의학박사 학위논문

Endocannabinoid System Regulates Inflammation and Insulin resistance via Resistin

리지스틴을 통해 Inflammation과 인슐린 저항성을 조절하는 Endocannabinoid system에 관한 연구

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Abstract

Endocannabinoid System Regulates Inflammation and Insulin resistance via Resistin

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Background

Obesity is recognized as a significant risk factor for cardiovascular disease and dysregulation of the endocannabinoid system. Endocannabinoid system has been regarded as a modulator of food intake that affects the regulation of obesity and insulin resistance. Resistin is an adipokine and involved in metabolic disorders and inflammation. Here, we hypothesized that the endocannabinoid system regulates the CB1R positive monocytes expressing resistin, thus modulating inflammation and insulin resistance in humans.

Methods and Results

In human atheromatous arteries, CB1R was co-localized with resistin and the levels of resistin expressions were significantly higher in sorted CB1R

positive cells. In CB1R positive cells, resistin expressions were increased by 2-AG and CB1 receptor blockade by SR141716 and AM251 reduced resistin expressions via the inhibition phosphorylation of p38 MAPKs. To test whether the endocannabinoid system regulates insulin resistance and inflammation *in vivo*, we used two animal models as humanized NOG mice and humanized resistin mice models. In both models, high fat diet increased insulin resistance, which was reversed by the treatment of CB1 receptor antagonists. Furthermore, the increase of CB1R, resistin and proinflammatory gene expressions induced by high fat diet was normalized in the normal diet in visceral fat tissues of both mice. Moreover, we confirmed that this process might be mediated by the infiltration of peripheral blood monocytes expressing resistin. Further studies including a migration assay and LC/MS/MS demonstrated that high fat diet increased the level of CB1 receptor ligand, promoting the migration and infiltration of CB1R positive monocytes.

Conclusion

Our study demonstrated that the endocannabinoid system modulates resistin expressions of human peripheral monocytes, thus regulating the infiltration of these cells into the tissues associated with insulin resistance and inflammation. These results provide an important insight into the pathophysiology of cardiometabolic disease and the development of new

drugs.

Key words : Endocannabinoid system, Resistin, human monocyte, Inflammation, Insulin resistance

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List of Figures

Contents

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality around the world. Obesity is associated with an increase in cardiovascular disease and related to cardiovascular disease risk factors.[1] The endocannabinoid system and its role in energy metabolism are associated with obesity and cardiovascular disease. Obesity increases endocannabinoid levels and leads to disorders of the endocannabinoid system in adipose tissues.[1-2]

The endocannabinoid system regulates energy balance, food intake and lipid and glucose metabolism. The endocannabinoid system consists of two membrane receptors, CB1 and CB2 receptors, and their endogenous ligands, endocannabinoids, as anandamide (AEA) and 2-arachidonoylglycerol (2-AG). CB1 receptors are mainly expressed in the brain and also present in peripheral tissues such as adipose tissue, liver, muscle and immune cells. CB2 receptors are predominantly expressed in immune cells but are also present in the brain.[1-3] Clinical trials with rimonabant (SR141716), a selective CB1R antagonist, have suggested a beneficial effect on obesity and cardiometabolic risk factors in humans. However, rimonabant has been withdrawn from the market due to its psychiatric side effects.[4-5] Recent studies have focused on the peripheral actions of CB1R antagonists or differential approaches because CB1R antagonists have beneficial metabolic effects.[6]

Resistin is a 12.5 kDa cysteine-rich polypeptide and discovered in

rodents as an adipose tissue-specific secreted protein that is down-regulated by antidiabetic thiazolidinediones (TZDs). Resistin is also known as found in inflammatory zone 3 (Fizz3) and adipocyte-secreted factor and linked to obesity and insulin resistance.[7-9] Although resistin has been implicated as a link between obesity and insulin resistance in rodents, there has been a lot of controversy regarding its role of resistin in insulin resistance. Because the main source of mouse resistin is adipose tissues but human resistin is monocytes and macrophages. In humans, recent studies suggest that resistin is involved in inflammation.[8-10]

In the present study, we hypothesized that there must be some relationship between the endocannabinoid system and resistin. In addition, there might be a mediator explaining the discrepancy between the role of resistin in mice and humans. Therefore, we investigated focused on the link between the endocannabinoid system and resistin and it involves high fat diet induced insulin resistance and inflammation *in vivo*.

Materials and Methods

Cell isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE healthcare, Sweden) according to the manufacturer's instructions and were washed with phosphate buffered saline (PBS). For CB1 receptor positive cells, PBMCs were stained with anti-CB1R (Abcam, MA, USA) and sorted on a BD FACSAria II cell sorter. The purity of CB1R positive cells was determined by flow cytometric analysis and cells were resuspended in EBM-2 with 1% FBS. To evaluate the effect of SR141716 (Sanofi-Aventis), cells were pretreated with or without 1μM SR141716 in 10μM 2-AG (Tocris Bioscience, UK).

RNA isolation and PCR

Total RNA was extracted using Trizol Reagent (Invitrogen, California, USA) according to the manufacturer's instruction. One microgram of total RNA was used for reverse transcription and was amplified by TaKaRa Ex-Taq. Real-time PCR was performed with SYBR Green mix (Applied Biosystems, Warrington, UK) using an ABI prism 7500 (Applied Biosystems, CA, USA). The cycling conditions consisted of 50℃ for 2min, 95℃ for 10min, and 95℃

15s, 60℃ for 1min for 40 cycles and the melting point was determined and dissociation curves were obtained to assure the specificity of the reaction. The primers were as follows: forward 5'-ctgtctcctcctcctccctg-3', reverse 5'-caggccaatgctgcttattg-3' for human resistin and forward 5'-gagtcaacggatttggtcgt-3', reverse 5'-gacaagcttcccgttctcag -3' for GAPDH.

Western blot

Cells were preincubated with 1μM SR141716 or 10μM AM251 and then treated with 10μM 2-AG. Western blot analysis was performed as previously.[14, 16] Cells were lysed with lysis buffers containing 50mM Tris (pH 7.2), 250 mM NaCl, 1% NP40, 0.05% Sodium Dodecyl sulfate (SDS), 2mM Ethylenediaminetetraacetic acid (EDTA), 0.5% Deoxycholic acid, 10mM β-glycerol phosphate, 100mM NaF, 1mM Orthovanadate and Protease inhibitor cocktail (Roche) and proteins were separated by SDS-polyacrylamide electrophoresis gel. Primary antibodies against human resistin (Abcam, MA, USA), phospho-p38, p38 (Cell signaling, MA, USA) and β-actin (Santa Cruz, CA, USA) were used.

Immunohistochemical staining

Immunohistochemistry was performed as previously described.[16] The paraffin-embedded samples were sectioned and blocked in 1% bovine serum albumin. The primary antibodies used were anti-CB1 receptor, anti-human resistin and anti-CD68 followed by Alexa Fluor 488, 555 and 633 secondary antibodies. The fluorescence image was obtained with a confocal microscope (Carl Zeiss LSM710).

Migration assay

The migration of cells was assayed using Transwell Inserts (Becton Dickinson, NJ, USA).[23-24] Cells suspended in EBM-2 medium were transferred to the insert and 2-AG in EBM-2 medium was placed in the lower wells. After incubation at 37℃, 5% CO_2 incubator, cells that migrated from the upper inserts to the lower wells were counted.

Mice

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) in Seoul National University Hospital. For humanized mice, NOG (NOD/Shi-*scid,IL-2Rγnull)* mice were irradiated with 2.4 Gy and

$1x10^5$ human CD34 positive cells were transplanted through tail vein injection.[19] After 8 weeks, mice were confirmed by FACS analysis as humanized mice. Humanized resistin mice were obtained from the University of Pennsylvania.[20] Humanized resistin mice (designated CD68hR) were confirmed by PCR of tail DNA with the primer forward 5′-ctgggttgctaaccatctcc-3′ and reverse 5′-caggccaatgctgcttattg-3′. Retn-/- (murine resistin knock out) mice were used as control mice. Mice were fed a normal chow or a high fat diet (60% fat, Research diets, NJ, USA) for 8 weeks and body weight was measured weekly. To evaluate the effect of SR141716, mice were injected intraperitoneally with SR141716 (10mg/kg).

GTT and insulin resistance

Glucose tolerance test (GTT) was performed after an overnight fast. Blood glucose concentrations were measured before and 15, 30, 45, 60, 90, and 120 minutes after an i.p. injection of glucose (2g/kg) using an Accu-check. Blood glucose level was measured at 0, 15, 30, 45, 60, 90 and 120 minutes. Insulin levels were measured by Ultra Sensitive Mouse Insulin ELISA Kit (ALPCO, NH, USA). The homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated as [G_0 (mmol/l) x I_0 (μU/ml)/22.5].[21] The quantitative insulin-sensitivity check index (QUICKI) was calculated as $1/[\log(I_0) + \log(G_0)]$.[22] I_0 is fasting insulin (μU/ml) and G_0 is fasting glucose

(mmol/l).

2-AG measurements

In measuring the levels of 2-AG, the extraction and quantification were performed as reported previously.[10-11] Briefly, tissues were homogenized with Heptane/Ethyl acetate (1:1, v/v) containing internal standards (1nmol 2-AG-d8, Cayman, MI, USA). The organic phase was evaporated and reconstituted with 0.1% formic acid and 50% acetonitrile for analysis by LC/MS/MS.

Statistical Analysis

All data are presented as mean±SEM. The statistical analysis was performed by Student *t*-test or ANOVA as appropriate. SPSS version 18.0 was used for the analysis. *P*-value of <0.05 was considered statistically significant.

Results

The endocannabinoid system regulates resistin expression in human monocytes

Both resistin and the endocannabinoid system are known to be associated with the process of atherosclerosis. To investigate whether the endocannabinoid system is relates to resistin, we evaluated resistin expression in human monocytes from peripheral blood (PBMCs). First we confirmed the resistin expressions by RT-PCR to determine endocannabinoid effects in PBMCs. Resistin expression was increased RNA levels by 2-AG, the endocannabinoid (Vehicle 1.01±0.07 vs. 2-AG+Vehicle 2.197±0.109, $P<0.0001$, n=3), and down regulated by SR141716, a CB1 antagonist, in a dose dependent manners (2-AG+0.1μM SR 1.917±0.126, 2-AG+1μM SR 1.4±0.1002, 2-AG+10μM SR 1.147± 0.0348, $P<0.0001$, n=3) (Figure 1). We then performed immunofluorescence analysis in human arteries. Interestingly, we found the cells expressing both CB1 receptor and resistin in the atheromatous plaque of the aorta derived from patients with aortic aneurysm in the CD68 positive area (Figure 2).

The CB1R positive fraction of human monocytes expresses and regulates resistin

We divided whole monocytes into CB1R positive and negative fractions by FACS sorters to examine the relationship between resistin and CB1R (Figure 3A). CB1R positive cells were expressed resistin and the levels of resistin were higher in CB1R positive cells than in CB1R negative cells (Figure 3B, 3C). Additional studies were carried out to determine whether endocannabinoid system regulates resistin expression in CB1R positive cells. The resistin expressions of CB1R positive cells were regulated by 2-AG and CB1R antagonists. 2-AG increased resistin expressions (Real-time PCR; Vehicle 1.0±0.007 vs. 2-AG+Vehicle 2.533±0.56, $P<0.0001$, n=5, WB; Vehicle 0.9775±0.022 vs. 2-AG+Vehicle 1.653±0.05, $P<0.0001$, n=4) but these were decreased by CB1R antagonists, 1μM SR141716 and 10 μM AM251 in CB1R positive cells (Real-time PCR; 2-AG+SR 0.993±0.2423, 2-AG+AM251 0.7532±0.4, $P<0.0001$, n=5, WB; 2-AG+SR 0.994±0.03, 2-AG+AM251 1.134±0.04, $P<0.0001$, n=4) (Figure 4A, 4B). Furthermore, inflammatory cytokines, TNFα, IL-1β and IL-6, were increased by 2-AG and reversed by CB1R antagonists in CB1R positive cell supernatants (TNFα; Vehicle 114.6±20.29, 2-AG+Vehicle 200.6±29.04, 2-AG+SR 106.4±13.01, 2-AG+AM251 115.6±12.33 $P<0.0001$, n=4, IL-1β; Vehicle 189.6±59.36, 2-AG+Vehicle 350.9±23.58, 2-AG+SR 188.6±57.29, 2-AG+AM251

192.9±58.88 P<0.0001, n=4, IL-6; Vehicle 1.332±0.2509, 2-AG+Vehicle 2.990±0.3252, 2-AG+SR 1.504±0.3748, 2-AG+AM251 1.304±0.1570 P<0.0001, n=4) (Figure 5A-5C).

The endocannabinoid system modulates resistin expression via p38 pathway

To understand the mechanism of the regulation of resistin expressions in CB1R positive cells by the endocannabinioid system, we screened several pathways. 2-AG activated the phosphorylation of p38 and was down regulated by SR141716 and AM251 in CB1R positive cells (Vehicle 1.0±0.001, 2-AG+Vehicle 2.716±0.149, 2AG+SR 1.809±0.115, 2-AG+AM251 1.203±0.02, P<0.0001, n=4) (Figure 6A). We also examined resistin expression levels in CB1R positive cells following treatment with SR141716, AM251 and SB203580, p38 inhibitor. The increased resistin expression by 2-AG were reduced by SR141716, AM251 and SB203580 (Vehicle 1.019±0.19, 2-AG+Vehicle 1.933±0.059, 2-AG+SR 0.747±0.101, 2-AG+AM251 0.89±0.01, 2-AG+SB 0.342±0.023, P<0.0001, n=4) (Figure 6B). These results suggest that the resistin expression in CB1R positive cells is regulated through p38 activation.

The endocannabinoid system regulates insulin resistance *in vivo*

To test whether the endocannabinoid system regulates insulin resistance *in vivo*, we studied with humanized NOG mice and humanized resistin mice as *in vivo* models. Because resistin expression differs in mice and humans. Humanized mice were the mice of which bone marrow is replaced with human monocytes. Therefore, with these mice, we can study the behavior of human monocytes *in vivo*. Humanized resistin mice were the mice which have human resistin in monocytes after deleting whole mouse resistin, resulting in mimicking the physiology of human resistin. Mice were fed a normal diet or a high fat diet with or without SR141716. SR141716 was injected intraperitoneally into mice for 8 weeks (Figure 7). To assess glucose homeostasis, we assessed glucose tolerance test in both models. The high fat diet mice had glucose intolerance compared to normal diet mice and the SR141716 treated mice showed normalized glucose intolerance (Figure 8A, 8D). In order to assess insulin sensitivity, HOMA-IR and QUICKI were calculated. HOMA-IR was higher and QUICKI was lower in the high fat diet mice compared to the normal diet mice, indicative of insulin resistance and the index was normalized in the high fat diet with SR141716 mice (Figure 8B, 8E). There was no significant difference in the control mice (Retn-/- mice)

(Figure 8G, 8H).

The endocannabinoid system regulates inflammation *in vivo*

Adipose tissue is known to be important for inflammation. Therefore, we investigated CB1R and resistin expressions in visceral fat and subcutaneous fat tissues. Immunofluorescence analysis revealed the higher infiltration of cells expressing both CB1R and resistin in visceral fat tissues of the high fat diet mice than normal diet mice. SR141716 treated mice attenuated the expressions compared to the high fat diet mice. In addition, this increased infiltration of cells was reversed by the treatment of CB1 receptor antagonist (Figure 9A, 9B). By contrast, there were no significant changes in subcutaneous fat tissues in both models (Figure 9C, 9D). We also quantified CB1R, resistin and proinflammatory genes in visceral fat tissues. The high fat diet led to an increase of CB1R and resistin levels in visceral fat tissues and these were reduced in SR141716 treated mice (Figure 9E-9J). The expression of proinflammatory genes, TNFα, IL-1β and IL-6, was increased by high fat diet in visceral fat tissues and reversed by SR141716 treatment in visceral fat tissues (TNFα; ND 1.0±0.008, HFD 1.546±0.06, HFD+SR 0.629±0.133, $P<0.0001$, n=4, IL-1β; ND 1.247±0.25, HFD 3.915±0.65, HFD+SR 1.632±0.08, P=0.01, n=4, IL-6; ND 1.0±0.01, HFD 4.716±0.72, HFD+SR 1.869±0.05, P=0.01, n=4, in humanized mice and TNFα; ND 1.225±0.13,

HFD 9.631±1.07, HFD+SR 5.136±1.51, P=0.001, n=4, IL-1β; ND 0.976±0.04, HFD 3.205±0.34, HFD+SR 1.692±0.06, P=0.0003, n=4, IL-6; ND 0.738±0.18, HFD 5.597±0.27, HFD+SR 1.937±0.85, P=0.003, n=4, in humanized resistin mice) (Figure 10A-10F). There was no significant different proinflammatory gene expression in control mice (Retn-/- mice) (Figure 10G-10I). Moreover, high fat diet increased adipocyte sizes and CLS (crown-like structures) as a mark of inflammation and reversed by SR141716 treatment in visceral fat tissues (Figure 10J-10K). These results suggested that the endocannabinoid system might regulate not only insulin resistance, but also inflammation.

High fat diet increases the level of CB1 receptor ligand, resulting in the increased migration of CB1R positive monocytes

To explain how high fat diet increased the infiltration of monocytes expressing CB1 receptors, we hypothesized that the high fat diet might increase the level of CB1 receptor ligands, and that the increased level of the ligands promotes the infiltration of CB1R positive cells. We evaluated 2-AG levels of tissues in both *in vivo* models by LC/MS/MS. The high fat diet increased 2-AG levels in visceral fat tissues and this was reversed by SR141716 treatment (humanized mice; ND 0.3708± 0.106, HFD 0.5657±

0.043, HFD+SR 0.3874± 0.07, P<0.05, n=4 and humanized resistin mice; ND 0.3045±0.05, HFD 0.7626±0.033, HFD+SR 0.30±0.027, P=0.003, n=4) (Figure 11C, 11D). In addition, 2-AG accelerated the migration of CB1R positive cells in a dose-dependent manner (Figure 11A). Moreover, transmigrated CB1R positive cells by 2-AG were reduced by the pretreatment of CB1R antagonists, SR141716 and AM251 (Figure 11B).

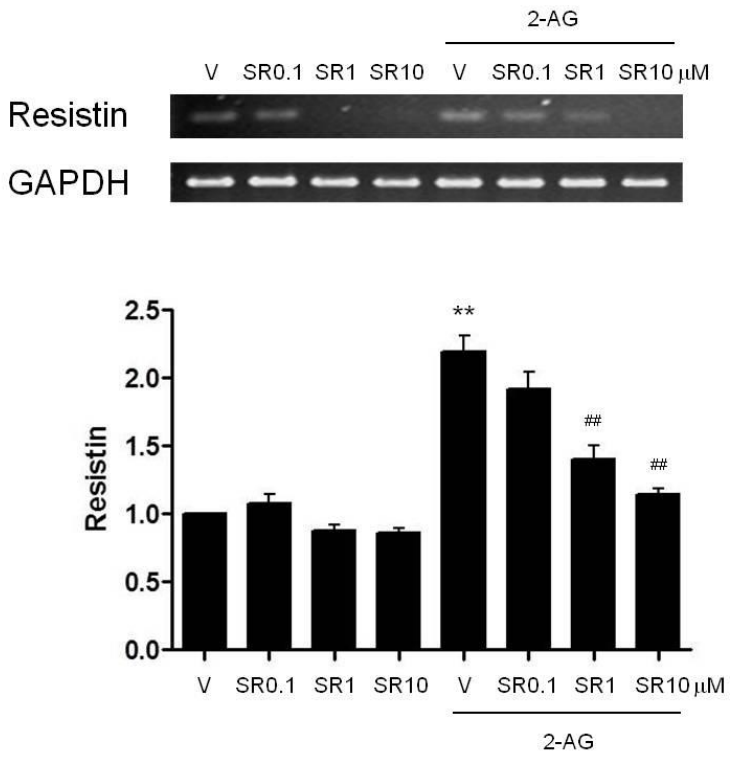


Figure 1. Resistin expressions in human peripheral blood mononuclear cells (PBMCs).

Human PBMCs were treated with 10μM 2-AG with or without 0.1μM, 1μM and 10μM SR141716. Cells were harvested and performed RT-PCR for resistin. All values are representative of 3 different experiments. Data are represented as mean±SEM. **p < 0.01 vehicle vs. 2-AG, ##p < 0.01 2-AG vs. 2-AG + SR141716.

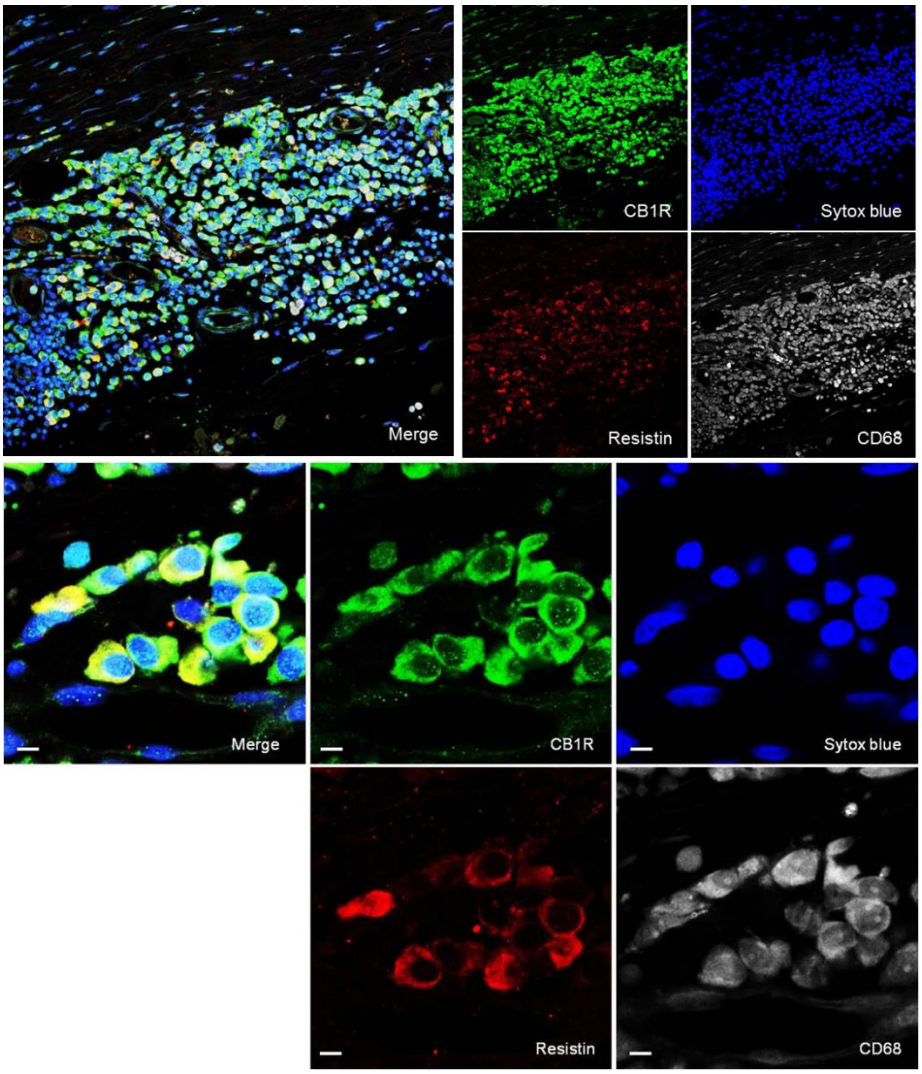


Figure 2. CB1R and resistin expressions in human arteries.

Immunofluorescence for CB1R and resistin in human atheromatous artery. Green = CB1R, Red = Resistin, Gray = CD68, Sytox blue = Nuclei. Scale bars: 10 μm.

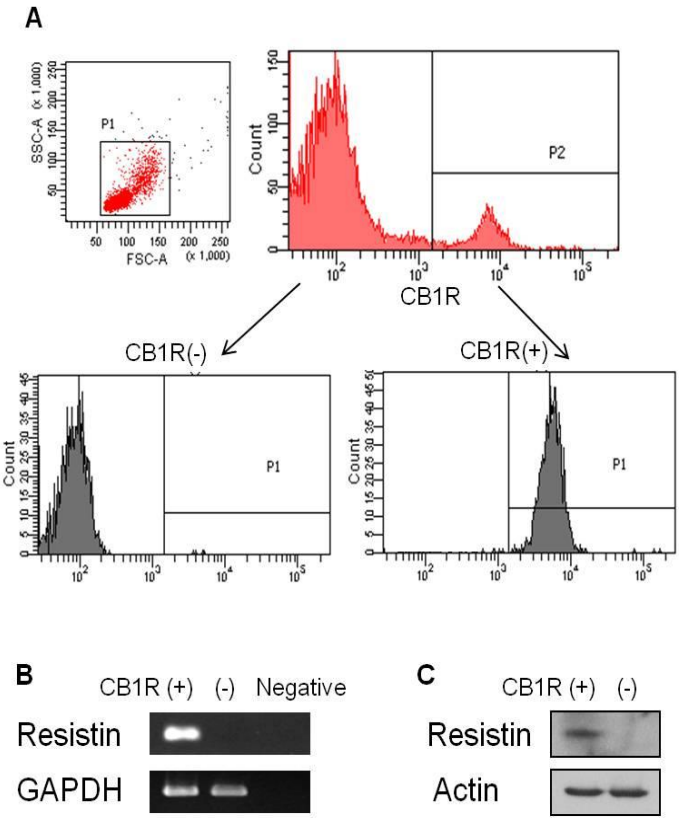


Figure 3. CB1R positive cell sorting and resistin expressions in CB1R positive cells.

(A) FACS sorting for CB1R positive cells and purities of the isolated cells confirmed by FACS analysis. Resistin expressions in sorted CB1R positive cells by RT-PCR (B) and western blot assay (C).

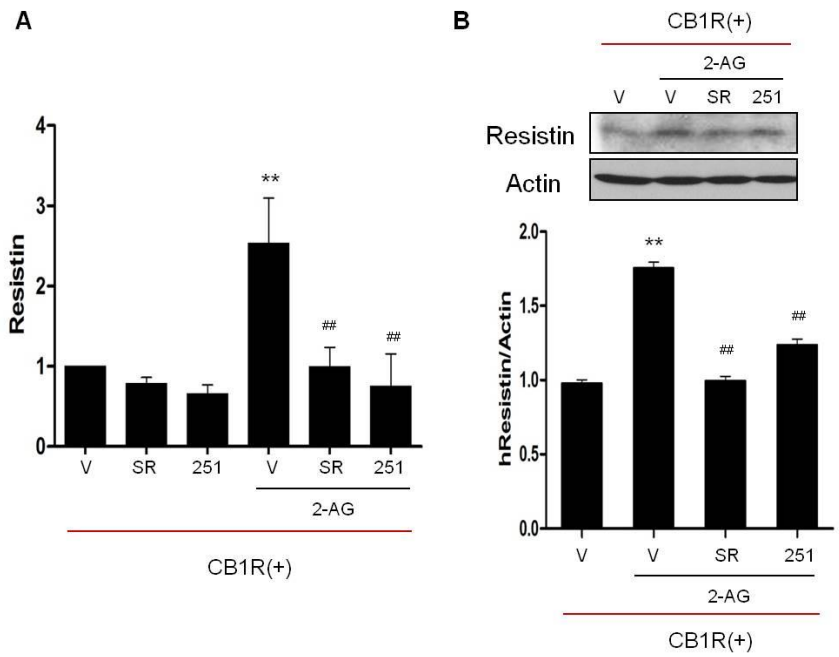


Figure 4. Resistin expressions regulated by the endocannabinoid system in CB1R positive cells.

Resistin expressions treated by 10μM 2-AG with or without 1μM SR141716 and 10μM AM251 in CB1R positive cells by Real-time PCR (A) and western blot and quantitation of the blot (B). All values are representative of 5 and 4 different experiments, respectively. Data are presented as mean±SEM. ** $p < 0.01$ vehicle vs. 2-AG, ## $p < 0.01$ 2-AG vs. 2-AG + SR141716 or AM251.

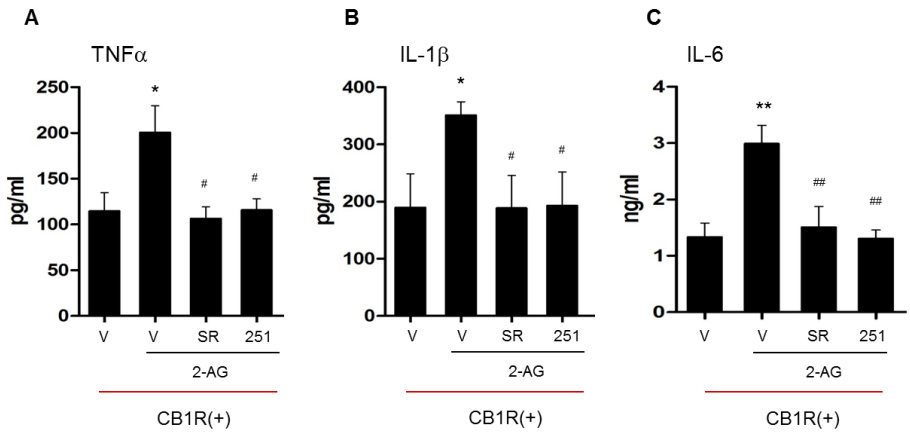
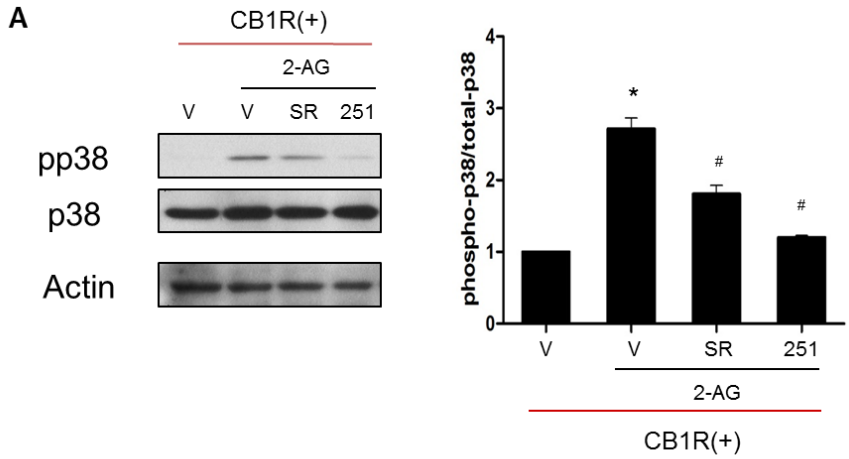


Figure 5. Inflammatory cytokines regulated by the endocannabinoid system in CB1R positive cells.

(A-C) Inflammatory cytokines were measured by ELISA in CB1R positive cell supernatant treated by 10μM 2-AG with or without 1μM SR141716 and 10μM AM251. All values are representative of 4 different experiments. Data are presented as mean±SEM. * $p < 0.05$, ** $p < 0.01$ vehicle vs. 2-AG, # $p < 0.05$, ## $p < 0.01$ 2-AG vs. 2-AG + SR141716 or AM251.


A
CB1R(+)
2-AG
V
V
SR
251
pp38
p38
Actin
phospho-p38/total-p38
4
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SR
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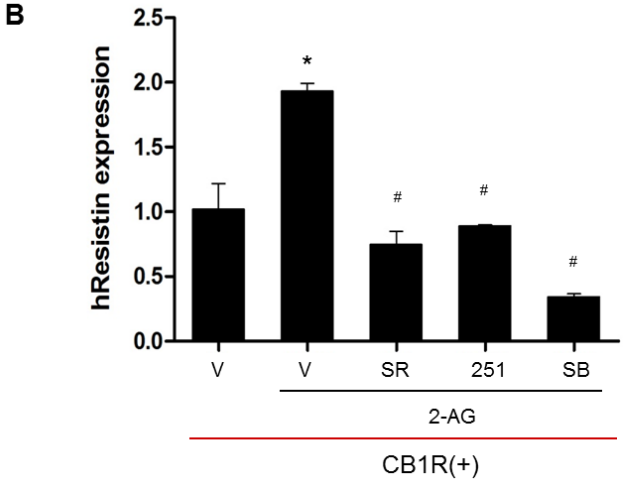
2-AG
CB1R(+)


B
hResistin expression
2.5
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251
SB
2-AG
CB1R(+)

Figure 6. Involvement of p38 MAPK in resistin expressions of CB1R positive cells.

(A) Western blot analysis of p38 phosphorylation in CB1R positive cells treated with 10μM 2-AG, 1μM SR141716, 10μM AM251 and 5μM SB203580. Quantitation of the blot is representative of 4 different experiments. (B) Real-time PCR for resistin in CB1R positive cells treated with 10μM 2-AG, 1μM SR141716, 10μM AM251 and 5μM SB203580. All values are representative of 4 different experiments. Data are represented as mean±SEM. * $p < 0.05$ vehicle vs. 2-AG, # $p < 0.05$ 2-AG vs. 2-AG + SR141716, AM251 or SB203580.

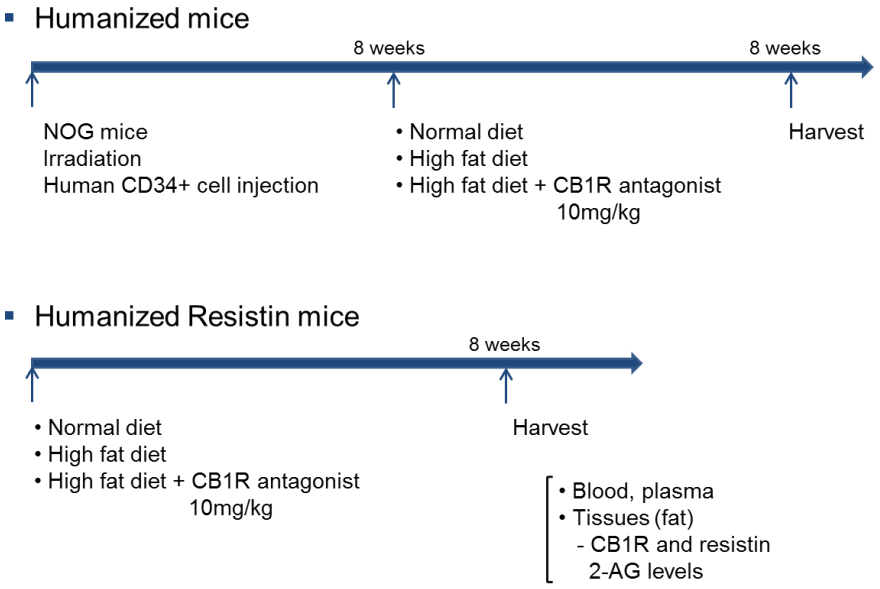
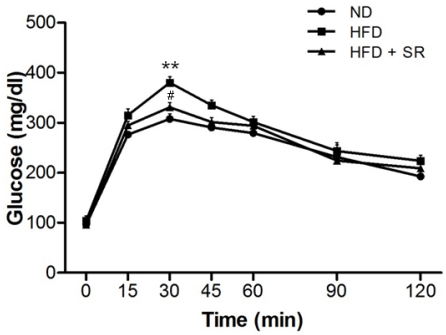


Figure 7. The schematic experiments *in vivo*.

- Humanized mice

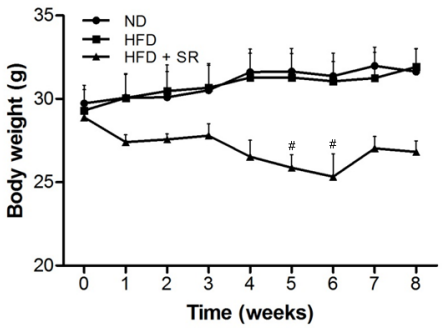
A Glucose-tolerance test



B

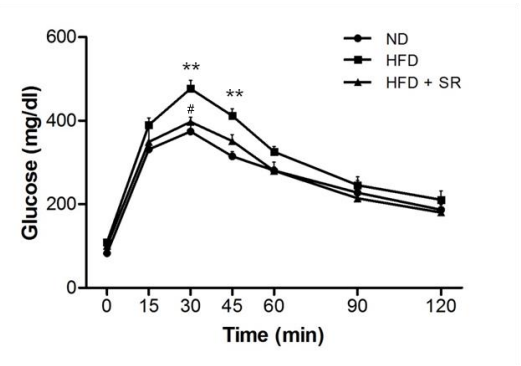
	ND	HFD	HFD + SR
Glucose (mg/dl)	107.0 ± 15.82	128.8 ± 16.85	106.0 ± 13.57
Insulin (ng/ml)	0.3519 ± 0.048	0.8283 ± 0.049	0.4128 ± 0.085
HOMA-IR	2.230 ± 0.3094	6.278 ± 0.05 *	2.592 ± 0.5343 #
QUICKI	0.34 ± 0.008	0.29 ± 0.002 *	0.33 ± 0.011 #

C Body weight



- Humanized resistin mice

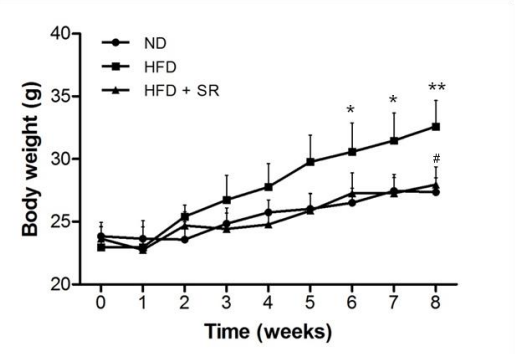
D Glucose-tolerance test



E

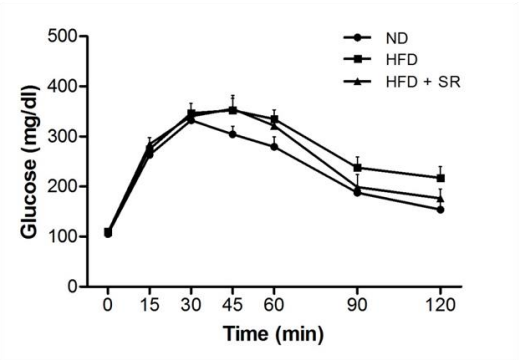
	ND	HFD	HFD + SR
Glucose (mg/dl)	106.5 ± 6.810	143.7 ± 9.0	113.9 ± 9.019
Insulin (ng/ml)	0.1852 ± 0.013	0.29 ± 0.003	0.1723 ± 0.025
HOMA-IR	1.17 ± 0.08	2.47 ± 0.028 *	1.16 ± 0.173 #
QUICKI	0.37 ± 0.004	0.33 ± 0.005 *	0.37 ± 0.008 #

F Body weight



- control mice (Retn-/-)

G Glucose-tolerance test



H

	ND	HFD	HFD + SR
Glucose (mg/dl)	105.0 ± 6.595	110.2 ± 5.006	109.0 ± 3.24
Insulin (ng/ml)	0.1538 ± 0.009	0.2137 ± 0.052	0.2004 ± 0.031
HOMA-IR	0.96 ± 0.061	1.632± 0.3979	1.495 ± 0.2383
QUICKI	0.39 ± 0.004	0.364± 0.012	0.366 ± 0.009

I Body weight

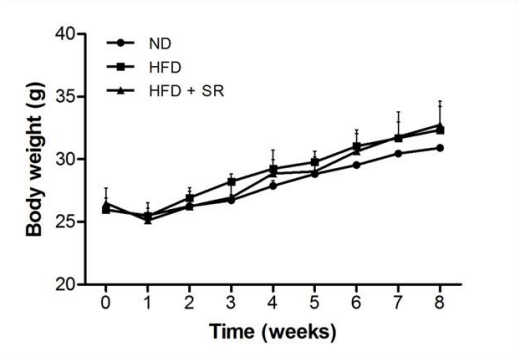
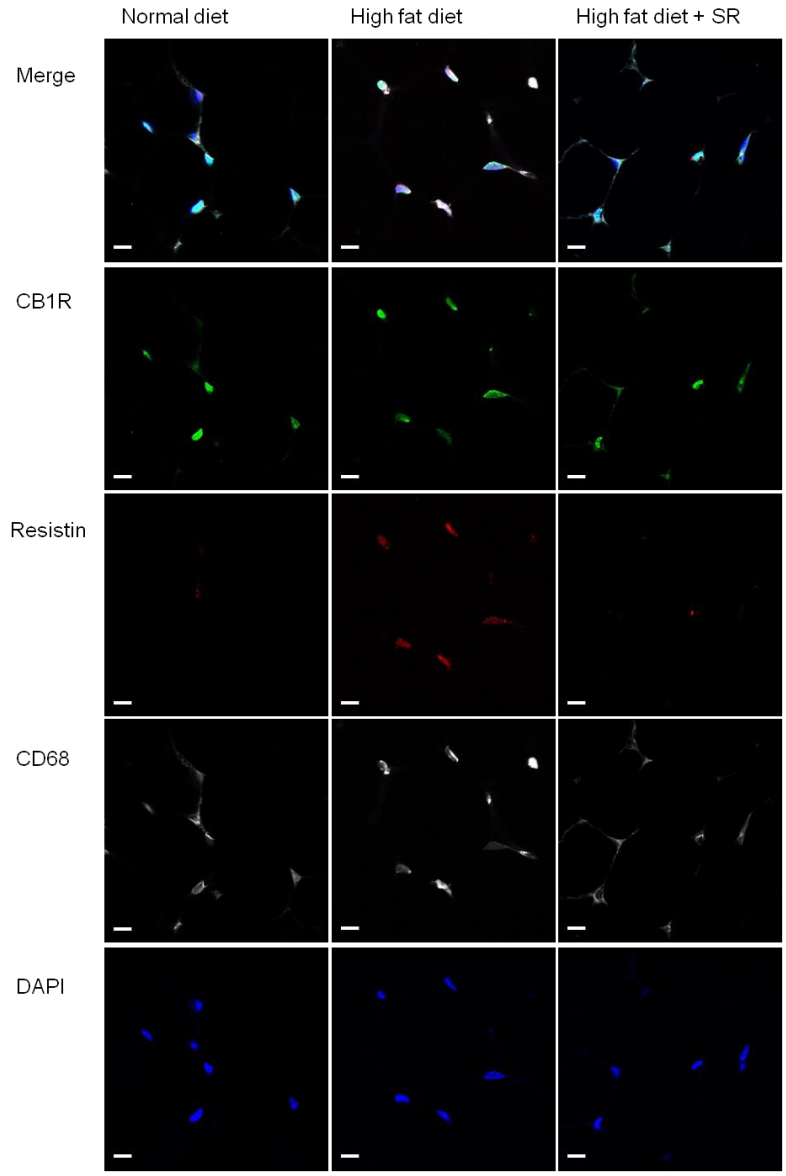


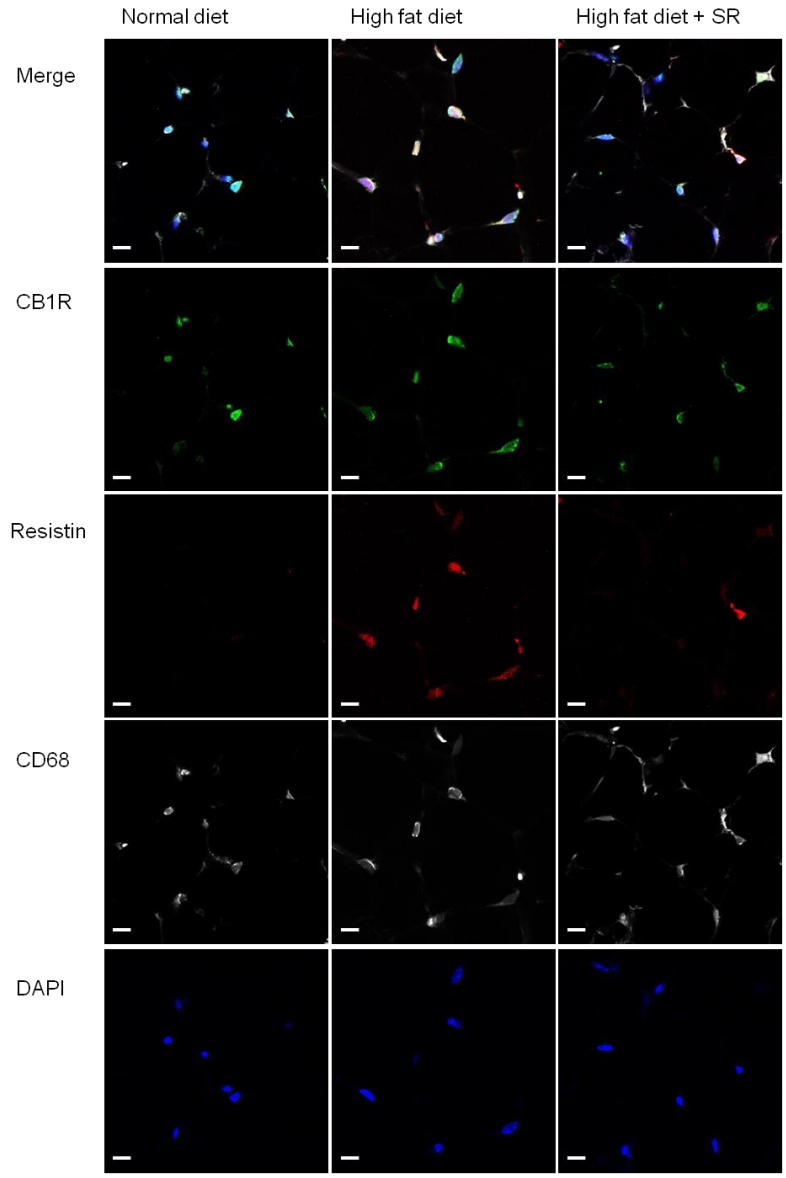
Figure 8. Glucose homeostasis in humanzied mice and humanized resistin mice.

(C)(F)(I) Body weight of mice fed with a normal diet, high fat diet and SR141716 i.p. injected high fat diet was measured weekly for 8 weeks. (A)(D)(G) Glucose-tolerance test was measured at 0, 15, 30, 45, 60, 90 and 120 min. (B)(E)(H) Glucose (mg/dl), Insulin (ng/ml) levels, HOMA-IR and QUICKI. All values are representative of 6-8 different experiments. Data are represented as mean±SEM. * $p < 0.05$, ** $p < 0.01$ normal diet vs. high fat diet, # $p < 0.05$ high fat diet vs. high fat diet with SR141716.

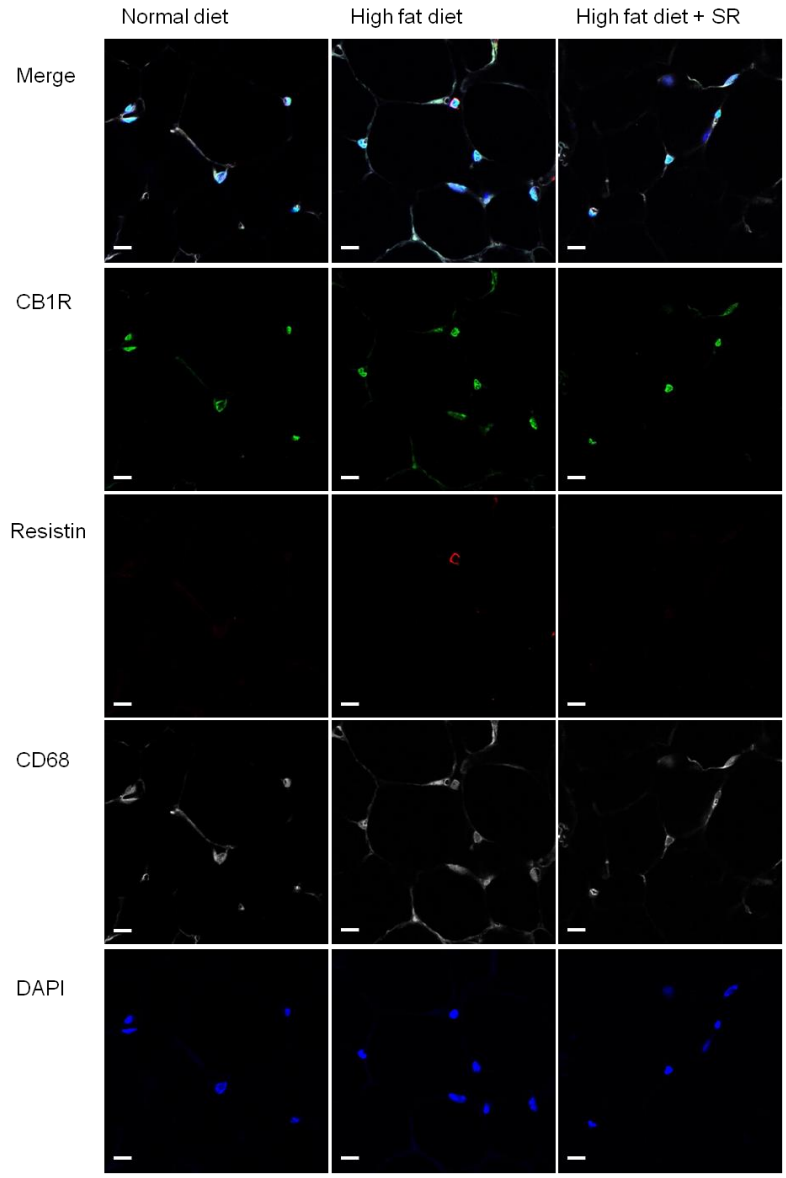
A Humanized mice



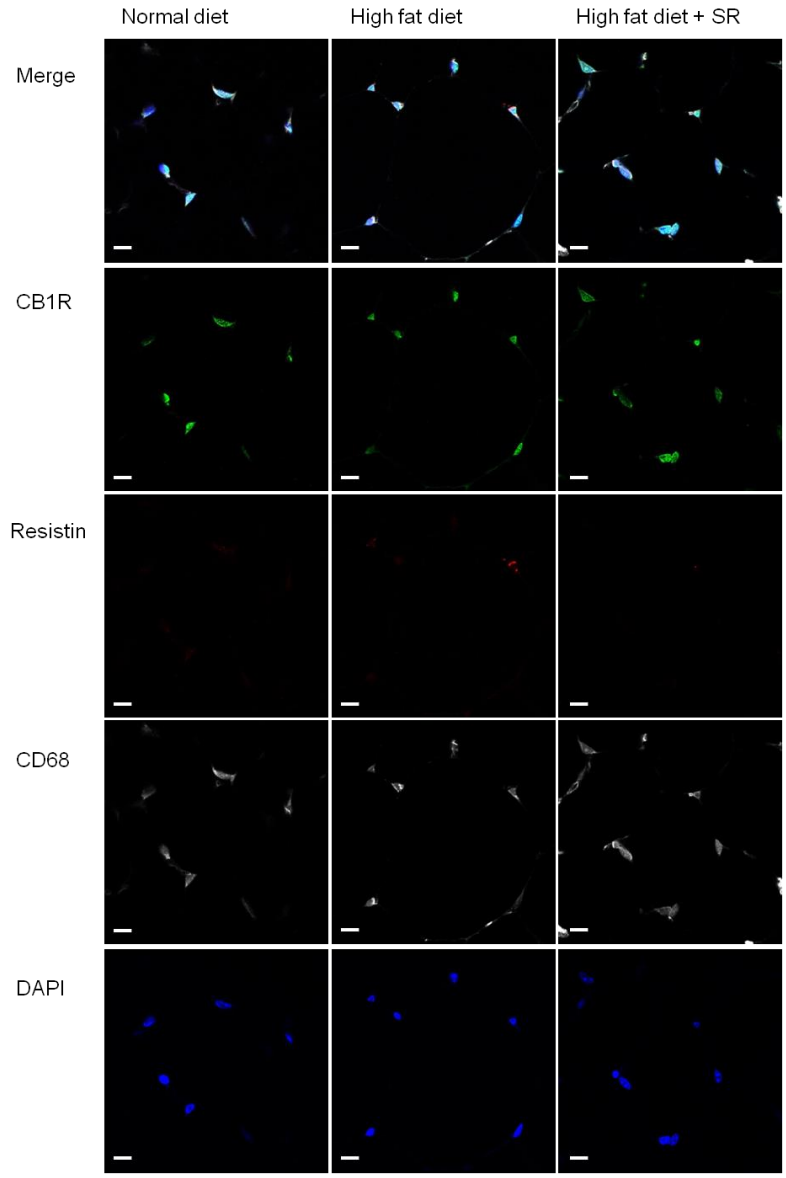
B Humanized resistin mice



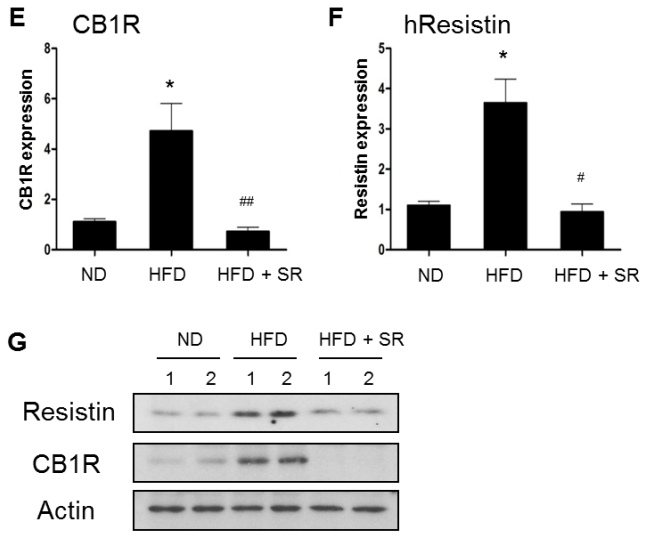
c Humanized mice



D Humanized resistin mice



- Humanized mice



- Humanized resistin mice

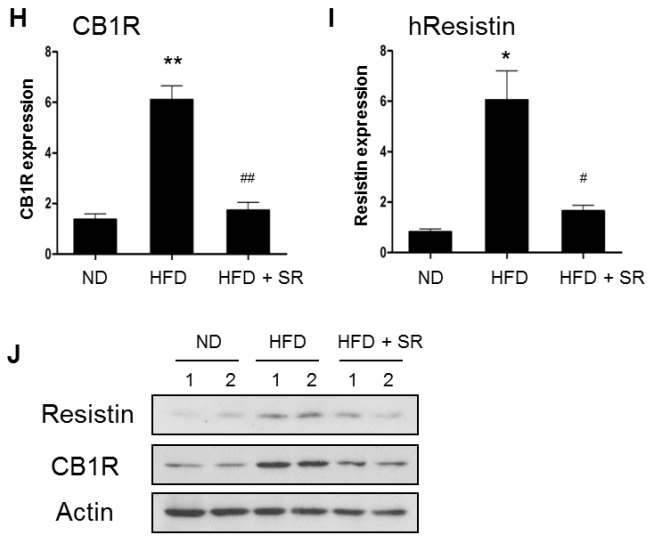
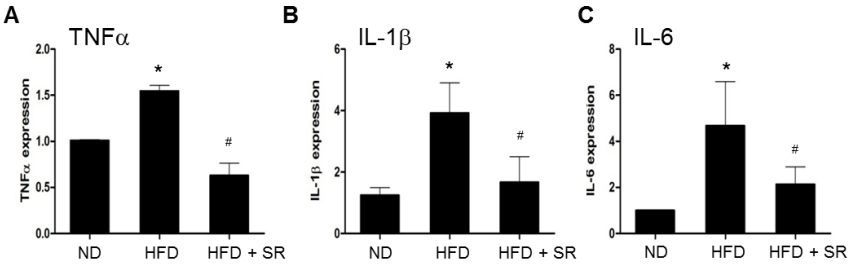


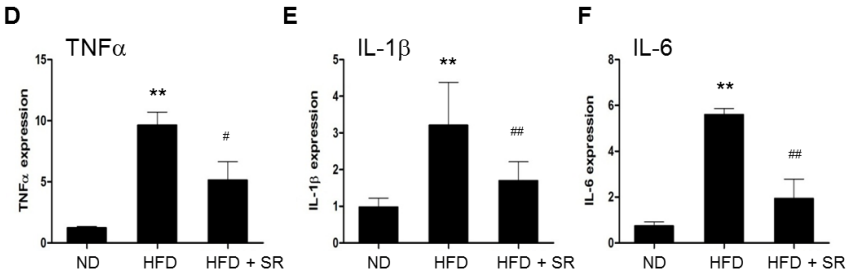
Figure 9. CB1R and resistin expressions in visceral and subcutaneous fat tissues *in vivo*.

Immunofluorescence staining for CB1R and resistin of fat tissues. (A-B) Visceral fat tissues of Humanized mice (A) and Humanized resistin mice (B). (C-D) Subcutaneous fat tissues of Humanized mice (C) and Humanized resistin mice (D). Real-time PCR and western blot of CB1R and resistin in visceral fat tissues of Humanized mice (E-G) and Humanized resistin mice (H-J). Mice were fed with normal diet and high fat diet with or without i.p. injected SR141716 for 8 weeks. Green = CB1R, Red = Resistin, Gray = CD68, DAPI = Nuclei. Scale bars: 10 μm.

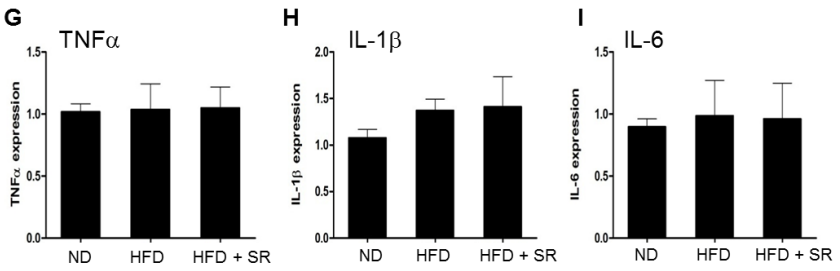
- Humanized mice



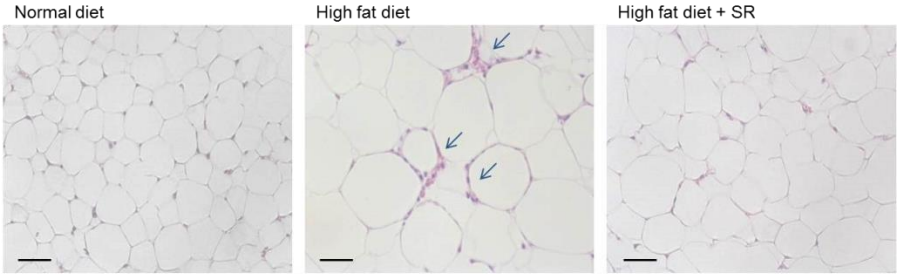
- Humanized resistin mice



- control mice (Retn-/-)



J Humanized mice



K Humanized Resistin mice

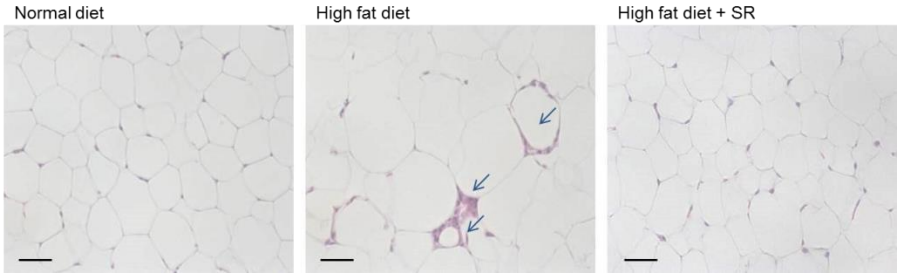
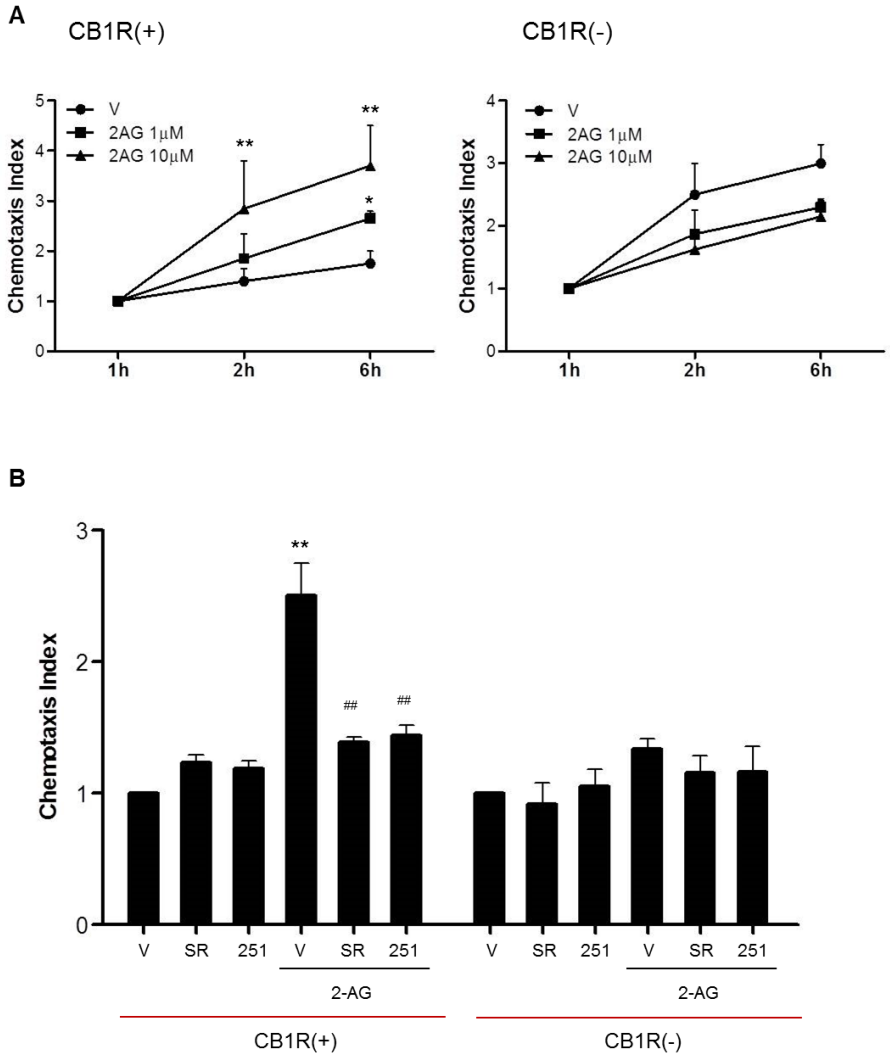
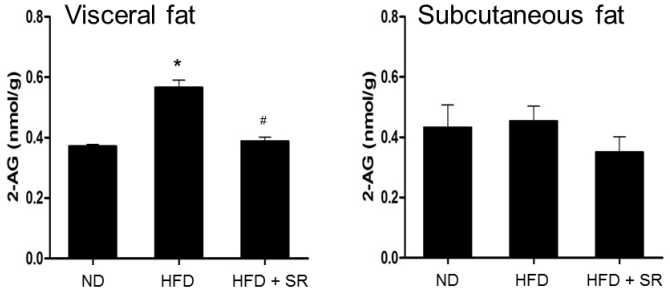


Figure 10. Proinflammatory gene expressions and CLS in visceral fat tissues *in vivo*.

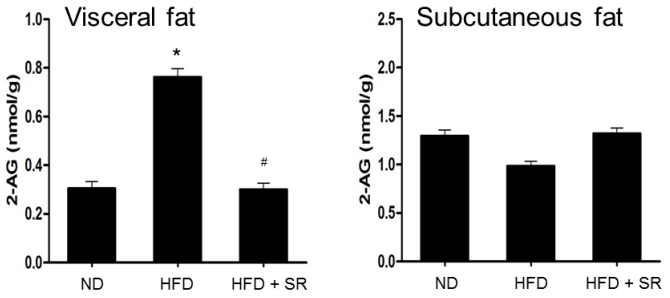
(A-I) Proinflammatory gene expressions by Real-time PCR in visceral fat tissues. (A)(D)(G) TNFα, (B)(E)(H) IL-1β and (C)(F)(I) IL-6 in humanized mice, humanized resistin mice and control mice, respectively. (J-K) Hematoxylin and eosin staining of visceral fat tissues. CLS (crown-like structures) are indicated by arrows. All values are representative of 5-8 different experiments. Data are represented as mean±SEM. * $p < 0.05$, ** $p < 0.01$ normal diet vs. high fat diet, # $p < 0.05$, ## $p < 0.05$ high fat diet vs. high fat diet with SR141716. Scale bars: 50 μm.


A
CB1R(+)
CB1R(-)
V
2AG 1μM
2AG 10μM
Chemotaxis Index
1h
2h
6h
**
*
B
Chemotaxis Index
V
SR
251
2-AG
CB1R(+)
CB1R(-)
##

C Humanized mice



D Humanized resistin mice



E control mice (Retn-/-)

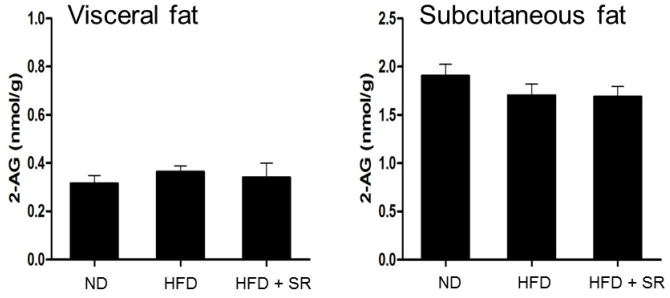


Figure 11. The migration of CB1R positive cells by 2-AG and 2-AG levels in fat tissues.

(A) CB1R positive cells and negative cells were added to the Transwell insets and 2-AG 1μM and 10μM was added to the lower compartment. Incubation was carried out for the indicated periods of time. (B) Cells were pretreated with vehicle, 1μM SR141716 and 10μM AM251 and then added to the Transwell inserts. The migration of the cells from the upper to lower compartment was measured. All values are representative of 4 different experiments. * $p < 0.05$ vehicle vs. 2-AG, # $p < 0.05$ 2-AG vs. 2-AG + SR141716 or AM251. (C-E) 2-AG levels in visceral and subcutaneous fat tissues of humanized mice (C), humanized resistin mice (D) and control mice (Retn-/-) (E) by LC/MS/MS. All values are representative of 5-8 different experiments. Data are represented as mean±SEM. * $p < 0.05$ normal diet vs. high fat diet, # $p < 0.05$ high fat diet vs. high fat diet with SR141716.

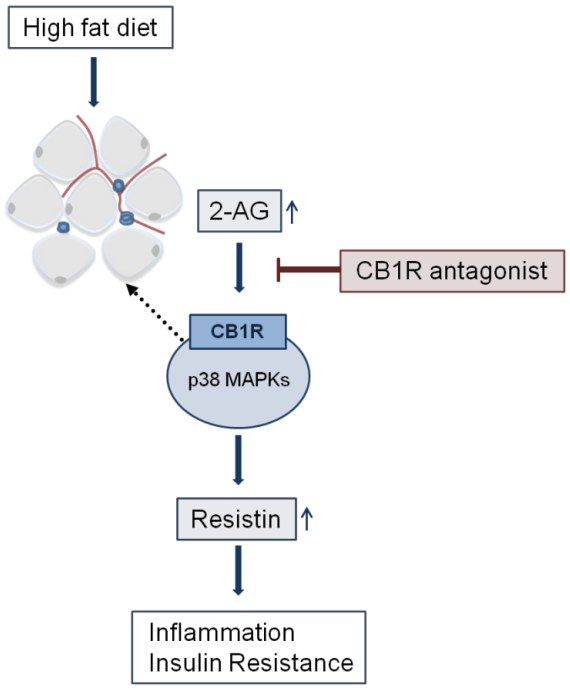


Figure 12. A schematic figure of resistin regulation by the endocannabinoid system.

The endocannabinoid system regulates resistin expression via the CB1 receptor and the infiltration of CB1R positive cells into the tissues increased endocannabinoid levels associated with the inflammation and insulin resistance.

Discussion

We showed that CB1R was expressed with resistin in human atheromatous arteries and resistin was expressed in CB1R positive cells. The resistin expression was increased by endocannabinoid and reduced by CB1R antagonists. These results indicate that endocannabinoid systems regulate resistin expressions.

In the current studies, CB1 receptors were expressed in the human atheromas, especially in the macrophage lesions[13-14] and resistin was expressed in atheromas around macrophages.[15-16] Thus we hypothesized that CB1 receptor might be crucial for resistin expressions. We found that resistin was colocalized with CB1R positive cells in atheromas and resistin expression was higher in CB1R positive cells than CB1R negative cells. The endocannabinoid system is activated by obesity and CB1R antagonists significantly reduce cardiometabolic risk factors.[4] In vitro studies showed that endocannabinoid 2-AG increased resistin levels and decreased by the CB1R antagonist, SR141716 and AM251. Based on the results from the CB1R specific antagonist, the reduced resistin expression should be mediated through CB1 receptors. From these results, we conclude that resistin is expressed in CB1R positive cells and regulated by endocannabinoid systems.

CB1 receptor is a member of the G protein-coupled receptors (GPCRs) superfamily and stimulation of CB1R leads to activation of mitogen-activated

protein kinases (MAPKs).[17-18] The present study indicated that 2-AG induced phosphorylation of p38 was attenuated by CB1R antagonists in CB1R positive cells. Moreover, increased resistin levels by 2-AG were inhibited by p38 inhibitor in CB1R positive cells. Therefore, resistin expressions regulated by endocannabinoid systems might involve through inhibition of p38 activation in CB1R positive cells.

Resistin, an adipokine that modulates insulin resistance is linked to obesity and cardiovascular disease. The association between resistin and insulin resistance in humans remains controversial because human resistin is secreted by monocytes and macrophages rather than adipocytes as in mice. In previous studies, a significant positive correlation between resistin and insulin resistance has been demonstrated in humanized resistin mice, transgenic mice that have macrophage specific expression of human resistin but lacks murine resistin.[20] In recent studies, resistin in humans has been expressed in atherosclerotic plaques as an inflammatory mediator.[14-16] We studied with humanized mice and humanized resistin mice for express human resistin to test human resistin contributes to insulin resistance and inflammation and whether it involves to the endocannabinoid systems. The present study found that CB1R activation by high fat diet enhances resistin expressions and it induces insulin resistance and inflammation. Through the specific inhibition of CB1 receptors using a CB1R antagonist, SR141716, we proved that high fat diet induced resistin expressions co-localized with CB1R were reduced by

SR141716. In addition, the decreased insulin sensitivity was normalized and increased proinflammatory gene levels were reduced by SR141716.

Several studies have reported that 2-AG potentiates human peripheral mononuclear cell migrations.[23-24] We examined the effect of 2-AG on the motility of CB1R positive cells. Transwell insert assays showed that 2-AG induced CB1R positive cell migrations and this was inhibited by CB1R antagonists. In humanized mice and humanized resistin mice models, 2-AG levels were increased by high fat diet and reduced by SR141716 in visceral fat tissues. Thus, these results indicated that the migration of CB1R positive cell by 2-AG was mediated through the CB1 receptors and the high fat diet induced CB1R positive cell infiltrations into the visceral fat tissues according to the increase of 2-AG levels. The migrated and activated CB1R positive cells by 2-AG express resistin, and that induces insulin resistance and inflammation. These results suggest that, in humans, the insulin resistance and inflammation might be provoked by the resistin secreted by monocytes infiltrating the tissues. This can explain why the human adipose tissue dose not secrete resistin and the relationship between resistin and insulin resistance and inflammation in humans.

The CB1 receptor antagonist, rimonabant was developed as a modulator of obesity. Randomized clinical trials showed a considerable reduction in body weight in subjects taking rimonabant. In these studies, rimonabant also increased the level of HDL, but decreased the level of TG. In addition,

patients treated with rimonabant exhibited the increased levels of adiponectin.[25-26] However, all these trials showed adverse events such as psychiatric disorders such as depression and anxiety, leading to discontinuation. Recently, many companies have tried to develop a CB1 receptor antagonist that does not cross the blood-brain barrier. Therefore, we expect that our findings could be applied to the treatment of cardiometabolic disease with the drugs developed in the future.

In summary, our results provide the first evidence that the endocannabinoid system regulates resistin expressions via the CB1 receptor of human peripheral monocytes, thus regulating the infiltration of these cells into the tissues associated with the insulin resistance and inflammation. Therefore, these findings might provide new insight into the relation between the endocannabinoid system and resistin. Furthermore, the regulation of resistin via CB1 receptor might provide beneficial effects in obesity related insulin resistance and inflammation as risk factors of cardiovascular disease.

References

1. Rosenson, R.S. (2009). Role of the endocannabinoid system in abdominal obesity and the implications for cardiovascular risk. Cardiology *114*, 212-225.

2. Pacher, P., and Steffens, S. (2009). The emerging role of the endocannabinoid system in cardiovascular disease. Seminars in immunopathology *31*, 63-77

3. Mach, F., and Steffens, S. (2008). The role of the endocannabinoid system in atherosclerosis. Journal of neuroendocrinology *20 Suppl 1*, 53-57

4. Mach, F., Montecucco, F., and Steffens, S. (2009). Effect of blockage of the endocannabinoid system by CB(1) antagonism on cardiovascular risk. Pharmacological reports : PR *61*, 13-21.

5. Fong, T.M., and Heymsfield, S.B. (2009). Cannabinoid-1 receptor inverse agonists: current understanding of mechanism of action and unanswered questions. International journal of obesity *33*, 947-955.

6. Kirilly, E., Gonda, X., and Bagdy, G. (2012). CB1 receptor antagonists: new discoveries leading to new perspectives. Acta physiologica *205*, 41-60.

7. Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S., and Lazar, M.A. (2001). The hormone resistin links obesity to diabetes. Nature *409*, 307-312.

8. Pang, S.S., and Le, Y.Y. (2006). Role of resistin in inflammation and inflammation-related diseases. Cellular & molecular immunology *3*, 29-34.

9. Bokarewa, M., Nagaev, I., Dahlberg, L., Smith, U., and Tarkowski, A. (2005). Resistin, an adipokine with potent proinflammatory properties. Journal of immunology *174*, 5789-5795.

10. Schwartz, D.R., and Lazar, M.A. (2011). Human resistin: found in translation from mouse to man. Trends in endocrinology and metabolism: TEM *22*, 259-265.

11. Starowicz, K.M., Cristino, L., Matias, I., Capasso, R., Racioppi, A., Izzo, A.A., and Di Marzo, V. (2008). Endocannabinoid dysregulation in the pancreas and adipose tissue of mice fed with a high-fat diet. Obesity *16*, 553-565.

12. Batetta, B., Griinari, M., Carta, G., Murru, E., Ligresti, A., Cordeddu, L., Giordano, E., Sanna, F., Bisogno, T., Uda, S., *et al.* (2009). Endocannabinoids may mediate the ability of (n-3) fatty acids to reduce ectopic fat and inflammatory mediators in obese Zucker rats. The Journal of nutrition *139*, 1495-1501.

13. Han, K.H., Lim, S., Ryu, J., Lee, C.W., Kim, Y., Kang, J.H., Kang, S.S., Ahn, Y.K., Park, C.S., and Kim, J.J. (2009). CB1 and CB2 cannabinoid receptors differentially regulate the production of reactive oxygen species by macrophages. Cardiovascular research *84*, 378-386.

14. Sugamura, K., Sugiyama, S., Nozaki, T., Matsuzawa, Y., Izumiya, Y., Miyata, K., Nakayama, M., Kaikita, K., Obata, T., Takeya, M., *et al.* (2009). Activated endocannabinoid system in coronary artery disease and antiinflammatory effects of cannabinoid 1 receptor blockade on macrophages. Circulation *119*, 28-36.

15. Jung, H.S., Park, K.H., Cho, Y.M., Chung, S.S., Cho, H.J., Cho, S.Y., Kim, S.J., Kim, S.Y., Lee, H.K., and Park, K.S. (2006). Resistin is secreted from macrophages in atheromas and promotes atherosclerosis. Cardiovascular research *69*, 76-85.

16. Cho, Y., Lee, S.E., Lee, H.C., Hur, J., Lee, S., Youn, S.W., Lee, J., Lee, H.J., Lee, T.K., Park, J., *et al.* (2011). Adipokine resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery. Journal of the American College of Cardiology *57*, 99-109.

17. Bosier, B., Muccioli, G.G., Hermans, E., and Lambert, D.M. (2010). Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities. Biochemical pharmacology *80*, 1-12.

18. Turu, G., and Hunyady, L. (2010). Signal transduction of the CB1 cannabinoid receptor. Journal of molecular endocrinology *44*, 75-85.

19. Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K.,

Ueyama, Y., Koyanagi, Y., Sugamura, K., Tsuji, K., *et al.* (2002). NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood *100*, 3175-3182.

20. Qatanani, M., Szwergold, N.R., Greaves, D.R., Ahima, R.S., and Lazar, M.A. (2009). Macrophage-derived human resistin exacerbates adipose tissue inflammation and insulin resistance in mice. The Journal of clinical investigation *119*, 531-539.

21. Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., and Turner, R.C. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia *28*, 412-419.

22. Katz, A., Nambi, S.S., Mather, K., Baron, A.D., Follmann, D.A., Sullivan, G., and Quon, M.J. (2000). Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. The Journal of clinical endocrinology and metabolism *85*, 2402-2410.

23. Kishimoto, S., Gokoh, M., Oka, S., Muramatsu, M., Kajiwara, T., Waku, K., and Sugiura, T. (2003). 2-arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB2 receptor-dependent

mechanism. The Journal of biological chemistry *278*, 24469-24475.

24. Kishimoto, S., Muramatsu, M., Gokoh, M., Oka, S., Waku, K., and Sugiura, T. (2005). Endogenous cannabinoid receptor ligand induces the migration of human natural killer cells. Journal of biochemistry *137*, 217-223.

25. Aronne, L.J., and Isoldi, K.K. (2007). Cannabinoid-1 receptor blockade in cardiometabolic risk reduction: efficacy. Am J Cardiol *100*, 18P-26P.

26. Topol, E.J., Bousser, M.G., Fox, K.A., Creager, M.A., Despres, J.P., Easton, J.D., Hamm, C.W., Montalescot, G., Steg, P.G., Pearson, T.A., *et al.* (2010). Rimonabant for prevention of cardiovascular events (CRESCENDO): a randomised, multicentre, placebo-controlled trial. Lancet *376*, 517-523.

국문초록

Cardiovascular disease는 미국을 포함한 선진국들에서 높은 사망률을 보이고 있다. Obesity는 cardiovascular morbidity와 mortality의 위험증가와 연관되어 있는데, insulin resistance, inflammation 등을 일으켜 cardiovascular disease를 일으킨다. 또한 obesity는 endocannabinoid level을 증가시켜 endocannabinoid system의 이상을 초래한다. Endocannabinoid system은 energy balance, food intake, lipid와 glucose metabolism 을 조절하며, overactivation되면 brain뿐만 아니라 peripheral tissue에 작용하여 cardiovascular disease의 위험을 증가시킨다. Resistin은 12.5 kDa adipokine으로 cysteine-rich secretory protein family이며, Thiazolidinediones(TZD)에 의해 downregulation되는 gene으로 발견되었고, adipocyte-derived polypeptide로 rodents에서 obesity와 insulin resistance와 관련해서 연구되었다. 그렇지만, resistin은 rodents와 다르게 human에서는 monocytes와 macrophages에서 주로 발현하고, adipose tissue에서는 거의 발현하지 않는다. 덕분에 human에서는 resistin이 어떤 역할을 하는지 명확히 알려져 있지 않은데, 최근 연구에서 resistin이 inflammatory process에 연관되어있다는 결과들이 나오고 있다. 따라서 본 연구에서는 endocannabinoid system과 resistin의 연관성을 살펴보고, insulin resistance와 inflammation을 조절할 수 있는

지 밝혀보고자 하였다.

Human atheromas 에서 CB1R과 resistin 이 coexpression 되었고, CB1R positive cell을 FACS sorting하였을 때 CB1R positive cell에서 resistin의 발현이 negative cell에 비해 현저히 높았다. 이렇게 resistin을 발현하는 CB1R positive cell에서 endocannabinoid인 2-AG에 의해서 resistin의 발현이 증가하고, CB1R antagonist인 SR141716 (Rimonabnat)과 AM251에 의해서 감소되었다. 또한, CB1R positive cell에서 endocannabinoid system에 의한 resistin 발현은 p38 pathway를 통해서 조절되었다. Endocannabinoid system이 resistin을 통해 insulin resistance와 inflammation을 조절할 수 있는지 알아보기 위해 Humanized NOG mice와 Humanized resistin mice를 이용해 in vivo실험을 하였다. High fat diet에 의해 insulin resistance가 증가되었고, 이는 SR141716을 처리하였을 때 감소되었다. 또한 High fat diet에 의해 증가된 CB1 receptor, resistin과 proinflammatory gene들이 SR141716에 의해 감소되었다. 이러한 결과들은 resistin을 발현하는 cell이 CB1 receptor ligand가 증가된 조직으로 이동하여 자극을 받아서 resistin을 발현하여 insulin resistance와 inflammation을 일으키고, 이는 CB1R antagonist에 의해 감소되는 것을 알 수 있었다.

이러한 결과들을 종합해봤을 때, endocannabinoid system이 CB1 receptor를 통해 resistin expression을 조절하며, 더 나아가 resistin을

expression하는 CB1R positive cell의 endocannabinoid level이 증가된 조직으로의 이동을 조절한다는 것을 알 수 있었다. 또한 이를 통해 inflammation과 insulin resistance를 막아 cardiovascular disease의 위험을 감소시킬 수 있음을 보여준다.

주요어 : Endocannabinoid system, Resistin, human monocyte, Inflammation, Insulin resistance

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