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The endocannabinoid system links gut microbiota to adipogenesis

Giulio G Muccioli[1,2,*], Damien Naslain[1,3], Fredrik Bäckhed[4], Christopher S Reigstad[4], Didier M Lambert[1,5], Nathalie M Delzenne[1,3] and Patrice D Cani[1,3,*]

[1] Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium, [2] Bioanalysis and Pharmacology of Bioactive Lipids laboratory, Brussels, Belgium, [3] Metabolism and Nutrition research group, Brussels, Belgium, [4] Department of Molecular and Clinical Medicine, Sahlgrenska Center for Cardiovascular and Metabolic Research/Wallenberg Laboratory, University of Gothenburg, Gothenburg, Sweden and [5] Medicinal Chemistry, Brussels, Belgium
* Corresponding authors. GG Muccioli, Université catholique de Louvain, LDRI, Bioanalysis and Pharmacology of Bioactive Lipids laboratory, CHAM7230, Avenue E. Mounier, 72, Brussels 1200, Belgium. Tel.: +32 2 764 72 31; Fax: +32 2 764 72 93; E-mail: giulio.muccioli@uclouvain.be or PD Cani, Université catholique de Louvain, LDRI, Metabolism and Nutrition research group, Avenue E. Mounier, PMNT 73/69, Brussels 1200, Belgium. Tel.: +32 2 764 73 97; Fax: +32 2 764 73 59; E-mail: patrice.cani@uclouvain.be

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Obesity is characterised by altered gut microbiota, low-grade inflammation and increased endocannabinoid (eCB) system tone; however, a clear connection between gut microbiota and eCB signalling has yet to be confirmed. Here, we report that gut microbiota modulate the intestinal eCB system tone, which in turn regulates gut permeability and plasma lipopolysaccharide (LPS) levels. The impact of the increased plasma LPS levels and eCB system tone found in obesity on adipose tissue metabolism (e.g. differentiation and lipogenesis) remains unknown. By interfering with the eCB system using CB_1 agonist and antagonist in lean and obese mouse models, we found that the eCB system controls gut permeability and adipogenesis. We also show that LPS acts as a master switch to control adipose tissue metabolism both *in vivo* and *ex vivo* by blocking cannabinoid-driven adipogenesis. These data indicate that gut microbiota determine adipose tissue physiology through LPS-eCB system regulatory loops and may have critical functions in adipose tissue plasticity during obesity.
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Introduction

Obesity and type II diabetes have reached epidemic proportions. Recent data have shown that these metabolic disorders are characterised by low-grade inflammation of unknown molecular origin (Hotamisligil and Erbay, 2008; Shoelson and Goldfine, 2009); therefore, it is of the utmost importance to identify the link between inflammation and adipose tissue metabolism, and plasticity. Emerging data have implicated gut microbiota (Ley *et al*, 2005; Turnbaugh *et al*, 2006; Cani *et al*, 2007a, b, 2008; Cani and Delzenne, 2009) and the endocannabinoid (eCB) system (Lambert and Muccioli, 2007; Di Marzo, 2008) as modulators of obesity and energy homeostasis.

During the past several years, our group and others have provided evidence that gut microbiota influence whole-body metabolism by affecting energy balance (Ley *et al*, 2005; Turnbaugh *et al*, 2006; Claus *et al*, 2008), gut permeability (Cani *et al*, 2008, 2009) and low-grade inflammation (Cani *et al*, 2007a, b) associated with obesity and related metabolic disorders. Recently, studies performed in gnotobiotic mice have emphasised the contributions of gut microbiota to fat storage (Backhed *et al*, 2004, 2007; Samuel *et al*, 2008).

Obesity is characterised by the massive expansion of adipose tissues and is associated with inflammation (Weisberg *et al*, 2003). It is possible that both this expansion and the associated inflammation are controlled by microbiota and lipopolysaccharide (LPS) (Cani *et al*, 2007a, 2008), a cell wall component of Gram-negative bacteria that is among the most potent inducers of inflammation. On the other hand, obesity is also characterised by greater eCB system tone, that is increased eCB plasma levels, altered expression of the cannabinoid receptor 1 (CB_1 mRNA) and increased eCB levels in adipose tissues (Engeli *et al*, 2005; Bluher *et al*, 2006; Matias *et al*, 2006; Cote *et al*, 2007; D'Eon *et al*, 2008; Starowicz *et al*, 2008; Di Marzo *et al*, 2009; Izzo *et al*, 2009). Several studies have suggested a close relationship between LPS and the eCB system. LPS controls the synthesis of eCBs both *in vivo* (Hoareau *et al*, 2009) and *in vitro* (Di Marzo *et al*, 1999; Maccarrone *et al*, 2001) through mechanisms that depend on

LPS receptor signalling (Liu *et al*, 2003). Although genetic and pharmacological impairments of CB_1 receptor have been shown to protect against the development of obesity, steatosis and related inflammation (Osei-Hyiaman *et al*, 2005, 2008; Gary-Bobo *et al*, 2007; DeLeve *et al*, 2008), the molecular link between eCB system activation and disorders associated with obesity remains elusive.

There is accumulating evidence that the eCB system, inflammation and obesity are interconnected (Scherer and Buettner, 2009); however, the convergent molecular mechanisms that may affect adiposity remain to be clarified. Here, we tested the hypothesis that gut microbiota and the eCB system control gut permeability and adipogenesis through an LPS-dependent mechanism under physiological and obesity-related conditions.

Results

Gut microbiota modulate colon CB1 receptor mRNA expression in normal and obese mice

To determine the contributions of gut microbiota to the regulation of the intestinal eCB system in both physiological and obese conditions, we investigated selective models (e.g. prebiotic treatment (Cani *et al*, 2007b, 2009; Martin *et al*, 2008) and a high-fat diet (HFD) (Cani *et al*, 2007a, 2008)) and drastic models (e.g. antibiotic treatment (Seki *et al*, 2007; Cani *et al*, 2008; Membrez *et al*, 2008) and germ-free mice (Backhed *et al*, 2004)) of gut microbiota modulation, in addition to mice bearing specific mutations in Myd88, an important gene involved in toll-like receptor (TLR)-mediated bacteria–host interactions. Tissue-specific changes in colonic CB_1 mRNA expression were observed in all five models (Figure 1A–C, E, G), whereas expression in the jejunum was unaffected (Figure 1D and F). In contrast, altered colonic expression of the second cannabinoid receptor CB_2 was not observed in any of the models tested (Supplementary Figure S1A–D), suggesting that gut microbiota selectively modulate colonic CB_1 mRNA expression.

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Devane *et al*, 1992; Mechoulam *et al*, 1995) are endogenous CB_1 and CB_2 ligands, and the main enzymes responsible for their degradation are fatty acid amide hydrolase (FAAH) (Cravatt *et al*, 1996) and monacylglycerol lipase (MGL) (Dinh *et al*, 2002), respectively. Consistent with the tissue-specific modulation of CB_1 mRNA expression, we found that FAAH and MGL expression levels were affected by gut microbiota in the colon (Supplementary Figure S2A, C, D and E), but not in the jejunum (Supplementary Figure S2B and F). We earlier showed that gut microbiota, at least in part, contribute to the systemic and hepatic inflammation associated with obesity (Cani *et al*, 2008, 2009) by increasing gut permeability, resulting in elevated LPS levels (defined as metabolic endotoxaemia). Obesity is also characterised by altered tone of the intestinal eCB system (Izzo *et al*, 2009). Thus, we hypothesised that the eCB system could link the development of gut permeability to the higher plasma LPS levels associated with obesity. To support these observations, we measured the intestinal AEA and 2-AG tissue content in genetically obese mice (B6.V-*Lep*^{ob}/J; *ob/ob*) and in *ob/ob* mice fed prebiotics. Consistent with the decrease in colonic CB_1 mRNA expression, we found that AEA content was reduced, whereas FAAH mRNA expression was increased (Figure 2A) in obese mice fed prebiotics. On the other hand, 2-AG content was not affected, despite a decrease in MGL mRNA expression (Figure 2A). Furthermore, these markers were not affected in the jejunum (Supplementary Figure S3A and B), strengthening the link between the eCB system (as indicated by CB_1 mRNA, FAAH mRNA and AEA levels) and gut microbiota. Accordingly, we found that the reduced plasma LPS levels in obese mice fed prebiotics (Figure 2B) correlated with both AEA levels and CB_1 mRNA expression in the colon (Pearson's correlation between LPS and CB_1 mRNA was r=0.46, P=0.04, whereas the correlation between LPS and AEA was r=0.43, P=0.05).

CB_1 receptor controls gut permeability

To clearly show that the eCB system regulates gut barrier function, we designed two *in vivo* experiments in obese and lean mice. In the first experiment, we blocked the CB_1 receptor with a specific, selective antagonist (SR141716A) in obese *ob/ob* mice with disrupted gut barrier and metabolic endotoxaemia (Brun *et al*, 2007; Cani *et al*, 2009) (Figure 2C and F; Ob-CT versus CT, $P<0.05$). As blocking CB_1 receptors also reduce food intake, we used pair feeding as a control. We found that obese mice treated with the CB_1 receptor antagonist (Ob-SR) for 12 days exhibited significantly reduced gut permeability as shown by their reduced plasma LPS levels (Figure 2C) compared with control (Ob-CT) or pair-fed (Ob-PF) mice. Moreover, we found that blocking the CB_1 receptor reduced plasma LPS levels through a mechanism linked to the improvement of gut barrier function. This improvement in gut barrier function was inferred from changes in the distribution and localisation of tight junction proteins (zonula occludens-1 (ZO-1) and occludin) (Figures 2D and 3). Consistent with the plasma LPS levels and plasma glucose levels, pair feeding did not alter the distribution of tight junction proteins (Figures 2D and 3; Supplementary Figure S4). Moreover, the discriminant analysis of gut permeability markers (plasma LPS, ZO-1 and occludin) shown by the canonical plot in Figure 2E supports the presence of separate clusters between Ob-SR and the other two groups (Figure 2E).

In addition, we confirmed the earlier findings that CB_1 receptor blockade significantly reduces body weight gain as compared with saline-treated mice (Supplementary Figure S4A), whereas pair-fed mice display an intermediate pattern. Moreover, CB_1 receptor antagonist treatment significantly decreased the total adiposity index and blood glucose levels, whereas no changes were observed after pair feeding (Supplementary Figure S4B and C). Finally, the reduced gut permeability was associated with decreased expression of the hepatic inflammatory markers TNF-α, PAI-1 and TLR4 mRNA (data not shown).

In a second set of experiments performed in lean wild-type mice, we mimicked the increased eCB system tone observed during obesity by chronic (4-week) infusion of a cannabinoid receptor agonist (HU-210) through mini-pumps implanted subcutaneously (s.c.). We found that cannabinoid agonist

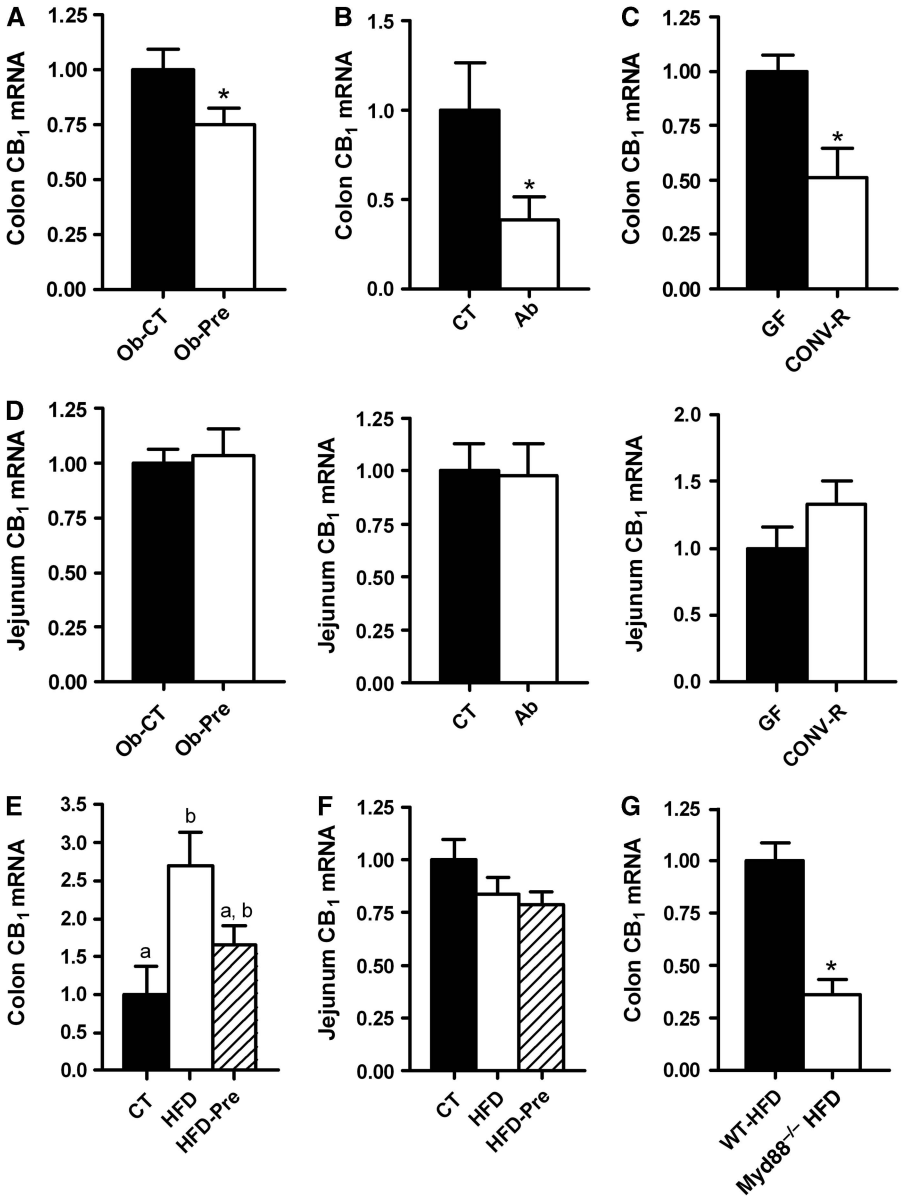

Figure 1 Gut microbiota selectively control colon CB_1 mRNA expression. CB_1 mRNA levels were selectively altered in the colons of mice according to the gut microbiota–host interaction model tested. (**A**) Colon CB_1 mRNA levels in *ob/ob* mice fed a normal chow diet (Ob-CT) or treated with prebiotics (Ob-Pre) for 5 weeks (n=10). (**B**) Colon CB_1 mRNA levels in lean wild-type mice (CT) or after antibiotic treatment (Ab) for 2 weeks (n=8–9). (**C**) Colon CB_1 mRNA levels in germ-free (GF) mice versus conventionally raised mice (CONV-R) (n=5). (**D**) Jejunum CB_1 mRNA levels in the same groups of mice described in panels A, B and C. (**E**) Colon CB_1 mRNA levels in mice fed a normal chow diet (CT) or a high-fat diet (HFD) enriched with or without prebiotics (HFD-Pre) for 14 weeks (n=7–8). (**F**) Jejunum CB_1 mRNA levels in CT, HFD and HFD-Pre mice (n=7–8). (**G**) Colon CB_1 mRNA levels in wild-type or *Myd88*$^{-/-}$ mice fed an HFD for 8 weeks (n=5). *$P<0.05$ as determined by a two-tailed Student's *t*-test. Data with different superscript letters were significantly different according to *post hoc* ANOVA one-way statistical analysis.

administration significantly increased plasma LPS levels. Furthermore, increased plasma fluorescein isothiocyanate-dextran levels were observed after oral gavage (Figure 2F and G). These sets of *in vivo* experiments strongly suggest that an overactive eCB system increases gut permeability.

Given that *in vivo* colonic CB_1 mRNA expression was consistently reduced in gut microbiota models known to improve gut permeability (Figure 1A, B and E) (Cani *et al*, 2007b, 2008, 2009) and that mice treated with a CB_1 receptor antagonist exhibited improved gut permeability (Figures 2C, D and 3), we hypothesised that the intestinal eCB system controls gut barrier function through a CB_1-dependent mechanism. Plasma LPS levels, however, have also been proposed as a factor involved in the development of gut barrier disruption (Kimura *et al*, 1997; Sheth *et al*, 2007). Thus, we used an *in vitro* cellular model of the intestinal epithelial barrier (Caco-2 cell monolayers) to investigate the specific functions of the eCB system and LPS in the development of gut permeability. When both LPS and the cannabinoid receptor agonist HU-210 were applied simultaneously, decreased

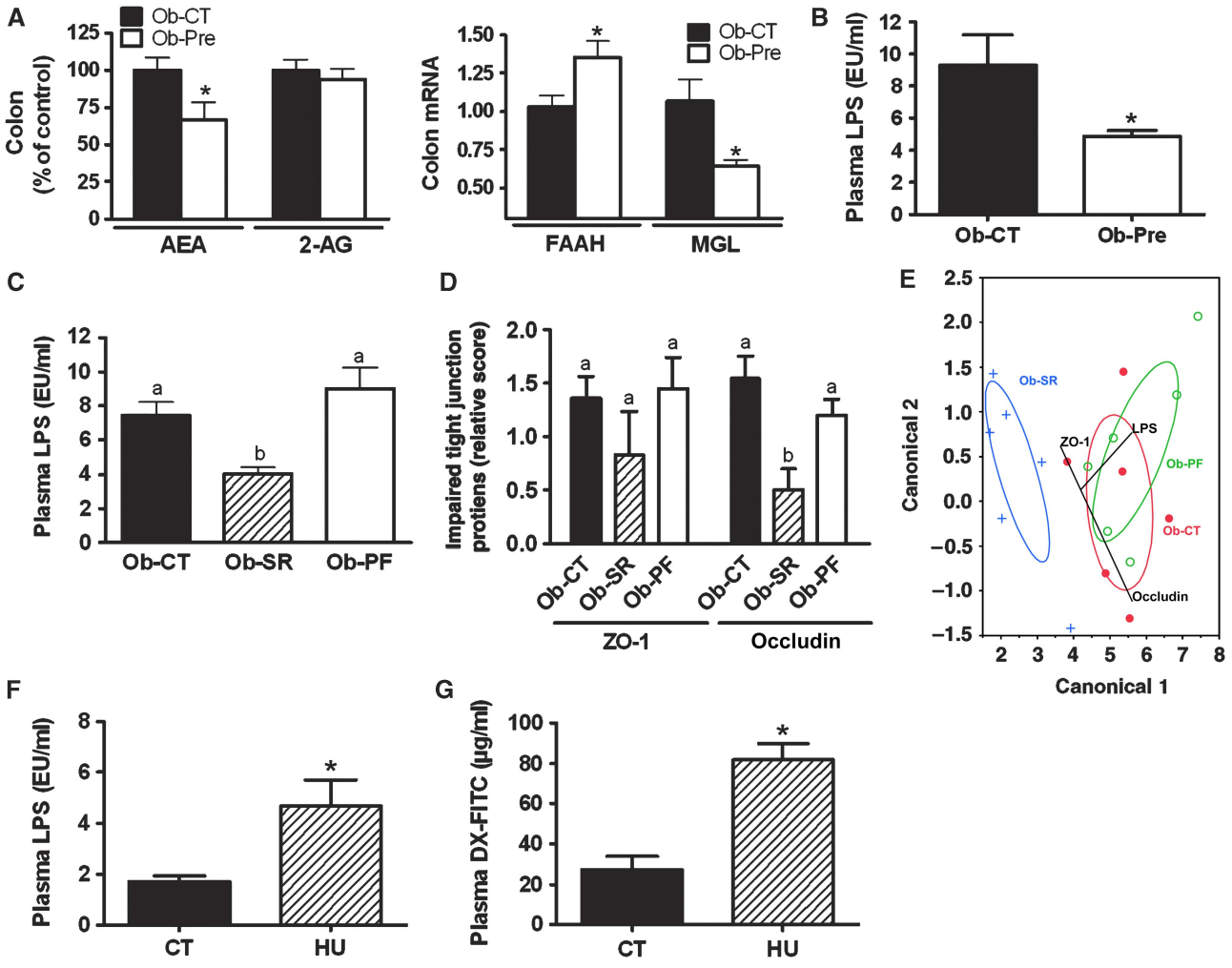

Figure 2 The eCB system controls gut permeability through a CB_1 receptor-dependent mechanism. (**A**) AEA and 2-AG levels (per cent of control values), and FAAH and MGL mRNA levels in the colons of *ob/ob* mice fed a normal chow diet (Ob-CT) or treated with prebiotics (Ob-Pre) for 5 weeks (n=10). (**B**) Plasma LPS levels in *ob/ob* mice fed a normal chow diet (Ob-CT) or treated with prebiotics (Ob-Pre) for 5 weeks (n=10). (**C**) Plasma LPS levels in obese *ob/ob* mice treated with vehicle (Ob-CT), CB_1 receptor antagonist or SR141716A (Ob-SR) (10 mg/kg//day) or pair fed (Ob-PF) for 12 days (n=6). (**D**) Relative score of tight junction (TJ) changes measured by immunohistological analysis of ZO-1 and occludin in the same mice as in panel C (n=6). (**E**) Canonical plot (*biplot*) showing the data and multivariate means in two dimensions that best separate the groups when one takes into account gut permeability markers. (**F**) Plasma LPS levels and (**G**) plasma levels of dextran-4000-FITC (FITC) in lean wild-type mice chronically infused with vehicle (CT) or the cannabinoid receptor agonist HU-210 (HU) (100 µg/kg/day) through mini-pumps implanted subcutaneously for 4 weeks (n=7). *$P<0.05$ as determined by a two-tailed Student's *t*-test. Data with different superscript letters were significantly different according to *post hoc* ANOVA one-way statistical analysis.

mRNA expression of two tight junction proteins, occludin and ZO-1, was observed (Figure 4A and B). Accordingly, the CB_1 receptor antagonist normalised occludin and ZO-1 mRNA expression (Figure 4A and B) and transepithelial electrical resistance (TEER) ($-9.8 \pm 4.4\%$ of the TEER measured before treatment and $7.3 \pm 5.2\%$ of the TEER measured before treatment, $P<0.01$, for LPS-HU and LPS-HU-SR1, respectively). In contrast, the CB_2 receptor antagonist SR144528 did not improve these parameters (Figure 4) (TEER=$-9.8 \pm 4.4\%$ of that measured before treatment and $-9.6 \pm 6.9\%$ of that measured before treatment, $P>0.05$, for LPS-HU and LPS-HU-SR2, respectively). Taken together, these *in vivo* and *in vitro* experiments support the hypothesis that the eCB system has an important function in the regulation of gut permeability through a CB_1 receptor-dependent mechanism.

Gut microbiota modulation decreases CB_1 expression in adipose tissue and fat mass and controls adipogenesis

Given that obesity is characterised by the dysregulation of eCB system tone in adipose tissue (Engeli *et al*, 2005; Bluher *et al*, 2006; Matias *et al*, 2006; Cote *et al*, 2007; D'Eon *et al*, 2008; Starowicz *et al*, 2008; Di Marzo *et al*, 2009; Izzo *et al*, 2009), we investigated this phenomenon in *ob/ob* mice compared with their lean littermates. In adipose tissues of the obese mice, there was a significant increase in the mRNA expression of *N*-acylphophatidylethanolamine phospholipase D (NAPE-PLD), the primary enzyme involved in AEA synthesis (Muccioli, 2010), and CB_1, along with reduced mRNA expression of FAAH (Figure 5A–C). Consistent with these findings, we found a significant

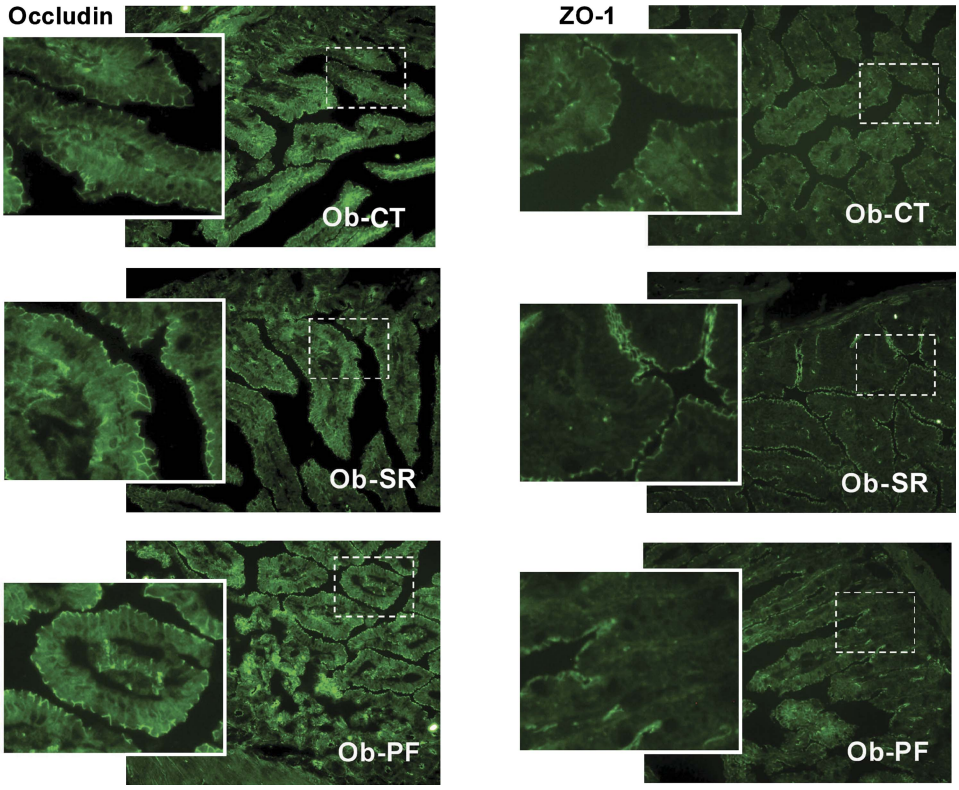

Figure 3 CB_1 receptor antagonist treatment changes the distribution of tight junction proteins. Representative immunofluorescence staining for occludin and ZO-1 in obese *ob/ob* mice treated with vehicle (Ob-CT), CB_1 receptor antagonist (SR141716A) (Ob-SR) (10 mg/kg/day) or pair fed (Ob-PF) for 12 days (n=6).

increase in AEA content (Figure 5D). Taken together, these data show for the first time that *ob/ob* mice display altered eCB system tone in adipose tissues.

Our laboratory and others have earlier shown that gut microbiota participate in the development of adipose tissue under both physiological and pathological conditions (Backhed *et al*, 2004, 2007; Cani *et al*, 2007a, 2008, 2009; Samuel *et al*, 2008). The molecular events linking the gut microbiota to adipogenesis, however, remain unidentified. Here, we confirm that changing the gut microbiota using prebiotics decreases fat mass development in obese mice (Figure 6A). Similar to the effects on colonic CB_1 receptor expression, changes in gut microbiota significantly decreased CB_1 mRNA expression in adipose tissues (Figure 6B). Given the recognised functions of AEA in metabolic processes controlled by the eCB system (Osei-Hyiaman *et al*, 2005; Buettner *et al*, 2008) and our present findings, we measured AEA levels and FAAH mRNA expression in adipose tissue. Prebiotics strongly decreased AEA levels and tended to increase FAAH mRNA levels (Figure 6C), further supporting the link between changes in gut microbiota and modulation of the eCB system.

Given that CB_1 receptor activation is known to increase lipogenesis-induced lipid accumulation in both liver (Osei-Hyiaman *et al*, 2005, 2008) and fat cells (Cota *et al*, 2003; Bouaboula *et al*, 2005; Gary-Bobo *et al*, 2006; Matias *et al*, 2006; Bellocchio *et al*, 2008; Pagano *et al*, 2008), we investigated whether reduced AEA levels and CB_1 mRNA expression during adipose tissue development could be linked to the diminution of adipogenesis and/or lipogenesis. To that end, we used qRT–PCR to determine the expression levels of the following adipogenic markers: CCAAT/enhancer-binding protein-α (C/EBP-α), peroxisome proliferator activated receptor-γ (PPAR-γ) and adipocyte fatty acid-binding protein (aP2, also known as FABP4, or AFABP). We also examined the following lipogenic markers: sterol regulatory element-binding protein-1c (SREBP-1c), a crucial transcription factor controlling the transcriptional regulation of both acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Osei-Hyiaman *et al*, 2005).

Strikingly, we found that the decreased fat mass, reduced CB_1 mRNA expression and reduced AEA levels observed after the modulation of gut microbiota in obese mice were associated with increases in markers of adipocyte differentiation (PPAR-γ, aP2, C/EBP-α) and lipogenesis (SREBP-1c, ACC, FAS) (Figure 6D and E). Similar results were obtained (also in obese mice) by blocking CB_1 receptor signalling (Figure 6F and G).

Under physiological conditions, on the other hand, activation of the eCB system by a cannabinoid receptor agonist (HU-210) increased adipogenesis in lean mice. Indeed, we found that activation of the cannabinoid receptors markedly increased the expression of genes involved in adipocyte differentiation (PPAR-γ, aP2, C/EBP-α) (Figure 7B) and lipogenesis (SREBP-1c, ACC, FAS), without affecting CB_1 mRNA levels (Figure 7C). Meanwhile, we observed a trend towards an increase in the adiposity index (Figure 7A). These effects were associated with smaller adipocyte size and an

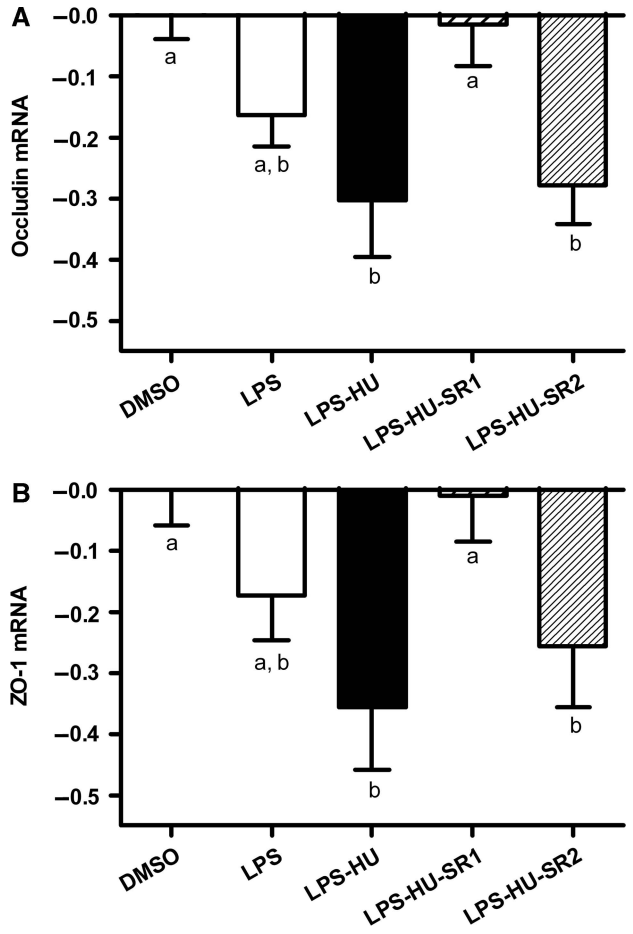

Figure 4 The eCB system controls gut permeability through a CB_1 receptor-dependent mechanism *in vitro*. The impact of the CB_1 and CB_2 receptors, and LPS on gut permeability was investigated in an *in vitro* colonic epithelial monolayer cell model (Caco-2) by measuring tight junction protein markers (**A**) Occludin and (**B**) ZO-1 mRNA expression levels in colonic cells treated with vehicle (DMSO) or LPS (200 μg/ml) or in combination with the cannabinoid receptor agonist HU-210 (HU) (1 μM), the CB_1 receptor antagonist SR141716A (SR1) (1 μM) or the CB_2 receptor antagonist SR144528 (SR2) (1 μM). The data represent the mean of three to four different experiments performed in triplicate. Data with different superscript letters were significantly different according to *post hoc* ANOVA one-way statistical analysis.

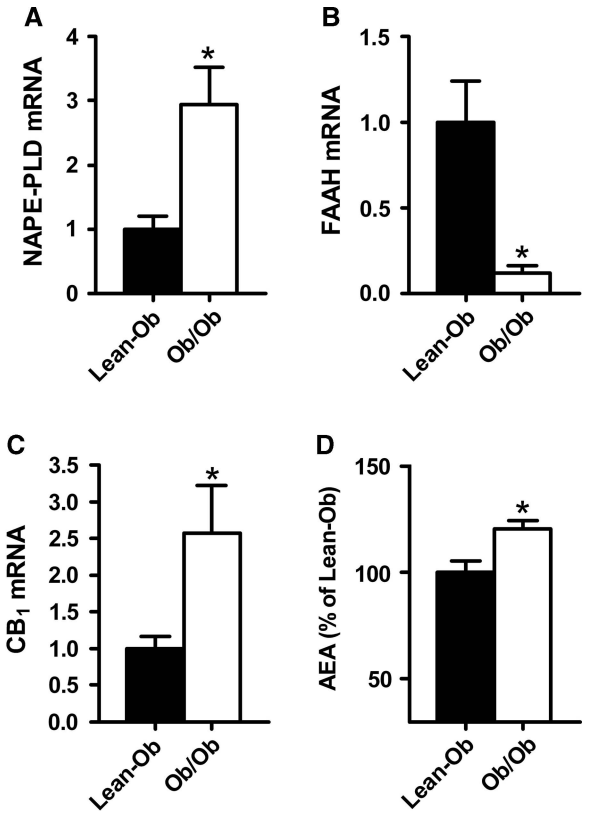

Figure 5 Obese mice are characterised by higher eCB tone in subcutaneous adipose tissues. (**A**) NAPE-PLD (*N*-acylphosphatidylethanolamine-phospholipase D) mRNA, (**B**) CB_1 mRNA, (**C**) FAAH mRNA and (**D**) AEA levels (per cent of lean values) in the subcutaneous adipose tissue of lean littermates (Lean-Ob) and obese *ob/ob* (Ob-Ob) mice (n=6). *$P<0.05$ as determined by a two-tailed Student's *t*-test.

increased number of adipocytes as determined by histological analysis (Figure 7D). Finally, a heat map profile examination and dendrogram analysis performed on the basis of adipocyte differentiation, lipogenesis and adipocyte size clearly showed two separate clusters between CT mice and those treated with the cannabinoid receptor agonist (Figure 7E). Consistent with the results obtained using the cannabinoid receptor agonist, inhibition of AEA degradation by administration of a potent FAAH inhibitor, URB597 (Kathuria *et al*, 2003), significantly increased AEA levels (CT: 100 ± 7.4%; URB597: 117.5 ± 4.9; $P<0.05$) and promoted adipogenesis (Supplementary Figure S6). This observation further supports the putative function of AEA and FAAH in regulating adipogenesis. These findings show that cannabinoid receptor activation induces adipogenesis *in vivo* under physiological conditions, thus confirming earlier data obtained *in vitro* (Matias *et al*, 2006; Pagano *et al*, 2007; Bellocchio *et al*, 2008).

Recent studies have proposed that obesity and its associated inflammation dysregulate adipose tissue metabolism by impairing adipogenesis (Gustafson *et al*, 2009; Isakson *et al*, 2009; McLaughlin *et al*, 2009). This phenomenon is associated with increased eCB system tone. Understanding the molecular mechanisms responsible for altered adipogenesis is essential to avoid the associated metabolic complications. Here, we show that selective changes in gut microbiota or CB_1 receptor antagonist reduce the inflammatory tone in obese mice by impacting the strength of the gut barrier and plasma LPS levels. This process may participate in the restoration of adipogenesis after reducing eCB system tone (e.g. with prebiotics or CB_1 receptor antagonists) in pathological situations such as obesity.

Stimulation of the eCB system leads to adipogenesis through an LPS-regulated mechanism

Our *in vivo* experiments suggest a link between gut microbiota, gut permeability, LPS, the eCB system and control of adipogenesis. Indeed, LPS receptor activation has been shown to decrease adipocyte differentiation and lipogenesis (Chung *et al*, 2006; Poulain-Godefroy and Froguel, 2007; Poulain-

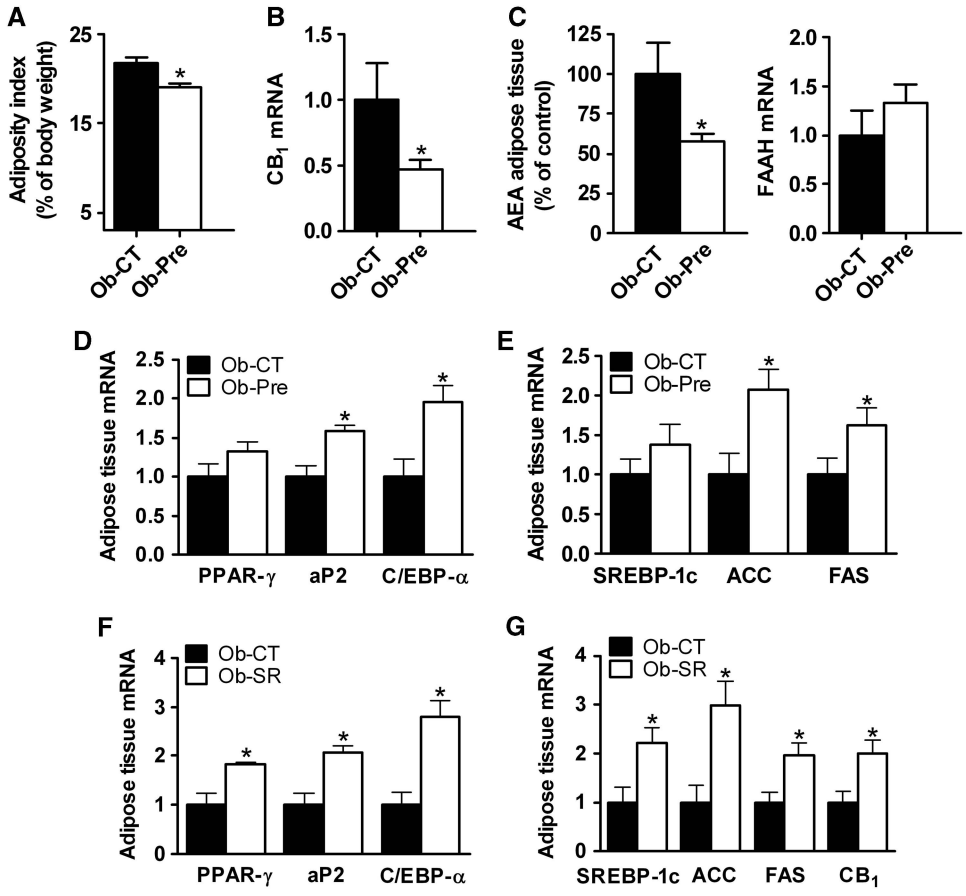

Figure 6 Changes in gut microbiota decrease adiposity and CB_1 mRNA expression, and regulate adipogenesis markers. (**A**) Adiposity index and (**B**) CB_1 mRNA expression levels in the adipose tissue of *ob/ob* mice fed a normal chow diet (Ob-CT) or treated with prebiotics (Ob-Pre) for 5 weeks (n=10). (**C**) White adipose tissue AEA (per cent of control values) and FAAH mRNA levels were measured in the same group of mice (n=10). (**D**) Adipocyte differentiation (PPAR-γ, aP2 and C/EBP-α) and (**E**) lipogenesis (SREBP-1c, ACC and FAS) mRNA expression levels in the adipose tissue of the same group of mice (n=10). (**F**) Adipocyte differentiation (PPAR-γ, aP2 and C/EBP-α) mRNA expression levels and (**G**) lipogenesis (SREBP-1c, ACC and FAS) mRNA expression levels in adipose tissue from obese *ob/ob* mice treated with vehicle (Ob-CT) or CB_1 receptor antagonist (SR141716A) (Ob-SR) (10 mg/kg/day) for 12 days (n=6). *$P<0.05$ as determined by a two-tailed Student's t-test.

Godefroy *et al*, 2008) and to activate the production of eCBs (Di Marzo *et al*, 1999; Maccarrone *et al*, 2001; Liu *et al*, 2003; Hoareau *et al*, 2009). Given that the amount of circulating LPS is under positive control by CB_1 through the regulation of eCB system tone in the gut and gut permeability, in this case, it is likely that LPS acts as an additional factor (dependent on the gut microbiota) in the control of adipogenesis and eCB system regulation. To evaluate this putative mechanism, we investigated the effects of cannabinoid receptor modulation and the involvement of LPS in the control of adipocyte differentiation and lipogenesis in cultured adipose tissue explants. Consistent with the *in vivo* data, activation of CB_1 receptors increased adipogenic markers in adipose tissue explants from wild-type mice (Figure 8A and B). In accordance with the hypothesis that LPS acts as a regulator of adipose tissue differentiation and lipogenesis, LPS decreased adipocyte differentiation markers, but had only a minor impact on lipogenesis markers (Figure 8A and B). Furthermore, LPS completely abolished the adipogenic effects (e.g. adipocyte differentiation and lipogenesis) of cannabinoid receptor activation. This latter observation shows that LPS acts as a master signal in eCB regulation of adipogenesis. Moreover, CB_1 mRNA levels were significantly increased by LPS (Figure 8C), supporting earlier observations that LPS increases eCB system tone (Di Marzo *et al*, 1999; Maccarrone *et al*, 2001; Liu *et al*, 2003; Hoareau *et al*, 2009).

To ascertain whether LPS could interfere with eCB system-induced activation of adipogenesis (HU) *in vivo*, we treated lean wild-type mice with LPS (HU-LPS) under the same experimental conditions as in Figure 7D. As before, increased LPS levels caused a decrease in adipogenesis markers (Figure 8D). Taken together, these data suggest that the eCB system regulates adipogenesis and that this regulation is controlled by LPS.

Given that increased LPS levels completely abolished the adipogenic effects of cannabinoid receptor activation, we wondered whether LPS could also act as a regulator of the well-characterised PPAR-γ-induced adipogenesis. In fact, LPS blunted PPAR-γ-induced adipogenesis (Figure 8E–G).

Discussion

Obesity is characterised by a massive expansion of adipose tissues, in addition to metabolic and inflammatory complications

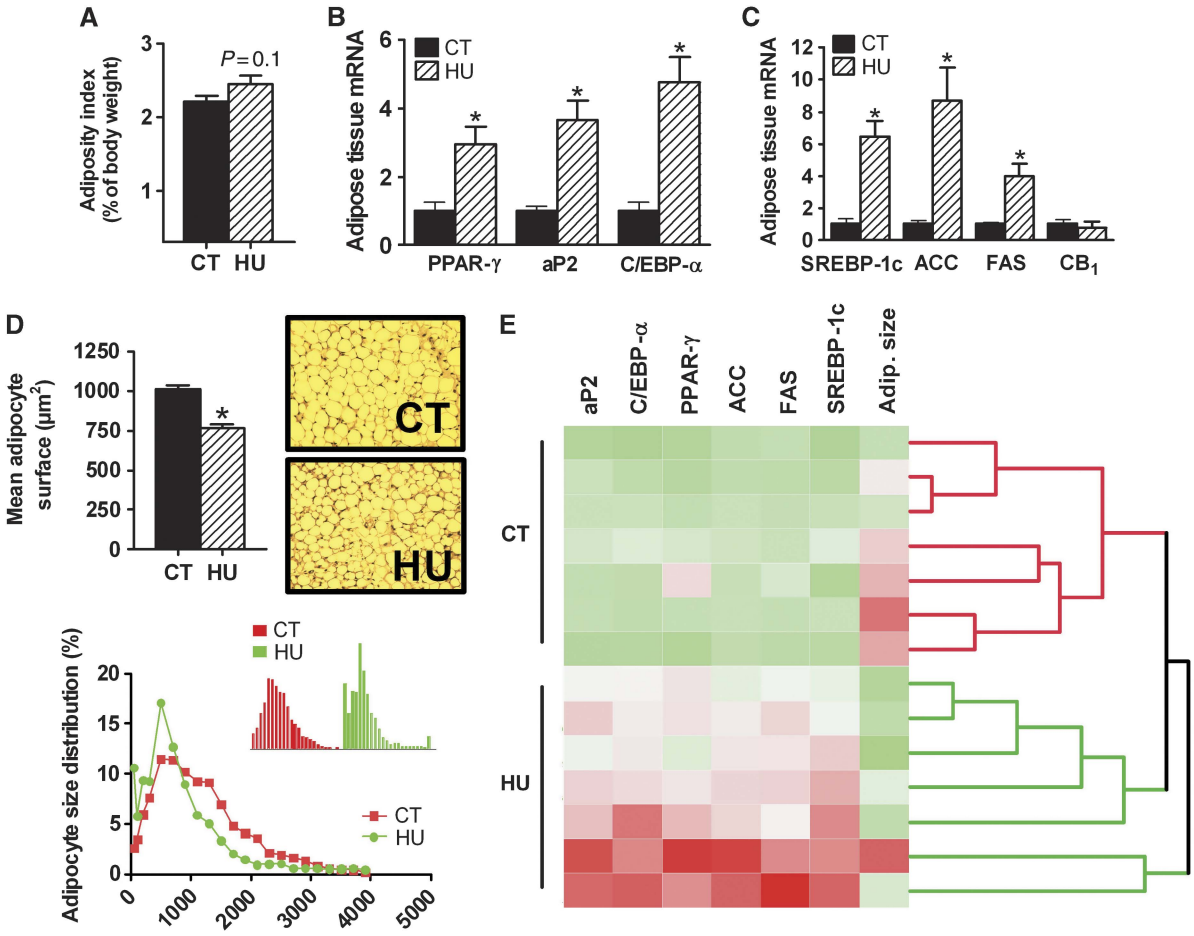

Figure 7 Stimulation of the eCB system leads to adipogenesis *in vivo* in lean mice. (**A**) Adiposity index and (**B**) adipocyte differentiation (PPAR-γ, aP2 and C/EBP-α) (**C**) lipogenesis (SREBP-1c, ACC and FAS) and CB_1 mRNA expression levels measured in lean wild-type mice chronically infused with vehicle (CT) or cannabinoid receptor agonist HU-210 (HU) (100 μg/kg/day) through mini-pumps implanted subcutaneously for 4 weeks (n=7). (**D**) Mean adipocyte surface area (μm^2), adipocyte size (μm^2) distribution and a representative haematoxylin and eosin-stained adipose tissue section (n=7) from the same group of mice. *$P<0.05$ as determined by a two-tailed Student's t-test. (**E**) Heat map profile and dendrogram analysis constructed on the basis of adipocyte differentiation mRNA levels, lipogenesis mRNA levels and adipocyte size markers.

(Hotamisligil and Erbay, 2008). Here, we characterise the crosstalk between gut microbiota and the regulation of adipogenesis by the eCB system and provide evidence that gut microbiota physiologically regulate the activity of the peripheral eCB system in intestinal and adipose tissue. The peripheral eCB system, in turn, controls gut barrier function and adipogenesis.

Obesity is characterised by dysregulated eCB system tone (Figure 5) (Engeli *et al*, 2005; Bluher *et al*, 2006; Matias *et al*, 2006; Cote *et al*, 2007; D'Eon *et al*, 2008; Starowicz *et al*, 2008; Di Marzo *et al*, 2009; Izzo *et al*, 2009), altered gut permeability and increased plasma LPS levels (Cani *et al*, 2008, 2009). Pharmacological blockade of the CB_1 receptor has been shown to reduce obesity associated with inflammation by an unresolved mechanism (Gary-Bobo *et al*, 2007; Caraceni *et al*, 2009). In this study, we evaluated the function of intestinal eCB system activation in the development of gut permeability, a major source of metabolic inflammation.

Evidence of a link between gut microbiota and the eCB system tone

Intestinal eCB system tone variations in response to gut microbiota modulation were observed in germ-free mice and in mouse models of bacterial–host interactions in colonic tissue, but almost no effects were seen in the small intestine. This reflects the higher microbial load found in the colon (Claus *et al*, 2008). The specific changes in gut microbiota or genetic disruptions of gut bacteria–host interactions selectively decreased CB_1 mRNA expression in the colon, without significant modulation of CB_2 mRNA expression. Interestingly, it has been shown that the administration of a very specific strain of bacteria, *Lactobacillus acidophilus* NCFM, increases CB_2 receptor expression in the colon in mice, whereas four other bacteria strains (well known as probiotics) belonging to the *Lactobacillus* and *Bifidobacterium* genera (*L. salivarius* Ls-33, *L. paracasei* Lpc-37, *B. lactis* Bi-07 and *B. lactis* Bi-04) and two *Escherichia coli* strains have no effect on CB_2 receptor

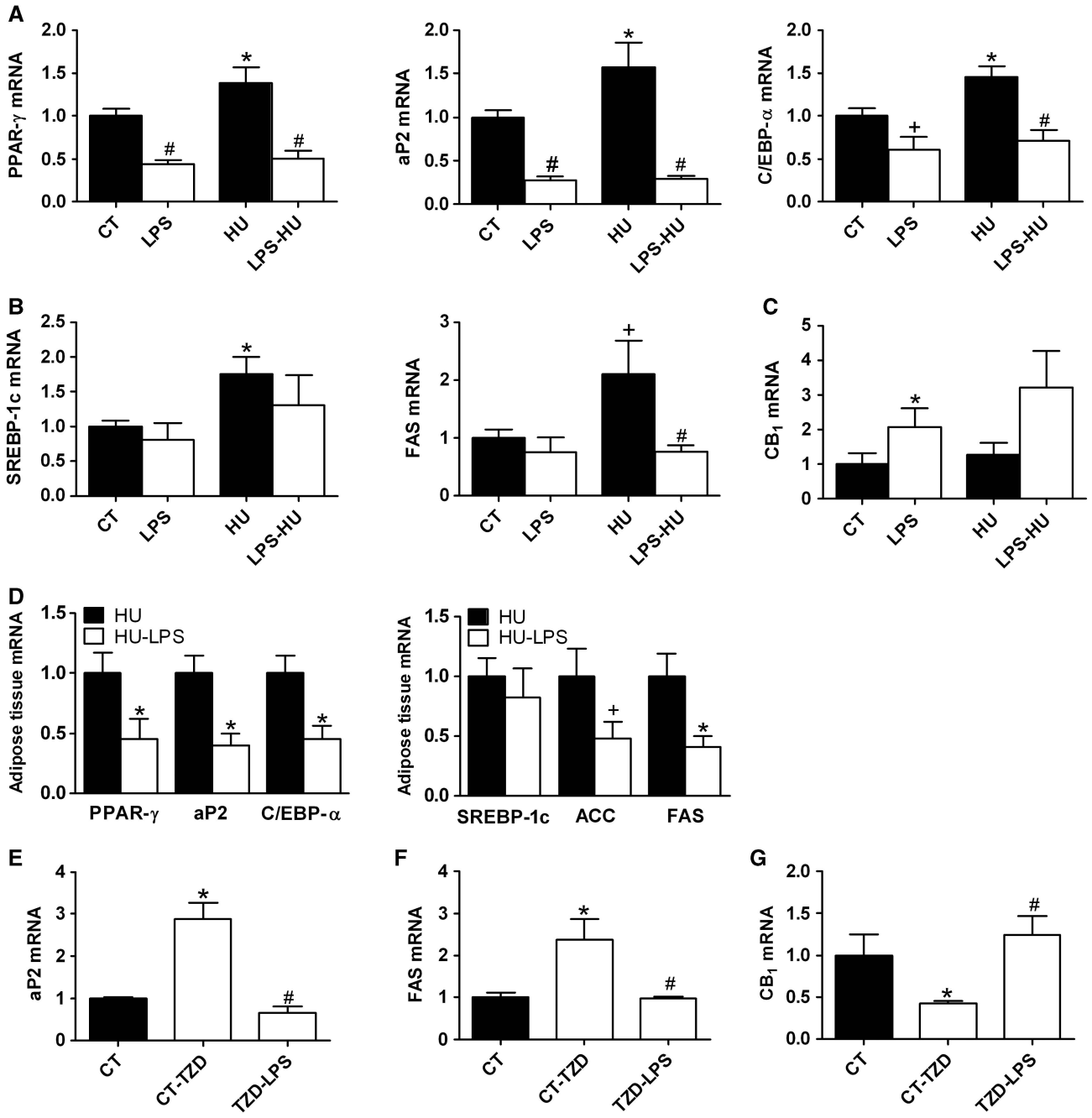

Figure 8 The eCB system directly regulates adipogenesis in cultures of adipose tissue explants, and LPS acts as a master switch both *in vitro* and *in vivo*. (**A**) mRNA expression levels of the adipocyte differentiation markers PPAR-γ, aP2 and C/EBP-α; (**B**) mRNA expression levels of the lipogenesis markers SREBP-1c and FAS and (**C**) mRNA expression levels of CB_1 in cultured adipose tissue explants exposed to vehicle (CT), LPS or cannabinoid receptor agonist HU-210 (HU) (100 nM) alone or in combination with LPS (100 ng/ml) for 24 h. (**D**) mRNA levels of the adipocyte differentiation markers PPAR-γ, aP2 and C/EBP-α and the lipogenesis markers SREBP-1c, ACC and FAS in lean wild-type mice chronically infused with cannabinoid receptor agonist (HU) (100 μg/kg/day) with or without the addition of LPS (HU-LPS) (300 μg/kg/day) through mini-pumps implanted subcutaneously for 4 weeks (n=7). (**E**) mRNA levels of an adipocyte differentiation marker (aP2), (**F**) a lipogenesis marker (FAS) and (**G**) CB_1 measured in cultured adipose tissue explants exposed to a PPAR-γ agonist (troglitazone, TZD) (10 μM) alone or in combination with LPS for 24 h. The data are the mean of four to five different determinations from 25 pooled mouse adipose depots. *Indicates $P<0.05$ for the drug effect; # indicates $P<0.05$ for the effects of LPS; and + indicates $P<0.07$ by a two-tailed, Student's t-test.

expression (Rousseaux *et al*, 2007). It is noteworthy that the lack of effect on CB_2 expression observed in our study could merely be explained by the fact that Rousseaux *et al* (2007) used a specific strain and did not investigate the modulation of gut microbiota. Given that they did not report effects on colonic eCB system tone (CB_1 and FAAH mRNA), we designed a similar experiment. Despite at least a 100-fold increase in the probiotic strains in the caecal content of the mice, we did not find any changes in CB_1 or FAAH mRNA expression in the colon (Supplementary Figure S5A and B). Still, CB_2 mRNA

expression tended to increase (Supplementary Figure S5C) (P=0.136). Taken together, our data support the hypothesis that gut microbiota participate in the regulation of the intestinal eCB system and also provide evidence that specific changes in gut microbiota known to reduce obesity and related metabolic disorders (Cani *et al*, 2007b, 2008, 2009; Membrez *et al*, 2008) are sufficient to decrease peripheral eCB system tone in two models of obesity (genetic and nutritional).

In this study, we confirmed that changes in gut microbiota after prebiotic ingestion reduce gut permeability in obese mice. Blocking the CB_1 receptor in obese mice also ameliorated gut barrier function as shown by improved distribution and localisation of tight junction proteins (ZO-1 and occludin). Importantly, this effect was dependent on CB_1 receptor blockade as pair feeding had no effect. Confirming these results, CB_1 activation increased gut permeability markers *in vivo* and *in vitro*. This demonstration that CB_1 receptors control gut permeability suggests a new eCB system-dependent mechanism in the pathogenesis of obesity-associated inflammation (systemic and hepatic).

Impact of eCB system tone on adipogenesis in obese and lean mice: the function of LPS

Numerous mechanisms have been suggested to explain the regulation of physiological and pathological adipose tissue development. Recently, gut microbiota have been suggested to modulate the onset of obesity (Ley *et al*, 2005; Turnbaugh *et al*, 2006; Cani *et al*, 2007a, b, 2008; Cani and Delzenne, 2009) and contribute to fat storage (Backhed *et al*, 2004, 2007; Martin *et al*, 2007; Samuel *et al*, 2008). Although it is clear that genetic or pharmacological blockade of the CB_1 cannabinoid receptor protects against the development of obesity (Osei-Hyiaman *et al*, 2005, 2008; Gary-Bobo *et al*, 2007; DeLeve *et al*, 2008), the molecular function of the eCB system in adipose tissue is still under investigation. Here, we identify several *in vivo* mechanisms by which the eCB system controls adipose tissue development through a putative gut microbiota-to-adipose tissue regulatory loop. An important result of these studies is the demonstration that peripheral (e.g. intestine and adipose tissue) eCB system tone (as indicated by CB_1 mRNA, FAAH mRNA and AEA levels) is under the control of gut microbiota. We can speculate that the altered profile of gut microbiota found in obesity (Ley *et al*, 2006; Cani *et al*, 2007a, b; Turnbaugh *et al*, 2009) is, in part, responsible for increased eCB system tone.

In conjunction with greater eCB system tone, increased inflammation and plasma LPS levels were observed in obese mice. In contrast, changes in gut microbiota after prebiotic feeding or CB_1 receptor blockade decreased inflammation and eCB tone. Hence, the greater eCB system tone found in obesity may participate in the regulation of adipogenesis not only directly by acting on adipose tissue, but also indirectly by increasing plasma LPS levels. The latter would consequently impair adipogenesis and promote inflammatory states. In accordance with this putative regulatory loop, it has been shown that adipogenesis-related genes are downregulated in the adipose tissue of obese and type II diabetic individuals (Yang *et al*, 2004; Dubois *et al*, 2006). Furthermore, recent studies have shown that the inflammatory tone associated with obesity leads to the dysregulation of adipogenesis (Gustafson *et al*, 2009; Isakson *et al*, 2009; McLaughlin *et al*, 2009). Here, both specific modulation of gut microbiota and CB_1 receptor blockade decreased plasma LPS levels and increased adipocyte differentiation and lipogenesis. One possible explanation for these surprising data could be as follows: plasma LPS levels might be under the control of CB_1 in the intestine (gut barrier function); therefore, under particular pathophysiological conditions (e.g. obesity), this could lead to higher circulating LPS levels. Furthermore, CB_1 receptor blockade might paradoxically increase adipogenesis because of the ability of CB_1 antagonist to reduce gut permeability and counteract the LPS-induced inhibitory effect on adipocyte differentiation and lipogenesis (i.e. a disinhibition mechanism). In summary, given that these treatments reduce gut permeability and, hence, plasma LPS levels and inflammatory tone, we hypothesised that LPS could act as a regulator in this process. This hypothesis was further supported *in vitro* and *in vivo* by the observation that cannabinoid-induced adipocyte differentiation and lipogenesis were directly altered (i.e. reduced) in the presence of physiological levels of LPS. Notably, whereas our data provide evidence that the consequences of obesity and gut microbiota dysregulation on gut permeability and metabolic endotoxaemia are clearly mediated by the eCB system, the changes observed in adiposity are likely the result of two system interactions: an LPS-dependent pathway and dysregulation of eCB system tone.

On the basis of our results, one might predict that restoring proper physiological levels of eCB and eCB system tone in obesity-related pathological situations would reduce gut permeability, low-grade inflammation and fat mass development. Still, some studies have shown no change, an increase or even a decrease in eCB system tone in different adipose depots (e.g. mesenteric, gonadal and subcutaneous) on the onset of obesity (Starowicz *et al*, 2008; Izzo *et al*, 2009; Sarzani *et al*, 2009; Bennetzen *et al*, 2010). These differences might be explained by the markers measured to evaluate eCB system tone (e.g. AEA, 2-AG, CB1 mRNA or proteins and FAAH mRNA) or by differences in the level of inflammation (e.g. systemic LPS levels). In the models tested in the present manuscript, we found a consistent increase in AEA and CB_1 mRNA expression and a decrease in FAAH mRNA expression. We also performed additional experiments in mice with diet-induced obesity treated for 3 or 8 weeks and found a two- and threefold increase, respectively, in CB_1 mRNA expression in subcutaneous adipose depots (PD Cani, 2009, personal communication). Furthermore, in two separate sets of experiments performed in obese and diabetic *db/db* mice, we observed a 2.5-fold increase in CB_1 mRNA expression in subcutaneous adipose tissue and a similar increase in mesenteric fat. On the other hand, FAAH mRNA levels were decreased by about 90% (PD Cani, 2009, personal communication). Therefore, given that in all of these models we observed both increased inflammatory markers and eCB system tone, it is likely that inflammation is responsible for the discrepancies found in the literature and that this is a consequence of enhanced LPS levels as shown in this study.

We propose the following model to illustrate how the eCB system links gut microbiota to adipogenesis (Figure 9).

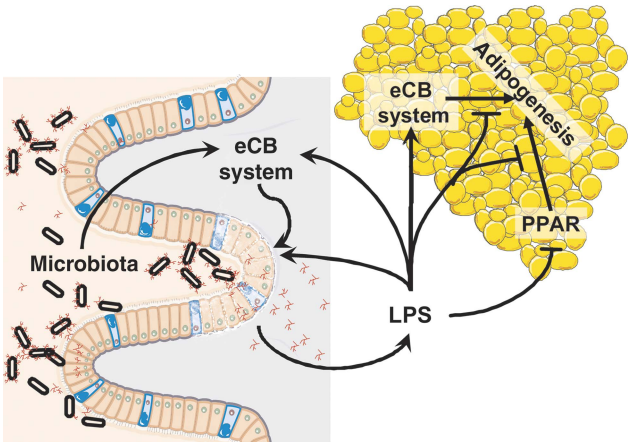

Figure 9 eCB system-LPS crosstalk participates in the regulation of adipogenesis by gut microbiota. Activation of the eCB system in the intestine (e.g. through gut microbiota) increases gut permeability, which enhances plasma LPS levels. This exacerbates gut barrier disruption and peripheral eCB system tone in both the intestine and adipose tissues. Increased fat mass results in enhanced eCB system tone. LPS inhibits both PPAR-induced and cannabinoid ligand-induced adipogenesis. Overall, the impairment of these regulatory loops within colon and adipose tissues found in obesity perpetuates the initial disequilibrium, leading to a vicious cycle. This cycle maintains the increased gut permeability, eCB system tone, adipogenesis and fat mass development that characterise obesity.

Activation of the eCB system in the intestine (e.g. through the gut microbiota) increases gut permeability, which enhances plasma LPS levels and exacerbates gut barrier disruption and peripheral eCB system tone in both the intestine and adipose tissues. Under the pathological conditions of obesity, the increased eCB tone and LPS levels participate in the dysregulation of adipogenesis, perpetuating the initial disequilibrium and leading to a vicious cycle (Figure 9).

In conclusion, we have identified a new pathophysiological mechanism linking gut microbiota to the eCB system in intestinal and adipose tissues with a major function in controlling adipogenesis. In addition, we provide evidence that adipogenesis is under the control of an LPS-eCB system regulatory loop. As obesity is commonly characterised by increased eCB system tone, higher plasma LPS levels, altered gut microbiota and impaired adipose tissue metabolism, it is likely that the increased eCB system tone found in obesity is caused by a failure or a vicious cycle within the pathways controlling the eCB system.

Modelling symbiotic systems biology such as gut microbiota–host interactions is the next great challenge in biological modelling, especially when aimed at trying to decipher the supersystem activities that will be crucial to understand gene–environment interactions and, thus, determine physiological and pathological consequences.

Materials and methods

Mice

We used male mice between 6 and 9 weeks of age with genetic compositions of C57/BL/6J, B6.V-Lep^{ob}/Jmice (from the Jackson Laboratory). We also used germ-free Swiss Webster and *Myd88*$^{-/-}$/C57BL/6 (bred in Pr. Backhed's laboratory, University of Gothenburg). Their diets consisted of a standard control diet (CT) (A04, Villemoisson sur Orge, France), an HFD or a control diet containing a mixture of prebiotics such as oligofructose (Pre) (Orafti, Tienen, Belgium) (Cani *et al*, 2007b, 2009). All animal use was approved by and performed in accordance with the local ethics committee. Housing conditions were as specified by the Belgian Law of 14 November 1993, regarding the protection of laboratory animals (agreement no. LA 1230314).

Tissue sampling

Mice were anesthetised using intraperitoneal (i.p.) ketamine and xylazine at concentrations of 100 and 10 mg/kg, respectively, after a 5-h fasting period. Blood samples and tissues were harvested for further analysis. Mice were killed by cervical dislocation. Epididymal, subcutaneous and visceral adipose depots were precisely dissected and weighed. The sum of the weights of these three adipose depots corresponded to the adiposity index. The intestinal segments (jejunum and colon) and adipose tissues were immersed in liquid nitrogen and stored at −80°C for further analysis.

Antibiotic treatment

Mice were treated with ampicillin (1 g/l; Sigma) and neomycin (0.5 g/l; Sigma) in their drinking water for 2 weeks (Cani *et al*, 2008).

Surgical procedures for implantation of the osmotic mini-pumps

Mice were implanted s.c. with an osmotic mini-pump (Alzet 2004, ALZA) as described earlier (Cani *et al*, 2006, 2007a).

In vivo pharmacological treatments

Mice were injected s.c. with HU-210 (100 μg/kg/day) (Tocris) using the osmotic mini-pumps for 4 weeks. The pumps also contained LPS from *E. coli* 055B:5 at a concentration of 300 μg/kg/day (Sigma) or a control vehicle (0.1% Tween/saline). Mice were injected i.p. with the CB_1 receptor antagonist/inverse agonist SR141716A (10 mg/kg/day) or vehicle for 12 days. Mice were injected i.p. with the fatty acid amid hydrolase inhibitor URB597 (3 mg/kg/day) and killed after 24 h.

Intestinal permeability *in vivo*

Intestinal permeability was measured in mice that had fasted for 6 h and received dextran-4000-FITC (Sigma) by gavage (500 mg/kg body weight, 125 mg/ml). Measurements were taken as described earlier (Cani *et al*, 2009).

Tight junction proteins (occludin and ZO-1) were assessed by immunohistochemistry as described earlier (Cani *et al*, 2009). All assessments were performed in duplicate in non-serial distant sections and analysed in a double-blind manner by two different investigators. Plasma LPS concentrations were determined using a kit based on a Limulus amaebocyte extract (LAL kit endpoint-QCL1000, Cambrex BioScience, Walkersville, MD). Determination of the non-inhibitory reaction and optimised sensitivity and specificity were performed as described earlier (Cani *et al*, 2009). An internal control for LPS recovery was included in each determination and calculation.

Measurement of AEA and 2-AG tissue levels

Tissues were homogenised in $CHCl_3$ (10 ml), and a deuterated standard (d-AEA and 2-AG; 200 pmol) was added. Methanol (5 ml) and H_2O (2.5 ml) were added, and the lipids were then extracted by vigorous mixing. After centrifugation, the organic layer was recovered, dried under a stream of N_2 and purified by solid-phase extraction using silica, followed by elution with an EtOAc-Acetone (1:1) solution (Muccioli *et al*, 2007; Muccioli and Stella, 2008). The resulting lipid fraction was analysed by HPLC-MS using an LTQ Orbitrap mass spectrometer (ThermoFisher Scientific) coupled to an Accela HPLC

system (ThermoFisher Scientific). Analyte separation was achieved using a C-18 Supelguard pre-column and a Supelcosil LC-18 column (3 μM, 4 × 150 mm) (Sigma-Aldrich). Mobile phases A and B were composed of MeOH-H_2O-acetic acid 75:25:0.1 (v/v/v) and MeOH-acetic acid 100:0.1 (v/v), respectively. The gradient (0.5 ml/min) was designed as follows: transition from 100% A to 100% B linearly over 15 min, followed by 10 min at 100% B and subsequent re-equilibration at 100% A. We performed MS analysis in the positive mode with an APCI ionisation source. The capillary and APCI vaporiser temperatures were set at 250 and 400°C, respectively. AEA and 2-AG were quantified by isotope dilution using their respective deuterated standards with identical retention. The calibration curves were generated as earlier described (Muccioli and Stella, 2008), and the data were normalised by tissue sample weight.

Caco-2 cell culture

Caco-2 cells were grown in flasks containing DMEM supplemented with foetal bovine serum (10%), L-glutamine (1%) and non-essential amino acids (1%) at 37°C in a 5% CO_2 atmosphere. For permeability testing purposes, the cells were seeded on the upper side of Transwell inserts (Costar) (1.6×10^5/1.12 cm^2) and grown for 21 days using the same media with the addition of penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μl/ml) (Invitrogen). The media in the upper and lower compartments were changed every other day. On day 21, basal TEER was measured using an Endohm™ tissue resistance chamber (World Precision Instruments, Sarasota, FL) connected to a Millicell®-RES (Millipore, Billerica, MA) ohmmeter before drugs were added (T=0 h). After a 24-h incubation in the presence of DMSO or LPS (*E. coli* 055:B5, 200 μg/ml) (Precourt *et al*, 2009) and drugs (1 μM HU-210, SR141716A, or SR144528), TEER was measured (T=24 h). Cells were recovered using TriPure reagent (Roche) for subsequent mRNA extraction. The TEER data are expressed as the per cent change from each individual baseline value.

Probiotic treatment

We orally administered probiotics belonging to the *Lactobacillus* and *Bifidobacterium* genera (*L. acidophilus* NCFM® *Bifidobacterium lactis* BI-07; Probactiol Plus, Metagenics, Ostende, Belgium) at doses of 1.2×10^9 colony-forming units (CFU) of each strain per day for 10 consecutive days (n=10 mice). Saline treatment was used as a control (n=10 mice).

Microbial analysis after probiotic treatment

The caecal contents of mice collected *post mortem* were stored at −80°C. Metagenomic DNA was extracted from the caecal content using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers and probes used to detect *Bifidobacterium* and *L. acidophilus* were based on 16S rRNA gene sequences: F-*Bifidobacterium* spp. TCGCGTCYGGTGTGAAAG, R-*Bifidobacterium* spp. CCACATCCAGCRTCCAC, F-*L. acidophilus* CCT TTCTAAGGAAGCGAAGGAT and R-*L. acidophilus* AATTCTCTTCTCGG TCGCTCTA. PCR amplification was carried out as follows: 10 min at 95°C followed by 45 cycles of 3 s at 95°C, 26 s at 58°C or 60°C (*L. acidophilus* or *Bifidobacterium*, respectively) and 10 s at 72°C. Detection was achieved with an STEP one PLUS instrument and software (Applied Biosystems, Foster City, CA) using MESA FAST qPCR MasterMix Plus for SYBR Assay (Eurogentec, Verviers, Belgium).

Each assay was performed in duplicate in the same run. The cycle threshold of each sample was then compared with a standard curve (performed in triplicate) made by diluting genomic DNA (fivefold serial dilution) (BCCM/LMG, Ghent, Belgium). The data are expressed as Log CFU/g of caecal content.

Adipose tissue explant cultures

Subcutaneous adipose depots from 25 mice were precisely dissected, and all visible vessels, particles and conjunctive tissue were removed. The fat tissue was then cut with scissors into small pieces (4 mm^3), pooled and placed in Krebs buffer (pH 7.4) containing 2% (wt/vol) free-fatty acid BSA, penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μl/ml) (Invitrogen). A total of 250 mg of adipose tissue was rinsed in phosphate-buffered saline and incubated in 100-mm Petri dishes containing 10 ml MEM with Earle's salts (Invitrogen) supplemented with 0.5% free-fatty acid-BSA, penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μl/ml) (Invitrogen). All conditions were repeated in four to five different dishes (n=4–5). The dishes were cultured for 24 h at 37°C in a 5% CO_2 atmosphere. The basal concentration of glucose in fresh media was 5 mmol/l, whereas the basal levels of cortisol and insulin were extremely low (∼0.5 nmol/l and 3 pmol/l, respectively). Different pharmacological agents were used in various combinations in accordance with the experimental protocols. LPS (*E. coli* 055:B5, 100 ng/ml) (Sigma), HU-210 (100 nM) (Tocris) and troglitazone (10 μM) (Sigma) were diluted in DMSO, which also served as a control. Cell viability did not change over the course of the experiment (data not shown). At the end of the experiment, the adipose material was rinsed in phosphate-buffered saline, collected, immediately frozen in liquid nitrogen and stored at −80°C until subsequent mRNA analysis.

RNA preparation and real-time qPCR analysis

Total RNA was prepared from tissues using TriPure reagent (Roche) as described earlier (Cani *et al*, 2008). cDNA was synthesised from 1 μg of total RNA using a reverse transcription kit (Promega Corp.). qPCR was performed with an STEP one PLUS instrument and software (Applied Biosystems) as described earlier (Cani *et al*, 2008). Primer sequences for the targeted mouse genes are shown in Supplementary Table 1.

Statistical analysis

The data are expressed as the mean ± s.e.m. Differences between two groups were assessed using an unpaired, two-tailed Student's *t*-test. Data sets involving more than two groups were assessed by ANOVA followed by a Bonferroni's *post hoc* test. Correlations were analysed using Pearson's correlation. Data with different superscript letters were significantly different (P<0.05) according to the *post hoc* ANOVA statistical analysis. Data were analysed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) and JMP 8.0.1 (SAS Campus Drive, Cary, NC). The results were considered statistically significant for P<0.05.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (http://www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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