Abnormal mitochondrial bioenergetics and heart rate dysfunction in mice lacking very-long-chain acyl-CoA dehydrogenase

Vernat J. Exil, Carla D. Gardner, Jeffrey N. Rottman, Harold Sims, Beatrijs Bartelds, Zaza Khuchua, Rekha Sindhal, Gemin Ni, and Arnold W. Strauss

Division of Cardiology, Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee; Washington University School of Medicine, St. Louis, Missouri; and Division of Cardiovascular Medicine, Department of Internal Medicine and Mouse Metabolic Phenotyping Center, Vanderbilt University School of Medicine, Nashville, Tennessee

Submitted 1 August 2005; accepted in final form 19 September 2005

Exil, Vernat J., Carla D. Gardner, Jeffrey N. Rottman, Harold Sims, Beatrijs Bartelds, Zaza Khuchua, Rekha Sindhal, Gemin Ni, and Arnold W. Strauss. Abnormal mitochondrial bioenergetics and heart rate dysfunction in mice lacking very-long-chain acyl-CoA dehydrogenase. Am J Physiol Heart Circ Physiol 290: H1289–H1297, 2006. First published September 30, 2005; doi:10.1152/ajpheart.00811.2005.—Mitochondrial very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is associated with severe hypoglycemia, cardiac dysfunction, and sudden death in neonates and children. Sudden death is common, but the underlying mechanisms are not fully understood. We report on a mouse model of VLCAD deficiency with a phenotype induced by the stresses of fasting and cold, which includes hypoglycemia, hypothermia, and severe bradycardia. The administration of glucose did not rescue the mice under stress conditions, but rewarming alone consistently led to heart rate recovery. Brown adipose tissue (BAT) from the VLCAD−/− mice showed elevated levels of the uncoupling protein isoforms and peroxisome proliferator-activated receptor-α. Biochemical assessment of the VLCAD−/− mice BAT showed increased oxygen consumption, attributed to uncoupled respiration in the absence of stress. ADP-stimulated respiration was 23.05 (SD 4.17) and 68.24 (SD 6.3) nmol O2/min·mg mitochondrial protein−1 for VLCAD+/+ and VLCAD−/− mice, respectively (P < 0.001), and carnobyl cyanide p-trifluoromethoxyphenylhydrazone-stimulated respiration was 35.9 (SD 3.6) and 49.3 (SD 9) nmol O2/min·mg mitochondrial protein−1 for VLCAD+/+ and VLCAD−/− mice, respectively (P < 0.20), but these rates were insufficient to protect them in the cold. We conclude that disturbed mitochondrial bioenergetics in BAT is a critical contributing factor for the cold sensitivity in VLCAD deficiency. Our observations provide insights into the possible mechanisms of stress-induced death in human newborns with abnormal fat metabolism and elucidate targeting of specific substrates for particular metabolic needs.

INBORN ERRORS OF METABOLISM affecting mitochondrial fatty acid oxidation are recessively inherited and account for a substantial number of cases of sudden infant death (4, 12, 16, 17, 25, 26). This report focuses on a mouse model of the human very-long-chain acyl-CoA dehydrogenase (VLCAD) enzyme deficiency and the stress-induced phenotype in mice exposed to fasting and cold stress. VLCAD catalyzes the first step in the β-oxidation pathway and is one of more than 16 different enzymes and transport proteins in the mitochondrial β-oxidation pathway. Inherited defects of VLCAD deficiency were first described by Bertrand et al. (5) in 1993. Diseases caused by mutations in the VLCAD gene affect children and young adults, but younger infants with null mutations present with the most severe form of the disease (1, 15, 21, 27, 30, 33). In the absence of stress, patients may have long-term survival, but physiological stressors such as fasting, exercise, and viral illnesses are typical triggers of disease crises. Acutely, patients present with nonketotic hypoglycemia, hypothermia, metabolic acidosis, and cardiac dysfunction. Profoundly ill patients can sometimes be rescued with supportive measures, including glucose infusion, aimed at restoring anabolism and reversal of metabolic acidosis. Despite these critical interventions, mortality is very high in the early-onset form of this disease.

There are a number of theorized mechanisms proposed as the causes of death in VLCAD deficiency. These include energy starvation, metabolic acidosis, generalized or organ-specific metabolic failure, lipotoxicity, and cardiac rhythm abnormalities. Previous work has shown that mice lacking the mitochondrial acyl-CoA dehydrogenases are cold sensitive (13, 14). Our laboratory (29) recently published data related to increased acylcarnitine levels in VLCAD-deficient mice exposed to cold stress and exercise. We found increased levels of long-chain acylcarnitines in the VLCAD-deficient mice in response to metabolic stressors that correlate with the clinical condition in human patients. This finding is significant, suggesting that the mouse model mimics some aspects of the human disease. In this article, we report the use of a mouse model of VLCAD deficiency to study the pathophysiological mechanisms of the stress-induced phenotypes to specifically test the hypothesized role of cardiac rhythm abnormalities in the mouse phenotype. In this study, we have assessed morphological and biochemical changes in liver and heart, when these mice were exposed to the stresses of fasting and cold. We systematically tested therapeutic/metabolic rescue of the mice in crises, using glucose replacement and heat administration. We also studied the possible molecular and biochemical elements at the basis of the stress-induced phenotype in the mouse.

MATERIALS AND METHODS

VLCAD-deficient mice. VLCAD-deficient mice were generated as described previously (11). The VLCAD gene was inactivated by homologous recombination such that exons 1 through 7 were replaced with a neomycin resistance gene. Male 129sv/C57BL6 VLCAD-deficient mice 2 mo of age were used in this study. These experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

INBORN ERRORS OF METABOLISMAffecting mitochondrial fatty acid oxidation are recessively inherited and account for a substantial number of cases of sudden infant death (4, 12, 16, 17, 25, 26). This report focuses on a mouse model of the human very-long-chain acyl-CoA dehydrogenase (VLCAD) enzyme deficiency and the stress-induced phenotype in mice exposed to fasting and cold stress. VLCAD catalyzes the first step in the β-oxidation pathway and is one of more than 16 different enzymes and transport proteins in the mitochondrial β-oxidation pathway.

Inherited defects of VLCAD deficiency were first described by Bertrand et al. (5) in 1993. Diseases caused by mutations in the VLCAD gene affect children and young adults, but younger infants with null mutations present with the most severe form of the disease (1, 15, 21, 27, 30, 33). In the absence of stress, patients may have long-term survival, but physiological stressors such as fasting, exercise, and viral illnesses are typical triggers of disease crises. Acutely, patients present with nonketotic hypoglycemia, hypothermia, metabolic acidosis, and cardiac dysfunction. Profoundly ill patients can sometimes be rescued with supportive measures, including glucose infusion, aimed at restoring anabolism and reversal of metabolic acidosis. Despite these critical interventions, mortality is very high in the early-onset form of this disease.

There are a number of theorized mechanisms proposed as the causes of death in VLCAD deficiency. These include energy starvation, metabolic acidosis, generalized or organ-specific metabolic failure, lipotoxicity, and cardiac rhythm abnormalities. Previous work has shown that mice lacking the mitochondrial acyl-CoA dehydrogenases are cold sensitive (13, 14). Our laboratory (29) recently published data related to increased acylcarnitine levels in VLCAD-deficient mice exposed to cold stress and exercise. We found increased levels of long-chain acylcarnitines in the VLCAD-deficient mice in response to metabolic stressors that correlate with the clinical condition in human patients. This finding is significant, suggesting that the mouse model mimics some aspects of the human disease. In this article, we report the use of a mouse model of VLCAD deficiency to study the pathophysiological mechanisms of the stress-induced phenotypes to specifically test the hypothesized role of cardiac rhythm abnormalities in the mouse phenotype. In this study, we have assessed morphological and biochemical changes in liver and heart, when these mice were exposed to the stresses of fasting and cold. We systematically tested therapeutic/metabolic rescue of the mice in crises, using glucose replacement and heat administration. We also studied the possible molecular and biochemical elements at the basis of the stress-induced phenotype in the mouse.

MATERIALS AND METHODS

VLCAD-deficient mice. VLCAD-deficient mice were generated as described previously (11). The VLCAD gene was inactivated by homologous recombination such that exons 1 through 7 were replaced with a neomycin resistance gene. Male 129sv/C57BL6 VLCAD-deficient mice 2 mo of age were used in this study. These experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Metabolic response to stress was assessed in four different groups of mice: 1) no intervention (n = 11: 3 VLCAD\(^{+/+}\), 3 VLCAD\(^{++/+}\), 5 VLCAD\(^{-/-}\)); 2) fasting alone (n = 15: 4 VLCAD\(^{+/+}\), 4 VLCAD\(^{++/+}\), 7 VLCAD\(^{-/-}\)); 3) exposure to cold (n = 14: 4 VLCAD\(^{+/+}\), 5 VLCAD\(^{++/+}\), 5 VLCAD\(^{-/-}\)); and 4) fasting in the cold (n = 31: 8 VLCAD\(^{+/+}\), 6 VLCAD\(^{++/+}\), 17 VLCAD\(^{-/-}\)).

Fasting was performed with animals caged individually with minimal bedding at room temperature and with free access to water. Cold stress involved placing the mice at 4°C in similar housing with free access to food and water. Fasting in the cold was performed with the mice placed at 4°C with free access to water. Cold stress was limited to 4 h or less, because our initial experiments showed that the combined stress was rapidly lethal. During these stresses, body temperature, spot tail-vein blood glucose, and single-lead electrocardiograms (ECG) were obtained on an hourly basis or if the body temperature, spot tail-vein blood glucose, and single-lead electrocardiograms (ECG) were obtained on an hourly basis or if the animal appeared visibly distressed. Experiments were suspended, and the animals were rescued or humanely euthanized if there was visible distress, a drop in the blood glucose to ≤50 mg/dL, a decrease in heart rate to ≤150 beats/min, or a fall in body temperature to 30°C.

Metabolic parameters and histology. Blood glucose levels were obtained from tail veins by using a glucometer and Glucostix strips (One Touch; Johnson & Johnson). During metabolic stress, serial temperature measurements were obtained with a rectal digital thermometer (Fisher Scientific). Liver glycogen was measured, upon death, using the modified Seifter method (18). Analysis of fatty acid metabolites in frozen plasma was performed using tandem mass spectrometry. Tissue morphology was determined in frozen sections (5 μm) of liver and heart by performing hematoxylin-eosin staining and oil red O staining.

**Rescue experiments, surface ECG, and echocardiograms.** Rescue experiments were conducted at room temperature with continuous heart rate and body temperature monitoring and administration of 10% dextrose at a dose of 0.5 mg/g body wt ip. Intraperitoneal glucose infusion was repeated after 5 min if no change in heart rate was observed; the total number of intraperitoneal injections was limited to two. For rewarming, the mice were placed prone on a 37°C warming pad with or without glucose administration. Upon completion of the rescue experiments, the mice were observed continuously until they were ambulatory. A surface ECG (lead I) was recorded via subcutaneous 27-gauge electrodes in each forelimb, amplified (0.1 mV/cm), and filtered between 0.05 and 400 Hz. Continuous heart rate monitoring was done throughout the rescue experiment. Transthoracic echocardiograms were performed in the unanesthetized mice as previously described (11).

**Mitochondrial fractions.** Male mice (n = 4 VLCAD\(^{-/-}\) and 4 VLCAD\(^{++/+}\)) were selected to study brown adipose tissue mitochondrial bioenergetics. Mitochondria were isolated from brown adipose tissue as described by Koivisto et al. (20) with minor modifications. Upon death of the mouse, brown adipose tissue was carefully dissected from the interscapular region in the mouse. To minimize protein degradation, we performed initial experiments at 4°C. Brown adipose tissue was immersed in mitochondrial homogenization buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, and 1 mM EGTA, pH 7.2) (31). Tissue was then finely minced with scissors and homogenized in a 15-ml Wheaton Scientific Teflon-to-glass homogenizer. Mitochondria were isolated by differential centrifugation. The resulting mitochondrial pellet was resuspended in 200 μL of homogenization buffer.
COLD SENSITIVITY AND HEART RATE DYSFUNCTION IN VLCAD-DEFICIENT MICE

Report

A

B

C

D

Fig. 2. Survival and metabolic changes in VLCAD-deficient mice upon exposure to cold and fasting. A: Kaplan-Meier analysis showing survival of VLCAD+/+, VLCAD+-/−, and VLCAD−/− mice subjected to fasting in the cold. B: Representative tracings of serial blood glucose during fasting in the cold, by genotype. C: Representative tracings of body temperature during fasting in the cold, by genotype. D: Liver glycogen measurements during fasting and cold, by genotype. *P < 0.02, VLCAD +/+ at baseline vs. VLCAD−/− at 4 h after stress exposure. **P < 0.001, VLCAD−/− at baseline vs. VLCAD−/− at 4 h after stress exposure. ***P < 0.001, VLCAD−/− at baseline vs. VLCAD−/− 4 h after stress exposure.

Respiration in isolated brown adipose tissue mitochondria from nonstressed mice. A YSI oxygen meter with a Clark-type electrode (Yellow Springs Instruments, Columbus, OH) was used in a chamber with continuous stirring were used. Respiration was measured in mitochondria isolated from the brown adipose tissue of VLCAD−/− and VLCAD−/+ control mice in the presence of oligomycin, an inhibitor of ATP synthase, and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupling agent. Oxygen consumption was measured with 0.2 mM ADP, 1 μM oligomycin, or 1 μM FCCP in 5 ml of medium containing 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 6 mM MgCl2, 4 mM KH2PO4, 5 mM malate, and 5 mM pyruvate at 25°C with continuous stirring. Mitochondrial oxygen consumption was expressed as nanomoles of O2 per minute per milligram of mitochondrial protein; ADP-stimulated, oligomycin-independent, and FCCP-uncoupled mitochondrial respirations were measured in mitochondria from VLCAD−/− and VLCAD−/+ mice. Approximately 0.35–0.40 mg of mitochondria was used in each experiment.

Protein isolation and immunoblotting. Brown adipose tissue was harvested from newborn and 2-mo-old mice representing each of the VLCAD genotypes (+/+, −/−, and −/+), and snap frozen in liquid nitrogen. Expression of VLCAD and uncoupling protein (UCP)-1, UCP-2, and UCP-3 was assessed by Western blot analysis, as we previously described (11), using the following peptide-specific rabbit polyclonal antibodies: VLCAD, UCP-1 (no. sc-6528; Santa Cruz Biochemicals, Santa Cruz, CA), UCP-2 (no. sc-6527; Santa Cruz Biochemicals), and UCP-3 (no. sc-7756; Santa Cruz Biochemicals). All primary antibodies were used at a dilution of 1:1,000 with Luminol-based detection using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and the Western blot chemiluminescence reagent ECL (Amersham Pharmacia Biotech UK). All results presented are characteristic of a minimum of three independent experiments.

RNA extraction and Northern blots. RNA extraction and Northern blotting were performed from brown adipose tissue, as previously described (11). RNA (10 μg per sample lane) was resolved by electrophoresis in a formaldehyde-agarose gel and then transferred to a nitrocellulose membrane. Random-primer probes, labeled with [α-32P]dCTP, were generated from 1 kb of the amino-terminal coding sequence of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ with the Prime-It II kit (Stratagene, La Jolla, CA).

Statistical analysis. Statistical comparisons reflect two-tailed t-tests or ANOVA with correction for multiple comparisons and were performed with the StatView software package (SAS Institute). Unless otherwise noted, data are expressed as means (SD). Significance was set at P < 0.05.

RESULTS

Phenotype of VLCAD-deficient mice upon exposure to stresses of fasting alone or cold alone. The VLCAD-deficient mice, progeny of those previously described (11), demonstrate long-term survival, with the ultrastructural and electrophysiological abnormalities in the absence of physiological stress as previously noted (11). Upon exposure to the stress of fasting alone, the VLCAD−/− mice and VLCAD−/+ mice were able to maintain blood glucose levels above 80 mg/dl (Fig. 1A) and normal body temperature. Analysis of fatty acid metabolites in plasma from the fasting VLCAD-deficient mice, performed using tandem mass spectrometry, showed normal concentrations of C-14 acylcarnitine species. Levels of C16 and C18
acylcarnitines were strikingly elevated in the VLCAD−/− mice compared with wild-type mice, as previously published (29). With cold stress alone, most of the mice of all three genotypes had normal blood glucose (Fig. 1B) and normal core temperatures, but one of five VLCAD−/− mice died after developing hypoglycemia and hypothermia.

Phenotype of VLCAD-deficient mice upon exposure to stresses of fasting and cold. There was a decrease in survival rate of the VLCAD−/− and VLCAD+/− mice after short exposure to cold and fasting, but 100% of the wild-type mice survived (Fig. 2A). Kaplan-Meier analysis confirmed a significant difference in survival among all three genotypes (VLCAD+/+ vs. VLCAD−/−, P < 0.0001; VLCAD+/− vs. VLCAD−/−, P < 0.0006) (Fig. 2A). VLCAD-deficient mice developed low blood sugar levels (Fig. 2B) and decreased body temperature during the combined physiological stresses of fasting and cold (Fig. 2C). Liver glycogen decreased from 11.3 (SD 2.3) to 4.3 (SD 0.67) μg/mg wet wt tissue in the VLCAD+/+ mice (VLCAD+/+ at baseline vs. VLCAD+/+ at 4 h after stress exposure, P < 0.02), from 10.7 (SD 0.58) to 0.95 (SD 0.09) μg/mg wet wt tissue in the VLCAD+/− mice (VLCAD+/− at baseline vs. VLCAD+/− at 4 h after stress exposure, P < 0.001), and from 10.7 (SD 0.58) to 0.6 (SD 0.78) μg/mg wet wt tissue in the VLCAD−/− mice (VLCAD−/− at baseline vs. VLCAD−/− 4 h after stress exposure, P < 0.0001) (Fig. 2D). Thus glycogen depletion, suggestive of higher glycogen utilization, was more pronounced in the VLCAD−/− mice compared with the VLCAD+/+ mice. Upon death, there was minimal fat infiltration in the livers of the wild-type mice (Fig. 3A). In contrast, autopsy revealed severe macrovesicular hepatic steatosis in the VLCAD−/− mice (Fig. 3B). Fat infiltration of cardiomyocytes was not present in the VLCAD+/+ mice (Fig. 3C), but microvesicular fat infiltrates were observed in the VLCAD−/− mice (Fig. 3D). Comparatively, organ lipidosis was more pronounced in the liver than in the heart of the affected mice. The hearts of the VLCAD−/− mice did not show fat infiltration of the cardiomyocytes 4 h after the initiation of the combined fasting and cold stress.

VLCAD-deficient mice can be rescued with rewarming but not with glucose infusion. The VLCAD-deficient mice developed severe bradycardia with the combination of fasting and cold stress. Typical ECG tracings showed sinus bradycardia without evidence of ventricular ectopy. Rescue efforts were attempted with glucose and heat treatment for ~30 min (Fig. 4). Intraperitoneal injection of glucose alone failed to rescue five of seven VLCAD−/− mice after fasting and cold exposure (see the initial course in Fig. 4, A and B). However, glucose administration in conjunction with rewarming (Fig. 4A) or rewarming alone (Fig. 4B) reversed the bradycardia and rescued seven of seven VLCAD-deficient mice (final course of

---

Fig. 3. Fat accumulation in tissues of stressed VLCAD-deficient mice. Oil red O staining was performed in liver tissue (A and B) and heart tissue (C and D). Hema-toxylin-eosin counterstaining of representative 5-μm frozen tissue sections was performed 4 h after exposure to the stresses of fasting and cold. There was trivial fatty infiltration of the hepatocytes in VLCAD+/+ mouse liver (A). Intracellular lipid vacuoles (arrows target representative vacuoles) indicate macrovesicular hepatic steatosis in VLCAD−/− mouse liver (B). Arrows in D indicate representative intercardiomyocyte lipid droplets in VLCAD−/− mouse heart, which are not present in VLCAD+/+ mouse heart (C). E. Quantitative estimate of the observed fat infiltration in liver and heart tissue for VLCAD+/+ and VLCAD−/− mice, plotted as the number of fat droplets per field at ×40 magnification. *P < 0.004, liver of VLCAD+/+ vs. VLCAD−/− mice. **P < 0.009, heart of VLCAD+/+ vs. VLCAD−/− mice.

---

AJP-Heart Circ Physiol • VOL 290 • MARCH 2006 • www.ajpheart.org
Fig. 4. Heart rate recovery with glucose infusion and rewarming in the stressed VLCAD+/− mice. A: representative beat-to-beat heart rate tracing during recovery in VLCAD-deficient mice. A 0- to 15-min echocardiogram was performed at room temperature. Glucose was provided without much response (left arrow). There was a steep rise in heart rate with rewarming initiated at 20 min (heat, right arrow). Bpm. Beats/min. B: similarly, a rapid recovery in heart rate was observed with rewarming alone (heat; arrow). C–E are representative single-lead electrocardiograms (ECG) showing different stages in the recovery process (i.e., heart rate recovery over time): C at 5 min, D at 12 min, and E at 57 min. F–H are representative M-mode echocardiograms performed during recovery. Note that the shortening frequency was taken in F at 5 min (2 heart beats appreciated), in G at 25 min (5 beats appreciated), and in H at 57 min (10 beats appreciated). The end-diastolic and end-systolic areas appear larger at the initiation of recovery and smaller after complete recovery.

Abnormal brown adipose tissue mitochondrial bioenergetics in VLCAD+/− mice. To test the thermogenic capacity of the BAT mitochondria in the VLCAD−/− and VLCAD+/− mice, we examined oxygen consumption in isolated mitochondria from brown adipose tissue. We measured ADP-stimulated, oligomycin-independent, and uncoupled oxygen consumption rates in mitochondria from the VLCAD+/− and VLCAD−/− mice. Mitochondria isolated from the VLCAD−/− mice were totally uncoupled (Fig. 5A). The VLCAD−/− mitochondria failed to respond to ADP stimulation and oligomycin inhibition and were not sensitive to FCCP. The slope of mitochondrial oxygen consumption remained identical in different states of respiration (Fig. 5A). In contrast, mitochondria isolated from the VLCAD+/− mice responded to ADP stimulation by increasing their respiration rate 26.3% [from 19 (SD 0) to 23 (SD 4.17) nmol O2·min−1·mg mitochondrial protein−1], a change inhibited by oligomycin (Fig. 5B). The mitochondrial uncoupler FCCP was able to further increase oxygen consumption in VLCAD+/− brown adipose tissue mitochondria by 78.9% [from 19.0 (SD 2.9) to 35 (SD 3.6) nmol O2·min−1·mg mitochondrial protein−1] (Fig. 5B). ADP-stimulated respiration was 23.05 (SD 4.17) nmol O2·min−1·mg mitochondrial protein−1 for VLCAD+/− mice and 68.24 (SD 6.3) nmol O2·min−1·mg mitochondrial protein−1 for VLCAD−/− mice (P < 0.001). FCCP-stimulated respiration was 35.9 (SD 3.6) nmol O2·min−1·mg mitochondrial protein−1 for the VLCAD+/− mice and 49.3 (SD 9) nmol O2·min−1·mg mitochondrial protein−1 for the VLCAD−/− mice (P < 0.20) (Fig. 5C). These findings suggest that brown adipose tissue mitochondria of the VLCAD−/− mice are maximally uncoupled in the absence of cold stress. Nevertheless, this uncoupling is ineffective and perhaps insufficient to prevent the observed cold sensitivity.

Expression of fatty acid-related genes in brown adipose tissue of VLCAD-deficient mice. We subsequently performed Western blot analysis of the expression of the uncoupling proteins (UCPs) in brown adipose tissue of the three VLCAD genotypes (22–24). Brown adipose tissue from the VLCAD−/− mice showed a 2.25-fold increase in UCP-1 levels, a 2.7-fold increase in UCP-2 levels, and a 2.5-fold increase in UCP-3 levels compared with wild-type controls before exposure to cold stress (Fig. 6, A and B). We also analyzed, under basal, non-stress conditions, the mRNA expression of transcription factors that regulate brown adipose tissue fatty acid metabo-
lism (3, 28). VLCAD brown adipose tissue had an increased level of PPAR-α and a decreased level of PPAR-γ (Fig. 6, C and D). Changes in the level of these transcription factors are also probably compensatory in nature but are not sufficient to protect in the cold.

In other animal models of cardiac lipidosis and contractile dysfunction [such as the Zucker diabetic (ZDF) rat], it has been hypothesized that contractile dysfunction occurs because of impairment in the PPAR-α system (36). In the heart, PPAR-α can alter the balance between the availability of fatty acids and their utilization. Consequently, we analyzed, in the nonstressed animals, the mRNA expression of transcription factors that regulate fatty acid metabolism in the heart. There was an 80% reduction in the mRNA expression of PPAR-α in the VLCAD deficient mouse heart (Fig. 6E). However, the expression of long-chain acyl-CoA dehydrogenase was unchanged (Fig. 6E).

DISCUSSION

Genetic defects affecting mitochondrial fatty acid oxidation represent a growing subset of clinically recognized inborn errors of metabolism and may account for a significant fraction of sudden infant death cases. Physiological stresses such as fasting, exercise, and viral illnesses are usual triggers of the observed phenotypes, through mechanisms that are not well understood. As a whole, the true incidence of defects in the mitochondrial fatty acid β-oxidation pathway is unknown. However, recent utilization of tandem mass spectrometry and expanded metabolic screening programs in the United States have made it possible to identify many of these infants at birth (8, 34). As the number of asymptomatic infants diagnosed with these disorders increases, a more comprehensive understanding of the spectrum of abnormal responses to stress is needed.

A critical feature of VLCAD deficiency in this murine model is the stress-induced phenotype, in which both the VLCAD deficient and VLCAD heterozygous mice are affected with characteristics similar to those of the human disease (1, 2, 5, 21): whereas the VLCAD deficient mice exhibit the most severe phenotype, the VLCAD heterozygous mice also are at increased risk, showing an intermediate but potentially lethal phenotype. In humans, VLCAD deficiency is inherited in a recessive fashion, and human heterozygous has no phenotype (1, 19, 30). A possible rationale for the discrepancy between heterozygous humans and heterozygous mice may reside in the fact that humans rarely become exposed to stress of the severity used in the mice and that testing of heterozygous humans who might be at increased risk is unethical. Nevertheless, our data suggest that human heterozygous patients may be significantly less able to tolerate certain stresses than normal individuals.

Mitochondria are critical for several physiological processes, including heat generation in mammalian organisms.
Oxidative phosphorylation is uncoupled when heat generation is needed to maintain the body temperature in hibernating animals, in animals exposed to cold, in small rodents, and in human newborn infants. This process, termed nonshivering thermogenesis, is unique to the brown adipose tissue, which is rich in thermogenin or UCP-1, a 33-kDa inner-mitochondrial protein (7, 22, 23). UCP-1 functions to abolish the proton gradient needed to produce ATP and in this way generates heat (7). In the VLCAD-deficient mice, before cold exposure, there are increased levels of UCP-1, UCP-2, and UCP-3. Changes in the biochemistry of the brown adipose are perhaps compensatory and adaptive. However, this adaptation fails in the cold. Cold sensitivity has been reported in UCP-1-deficient mice (10), in obese mice (Lep/Lep and Lepr/Lepr) (32), and in other mouse models of fatty acid oxidation deficiency (14). This is the first report in which bradycardic arrest is profiled as the main cause of death with cold sensitivity. In our model, UCP levels are increased and leptin levels are strikingly elevated before cold exposure (data not shown). These findings suggest that the molecular events controlling thermogenesis and cold sensitivity are not uniform. In the VLCAD-deficient mice, abnormal mitochondrial bioenergetics, as evident in altered mitochondrial oxygen consumption and terminally uncoupled mitochondria, are at the basis of the cold sensitivity. The failure of glucose alone, without rewarming, to afford significant rescue may be due to either an impaired ability to reinitiate the required metabolic activity or, more likely, the nonfungibility of different energy substrates for purposes of thermogenesis.

This article broadens the set of arrhythmias associated with this animal model of VLCAD deficiency. In a recent publication (6), the analysis of 107 patients diagnosed with an inherited fatty acid oxidation disorder revealed that arrhythmia was the predominant presenting symptom. In children with VLCAD deficiency, ventricular arrhythmias are a common occurrence and are postulated to be a major cause of death. Although we (11) have previously shown that ventricular tachycardia is inducible in the nonstressed VLCAD-deficient mouse, profound bradycardia is the predominant finding upon exposure to the physiological stresses of fasting and cold. It is unclear presently whether this can be extrapolated to human patients with VLCAD deficiency. Nevertheless, these observations may be potentially relevant to other models of altered fat metabolism and cold sensitivity and may reflect metabolic conditions required for the maintenance of appropriate heart rate. Moreover, our studies show that there may be multiple independent mechanisms leading to cardiac death in VLCAD
deficiency in mice and, perhaps, in humans. In the mouse, ventricular tachycardia is a prominent feature of VLCAD deficiency in the absence of stress, but upon exposure to physiological stress, bradycardia leading to asystole is the main cardiac finding.

Potential contributing factors to the bradycardic arrest in the cold include hypoglycemia, cardiac lipidosis (lipotoxicity), and hypothermia. Because glucose infusion alone did not reverse the bradycardia, it is unlikely that hypoglycemia alone could account for the low heart rate. In other animal models of lipotoxic heart disease, it has been shown that lipotoxicity is toxic to the myocardium and may contribute to cardiac dysfunction (35, 36). Fat infiltration of the cardiomyocytes is hypothesized to change heart automaticity or to lead to degeneration of adjacent myocardial cells, through mechanisms that as yet remain unclear (35). Cardiac lipidosis also can lead to cell dysfunction and death through apoptosis (called lipoapoptosis) (9, 26). Impairment of the PPAR-α system in the heart of the VLCAD−/− mice also may be an added aspect in the observed cardiac lipidosis and heart rate dysfunction. Consequently, cardiac lipidosis in the VLCAD−/− mice may predispose to heart dysfunction in the cold. However, cardiac lipotoxicity cannot solely explain the bradycardia and recovery. Because recovery consistently occurred with rewarming alone, it appears then that uncoupling thermogenesis has a direct role in the observed heart rate recovery. Our current hypothesis is that perhaps with cooling, heat generation becomes a local phenomenon. In the heart under stress, heat generation may be favored over ATP production, leading to a decrease in ATP-dependent physiological processes such as heart rate and contractility (Fig. 7).

There are several limitations to our report. Reported heart rate testing was limited to continuous ECG monitoring during recovery experiments. For mice that died in the initial experiments, we cannot exclude other types of arrhythmias, although none appeared in later experiments. Similarly, the bradycardic response may perhaps be due to the hypoglycemia, hypothermia, cardiac lipidosis, and/or other complex molecular events, including potential effects of ion channels that affect heart rate or contractility under these physiological stresses. Future studies are needed to evaluate the effects of hypoglycemia alone or hypothermia alone in heart rate dysfunction using both the VLCAD−/− and VLCAD+/+ mice. In addition, future studies are needed to assess the molecular mechanisms involved in the observed recovery, an aspect of our research that is of potential clinical significance to our patient population.

In summary, in the absence of stress, the VLCAD−/− mice, like affected humans, appear asymptomatic. Exposure to the stresses of fasting and cold, however, triggered hypoglycemia, hypothermia, liver and heart steatosis, and severe bradycardia in our mice, replicating features of human patients with VLCAD deficiency. In our model, both the VLCAD−/− and VLCAD+/− genotypes were affected. Bioenergetics of brown adipose tissue mitochondria are abnormal in the VLCAD-deficient mice compared with the wild-type controls. Biochemical changes in the brown adipose tissue are probably adaptive in nature, but such compensation is not sufficient to prevent the hypothermia and heart rate dysfunction upon exposure to the combined physiological stresses of fasting and cold. Our studies provide the first insights into the possible type of cardiac death in the setting of fatty acid β-oxidation enzyme defects and a stress-induced phenotype. Finally, they afford a unique tractable model for the study of tissue and role-specific targeting of substrates for energy generation in a mouse model of this human disease.

ACKNOWLEDGMENTS

We thank Jack Baty, Division of Biostatistics, Washington University School of Medicine, for assistance. We also thank Marilyn Reel for editorial assistance.

GRANTS

This work was supported by fellowship grants from the Robert Wood Johnson Foundation, the Vanderbilt Physician-Scientist Program, and the American Heart Association-South East Affiliate (to V. J. Exil), the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences (to B. Bartelds), the National Heart, Lung, and Blood Institute (to A. W. Strauss), and the National Institute of Diabetes and Digestive and Kidney Diseases Mouse Metabolic Phenotyping Center (to J. N. Rottman).

REFERENCES


