A subcutaneous adipose tissue–liver signalling axis controls hepatic gluconeogenesis

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The search for effective treatments for obesity and its comorbidities is of prime importance. We previously identified IKK-ε and TBK1 as promising therapeutic targets for the treatment of obesity and associated insulin resistance. Here we show that acute inhibition of IKK-ε and TBK1 with amlexanox treatment increases cAMP levels in subcutaneous adipose depots of obese mice, promoting the synthesis and secretion of the cytokine IL-6 from adipocytes and preadipocytes, but not from macrophages. IL-6, in turn, stimulates the phosphorylation of hepatic Stat3 to suppress expression of genes involved in gluconeogenesis, in the process improving glucose handling in obese mice. Preliminary data in a small cohort of obese patients show a similar association. These data support an important role for a subcutaneous adipose tissue–liver axis in mediating the acute metabolic benefits of amlexanox on glucose metabolism, and point to a new therapeutic pathway for type 2 diabetes.
Accumulating evidence indicates that the chronic low-grade inflammation associated with obesity plays an important role in the development of metabolic disease. Obesity induces an increase in the NF-κB pathway\(^1\)–\(^3\), which leads to induction of the non-canonical IKKs, IKK-\(\varepsilon\) and TBK1 in adipose tissue and liver\(^4\)–\(^7\). Once induced, these kinases can initiate a counter-inflammatory programme by phosphorylating and activating phosphodiesterase 3B (PDE-3B) in adipocytes, thus producing reduced levels of cAMP and resistance to stimulation by catecholamines such as epinephrine and norepinephrine\(^8\). This resulting catecholamine resistance reduces energy expenditure and thermogenesis, and contributes to the development of obesity in humans and mice\(^8\)–\(^12\). Our previous studies demonstrated that inhibition of IKK-\(\varepsilon\)/TBK1 with the dual-specificity inhibitor amlexanox improved catecholamine and insulin resistance, and resulted in significant weight loss and reversal of metabolic disease in obese mice, but had no effect on normal-chow fed mice in which the kinases are not elevated\(^13\)–\(^15\).

While improved catecholamine sensitivity may account for the dramatic weight loss observed after IKK-\(\varepsilon\) and TBK1 inhibition in obese mice, the mechanism of improved insulin sensitivity and glucose handling remains uncertain. A euglycemic–hyperinsulinemic clamp revealed that the insulin-sensitizing effects of amlexanox were mainly attributed to suppression of hepatic glucose production\(^15\). Moreover, insulin sensitization was observed prior to significant weight loss in obese mice. However, it was not clear whether the effects of amlexanox in the liver were mediated through direct inhibition of hepatic IKK-\(\varepsilon\)/TBK1 or another indirect mechanism. Here we describe one indirect pathway by which inhibition of IKK-\(\varepsilon\) and TBK1 in the subcutaneous adipose tissue affects reduced hepatic glucose production. Specifically, inhibition of IKK-\(\varepsilon\) and TBK1 by amlexanox stimulates the secretion of interleukin-6 (IL-6) from adipocytes as well as preadipocytes in the subcutaneous adipose tissue via a cAMP/p38-dependent pathway. The resulting increase in serum IL-6 is responsible for the activation of hepatic Stat3, which suppresses expression of G6pc to reduce hepatic glucose output.

**Results**

Direct versus indirect effects of amlexanox in the liver. To determine the mechanism by which amlexanox improves hepatic insulin sensitivity, we performed RNA sequencing analysis of hepatic gene expression a few hours after *in vivo* amlexanox treatment, and identified over 1,700 differentially expressed genes (GEO accession code GSE57054). Pathway analysis of these differentially regulated genes revealed that the top two most enriched pathways were the adipocytokine signalling pathway and the Jak/Stat signalling pathway, presumably activated by a tissue-derived ligand (Supplementary Table 1).

Amlexanox treatment induces IL-6 signalling. The preponderance of Jak/Stat responsive genes in the RNA sequencing analysis led us to hypothesize that early effects of amlexanox in the liver might, at least partially, be due to the increased appearance of cytokines in the circulation. We thus screened serum from amlexanox-treated obese mice for a series of cytokines known to be upstream of the Jak/Stat pathway, and identified one, IL-6. Time course studies revealed that serum IL-6 levels were significantly elevated soon after amlexanox treatment, peaking 4 h after gavage with the drug (Fig. 1a,b). Increased serum levels of leukemia-inducible factor were also observed with amlexanox treatment; this IL-6 family ligand may also contribute to the effects of amlexanox on Jak/Stat signalling (Supplementary Fig. 1). No changes in other IL-6 family ligands, including leptin, were observed. To identify the source of IL-6, we performed gene expression analysis in various tissues from mice treated for 4 h with amlexanox. No difference was observed in muscle, liver or epididymal fat. IL6 messenger RNA (mRNA) levels (Fig. 1c). However, there was a significant increase in IL6 expression in the inguinal adipose tissue. Consistent with preferential induction of IL6 expression in subcutaneous fat, we also observed increased expression in the dorsal subcutaneous depot, but not in the

![Figure 1](https://example.com/figure1.png)

**Figure 1** | Inhibition of IKK-\(\varepsilon\)/TBK1 by amlexanox results in IL-6 secretion from subcutaneous adipose tissue. (a) Time course of IL-6 secretion after amlexanox administration (\(n = 4\) mice per time point). (b) Serum IL-6 levels 4 h after amlexanox treatment (\(n = 8\) mice per treatment). (c) IL6 gene expression across tissues. BAT, brown adipose tissue (\(n = 8\) mice per treatment); WAT, white adipose tissue. (d) Relative IL6 expression in different fat pads (\(n = 8\) mice per treatment). * indicates a \(P\) value < 0.05 (heteroscedastic t-test (a,b), students t-test (c,d)) amlexanox versus vehicle. Error bars presented as s.e.m.
perirenal visceral fat (Fig. 1d). The precise mechanism regulating the depot specificity of IL-6 expression is unclear, as similar differences are not observed when the tissues are treated ex vivo.

**Amlexanox induces IL-6 expression specifically in adipocytes.** To determine which cell type within the subcutaneous fat was responsible for amlexanox-stimulated IL-6 secretion, we fractionated the tissue and measured IL-6 expression levels in the various cell types. Increased IL-6 expression was observed both in mature adipocytes and the stromal vascular cells (SVC) (Fig. 2a). Emr1 expression was restricted to the SVC, indicating that there was negligible macrophage contamination of the mature adipocyte fraction from the inguinal white adipose tissue (Fig. 2b). As expected, Adipoq expression was exclusive to the mature adipocytes (Fig. 2c). We further sorted the SVC to evaluate the amlexanox-induced IL-6 expression in preadipocytes versus immune cells (Fig. 2d,e). Emr1 expression was exclusive to the CD45⁺ cells, confirming the sorting of macrophages into this population (Fig. 2f). In addition, enriched expression of Pdgfra confirmed the identity of the preadipocyte population (Fig. 2g). Amlexanox induced IL-6 expression specifically in the preadipocyte population but not in CD45⁺ cells (Fig. 2h). Importantly other cytokines such as Tnfα were specifically expressed in the CD45⁺ immune cells (Fig. 2i). These data indicate that amlexanox specifically directs IL-6 expression in adipocytes as well as preadipocytes, but interestingly not in immune cells such as macrophages.

**Induction of IL-6 expression in adipocytes via cAMP signalling.** In adipocytes, IKK-ε and TBK1 phosphorylate and activate PDE-3B, thereby reducing intracellular cAMP levels and contributing to catecholamine resistance, which has been well-described in obese rodents and patients. Inhibition of IKK-ε/TBK1 by amlexanox in vivo and in vitro relieves this inhibition and restores catecholamine sensitivity. Downstream of cAMP, p38 activation leads to increased expression of Ucp1, which likely contributes to increased energy expenditure in amlexanox-treated mice. Interestingly, the Il6 gene is also reported to be a downstream target of cAMP signalling. Consistently, treatment of 3T3-L1 adipocytes with CAY-10576 also increased the expression of Il6 in response to cAMP signalling, confirming that this effect is due to inhibition of IKK-ε/TBK1, and not a non-specific effect of amlexanox.

**Amlexanox-induced IL-6 signalling is p38-dependent in vivo.** We also explored the role of the cAMP/p38 pathway in vivo. Similar to the induction of Il6 expression, amlexanox treatment specifically increased cAMP levels in inguinal adipose tissue (Fig. 6a). To demonstrate that the stimulatory effect of amlexanox on IL-6 secretion was downstream of this pathway, we pretreated mice by oral gavage with SB203,580. The stimulatory effect of amlexanox on serum IL-6 levels was inhibited by p38 blockade (Fig. 6b). Consistent with p38-dependent induction of IL-6 in inguinal adipose tissue by amlexanox, we observed a significant increase in Il6 mRNA levels as well as IL-6 precursor protein levels, both of which were completely blocked by administration of SB203,580 (Fig. 6c–e and Supplementary Fig. 5).

**Hepatic Stat3 activation is observed with amlexanox treatment.** Next, we investigated whether the increase in serum IL-6 after amlexanox treatment was associated with increased Stat3 activity in the liver. Following administration of amlexanox to obese mice, we assayed phosphorylation of Stat3 at tyrosine 705, which mediates dimerization and subsequent nuclear localization and transcriptional regulation. Hepatic Stat3 phosphorylation was increased 4 h after amlexanox treatment, coincident with the increase in serum IL-6 levels (Figs 7a,b and 1a, respectively, Supplementary Fig. 6). As expected, Stat3 phosphorylation was specifically associated with nuclear localization and DNA binding in livers from the amlexanox-treated mice (Fig. 7c,d, respectively, Supplementary Fig. 7). Furthermore, amlexanox treatment significantly enriched Stat3 occupancy at the promoter of Socs3, a well-known Stat3 target gene (Fig. 7e).

**Amlexanox-induced IL-6 expression is cAMP/p38 dependent.** Treatment of 3T3-L1 adipocytes with amlexanox alone for 2 h significantly increased Il6 expression (Fig. 4a). The amlexanox-induced increase in Il6 expression correlated with secretion of IL-6 from 3T3-L1 adipocytes into the cell culture media 4 h after treatment (Fig. 4b). As predicted by the proposed mechanism involving reduced PDE-3B activity, amlexanox induced a significant increase in cellular cAMP levels in adipocytes (Fig. 4c), confirming that the drug increases IL-6 secretion from adipocytes via increased cAMP signalling.

**Induction of IL-6 by cAMP has been previously shown to be dependent on p38** (refs 18–20). Consistent with this notion, p38 phosphorylation increased after amlexanox treatment (Fig. 4d and Supplementary Fig. 2). Notably, the stimulation of p38 phosphorylation by amlexanox was rapid, occurring over a time course similar to its ability to relieve the feedback inhibition of TBK1 phosphorylation. The induction of Stat3 phosphorylation in these adipocytes was not observed until after 4 h of treatment, a lag consistent with the time course of secretion of IL-6 into the media. Further supporting that Il6 induction by amlexanox is mediated by a p38-dependent pathway downstream of cAMP, pretreatment of adipocytes with the p38 inhibitor SB203,580 completely blocked the effect of amlexanox to increase both Il6 expression and IL-6 secretion (Fig. 4e,f). The dependency of amlexanox-induced IL-6 secretion in adipocytes on p38 was confirmed by short interfering RNA-mediated knockdown of p38 (Mapk14) prior to amlexanox treatment (Fig. 5 and Supplementary Fig. 3). To verify that these effects of amlexanox were mediated by its inhibition of IKK-ε/TBK1, we utilized another structurally unrelated inhibitor of these kinases CAY-10576. Treatment of 3T3-L1 adipocytes with CAY-10576 also increased the expression and secretion of IL-6 in a p38-dependent manner (Supplementary Fig. 4), confirming that this effect is due to inhibition of IKK-ε/TBK1, and not a non-specific effect of amlexanox.

**Activation of hepatic Stat3 is dependent on IL-6 signalling.** Pre-treatment with SB203,580 dose dependently decreased...
Figure 2 | Cellular specificity of amlexanox induced Il6 expression within fat. (a) Il6 expression in mature adipocytes, SVC and whole inguinal adipose tissue (n = 8 mice fractionated individually per treatment). (b) Relative Emr1 (F4/80, a macrophage marker) and (c) Adipoq (a mature adipocyte marker) expression in the different inguinal adipose tissue fractions (n = 8 mice per treatment). (d,e) Gating strategy for fluorescence-activated cell sorting (FACS) of SVC. (f) Relative Emr1 and (g) Pdgfra (a preadipocyte marker) expression in different inguinal stromal cell fractions (n = 4 groups of 2). (h) Il6 and (i) Tnfa expression in preadipocytes (CD45−, CD31−, Sca1+) and immune cells (CD45+ cells, both CD11c+ and CD11c− populations) (n = 4 separations per treatment each consisting of pooled inguinal fat from 2 mice). * indicates a P value < 0.05 (heteroscedastic t-test) amlexanox versus vehicle. Error bars presented as s.e.m.
Post-hoc analysis) vehicle versus amlexanox (or two-way ANOVA plus Tukey–Krammer signalling (10).

Suppression of G6pc development and on responses to diet. Instead of genetic knockout of Il6 (Supplementary Fig. 10). We opted to use neutralizing antibodies stimulating of Stat3 phosphorylation in the amlexanox-treated control-treated mice, the IL-6 neutralizing antibody blocked the IgG control antibody had no effect on the amlexanox or vehicle phosphorylation (Fig. 7h and Supplementary Fig. 9). While the which efficiently blocked amlexanox-stimulated hepatic Stat3 phosphorylation, we utilized an IL-6 neutralizing stimulation to hepatic Stat3 phosphorylation, coordinat-

Figure 3 | Induction of Il6 by β-adrenergic signalling in adipocytes. (a) Relative induction of Il6 expression after pharmaceutical activation of β-adrenergic signalling (10 μM isoproterenol and CL316,243) or adenyl cyclase (50 μM forskolin). (b) Relative induction of Il6 by 10 μM CL316,243 after overexpression of green fluorescent protein control, IKK-ε wild type (WT) or IKK-ε kinase dead mutant (KD). (c) Expression of Il6 in cells treated with 10 μM CL316,243 after 1 h pretreatment with 100 μM amlexanox or vehicle control. (d) Relative Il6 expression in 3T3-L1 fibroblasts after 2 h amlexanox treatment. (e) Relative Il6 expression and (f) IL-6 secretion in RAW264.7 cells after 2 h of amlexanox treatment. (g) Relative Il6 expression and (h) IL-6 secretion in RAW264.7 cells after 2 h of 0.5 μg ml⁻¹ isopolysaccharide (LPS) treatment, with 1 h pretreatment with 2 μM Compound VIII. (i) Relative Il6 expression and (j) IL-6 secretion in RAW264.7 cells after 2 h of 50 μM forskolin treatment. * indicates P value < 0.05 (student’s t-test (a,b,d) or two-way ANOVA plus Tukey-Krammer post-hoc analysis) vehicle versus amlexanox (c) or Compound VIII versus control (g,h). Error bars presented as s.e.m.

amlexanox-stimulated hepatic Stat3 phosphorylation, coordinat-

Suppression of G6pc expression by Stat3. In the liver, Stat3 activity is associated with suppression of gluconeogenic gene expression, specifically G6pc (refs 21–23). Increased binding of Stat3 to the G6pc promoter at all four previously characterized binding regions was observed in liver derived from amlexanox-treated mice (Fig. 8a–d). Amlexanox treatment specifically increased DNA binding by Stat3, but not FoxO1 or CREB, indicating that the latter transcription factors are likely not involved in the regulation of G6pc expression by the drug (Fig. 8e). Consistent with the indirect effects of amlexanox in the liver and lack of effect on CREB, amlexanox did not increase cAMP levels in liver (Fig. 8f). We verified that treatment with amlexanox results in reduced gluconeogenesis in obese mice after only 3 days of treatment, by performing a pyruvate tolerance test (Fig. 8g). Consistent with Stat3 activation by IL-6, the reduction in hepatic glucose production was associated with reduced expression of G6pc, and increased expression of Socs3 (Fig. 8h,i). Furthermore, treatment with an IL-6 neutralizing antibody completely blocked the anti-gluconeogenic effects of amlexanox treatment, as determined by a pyruvate tolerance test (Fig. 9a,b). The IL-6 neutralizing antibody also blocked the suppression of
IL-6 secretion from adipocytes by a cAMP/p38-dependent pathway in vitro. 

(a) *Il6* expression in 3T3-L1 adipocytes after amlexanox treatment (n = 3 wells per treatment per time point). 
(b) IL-6 secretion from 3T3-L1 adipocytes after amlexanox treatment (n = 3 wells per treatment per time point). 
(c) cAMP in 3T3-L1 adipocytes treated with amlexanox for 5, 15, 30, 60 and 120 min (n = 3 wells per treatment per time point). 
(d) Immunoblot analysis of Stat3, TBK1 and p38 phosphorylation in 3T3-L1 adipocytes treated with amlexanox for indicated times. RalA serves as loading control. 
(e) *Il6* expression in 3T3-L1 adipocytes after amlexanox treatment with and without pre-treatment with SB203,580 (n = 3 wells per treatment per time point). 
(f) IL-6 secretion from 3T3-L1 adipocytes after amlexanox treatment with and without pre-treatment with SB203,580 (n = 3 wells per treatment per time point). 

* indicates P value < 0.05 (student’s t-test a–c or two-way ANOVA plus Tukey-Krammer post-hoc analysis e,f) amlexanox treated versus vehicle control. 
# indicates P value < 0.05 (two-way ANOVA plus Tukey-Krammer post-hoc analysis) amlexanox, SB203,580 treated versus amlexanox only. Error bars presented as s.e.m.

### Long-term benefits could be mediated by reduced inflammation.

The exact role of IL-6-mediated suppression of gluconeogenesis in the long-term insulin-sensitizing effects of amlexanox remains to be fully elucidated. IL-6 signalling has previously been shown to have anti-inflammatory effects in liver as well as inflammatory cells. Therefore, it is possible that IL-6 stimulates the resolution of chronic low-grade inflammation, as seen with long-term amlexanox treatment. Interestingly, serum IL-10 levels increase after both short and long-term amlexanox treatment. Potential relevance to human disease. To explore whether a similar crosstalk occurs in humans, we investigated the relationship between circulating IL-6 levels and measures of insulin sensitivity in a small cohort of obese-diabetic patients treated with amlexanox in a 12-week open-label clinical trial. In the mice, the most robust effects on IL-6 signalling over the first 24 h of drug treatment. However, the earliest serum withdrawal in this clinical trial was 1 week after initiation of treatment. In addition, differences in the dosage of amlexanox in this clinical trial further obfuscate direct comparison with the IL-6 response in the animal studies. Nevertheless, a relatively small but statistically significant increase in serum IL-6 levels was observed after the sixth week of treatment, in these patients (Fig. 10a). Increased serum IL-6 levels were also observed during the first 2 weeks of treatment in four of the six patients (Fig. 10b). The relative increase in serum IL-6 in response to amlexanox treatment varied from patient to patient, with some patients showing a more pronounced response than others, even after baseline normalization (Fig. 10c).

Although fasting blood glucose levels and the homeostasis model assessment-estimated insulin resistance (HOMA-IR) score tended to be lower after amlexanox treatment, these results did not reach statistical significance in this small cohort (Supplementary Fig. 11). Hypothesizing that the insulin-sensitizing effect of amlexanox treatment in humans is associated with the relative IL-6 response, we performed a linear regression analysis to determine whether the IL-6 response correlated with improvements in the HOMA-IR score, as a measure of insulin sensitivity and amlexanox treatment (Fig. 9e).
sensitivity\textsuperscript{26–28}. To quantify the IL-6 responsiveness for each patient, we calculated the area under the curve for serum IL-6 levels after normalization to baseline for each patient. We calculated the HOMA-IR score at the end of the 12-week study and correlate with insulin resistance\textsuperscript{30–33}, and reports suggest that the cytokine might contribute to insulin resistance through induction of Socs3 in hepatocytes\textsuperscript{34,35}. However, other studies have suggested a beneficial effect of the cytokine\textsuperscript{36–38}. Differences in the chronic versus acute metabolic effects of IL-6 may explain why contradictory results have been observed in genetic mouse models of disrupted IL-6 signalling. It is acutely secreted from skeletal muscle in response to exercise, and studies have indicated that it is produced from brown or white fat depots in response to

**Discussion**

The role of IL-6 in regulation of glucose homoeostasis has been controversial\textsuperscript{29}. IL-6 levels are chronically increased in obesity and correlate with insulin resistance\textsuperscript{30–33}, and reports suggest that the cytokine might contribute to insulin resistance through induction of Socs3 in hepatocytes\textsuperscript{34,35}. However, other studies have suggested a beneficial effect of the cytokine\textsuperscript{36–38}. Differences in the chronic versus acute metabolic effects of IL-6 may explain why contradictory results have been observed in genetic mouse models of disrupted IL-6 signalling. It is acutely secreted from skeletal muscle in response to exercise, and studies have indicated that it is produced from brown or white fat depots in response to

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**Figure 5 | Knockdown of p38 blocks amlexanox-stimulated IL6 expression in adipocytes.** Validated stealth RNAi to Mapk14 (1320001) and control (12935300) from Life Technologies was electroporated into 3T3-L1 adipocytes on day 1 of 10% FBS only media, and then 4 days after electroporation, vehicle and amlexanox treatments were administered after a 3-h serum starve. (a) Immunoblots for total and phosphorylated p38 protein. (b) quantification of immunoblots in a. (c) Further validation of p38 knockdown using Q-PCR to measure Mapk14 mRNA levels. (d) mRNA levels of Il6 after 4 h of amlexanox treatment in control and p38 knockdown cells (n = 3 wells per condition). * indicates P value <0.05 p38 KD versus control, # indicates P value <0.05 amlexanox versus vehicle (two-way ANOVA plus Tukey-Krammer post-hoc analysis). Error bars represent s.e.m.

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**Figure 6 | IL-6 secretion from adipocytes by a cAMP/p38-dependent pathway in vivo.** (a) cAMP levels in adipose tissue 4 h after amlexanox treatment in vivo (n = 8 mice per treatment). (b) Serum IL-6 levels 4 h after amlexanox treatment following SB203,580 pre-treatment (n = 6 amlexanox-treated and 5 vehicle control mice per SB203,580 dose). (c) Il6 expression and (d) IL-6 precursor protein levels in inguinal adipose tissue 4 h after amlexanox treatment following SB203,580 pre-treatment, quantification in left panel (n = 6 mice per treatment). (e) quantification of immunoblots in d. * indicates P value <0.05 (student’s t-test a or two-way ANOVA plus Tukey-Krammer post-hoc analysis b-e) amlexanox treated versus vehicle control. # indicates P value <0.05 (two-way ANOVA plus Tukey-Krammer post-hoc analysis) amlexanox, SB203,580 treated versus amlexanox only. Error bars represent s.e.m.
sympathetic activation. Adding further complexity to the metabolic effects of IL-6, our results indicate that IL-6 may also represent a crucial player in a subcutaneous adipose tissue–liver axis.

Taken together, these data indicate a new, previously unappreciated crosstalk between subcutaneous adipose tissue and liver in the control of glucose homoeostasis. These findings suggest that activation of the inflammatory programme in obesity induces expression of IKK-ε/TBK1 in both adipocytes and preadipocytes of subcutaneous fat, initiating a process of catecholamine resistance that reduces energy expenditure, but also suppresses the catecholamine-induced increase in adipose IL-6 expression, thereby contributing to elevated hepatic glucose production in obesity. Acute inhibition of IKK-ε/TBK1 by amlexanox reverses this catecholamine resistance, raising cAMP levels in subcutaneous adipose tissue, and in the process dramatically increasing IL-6 expression and subsequent activation of Stat3 in the liver to suppress gluconeogenesis. These studies reinforce the notion of IKK-ε/TBK1 inhibition as a promising
therapeutic strategy for the treatment of insulin resistance in obese patients.

**Methods**

**Reagents.** All chemicals were obtained from Sigma-Aldrich unless otherwise stated. TBK1 (3013), phosphorilated TBK1 (Ser172-Ser181) diluted 1:500, Stat3 (9139), phosphorilated Stat3 (Tyr705-9131), p38 (9212), phosphorilated p38 (Thr180/Tyr182-9211), tubulin (2128)-specific antibodies were purchased from Cell Signalling. HDAC1 (H-51) and Stat3 (C-20)-specific antibodies were purchased from Santa Cruz. RalA (61022)-specific antibody was obtained from BD Bioscience and used at a dilution of 1:20,000. IL-6 antibody was obtained from Abcam (ab6672). All antibodies were diluted 1:1,000 unless otherwise noted. EDTA-free protease inhibitor tablet was purchased from Roche Diagnostics. For *in vitro* experiments SB203,580 (1 mg ml⁻¹) was purchased from Calbiochem-EMD. For *in vivo* experiments SB203,580 powder was purchased from Selleck Chemicals.

**Animals.** We fed wild-type male C57Bl/6 mice a high fat diet consisting of 45% of calories from fat (D12451 Research Diets Inc.) starting at 8 weeks of age. Amlexanox was administered by daily oral gavage at a dose of 25 mg kg⁻¹. SB203,580 was administered by oral gavage at the indicated doses 30–60 min prior to amlexanox administration. Mice were preconditioned to the oral gavage for a minimum of 1-week daily gavage with an equivalent volume of saline. Neutralizing antibodies (R&D AB-406-NA and AB-108-C) were administered by intraperitoneal injection just prior to oral gavage. During metabolic studies, such as pyruvate tolerance tests, ear tag numbers were used to identify individual animals and the researcher performing the test was blinded to the treatment group assignment during data collection. Cohorts of 20–44 mice were used in up to 20 in-house breeding cages, and constructed to minimize the birthdate range. Mice were assigned to treatment groups, such that prior to treatment the mean body weight, as well as the s.d. of the body weight, was equal across treatment groups, using a method similar to block randomization. Additional consideration was given to housing, such that each cage contained multiple treatment groups, to avoid confounding by cage effects. Sample size was determined based on the available cohorts and the number of treatment groups required. Mice were housed in a specific pathogen-free facility with a 12-h light, 12-h dark cycle and given free access to food and water, except when food was restricted during fasting. All animal use was in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Pyruvate tolerance test.** Fasting blood glucose levels from tail blood were measured after a 16-h fast, using the OneTouch Ultra glucometer (Lifescan). Mice were then given an intraperitoneal injection of sodium pyruvate (P2256) at a dose of 1.5 g kg⁻¹ body weight. Blood glucose levels were measured again at 15, 30, 45, 60, 90 and 120 min post injection.

**3T3-L1 differentiation.** 3T3-L1 fibroblasts (American Type Culture Collection) were cultured in DMEM containing 10% FBS. Once grown to confluence, adipocyte differentiation was initiated using a three component cocktail containing insulin and finally differentiation was completed in the culture media. Only cultures in which >90% of cells displayed adipocyte morphology were used. Fully differentiated adipocytes, which had been cultured in the FBS only media for 1 week, were serum starved for 3 h in media containing 0.5% FBS, then treated with 100 μM amlexanox or vehicle control. Where applicable, cells were pretreated for 30 min with 5 mM SB203,580 or vehicle control.

**IL-6 measurement.** Mouse IL-6 levels were quantified using Mouse IL-6 Quantikine ELISA from R&D (DSMAB90B), with 50 μl of serum or cell culture media. Human IL-6 serum levels were quantified using the Human IL-6 Quantikine ELISA Kit (D6050) with 100 μl serum.

**cAMP measurement in cells and tissues.** Tissue cAMP levels were determined using the cAMP enzyme immunoassay kit (Sigma CA200) according to the manufacturer’s instructions. Cellular cAMP levels were determined using the cAMP enzyme immunoassay kit (Sigma CA200) according to the manufacturer’s instructions.

**Western blot analysis.** We homogenized tissues in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 1 mM Na3VO4, 5 mM NaF, 1 mM phenylmethanesulfonylfluoride, 25 mM glycerol 2-phosphate and freshly added protease inhibitor tablet) and then incubated them for 1 h at 4°C. Cell lysates were produced in an SDS lysis buffer (100 mM Tris pH 7.5, 130 mM NaCl, 1% NP-40, 0.1% SDS, 0.2% sodium deoxycholate, 1 mM Na3VO4, 1 mM NaF, 100 mM Na2VO3, 1 mM
phenylmethanesulfonylfluoride, 25 mM glycerol 2-phosphate and freshly added protease inhibitor tablet) and sonicated for three 5 s pulses at an output power of 6.

We centrifuged crude lysates at 14,000 g for 15 min twice and determined the protein concentration using Bio-Rad Protein Assay Dye 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 specific antibodies and visualized on film using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and Western Lightning Enhanced Chemiluminescence (Perkin Elmer Life Sciences).

**SVC isolation and fluorescence-activated cell sorting.** The stromal vascular fraction was separated from adipocytes by centrifugation after digestion. Excised white adipose tissue was digested in RPMI with 0.5% BSA and 1 mg ml⁻¹ type II

**Figure 9 | Amlexanox reduces hepatic glucose production in an IL-6-dependent manner.** Pyruvate tolerance test in mice pretreated with either IL-6 neutralizing antibody (NA) or serotype-matched IgG control antibody (n = 6 mice per treatment) before either (a) amlexanox or (b) vehicle control treatment. Hepatic expression of (c) G6pc and (d) Socs3 in amlexanox-treated and vehicle control mice after pretreatment with either IL-6 NA or serotype-matched IgG control antibody (n = 6 mice per treatment). (h) Serum IL-10 levels 4 h after amlexanox or vehicle control treatment. * indicates P value < 0.05 (student’s t-test a,b,e and ANOVA plus Tukey–Krammer post-hoc analysis c,d) IL-6 neutralizing antibody versus IgG control and amlexanox treated versus vehicle control (e and as indicated in c,d) or error bars represent s.e.m.

**Figure 10 | Improved HOMA-IR correlates with IL-6 response in human subjects treated with amlexanox.** (a) Average serum IL-6 levels in human subjects over the course of 12 weeks of amlexanox treatment (n = 6). * indicates P value < 0.05 (paired t-test) indicated time point versus baseline. Error bars represent s.e.m. (b) Individual serum IL-6 levels in human subjects over the course of 12 weeks of amlexanox treatment (n = 6). (c) Individual baseline normalized IL-6 levels over the course of 12 weeks of amlexanox treatment (n = 6). (d) Linear regression of change in HOMA-IR score with baseline normalized IL-6 area under curve (AUC). Y-axis: % basal HOMA-IR = (HOMA-IR after 12 weeks of treatment)/HOMA-IR at baseline) × 100%. X-axis: AUC (% basal serum IL-6 over the 12 weeks of treatment—such that a value of 1,200 indicates no response and a value of 1,800 corresponds with an average 1.5-fold increase in serum IL-6 levels over the 12 weeks of treatment).
collagenase for 30 min at 37 °C with gentle agitation. Then the cell suspension was filtered through a 100 μm filter and centrifuged at 700g for 5 min to separate floating adipocytes from SVEC pellet, and RPMI with 0.05% BSA was used to wash cells. The following antibodies were used for staining: anti-CD45 (30-F11), anti-CD31 (390), anti-CD11c (N418) and anti-Sca-1 (1D7), all from eBioscience. Sided SVC were sorted by flow cytometry on a MoFlo Astrios cell sorter (Beckman Coulter) with the help of the University of Michigan’s Flow Cytometry Core.

**Gene expression analysis.** RNA extractions from tissues were performed using the NReasy Lipid Tissue Kit (Qiagen) while RNA extractions from cells utilized the NReasy Kit. We used the SuperScript First-Strand Synthesis System for reverse transcription–PCR (Invitrogen) with a 3:1 mixture of random hexamers/oligo dT primer for reverse transcription. Real-time PCR amplification 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 and quantified using an internal standard curve and Arb as the control gene. The sequences of all primers used in this study are listed in Supplementary Table 2.

**RNA sequencing.** Total RNA from mouse livers was isolated using Trizol (Invitrogen) and the NReasy mini kit according to the manufacturer’s instructions. Biological triplicates of ameloxan-treated and non-treated tissues were used to prepare sequencing libraries from 100–500 ng total RNA using the TrueSeq RNA Sample Preparation Kit v2 (Illumina) according to the manufacturer’s protocol. Sample preparation. Briefly, mRNA was purified, fragmented and used for first- and second-strand complementary DNA synthesis followed by adenylation of 3’ ends. Samples were ligated to unique adapters and subjected to PCR amplification. Libraries were then validated using the 2100 BioAnalyzer (Agilent), normalized and pooled. Sequencing on the Illumina HiSeq 2000 using bar-coded multiplexing and a 100 bp read length.

**Data analysis.** Read alignment and junction mapping was accomplished using TopHat2 v2.0.4 using a 25 bp 5’ segment seed for initial mapping followed by differential gene expression analysis using Cuffdiff v2.0.2 to map reads to the reference annotation (ref. 43). Mean normalization of sequencing read yield per replicate sample was 24.2 M. Data were expressed as fragments per kilobase of exon per million fragments mapped. Volcano plots were generated from Cuffdiff output using CummeRbund v2.0.0 (ref. 43).

**RNA-seq library construction.** Single-cell complementary DNA size distribution and contamination were assessed on a capillary electrophoresis-based Fragmentanalyser (Advanced Analytical). Illumina libraries were constructed in 96-well plates using the Illumina Nextera XT DNA Sample Preparation kit as described previously using the protocol supplied by Fluidigm. For each C1 experiment, a bulk RNA control (about 200 cells) and a no-cell negative control were processed in parallel in PCR tubes, using the same reagent mixes as used on chip. Libraries were quantified by Bioanalyzer, using High Sensitivity DNA analysis kit, and also fluorometrically using Qubit dsDNA HS Assay kits and a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies).

**DNA sequencing.** Single-cell Nextera XT (Illumina) libraries of one experiment were pooled and 100 bp paired-end were sequenced on Illumina HiSeq 2000 to a depth of (2–6) × 106 reads (three replicate experiments of distal mouse lung epithelial cells at E18.5, one experiment at E14.5 and one experiment on adult A2T2 cells) or 150 bp paired-end on Illumina MiSeq (one experiment at E16.5) to a depth of 100,000–550,000 reads with v3 chemistry. CASAVA 1.8.2 was used to separate the data for each single cell by using unique barcode combinations from the Nextera XT preparation and to generate .fastq files.

**KEGG pathway analysis.** Normalized relative fold expression levels from RNA-Seq were calculated for selected genes and differentially expressed genes found in Supplementary Table 3.

**Transcription factor DNA binding assays.** Nuclear extracts from liver were prepared using a nuclear extraction kit (Active Motif 40010). Ten nanograms of nuclear extracts were incubated with 100 fmol of specific transcription factor DNA binding activity using TransAM kits (Active Motif 45196, 46396 and 42092). These kits utilize response element DNA affixed to the plate and specific primary antibodies to quantify the relative DNA binding activity of specific transcription factors.

**Clinical trial.** Six obese type-2 diabetic patients (body mass index range 31 to 39 kg m–2), two males and four females, ages 24 to 68 years were enrolled in clinical trial NCT01842282. After patients gave informed consent, they were instructed to take ameloxan tablets (Takeda) in an open-label manner at a dosage of 25 mg three times daily. After 2 weeks, the dosage was increased to 50 mg tablets three times daily. Blood collection was performed at baseline and 1, 2, 4, 6 and 12 weeks of treatment, serum samples were frozen at −80 °C for future combined analysis. Patients were provided instructions for a standardized meal (600 kcal, 55% carbohydrates, 30% fat and 15% protein) and then instructed to fast overnight after 2200 hours prior to the baseline and 12-week visits, such that HOMA-IR could be calculated from measurements of fasted blood glucose and serum insulin. The study was reviewed and approved by the University of Michigan Medical School Institutional Review Board (IRBMED; HUM00065177).

**Statistics.** Averaged values are presented as the mean ± s.e.m. Equality of variance was determined using an F-test. Data were determined to be normally distributed as the maximum and minimum values in each data set were < 3 s.d. from the mean. When testing the two groups derived from separate experiments, the F-test was used. When more than two groups were investigated, we first performed an analysis of variance (ANOVA) to establish that not all groups were equal. Following a statistically significant F-test, group comparisons were performed between the Tukey–Krammer post-hoc analysis. Linear regression was used to determine correlation significance, and displayed as the best fit line with a 95% confidence band. Linear regression, ANOVA and Tukey–Krammer tests were performed using Prism version 6.0 (ref. 46).

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Author contributions

A.R.S. and S.M.R. wrote the manuscript. S.M.R. created figures. S.M.R. performed experiments in Figures 1a–c; 3a–d; 4; 5; 6; 7a,b,e–j; 8–i; 9; 10 and Supplementary Figs 1–6. A.R.S. and S.M.R. performed experiments in Figures 7c,d; 8a–d and Supplementary Figs 7, B.F.Z. and C.N.L. performed experiments in Figure 2d–i. L.C. and R.M.E. performed experiments in Fig. 3e–j. M.U. performed experiments in Figure 3a,b. B.P. performed SVC isolation, RNA isolation and Q-PCR, as well as editing. X.P. maintained the clinical trial and provided samples to S.M.R. for IL-6 measurement and correlation analysis in Fig. 10.

Additional information

Accession codes: The RNA sequencing data have been deposited in NCBI’s Gene Expression Omnibus under accession code GSE57054.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: Alan Saltiel is a named inventor on patents pertaining to the therapeutic use of ameloxan. The other authors declare no competing financial interests.

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