Induction of Cardiac Angptl4 by Dietary Fatty Acids Is Mediated by Peroxisome Proliferator-Activated Receptor β/δ and Protects Against Fatty Acid–Induced Oxidative Stress

Anastasia Georgiadi, Laetitia Lichtenstein, Tatjana Degenhardt, Mark V. Boekschoten, Marc van Bilsen, Beatrice Desvergne, Michael Müller, Sander Kersten

Rationale: Although dietary fatty acids are a major fuel for the heart, little is known about the direct effects of dietary fatty acids on gene regulation in the intact heart.

Objective: To study the effect of dietary fatty acids on cardiac gene expression and explore the functional consequences.

Methods and Results: Oral administration of synthetic triglycerides composed of one single fatty acid altered cardiac expression of numerous genes, many of which are involved in the oxidative stress response. The gene most significantly and consistently upregulated by dietary fatty acids encoded Angiopoietin-like protein (Angptl)4, a circulating inhibitor of lipoprotein lipase expressed by cardiomyocytes. Induction of Angptl4 by the fatty acid linolenic acid was specifically abolished in peroxisome proliferator-activated receptor (PPAR)β/δ−/− mice and was blunted on siRNA-mediated PPARβ/δ knockdown in cultured cardiomyocytes. Consistent with these data, linolenic acid stimulated binding of PPARβ/δ but not PPARα to the Angptl4 gene. Upregulation of Angptl4 resulted in decreased cardiac uptake of plasma triglyceride-derived fatty acids and decreased fatty acid–induced oxidative stress and lipid peroxidation. In contrast, Angptl4 deletion led to enhanced oxidative stress in the heart, both after an acute oral fat load and after prolonged high fat feeding.

Conclusions: Stimulation of cardiac Angptl4 gene expression by dietary fatty acids and via PPARβ/δ is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently fatty acid–induced oxidative stress. (Circ Res. 2010;106:1712-1721.)

Key Words: peroxisome proliferator-activated receptor ■ Angptl4 ■ fatty acids ■ gene expression ■ cardiac oxidative stress

Cardiac contractility is dependent on the adequate delivery of oxygen and energy substrates to the heart followed by their efficient metabolic degradation to yield ATP. The energy requirements of the contracting heart are primarily met by fatty acid oxidation, with the remainder of energy coming from glucose and lactate. Although fatty acids are thus of major importance to the heart, excessive uptake of fatty acids causes lipid overload or lipotoxicity and may compromise cardiac function, possibly leading to cardiomyopathy. Consequently, cardiac uptake of fatty acids needs to be well adjusted to fatty acid utilization. Because most of the fatty acids taken up by the heart are derived from lipoprotein lipase (LPL)-dependent hydrolysis of circulating triglyceride-rich lipoproteins, the activity of LPL needs to be carefully regulated via specific activators and inhibitors, especially after a fatty meal.

Besides serving as a major fuel for the heart and a potential lipotoxic substrate, fatty acids are able to regulate gene expression. In vitro experiments in rat cardiomyocytes have shown that fatty acids increase expression of uncoupling protein 2, carnitine palmitoyltransferase 1, fatty acid transporter Cd36, fatty acid binding protein 3, acyl-coenzyme (Co)A synthetase long-chain family member 1, acyl-CoA thioesterase, and long chain acyl-CoA dehydrogenase. As these genes all represent target genes of peroxisome proliferator-activated receptor (PPAR)α, they suggest an important role of PPARα in fatty acid–dependent gene regulation in the heart. However, little is known about the direct...
effects of dietary fatty acids on gene expression in the intact heart. In addition, it is unclear what pathways are activated by fatty acids besides their own catabolism.

Here we studied the comprehensive effects of dietary fatty acids on cardiac gene expression in vivo by giving mice a single oral bolus of synthetic triglyceride composed entirely of one single fatty acid, which were either linolenic acid (C18:3), linoleic acid (C18:2) or oleic acid (C18:1). Subsequent microarrays analysis yielded Angptl4 as the gene most highly induced in the heart after oral fat administration. The collective data suggest that induction of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and is part of a feedback mechanism aimed at protecting cardiomyocytes against lipid overload and consequently fatty acid–induced oxidative stress, eg, lipotoxicity.

Methods

Materials

GW501516 was purchased from Alexis (Axxora, Raamsdonkveer, The Netherlands). Wy14643 was obtained from Eagle Picher Technologies laboratories (Lenexa, Kan). Trilinolein (9c,12c) and trilinolein (9c,12c,15c) were from Larodan Free Chemicals (Malmo, Sweden). SYBR green was from Eurogentec (Seraing, Belgium), and all other chemicals were from Sigma (Zwijndrecht, The Netherlands).

Animals

Pure-bred Sv129 PPARα−/− mice (129S4/SvJae) and corresponding wild-type mice (129S1/SvImJ) were purchased from Jackson Laboratory (Bar Harbor, Maine). The Angptl4−/− and transgenic mice were on C57Bl/6 background and have been previously described.13,14 The PPARβ/δ−/− mice were on a mixed background (Sv129/C57Bl/6) and have been previously described.15 Males mice were used at 2.5 to 4 months of age.

Mice were anesthetized with a mixture of isoflurane (1.5%), nitrous oxide (70%), and oxygen (30%). Blood was collected via orbital puncture into EDTA tubes. After euthanasia, the hearts were excised and stored in −80°C. The animal studies were approved by Animal Ethics Committee of Wageningen University and the University of Lausanne, Switzerland.

Oral Lipid Load

Starting at 5:00 AM, the animals were fasted for 4 hours, followed by an intragastric gavage of 400 μL of synthetic triglyceride (triolein, trilinolein, and trilinolein). The control group received only carboxymethylcellulose (CMC). The mice were killed 6 hours thereafter. Four to 5 mice per group were used.

High-Fat Diet

Angptl4−/−, +/+ , and transgenic mice on C57Bl/6 background received a low-fat diet (LFD) or high-fat diet (HFD) for 8 weeks, providing 10 or 45 energy percent in the form of triglycerides, respectively (D12450B or D12451, Research Diets, New Brunswick). The major source of fat in the diet was palm oil, with 5 energy percent provided as soybean oil.

Cell Culture

Neonatal cardiomyocytes were isolated and cultured as described using differential plating to separate myocytes from nonmyocytes.16 The experiments were approved by the Animal Ethics Committee of Maastricht University. Neonatal cardiomyocytes were incubated with 1 μmol/L GW501516 or 62.5 μmol/L, 125 μmol/L, or 250 μmol/L linolenic for 6 hours as previously described.17 In a second experiment, cardiomyocytes were incubated with 1 μmol/L GW501516, 10 μmol/L Wy14643, or 250 μmol/L linolenic acid for 24 hours.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>4HNE</td>
<td>4-hydroxynonenal</td>
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<tr>
<td>Angptl4</td>
<td>angiopoietin-like protein 4</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>CMC</td>
<td>carboxymethylcellulose</td>
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<td>CoA</td>
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<td>LFD</td>
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<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
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<tr>
<td>PPARE</td>
<td>peroxisome proliferator-activated receptor response element</td>
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<tr>
<td>Tg</td>
<td>transgenic</td>
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<td>TSS</td>
<td>transcription start site</td>
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<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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Plasma Lipid Parameters

Plasma was obtained from blood by centrifugation for 10 minutes at 10,000 g. The plasma free fatty acids and triglyceride concentration were determined using kits from Instruchem (Delfzijl, The Netherlands).

Results

Dietary Fatty Acids Have a Major Impact on Cardiac Gene Expression

To study the acute effects of dietary fatty acids on cardiac gene expression in vivo, SV129 mice were given a single oral gavage of synthetic TGs composed entirely of either C18:1, C18:2, or C18:3,18 thus mimicking a postprandial lipid challenge. Animals receiving carboxymethylcellulose were used as control to study the absolute effect of dietary fatty acids. Expression profiling was carried out on individual mouse hearts collected 6 hours after the gavage using Affymetrix Mouse Genome 430 2.0 Arrays. Pathway analysis using Ingenuity revealed that the predominant pathway affected by the oral fat load was nuclear factor-like 2 (Nrf2)-related oxidative stress, indicating that the fatty acids induced oxidative stress (Online Figure I). This was supported by examination of the top 20 of upregulated genes, most of which were involved in the oxidative stress response, including uncoupling protein 3 (Ucp3), heme oxygenase 1 (Hmox1), FK506 binding protein 5 (Fkbp5), lipocalin 2 (Lcn2), glutathione S-transferase A3 (Gsta3), and metallothionein 2 (Mt2) (Figure 1). A large correspondence in gene regulation between the fatty acids was observed, especially between C18:2 and C18:3. Indeed, scatter plot analysis indicated that the effects of C18:2 and C18:3 on cardiac gene expression were remarkably similar, whereas effects of C18:1 were somewhat different (Online Figure II). Therefore, the remainder of the present article focuses on effects of C18:3.

Apart from genes involved in the oxidative stress response, various genes involved in lipid metabolism were also induced by the fatty acids. Interestingly, the gene most significantly and consistently upregulated by each of the dietary fatty acids was Angptl4 (Figure 1), which encodes a secreted protein...
involved in the regulation of plasma triglyceride levels. Previous studies have shown that Angptl4 potently inhibits LPL and accordingly plasma triglyceride clearance by converting active LPL-dimers into inactive LPL-monomers. Although Angptl4 is known to be expressed in heart, the specific cardiac cell types that produce Angptl4 remain unclear. Accordingly, we performed immunohistological staining of Angptl4 in human heart samples. The results reveal the presence of Angptl4 protein in cardiomyocytes and vascular smooth muscle cells but not endothelial cells and fibroblasts (Figure 2A). Significant production of Angptl4 by cardiomyocytes was confirmed by the relatively low Ct values for amplification of Angptl4 cDNA from rat cardiomyocytes (Ct 22 to 23, data not shown).

**Regulation of Angptl4 by Dietary PUFA Is Entirely Mediated by PPARβ/δ**

Long-chain fatty acids are bona fide ligands for PPARs. The previous demonstration that Angptl4 is a direct PPAR target gene prompted us to investigate the role of PPARs in Angptl4 gene regulation by dietary fatty acids. We first determined the relative expression of PPARs in mouse heart. All three PPARs are expressed in mouse heart, with the highest expression of PPARβ/δ (Figure 1).
In cultured neonatal rat cardiomyocytes, expression of PPARs (Online Figure III). PPAR efficiency of 100% for all PPARs (Online Figure III). PPAR expression was calculated as 1/2^\text{CtPPAR}

or negative control Rplp0 was observed (Figure 3F). These results demonstrate that the induction of cardiac Angptl4 gene expression by dietary C18:3 is mediated by PPARβ/δ.

The fatty acid- and PPARβ/δ-mediated induction of cardiac Angptl4 expression likely occurred in cardiomyocytes, as treatment of rat neonatal cardiomyocytes for 6 hours with C18:3 dose-dependently increased Angptl4 mRNA, which at the highest concentration was equivalent to that obtained using GW501516 (Figure 3G). To further investigate the specific role of PPARβ/δ in Angptl4 upregulation by fatty acids in cardiomyocytes, we knocked-down PPARα or PPARβ/δ in the cardiomyocyte cell line H9c2, which expresses both receptors, using siRNA and studied the effect on Angptl4 gene induction by C18:3 (Figure 3H). We observed that knock-down of PPARβ/δ almost entirely abolished the induction of Angptl4 gene expression by C18:3, whereas knock-down of PPARα had little to no effect (Figure 3I).

Our results do not imply that Angptl4 is an exclusive target gene of PPARβ/δ under any type of circumstances. Indeed, we find that in rat neonatal cardiomyocytes, Angptl4 is induced to a similar extent by synthetic PPARα and PPARβ/δ agonists, as are other cardiac PPAR targets such as Acs1 and Acox1 (Figure 3J). Instead, our data suggest that the stimulatory effect of dietary fatty acids on cardiac Angptl4 expression is mediated specifically by PPARβ/δ.

**Induction of Angptl4 Protects Against Fatty Acid–Induced Oxidative Stress**

To study the effect of Angptl4 on the metabolic response to dietary fat, we performed the oral fat load with C18:3 triglyceride in wild-type, Angptl4−/− and Angptl4 transgenic (Angptl4-Tg) mice. In agreement with inhibition of LPL by was entirely abolished in the PPARβ/δ−/− mice, whereas it was retained in the PPARα−/− mice (Figure 3A). In contrast, upregulation of Ucp3, another well-characterized PPAR target gene, was retained in PPARβ/δ−/− mice and completely abolished in PPARα−/− mice (Figure 3B). No compensatory increase in PPARβ/δ and PPARα expression was observed in PPARα−/− and PPARβ/δ−/− mice, respectively (Online Figure IV).

To examine whether regulation of Angptl4 by PPARβ/δ and not PPARα was supported by binding of PPARβ/δ to the Angptl4 gene, we performed chromatin immunoprecipitation (ChIP). Previously, we located the response element responsible for PPAR-mediated upregulation to intron 3 of the Angptl4 gene.25 Consistent with data on Angptl4 gene regulation, ChIP on hearts of wild-type mice six hours after oral gavage of C18:3 showed enhanced binding of PPARβ/δ but not PPARα to the intronic PPRE (Figure 3C).

Nuclear receptors and other transcription factors bound to such distal sites likely contact the basal transcription machinery via DNA looping, and accordingly binding of PPAR to distant PPREs can be demonstrated by showing cross-linking of PPAR to the transcriptional start site (TSS).26,27 Indeed, oral gavage of C18:3 enhanced binding of PPARβ/δ but not PPARα to TSS of the Angptl4 gene (Figure 3D), whereas C18:3 enhanced binding of PPARα but not PPARβ/δ to the TSS of the Ucp3 gene (Figure 3E). No binding of either PPARα or PPARβ/δ to the negative control gene Rplp0 was observed (Figure 3F). These results demonstrate that the induction of cardiac Angptl4 gene expression by dietary C18:3 is mediated by PPARβ/δ.

**Induction of Angptl4 Protects Against Oxidative Stress**

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Angptl4, the postprandial increase in plasma triglyceride was dramatically increased in Angptl4-Tg mice, whereas it was entirely blunted in Angptl4/H11002/H11002 mice (Figure 4A). Consistent with LPL inhibition, Angptl4 overexpression markedly reduced cardiac fatty acid uptake from [3H]triolein-labeled very-low-density lipoprotein (VLDL)-like particles (Figure 4B). These results suggest that upregulation of Angptl4 by dietary fatty acids will lead to reduced cardiac uptake of fatty acids via inhibition of LPL, thereby suppressing the stimulus that led to induction of Angptl4 expression.

To examine whether the inhibitory effect of Angptl4 on cardiac fatty acid uptake is associated with reduced fatty acid-induced oxidative stress, expression of Fkbp5, Lcn2, and Gsta3 was determined 6 hours after oral gavage with either control treatment (CMC) or C18:3 triglyceride in wild-type, Angptl4/H11002/H11002, and Angptl4-Tg mice. All three genes represent markers of oxidative stress.28–30 Consistent with a protective role of Angptl4 against fatty acid-induced oxidative stress, the magnitude of induction of Fkbp5, Lcn2, and Gsta3 by C18:3 was dependent on Angptl4 genotype and inversely correlated with Angptl4 expression (Figure 5A and 5B). Expression of Fkbp5, Lcn2, and Gsta3 after the oral fat load was not related to plasma free fatty acid (FFA) levels, which followed an opposite pattern (Figure 5C).

Figure 3. PPARβ/δ but not PPARα mediates the induction of Angptl4 expression by dietary linolenic acid. Wild-type, PPARα−/−, and PPARβ/δ−/− mice were given a single oral gavage of 0.5% CMC (open bars) or synthetic triglycerides composed entirely of C18:3 (closed bars). mRNA expression levels of Angptl4 (A) and Ucp3 (B) were determined in mouse heart using real-time PCR. Results are expressed as fold change compared to the wild-type control mice. C through F, ChIP was performed on hearts of wild-type mice given an oral gavage of either CMC or C18:3. Chromatin was precipitated using antibodies against PPARα or PPARβ/δ. Rabbit IgG was used as a specificity control. Real-time quantitative PCR was performed on reverse–cross-linked chromatin templates using primers specific to the known PPRE in intron 3 of the Angptl4 gene (C), the TSS of Angptl4 (D), the TSS of Ucp3 (E), and the negative control gene RpLp0 (F). G, Angptl4 expression in rat neonatal cardiomyocytes incubated with increasing concentrations of linolenic acid (0, 62.5, 125, and 250 μmol/L) or GW501516 (1 μmol/L) for 6 hours. H, Expression of PPARα and PPARβ/δ in H9c2 cardiomyocytes transfected with siRNA against PPARα and PPARβ/δ. I, Fold induction of Angptl4 expression by 6 hours of linolenic acid treatment (250 μmol/L) in H9c2 cardiomyocytes transfected with siRNA against PPARα and PPARβ/δ. J, Expression of Angptl4 and known PPAR targets Acs1 and Acox1 in rat neonatal cardiomyocytes incubated for 24 hours with linolenic acid (250 μmol/L), GW501516 (1 μmol/L), or Wy14643 (10 μmol/L). Error bars represent SEM. Statistical significance was determined with a Student’s t test (P<0.05).
endoplasmic reticulum stress marker Herpud1 mimicked the pattern of oxidative stress markers (Figure 5A).

Finally, we examined whether Angptl4 may exert a similar effect in the context of a chronic fat overload. To that end, we measured expression of the oxidative stress markers and performed immunohistochemical and quantitative analysis of 4-HNE protein adducts in wild-type and Angptl4−/− mice fed a HFD for 8 weeks. 4-HNE is one of the major biologically active aldehydes formed during inflammation and oxidative stress. Formation of 4-HNE protein adducts is a marker for lipid peroxidation.

Although high fat feeding did not influence expression levels of Fkbp5, Lcn2 and Gsta3, expression was significantly higher in Angptl4−/− mice fed HFD compared to wild-type mice fed HFD (Figure 6A). A similar trend was observed for Herpud1. Furthermore, lipid peroxidation was increased in Angptl4−/− mice fed HFD, as shown by enhanced 4-HNE staining (Figure 6B). These results were supported by quantitative analysis of 4HNE protein adducts (Figure 6C) and MDA adducts (Figure 6D), which were significantly increased in Angptl4−/− mice fed HFD compared to wild-type mice fed HFD. These data indicate that Angptl4 protects against oxidative stress in the context of a chronic fat overload. No differences in cardiac triglyceride levels were observed between wild-type and Angptl4−/− mice (Figure 6E).

**Discussion**

In the present article we show that the gene most significantly and consistently upregulated by short term treatment with dietary fatty acids is Angptl4. Induction of Angptl4 by dietary fatty acids is mediated by PPARγ and confers a protective effect against fatty acid-induced oxidative stress by restricting cardiac fatty acid uptake via inhibition of LPL. Overall, our data suggest that upregulation of Angptl4 by fatty acids is part of a feedback mechanism aimed at preventing myocardial fatty acid accumulation, thereby minimizing lipid-induced oxidative stress and lipotoxicity (Figure 7). Although our follow-up studies only included linolenic acid, the results are likely generalizable to other dietary fatty acids.

Lipotoxicity describes the untoward consequences of fat overload in a particular tissue and may be related to fatty acid-induced oxidative stress, accumulation of lipotoxic intermediates such as ceramides and fatty acyl-CoA, and excess storage of triglycerides.31 Chronic lipotoxicity in the heart has
been shown to promote cardiomyopathy in several animal models. Most of these models are characterized by a mismatch between myocardial fatty acid uptake and utilization, as in mice with heart-specific overexpression of acyl-CoA synthetase, fatty acid transport protein 1, or lipoprotein lipase. Although triglycerides are unlikely to be the actual culprit in cardiac lipotoxicity, they may be guilty by association as their levels may be positively correlated with lipotoxic intermediates. However, we did not see increased cardiac triglyceride levels in Angptl4−/− mice compared to wild-type mice after 8 weeks of high fat feeding, suggesting that the increase in fatty acid uptake is limited or that the incoming fatty acids are efficiently oxidized. In the present study, it was not possible to investigate the effect of Angptl4 deletion on cardiac lipid storage, oxidative stress, and parameters of cardiac dysfunction after a more prolonged period of HFD, as a cachectic phenotype progressively emerges after 12 weeks of HFD (L.L. et al, manuscript in preparation).

In a previous study, heart-specific Angptl4 overexpression reduced cardiac LPL activity and reversed the excessive lipid storage in hearts of lipotoxic acyl-CoA synthetase transgenic mice. In agreement with these data, we find that Angptl4 overexpression reduced cardiac fatty acid uptake and protects against fatty acid-induced oxidative stress. In contrast, Angptl4 deletion aggravated oxidative stress both acutely and after chronic HFD. Because we used whole body Angptl4 overexpression and deletion models, strictly we cannot rule out that the observed effects may be related to changes in extracardiac Angptl4 expression. However, the current literature mainly supports a paracrine function of Angptl4, and the role of Angptl4 as an endocrine factor remains somewhat uncertain.

Angptl4 was discovered by screening for target genes of PPARα and PPARγ in liver and adipose tissue, respectively. It is member of a family of angiopoietins and angiopoietin-like proteins and is produced by a variety of organs. Numerous studies using Angptl4 transgenic or knockout mice have invariably shown a stimulatory effect of Angptl4 on plasma triglyceride levels, which is achieved by inhibiting LPL activity. These data have established Angptl4 as an important regulator of plasma triglyceride levels. The present data suggest that Angptl4 is upregulated by dietary fatty acids to inhibit local LPL activity and consequently reduce fatty acid uptake and lipid-induced oxidative stress.

To study the role of PPARs in cardiac gene regulation by dietary fatty acids, we ideally should have used cardiomyocyte-specific PPAR−/− mice but unfortunately we did not have access to these animals. The PPAR-dependent upregulation of Angptl4 and Ucp3 by dietary linoleic acid underscores the importance of TG-rich lipoproteins as source of PPAR ligands in the heart, which are liberated via LPL.
Remarkably, upregulation of Angptl4 by C18:3 in intact heart and cultured cardiomyocytes was entirely mediated by PPARβ/δ and not PPARα. These data were supported by ChIP data showing C18:3-induced binding of PPARβ/δ and not PPARα to the Angptl4 gene in the intact heart. Because we did not study PPARγ, strictly we cannot rule out a role for PPARγ in mediating the effect of (dietary) fatty acids on cardiac Angptl4 expression. However, similar results were obtained in cultured cardiomyocytes, which express little PPARγ, the data favor a specific role of PPARβ/δ in the regulation of cardiac Angptl4 by C18:3. It is unclear what mechanism may underlie the differential role of PPARβ/δ versus PPARα in mediating induction of Angptl4 and Ucp3 by C18:3, respectively. Induction of Ucp3 by linoleic acid via PPARα rules out a PPARβ/δ-specific ligand activity that is generated by linolenic acid in heart. Gel shift and transactivation studies have failed to provide convincing evidence for the existence of response elements or promoters that are specifically or selectively bound or regulated by a particular PPAR isotype. Indeed, in vitro experiments have revealed that all three PPARs are intrinsically able to (trans)activate the human and mouse Angptl4 gene. However, the situation may be different in vivo in the absence of PPAR overexpression or when PPARs are activated via endogenous ligands rather than via high-affinity synthetic agonists. Thus, the dominant receptor in the regulation of a particular PPAR target is likely context- and tissue-dependent and additionally depends on whether PPAR is activated via endogenous or synthetic agonists. When as in cardiomyocytes two or more PPARs are expressed in the same cell and are simultaneously activated, it is possible that specific binding of one PPAR isotype to a particular PPRE is promoted via interactions with another protein that binds adjacent to the PPRE and is expressed in a tissue-specific manner. In this context, it is interesting to mention that recent genome wide profiling of PPARα and PPARγ binding sites revealed colocalization of PPAR binding with other transcription factor binding sites and demonstrated interplay between PPARs and other transcription factors in PPAR-mediated gene regulation.

Recently, targeted PPARβ/δ overexpression in the heart was shown to have a clear differential effect on cardiac metabolism compared to PPARα overexpression. In contrast to PPARα, PPARβ/δ overexpression did not impact fatty acid transport and failed to induce myocardial lipid accumulation. Based on the data presented here it can be hypothesized that PPARβ/δ is neutral toward cardiac lipid storage by inducing Angptl4 expression, which in turn feeds back on fatty acid uptake.

Multiple studies support an effect of Angptl4 on endothelial function, mostly pointing to an antiangiogenic activity of Angptl4. Our immunohistochemical results indicate that Angptl4 is absent from vascular endothelial cells in the heart, whereas it is abundantly present in cardiomyocytes. These data are in line with previous studies showing that Angptl4 is absent from a number of different endothelial cells, yet is dramatically induced under hypoxic conditions. Hypoxia also upregulates Angptl4 in cardiomyocytes. Induction of Angptl4 by hypoxia and the associated inhibition of fatty acid uptake may be an adaptive mechanism to shift fuel use toward glucose, which requires less oxygen for oxidation.

In conclusion, our data show that an acute oral load of triglycerides stimulates an oxidative stress response in the heart. The concomitant upregulation of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently fatty acid–induced oxidative stress, one of the hallmarks of lipotoxic cardiomyopathy.

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Disclosures
None.

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**Novelty and Significance**

**What Is Known?**

- Little is known about the impact of dietary fat on gene expression in the heart.
- Peroxisome proliferator-activated receptors (PPARs) mediate the effects of fatty acids on gene expression in several tissues.
- The secreted protein angiopoietin-like protein (Angptl)4 is an inhibitor of plasma triglyceride hydrolysis.

**What New Information Does This Article Contribute?**

- Dietary fat induces the expression of Angptl4 in the heart.
- Induction Angptl4 by fat is mediated by PPARβ/δ, and it protects the heart against lipid overload and oxidative stress.

Dietary fat is the major fuel for the heart, but little is known about its impact on the expression of cardiac genes. We studied, for the first time, the effect of specific fatty acids on whole genome expression in the heart. We found that the gene most highly induced by dietary fat was Angptl4. This gene codes for a secreted inhibitor of plasma triglyceride hydrolysis. Angptl4 was found to be produced by cardiomyocytes, and its induction by fat was mediated by the transcription factor PPARβ/δ, but not PPARα. Induction of Angptl4 was associated with a decrease in lipid uptake from the blood. Myocardial lipid overload and oxidative stress were also decreased. These findings suggest that Angptl4 is a major modulator of fat uptake in the heart and that it participates in a novel feedback mechanism that involves PPARβ/δ. Angptl4 protects the heart against lipid overload and fatty acid–induced oxidative stress after ingestion of dietary fat.
Induction of Cardiac Angptl4 by Dietary Fatty Acids Is Mediated by Peroxisome Proliferator-Activated Receptor β/δ and Protects Against Fatty Acid–Induced Oxidative Stress
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**Supplement Material**

**Detailed Methods**

*RNA isolation and qRT-PCR:*

Total RNA was isolated with TRIzol Reagent (Invitrogen, Breda, the Netherlands). 1µg of total RNA for the *in vivo* studies and 350ng of total RNA for the *in vitro* experiment was reverse transcribed using iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on BioRad MyIQ or iCycler machine using Platinum Taq polymerase (Invitrogen, Breda, the Netherlands). PCR primer sequences were taken from the PrimerBank and ordered from Eurogentec (Seraing, Belgium). Sequences of the primers used are presented on Online Table I. To compare expression of the three PPAR isotypes in adult mouse heart and in rat neonatal cardiomyocytes, primers were used that yielded amplicons of equal length. A standard curve was included to confirm an amplification efficiency of 100%±2 for all PPARs and for the 18S control gene. PPAR expression was calculated as 1/(2^(CtPPAR-Ct18S)), allowing for direct comparison between the PPAR isotypes.

*Chromatin immunoprecipitation assay (ChIP):*

Wildtype C57Bl/6 mice were fasted for 4 hours followed by an oral gavage of Trilinolenin (n=3). Six hours thereafter the mice were killed by cervical dislocation and the hearts excised. The fresh hearts were cut into half and placed into PBS containing 1% formaldehyde. Cross-linking was stopped after 15 min by adding glycine to a final concentration of 0.125M for 5 min at room temperature. The samples were centrifuged for 5 min at 700 g at 4 °C to collect the heart pieces, the supernatant was removed and washed once again with ice-cold PBS. Fresh PBS containing protease inhibitors (Roche, Almere, Netherlands) was added and the tissue was disaggregated with a homogenizer Ultra Turrax T25 basic (Ika Werke, Staufen, Germany). The tissue was distributed into 3 tubes (2 mL each), centrifuged for 5 min at 700 g at 4°C. After the supernatant was removed, heart homogenate was resuspended in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1, protease inhibitors) and the lysates were sonicated with a Bioruptor™ (Diagenode, Liège, Belgium) to achieve a DNA length of 300-800 bp. After removal of cellular debris by centrifugation, supernatants were diluted 1:10 in ChIP dilution buffer (150mM NaCl, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 7.5, protease inhibitors). Chromatin was incubated overnight at 4°C with 2µg antibody, 25µl BSA (100 mg/ml) and 2.4µl sonicated salmon sperm (10 mg/ml). The following antibodies were used: anti-PPARα (sc-9000), and anti-PPARβ/δ (sc-7197). All were obtained by Santa Cruz Biotechnologies (Heidelberg, Germany). Immunocomplexes were collected with 25µl MagaCell® Protein A Magnetic beads (Isogen Life Science) for 1hour at room temperature, and subsequently washed with 700µL of the following buffers: ChIP was buffer 1 (150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, protease inhibitors) two times, ChIP wash buffer 2 (500mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, protease inhibitors), ChIP wash buffer 3 (250 mM LiCl, 1% NP40, 1% Deoxycholate, 1mM EDTA, 10 mM Tris-HCl pH 8), two times TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8). Elution of immunocomplexes were carried out in 250µL elution buffer (10 mM EDTA, 0.5% SDS, 25mM Tris-HCl pH 7.5) at 64°C for 30 min. After collection of supernatant, elution was repeated with 250µl elution buffer at room temperature for 2 min. After combining the supernatants, cross-linking was reversed at 64°C overnight with 2.5µl Proteinase K (20 mg/ml) for digestion of any remaining proteins. Genomic DNA fragments were recovered by phenol-chloroform extraction with phase lock gel (Eppendorf, Wesseling-Berzdorf, Germany), followed by salt-ethanol precipitation. Samples were diluted in sterile H2O, and analyzed with qPCR.
The ChIP data are normalized against IgG to account for non-specific immunoprecipitation. A fold-enrichment value of 1 represents baseline thus no enrichment and no specific precipitation.

Primers were chosen to study binding of PPARs to the transcriptional start site of the Angptl4 and Ucp3 genes, and to the previously identified PPRE within intron 3 of the Angptl4 gene. The ribosomal phosphoprotein P0 (Rplp0) was used as negative control for PPAR binding. The sequences of primers used in ChIP were as follows:

Ucp3-TSS: (For: 5'-GAGCCCCAGGTCACGGAAG-3', Rev: 5'-CTGTGCGTCTAGCAGGTTG-3'),
Angptl4-TSS: (For: 5'-CCAGCAAGTTCATCTCGTC-3', Rev: 5'-TCCCTCCACTCCACACC-3'),
Angptl4-PPRE: (For: 5'-TCTGGGTCTGCCCATCCTGCG-3', Rev: 5'-GTGTGTGTGTGGATACGGCTAT-3'),
Rplp0 (For: 5'–CGAGGACCGCCCTGGTTCTC-3', Rev: 5'–GTCACTGGGGAGAGAGG-3').

In Vivo Clearance of very low density lipoprotein (VLDL)-Like Emulsion Particles:
Tissue uptake of $[^3]H$-labeled TG packaged into VLDL-like emulsion particles was measured as previously described. The data shown represent the percentage of injected radioactivity taken up by the heart after 30 min.

Immunohistochemistry:
Deep frozen tissues (-80°C) were cryosectioned (5µm) with a cryostat (Leica, CM1900 UV). Immunostaining of protein adducts of the lipid peroxidation byproduct 4-hydroxy-2-nonenal (4-HNE) was performed on freshly cut frozen sections using a rabbit polyclonal antibody (Calbiochem, San Diego, CA, USA). All steps were carried out at room temperature. The tissue was fixed in 70% ethanol for 3 min and then rinsed in PBS 1X for 3 min. To block endogenous peroxidase activity, slides were incubated with 3% H$_2$O$_2$ for 10 min. Incubation with the primary antibody (rabbit anti 4-HNE protein-adducts, 1:50 dilution in PBS 1X) was performed for 1h. After rinsing with PBS 1X, tissue was incubated for 45 min with the secondary antibody (Dako EnVision+® System Labelled Polymer-HRP AntiRabbit). Visualization of the complex was carried out using AEC substrate chromogen (Dako Cytomation) for 15 minutes. Sections were mounted with Kaiser’s glycerol gelatin mounting medium (Merck KGaA, Darmstadt, Germany). Normal rabbit serum was used as a negative control (Vector Laboratories).

Immunostaining of Angptl4 in human heart was performed using an antibody directed against the C-terminus of Angptl4. Five-micrometer sections of paraffin-embedded human heart were mounted on Superfrost microscope slides. These sections were dewaxed in xylene and rehydrated in a series of graded alcohols. To block endogenous peroxidase activity, slides were incubated with 3% H$_2$O$_2$ for 20 min. Antigen retrieval was performed by placing the slides in citrate buffer (pH 6.0) and heat them in a microwave oven 5 min 700 W (without lid) and 4 times 5 min 500 W (with lid). After cooling down to room temperature, the sections were briefly washed with PBS. Prior to staining, a 20 min preincubation was performed using 20% normal goat serum (Vector Laboratories, Burlingame, CA, USA). Incubation with the primary antibody (1:50) was performed for 1h. After rinsing with PBS 1X, tissue was incubated for 45 min with the secondary antibody (Dako EnVision+® System Labelled Polymer-HRP AntiRabbit). Visualization of the complex was carried out using AEC substrate chromogen (Dako Cytomation) for 15 minutes. After counterstaining with Meyer’s hematoxylin, sections were mounted with DePex mounting medium (Gurr, BDH, Poole, Dorset, UK). Negative control staining was performed using only the secondary antibody.
Tissue homogenization and quantification of oxidative stress:
The extent of lipid peroxidation in heart homogenates was determined by measuring the levels of hydroxynonenal-histidine (HNE-His) protein adducts and malondialdehyde (MDA) adducts. 25mg of heart tissue were homogenized in 250µL of tissue homogenation buffer (1mM EDTA, PBS 1X pH 7.4 containing protease inhibitors). Heart tissue was homogenized over ice by needle sonication for 15sec at 40V. Heart homogenates were centrifuged at 1600xg for 10min at 4ºC. Protein content was determined in tissue supernatants by BCA Protein assay reagent. Starting from a protein concentration of 10 µg/mL 4-HNE-His protein adducts and MDA adducts content were quantified using the Oxiselect HNE-His Adduct ELISA kit (Cell Biolabs Inc., San Diego, USA) and MDA Adduct ELISA kit (Cell Biolabs Inc.), respectively. The quantity of HNE-His protein adducts was determined by using a standard curve containing known amounts of HNE-BSA (0-10µg/ml). For the quantification of MDA adducts a standard curve of known amounts of MDA-BSA (0-120pmol/mg) was used.

Tissue triglyceride content:
Triglycerides content was measured in tissue homogenates with Triglyceride LiquiColor® Test (Mono) HUMAN GmbH, kit (Instruchemie, Delfzijl, The Netherlands). 5% tissue homogenates were prepared by needle sonication over ice in tissue homogenization buffer consisting of 10mM Tris, 2mM EDTA, 0.25M sucrose pH 7.5.

Affymetrix microarray and pathway analysis:
Expression profiling was carried out on individual mouse hearts. Total RNA (5 µg) was labeled using the Affymetrix One-cycle Target Labeling Assay kit (Affymetrix, Santa Clara, CA). The correspondingly labeled RNA samples were hybridized on Affymetrix Mouse Genome 430 2.0 Arrays, washed, stained and scanned on an Affymetrix GeneChip 3000 7G scanner. Packages from the Bioconductor project, integrated in an in-house developed on-line management and analysis database for multiplatform microarray experiments, were used for analyzing the scanned arrays. Probesets were redefined according to Dai et al. as the genome information utilized by Affymetrix at the time of designing the arrays is not current anymore, resulting in unreliable reconstruction of expression levels. In this study probes were reorganized based on the Entrez Gene database, build 36, version 2 (remapped CDF v10). Expression estimates were obtained by GC-robust multi-array (GCRMA) analysis, employing the empirical Bayes approach for background adjustment, followed by quantile normalization and summarization. Differentially expressed probesets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularisation of standard errors. A probeset was found to be significantly changed after treatment if p<0.05. For the pathway analysis ingenuity software version 6.5 was used.

siRNA-mediated silencing of PPARα and PPARβ/δ in H9c2 cardiomyocytes:
H9c2 cardiomyoblasts at passage number 18 were seeded at a density of 40000 cells/well in 6-well plates and subsequently grown for 24h in DMEM, antibiotic-free medium containing 10% FCS. After 24h, cells were transfected for 72h with siRNA molecule (100pmol/ml) according to the DharmaFECT 1 siRNA Transfection Protocol Thermo Scientific for H9c2. The siRNA oligos used were selected from a set of 4 individual sequences (ON-TARGETplus Set of 4) designed by Dharmacon that we tested for efficient silencing of PPARα and PPARβ/δ expression in H9c2 cells. The final target sequence used for PPARα was 5’-UCACCGAGCUCACGGAAUU-3’ and for PPARβ/δ 5’-CAUGAGUUCUUUGCCGAGUA-3’. Transfection medium was replaced with DMEM, antibiotic-free medium containing 10% FCS 48h after the siRNA transfection. After 72h, linolenic acid (250 µM) was added for 6h,
followed by harvesting of the cells. Cell viability was assessed with trypan blue and exceeded 80%.
Online Figure I: Induction of oxidative stress response 6 hours after the oral ingestion of linolenic acid (A), linoleic acid (B) or oleic acid (C).

Bars show the percentage of upregulated genes (red) and downregulated genes (green) out of the total number of eligible genes for each pathway, based on the Ingenuity knowledge database. The white part represents the percentage of genes that do not overlap with the experimental dataset. The pathways are displayed from the direction of the most significantly regulated to least significantly regulated based on Fisher’s Exact Test p-value (cut off p<0.05). The –log(p-value) is displayed on the top of each pathway (yellow square).
Online Figure II: Similarity in cardiac gene regulation between fatty acids.
Graphs show fold-change in gene expression after treatment with C18:3TG (y-axis) plotted against fold-change in gene expression after treatment with C18:2TG (A) or C18:1TG (B) (x-axis). Analysis shows more significant similarity in gene regulation between C18:3 and C18:2, compared to between C18:3 and C18:1.
Online Figure III: Equal efficiency of amplification of mPPARα, mPPARβ/δ, and mPPARγ in mouse heart.
A standard curve was generated from cDNA prepared from mouse heart. cDNA was amplified by qPCR using primers specific for the three PPAR isotypes. Similar efficiencies of amplification were obtained using cDNA from rat neonatal cardiomyocytes.
Online Figure IV: No compensatory increase in PPARβ/δ and PPARα expression in PPARα-/- and PPARβ/δ-/- mice, respectively.
Wildtype, PPARα-/-, and PPARβ/δ-/- were given a single oral gavage of 0.5% CMC (open bars) or synthetic TG composed entirely of C18:3 (closed bars). mRNA expression levels of PPARβ/δ (left panel) and PPARα (right panel) were determined in mouse heart using real-time PCR. Results are expressed as fold-change compared to the WT control mice. Error bars represent SEM.
### Online Table I: Primer sequences used for qPCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward sequence</th>
<th>Reversed sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse PPAR(\alpha)</td>
<td>TATTCGGCTGAAGCTGGTGTAC</td>
<td>CTGGCATTTGTCGGGTCTT</td>
</tr>
<tr>
<td>mouse PPAR(\beta/\delta)</td>
<td>TGAGCCCAAGTTCGAGTTTG</td>
<td>CGGCTCCACACAGAATGATG</td>
</tr>
<tr>
<td>mouse PPAR(\gamma)</td>
<td>CAC AAT GCC ATC AGG TTT GG</td>
<td>GCT GGT CGA TAT CAC TGG AGA</td>
</tr>
<tr>
<td>rat PPAR(\alpha)</td>
<td>CACCCCTTCTCAGGCTTCAAG</td>
<td>GCCTGTCCCACATATTGG</td>
</tr>
<tr>
<td>rat PPAR(\beta/\delta)</td>
<td>AACAGATCAGCGTGAGTGTG</td>
<td>TGAGGAAGAGGCTGTAGAAGT</td>
</tr>
<tr>
<td>mouse Angptl4</td>
<td>GTCGCAAGACTCAGCTCAAGGG</td>
<td>CAAAGAGGCTAGAAGTGTT</td>
</tr>
<tr>
<td>mouse PPAR(\gamma)</td>
<td>CAC AAT GCC ATC AGG TTT GG</td>
<td>GCT GGT CGA TAT CAC TGG AGA</td>
</tr>
<tr>
<td>mouse Ucp3</td>
<td>TCGTGAGATGGTGAGCTTAG</td>
<td>CCAAGGCGAGACAAAGT</td>
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<tr>
<td>mouse Gsta3</td>
<td>TCGAGATGAGGGATGAACTT</td>
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<tr>
<td>mouse Lcn2</td>
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<td>TGAGGAAGGAGGAGCTG</td>
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<tr>
<td>mouse Acs1</td>
<td>ACCACCTTCTGAGGCTCAC</td>
<td>TGAGATCGTGTAGTACCC</td>
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<td>mouse Acox1</td>
<td>TGAAATGGCCAGGTACTG</td>
<td>ATCTGGTTGAGGCTAGT</td>
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<tr>
<td>mouse Cyclophilin</td>
<td>TGTCTGGAACTTTGCTGCAA</td>
<td>CAGACGCCACTGTCGCTT</td>
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<td>mouse 36B4</td>
<td>ATGGTGACTAAGCAGCGTCCTG</td>
<td>GCCCTGACCTTTTCAGTAAG</td>
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Supplemental references


