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Myocardial Dysfunction and Male Mortality in Peroxisome Proliferator-Activated Receptor Alpha Knockout Mice Overexpressing Lipoprotein Lipase in Muscle

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SUMMARY: Free fatty acids (FFA) are liberated from triglyceride-rich lipoproteins by lipoprotein lipase (LPL) and are considered to be a principal energy source for the heart. The peroxisome proliferator-activated receptor alpha (PPARα) is a key regulator of FFA catabolism. To investigate its role in cardiac muscle metabolism, transgenic mice overexpressing LPL in skeletal and cardiac muscle were bred on a PPARα knockout background. Fifty-five percent of male animals lacking PPARα and overexpressing LPL died within 4 months after birth. In contrast, females of this genotype stayed alive. Deceased animals exhibited cardiopulmonary congestion but had no increase of neutral lipids in the heart. Changes in plasma glucose, FFA, lactate, and triglycerides did not clearly account for gender-specific differences in mortality; however, they indicated a critical role for PPARα during fasting. Analysis of cardiac function revealed that in isolated perfused hearts, left ventricular developed pressure (a measure of contractility) was markedly lower in PPARα knockout mice overexpressing LPL compared with controls. Glucose uptake of isolated perfused hearts was significantly higher in PPARα knockout mice with both normal or increased LPL expression. However, uptake of FFA was not different among genotypes. In contrast, fasted FFA levels were significantly lower in cardiac muscle of PPARα knockout mice with normal LPL expression ($-$26%) and PPARα knockout mice overexpressing LPL ($-$38%) compared with controls. Our results indicate a critical role for PPARα in myocardial pump function and suggest that mouse models combining different genetic effects such as PPARα knockout mice overexpressing muscle LPL may be useful to study cardiomyopathies. (*Lab Invest 2003, 83:259–269*).

Lipoprotein lipase (LPL) catalyzes the hydrolysis of triglycerides (TG) associated with circulating chylomicrons and very low-density lipoprotein particles (for review see Olivecrona and Bengtsson-Olivecrona, 1987, 1993). The highest levels of LPL expression and enzyme activity are found on the luminal side of capillaries in striated muscle and adipose tissue. In muscle, the liberated free fatty acids (FFA) are mainly used for β-oxidation and energy production, whereas in adipose tissue, FFA are reesterified and stored as intracellular TG within lipid droplets. It has been proposed that imbalances in partitioning FFA after a deregulation of LPL expression could lead to obesity or weight loss (Greenwood, 1985; Zechner, 1997). Recently, induced mutant mouse lines were generated that lack LPL or that express the enzyme in single tissues. Total LPL deficiency in mice leads to lethal hypertriglyceridemia within 18 hours of life (Weinstock et al, 1995). Transgenic mice that specifically overexpress LPL in both skeletal and cardiac muscle on normal LPL background show, proportional to the level of LPL overexpression, decreased plasma TG levels, increased muscle FFA concentrations, proliferation of mitochondria and peroxisomes, and, at high levels of LPL overexpression, peripheral myopathy (Hoefler et al, 1997; Levak-Frank et al, 1995). Exclusive LPL expression in skeletal muscle (Levak-Frank et al, 1997), in cardiac muscle (Levak-Frank et al, 1999), or in liver (Merkel et al, 1998) on a LPL null background rescues LPL null mice from neonatal death. These data not only provide strong evidence for a vital role of LPL as a gatekeeper for FFA entry into tissues but also prove that FFA can be successfully targeted to a given tissue, thereby permitting the investigation of the effects of excess FFA in a specific tissue.

Peroxisome proliferator-activated receptors (PPARs) are a group of transcription factors that belong to the nuclear receptor superfamily and are believed to be chief regulators of FA catabolism and storage (Dreyer et al,

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1992; Tontonoz et al, 1994). To date three different PPARs (α, δ, γ) have been identified in mammals, each encoded by a separate gene and showing a distinct tissue distribution (for review see Kliewer et al, 1994; Schoojans et al, 1996). PPARs can be activated by a variety of FA, FA derivatives (eg, prostaglandins, eicosanoids), and FA analogues including the hypolipidemic fibrate class of drugs. PPAR activation involves its heterodimerization with the 9-cis retinoic acid receptor and binding to specific sites called peroxisome proliferator response elements. This process alters transcription of a large number of distinct target genes (for review see Schoojans et al, 1996) encoding enzymes involved in peroxisomal and mitochondrial β-oxidation, ketone body synthesis, and the production of FA binding proteins, apolipoproteins, and LPL. Considering that increased FFA utilization is observed in muscle under fasting conditions, a critical role of PPARα in the cellular fasting response was recently demonstrated in PPARα knockout mice (Kersten et al, 1999; Leone et al, 1999). Short-term starvation caused hepatic steatosis, myocardial lipid accumulation, and hypoglycemia, with an inadequate ketogenic response, a phenotype that bears remarkable similarity to that of humans with genetic defects in mitochondrial FA oxidation enzymes. PPARα activation also leads to peroxisome proliferation, which is lacking in animals with a targeted disruption in the PPARα gene (Lee et al, 1995).

By crossing PPARα knockout mice with mice that overexpress LPL in skeletal and cardiac muscle on a normal LPL background, we aimed to elucidate the role of PPARα in FFA metabolism of these tissues. We analyzed morphologic, metabolic, and functional effects in PPARα knockout mice overexpressing LPL in muscle.

Results

Generation of PPARα Knockout Mice Overexpressing LPL Specifically in Muscle

To elucidate the role of PPARα in FA metabolism in muscle, mouse lines were generated that express human LPL in skeletal and cardiac muscle on a normal LPL background and lack PPARα (PPAR$-/-$ mLPL-high; for detailed nomenclature of mouse lines see the first paragraph of "Materials and Methods"). To check for tissue-specific expression of the human LPL transgene, activities in skeletal and cardiac muscle were analyzed in PPAR$-/-$ mLPL-high mice and found to be similar to those previously described for transgenic LPL mice expressing wild-type PPARα (Levak-Frank et al, 1995).

Growth Curves

Figure 1 shows the development of body weight of the three experimental groups. Male PPAR$-/-$ mLPL-high mice were lighter than controls from 4 weeks of age onward, whereas PPAR$-/-$ males only initially (at 4 weeks) fell behind control weight (Fig. 1A). The effect of muscle LPL overexpression on body weight was less pronounced in female PPAR$-/-$ mice (Fig. 1B) but still noticeable.

Life Span

The life spans of male and female PPAR$-/-$ and PPAR$-/-$ mLPL-high mice are shown as a Kaplan-Meier plot in Figure 2. Fifty-five percent of male animals lacking PPARα and overexpressing LPL in muscle (PPAR$-/-$ mLPL-high) died prematurely within 4 months after birth, whereas the remaining 45% died between 7 and 11 months of age. Interestingly, even male heterozygous PPARα knockout mice overexpressing LPL (PPAR$+/-$ mLPL-high) survived for only 6 to 8 months ($n = 25$, data not shown). In contrast, females of all three genotypes and males from the PPAR$-/-$ group survived for more than 12 months. All male PPAR$-/-$ mLPL-high mice died without obvious signs of prior sickness.

Gross Morphologic and Microscopic Features in Deceased Animals

After dissection, PPAR$-/-$ mLPL-high males did not show gross abnormalities except for a reduced skeletal muscle mass, which had already been observed in transgenic mice overexpressing muscle LPL on a normal PPARα background (Levak-Frank et al, 1995). Likewise, histologic changes in skeletal muscle were also comparable. We observed acute cardiopulmonary congestion and steatosis of the liver but no other obvious histopathologic changes in deceased PPAR$-/-$ mLPL-high mice.

Histology of Heart Specimens

The acute cardiopulmonary congestion prompted us to investigate heart specimens in more detail. Light and electron microscopy of ventricle cross sections did not reveal signs of myopathic changes nor changes in mitochondrial morphology. No obvious lipid storage, which had been reported in etomoxir-treated PPAR$-/-$ mice (Djouadi et al, 1998), could be detected. We also checked for possible minor neutral lipid accumulation in cardiac tissue by performing Oil-Red-O staining on frozen sections. However, no neutral lipid accumulation was found except for some minor lipid deposits in the vicinity of lymphatic vessels (Fig. 3A). Similar results were obtained when antibodies against mouse adipocyte differentiation-related protein, a marker for steatosis (Heid et al, 1998), were used (Fig. 3B).

Plasma Parameters

Because cardiac function is dependent on a sufficient energy supply provided by glucose and FA metabolism, we analyzed plasma concentrations of glucose, FFA, TG, total cholesterol, and lactate in fed and fasted control, PPAR$-/-$, and PPAR$-/-$ mLPL-high mice (Fig. 4). Glucose levels are shown in Figure 4A. PPAR$-/-$ mice generally exhibited lower levels than

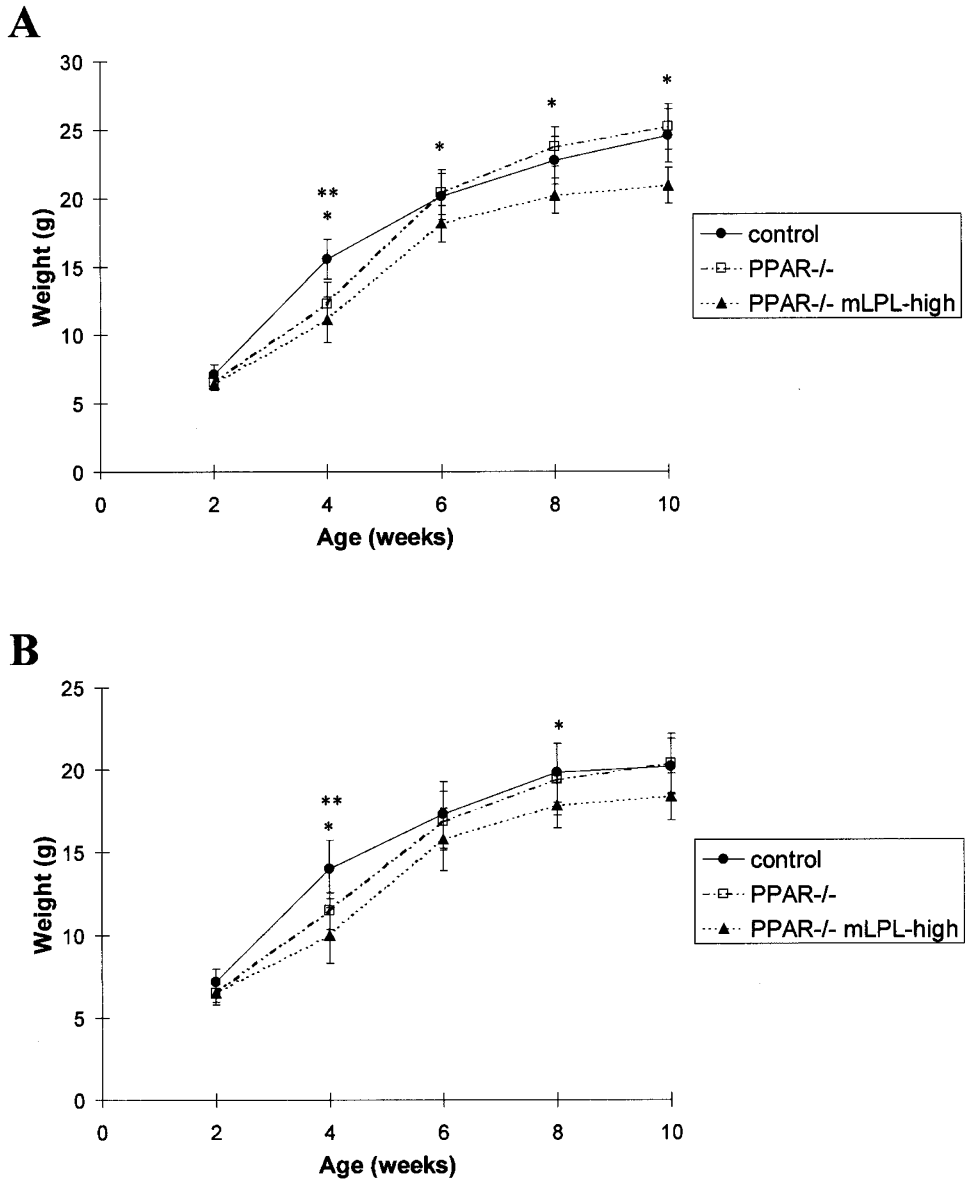

Figure 1.

Growth curves of male (A) and female (B) peroxisome proliferator-activated receptor alpha (PPARα) knockout mice with normal lipoprotein lipase (LPL) expression (PPAR$-/-$) or muscle-specific LPL overexpression (PPAR$-/-$ mLPL-high) compared with control mice. Ten to fifteen mice per genotype and gender were weighed every second week beginning 2 weeks after birth. Values are presented as means $\pm$ SD. A statistical difference at the $p < 0.005$ level is indicated by an *asterisk* comparing PPAR$-/-$ mLPL-high animals with controls and by a *double asterisk* comparing PPAR$-/-$ mice and controls.

controls; however, statistical significance (indicated by *solid dots* above the columns) was reached only in PPAR$-/-$ mLPL-high males (28% reduction in fasted, 17% reduction in fed animals) and PPAR$-/-$ females (22% reduction in fasted, 10% reduction in fed animals). In the fed state, male PPAR$-/-$ mice also had significantly higher levels compared with females (indicated by *solid triangles*). As expected, glucose levels were significantly lower in fasted compared with fed animals (see *asterisks* above columns). Glucose levels in mLPL-high mice did not show gender-specific differences and were similar to levels in control animals ($n = 5$, data not shown).

FFA levels are depicted in Figure 4B. The most obvious differences between PPAR$-/-$ and control animals were noted in fasted mice. Both PPAR$-/-$ and PPAR$-/-$ mLPL-high males exhibited 2.0-fold higher FFA levels than controls. The respective female animals exhibited an even higher increase (2.2-fold both in PPAR$-/-$ and PPAR$-/-$ mLPL-high) compared with controls. The difference between females and males was also significant. In contrast, evaluation of FFA levels reported earlier for mLPL-high mice (Levak-Frank et al, 1995) did not show gender-specific differences. In the fed state, only minor differences were noted. Comparing the nutritional state, fasted PPAR$-/-$ and PPAR$-/-$ mLPL-high mice of both genders displayed much higher plasma FFA concentrations than fed animals.

Plasma TG levels are illustrated in Figure 4C. In fasted animals, muscle LPL overexpression led to a

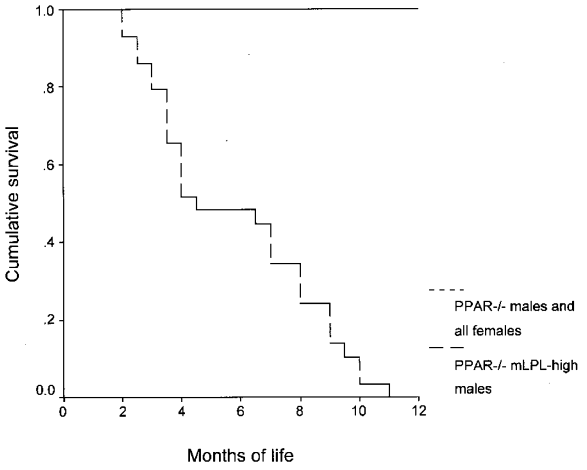

Figure 2.
Kaplan-Meier plot showing the cumulative survival of male and female PPARα knockout mice (PPAR$-/-$) and PPARα knockout mice overexpressing LPL specifically in muscle (PPAR$-/-$ mLPL-high). Data for both genders are shown ($n = 29$ for each experimental group).

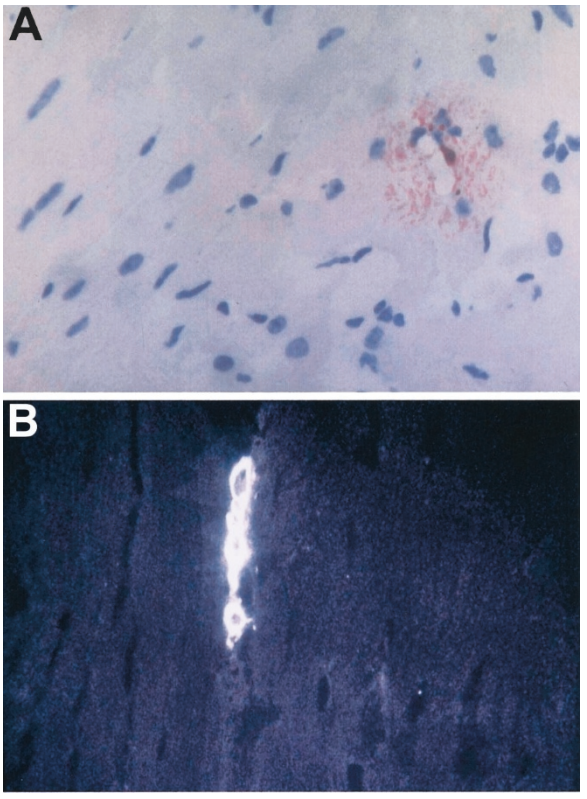

Figure 3.
Histologic examination of lipid stores. A, Light microscopy of Oil-Red-O-stained heart sections (magnification, $\times$40). B, Immunofluorescence analysis of heart sections stained with antibodies against a peptide corresponding to the N-terminus of mouse adipocyte differentiation-related protein (magnification, $\times$60).

significant decrease in plasma TG levels in males (54% decrease) and in females (34% decrease) compared with controls. In the fed state, we observed a similar trend in males (22% decrease; p = NS) and a significant reduction in females (40% decrease). Gender-specific differences were only detected for fasted PPAR$-/-$ mLPL-high animals: male mice had 30% lower TG values than females. In addition, PPAR$-/-$ mLPL-high animals had significantly reduced fasting TG levels (51% decrease in male and 17% in female mice). Only minor differences were found in total cholesterol levels between the three experimental groups (Fig. 4D).

Mean plasma lactate concentrations in mice fasted overnight were 4.0 (control), 2.6 (PPAR$-/-$ mLPL-high), and 3.2 (PPAR$-/-$) mM ($n = 10$ in all cases), ie, 35% and 20% less in the transgenic groups than in controls ($p < 0.05$). There was no gender-specific difference. Nonfasted values were not determined.

FFA Concentrations in Tissues

To assess the effect of LPL overexpression and the role of PPARα, FFA concentrations were determined in skeletal and cardiac muscle after an overnight fasting period. The FFA concentrations in controls and skeletal muscle of PPAR$-/-$ mLPL-high animals were 0.20 ± 0.01 and 0.77 ± 0.45 μmol/g wet weight, respectively (3.8-fold increase; $p < 0.05$, $n = 4$). There was no significant difference between PPAR$-/-$ and control. In contrast, in cardiac muscle, the respective concentrations were 1.29 ± 0.13 (control), 0.81 ± 0.11 (PPAR$-/-$ mLPL-high), and 0.97 ± 0.16 (PPAR$-/-$) μmol/g wet weight, corresponding to a reduction by 38% and 26% compared with control ($p < 0.05$, $n = 4$).

Functional Tests on Isolated Hearts

To test whether cardiac dysfunction might account for the observed cardiopulmonary congestion and sudden death of male PPAR$-/-$ mLPL-high animals, we studied myocardial function in all four experimental groups. The effect of norepinephrine and strophanthin on left ventricular developed pressure (LVDevP) in hearts from male and female mice is shown in Figure 5. Neither PPARα deficiency per se nor muscle LPL overexpression alone had an effect on baseline LVDevP in either gender (Fig. 5, A to D). Likewise, the positive inotropic potency was similar in all groups for both agents. However, overexpression of LPL in the absence of PPARα depressed LVDevP by about 23% (see baseline in Fig. 5, A to D) and considerably reduced contractility in both genders. The mean effective concentration (EC_{50}) of norepinephrine (males) was 3.7×10^{-7} M in the PPAR$-/-$ mLPL-high group and 1.5×10^{-8} M, 4.5×10^{-8} M, and 1.8×10^{-8} M in the other groups (25-, 8-, and 21-fold difference, $p < 0.05$ PPAR$-/-$ mLPL-high vs the other three groups). For strophanthin, the respective mean potency difference versus the other three groups was 7-fold ($p < 0.05$, Fig. 5, A and C). Neither acetylcholine nor S-nitroso-N-acetyl-DL-penicillamine affected contractility ($n = 3$, not shown). Likewise, norepinephrine was considerably less potent in female PPAR$-/-$ mLPL-high hearts than hearts from the other three groups (27-, 70-, and 19-fold difference; $p < 0.05$). For

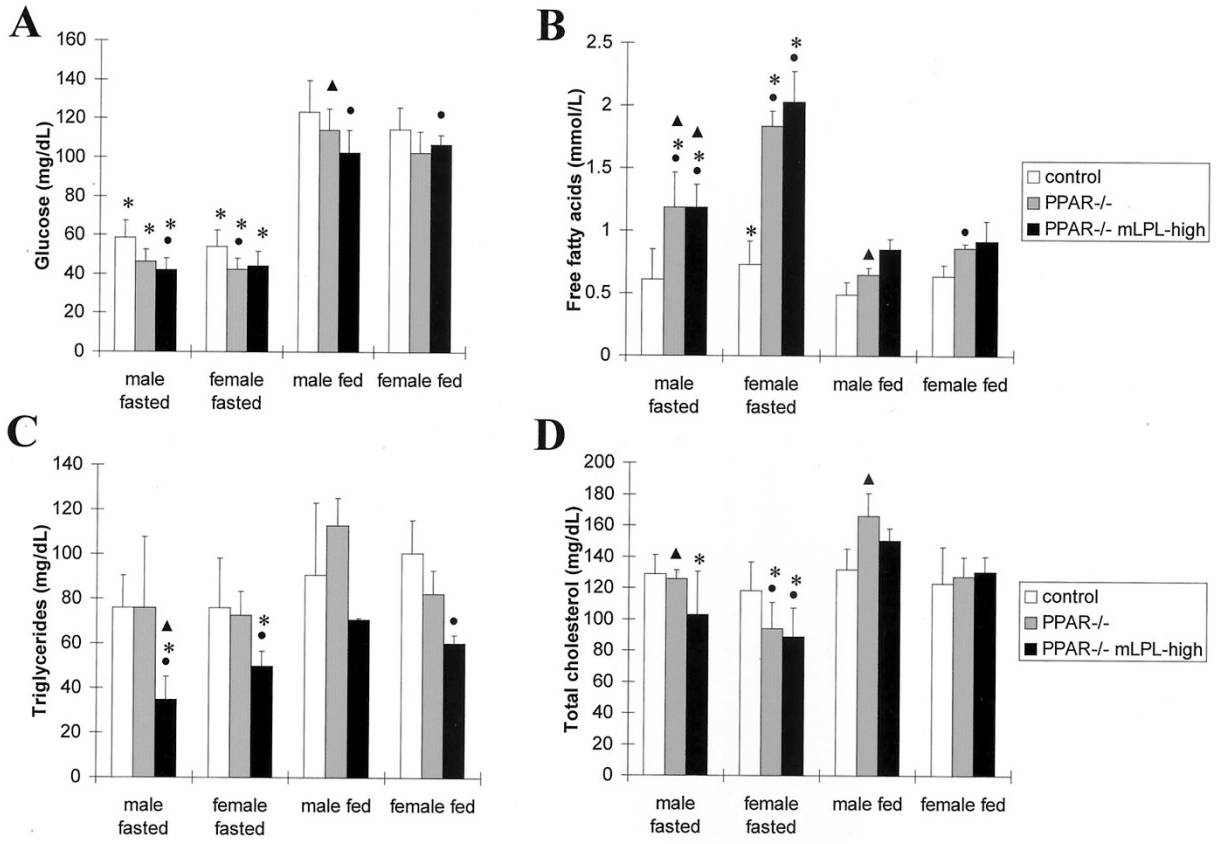

Figure 4.

Plasma glucose (A), free fatty acids (FFA) (B), triglycerides (TG) (C), and total cholesterol (D) levels of 12-week-old control, PPAR−/−, and PPAR−/− mLPL-high mice after overnight fasting and feeding. *Filled circles* indicate a statistical difference at the $p < 0.05$ level comparing PPAR−/− and PPAR−/− mLPL-high mice with controls. Gender-specific differences of statistical significance ($p < 0.05$) are denoted as *triangles*. *Asterisks* demonstrate a statistical difference of $p < 0.05$ comparing fasted with fed state. Glucose, $n = 5$ to 10 (fed and fasted); FFA, TG, and total cholesterol, $n = 5$ to 10 (fasted), $n = 3$ to 7 (fed).

strophanthin the respective potency differences were 2- to 3-fold ($p < 0.05$, Fig. 5, B and D). The maximal LVDevP (B_{max}) values were consistently lower in the PPAR−/− mLPL-high group than in the three other groups, and this was largely attributable to the difference in baseline contractility as apparent from the parallel course of the curves in Figure 5. Neither EC_{50} nor maximal LVDevP values were different between hearts from male and female animals. Analysis of the rate of rise and fall of dP/dt gave a similar picture as obtained for LVDevP ($n = 3$, data not shown).

Baseline heart rate was similar in all four experimental groups and in both genders (465 ± 5 beats/minute, $n = 24$; p = NS). Norepinephrine increased heart rate to 541 ± 11 (male) and 560 ± 12 (female) beats/minute, and strophanthin decreased it to 365 ± 11 (male) and 360 ± 10 (female) beats/minute, respectively ($n = 12$ in each case; $p < 0.05$ vs baseline). The differences between genders were not significant (p = NS). Acetylcholine and S-nitroso-N-acetyl-DL-penicillamine had no effect on heart rate (male mice, data not shown).

Baseline coronary flow was not different in the four groups or between genders (2.2 ± 0.05 mL/min; $n = 24$). The effect of norepinephrine was biphasic, ie, after each dose flow was reduced for ∼20 seconds (not shown), followed by an increase that was ∼30% less pronounced in male hearts overexpressing LPL ($n = 3$ in each group; $p < 0.05$, Fig. 6A). The coronary relaxant potency was 14-fold less in PPAR−/− mLPL-high hearts ($p < 0.05$ vs each of the other groups). A similar difference was noted in hearts from female mice (Fig. 6B). As expected, strophanthin constricted the coronary circulation (coronary flow, 1.0 ± 0.06 mL/min, $n = 12$), and acetylcholine and S-nitroso-N-acetyl-DL-penicillamine increased it, with little difference between experimental groups ($n = 3$ male animals, data not shown).

Effects of FFA on Heart Function

To investigate possible metabolic causes for contractile dysfunction in PPAR−/− mLPL-high hearts and to compensate for possible differences in the provision of FFA, the perfusate was supplemented with [^{3}H]triolein-containing very low density lipoprotein (VLDL) at a concentration of 0.4 mg VLDL per milliliter of perfusate for 45 minutes. LVDevP was similarly decreased in all four groups (mean, −19%), indicating a negative effect of FFA on contractility in all groups (Fig. 7).

FFA and Glucose Uptake Studies in Isolated Hearts

Uptake of glucose and FFA during perfusion was determined using [^{14}C]deoxyglucose and [^{3}H]triolein, respectively. In controls, glucose uptake was 1124 ±

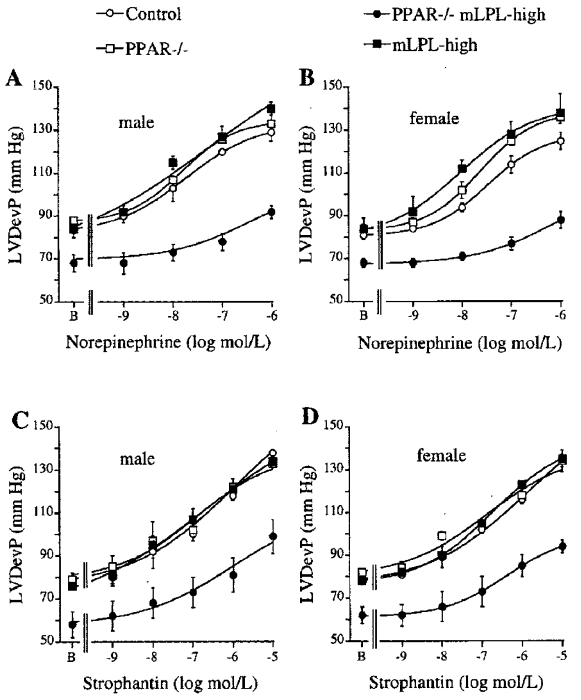

Figure 5.

Effect of norepinephrine (A and B) and strophanthin (C and D) on cardiac contractility in hearts derived from male (A and C) or female (B and D) mice. Hearts were perfused to establish baseline conditions (B), followed by infusion of norepinephrine or strophanthin through a sideline at the final concentrations indicated. Contractility was measured in terms of left ventricular developed pressure (LVDevP) and was significantly lower in hearts of PPARα knockout mice overexpressing LPL (PPAR$-/-$ mLPL-high, *solid circles*) than in the other three groups. Data are means $\pm$ SEM of three hearts in each group.

89 nmol/minute per gram wet weight and similar to mLPL-high mice (1107 $\pm$ 88 nmol/minute per gram; p = NS). However, uptake was significantly increased in PPAR$-/-$ and PPAR$-/-$ mLPL-high hearts (1556 $\pm$ 76 and 1603 $\pm$ 81 nmol/minute per gram wet weight; $p < 0.05$, $n = 5$), indicating a PPARα-dependent effect. FFA uptake was higher in PPAR$-/-$ mLPL-high hearts (5.2 $\pm$ 0.3% of supplied triolein; $n = 5$); however, there was no significant difference compared with the other experimental groups (control 4.3 $\pm$ 5%, $n = 4$; PPAR$-/-$ 4.2 $\pm$ 0.5%, $n = 5$; mLPL-high 4.3 $\pm$ 0.5%, $n = 3$).

Lactate Production in Isolated Hearts

The lactate concentrations measured in coronary effluent were 47 $\pm$ 3 nM in control hearts and tended to be lower in PPAR$-/-$ hearts (38 $\pm$ 4 nM), but the difference (19%) was not significant ($n = 5$). In mLPL-high and PPAR$-/-$ mLPL-high hearts, the corresponding values were 49 $\pm$ 5 and 47 $\pm$ 4 nM (p = NS vs control). In hearts stimulated at 9.2 Hz (550 beats/min) to increase lactate production, coronary efflux of the metabolite was increased, but there was still no significant difference among groups (n = 3–5; data not shown).

Discussion

We previously showed that tissue-specific overexpression of LPL allows study of the effects of increased FFA import into skeletal muscle, which leads to gene dosage-dependent peripheral myopathy and proliferation of peroxisomes and mitochondria (Levak-Frank et al, 1995). In addition, mRNA levels and protein concentration of acyl-CoA oxidase, the rate-limiting enzyme in peroxisomal β-oxidation, were increased (Hoefler et al, 1997). The present study significantly extends these results by showing that additional disruption of the PPARα gene causes myocardial dysfunction and premature death. The following discussion focuses on possible causes for these observations.

Reduced Life Span of PPAR$-/-$ mLPL-High Male Animals

Targeted disruption of PPARα led to a significant reduction in the life span of LPL-overexpressing male mice. Although the average life expectancy of transgenic mice overexpressing muscle LPL on a normal PPARα background was 8 to 12 months (Levak-Frank et al, 1995), many male animals with additional disruption of PPARα died within 4 months after birth, and the remaining ones survived $<$12 months. Unexpectedly, life expectancy of female mice of the identical genotypes was not reduced, possibly because of better availability of FFA as indirectly suggested by the higher plasma levels in female PPAR$-/-$ mice, irrespective of LPL expression (see Fig. 4B). The tendency toward a slower growth rate observed in the male PPAR$-/-$ mLPL-high animals might be an early indication for their higher mortality. A gender-specific effect has been reported in male PPARα knockout animals after inhibition of mitochondrial FA import by short-term treatment with etomoxir (Djouadi et al, 1998). In their model, inhibition of mitochondrial FA import caused massive hepatic and cardiac lipid accumulation, hypoglycemia, and death in all male mice but only in 25% of female PPARα $-/-$ mice. In our model (PPAR$-/-$ mLPL-high animals), however, histologic examination, Oil-Red-O staining, and immunohistochemical analyses using antibodies against adipocyte differentiation-related protein did not show lipid storage in cardiac muscle. These observations indicate that in our PPAR$-/-$ mLPL-high animal model, dysfunction of the heart is not caused by excessive cardiac lipid storage. This model might, therefore, be more relevant to the study of human cardiomyopathy in diabetes or mitochondrial disorders, in which no massive lipid accumulation is observed (Roe and Coates, 1995).

Plasma Metabolites

Our analyses of plasma glucose, FFA, TG, total cholesterol, and plasma lactate yielded differences mainly in the fasted state. Lack of PPARα (irrespective of LPL expression) seems to cause a reduction in glucose and an increase in FFA levels. Glucose levels were

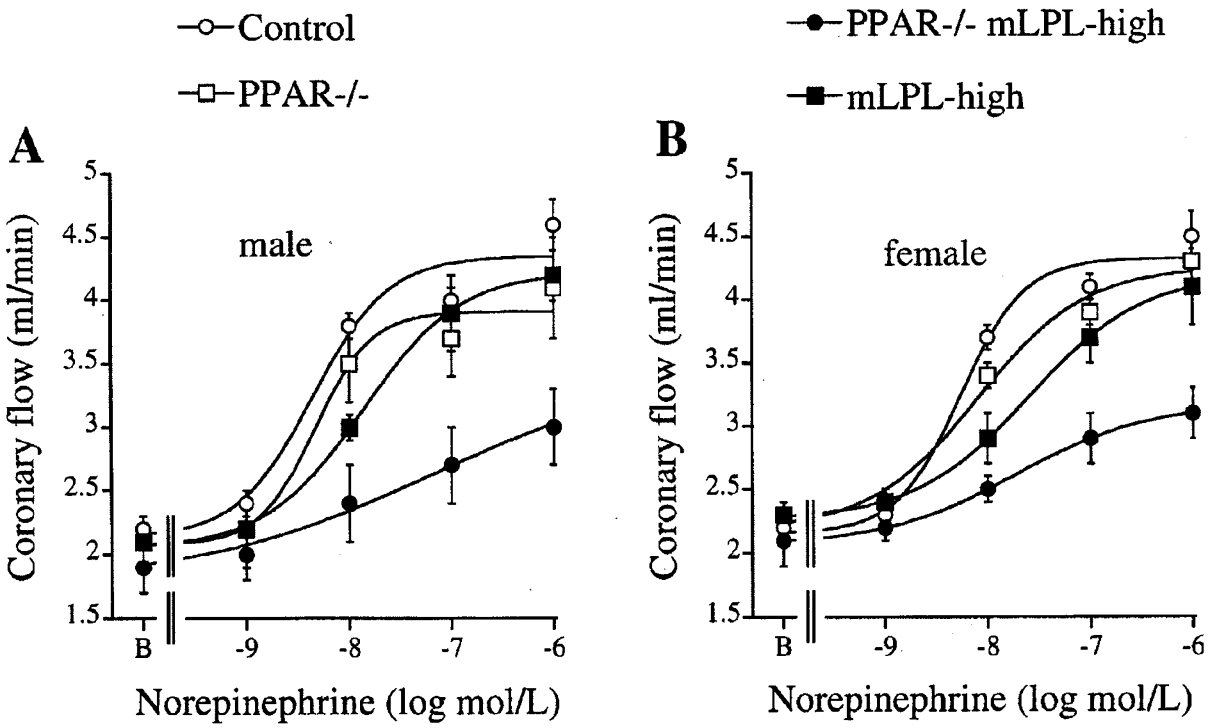

Figure 6.

Effect of norepinephrine on coronary flow. Hearts were perfused to establish baseline conditions (B), followed by infusion of norepinephrine through a sideline at the final concentrations indicated. Coronary flow was significantly lower in hearts of PPARα knockout mice overexpressing LPL (PPAR−/− mLPL-high, *solid circles*) than in the other three groups. Data are means ± SEM of three hearts derived from male (A) or female (B) mice. The initial constricting phase preceding the coronary relaxation is not shown.

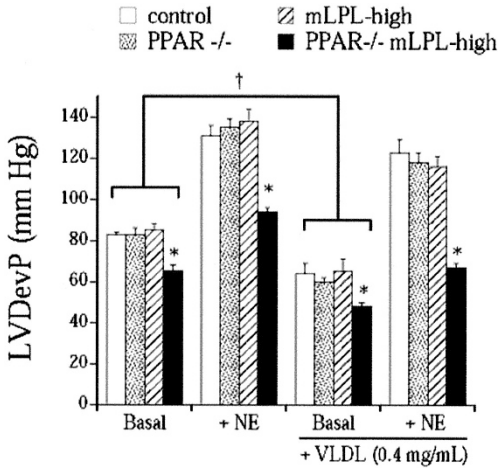

Figure 7.

Effect of VLDL on heart function (male animals). The perfusate was supplemented with VLDL containing [^{3}H]triolein (0.4 mg VLDL/mL perfusate) and recirculated during 45 minutes. Between 30 and 45 minutes, hearts were stimulated with norepinephrine (0.1 μM). The data show the LVDevP for the four experimental groups at baseline, after norepinephrine challenge, and after VLDL addition in the absence and presence of norepinephrine, respectively. Data are mean ± SEM; $n = 5$ except for mLPL-high ($n = 3$). $^*p < 0.05$ vs control; †$p < 0.05$ for effect of VLDL.

reduced in all animals lacking PPARα, but this effect reached significance only in PPAR−/− mLPL-high males and PPAR−/− females. Our data are in line with similar previous observations (Kersten et al, 1999; Leone et al, 1999), indicating a critical role for PPARα in the cellular fasting response. However, this effect is unlikely to account for reduced cardiac function observed in PPAR−/− mLPL-high mice, because the drop in glucose levels was not different from PPAR−/− animals having normal LPL activity. The lack of clear-cut gender-specific differences excludes reduced glucose levels as the primary cause for male mortality in our model.

Plasma lactate was significantly reduced in PPAR−/− and PPAR−/− mLPL-high animals compared with controls, indicating a PPARα-dependent effect (compare Kersten et al, 1999). However, plasma lactate cannot explain the reduced cardiac function because the latter is reduced only in the PPAR−/− mLPL-high group; nor do the lower lactate levels seem to be related to the observed male mortality, because there was no gender-specific difference.

A reduction in plasma TG was observed in PPAR−/− mLPL-high animals irrespective of nutritional state and gender and has been mainly attributed to a reduction in VLDL (Levak-Frank et al, 1995). Lack of PPARα did not affect TG levels in the present study. The decrease in TG probably reflects increased uptake of FFA in skeletal muscle, as indicated by some 4-fold higher FFA levels in this tissue. Plasma FFA levels were significantly higher in animals lacking PPARα compared with controls, irrespective of LPL expression, which is in agreement with recent findings (Kersten et al, 1999; Leone et al, 1999). Probably, the observed increase in plasma FFA in PPAR−/− ani-

mals results from the inability to increase mitochondrial or peroxisomal β-oxidation because of the lack of PPARα. Because transcription of FA transporter genes is stimulated by PPARα (Kersten et al, 1999), elevated plasma FFA levels might result from decreased FA uptake, but this is unlikely because we observed no differences in triolein uptake between PPAR$-/-$ and control hearts.

Cardiac Function

In view of the histopathologic finding of an acute cardiopulmonary congestion in PPAR$-/-$ mLPL-high mice, we analyzed cardiac function in isolated hearts. Hearts from PPAR$-/-$ mice overexpressing LPL in muscle had a lower basal contractility (see baseline LVDevP in Fig. 5), and the inotropic effect of norepinephrine and strophanthin was considerably lower in hearts from this genotype. No such changes were observed in any of the three other genotypes. The displacement to the right of the pressure-concentration curves is reminiscent of the picture in diabetic cardiac dysfunction, in which LVDevP and cardiac output is reduced (Rodrigues et al, 1997). The myocardial dysfunction observed in PPAR$-/-$ mLPL-high (but not in PPAR$-/-$) mice is probably not directly related to increased FFA plasma levels because no significant differences in FFA levels were found when comparing PPAR$-/-$ and PPAR$-/-$ mLPL-high mice. Importantly, we observed that cardiac FA uptake is not enhanced in PPAR$-/-$ hearts and that cardiac FFA content was considerably (26%) lower in this genotype. Furthermore, LPL overexpression resulted in a massive (3.8-fold) increase of FFA content in skeletal muscle. The simultaneous occurrence of these effects might cause a further reduction of FFA in cardiac muscle, which is what we observed in PPAR$-/-$ mLPL-high animals (-16%). As a final consequence, cardiac muscle would be deprived of primary energy, resulting in reduced myocardial contractility (Fig. 5).

We also documented coronary and electric function in hearts from the four genotypes and found that PPAR$-/-$ mLPL-high mice also showed impaired metabolic coronary dilatation after norepinephrine application, whereas no such effect was seen in hearts from PPAR$-/-$ or mLPL-high mice. Thus, LPL overexpression on a PPARα $-/-$ background also impairs coronary function, in comparison to chemically induced diabetes, in which depressed cardiac contractility is not accompanied by alterations in coronary flow or major vessel disease (Rodrigues et al, 1997). As expected, norepinephrine increased and strophanthin decreased spontaneous heart rate in a concentration-dependent manner, but there were no differences among the four experimental groups. Likewise, there were no differences between any of the hemodynamic parameters between hearts derived from male and female mice, respectively. Therefore, the much shorter survival of male PPAR$-/-$ mLPL-high mice is unlikely to result from a gender-specific hemodynamic defect.

Conclusions and Perspectives

LPL overexpression in muscle tissue of male mice reduces their life span, and additional disruption of PPARα shortens it even further. However, only the combination of both manipulations results in myocardial and coronary dysfunction. These effects are accompanied by metabolic changes that are likely to affect heart function, namely an LPL dependent and PPARα $-/-$ dependent deprivation of FA. However, this deprivation is not compensated for by heightened FA transport rate into myocardial cells. The functional impairments do not seem to be related to excessive lipid accumulation after disruption of PPARα. This suggests that in situations in which FFA are the primary source of energy production (such as fasting and diabetes), cardiomyopathy can also be caused by pathogenic mechanisms other than lipid accumulation. Therefore, combining genetic modifications such as overexpression of LPL and knockout of PPARα may be a valuable novel approach for the elucidation of physiologic modulations, pathologic changes, and interactions in cellular lipid metabolism.

Materials and Methods

Generation of PPARα Knockout Mice Overexpressing LPL Specifically in Muscle

Two crosses were necessary to produce mice that overexpress LPL specifically in cardiac and skeletal muscle on a PPARα $-/-$ background. First, transgenic mice carrying approximately 10 copies of a human LPL minigene under the control of the muscle creatine kinase promoter (= *m*LPL-high for overexpressing *m*uscle LPL, previously described in Hoefler et al, 1997) were crossbred with PPARα knockout mice (Lee et al, 1995) to obtain animals that are heterozygous for the LPL transgene while carrying only one intact copy of the PPARα gene (PPAR$+/-$ mLPL-high). These mice were then crossed with PPARα knockout mice to produce both PPARα knockout mice with normal LPL tissue expression (PPAR$-/-$) and PPARα knockout mice heterozygous for the LPL transgene (PPAR$-/-$ mLPL-high). To guarantee continuous heterozygosity of the LPL transgene, PPARα knockout mice with normal LPL expression (PPAR$-/-$) were always bred with PPARα knockout mice heterozygous for the LPL transgene (PPAR$-/-$ mLPL-high). Controls were bred considering the different background strains (50% SV129, 25% Bl6, and 25% CBA).

Genotyping for the Human LPL Transgene and the Disruption of the PPARα Gene

To detect the human LPL transgene by PCR analysis, a 410-bp fragment near the 5' end of the integrated human LPL cDNA (for detailed position see Levak-Frank et al, 1997) was amplified using the primers LPL106 5' GTTACCGTCCAGCCATGGATCACC 3' and LPL193 5' CCTCAAGGGAAAGCTGCCCAC 3'. Five hundred nanograms of tail tip DNA was amplified

in a 50-μL PCR reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 0.5 μM of the primers LPL106 and LPL193, 0.5 mM dNTPs, and 2.5 U of Ampli TAQ DNA polymerase (Perkin Elmer Cetus, Norwalk, Connecticut). After a hot start (94° C for 5 minutes), *Taq* polymerase was added to the PCR overlaid with mineral oil, and the mixture was cycled 35 times at 94° C for 1 minute, 64° C for 1 minute, and 72° C for 90 seconds followed by a final extension for 10 minutes at 72° C. To screen for the targeted disruption of the PPARα gene, we used primers (PPGT1 5' GAGAAGTTGCAGGAGGGGATTGTG 3' and PPGT2 5' CCCATTTCGGTAGCAGGTAGTCTT 3') flanking the region where exon 8 of the PPARα gene is disrupted because of insertion of the phosphoribosyltransferase II gene (for details see Lee et al, 1995). A single band of approximately 1500 bp was detected when both alleles of the PPARα gene were disrupted (PPAR−/−) and a single band of 410 bp when the PPARα gene was intact on both chromosomes (PPAR+/+). Five hundred nanograms of tail tip DNA was amplified in a 50-μL PCR reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM dNTPs, and 1 μM of the primers PPGT1 and PPGT2. Cycling conditions were as above, except that an annealing temperature of 60° C was used.

Analysis of LPL Activity

LPL activity was analyzed in heart and skeletal muscle. Tissue specimens were removed from animals immediately after decapitation and put into ice-cold tubes containing 1 mL of DMEM medium with 2% BSA and 2 U/mL heparin. The tissue was minced with scissors and incubated in medium for 1 hour at 37° C. Enzyme activity was assayed as described earlier (Zechner, 1990). To differentiate between human and mouse LPL activity in tissue extracts, the assay mixture was preincubated for 1 hour at 4° C in the presence or absence of 0.1 μg of mAb 5D2 (kindly provided by Dr. J. D. Brunzell, University of Washington, Seattle, Washington) (Babirak et al, 1989). This antibody specifically inhibits the human enzyme in LPL activity determinations.

Animals

All animals were fed a regular mouse chow (4% fat, 20% protein) and given free access to food and water. Pups were weaned at 4 weeks of age. Total body weights were measured every second week beginning at 2 weeks after birth.

Oil-Red-O Staining

Tissue lipid distribution was analyzed by Oil-Red-O (lipid) staining of specimens from mice aged 12 weeks that had been killed by decapitation after anesthesia with methoxyflurane (Forane; Abbott, Abbott Park, Illinois). Hearts were excised and frozen in 5-methylbutane precooled in liquid nitrogen. Cryocut sections (3-μm–thick transverse) were stained with Oil-Red-O and mounted in gelatin-glycerol.

Immunofluorescence Microscopy

Immunofluorescence staining was performed on 3-μm-thick transverse cryocut sections from the same heart specimens used for Oil-Red-O staining. After fixation with acetone/methanol at −20° C for 10 minutes, sections were incubated with rabbit antibodies against a peptide corresponding to the N-terminus of mouse adipophilin (adipocyte differentiation-related protein) diluted 1:200 in PBS for 1 hour. After several washes in PBS, the slides were incubated with a secondary FITC-conjugated swine antibody to rabbit Ig (F205; Dako, Glostrup, Denmark) diluted 1:50 for 30 minutes. Specimens were analyzed in an MRC 600 laser-scanning confocal device (BioRad, Richmond, California) attached to a Zeiss Axiophot (Jena, Germany). The fluorescent images were collected using the confocal photomultiplier tube as full frame (768 × 512 pixels). For detection of FITC-labeled antibody, an excitation wavelength of 488 nm was used from a krypton/argon laser.

Plasma Parameters

Generally, blood was obtained from mice aged 12 weeks by decapitation under methoxyflurane (Forane; Abbott) anesthesia. In animals fasted overnight, blood was taken from the retro-orbital plexus after methoxyflurane anesthesia. Blood was centrifuged in EDTA-spiked tubes, and plasma parameters were determined enzymatically according to the suppliers protocols: TG (Triglycerid GPO-Trinder 20; Sigma, Vienna, Austria), FFA (NEFA C; Wako Chemicals, Neuss, Germany), and total cholesterol (CHOL MPR1; Roche Diagnostics, Vienna, Austria). Glucose levels (fed and fasted) were determined in the blood of tail veins using a standard blood glucometer (Glucose SuperG analyzer; Diasys Diagnostic Systems, Dreihausen, Germany). For the quantitative determination of nonesterified (free) fatty acids in serum and tissues, the NebraskaFA C test kit (Wako Chemicals, Neuss, Germany) with an in vitro enzymatic colorimetric method was used. For quantification in tissue, wet tissue weight was determined, followed by homogenization of the tissue in 1 mL of 0.9% NaCl. Plasma lactate levels were measured using standard methods.

Preparation and Labeling of VLDL

Human VLDL was prepared by ultracentrifugation as previously described (Steyrer et al, 1990). Lipoprotein aggregates were removed by filtration through a 0.8-μm syringe filter. To label VLDL, 0.5 mCi of [9,10-^{3}H (N)]-Triolein (NEN/Perkin Elmer, Boston, Massachusetts) was dissolved in 0.3 mL of toluene and added slowly to the VLDL solution (25 mg in 30 mL saline) under vigorous vortexing. The mixture was dialyzed overnight at 4° C against PBS, pH 7.4.

Functional Tests on Isolated Hearts

Mice aged ∼12 weeks belonging to the four experimental groups were anesthetized with urethane (1 gm/kg

body weight, ip) and heparinized (1000 U/kg, ip). During preparation the animals were ventilated with room air at a rate of 60 strokes/minute with a tidal volume of 0.5 mL. The heart was removed, placed in chilled perfusion medium, and attached to a Langendorff perfusion system (ISOHEART #3; Harvard Apparatus/Hugo Sachs Instruments, March-Hugstetten, Germany). Retrograde perfusion was done at constant pressure of 80 mm Hg (Brooks and Apstein, 1996) with a modified Krebs-Henseleit bicarbonate buffer. Cardiac parameters were monitored continuously and included coronary perfusion pressure, coronary flow, and left ventricular peak systolic pressure. Left ventricular end diastolic pressure was set at 0 mm Hg and maintained throughout the experiment. LVDevP was calculated from the difference between left ventricular peak systolic pressure and left ventricular end diastolic pressure. The maximum rate of rise and fall of left ventricular pressure (+dP/dt, −dP/dt) and heart rate were obtained from the pressure signal using a differentiator and heart rate module, respectively. All parameters were obtained online using the PLUGSYS data acquisition setup (Harvard Apparatus/Hugo Sachs Instruments; for all details, see Brunner et al, 2001).

The experimental protocol consisted of 30 minutes of equilibration, followed by application of two drugs with positive inotropic action in succession (total duration, including equilibration, 130 minutes), followed by application of two vascularly active drugs in some groups of hearts (male mice; total duration 210 minutes). The drugs were injected through a sideline just above the heart in noncumulative fashion. The following drugs were used: norepinephrine (1×10^{-9} to 1×10^{-6} M), strophanthin (1×10^{-9} to 1×10^{-5} M), acetylcholine, and S-nitroso-N-acetyl-DL-penicillamine (Tocris Cookson, Bristol, UK) (1×10^{-9} to 1×10^{-7} M in both cases).

Metabolic Studies

In another set of male animals aged 3 to 4 months (n = 3–5 per group), a separate protocol was used to document possible differences in cardiac glucose and FFA uptake and lactate release. After equilibration, coronary effluent (1 mL) was collected for lactate determination under basal and stimulated conditions (9.2 Hz, 550 beats/min), followed by (i) application of norepinephrine (0.1 μM, 15 minutes; determination of cardiac function), (ii) washout of norepinephrine, (iii) spiking of the perfusing buffer (25 mL) with 20 μL ^{14}C-2-deoxyglucose solution (containing ~300,000 dpm) and 0.4 mL of a human VLDL solution (containing $\sim 1.5 \times 10^6$ dpm [^{3}H]triolein) (both from New England Nuclear, Vienna, Austria) and recirculation for 45 minutes, with norepinephrine present during the last 15 minutes of recirculation (final VLDL concentration in the perfusate, 0.4 mg/mL; specific activity in the perfusate, 150 dpm/μg protein). Functional parameters were recorded throughout. Finally, hearts were perfused with normal buffer without additions (10 minutes) to remove radiolabels from inside the coronary vessels, and the hearts were quickly removed and dissolved in 0.5 N NaOH to determine the respective uptake rates. Lactate concentration in coronary effluents was measured as above.

Calculations and Statistics

Body weights were compared among groups at each time point using the two-tailed Student's t test (n = 10–15 determinations). Likewise, plasma parameters for the three groups and experimental conditions such as feeding state and gender were similarly analyzed. Results are given as mean ± SD of the following number of analyses: FFA, TG, and total cholesterol, n = 5 to -10 (fasted) and n = 3 to 7 (fed); glucose, n = 5 to 10 (fed and fasted). For the statistical analysis of cardiac function, concentration effect-curves were fitted to a Hill model as described previously (Brunner et al, 1995). Individual curves were analyzed giving estimates for EC_{50} and the maximum effect (E_{max}) obtained for each drug. Uptake rates were determined as nmol/minute per gram wet weight (2-deoxyglucose) and as percent of applied [^{3}H]triolein, respectively. The functional data are reported as mean ± SEM (n = 3–5 as indicated). A probability of $<5\%$ was considered significant.

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