Angiogenesis Inhibitor, TNP-470, Prevents Diet-Induced and Genetic Obesity in Mice

Ebba Bräkenhielm, Renhai Cao, Bihu Gao, Bo Angelin, Barbara Cannon, Paolo Parini, Yihai Cao

Abstract—Adipose tissue growth has been proposed to involve recruitment of new blood vessels. Here, we test the hypothesis that delivery of an angiogenesis inhibitor in mice may prevent diet-induced obesity, the most common type of obesity in humans. We show that systemic administration of a selective angiogenesis inhibitor, TNP-470 (AGM-1470), prevents obesity in high caloric diet-fed wt mice as well as in genetically leptin-deficient ob/ob mice. Inhibition of obesity in mice by TNP-470 involves a reduction of vascularity in the adipose tissue. This therapeutic strategy appears to selectively affect the growth of adipose tissue as measured by the ratio between total fat and lean body mass. Interestingly, the treatment with TNP-470 results in decreased serum levels of low-density lipoprotein cholesterol. Furthermore, insulin levels are reduced, which indicates increased insulin sensitivity, suggesting that angiogenesis inhibitors may prevent the development of type II diabetes. Our findings suggest that similarly to growth and organogenesis in other tissues, adipose tissue growth is dependent on angiogenesis. Our observations may have conceptual implications for the prevention of obesity and related disorders. (Circ Res. 2004;94:1579-1588.)

Key Words: neovascularization ■ adipogenesis ■ obesity ■ TNP-470 ■ leptin

Obesity has become a worldwide major public health problem. Approximately 30% of the population in the US is estimated to be obese. The obese population has an increased risk for diabetes, dyslipidemia, cardiovascular disease, cancer, and sleep-breathing disorders. The causes of increased human obesity are directly linked to a high dietary fat intake and to reduced physical exercise. In fact, obesity resulting from inappropriate food-intake is the most common cause of human obesity, although genetic factors may also play a role.

The inappropriate growth of adipose tissue by increasing both the number and size of adipocytes leads to obesity. Several hormones and cytokines, such as leptin and neuropeptide Y, have been found to be critical components controlling adipogenesis in the body. Inactivating mutations of either leptin (ob/ob) or its functional receptors (db/db) result in genetic obesity in mice and humans. Leptin have been shown to stimulate angiogenesis. These findings suggest that the targets of adipogenesis regulatory hormones are located both in the central nervous system and in peripheral tissues. Tissue growth and organ regeneration are angiogenesis-dependent. Several studies show that adipogenesis and angiogenesis are tightly correlated during fat mass deposit. We hypothesized that adipogenesis is concomitantly accompanied by new blood vessel growth, and thus suppression of angiogenesis would prevent adipogenesis and obesity independent of the obesity cause. To test this hypothesis, we chose a well-characterized angiogenesis inhibitor, TNP-470 (AGM-1470), to treat high-fat diet-fed C57Bl/6 wt and ob/ob mice. TNP-470 is a synthetic analog of fumagillin, which selectively inhibits endothelial cell growth and angiogenesis. The angiostatic mechanism of TNP-470 involves suppression of methionine aminopeptidase (MetAP-2) in endothelial cells.

Methods

Animals

All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Ethics Board. An expanded Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Antiangiogenic Therapy

Ob/ob mice and C57Bl/6 mice, fed a high-fat or a standard diet, were subcutaneously injected with TNP-470 at the dose of 20 mg/kg every other day. Control animals were injected with the same amount of ethanol in phosphate-buffered saline. In the second ob/ob mouse experiment, a dose of 15 mg/kg TNP-470 was used. Pair-feeding was performed as previously described (for further information see supplemental methods section).

Histological Analysis

Immunohistochemical analysis was performed as previously described (see supplemental online Methods section). Adipocyte size and number were calculated as previously described.
**Mouse Corneal Assay**

The corneal angiogenesis assay was performed as previously described (for further information see online Methods section). 21 Ob/ob mice were 13-weeks-old and wt mice were 9-weeks-old at the time of implantation.

**Analysis of Serum and Lipoprotein Lipids and Carbohydrates**

Total cholesterol, triglycerides, glucose, free fatty acids, insulin, corticosterone levels in serum, and lipoprotein cholesterol and triglyceride contents were determined as previously described (see online Methods section).22

**Calorimetry**

Whole-animal oxygen consumption measurements were performed by indirect calorimetry and body composition was measured as previously described (see online Methods section).23,24

**Cell Culture**

3T3-L1 mouse preadipocytes and bovine capillary endothelial cells were maintained and assayed as previously described (see online Methods section).25,26

**Statistics**

The significance of differences between groups was tested by 2-tailed Student t test, or by 1-way ANOVA, followed by post-hoc comparisons according to least significant difference test (see online Methods).27

**Results**

**Antiobesity Effect of TNP-470 in ob/ob Mice**

Young (4- to 5-week-old) ob/ob mice that had not yet developed pronounced obesity (body weight 15 to 25 grams) were systemically treated with TNP-470. The drug was administered at a dose of 20 mg/kg every other day and was in a dose range previously proven effective in antiangiogenic treatment of cancer in mice.28 Body weight, body length, and food consumption were monitored. In the first set of experiments, the TNP-470-treated group (n=6) did not show any significant increase in body weight over the 12-week treatment period (Figure 1A and online Figure IA). These mice continued to grow in body length at a slightly slower rate than the nontreated control group (n=6), resulting in decreasing body mass index (BMI) scores over time (online Figure IB). After 1 week of treatment, both body weight and BMI of TNP-470–treated mice were lower than those of control animals (P<0.01). In contrast, mice in the nontreated control group continuously gained body weight from week 1 until the termination of the experiment. By week 8, mice in the nontreated group were severely obese, with an average body weight of 40 grams and a BMI of >0.55 g/cm² (online Figure IA and IB). During the experiment, we observed a reduced food intake in TNP-470–treated ob/ob mice (Figure 1B).

Encouraged by these initial results, we performed further studies by including, as an appropriate control, a group of mice pair-fed to the TNP-470–treated animals. In this second set of experiments, we were able to validate our initial finding that body weight and BMI of TNP-470-treated mice were lower than those of control animals (P<0.01). In contrast, mice in the nontreated control group continuously gained body weight from week 1 until the termination of the experiment. By week 8, mice in the nontreated group were severely obese, with an average body weight of >40 grams and a BMI of >0.55 g/cm² (online Figure IC and online Figure IB). The growth in body length of TNP-470–treated ob/ob mice was slightly inhibited by week 5 as compared with both control groups (Figure 1D, P<0.01). Because the growth of body length also may require angiogenesis, the inhibitory effect of TNP-470 was not surprising.

**Figure 1.** Prevention of obesity in ob/ob mice by TNP-470. Five-week-old male C57Bl/6 ob/ob mice were treated with TNP-470 at a dose of 20 mg/kg or received no treatment (A). Five-week-old male C57Bl/6 ob/ob mice were treated with TNP-470 at a dose of 15 mg/kg. Ad libitum-fed and pair-fed mice were used as controls. Food intake was measured daily (B). Body weight was measured every other day (C) and body length once per week (D). Autopsy examination of adipose tissue distribution was performed at week 8 (E). Subcutaneous, perigonadal, and omental fat depots and liver weights were measured (F). Total fat/total body weight (G), lean mass/total body weight (H), and the correlation between total fat mass versus body weight (I) were calculated. Black bars=ad libitum-fed mice (A), blue bars=pair-fed mice (P), red bars=TNP-470–treated animals (T). For comparisons with pair-fed group: *P<0.05, **P<0.01, ***P<0.001; comparisons with ad libitum-fed group: #P<0.05.
Figure 2. Adipose tissue vascularity in ob/ob mice. After 12 (A and B) and 16 (D and E) weeks of treatment, subcutaneous adipose tissue sections were stained with an anti-CD31 antibody. The immunofluorescence images were randomly counted (20×) (F). ***P<0.001, #P<0.05 as compared with ob/ob control group.

Autopsy examination of TNP-470-treated ob/ob mice after 8-week treatment revealed that the depots of subcutaneous and omental fat were dramatically decreased compared with ad libitum-fed and pair-fed control mice (Figure 1E). Dissection of total subcutaneous, perigonadal, and omental adipose tissues showed that the fat depots from the TNP-470–treated mice were significantly reduced compared with both control groups (P<0.01) (Figure 1F and 1G). Liver weights were also significantly reduced in the TNP-470–treated mice (Figure 1F). The percentage of total body fat (total body fat/total body weight) was significantly reduced (>10%) in TNP-470–treated mice as compared with both control groups (P<0.01) (Figure 1H). As a result, a relative increased ratio of lean body mass/body weight was detected in the TNP-470–treated group (P<0.01) (Figure 1I). Reduction of body weight by TNP-470 was thus well-correlated with reduction of adipose tissue mass (Figure 1I). These data demonstrate that the growth of adipose tissue was selectively inhibited by TNP-470. It should be emphasized that mice treated with TNP-470, at a dose of 20 mg/kg every other day, did not experience any toxic side effects as previously reported.28

Inhibition of Neovascularization in Adipose Tissue
To study whether the antiangiogenic effect might be involved in the antiobesity activity of TNP-470, we examined the degree of vascularization of adipose tissues of TNP-470–treated and nontreated ob/ob animals. We found that adipose tissue of nontreated animals was highly vascularized with an average vascular density of >300 microvessels/mm² as revealed by an anti-CD31 antibody (Figure 2A and 2D). This vascular density is nearly 2-fold higher than that detected in tumor tissues,29 a finding in agreement with previous observations that adipose tissue is highly vascularized.30,31 However, a significant reduction of vascularization was detected in the adipose tissue of the TNP-470–treated mice as compared with the nontreated obese animals (Figure 2B, 2E, and 2F) (P<0.001). Notably, the average size of adipocytes in subcutaneous fat deposits of the TNP-470–treated group was smaller than that in the control group (Figure 2A, 2B, 2D, and 2E). Control adipose tissue from nonobese wt mice also contained a high density of microvessels (Figure 2C). However, the number of microvessels in adipose tissue of nonobese mice was significantly less than that of obese mice (Figure 2F), suggesting that active angiogenesis occurs in growing adipose tissues. Thus, the antiobesity effect of TNP-470 correlated with a reduction of neovascularization in the adipose tissue.

Inhibition of Corneal Neovascularization
To further investigate if ob/ob mice responded to the antiangiogenic effect of TNP-470, and to evaluate the systemic efficacy of TNP-470 treatment at a dose of 20 mg/kg every other day, we performed the mouse corneal angiogenesis assay in ob/ob mice and in wt C57Bl/6 mice. Vascular endothelial growth factor (VEGF) or fibroblast growth factor-2 (FGF-2) was implanted into corneas of mice as previously described.9,21 FGF-2 or VEGF induced robust angiogenic responses in corneas of both control groups (n=4) (Figure 3A to 3D). TNP-470 treatment almost completely prevented corneal neovascularization induced by either FGF-2 or VEGF in ob/ob mice (n=4) (Figure 3G and 3H). Similarly, FGF-2–induced and VEGF-induced corneal angiogenesis were potently inhibited by systemic administration of TNP-470 in C57Bl/6 mice (Figure 3E and 3F). The inhibitory effect of TNP-470 on corneal neovascularization in ob/ob mice was comparable with that in wt C57Bl/6 mice (Figure 3I). The measured areas of corneal neovascularization in both TNP-470–treated groups were significantly smaller than those in control groups (P<0.0001) (Figure 3I). This demonstrated that systemic administration of TNP-470 produced potent antiangiogenic effects against both VEGF- and FGF-2-induced angiogenesis in ob/ob as well as in wt C57Bl/6 mice.

Lipid and Carbohydrate Metabolism in ob/ob Mice
Analysis of triglycerides in serum showed no significant differences between the groups of ob/ob mice (Figure 4C).
However, triglyceride content in very-low-density lipoprotein (VLDL) particles was higher in the TNP-470–treated mice as compared with ad libitum-fed and pair-fed animals (Figure 4A). Analysis of serum cholesterol showed a slight reduction in TNP-470–treated animals when compared with their pair-fed controls (Figure 4D). A further reduction (≈35%) was observed when the TNP-470–treated ob/ob mice were compared with the ad libitum-fed animals (P<0.01). Analysis of lipoprotein cholesterol pattern demonstrated that the reduction in total serum cholesterol was caused by a decrease in cholesterol confined to the LDL particles (Figure 4B).

A tendency for a reduction in serum glucose was observed in the TNP-470 group (Figure 4E). This was associated with reductions of insulin levels by 75% (P<0.05) and 84%
(P<0.001), when the TNP-470-treated mice were compared with pair-fed and ad libitum-fed controls, respectively (Figure 4F). No differences were observed for free fatty acids (FFA) among the groups (Figure 4G), nor were corticosterone levels affected by TNP-470 (data not shown). However, the product of insulin times FFA, an indirect measurement of insulin resistance, was significantly lower in TNP-470–treated group as compared with both controls (Figure 4H), indicating an increased insulin sensitivity.

**Antiobesity Effect in High-Fat Diet-Fed wt Mice**

Because genetically related obesity represents only a small proportion of the cause of human obesity, most cases are probably caused by inappropriate intake of dietary fat. Thus, we investigated if TNP-470 treatment could prevent obesity in mice fed a high-fat diet (45 kcal% fat in food). Unlike in ob/ob mice, TNP-470 had little influence on food intake in these wt mice (Figure 5A). Systemic treatment of C57Bl/6 mice (n=6) with TNP-470 at the dose of 20 mg/kg significantly prevented body weight gain by 2 weeks of treatment as compared with controls (P<0.05) (Figure 5B). The antiobesity effect became pronounced after 4 weeks of treatment (P<0.01). This effect appeared to be continuously increased during extended treatments with TNP-470 as seen by week 10 (P<0.001). In contrast, body length was nearly identical in treated and control groups (Figure 5C).

Autopsy examination of high-fat diet-fed wt mice after 16-week treatment with TNP-470 revealed that the depots of subcutaneous, perigonadal, and omental fat were dramatically decreased as compared with control mice (P<0.001) (Figure 5D and 5E). Among these depots, the omental adipose tissue showed the most remarkable reduction (>5-fold decrease). Liver weights were slightly but significantly reduced in the TNP-470–treated mice (Figure 5D). The percentage of total body fat was significantly reduced (63%) in TNP-470–treated wt mice as compared with the control group (P<0.001) (Figure 5E). As a consequence of shifting this ratio, a relative increased percentage of lean body mass was detected in the TNP-470–treated group (P<0.001) (Figure 5F).

**Measurement of Energy Expenditure in High-Fat Diet-Fed Mice**

To investigate if the lower fat accumulation was caused by increased energy expenditure in TNP-470–treated high-fat diet-fed mice, we measured metabolic rates after 14-week treatment. Indirect calorimetric analysis (O2 consumption) showed no significant differences in basal resting metabolic rate at 30℃ in TNP-470–treated mice as compared with controls (0.52±0.04 versus 0.61±0.06 mL O2/min per mouse, respectively) (online Table I). Further, the metabolic activity at rest during daytime at the habituated temperature (23℃) was also not increased by TNP-470 treatment as

![Figure 5. Prevention of obesity by TNP-470 in high fat diet-fed wt mice. Five-week-old male C57Bl/6 mice were fed a high-fat diet. Mice were systemically treated with TNP-470 at a dose of 20 mg/kg or with vehicle alone for 12 weeks (A to C). Food intake in the high-fat diet-fed mice with or without TNP-470 treatment (A). Mouse body weight was measured twice every week (B). Body length was measured weekly (C). Autopsy examination of adipose tissue distribution was performed after 16-week treatment. Subcutaneous, perigonadal, and omental fat depots and liver weights were measured (D). Total fat/total body weight (E) and lean mass/total body weight (F) ratios were calculated. For comparisons: *P<0.05, **P<0.01, ***P<0.001.](image)
compared with controls (0.83 ± 0.06 versus 0.99 ± 0.07 mL O₂/min per mouse, respectively), nor was the 1-hour total metabolism at 23°C increased (online Table I). Thus there were no detectable increases in energy expenditure in the TNP-470–treated mice that might have contributed to the reduction of body weight gain. Analyses of brown adipose tissue capacity, such as noradrenaline-induced increase in oxygen consumption and protein levels of uncoupling protein (UCP)-1 in brown adipose tissue, also showed that no increase in thermogenic capacity had been induced by TNP-470 treatment (online Table I).

**Lipid and Carbohydrate Metabolism in High-Fat Diet-Fed wt Mice**

Blood samples were analyzed after 12- and 16-week treatment and showed that serum levels of triglycerides were slightly reduced in the TNP-470–treated mice as compared with controls (Figure 6C). This reduction was mainly caused by a decrease in VLDL triglyceride content (Figure 6A). Moreover, a remarkable reduction (~45%) in serum cholesterol was found at both time points (Figure 6C) and was related mainly to a decrease in LDL cholesterol (Figure 6B). Similar to ob/ob mice, serum levels of FFA seemed to be unaffected by TNP-470 treatment in high-fat diet-fed mice (Figure 6C).

Interestingly, serum glucose levels were lower in TNP-470–treated mice as compared with controls (Figure 6D). Measurement of serum insulin revealed a remarkable reduction (~80% to 90%) in TNP-470–treated mice at both time points (Figure 6E). The product of insulin times FFA was reduced by ~90% in TNP-470–treated mice as compared with control mice (Figure 6F). Although these control wt mice, unlike the ob/ob animals, did not display high levels of insulin, the observed reduction of the insulin-times-FFA product during TNP-470 treatment is suggestive of increased insulin sensitivity. In addition to reduction of serum levels of lipids, cholesterol and triglyceride contents in livers of TNP-470–treated mice were significantly decreased (Figure 6G and 6H). There seemed to be a tendency (P=0.055) of decreased triglyceride content in skeletal muscles of TNP-470–treated animals, but no difference of cholesterol was found between the 2 groups (Figure 6G and 6H).

**Reduction of Adipose Tissue Neovascularization**

To correlate the decrease in adipose tissue with reduced vascularization, we performed histological analysis of subcutaneous adipose depots. We found a ~10-fold decrease in adipocyte size in TNP-470–treated high-fat diet-fed mice as compared with controls (online Figure IIA). As a result of the
decreased in adipocyte size, the average number/mm² of adipocytes was slightly increased (online Figure IIB). The vascular density in the TNP-470–treated samples was significantly reduced (online Figure IIC). However, this reduction was underestimated by the occurrence of significantly smaller adipocytes in this group. Thus, we compared the ratio between microvessel number and adipocyte number in both groups. A highly significant difference of vascularity was found between the 2 groups (online Figure IID). These data thus demonstrate that inhibition of neovascularization in the adipose tissue by TNP-470 contributed to the antiobesity effect.

Effects of TNP-470 on Body Weight and Lipid Metabolism in wt Lean Mice
To investigate the effect of TNP-470 treatment in lean mice, we studied wt C57Bl/6 mice fed a standard animal diet. After 2-week treatment, food intake in the TNP-470–treated mice was slightly reduced (~10%) as compared with ad libitum-fed mice. However, this reduction disappeared after 4 weeks, suggesting only transient effects of TNP-470 on suppression of food intake in wt mice (Figure 7A). Nevertheless, we included pair-fed controls in this experiment. After 7-week treatment, the body weight in TNP-470–treated mice was slightly, but not significantly, reduced as compared with pair-fed animals (n=6/group) (Figure 7B). The control ad libitum-fed mice weighed significantly more than the TNP-470–treated group already after 3 weeks (Figure 7B). It is possible that the decrease in body weight in the TNP-470–treated group was caused by the aforementioned transient decrease in food intake. However, we cannot completely exclude the possibility that TNP-470 treatment may also directly reduce body weight gain in wt mice fed a normal diet. In contrast, body length was only slightly, and not significantly, decreased in TNP-470–treated mice as compared with control groups (Figure 7C).

Autopsy analyses revealed that subcutaneous and omental fat depots in TNP-470–treated mice were not reduced as compared with the ad libitum-fed group, despite the slight but significantly increased fat depots found in the pair-fed group (Figure 7D and 7E). Liver weights were similar between the groups. Lean mass was slightly reduced in the pair-fed group as compared with ad libitum-fed controls and TNP-470–treated mice, respectively (Figure 7F). Interestingly, serum
lipid profiles including levels of triglycerides, cholesterol, and FFA did not differ between the TNP-470–treated group and control animals (online Figure IIIA). However, we found that serum insulin levels seemed to be decreased in the TNP-470–treated group (online Figure IIIB).

In conclusion, our data indicate that systemic treatment with TNP-470 prevents both genetically-related and high-fat diet-induced obesity in mice but does not affect lean mice beyond slight effects on food intake reduction. Interestingly, the inhibition of food intake by TNP-470 seemed to be most pronounced in the hyperphagic ob/ob mice, because food intake levels in wt C57Bl/6 lean mice and in high-caloric diet-fed mice were only slightly affected by this drug (Figures 1B, 5A, 7A, and online Figure IV).

No Direct Effects on Preadipocytes by TNP-470

To investigate the potential direct inhibitory effects of TNP-470 on preadipocytes, we performed in vitro analyses of cell proliferation and differentiation. We found that TNP-470 did not arrest 3T3-L1 preadipocyte proliferation, even at concentrations up to 1 µmol/L (Figure 8A). This finding is in agreement with a previous report. In contrast, TNP-470, as expected, exhibited a potent inhibitory effect on bovine endothelial cell growth with an IC50 near 100 pM (Figure 8B). Thus, it is unlikely that TNP-470 would directly act on preadipocytes in vivo, especially as the concentration of TNP-470 in the blood after systemic delivery is estimated to be considerably lower (nM range). Further, we found that exposure of 3T3-L1 cells to TNP-470 did not prevent preadipocyte differentiation, because the treated cells accumulated intracellular lipid droplets to similar degrees as controls, even in the presence of TNP-470 concentrations up to 10 µmol/L (Figure 8C). Thus we conclude that inhibition of angiogenesis could play a critical and indirect role in suppression of adipogenesis.

Discussion

Current antiobesity approaches include restriction of food intake, increasing physical exercise, medication, and surgical intervention. These approaches aim to affect lipid metabolism, endocrine balance, energy expenditure rates, or degree of adipocyte maturation. However, some of these strategies may be valid only in certain types of obesity. For example, leptin is only effective in the treatment of monogenic obesity caused by functionally deficient leptin because of genetic mutations. Like other tissues in the body, the expansion of adipose tissue represents an imbalanced angiogenic phenotype. Several angiogenic factors including VEGF and leptin are expressed at high levels in expanding adipose tissues. A recently identified angiogenesis inhibitor, adiponectin, specifically produced by adipose tissues, is secreted at reduced levels in growing adipose tissues. Our present work showing that an angiogenesis inhibitor prevents obesity, both in ob/ob and wt mice, establishes a novel concept in the prevention of obesity irrespective of cause. Consistent with our findings, another group recently reported that several angiogenesis inhibitors, including angiotatin and endostatin could prevent genetically related obesity in mice. Our present data demonstrate that the angiogenesis inhibitor TNP-470 also prevents diet-induced obesity, the most common type of obesity in humans.

Although TNP-470 is a selective angiogenesis inhibitor in clinical trials for the treatment of cancer, high dosages of this agent may affect other systems in the body. Thus, we cannot exclude the possibility that TNP-470 may prevent obesity through mechanisms other than antiangiogenesis. For example, TNP-470 affects food intake, even though this reduction seems to mostly affect ob/ob mice, with only a slight or very slight effect in wt mice, depending on their dietary regimen. It is not clear why TNP-470 differentially influences food intake in different strains of mice. Nevertheless, the influence
on food intake by TNP-470 can only partially explain its antiobesity effect because pair-fed control animals were significantly more obese than the TNP-470-treated ob/ob mice. It should be emphasized that food intake is regulated according to body size.

TNP-470 selectively inhibits endothelial cell proliferation but not preadipocyte proliferation or differentiation in vitro, supporting the hypothesis that the antiangiogenic activity may play an important role in the prevention of obesity. In agreement, we observed a decreased vascularization in adipose tissue of TNP-470–treated mice. In general, the adipose tissue is more remarkably plastic than any other tissue in that it can rapidly expand or regress throughout life. Because angiogenesis occurs only in actively growing tissues, it is not surprising that an angiogenesis inhibitor selectively suppresses the growth of adipose tissues without affecting quiescent vasculatures in adult mice. TNP-470 does not affect the quiescent nonproliferation vascular beds in other tissues.

Ob/ob mice are known to have a particular dyslipidemia characterized by decreased triglyceride levels in VLDL particles and an increased content of cholesterol in LDL and high-density lipoprotein. Interestingly, TNP-470 normalized this altered lipoprotein profile, without affecting high-density lipoprotein cholesterol levels. As observed for adipose tissue, only a minor part of the effects on lipoproteins could be explained by a reduction of food intake induced by TNP-470 in ob/ob mice. Comparing the ad libitum-fed, the pair-fed, and the TNP-470–treated mice, it seems that the effect of TNP-470 on VLDL triglycerides is independent of serum FFA levels. Furthermore, the degree of hypercholesterolemia seems to be related to the severity of obesity and diabetic phenotype in ob/ob mice. Thus, normalization of fat mass and increased insulin sensitivity by TNP-470 could ameliorate the lipoprotein profile. In nondiabetic high-fat diet-fed wt mice, TNP-470 also decreased insulin levels and improved lipoprotein profiles. However, we cannot exclude a possible direct effect of TNP-470 on hepatic triglyceride and cholesterol metabolism. This very interesting question remains to be further explored.

In conclusion, because development of obesity, cancer, and diabetic complications are all dependent on angiogenesis and the cause of these pathological conditions can be related, antiangiogenic therapy may prove to be multibeneficial in these disorders. Thus, angiogenesis inhibitors used alone or in combination with other therapies may become an important new strategy in the prevention of obesity.

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Methods

Reagents and Animals
TNP-470 was obtained from Takeda Chemicals (Osaka, Japan). Four-week-old male C57Bl/6 ob/ob mice were obtained from B&D Inc. (Copenhagen, Denmark). Five-week-old C57Bl/6 male mice were obtained from the breeding facility of the Microbiology and Tumor Biology Center at the Karolinska Institutet (Stockholm, Sweden). Animals were acclimatized and caged in groups of six or less. High fat diet (45 kcal% fat, D12451) was purchased from Research Diets Inc. (New Brunswick, NJ). All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Ethics Board.

Antiangiogenic therapy
TNP-470 was prepared as a fresh solution immediately before use and dissolved in 100% ethanol, followed by dilution in phosphate-buffered saline (PBS) to a final concentration of 3 mg/ml containing 3% ethanol. Ob/ob mice, high fat diet-fed and standard diet-fed C57Bl/6 mice were subcutaneously injected (20 mg/kg) with TNP-470 every other day throughout the experiments. Control animals were injected with the same amount of ethanol in PBS. In the second ob/ob mouse experiment a dose of 15 mg/kg TNP-470 was used, and injections were repeated 3 times per week. Pair-feeding was carried out as previously described1. Briefly, pair-fed animals were restricted to the daily food intake levels of TNP-470-treated mice. Body weight was
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determined every other day, body length measured once a week, and food consumption was determined daily. By the end of the treatment, subcutaneous, perigonadal and omental fat, skeletal muscles, and livers of TNP-470-treated and control groups of mice were dissected and weighted.

Histological analysis

By the end of week 12 or 16, TNP-470-treated- and control- ob/ob or high fat diet–fed C57Bl/6 mice were sacrificed and subcutaneous fat was immersed in 3% paraformaldehyde at +4°C overnight, followed by washing with 30% sucrose. Tissues were frozen in Tissue Tek OCT (Histolab, Gothenburg, Sweden) on dry ice and stored at –80°C until sectioning. Cryosections of 12 µm were cut using a cryostat, stained according to standard immunohistochemical protocols with a monoclonal rat anti-mouse CD31 antibody (Pharmingen, San Diego, CA), and detected using either FITC-labeled anti-rat-IgG secondary antibodies or using NEN TSA amplification (PerkinElmer Lifesciences Inc., Boston, MA) protocol for biotinylated primary antibodies as previously described2. The signals were examined by fluorescence or light microscopy. Adipose microvessels were randomly counted in at least 8 sections at 20x magnification. Adipocyte size and number in subcutaneous adipose depots were calculated as previously described3. Briefly, fat cell number was counted and adipocyte diameters were measured digitally in histological light microscopic images (20x) of adipose tissue sections (n>6 sections/ animal; 4-6 animals/ group) using Adobe Photoshop 7.0, and cell volumes were calculated according to the formula: \((\pi \times r^3)/3\).
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Whole mount preparation
By the end of week 16, TNP-470-treated- and control- high fat diet–fed C57Bl/6 mice were sacrificed, and subcutaneous fat was collected and immersed in 3% paraformaldehyde at +4°C overnight, followed by washing in PBS. Small tissue pieces were cut and stained according to standard immunohistochemical protocols with a monoclonal rat anti-mouse CD31 antibody (Phar mingen, San Diego, CA) and detected using FITC-labeled anti-rat-IgG secondary antibodies. The signals were examined under a Zeiss confocal microscope. Three-dimensional images were created by serial scanning of 5 µm sections to compile 40 µm thick samples.

Mouse corneal assay
Ob/ob and wt mice were systemically treated every other day with TNP-470 at a dose of 20mg/kg every other day, 1 day prior to implantation of angiogenic factors in corneas and throughout the study. The corneal angiogenesis assay was performed as previously described4. Briefly, micropellets (0.35 x 0.35 mm) of sucrose aluminum sulfate (Bukh Meditec, Copenhagen, Denmark) coated with hydron polymer type NCC (IFN Sciences, New Brunswick, NJ) containing approximately 60 ng FGF-2 or 120 ng VEGF were implanted into the corneal pockets of TNP-470-treated- and non-treated-ob/ob mice or standard diet-fed wt C57Bl/6 mice. The pellet was positioned 1.0-1.2 mm from the corneal limbus. After implantation, erythromycin/ophthalmic ointment was applied to each eye. The corneal neovascularizations were examined using a slit-lamp microscope on day 5 after pellet implantation. Areas of corneal neovascularizations were measured as described previously2,4.
Analysis of serum and lipoprotein lipids and carbohydrates

After 8-week-treatment in ob/ob mice, 12- or 16-week-treatment in high fat diet fed wt mice, and 7-week-treatment in standard-diet fed wt mice, ad libitum-fed (n=6), TNP-470-treated- (n=6), and pair-fed (n=6) mice were sacrificed and blood was drawn by cardiac puncture under light methoxyfluorane anesthesia and kept on ice before further analyses. In C57Bl/6 groups, pooled serum samples were analyzed. Total cholesterol, triglyceride, and glucose were determined individually on serum, using a Monarch automated analyser (ILS Laboratories Scandinavia AB, Sollentuna, Sweden). Serum free fatty acid content was determined using a commercially available kit (Wako Chemicals GmbH, Neuss, Germany). Insulin and corticosterone in serum were determined by a RIA assay (Linco Research Inc., St. Charles, MO, and IDS Limited, Boldon, UK, respectively). Size-fractionation of lipoproteins was performed on 10 µl of pooled sera from each group, using a micro-FPLC column (30 x 0.32 cm Superose 6B, Amersham Pharmacia Biotech AB, Uppsala, Sweden) at a flow rate of 40 µl/min, as previously described. After FPLC separation, cholesterol and triglyceride content in lipoproteins were determined online using commercially available kits (Roche Molecular Biochemicals, Indianapolis, IN). Skeletal muscle and liver samples from high fat diet fed wt mice following 16-week-treatment were similarly analysed for total cholesterol and triglyceride content.
Calorimetry

Whole animal oxygen consumption measurements were performed in an indirect calorimetry apparatus (Somedic, Hörby, Sweden) as previously described. Resting metabolic rates at 23 °C or 30 °C were defined as the lowest, stable oxygen consumption observed for at least 10 minutes (n=6 animals/group). Measurements were performed for 3 hours at each temperature. Total metabolism, including physical activity, was determined for 1 h at 23 °C. In order to estimate the animal’s non-shivering thermogenic capacity, metabolic responses, induced by injection of noradrenaline (NE, 1 mg/kg, s.c; Sigma Aldrich, Stockholm, Sweden), were measured at 33 °C under anesthesia with sodium pentobarbital (90mg/kg, i.p; Apoteksbolaget, Stockholm, Sweden). ∆NE was calculated as the maximal increase in oxygen consumption induced by NE during 1 h.

Body composition

Body composition was measured in anesthetized mice using a dual energy X-ray absorptiometry (DEXA) Scanner from Lunar/GE Medical Systems (PIXImus2, distributed by Faxitron X-Ray Corporation, Wheeling, IL) as previously described. Before each set of experiments, the DEXA scanner was calibrated for bone mineral density and percent fat content using the “phantom mouse” unit supplied with the machine. This calibration procedure is part of the quality control process and measurements for bone mineral density, and percent fat must be within 2% of the expected value in order to proceed with animal scans. The mice were anesthetized with a Hypnorm, Dormicum, and water mix (1:1:2) and placed ventral side down on disposable
plastic trays such that the entire body and tail were measured in the scan. Trays were positioned in the DEXA scanner so that the head was always oriented toward the left from the investigator’s point of view. After completion of the scan, mice were returned to their home cage at 30 °C until they recovered from the anesthesia. DEXA scans were analyzed using the PIXImus2 software (version 1.46.007). The head region was excluded from the analysis. Values (calculated by the software) for total tissue mass (g), total body area (cm²), fat content (g), lean content (total tissue mass minus fat content, g), and percent fat (fat content divided by total tissue mass) were obtained. Body weight (g) was measured just prior to scanning.

**Cell Culture**

3T3-L1 mouse preadipocytes and bovine capillary endothelial (BCE) cells were maintained as previously described. For proliferation studies approximately 10 000 cells/well were seeded in 24-well-plates in growth medium containing 5% serum. For BCE cells, the positive control and all samples included 2 ng/ml FGF-2. TNP-470 was diluted in DMSO and added to the wells following 1 h of cell attachment after seeding. Negative controls contained the same concentration of DMSO (final conc. <0.1% DMSO). Following a 72-h-incubation, cells were resuspended in trypsin and counted with a Coulter counter. For differentiation experiments with 3T3-L1 cells a standard protocol was used. Briefly, following 2 days of cell confluence, the preadipocytes were induced to differentiate by a combination of isobutylmethylxanthine, dexamethasone, and insulin. TNP-470 was added at different concentrations, and the medium was changed every other day. After
6 days, the preadipocytes had begun to accumulate lipid droplets and to differentiate into mature poly- or unilocular adipocytes. Light microscopic images (20x) were taken to document the degree of differentiation.

**Statistics**

Data are presented as mean ± SEM. The significance of differences between groups was tested by two-tailed student’s $t$ test, or where appropriate by one-way ANOVA (repeated measurements or fully randomized designs), followed by post-hoc comparisons according to LSD test (Statistica software, Stat Soft, Tulsa, OK). To stabilize the variances, data were logarithmically transformed when the assumption for no correlation between means and variances was violated.
Figure Legends

**Supplemental Fig. 1 Anti-obesity effect in ob/ob mice**

Five-week-old male C57Bl/6 ob/ob mice were treated every other day with TNP-470 (20 mg/kg) or received no treatment for 12 weeks. Mouse body weight was measured twice every week (A). BMI was calculated according to the formula: weight/length$^2$ (g/cm$^2$) (B). Five-week-old male C57Bl/6 ob/ob mice were treated 3 times per week with TNP-470 (15 mg/kg) for 8 weeks. Ad libitum-fed and pair-fed mice were used as controls. BMI was calculated (C).

**Supplemental Fig. 2 Histological analyses of adipose tissue in high fat diet-fed wt mice.** Five-week-old male C57Bl/6 mice were fed a high fat-diet. Mice were systemically treated with TNP-470 at a dose of 20 mg/kg or with vehicle alone for 16 weeks. Adipocyte volume was determined in the treated and control groups (A). The number of adipocytes (B) and microvessels (C) per area were analyzed. Correlation between the number of microvessels and the number of adipocytes per area (D). * p<0.05, ** p<0.01, *** p<0.001. Examples of histological sections; whole mount CD31 immunofluorescence staining of adipose tissue from control (E) and TNP-470-treated (F) mice (20x, 40 µm thick section). Scale bar=50 µm. Light microscopic images of adipose tissue from control (G) and TNP-470-treated (H) groups (20x). Scale bar=100 µm.

**Supplemental Fig. 3 Metabolic analyses in standard diet-fed wt mice.**

The effects on lipid and carbohydrate metabolism of 7-week-treatment with
Angiogenesis inhibitor, TNP-470, prevents both diet-induced and genetic obesity in mice
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TNP-470 were determined. Total serum triglyceride (TG), cholesterol (Chol.), and free fatty acid (FFA) levels (A). Insulin serum levels (B). Black bars= ad libitum fed (A); red bars= pair-fed (P); white bars= TNP-470-treated (T).

Supplemental Fig. 4 Effects of TNP-470 on food consumption. Comparisons of food intake levels in ob/ob and wt strains of mice on different dietary regimens at a fixed body weight (25g/mouse). Controls (ad libitum-fed)= black bars; TNP-470-treated animals= red bars. Data is presented as means of values (+SEM) (n=6/group).

Supplemental Fig. 5 Studies of UCP-1 expression in subcutaneous WAT samples. In order to exclude the possibility that the white adipose tissue (WAT) samples contained any brown adipocytes we performed a Western blot analysis to determine the presence or absence of the brown adipose tissue (BAT)-specific marker uncoupling protein (UCP)-1 in WAT samples from control and treated groups of high fat diet-fed wt animals. Interscapular-derived BAT served as a positive control.

Supplemental Table 1 Metabolic measurements in high fat diet-fed wt mice. Five-week-old male C57Bl/6 mice were fed a high fat-diet. Mice were systemically treated with TNP-470 (20 mg/kg) or with vehicle alone. After 14 weeks, body weight, body composition, and body temperature were determined. Oxygen consumption levels were determined at the habituated temperature (23°C). For determination of resting basal metabolic rate the oxygen consumption at 30°C was measured. Total metabolic expenditure,
including physical activity, was determined for 1 h at 23 ºC. The non-shivering thermogenic capacity was measured as the maximal increase in oxygen consumption during noradrenaline-induced activation (ΔNE). At autopsy examination after 16-week-treatment, BAT samples were retrieved, and total BAT protein, total mRNA or protein UCP-1 levels were measured. Values are given as mean ± SEM (n=4-6 animals/group).

References


Angiogenesis inhibitor, TNP-470, prevents both diet-induced and genetic obesity in mice
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<td>body weight</td>
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<td>lean body mass</td>
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<td>food intake</td>
<td>(g/mouse/day)</td>
<td>1.8 ±0.2</td>
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<td>body temperature</td>
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<td>O₂ consumption at 23°C</td>
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<td>∆NE</td>
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<td>100 ±15</td>
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university Press; 1980.
Fig. S2, Brakenhielm et al. 2004

A

Adipocyte volume (ul)

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B

# of Adipocytes / mm²

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C

Vessel counts / mm²

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D

Ratio: Vessels / Adipocytes

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E

Image E

F

Image F

G

Image G

H

Image H
Fig. S5, Brakenhielm et al. 2004

WAT

BAT

control

TNP-470-treated

UCP-1

32 kDa