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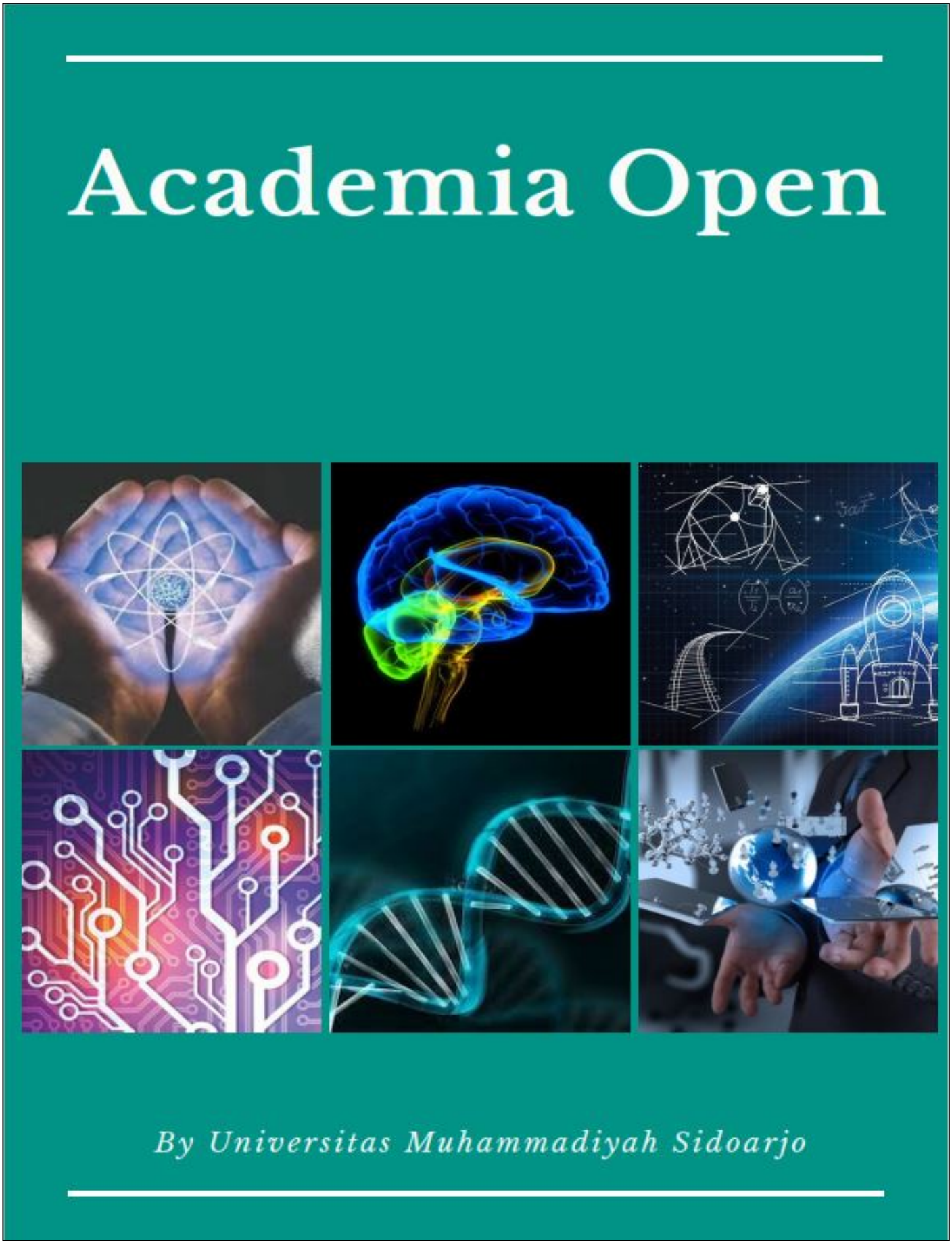

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Modulation of p53 and p21 Genes Expression in Cardiovascular Cultured Cells

Modulasi Ekspresi Gen p53 dan p21 dalam Sel Kultur Kardiovaskular

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Abstract

This study explores the role of the p53 and p21 genes, central to the cell cycle arrest pathway, in cardiovascular diseases, focusing on their modulation by endocannabinoid ligands. Previous research has established the significance of CNR1 in cardiovascular regulation; however, its interaction with p53 pathways remains underexplored. We aimed to investigate the effects of Anandamide and Rimbonant on the expression levels of p53 and p21 in smooth muscle cells at both mRNA and protein levels. Our results indicate that Anandamide significantly increases p53 mRNA (3.191±0.38, Pv≤0.01) and protein (31.37±2.60) levels, while Rimbonant shows a decrease after 1 hour of treatment. Similarly, p21 expression was upregulated by Anandamide and downregulated by Rimbonant. These findings suggest that manipulating p53 activity through CNR1 ligands could potentially mitigate cardiovascular disease risks, warranting further investigation into their pharmacological applications.

Highlights:

- Gene Modulation: Anandamide boosts p53 and p21 expression.
- Rimbonant Effects: Rimbonant reduces these gene levels.
- Therapeutic Potential: Indicates possibilities for cardiovascular disease treatment.

Keywords: P53 Gene, Cardiovascular Disease, Endocannabinoids, Anandamide, Rimbonant

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Introduction

Transcription factor P53 gene regulates innumerous number of genes to maintain the normal function of cells during transpression and transactivation. Increasing p53 expression level can cause cell cycle arrest, metabolism alteration and apoptosis [1]. These functions resulted in DNA damage, ribosomal dysfunction, oncogene expression, hypoxia and oxidative stress.

It has been observed increasing in activity and expression level of p53 in dog model that coupled with increasing in cell death program [2]. Increasing p53 expression was investigated in heart failure samples of human. P53 deletion decreased the function of heart and triggered hypertrophy . P53 has primary mechanisms to implicate the pathogenesis of cardiac diseases. Post transcriptional alterations including ubiquitination, glycosylation, acetylation and phosphorylation controlled p53 proteins regulation. MDM2/MDM4 promote ubiquitination of p53 protein to export and degrade p53 proteasomal. When stress stimulation occurs, siruin-1 de-acetylate and activate p53 at k382, k379, k373 to promote the transcription of p53, p300, CBP and PCAF were also stimulate to enhance the transcription of p53 at k370, k373, k382 via the acetylation leading activation and stabilization of p53. These an examples to promote p53 expression via acytelation and deacytelation processing.

P21 gene expression dependent on p53 gene, p21 performs some functions of p53 and consider as a downstream mediator. When DNA damage occurred in the cells or exposed to chemical radiation, the expression of p21 and p53 were highly increased in wild type cells of p53 while cells that have a deficiency in p53 expression usually do not express 21 at high level causing inhibition of cell cycle arrest. The classical pathway of p53 and p21 requires to regulate the arrest of cell cycle [3]. Recently, Alebady *et al*. indicated that triggered cell death program, which reflected via the high level of p53 and p21 that required to regulate the arrest of cell cycle.

Endocannabinoids ligands are lipid components created from phospholipids that located in the cell membrane, they stimulate the cannabinoid receptors, which include CNR1 and CNR2 receptors. CNR1 vastly expressed in the nervous system and expressed in peripheral system, including the cardiac tissues, coronary artery of human, myocardium and smooth muscle cell. CNR1 encoded by CNR1 gene that located in chromesome 4 in mice and chromosome 6 in human. This gene implicated in apoptosis, inflammation, oxidative stress and metabolic dysregulation. CNR1 plays role in the function regulation of cardiovascular system. Knockout CNR1 can lead to chronic failure of heart and cause stroke. CNR1 receptor expression has been increased in coronary atherectomy specimens of human and its expression was decreased in fibrous plaques compared with atheromatous plaques that riches in lipid. Experimental and clinical studies indicated that systemic and local elevation levels of endocannabinoids ECS were positively incorporates with atherosclerosis and suggested that endocannabinoids could be biomarkers of vascular disease or risk factors of ongoing cardiovascular disease, increasing levels of ECS was estimated in samples of coronary artery disease. Endocannabinoids in the heart and vessels act by stimulating CNR1 and CNR2. Anandamind that is also called N-arachidonoglethanolamide (AEA) is an endogenous stimulator and has a high affinity of CNR1. Overstimulation of CNR1 via endocannabinoids activators can lead to liver cirrhosis hypotension and myocardial infraction [4].

CNR1 activation increases pro-inflammatory, pro-fibrogenic and pro-oxidative in states of cardiac disease. These increasing can lead to accumulate collagen inflammatory cytokines, reactive oxygen species and apoptotic. Additionally, increasing p53 expression level can cause cell cycle arrest, metabolism alteration and apoptosis [5].

According to implicate CNR1 and P53 genes in cardiovascular disease and their role in maintaining the normal function of cells, cultured cardiovascular smooth muscle cells were utilized to stimulate CNR1 and to investigate p53 and P21 expression levels if were modulated and to highlight their possibility of utilizing p53 as a pharmacological target to treat cardiovascular diseases.

Methods

A. Cell Culture

Human vascular smooth muscle cells (hVSMCs) were cultured in 231medium (Thermo Fisher), which supplemented with 5% of SMGS to induce a normal proliferation of cells and 10% FBS in optimal conditions including 5% CO2 humidified atmosphere at 37°C.

B. CNR1 Activator/Inhibitor Treatment

When cultured cells reached up to 80% confluence in 6 well plates, CNR1 activator (Anandamide) was added to the cells at (1µM) concentration for 24 hours. (10µM) of inhibitor (rimonabant) was incubated with activated cells for 1 hour.

C. Real Time PCR

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Following hVSMCs treatment, total mRNA was extracted utilizing Trizol (Invitrogen), then was treated to discriminate any genomic DNA using DNaseI (Invitrogen), followed Iscriptc DNA kit from (Bio-Rad) was used to reverse transcribed. qPCR was conducted utilizing SYBR green one step kit (Invitrogen), following primers were used to measure interested gene expression level :

Gene	Primer Sequence
P53	F 5' (CCC CTC CAT CCT TTC TTC TC) 3'R 5'(ATG AGC CAG ATC AGG GAC TG)
P21	F 5'(GAC CAG CAT GAC AGA TTT C)R 5'(TGA GAC TAA GGC AGA AGA TC)
β-actin	F 5'(ACT CCT ATG TGG GCA ACG AG)R 5'(AGG TGT GGT GCC AGA TCT C)

Table 1. *Primers sequence of p53 , p21 and β-actin*

D. Protein A ssessment

Media of growth was removed and the cells were rinsed two times with PBS. 1.5 mL of extraction buffer was added directly to the confluent plate. Scraped cells were transferred into a tube and the lysate kept on ice for 15 min. Then, lysate was centrifuged at 18,000 rpm for 20 min. supernatants were collected into a clean tube. ELISA kit (ab156027) was applied to assess p53 protein level. While (ab214658) ELISA Kit was applied to assess p21protein level.

E. Statistical Analysis

Computational program Graphpad (prism 8.3) was used to analyze resulted values including ANOVA (One way) analysis was performed to compare one factor between multiple groups. Significant statistic of values was presented at pv≤0.05. values were viewed as mean ±SEM. Each experiment was conducted at least with three times.

Results and Discussion

A. Results

1. mRNA level of p53 and p21 genes

qPCR assay was used to measure the effect of CNR1 activation and inhibition on mRNA level of p53 and p21 genes. Following 24 hours of exposure to Anandamide with 1μM hVSMCs was significantly expressed p53 more than three times (3.19±0.38, pv<0.001), while after exposed these cells to Ribombant, p53 expression was decreased up to two times (2.11±0.15,pv≤0.01) as showed in Table (2), Figure (1,A).

p21 mRNA expression represented significant increase after exposing to Anandamide (1μM) for 24 hours at (1.90±0.17, pv≤0.01). Then hCVMCs were exposed to antagonist of CNR1 with (10μM) and p21 expression was significantly decreased up to (1.14±0.23, pv≤0.05) compared with cells that only exposed to agonist of CNR1 Figure (1,B).

Treatment groups	mRNA Folding changing	
	P53	P21
Control	1.000 ± 0.013a	1.000 ± 0.021a
Anandamide (1μ)	3.191 ± 0.3805b	1.907 ± 0.1793b
Anandamide (1μ) + Rimonabant (10μ)	2.118 ± 0.1500ac	1.140 ± 0.2328ac

Table 2. ***F olding change of p53 and p21 mRNA levels between treated groups***

Variable data exhibited as Mean±S.E. In same column, the similar litters showed non statistically significant differ. While different litters presented as statistically significant differ when pv equal or less from 0.05.

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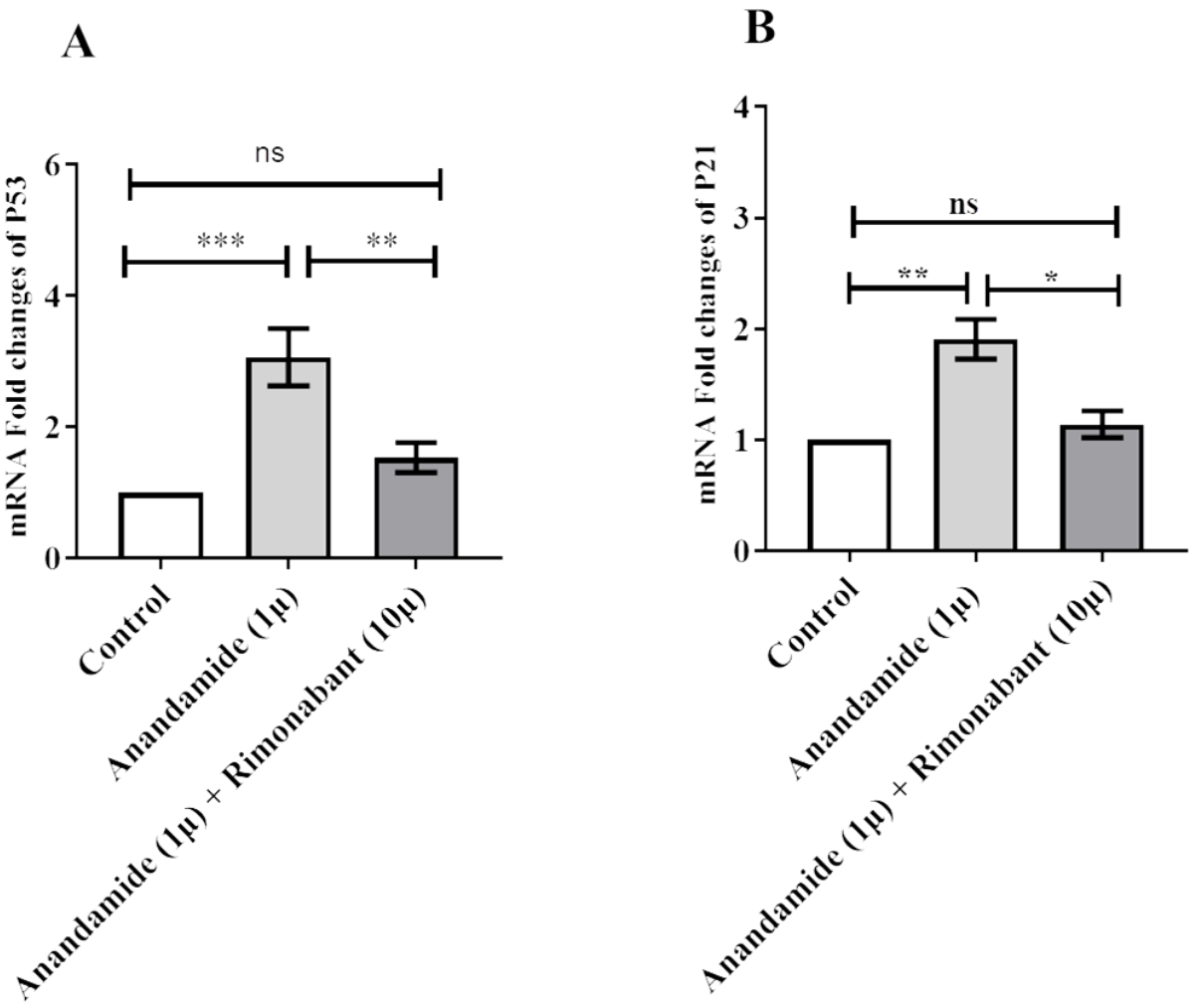

Figure 1. ***qPCR data analysis of mRNA levels. A-fold change of mRNA level of p53 gene***

B-fold change of p21 gene. T he data of expression were normalized with β-actin as a n interior control. Data expressed as Mean±S.E. n=9 replicates. Significance was detected by p value less than 0.05.

2. Protein expression of p53 and p21 in cultured cells

In addition to qPCR quantification of p53 and p21 mRNA levels, protein expression was quantified to confirm endocanibinoids ligands modulate protein expression of p53 and p21. At 24 hours of exposing to agonist of CNR1, was a significant change of p53 gene protein expression (31.37±2.60, pv≤0.01) compared with the control which expressed at (10.31±0.60). When activated cells with anandamide, which treated with CNR1 antagonist (10µM) for 1 hour a significant decreased in p53 protein expression was observed at (17.07±1.60,pv≤0.05) compared with cells that activated with anandamide Table(3) as shown Figure (2).

In addition to measure p53 protein expression in this assay, protein level of p21 that downstream regulated of p53 gene was assessed, to demonstrate whether modulation of p21protein level corresponds with change of p53 alteration. As well as to investigate endocanibinoids ligands exposer affected p21 protein expression level or this alteration restricted to mRNA level only. hCVMCs treated for 24 hours with anandamid showed a highly increasing (26.74±0.34, pv≤0.05) compared with control cells (13.40±0.12) Table (3). In contrast, incubating hCVMCs with antagonist CNR1 (rimonabant) for 1 hour decreased p21 protein expression as shown Figure (2,B) compared with cells that treated with anandamide only (26.74±0.34).

Treatment groups	Quantitative protein expression	
	P53	P21
Control	10.31 ± 0.600a	13.40 ± 0.1223a
Anandamide (1µ)	31.37 ± 2.600b	26.74 ± 0.3417 b

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Anandamide (1µ) + Rimonabant (10µ)	17.07 ± 1.600ac	15.73 ± 0.1798ac

Table 3. ***Q uantitative p53,p21 proteins expression***

In same column, same litters refers to significant value while differ litters represent non –significant change. Data were expressed as mean ± S.E.

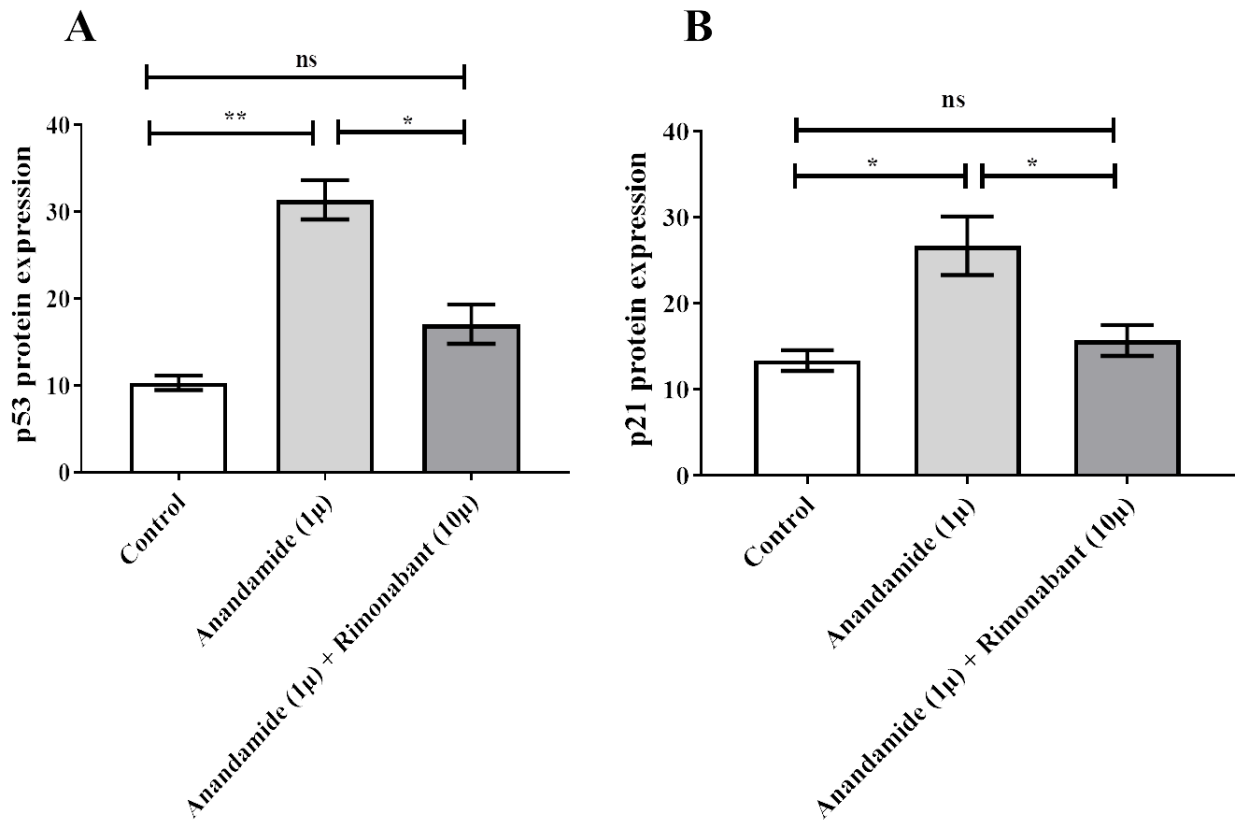

Figure 2. ***p53,p21 protein expression in h VSMCs which treated with AEA and rimonabant***

A- p53 protein expression B- p21 protein expression that normalized to β-actin that served as a c ontrol. Th is experiment was conducted three times and data were presented as mean ± S.E. Significance is represented by p value ≤0.05.

B. Discussion

In the last decades, tumor suppressor (p53) gene has drawn the attention of scientists on its impact in cardiovascular function [6]. It is determined to play a regulator role and could be implicated in the cardio remodeling, pulmonary hypertension or atherosclerosis. Several stressors factors including telomere dysfunction, oxidative stress and DNA damage induce several genes including p53 and causing cellular senescence. Even though, senescence is a protective mechanism to reduce tumors developing, accumulating cellular senescent could cause cell aging of vascular. Aging of endothelial cells of vascular plays a vital role in CVDs progression [7].

P53 implicated in regulating pathway of cellular senescence with significant increasing of inflammatory signaling of older adults arteries. P53 has several mechanisms to increase the progression of CVDs. In current work, hVSMCs was cultured to evaluate the expression level of p53 and its regulator p21 gene in mRNA and protein levels. Knockout p53 in artery mouse module decreased VSMCs apoptosis [8]. p53 expressed in hVSMCs, therefor we chose this type of cell lines as a model to perform current project and test our hypothesis does endocanabinoids ligands affected p53 and p21 expression in hVSMCs, when some studies provide evidence in involving cannabinoids receptors in CVDs. A significant expression of p53 and p21 when incubated with anandamide for 24 hours, it has been detected in current study. These finding in agreement with the study of Downer *et al.,*. They reported that cannabanoids ligand activated via Δ-tertiary cannabinoid type of endocannabanoids ligands. Increased p53 expression in cultured cells of cortical neuronsaud, they demonstrate the role of CNR1 and down stream signiling of (JNK) in THC to induce p53 phosphorelation that lead to increase Bax expression, BC1-2 phosphorylation and

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DNA fragmentation [9]. In curent study we find using rimonabant to block CB1 receptor decreased p53 and p21 expression in cultured cells. These findings support the study of [10], which incuding p53 expression was downregulated acording to use pharmalogical blocked (AM6545), in contrast, p53 expression was increased when they used agonist (Hu-210) with (10μM) to activate CB1receptor in HEPG2 cell line and they assesed p21 mRNA that known to be regulated by p53 gene and explain this elevation of p53 expression related to its acetylation that increased p53 stability, its coupled multiple proteins and p53 binding with promoter low affinity [11]. P53 acetylation directly impact its activity of transcription that resulted in altering p53 coupling with several responsed elements in target gene (Reed and Quelle, 2014). Previous studies lead to arrest the cell cycle at G0/G1 stage via activiting ATM/ATR signal leading to downregulation of p53 and downregulation p21 via decreasing CDK2 level [12]. In addition, cannabidiol significantly increased p53 and ATM protein expression and decreased p21 protein level in SGC-79 of cell line causing the arrest of cell cycle in G0/G1 and cell apoptosis [13]. Cannabinol induces apoptosis via downregulation of p27 and p21 through downregulation of CDK1,CDK2 and cyclin E1 [14]. The main limitation of this study is that endocannabinoids affect was examend only in hVSMSc cell line. Thus, further cell lines are requirred to examin the hypotheis invitro. In vivo, sample of patients suffer from cardiovascular diseases are required to exam the hypothesis of this project. Interestingly, all obtaind data from this work suggest the positive regulation of endocanibinoidal legand to p53 and p21 mRNA and protein levels in hVSMCs.

Conclusions

This study explores the regulatory effects of endocannabinoids on p53 and p21 gene expression at both mRNA and protein levels in human vascular smooth muscle cells (hVSMCs). Utilizing Anandamide and Rimonabant to modulate CNR1 receptor activity, we observed a significant upregulation in p53 and p21 expressions following Anandamide exposure, and a reduction when treated with Rimonabant. These findings suggest that p53 and p21, through their role in cell cycle arrest and apoptosis, could be pivotal in cardiovascular pathologies, potentially serving as pharmacological targets for cardiovascular disease treatment. However, the results being limited to in vitro hVSMC lines underscore the need for broader validation across multiple cell lines and in vivo patient samples to substantiate the therapeutic potential of targeting the endocannabinoid system in cardiovascular diseases. Further research should also investigate the mechanistic pathways of CNR1 interactions with p53 and p21, which could elucidate novel interventions for cardiovascular dysfunctions.

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