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Glial Cell Line-Derived Neurotrophic Factor induced Mice Liver Defatting: A Novel Strategy to Enable Transplantation of Steatotic Livers

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Abstract

Moderate macrovesicular steatosis (>30%), which is present in almost 50% of livers considered for transplantation increases the risk of primary graft dysfunction. Our previously published data showed that glial cell line-derived neurotrophic factor (GDNF) is protective against high-fat diet (HFD)-induced hepatic steatosis in mice. Hence, we hypothesized that perfusion of steatotic livers with GDNF may reduce liver fat content prior to transplantation. Livers from 8 weeks regular diet (RD) and HFD-fed mice were perfused ex-vivo for 4 hours with either vehicle, GDNF, or a previously described defatting cocktail. Liver's residual fat was quantified colorimetrically using a triglyceride assay kit, and by Oil Red-O and Nile Red/Hoechst staining. Liver tissue injury was assessed using an LDH activity assay. In vitro induction of lipolysis in HepG2 cells was assessed by measuring glycerol and free fatty acid release. Oil Red-O staining showed significantly more steatosis in liver from HFD-fed mice compared with RD-fed mice (P<0.001). HFD Livers perfused with GDNF had significantly less steatosis than those not perfused (P=0.001) or perfused with vehicle (P<0.05). GDNF is equally effective in steatotic liver defatting compared to the defatting cocktail; however, GDNF induces less liver damage than the defatting cocktail. These observations were consistent with data obtained from assessment of liver triglyceride content. Assessment of liver injury revealed significant hepatocyte injury in livers perfused with the control defatting cocktail but no evidence of injury in livers perfused with either GDNF or vehicle. In vitro, GDNF reduced triglyceride accumulation in HepG2 cells and stimulated increased triglyceride lipolysis.

Conclusion—GDNF can decrease mice liver fat content to an acceptable range and could be a potential defatting agent prior to liver transplantation.
Keywords
High Fat Diet; Liver transplantation; Triglycerides; Fatty Liver Disease; Liver infusion

Introduction
Orthotopic liver transplantation (OLT) is a successful therapeutic modality for the treatment of end-stage liver disease, but is limited by donor scarcity (1). Macropiduncular steatosis of triglycerides (TG) in 30% or more of the donor livers increases the risk of primary graft dysfunction (2) and is a common cause of donor ineligibility (3). Macrovesicular steatosis is present in nearly 50% of the livers rejected from the donor pool (4). Several strategies have been developed to recover donor livers that are not readily suitable for transplantation (5). While many have focused on protecting the fatty liver from post transplantation injury (6), so far no approach has been successful in clinical practice. Thus, techniques to salvage macrosteatotic livers can help close the gap between supply and demand in liver transplantation by enhancing donor supply (7).

Various mechanisms of targeting the downstream effects of macrosteatosis have been proposed in animal studies (7–9). One such method is to perfuse the fatty livers with compounds that promote lipid droplet breakdown and accelerate lipid metabolism (10). Several compounds have been used prior to transplantation to increase lipid export and/or oxidation, specifically aimed at reducing the fat content of steatotic livers to a normal range, but success rates of defatting are still not promising and the agents need to be used in combination to achieve maximum defatting effect. Among the compounds showing promise are the peroxisome proliferators-activated receptors (PPARs), visfatin and forskolin (11–13).

Pegorier et al, studied the effects of pancreatic hormones and cyclic AMP on the induction of ketogenesis and long-chain fatty acid oxidation in primary cultures of hepatocytes from fetal and newborn rabbits (13). They showed that glucagon, forskolin and cyclic AMP enhance lipolysis through oleate oxidation. In another study, the effects of bezafibrate, a peroxisome proliferator-activated receptor (PPAR) pan-agonist, and GW501516, a PPARδ agonist, were evaluated on a mouse model of non-alcoholic steatohepatitis (NASH). Bezafibrate and GW501516 inhibited the diet-induced elevations of hepatic triglyceride and thiobarbituric acid-reactants contents, as well as histopathological increase in fatty droplets within hepatocytes. It suggested that bezafibrate and GW501516 might improve hepatic steatosis via improvement in fatty acid beta-oxidation and a direct prevention of inflammation (11).

We have previously shown that GDNF enhances β-cell mass and improves glucose control in rodents (14). We also recently showed that GDNF transgenic (Tg) mice that overexpress GDNF are resistant to HFD-induced weight gain and hepatic steatosis. GDNF Tg mice exhibit significantly higher energy expenditure than WT mice and have increased expression in skeletal muscle and brown adipose tissue of peroxisome proliferator activated receptor-α and β1- and β3-adrenergic receptor genes, which are associated with increased lipolysis and enhanced lipid β-oxidation (15). We also showed that GDNF enhances β-adrenergic-mediated cAMP release and suppresses lipid accumulation in adipocytes in vitro (15).

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In this study we hypothesized that GDNF can be used to lower fat content in steatotic livers before transplantation to improve the chances of liver graft survival after transplantation. We compared the defatting efficacy of GDNF to that of a previously described defatting cocktail (visfatin, forskolin, GW7647, GW501516, hypericin, and scoparone) (11–13) in \textit{ex vivo} perfused fatty livers, and on lipid metabolism in steatotic cultured HepG2 cells.

**Material and Methods**

GW7647 and forskolin were purchased from Sigma Chemical (St. Louis, MO). Scoparone was purchased from Calbiochem (La Jolla, CA). Visfatin, hypericin, and GW501516 were purchased from Axora Alexis (San Diego, CA). Recombinant rat GDNF was prepared as previously described (16).

**Animal Model**

Male C57BL/6 mice (The Jackson laboratory, Bar Harbor, Maine, USA) aged 4–6 weeks were used. 5 groups of mice (n = 4 for each group) was maintained on a high-fat diet (HFD; TD.06414; Harlan, Madison, WI containing 60% of calories from fat) for 8 weeks, while another 3 groups were maintained on a regular rodent diet (RD; TD. 2018, 18% calories from fat). All experimental procedures followed National Research Council guidelines and were approved by the Institutional Animal Care and Use Committees (IACUC) at Emory University.

**Preparation of defatting media**

Medium consisting of Eagle’s Minimum Essential Medium (EMEM; ATCC 30-2003) supplemented with 3% bovine serum albumin (Fraction V, Sigma Chemical Co.), 1.1 mM lactic acid, and 0.1 mM pyruvic acid was adjusted to pH 7.4. A cocktail of various defatting agents (10mM forskolin; 1mM GW7647; 10 mM hypericin; 10 mM scoparone; 0.4 ng/mL visfatin; 1 mM GW501516) was added to the medium/perfusate to prepare basal medium for \textit{ex vivo} perfusions (10). To prepare vehicle controls, the defatting agents were replaced by an equivalent amount of dimethylsulfoxide (DMSO) as the solvent for the defatting cocktail. To make the final concentration of 12.5 ng/ml for GDNF, 50 μl of GDNF in PBS, from the GDNF stock of 100 μg/ml was added to 400 ml of the final perfusate. For \textit{ex vivo} perfusions, perfusate was equilibrated with a humidified 95% O\textsubscript{2}/5% CO\textsubscript{2} gas mixture.

**Liver perfusion**

Mice liver perfusions were performed according to a modification of a protocol described by Mortimore (17). Mice were subjected to laparotomy to expose their portal vein, common bile duct (CBD) and inferior vena cava (IVC) by retracting the bowel to the left. The suprahepatic vena cava was ligated and the liver was perfused \textit{in situ} via the IVC, in an unclosed circuit, with the outflow being directed towards the portal vein and discarded afterwards. After an initial perfusion for 10 min with Minimum Essential Medium (MEM) to wash out the blood, livers were perfused for 4 hours at a constant flow rate of 100 mL/h with different perfusion cocktails at a constant temperature of 37°C. Livers from RD-fed and HFD-fed mice (n = 4) were perfused with vehicle, while another set of RD-fed and HFD-fed group (n = 4) were perfused with GDNF-containing medium. Four livers from the HFD-fed
group were perfused with medium containing the defatting cocktail. Finally, two groups of livers from the RD-fed and the HFD-fed groups were left un-perfused to serve as references. Figure 1 shows the gross changes in HFD-fed mouse liver before and after ex vivo perfusions.

**Oil Red-O Staining**

Liver tissue from the left lateral lobe was frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, U.S.A.) and sections (5 μm thick) were made using standard protocol. Oil Red-O staining was performed using a modification of a previously published protocol (http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_ManualsProductInstructions_Oil_Red_O_Stain_for_In_Vitro_Adipogenesis.pdf). A blinded investigator performed all histological evaluations. Total fat content was quantified using morphometry (ImageJ, version 1.49). In the ImageJ program, images captured by microscope (10–15 fields per sample, 10x and 20x) in a random fashion and were converted to black and white images using the “Image-type” submenu. Subsequently, the threshold function was used either as default or auto settings to select only the dark area within the image (i.e., image area containing fat droplets). The total fat droplet area was then determined using the “Analyze- Analyze particles” submenu. The summary information includes a count of objects and total particle area. To eliminate the possible effect of water content and cell volume, we normalized Oil-Red-O staining measurements to the number of cells by reporting the data as Oil-red-O staining area per 1000 cells. These procedures are described on the ImageJ homepage (http://rsb.info.nih.gov/ij/).

**Liver triglyceride content analysis**

Liver tissue (100 mg) were homogenized in 1 ml of a 5% NP-40 solution. The samples were slowly heated to 80–100°C in a water bath for 5 minutes, cooled down to room temperature and centrifuged for 2 minutes at 14000 rpm to remove insoluble material. The resulting solution was assayed for TG content using a commercial kit (Abcam Triglyceride Quantification Assay kit-ab65336) following the manufacturer’s instructions (18).

**Liver injury/hepatocyte viability measurement**

Using Cleaved caspase-3 staining on liver OCT sections, and a LDH activity assay, measured by a colorimetric assay kit (Abcam Inc., Cambridge, MA) according to the manufacturer’s protocol, the viability of cells and possibility of perfusion-induced liver injury was assessed (19, 20).

**Cell Culture**

The HepG2 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C in a 5% CO2 incubator in Minimum Essential Medium (ATCC) supplemented with fetal bovine serum (10%) according to recommended procedure. To assess the effects of GDNF on steatosis, HepG2 cells were cultured for 2 days in MEM supplemented with 1 mM palmitic acid and 4% bovine serum albumin followed by 2 days in fresh medium containing GDNF (100 ng/ml) or vehicle. Free fatty acids and glycerol released into the medium were measured using a dual glycerol and free fatty acids
detection lipolysis assay kit (Zen-Bio, Research Triangle Park, NC) (21). HepG2 cells were also fixed in 4% paraformaldehyde, stained with the lipid-specific Nile Red Stain-derived AdipoRed Assay Reagent (Lonza Walkersville, Walkersville, MD) (22), and counter-stained with 1 μg/mL of nuclei-specific Hoechst-33342 stain (Invitrogen). In accordance to the manufacturer’s instructions. Fluorescence images were obtained with an Olympus IX-80 microscope and image analysis method was used to quantify lipid droplet size distribution.

**Statistical analysis**

Student *t*-test was performed to compare two groups and One-way ANOVA followed by Fisher’s LSD post-hoc test was performed for more than two groups, using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California, USA).

**Results**

**Ex Vivo GDNF perfusion induces liver defatting**

Livers from a group of RD and HFD mice were harvested after 8 weeks of feeding and assessed for fat content by quantitative Oil Red-O staining to determine the extent of liver steatosis. Livers from HFD-fed mice (n = 4) had significantly higher fat content than livers from RD-fed mice (P< 0.001). The remaining livers were then perfused either with vehicle (negative control), GDNF, or defatting cocktail (positive control) and liver fat was then assessed, as described in methods section. Livers from HFD-fed mice perfused with GDNF had significantly less fat than HFD-fed mice non-perfused livers and HFD-fed mice livers perfused with vehicle, based on quantitative measurement of Oil Red-O area stained per 1000 cells. [Figure 2 A & B] Assessment of intrahepatic TG content also showed that perfusion of liver with GDNF resulted in a significantly larger reduction in liver TG content than vehicle (P=0.001) [Figure 3].

We then assessed if the perfusion process could result in hepatocyte injury. Using Cleaved caspase-3 staining on liver OCT sections, we did not observe significant increase in apoptosis in the HFD-fed mice liver tissue perfused with either vehicle, GDNF or defatting cocktail compared with non-perfused livers from HFD-fed mice (data not shown). In addition LDH activity, measured in liver tissues supernatants was not altered by perfusion of vehicle or GDNF in HFD-fed mice liver tissue; however it was increased in HFD-fed mice livers perfused with defatting cocktail solution [Figure 4]. This indicates that the defatting cocktail was associated with liver damage, which did not happen after GDNF perfusion.

**GDNF induces lipolysis in HepG2 cells**

Based on the results obtained with *ex vivo* experiments, we sought to understand the mechanism of GDNF resulting in defatting. To do this, we assessed the effects of GDNF *in vitro*. An experimental protocol was set up as described in Figure 5. We cultured HepG2 cells in a standard hepatocyte culture medium for one week, and then switched to fatty acid (palmitic acid)-supplemented medium for 48h. Following this, vehicle or GDNF (100ng/mL) was added to the medium and cells were cultured for an additional 48h, and intracellular lipid content was assessed. Nile Red lipid staining showed more intense staining in cells cultured in the presence of palmitate, compared to the vehicle. Adding
GDNF to the medium reduced the triglyceride accumulation compared with the medium with vehicle alone [Figure 6A]. Quantification of stained area showed that HepG2 cells that were cultured in the medium supplemented with palmitate, had significantly higher lipid droplet area compared with cells cultured in medium alone; and that GDNF significantly lowered lipid droplet area in both vehicle and palmitate treated HepG2 cells [Figure 6B].

To further assess the mechanism of lipid content reduction by GDNF, the levels of products of lipolysis such as free fatty acid (FFA) and glycerol were measured in the culture media. FFA levels were significantly higher in the presence of GDNF compared with the vehicle treated cultures after 48h. GDNF increased lipolysis of TG and release of FFA and glycerol into the media by nearly 1.5 and 2 fold after 48h of incubation, respectively, compared with vehicle. These results corroborate the \textit{ex vivo} data, suggesting that GDNF is responsible for lipid clearance [Figure 7A & B].

\section*{Discussion}

In this study, we report that GDNF stimulates lipolysis, resulting in a defatting process of steatotic HepG2 cells as well as the perfused livers. Defatting agents including GDNF were studied using both cultured HepG2 cells (pre-incubated in fatty acid-supplemented medium) and steatotic liver perfusion technique, that was previously shown to be effective with other defatting agents (10, 23, 24).

It has been recently shown that GDNF enhances \(\beta\)-cell mass and improves glucose control in rodents. In previous studies, we investigated the ability of GDNF to protect against high-fat diet (HFD)-induced obesity. GDNF transgenic (Tg) mice that overexpress GDNF resisted the HFD-induced weight gain, insulin resistance, dyslipidemia, hyperleptinemia, and hepatic steatosis seen in WT mice despite similar food intake and activity levels. They exhibited significantly higher energy expenditure than WT mice and increased expression in skeletal muscle and brown adipose tissue of peroxisome proliferator activated receptor-\(\alpha\) and \(\beta\)1- and \(\beta\)3-adrenergic receptor genes, which are associated with increased lipolysis and enhanced lipid \(\beta\)-oxidation. We were also able to show that \textit{in vitro}, GDNF enhanced \(\beta\)-adrenergic-mediated cAMP release in brown adipocytes and suppressed lipid accumulation. Through this we demonstrated a novel role for GDNF in the regulation of high-fat diet-induced obesity by increasing energy expenditure, showing that GDNF and its receptor agonists may be potential targets for the treatment or prevention of obesity (15). However, in the current study we focus mainly on \textit{ex vivo} perfusion of liver with GDNF for a limited time and under special circumstances, to investigate the potential effects of GDNF on defatting the steatotic livers. Alongside, we assess the same effect \textit{in vitro} on HCC cell line to confirm if there is any increased lipolysis induced by GDNF.

Animal studies have demonstrated the feasibility of macrosteatosis reduction of explanted steatotic livers via machine perfusion. This process was accelerated by introducing agents that promote lipid metabolism (10, 25, 26). It was shown that a combination of peroxisome proliferator activated-receptor \(\alpha\) ligand GW7647 (GW7), peroxisome proliferator-activated receptor \(\alpha\) ligand GW501516 (GW5), pregnane X receptor ligand hypericin (HPC), the constitutive androstane receptor ligand (Scoparon), glucagon mimetic cAMP activator

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Forskolin, and the insulin-mimetic adipokine, visfatin makes an effective defatting cocktail, which results in significant decrease in intracellular lipid content by more than 50% during 3 h liver perfusions (10). In our study we showed that GDNF is significantly effective in inducing lipolysis and can reduce the hepatocyte lipid content by up to 40% during a 4 hour perfusion period. Similarly, GDNF is also effective in vitro by regulating the lipolysis. These findings support our hypothesis regarding the role of GDNF in reduction of liver lipid storage through lipolysis.

We have previously shown that GDNF can protect against high-fat-induced hepatic steatosis in mice (15). GDNF signals through a multicomponent receptor consisting of the Ret receptor that it shares with other GDNF family members, and a specific co-receptor, GDNF family receptor α1 (GFRα1), to activate the PI3K/Akt and MAPK (mitogen-activated protein kinases) signaling pathways (27). We recently showed the expression of GFRα1 receptor on hepatocytes (15). Our results suggest that the mechanism of GDNF regulation of fat storage involves both peripheral and central mechanisms. We have previously shown that GDNF promotes enhanced energy expenditure in brown fat by increasing brown adipocyte differentiation and function evidenced by increased expression of the genes coding for PRDM16, CycS, and the 1- and 3-adrenergic receptors in vitro and in vivo and the ability of GDNF to stimulate increased cAMP release in brown adipocytes (15). In white adipose tissue (WAT) there is increased expression of PPARα and PGC1-α, both genes known to increase mitochondrial oxidation and subsequent energy expenditure. Similarly, in skeletal muscle there is also increased expression of PPARα in mice. GDNF also reduces fat accumulation in WAT by reducing the expression of genes involved in adipogenesis such as PPARα and fatty acid transport such as CD36 and FABP4. In vitro, GDNF suppresses the genes related to fatty acid synthesis including PPARα, FABP4, Srebf1, and FASN. (15).

The potential mechanisms for reduction of intracellular lipids are secretion of TG through very low-density lipoprotein (VLDL) and fatty acid oxidation. In both cases, the stored TG undergoes lipolysis followed by re-esterification and assembly with Apo-lipoproteins into TG-rich VLDL, or complete hydrolysis into glycerol and fatty acids (10, 28). Triacylglycerol hydrolase is responsible for TG hydrolysis in hepatocyte cell line, and has been shown to play a major role in TG lipolysis and re-esterification (28). We hypothesize that GDNF stimulates lipolysis in vitro in HepG2 cells potentially through β-adrenergic-mediated signaling. We have previously described the potential mechanism through which GDNF is able to enhance β-adrenergic signaling in adipocytes to induce lipolysis and lipid β-oxidation to protect against HFD-induced obesity (15).

By using GDNF, we showed an effective method that can significantly reduce the lipid content in liver by 40% in only 4 hours of normothermic perfusion. Since there is a strong correlation between increased degree of steatosis and graft primary non-function (29), rapid defatting via normothermic perfusion with GDNF may make it possible to salvage moderately (30–60%) or perhaps by more dosage adjustments, even severely (>60%) steatotic livers for transplantation. Ultimately, surgeons require proof that defatted livers are indeed similar to normal lean livers before this approach will gain wide acceptance. It has been shown that even with 75% reduction in fat content, hepatocytes are 83% viable and functional (30). Similarly, in the present study we assessed the impact of accelerated
Macrosteatosis reduction on the viability and recovery of liver functions by using Cleaved caspase-3 staining and LDH activity assay. Our findings revealed that effective fat content reduction in the steatotic livers, did not impact the viability of hepatocytes. However, further studies are warranted to assess the short and long term functionality of steatotic livers.

In conclusion, we showed for the first time that GDNF can be used to significantly reduce the intracellular fat content of perfused livers and cultured HepG2 cells within a few hours. GDNF is equally effective in steatotic liver defatting compared to the defatting cocktail; however, GDNF induces less liver damage than the defatting cocktail. While still many challenges need to be overcome, liver defatting is potentially a promising approach that may enable the successful recovery of a large number of livers that would otherwise be discarded.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CBD</td>
<td>Common bile duct</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>EMEM</td>
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<tr>
<td>FFA</td>
<td>Free fatty acid</td>
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<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<td>GFRα1</td>
<td>GDNF, family receptor α1</td>
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<td>High-fat diet</td>
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<tr>
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<td>Hypericin</td>
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<td>Mitogen-activated protein kinases</td>
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<td>OLT</td>
<td>Orthotopic liver transplantation</td>
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<tr>
<td>PPARs</td>
<td>Peroxisome proliferator-activated receptors</td>
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<td>PBS</td>
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References


Figure 1. Gross view of the perfusion of HFD-fed mouse livers before (A) and after (B) perfusion Photographs show livers of mice fed a HFD for 8 weeks and perfused with vehicle for 4h.
Figure 2. GDNF perfusion reduces hepatic macrosteatosis
A. Representative images showing Oil Red-O-stained perfused livers from HFD-fed mice.
B. Plot of Oil Red-O-stained area per 1000 cells. Livers were perfused with vehicle, defatting cocktail or GDNF (n=4 mice in all HFD/RD fed groups). Data is presented as means + SEM. [* P<0.05, ** P<0.01, *** P<0.001]
Figure 3. GDNF perfusion reduces hepatocyte triglyceride content
Triglyceride content of non-perfused and perfused livers from HFD-fed mice was assessed as described in the Methods section (n=4 mice in all HFD/RD fed groups). Plotted are means + SEM. [* P<0.05, ** P<0.01]
Figure 4. Total LDH activity in RD/HFD fed mice liver tissues
LDH activity was assessed in liver tissue with and without 4h of perfusion, in RD and HFD-fed mice, as described in the Methods (n=4 mice in all HFD/RD fed groups). Plotted are means + SEM. [* P<0.05]
Figure 5. Experimental timeline for the in vitro experiments
HepG2 cells were cultured as shown and treated with steatosis inducing or reducing agents.
Figure 6. GDNF reduces hepatocyte lipid content in vitro

A. HepG2 cells were cultured in medium supplemented with 1 mM palmitate or without palmitate (vehicle) in the presence or absence of GDNF (100 ng/ml) and Nile-Red staining performed. B. Comparison of Nile-Red staining area for HepG2 cells cultured in medium supplemented with 1 mM palmitate or without palmitate (vehicle) in the presence or absence of GDNF (100 ng/ml). Plotted are means + SEM. [** P<0.01].
Figure 7. GDNF induces lipolysis in HepG2 cells
HepG2 cells were cultured in medium supplemented with 1 mM palmitate in the presence of GDNF (100 ng/ml) or absence of GDNF (vehicle). The FFA (A) and glycerol (B) concentration was measured in the medium. Plotted are means + SEM. [* P<0.05, ** P<0.01]