RESEARCH ARTICLE

The lysyl oxidase inhibitor β-aminopropionitrile reduces body weight gain and improves the metabolic profile in diet-induced obesity in rats

María Miana1,2,*, María Galán3,*, Ernesto Martínez-Martínez1,2,4, Saray Varona3, Raquel Jurado-López1,2, Belén Bausa-Miranda1,2, Alfonso Antequera5, María Luaces6, José Martínez-González3, Cristina Rodríguez3,*,‡ and Victoria Cachofeiro1,2,‡

ABSTRACT

Extracellular matrix (ECM) remodelling of the adipose tissue plays a pivotal role in the pathophysiology of obesity. The lysyl oxidase (LOX) family of amine oxidases, including LOX and LOX-like (LOXL) isoenzymes, controls ECM maturation, and upregulation of LOX activity is essential in fibrosis; however, its involvement in adipose tissue dysfunction in obesity is unclear. In this study, we observed that LOX is the main isoenzyme expressed in human adipose tissue and that its expression is strongly upregulated in samples from obese individuals that had been referred to bariatric surgery. LOX expression was also induced in the adipose tissue from male Wistar rats fed a high-fat diet (HFD). Interestingly, treatment with β-aminopropionitrile (BAPN), a specific and irreversible inhibitor of LOX activity, attenuated the increase in body weight and fat mass that was observed in obese animals and shifted adipocyte size toward smaller adipocytes. BAPN also ameliorated the increase in collagen content that was observed in adipose tissue from obese animals and improved several metabolic parameters – it ameliorated glucose and insulin levels, decreased homeostasis model assessment (HOMA) index and reduced plasma triglyceride levels. Furthermore, in white adipose tissue from obese animals, BAPN prevented the downregulation of adiponectin and glucose transporter 4 (GLUT4), as well as the increase in suppressor of cytokine signaling 3 (SOCS3) and dipeptidyl peptidase 4 (DPP4) levels, triggered by the HFD. Likewise, in the TNFα-induced insulin-resistant 3T3-L1 adipocyte model, BAPN prevented the downregulation of adiponectin and GLUT4 and the increase in SOCS3 levels, and consequently normalised insulin-stimulated glucose uptake. Therefore, our data provide evidence that LOX plays a pathologically relevant role in the metabolic dysfunction induced by obesity and emphasise the interest of novel pharmacological interventions that target adipose tissue fibrosis and LOX activity for the clinical management of this disease.

KEY WORDS: Lysyl oxidase, Extracellular matrix, Adipose tissue, Fibrosis, Obesity, Insulin resistance

INTRODUCTION

In recent years, there has been increasing evidence that the extracellular matrix (ECM) plays a key role in adipose tissue development and function, and fibrosis is now recognised as a crucial player in adipose tissue dysfunction in obesity. In fact, early deposition of connective tissue occurs in subcutaneous white adipose tissue after moderate weight gain and further increases with obesity (Mutch et al., 2009; Divoux et al., 2010; Henegar et al., 2008; Alligier et al., 2012). Interestingly, data from animal models of obesity suggest that this abnormal ECM remodelling of white adipose tissue could contribute to local and systemic metabolic alterations (Halberg et al., 2009; Khan et al., 2009).

Fibrosis arises from the imbalance between ECM synthesis and degradation that culminates in an excessive accumulation of ECM components. Lysyl oxidase (LOX) activity is crucially involved in ECM synthesis and processing. To date, five isoenzymes belonging to the LOX family have been identified, including LOX, LOX-like1 (LOXL1), LOXL2, LOXL3 and LOXL4. They are copper-dependent amine oxidases that exhibit a high degree of homology in the C-terminal domain, which contains the catalytic site. LOX, the archetypal member of this family and the best characterised, catalyses the covalent cross-linking of collagens and elastin fibres (Rodríguez et al., 2008a,b). This enzyme oxidises lysine and hydroxylysine residues in these substrates, leading to the synthesis of highly reactive peptidylsemialdehydes that can spontaneously condense to form the intra- and intermolecular covalent cross-linkages that are responsible for ECM stability. The disturbance of LOX activity induces connective tissue abnormalities related to pathological processes, including cardiovascular diseases, as we have previously described (Miana et al., 2011; Orriols et al., 2014; Raposo et al., 2004; Rodríguez et al., 2002, 2009; Stefanon et al., 2013).

Changes in LOX activity could be involved in the disturbance of adipocyte function in obesity. In fact, LOX expression is upregulated in the white adipose tissue from ob/ob mice (Halberg et al., 2009), and it is inhibited during adipocyte differentiation (Dimaculangan et al., 1994). Furthermore, LOX participates in the commitment of pluripotent stem cells to the adipocyte lineage (Huang et al., 2009). However, the potential role of LOX activity in human obesity has been poorly characterised. A transcriptomic study revealed an increase in LOX expression in subcutaneous white
TRANSITIONAL IMPACT

Clinical issue
Obesity is one of the most serious public health challenges of the 21st century. The risk of a number of major diseases, including type 2 diabetes mellitus, ischemic heart disease, ischemic stroke and several common forms of cancers, is dramatically increased in obese individuals. The epidemic proportions achieved by obesity makes it mandatory to reach a deeper understanding of its underlying pathophysiologial mechanisms, which could provide novel therapeutic targets. In recent years, fibrosis has been recognised as a crucial player in adipose tissue dysfunction in obesity. Lysyl oxidase (LOX) activity, which governs extracellular matrix maturation, is essential for tissue fibrosis. However, its contribution to adipose tissue dysfunction in obesity has not been clearly established.

Results
This study analyses the role of LOX in adipose tissue remodelling by using three experimental systems: adipose tissue samples from obese individuals that had been referred for bariatric surgery (weight loss surgery), an animal model of diet-induced obesity and cell-based studies. The authors demonstrate that LOX is the main lysyl oxidase isoenzyme expressed in human adipose tissue and that it is upregulated in samples from both obese individuals and rats fed a high-fat diet. In obese rats, the inhibition of LOX activity through β-aminopropionitrile (BAPN), a specific and irreversible inhibitor with obesity. For this purpose, we have evaluated the impact of adipose tissue fibrosis. However, its contribution to adipose tissue dysfunction in obesity has not been clearly established.

Implications and future directions
The results reported in this study demonstrate the upregulation of the adipose tissue expression of LOX in human obesity and provide evidence that LOX inhibition prevents adipose tissue dysfunction, reduces bodyweight gain and improves metabolic disturbances in diet-induced obesity. These findings uncover the pathologically relevant contribution of LOX to the metabolic dysfunction induced by obesity and emphasise the potential of novel pharmacological interventions targeting adipose tissue fibrosis and LOX activity for the clinical management of obesity. Future studies will help to further clarify the mechanisms underlying the beneficial consequences of LOX inhibition in obesity.

BAPN ameliorates the increase in body weight, adiposity and adipose tissue fibrosis in HFD-fed rats
To investigate whether LOX contributes to the adipose tissue dysfunction associated with obesity, rats subjected to a HFD were treated with BAPN, an irreversible and specific inhibitor of LOX activity. As shown in Fig. 3A, the HFD induced a significant increase in body weight that reached a significant difference from the third week onwards. After three weeks of treatment, BAPN significantly prevented the rise in body weight in HFD rats, but not in animals that were fed a standard diet (Fig. 3A). These differences were sustained until the end of the study (Table 1, Fig. 3A). Similarly, BAPN reduced the increase in the weight of white adipose tissue (both epididymal and lumbar) in obese animals (Table 1) and attenuated their enhanced adiposity (Table 1). It should be noted that the changes

Results
This study analyses the role of LOX in adipose tissue remodelling by using three experimental systems: adipose tissue samples from obese individuals that had been referred for bariatric surgery (weight loss surgery), an animal model of diet-induced obesity and cell-based studies. The authors demonstrate that LOX is the main lysyl oxidase isoenzyme expressed in human adipose tissue and that it is upregulated in samples from both obese individuals and rats fed a high-fat diet. In obese rats, the inhibition of LOX activity through β-aminopropionitrile (BAPN), a specific and irreversible inhibitor with obesity. For this purpose, we have evaluated the impact of adipose tissue fibrosis. However, its contribution to adipose tissue dysfunction in obesity has not been clearly established.

Implications and future directions
The results reported in this study demonstrate the upregulation of the adipose tissue expression of LOX in human obesity and provide evidence that LOX inhibition prevents adipose tissue dysfunction, reduces bodyweight gain and improves metabolic disturbances in diet-induced obesity. These findings uncover the pathologically relevant contribution of LOX to the metabolic dysfunction induced by obesity and emphasise the potential of novel pharmacological interventions targeting adipose tissue fibrosis and LOX activity for the clinical management of obesity. Future studies will help to further clarify the mechanisms underlying the beneficial consequences of LOX inhibition in obesity.

BAPN ameliorates the increase in body weight, adiposity and adipose tissue fibrosis in HFD-fed rats
To investigate whether LOX contributes to the adipose tissue dysfunction associated with obesity, rats subjected to a HFD were treated with BAPN, an irreversible and specific inhibitor of LOX activity. As shown in Fig. 3A, the HFD induced a significant increase in body weight that reached a significant difference from the third week onwards. After three weeks of treatment, BAPN significantly prevented the rise in body weight in HFD rats, but not in animals that were fed a standard diet (Fig. 3A). These differences were sustained until the end of the study (Table 1, Fig. 3A). Similarly, BAPN reduced the increase in the weight of white adipose tissue (both epididymal and lumbar) in obese animals (Table 1) and attenuated their enhanced adiposity (Table 1). It should be noted that the changes
in body weight triggered by BAPN in HFD-fed rats were not related to differences in food intake (Table 1).

The changes in adipose tissue mass elicited by BAPN in HFD-fed rats prompted us to determine whether LOX inhibition could modulate the adipocyte area. Histological analysis of epididymal adipose tissue revealed an increased adipocyte area in the HFD group. A trend towards an attenuation of this parameter was observed in obese animals that had been treated with BAPN (Fig. 3B,C). Furthermore, a shift toward smaller adipocytes was detected in BAPN-treated obese animals compared with HFD-fed rats (Fig. 3D). Interestingly, LOX inhibition prevented the increase in pericellular collagen content observed in obese rats, as analysed by using Picrosirius red staining (Fig. 3E,F).

**BAPN improves insulin signalling in adipose tissue from obese animals**

In order to understand how BAPN improves insulin sensitivity in obese animals, we analysed the levels of proteins involved in the control of insulin sensitivity in epididymal adipose tissue. The reduction in both glucose transporter 4 (GLUT4) and adiponectin expression observed in the HFD group was normalised through the inhibition of LOX activity (Fig. 4A,B). Furthermore, the increase in the protein levels of DPP4 and suppressor of cytokine signaling 3 (SOCS3) triggered by the HFD was completely prevented by BAPN (Fig. 4C,D).

**BAPN normalises the expression of GLUT4 and adiponectin, and improves glucose uptake in an in vitro model of insulin resistance**

To determine whether BAPN directly affects adipocyte function, we performed in vitro experiments in the TNFα-induced insulin resistance model in differentiated 3T3-L1 adipocytes. As shown in Fig. 5 and as previously described (Stephens et al., 1997; Sethi and Hotamisligil, 1999), TNFα reduced expression of GLUT4 and adiponectin and increased SOCS3 protein levels in these cells. Interestingly, these effects were prevented by BAPN (Fig. 5A-C). Accordingly, BAPN normalised the TNFα-induced decrease in
insulin-stimulated glucose uptake observed in differentiated 3T3-L1 adipocytes (Fig. 5D).

DISCUSSION
It is becoming apparent that fibrosis is an essential factor in adipose tissue dysfunction in obesity (Divoux et al., 2010; Halberg et al., 2009; Khan et al., 2009). ECM remodelling impacts on the metabolic function of adipose tissue, and the control of LOX activity, a major player in ECM maturation, could be crucial in this context. The research presented here reveals that LOX is the main lysyl oxidase isoenzyme expressed in adipose tissue and that its expression is upregulated in the adipose tissue of morbidly obese patients and obese animals. More interestingly, we observed that inhibition of LOX activity protects against diet-induced obesity, thereby limiting weight gain, attenuating the disturbances in adipose tissue and improving the insulin resistance observed in obese animals.

Increased expression of ECM components in adipose tissue has been repeatedly reported in murine models of obesity and in obese subjects associated with either body mass index (BMI), insulin sensitivity and/or resistance, and/or inflammation (Abdennour et al., 2014; Divoux et al., 2010; Halberg et al., 2009; Khan et al., 2009; Tam et al., 2012; Walker et al., 2014). A transcriptomic analysis of adipose tissue from obese individuals revealed the pathological relevance of ECM. However, the increase in the expression of LOX and other matrix components observed in these microarray studies was not subsequently validated using other techniques (Henegar et al., 2008). Accordingly, we found that LOX mRNA levels are increased in adipose tissue samples from obese individuals, which was confirmed by western blotting and immunohistochemistry. Furthermore, obese individuals exhibited a marked fibrosis of adipose tissue, a pathological process that reduces tissue plasticity and alters adipocyte function (Khan et al., 2009; Virtue and Vidal-Puig, 2010). In agreement with previous reports in the ob/ob model (Halberg et al., 2009), we also observed an upregulation of LOX in the adipose tissue of rats fed a HFD that was associated with enhanced pericellular fibrosis. The strong correlation of LOX expression with pericellular fibrosis in the adipose tissue from both humans and rats, as well as the ability of BAPN to limit pericellular fibrosis in HFD-fed animals support the notion that LOX makes a relevant contribution to the fibrotic response in obesity.

LOX and LOXL isoenzymes differ from other copper amino oxidases in their quinone cofactor, the lysine tyrosylquinone (LTQ), which confers particular catalytic properties to these enzymes (Rodriguez et al., 2008b). BAPN is a well-known specific and irreversible inhibitor of LOX activity (Tang et al., 1983; Rodriguez et al., 2008b) that targets the active site of LOX or LOXL isoenzymes. BAPN is a useful tool to study cellular processes involving LOX and LOXL isoenzymes; however, its pharmacological use as a systemic drug is not indicated. It has been observed that prolonged administration of BAPN evokes a weak neurotoxicity and reduces the mechanical strength of bones (Ahsan et al., 1999; Spencer and Schaumburg, 1983; Turecek et al., 2008). Further, lathyrism, a disease characterised by extensive disruption of connective tissue, is caused by the excessive and long-term consumption of certain legumes of the genus Lathyrus, which contain high amounts of BAPN and related compounds (Dasler, 1954). Nevertheless, owing to the lack of viable LOX-deficient mice (Maki et al., 2002; Hornstra et al., 2003), this inhibitor has been extensively used in short-term in vivo studies to support the involvement of lysyl oxidases in multiple biological processes, including tumour progression and metastasis (Levental et al., 2009; Erler et al., 2006), pulmonary arterial hypertension (Nave et al., 2014) and arterial stiffening (Kothapalli et al., 2012). Similarly, we have observed that BAPN prevents the increase in both body weight and fat-pad weight found in obese animals without affecting food intake. These changes were accompanied by a shift toward smaller adipocytes. Likewise, in mice of an ob/ob background, the knockdown of ECM components, such as collagen VI, limited the gain of body weight and reduced the increase in fat mass (Khan
Therefore, the composition and structure of ECM seems to modulate the metabolism of fat tissue in different animal models.

Dysfunction of adipose tissue metabolism has a negative impact on lipid and glucose homeostasis and plays a crucial role in the development of insulin resistance in obesity (Goossens, 2008; Guilherme et al., 2008; Trayhurn, 2013). As in obese individuals, HFD-fed animals also exhibited a decline in insulin sensitivity, as indicated by an increase in HOMA index. This was associated with altered expression of several mediators involved in the control of metabolic functions in the adipose tissue, including adiponectin, GLUT4, SOCS3 and DPP4, as previously described in both humans and animal models (Emanuelli et al., 2001; Kadowaki et al., 2006; Kanatani et al., 2007; Kern et al., 2003; Lamers et al., 2011; Palanivel et al., 2012; Sell et al., 2013; Shepherd and Kahn, 1999; Wang et al., 2000), and with a significant hypertriglyceridemia, a sign frequently observed in the setting of obesity linked to insulin resistance (Eckel, 2011; Taskinen et al., 2011).

More interestingly, we have observed that inhibition of LOX activity attenuates insulin resistance in obese animals. In fact, BAPN reduced insulin and glucose levels and consequently HOMA index, prevented the upregulation of plasma triglycerides and ameliorated the changes in protein expression observed in the adipose tissue from HFD rats. Inhibition of LOX activity reversed the decrease in GLUT4 and adiponectin levels, and the increase in both SOCS3 and DPP4 expression. In view of the low expression of LOXL isoenzymes in human adipose tissue, these data suggest that the upregulation of LOX observed in obesity has multiple consequences for the physiology of adipose tissue. This functional repercussion of LOX upregulation in systemic and adipose tissue metabolism has been suggested previously in a transgenic mouse model that specifically overexpresses HIF-1α in the adipose tissue.

Fig. 3. Effects of lysyl oxidase inhibition on body weight evolution, adipocyte size and distribution and total collagen content in epididymal adipose tissue from control and obese rats. (A) Body weight evolution along the study. (B) Adipocyte area of epididymal adipose tissue analysed in haematoxylin-eosin-stained sections from control rats fed a normal chow (CT) and rats fed a high-fat diet (HFD) treated with vehicle or with the inhibitor of LOX activity (β-aminopropionitrile, BAPN, 100 mg/kg/day). (C) Representative microphotographs of sections stained with haematoxylin and eosin and then examined by using light microscopy (magnification 40×). (D) Size distribution of adipocytes from epididymal adipose tissue of the animals described in B. (E) Quantification of collagen volume fraction (CVF) normalised for the number of adipocytes analysed in epididymal adipose tissue sections stained with Picrosirius Red. (F) Representative microphotographs of sections stained with Picrosirius Red that were examined by using light microscopy (magnification 40×). Scale bars: 50 µm. Values are mean±s.d. of six to eight animals. *P<0.05, **P<0.01, ***P<0.001 vs control group. †P<0.05, ††P<0.01 vs HFD group.
Table 1. Effect of the inhibition of lysyl oxidase activity with β-aminopropionitrile (BAPN; 100 mg/kg/day) on general characteristics and metabolic parameters in rats fed a normal chow (CT) and rats fed a high-fat diet (HFD)

<table>
<thead>
<tr>
<th></th>
<th>CT</th>
<th>BAPN</th>
<th>HFD</th>
<th>HFD+BAPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>320±24</td>
<td>304±15</td>
<td>374±11</td>
<td>332±24</td>
</tr>
<tr>
<td>Fl (g/day)</td>
<td>20±5</td>
<td>23±2</td>
<td>13±3</td>
<td>14±2</td>
</tr>
<tr>
<td>EAT (mg/cm/tibia)</td>
<td>0.09±0.03</td>
<td>0.07±0.03</td>
<td>0.25±0.05*</td>
<td>0.19±0.04**</td>
</tr>
<tr>
<td>LAT (mg/cm/tibia)</td>
<td>0.11±0.03</td>
<td>0.09±0.03</td>
<td>0.3±0.05***</td>
<td>0.23±0.05***</td>
</tr>
<tr>
<td>Adiposity (%)</td>
<td>2.3±0.8</td>
<td>2.4±0.8</td>
<td>6.1±1.3**</td>
<td>4.4±1.0*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>97±10</td>
<td>91±9</td>
<td>115±6*</td>
<td>97±8</td>
</tr>
<tr>
<td>Insulin (pg/ml)</td>
<td>64±20</td>
<td>50±13</td>
<td>184±91</td>
<td>79±39</td>
</tr>
<tr>
<td>HOMA index</td>
<td>2.1±0.6</td>
<td>1.6±0.5</td>
<td>7.6±3.8*</td>
<td>2.4±1.1†</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>50±11</td>
<td>52±7</td>
<td>71±7*</td>
<td>51±10†</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>55±7</td>
<td>55±11</td>
<td>61±7</td>
<td>53±6</td>
</tr>
</tbody>
</table>

**BW, body weight; EAT, epididymal adipose tissue; Fl, food intake; HOMA index, the homeostasis model assessment; LAT, lumbar adipose tissue; TG, triglycerides; TC, total cholesterol. Data values represent mean±s.d. of eight animals. *P<0.05, **P<0.01, ***P<0.001 compared with control group.†P<0.05, ††P<0.01 compared with HFD group.**

...cellular functions, including the control of epithelial-to-mesenchymal transition, cell proliferation, migration, adhesion and transformation (Huang et al., 2009). The molecular mechanisms underlying the consequences of LOX inhibition in local and systemic metabolism are unknown and, in view of the complex biology of this enzyme, further research is warranted.

In summary, this study demonstrates that LOX could play a relevant role in the metabolic dysfunction induced by obesity and suggests that the inhibition of LOX activity could be a valuable strategy to ameliorate obesity-related metabolic disturbances. These aspects highlight the benefits as well as the complexity of targeting fibrosis as a potential therapeutic intervention in obesity.

**MATERIALS AND METHODS**

**Subjects**

Visceral adipose tissue was obtained from five individuals that had been referred for bariatric surgery (all women). Inclusion criteria were: age≥18 years and universally accepted indications: long-term obesity (more than 4 years); BMI≥40 despite other weight loss strategies or BMI≥35 in the presence of obesity-related comorbidities (diabetes mellitus, obesity hypoventilation syndrome, obstructive sleep apnoea syndrome and hypertension). Exclusion criteria were: age>60 years and unacceptable surgical risk. Visceral adipose tissue was also obtained from five normoweight BMI≤25 individuals that had been referred to non-bariatric surgery who were included as controls (40% women). The BMI was calculated according to the formula: weight (kg)/square of the height (metres). In accordance with institutional guidelines, all individuals gave informed consent. The study was performed in accordance with the Declaration of Helsinki, and the Ethical Committee of the Fuenlabrada University Hospital approved the protocol.

**Animals**

Male Wistar rats of 150 g (Harlan Ibérica, Barcelona, Spain) were fed either a HFD (33.5% fat; Harlan Teklad no. TD.03307, Haslett, MI, USA; n=16) or a standard diet (3.5% fat; Harlan Teklad no. TD.2014, Haslett, MI, USA; n=16) for 6 weeks. Half of the animals of each group received the irreversible inhibitor of LOX activity BAPN (100 mg/kg/day; Sigma-
TaqMan™ (Applied Biosystems, Thermo Fisher Scientific Inc, Waltham, MA, USA) and the following primers were reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific Inc, Waltham, MA, USA). Quantification of mRNA levels was performed by real-time PCR using the TaqMan™ (Applied Biosystems, Thermo Fisher Scientific Inc, Waltham, MA, USA) for rat (Rn00566984_m1; RefSeq: NM_00173746_m1; RefSeq: NM_005576.2), human LOXL2 (Hs00184700_m1; RefSeq: NM_002317.5), human LOX (Hs00184700_m1; RefSeq: NM_002317.5). The amount of BAPN was measured using real-time PCR with SYBR Green (www.Biemers.net, Donau, Germany): 5′-GTCCTTCAGCAACAACAAAC-3′ and 5′-GGGTACTTGTCAGGCAAGT-3′ (positions 2331 to 2434 corresponding to RefSeq: NM_022536.2; amplicon length: 101).

**Morphological and histological evaluation**

Vascular and epididymal adipose tissue samples were dehydrated, embedded in paraffin and cut into 3-µm-thick sections. Sections were stained with Picrosirius Red in order to detect collagen fibres. The area of pericellular fibrosis was identified as the ratio of collagen deposition to the total tissue area after excluding the vessel area from the region of interest. This value was normalised by the number of adipocytes. Adipocytes (80-100 per subject or animal) with intact cellular membranes were chosen for determination of the fibrosis. Cross-sectional area in hematoxylin-eosin stained sections. For each sample, 10-15 fields were analysed using a 40× objective (Leica DM 2000; Leica Camera AG, Wetzlar, Germany) and quantified (Leica Q550 IWB; Leica Camera AG, Wetzlar, Germany).

**Real-time PCR**

Total RNA was isolated using Ultraspec™ (Biotecx, Houston, TX, USA) and was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific Inc, Waltham, MA, USA). Quantification of mRNA levels was performed by using real-time PCR and an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Thermo Fisher Scientific Inc, Waltham, MA, USA) and TaqMan™ gene expression assays-on-demand (Applied Biosystems, Thermo Fisher Scientific Inc, Waltham, MA, USA) for rat (Rat00566984_m1; RefSeq: NM_017061.2) and human LOX (Hs00184700_m1; RefSeq:NM_002317.5), human LOXL1 (Hs00173746_m1; RefSeq: NM_005576.2), human LOXL2 (Hs00158757_m1; RefSeq: NM_002318.2), human LOXL3 (Hs00261671_m1; RefSeq: NM_032603.2) and human LOXL4 (Hs00260059_m1; RefSeq: NM_00260059.1). Rat BMP1 mRNA was determined by using real-time PCR with SYBR Green PCR Master mix (Applied Biosystems, Thermo Fisher Scientific Inc, Waltham, MA, USA) and the following primers (www.Biemers.net, Donau, Germany): 5′-GTCCTTCAGCAACAACAAAC-3′ and 5′-GGGTACTTGTCAGGCAAGT-3′ (positions 2331 to 2434 corresponding to RefSeq: NM_022536.2; amplicon length: 101).

**ΔΔCt method**

In order to appropriately apply this method, we checked that the target and the reference genes were amplified with comparable efficiencies and that the expression of β-actin was expressed as mean±s.d. of the counts of [3H]2-deoxyglucose normalised to the amount of total protein. Values are mean±s.d. of four assays. *P<0.05, **P<0.01 vs unstimulated cells (TNFα=0); †P<0.05, ††P<0.01 vs cells stimulated with the same concentration of TNFα in the absence of BAPN.

**Fig. 5. LOX inhibition influences glucose uptake and the expression of factors involved in the control of insulin sensitivity in an in vitro model of insulin resistance.** Protein levels of (A) glucose transporter type 4 (GLUT4), (B) adiponectin (Adipo) and (C) suppressor of cytokine signaling 3 (SOCS3). 3T3-L1 adipocytes were stimulated with or without TNFα (1.15 and 2.87 nM, equivalent to 20 and 50 ng/ml, respectively) for 72 h in the presence of either vehicle or β-aminopropionitrile, an inhibitor of LOX activity (BAPN, 200 µM). Data that were normalised to the expression of β-actin are expressed as mean±s.d. of four assays in arbitrary units. (D) Insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Bars on graphs represent the mean±s.d. of the counts of [3H]2-deoxyglucose normalised to the amount of total protein. Values are mean±s.d. of four assays. *P<0.05, **P<0.01 vs unstimulated cells (TNFα=0); †P<0.05, ††P<0.01 vs cells stimulated with the same concentration of TNFα in the absence of BAPN.
Immunohistological evaluation
Sections were deparaffinised in xylene and rehydrated in graded ethanol solutions. After blocking, slides were incubated with antibodies against LOX (Abcam, Cambridge, UK). Afterwards, samples were incubated with a biotinylated secondary antibody (Vector Laboratories Inc, Burlingame, CA, USA), washed and treated with the Vectastain (ABC) avidin-biotin peroxidase complex (Vector Laboratories Inc, Burlingame, CA, USA). Colour was developed using 3,3′-diaminobenzidine (DAB) and sections were counterstained with haematoxylin. Negative controls, in which the primary antibody was omitted, were included to test for non-specific binding (Orriols et al., 2014).

Cell culture and differentiation
Murine 3T3-L1 preadipocytes were cultured to confluence in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Thermo Fischer Scientific Inc, Waltham, MA, USA) that was supplemented with 10% (v/v) calf serum (Biological Industries, Kibbutz Beit-Haemek, Israel). At 2 days post-confluence (designated day 0), cells were induced to differentiate with DMEM containing a standard induction cocktail of 10% (v/v) fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel), 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 100 nM insulin (all from Sigma-Aldrich, St Louis, MO, USA). After 48 h, this medium was replaced with DMEM supplemented with 10% FBS and 100 nM of insulin. This medium was changed every 48 h. As a model of insulin resistance, murine TNFα (20 or 50 ng/ml, equivalent to 1.15 and 2.87 nM, respectively, Sigma-Aldrich, St Louis, MO, USA) was added to the cell culture medium 7 days after the induction of differentiation, when more than 95% of the cells had the morphological and biochemical properties of adipocytes. For treatment with TNFα (72 h), fully differentiated 3T3-L1 adipocytes were treated every 24 h with the cytokine as previously described (Stephens et al., 1997). BAPN (200 μM) was added to TNFα-treated cells during the last 24 h of incubation. This treatment did not induce cytotoxicity, as analysed by using the XTT-based assay for cell viability (Roche Diagnostics, Indianapolis, IN, USA).

Preparation of whole cell and tissue extracts
3T3-L1 preadipocytes were washed with PBS and cell monolayers were harvested in a non-denaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM Na3VO4, 1 μg/ml leupeptin and 1 mM DTT. Samples were extracted for 30 min on ice and centrifuged at 16,000 g at 4°C for 15 min. Supernatants were analysed for protein content using the BCA kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). Total protein from epidydimal adipose tissue was obtained by homogenisation in lysis buffer and centrifugation for 5 min at 12,700 g (4°C). The tissue extract was then separated from the fat and cellular debris, and analysed for protein content.

Western blotting
Cell and tissue lysates were separated using SDS-PAGE and transferred to 0.45-μm polyvinylidene difluoride membranes (Immobilon, Merck Millipore, Darmstadt, Germany). Blots were incubated with antibodies against GLUT4 (Santa Cruz Biotechnology Inc, Heidelberg, Germany), adiponectin (Chemicon, Merck Millipore, Darmstadt, Germany), DPD4 (Abcam, Cambridge, UK), SOCS3 (Cell Signaling Technology Inc, Danvers, MA, USA) and LOX (Abcam, Cambridge, UK). Bound antibodies were detected after incubation with a horseradish peroxidase (HRP)-conjugated IgG and then using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific Inc, Waltham, MA, USA). Equal loading of protein in each lane was verified by Ponceau staining and by western blotting for β-actin (Sigma).

Glucose uptake measurements
Fully differentiated 3T3-L1 adipocytes were insulin-deprived and pre-treated with BAPN in the presence or absence of TNFα for 24 h. Adipocytes were serum-deprived for 3 h in DMEM supplemented with 2% of fatty-acid-free bovine serum albumin (BSA) with or without TNFα and BAPN. Serum-free medium was then removed and the cells were washed with 1 ml of Krebs-Ringer-HEPES (KRH) buffer pH 7.4 plus 0.2% BSA. Glucose uptake was initiated with 0.9 ml of KRH buffer containing 100 mM insulin for 30 min followed by the addition of 100 μM 2-deoxy-D-glucose and 1 μCi/ml of [3H]-2-deoxy-D-glucose/ml. After 10 min, cells were washed with an ice-cold solution of 50 mM n-glucose in PBS three times. Cells were lysed with a buffer containing 0.5 N NaOH and 0.1% sodium dodecyl sulfate (SDS), and the radioactivity retained by the cell lysate was measured using a liquid scintillation counter (LS 6500, Beckman Coulter, Fullerton, CA, USA). Measurements were made in triplicate and corrected for nonspecific diffusion. Counts of [3H]-2-deoxyglucose were normalised to protein levels.

Statistical analysis
Data are expressed as means and standard deviations. Normality of distributions and homogeneity of the variance were verified by means of either the Kolmogorov–Smirnov or Levene’s test. Either Pearson or Spearman correlation analysis was used to examine the association among different variables according to whether they were normally distributed or not, respectively. Differences between two groups were analysed by either unpaired Student’s t-test or Mann–Whitney as parametric and non-parametric tests, respectively. Specific differences between more groups were analysed using Kruskal–Wallis followed by Dunn’s test for non-normal distribution variables. For normal distribution variables, one-way analysis of variance followed by Newman–Keuls or Tamhane tests were used whether the variance was homogenous or not, respectively. Data analysis was performed using the statistical program SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The predefined significance level was α=0.05.

Acknowledgements
We thank Encarna Muñoz-Ferrero, Silvia Aguiló and Blanca Martínez for their technical help, Anthony DeMarco for his help in editing the manuscript and Cristina Fernández for her help in the statistical analysis of the data.

Competing interests
The authors declare no competing or financial interests.

Author contributions
V.C. and C.R. designed the study, performed experiments and wrote data analysis and wrote the manuscript. M.M. and M.G. performed experiments and data analysis and helped to write the manuscript. E.M.-M., S.V., R.J.-L. and B.B.-M. performed experiments and contributed to the discussion of the manuscript. J.M.-G. contributed to the discussion and the writing of the manuscript. A.A. and M.L. were responsible for the clinical study and contributed to discussion of the manuscript. V.C. and C.R. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Funding
This work was supported by Plan Estatal I+D+I 2013-2016 [PI12/01729, PI12/01952] and Red de Investigación Cardiovascular [RD12/0042/0033 and RD12/0042/0053] of the Instituto de Salud Carlos III (ISCIII), as well as the Ministerio de Economía y Competitividad (MINECO) [SAF2012-40127]. The study was cofunded by Fondo Europeo de Desarrollo Regional (FEDER), a way to make Europe. M.G. was supported by the Sara Borrell Program from ISCIII (CD12/00589).

References


