Cannabinoid Receptor Type 2 (CB2) Dependent and Independent Effects of WIN55,212-2 on Atherosclerosis in Ldlr-null Mice

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Abstract: *Purpose*: WIN55,212-2, a potent synthetic agonist of cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2), reduces atherosclerosis in apolipoprotein E (ApoE) null mice. Although pharmacologic evidence suggests the anti-atherosclerotic effects of WIN55,212-2 are mediated *via* CB2, this remains to be confirmed by genetic studies. Therefore, in this study, we investigated the effects of WIN55,212-2 on development of atherosclerosis in low-density lipoprotein receptor (Ldlr) null mice with and without homozygous deletion of the CB2 gene.

Methods: After 6 weeks on an atherogenic diet, groups of $CB2^{+/+}$ and $CB2^{-/-}$ Ldlr-null mice received a daily intraperitoneal injection of WIN55,212-2 or vehicle. After two weeks, plasma lipid levels and atherosclerosis in the aortic root were quantified.

Results: Plasma cholesterol and triglyceride levels did not differ between $CB2^{+/+}$ and $CB2^{-/-}$ mice and WIN55,212-2 had no effect on total cholesterol levels in either genotype. However, triglyceride levels in both $CB2^{+/+}$ and $CB2^{-/-}$ mice were significantly lowered by WIN55,212-2. The size of aortic root lesions did not differ significantly between $CB2^{+/+}$ and $CB2^{-/-}$ mice with or without WIN55,212-2 treatment. However, WIN55,212-2 treatment significantly lowered lesional macrophage accumulation in $CB2^{+/+}$ mice, and lesional smooth muscle content in both $CB2^{+/+}$ and $CB2^{-/-}$ mice. Lesional apoptosis was also greater in $CB2^{+/+}$ mice compared to $CB2^{-/-}$mice, and only reduced by WIN55,212-2 in $CB2^{+/+}$ mice. Collagen content and elastin fiber fragmentation were unaffected by genotype or WIN55,212-2.

Conclusions: WIN55,212-2 treatment does not alter lesion size in Ldlr null-mice, but does modify lesion cellularity *via* CB2-dependent and CB2-independent mechanisms.

Keywords: Atherosclerosis, Cannabinoid Receptor, WIN55,212-2.

INTRODUCTION

Atherosclerosis is the underlying cause of myocardial infarction and stroke, the primary causes of mortality and morbidity in the developed world [1]. Originally characterized as a dysfunction of lipid metabolism, atherosclerosis is now widely recognized as a chronic, progressive inflammatory condition of the arterial wall [2]. Currently, anti-atherosclerosis therapies target cardiovascular disease risk factors, such as hypercholesterolemia with lipid-lowering drugs (statins) and hypertension with beta-blockers. Unfortunately, these therapies have only slightly decreased mortality rates. The development of novel therapeutic strategies aimed at controlling or reducing vascular wall inflammation in atherosclerosis has therefore gained much interest.

In addition to their well-known psychotropic effects, cannabinoids exert immunosuppressive and anti-inflammatory effects, leading to the suggestion that cannabinoids may prove beneficial in the treatment of inflammatory disorders [3-6] and atherosclerosis [7-12]. Cannabinoids produce their effects primarily through binding of two G-protein coupled receptors, cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2). Psychotropic effects of cannabinoids result from activation of CB1, which is expressed predominantly in the central nervous system but also found in peripheral tissues including vasculature [13], heart [14], adipocytes [15], skeletal muscle [16], and liver [17]. The anti-inflammatory and immunosuppressive effects of cannabinoids arise from activation of CB2, which is prominently found in peripheral immune cells, but is also present in the brain [18], adipocytes [19], pancreas [20], and endothelial cells [21-23].

Cannabinoids and cannabinoid receptor agonists have been demonstrated to provide CB2-dependent beneficial effects in multiple animal models of inflammatory conditions, including hepatic and cardiac ischemia-reperfusion, gastrointestinal diseases, and neurodegenerative diseases [24-26]. However, the functions of CB2 in atherosclerosis are not fully understood and studies examining the effect of cannabinoids on atherosclerosis in murine models

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have shown variable results. Administration of Δ^9-tetrahydrocannabinol (THC) [8], a plant-derived cannabinoid, or WIN55,212-2 [9, 10], a synthetic cannabinoid and mixed CB1/CB2 agonist, were found to reduce atherosclerosis in ApoE-null mice by mechanisms that are sensitive to co-administration of a CB2-selective antagonist. The anti-atherosclerotic effects of WIN55,212-2 were attributed to the ability of WIN55,212-2 to diminish the inflammatory response [9] and reduce monocyte recruitment *via* down regulation of adhesion molecule expression in a CB2-antagonist sensitive manner [10]. However, in other studies, administration of a CB2-selective agonist, JWH133, or a CB2-selective antagonist, SR144528 [27], did not affect lesion progression in Ldlr-null mice [28], but reduced atherosclerosis in ApoE-null mice [12].

Previously, we found CB2 gene deletion in Ldlr-null mice did not affect the size of early atherosclerotic lesions, but did increase macrophage and smooth muscle cell content, decrease lesional apoptosis and alter the extracellular matrix composition of lesions, indicating that CB2 has a protective role in modulating processes within atherosclerotic lesions [7]. Subsequently, other independent investigations concluded that CB2 gene deficiency (systemic and hematopoietic-specific) increases lesional macrophage infiltration in both ApoE-null [12] and Ldlr-null mice [11, 28], supporting an anti-atherosclerotic function of CB2.

Together, these prior studies provide good experimental evidence for the potential to develop anti-atherosclerotic therapies pharmacologically targeting CB2. However, absolute confirmation of a role for CB2 in mediating the effects of an exogenous cannabinoid requires studies conducted in CB2-deficient models. Therefore, in the current study we utilized wild type Ldlr-null mice and CB2-deficient Ldlr-null mice to assess the effects of WIN55,212-2 on the development of early atherosclerotic lesions.

METHODS

Animals and Treatment Protocols

CB2-deficient, Ldlr null ($CB2^{-/-}$/$Ldlr^{-/-}$) mice and wild type Ldlr-null ($CB2^{+/+}$/$Ldlr^{-/-}$) mice were as previously described [7]. At 8 weeks of age, mice were placed on an atherogenic diet (21% fat, 0.15% cholesterol; Harlan Teklad, Madison, WI) for a total of 8 weeks. After 6 weeks on the atherogenic diet, the mice received a daily intraperitoneal (i.p.) injection of [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (WIN55,212-2) (Cayman Chemical, Ann Arbor, MI) for two weeks. Control mice received i.p. injections of an equivalent volume of vehicle (50% DMSO in saline).

All mice were housed at the Animal Research Facility at East Tennessee State University in a pathogen-free, humidity-and temperature controlled room. Mice were maintained on a standard chow diet (Ralston Purina, St Louis, MO) with water provided *ad libitum*. All animal procedures were approved by and conducted in accordance with the guidelines administered by the Institutional Animal Care and Usage Committee of East Tennessee State University and in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals published by the National Institutes of Health.

Physiological Parameters

Blood samples were collected following an overnight fast by cardiac puncture under anesthesia. Blood EDTA plasma was separated by centrifugation at 2000xg for 10 min and stored at 4°C. Total plasma cholesterol and triglycerides levels were determined using assay kits (Pointe Scientific, Inc., Canton, MI) according to the manufacturer's protocols. Lipoprotein profiles were determined by subjecting equivalent aliquots of pooled plasma to separation by fast protein liquid chromatography (FPLC) as previously described [29].

Analysis of Atherosclerosis

Anesthetized mice were euthanized by cardiac perfusion with 10mL of ice-cold phosphate buffered saline (PBS) and the hearts with proximal aortas were excised and snap frozen in OCT-embedded medium. Serial cross-sections of the aortic root were collected beginning at the first sign of aortic valve leaflets and the extent of atherosclerosis determined by computer-assisted morphometric analysis after staining with oil-red O as previously described [7, 29].

Histological and Immunohistochemical Staining

Collagen and elastin fiber composition of lesions was determined using Masson's trichrome staining and Verhoeff-van Gieson staining kits according to the manufacturer's directions (Richard-Allan Scientific, Kalamazoo, MI). Elastin fiber breaks, defined by interruption in the elastin fiber together with the reappearance of elastin fiber, were quantified visually under high-power microscopy as previously described

[7]. Macrophages and SMC content was determined by immunostaining with MAB1852 antibody (Chemicon International, Temecula, CA) (diluted 1:1000) and smooth muscle α-actin antibody (Neo-Markers, Fremont, CA) (diluted 1:200), respectively, as described previously [7]. Briefly, cryosections were fixed in acetone, dried, and pretreated with 1% H_2O_2 for 30 min. The sections were then rinsed in PBS and blocked in normal goat serum for 1 h prior to overnight incubation with primary antibodies. Sections were rinsed and then developed using a Vector Labs Elite ABC kit (Burlingame, CA) and counterstained with methyl green. Immunopositive staining areas were quantified using Image J software (NIH, Bethesda, MD). A threshold for staining that discriminated between positive and negative lesions areas was defined by sampling and applied to all specimens examined. Data were computed as the percentage of the total intimal lesion area staining positive. All evaluations were performed by a trained observer in blinded fashion.

Analysis of Lesional Apoptosis

Frozen cryosections were thawed and air dried for 2h, rehydrated with ethanol and pretreated with 3% citric acid prior to *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using an *in situ* Cell Death Detection kit (Roche Applied Sciences, Indianapolis, IN) as previously described [7]. TUNEL enzyme was diluted 1:150 in TUNEL dilution buffer. TUNEL-positive nuclei were quantitated independently by two observers in blinded fashion.

Statistical Analysis

Data were analyzed by Student's t-test or analysis of variance (ANOVA) followed by the Bonferroni test for comparisons between groups, as appropriate. $P<0.05$ were considered statistically significant. All data analyses were performed using Sigma Plot software (Systat Software, Inc., San Jose, CA).

RESULTS

WIN55,212-2 Decreases Plasma Triglycerides Independent of CB2 in Ldlr-null Mice

Ldlr-null mice with CB2 ($CB2^{+/+}$) or lacking CB2 ($CB2^{-/-}$) were fed an atherogenic diet for 6 weeks to initiate atherogenesis and then subjected to daily i.p. injections of WIN55,212-2 (1 or 3 mg/kg/day) for two weeks while continuing on the atherogenic diet. After 8 weeks of atherogenic diet, the mean body mass of $CB2^{+/+}$ and $CB2^{-/-}$ mice did not differ significantly and was unaffected by WIN55,212-2 treatment (Table **1**).

Table 1: Effect of WIN55,212-2 on Body Mass of $CB2^{+/+}$ and $CB2^{-/-}$/$Ldlr^{-/-}$ Mice

	$CB2^{+/+}$	$CB2^{-/-}$
Vehicle only	28.6±1.3	28.5±0.8
1 mg/kg/day	28.2±0.6	29.6±1.0
3 mg/kg/day	28.5±1.1	26.9±0.5

Data are mean weight in gm±SEM. n≥8 for all groups.

Total plasma levels of cholesterol and triglycerides were elevated in response to the atherogenic diet to a similar extent in untreated $CB2^{-/-}$ and $CB2^{+/+}$ mice (Figure **1**). Treatment with either 1 or 3 mg/kg/day WIN55,212-2 did not affect the plasma total cholesterol levels in either genotype (Figure **1A**). In contrast, $CB2^{-/-}$ mice treated with 1 mg/kg/day WIN55,212-2 displayed significantly lower plasma triglyceride levels compared to vehicle-injected $CB2^{-/-}$ mice (199±29 vs. 305±42 mg/dL, p=0.01, Figure **1B**). The lower dose of WIN55,212-2 also produced a modest, but not statistically significant, decrease in plasma triglyceride levels in $CB2^{+/+}$ mice compared to vehicle-injected controls (282±35 vs. 342±33 mg/dL, p= 0.22, Figure **1B**). Treatment with a higher dose (3 mg/kg/day) of WIN55,212-2 significantly reduced plasma triglyceride levels in both genotypes to approximately the same extent ($CB2^{+/+}$ 212±29 vs. 342±33 mg/dL, p=0.026; $CB2^{-/-}$ 167±16 vs. 305±42 mg/dL, p=0.003, Figure **1B**). The distribution of cholesterol among lipoproteins in plasma of $CB2^{+/+}$ mice was not altered by 3 mg/kg/day WIN55,212-2 (Figure **1C**), but the distribution of triglycerides was moderately decreased in the very low density lipoprotein (VLDL) fractions compared to vehicle-injected controls (Figure **1D**). Plasma from $CB2^{-/-}$ mice treated with WIN55,212-2 also showed a similar reduction in triglycerides in the VLDL fraction (data not shown).

Atherosclerosis was quantified by computer-assisted morphometric analysis of oil red-O stained aortic root cross sections (Figure **2**). Under all conditions, aortic root lesions of $CB2^{-/-}$ mice were slightly smaller on average than those of similarly treated $CB2^{+/+}$ mice (Figure **2B**); however, the differences did not reach statistical significance. Treatment with 1 mg/kg/day WIN55,212-2 produced no significant effect on the mean lesion area of $CB2^{+/+}$ mice compared to vehicle-injected controls (70977± 8406 vs. 74125 ± 6659 μm^2, p=0.77), or $CB2^{-/-}$ mice

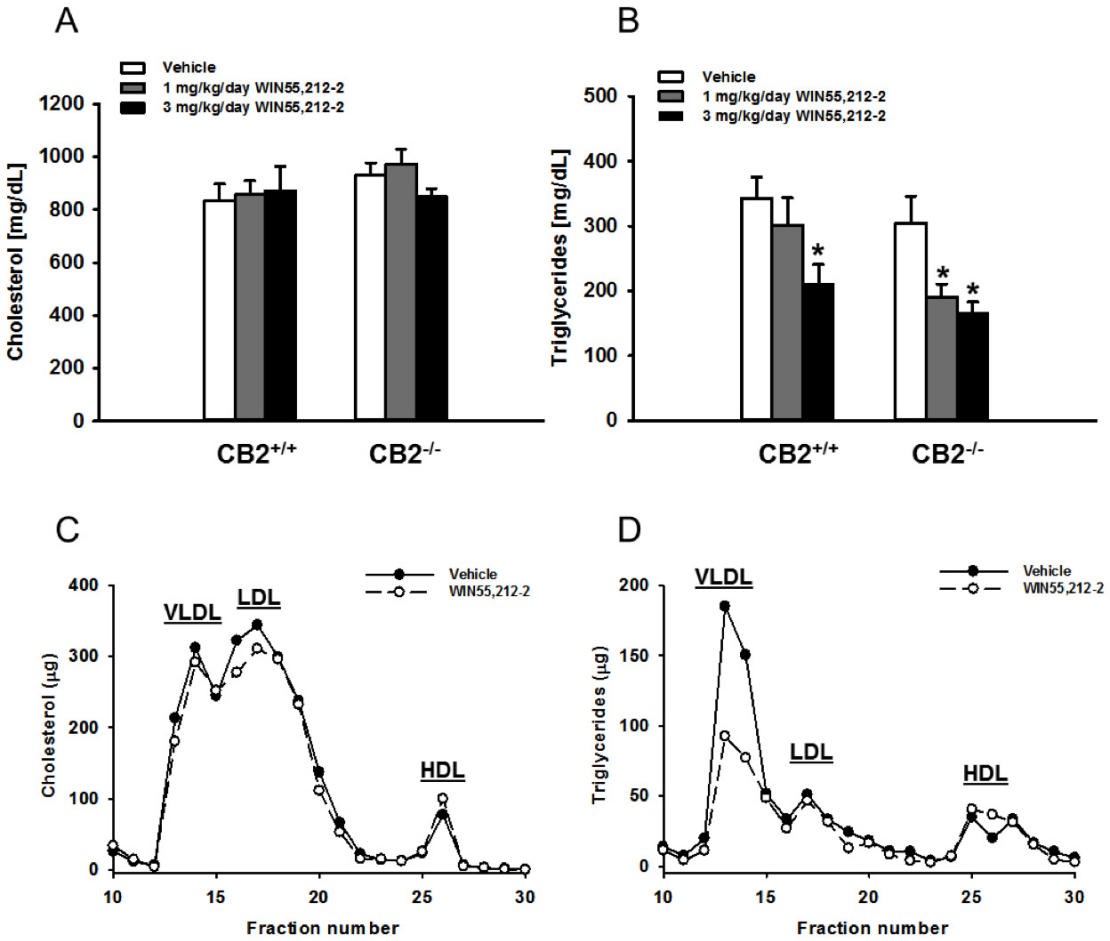

Figure 1: Diet-induced hyperlipidemia in $CB2^{+/+}$ and $CB2^{-/-}$/$Ldlr^{-/-}$ mice treated with and without WIN55,212-2. Total cholesterol (**A**) and triglyceride levels (**B**) in plasma of $CB2^{-/-}$ and $CB2^{+/+}$ Ldlr-null mice after 8 weeks of an atherogenic diet and treatment with WIN55,212-2 (1 or 3 mg/kg/day) or vehicle alone. (**C**) Total cholesterol and (**D**) triglyceride levels in fractions of pooled plasma samples from randomly selected Ldlr-null mice (n=6) treated with and without 3 mg/kg/day WIN55,212-2 after separation by FPLC. The values are expressed as the mean±SEM. **P*<0.05, compared with vehicle-injected controls, n≥9 for each group.

(52665±4667 vs. 61428±4880µm^2, p=0.021). Treatment with 3 mg/kg/day WIN55,212-2 also had no effect on aortic root lesion area in $CB2^{-/-}$ mice (56437 ± 6058 vs. 61428±4880µm^2, p=0.52), or $CB2^{+/+}$ mice (88274±12382 vs. 74125±6659 µm^2, p=0.30) compared to vehicle-injected controls (Figure **2B**).

WIN55,212-2 Reduces Lesional Macrophage and Smooth Muscle Cell Content

Immunohistochemical staining of aortic root lesions with a monocyte/macrophage specific antibody (MOMA2) was performed to determine the effect of WIN55,212-2 on lesional macrophage infiltration in $CB2^{+/+}$ and $CB2^{-/-}$ mice (Figure **3A**). Quantification of MOMA2-positive areas showed lesional macrophage content to be slightly greater, but not significantly different, in $CB2^{-/-}$ mice compared to similarly treated $CB2^{+/+}$ mice (Figure **3B**). WIN55,212-2, at 1 mg/kg/day, did not significantly alter lesional macrophage infiltration in either $CB2^{-/-}$ or $CB2^{+/+}$ mice; however, WIN55,212-2 at 3 mg/kg/day produced a small (~8%), but significant, reduction in the lesional macrophage content of $CB2^{+/+}$ mice (83.9±1.2 vs. 76.3±1.6%, p=0.001). In contrast, treatment of $CB2^{-/-}$ mice with WIN55,212-2 at either 1 or 3 mg/kg/day did not significantly alter lesional macrophage content compared to vehicle-injected controls (87.0±1.1 vs. 88.2±0.8 and 84.4±1.3%, p=0.51 and p=0.14, respectively).

The effect of WIN55,212-2 on lesional SMC content was assessed by immunohistochemical staining with a smooth muscle α-actin antibody (Figure **3A**). Digital quantification revealed no significant difference between the SM α-actin immunoreactivity in lesions of untreated $CB2^{+/+}$ and $CB2^{-/-}$ mice (Figure **3C**). Treatment with 1 mg/kg/day WIN55,212-2 did not affect

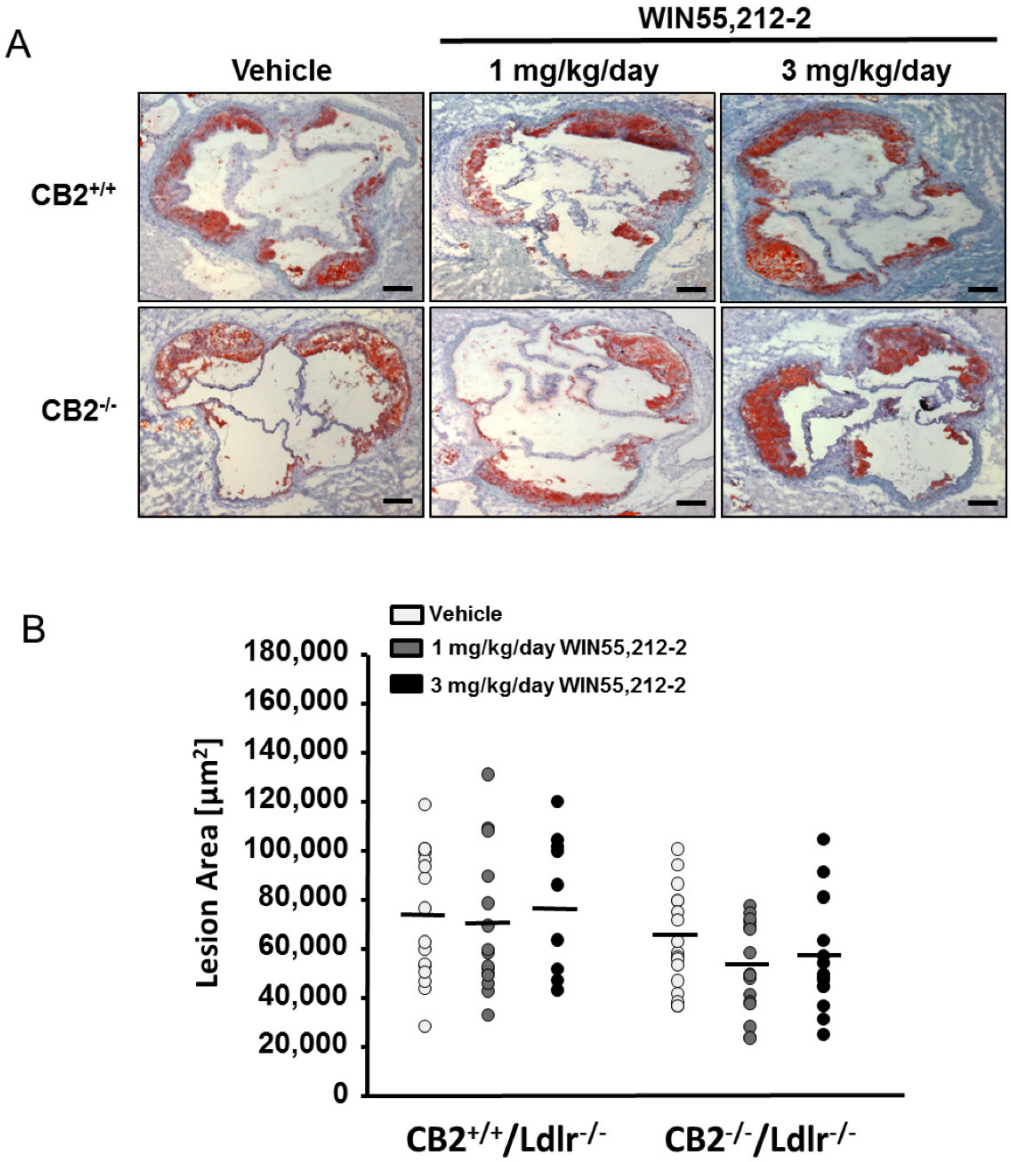

Figure 2: Atherosclerotic burden in the aortic root of $CB2^{+/+}$ and $CB2^{-/-}$/$Ldlr^{-/-}$ mice. (**A**) Representative photomicrographs of oil red-O stained aortic root cross sections from $CB2^{+/+}$ and $CB2^{-/-}$ mice following i.p. injections of WIN55,212-2 or vehicle are shown. (**B**) Quantitation of the lesion area in aortic root sections. Dots represent the mean lesion area per mouse; lines indicate the mean lesion area for each group. n≥9 for each group. Scale bars: 100 µm.

the SM α-actin immunoreactivity in lesions in either genotype; however, 3 mg/kg/day WIN55,212-2 significantly decreased the lesional SM α-actin positive area in both $CB2^{+/+}$ mice (2.6±0.55 vs. 6.9±1.6%, p=0.044) and $CB2^{-/-}$mice (2.8±0.59, vs. 7.1±1.2% p=0.007) compared to vehicle-injected controls (Figure **3C**).

WIN55,212-2 Decreases Lesional Apoptosis in Wild Type but not CB2-Deficient Ldlr-null Mice

In situ TUNEL staining of aortic root cross sections revealed lesions from vehicle-injected $CB2^{-/-}$ mice contained significantly fewer (~40%) apoptotic cells compared to lesions from vehicle-injected $CB2^{+/+}$ mice (6.5±0.97 vs. 11.2±1.9, p=0.05, Figure **4**). WIN55,212-2 treatment reduced the number of TUNEL-positive nuclei in aortic root lesions of $CB2^{+/+}$ mice in a dose-dependent manner; with 1 mg/kg/day WIN55,212-2 producing a small (~25%) decrease (8.5±1.2 vs. 11.2±1.9, p=n.s.) and 3 mg/kg/day WIN55,212-2 producing a significant (~53%) decrease (5.3 ± 0.95 vs. 11.2±1.9, p=0.03) compared to vehicle-injected controls. In contrast, the mean number of TUNEL-positive nuclei present in lesions of $CB2^{-/-}$ mice was unaffected by WIN55,212-2 treatment at either 1 or 3 mg/kg/day (6.6±1.4 and 6.9±1.1, respectively,

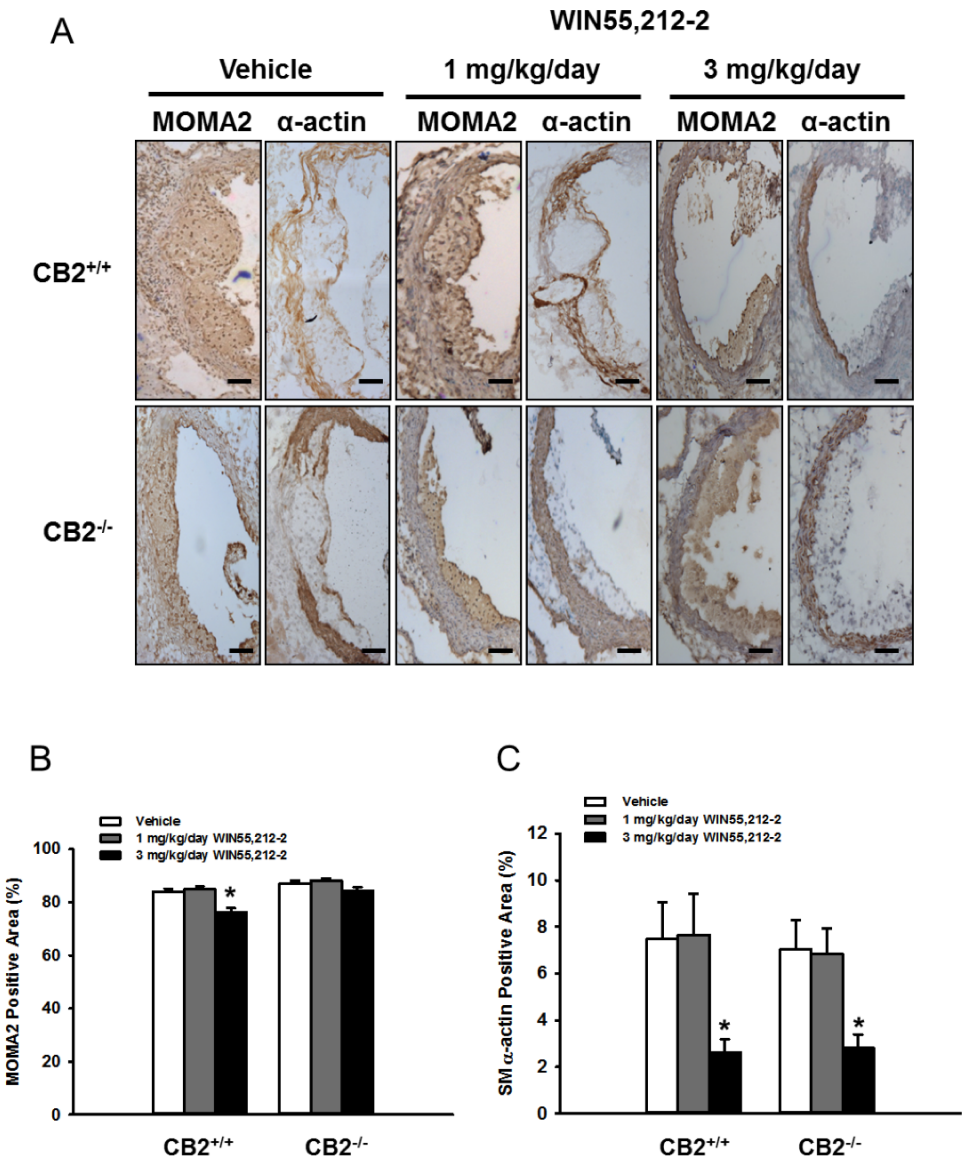

Figure 3: Immunostaining for macrophages and smooth muscle cells in aortic root lesions of $CB2^{+/+}$ and $CB2^{-/-}$ /$Ldlr^{-/-}$ mice treated with and without WIN55,212-2.

(**A**) Representative photomicrographs of aortic root lesions after staining for macrophages (MOMA2 antibody) or SMC (α-actin antibody) as indicated. The percentage of the total lesion area staining positive for macrophages (**B**) and SMCs (**C**) was digitally quantified and expressed as the mean±SEM. **P*<0.001 compared with vehicle-injected controls. n ≥ 9 for each group. Scale bars: 50 μm

compared to 6.5 ± 0.95 for vehicle-injected $CB2^{-/-}$ mice).

WIN55,212-2 does not Alter the Extracellular Matrix Composition of Lesions in Ldlr-null Mice

The effect of WIN55,212-2 on collagen content, visualized by Masson's trichrome staining, and elastin fiber fragmentation, visualized by Verhoeff-van Gieson staining, was evaluated as surrogate parameters of lesion stability and vulnerability to rupture. Digital quantitation of the collagen present in aortic root lesions of $CB2^{-/-}$ and $CB2^{+/+}$ mice after 8 weeks of an atherogenic diet revealed no appreciable difference in the presence or absence of WIN55,212-2 treatment (Figure **5A** and **5B**). Although both genotypes displayed a slight trend towards reduced elastin fiber fragmentation with increasing WIN55, 212 treatment, quantitation of the number of elastin fiber breaks per lesion identified no statistically significant difference between $CB2^{-/-}$ and $CB2^{+/+}$ mice in the presence or absence of WIN55,212-2 treatment (Figure **5C** and **5D**).

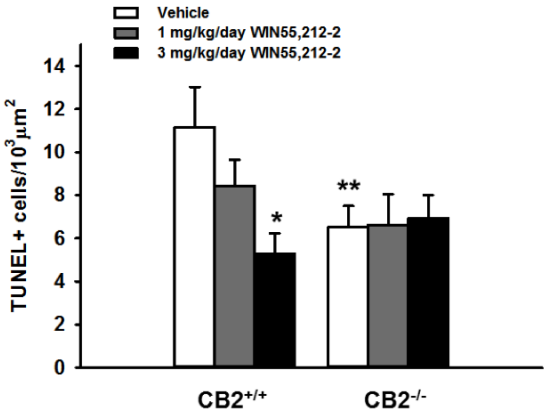

Figure 4: *In situ* TUNEL analysis of the effect of WIN55,212-2 on lesional apoptosis.

Quantitative analysis of TUNEL-positive nuclei in aortic root lesions of CB2$^{+/+}$ and CB2$^{-/-}$/Ldlr$^{-/-}$ mice treated with and without WIN55,212-2. Values are the mean number of TUNEL-positive nuclei per lesion area ± SEM, * *P*< 0.05 compared to vehicle-injected controls.** *P*< 0.05 compared to vehicle-injected CB2$^{+/+}$ mice. n≥6 in each group.

DISCUSSION

Studies have suggested that administration of the synthetic cannabinoid WIN55,212-2 reduces progression of atherosclerosis in ApoE knockout mice fed a high fat diet due to stimulation of the CB2 receptor [9, 10]. However, the evidence supporting CB2-dependent effects in these studies was indirect and lacked confirmation in mice genetically deficient in CB2. Furthermore, the ability of WIN55,212-2 to reduce progression of atherosclerosis in other murine models of atherosclerosis has not been established. In the present study, we investigated the effect of WIN55,212-2 on high fat diet-induced atherosclerosis in Ldlr-null mice and Ldlr-null mice lacking CB2.

The observation that CB2 deficiency did not alter aortic root lesion size in Ldlr-null mice is consistent with our previous study [7], as well as recent studies from other investigators demonstrating that systemic or hematopoietic CB2 deficiency did not affect aortic root lesion area in Ldlr-null mice [11, 28] or ApoE-null mice [12]. The finding that lesion burden in Ldlr-null mice was not affected by WIN55,212-2 treatment was unexpected and is in apparent contradiction to studies demonstrating that administration of CB1/CB2 agonists regresses atherosclerosis in ApoE-null mice *via* mechanisms sensitive to CB2-selective

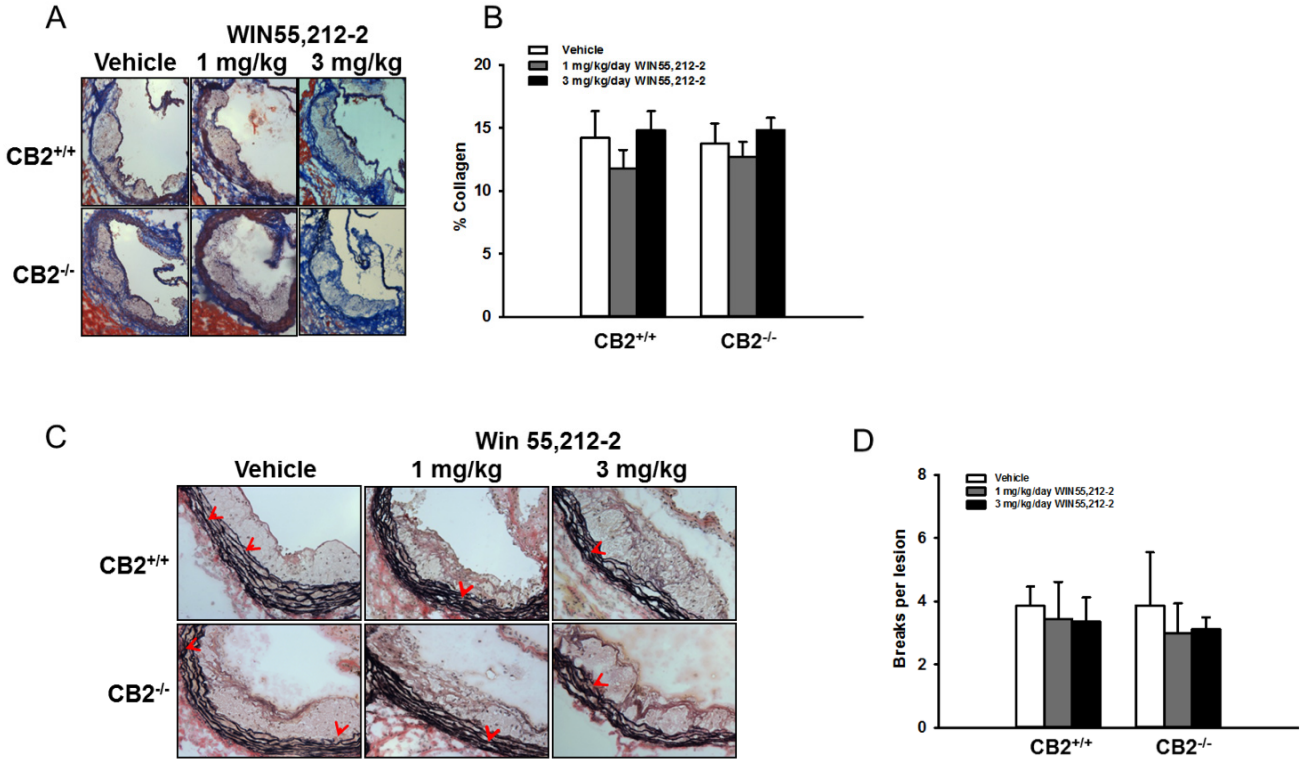

Figure 5: Effect of WIN55,212-2 on the extracellular matrix composition of lesions. Representative photomicrographs of aortic root lesions from CB2$^{+/+}$ and CB2$^{-/-}$/Ldlr$^{-/-}$ mice treated with and without WIN55,212-2 following Masson's trichrome staining for collagen (**A**) or Verhoeff–van Gieson staining for elastin (**C**). Arrows indicate breaks in elastin fibers of the inner elastic lamina. (**B**) Quantitative analysis of the collagen stained area in lesions from each group as a percentage of the total lesion area. (**D**) Quantitation of the number of elastin fiber breaks visible in the inner elastic lamina of lesions from each group (**D**). Values are the mean±SEM. n ≥ 6 for each group.

pharmacological blockade [8-10], as well as a study [12] showing that administration of a CB2-selective agonist, JWH-133, reduced lesion burden in wild type, but not CB2-deficient ApoE-null mice. However, they are consistent with a study by Willecke *et al*. [28] demonstrating no effect of JWH-133 on lesion size in Ldlr-null mice. These conflicting results may be partly explained by several factors including the atherosclerotic genetic background (Ldlr-null vs. ApoE-null), the composition of the atherogenic diets (Western vs. high fat diet), and/or the methods for lesion quantitation. Based on the fact that all studies to date demonstrating a cannabinoid-induced reduction in lesion size were conducted in the ApoE-null mice model and those showing no effect utilized the Ldlr-null mice model raises the possibility that differences between the murine models is, at least partly, responsible for the divergent results. In support of this, ApoE-null and Ldlr-null mice are well known to have differences in a number of parameters affecting the pathophysiology of atherosclerosis [30]. In addition, since aortic root lesion development is faster in Ldlr-null mice compared to ApoE-null mice it is also possible that subtle anti-atherosclerotic effects of CB2-stimulation on lesion size are masked by the more accelerated atherosclerosis of the Ldlr-null mouse model.

The observation that 1 mg/kg/day WIN55,212-2 did not affect diet-induced hyperlipidemia in Ldlr-null mice is in agreement with other studies using the same or smaller doses of WIN55,212-2 in ApoE-null mice [9, 10]. However, the observation that 3 mg/kg/day WIN55,212-2 significantly reduces plasma triglyceride levels in the VLDL fraction of Ldlr-null mice is novel. The fact that diet-induced hypercholesterolemia was unaffected by WIN55,212-2 suggests that the effect is specific for triglyceride metabolism. The mechanism by which WIN55,212-2 reduces plasma triglycerides is currently unknown; however, the observation that triglycerides were similarly reduced in Ldr-null mice lacking CB2 indicates a CB2-independent mechanism. Administration of SR141716 (Rimonabant), a CB1-specific antagonist, reduces plasma triglyceride levels in mice [31] and humans [32-35] *via* effects on the liver. As CB1 in the central nervous system undergoes down-regulation after chronic exposure to cannabinoids [36-38], the prolonged WIN55,212-2 treatment may have caused a down-regulation of hepatic CB1 resulting in lower triglycerides. Alternatively, WIN55,212-2 may be acting *via* signaling pathways independent of cannabinoid receptors that regulate triglyceride metabolism. In support of this suggestion, WIN55,212-2 has been shown to activate peroxisome proliferation activator nuclear receptors (PPARs), including PPARα and PPARγ [39]. PPARα agonists are used in the treatment of dyslipidemia and are known to reduce plasma triglyceride levels [40].

The production and clearance of apoptotic macrophages within lesions are important processes in the formation and progression of atherosclerosis [29, 41-44]. In prior studies, we noted that CB2 deficiency in macrophages was associated with reduced susceptibility to apoptosis, *in vitro* [45] and *in vivo* within atherosclerotic lesions [7], suggesting that it may be possible to modulate lesional macrophage apoptosis by pharmacologically targeting CB2. The fact that WIN55,212-2 altered lesional apoptosis in a dose-dependent and CB2-dependent manner are consistent with this suggestion; however, it was somewhat surprising that WIN55,212-2 decreased lesional apoptosis. The reason for this unexpected result is not clear, but may be related to peculiarities of macrophage biology in the setting of systemic CB2 deficiency. Several other studies have shown anti-apoptotic effects of WIN55,212-2 in murine models of ischemia reperfusion injury *via* activation of CB2 [46, 47]. Alternatively, it is possible that WIN55,212-2 treatment resulted in CB2 receptor internalization [48], which would mimic CB2 deficiency in lesional macrophages and reduce apoptosis. In support of this, CB2 internalization in response to activation with an exogenous cannabinoid has been described [49].

CB2 agonists ameliorate atherosclerosis in ApoE-null mice in part by reducing lesional macrophage content [8-10]. In a prior study, we found that macrophage content is not affected by CB2 deficiency in Ldlr-null mice fed an atherogenic diet for 8 weeks, but increases significantly after 12 weeks [7], suggesting that CB2 activation may also lessen lesional macrophage accumulation in Ldlr-null mice. In contrast, Willecke *et al*. [28] reported that a CB2-selective agonist, JWH-133, had no effect on lesional macrophage content in Ldlr-null mice. Here, we observed that WIN55,212-2 treatment significantly reduced macrophage content in Ldlr-null mice but not CB2-deficient Ldlr-null mice. This result further strengthens the conclusions from prior studies conducted in ApoE-null mice that CB2 activation alters macrophage lesion accumulation.

In vitro studies have shown that cannabinoids impair TNFα-induced VSMC proliferation and migration

by a mechanism sensitive to CB2-selective blockade [50]. In this study, we observed that 3 mg/kg/day WIN55,212-2 significantly reduced lesional VSMC content in wild type and CB2-null mice. These results indicate that CB2 activation is not responsible for the effects of WIN55,212-2 on lesional VSMC content in Ldlr-null mice. Consistent with this, Willecke *et al*. reported no effect of CB2-selective stimulation with JWH-133 on lesional VSMC content in Ldlr-null mice [28]. In further support of a CB2-independent mechanism, WIN55,212-2 has been shown to be a PPARγ agonist [51] and PPARγ is known to regulate VSMC proliferation and migration [52, 53].

After 12 weeks of atherogenic diet, extracellular matrix (ECM) components of lesions in CB2-deficient Ldlr-null mice show evidence of degradation [7], and CB2 activation is correlated with reduced matrix metalloproteinase 9 (MMP9) in mouse lesions [54], suggesting that CB2-dependent mechanisms inhibit ECM degradation in more advanced lesions. The observation that WIN55,212-2 did not affect collagen content and elastin fiber fragmentation support this suggestion and are consistent with prior results showing a lack of CB2 modulation of ECM composition in very early lesions [7]. At present, it is unclear if CB2 activation will inhibit ECM degradation in more advanced lesions.

CONCLUSION

Our results show that WIN55,212-2 affects plasma triglyceride levels and the cellular composition of atherosclerotic lesions in Ldlr-null mice, lending further support to the suggestion that WIN55,212-2, and other cannabinoids, may have novel therapeutic utility in the treatment of atherosclerosis. The effects of WIN55,212-2 on lesional apoptosis and macrophage accumulation were dependent upon CB2, while the effects on plasma triglycerides and lesional SMC content were independent of CB2.

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