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Hepatic Cannabinoid Receptor-1 Mediates Diet-Induced Insulin Resistance via Inhibition of Insulin Signaling and Clearance in Mice

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Abstract

BACKGROUND & AIMS—Obesity-related insulin resistance contributes to cardiovascular disease. Cannabinoid receptor-1 (CB_1) blockade improves insulin sensitivity in obese animals and people, suggesting endocannabinoid involvement. We explored the role of hepatic CB_1 in insulin resistance and inhibition of insulin signaling pathways.

METHODS—Wild-type mice and mice with disruption of *CB_1* ($CB_1^{-/-}$ mice) or with hepatocyte-specific deletion or transgenic overexpression of *CB_1* were maintained on regular chow or a high-fat diet (HFD) to induce obesity and insulin resistance. Hyperinsulinemic-euglycemic clamp analysis was used to analyze the role of the liver and hepatic CB_1 in HFD-induced insulin resistance. The cellular mechanisms of insulin resistance were analyzed in mouse and human isolated hepatocytes using small interfering or short hairpin RNAs and lentiviral knockdown of gene expression.

RESULTS—The HFD induced hepatic insulin resistance in wild-type mice, but not in $CB_1^{-/-}$ mice or mice with hepatocyte-specific deletion of CB_1. $CB_1^{-/-}$ mice that overexpressed CB_1 specifically in hepatocytes became hyperinsulinemic as a result of reduced insulin clearance due to down-regulation of the insulin-degrading enzyme. However, they had increased hepatic glucose production due to increased glycogenolysis, indicating hepatic insulin resistance; this was further increased by the HFD. In mice with hepatocytes that express CB_1, the HFD or CB_1 activation induced the endoplasmic reticulum stress response via activation of the Bip-PERK-eIF2α protein translation pathway. In hepatocytes isolated from human or mouse liver, CB_1 activation caused endoplasmic reticulum stress-dependent suppression of insulin-induced phosphorylation of akt-2

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Conflicts of interest
The authors disclose no conflicts.

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via phosphorylation of IRS1 at serine-307 and by inducing the expression of the serine and threonine phosphatase Phlpp1. Expression of CB_1 was up-regulated in samples from patients with nonalcoholic fatty liver disease.

CONCLUSIONS—Endocannabinoids contribute to diet-induced insulin resistance in mice via hepatic CB_1-mediated inhibition of insulin signaling and clearance.

Keywords

NASH; Signal Transduction; Mouse Model; Liver Disease

Insulin resistance is a major public health concern because it can lead to type 2 diabetes, dyslipidemias, and arterio-sclerotic heart disease. The liver plays a central role in obesity-related insulin resistance,[1] reflected in impaired insulin suppression of hepatic glucose production by down-regulating gluconeogenesis[2] and inhibiting glycogenolysis.[3,4] Insulin also promotes de novo hepatic lipogenesis through activation of the transcription factor SREBP1c,[5] which is not reduced in insulin-resistant states, but rather increased due to compensatory hyperinsulinemia.[6]

Endocannabinoids and cannabinoid receptor-1 (CB_1) contribute to obesity and its metabolic consequences, as indicated by the effectiveness of CB_1 antagonists in reducing body weight and improving insulin resistance and dyslipidemia in subjects with the metabolic syndrome.[7–10] Furthermore, CB_1-deficient ($CB_1^{-/-}$) mice are resistant to high-fat diet (HFD)-induced obesity, fatty liver, insulin resistance, and dyslipidemia despite a caloric intake similar to that in controls.[11,12] Whereas hepatocyte-specific CB_1 knockout ($hCB_1^{-/-}$) mice do become obese on HFD, they remain insulin sensitive, suggesting the involvement of hepatic CB_1 in whole body insulin resistance.13 Here we explored the involvement of hepatic CB_1 in HFD-induced hepatic insulin resistance using a euglycemic hyperinsulinemic clamp. Parallel experiments in $hCB_1^{-/-}$ mice and mice with hepatocyte-specific transgenic overexpression of CB_1 on a global CB_1 knockout background ($htgCB_1^{-/-}$ mice) indicated that hepatic CB_1 activation is both necessary and sufficient to account for diet-induced hepatic insulin resistance, independent of body weight. The results further indicate that CB_1-induced insulin resistance is secondary to endoplasmic reticulum (ER) stress, triggered by activation of the Bip/PERK/eIF2α protein translation pathway, with CB_1 inducing serine-307 phosphorylation of IRS1 and activation of the serine/threonine phosphatase Phlpp1, which reverses insulin-induced akt-2 phosphorylation. Finally, we show that the hyperinsulinemia that accompanies CB_1-induced insulin resistance is due to decreased insulin clearance, secondary to down-regulation of the insulin-degrading enzyme (IDE) in the liver.

Materials and Methods

Mice

Male C57Bl/6J mice and genetically modified strains backcrossed 10 times to a C57BL/6J background were bred from heterozygote pairs to allow for littermate controls. All experiments were approved by the institutional animal care and use committee. $CB_1^{-/-}$ and $hCB_1^{-/-}$ mice were generated as described.[13,14] A DNA construct containing the mouse albumin promoter and the CB_1 receptor coding sequence was injected into fertilized $CB_1^{-/-}$ oocytes to generate mice with transgenic expression of CB_1 in hepatocytes on a global $CB_1^{-/-}$ background ($htgCB_1^{-/-}$ mice). Mice aged 8 –12 weeks were placed on standard chow (STD; NIH-31 rodent diet) or an HFD (TD97070; Harlan Teklad, Frederick, MD) containing 33.5% fat (60% of calories) 26.5% carbohydrate, and 27.4% protein for 14 –16 weeks.

Intraperitoneal Glucose Tolerance and Insulin Sensitivity Tests

Mice fasted overnight were injected intraperitoneally with 2 g/kg glucose. Tail blood was collected at indicated time points and glucose levels were determined using a glucometer (Elite; Bayer, Pittsburgh, PA). One week later, mice fasted for 6 hours before the test received an intraperitoneal injection of insulin (0.75 U/kg; Eli Lilly, Indianapolis, IN) and blood glucose levels were determined.

Hyperinsulinemic Euglycemic Clamp

Clamps and glucose tracer analyses in conscious, cannulated mice were performed as described.[15]

Plasma Hormone Levels

Total plasma insulin was analyzed by the Milliplex Map Mouse Endocrine Panel, human insulin levels were assessed via the Ultrasensitive Human Insulin Assay, and C-peptide in plasma was measured with the Mouse C-Peptide Multiplex Assay (Millipore, Billerica, MA).

Culture of Primary Hepatocytes and Gene Knockdown

Primary mouse hepatocytes were prepared by collagenase perfusion as described.[16] After overnight attachment, cells were transfected with Phlpp1 small interfering RNA (siRNA; Qiagen, Valencia, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or infected with GFP– eIF2α–short hairpin RNA (shRNA) lentivirus at a multiplicity of infection of 50 for 48 hours. Cells were serum starved for 4 hours and subjected to various treatments. The degree of knockdown relative to mock-transfected cells (60%–70%) was verified by real-time polymerase chain reaction (PCR) of target messenger RNA (mRNA). Primary human hepatocytes were isolated by collagenase digestion of human donor livers not used for transplant.[17]

Reverse-Transcription PCR

CB_1R mRNA expression was assessed by reverse-transcription PCR and normalized with β-actin as described previously.[18] Primer sequences are provided in Supplementary Table 1.

Quantitative Real-Time PCR

We used a model PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA) and iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA). Predesigned mouse GAPDH, phosphoenolpyruvate carboxykinase (PEPCK), G6pc, and Glut4 primers were purchased from Qiagen (Valencia, CA). Gene expression values were calculated based on the $\Delta\Delta$Ct method.

Western Blotting and Immunoprecipitation

Protein was extracted from frozen brain or liver samples or from cultured hepatocytes. Western blotting was performed as described,[18] and immunoprecipitation was performed using the Pierce (Rockford, IL) Seize Classic (G) Immunoprecipitation Kit.

Tissue Uptake of 2-Deoxyglucose

$CB_1^{-/-}$ and $htgCB_1^{-/-}$ mice were injected intravenously with 0.2 nmol/g body weight of 2-[1-^{14}C]deoxyglucose (57.7 mCi/mmol; Perkin Elmer, Waltham, MA) and 0.75 U/kg insulin in 150 μL saline and killed 25 minutes later. Tissue samples were collected, weighed, and digested in NCSII solubilizer (Amersham, Piscataway, NJ), and radioactivity was determined by liquid scintillation spectrometry.

Guanosine 5′-[γ-thio]Triphosphate Binding

Agonist-stimulated [^{35}S]guanosine 5′-[γ-thio]triphosphate (GTP[γS]) binding was measured as described.[19]

G6-Pase, PEPCK, Glycogen Phosphorylase, and IDE Activity

Details of the enzyme assays are provided in Supplementary Materials and Methods.

Results

Anandamide Causes CB_1-Mediated Glucose Intolerance and Whole Body Insulin Resistance

To explore the role of endocannabinoids in glucose homeostasis, we examined the effect of acute in vivo treatment with anandamide on whole body glucose tolerance and insulin sensitivity in mice kept on STD. In wild-type mice, anandamide (10 mg/kg intraperitoneally) caused acute glucose intolerance and insulin resistance, with no such effects observed in $CB_1^{-/-}$ mice (Figure 1*A*). However, the previously described effects of anandamide reappeared in $htgCB_1^{-/-}$ mice, in which the overexpression of hepatic CB_1 matched the HFD-induced increase in hepatic CB_1 in wild-type mice,13 and resulted in increased CB_1 agonist efficacy quantified by GTPγS binding (Figure 1*C*). $HtgCB_1^{-/-}$ mice had elevated fasting blood glucose and insulin levels relative to $CB_1^{-/-}$ littermates, while the rest of their hormonal/metabolic profile and body composition were similar (Supplementary Figure 1). We next tested the effect of HFD on glucose tolerance and insulin resistance. $HtgCB_1^{-/-}$ mice on HFD remained lean and had a moderate increase in liver triglyceride levels (Supplementary Figure 1). Again, wild-type and $htgCB_1^{-/-}$ mice, but not $CB_1^{-/-}$ mice, developed marked HFD-induced glucose intolerance/insulin resistance (Figure 1*B*), indicating that activation of hepatic CB_1 induces glucose intolerance and insulin resistance and mediates the similar effects of HFD.

Hepatic CB_1 Are Necessary for Diet-Induced Hepatic Insulin Resistance

To assess the contribution of the liver to CB_1-mediated changes in glucose homeostasis, we conducted euglycemic hyperinsulinemic clamps in wild-type, $CB_1^{-/-}$, $hCB_1^{-/-}$, and $htgCB_1^{-/-}$ mice on STD or HFD. Wild-type mice became obese on HFD and displayed hepatic insulin resistance, as indicated by the marked reduction in glucose infusion rates, resulting from the reduced ability of insulin to suppress hepatic glucose production. Whole body glucose uptake tended to decrease in mice receiving HFD, but this did not reach statistical significance (Figure 2). $CB_1^{-/-}$ mice receiving HFD remained lean and insulin sensitive, with hepatic glucose production, glucose infusion rate, and whole body glucose uptake that were similar to their controls receiving STD. Interestingly, $hCB_1^{-/-}$ mice receiving HFD also remained insulin sensitive despite becoming as obese as wild-type mice,[13] whereas $ht\text{-}gCB_1^{-/-}$ mice receiving HFD were as insulin resistant as wild-type mice receiving HFD despite remaining lean, with marked reduction of glucose infusion rate and a modest decrease in whole body glucose uptake (Figure 2 and Supplementary Figure 1). This indicates that hepatic insulin resistance depends on CB_1 activity and is independent of adiposity.

Hepatic CB_1 Are Sufficient to Induce Hepatic Insulin Resistance Associated With Reduced Insulin Clearance

$HtgCB_1^{-/-}$ mice on STD had elevated fasting blood glucose relative to their $CB_1^{-/-}$ littermates (Figure 3*A*). When subjected to hyperinsulinemic clamp, basal hepatic glucose production and plasma insulin levels were significantly higher than in $CB_1^{-/-}$ littermates (Figure 3*A* and *B* and Supplementary Figure 2), indicating hepatic insulin resistance.

Unexpectedly, plasma insulin levels remained significantly elevated in htg$CB_1^{-/-}$ compared with $CB_1^{-/-}$ mice near the end of the clamp, suggesting reduced insulin clearance in the former. This was confirmed by the finding that plasma levels of the infused insulin, determined with an assay selective for human insulin, were similarly ~3 times higher in the htg$CB_1^{-/-}$ mice (Figure 3*C*), whereas plasma levels of C-peptide were similar in the 2 strains, the latter indicating similar insulin secretion rates (Figure 3*D*). The hepatic levels of IDE were about half of that in $CB_1^{-/-}$ littermates, and there was a parallel reduction in IDE catalytic activity in htg$CB_1^{-/-}$ mice (Figure 3*E*), whereas levels of p-CEACAM1, another regulator of insulin degradation,[20] were similar in the 2 strains (Figure 3*E*). This suggests that reduced insulin degradation by IDE contributes to the moderate hyperin-sulinemia of htg$CB_1^{-/-}$ mice.

Regulation of IDE by hepatic CB_1 was further indicated by the HFD-induced down-regulation of IDE expression in wild-type and htg$CB_1^{-/-}$, but not in $CB_1^{-/-}$, mice (Figure 4*A*) and by the ability of anandamide to down-regulate IDE expression in vivo in wild-type mice (Figure 4*B*) or in vitro in HepG2 cells (Figure 4*C*). CB_1-induced down-regulation of IDE expression was associated with increased serine-307 phosphorylation of IRS1 (Figure 4*A–C*), and it involved pertussis toxin-sensitive activation of $G_{i\alpha}$ (Figure 4*C*).

CB_1-Mediated Hepatic Insulin Resistance Manifests in Increased Glycogenolysis

The activity of the gluconeogenic enzymes glucose-6-phosphatase and PEPCK were similar in liver extracts from htg$CB_1^{-/-}$ and $CB_1^{-/-}$ mice, whereas glycogen phosphorylase *a* activity was increased by ~70% in the former (Supplementary Figure 3), which suggests that glycogenolysis is a primary source of the increased hepatic glucose production. Indeed, hepatic glycogen at the end of the clamp was significantly lower in htg$CB_1^{-/-}$ than in $CB_1^{-/-}$ mice (Supplementary Table 1), most likely due to a compensatory increase in GLUT4 expression and a corresponding increase in glucose uptake in skeletal muscle and kidney, as revealed by [^{14}C]2-deoxyglucose uptake (Supplementary Figure 4). Thus, overexpression of hepatic CB_1 leads to increased hepatic glucose production due to increased glycogenolysis, which is partially offset by a compensatory increase in glucose uptake, primarily into skeletal muscle.

CB_1-Induced Hepatic Insulin Resistance is ER Stress Dependent

To test whether CB_1-mediated insulin resistance may be via increased ER stress, we analyzed the hepatic level of the molecular chaperone Bip and the phosphorylation status of its downstream targets PERK[21] and the α subunit of translation initiation factor-2 (eIF2α), key indicators of ER stress.[22] The phosphorylation status of c-Jun and serine-307 of IRS1 was also monitored as indicators of the activation of c-Jun NH_2-terminal kinase 1 (JNK1), which has been also linked to inhibition of insulin signaling.[23,24] In agreement with earlier findings,[25] HFD in wild-type mice markedly increased Bip protein levels and the phosphorylation of PERK, eIF2α, c-Jun, and IRS1 (serine-307) in the liver. There was a similar HFD-induced increase in Bip, p-PERK, p-eIF2α, p-c-Jun, and p-serine-307IRS1 in the liver of htg$CB_1^{-/-}$, but not $CB_1^{-/-}$, mice (Figure 4*A*), whereas markers of 2 alternative pathways of ER stress, ATF6 and the spliced form of XBP1, were unaffected by genotype or HFD (not shown). To test whether activation of hepatic CB_1 induces ER stress, we treated wild-type mice with anandamide (10 mg/kg intraperitoneally) and killed them at various times posttreatment. Anandamide caused a rapid increase in Bip, p-c-Jun, and p-IRS1 that peaked at 30 – 60 minutes and a slower, more prolonged increase in p-PERK and p-eIF2α (Figure 4*B*).

The chemical chaperone 4-phenyl butyric acid (PBA) reverses HFD-induced ER stress and insulin resistance.[26] In agreement with those findings, daily oral treatment of HFD-fed mice

with 1 g/kg PBA for 20 days reversed the glucose intolerance (Figure 5*A*) and insulin resistance (Figure 5*B*) and also reversed the HFD-induced decrease in IDE expression (Figure 5*C*), thus providing additional evidence for the role of IDE in insulin resistance and its regulation by ER stress.

CB_1 Inhibits Insulin-Induced akt-2 Phosphorylation in Mouse and Human Hepatocytes in an ER Stress-Dependent Manner

Protein kinase B/akt-2 plays an obligatory role in insulin-induced hypoglycemia by down-regulating gluconeogenic gene expression[6] and increasing the activity of glycogen synthase.[15] Exposure of human or mouse isolated hepatocytes to 10 nmol/L insulin increased phosphorylation of akt-2 at the thre308 and ser473 sites, which was concentration-dependently inhibited by anandamide or the synthetic cannabinoid WIN55,212-2 in human hepatocytes and in hepatocytes from wild-type or htg$CB_1^{-/-}$, but not $CB_1^{-/-}$, mice (Figure 6*A*). The role of CB_1 was further verified by blocking the effect of anandamide with the CB_1 antagonist SR141716 (Figure 6*B*). The inhibition of akt-2 phosphorylation by anandamide is ER stress dependent. In mouse hepatocytes, shRNA knock-down of eIF2α by 61.6% ± 7.1% resulted in marked suppression of serine-307 phosphorylation of IRS1 (Figure 6*C*), whereas insulin-induced akt-2 phosphorylation was enhanced and was resistant to inhibition by anandamide or WIN 55,212-2 (Figure 6*D*).

Anandamide Inhibits Insulin Signaling via Activation of Phlpp1

Anandamide inhibition of insulin-induced akt-2 phosphorylation in wild-type mouse hepatocytes was blocked by a phosphatase inhibitor cocktail (Figure 7*A*), suggesting that anandamide promotes dephosphorylation of akt-2 by a phosphatase. The *PH* domain leucine-rich repeat protein phosphatase-1 (Phlpp1) has been implicated in the dephosphorylation of akt-2.[27,28] In mouse hepatocytes with an siRNA-induced, 68.8% ± 1.3% knockdown of Phlpp1, insulin-induced akt-2 phosphorylation was enhanced and the inhibitory effect of anandamide was blunted (Figure 7*B*). Hepatic levels of Phlpp1 protein were increased by HFD in a CB_1-dependent manner (Figure 4*A*) and were also increased following in vivo treatment with anandamide (Figure 4*B*). In wild-type mouse hepatocytes, anandamide increases Phlpp1 expression via the ER stress response, as indicated by its blunted effect in cells with shRNA-mediated knockdown of eIF2α (Figure 7*C*). CB_1 is a G_i/G_o-coupled receptor, and the action of anandamide involves activation of $G_{i\alpha}$ (Figure 7*D*).

Up-regulation of Hepatic CB_1 in Human Nonalcoholic Fatty Liver Disease

The effects of cannabinoids observed in human hepatocytes predicated the presence of CB_1 in human liver. Indeed, CB_1 mRNA could be detected by real-time PCR in liver biopsy tissue from 5 patients with no liver pathology, and a 34.2-fold ± 9.7-fold increase ($P < .05$) in CB_1 mRNA relative to these controls was observed in tissue from 26 patients with nonalcoholic fatty liver disease. Increased expression of CB_1 protein in fatty versus nonfatty livers could also be documented by immunohistochemistry (Supplementary Figure 5).

Discussion

Increased activity of the endocannabinoid/CB_1 system has emerged as a pathogenic factor in visceral obesity.[29,30] A common complication of obesity is insulin resistance, and CB_1 blockade not only reduces weight and adiposity, but also improves insulin sensitivity both in obese subjects[8] and animals,[31–33] suggesting a role for endocannabinoids in glycemic control. Mice with selective deletion of CB_1 in hepatocytes become obese on HFD but remain glucose tolerant and insulin sensitive, whereas mice that express CB_1 only in hepatocytes remain lean on HFD but are insulin resistant, suggesting a weight-independent effect of hepatic CB_1 on insulin sensitivity.13 Here we provide in vivo and in vitro evidence

that endocannabinoids acting via hepatic CB_1 contribute to diet-induced hepatic insulin resistance by inhibiting both insulin signaling and clearance. Hepatic CB_1 activate the serine/threonine phosphatase Phlpp1 via the Bip/PERK/eIF2α ER stress pathway, which suppresses insulin signaling by counteracting insulin-induced akt-2 phosphorylation. Hepatic CB_1 also induce serine-307 phosphorylation of IRS1, which inhibits insulin signaling.[34] Furthermore, activation of hepatic CB_1 suppresses insulin clearance via reducing the expression of the IDE.

HFD-induced hepatic insulin resistance, as revealed by a euglycemic hyperinsulinemic clamp, is dependent on the presence of CB_1 in hepatocytes (Figure 2), activation of which suppresses insulin signaling, resulting in increased glucose production. Hepatic CB_1 are not only necessary but also sufficient to mediate hepatic insulin resistance; $CB_1^{-/-}$ mice with selective hepatic reexpression of CB_1 are hyperglycemic and hyperinsulinemic relative to their $CB_1^{-/-}$ littermates. These mice are insulin resistant even on normal diet, probably due to the transgenic overexpression of hepatic CB_1 to levels similar to those in normal mice on HFD[13,35] (Figure 1*C*), and their insulin resistance is further increased by HFD (Figure 2), likely mediated by the parallel increase in hepatic anandamide (Supplementary Figure 1). The other endocannabinoid, 2-arachidonoyl glycerol, was elevated in the liver of a different experimental model on HFD; however, the magnitude of glucose intolerance was somewhat mitigated in those $ApoE^{-/-}$ mutants compared with wild-type mice on the same HFD.[36] The finding of a robust up-regulation of hepatic CB_1 expression in people with nonalcoholic fatty liver disease (Supplementary Figure 5), along with evidence for CB_1-mediated inhibition of insulin signaling in human hepatocytes (Figure 6*A*), suggests a similar regulatory function of CB_1 in the human liver. Indeed, marijuana smoking was shown more than 30 years ago to induce insulin resistance,[37] and cannabis also induces insulin resistance in rodents.[38] The key role of hepatic CB_1 in insulin resistance is further indicated by the finding that transgenic reexpression of hepatic CB_1 in $CB_1^{-/-}$ mice rescues the ability of acute in vivo treatment with anandamide to induce insulin resistance (Figure 1*A*).

The increased hepatic glucose production of $htgCB_1^{-/-}$ mice could be attributed to increased glycogenolysis rather than gluconeogenesis, based on ex vivo measurement of glycogen phosphorylase versus glucose-6-phosphatase and PEPCK activities, respectively (Supplementary Figure 3). Nevertheless, a possible contribution of gluconeogenesis cannot be excluded, because regulation may also occur at the level of fructose 2,6-biphosphatase. Indeed, in a recent study in rat and human isolated hepatocytes, direct activation of CB_1 receptors by 2-arachidonoylglycerol was found to increase glucose-6-phosphatase and PEPCK gene expression as well as glucose production.[39] However, the relative contribution of gluconeogenesis and glycogenolysis to regulated glucose production may be different under in vitro and in vivo conditions. Also, enzyme activities were not measured and the possible contribution of glycogenolysis to the observed increase in glucose production was not explored in the previously described study. Future studies of glucose fluxes could provide a more definitive answer in this regard.

Although diet-induced glucose intolerance is predominantly attributable to hepatic insulin resistance, CB_1 can also inhibit glucose uptake into skeletal muscle[40,41] and adipose tissue[42,43] and inhibit insulin signaling in pancreatic beta cells.[44] The relative contributions of these targets to glycemic control by endocannabinoids may be different in rodents and humans and remain to be further explored.

The importance of reduced insulin clearance and IDE as pathogenic factors in diabetes is suggested by reports of an association of type 2 diabetes with loss of function mutations in IDE[45] or with polymorphisms in the IDE gene.[46,47] Plasma levels of both endogenous and exogenous insulin were higher and hepatic IDE protein and activity levels were lower in

htg$CB_1^{-/-}$ than in $CB_1^{-/-}$ mice (Figure 3*E*). The HFD-induced decrease in IDE depends on the presence of CB_1 in hepatocytes (Figure 4*A*), and the role of CB_1 in regulating IDE expression is further indicated by anandamide down-regulation of IDE in the liver in vivo and in HepG2 cells. Furthermore, obesity-related insulin resistance and reduced IDE expression are both reversed by the chemical chaperone PBA,[26] which links ER stress to IDE expression. These findings represent the first direct link among a specific diabetogenic signal, hepatic CB_1 activation, and reduced insulin clearance via down-regulation of IDE.

It is well established that obesity is accompanied by activation of the ER stress response in the liver,[25,48,49] which inhibits insulin signaling.[25] Activation of CB_1 can also induce ER stress, as documented recently in human glioma cells.[50] Because obesity leads to activation of the endocannabinoid/CB_1 system,12,29,35 we hypothesized that the ER stress response in obesity may be mediated by endocannabinoids acting via hepatic CB_1. This hypothesis is now supported by both in vivo and in vitro evidence.

Under in vivo conditions, HFD induces the expression of the chaperone Bip and increases the phosphorylation of its downstream targets PERK and eIF2α, a key pathway of ER stress involved in suppressing protein translation,[51] whereas ATF6 and XBP1, key components of 2 additional arms of the ER stress response involved in protein folding and degradation,[51] appear unaffected. The effects of HFD on the Bip/PERK/eIF2α pathway are CB_1 dependent, because they are detectable in the liver of wild-type and htg$CB_1^{-/-}$, but not $CB_1^{-/-}$, mice (Figure 4*A*). CB_1 involvement is further supported by the in vivo action of anandamide to induce Bip expression and PERK and eIF2α phosphorylation (Figure 4*B*).

In primary cultured mouse hepatocytes, shRNA knock-down of eIF2α abrogated the ability of CB_1 agonists to inhibit insulin-induced akt-2 phosphorylation (Figure 6*D*) or to induce the expression of Phlpp1 (Figure 7*C*), a serine/threonine phosphatase involved in the dephosphorylation of akt-2.[27,28] Furthermore, HFD-induced serine-307 phosphorylation of IRS1, which negatively regulates akt,[25] depends on the presence of CB_1 in hepatocytes (Figure 4*A*), and anandamide directly increases IRS1 serine phosphorylation (Figure 4*B* and *C*). These findings outline the signaling pathway engaged by hepatic CB_1 to inhibit the antiglycemic action of insulin: activation of hepatic CB_1 triggers ER stress, which inhibits insulin signaling by suppressing p-akt-2 via serine phosphorylation of IRS1 and at the same time promoting the dephosphorylation of p-akt-2 via activation of Phlpp1.

The possible role of JNK1 in hepatic ER stress and insulin resistance is complex. The protective role of germline deletion of JNK1 against obesity and insulin resistance[23,24] could be attributed to loss of JNK1 activity in the brain,[52] whereas selective hepatic deletion of JNK1 has the opposite effect, that is, an increase in insulin resistance.[53] Thus, activation of JNK by CB_1 (Figure 4*A* and *B*) is unlikely to contribute to the parallel development of hepatic insulin resistance. This is further suggested by our observation that pretreatment of mice with the JNK inhibitor BI-78D3[54] failed to prevent anandamide-induced glucose intolerance (not shown).

The ability of a peripherally restricted CB_1R antagonist to reverse obesity-related insulin resistance has highlighted the importance of an overactive peripheral endocannabinoid system in the metabolic consequences of obesity[33] and is also compatible with the role of hepatic CB_1 in this effect, as shown here. The present findings identify hepatic CB_1 as a potential novel molecular target for the pharmacotherapy of insulin resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

CB_1	cannabinoid receptor-1
ER	endoplasmic reticulum
GTP[γS]	[^{35}S]guanosine 5′-[γ-thio]triphosphate
HFD	high-fat diet
IDE	insulin-degrading enzyme
JNK	c-Jun NH_2-terminal kinase 1
PBA	4-phenyl butyric acid
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
shRNA	short hairpin RNA
siRNA	small interfering RNA
STD	standard chow

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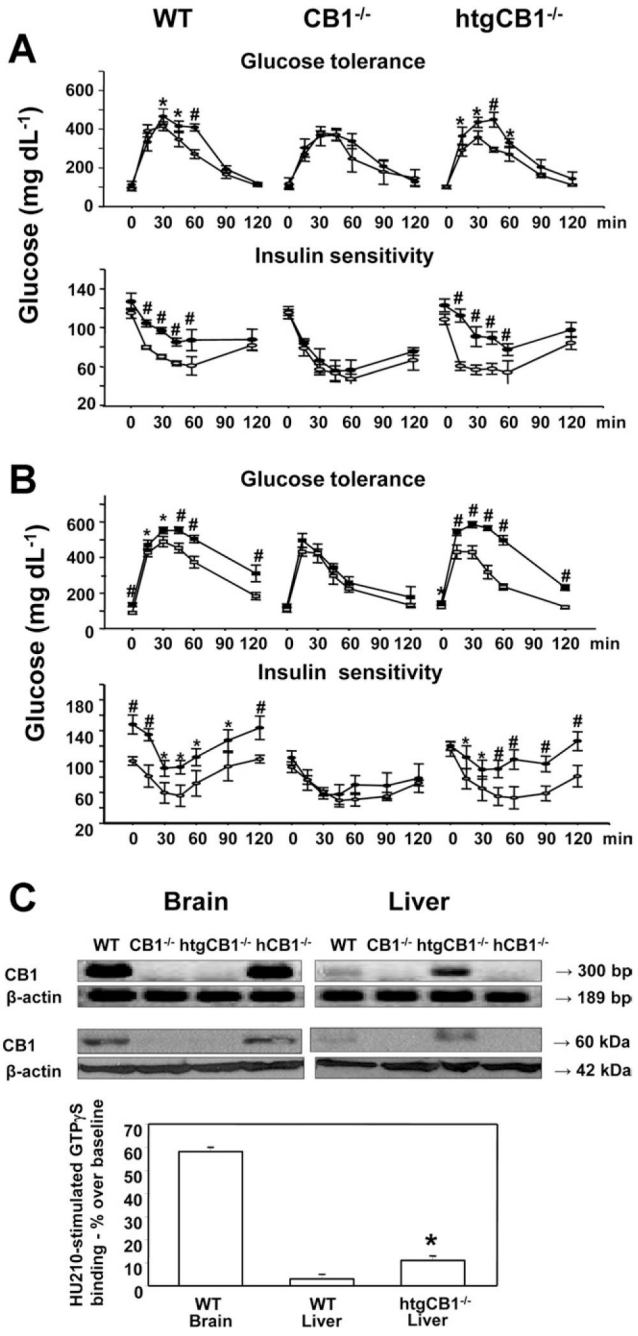

Figure 1.

(*A*) Anandamide induces whole body glucose intolerance and insulin resistance via activation of hepatic CB_1. Male wild-type, $CB_1^{-/-}$, and $htgCB_1^{-/-}$ mice were subjected to an intraperitoneal glucose tolerance test (GTT) and a week later an insulin sensitivity test (IST). Overnight fasted mice received an intraperitoneal injection of vehicle (*open symbols*) or 10 mg/kg anandamide (*filled symbols*). Ten minutes later, the animals received 2 g/kg glucose intraperitoneally, followed by blood glucose measurements at the indicated time points. One week later, each mouse received an intraperitoneal injection of vehicle or 10 mg/kg anandamide. Ten minutes later, 0.75 U/kg insulin was injected and blood glucose monitoring was continued for an additional 2 hours. Note that anandamide caused acute

glucose intolerance and insulin resistance in wt and htg$CB_1^{-/-}$, but not in $CB_1^{-/-}$, mice. N = 6 – 8 animals/group. *$P < .05$ or #$P < .005$ from corresponding value in vehicle-pretreated mice. (*B*) GTT and IST were conducted as previously described in wild-type (wt), $CB_1^{-/-}$, and htg$CB_1^{-/-}$ mice on STD (*open symbols*) or HFD (*solid symbols*) for 14 –16 weeks. N = 6 – 8 animals/group, significance indicated as in *A*. (*C*) CB_1 receptor expression profile in wt, $CB_1^{-/-}$, htg$CB_1^{-/-}$, and h$CB_1^{-/-}$ mice. (*Top*) CB_1 mRNA expression in brain and liver using reverse-transcription PCR. (*Middle*) Western blotting of CB_1 protein using a CB_1 receptor N-terminal antibody (Cayman) or a β-actin antibody for loading control. (*Bottom*) GTPγS binding stimulated by the cannabinoid agonist HU-210 (100 nmol/L) in membranes from wild-type mouse brain and liver and htg$CB_1^{-/-}$ liver. Similar results were obtained in 3 independent experiments. Note that CB_1 overexpression in ht-g$CB_1^{-/-}$ hepatocytes is associated with increased CB_1-stimulated GTPγS binding.

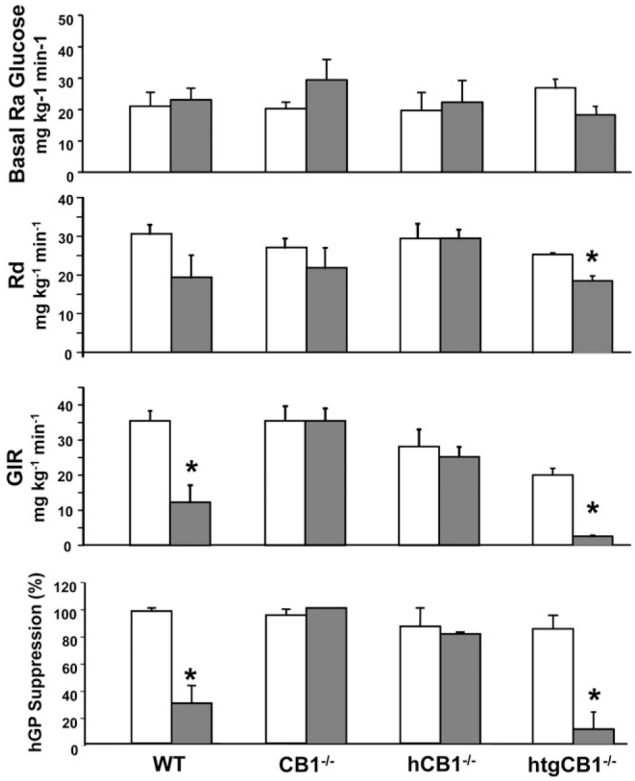

Figure 2.

HFD induces hepatic insulin resistance via activation of hepatic CB_1. Hyperinsulinemic euglycemic clamps were performed in wild-type, $CB_1^{-/-}$, $hCB_1^{-/-}$, and $htgCB_1^{-/-}$ mice 5 hours after withdrawal of food, as described in Materials and Methods. The mice had been maintained on HFD (*filled columns*) or STD (*open columns*) for 14 –16 weeks before the clamps. Note that HFD results in suppression of glucose infusion rate (GIR) and hepatic glucose production (hGP), indicating decreased insulin-mediated suppression of hepatic glucose production in mice with (wild-type [WT] and $htgCB_1^{-/-}$) but not those without hepatic CB_1 ($CB_1^{-/-}$ or $hCB_1^{-/-}$). Rd, whole body glucose uptake. Means ± SE from 3– 6 animals/group are shown. $*P < .05$ relative to corresponding STD values.

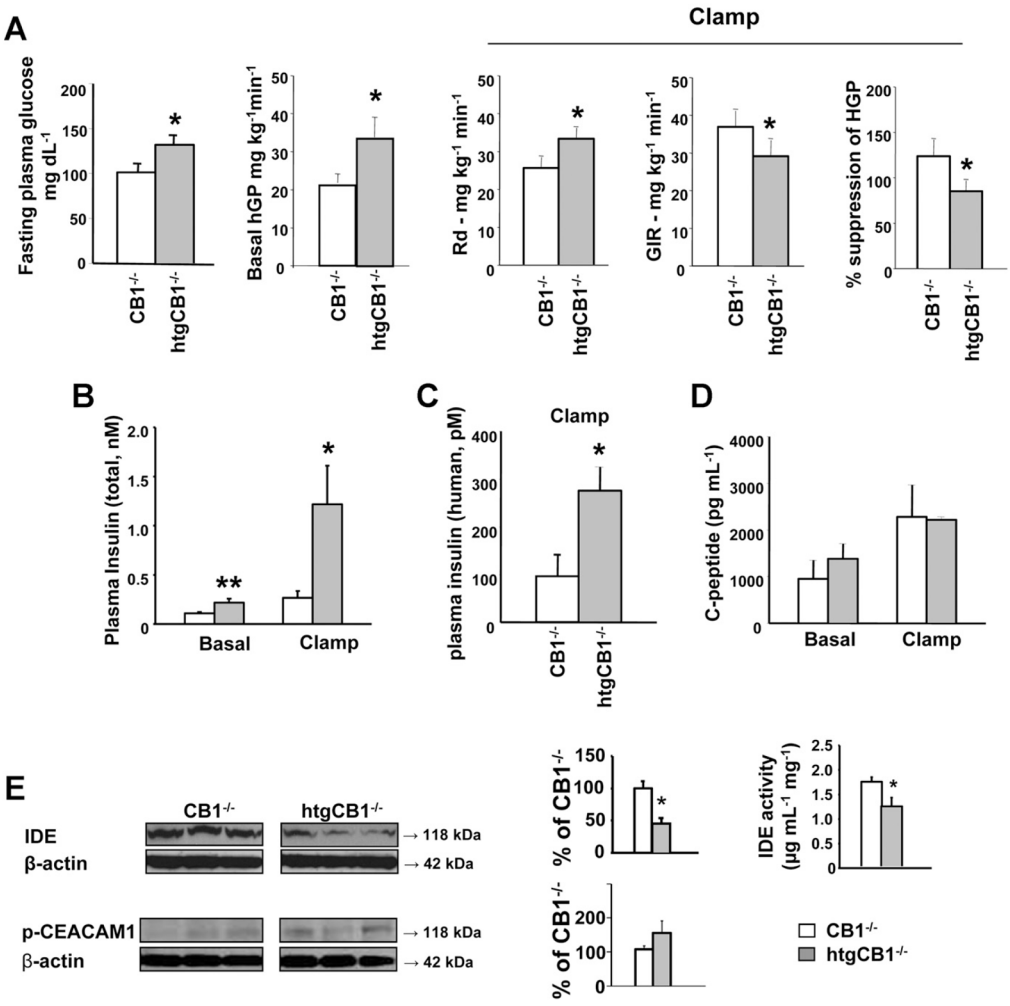

Figure 3.

Selective overexpression of CB_1 in the liver is associated with hepatic insulin resistance and reduced insulin clearance. (*A*) $HtgCB_1^{-/-}$ mice on STD (*filled columns*) are hyperglycemic and show hepatic insulin resistance, compensated by increased whole body glucose uptake, relative to $CB_1^{-/-}$ littermates (*open columns*). (*B*) Plasma insulin before (basal) and 120 minutes after initiation of euglycemic/hyperinsulinemic clamp. $^{*}P < .05$, $^{**}P < .01$ relative to corresponding value in $CB_1^{-/-}$ mice, n = 7 ($CB_1^{-/-}$) or 8 ($htgCB_1^{-/-}$). (*C*) Plasma levels of human insulin, measured in the same "clamp" samples, $^{*}P < .05$. (*D*) Plasma levels of C-peptide in same basal and clamp samples. (*E*) IDE and p-CEACAM1 levels and IDE activity in livers from $CB_1^{-/-}$ and $htgCB_1^{-/-}$ mice.

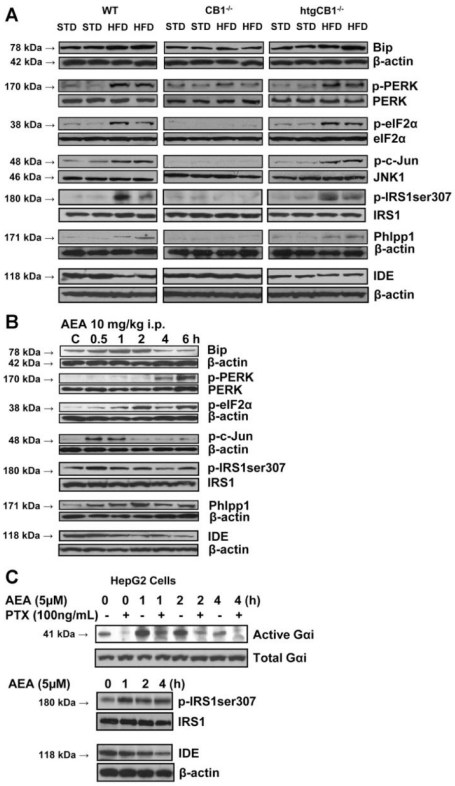

Figure 4.

Both HFD and acute treatment with anandamide inhibit hepatic insulin signaling via CB_1 receptor-induced ER stress response, through serine-307 phosphorylation of IRS1 and activation of Phlpp1. (*A*) HFD increases Bip levels; induces PERK, eIF2α, c-Jun, and IRS1 (serine) phosphorylation; increases Phlpp1; and decreases IDE protein in the liver of wild-type and htg$CB_1^{-/-}$, but not $CB_1^{-/-}$, mice (Western blots). (*B*) Acute treatment of wild-type mice on STD with anandamide (10 mg/kg intraperitoneally) induces hepatic Bip expression, PERK, eIF2α, c-Jun, and IRS1 (serine) phosphorylation and increases Phlpp1 and decreases IDE expression. (*C*) In HepG2 cells, anandamide causes pertussis toxin (PTX)-sensitive activation of Giα, serine-307 phosphorylation of IRS1, and inhibition of IDE expression in a time-dependent manner.

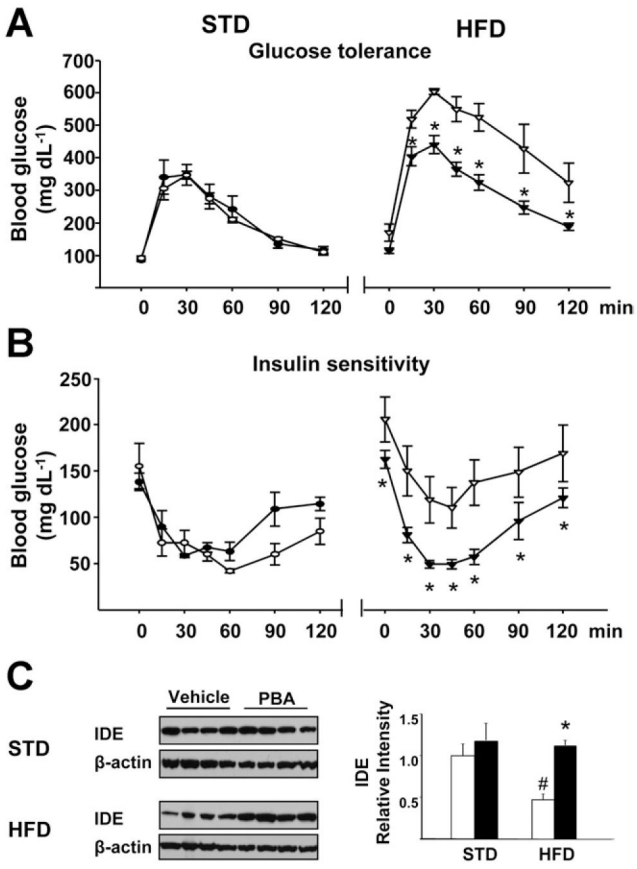

Figure 5.

In vivo treatment of mice with the chemical chaperone PBA (1 g · kg^{-1} · day^{-1} for 20 days, *solid symbols*) reverses (*A*) obesity-induced glucose intolerance, (*B*) insulin resistance, and (*C*) down-regulation of IDE in the liver. **P* < .05 from corresponding value in vehicle-treated mice; $^{\#}P$ < .05 relative to corresponding STD values.

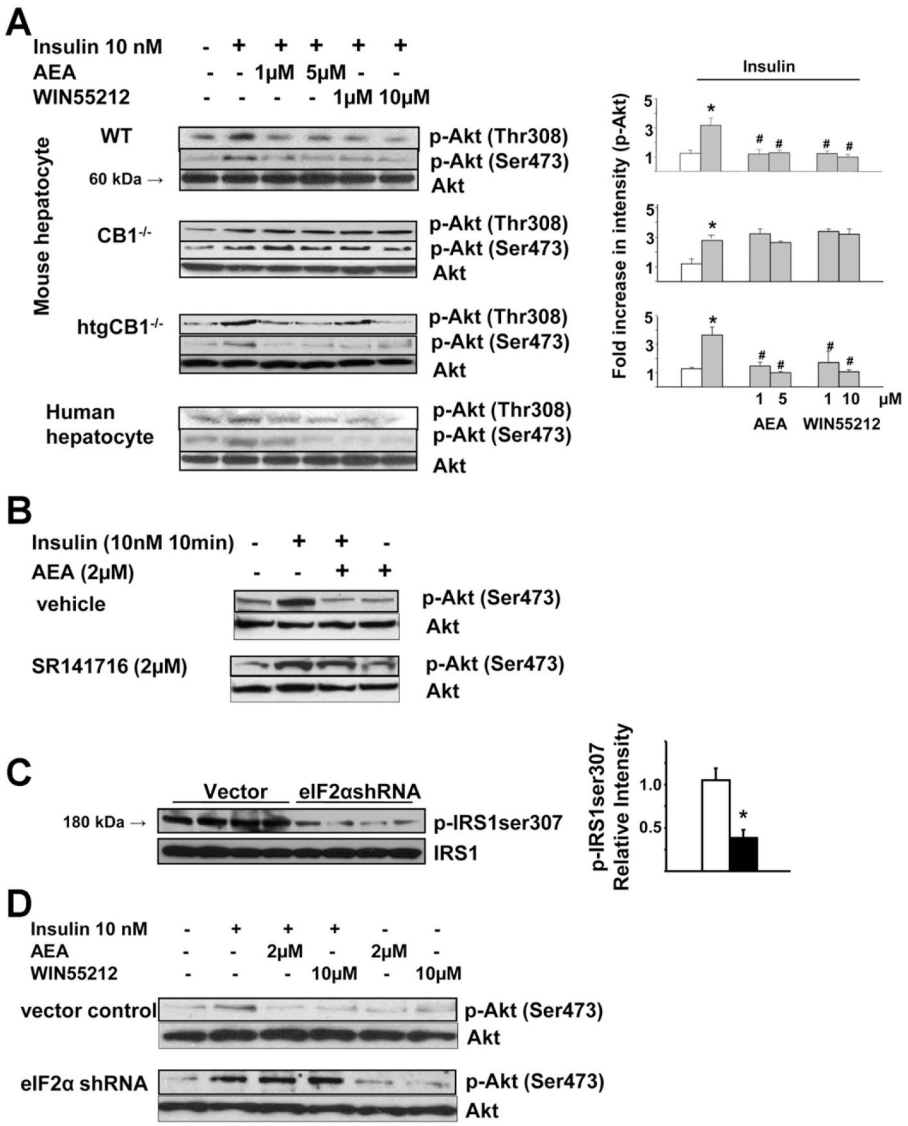

Figure 6.

CB_1-mediated inhibition of akt-2 phosphorylation is ER stress dependent. (*A*) Insulin-induced phosphorylation of akt-2 is inhibited by anandamide or WIN55,212-2 in isolated hepatocytes obtained from wild-type or htg$CB_1^{-/-}$ mice or from human livers, but not in hepatocytes from $CB_1^{-/-}$ mice (quantified by densitometry, $^{*}P < .05$ relative to control cells (*first lanes, open columns*); $^{\#}P < .05$ relative to cells treated with insulin only. (*B*) Anandamide inhibition of insulin-induced akt-2 phosphorylation is abolished by the CB_1 antagonist rimonabant. (*C*) shRNA knockdown of eIF2α prevents serine-307 phosphorylation of IRS1 in HepG2 cells. (*D*) shRNA knockdown of eIF2α prevents CB_1 agonist-induced inhibition of akt-2 phosphorylation. Each blot in *A*–*D* has been replicated 3 times with similar results.

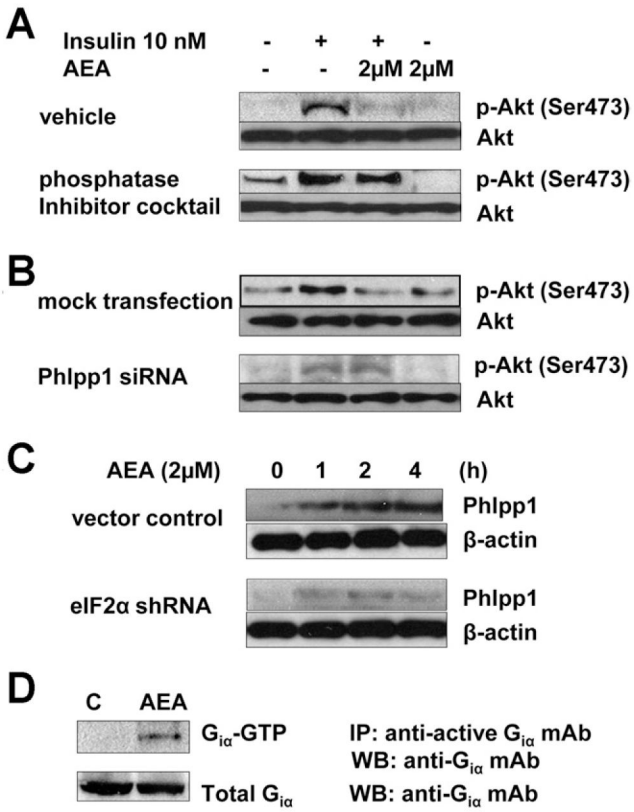

Figure 7.

Anandamide inhibits insulin-induced akt-2 phosphorylation in mouse hepatocytes via ER stress-dependent activation of Phlpp1. Anandamide inhibition of insulin-induced akt-2 phosphorylation is abrogated by (*A*) a phosphatase inhibitory cocktail or by (*B*) siRNA knockdown of Phlpp1 expression. (*C*) Anandamide induction of Phlpp1 is inhibited by shRNA-mediated knockdown of eIF2*a*. (*D*) Anandamide activates $G_{i\alpha}$ in hepatocytes. Each blot in *A*–*D* has been replicated 3 times with similar results.