Liver and Muscle Contribute Differently to the Plasma Acylcarnitine Pool During Fasting and Exercise in Humans

G. Xu, J. S. Hansen, X. J. Zhao, S. Chen, M. Hoene, X. L. Wang, J. O. Clemmesen, N. H. Secher, H. U. Häring, B. K. Pedersen, R. Lehmann, Cora Weigert, and Peter Plomgaard

Key Laboratory of Separation Science for Analytical Chemistry (G.X., X.J.Z., X.L.W.), Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China; Department of Clinical Biochemistry (J.S.H., P.P.), Rigshospitalet, Copenhagen, Denmark; The Centre of Inflammation and Metabolism and the Centre for Physical Activity Research (J.S.H., B.K.P., P.P.), Department of Infectious Diseases and CMRC, Rigshospitalet, Copenhagen, Denmark; Department of General Surgery and Laboratory of General Surgery (S.C.), Xinhua Hospital, affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China; Institute of Biliary Tract Diseases Research (S.C.), Shanghai Jiao Tong University School of Medicine, Shanghai, China; Division of Endocrinology (M.H., H.U.H., R.L., C.W.), Diabetology, Angiology, Nephrology, Pathobiochemistry and Clinical Chemistry, Department of Internal Medicine IV, University Tuebingen, Germany; Department of Hepatology (J.O.C.), Rigshospitalet, Copenhagen, Denmark; Department of Anaesthesiology (N.H.S.), The Copenhagen Muscle Research Centre, Rigshospitalet, Copenhagen, Denmark; Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Zentrum München at the University of Tuebingen (H.U.H., R.L., C.W.), Tuebingen, Germany; and German Center for Diabetes Research (H.U.H., R.L., C.W.), Germany

Background: Plasma acylcarnitine levels are elevated by physiological conditions such as fasting and exercise but also in states of insulin resistance and obesity.

Aim: To elucidate the contribution of liver and skeletal muscle to plasma acylcarnitines in the fasting state and during exercise in humans.

Methods: In 2 independent studies, young healthy males were fasted overnight and performed an acute bout of exercise to investigate either acylcarnitines in skeletal muscle biopsies and arterial-to-venous plasma differences over the exercising and resting leg (n = 9) or the flux over the hepato-splanchnic bed (n = 10).

Results: In the fasting state, a pronounced release of C2- and C3-carnitines from the hepato-splanchnic bed and an uptake of free carnitine by the legs were detected. Exercise further increased the release of C3-carnitine from the hepato-splanchnic bed and the uptake of free carnitine in the exercising leg. In plasma and in the exercising muscle, exercise induced an increase of most acyl-carnitines followed by a rapid decline to preexercise values during recovery. In contrast, free carnitine was decreased in the exercising muscle and quickly restored thereafter. C8-, C10-, C10:1-, C12-, and C12:1-carnitines were released from the exercising leg and simultaneously; C6, C8, C10, C10:1, C14, and C16:1 were taken up by the hepato-splanchnic.

Conclusion: These data provide novel insight to the organo-specific release/uptake of acylcarnitines. The liver is a major contributor to systemic short chain acylcarnitines, whereas the muscle tissue releases mostly medium chain acylcarnitines during exercise, indicating that other tissues are contributing to the systemic increase in long chain acylcarnitines. **(*J Clin Endocrinol Metab* 101: 5044–5052, 2016)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received April 10, 2016. Accepted September 15, 2016.
First Published Online September 20, 2016

Abbreviation: CoA, coenzyme A.

Acylcarnitines are important intermediates in the transport of long chain acyl-coenzyme A (CoA) into the mitochondrion for subsequent β-oxidation. In the mitochondrial matrix, the carnitine esters are reconverted to acyl-CoA. From inherited diseases affecting fatty acid oxidation, it is revealed that the process is bidirectional; acylcarnitines can be formed in the mitochondrial matrix from acyl-CoA and carnitine and are exported and released into plasma (1). Almost all acyl-CoA can be found as its respective carnitine ester in the circulation with a pronounced increase in physiological and pathophysiological conditions (1). This may be due to inherited enzymatic defects and conditions of increased free fatty acids oxidation as exercise (2) or fasting (3). Plasma acylcarnitines attract interest, because they may represent biomarkers of metabolically acquired conditions such as diabetes (4), obesity (5, 6), insulin resistance (7, 8), and cardiovascular diseases (9).

Both in vitro (10) and in vivo (11) animal studies suggest tissue specific differences in acylcarnitine fluxes as mitochondria isolated from different tissues release different species of acylcarnitines (10). In mice, there is no clear association between tissue and circulating levels of acylcarnitines (12). One strategy to identify the origin of acylcarnitine uptake or release is by studying arterial to venous differences. This approach has been applied in pigs after an overnight fast and during a meal. It revealed that different tissues take up or release specific acylcarnitine species and a particular importance of the liver for C2-carnitine metabolism (11). Human data on the uptake or release of individual acylcarnitine species over tissues/organs are lacking. In response to exercise particularly medium and long chain acylcarnitines increase in plasma with a rapid decline afterwards, whereas short chain species C2- and C4-OH-carnitine remain high in the recovery phase (13, 14). The contracting muscle tissue is considered to be the main contributor to the elevated systemic acylcarnitine concentrations due to the increase in glucose and fatty acid oxidation during exercise. In the muscle tissue, particularly C2-carnitine increases in response to high intensity exercise, whereas several short, medium, and long chain acylcarnitines are increased in muscle 24 hours after the last exercise session of a 6-month training intervention (2, 15, 16). Moreover, primary human myotubes have been shown to release excess short, medium, and long chain acylcarnitines (17). However, other organs/tissues as the liver and heart may also contribute to the circulating levels of acylcarnitines during exercise.

The aim of the present study was to investigate the leg and hepato-splanchnic fluxes of acylcarnitines in humans after an overnight fast and when stimulated by an acute bout of exercise. For this, plasma samples from 2 existing independent exercise studies, where arterial-to-venous differences over the hepato-splanchnic bed (18) and the leg (19), respectively, had been investigated, were analyzed for acylcarnitine concentrations by targeted ultra performance liquid chromatography-mass spectrometry. Determining the contribution of different tissues to the plasma acylcarnitine profile is important to understanding not only their physiological regulation but also their role as biomarkers in acquired metabolic conditions such as obesity and type 2 diabetes and in associated disorders such as cardiovascular disease.

Methods and Materials

Experimental design

The effect of exercise on leg acylcarnitine flux and muscle acylcarnitine content was addressed by investigating plasma and muscle tissue samples from a 1-legged knee extensor study where the exercising leg was compared with the counter lateral resting leg (19). Arterial-to-venous differences over both legs were combined with analyses of biopsies from both the resting and exercising muscle. Briefly, after an overnight fast, 9 healthy male subjects (age, 20.9 ± 0.5 y and Body Mass Index, 22.6 ± 0.8 kg/m^2) performed 1-legged knee-extensor exercise for 2 hours at 50% of maximum workload on a modified Krogh ergometer, whereas the contralateral leg was resting. The maximum workload was determined 1 week before the experimental day. Catheters were inserted retrograde in both femoral veins as well as 1 femoral artery. The retrograde placement avoids a contribution from the veins draining the lower abdominal adipose tissue (vena epigastrica superficialis) and leg cutaneous and subcutaneus adipose tissues (vena saphena magna), which is important for the study of lipid metabolism (20). The subjects were fasting until 3 hours after the exercise bout. Blood flow was determined for both the resting and exercising leg using ultrasound Doppler in 3 subjects, and the mean value was used to calculate net skeletal muscle release/uptake for all subjects. Three blood samples were drawn simultaneously to each time point. Percutaneous needle biopsies from the vastus lateralis muscle of both legs were obtained at baseline after 120 minutes, 300 minutes, and 24 hours for acylcarnitine analysis.

The hepato-splanchnic exercise study has also been described in detail previously (18). In brief, 10 healthy male subjects (age, 22.9 ± 0.8 y and Body Mass Index, 22.6 ± 0.5 kg/m^2) reported to the laboratory after an overnight fast. Catheters were inserted in the brachial artery of the nondominant arm and a liver vein via the right femoral vein. The subjects performed a 2-hour cycling exercise at 60% maximal O_2 uptake with both legs in semisupine position. One week before the experimental day, the subjects performed an incremental exercise test on a cycle ergometer (Monark Ergomedic 839 E; Monark Ltd) and maximal O_2 uptake was determined to by indirect calorimetry (mean 51.0 ± 1.2 L/kg·min) (Quark b^2; CosMed) (for further details see Ref. 18). Blood samples were obtained from both catheters at each time point, and hepatic blood flow was measured by indocyanine green clearance. In both studies, blood was collected in EDTA-containing tubes, placed on ice, immediately spun, and aliquots were stored at −80°C until analysis. Samples obtained at base-

line, 60, 120, 180, 240, 300, and 360 minutes were used for acylcarnitine analysis. Data on follistatin (21) and fibroblast growth factor 21, including blood flow (18), have been published for both studies.

Quantification of acylcarnitines in plasma and muscle biopsy samples

Analysis of acylcarnitines was performed by ultra performance liquid chromatography-mass spectrometry. Plasma samples were deproteinized with acetonitrile containing isotope labeled internal standards (Ten Brink), run to dryness in a vacuum centrifuge, and reconstituted in acetonitrile/water (4:1). Freeze-dried muscle biopsy samples were extracted with methyl-tert-butyl ether, and a mix of the 2 phases was used for analysis (22). Quantification of acylcarnitines in plasma and in muscle tissue was performed by ultra performance liquid chromatography (Waters Corp) coupled with a Q Exactive HF MS (Thermo Fisher). For chromatographic separation, an Acquity BEH C8 column (2.1 × 100 mm, 1.7 μm) was used, the column temperature was maintained at 50°C, and the injection volume was 5 μL. The mobile phase (A) was water with 0.1% formic acid and (B) was acetonitrile. From the start to 1 minute, buffer B was kept at 10% and linearly increased to 40% in 4 minutes, then continuously changed to 100% over 12 minutes and kept for 5 minutes, then reduced to 10% and maintained for 3 minutes. The flow rate was kept at 0.35 mL/min. Mass spectrometric detection was performed in positive ion mode. The mass spectrometric parameters were set as resolution 120 000, capillary temperature 300°C, and spray voltage 3.5 kV. Integrated peak areas of samples and of appropriate internal standards d_3-L-carnitine, d_3-C2-carnitine, and d_3-C10-carnitine were used for quantification. Acylcarnitines containing double bonds are denoted CX:Y, where X is the number of carbon atoms in the acyl chain and Y the number of double bonds.

Calculations of flux

Leg and hepato-splanchnic fluxes (release or uptake) of acylcarnitines were calculated by multiplying the arterial-to-venous difference by the plasma flow:

The leg blood flow was measured by ultrasound Doppler technique whereas the hepatic blood flow was measured using indocyanine green clearance. The blood flow was converted into a plasma flow by adjusting with the hematocrit, as detailed in Ref. 18. In the 1-legged exercise study, the leg flux during fasting was calculated from both legs using the preexercise time point (t = 0) (Figure 1, A and B).

Statistics

Data are presented as mean ± SEM. To evaluate an uptake or release over the leg or hepato-splanchnic bed in the fasting state, the flux was evaluated at time point "0 minutes" using a *t* test compared with 0. The difference between the exercising and resting legs was evaluated by a two-way ANOVA. The group effect was evaluated using the slice function in the mixed model. Significant effect of time was evaluated by a one-way ANOVA followed by a Dunnetts post hoc test. A similar approach was applied on both leg fluxes and muscle acylcarnitine content. The hepato-splanchnic fluxes and the arterial concentrations were evaluated by a one-way ANOVA with a post hoc test (Dunnetts).

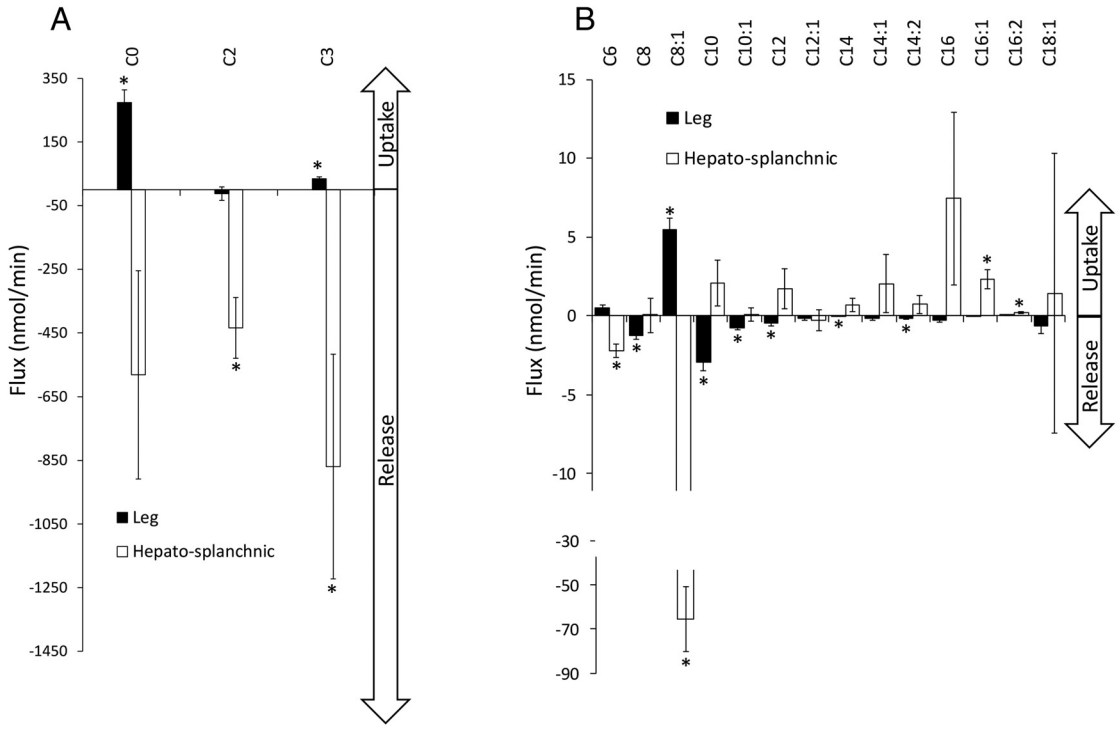

Figure 1. Comparison of the acylcarnitine fluxes (nmol/min) over the hepato-splanchnic bed and leg after an overnight fast in humans. A, C0 (free carnitine), C2, and C3. B, C6–C18. From the 1-legged exercise study, the presample values (0 min) from both legs were included. Black bars (■) indicate leg, and open bars (□) indicate the hepato-splanchnic bed. A positive value equals an uptake, whereas a negative value equals a release. *, significant difference from 0.

The statistical analyses were performed using SAS 9.4 (Institute, Inc), and a $P < .05$ was considered statistically significant.

Results

Leg and hepato-splanchnic fluxes of acylcarnitines after overnight fast

After overnight fast, the hepato-splanchnic bed released pronounced amounts of C2- and C3-carnitines, whereas there was a slight uptake of C3 by the leg (Figure 1A). The release of free carnitine from the hepato-splanchnic bed did not reach significance, whereas there was a clear uptake by the leg. The fluxes of other acylcarnitines were small, with a release of some medium and long chain acylcarnitines by the leg (C8, C10, C10:1, C12, C14, and C14:2), whereas C8:1-carnitine was taken up (Figure 1B). The flux over the hepato-splanchnic bed revealed a different pattern with a release of C6- and C8:1- and an uptake of C16:1- and C16:2-carnitine (Figure 1B).

Divergent leg and hepato-splanchnic bed fluxes of medium chain acylcarnitines during exercise

Except for C8:1, the arterial concentration of all medium chain acylcarnitines showed an increase with exercise in both the 1-legged exercise and the hepato-splanchnic bed studies (Figure 2, top panel, and Supplemental Tables 1 and 2). In contrast to the resting leg, the exercising leg released C6-, C8-, C10-, C10:1-, C12-, and C12:1-carnitine. Several of these acylcarnitines showed an uptake by the hepato-splanchnic bed (Figure 2). The hepato-splanchnic uptake of C6-carnitine returned to a release in the recovery phase. C8:1-carnitine showed a different regulation compared with the other medium chain acylcarnitines. It was released from the hepato-splanchnic circulation, but the release was not influenced by exercise. In contrast, the uptake of C8:1 in the exercising leg was increased (Figure 2). The flux over the resting leg was unaltered during exercise. The systemic concentration of free fatty acids increased from 646 ± 138 before exercise to 1164 ± 167 μmol/L immediately after exercise in the 1-legged exercise study and from 448 ± 48 to 1348 ± 143 μmol/L immediately after exercise in the hepato-splanchnic study (data not shown).

No pronounced change in the hepato-splanchnic and leg fluxes of long chain acylcarnitines

All carnitine esters with an acyl chain from C14 to C18 increased systemically with exercise (Supplemental Tables 1 and 2). However, no release from the resting or exercising leg was observed with exercise. Instead, there was an uptake of C16:1- and C16-OH-carnitine in the exercising leg. Over the hepato-splanchnic bed, C14- and C16:1-carnitine showed an increased uptake with exercise.

Release of free carnitine (C0) and C2- and C3-carnitines from the hepato-splanchnic bed during exercise

In contrast to the other acylcarnitines, the systemic concentration of C2-carnitine remained increased during the recovery phase (Figure 3). C3-carnitine increased during exercise but decreased to resting levels and below during the recovery. A constant release of C2-carnitine from the hepato-splanchnic bed was found, which was not affected by exercise (Figure 3). No uptake or release of C3-carnitine by the exercising leg was detected, but an increased release by the hepato-splanchnic bed was. Other short chain acylcarnitines with an exercise-induced increase in arterial plasma were C4- and C5-carnitines (Supplemental Tables 1 and 2), whereas C4-OH-carnitine was increased only in the recovery phase. The arterial concentration of free carnitine (C0) was only modestly influenced by exercise (Figure 3). The exercising leg showed an increased uptake of free carnitine, whereas the flux over the resting leg was unchanged.

Regulation of acylcarnitine concentrations within skeletal muscle tissue during exercise

An increase of almost all detected short, medium, and long chain acylcarnitines was observed in the exercising leg (Figure 4 and Supplemental Table 3). A strikingly high change compared with preexercise values was found for C3-carnitine (34-fold), C4-carnitine (59-fold), and C5-carnitine (26-fold). Hydroxylated acylcarnitines C4:0-OH, C5:0-OH, and C6:0-OH were also increased in the recovery phase (Supplemental Table 3). In the resting leg, a decrease was observed for C2-carnitine immediately after exercise. Free carnitine (C0) was reduced in the exercising leg and returned to preexercise values, whereas no change occurred in the resting leg (Figure 4). Only C18:1- and C18:2-carnitines were not changed by exercise in muscle tissue. On the day after exercise, the acylcarnitine levels in both legs were not different from the preexercise values.

Discussion

The main finding in the present study is that both at rest and during exercise, the hepato-splanchnic bed and the leg muscle contribute differently to the acylcarnitine pool in plasma (Figure 5). The results obtained from the muscle biopsies demonstrate that alterations in acylcarnitine tissue concentrations do not necessarily reflect an altered uptake or release. Even though most acylcarnitines in-

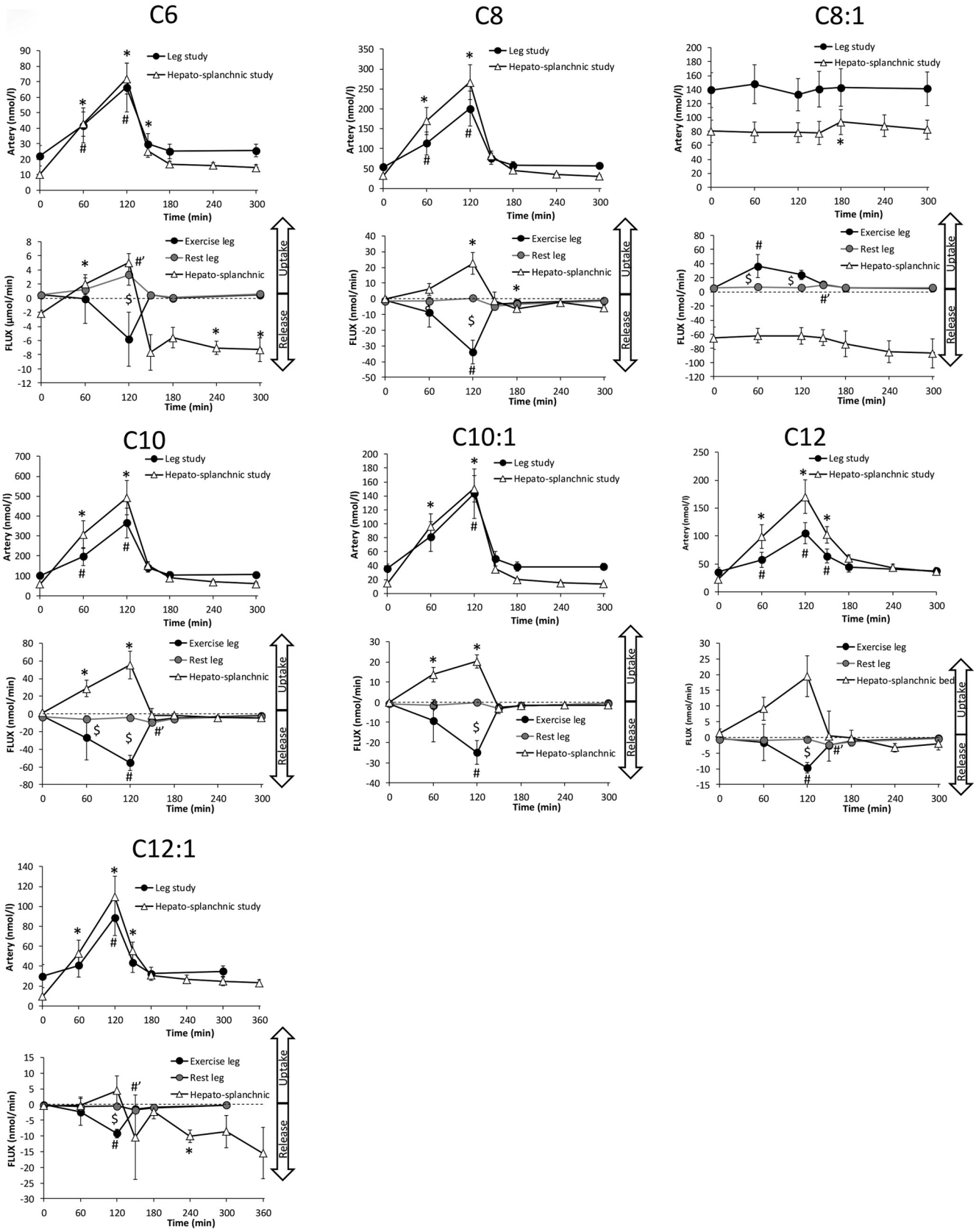

Figure 2. Medium chain acylcarnitine response to exercise in humans. Top graphs depict the systemic response (arterial concentration; nmol/L) to exercise: 1-legged exercise study (●) and hepato-splanchnic study (Δ), where 0–300 minutes are included. Bottom graphs illustrate the fluxes (nmol/min) over the resting leg (◍), exercising leg (●), and hepato-splanchnic bed (Δ). $, significant difference between legs by a two-way ANOVA. A significant change from (0 min) by a one-way ANOVA with a Dunnetts post hoc test is indicated by * for the hepato-splanchnic study and # and #′ for the exercising and resting le, respectively. $P < .05$ was considered significant.

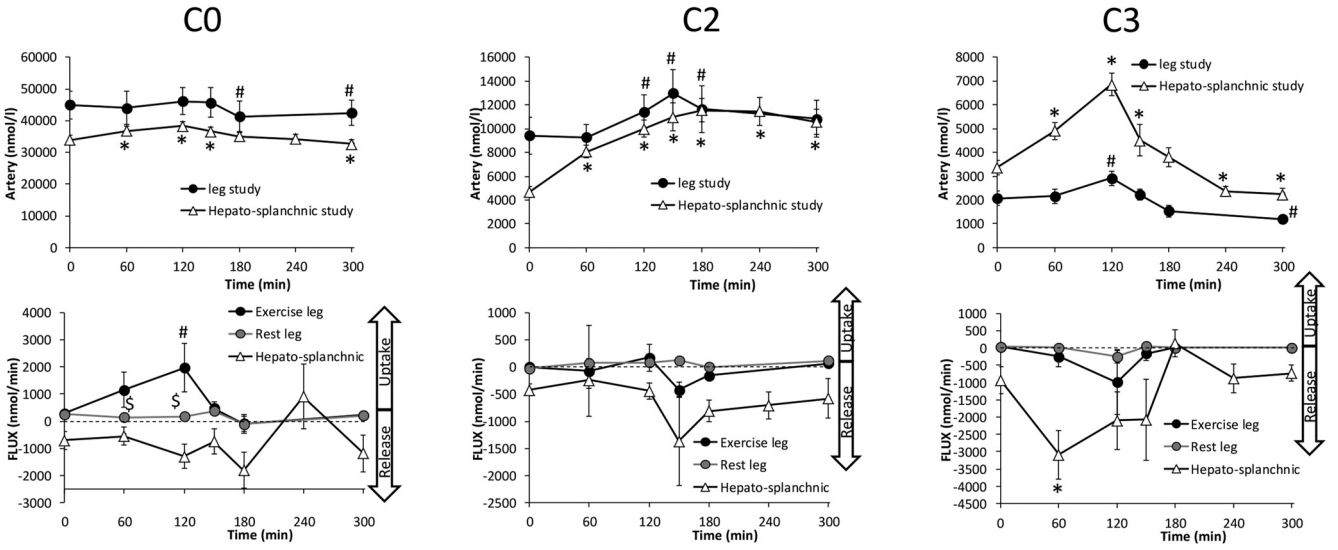

Figure 3. C0 (free carnitine) and C2- and C3-carnitine responses to exercise in humans. Top graphs depict the systemic response (arterial concentration; nmol/L) to exercise: 1-legged exercise study (●) and hepato-splanchnic study (Δ). Bottom graphs illustrate the fluxes (nmol/min) over the resting leg (◍), exercising leg (●), and hepato-splanchnic bed (Δ), where 0–300 minutes are included. A significant change from (0 min) by a one-way ANOVA with a Dunnetts post hoc test is indicated by * for the hepato-splanchnic and # for the 1-legged exercise studies, respectively. $P < .05$ was considered significant.

creased both in plasma and in the working muscle during exercise, only medium chain acylcarnitines were released from the leg. Moreover, the data provide evidence that the increase in skeletal muscle tissue acylcarnitines is determined only by the muscle contraction and the subsequent demand for fuel and not by a humoral stimulus, because no change was detectable in the resting leg.

It has been reported by several groups that exercise increases plasma acylcarnitines (2, 13, 14, 23, 24). The present study adds to these findings by demonstrating that medium chain C6- to C12-carnitines are released from the exercising leg. One human study from the premetabolomics era found no release of total acylcarnitines measured over the leg during exercise using an enzymatic assay (23), corresponding to the fact that the acylcarnitine species released from the leg in the present study are low in abundance. The hepato-splanchnic bed contributes to the plasma acylcarnitine pool during exercise with release of C2- and C8:1-carnitine and enhanced release of C3-carnitine. These data suggest that tissues other than skeletal muscle and liver are the source of the elevated levels of other acylcarnitine species after exercise, in particular of long chain acylcarnitines. For example, the working heart uses preferentially fatty acids for ATP production (25) and thus may contribute to increased plasma acylcarnitines. During exercise, the glomerular filtration rate is reduced (26) and a reduced excretion of acylcarnitines would be expected, leading to an accumulation in plasma.

Exercise increased the muscle concentration of all classes of acylcarnitines, including those with odd-numbered and hydroxylated acyl chains, only in the exercising leg and not in the resting leg, indicating that the muscle contraction per se and thus high demand for ATP generation leads to a huge increase in substrate oxidation. Moreover, increased acylcarnitine formation may prevent CoA trapping, which is not a mitochondrial function per se (24). Not only medium and long chain acylcarnitines originating from the oxidation of abundant even-numbered fatty acids were increased in muscle tissue of the exercising leg. The increase in C3 and C5 acylcarnitines reflects enhanced oxidation of odd-chain fatty acids or of branched chain amino acids. The increase in C4-OH is mainly derived from the ketone body β-hydroxybutyrate (27) and also found in muscle tissue of fasted humans and mice (12, 27). Notably, the leg takes up β-hydroxybutyrate as demonstrated in resting healthy humans during an experimental increase of β-hydroxybutyrate levels (28). Thus, the muscle acylcarnitine pattern reflect the high demand for ATP generation during exercise, leading to enhanced oxidation of glucose, lipids, and amino acids.

The decrease in free carnitine only in the exercising muscle tissue indicates a shift from free carnitine to carnitine esters. The free carnitine pool in the exercising muscle was restored after 3 hours of recovery, and all elevated acylcarnitines were back to preexercise levels without a detectable net release from the leg. The increase in muscle free carnitine from 8.9 to 16.5 nmol/mg tissue can to a large extend be attributed to the decrease in the total amount of acylcarnitines (from 8.6 to 2.4 nmol/mg tissue). This shift back to free carnitine is well in line with an acyl-CoA/acylcarnitine system buffering the huge increase in acyl-CoA during high oxidation

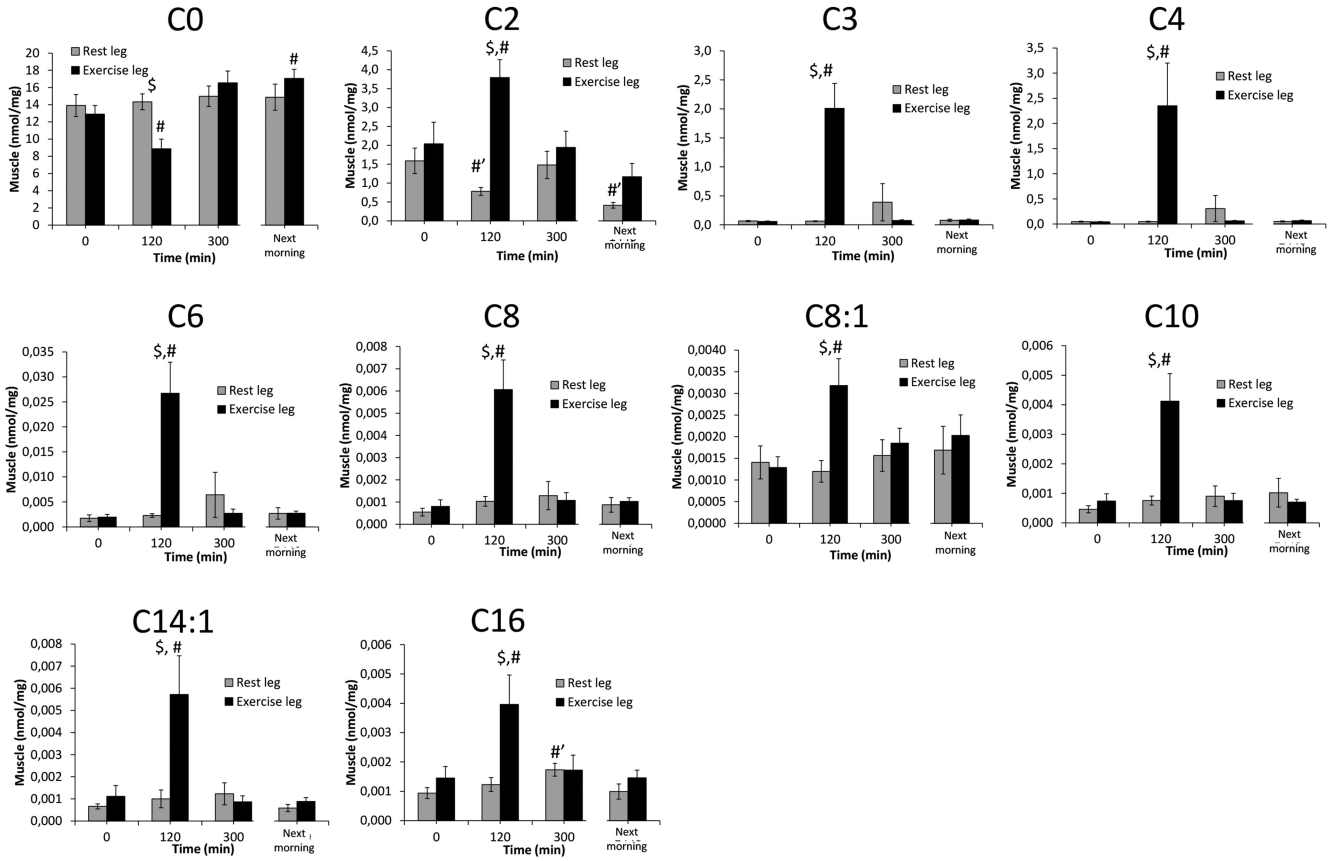

Figure 4. Acylcarnitine changes in the muscle tissue. Acylcarnitine muscle content (nmol/mg dry weight muscle) from the resting leg (▒) and exercising leg (■) before (0 min), immediately after exercise (120 min), 3 hours into recovery after exercise (300 min), and the next morning, where the subjects reported to the lab after an overnight fast (next morning). $, significant difference between groups. A significant change from (0 min) by one-way ANOVA with a Dunnetts post hoc test is indicated by # and #′ for the exercising and resting legs, respectively. $P < .05$ was considered significant.

rates of fatty acids and other substrates to maintain free HS-CoA (24). After exercise the acyl-chains buffered as acylcarnitines can be reesterified to acyl-CoA and enter their biochemical pathways, resulting in the preexercise level of free carnitine. The increased uptake of carnitine in the leg can also contribute to the recovery of the free carnitine pool after exercise. Notably, the muscle itself appears not to be able to synthesize carnitine (29).

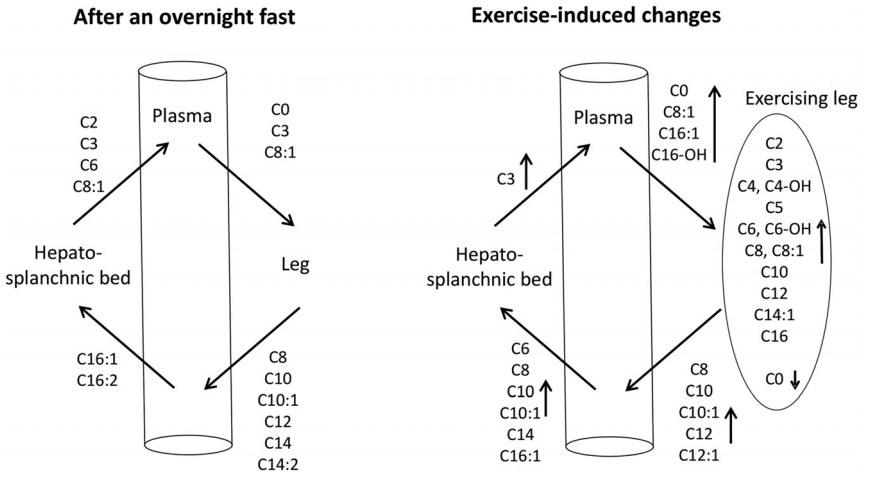

Figure 5. Schematic presentation of the acylcarnitine fluxes after an overnight fast (left side) and of the exercise-induced changes (right side). For the latter, fluxes at 60 or 120 minutes of exercise were compared with 0 minutes (immediately before acute exercise). Acylcarnitine changes within the muscle tissue of the exercising leg are included on the right side. ↑, increase by exercise; ↓, decrease by exercise.

C2- and C4-OH-carnitines differed in their kinetics from most other acylcarnitines, because their systemic plasma levels increased in the recovery phase. Both carnitine esters could reflect a prolonged acetyl-CoA production leading to formation of ketone bodies. Notably, the subjects in both studies fasted during the recovery, which explains the reliance on fatty acid oxidation in confirmation of previous results (14). Another noticeable acylcarnitine was C8:1, which shows a different pattern in plasma and over the hepato-splanchnic bed compared with other medium chain acylcarnitines. C8:1 was constantly released from the hepato-splanchnic

bed and taken up by the leg with a further increase during exercise, but systemic levels were unchanged or only modestly influenced by exercise. Similarly, we did not observe an increase in systemic C8:1-carnitine levels after 60 minutes of exercise in a previous study (14). It is difficult to speculate about the regulation of the metabolic route that builds C8:1, which is apparently different from the formation of the other medium chain acylcarnitines. C8:1-carnitine has been suggested to be derived from fatty acid degradation by gut bacteria (30), which could be in line with its constant release from the hepato-splanchnic bed being unaffected by the exercise bout in our study.

The data also indicate that elevated tissue levels of acylcarnitines must be carefully interpreted because both an increased production and an increased extraction from plasma can contribute. The observed accumulation of C8:1 and C16 in the muscle tissue of the exercising leg can also be a result of the increased uptake of these acylcarnitines in the exercising leg.

The plasma acylcarnitine profile is used to detect inborn errors in fatty acid oxidation (31). Similar acylcarnitine profiles might also uncover metabolic impairments, eg, a reduced flexibility to switch between the oxidation of different substrates during an acute metabolic challenge like exercise. Because dysregulated mitochondrial oxidation may contribute to the pathogenesis of type 2 diabetes (32), we have, in a previously published study, compared the plasma acylcarnitine profiles in patients with type 2 diabetes with values in a matched group of healthy controls during exercise (14). Notably, no difference in the exercise-induced increase in plasma acylcarnitines between the 2 groups was found (14), suggesting that the mitochondrial impairment observed with type 2 diabetes (32) is overruled by an acute bout of exercise. This also emphasizes that plasma acylcarnitine analysis should be done in a very standardized way, taking physical activity and food intake into account.

A limitation is that the acylcarnitine flux detected over the hepato-splanchnic bed and leg was induced by different types of exercise. The relative increases in C2- and C3-carnitines and in nonesterified fatty acids appear to be more pronounced in the hepato-splanchnic study, presumably reflecting the greater metabolic challenge when exercising with 2 legs. Because the respiratory exchange ratios were not determined in both studies, it could only be speculated whether the difference in exercise modalities influenced the fuel selection during exercise. Notably, the systemic increase in several acylcarnitine species is comparable. More studies are needed to elucidate the impact of exercise-induced hormones such as glucagon, cortisol, GH, and epinephrine as well as dietary and training status on acylcarnitine metabolism.

Another limitation is that the hepato-splanchnic bed is a mix of blood from several organs, including liver, gut, pancreas, and spleen, although the liver can be expected to have the highest metabolic activity during an exercise bout. Schooneman et al showed the flux of carnitine and of 5 acylcarnitines (C2, C3, C4-OH, C16, and C18:1) over liver, muscle, kidney, and gut in fasting pigs (11). The porcine model allowed portal vein catheterization, which makes calculations of flux over the liver possible. After an overnight fast, both C2- and C3-carnitines are released from the liver. Thus, acylcarnitine fluxes in the fasting state are comparable in humans and pigs with a pronounced net release of C2- and C3-carnitines from the liver. In contrast to the porcine model, we found a clear uptake of free carnitine by the leg. When studying lipid metabolism of the leg by arterial to venous differences, it is important to sample pure leg blood and avoid contamination of blood leaving the leg with blood from the subcutaneus adipose tissue (20). In the presented study, we followed the recommendation by van Hall et al and inserted the catheters retrograde in the femoral vein (20). This difference to the leg blood sampling in the porcine model may explain the different results for leg flux of free carnitine and C2-carnitine.

In conclusion, fluxes of acylcarnitines in humans are tissue dependent during fasting and exercise. An increased tissue or plasma level of total or specific acylcarnitines should be interpreted with caution when predicting tissue release or uptake. To understand the information carried by a plasma biomarker, it is important to address which tissue(s) is/are responsible for its production, release, and clearance.

Acknowledgments

Address all correspondence and requests for reprints to: Peter Plomgaard, MD, PhD, Department of Clinical Biochemistry and Centre for Physical Activity Research (CFAS), Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. E-mail: plomgaard@dadlnet.dk; or Cora Weigert, PhD, Division of Pathobiochemistry and Clinical, Chemistry, University Tuebingen, Otfried-Müller-Straße 10, 72076 Tuebingen, Germany. E-mail: cora.weigert@med.uni-tuebingen.de.

The Centre for Physical Activity Research (CFAS) is supported by a grant from TrygFonden. The Copenhagen Muscle Research Centre is supported by a grant from the Capital Region of Denmark. During the study period, the Centre of Inflammation and Metabolism (CIM) was supported by a grant from the Danish National Research Foundation (DNRF55). CIM/CFAS is a member of DD2, the Danish Center for Strategic Research in Type 2 Diabetes (Danish Council for Strategic Research Grants 09-067009 and 09-075724). This study was also supported by a grant from the Danish Diabetes Academy supported by the Novo Nordisk Foundation (C.W.); by the Augustinus Founda-

tion, and in part by grants from the German Federal Ministry of Education and Research to the German Centre for Diabetes Research (01GI0925); by grants of the Sino-German Center for Research Promotion (GZ 753 by Deutsche Forschungsgemeinschaft and National Natural Science Foundation of China [to G.X. and R.L.] and LE 1391/1-1 by Deutsche Forschungsgemeinschaft [to R.L.]); and by the key foundations (21435006) and the creative research group project (21321064) from the National Natural Science Foundation of China (to G.X.).

Disclosure Summary: The authors have nothing to disclose.

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