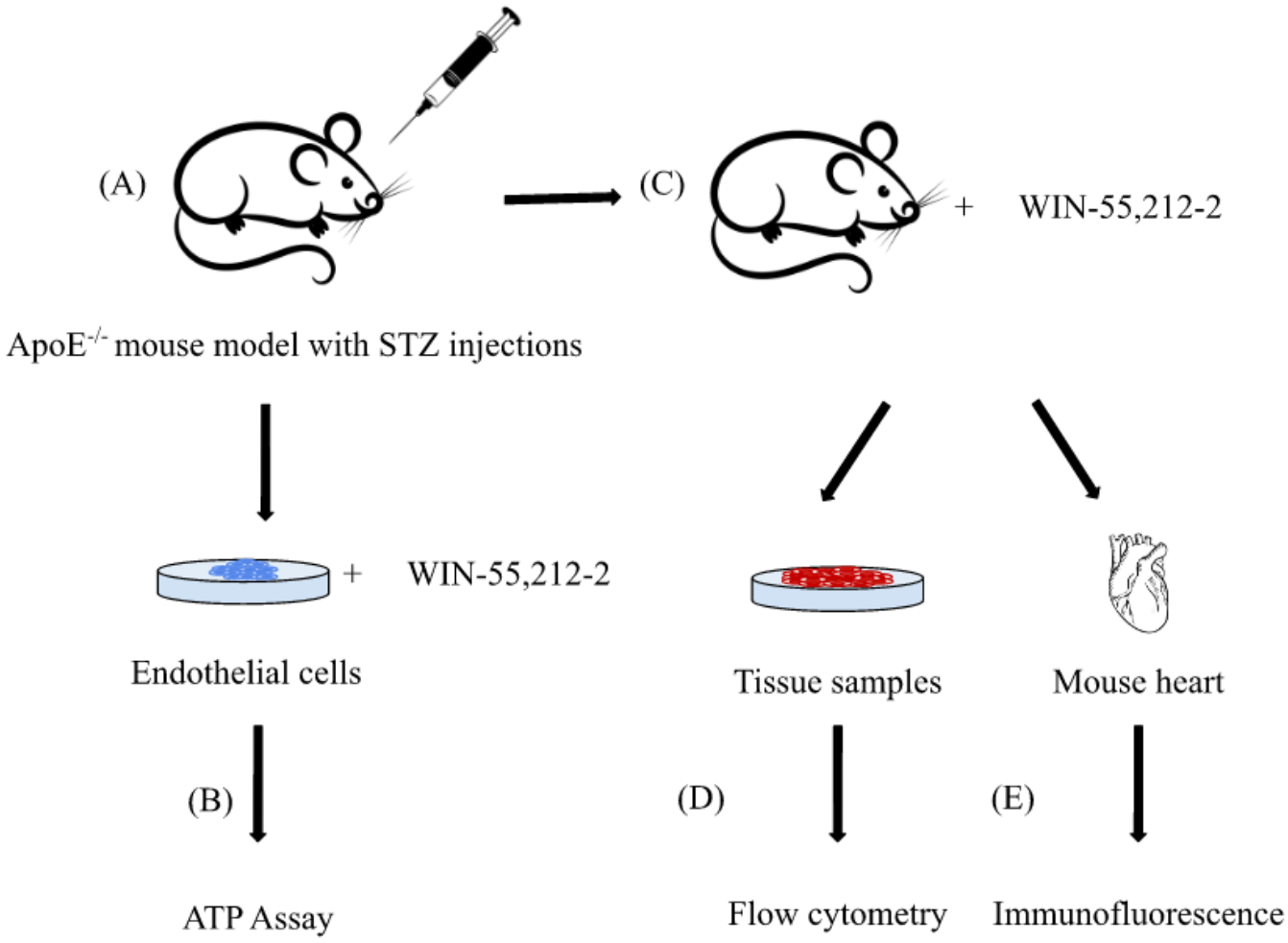

Figure 1. A depiction of the methods used to assess WIN's impact on EH activity. (A) ApoE$^{-/-}$ mice will be injected with STZ to induce hyperglycemia. (B) ECs will be isolated and treated with WIN. An ATP assay will be used to measure ATP release. (C) ApoE$^{-/-}$ mice will be treated with WIN. (D) Tissue samples will be collected, and flow cytometry will be conducted to assess immune cell and cytokine changes. (E) Immunofluorescence of aortic sinus slides will be conducted to assess changes in the immune response (Figure created with Microsoft PowerPoint using ClipartALL [Image]. Syringe Clipart, Openclipart [Image]. Mouse Outline clipart, and Seamartini [Image]. Isolated human heart outline sketch).

Results

We predict that WIN would inhibit the prolonged opening of EH activity, reducing the concentration of extracellular ATP. There should be fewer pro and anti-inflammatory cytokines in the WIN-treated tissue sample than in the control group. Finally, we expect a reduced MAC3 expression in the aortic sinus of the experimental group compared to the untreated group. There is the possibility that no difference can be found between the control and WIN-treated groups. This can happen for various reasons – especially random or systematic errors. Repetition of this study, as well as ensuring all devices and technologies are functioning correctly, are, therefore, necessary to confirm the accuracy of the conclusions drawn.

Discussion

The primary purpose of assessing the results would be to confirm an alternative hypothesis in which there is a difference between the control and experimental group. First, the luminescence of the cultured EC samples tested with the ATP viability assay will be plotted, and the extracellular ATP concentration will be estimated from the curve. Then, flow cytometry analysis will include the cytogram to determine the percentage of cytokines in the plasma sub-population. Finally, quantitative tissue absorption studies, including the Aperio Positive Pixel Count FL Algorithm, must be performed to observe the expression of MAC3 in the aortic sinus slides.

Conclusions

This publication intends to exemplify another mechanism for reducing atherosclerotic plaque. With atherosclerosis as the primary cause of cardiovascular diseases – which kills 1 in 4 people every year in the United States – it is vital that we find a way of slowing the disease's progression as a preventative course of action [11]. Several cardiovascular-related deaths can therefore be avoided in the future. Furthermore, regulating EH activity can inhibit other conditions, including cell death [12]. Overall, such a novel therapeutic strategy for CVDs – especially in people with diabetes – is crucial to the healthcare system, where there is already a significant burden from these conditions.

If there were a success with WIN in reducing hemichannel activity and hindering atherosclerotic plaque development, the next step might include a human trial. From an ethical standpoint that likewise employs the scientific method, this experiment must be repeated several times to confirm the results are accurate. Other animal models may be used to verify and validate our results. While the small animal models are great representatives of atherosclerosis, large animal models are typically employed for further exploration into the condition's prevention and treatment, which may be better translated to humans [13]. Gene-modified minipigs serve as one example of the future of this investigation. These minipigs can develop atherosclerosis and are similar to humans in their predisposition to lesions and histopathology [13]. Several routes – especially animal models – can be taken to ensure WIN-efficacy and elicit its eventual clinical application to manage atherosclerosis.

List of Abbreviations Used

ApoE$^{-/-}$: Apolipoprotein E Knockout
CBD: Cannabidiol
CVD: Cardiovascular disease
EC: Endothelial Cell
EH: Endothelial Hemichannel
STZ: Streptozotocin
WIN: WIN-55,212-2

Conflicts of Interest

The author declares that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study would require ethical approval for animal treatment if the experiment is carried out. Participant consent is unnecessary as the study does not include a human trial.

Authors' Contributions

SMB: contributed to study design and planning, drafted the manuscript, and gave final approval of the version to be published

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