An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice

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Emerging evidence suggests that inflammation provides a link between obesity and insulin resistance. The noncanonical IκB kinases IKK-ε and TANK-binding kinase 1 (TBK1) are induced in liver and fat by NF-κB activation upon high-fat diet feeding and in turn initiate a program of counterinflammation that preserves energy storage. Here we report that amlexanox, an approved small-molecule therapeutic presently used in the clinic to treat aphthous ulcers and asthma, is an inhibitor of these kinases. Treatment of obese mice with amlexanox elevates energy expenditure through increased thermogenesis, producing weight loss, improved insulin sensitivity and decreased steatosis. Because of its record of safety in patients, amlexanox may be an interesting candidate for clinical evaluation in the treatment of obesity and related disorders.

Although the molecular events underlying the relationship between obesity and insulin resistance remain uncertain1–4, numerous studies have implicated an inflammatory link5–7. Obesity produces a state of chronic, low-grade inflammation in liver and fat accompanied by the local secretion of cytokines and chemokines that attenuate insulin action. Knockout or pharmacological inhibition of inflammatory pathways can disrupt the link between genetic- or diet-induced obesity and insulin resistance, suggesting that local inflammation is a key step in the generation of cellular resistance to important hormones that regulate metabolism8–11.

The nuclear factor-κB (NF-κB) transcriptional program is activated in obese fat and liver and, to a lesser extent, in muscle and seems to have an important role in the development of insulin resistance12–15. NF-κB activation is triggered by phosphorylation of the regulatory protein IκB. Four different IκB kinases (IKKs) have been identified: IKK-α, IKK-β, IKK-ε and TBK1. A role for IKK-α and IKK-β in NF-κB activation has been firmly established, but whether TBK1 and IKK-ε regulate the pathway remains uncertain16–18. However, the genes encoding both of these kinases contain κB regulatory sites in their promoter regions, and activation of the NF-κB transcriptional pathway induces their expression19, suggesting that they may act downstream of this transcriptional pathway.

We recently reported that expression of both Tbk1 and Ikbke mRNA and TBK1 and IKK-ε protein are increased during a high-fat diet (HFD) in adipose tissue, and the expression of IKK-ε is increased in liver15. Moreover, deletion of the Ikbke gene rendered mice partially resistant to the HFD-dependent development of obesity, insulin resistance, hepatic steatosis and inflammation, leading us to search for small-molecule inhibitors of these kinases as a potential therapeutic option to treat these conditions. We report here the identification of one such compound, amlexanox, which had been previously developed for the treatment of asthma, allergic rhinitis and aphthous ulcers but has an unclear mechanism of action20,21. Administration of this selective TBK1 and IKK-ε inhibitor to obese mice produces reversible weight loss and improved insulin sensitivity, reduced inflammation and attenuated hepatic steatosis without affecting food intake. These data suggest that IKK-ε and TBK1 are part of a counterinflammatory process that sustains energy storage in the context of insulin resistance22. Disruption of this process by amlexanox thus increases adaptive energy expenditure and restores insulin sensitivity. Because of the apparent safety of this drug in patients, we propose that it undergo study for the treatment of obesity, type 2 diabetes and nonalcoholic fatty liver disease in patients.

RESULTS

Obesity increases IKK-ε and TBK1 activity through NF-κB

In a previous study15 we reported that the mRNA levels of both Ikbke and Tbk1 are elevated in white adipose tissue (WAT) from

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HFD-fed mice as compared to normal-chow diet (NCD) controls, whereas *Ikbke* mRNA is elevated in liver. Immune complex assays revealed that both IKK-ε and TBK1 kinase activity were elevated in livers from HFD-fed mice (Fig. 1a). In the adipose tissue, we also found higher kinase activity, even when normalized to the elevated protein expression. We found this elevated expression after 6–7 weeks of HFD feeding, directly correlating with the onset of inflammatory macrophage infiltration in adipose tissue and liver (data not shown).

We modeled inflammation in 3T3-L1 cells by treating differentiated adipocytes with the inflammatory cytokine tumor necrosis factor α (TNF-α)23. Chronic TNF-α treatment increased the mRNA levels of both kinases (Fig. 1b). These effects were prevented by pretreatment of cells with the IKK-β inhibitor compound VIII23. Furthermore, TNF-α produced a dose-dependent increase in IKK-ε protein expression in these cells, as well as downregulation of peroxisome proliferator-activated receptor-γ (PPAR-γ). TNF-α also produced increased phosphorylation of TBK1 at Ser172, which is associated with activation in vivo.

To investigate whether inflammatory signals are responsible for the induction of these kinases in vivo, we treated HFD-fed mice with the PPAR-γ agonist rosiglitazone or the ω-3 fatty acid EPA and docosahexaenoic acid (DHA). Both rosiglitazone and ω-3 fatty acids reduce the inflammation and insulin resistance associated with obesity25. Diet supplemented with ω-3 fatty acids markedly attenuated the HFD-induced elevation in the expression of IKK-ε mRNA and protein expression in both WAT and liver (Fig. 1d,e). Treatment of obese mice with rosiglitazone also reduced expression of *Ikbke* in adipocytes as well as in the stromal vascular fraction of WAT (Fig. 1f). Because neither rosiglitazone nor ω-3 fatty acid diet supplementation caused weight loss25, the elevated expression of these kinases from high-fat feeding is probably the result of inflammation rather than a direct result of obesity.

**Amlexanox is an inhibitor of IKK-ε and TBK1**

Because deletion of the *Ikbke* gene in mice produced partial resistance to the effects of a HFD15, we screened a library of 150,000 chemical compounds to search for inhibitors of IKK-ε using recombinant baculoviral-expressed, purified full-length IKK-ε with myelin basic protein (MBP) as a substrate and identified amlexanox as a high-affinity inhibitor (Fig. 2a). This previously discovered drug of unknown mechanism is used to treat asthma and allergic rhinitis in Japan and aphthous ulcers in the United States20,21.

Dose-response experiments revealed that amlexanox blocked IKK-ε activity with a half-maximal inhibitory concentration (IC50) of approximately 1–2 μM (Fig. 2b). Amlexanox also blocked the activity of TBK1 at approximately the same concentrations but had no effect on IKK-α or IKK-β and at these concentrations did not block any other kinases from a broad panel representing most kinase families. TBK1 and IKK-ε share an overall 65% sequence similarity and are 72% identical in the ATP-binding region. Inhibition of IKK-ε or TBK1 (data not shown) by amlexanox was competitive for its substrate ATP (Fig. 2c), indicating that it interacts with the enzymes in the ATP-binding site. This is consistent with a model of the compound docked in the presumed ATP-binding pocket of TBK1 (Fig. 2d) on the basis of its published structure26.

Amlexanox increased the phosphorylation of TBK1 at Ser172 in 3T3-L1 adipocytes and blocked polynosinonic:polycytidylic acid (poly I:C)-stimulated phosphorylation of interferon responsive factor-3 (IRF3), a presumed substrate of IKK-ε and TBK1 (ref. 27 and Fig. 2e). Reduced IRF3 phosphorylation was also produced by the previously identified IKK-ε and TBK1 inhibitor Cay10576 (cayman)28. Furthermore, cayman treatment of RAW264.7 macrophages stimulated with lipopolysaccharide (LPS) or poly I:C also blocked the phosphorylation of IRF3 and stimulated the phosphorylation of TBK1 at Ser172 (Fig. 2f). The phosphorylation of TBK1 was also increased in peritoneal macrophages derived from *Ikbke* knock-out mice (data not shown), which was probably caused by blockade of feedback inhibition of the pathway29, as evidenced by higher IKK-β phosphorylation in cayman-treated RAW264.7 cells.

**Amlexanox produces reversible weight loss in obese mice**

We fed C57BL/6 mice a diet of 45% fat and gavaged them daily with a vehicle control or with 25 or 100 mg per kg body weight amlexanox and monitored their body weight (Fig. 3a). Serum concentrations of the drug exceed 5 μM at these doses30. Treatment of mice with...
Amlexanox is a specific inhibitor of IKK-ε and TBK1. (a) Diagram of the chemical structure of amlexanox. (b) Dose-response relationship of amlexanox inhibition of IKK-ε and TBK1 activity as determined by MBP phosphorylation showing an IC$_{50}$ of approximately 1–2 µM. Results were replicated in more than three experiments. (c) Lineweaver-Burk plot showing competition of amlexanox with ATP for inhibition of IKK-ε. Results are representative of three experiments. (d) Model of amlexanox binding to the ATP-binding site of TBK1. Top, surface model showing the binding of amlexanox in the active site of TBK1; bottom, hydrogen bonding of amlexanox (cyan) in the active site of TBK1. (e) TBK1 phosphorylation (pTBK1) at Ser172 and IRF3 phosphorylation at Ser396 (pIRF3) in 3T3-L1 adipocytes treated with amlexanox or cayman and with or without poly I:C. Results were replicated in more than three experiments. RalA was used as a loading control. (f) IRF3, IKK-β (pIKK-β) and TBK1 phosphorylation in RAW264.7 cells treated with or without cayman, LPS and poly I:C. Results were replicated in multiple experiments. RalA was used as a loading control.

**Figure 2** Amlexanox is a specific inhibitor of IKK-ε and TBK1. (a) Diagram of the chemical structure of amlexanox. (b) Dose-response relationship of amlexanox inhibition of IKK-ε and TBK1 activity as determined by MBP phosphorylation showing an IC$_{50}$ of approximately 1–2 µM. Results were replicated in more than three experiments. (c) Lineweaver-Burk plot showing competition of amlexanox with ATP for inhibition of IKK-ε. Results are representative of three experiments. (d) Model of amlexanox binding to the ATP-binding site of TBK1. Top, surface model showing the binding of amlexanox in the active site of TBK1; bottom, hydrogen bonding of amlexanox (cyan) in the active site of TBK1. (e) TBK1 phosphorylation (pTBK1) at Ser172 and IRF3 phosphorylation at Ser396 (pIRF3) in 3T3-L1 adipocytes treated with amlexanox or cayman and with or without poly I:C. Results were replicated in more than three experiments. RalA was used as a loading control. (f) IRF3, IKK-β (pIKK-β) and TBK1 phosphorylation in RAW264.7 cells treated with or without cayman, LPS and poly I:C. Results were replicated in multiple experiments. RalA was used as a loading control.

either dose of amlexanox prevented the weight gain produced by a HFD; drug-treated mice maintained weights equivalent to those of vehicle-treated mice on a control diet through 12 weeks. There was no effect of the drug on food intake, either at the beginning or end of the study (Fig. 3b).

We also evaluated amlexanox in mice with established obesity (Fig. 3c and Supplementary Fig. 1). Treatment with amlexanox produced a 10-g weight loss in HFD-fed obese mice after only 4 weeks of treatment with no further effect thereafter. Moreover, escalation of the dose at this time point had no further effect on weight (data not shown). Withdrawal of the drug after 8 weeks of treatment restored weight gain, and the mice returned to their original weights after 6–8 weeks (Fig. 3c). Weight loss during the treatment phase was accompanied by more than a 6-g reduction in overall mass of adipose tissue, as well as an 80% decrease in fasting leptin concentration, whereas the concentrations of serum triglycerides, free fatty acids and cholesterol were unchanged (Fig. 3d and data not shown).

To determine whether the effects of amlexanox could be seen in another model of obesity, we treated ob/ob mice with 100 mg per kg body weight of amlexanox or vehicle control and monitored their body weight (Fig. 3e). Although the drug had no effect on food intake, amlexanox produced a 7- to 8-g weight loss after 4 weeks of treatment. Amlexanox also caused a significant decrease in adipose tissue mass in these mice and an increase in circulating adiponectin concentrations (Fig. 3f,g).

Because amlexanox had no effect on food intake, we reasoned that the weight loss was the result of increased energy expenditure. We thus used metabolic cages to monitor energy expenditure in diet-induced obese (DIO) mice treated with or without amlexanox. Four-week treatment of DIO mice with 25 mg per kg body weight amlexanox resulted in significantly ($P < 0.001$) higher oxygen consumption as compared to treatment with vehicle control, which is consistent with an increase in energy expenditure (Fig. 3h). Exhaled carbon dioxide was also significantly ($P = 0.001$) higher in the amlexanox-treated mice, and their respiratory exchange ratio remained unchanged compared to control-treated mice (Supplementary Fig. 2), although amlexanox had no effect on NCD-fed mice, in which neither IKK-ε nor TBK1 was induced. Together, these data suggest that amlexanox might induce an increase in thermogenesis in obese mice. Amlexanox treatment produced an approximate 1 °C increase in body temperature compared to HFD-fed vehicle-treated mice, restoring the temperatures to those of NCD-fed mice (Fig. 3i).

Amlexanox improves insulin sensitivity in obese mice

To assess in more detail the metabolic impact of amlexanox treatment, we treated DIO mice with 25 mg per kg body weight amlexanox or vehicle control for 8 weeks either before or after obesity was established and then assessed their metabolic parameters. Mice treated with amlexanox concurrently with a HFD had improved glucose tolerance after obesity was established, with approxiately 30–40% reduction in the area under the curve for glucose (Fig. 4a). Treatment of mice with amlexanox after established DIO reversed the elevations in fasting serum insulin concentrations caused by the HFD, suggesting improved insulin sensitivity (Fig. 4b). Insulin tolerance tests revealed that amlexanox treatment significantly improved insulin sensitivity in mice with established DIO, as indicated by a restoration...
Amlexanox reverses hepatic steatosis in obese mice

We treated DIO mice with 25 mg per kg body weight amlexanox or vehicle control for 8 weeks and examined their livers postmortem (Fig. 5a). The hepatomegaly normally observed in HFD-fed mice was significantly reduced in amlexanox-treated mice (Student’s t test). Moreover, although the basal phosphorylation of hormone-sensitive lipase (HSL) was higher in adipose tissue from amlexanox-treated mice than vehicle-treated mice, the dephosphorylation of HSL produced by insulin was enhanced after amlexanox treatment compared to vehicle-treated mice on HFD (Supplementary Fig. 3h). Phosphorylation of Akt in response to insulin was also enhanced in amlexanox-treated mice compared to vehicle-treated controls, indicating improved insulin sensitivity in VAT (Supplementary Fig. 3i).

Amlexanox reverses hepatic steatosis in obese mice

We treated DIO mice with 25 mg per kg body weight amlexanox or vehicle control for 8 weeks and examined their livers postmortem (Fig. 5a). The hepatomegaly normally observed in HFD-fed mice was significantly reduced in amlexanox-treated mice (Student’s t test). Moreover, although the basal phosphorylation of hormone-sensitive lipase (HSL) was higher in adipose tissue from amlexanox-treated mice than vehicle-treated mice, the dephosphorylation of HSL produced by insulin was enhanced after amlexanox treatment compared to vehicle-treated mice on HFD (Supplementary Fig. 3h). Phosphorylation of Akt in response to insulin was also enhanced in amlexanox-treated mice compared to vehicle-treated controls, indicating improved insulin sensitivity in VAT (Supplementary Fig. 3i).
was largely reversed by the drug, with a greater than 20% reduction in liver weight (Fig. 5b). Moreover, triglyceride content in the liver was more than 50% lower in the amlexanox-treated mice compared to mice treated with vehicle control. Hepatic glycogen content, which is elevated in HFD-fed mice, was reduced by amlexanox (Fig. 5d). Large lipid droplets were apparent by H&E staining in the livers from vehicle-treated control DIO mice but were largely disseminated by treatment with amlexanox, which is consistent with the marked reductions in triglycerides, glycogen and liver weight (Fig. 5b). These beneficial reductions in hepatic lipids were reversed in livers from mice that were taken off amlexanox treatment and continued on a HFD (Fig. 5e). Steatosis was also resolved in ob/ob mice treated with the drug (Supplementary Fig. 4a–c).

To further investigate the hepatosteatotic phenotype, we measured the expression of key metabolic and inflammatory genes in the livers of NCD-fed, HFD-fed and amlexanox-treated HFD-fed (control) mice. Mice treated with amlexanox had lower expression of certain key lipogenic genes compared to control mice, including those encoding fatty acid synthase (Fasn), malic enzyme 1 (Mel1), acetyl CoA carboxylase (Acaca) and stearyl CoA desaturase 1 (Scd1) (Fig. 5f). Indicative of a higher lipid load, HFD was associated with higher expression of Ppara, Pparg, Cd36, Fabp4 (encoding fatty acid binding protein 4) and Lpl (encoding lipoprotein lipase). The expression of these genes was lower in amlexanox-treated and NCD-fed mice. Gluconeogenic gene expression was modestly higher in HFD-fed mice and was not lower with amlexanox treatment. Glycolytic genes, the expression levels of which were higher in HFD-fed mice relative to NCD-fed mice, were expressed at lower levels in amlexanox-treated mice (Fig. 5g). In addition, we found lower levels of HFD-induced expression of several inflammatory genes, including Tnfa, Cccl10 and Ccl5 (also known as Rantes), which encode proinflammatory cytokines (Fig. 5h), Ccl2 (also known as Mcp1) and Ccl3 (also known as Mip-1α), which encode chemotactic factors, and Emr1 and Igax (also known as F4/80 and Cd11c), which encode markers of macrophage infiltration. Expression of Cybb, encoding cytochrome b-245, β-polypeptide, which is involved in the microbial oxidase system of phagocytes, was lower in the drug-treated group. Genetic measures of hepatosteatosis were similarly lower in ob/ob mice (Supplementary Fig. 4d,e).

Amlexanox reduces adipose tissue inflammation in obese mice
Infiltration of inflammatory macrophages into WAT was markedly lower in amlexanox-treated mice compared to vehicle-treated controls, as assessed by H&E staining (Fig. 6a). This amlexanox-dependent reduction in the appearance of macrophages in crown-like structures was accompanied by a marked attenuation in the levels of miRNAs encoding key inflammatory genes in adipose tissue, including Tnfa, Cc12, Cc13, Igax and Emr1 (Fig. 6b). We also measured the concentration of cytokines in the serum of control and amlexanox-treated mice. Although the drug had no effect on the circulating concentrations of Mcp1 or Rantes, the concentrations of TNF-α, IL-1α and Mip-1α were lower (Fig. 6c). Serum concentrations of the anti-inflammatory cytokine IL-10 (ref. 34) were higher in amlexanox-treated mice compared to controls. We found higher expression of fat
cell–enriched proteins such as Slc2a4 and Pparg, which are indicative of improved insulin sensitivity, in WAT from amlexanox–treated mice (Fig. 6b). The expression of uncoupling protein 1 (UCP1) was also higher in the adipose tissue of amlexanox–treated DIO mice as compared to HFD–fed vehicle–treated controls (Fig. 6d).

To evaluate whether amlexanox might control energy metabolism by directly affecting inflammation, we examined the effects of the drug on primary macrophages or the cultured RAW267.2 macrophage cell line. The addition of amlexanox did not affect macrophage migration in a wound–healing assay performed in RAW267.2 cells (Supplementary Fig. 5a). Bone marrow–derived and peritoneal macrophages from Ikbke knockout mice were slightly more responsive to the effects of LPS in inducing the expression of various cytokines (Supplementary Fig. 5b–d). We also evaluated the effect of these inhibitors on poly I:C– or TNF–α–stimulated NF–κB activation in adipocytes and found a slight increase in the activation of NF–κB after pretreatment with the IKK–ε and TBK1 inhibitors (Supplementary Fig. 5e). Moreover, bone marrow transplant from Ikbke–deficient mice into wild–type mice on a HFD did not influence weight or insulin sensitivity (data not shown). Together with the increase in TBK1 phosphorylation induced by the inhibitors described in Figure 2, these data indicate that inhibition of IKK–ε and TBK1 does not directly block the proinflammatory effects of cytokines or LPS on inflammatory signaling or the NF–κB pathway but, rather, may prevent feedback inhibition that is a consequence of elevated expression of TBK1 and IKK–ε. Thus, these noncanonical kinases do not act as IκB kinases and are not directly proinflammatory. However, IKK–ε and TBK1 may attenuate the inflammatory response without eliminating it. Thus, the induction of these ‘counterinflammatory’ kinases may contribute to the generation of low–grade, sustained inflammation in obesity by preventing its resolution.

**Amlexanox promotes energy expenditure in adipose tissue**

To discern whether amlexanox directly influences phosphorylation in vivo, we first examined a number of proteins known to undergo hyperphosphorylation in states of obesity. As previously described, phosphorylation of proteins in the mammalian target of rapamycin complex1 (mTORC1) pathway such as S6K and S6 were elevated in response to a HFD; this increase was largely attenuated in amlexanox–treated DIO mice (Fig. 6d and Supplementary Fig. 6a,b).
Amlexanox also blocked induction of IKK-ε and TBK1 protein by a HFD in adipose tissue. This finding was consistent with the fact that overexpression of wild-type but not kinase-inactive TBK1 in cells enhanced the stimulation of S6K phosphorylation by insulin, whereas knockdown of Ikba or Tbk1 in 3T3-L1 adipocytes lowered the amount of insulin-stimulated S6 phosphorylation (Supplementary Fig. 6c). In adipose tissue from HFD-fed mice, HSL phosphorylation was lower, which is consistent with the well-established desensitization of the β-adrenergic pathway in this tissue during obesity36. Amlexanox treatment prevented the reduction in HSL phosphorylation associated with a HFD (Fig. 6d and Supplementary Fig. 6a,b).

In vehicle-treated control mice on a HFD, brown adipose tissue features of white fat. Brown fat reverted to its normal appearance in amlexanox-treated mice (Fig. 6e). After withdrawal of amlexanox treatment, lipids again accumulated in the BAT, reverting to the HFD phenotype of control obese mice (Supplementary Fig. 6f). UCP1 protein expression was also reduced in BAT treated with amlexanox or vehicle control. With amlexanox treatment, there was also an increase in the expression of cytochrome oxidases and Elovl3, which are involved in lipid metabolism in the BAT. Amlexanox treatment reduced IKK-ε and TBK1 protein expression in BAT (Fig. 6g and Supplementary Fig. 6h). We also found reduced signaling through the mTORC1 pathway in BAT of amlexanox-treated mice as compared to controls. Ex vivo treatment with amlexanox acutely stimulated lipid oxidation and oxygen consumption in BAT explants from HFD-fed mice, supporting a direct effect of amlexanox on energy expenditure (Fig. 6h,i).

To evaluate the roles of IKK-ε and TBK1 in mediating the effects of the drug, we treated Ikbek knockout mice with amlexanox and monitored their weight loss and insulin sensitivity. The weight loss produced by amlexanox was equivalent between wild-type and Ikbek knockout mice, indicating that the upregulation of TBK1 in these mice may compensate for IKK-ε inhibition (Supplementary Fig. 7a). We also monitored insulin sensitivity by intermittent insulin tolerance tests over several weeks of treatment (Supplementary Fig. 7b). Although Ikbek knockout mice were more insulin sensitive than their wild-type littermates, the drug produced an additional improvement, achieving the insulin sensitivity observed in wild-type mice treated with amlexanox. Improvements in insulin sensitivity preceded weight loss, indicating that improved insulin sensitivity may not be secondary to weight loss.
to weight loss. Taken together with the data in Figure 4, which show that the effects of amlexanox are comparable to those of other insulin-sensitizing drugs such as the thiazolidinediones37, these results suggest that amlexanox is a bona fide insulin sensitizer.

As Tbk1 knockout mice are not viable, we used mouse embryonic fibroblasts (MEFs) to further investigate the importance of these kinases in the specificity of amlexanox. Treatment of wild-type MEFs with amlexanox produced a dose-dependent increase in the phosphorylation of TBK1 and the P65 NF-κB subunit, presumably because of feedback inhibition27. Both effects were absent in Ikbke and Tbk1 double-knockout MEFs (Supplementary Fig. 7c–e). However, MEFs with single knockout of either Ikbke or Tbk1 retained responsiveness to amlexanox. In Ikbke knockout MEFs, we found TBK1 phosphorylation and degradation of IkBα after amlexanox treatment, whereas Tbk1 knockout MEFs showed robust phosphorylation of IKK-β in response to amlexanox treatment. Amlexanox also enhanced oxygen consumption in wild-type but not Ikbke and Tbk1 double-knockout MEFs, indicating that the energy expenditure-promoting effect of amlexanox is mediated specifically through inhibition of both IKK-ε and TBK1.

**DISCUSSION**

Compelling evidence has accrued over the past few years that both genetic and dietary forms of rodent obesity are accompanied by the generation of low-grade inflammation in key metabolic tissues, which serves as an important link between obesity and sustained insulin resistance7–11. Almost all rodent models of obesity-induced insulin resistance are associated with chronic inflammation, and a strong correlation between insulin resistance and inflammatory markers in studies of several patient groups has been shown38–41. This inflammation is generally low grade and is accompanied by macrophage switching from a type 2 to a type 1 polarization state34,42,43, as well as the presence of additional inflammatory cells44–47. Inhibition of inflammatory signaling by knockout of key pathways in obese mice can disrupt the link between obesity and insulin resistance11,48–55. Likewise, different anti-inflammatory pharmacological and genetic approaches can also reduce insulin resistance in rodents and patients56–58. Cultivation studies and neutralizing antibodies directed against different proinflammatory cytokines have demonstrated in vitro that inflammatory signals derived from M1-polarized macrophages can desensitize cells to insulin through different mechanisms23.

Although the molecular links between inflammation and insulin resistance are not yet completely understood, it is clear that the NF-κB program has a key role13. Disruption of NF-κB signaling by targeted knockout of the canonical IκB kinase IKK-β gene or pharmacological inhibition of this pathway can restore insulin sensitivity in obese states. However, the roles of the noncanonical IKKs, TBK1 and IKK-ε in this process are less well understood. Our previous studies indicated that deletion of the Ikbke gene partially restored insulin sensitivity and reduced weight in mice fed a HFD despite having no apparent effect on NF-κB signaling, although the relevant mechanism was not defined35. Here we describe amlexanox, a newly identified chemical inhibitor of IKK-ε and TBK1 that reproduces many of these effects. Administration of this compound to mice reduced weight, insulin resistance, inflammation and steatosis in three different obese mouse models. These effects are apparent within 3–4 weeks after starting drug therapy and are reversible. They are probably attributed to increased triglyceride hydrolysis and oxidation, along with reduced lipogenesis and glycogenesis. Signaling pathways that modulate these processes are attenuated in vitro by the addition of amlexanox or another structurally unrelated inhibitor to cultured adipocytes.

The precise mechanisms by which amlexanox produces these beneficial effects in obese rodents have not yet been completely elucidated. Although amlexanox is known to be a mast cell stabilizer of unknown mechanism20, and depletion of mast cells may have beneficial metabolic effects29, most of the in vivo and in vitro evidence points to a role for the drug in increasing expenditure of energy while reducing its storage in adipocytes and hepatocytes. Furthermore, the lack of a phenotype in wild-type mice reconstituted with Ikbke knockout bone marrow indicates that the role of IKK-ε in bone marrow-derived cells such as mast cells and macrophages is less important than its role in other cell types such as adipocytes and hepatocytes. Although IKK-ε and TBK1 expression is elevated as part of the inflammatory program downstream of NF-κB, the kinase targets of the drug do not seem to be direct participants in the increased inflammatory program.

In fact, the reduced inflammation observed in vivo with amlexanox treatment may be an indirect effect of improved metabolic disease or, perhaps, of elimination of a feedback pathway that maintains inflammation at low levels such that inflammation is permitted to resolve. Moreover, despite the fact that administration of amlexanox to obese mice restores insulin sensitivity, these compounds are not direct insulin sensitizers in vitro. Phosphorylation analyses indicate that IKK-ε and TBK1 may co-opt signaling pathways that drive energy conservation in the context of insulin resistance, ensuring that energy expenditure is kept in check by producing catecholamine resistance in adipose tissue, in the process reducing HSL phosphorylation and UCP1 expression while also increasing mTOR signaling to drive lipogenesis and glycogen synthesis. Thus, together these data suggest that expression of TBK1 and IKK-ε by obesity-dependent inflammation may conserve energy during the transition from an insulin-dependent to an insulin-independent state of nutrient storage. Moreover, it is probable that the improvement in insulin resistance produced by treatment with amlexanox results from reducing excessive energy storage while preventing homeostatic feedback loops (such as mTOR) that block insulin receptor signaling60.

Amlexanox seems to be a relatively safe drug with a long history of use in patients. As such, there might be an interesting opportunity for repurposing this agent for diabetes and obesity. Future clinical studies will be needed to explore this possibility and validate the hypothesis that these noncanonical IκB kinases contribute to counterinflammatory actions that sustain metabolic disease.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
A.R.S. and S.M.R. wrote the manuscript. S.M.R. created figures. S.M.R. produced the data in Figures 1a–d, 2a–d, 3a–c, 4a–h, 5a–e and 6a–c and in Supplementary Figures 1–3, 5c–e and 6a–c. B.C.H. produced the data in Figures 3a–d–f–h, 4a–e, 5a–e and 6a–b and in Supplementary Figures 2a–b, 3a and 5d–f. S.J.D. and M.J.I. performed the screen identifying amlexanox as an IKKe inhibitor. L.C. and S.J.D. produced the data in Figures 1b and 2b,c,f. M.U. produced the data in Figures 1a and 2e and in Supplementary Figures 5c–e and 6c–e. J.R.R. produced the images in Figure 1d. J.M. produced the data in Figure 1c. N.M.W. produced the data in Supplementary Figure 4a–d. I.H. produced the data in Supplementary Figure 6e. M.D.R., T.Y.T., C.L. and R.M.E. contributed data not shown, which was instrumental in generating Figure 5f–h. D.O. produced the data in Figure 1d and the samples for Figure 1e. P.L. produced the data in Figure 1f under the guidance of J.M.O.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. All chemicals were obtained from Sigma-Aldrich unless otherwise stated. Antibodies to IKK-α (2690), TBK1 (3013), pTBK1 (Ser172; 5483), IKK-α and IKK-β (2684), pIKK-α and pIKK-β (Ser176; 2687), AKT (9272), PAKT (Ser473; 9272), p65 (2708, diluted 1:250), p65 (Thr389; 9205), p65 (Ser217), p56 (Ser235 and Ser236; 2211), IRF3 (4302), IRF3 (Ser396; 4947), HSL (4107), pHSL (Ser563; 4139, or Ser660; 4126) and PPAR-γ (2443) were purchased from Cell Signaling. RALA-specific antibody (610222) was obtained from BD Bioscience. UCP1-specific antibody (UCP11-A, diluted 1:250 for FAT detection) was obtained from Alpha Diagnostics. All antibodies were diluted 1:1,000 unless otherwise noted. Enhanced chemiluminescence reagents were purchased from NEN, Inc. EDTA-free protease inhibitor tablet was purchased from Roche Diagnostics.

Animals and animal care. We fed wild-type male C57BL/6 mice a HFD consisting of 45% of calories from fat (D12451 Research Diets Inc.) starting at 8 weeks of age for 12–24 weeks, and we maintained NCD-fed male C57BL/6 controls on normal Chow diet consisting of 4.5% fat (5002 Lab Diet). We fed the C57BL/6 mice diets containing or -3 fatty acids as previously described. Rosiglitazone treatment was administered for 3 weeks by addition of the compound to the diet in mice that had been on HFD for 16 weeks. Each mouse consumed on average 3.5 mg per kg body weight rosiglitazone per day. Amlexanox treatment was begun at 20 weeks of age after 12 weeks of HFD feeding at 8 weeks of age. For the treatment groups, 25 mg per kg body weight amlexanox treatment was administered for 3 weeks by addition of the compound to the diet. Each mouse consumed on average 1.2 g per kg body weight amlexanox (100–300 mg per kg body weight for C57BL/6 mice or 1.2 g per kg body weight (ob/ob) mice). For insulin tolerance tests, mice were fasted for 3 h and then given an intraperitoneal injection of insulin (1.2 units per kg body weight for C57BL/6 mice and 2.0 units per kg body weight for ob/ob mice). We measured the basal blood glucose level and then at 15, 30, 45, 60, 90, 120 and 180 min from tail blood using the OneTouch Ultra glucometer (Lifescan).

Food intake. The remaining weight of food provided was determined daily for singly housed mice. Daily food consumption was calculated from a 3-d average.

Energy expenditure and respiratory quotient. C57BL/6 mice in the amlexanox treatment group were placed in metabolic cages. The University of Michigan Metabolic Phenotyping Core measured oxygen consumption (VO2), carbon dioxide production (VCO2) and spontaneous motor activity during 3 consecutive days using the Comprehensive Laboratory Monitoring System (Columbus Instruments), an integrated open-circuit calorimeter equipped with an optical beam activity monitoring system. Values presented are normalized to body weight. The respiratory quotient was calculated by dividing the carbon dioxide production by the oxygen consumption. We used the mean values for the light and dark cycles to analyze statistical significance.

Body composition. The University of Michigan Metabolic Phenotyping Core used nuclear magnetic resonance analysis to quantify body fat, lean body mass and fluid content in ob/ob mice and C57BL/6 mice in the amlexanox treatment group.

Hyperinsulinemic-euglycemic clamp. C57BL/6 mice were placed on HFD starting at 8 weeks of age, and starting at 16 weeks of age, the mice were gavaged daily with 50 mg per kg body weight amlexanox or vehicle control. After 4 weeks of treatment, the right jugular vein and carotid artery were surgically catheterized, and mice were given 5 d to recover from the surgery. During this recovery period, amlexanox was delivered in drinking water. On the day of the clamp, a final oral gavage treatment was performed. After a 5- to 6-h fast, hyperinsulinemic clamp studies were performed on conscious mice using the protocol adopted from the Vanderbilt Mouse Metabolic Phenotyping Center by the University of Michigan Animal Phenotyping Core consisting of a 90-min equilibration period followed by a 120-min experimental period (t = 0 to t = 120 min). Insulin was infused at 4.0 mU per kg body weight per min. This experiment was performed at the University of Michigan Metabolic Phenotyping Core. We measured serum free fatty acid concentrations using the colorimetric NEFA-HR(2) kit (Wako).

Core body temperature. We performed rectal temperature measurements using a YSI 4600 Precision thermometer (YSI, Inc.).

Blood chemistry analysis. Blood glucose was measured by OneTouch Ultra Glucometer. Plasma from mice fasted for 6 h was isolated from whole blood collected into heparinized tubes. We used an insulin ELISA kit (Crystal Chem Inc.) to measure serum insulin concentrations. Leptin and adiponectin concentrations were measured by ELISA kits purchased from Cayman Chem Inc. We quantified serum cytokine concentrations using luminescent technology in a multianalyte plate panel purchased from Millipore. Additionally, we measured TNF-α concentrations using ELISA kits purchased from R&D Systems.

Glucose and insulin tolerance tests. For glucose tolerance tests, after a 6-h fast, we gavaged mice with glucose at a dose of 1.5 g per kg body weight (C57BL/6 mice) or 1.2 g per kg body weight (ob/ob mice). For insulin tolerance tests, mice were fasted for 3 h and then given an intraperitoneal injection of insulin (1.2 units per kg body weight for C57BL/6 mice and 2.0 units per kg body weight for ob/ob mice). We measured the basal blood glucose level and then at 15, 30, 45, 60, 90, 120 and 180 min from tail blood using the OneTouch Ultra glucometer (Lifescan).

Tissue lipid content. We isolated liver lipids by ethanol KOH saponification and BAT lipids by chloroform extraction and then quantified triglyceride concentrations using the Triglyceride Reagent kit (Thermo Scientific).

Lever glycogen content. We digested liver tissue in a 30% potassium hydroxide solution and then precipitated glycogen using 70% ethanol. After three washes (resuspend pellet in water, then add ethanol to 70% and centrifuge down the pellet) to remove any traces of glucose, we digested glycogen by addition of amyloglucosidase. Released glucose was quantified using a colorimetric kit (Wako).

Stromal vascular fraction (SVF) and adipocyte isolation. We digested excised WAT in PBS containing 1% BSA and 1 mg ml−1 type I collagenase for 30 min at 37 °C with gentle agitation. The cell suspension was filtered through a 100-µm filter and then centrifuged at 700 g for 5 min to separate floating adipocytes from the SVF pellet. We washed floating adipocytes twice with PBS containing 1% BSA and collected the SVF pellets after each wash.

Western blot analysis. We homogenized tissues in lysis buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 250 mM sucrose, 1% NP-40, 2 mM dithiothreitol (DTT), 1 mM sodium vanadate, 100 mM NaF, 10 mM Na3PO4 and freshly added protease inhibitor tablet) and then incubated them for 1 h at 4 °C. We centrifuged crude lysates at 14,000g for 15 min twice and determined the protein concentration using BioRad Protein Assay Reagent. Samples were diluted in SDS sample buffer. Bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Individual proteins were detected with the specific antibodies and visualized on film using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) and Western Lightning Enhanced Chemiluminescence (Perkin Elmer Life Sciences). Alternatively, infrared fluorescent secondary antibodies were used for detection and quantification of specific protein on the Odyssey CLx imager (LI-COR).

Histochrome. Tissues were fixed in formalin for 3 d. The University of Michigan Cancer Center Research Histology Laboratory performed histology. A 1:100 dilution of the UCP1-specific antibody (UCP11, Alpha Diagnostics) at 10 µg ml−1 was used for immunohistochemical detection of UCP1 protein.

Gene expression analysis. We rinsed isolated mouse tissues in PBS and then froze them in liquid nitrogen and stored them at −80 °C until extraction.
We extracted total RNA from liver, WAT and BAT tissues, as well as differentiated 3T3-L1 cells, using the RNeasy Lipid Tissue Kit (Qiagen) according to the manufacturer's instructions with the inclusion of a DNase digestion step. We extracted total RNA from bone marrow–derived macrophages and SVF cells using the RNeasy Kit (Qiagen) with a DNase step. We used the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) with random primers for reverse transcription. Real-time PCR amplification of the complementary DNA was performed on samples in triplicate with Power SYBR Green PCR Master Mix (Applied Biosystems) using the Applied Biosystems 7900HT Fast real-time PCR System. We chose Adrp (also known as Plin2) and GAPDH as the internal controls for normalization after screening several candidate genes; their expression was not substantially affected by experimental conditions. The sequences of all primers used in this study are listed in Supplementary Table 1. Data were analyzed using the 2−ΔΔCT method, and the control sample value was normalized to 1. Other data were quantified using an internal standard curve. Statistical significance was determined using the unpaired heteroscedastic Student's t test with one averaged sample value per mouse.

**Lipid oxidation rate.** We excised intrascapular BAT and placed it in DMEM with 2% BSA with or without 50 μM amlexanox then incubated it at 37 °C for 1 h, after which we changed the medium to DMEM with 2% BSA, 0.25 mM carnitine, 0.2 mM palmitic acid and trace levels of 3H-palmitic acid and incubated it for 1 more hour at 37 °C and then collected the medium and isolated the aqueous faction. Lipid oxidation was determined by the conversion of 3H-palmitic acid to 3H2O.

**IKK-ε and TBK1 in vitro kinase assays.** We performed in vitro kinase assays by incubating purified kinase (IKK-ε or TBK1) in kinase buffer containing 25 mM Tris (pH 7.5), 10 mM MgCl2, 1 mM DTT and 10 μM ATP for 30 min at 30 °C in the presence of 0.5 μCi γ-[32P]-ATP and 1 μg MBP per sample as a substrate. We stopped the kinase reaction by adding 4× SDS sample buffer and boiling for 5 min at 95 °C. Supernatants were resolved by SDS-PAGE, transferred to nitrocellulose and analyzed by autoradiography using a Typhoon 9410 phosphorimager (GE Lifesciences). We quantified the bands using ImageQuant.

**IKK-ε and TBK1 immunocomplex kinase assay.** We collected liver and WAT from C57BL/6 mice on NCD or HFD and homogenized the tissues using a Dounce homogenizer with lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 1 mM DTT and 1 mM PMSF in the presence of protease inhibitors (Roche Diagnostics). We incubated tissue cell lysates for 1 h at 4 °C and cleared them by spinning at 13,000 r.p.m. for 15 min at 4 °C in a table-top centrifuge. Each 1 mg of lysate was subjected to immunoprecipitation using 5 μl of undiluted rabbit polyclonal antibody to TBK1 or IKK-ε for 1.5 h at 4 °C. We harvested immunocomplexes by incubation with ProtA beads (Roche Diagnostics) for 2 h at 4 °C. We washed immunoprecipitates once with lymph buffer and three times with wash buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (pH 7.4), 50 mM NaCl, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM NaF, 10 mM MgCl2 and 1 mM DTT. We performed an in vitro kinase assay using the immunoprecipitated kinases as described above. Relative amounts of MBP phosphorylation were detected by autoradiograph and normalized to the amounts of IKK-ε or TBK1 kinase detected in the immunoprecipitate by immunoblotting.

**Cell culture.** 3T3-L1 fibroblasts (American Type Culture Collection) were cultured in 10% neonatal calf serum in DMEM for proliferation and 10% FBS in DMEM for differentiation. We grew the cells to confluence and then induced differentiation 2 d later with 500 μM 3-isobutyl-1-methylxanthine, 250 mM dexamethasone and insulin (1 μg ml−1) for 3 d followed by insulin-only treatment for 2 d. We routinely used cells within 7 d after completion of the differentiation process and only used cultures in which >90% of cells showed adipocyte morphology. We serum starved 3T3-L1 adipocytes with 0.5% FBS in DMEM before treatment. TNF-α treatments (50 ng ml−1, unless otherwise noted) were performed during the 24 h before harvest and after pretreatment with the IKK-β inhibitor compound VIII (EMD Biosciences) for 1 h where indicated. We pretreated 3T3-L1 adipocytes 1 h with amlexanox at the given concentrations and then treated them with 20 μg ml−1 of poly I:C for 1 h. Alternatively, we treated 3T3-L1 adipocytes with 50 μM forskolin for 15 min after a 30-min amlexanox pretreatment. We treated cells with or without 10 mM of insulin for 15 min. We determined glycerol release into the supernatant (DMEM without phenol red) using a colorimetric assay. We serum starved RAW264.7 cells with 0.5% FBS in DMEM medium and pretreated them with or without Cay10576 (Cayman Chemical). The cells were then treated with LPS (0.5 μg ml−1) or poly I:C (50 μg ml−1) for 1 h. We harvested the cells for total RNA and analyzed them by real-time PCR. We also resolved cell lysates by SDS-PAGE and analyzed them by immunoblotting using the indicated antibodies. Wild-type and Ikbke Tbk1 double-knockout and Ikbke or Tbk1 single-knockout MEFs were kindly provided by S. Akira. Cells were cultured in DMEM with 10% FBS and serum starved in 0.5% FBS for 12 h before treatment. TNF-α treatment (50 ng ml−1) was performed for 15 min at the end of a 2-h pretreatment with amlexanox.

**Oxygen consumption rate.** The Michigan Metabolic Phenotyping Core and Michigan Nutritional Obesity Research Center measured oxygen consumption in cells and ex vivo tissue using an extracellular flux analyzer (model XF24-3, Seahorse Bioscience).

**Molecular modeling of amlexanox in the TBK1 ATP-binding site.** We modeled amlexanox into the known structure of the TBK1 catalytic domain (Protein Data Bank ID codes 4EUT and 4EUU and ref. 26). The best fit of amlexanox in the TBK1 ATP-binding site was obtained using AutoDock Vina.

**Statistical analyses.** Averaged values are presented as the mean ± s.e.m. When comparing two groups, we determined statistical significance using Student's t test. When more than two groups were investigated, we first performed an analysis of variance (ANOVA) to establish that not all groups were equal. After a statistically significant ANOVA result, we performed between-group comparisons using the Tukey-Kramer post hoc analysis. ANOVA and Tukey-Kramer tests were performed using Stata version 12.0.

**Additional methods.** Detailed methodology is described in the Supplementary Methods.