Cafeteria diet-induced insulin resistance is not associated with decreased insulin signaling or AMPK activity and is alleviated by physical training in rats

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individually and kept on a 12:12-h light-dark cycle at 22°C. In all experimental groups, daily food intake and body weight were registered. This study protocol was approved by the Animal Ethics Committee of the University of Leuven (Belgium).

Study Protocol

Rats were divided randomly into one of three 12-wk intervention groups distinguished by dietary composition and application of exercise training. Group 1 [chow-fed controls (CON); n = 17] was used as a control group fed a normal pellet diet containing 11% fat, 26% protein, and 63% carbohydrate. Groups 2 (n = 13) and 3 (n = 14) were fed a “cafeteria-style” diet (CAF) (26) containing 330 g/kg ground Muracon-G pellets (Carfil Quality, Oud-Turnhout, Belgium), 330 g/kg Nestlé full-fat sweetened condensed milk, 70 g/kg sucrose, and 270 g/kg water. This cafeteria diet provided 15% of its energy content as protein, 69% as carbohydrate and 16% as fat (37). This diet was designed to be highly palatable by preparing it as a paste that the rats seemed to like, and thus they ate relatively large amounts of it. Furthermore, group 3 was subjected to exercise training during the last 4 wk of the 12-wk diet period (CAFTR). The rats performed 1 h of swimming exercise 5 times/wk in water maintained at 32–35°C. To ensure continuous swimming, each rat had a load attached to the tail equivalent to 2% of its body weight.

Intravenous Glucose Tolerance Test

Following the above-described 12-wk period of interventions, rats were anesthetized by an intraperitoneal injection (0.2 ml/100 g body wt) of a mixture containing 50% Ketalar (50 mg/ml ketamine; Pfizer, Brussels, Belgium), 25% Rompun (2% xylazine; Bayer, Leverkusen, Germany), and 25% atropine (0.5 mg/ml) and prepared surgically for an intravenous glucose tolerance test (IVGTT) by insertion of a catheter into the left vena jugularis. Rats were then allowed to recover from the anesthesia and were subjected to the IVGTT the next day, which for the exercise-trained group was 24 h after the last swimming bout. After an overnight fast (16–18 h), a jugular catheter with 1 g glucose/kg body wt was injected into conscious rats, using a 30% (wt/vol) glucose solution in 0.9% (wt/vol) saline. For blood glucose determination, tail blood samples (25 µl) were collected in Na-heparinized capillaries before the glucose injection and 5, 10, 15, 30, 60, 90, and 120 min afterward. Blood glucose concentration was measured immediately after, using an Analox GM7 (Analox Instruments, London, UK) glucose analyzer. Plasma insulin was assayed by a sensitive rat insulin RIA kit (Linco Research).

Hindlimb Perfusion Procedure

Rats were allowed to recover from the IVGTT and received their usual diet for 4 nights, during which daily food intake was registered. The exercise-trained group resumed swimming (2 bouts) after the IVGTT and had its last bout 48 h before perfusion. Following an overnight fast, rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Hindlimb perfusion was performed as described by Wojtaszewski et al. (38).

All perfusions were carried out using a cell-free perfusate consisting of Krebs-Ringer bicarbonate buffer solution, 4% bovine serum albumin (fraction V; Sigma), 0.15 mM pyruvate, and 4.2 IU/ml heparin and no (basal glucose transport) or 200 µU/ml insulin (insulin-stimulated glucose transport). During perfusion the arterial perfusate was continuously gassed with a mixture of 95% O2 and 5% CO2, yielding average O2 pressure and pH of 630 ± 13 mmHg and 7.33 ± 0.02, respectively. The temperature of the arterial perfusate was 35°C. Following surgical preparation, the hindlimbs were allowed to recover for 15 min with perfusate recirculation at a flow of 20 ml/min. The initial 150 ml of glucose-free perfusate passing through the hindlimb was discarded. Thereafter, the glucose-free recovery perfusate was exchanged for perfusate containing 8 mM 2-deoxy-o-glucose and 1 mM mannitol, including 2-[2,6-3H]deoxy-o-glucose (specific activity 51.0 mCi·mmol−1·l−1; Amersham Radiochemicals, GE Healthcare, Uppsala, Sweden) and α-1-[14C]mannitol (specific activity 87.0 mCi·mmol−1·l−1; Amersham Radiochemicals, GE Healthcare) yielding activities of 0.075 µCi/ml for 1H and 0.050 µCi/ml for 14C. The isotope reached the hindlimbs exactly at the end of the 15-min recovery period. To secure a constant specific activity for 2-deoxy-o-glucose in the arterial perfusate, one-way perfusion at a flow of 20 ml/min was started. Following 10 min of isotopic perfusion, circulation was stopped. Immediately after this, the medial superficial part and the deep part of the medial head of m. gastrocnemius were dissected from both hindlimbs, freed from any visible connective tissue and blood, and snap-frozen with aluminum clamps cooled in liquid nitrogen and stored at −80°C. These two muscle parts were selected because they represent primarily white glycolytic and red oxidative and glycolytic fibers, respectively (2).

Table 1. Body mass and epididymal fat pad mass in control rats and rats receiving cafeteria diet in the absence or presence of exercise training

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CAF</th>
<th>CAFTR</th>
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<tbody>
<tr>
<td></td>
<td>0 wk</td>
<td>12 wk</td>
<td>0 wk</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>218 ± 3 (n = 9)</td>
<td>474 ± 16* (n = 9)</td>
<td>217 ± 1 (n = 13)</td>
</tr>
<tr>
<td>Epididymal fat pad mass, g</td>
<td>7.3 ± 0.8 (n = 3)</td>
<td>15.2 ± 1§§ (n = 10)</td>
<td>10.6 ± 0.9* (n = 10)</td>
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</table>

Values are means ± SE. CON, control rats; CAF, cafeteria diet-fed rats in the absence of training; CAFTR, cafeteria diet-fed rats in the presence of training. *P < 0.01 between 0 and 12 wk; †P < 0.05, different compared with CON; ‡P < 0.01, different compared with CON; §§P < 0.01 different compared with CAFTR. Body mass data have been published elsewhere (37).
samples from the right hindlimb were freeze-dried for 36 h at 50°C.

After an overnight incubation at 4°C, the immunoprecipitate was washed twice in Tris-buffered saline (pH 7.5), 10 mM leupeptin, 2 mM Na3VO4, and 3 mM benzamidine. Homogenates were rotated end over end for 1 h at 4°C before being centrifuged for 30 min (17,500 × g) during a 120-min IVGTT. Values are means ± SE; *P < 0.05, different compared with CON; ‡P < 0.01, different compared with CAFTR; §P < 0.01, different compared with CAFTR. Daily energy intake data have been published elsewhere (37).

Skeletal Muscle Analysis

Muscle glycogen. For determination of glycogen content, muscle samples from the right hindlimb were freeze-dried for 36 h at −50°C. Muscle glycogen content of the freeze-dried samples (2–3 mg) was determined fluorometrically by the hexokinase method after acid hydrolysis (19a).

Homogenization. In brief, 20 mg of freeze-dried muscle was homogenized in ice-cold buffer containing 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 8.0), 2 mM PMSF, 1 mM CaCl2, 1 mM MgCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, and 3 mM benzamidine. Homogenates were rotated end over end for 1 h at 4°C before being centrifuged for 30 min (17,500 g, 4°C). Protein content in the supernatant was measured by the bicinchoninic acid method (Pierce).

Immunoblotting. Total protein and phosphorylation of relevant proteins were determined in muscle lysates by SDS-PAGE, followed by immunoblotting. Phosphorylation of Akt Ser473, IR Tyr1158, JNK Thr183/Tyr185, and AMPK Thr172 as well as GSK-3β Ser21/9 was measured using phosphospecific antibodies from Cell Signaling Technology (Beverly, MA). Total protein levels of Akt1 and Akt2 were measured using antibodies from Cell Signaling Technology. Phosphorylation of Akt Thr308 was measured using a phosphospecific antibody kindly provided by Dr. Ken Siddle (University of Cambridge, Cambridge, UK). After an overnight hydrolysis (19a).

Insulin receptor substrate-1 associated phosphatidylinositol-3 kinase activity

Insulin receptor substrate-1 (IRS-1) was immunoprecipitated from 300 μg of lysate with a specific antibody kindly provided by Dr. Ken Siddle (University of Cambridge, Cambridge, UK). After an overnight hydrolysis (19a).

Insulin receptor substrate-1 associated phosphatidylinositol-3 kinase activity

Akt1 was immunoprecipitated from 100 μg of lysate from CON, CAF, and CAFTR samples with an Akt1 antibody from Cell Signaling Technology. After an overnight incubation at 4°C, the immunoprecipitate was washed twice in Tris-buffered saline (pH 7.5) with 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM EDTA (pH 8.0), 2 mM PMSF, 1 mM CaCl2, 1 mM MgCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, and 3 mM benzamidine.

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Immunoprecipitation of Akt

Akt1 was immunoprecipitated from 100 μg of lysate from CON, CAF, and CAFTR samples with an Akt1 antibody from Cell Signaling Technology. After an overnight incubation at 4°C, the immunoprecipitate was washed twice in Tris-buffered saline (pH 7.5) with 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM EDTA (pH 8.0), 2 mM PMSF, 1 mM CaCl2, 1 mM MgCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, and 3 mM benzamidine.
incubation at 4°C, the immunoprecipitate was washed twice in PBS (pH 7.5) with 1% NP-40 and 100 mM Na2VO4 and twice in Tris-buffered saline (pH 7.5) with 100 mM NaCl, 1 mM EDTA, and 100 mM Na2VO4 and left with 50 µl of the last wash buffer. Ten microliters of 100 mM MgCl2 and 10 µg of 1-α-phosphatidylinositol (PI; Sigma-Aldrich) were added, and the samples were left at room temperature for 15 min. The reaction was started by the addition of 10 µl of reaction mixture [8.25 mM Tris (pH 7.5), 825 µM EDTA, 6 mM MgCl2, 440 µM ATP, and 100 pM33P-ATP (0.6 µCi); PerkinElmer]. The reaction ran for 15 min at 30°C and was stopped by the addition of 10 µl of 5 N HCl. Then 180 µl of methanol-chloroform (1:1) was added, and samples were shaken vigorously for 1 min and centrifuged for 90 s. Eighty microliters of the lower organic fraction was transferred to a new tube, and 50 µl were spotted onto a TLC Silica gel (Merck). The chromatographic separation ran for 45 min, after which the TLC gel was dried and exposed in a PhosphorImager Cassette for 48 h before scanning in a Molecular Dynamics STORM scanner (Struers Kebo Laboratory).

Statistical Analysis

Data are expressed as means ± SE. Statistical evaluations were performed by two-way ANOVA with repeated measurements using the Bonferroni test for post hoc comparisons when appropriate. Differences between groups were considered statistically significant if P < 0.05.

RESULTS

Some of the basic descriptive data from these rats have been published before (37). For clarity, a few data are repeated here, and in the tables and figures it is clearly stated whether data have been published before.


There were no detectable differences in animal body weights prior to the interventions. During the 12 wk animals in all groups gained weight, and after 12 wk the CAF group was
significantly heavier (~10%, $P < 0.01$) than the two other groups (Table 1). Twelve weeks of cafeteria diet feeding led to development of significantly larger epididymal fat pads in both the CAF and the CAFTR groups (105 and 43%, respectively, $P < 0.01$, $P < 0.05$) compared with CON, although CAFTR has smaller pads than CAF ($P < 0.01$) (Table 1). The average daily energy intake during the 12 wk was significantly larger (~12%, $P < 0.05$) in the CAF group compared with the two other groups (Table 2). After 12 wk of intervention there was no difference in fasting glucose between groups, but fasting insulin was significantly higher in the CAF group compared with CON and CAFTR (180 and 68%, respectively, $P < 0.01$; Table 2).

In Vivo Glucose Tolerance: IVGTT

During an IVGTT, fasting plasma glucose was similar among groups, whereas the glucose level in the CAF group

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**Fig. 4.** A: Akt1 and Akt2 Ser473 and Thr308 phosphorylation in immunoprecipitates (IP) of Akt1 in lysates obtained from CON, CAF, and CAFTR rats. IP, post-IP, and lysate from CON, CAF, and CAFTR samples are loaded, and the protein content of Akt1 and Akt2 as well as phosphorylation on Ser473 and Thr308 is shown. Akt phosphorylation on Ser473 (B and C) and Thr308 (D and E) in the basal (black bars) and insulin-stimulated (open bars) state in RG (B–D) and WG (C–E) muscle from a perfused hindlimb. Muscles were obtained from CON rats and rats receiving cafeteria-style diet in the absence and in the presence of training interventions during the last 4 wk of the 12-wk diet period. Values are means ± SE; $n = 5–9$. **$P < 0.01$, different compared with basal.
throughout the IVGTT was elevated (37) as well as when expressed as area under the curve (AUC; \(P < 0.05\)). Plasma insulin at all time points as well as when it was expressed as AUC was significantly higher (\(P < 0.01\)) in the CAF group compared with the two other groups (Fig. 1).

**In Vitro Glucose Transport**

Glucose transport in perfused muscle was similar between groups in the absence of insulin in both red (RG) and white (WG) gastrocnemius muscle (Fig. 2). In response to insulin, glucose uptake increased about fourfold (\(P < 0.05\)) in RG in the CON group, whereas no significant response was observed in the CAF group. Interestingly, the insulin response was rescued in the CAFTR group in RG (\(P < 0.05\)). In WG the insulin response was less than in RG, and like in RG there was no response in the CAF group. In contrast to the results in RG, glucose transport only tended to be rescued by exercise in the CAFTR group (Fig. 2).

**Insulin Receptor Phosphorylation and PI 3-Kinase Activity**

Basal phosphorylation of the insulin receptor (IR) in RG was equal among groups. Upon insulin stimulation, IR was activated via phosphorylation on Tyr\(^{1151}\), which is one of the major autophosphorylation sites. Phosphorylation of IR increased \(\sim 1.5\)-fold (\(P < 0.01;\) Fig. 3) and was unaffected by the interventions.

IRS-1-associated PI 3-kinase activity in RG increased approximately onefold (\(P < 0.01;\) Fig. 3) upon insulin stimulation without any differences among groups in either the basal or the insulin-stimulated state.

**JNK Phosphorylation**

Phosphorylation of JNK Thr\(^{183}/\text{Tyr}^{185}\) in RG was not different among groups after 12 wk of interventions (Fig. 3).

**Akt Isoforms**

All Akt1 protein was immunoprecipitated with the Akt1 antibody, whereas all Akt2 was left in the postimmunoprecipitate. Both Ser\(^{473}\) and Thr\(^{308}\) phosphorylation were found solely in the postimmunoprecipitate, suggesting that only Akt2 was phosphorylated in the lysates (Fig. 4A). Therefore, phosphorylation of the two sites on Akt is expressed as phosphorylated protein relative to total Akt2 content.

**Akt Phosphorylation**

Basal phosphorylation of Akt on Ser\(^{473}\) and Thr\(^{308}\) was equal in all groups in both RG (Fig. 4, B and D) and WG (Fig. 4, C and E). Upon insulin stimulation, Akt was activated in terms of phosphorylation on both Ser\(^{473}\) and Thr\(^{308}\) in all groups. In RG, there was a tendency toward a minor reduction of Ser\(^{473}\) phosphorylation (\(P = 0.051\)) in the CAF group that was fully rescued by exercise in the CAFTR group. Phosphorylation of Thr\(^{308}\) in RG was not affected; this was also the case for both phosphorylation sites in WG.

**GSK-3 and TBC1D4 Phosphorylation**

Basal GSK-3-\(\beta\) Ser\(^{9}\) in RG did not differ among groups. In response to insulin stimulation, phosphorylation leading to inhibition increased by \(\sim 65\%\) (\(P < 0.01;\) Fig. 5A) in all groups. In RG, TBC1D4 Thr\(^{442}\) phosphorylation was similar in all groups in the basal state. Upon insulin stimulation, phosphorylation level increased by \(\sim 70\%\) (\(P < 0.01;\) Fig. 5B), with no statistical difference among groups.

**GLUT4, Munc18c, and Syntaxin 4 Protein Content**

Neither content of GLUT4, Munc18c, nor syntaxin 4 protein in RG differed among groups after 12 wk of interventions (Fig. 6). Since basal and insulin-stimulated samples did not differ, these are presented combined.

**HKII and AMPK Protein Content and Phosphorylation**

After \(4\) wk of exercise training the protein levels of HKII and AMPK\(\alpha 2\) were increased in RG in the CAFTR group by \(\sim 36\%\) (\(P < 0.05;\) Fig. 7A) and \(43\%\) (\(P < 0.01;\) Fig. 7C), respectively, compared with both CON and CAF. There was no difference in AMPK\(\alpha 1\) protein levels among groups (Fig. 7B). There was no effect of CAF diet on AMPK expression. Despite the increase in AMPK\(\alpha 2\) expression in the CAFTR group, the phosphorylation level of AMPK on Thr\(^{172}\) was not different among groups (\(P = 0.172;\) Fig. 7D). Since basal and insulin-stimulated samples did not differ, these are presented combined.
DISCUSSION

The present study shows that CAF feeding leads to development of obesity and insulin resistance on the whole body and muscle level and that exercise training almost completely reverses these deleterious effects of the cafeteria diet. Surprisingly, CAF-induced insulin resistance was not accompanied by marked impairment of insulin signaling, activation of JNK, or altered expression or phosphorylation of AMPK in skeletal muscle.

Previously, CAF feeding in rats was shown to increase body weight considerably due to an increased daily energy intake and impair whole body glucose tolerance (25, 26). The impact of CAF feeding on insulin signaling toward an increased glucose uptake in skeletal muscle has not previously been addressed.

In contrast to previous studies where HFD is accompanied by activation of various stress-activated kinases and massive increases in IRS-1 serine phosphorylation, ~50% reduced IRS-1 tyrosine phosphorylation, and ~30% reduction in Akt serine phosphorylation/activity (36, 42), we observed no attenuations in the proximal part of the insulin-signaling cascade in terms of IR phosphorylation and IRS-1-associated PI 3-kinase activity in this study, whereas a minor defect was present on Akt phosphorylation. Since two downstream targets of Akt in the insulin-signaling cascade, GSK-3 and TBC1D4, appear to be regulated normally in all animals, the tendency toward reduced phosphorylation of Akt in the CAF group does not seem to play that important a role concerning regulation of insulin-stimulated glucose transport, in agreement with other studies showing that only very limited Akt phosphorylation is necessary to elicit a full response of glucose transport (10). Thus impaired insulin action caused by 12 wk of CAF seems to be due to defects distal to TBC1D4. This indicates that high-fat feeding apparently induces insulin resistance via different molecular mechanisms compared with insulin resistance induced by CAF feeding. CAF-induced obesity and insulin resistance seems to be a more relevant model that better resembles obesity and insulin resistance in a Western population since a normal Western diet does not contain 60% fat, as is often used in high-fat feeding (14a).

Previously, GLUT4 protein expression, as well as insulin-stimulated GLUT4 translocation to the plasma membrane, has been reported to be reduced in HFD (12, 18, 42). However, we found no effect of CAF feeding on GLUT4 protein expression. The diet-induced defect causing the reduced insulin-stimulated glucose transport might then be related to recruitment of GLUT4 to the plasma membrane and/or the docking/fusion process with the membrane. Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are central in fusion of transport vesicles with their target membrane. Munc18 proteins are thought to be involved in regulating conformation of syntaxins and, therefore, SNARE complex formation. In adipocytes, Munc18c has been shown to inhibit docking/fusion of GLUT4 vesicles by blocking binding of the v-SNARE VAMP2 to the t-SNARE syntaxin 4 (1, 3a, 34, 35). Furthermore, knockout of Munc18c in mice has been shown to increase insulin-stimulated GLUT4 exocytosis (13), whereas overexpression of Munc18c in adipocytes has been shown to inhibit insulin-stimulated GLUT4 translocation (35). In the present study, we found no effect of either CAF feeding or exercise training on Munc18c or syntaxin 4 protein expression, suggesting that these two proteins are not involved in the decreased insulin action after CAF.

Exercise training has previously been shown to protect against the deleterious effects of HFD on insulin-stimulated glucose disposal (15, 17, 18, 33). A period of exercise training in high-fat-fed rodents has been shown to reduce the diet-induced serine phosphorylation of IRS-1 and reverse the re-

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**Fig. 6.** Protein content of GLUT4 (A), Munc18c (B), and syntaxin 4 (C) in RG muscle obtained from CON, CAF, and CAFTR rats. Values are means ± SE of combined basal and insulin-stimulated samples; n = 11–13.
duction in IRS-1-associated PI 3-kinase activity and the reduction in both phosphorylation and activity of cytosolic Akt2 (18, 42). However, the molecular mechanism behind the effect of exercise is unresolved. One possibility is that activation of AMPK during exercise (40) plays a major role as suggested (28). Supporting such a contention, obese Zucker rats as well as ob/ob mice treated with the AMPK-activating agent AICAR improved their metabolic status markedly (27, 30). Furthermore, incubation with palmitate has been shown to impair in vitro glucose transport in incubated rat extensor digitorum longus muscle, where AICAR treatment ameliorated palmitate-induced insulin resistance (24). Further supporting a possible role of AMPK in exercise-induced reversal of insulin resistance are findings in humans that showed that endurance training led to an increase in AMPK expression accompanied by increases in both α1- and α2-associated AMPK activity and phosphorylation (24). Further supporting a possible role of AMPK in exercise-induced reversal of insulin resistance are findings in humans that showed that endurance training led to an increase in AMPK expression accompanied by increases in both α1- and α2-associated AMPK activity and phosphorylation (24). Previous studies have shown conflicting data concerning activation, phosphorylation, and expression of AMPK following HFD. Some show an increased activation and phosphorylation of AMPKα2 but not of AMPKα1 (18), whereas others show a decrease in AMPKα2 but not of AMPKα1 protein expression accompanied by a decreased phosphorylation of AMPK (19). In the present study we found no effect of CAF on either AMPK expression or phosphorylation.

In conclusion, we have provided novel data to demonstrate that excess intake of a palatable low-fat diet induces obesity and insulin resistance both on a whole body level and in skeletal muscle. Interestingly, CAF-induced insulin resistance was not accompanied by marked impairment of insulin signaling and does not seem to be related to low-grade inflammation, as has been described after HFD. Thus the present results indicate that the mechanism by which insulin resistance is induced by a CAF is different from insulin resistance induced by a high-fat diet and is furthermore reversed by exercise training.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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