Mechanisms underlying the onset of oral lipid-induced skeletal muscle insulin resistance in humans

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ABSTRACT
Several mechanisms, such as innate immune responses via toll-like receptor-4, accumulation of diacylglycerols (DAG)/ceramides and activation of protein kinase C (PKC), are considered to underlie skeletal muscle insulin resistance. Here, we examined initial events occurring during the onset of insulin resistance upon oral high-fat loading compared to lipid and low-dose endotoxin infusion.

Sixteen lean insulin-sensitive volunteers received intravenous fat (iv fat), oral fat (po fat), intravenous endotoxin (LPS) and intravenous glycerol as control (con). After 6 hours, whole-body insulin sensitivity was reduced by iv fat, po fat and LPS to 60%, 67% and 48%, respectively (all \( P<0.01 \)), which was due to decreased nonoxidative glucose utilization, while hepatic insulin sensitivity was unaffected. Muscle PKC\( \theta \) activation increased by 50% after iv and po fat, membrane Di-C18:2 DAG species doubled after iv fat and correlated with PKC\( \theta \) activation after po fat, whereas ceramides were unchanged. Only after LPS, circulating inflammatory markers (TNF-\( \alpha \), IL-6, IL-1ra), their mRNA expression in subcutaneous adipose tissue and circulating cortisol were elevated.

Oral fat ingestion rapidly induces insulin resistance by reducing nonoxidative glucose disposal, which associates with PKC\( \theta \) activation and a rise in distinct myocellular membrane DAG, while endotoxin-induced insulin resistance is exclusively associated with stimulation of inflammatory pathways.
Insulin resistance is frequently associated with obesity and precedes the onset of type-2 diabetes by decades (1). Although the pathogenesis of common insulin resistance is not yet clarified, activation of the innate immune system and abnormal lipid and/or energy metabolism are considered as key mechanisms (2;3).

Particularly, circulating fatty acids (FA) and triglycerides associate with obesity, insulin resistance and can predict type-2 diabetes (3-5). Employing intravenous lipid infusion in humans made it possible to detect that FA elevation induces insulin resistance (6), which is due to a reduction of insulin-stimulated muscle glucose transport (6;7). While numerous studies mainly in rodents showed that long-term high-fat diet also results in insulin resistance (3), data on the initiation of insulin resistance after a single high-fat meal are scarce (8). Moreover, the mechanisms underlying lipid-induced insulin resistance are also under debate. Cellular accumulation of lipid metabolites such as diacylglycerols (DAG) can activate novel protein kinase C (PKC) isoforms, which cause serine phosphorylation of insulin receptor substrate-1 (3). Alternatively, binding of FA to muscle toll-like receptor-4 (TLR-4) can give rise to ceramides, which impair insulin-stimulated AKT activation (9). FA - like the bacteria-derived lipopolysaccharide (LPS) - may bind to TLR-4 on macrophages and adipocytes and thereby activate the innate immune system and proinflammatory pathways (10). Indeed, deficiency of TLR-4 (11) or CD14 (12) largely protects mice from lipid-induced insulin resistance, suggesting a causal role of the CD14-TLR4 receptor complex for high-fat diet and LPS-induced insulin resistance. Of note, ingestion of high-fat meals and infusion of low-dose LPS can raise circulating cytokines (13;14), which in turn could activate different cellular pathways and raise lipid metabolites (2;3;9). These divergent and complex findings leave the primary mechanism by which fat loading induces insulin resistance in humans unresolved.

This study examined the initial events associated with the onset of insulin resistance upon a high-fat load applied either by the oral or the intravenous route compared with induction of a similar degree of insulin resistance by low-dose LPS infusion in healthy humans.
RESEARCH DESIGN AND METHODS

Volunteers. All participants gave their written informed consent before inclusion into the study (Clinicaltrials.gov Identifier number: NCT01054989), which was performed according to the Declaration of Helsinki and approved by the local institutional ethics board. Inclusion criteria were an age of 20-40 years and a BMI of 20-25 kg/m². Exclusion criteria were dysglycemia, menstrual irregularities, family history of diabetes, a history of smoking and other acute or chronic diseases including cancer. Humans taking any medication affecting insulin sensitivity, lipid metabolism or immune system were also excluded. Screening included a standardized 75-grams oral glucose tolerance test, routine lab tests, bioimpedance analysis to obtain lean body mass and a questionnaire on habitual physical activity. Females were studied between days 5-9 of their menstrual cycle. All participants were assigned to four study days at least 4 weeks apart in random order and were instructed to maintain normal physical activity and a carbohydrate-rich diet for 3 days before all study days.

Experimental design. Participants arrived at the Clinical Research Center in the morning after 10-hours overnight fasting (Figure 1). Two intravenous catheters were inserted to contralateral forearm veins. From -120 min, a continuous infusion [0.036 mg·(kg bodyweight)⁻¹·min⁻¹] of D-[6,6-²H₂]glucose (99% enriched in ²H glucose; Cambridge Isotope Laboratories, Andover, USA) was given after a priming bolus [0.36 mg·(kg bodyweight)⁻¹·min⁻¹·(mg/dl fasting plasma glucose)] for 5 min (15). At zero time, participants received one of four interventions: (i) po fat: 100 ml of soy bean oil (61% polyunsaturated, 23% monounsaturated and 16% saturated fatty acids; Sojola, Vandemoortele Deutschland GmbH; Dresden, Germany) consumed within 10 minutes, (ii) LPS: 10-min infusion of LPS (0.5 ng/kg bodyweight; National Reference Endotoxin, Escherichia coli O:113; USP, Rockville, MD, USA), (iii) iv fat: infusion of Intralipid® (1.5 ml/min; Fresenius Kabi GmbH, Bad Homburg, Germany) over 360 min or (iv) control: 2.5% glycerol infusion dissolved in 0.9% saline (1.5
ml/min; Fresenius Kabi GmbH) for 6 hours. Each intervention was followed by a hyperinsulinemic-euglycemic clamp (10-min insulin bolus: 40 U/h; continuous insulin infusion: 40 mU·m⁻²·min⁻¹; Insuman® Rapid; sanofi-aventis; Frankfurt; Germany). Plasma glucose was adjusted with 20% glucose (B. Braun AG, Melsungen, Germany) enriched with 2% D-[6,6-²H₂]glucose to 5.0 mmol/l according to previously established protocols (15). Vital function (heart rate, blood pressure, body temperature) was monitored every 30 minutes. Only endotoxin administration led to mild flu-like symptoms with a maximum after 3 hours, while other interventions had no side effects.

**Indirect calorimetry.** Indirect calorimetry was performed in the canopy mode using Vmax Encore 29n (CareFusion, Höchberg, Germany) during baseline, end of intervention and steady-state clamp conditions for 20 min after 10-min adaptation. Oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were measured and respiratory quotient (RQ) and resting energy expenditure (REE) calculated using abbreviated Weir equations. Substrate oxidation was calculated with fixed estimated protein oxidation rate (Pox) of 15% of REE:

- Carbohydrate oxidation rate (mg·kg⁻¹·min⁻¹) = [(4.55·VCO₂) – (3.21·VO₂) – 0.459·Pox]·1000/kg bodyweight
- Lipid oxidation rate (mg·kg⁻¹·min⁻¹) = [(1.67·VO₂) – (1.67·VCO₂) – 0.307·Pox]·1000/kg bodyweight

Nonoxidative glucose disposal was calculated from the difference between Rd and carbohydrate oxidation.

**Muscle biopsy.** The biopsy was obtained at the end of intervention period from the vastus lateralis muscle using a modified Bergström needle with suction under local anesthesia as described (17). Samples were immediately blotted free of blood, fat and connective tissue, frozen in liquid nitrogen and stored at -80°C. For DAG extraction, muscle tissue was homogenized in buffer A (20 mmol/l Tris-HCl, 1 mmol/l EDTA, 0.25 mmol/l EGTA, 250 mmol/l sucrose, 2 mmol/l PMSF) containing a protease and phosphatase inhibitor mixture.
(Roche) in Thickwall Polycarbonate tubes and centrifuged (40,000 g, 4°C, 1 h). The supernatants containing the cytosolic fraction were collected, the pellet was resuspended in 500 µl buffer A as membrane fraction and enrichment of membrane and cytosolic proteins was checked by Western blotting (Figure 6C). DAG and ceramide levels were measured using mass spectrometry as described (18). Total DAG content is expressed as the sum of individual species. Measurement of membrane translocation of PKCθ was performed as described (19). Both membrane and cytosolic proteins were detected on the same film with enhanced chemiluminescence at the same exposure time. PKCθ translocation was expressed as the ratio of arbitrary units of membrane bands over cytosol bands (Figure 6B). Membrane band density was corrected by sodium potassium ATPase band density (Abcam, Cambridge MA, USA), and cytosolic band density was corrected by GAPDH band density (Cell Signaling, Denvars MA, USA).

Subcutaneous adipose tissue biopsy. The biopsy was obtained before and at the end of the intervention period from abdominal subcutaneous adipose tissue by needle suction technique under local anesthesia. Fat tissue was immediately blotted free of blood, frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated according to the protocol of the miRNeasy Mini Kit (QIAGEN, Hilden, Germany) including on-column DNase digestion. RNA quantity and quality were determined by Nanodrop (Peqlab, Erlangen, Germany) and RNA 6000 Nano Kit (Agilent, Böblingen, Germany). For reverse transcription, we used the miScript Reverse Transcription Kit (QIAGEN), for RT-PCR, QuantiTect SYBR® Green PCR Kit and QuantiTect Primer Assay (QIAGEN). Details are given in Online Supplementary Material. For relative quantification of gene expression, we used the comparative C_T (threshold cycle) method and determined the threshold to be 0.4 and the baseline to be between cycle 3 and 15.
Metabolites and hormones. Blood samples were immediately chilled, centrifuged and supernatants stored at -20°C until analysis. Venous blood glucose concentration was measured immediately using the glucose oxidase method, which offers excellent performance (EKF biosen C-Line glucose analyzer, EKF diagnostic GmbH, Barleben, Germany; (20). Serum triglycerides, liver enzymes and cholesterol were analyzed enzymatically on a Hitachi analyzer (Roche Diagnostics, Mannheim, Germany). Plasma chylomicron content was determined from the triglyceride concentration in the first fraction of density gradient ultracentrifugation as described (21). FA were assayed microfluorimetrically (intraassay CV <1%, interassay CV 2.4%; Wako, Neuss, Germany) after prevention of in vitro lipolysis using orlistat (22). Serum C-peptide, insulin and plasma glucagon were measured by RIA (intraassay CV for all <1%, interassay CV 6-7%, 5-9% and 5-10%, respectively; Millipore, St. Charles, USA). Cortisol was measured using fluorescence polarization immunoassay on the Axsym analyzer (intraassay CV 4.0%, interassay CV 5.9%; Abbott, Abbott Park, USA). GLP-1 and GIP were measured using ELISA (GLP-1: interassay CV 10%; TECOmedical, Sissach, Switzerland; GIP: interassay CV 12%; Millipore, St. Charles, USA). Serum inflammatory markers were assayed using the Quantikine HS (TNF-α, IL-6) and Quantikine (IL-1ra) ELISA kits (R&D Systems, Wiesbaden, Germany) as described (23) with mean intra-assay and inter-assay CVs of 6.7%, 5.2%, 3.9% and 8.8%, 15.5%, 10.9%, respectively, and limits of detection of 0.25 pg/ml, 0.08 pg/ml and 14 pg/ml, respectively.

Fluorescence activated cell sorting (FACS). The distribution of leukocyte subfractions from heparinized blood samples was determined before and after 300 min. Upon lysis of erythrocytes using BD Lysing solution granulocytes, monocytes and lymphocytes were differentiated by their characteristic FSC/SSC signals on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) using Cell Quest software (BD Biosciences).
Gas chromatography-mass spectrometry. Determination of atom percent enrichment (APE) of $^2$H was done as described (24) after deproteinization. Briefly, 100 µl KF-EDTA plasma was diluted with an equal amount of water and deproteinized after adding 300 µl of 0.3 N ZnSO$_4$ solution followed by 300 µl of 0.3 N Ba(OH)$_2$ solution. After vortexing for 20 minutes, samples were centrifuged (21,000 g, room temperature). Then, 400 µl were evaporated under a stream of nitrogen 5.0 at 37°C and both endogenous and infused [6,6-$^2$H$_2$] glucose were derivatized with HOX (100 µl of 2% solution in pyridine, 60 min at 90°C, cooling for 5 min) and acetic anhydride (200 µl, 60 min at 90°C, 5-min cooling) to the aldonitrile-pentaacetate. The analyses were performed on a Hewlett-Packard 6890 gas chromatograph equipped with a 25-m CPSil5CB capillary column (0.2 mm i. d., 0.12 µm film thickness; Chrompack/Varian, Middelburg, NL) and interfaced to a Hewlett Packard 5975 mass selective detector. Selected ion monitoring was used to determine enrichments of the fragments C3 to C6 with the average mass units 187 for the endogenous glucose and 189 for the [6,6-$^2$H$_2$]glucose. Intra- and inter-assay CVs were 0.6% and 1.0.

Calculation of whole-body glucose turnover and endogenous glucose production (EGP). Rates of EGP were determined from the tracer infusion rate of D-[6,6-$^2$H$_2$] glucose and its enrichment to the hydrogens bound to carbon 6 divided by the mean percent enrichment of plasma D-[6,6-$^2$H$_2$]glucose. Because tracer-to-tracee ratios were constant after 300 min (Supplementary Figure 1B), steady-state equations were appropriate for calculation of basal EGP over the last hour of intervention (300-360 min) and insulin-suppressed EGP during the last 30 min of clamp test. Whole-body glucose disposal (M-value) was calculated from glucose infusion rates during the last 30 min of the clamp.

Statistical analyses. Results are presented as means±SEM and were compared using two-tailed Student’s $t$-test for paired analysis (comparisons before and after intervention for
circulating immune cells and adipose tissue gene expression) or using repeated measures ANOVA with the Dunnett post-hoc testing. Incremental area under the curve (AUC) was calculated using the trapezoid method. Statistical significance of differences was defined at p<0.05. Calculations were performed using GraphPad Prism version 4.03 (GraphPad Software Inc., LaJolla, USA).
RESULTS

Metabolites and hormones. Sixteen lean young insulin-sensitive volunteers (Table 1) underwent four different interventions (intravenous fat [iv fat], oral fat [po fat], endotoxin [LPS] and glycerol i.v. serving as control [con]) in random order at least 4 weeks apart (Figure 1). Plasma triglycerides rose continuously to 3.7±0.7 mmol/l (AUC P<0.01 vs. control) during iv fat infusion and remained unchanged after po fat or LPS (Figure 2A), while triglyceride content in chylomicrons increased from 1.2±0.3 mmol/l to 2.0±0.3 mmol/l after po fat (P<0.05 vs. baseline). Plasma free FA increased after iv fat by 178% to 0.79±0.04 mmol/l at 360 min (AUC P<0.01 vs. control), but not after po fat (Figure 2B). Plasma FA started to increase 90 min following endotoxin application to 0.84±0.04 mmol/l (AUC P<0.01 vs. control). During the clamp, plasma FA decreased rapidly but remained higher after intravenous and oral fat (0.23±0.02 mmol/l and 0.10±0.02 mmol/l; both P<0.01 vs. control). Plasma insulin concentrations were constant during the intervention period and increased during clamp after po fat in the presence of comparable clamp-induced glycemia (P<0.05; Figure 3A, Suppl. Figure 1A). In parallel, steady-state plasma C-peptide levels were also greater after po fat compared to control (4.7±0.6 ng/dl vs. 2.3±0.3 ng/dl, P<0.01). Only upon po fat, glucagon-like peptide-1 (GLP-1) and gastric inhibitor peptide (GIP) rose immediately and remained elevated until the end of intervention period (Figure 3 C/D). Plasma glucagon rose after po fat and remained higher until the clamp (Figure 3B).

Tissue-specific insulin sensitivity. Intravenous and oral fat as well as endotoxin similarly reduced whole-body insulin sensitivity compared to control to 60%, 67% and 48%, respectively (all P<0.01; Figure 4A). Hepatic insulin sensitivity, assessed from insulin-mediated suppression of EGP, was not different between all studies (EGP suppression by 42±8% [iv fat], 67±13% [po fat], 54±7% [LPS] vs. 69±15% after control).

Energy expenditure and substrate oxidation. REE was 1579±221 kcal/day and RQ was 0.83±0.06%. At 6 hours, lipid oxidation rates were 40% higher (P<0.05) after iv, but not after
po fat compared with control (Figure 4B). During insulin stimulation, lipid oxidation rates fell by 39±7%, 48±15%, 94±18% and 106±22% during iv fat, po fat, LPS and control (all P<0.001 vs. fasting), but remained higher after iv fat (P<0.01 vs. control). Oxidative glucose utilization was not different between the studies, but iv fat, po fat and LPS decreased nonoxidative glucose utilization by 30% (P<0.05), 33% (P<0.05) and 56% (P<0.01) compared to control (Figure 4C).

**Systemic and adipose tissue inflammation.** After endotoxin, the fractional contribution of granulocytes to blood leukocytes rose by 44% (P<0.0001), while that of lymphocytes fell by 75% (P<0.0001) (Table 2). Oral fat induced minor changes of the granulocyte and monocyte fractions. After endotoxin, plasma TNF-α and IL-6 transiently rose with peaks at 120 min and 180 min, respectively. Plasma cortisol and IL-1ra peaked at 240 min (Figure 5). Neither fat - regardless of the route of administration - nor control conditions affected systemic concentrations of immune markers. In subcutaneous adipose tissue, the mRNA expression of the related genes showed similar behavior and was increased only after endotoxin (Table 2).

**Muscle DAG, ceramides and PKC.** In biopsies obtained from vastus lateralis muscle at 5 hours, the membrane/cytosol PKCθ translocation reflecting its activation was 50% greater after iv and po fat, but unchanged after endotoxin when compared to control (P<0.05 for differences between groups; Figure 6A, B). Total cytosolic and membrane DAG as well as ceramides were unchanged (Figure 6C), while the membrane Di-C18:2 DAG species doubled after iv fat (p<0.01 vs. control; Figure 6D). After po fat, total membrane DAG and Di-C18:2 DAG species exhibited a tight positive correlation with muscle PKCθ translocation (r=0.77 and r=0.86, respectively, both P<0.05), while iv fat and LPS did not have any effect.
DISCUSSION

This study shows that one single oral fat load decreases whole-body insulin sensitivity to an extent comparable to that induced by intravenous fat or low-dose endotoxin administration. This effect associates with reduced insulin-stimulated nonoxidative glucose utilization by skeletal muscle and activation of PKCθ, whereas systemic or local adipose tissue inflammation does not seem to be involved.

Previous studies on insulin action after oral fat loading found either no or only minor decrease of insulin sensitivity in humans (25-27). This is most likely attributable to the different study designs, which employed lipid meals ingested over the course of several hours or up to few weeks or offered mixed meals also containing protein and/or carbohydrates. An hourly intake of oil enriched in polyunsaturated (78%), monosaturated (78%) or saturated fatty acids (50%) over 12 hours increased plasma FA starting at 8 hours after the first dose and resulted in higher glucose infusion rates during clamps after ingestion of polyunsaturated, but not monosaturated or saturated FA after 24 hours (28). In contrast, the present study demonstrates a profound reduction of insulin sensitivity at 6-8 hours after single ingestion of soybean oil rich in polyunsaturated FA (61%), which was used in order to match for the composition of the intravenous fat solution known to rapidly induce insulin resistance in humans (6;7). The seemingly contradictory findings probably result from different time intervals between fat ingestion and assessment of insulin sensitivity, differences in timing of fat application, its composition and the absolute plasma FA concentration achieved (26;29). Of note, the aim of the current study was to analyze whether the mechanisms proposed from the lipid infusion models are also involved after ingestion of identically composed pure fat and to set the stage for further studies analyzing mechanisms involved in the interaction of oral fat with other macronutrients under real-life conditions.

The present study also found that neither intravenous nor oral lipids immediately affect hepatic insulin sensitivity. Over a longer period of 5 days a hypercaloric mixed diet
containing 60% fat equally composed of poly-, mono- and saturated FA combined with carbohydrates and proteins affected hepatic glucose metabolism by increased fasting EGP and decreasing hepatic insulin sensitivity (25), but it does not allow conclusions on the effect of fat per se. On the other hand, the glycerol infusion used as a control to account for increased glycerol delivery during lipid application may have stimulated gluconeogenesis and resulted in the incomplete (approximately 75%) insulin-induced suppression of EGP (24). Thus, the design allowed assessing the net hepatic effect of FA and endotoxin application per se at the expense of underestimating total, i.e. glycerol-mediated, hepatic insulin resistance. As this study was primarily designed to examine effects on muscle insulin sensitivity, the use of higher insulin doses may have prevented to detect differences in hepatic insulin sensitivity. In addition, incretin-mediated changes in portal insulin and glucagon concentrations may have masked effects of oral lipid ingestion on liver glucose metabolism, which is sensitive to small changes in portal insulin and glucagon (30). Incretins can also increase lipoprotein lipase activity in adipose tissue (31), thereby further increasing the release and uptake of FA from chylomicrons and reducing the spillover, i.e. the release into the blood (32), which is in line with the observation of elevated chylomicrons, but not FA in the present study.

The reduction of insulin-stimulated muscle glucose disposal upon fat or endotoxin application was accounted for by lower nonoxidative glucose disposal without changes in glucose oxidation. This extends the concept originally derived from lipid infusion studies to oral fat ingestion and endotoxin exposure, in that diminished muscle glucose uptake is not due to substrate (glucose-FA) competition for oxidation, but results from impaired glucose transport/phosphorylation followed by lower nonoxidative glucose storage (3;6;29). Furthermore, membrane PKCθ was activated in skeletal muscle at 5 hours after oral lipid ingestion and lipid infusion. While an increase in membrane PKCθ translocation has been shown in animal models after 3-weeks high-fat diet (33) and 3 hours after lipid infusion (18;34), PKCθ-knockout mice are protected (35). In humans, PKC isoforms βII and δ, but not
θ were elevated in muscle 6 hours after combined insulin and lipid infusion (36), and this has not been reported after high-fat meals in humans before. Thus, basal muscle PKCθ activation may represent the key signaling event in lipid-induced insulin resistance by serine phosphorylation of insulin receptor substrate (IRS)-1 at residues 307/312 (18) or 1101 (37) or alternatively via serine phosphorylation of 3-phosphoinositide-dependent kinase-1 (PDK-1), which in turn inactivates Akt (38).

Although muscle PKCθ is a target of DAG, total DAG concentrations were not different between all interventions at 5 hours, which is in line with one (39) but not with other studies reporting increased total DAG at 5-6 h hours of a parallel lipid and insulin-glucose infusion in humans (36) and mice (18;40). Other studies associated PKCθ or other PKC isoforms with increased DAG concentrations in cross-sectional analyses (36;41) or after high fat diet in mice (42). Nevertheless, total DAG mass might be less relevant for insulin sensitive pathways than its distribution in specific subcellular compartments (41;43;44). Here we show that myocellular membrane Di-C18:2 DAG content was increased at 5 hours of lipid infusion, which reflects the 53% content of linoleic acid in the infusion. Of note, desaturation of both fatty acids moieties of total DAG was also found in muscles of obese insulin resistant humans without lipid infusion (43), whereas others hold Di-C18:0 subspecies responsible (41). Nevertheless, soybean oil infusion in rats exclusively raises myocellular 18:2-fatty acyl coenzyme A within 2 hours prior to the transient increase in DAG between 2-4 hours and ultimately followed by PKCθ translocation in rats (18). This time sequence of events also holds true for lipid infusion in humans (44) and is probably responsible for a peak in DAG species occurring earlier after oral fat ingestion than during continuous lipid infusion, therefore we may have missed the DAG peak in our oral fat study.

Total concentrations of ceramide species remained unchanged not only after lipid, but also after endotoxin application. This is similar to some studies in humans (36;39) and mice (18),
but not all studies (45;46). The relatively low content of saturated FA (16%) in soybean oil, which are key to de novo synthesis of ceramides also after TLR-4 activation (9), could contribute to the divergent results, but the present study cannot exclude a transient increase in ceramides. In humans, high fat diets generally contain more saturated FA, which might affect the mechanisms causing insulin resistance and could also involve ceramides or inflammatory processes (2;9). Moreover, chronic overnutrition usually leads to greater protein intake with augmented availability of amino acids, which cause insulin resistance via other pathways (17).

Oral and intravenous fat did not result in detectable inflammatory processes, which are known to relate to insulin resistance in humans (47). The minor changes in leukocyte fractions upon oral fat are in line with those found upon endotoxin and could result from mild endotoxin spillover, which cannot be confirmed by measuring plasma endotoxin levels, although recent studies suggested an increase in LPS and cytokines after meals enriched in saturated fat (12), indicating that insulin resistance by mainly unsaturated FA occurs independently of TLR-4 activation. Application of LPS O:113 induced an equal degree of peripheral insulin resistance in association with pronounced proinflammatory response including mild clinical signs of increased body temperature (48). Nevertheless, application of other LPS subtypes of gram-negative bacteria might have caused different effects. The observed rise in TNFα and IL-6 concentration and adipose tissue expression is comparable to a recent study (14) using combined lipid and endotoxin administration. Here we further describe a subsequent rise in the anti-inflammatory cytokine IL-1Ra, which could have attenuated the endotoxin effect on insulin sensitivity. Although plasma FA concentrations transiently rose after endotoxin infusion to similar levels as during lipid infusion for about 2 hours, this was neither associated with elevation of myocellular lipid intermediates nor with PKCθ translocation. Probably, FA contribute to a more pronounced activation of inflammatory pathways such as nuclear factor
κB (NFκB) p65 binding and c-Jun-N-terminal kinase (JNK) that mostly mediate endotoxin-induced insulin resistance (47). Additional lipid-induced mechanisms such as reduced blood flow as reported in a mouse model (49) are possible, but rather unlikely to occur in humans under these conditions (50).

In conclusion, this study showed that: (i) oral fat ingestion rapidly induces insulin resistance, which is primarily accounted for by reduced muscle nonoxidative glucose disposal, (ii) lipid-induced insulin resistance is due to PKCθ translocation and likely mediated by distinct myocellular membrane DAG, but neither by local nor systemic inflammation in humans and (iii) endotoxin-induced insulin resistance also starts in skeletal muscle, occurs independently of PKCθ and is exclusively associated with adipose tissue inflammation.
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REFERENCES

1. Tabak, AG, Jokela, M, Akbaraly, TN, Brunner, EJ, Kivimaki, M, Witte, DR: Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study. *Lancet* 373:2215-2221, 2009

2. Gregor, MF, Hotamisligil, GS: Inflammatory mechanisms in obesity. *Annu Rev Immunol* 29:415-445, 2011

3. Samuel, VT, Shulman, GI: Mechanisms for insulin resistance: common threads and missing links. *Cell* 148:852-871, 2012

4. Jensen, MD, Hayden, MW, Rizza, RA, Cryer, PE, Miles, JM: Influence of body fat distribution on free fatty acid metabolism in obesity. *J Clin Invest* 83:1168-1173, 1989

5. Paolisso, G, Tataranni, PA, Foley, JE, Bogardus, C, Howard, BV, Ravussin, E: A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. *Diabetologia* 38:1213-1217, 1995

6. Roden, M, Price, TB, Perseghin, G, Petersen, KF, Rothman, DL, Cline, GW, Shulman, GI: Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97:2859-2865, 1996

7. Roden, M, Krssak, M, Stingl, H, Gruber, S, Hofer, A, Furnsinn, C, Moser, E, Waldhaeusl, W: Rapid impairment of skeletal muscle glucose transport/phosphorylation by free fatty acids in humans. *Diabetes* 48:358-364, 1999

8. Giacco, R, Clemente, G, Busiello, L, Lasorella, G, Rivieccio, AM, Rivellese, AA, Riccardi, G: Insulin sensitivity is increased and fat oxidation after a high-fat meal is reduced in normal-weight healthy men with strong familial predisposition to overweight. *Int J Obes Relat Metab Disord* 28:342-348, 2004

9. Bikman, BT, Summers, SA: Ceramides as modulators of cellular and whole-body metabolism. *J Clin Invest* 121:4222-4230, 2011

10. Medzhitov, R: Recognition of microorganisms and activation of the immune response. *Nature* 449:819-826, 2007

11. Shi, H, Kokoeva, MV, Inouye, K, Tzameli, I, Yin, H, Flier, JS: TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 116:3015-3025, 2006

12. Cani, PD, Amar, J, Iglesias, MA, Poggi, M, Knauf, C, Bastelica, D, Neyrinck, AM, Fava, F, Tuohy, KM, Chabo, C, Waget, A, Delmee, E, Cousin, B, Sulpice, T, Chamontin, B, Ferrieres, J, Tanti, JF, Gibson, GR, Castella, L, Delzenne, NM, Alessi, MC, Burcelin, R: Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56:1761-1772, 2007

13. Nappo, F, Esposito, K, Cioffi-Meltz, M, Giugliano, G, Molinari, AM, Paolisso, G, Marfella, R, Giugliano, D: Postprandial endothelial activation in healthy subjects and in type 2 diabetic patients: role of fat and carbohydrate meals. *J Am Coll Cardiol* 39:1149-1150, 2002

14. Krogh-Madsen, R, Plomgaard, P, Akerstrom, T, Moller, K, Schmitz, O, Pedersen, BK: Effect of short-term intralipid infusion on the immune response during low-dose endotoxemia in humans. *Am J Physiol Endocrinol Metab* 294:E371-E379, 2008

15. Weickert, MO, Loeffelholz, CV, Roden, M, Chandramouli, V, Brehm, A, Nowotny, P, Osterhoff, MA, Isken, F, Spranger, J, Landau, BR, Pfeiffer, AF, Mohlig, M: A Thr94Ala mutation in human liver fatty acid-binding protein contributes to reduced hepatic glycogenolysis and blunted elevation of plasma glucose levels in lipid-exposed subjects. *Am J Physiol Endocrinol Metab* 293:E1078-E1084, 2007

16. Frayn, KN: Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 55:628-634, 1983

17. Tremblay, F, Krebs, M, Dombrowski, L, Brehm, A, Bernroider, E, Roth, E, Nowotny, P, Waldhaeusl, W, Marette, A, Roden, M: Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. *Diabetes* 54:2674-2684, 2005

18. Yu, C, Chen, Y, Cline, GW, Zhang, D, Zong, H, Wang, Y, Bergeron, R, Kim, JK, Cushman, SW, Cooney, GJ, Atchison, B, White, MF, Kraegen, EW, Shulman, GI: Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277:50230-50236, 2002

19. Kumashiro, N, Erion, DM, Zhang, D, Kahn, M, Beddow, SA, Chu, X, Still, CD, Gerhard, GS, Han, X, Dziura, J, Petersen, KF, Samuel, VT, Shulman, GI: Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. *Proc Natl Acad Sci U S A* 108:16381-16385, 2011
20. Nowotny, B, Nowotny, PJ, Strassburger, K, Roden, M: Precision and accuracy of blood glucose measurements using three different instruments. *Diabet Med* 29:260-265, 2012

21. Redgrave, TG, Carlson, LA: Changes in plasma very low density and low density lipoprotein content, composition, and size after a fatty meal in normo- and hypertriglyceridemic man. *J Lipid Res* 20:217-229, 1979

22. Brehm, A, Krssak, M, Schmid, AI, Nowotny, P, Waldhaeusl, W, Roden, M: Increased lipid availability impairs insulin-stimulated ATP synthesis in human skeletal muscle. *Diabetes* 55:136-140, 2006

23. Herder, C, Schneitler, S, Rathmann, W, Haastert, B, Schneitler, H, Winkler, H, Bredahl, R, Hahnloser, E, Martin, S: Low-grade inflammation, obesity, and insulin resistance in adolescents. *J Clin Endocrinol Metab* 92:4569-4574, 2007

24. Stingl, H, Krssak, M, Krebs, M, Bischof, MG, Nowotny, P, Furnsinn, C, Shulman, GI, Waldhaeusl, W, Roden, M: Lipid-dependent control of hepatic glycogen stores in healthy humans. *Diabetologia* 44:48-54, 2001

25. Brons, C, Jensen, CB, Storgaard, H, Hiscock, NJ, White, A, Appel, JS, Jacobsen, S, Nilsson, E, Larsen, CM, Astrup, A, Quistorff, B, Vaag, A: Impact of short-term high-fat feeding on glucose and insulin metabolism in young healthy men. *J Physiol* 587:2387-2397, 2009

26. Galgani, JE, Uauy, RD, Aguirre, CA, Diaz, EO: Effect of the dietary fat quality on insulin sensitivity. *Br J Nutr* 100:471-479, 2008

27. Lopez, S, Bermudez, B, Ortega, A, Varela, LM, Pacheco, YM, Villar, J, Abia, R, Muriana, FJ: Effects of meals rich in either monounsaturated or saturated fat on lipid concentrations and on insulin secretion and action in subjects with high fasting triglyceride concentrations. *Am J Clin Nutr* 93:494-499, 2011

28. Xiao, C, Giacca, A, Carpentier, A, Lewis, GF: Differential effects of monounsaturated, polyunsaturated and saturated fat ingestion on glucose-stimulated insulin secretion, sensitivity and clearance in overweight and obese, non-diabetic humans. *Diabetologia* 49:1371-1379, 2006

29. Roden, M: How free fatty acids inhibit glucose utilization in human skeletal muscle. *News Physiol Sci* 19:92-96, 2004

30. Roden, M, Perseghin, G, Petersen, KF, Hwang, JH, Cline, GW, Gerow, K, Rothman, DL, Shulman, GI: The roles of insulin and glucagon in the regulation of hepatic glycogen synthesis and turnover in humans. *J Clin Invest* 97:642-648, 1996

31. Kim, SJ, Nian, C, McIntosh, CH: GIP increases human adipocyte LPL expression through CREB and TORC2-mediated trans-activation of the LPL gene. *J Lipid Res* 51:3145-3157, 2010

32. Bell, KS, Schmitz-Peiffer, C, Lim-Fraser, M, Biden, TJ, Cooney, GI, Kraegen, EW: Acute reversal of lipid-induced muscle insulin resistance is associated with rapid alteration in PKC-theta localization. *Am J Physiol Endocrinol Metab* 279:E1196-E1201, 2000

33. Griffin, ME, Marcucci, MJ, Cline, GW, Bell, K, Barucci, N, Lee, D, Goodyear, LJ, Kraegen, EW, White, MF, Shulman, GI: Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48:1270-1274, 1999

34. Kim, JK, Fillmore, JJ, Sunshine, MJ, Albrecht, B, Higashimori, T, Kim, DW, Liu, ZX, Soos, TJ, Cline, GW, O'Brien, WR, Littman, DR, Shulman, GI: PKC-theta knockout mice are protected from fat-induced insulin resistance. *J Clin Invest* 114:823-827, 2004

35. Itani, SI, Ruderman, NB, Schmiede, F, Boden, G: Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* 51:2005-2011, 2002

36. Li, Y, Soos, TJ, Li, X, Wu, J, Degennaro, M, Sun, X, Littman, DR, Birnbaum, MJ, Polakiewicz, RD: Protein kinase C Theta inhibits insulin signaling by phosphorylating IRS1 at Ser(1101). *J Biol Chem* 279:45304-45307, 2004

37. Wang, C, Liu, M, Riojas, RA, Xin, X, Zeng, R, Wu, J, Dong, LQ, Liu, F: Protein kinase C theta (PKCtheta)-dependent phosphorylation of PDK1 at Ser504 and Ser532 contributes to palmitate-induced insulin resistance. *J Biol Chem* 284:2038-2044, 2009

38. Hoeg, LD, Sjoberg, KA, Jeppesen, J, Jensen, TE, Froisig, C, Birk, JB, Bisiani, B, Hiscock, N, Pilegaard, H, Wojtaszewski, JF, Richter, EA, Kiens, B: Lipid-induced insulin resistance affects
women less than men and is not accompanied by inflammation or impaired proximal insulin signaling. *Diabetes* 60:64-73, 2011

40. Holland, WL, Bikman, BT, Wang, LP, Yuguang, G, Sargent, KM, Bulchand, S, Knotts, TA, Shui, G, Clegg, DJ, Wenk, MR, Pagliassotti, MJ, Scherer, PE, Summers, SA: Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. *J Clin Invest* 121:1858-1870, 2011

41. Bergman, BC, Hunerdosse, DM, Kerege, A, Playdon, MC, Perreault, L: Localization and composition of skeletal muscle diacylglycerol predicts insulin resistance in humans. *Diabetologia* 55:1140-1150, 2012

42. Lee, HY, Choi, CS, Birkenfeld, AL, Alves, TC, Jornayvaz, FR, Jurczak, MJ, Zhang, D, Woo, DK, Shadel, GS, Ladiges, W, Rabinovitch, PS, Santos, JH, Petersen, KF, Samuel, VT, Shulman, GI: Targeted expression of catalase to mitochondria prevents age-associated reductions in mitochondrial function and insulin resistance. *Cell Metab* 12:668-674, 2010

43. Amati, F, Dube, JJ, Alvarez-Carnero, E, Edreira, MM, Chomentowski, P, Coen, PM, Switzer, GE, Bickel, PE, Stefanovic-Racic, M, Toledo, FG, Goodpaster, BH: Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes* 60:2588-2597, 2011

44. Szendroedi J, Yoshimura T, Phielix E, Marcucci M, Zhang D, Herder C, Nowotny P, Shulman GI, Roden M: Role of diacylglycerol activation of PKCtheta in lipid-induced muscle insulin resistance in humans (Abstract). *Diabetes* 61: Suppl.1, 2012

45. Straczkowski, M, Kowalska, I, Nikolajuk, A, Dzienis-Straczkowska, S, Kinalska, I, Baranowski, M, Zendzian-Piotrowska, M, Brzezinska, Z, Gorski, J: Relationship between insulin sensitivity and sphingomyelin signaling pathway in human skeletal muscle. *Diabetes* 53:1215-1221, 2004

46. Watt, MJ, Hevener, A, Lancaster, GI, Febbraio, MA: Ciliary neurotrophic factor prevents acute lipid-induced insulin resistance by attenuating ceramide accumulation and phosphorylation of c-Jun N-terminal kinase in peripheral tissues. *Endocrinology* 147:2077-2085, 2006

47. Glass, CK, Olefsky, JM: Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab* 15:635-645, 2012

48. Suffredini, AF, Hochstein, HD, McMahon, FG: Dose-related inflammatory effects of intravenous endotoxin in humans: evaluation of a new clinical lot of Escherichia coli O:113 endotoxin. *J Infect Dis* 179:1278-1282, 1999

49. Mulligan, KX, Morris, RT, Otero, YF, Wasserman, DH, McGuinness, OP: Disassociation of muscle insulin signaling and insulin-stimulated glucose uptake during endotoxemia. *PLoS One* 7:e30160, 2012

50. Szendroedi, J, Frossard, M, Klein, N, Bieglmayer, C, Wagner, O, Pacini, G, Decker, J, Nowotny, P, Muller, M, Roden, M: Lipid-Induced Insulin Resistance Is Not Mediated by Impaired Transcapillary Transport of Insulin and Glucose in Humans. *Diabetes* Aug 13, [Epub ahead of print]: 2012
Tables

Table 1. Volunteers’ characteristics

| Characteristic                          | Value     |
|----------------------------------------|-----------|
| n (male/females)                       | 16 (11/5) |
| Age [years]                            | 24±1      |
| BMI [kg/m²]                            | 22.7±0.3  |
| Waist circumference [cm]               | 81±2      |
| Lean body weight [kg]                  | 59±2      |
| Body fat [%]                           | 21±1      |
| Systolic blood pressure [mmHg]         | 127±3     |
| Diastolic blood pressure [mmHg]        | 74±2      |
| Triglycerides [mmol/l]                 | 0.93±0.08 |
| LDL-cholesterol [mmol/l]               | 2.63±0.14 |
| HDL-cholesterol [mmol/l]               | 1.44±0.11 |
| Fatty acids (FA) [mmol/l]B             | 0.49±0.05 |
| Alanine aminotransferase (ALT) [U/l]   | 22±2      |
| Aspartate aminotransferase (AST) [U/l] | 26±1      |
| Fasting blood glucose [mmol/l]         | 4.37±0.06 |
| 2-hours blood glucose [mmol/l]         | 4.43±0.22 |

A Values given are means±SEM; B Value obtained during control intervention.
Table 2. Parameters of systemic and tissue-specific inflammation

|                       | IV FAT<sup>C</sup> | PO FAT<sup>C</sup> | LPS<sup>C</sup> | CON<sup>C</sup> |
|-----------------------|--------------------|--------------------|----------------|---------------|
|                       | Before             | After              | Before         | After         | Before         | After         |
| Percent contribution to circulating leukocytes<sup>A</sup> |                    |                    |                |               |                |               |
| Granulocytes          | 62±3               | 59±3               | 60±2           | 63±2**        | 60±3           | 86±1***       | 57±3          | 56±3          |
| Lymphocytes           | 31±2               | 34±2               | 38±2           | 30±2          | 33±3           | 81***         | 35±3          | 37±3          |
| Monocytes             | 7.0±0.5            | 7.3±1.0            | 7.4±0.5        | 6.1±0.5**     | 7.7±0.6        | 6.7±0.6       | 7.4±0.5       | 6.6±0.6*      |
| Adipose tissue mRNA expression<sup>B</sup> |                    |                    |                |               |                |               |               |               |
| TNF-α                 | 11±2               | 14±5               | 10±1           | 8±2           | 15±4           | 23±4*         | 14±3          | 16±5          |
| IL-6                  | 0.8±0.2            | 0.8±0.1            | 0.7±0.3        | 0.7±0.1       | 1.9±0.9        | 6.3±1.3*      | 0.7±0.1       | 1.0±0.3       |
| TLR-2                 | 21±5               | 28±8               | 15±3           | 12±2          | 23±4           | 66±13**       | 23±6          | 25±8          |
| TLR-4                 | 31±4               | 38±7               | 37±4           | 38±7          | 36±7           | 75±12**       | 34±3          | 45±6          |

<sup>A</sup>Values given are means±SEM as % of all leukocytes; <sup>B</sup>Values given are means±SEM as relative gene expression units; <sup>C</sup>*: P<0.05; **: P<0.01; ***: P<0.0001 after vs. before intervention using Student’s t-test. TNF-α: Tumor necrosis factor α; IL: Interleukin; TLR: Toll-like receptor.
FIGURE LEGENDS

Fig. 1. Study protocol. One out of four interventions was started at 0 min at each study day: either 1) iv fat: infusion of Intralipid® (1.5 ml/min) over 360 min, 2) po fat: 100ml of soy bean oil consumed within 10 minutes, 3) LPS: short-term infusion of LPS (0.5 ng/kg bodyweight) or 4) control: infusion 2.5% glycerol solution (1.5 ml/min) for 6 hours.

Fig. 2. Time course of total and chylomicron triglycerides (A) and FA (B) during iv fat (red), po fat (blue), LPS (green), control (black) and triglyceride content in chylomicron fraction after po fat as broken line (blue).
***, p<0.01 for area under the curve (AUC) during intervention vs. AUC during CON. ##, p<0.01 vs. CON at 480 min using repeated measures ANOVA and post-hoc Dunnett testing.

Fig. 3. Time course of plasma insulin (A), glucagon (B), GLP-1 (C) and GIP (D) during iv fat (red), po fat (blue), LPS (green), control (black).
**, P<0.01 for area under the curve (AUC) during po fat vs. AUC during control. #, P<0.05 po fat vs. control for mean of min 450 and 480 and P<0.01 vs. FAT IV and LPS, respectively, and §, P<0.05 po fat vs. control at using repeated measures ANOVA and post hoc Dunnett testing.

Fig. 4. Whole-body insulin sensitivity (A) and rates for lipid oxidation (C) and oxidative/non-oxidative glucose utilization (D).
The M-value was obtained during steady state conditions of the hyperinsulinemic-euglycemic clamp test. Lipid oxidation and glucose utilization were assessed after intervention and during steady state conditions of hyperinsulinemic-euglycemic clamp. Iv fat (red), po fat (blue), LPS (green), control (black), hatched bars are given for non-oxidative glucose utilization. Mean±SEM are given; *, P<0.05 and **, P<0.01 vs. control analyzed by repeated measures ANOVA and post-hoc Dunnett testing.

Fig. 5. Time courses of systemic immune regulating proteins & hormones.
TNF-α (A), Cortisol (B), IL-6 (C) and IL-1ra (D). Iv fat (red), po fat (blue), LPS (green), control (black). **, P<0.01 for area under the curve (AUC) during intervention vs. AUC during control visit.

Fig. 6. Muscle PKCθ translocation (A), total cytosolic and membrane DAG, total ceramides (C), C18 DAG and C18 ceramide levels (D).
Representative blot of cytosolic/membrane PKCθ using enhanced chemiluminescense and respective control proteins (sodium potassium ATPase [NaKATPase] for membrane and GAPDH for cytosolic band density) for correction (B), enrichment of cytosolic/membrane markers after DAG fractionation is depicted as insert in (C). Biopsies were taken after 5 hours after intervention: iv fat (red), po fat (blue), LPS (green), control (black). #, P<0.05 for overall difference between groups analyzed by repeated measures ANOVA; **, P<0.01 vs. CON using repeated measures ANOVA.
Figure 1

170x97mm (232 x 232 DPI)
Figure 2

A

Triglycerides [mmol/l]

Time [min]

B

Free fatty acids [mmol/l]

Time [min]

90x147mm (200 x 200 DPI)
Figure 5

A

TNF-α [pg/mL]

Time [min]

B

Cortisol [pg/dl]

Time [min]

C

IL-6 [pg/mL]

Time [min]

D

IL-1ra [ng/mL]

Time [min]

165x142mm (200 x 200 DPI)
Figure 6

(A) PKC theta translocation (AU)

(B) Western blot analysis

- Cyt. PKCθ
- GAPDH
- Mem. PKCθ
- NaKATPase

(C) Cytosol vs. Membrane

(D) Ceramide levels

246x225mm (150 x 122 DPI)
Online Supplementary Material

Subcutaneous adipose tissue RT PCR. Master mix consisted of 4 µl 5x miScript RT Puffer, 1 µl RNase Inhibitor 10 U/µl (Invitrogen, Carlsbad, USA) and 1 µl miScript Reverse Transcriptase Mix per sample. The total volume of 20 µl contained 500 ng RNA in RNase-free water and was incubated for 60 min. at 37°C and for 5 min. at 95°C. Master Mix consisted of 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix and 2.5 µl QuantiTect Primer per sample and was added to 10 µl cDNA diluted in RNase-free water. For detecting expression levels of the target genes, we used 10 ng cDNA for IL-6, 25 ng cDNA for TNFα and 5 ng cDNA for TLR2 and TLR4 as well as 0.05 ng cDNA for the reference gene PPIA. The RT-PCR started with an initial activation step of 95°C for 15 min. The 3-step cycling consisted of denaturation (94°C for 15 s), annealing (55°C for 30 s) and extension (72°C for 30 s) and was repeated 39 times. Finally we performed a melting curve analysis and incubated the samples at 95°C for 15 s, at 50°C for 20 s with a final increase by 0.3°C until reaching 95°C for 15 s.
Supplementary Figure Legends

Suppl. Fig. 1 Time courses of blood glucose levels (A) and glucose tracer enrichment (B). iv fat (red), po fat (blue), LPS (green), control (black). Tracer enrichment is shown as incremental tracer-to-tracee ratio.
Supplemental figure 1

A  Blood glucose

B  Glucose tracer enrichment

90x147mm (200 x 200 DPI)