Activated Endocannabinoid System in Coronary Artery Disease and Antiinflammatory Effects of Cannabinoid 1 Receptor Blockade on Macrophages

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Background—Cannabinoid 1 (CB1) receptor blockade with rimonabant represents a clinical therapeutic strategy for obesity. Recently, the role of the endocannabinoid system has been described in peripheral organs. We sought to determine whether the endocannabinoid system could be involved in human atherosclerosis and whether CB1 receptor blockade could modulate proinflammatory activity in macrophages.

Methods and Results—mRNA expression levels of CB1 receptor in coronary atherectomy samples were significantly higher in patients with unstable angina than in those with stable angina (3.62±2.96-fold; n=7; P<0.05). Immunoreactive area analysis of the coronary artery showed that CB1 receptor expression was greater in lipid-rich atheromatous plaques than in fibrous plaques, especially in CD68 macrophages (9.5±1.2% versus 0.6±0.6%; n=5; P<0.01). Levels of blood endocannabinoids were significantly higher in patients with coronary artery disease (n=20) than those without coronary artery disease (n=20) (median [interquartile range]: anandamide, 1.048 pmol/mL [0.687 to 1.387 pmol/mL] versus 0.537 pmol/mL [0.468 to 0.857 pmol/mL], P<0.01; 2-arachidonoyl glycerol, 13.30 pmol/mL [6.65 to 16.21 pmol/mL] versus 7.67 pmol/mL [6.39 to 10.03 pmol/mL], P<0.05). In cultured macrophages, expression of CB1 receptor was significantly increased during monocyte-macrophage differentiation (1.78±0.13-fold; n=6; P<0.01). CB1 receptor blockade in macrophages induced a significant increase in cytosolic cAMP (29.9±13.0%; n=4; P<0.01), inhibited phosphorylation of c-Jun N-terminal kinase (−19.1±12.6%, n=4; P<0.05), and resulted in a significant decrease in the production of proinflammatory mediators (interleukin-1β, −28.9±10.9%; interleukin-6, −24.8±7.6%; interleukin-8, −22.7±5.2%; tumor necrosis factor-α, −13.6±4.8%; matrix metalloproteinase-9, −16.4±3.8%; n=4 to 8; P<0.01).

Conclusions—Patients with coronary artery disease demonstrated the activation of the endocannabinoid system with elevated levels of blood endocannabinoids and increased expression of CB1 receptor in coronary atheroma. CB1 receptor blockade exhibited antiinflammatory effects on macrophages, which might provide beneficial effects on atherogenesis. (Circulation. 2009;119:28-36.)

Key Words: atherosclerosis ▪ inflammation ▪ macrophages ▪ obesity ▪ receptors, cannabinoid

Overweight and obesity (particularly visceral obesity) are quickly reaching global epidemic proportions and are associated with the metabolic syndrome, correlating with an increased risk of cardiovascular morbidity and mortality.1–3 However, lifestyle intervention programs aimed at reducing the risk of cardiovascular disease in obese patients do not appear to provide long-lasting success.4 Consequently, anti-obesity pharmacotherapy has been recognized as an important adjunctive therapy to lifestyle modification to prevent cardiovascular complications.

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The recently discovered endocannabinoid system contributes to the physiological regulation of energy balance, food intake, and metabolism of lipids and glucose.5 This system consists mainly of 2 endogenous ligands, anandamide and 2-arachidonoyl glycerol (2-AG), and 2 types of cannabinoid receptors, cannabinoid 1 (CB1) receptor and cannabinoid 2 (CB2) receptor. CB1 receptors are expressed extensively in the brain and
peripheral tissues, including adipose tissue, liver, and skeletal muscle.6,7

Rimonabant, the first commercially available CB1 receptor blocker, is reported to produce a dose-dependent reduction in food intake. Several clinical trials have demonstrated the efficacy of rimonabant in significantly decreasing body weight and waist circumference and improving multiple cardiometabolic risk factors.8–11 Therefore, CB1 receptor blockade represents a new therapeutic strategy for improving obesity and its associated metabolic abnormalities.

The beneficial effects of CB1 receptor blockade on cardiovascular risk factors in clinical trials were greater than those that could be estimated from weight loss alone.9 It has been suggested that CB1 receptor blockade promotes additional weight-independent effects, including adiponectin secretion from adipocytes, as well as regulation of lipolysis and energy balance.12,13 These studies highlight the potential importance of the peripheral endocannabinoid system, particularly in the pathogenesis of obesity-related cardiovascular diseases.

Inflammation plays an important role throughout all phases of atherosclerosis from the development of fatty streaks to advanced atheroma and macrophage accumulation, ultimately contributing to plaque progression, plaque rupture, and acute coronary syndromes.14 Approaches designed to reduce the inflammatory activity of macrophages in atherogenesis may have additional therapeutic value in preventing and treating coronary artery disease (CAD).15 We sought to show that CB1 receptor blockade could have additional therapeutic value in atherogenesis by decreasing the levels of high-sensitivity C-reactive protein (hsCRP) by CB1 receptor blockade in a large clinical trial.9 Furthermore, a recent clinical study using coronary intravascular ultrasound showed that a CB1 receptor antagonist failed the primary end point of coronary atheroma reduction, but the secondary end point (total atheroma volume) was significantly reduced by a CB1 receptor blocker, suggesting that CB1 receptor blockade may have some potential benefit in antiatherogenic therapy.16

Thus, we hypothesized that the endocannabinoid system is involved in human atherosclerosis and that CB1 receptor blockade might modulate the proinflammatory processes associated with atherogenesis. Here, we investigated the existence and activation of the endocannabinoid system in patients with CAD and the antiinflammatory effects of CB1 receptor blockade on human macrophages.

Methods

An expanded Methods section is available in the online-only Data Supplement.

Immunohistochemistry and Histological Quantification

A total of 15 fresh-frozen human coronary artery specimens were studied. Normal skin samples were used as positive controls for CB1 and CB2 receptor.17 Immunohistochemistry was performed by an indirect immunoperoxidase method with the following antibodies: anti–CB1 receptor (1:500 dilution; PA1–743; Cayman, 101550) for cannabinoid receptors has been verified previously.17,18 The serial sections were examined with computer-assisted image analysis software (Lumina Vision, Mitani Co, Fukui, Japan) used to determine the CB1 receptor–positive areas. Data are presented as the percentage of the CB1 receptor–positive area to plaque area.

Direct Coronary Atherectomy Samples and Measurement of Endocannabinoid Levels in Human Blood

Direct coronary atherectomy (DCA) samples were obtained from 14 patients who underwent percutaneous coronary intervention (7 patients with unstable angina pectoris and 7 patients with stable angina pectoris). Unstable angina pectoris was classified as class IIB-Tpos angina according to the classification of unstable angina pectoris by Hamm and Braunwald.19 Unstable angina pectoris was defined as chest pain at rest with documented transient ST-segment depression or elevation of 0.1 mV in at least 2 continuous ECG leads. We determined the culprit coronary lesions from changes in ECG synchronized with their attacks and echocardiography.

We consecutively measured blood endocannabinoids levels in patients who were scheduled for coronary angiography. Exclusion criteria were inflammatory disease, collagen disease, active infection, and cancer. We recruited 20 patients with CAD defined as the presence of ≥1 stenotic coronary arteries with >50% stenosis on coronary angiography. We included 20 patients without CAD who were age matched to the patients with CAD. All of the patients without CAD were confirmed to be free of CAD by their clinical history, coronary angiography, and exercise stress tests. Endocannabinoids (anandamide, 2-AG) were detected with a liquid chromatography tandem mass spectrometry system and software (Q-Trap and Analyst, version 1.3; Applied Biosystems, Foster City, Calif). Written informed consent was obtained from all patients in this study. The study was in agreement with the guidelines approved by the ethics committee of Kumamoto University Graduate School of Medical Sciences.

Cell Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from the donated buffy coat and blood samples from normal volunteers as described previously.20 The cells were cultured with 10 ng/mL macrophage colony-stimulating factor (M-CSF) and 10 ng/mL granulocyte M-CSF under standard culture conditions. After 4 days of culture, the PBMCs were used as human PBMC-derived macrophages in the experiments. The human monocytic cell line THP-1 was purchased from the American Type Culture Collection (Manassas, Va). These cells were cultured with same protocol and then used as THP-1 cell–derived macrophages after the 4-day culture.

Statistical Analysis

Data are presented as mean ± SD of the indicated number of samples. The mean values for >3 groups were compared by ANOVA. The difference between 2 mean values was analyzed with the unpaired Student t test and Mann-Whitney U test. Frequencies between the 2 groups were compared by use of χ² analysis. A value of P<0.05 was considered statistically significant. Correlations between blood levels of endocannabinoids and age, body weight, body mass index, and continuous blood parameters were examined by simple regression analysis.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript written.

Results

Human Coronary Atheroma Expressed CB1 Receptors, Especially in Lesional Macrophages

The DCA samples taken from patients with unstable angina pectoris had significantly higher levels of CB1 receptor


mRNA expression compared with those taken from patients with stable angina pectoris (3.6 ± 2.96-fold versus stable angina pectoris; n = 7; P < 0.05; Figure 1A). No significant difference in coronary risk factors was observed between the 2 groups (data not shown). The majority of CB1 receptor–positive cells in the advanced atheroma were simultaneously stained with CD68 (Figure 1C and 1D), indicating that macrophages in human atheroma expressed CB1 receptors. Endothelial cells (factor VIII positive) and few intimal smooth muscle cells (α-smooth muscle active positive) also expressed CB1 receptors (Figure 1I and 1J), although the immunoreactivity was weak. According to the American Heart Association histological criteria, the CB1 receptor–positive area was significantly larger in lipid-rich atheromatous plaques (type IV or Va; Figure 1C) than fibrous plaques (type Vc; Figure 1I) (percentage of CB1 receptor–positive area to plaque area: atheromatous plaque, 9.5 ± 1.2%; n = 5; fibrous plaque, 0.6 ± 0.6%; n = 5; P < 0.01; Figure 1B). The area of diffuse intimal thickening also was weakly positive for CB1 receptors at a level similar to that observed in fibrous plaques (Figure 1K). CB1 receptors were expressed to a greater extent than CB2 receptors in advanced atheroma (Figure 1C and 1F).

Figure 1. Expression of cannabinoid receptors in human coronary atherosclerotic plaques. A, Expression of CB1 receptor mRNA in DCA samples. Relative mRNA levels were quantified by real-time polymerase chain reaction analysis corrected for GAPDH mRNA levels used as internal control and expressed as a fold of the mean value measured in samples taken from stable angina. Bars represent mean ± SD. B, Quantitative comparison of CB1 receptor immunoreactive area in various coronary plaques (n = 5 each). CB1 receptor–positive areas were quantified by computer-assisted image analysis software. Data represent the mean ± SD percentage of CB1 receptor–positive area relative to plaque area. C, Distribution of CB1 receptor immunostaining (brown) in coronary artery plaque section (atheromatous area) of a representative case. D, Expression of CD68 (brown) in serial plaque section. E, Positive control for CB1 receptors in human skin. F, Expression of CB2 receptors; representative immunostaining (brown) of atheromatous area. G, Negative control for CB1 and CB2 receptors. H, Positive control for CB2 receptors in human skin. I, Expression of CB1 receptors in the fibrous plaque area; arrows and arrowheads indicate representative immunostaining (brown) of endothelial cells (arrows) and smooth muscle cells (arrowheads). J, Expression of smooth muscle α-actin (α-SMA) (brown) in the serial fibrous plaque area. K, Expression of CB1 receptors (brown) in intimal thickening area; arrows and arrowheads indicate representative immunostaining (brown) of endothelial cells (arrows) and smooth muscle cells (arrowheads). Original magnification: C, D, F, G, I, and J, ×200; E, H, and K, ×100. Scale bars represent 125 μm in C, D, F, G, I, and J and 251 μm in E, H, and K.

Cultured Human Macrophages Expressed CB1 Receptors, and the Expression Was Enhanced by Differentiation Process and Stimuli Contained in Atheroma

Reverse transcriptase polymerase chain reaction revealed that monocytes, PBMC-derived macrophages, and THP-1 cell–derived macrophages (days 1 and 4) expressed CB1 receptor mRNA (Figure 2A). Western blot analysis showed that human monocytes and macrophages expressed CB1 receptor protein; the levels of CB1 receptor protein were higher in macrophages than in monocytes (Figure 2B). In PBMC–derived macrophages, CB1 receptor mRNA expression was significantly increased during monocyte-macrophage differentiation (3.88 ± 2.95 fold; n = 6; P < 0.05; Figure 2C). CB1 receptor mRNA expression on THP-1 cell–derived macrophages also was significantly increased during differentiation in the in vitro culture (1.78 ± 0.13 fold; n = 6; P < 0.01; Figure 2D). The CB1 receptor mRNA expression was significantly
correlated with the augmentation of class A macrophage scavenger receptor mRNA expression (an established marker of macrophage differentiation: r = 0.93; n = 20; P < 0.01). In contrast, CB2 receptor mRNA expression on human macrophages significantly decreased during the in vitro culture (PBMC-derived macrophages, 0.22 ± 0.04-fold, n = 6; THP-1 cell–derived macrophages, 0.23 ± 0.23-fold, n = 6; P < 0.01). Furthermore, CB1 receptor mRNA expression in macrophages was significantly augmented by M-CSF and oxidized low-density lipoprotein (OxLDL) in a concentration-dependent manner (M-CSF 10 ng/mL, 27.7 ± 5.3%, n = 6; OxLDL 50 μg/mL, 42.2 ± 12.7%, n = 6; P < 0.01) (Figure 2E and 2F).

Blood Endocannabinoid Levels Were Increased in Patients With CAD
The blood levels of 2 endocannabinoids were significantly higher in patients with CAD (n = 20) compared with those without CAD (n = 20) (median [interquartile range]: anandamide, 1.048 pmol/mL [0.687 to 1.387 pmol/mL] versus 0.537 pmol/mL [0.468 to 0.857 pmol/mL], P < 0.01; 2-AG, 13.30 pmol/mL [6.65 to 16.21 pmol/mL] versus 7.67 pmol/mL [6.39 to 10.03 pmol/mL], P < 0.05; Figure 3A and 3B). The clinical characteristics of the 2 groups are shown in Table 1. The level of anandamide was significantly associated with body mass index, triglycerides, hsCRP, and glycosylated hemoglobin A1c (HbA1c); the level of 2-AG was significantly associated with hsCRP, HbA1c, and fasting glucose.

Endocannabinoids Were Augmented in Cultured Human Macrophages
During monocyte-macrophage differentiation, mRNA levels of an endocannabinoid-biosynthesizing enzyme (N-acylphosphatidyl-ethanolamine-selective phospholipase D) were significantly increased and the mRNA levels of an endocannabinoid-degrading enzyme (fatty acid amide hydrolase) were significantly reduced (N-acyl-phosphatidyl-ethanolamine-selective phospholipase D, 45.0 ± 9.6%; fatty

Figure 3. Comparison of blood endocannabinoid levels between patients with CAD and those without CAD. A, Box-and-whisker plot showing blood anandamide (AEA) levels in patients with CAD (n = 20) and those without CAD (n = 20). B, Box-and-whisker plot showing blood 2-AG levels in the same groups. In these plots, lines within boxes represent median values: the top and bottom lines of the boxes represent the 25th and 75th percentiles, respectively; the top and bottom bars outside the boxes represent the 90th and 10th percentiles, respectively.
Table 1. Clinical Characteristics of Patients With CAD and Those Without CAD

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients Without CAD (n=20)</th>
<th>Patients With CAD (n=20)</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>Age (mean), y</td>
<td>61.7±14.9</td>
<td>63.0±11.6</td>
<td>0.76</td>
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<tr>
<td>Men/women, n</td>
<td>11/9</td>
<td>15/5</td>
<td>0.32</td>
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<tr>
<td>Smoking, n (%)</td>
<td>1 (5)</td>
<td>5 (25)</td>
<td>0.16</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>12 (60)</td>
<td>16 (80)</td>
<td>0.30</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.1±3.3</td>
<td>24.7±4.3</td>
<td>0.19</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>60.8±12.3</td>
<td>65.6±16.9</td>
<td>0.31</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>84.1±10.5</td>
<td>88.4±8.7</td>
<td>0.17</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>190.6±30.0</td>
<td>178.7±36.6</td>
<td>0.27</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>112.9±21.9</td>
<td>107.9±34.2</td>
<td>0.59</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>59.0±15.3</td>
<td>49.4±19.0</td>
<td>0.09</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>109.5 (77.5–136.5)</td>
<td>146.0 (85.8–215.8)</td>
<td>0.20</td>
</tr>
<tr>
<td>hsCRP, mg/dL</td>
<td>0.05 (0.02–0.14)</td>
<td>0.30 (0.13–0.83)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.2±0.4</td>
<td>5.7±1.0</td>
<td>0.09</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>100.6±25.0</td>
<td>107.9±27.7</td>
<td>0.40</td>
</tr>
<tr>
<td>Medications, n (%)</td>
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<td></td>
<td></td>
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<tr>
<td>Aspirin</td>
<td>6 (30)</td>
<td>20 (100)</td>
<td>&lt;0.001</td>
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<tr>
<td>Statins</td>
<td>8 (40)</td>
<td>17 (85)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>0 (0)</td>
<td>6 (30)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ARBs</td>
<td>7 (35)</td>
<td>14 (70)</td>
<td>0.06</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>14 (70)</td>
<td>12 (60)</td>
<td>0.74</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>2 (10)</td>
<td>13 (65)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nitrates</td>
<td>1 (5)</td>
<td>5 (25)</td>
<td>0.18</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; HDL, high-density lipoprotein; ACE, angiotensin-converting enzyme; and ARB, angiotensin II receptor antagonist. Data are presented as mean±SD and median (interquartile range) when appropriate.

Table 2. Correlation Between Blood Endocannabinoids and Clinical Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AEA</th>
<th>P</th>
<th>2-AG</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>0.06</td>
<td>0.70</td>
<td>0.11</td>
<td>0.51</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>0.43</td>
<td>&lt;0.01</td>
<td>−0.04</td>
<td>0.81</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>0.26</td>
<td>0.10</td>
<td>−0.11</td>
<td>0.50</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>0.27</td>
<td>0.10</td>
<td>−0.10</td>
<td>0.55</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>−0.03</td>
<td>0.79</td>
<td>−0.23</td>
<td>0.16</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>0.03</td>
<td>0.59</td>
<td>−0.27</td>
<td>0.10</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>−0.33</td>
<td>0.14</td>
<td>&lt;0.01</td>
<td>0.98</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>0.41</td>
<td>0.02</td>
<td>0.02</td>
<td>0.91</td>
</tr>
<tr>
<td>hsCRP, mg/dL</td>
<td>0.39</td>
<td>0.03</td>
<td>0.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>0.30</td>
<td>0.02</td>
<td>0.61</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>−0.06</td>
<td>0.67</td>
<td>0.38</td>
<td>&lt;0.05</td>
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</table>

AEA indicates anandamide; BMI, body mass index; and HDL, high-density lipoprotein. n=40.

Other CB1 Receptor Antagonists Suppress Lipopolysaccharide-Induced Production of Inflammatory Cytokines, and CB1 Receptor Knockdown Abolishes the Effects of Rimonabant in Cultured Human Macrophages

Other CB1 receptor antagonists, AM251 (1.0 μmol/L) and AM281 (1.0 μmol/L), resulted in equal reduction in lipopolysaccharide-induced IL-1β production compared with rimonabant 1.0 μmol/L in cultured human macrophages (rimonabant, −23.8±7.3%; AM251, −29.0±5.0%; AM281, −23.3±6.8%; n=6; P<0.01; Figure 5A). When we ablated CB1 receptor expression by RNA interference (RNAi) on cultured human macrophages, rimonabant failed to suppress lipopolysaccharide-induced IL-6 production compared with negative control RNAi (CB1 receptor RNAi, −4.7±0.7% versus negative control RNAi, −25.8±4.5%; n=4; P<0.05; Figure 6B).

CB1 Receptor Blockade With Rimonabant Increased cAMP Levels and Modulated the Activation of c-Jun N-Terminal Kinase in Cultured Human Macrophages

Exposure to rimonabant 1.0 μmol/L for 5 and 15 minutes caused a significant increase in the levels of cytosolic cAMP (5 minutes, 29.9±13.0%; 15 minutes, 20.2±11.0%; n=4; both P<0.01; Figure 6A). Results from Western blot analysis showed that pretreatment with rimonabant 1.0 μmol/L for 24 hours significantly attenuated the phosphorylation of c-Jun N-terminal kinase (JNK) at 15 minutes after lipopolysaccharide stimulation (−19.1±12.6%; P<0.05; Figure 6B and 6C).

Discussion

We showed that CB1 receptors were expressed in the human coronary atheroma, particularly in lesional macrophages, and that endocannabinoid system was activated in patients with CAD. CB1 receptor blockade significantly reduced the production of the proinflammatory mediators from human
macrophages. These results indicate the presence and activation of the endocannabinoid system in human atherosclerosis and suggest that CB1 receptor blockade modulates the inflammatory state in atheromata through its antiinflammatory effects on macrophages.

This study demonstrated the increased levels of CB1 receptor mRNA expression in DCA samples from patients with unstable angina pectoris compared with those with stable angina pectoris. Immunohistochemistry showed that the expression of CB1 receptors was greater in atheromatous plaques than in fibrous plaques. CD68-positive plaque macrophages expressed CB1 receptors, indicating that these cells could play a central role in the endocannabinoid system in atheromata. We also demonstrated that the blood levels of endocannabinoids in patients with CAD were significantly higher than in those without CAD. These findings indicate the presence of the endocannabinoid system in human atherosclerotic lesions and the activated endocannabinoid system in patients with CAD.

During atherogenesis, circulating monocytes migrate into the arterial wall and mature into lesional macrophages, and functional properties and phenotypes of macrophage could depend on the local microenvironment and factors in atheromata. The present study found that CB1 receptor expression was significantly increased during the monocyte-macrophage differentiation process and was significantly augmented by M-CSF and OxLDL in vitro. These data suggest that CB1 receptor expression might be increased during the differentiation of human macrophages and that mature macrophages could overexpress CB1 receptors via stimulation by M-CSF and OxLDL in atherosclerotic lesions.

In atherogenesis, macrophages can play critical roles in plaque activation via inflammation and in the pathogenesis of acute coronary syndromes. Inflammatory responses mediated by inflammatory cytokines are presumably important in all stages of atherosclerosis. Inflammatory cytokines induce the expression of MMPs from macrophages, and MMPs could be involved in the pathogenesis of plaque rupture through degradation of the extracellular matrix of fibrous caps. Atheromata with large lipid cores have been recognized as vulnerable plaques, and we found CB1 receptor overexpression in the lipid-rich atheroma. Indeed, data from the present study showed that CB1 receptor blockade significantly reduced the production of inflammatory cytokines and...

Figure 4. Effects of CB1 receptor blockade on the production of inflammatory mediators from cultured lipopolysaccharide-stimulated human macrophages. After a 4-day culture, macrophages were treated with vehicle or rimonabant (0.01 to 1.0 μmol/L). After 24 hours, the media were changed, and rimonabant was added at the indicated concentrations, along with 20 ng/mL lipopolysaccharide. A, IL-1β; B, IL-6; C, IL-8; D, tumor necrosis factor-α (TNF-α); E, MMP-9; F, IL-10 in the cell culture supernatants were determined by ELISA (n=4 to 8). Representative samples of 3 experiments with similar results. Data are mean of the percentage reduction vs lipopolysaccharide alone. Bars represent mean±SD.
MMP-9 from cultured activated human macrophages. Reduction of the inflammatory activity of macrophages by a CB1 receptor antagonist (through inhibition of the production of inflammatory cytokines and MMP-9) could improve plaque vulnerability and modulate the atherogenic process.

Several clinical trials indicated that CB1 receptor blockade could decrease the levels of hsCRP in overweight patients with dyslipidemia.9 Recently, in obese patients who received near-optimal evidence-based therapies with 90% of patients on aspirin and 80% on statin, the Strategy to Reduce Atherosclerosis Development Involving Administration of Rimonabant–The Intravascular Ultrasound Study suggests that CB1 receptor blockade may favorably influence the progression of atherosclerosis.24 These clinical data and our present results may propose the potential benefits of CB1 receptor blockade on atherogenesis, in part, by altering the composition and inflammatory activity of atherosclerotic plaques.

Patients with obesity or type 2 diabetes–related hyperglycemia exhibit higher concentrations of endocannabinoids in visceral fat or serum, indicating that the endocannabinoid system is upregulated in patients with obesity and those with multiple cardiovascular risk factors.25,26 The present findings also demonstrated that the levels of endocannabinoids were significantly elevated in patients with CAD and that endocannabinoids were significantly associated with cardiometabolic risk factors. Endocannabinoids are lipid derivatives and are not stored in cells but produced on demand in situations of cellular stress or damage.27 In addition, endocannabinoids are degraded rapidly by the intracellular enzyme. Endocannabinoid availability is regulated by these enzymes.28 In our experiments, cultured macrophages exhibited an increase in an endocannabinoid-biosynthesizing enzyme and a decrease in an endocannabinoid-degrading enzyme during macrophage differentiation. The activated macrophage can secrete anandamide and 2-AG,29 which may modulate several macrophage functions via the paracrine and autocrine pathways.

On the basis of the results from CB1 receptor ablation by RNAi or blockade with other CB1 receptor antagonists in the present study, the antiinflammatory effects of rimonabant should be mediated through CB1 receptors. The CB1 receptor belongs to the G protein–coupled group of receptors, and G–mediated signals are conventionally regarded as having a suppressive effect on cytosolic cAMP levels and stimulating the mitogen-activated protein kinase cascade,.

Figure 5. Effect of CB1 receptor knockdown or CB1 receptor antagonists on lipopolysaccharide (LPS)-induced production of inflammatory cytokines in cultured human macrophages. A, Lipopolysaccharide-induced production of IL-1β in the cell culture supernatants of cultured human macrophages treated with vehicle or 1.0 μmol/L rimonabant, 1.0 μmol/L AM251, or 1.0 μmol/L AM281 (n=6 each). B, Lipopolysaccharide-induced production of IL-6 in the cell culture supernatants collected from cultured human macrophages with or without 1.0 μmol/L rimonabant after pretreatment of CB1 receptor RNAi or negative control RNAi (n=4 each). Representative samples of 3 experiments with similar results. Data are mean of the percent reduction vs lipopolysaccharide alone. Bars represent mean±SD.

Figure 6. Effects of CB1 receptor blockade on cytosolic cAMP levels and JNK phosphorylation in cultured human macrophages. A, Cultured human macrophages were incubated with 1.0 μmol/L rimonabant (black bars) or vehicle (white bars) for 5 and 15 minutes at 37°C (n=4 each). Bars represent mean±SD. B, Effects of pretreatment with rimonabant for 24 hours on JNK activation at 5 and 15 minutes after lipopolysaccharide stimulation were quantified by Western blots (n=4 each). Top, Immunoblot of antiphosphorylated JNK (upper band 54 kDA, lower band 46 kDA); bottom, immunoblot of anti-JNK (upper band 54 kDA, lower band 46 kDA) as control (representative sample). Relative values were demonstrated vs control. Bars represent mean±SD. P-JNK indicates phosphorylated JNK.
including JNK, which has prominent roles in the control of cell growth and inflammation. The present study indicated that the intracellular cAMP levels were increased and lipopolysaccharide-induced phosphorylation of JNK was attenuated by CB1 receptor blockade in human macrophages. Therefore, the antiinflammatory actions of CB1 receptor blockade in atherosclerotic lesions may act, in part, through elevating intracellular cAMP levels and inhibiting phosphorylation of JNK in macrophages.

However, the CB2 receptor also was shown to be expressed in human coronary atheroma; the human monocyte-macrophage expression of CB2 receptors was decreased after the differentiation process in in vitro cultures in the present study. Although several CB2 receptor agonists have been developed as antiinflammatory drugs, they have not acquired clinical application.31 One interesting report has suggested that CB2 receptor stimulation may promote antiatherogenic effects by inhibiting inflammation.32 It is suggested that CB1 receptor blockade with a specific CB1 receptor antagonist might enhance the endogenous activation of CB2 receptors, leading to the additional antiinflammatory effects on atherogenesis.

Study Limitations
The present study has several limitations. The number of DCA samples and the number of patients with measured blood endocannabinoid levels in our analysis were small. We could not obtain new DCA samples because this procedure is no longer in use in our clinic. The correlation analysis between blood endocannabinoid levels and cardiometabolic risk factors might have a type II error because of the small sample size. It is necessary to evaluate blood endocannabinoid levels in a large population in future studies.

Conclusions
The results of this study demonstrated the activation of the endocannabinoid system in patients with CAD and the increased expression of the CB1 receptor in coronary atheroma, particularly in lesional macrophages, and further indicated that the CB1 receptor antagonist exhibited antiinflammatory effects on human macrophages. CB1 receptor blockade might provide antiatherosclerotic properties through modulation of the endocannabinoid system in atheroma in patients with CAD, in addition to its original effect on weight reduction.

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Disclosures
None.

References
Clinical Perspective

Obesity is associated with an increased risk of cardiovascular morbidity and mortality. The recently discovered endocannabinoid system contributes to the physiological regulation of energy balance and food intake. In several clinical trials, modification of this system by blockade of the cannabinoid 1 (CB1) receptor, one of the receptors in the endocannabinoid system, is reported to reduce body weight and to improve multiple cardiometabolic risk factors. Furthermore, a recent clinical study using coronary intravascular ultrasound showed that a CB1 receptor antagonist may favorably influence the progression of atherosclerosis. In this study, we investigated the relationship between the endocannabinoid system and atherogenesis. Results indicated the presence and activation of the endocannabinoid system in patients with coronary artery disease and human atherosclerosis. We demonstrated an increased expression of CB1 receptor in coronary atheromata, particularly in lesional macrophages. Our data further indicate that the CB1 receptor antagonist exhibited antiinflammatory effects on human macrophages. They suggest that CB1 receptor blockade modulates the inflammatory state in atheromata through its antiinflammatory effects on macrophages. Our study highlights the potential role of the CB1 receptor in atherogenesis and suggests that studies are needed to test the effect of CB1 receptor blockade in vascular disease.
Activated Endocannabinoid System in Coronary Artery Disease and Antiinflammatory Effects of Cannabinoid 1 Receptor Blockade on Macrophages

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SUPPLEMENTAL MATERIAL.

[Supplementary Methods]

Methods

Reagents

Selective CB1 receptor antagonists AM251 and AM281 were purchased from Tocris (Ellisville, Missouri) and rimonabant (SR141716A) was kindly provided by sanofi-aventis R&D (Chilly Mazarin, France). Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) were acquired from Sigma Aldrich Co. (St. Louis, MO). Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) were purchased from R&D Systems, Inc. (Minneapolis, MN). Oxidized low density lipoprotein (OxLDL) was from Biomedical Technologies Inc. (Stoughton, MA).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from the cultured cells using RNA easy mini kit (Qiagen, Bulimba, Germany), and cDNA synthesized using the Quantitect Reverse Transcription Kit (Qiagen). RT-PCR was performed in a DNA thermal cycler for 40 cycles (1 minute at 94°C, 30 seconds at 62°C, and 1 minute at 72°C). Oligonucleotide primers (Hokkaido bioscience) were as follows:
human CB1 receptor,

5’-CGTGGGCAGCCTGTTCTCA-3’(sense) and
5’-GAGCATACTGCAGAATGCAAACACC-3’(anti-sense);

human glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

5’-GAAGGTGAAGGTCGGAGTC-3’(sense) and
5’-GAAGATGGTGATGGGATTTC-3’(anti-sense).

**Quantitative Real-Time RT-PCR**

The primers and the TaqMan probe sets for human CB1 receptor (Hs00275009), CB2 receptor (Hs00361490), GAPDH (Hs99999905), class A macrophage scavenger receptor (SR-A; Hs00234007), fatty acid amide hydrolase (FAAH; Hs00757813) and N-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD; Hs01113995) were purchased from Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA). Real-time RT-PCR was carried out using a TaqMan Universal Master Mix kit (Applied Biosystems) with an ABI Prism 7900 sequence detection system (Applied Biosystems).

**Western Blotting**

Aliquots with equal protein contents (10 μg) were separated by standard electrophoresis and
transferred onto polyvinylidene difluoride membranes. Membranes were blocked using 5% nonfat dry milk and 0.1% Tween-20 in Tris buffer saline (TBST) for 1 hr at room temperature. The primary antibody (dilution 1:1000 goat anti-CB1 receptor antibody ab40860-100, abcam, Cambridge, UK) incubated with membranes for overnight at 4°C. For detecting the activation of c-Jun N-terminal kinase (JNK), nonspecific binding was reduced by SuperBlock Blocking Buffer (Thermo scientific, Rockford, IL) in TBST for 1 hr at room temperature, and the primary antibodies (dilution 1:1000 anti-phosphorylated JNK antibody #9251 and anti-JNK antibody #9252 Cell Signaling Technology, Danvers, MA) incubated for overnight at 4°C. Secondary antibody incubated with membranes for 1 hr at room temperature. Blots were incubated in chemiluminescence reagent (ECL plus, GE Healthcare, Buckinghamshire, UK) and visualized by exposure to X-ray film.

**Small interfering RNA (siRNA)-mediated RNA Interference (RNAi) on Macrophages**

THP-1 cells were plated on RepCell (CellSeed Inc. Tokyo, Japan) and differentiated into macrophages as protocol described in cell culture part. After 4-days culture, the cells were detachment and harvested by temperature downing. These cells were re-suspended in solution V according to manufacturer’s protocol (Amaza Biosystems, GmbH, Köln, Germany). The cells were combined with siRNA (Silencer Select ID: s3261, Ambion, Austin, TX), and electroporated by a Nucleofector 2 instrument (Amaza Biosystems). The transfected cells
were re-plated and incubated for 24 hours. The extent of the mRNA knock-down was verified by TaqMan real time PCR analysis.

**Measurement of Cytokines and Matrix Metalloproteinase (MMP)-9 from Macrophages**

On day 4, the medium was changed to RPMI-1640 containing 2% fetal bovine serum (FBS), and rimonabant, AM251, AM281 or vehicle added to THP-1 cell-derived macrophages at concentrations of 0, 0.01, 0.1, 0.5, or 1.0 μmol/L. After 24 hours, the cultured medium was substituted for fresh RPMI-1640 containing 2% FBS and the concentrations of CB1 receptor antagonist described above. Macrophages were then stimulated with 20 ng/mL LPS and further incubated at 37°C for 24 hours. Culture media levels of interleukin (IL)-1β, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)-α and MMP-9 were determined using an ELISA according to the instructions provided by the manufacturers (IL-1β, IL-6, IL-8 and IL-10, R&D systems; TNF-α, BioSource; MMP-9, GE Healthcare). Results were calculated as the percentage change versus LPS alone.

**Direct Cyclic Adenosine Monophosphate (cAMP) Assay**

THP-1 cell derived macrophages were cultured with rimonabant 1.0 μmol/L or vehicle. Reactions were halted by cell lysis in 0.1 mol/L HCl/1% Triton X-100. The intracellular cAMP levels were measured using Correlate-EIA™ Direct cAMP assays (Assay Designs,
Ann Arbor, MI).