Diabetic cardiomyopathy is a secondary complication of diabetes with an unclear etiology. Based on a functional genomic evaluation of obesity-associated cardiac gene expression, we previously identified and cloned the gene encoding apolipoprotein O (APOO), which is overexpressed in hearts from diabetic patients. Here, we generated APOO-Tg mice, transgenic mouse lines that expresses physiological levels of human APOO in heart tissue. APOO-Tg mice fed a high-fat diet exhibited depressed ventricular function with reduced fractional shortening and ejection fraction, and myocardial sections from APOO-Tg mice revealed mitochondrial degenerative changes. In vivo fluorescent labeling and subcellular fractionation revealed that APOO localizes with mitochondria. Furthermore, APOO enhanced mitochondrial uncoupling and respiration, both of which were reduced by deletion of the N-terminus and by targeted knockdown of APOO. Consequently, fatty acid metabolism and ROS production were enhanced, leading to increased AMPK phosphorylation and \( Ppara \) and \( Pgc1a \) expression. Finally, we demonstrated that the APOO-induced cascade of events generates a mitochondrial metabolic sink whereby accumulation of lipotoxic byproducts leads to lipoapoptosis, loss of cardiac cells, and cardiomyopathy, mimicking the diabetic heart–associated metabolic phenotypes. Our data suggest that APOO represents a link between impaired mitochondrial function and cardiomyopathy onset, and targeting APOO-dependent metabolic remodeling has potential as a strategy to adjust heart metabolism and protect the myocardium from impaired contractility.
Apolipoprotein O is mitochondrial and promotes lipotoxicity in heart

Annie Turkieh, Céline Caubère, Manon Barutaut, Franck Desmoulin, Romain Harmancey, Michel Galinier, Matthieu Berry, Camille Dambrin, Carlo Polidori, Louis Casteilla, François Koukoui, Philippe Rouet, and Fatima Smih

1INSERM U1048 Equipe no. 7, Obésité et insuffisance cardiaque: approches moléculaires et cliniques, Toulouse, France.
2Hôpital Rangueil, Toulouse, France.
3University of Camerino, Camerino, Italy.
4INSERM U1031 Equipe no. 1, Toulouse, France.

Introduction

Diabetic cardiomyopathy is a secondary complication of diabetes with an unclear etiology. Based on a functional genomic evaluation of obesity-associated cardiac gene expression, we previously identified and cloned the gene encoding apolipoprotein O (APOO), which is overexpressed in hearts from diabetic patients. Here, we generated APOO-Tg mice, transgenic mouse lines that expresses physiological levels of human APOO in heart tissue. APOO-Tg mice fed a high-fat diet exhibited depressed ventricular function with reduced fractional shortening and ejection fraction, and myocardial sections from APOO-Tg mice revealed mitochondrial degenerative changes. In vivo fluorescent labeling and subcellular fractionation revealed that APOO localizes with mitochondria. Furthermore, APOO enhanced mitochondrial uncoupling and respiration, both of which were reduced by deletion of the N-terminus and by targeted knockdown of APOO. Consequently, fatty acid metabolism and ROS production were enhanced, leading to increased AMPK phosphorylation and Pparα and Pgc1α expression. Finally, we demonstrated that the APOO-induced cascade of events generates a mitochondrial metabolic sink whereby accumulation of lipotoxic byproducts leads to lipoapoptosis, loss of cardiac cells, and cardiomyopathy, mimicking the diabetic heart–associated metabolic phenotypes. Our data suggest that APOO represents a link between impaired mitochondrial function and cardiomyopathy onset, and targeting APOO-dependent metabolic remodeling has potential as a strategy to adjust heart metabolism and protect the myocardium from impaired contractility.

Diabetic cardiomyopathy is a secondary complication of diabetes with an unclear etiology. Based on a functional genomic evaluation of obesity-associated cardiac gene expression, we previously identified and cloned the gene encoding apolipoprotein O (APOO), which is overexpressed in hearts from diabetic patients. Here, we generated APOO-Tg mice, transgenic mouse lines that expresses physiological levels of human APOO in heart tissue. APOO-Tg mice fed a high-fat diet exhibited depressed ventricular function with reduced fractional shortening and ejection fraction, and myocardial sections from APOO-Tg mice revealed mitochondrial degenerative changes. In vivo fluorescent labeling and subcellular fractionation revealed that APOO localizes with mitochondria. Furthermore, APOO enhanced mitochondrial uncoupling and respiration, both of which were reduced by deletion of the N-terminus and by targeted knockdown of APOO. Consequently, fatty acid metabolism and ROS production were enhanced, leading to increased AMPK phosphorylation and Pparα and Pgc1α expression. Finally, we demonstrated that the APOO-induced cascade of events generates a mitochondrial metabolic sink whereby accumulation of lipotoxic byproducts leads to lipoapoptosis, loss of cardiac cells, and cardiomyopathy, mimicking the diabetic heart–associated metabolic phenotypes. Our data suggest that APOO represents a link between impaired mitochondrial function and cardiomyopathy onset, and targeting APOO-dependent metabolic remodeling has potential as a strategy to adjust heart metabolism and protect the myocardium from impaired contractility.

In order to uncover how changes in APOO expression relate to modifications of cardiac function, we performed in silico, in vitro, and in vivo manipulations using (a) cardiac myoblasts, (b) 3 independent cardiac-specific transgenic mouse lines expressing APOO at physiological levels (referred to herein as APOO-Tg mice), (c) in vivo transfected mouse liver, and (d) human heart samples. We showed that APOO localizes to mitochondria in cardiomyocytes and enhances uncoupling. This leads to a specific cascade of mal-adaptive events leading to cardiomyopathy. We demonstrated the

Authorship note: Annie Turkieh and Céline Caubère contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2014;124(5):2277–2286. doi:10.1172/JCI74668.
The Journal of Clinical Investigation

The onset of cardiomyopathy. Our present findings outline the inter

original link between impaired mitochondrial function and the

function and cardiac metabolism. The APOO model represents an

importance of APOO as a new signal regulator of mitochondrial

expression revealed enrichment in various meta-

and lowest

expression ratios between microarrays with the highest

Figure 1

Characterization of APOO-Tg mice. (A) APOO mRNA levels in WT (n = 38) and APOO-Tg (n = 32) mice. (B) APOO (55 kDa) Western blot analysis of WT

and APOO-Tg mouse heart extracts. Calreticulin (48 kDa) was used as loading control and detected with calreticulin antibody on the same membrane

after dehybridization. Lanes were run on the same gel but were noncontiguous. (C–E) WT and APOO-Tg mice were aged 18–20 weeks and fed HFD for

9–10 weeks. (C) PR interval, measured from the beginning of the P wave to the beginning of the QRS complex. (D) Representative echocardiographic images. (E) TEM of myocardial sections (longitudinal). Arrowheads denote cristae in markedly altered mitochondria (aM), nM, normal mitochondria; nZB, normal Z bands. Original magnification, ×10,000 (left and center), ×15,000 (right). *P < 0.05, ***P < 0.001. Data represent mean ± SEM.

importance of APOO as a new signal regulator of mitochondrial function and cardiac metabolism. The APOO model represents an original link between impaired mitochondrial function and the onset of cardiomyopathy. Our present findings outline the interplay between mitochondrial dysfunction and lipotoxicity.

Results

APOO is mitochondrial and associates with heart dysfunction in mice and humans. Examination of publicly available human heart microarray datasets (from patients undergoing cardiac transplantation and from “normal” organ donors; see Methods) revealed that APOO mRNA levels fluctuated between 1 and 5 AU. Pathway analysis using synthetic expression ratios between microarrays with the highest and lowest APOO expression revealed enrichment in various metabolic pathways, the most significant being oxidative phosphorylation and mitochondrial dysfunction (Supplemental Figure 1, A–D; supplemental material available online with this article; doi:10.1172/JCI74668DS1). We generated transgenic mouse lines constitutively expressing human APOO in heart at physiological levels (<3-fold endogenous APOO; Figure 1, A and B). Under a HFD, the APOO-Tg mice exhibited depressed ventricular function. Specifically, these mice displayed a lengthening of the PR interval and reductions in ejection fraction (56.65% ± 1.74% vs. 63.41% ± 2.11%, P < 0.05), fractional shortening (25.45% ± 1.08% vs. 29.77% ± 1.320%, P < 0.05), and LV posterior wall thickness (48.47% ± 4.70% vs. 65.05% ± 3.89%, P < 0.05) (Figure 1, C and D). Transmission electron microscopy (TEM) analysis of longitudinal myocardial sections revealed mitochondrial degenerative changes, such as loss or discontinuity of cristae and swelling (Figure 1E and Supplemental Figure 2A). Irreversible disorganization and degeneration in the myocardium, like myofibrillar disarray and decreased myofibrillar content, were evident. In some cardiomyocytes, a widening of intercellular junctions was also observed. In severely affected areas, we observed intercellular dissociation and ultrastructural evidence of marked loss of contractile elements, which produced a diffuse pattern in defective myocytes that led to cell death (Supplemental Figure 2, B–E), correlating with the alteration of hemodynamic parameters. In silico investigation of the APOO sequence revealed a putative N-terminal mitochondrial “address label.” We then designed expression vectors and generated different cardiac myoblast transfectants expressing human APOO (referred to herein as APOO cells), as well as APOO cells in which APOO expression was diminished by transfection of APOO shRNA vectors (shAPOO; Supplemental Figure 3, A–C) and APOO cells with N-terminal deletion of 40 aa of APOO. In vivo fluorescent labeling, as well as studies with protein extracts and subcellular fractions from APOO cells, revealed a mitochondrial localization for this protein (Figure 2, A–C). Deletion of the 40 N-terminal residues changed APOO distribution from the mitochondria to the cytoplasm (Figure 2, D and E). Furthermore, hydrodynamics-based in vivo mouse liver transfection by rapid tail vein injection of pTT-APOO expression vector led to a rise in APOO expression, as evidenced by PCR amplification of the transfected expression vector and an increased level of APOO protein in the mitochondria (Supplemental Figure 4, A–C).

APOO promotes mitochondrial uncoupling and respiration. APOO cells displayed a 2-fold increase in basal oxygen consumption that was dependent on mitochondrial localization of APOO and ablated by shAPOO treatment (Figure 3A). Antimycin, an inhibitor of the quinone cycle, almost completely inhibited oxygen consumption (Figure 3B), which indicates that most of the respiration being measured is mitochondrial. Basal oxygen consumption was partly inhibited by oligomycin in APOO cells, and addition of the uncoupler carbonyl cyanide m-chlorophenyl hydrazone led to a 2-fold increase in oxygen consumption (Figure 3B), which suggests that these cells have enhanced electron transport activity. Respiration coupling (RC) calculations revealed decreased RC in APOO cells (Figure 3C). We observed equivalent results with isolated mitochondria from hydrodynamics-based in vivo transfected mouse liver, which displayed a significant increase in oxygen consumption (Supplemental Figure 4D). These effects were also associated with increased intracellular ROS (Figure 3D). Thus, APOO has 2 distinct effects on mitochondrial function: an increase in uncoupling and an increase in total respiration.
We observed a significant increase in cytochrome C oxidase activity, a mandatory component of the respiratory chain, in APOO cells and APOO-Tg hearts (Figure 3E and Supplemental Figure 5). We also demonstrated a significant increase in the expression of oxidative phosphorylation genes in APOO cells, which was reduced by both N-terminal deletion of APOO and shAPOO treatment (Figure 3, F and G).

APOO enhances fatty acid metabolism. APOO is highly expressed in mitochondria-enriched tissues that mainly use fatty acids as an energy source (6). Therefore, we assumed that the APOO-induced increase in electron transport chain flux would increase the mitochondrial use of long-chain fatty acids (LCFAs) and induce a mitochondrial metabolic sink. Indeed, in order to provide mitochondria with LCFAs, cells would ultimately increase fatty acid uptake at the plasma membrane.

We analyzed whether APOO could induce the expression of genes involved in fatty acid metabolism. Long-chain acyl-CoA synthetase (ACSL) and FATP4 catalyze LCFA esterification, allowing lipid channeling. In APOO cells, fatty acid transporter (CD36 and FATP4) expression and ACSL activity were strongly increased, effects that were significantly reversed by treatment with either shAPOO or the ACSL inhibitor triacsin C (Figure 4, A–C). Accordingly, we measured rapid accumulation of green fluorescent BODIPY-palmitate in APOO-expressing cells and found that basal intracellular fatty acids increased 120% in these cells (Figure 4, D and E). Deletion of APOO 40 N-terminal residues deviated the intracellular fatty acids increased 120% in these cells (Figure 4, F and G). Similarly, hearts from APOO-Tg mice displayed a significant increase in fatty acid transporter expression and ACSL expression and activity (Figure 5, A–D). In human atrial appendage samples, APOO expression positively correlated with the expression of CD36, FATP4, and ACSL3 (Figure 5, E–G). Human heart microarray data mining (see Methods) were in accordance with these results and showed that CD36 and FATP2 expression were also increased in the hearts of patients with elevated APOO expression (Supplemental Figure 6, A and B). These data suggest that slight APOO overexpression affects not only mitochondrial uncoupling and respiration, but also fatty acid metabolism.

APOO increases lipotoxicity. When excessive fatty acid uptake exceeds mitochondrial fatty acid oxidative capacity, toxic lipid storage increases, resulting in lipotoxicity (13). Palmitate treatment of APOO cells induced a dramatic intracellular accumulation of diglycerides and did not significantly affect triglyceride levels compared with control cells. Deletion of the 40 N-terminal aa of APOO significantly reduced cellular diglyceride levels (Figure 6, A and B). Likewise, while triglyceride levels remained the same in controls, diglycerides increased 1.6-fold in APOO-Tg hearts and 2.5-fold in transfected liver (Figure 6, C and D, and Supplemental Figure 4, E and F). In human heart samples, APOO mRNA levels correlated with diglyceride levels, but not with triglyceride levels (Figure 6, E and F).

We hypothesized that APOO overexpression promotes apoptosis and found positive correlations between mRNA levels of APOO and the proapoptotic factor BAX in human heart (Figure 7A). Even with modest overexpression of APOO in hearts from HFD-fed APOO-Tg mice, Bax/Bcl2 expression ratio and caspase-3 activity were enhanced (Figure 7, B and C). These results were confirmed in vitro with APOO cells, in which increased Bax expression and...
caspase-3 activity were significantly reversed by shAPOO treatment (Figure 7, D and E). This treatment “cured” APOO cells that afterward presented characteristic morphological amelioration, including reduced cell body condensation and cytoplasmic vacuolization. Moreover, APOO overexpression dramatically amplified the apoptotic effect of increasing doses of palmitate, which moderately increased caspase-3 activity in control cells (Figure 7F). LCFA uptake exceeds mitochondrial fatty acid oxidative capacity and leads to lipotoxicity, especially in the presence of saturated LCFA such as palmitate. Excess palmitate generates toxic lipid byproducts, such as diglycerides. This toxicity can be diminished by the addition of unsaturated lipids, such as oleate (14–16), which compels palmitate to produce nontoxic triglycerides. Indeed, APOO cells treated with both palmitate and oleate displayed a 12-fold decrease in diglyceride/triglyceride ratio and a 2-fold decrease in caspase-3 activity (Figure 7, G–I).

These changes should be associated with an increase in expression of PPARα, a transcription factor known to be involved in lipid uptake and β-oxidation (17). Indeed, activity of PPARα was increased in diabetic hearts, and Ppara overexpression in mice led to cardiac dysfunction. Expression of Ppara was enhanced in APOO cells and in vivo transfected mice (Figure 8, A and B). APOO expression was also associated with an increase in PPARα mRNA levels in human atrial appendage samples and in hearts from APOO-Tg mice (Figure 8, A and B). Accordingly, we found that AMPK was activated in APOO versus control cells (Supplemental Figure 7C).

These results suggest a potential link between pathological APOO overexpression and the induction of mitochondrial dysfunction, which should ultimately trigger mitochondrial biogenesis. Expression of APOO and PPARγ coactivator 1α (PGC-1α), a master regulator of mitochondrial biogenesis (18), were tightly correlated in human right atrial appendage samples from patients undergoing heart surgery and in hearts from APOO-Tg mice (Figure 8, C and D). The increased mitochondrial synthesis may be an adaptive response that balances the mitochondrial alteration or degradation in autophagosomal vacuoles and multilamellar bodies observed in APOO-Tg hearts and APOO cells (Figure 9, A–D) and attempts to restore falling cellular energy. In support of this, the ATP/AMP ratio was decreased in APOO cells (Supplemental Figure 7D). Ultrastructural analyses have revealed mitochondrial damage in mouse models of the metabolic syndrome and in models of type 1 and type 2 diabetes mellitus. We believe that the pathological overexpression of APOO induces a mitochondrial metabolic sink and drives the cell into a vicious cycle that ends in mitochondrial alteration, apoptosis, and cell death in the presence of high extracellular concentration of saturated lipid. This hypothesis could explain the myofibrillar and cardiomyocyte loss observed in APOO-Tg hearts (Supplemental Figure 7C). We propose APOO as a central molecule in lipid and mitochondrial homeostasis.

Discussion

Analysis of numerous human myocardial samples revealed that APOO mRNA levels slightly fluctuated between 1 and 5 AU and correlated with mitochondrial dysfunction. Pursuant to this, we were unable to generate cardiac-specific transgenic mice overexpressing APOO more than 5-fold, which suggests that APOO levels higher than this are extremely deleterious in mouse heart. APOO is thus different from other mitochondrial proteins, like monoamine oxidase A, that can be overexpressed greater than 600-fold in transgenic mouse heart (19). Either Apo gene deletion in yeast (8) or slight overexpressions in mammalian cells (present study) are deleterious for mitochondria. Our results

![Figure 3](https://example.com/figure3.png)

**Figure 3**

Role of APOO in respiration and oxidative stress in cardiac myoblasts. (A) Basal oxygen consumption in control cells, APOO cells, APOO Δ1–40 cells, or APOO cells transfected with shAPOO (n = 5). (B) Oxygen consumption of control and APOO cells treated with 1.5 μg/ml oligomycin (Oligo), 2 μM carbonyl cyanide m-chlorophenyl hydrazine (CCCP), or 1 μM antimycin (Ant) (n = 5). (C) RC index in control and APOO cells. (D) ROS, measured in the presence of increasing doses of 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), in control and APOO cells (n = 4). (E) Cytochrome C oxidase activity in control and APOO cells (n = 4). (F and G) Expression levels of (F) mitochondrial complex I and (G) mitochondrial complex III genes in control cells, APOO cells, APOO cells subsequently transfected with shAPOO, and APOO Δ1–40 cells. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001. Data represent mean ± SEM.
provided evidence that a basal level of APOO expression is needed for optimized mitochondrial function and that tight regulation of APOO expression is essential for maintaining normal heart health and homeostasis. In recent studies in yeast (8) and *Caenorhabditis elegans* (9), Apoo orthologs have been proposed to be mitochondrial. Interestingly, mutation in the Apoo ortholog of *C. elegans* led to cristae disorganization, similar to that observed upon modest APOO overexpression in mouse heart. We have previously shown that APOO colocalized with perilipins in cardiac myoblasts (6). This is not in discrepancy with the present work, since perilipins also localize to mitochondria (20).

APOO enhances mitochondrial uncoupling. Consequently, electron transport chain activity and oxygen consumption increase. Previous studies in animal models (21, 22) and in humans (23) suggested that mitochondrial uncoupling plays a key role in the development of cardiomyopathy in obesity and diabetes. However, in *ob/ob* heart studies, mitochondrial uncoupling was not associated with increased protein levels of the...
uncoupling proteins (UCPs). Thus, the observed uncoupling in these hearts is independent of changes in the expression of mitochondrial UCPs (24), which suggests the uncoupling may reflect activation of allosteric modification of the UCPs and/or unknown mitochondrial uncoupling mechanisms. Given our present findings, it is tempting to propose that APOO is implicated in this process. In diabetic heart, increased myocardial oxygen consumption is associated with increased fatty acid oxidation in animal models (25, 26) and humans (27). Indeed, obesity and diabetes are forerunners to secondary organ failure through excessive ectopic lipid deposition. This lipotoxicity manifests as cardiomypathy, myopathy, fatty liver, and diabetes (28). The exact nature of the signal that leads to enhanced and sustained fatty acid uptake in the cardiomyocyte remains uncertain. In cardiomyocytes overexpressing APOO, a specific cascade of events originated with APOO-induced uncoupling. To compensate for the resulting proton leak, the electron transport chain activity increased and depleted the mitochondrial NADH and FADH substrates, generating a mitochondrial metabolic sink. To supplement mitochondria with LCFA, the cardiomyocyte elevated the number of fatty acid transporters. In diabetic heart, fatty acid oxidation and lipid accumulation are considerably increased, and the cardiomyocyte dies by lipoapoptosis (13, 29). The APOO-induced mitochondrial metabolic sink could be an elucidation for the lipid overload. Saturated fatty acids like palmitate exacerbated this toxicity, since more diglycerides are formed. Available data indicate that fatty acid transporters, intracellular fatty acids, and diglycerides have an important role in the etiology of cardiac disease, especially diabetic cardiomyopathy (28, 30–34). In agreement with our present findings, different studies indicate that injury originates inside the myocyte and is not simply secondary to systemic changes associated with hyperlipidemia (35).

Animal lipotoxicity models display evident mitochondrial dysfunction. In these models, lipid accumulation has been proposed to precede the reduction in mitochondrial function. However, converse mechanisms have been proposed in which mitochondrial dysfunction plays a more causative role (36–39). In our models, lipotoxicity appears to be a consequence and not a cause of mitochondrial dysfunction, and our hypothesis implies that mitochondrial failure may be an initial event in diabetic cardiomyopathy.

We also showed that ROS are produced in APOO cells. Several studies have shown that ROS are increased in both type 1 and type 2 diabetes and suggest that increased ROS production is a contributing factor to the development and progression of diabetic cardiomyopathy (40). In support of this, it was reported that overexpression of the mitochondrial antioxidant enzyme manganese superoxide dismutase in the heart of a mouse model of type 1 diabetes mellitus partially reversed altered mitochondrial morphology and function and maintained cardiomyocyte function (41). Moreover, several studies have shown that ROS-mediated mitochondrial biogenesis is likely to occur via the upregulation and actions of PGC-1α. More recently, it was demonstrated that gene expression of Pgc1α and its target gene, Ppara, are regulated by ROS via several signaling kinases in response to various stimuli, including phosphorylation and activation of AMPK (42). Elevated ROS leads to activation of AMPK, likely via a decline in ATP levels. AMPK activation, in turn, increases mRNA expression of Ppara (43–45). APOO overexpression reduces ATP levels and activates AMPK, which could explain the high correlation we observed between the expression of APOO and that of PGC1α and PPARα in human and mouse heart. Conversely, AMPK and PPARα can be directly activated by intracellular fatty acids or their derivatives (46). ApoO cells increased intracellular fatty acid levels by 120%. Inhibiting APOO targeting to the mitochondrion or treating APOO cells with shAPOO normalized the intracellular levels of fatty acids, which suggests that AMPK and PPARα could be downstream effectors of APOO. The lipid-enhancing effect of APOO is probably achieved by activating cell respiration that results in increasing fatty acid transporter expression, lipotoxicity, and apoptosis.

High or irreversible uncoupling damages mitochondria and contributes to the pathogenesis of a panel of clinically distinct disorders (47). In addition to sustained or irreversible uncou-
pling, which underlies cell death, it has also been shown that transient and mild uncoupling occurs incessantly in intact cells and in ex vivo beating hearts (48, 49). The baseline or homeostatic function of the uncoupling has been widely studied, and although there is no general agreement, there is a strong conviction that mild uncoupling may be involved in modulating mitochondrial ROS production. Indeed, several studies indicate that physiological levels of ROS are required for the regulation of diverse cellular processes (50–52). Thus, a picture is emerging that uncoupling is a double-edged sword exerting both protective and harmful effects. These findings raise the intriguing possibility that basal APOO level participates in maintaining the inner mitochondrial membrane leak.

In conclusion, we have shown for the first time that APOO induces a rise in respiration, intracellular lipids, and apoptosis. This could be diminished by blocking APOO transfer to the mitochondria or by treating the cells with shAPOO. These treatments not only restored the respiration rate, but also reduced fatty acid transporter expression, lipid accumulation, and apoptosis, as indicated by the decreased activity of caspase-3 and expression of fatty acid transporter genes. Apoop gene expression is upregulated in heart from animal models after 9 weeks of HFD, i.e., in the early phases of metabolic stress (7). APOO could be a trigger molecule for diabetic cardiomyopathy. Taken together, our findings suggest that partial inhibition of cardiac APOO in individuals predisposed to diabetic cardiomyopathy may represent a valid strategy by which to adjust heart metabolism and protect the myocardium from impaired contractility and function.

Methods
Further information can be found in Supplemental Methods.

Animal studies
Generation of APOO-Tg mice. The Amhc promoter–APOO transgene was constructed from a 5.5-kb BamHI–Sall fragment containing the murine Amhc promoter (53) and a Sall–HindIII cDNA fragment containing the human APOO coding sequence (6). The Amhc promoter–APOO transgene was linearized with NotI, purified by electroelution, concentrated on an elutip-d column (Schleicher and Schuell), and used for nuclear injection in fertilized eggs of B6D2/F1 hybrid females. The microinjected oocytes were then reimplanted in B6CBA/F1 hybrid pseudopregnant foster mothers. 3 transgenic mouse lines were generated and crossed with C57BL/6J mice. Genomic DNA was extracted using DNAeasy blood and tissue kit in a QIAcube apparatus (Qiagen). Offspring were followed by PCR using primers rtiMHCP1F (5′-CCTAGCCCACACCAGAAATGA-3′) and rtiMHCP1R (5′-CCCCACGGACCTCTGAATTA-3′) and Dynazyme II enzyme (Ozyme) as recommended. PCR was performed at least 3 times per mouse, and PCR products were analyzed by acrylamide gel electrophoresis. Animals were...
housed at the Toulouse I2MC animal core facility in a room lit 12 hours per day (6am–6pm) at an ambient temperature of 22°C ± 1°C.

9- to 10-week old transgenic and nontransgenic littermate male mice were fed a HFD (catalog no. D12492; Research Diet) for 9 weeks.

**Cellular models**

**Cell culture and transfection of H9c2 cardiac myoblasts.** H9c2 cells were obtained from the European Collection of Cell Cultures and cultured in DMEM (Life Technologies). Cells were grown in a 5% CO2 incubator at 37°C with saturating humidity. H9c2 cardiomyoblasts were transfected by electroporation, and pools of transfectants were selected as previously described (54).

**Generation of APOO-overexpressing cells.** To overexpress human APOO (pTT-APOO), the APOO coding sequence was amplified using primers hAPOO5BamPTT (5′-CGCGGATCCGCCAGTTTGGTGTA-3′) and hAPOO3BamPTT (5′-CGCGGATCCCTAGTTCCAGGTGAATTCTTCA-3′) and cloned into the BamH1 site of pTT expression vector (55). To localize APOO in H9c2 cardiac cells, these cells were transfected with pSNAP-APOO vector. pSNAP-APOO was generated by PCR amplification of pTT-APOO using primers SnapApoEcOR-F (5′′-AAGATATCATGTTCAAGGTAATTCAGAGG-3′′) and SnapApoEcorV-R (5′′-TTGATATCCTTAGTTCCAGGTGAATTCTT-3′′) and cloned into the EcoRV site of pSNAP-tag (Ozyme). All primers used herein were synthesized by Eurogentec. All restriction enzymes used were from New England Biolabs. All APOO sequences within the expression vectors were verified by DNA double-strand sequencing using ABI PRISM BigDye Terminator version 3.1 Ready reaction cycle sequencing kit (Life Technologies) and loaded on an ABI 3130XL DNA sequencing instrument (Life Technologies).

**Knockdown of APOO overexpression.** shRNA used to knock down APOO gene expression and control shRNA were from MISSION shRNA set (Sh2, TRCN 72707; Sh4, TRCN 72704; Sh5, TRCN 72705; Sigma-Aldrich) and used as recommended by generating pools of stable

---

**Figure 8**

APOO associates with increased PPARα and PGC-1α expression. (A and B) Correlation between APOO and PPARα mRNA levels in human atrial heart appendage samples (A; n = 34) and in hearts from 3 APOO-Tg mouse lines (B; n = 70). (C and D) Correlation between APOO and PGC1A mRNA levels in human atrial heart appendage samples (C; n = 29) and in APOO-Tg mouse hearts (D; n = 70).

**Figure 9**

Mitochondrial alteration and degradation in cardiac myoblasts overexpressing APOO. TEM analysis of (A) control and (B–D) APOO cells treated for 24 hours with 100 μM palmitate. Boxed regions in B are shown at higher magnification in C and D. APOO cells demonstrated degradation of mitochondria in autophagosomal vacuoles and multilamellar bodies. mf, myelin figure; N, nucleus. Original magnification, ×6,000 (A and B); ×50,000 (C); ×35,000 (D).
transfectants. Empty vector control (no shRNA insert) was also transfected and had no significant effect.

Production and purification of recombinant HIS-tagged APOO. To produce recombinant His6-tagged APOO fusion protein, the APOO coding sequence was amplified using primers CherryApoF (5′-GGGAAATTC-CATATGTTCAAGGTATCCAGGTTCC-3′) and CherryApoR (5′-ATAGTTTACGCGCCCTGTACGGTAACTTCT-3′). The APOO fragment was then cloned into the NdeI/NotI sites of pSCodonL2 (Eurogentec) expression vector. pSCodonL2-APOO plasmid was then transfected into SE1 E. coli cells (Eurogentec). Cells were grown in 2-L Luria-Bertani medium with 50 μg/ml ampicillin (Euromedex) at 37°C with shaking at 200 rpm in an orbital shaker (New Brunswick Scientific) until A600nm reached 0.5. Production of recombinant His6-tagged APOO fusion protein was induced by 1 mM isopropyl β-D-thiogalactoside for 3 hours. Cells were pelleted at 5,000 g for 15 minutes and frozen in liquid nitrogen. Recombinant APOO was purified using nickel–nitrilotriacetic acid–agarose in batch using an artificial chaperone-assisted refolding method as previously described (6).

Functional genomics
Bioinformatics. Microarray expression data from human heart samples were downloaded from GEO (accession no. GSE11445). This series consisted of 107 myocardial samples collected from patients undergoing cardiac transplantation whose failure arose from different etiologies (e.g., idiopathic dilated cardiomyopathy, ischemic cardiomyopathy, valvular cardiomyopathy, and hypertrophic cardiomyopathy) and from "normal" organ donors whose hearts could not be used for transplants. Arrays were intensity normalized, and hierarchical clustering was applied (average group linkage, Pearson correlation, threshold r = 0.8) to identify groups of coexpressed genes. APOO expression levels were plotted for the 107 human hearts tested and used to define relevant molecular pathways using both Toppgene (56) and Ingenuity Pathway Analysis (Ingenuity Systems).

Human heart samples
After ethical committee approval, all patients in this study gave written consent for sample collection and molecular analysis prior to inclusion. Patients were carefully selected by physicians from the Department of Cardiology, Toulouse University Hospital, prior to coronary bypass surgery.

Hydrodynamics-based in vivo transfection of the mouse liver
DNA was administered as described previously, with minor modifications, by a single hydrodynamic injection of 50 μg plasmid in 2 ml isotonic NaCl in the tail vein of 20- to 24-g mice over 6–8 s (57).

Echocardiography
Echocardiograms were performed using the Vivid 7 PRO echocardiographic system (GE Medical System) equipped with an i13 L14-MHz linear-array transducer. Images were obtained from chest-shaven rats lightly anesthetized by 1%–2% isoflurane (Baxter) lying on their back with transducers placed on the left hemithorax. 2D parasternal long- and short-axis images of the LV were obtained, and 2D targeted M-mode tracings were recorded at a sweep speed of 200 mm/s. All measurements were performed according to the recommendations of the American Society of Echocardiography. The leading-edge method was applied to 3 consecutive cardiac cycles (n) with the roundness of the LV cavity (2D image) as a criterion that the image was on axis. Great effort was taken to achieve good image quality and to visualize the endocardial and epicardial borders of the heart by gently moving and orientating the transducer. Fractional shortening (a measure of LV systolic function) was calculated as (EDD − ESD)/EDD, where EDD and ESD are end-diastolic and end-systolic diameters, respectively, and expressed as a percentage.

ECG
Surface ECGs were recorded using an ADI system (ADIInstruments Ltd.).

Statistics
All results are depicted as mean ± SEM. Multiple comparisons were analyzed by ANOVA — followed, when appropriate, by Dunnett post-hoc test — using Statview 4.5 software (Abacus Concepts). Single comparisons were performed by 2-tailed unpaired Student's t test. A P value of 0.05 or less was considered significant.

Study approval
All experimental procedures on mice were performed according to INSERM and Genotoul Anexpol animal core facility guidelines for the care and use of laboratory animals. Human heart (right appendage) samples were collected after approval from the “Comité de Protection des Personnes (CPP) Sud Ouest et Outre Mer I et II” (no. DC 2008-452) and informed signed consent of the patients.

Acknowledgments
We thank P. Romanienko for reading the manuscript; A. Galnier for aiding with COX activity measurement; M. Andre, P. Guillou, S. Eddery, and P. Decaens for technical assistance; T. Picard for bioinformatics analyses; and the MetaSys and Genotoul networks for electron microscopy, cellular imaging, lipidomics, metabolomics, animal, and biocomputing facilities. This study was supported by INSERM and INSERM/Paul Sabatier University grant.

Received for publication December 9, 2013, and accepted in revised form February 20, 2014.

Address correspondence to: Fatima Smih, INSERM U1048 Equipe no. 7, 1 avenue Jean Poulhiés, BP 84225, 31432 Toulouse Cedex 4, France. Phone: 33.5.31.22.41.18; Fax: 33.5.61.32.29.52; E-mail: Fatima.smith-rouet@insERM.fr.


