Cyanidin-3-*O*-β-Glucoside and Protocatechuic Acid Exert Insulin-Like Effects by Upregulating PPARγ Activity in Human Omental Adipocytes

Beatrice Scazzocchio,[1] Rosaria Varì,[1] Carmelina Filesi,[1] Massimo D'Archivio,[1] Carmela Santangelo,[1] Claudio Giovannini,[1] Annunziata Iacovelli,[2] Gianfranco Silecchia,[3] Giovanni Li Volti,[4,5] Fabio Galvano,[4] and Roberta Masella[1]

OBJECTIVE—Insulin resistance (IR) represents an independent risk factor for metabolic, cardiovascular, and neoplastic disorders. Preventing/attenuating IR is a major objective to be reached to preserve population health. Because many insulin-sensitizing drugs have shown unwanted side effects, active harmless compounds are sought after. Dietary anthocyanins have been demonstrated to ameliorate hyperglycemia and insulin sensitivity. This study aimed at investigating whether cyanidin-3-*O*-β-glucoside (C3G) and its metabolite protocatechuic acid (PCA) might have a role in glucose transport activation in human omental adipocytes and 3T3-L1 cells.

RESEARCH DESIGN AND METHODS—In cells treated with 50 μmol/L C3G and 100 μmol/L PCA, [^{3}H]-2-deoxyglucose uptake, GLUT4 translocation by immunoblotting, adiponectin secretion, and peroxisome proliferator–activated receptor-γ (PPARγ) activation by enzyme-linked immunosorbent assay kits were evaluated. Parallel experiments were carried out in murine adipocyte 3T3-L1. To define the role of PPARγ in modulating polyphenol effects, small interfering RNA technique and PPARγ antagonist were used to inhibit transcription factor activity.

RESULTS—C3G and PCA increased adipocyte glucose uptake ($P < 0.05$) and GLUT4 membrane translocation ($P < 0.01$). Significant increases ($P < 0.05$) in nuclear PPARγ activity, as well as in adiponectin and GLUT4 expressions ($P < 0.01$), were also shown. It is interesting that PPARγ inhibition counteracted the polyphenol-induced adiponectin and GLUT4 upregulations, suggesting a direct involvement of PPARγ in this process.

CONCLUSIONS—Our study provides evidence that C3G and PCA might exert insulin-like activities by PPARγ activation, evidencing a causal relationship between this transcription factor and adiponectin and GLUT4 upregulation. Dietary polyphenols could be included in the preventive/therapeutic armory against pathological conditions associated with IR. ***Diabetes*** **60:2234–2244, 2011**

From the [1]Department of Veterinary Public Health and Food Safety, National Institute of Health, Rome, Italy; [2]Fabia Mater Hospital, Rome, Italy; the [3]Department of Surgery P. Stefanini, University of Rome La Sapienza, Rome, Italy; the [4]Department of Biological Chemistry, Medical Chemistry, and Molecular Biology, University of Catania, Catania, Italy; and the [5]Department of Cardiac Surgery, IRCSS, S. Donato Hospital, Milan, Italy.

Corresponding author: Roberta Masella, roberta.masella@iss.it.

Received 15 October 2010 and accepted 15 June 2011.

DOI: 10.2337/db10-1461

The prevalence of type 2 diabetes is estimated to reach >300 million cases by year 2030 (1). Metabolic syndrome, which is often a precursor to diabetes and cardiovascular diseases, is characterized by insulin resistance (IR), increased fasting glucose, decreased HDL, hypertension, and obesity (specifically, visceral obesity) (2). Furthermore, metabolic syndrome as well as atherosclerosis, type 2 diabetes, and obesity is associated with increased circulating oxidized LDL (oxLDL) (3–6). In cultured cells, oxLDLs have been demonstrated to lower insulin sensitivity (7) and to impair the insulin-dependent GLUT4-mediated uptake of glucose (8–10).

Many studies have shown that adipocytes play an important role in the development of obesity-associated pathologies and IR, mostly by synthesizing and secreting biologically active molecules called adipocytokines (11), such as adiponectin, which is able to improve insulin sensitivity of target cells (12). Serum levels of adiponectin protein correlate with systemic insulin sensitivity (13) and are decreased in insulin-resistant, diabetic, and obese subjects (14).

Adiponectin is regulated by peroxisome proliferator–activated receptor-γ (PPARγ) (15). PPARγ is a ligand-activated nuclear hormone receptor that controls glucose and lipid metabolism (16,17), as well as the transcription of proteins involved in glucose and fatty acid cellular uptake. For these reasons, it represents a main target for antidiabetic drugs, such as thiazolidinediones (TZDs) (18). In addition to their insulin-sensitizing effects, TZDs have a number of side effects, such as promoting adipogenesis, causing body weight gain, and increasing risk for bone fracture and cardiovascular diseases. Hence, ligands for PPARγ that do not procure these unwanted side effects are being sought.

Recently, great interest has arisen regarding evidence that the consumption of a diet rich in vegetables and fruit can exert beneficial healthy effects, likely because of the high content in fiber, mineral salts, vitamins, and polyphenols (19–22). Among polyphenols, anthocyanins (ACNs) are flavonoids of great nutritional interest because their daily intake (180–250 mg/day) is much higher than that of other polyphenols (23). ACNs are absorbed in animals and humans (24–26) and rapidly metabolized, ultimately leading to the formation of phenolic acids and aldehydes (27). In particular, at physiological pH (such as in the bloodstream), ACNs easily convert to protocatechuic acid (PCA), which is also abundantly formed and absorbed in the large intestine after microbial metabolization (28). Owing to the potential benefits for human health (29), many studies have focused on cyanidin-3-*O*-β-glucoside (C3G), the best known and

most investigated ACN, highlighting its potential activities in free radical scavenging and prevention of oxLDL generation, exerting beneficial effects on cardiovascular diseases, obesity, and inflammation (30–32).

Some compelling studies have reported that ACNs improve insulin sensitivity and glucose uptake in diabetic rats (33) and effectively upregulate the signaling pathway of PPARγ in mouse peritoneal macrophages (34), strongly suggesting that they could be successfully used as insulin-sensitizing agents. However, the molecular mechanism of action and the effectiveness of C3G and PCA in exerting protective effects against IR are still poorly understood.

Finally, most of the research on adipocytes has been conducted on the murine cell line 3T3-L1, which is considered a suitable model for studying the pathophysiology of adipocytes and, in particular, for assessing the response to insulin (10). Conversely, to the best of our knowledge, only a few studies have been specifically carried out on human omental adipocytes.

This study investigated the effects of C3G and PCA on glucose uptake machinery in adipocytes by evaluating their ability to reverse the oxLDL-induced impairment of adipocyte response to insulin and the molecular events underlying their effects. Specifically, we demonstrated that C3G and PCA enhanced glucose uptake and GLUT4 translocation in both insulin-stimulated human omental adipocytes and 3T3-L1 cells. Notably, the polyphenols elicited the same response in the absence of insulin, showing insulin-like activity; specifically, they upregulated PPARγ activity and the expression and secretion of its target gene adiponectin. These findings support the hypothesis that C3G, and its main metabolite PCA, might play a role in the therapeutic armory against disease states associated with IR, such as type 2 diabetes and obesity.

RESEARCH DESIGN AND METHODS

Plasma LDL isolation and oxidation. LDLs (1.019–1.063 g/mL) were isolated by density gradient ultracentrifugation from fresh pooled plasma of healthy volunteers as described elsewhere (35). The protein content was measured by Lowry method (8). Native LDLs (nLDLs) were oxidized as previously described (10).

Isolation of human omental adipocytes. Human omental adipocytes were collected from anesthetized individuals undergoing abdominal surgery or laparoscopy for benign conditions (i.e., gallbladder disease without icterus, umbilical hernia, or uterine fibromatosis) (11 females and 9 males, age 50–70 years, BMI 20.0–26.9, waist circumference ≤107 for males and ≤92 for females). Exclusion criteria were steroidal and nonsteroidal anti-inflammatory therapies, hormonal substitutive or contraceptive therapy, hormonal therapy for any thyroid dysfunctions, drug or alcohol abuse, diabetes, chronic renal failure, cancer, pregnancy, and mental disability.

The omental sampling was performed at the same site (great omentum) with the patient in anti-Trendelenburg position (25° head up) with the surgeon standing between the legs. The biopsies were obtained by monopolar electrocautery or harmonic scalpel. The standard sampling was considered 2 × 2 cm, avoiding bleeding and other possible contamination. The omentum was collected into an endobag and extracted through the umbilical trocar to avoid crash and contamination. The study protocol has been approved by the ethics committee of the La Sapienza University. All the subjects were volunteers and gave their informed consent according to the Italian law on this matter (Legislative Decree of the Italian Ministry of Health, 25 January 2001, published in the *Official Gazette* of 3 April 2001).

From 5 to 10 grams of omental biopsies were microdissected, rinsed several times in 0.9% NaCl, and digested with 5 mL of Krebs-Ringer solution (0.12 mol/L NaCl, 4.7 mol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, and 1.2 mmol/L KH_2PO_4) containing 20 mmol/L HEPES pH 7.4, 3.5% fatty acid–free BSA, 200 nmol/L adenosine, 2 mmol/L glucose, and collagenase (type 1) for 1 h (1 mg/g adipose tissue) at 37°C in shaking water bath (36). After collagenase digestion, the adipocytes were separated from tissue debris by filtering through sterile nylon mesh (250 μm). Cells were then washed three times with Krebs-Ringer solution containing 20 mmol/L HEPES, pH 7.4, 1% fatty acid–free BSA, 200 nmol/L adenosine, and 2 mmol/L glucose and resuspended in 199-medium containing 1% fatty acid–free BSA and 25 mmol/L HEPES. Floating fraction of isolated omental adipocytes from different individuals was used for the experiments described below.

3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes (American Type Culture Collection) were induced to differentiate according to Masella et al. (8). The cells were used for the experiments on day 14, when >90% of cells presented the adipocyte phenotype (8).

Treatment of adipocytes with oxLDL. Different oxLDL concentrations (25–200 mg protein/L) were used to test both oxLDL cytotoxicity and their effects on glucose uptake to determine the best concentration. The 100 mg/L concentration was chosen because it effectively reduces the glucose uptake by 50% in human and murine adipocytes without showing any sign of cytotoxicity, as assessed by Neutral Red assay, or affecting the morphology or the metabolism of adipocytes, as determined by the expression of leptin and adipocyte protein 2 (aP2) and by the incorporation of [^{3}H]Uridine, which were both comparable to the controls (data not shown). Under all the experimental conditions described below, adipocytes, untreated and treated with nLDL (100 mg/L), were used as controls. Because we obtained wholly overlapping results, we report data for untreated cells only.

Treatment of adipocytes with C3G and PCA. Adipocytes were incubated with C3G (Polyphenols Laboratories AS, Sandnes, Norway) or PCA (Sigma-Aldrich, St. Louis, MO) at concentrations of 50 and 100 μmol/L, respectively, for human omental adipocytes and 10 and 100 μmol/L, respectively, for 3T3-L1 18 h before the addition of nLDL or oxLDL for 4 or 18 h. To define the experimental conditions, we carried out preliminary trials, incubating the cells with different concentrations of the polyphenols (1–150 μmol/L) for different times before oxLDL addition and determining the percentage of glucose internalized in the cells after insulin stimulation. On the basis of the data obtained (not shown), the time and the lowest concentration of the two polyphenols able to provide a 50% recovery of glucose uptake in oxLDL-treated cells were used in all the experiments. To define the effect of the polyphenols on PPARγ, we assessed the mRNA expression and activity of the transcription factor in cells incubated for 2 or 18 h with C3G or PCA. In the experiments intended to evaluate the specific involvement of PPARγ in the activation of its target genes, the cells were treated with 10 μmol/L GW9662, a PPARγ antagonist, 30 min before the treatment with polyphenols.

Glucose uptake assay. Glucose transport was measured as described elsewhere (37). Briefly, human and 3T3-L1 adipocytes, plated in low-glucose Dulbecco's modified Eagle's medium (1,000 mg/L D-(+)-glucose), were serum starved for 18 h and stimulated with 20 nmol/L insulin for 15 min. [^{3}H]-2-DG (2-deoxyglucose) (1 μCi/well) was added to the cells, and 45 min was allowed for its uptake by the cells. The reaction was stopped by ice-cold PBS in 3T3-L1 cells and by rapid centrifugation at 8,000 rpm for 5 min through 300 μL cushion of silicon oil in human omental adipocytes. The total incorporated radioactivity was determined in a liquid scintillation counter. The results were corrected for aspecific absorption (37). Aspecific absorption was always <10% of total uptake. Results were normalized for protein content.

Protein determination by immunoblotting analysis. To evaluate GLUT4 in plasma membranes (PM) and in low-density microsome (LDM) fractions enriched in the intracellular GLUT4 storage vesicles (38), the cells were fractionated according to McKeel and Jarett (39). Whole-cell extracts were prepared from cells as previously described (8). Nuclear protein extracts were prepared by the Nuclear/Cytosol Fractionation Kit (Medical & Biological Laboratories, Watertown, LA) according to the manufacturer's instructions. Immunoblotting analyses were carried out using specific antibodies for PPARγ and GLUT4 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were treated with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) followed by enhanced chemiluminescence detection (Amersham Bio-sciences, Buckinghamshire, U.K.). Equal loading of proteins was verified by immunoblotting with a goat anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. Densitometric analysis was performed using a molecular imager FX (Bio-Rad, Hercules, CA).

mRNA determination by quantitative real-time PCR. Total RNA was isolated with TRIZOL reagent (Invitrogen-Life Technologies, Carlsbad, CA) as reported elsewhere (8). Quantitative real-time PCR (RTq-PCR) was carried out with gene-specific TaqMan MGB probes and primers (Applied Biosystems, Carlsbad, CA) in an ABI 7700 sequence detector (Applied Biosystems). PPARγ, GLUT4, adiponectin, and endogenous controls TATA-box binding protein (TBP) and GAPDH were purchased from Applied Biosystems as predesigned assays. All gene expression assays have a FAM reporter dye at 5′ end of TaqMan MGB probe and a nonfluorescent quencher at 3′ end of the probe.

Expression of *PPARγ*, *GLUT4*, and *adiponectin* genes were determined as the amount of individual mRNA relative to mRNA for TBP (murine adipocytes) or GAPDH (human adipocytes) using the comparative C_T method described in the ABI 7700 sequence detection system, user bulletin number two.

Assessment of PPARγ activity. PPARγ activity was determined in nuclear extracts with the TransAM ELISA Kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions.

Evaluation of adiponectin secretion. The release of adiponectin was evaluated in the culture media by ELISA Kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

PPARγ silencing by small interfering RNA. PPARγ expression was inhibited with specific small interfering RNA (siRNA) reagents (mouse PPARγ siGENOME SMARTpool siRNA; Dharmacon, Lafayette, CO) as previously described (8). Scrambled nontargeting siRNA was used as negative control. At selected time points after transfection, mRNA and protein were extracted to assess PPARγ, GLUT4, and adiponectin mRNA expressions and GLUT4 protein in PM.

Statistical analysis. The results are expressed as means ± SEM of at least four independent experiments performed in duplicate. In the human studies, depending on the amount of adipocytes isolated from each subject, each experiment was performed in at least 4 different individuals (men-to-women 1:1) randomly chosen from the 20 recruited subjects. Comparisons between two groups were carried out by Student *t* test. ANOVA followed by Student-Newman-Keuls multiple comparison test were used when >2 groups were compared. Differences were considered significant when $P < 0.05$.

RESULTS

Impairment of glucose uptake in human omental adipocytes by oxLDL. We have previously demonstrated that oxLDLs are able to affect cell sensitivity toward insulin, inhibiting glucose uptake by interfering with the cell response to insulin in 3T3-L1 adipocytes (10). Similar results were obtained in insulin-stimulated human omental adipocytes treated with oxLDL (100 mg/L). Indeed, these cells showed a strong decrease (−40%) in glucose uptake and a concomitant reduction of GLUT4 protein (−60%) in the PMs after oxLDL treatment (Fig. 1*A* and *B*).

Effects of PCA and C3G on glucose transport and GLUT4 translocation. In the present article, we evaluated whether polyphenols, specifically PCA and C3G, could counteract the detrimental effects induced by oxLDL in adipocyte cell models. To this end, glucose uptake was evaluated in oxLDL-treated adipocytes, previously incubated with each polyphenol for 18 h, as described in RESEARCH DESIGN AND METHODS. Our results evidenced that both the polyphenols were able to counteract the drop in glucose uptake of human as well as murine insulin-treated adipocytes (Fig. 1*A* and *C*, respectively). In addition, they reversed the impairment of GLUT4 translocation (Fig. 1*B* and *D*) induced by oxLDL.

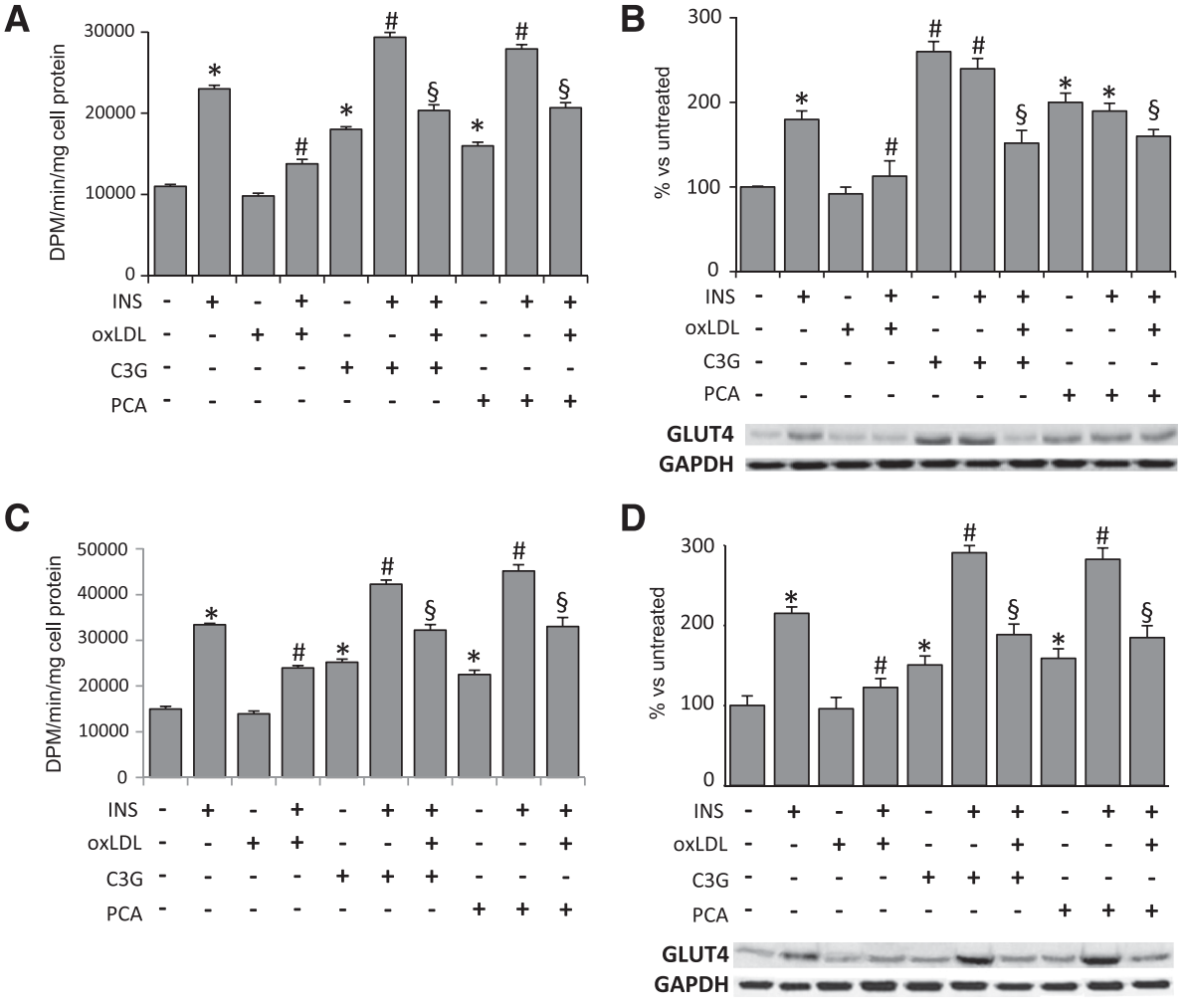

FIG. 1. Effects of C3G and PCA on the impairment of glucose uptake by oxLDL in human and 3T3-L1 adipocytes. Human and murine adipocytes were serum starved in low-glucose medium for 18 h, incubated with 50 μmol/L and 10 μmol/L C3G, respectively, or with 100 μmol/L PCA for a further 18 h, and then treated with 100 mg/L oxLDL for 4 h. The rate of glucose uptake was determined after the addition of [^{3}H]-2-DG (2-deoxyglucose) in cells with or without 20 nmol/L insulin stimulation. Glucose uptake in human (*A*) and murine (*C*) adipocytes was expressed as radioactivity per minute per milligram of cell proteins (DPM/min/mg cell proteins). Data are means ± SEM of four independent experiments. PM fractions from human (*B*) and 3T3-L1 (*D*) adipocytes, isolated as described in RESEARCH DESIGN AND METHODS, were resolved by SDS-PAGE and analyzed using antibodies against GLUT4. Results were normalized to GAPDH protein content. Representative blots are shown. ANOVA, $P < 0.0001$; post hoc test, *$P < 0.001$ compared with unstimulated control cells, #$P < 0.05$ compared with insulin-stimulated cells, and §$P < 0.05$ compared with oxLDL-treated cells stimulated with insulin.

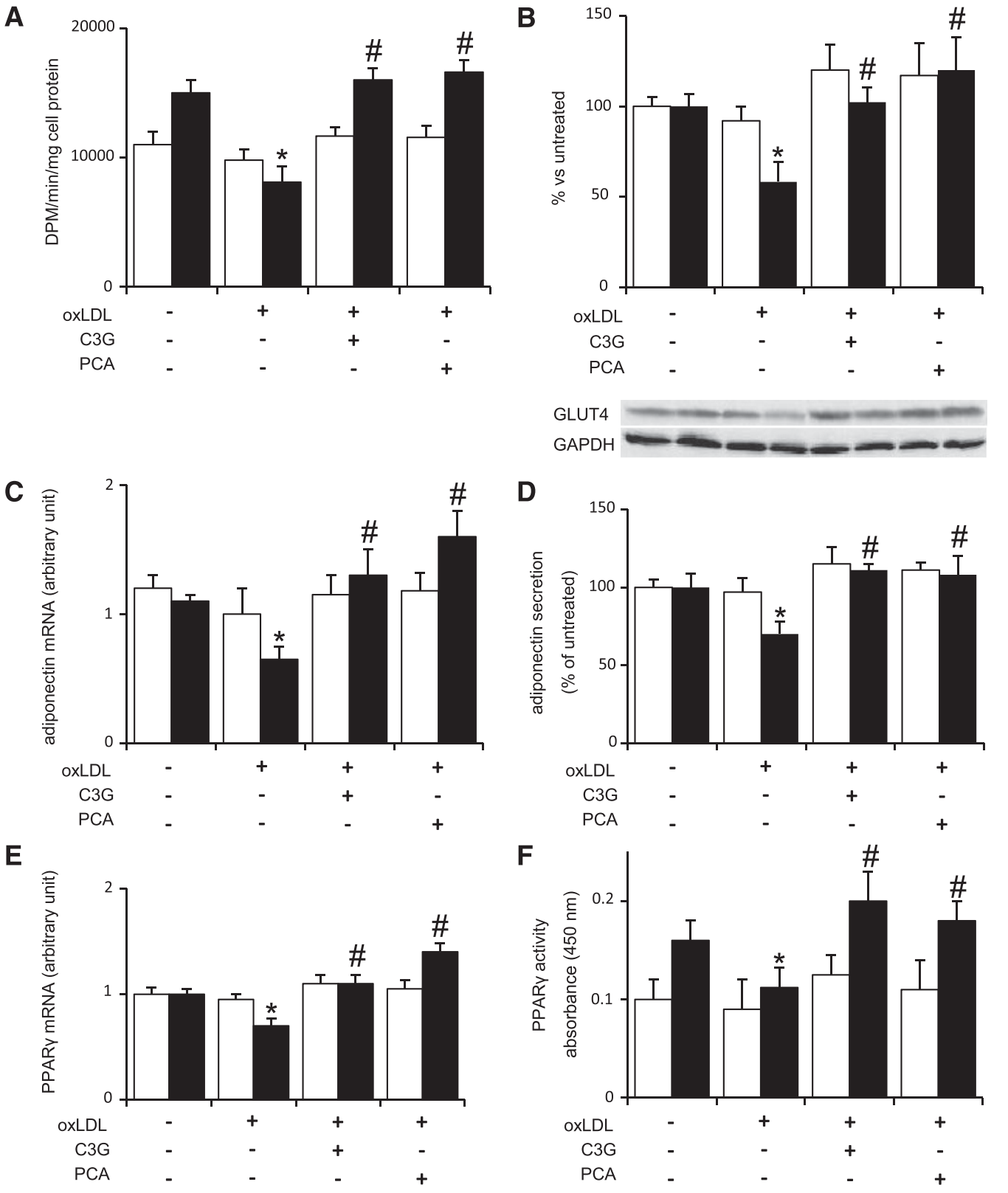

FIG. 2. Effects of oxLDL and polyphenols on the basal glucose transport machinery. Human omental adipocytes were serum starved in low-glucose medium for 18 h, incubated with 50 μmol/L C3G or with 100 μmol/L PCA for a further 18 h, and then treated with 100 mg/L oxLDL for 4 h (white bars) and 18 h (black bars). The rate of glucose uptake (*A*), determined after the addition of [^{3}H]-2-DG (2-deoxyglucose), was expressed as radioactivity per minute per milligram of cell proteins (DPM/min/mg cell proteins). PM fractions (*B*), isolated as described in RESEARCH DESIGN AND METHODS, were resolved by SDS-PAGE and analyzed using antibodies against GLUT4. The results were normalized to GAPDH protein content. Representative blots are shown. Adiponectin (*C*) and PPARγ (*E*) mRNAs were assessed by RTq-PCR. The values indicate the expression of the target gene normalized to GAPDH RNA by using the comparative C_T method as described in RESEARCH DESIGN AND METHODS. Adiponectin release (*D*) was evaluated in the culture media by enzyme-linked immunosorbent assay kit, and PPARγ activity (*F*) was determined in nuclear extracts as described in RESEARCH DESIGN AND METHODS. Data are means ± SEM of four independent experiments. ANOVA, $P < 0.0001$; post hoc test, *$P < 0.05$ compared with unstimulated cells and #$P < 0.05$ compared with oxLDL-treated cells.

Notably, the levels of both glucose uptake and GLUT4 translocation were increased in insulin-stimulated adipocytes treated with polyphenols with respect to those treated with insulin alone (up to 40% at 50 μmol/L and 100 μmol/L of C3G and PCA, respectively). This increase might be the result of an enhancement of cell sensibility to insulin as well as to an additive effect (i.e., the polyphenols could improve glucose uptake and GLUT4 translocation by activating other factors than those specifically involved in insulin pathways). However, we have no evidence to support any hypothesis.

To better define the possible effects of oxLDL on the basal uptake of glucose, we determined glucose uptake and GLUT4 translocation in unstimulated human omental adipocytes after 4 and 18 h of incubation with oxLDL alone and in the presence of polyphenols. The glucose transport machinery appeared not to be affected by 4-h oxLDL treatment, whereas 18-h treatment caused the lowering of glucose uptake and GLUT4 translocation. Both the polyphenols were able to counteract such decrease (Fig. 2*A* and *B*).

On the basis of this finding, we carried out an in-depth study to define the effects of the polyphenols on the efficiency of the glucose transport mechanism. Thus, human omental adipocytes and 3T3-L1 were incubated with different concentrations of C3G (1–100 μmol/L) or PCA (1–150 μmol/L) for 18 h. Then, 2-deoxy-D-[^{3}H]glucose transport was assessed in either insulin-stimulated or unstimulated cells. The results indicate that C3G and PCA treatments were associated with an enhancement of glucose uptake in the adipocytes not only in the insulin-stimulated cells but also in the unstimulated cells (Fig. 3). It is worth noting that the glucose uptake in adipocytes not stimulated with insulin increased by up to 60 and 40% with 50 μmol/L C3G or 100 μmol/L PCA, respectively (Fig. 3*A* and *B*). A similar trend was also observed in 3T3-L1 cells in which glucose uptake increased by up to 60% with 10 μmol/L C3G or 100 μmol/L PCA (Fig. 3*C* and *D*). Thus, the polyphenols exhibited a significant insulin-like activity.

To elucidate the mechanism responsible for the insulin-like activity of the polyphenols, we evaluated whether C3G and PCA exhibited a direct effect on the glucose transporter GLUT4. In particular, we determined its expression, as protein and mRNA, in adipocytes treated with each polyphenol. As shown in Fig. 4*A*, in human omental adipocytes, C3G and PCA were able to upregulate GLUT4 mRNA, as also demonstrated in 3T3-L1 adipocytes (Fig. 4*C*). In parallel, we assessed GLUT4 protein levels in whole-cell lysates and PMs. We found that in human omental adipocytes, as well as in 3T3-L1, GLUT4 was upregulated in whole-cell lysates (~30% in both human and murine cells) but especially in PMs (up to 170 and 50% in human and murine adipocytes, respectively) (Fig. 4*B* and *D*). Notably, in human omental adipocytes, we also determined GLUT4 in the storage vesicles (LDM), showing that the

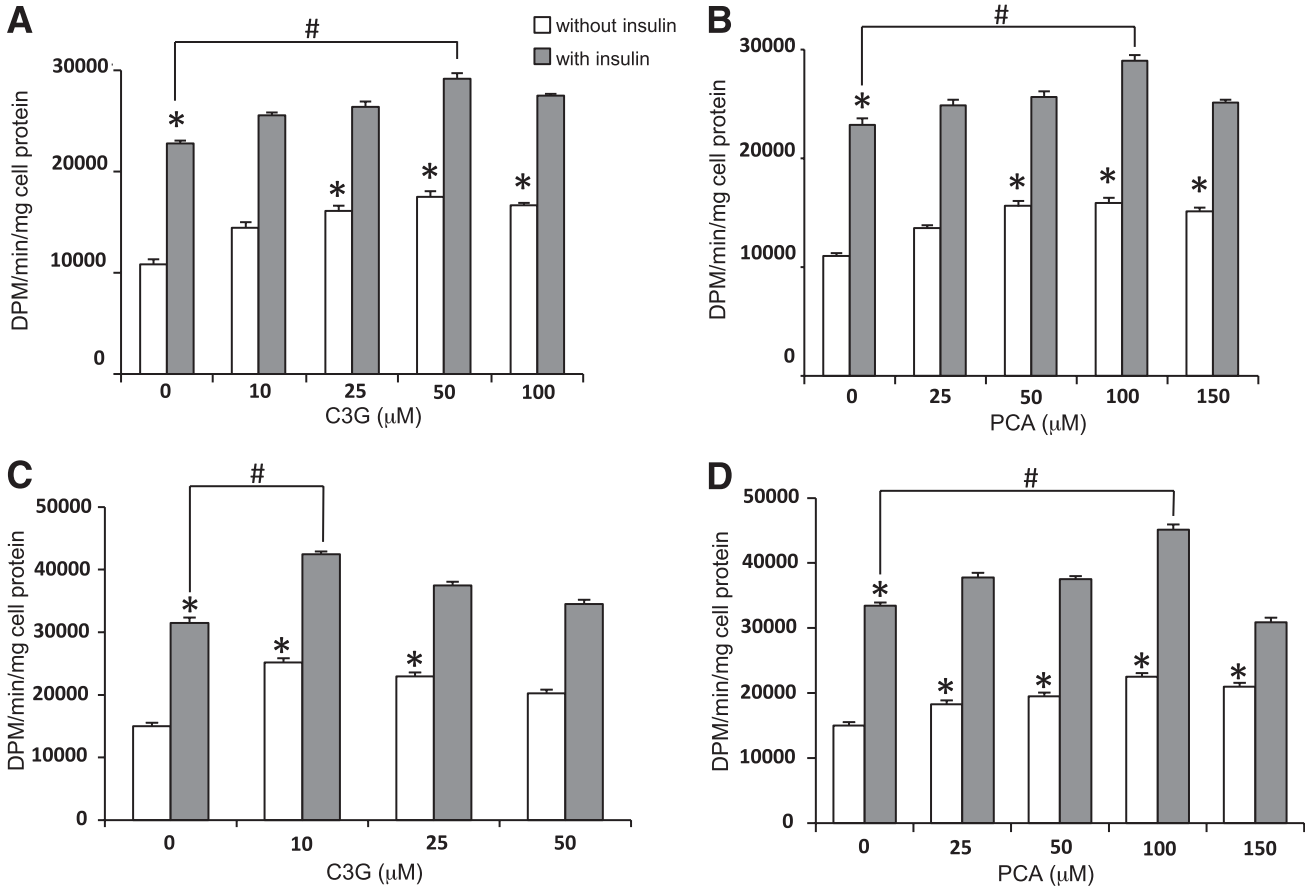

FIG. 3. Dose-response curve of C3G and PCA on glucose uptake. Human (*A* and *B*) and 3T3-L1 (*C* and *D*) adipocytes, serum starved (18 h) in low-glucose medium, were incubated with different concentrations of polyphenols for 18 h. The rate of glucose uptake was determined after the addition of [^{3}H]-2-DG (2-deoxyglucose) in cells stimulated (gray bars) or not (white bars) with 20 nmol/L insulin for 15 min. Glucose uptake was expressed as radioactivity per minute per milligram of cell proteins (DPM/min/mg cell proteins). Data are means ± SEM of four independent experiments. ANOVA, $P < 0.0001$; post hoc test, *$P < 0.05$ compared with unstimulated cells without polyphenol treatment and #$P < 0.05$ compared with insulin-stimulated cells without polyphenol treatment.

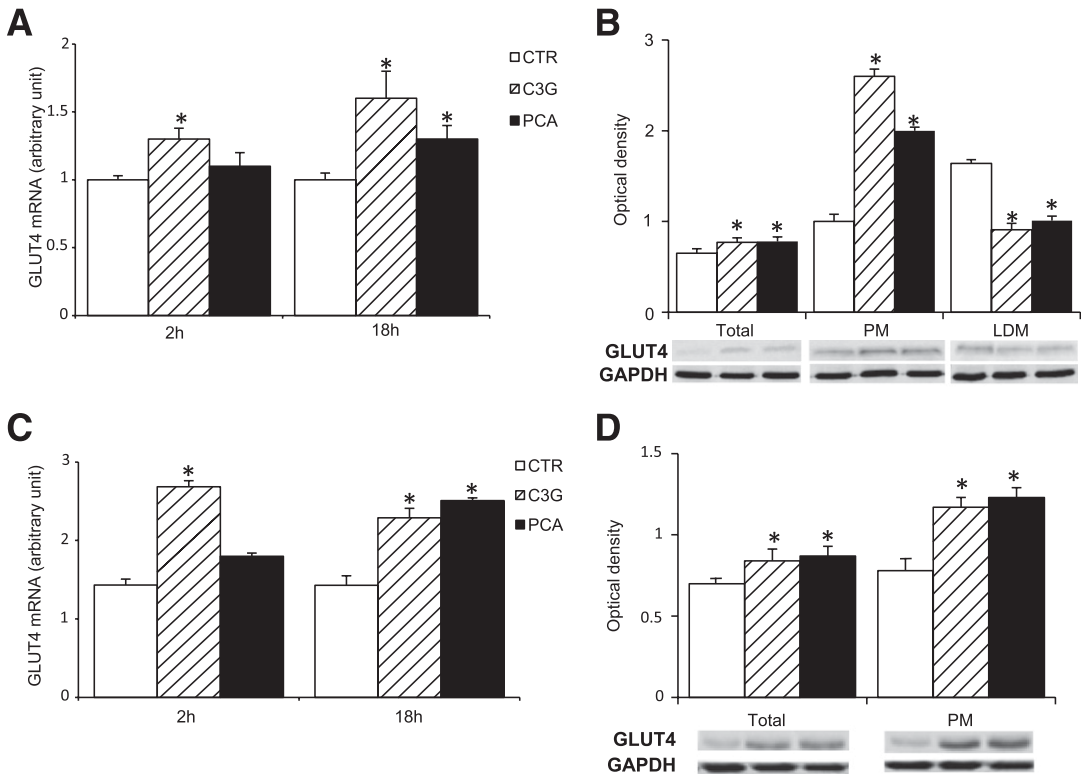

FIG. 4. Gene and protein expressions of GLUT4 in polyphenol-treated adipocytes. Human and 3T3-L1 adipocytes were serum starved for 18 h and incubated with 50 μmol/L and 10 μmol/L C3G (striped bars), respectively, or with 100 μmol/L PCA (black bars) for 2 or 18 h. GLUT4 mRNA was assessed in human (*A*) and murine (*C*) adipocytes by RTq-PCR. The values indicate the expression of target gene normalized to TBP (murine adipocytes) or GAPDH (human adipocytes) RNA by using the comparative C_T method as described in RESEARCH DESIGN AND METHODS. GLUT4 protein expression was determined at 18 h of polyphenol treatment in *1*) whole-cell lysates (Total), PMs, and storage vesicles (LDM) prepared from human adipocytes (*B*), and *2*) whole-cell lysates (Total) and PMs from 3T3-L1 cells (*D*). Results were normalized to GAPDH protein content. Data are expressed as means ± SEM of four independent experiments. *$P < 0.05$ compared with matched control cells (CTR, white bars). Representative blots are shown.

upregulation of GLUT4 in the PMs was accompanied by a significant decrease in the LDM fractions (Fig. 4*B*).

These findings indicated that polyphenols increased glucose uptake by significantly inducing GLUT4 expression and mostly, GLUT4 translocation.

PCA and C3G mediate induction of adiponectin gene expression. Adiponectin has been shown to have some insulin-sensitizing properties (13,40) and to be decreased in serum of insulin-resistant, diabetic, and obese subjects (14). Thus, we hypothesized that oxLDL could affect adiponectin production/secretion and that the polyphenols could counteract this effect. To verify our hypothesis, human omental adipocytes were incubated for 4 and 18 h with oxLDL alone or in the presence of the phenolic compounds. After 4 h of oxLDL treatment, both in presence or absence of polyphenols, the levels of adiponectin expression and secretion were not substantially changed (Fig. 2*C* and *D*). On the contrary, at 18 h, oxLDL reduced adiponectin mRNA levels by 50% (Fig. 2*C*) and adiponectin secretion by 30% (Fig. 2*D*), whereas both polyphenols prevented such reductions. Worthy of note is the finding that polyphenols were able to upregulate both mRNA expression and secretion of adiponectin by themselves (Fig. 5*A* and *B*).

A similar increase in adiponectin expression and secretion was also demonstrated in 3T3-L1 cells after polyphenol treatment (Fig. 5*C* and *D*).

PCA and C3G induce PPARγ expression and activity. To identify a possible mechanism responsible for the different modulation of GLUT4 and adiponectin observed in oxLDL- or polyphenol-treated adipocytes, we assessed whether PPARγ, the master regulator of mature adipocyte genes (15), could be involved.

PPARγ mRNA expression and activity. For this purpose, we carried out experiments to determine PPARγ mRNA after treatment with oxLDL and/or polyphenols. RTq-PCR analysis showed a significant decrease in PPARγ mRNA expression ($P < 0.001$) in human omental adipocytes within 18 h of treatment with oxLDL that was counteracted by polyphenols (Fig. 2*E*). Furthermore, we determined the activation status of PPARγ in nuclear extracts of cells treated with oxLDL and/or polyphenols, further demonstrating that oxLDL negatively affected PPARγ activation, whereas C3G and PCA were able to counteract the oxLDL-induced detrimental action (Fig. 2*F*). It is interesting that the polyphenols elicited per se both the rise of *PPARγ* gene transcription (Fig. 6*A*) and an early and prolonged increase in PPARγ activity (Fig. 6*B*). In 3T3-L1 cells, C3G and PCA also upregulated *PPARγ* gene expression and activity (Fig. 6*C* and *D*).

PPARγ silencing. To further demonstrate the involvement of PPARγ in the insulin-like activity exerted by C3G and PCA, we silenced PPARγ expression in 3T3-L1 by using the small interfering mRNA technique and the PPARγ antagonist GW9662. In the transfected cells, as well as in the PPARγ inhibitor–treated cells, we found that the significant upregulation of adiponectin and GLUT4 mRNAs induced by polyphenols were counteracted (Fig. 7*A* and *B*), providing additional evidence for the causal relationship between PPARγ activation and polyphenol-induced upregulation of adiponectin and GLUT4. We also demonstrated

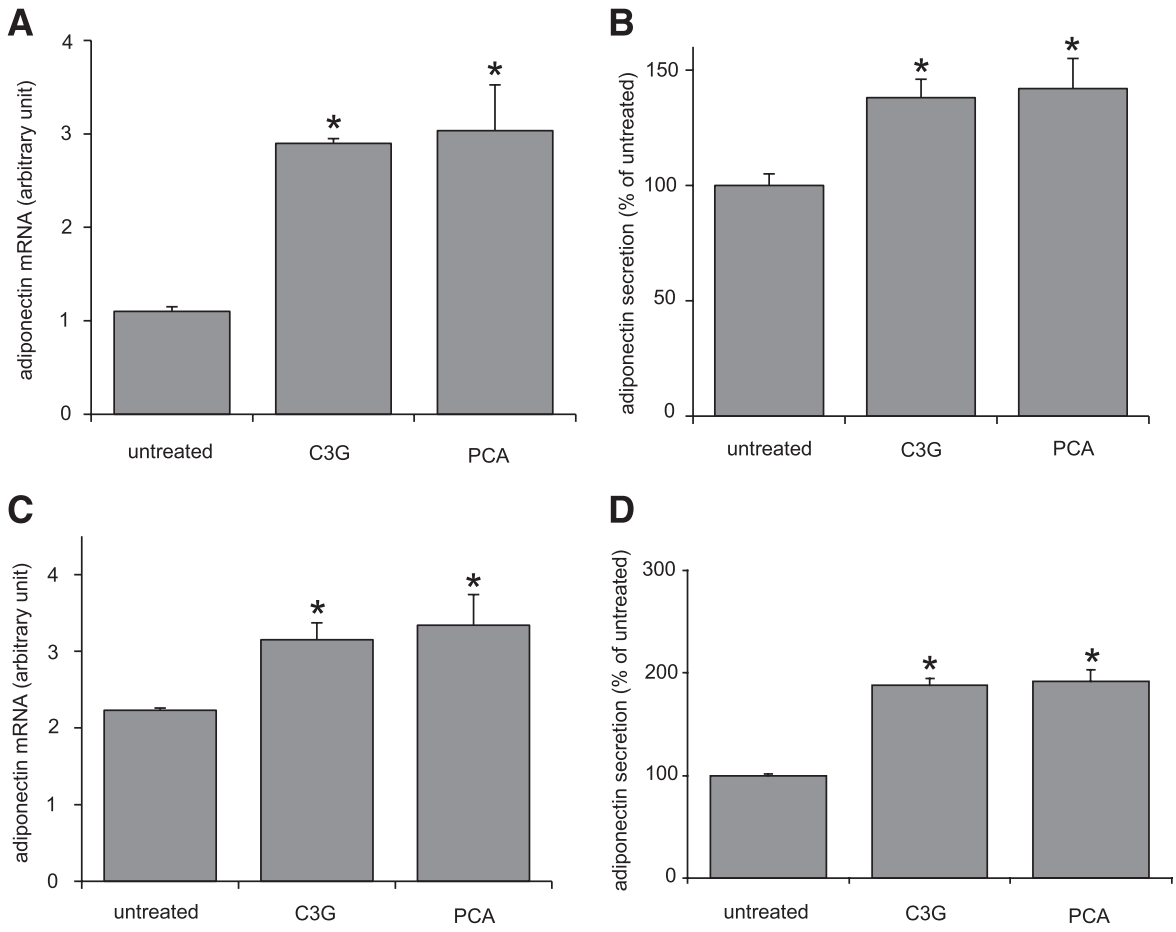

FIG. 5. Gene expression and secretion of adiponectin in polyphenol-treated adipocytes. Human and murine adipocytes, serum starved for 18 h and incubated with 50 μmol/L and 10 μmol/L C3G, respectively, or with 100 μmol/L PCA for 18 h. Adiponectin mRNA was assessed in human (*A*) and murine (*C*) adipocytes by RTq-PCR. The values indicate the expression of the target gene normalized to TBP (murine adipocytes) or GAPDH (human adipocytes) RNA by using the comparative C_T method as described in RESEARCH DESIGN AND METHODS. Adiponectin release was evaluated in the culture media of human (*B*) and murine (*D*) adipocytes by enzyme-linked immunosorbent assay kit as described in RESEARCH DESIGN AND METHODS. Data are means ± SEM of four independent experiments. *$P < 0.001$ compared with untreated cells (CTR).

that in the absence of PPARγ activity, GLUT4 translocation to the PM did not occur (Fig. 7*C*). This was a conclusive demonstration of the direct involvement of PPARγ in the regulation of the glucose transport mechanism by C3G and PCA.

DISCUSSION

In the current study, we demonstrated for the first time that C3G and its main metabolite PCA were able to completely counteract the oxLDL-induced impairment of glucose transport mechanism in human omental and murine adipocytes. More important, we provided evidence for an insulin-like activity of the polyphenols that were able to regulate the internalization of glucose. Maintenance of glucose homeostasis by strict hormonal control is of utmost importance to human physiology. Failure of this control, with defects in both insulin action and insulin secretion, can result in metabolic syndrome, a multisymptom disorder of energy homeostasis. The disturbance of glucose metabolism is often related to the increase of fat mass, especially in the abdominal area, which in turn results in inflammation, exacerbated oxidative stress at the whole body level with increased circulating oxLDL levels, and malfunction in several organs, including adipose tissue (3).

IR seems to underlie the early stages of development of metabolic syndrome and, thus, approaches to improve insulin action have been and remain key targets for potentially slowing or ultimately preventing type 2 diabetes (41,42). The potential of polyphenols in controlling glycemia is currently under intensive study. However, although indications for positive effects of ACNs on glucose homeostasis have been obtained in vitro and in animal studies (43–45), definitive conclusions in humans, especially at the molecular mechanistic level, are still lacking. Furthermore, the biological properties of ACNs have nearly always been studied in vitro by using their native form, which appears quite inappropriate because of their in vivo extensive and rapid biotransformation after ingestion (28). Indeed, the native forms of ACN are poorly present in the bloodstream (28), and they might be metabolites such as PCA, which likely reach tissues and may exert biological effects. So far, PCA has been poorly investigated because of its low concentration in foods. However, in our opinion, it deserves great nutritional interest as the main human metabolite of C3G, which is in turn the most representative dietary ACN. In this regard, our study represents a novel approach in this field of research because for the first time, the properties of both C3G and PCA were evaluated in an innovative ex vivo model of human omental adipocytes. Actually, few studies

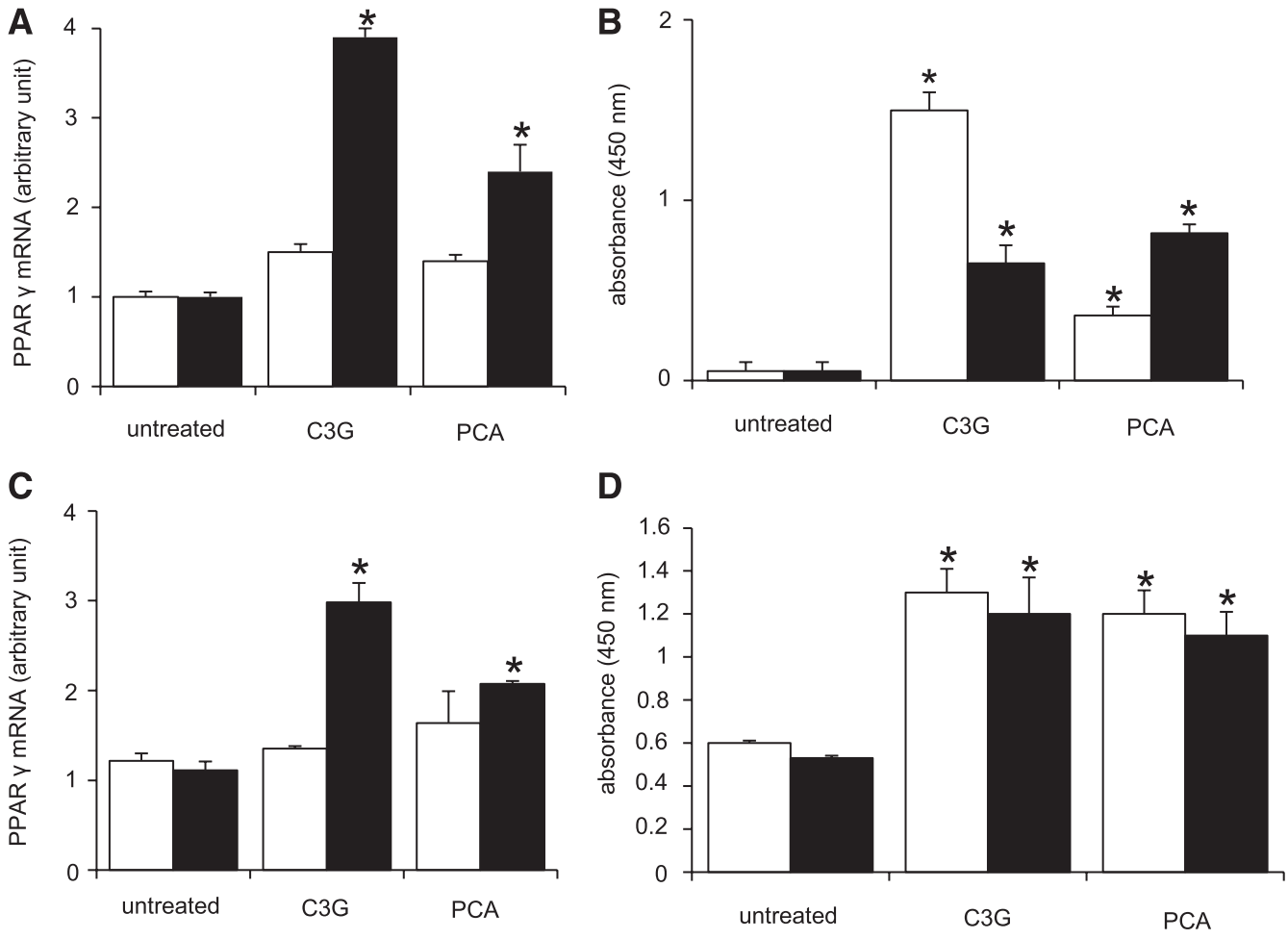

FIG. 6. Gene expression and activity of PPARγ in polyphenol-treated adipocytes. Human and 3T3-L1 adipocytes were serum starved for 18 h and incubated with 50 μmol/L and 10 μmol/L C3G, respectively, or with 100 μmol/L PCA for 2 h (white bars) or 18 h (black bars). PPARγ mRNA was assessed in human (*A*) and murine (*C*) adipocytes by RTq-PCR. The values indicate the expression of the target gene normalized to TBP (murine adipocytes) or GAPDH (human adipocytes) RNA by using the comparative C_T method as described in RESEARCH DESIGN AND METHODS. PPARγ activity was determined in nuclear extracts of human (*B*) or murine (*D*) adipocytes. Data are means ± SEM of four independent experiments. *$P < 0.001$ compared with time-matched untreated cells.

have specifically investigated ACN and phenolic acid bioavailability in humans (28,46,47). PCA was first identified as human C3G metabolite by Vitaglione et al. (28), accounting for almost 73% of ingested C3G. Details of the study show that after ingestion of 1 L of blood orange juice containing 71 mg C3G, the serum maximal concentrations of C3G and PCA were 1.9 nmol/L and 492 nmol/L, respectively. However, it should be considered that bioavailability can be affected by "chronic" exposure to the polyphenols, as that achievable by daily consumption of ACN-rich food. Furthermore, the polyphenols might concentrate at cellular level in the tissue microenvironment. From this point of view, the polyphenol concentrations tested in our experiments, although higher than that reported after ingestion of food rich in ACN, can provide significant information. Besides, from a pharmacological point of view, our study offers a new clue to the possible use of PCA as a hypoglycemic agent.

Finally, this study provides strong evidence of the molecular mechanism that enabled the two polyphenols to exert some insulin-like effects.

It is worth noting that C3G and PCA were able to positively modulate adipocyte glucose uptake largely by inducing GLUT4 translocation as demonstrated by the increase of GLUT4 level in PMs and the concomitant decrease in the storage vesicles. Adiponectin has been described as a principal player in modulating both glucose and lipid metabolism in skeletal muscle and liver by acting as an insulin sensitizer (13). Low levels of plasma adiponectin are associated with several pathological conditions that represent risk factors for cardiovascular disease (13,48). It is worthy of note that oxLDL determined a significant decrease in adiponectin in human omental adipocytes. Because adiponectin has often been considered a good target for developing therapeutic strategies (12), our finding that both C3G and PCA were able not only to counteract the adiponectin decrease induced by oxLDL but also to significantly stimulate the expression and secretion of adiponectin by adipocytes is therefore of particular interest. Furthermore, the significant increase in adiponectin mRNA expression induced by C3G was consistent with the previous data obtained by Tsuda et al. in rat (49) and human adipocytes (50).

PPARγ is the most extensively studied and clinically validated gene for therapeutic utility in type 2 diabetes (18) because it is a main metabolic regulator of peripheral organs and tissues, such as adipose tissue (17). Upregulation of PPARγ expression/activity by TZDs (16) and ACNs has been reported to improve insulin sensitivity and glucose

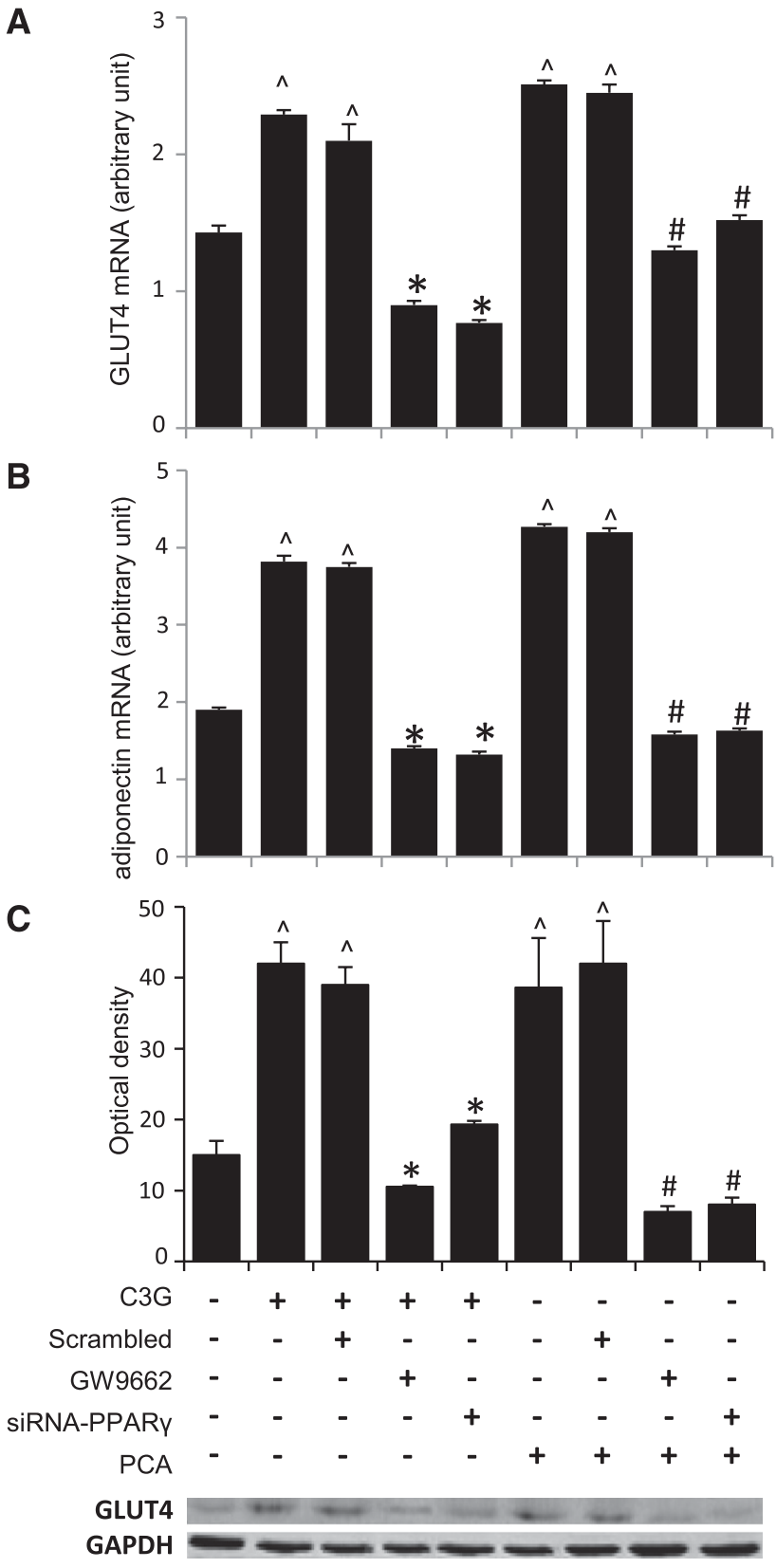

FIG. 7. Effects of PPARγ silencing on GLUT4 and adiponectin expression in polyphenol-treated 3T3-L1 adipocytes. To evaluate the effect of PPARγ inhibition on polyphenol-induced PPARγ target gene overexpressions, cells were transfected with anti-PPARγ-siRNA (100 nmol/L) for 18 h or incubated with 10 μmol/L GW9662 for 30 min before the addition of 10 μmol/L C3G or 100 μmol/L PCA. Cells transfected with scrambled siRNA were used as negative control. RTq-PCR determination of GLUT4 (*A*) and adiponectin (*B*) mRNAs were evaluated. The values indicate the expression of target genes normalized to TBP RNA by using the comparative C_T method as described in RESEARCH DESIGN AND METHODS. *C*: Immunoblotting of GLUT4 in PM fractions prepared as described in RESEARCH DESIGN AND METHODS. Representative blots are shown. Results were normalized to GAPDH protein content. Data are means ± SEM of four independent experiments. ^*P* < 0.001 compared with untreated cells, **P* < 0.001 compared with C3G-treated cells, and #*P* < 0.001 compared with PCA-treated cells.

uptake in human adipocytes (50) and in animal models of diabetes (33).

We hypothesized that oxLDL and polyphenols could affect GLUT4 and adiponectin expressions by differently modulating PPARγ. Our results allowed us to strongly support this hypothesis. Indeed, oxLDL significantly reduced the expression and activity of PPARγ in human omental adipocytes, as already reported in 3T3-L1 (10), whereas the polyphenols were able to counteract such decrease. It is interesting that in untreated cells, both C3G and PCA significantly increased the expression of *PPARγ* gene and especially its activity with respect to basal values. PPARγ activity remained higher during the entire experimental period, likely through the promotion of its binding to the oligonucleotide at its consensus binding site.

Finally, our data strongly suggest that PPARγ plays a key role in the activation of its target genes by C3G and PCA. In fact, the silencing of PPARγ overrode the increase in GLUT4 and adiponectin and the translocation of GLUT4 on the PM induced by the two polyphenols.

In conclusion, we demonstrated for the first time that C3G and PCA exert insulin-like activity in human omental adipocytes. The increase in glucose uptake was associated with enhanced GLUT4 translocation and adiponectin secretion, which were probably caused by the increased activity of PPARγ induced by the polyphenols. We also confirmed that the 3T3-L1 cell line represents a suitable model for the study of human adipocyte biology because they showed the same response to polyphenol treatment as human adipocytes.

Altogether, our data provide new evidence on the biological activity of C3G and PCA, supporting a possible use of these polyphenols as dietary bioactive compounds against the IR condition linked to the occurrence of metabolic syndrome.

ACKNOWLEDGMENTS

This study was partially supported by the Provincia Regionale di Catania through the Antioxidant Properties of Sicilian Pigmented Oranges Project.

No potential conflicts of interest relevant to this article were reported.

B.S. provided research data, contributed to discussion, and wrote the manuscript. R.V. contributed to research data and discussion. C.F. contributed to research data. M.D., C.S., and C.G. contributed to discussion and reviewed the manuscript. A.I. and G.S. provided the human biopsies and reviewed the manuscript. G.L.V. and F.G. contributed to discussion and reviewed the manuscript. R.M. contributed to discussion and wrote the manuscript.

The authors wish to thank Professor Gabriella Girelli, Director of Centro Trasfusionale, University of Rome La Sapienza, for providing human plasma and Antonietta Pucciarmati, Department of Veterinary Public Health and Food Safety, Italian National Institute of Health, for technical assistance.

REFERENCES

1. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27:1047–1053
2. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet 2005; 365:1415–1428
3. Holvoet P, Lee DH, Steffes M, Gross M, Jacobs DR Jr. Association between circulating oxidized low-density lipoprotein and incidence of the metabolic syndrome. JAMA 2008;299:2287–2293
4. Tsuzura S, Ikeda Y, Suehiro T, et al. Correlation of plasma oxidized low-density lipoprotein levels to vascular complications and human serum paraoxonase in patients with type 2 diabetes. Metabolism 2004;53:297–302
5. Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. Circulation 1998;98:1487–1494
6. Weinbrenner T, Schröder H, Escurriol V, et al. Circulating oxidized LDL is associated with increased waist circumference independent of body mass index in men and women. Am J Clin Nutr 2006;83:30–35; quiz 181–182
7. Mazière C, Morlière P, Santus R, et al. Inhibition of insulin signaling by oxidized low density lipoprotein. Protective effect of the antioxidant vitamin E. Atherosclerosis 2004;175:23–30
8. Masella R, Varì R, D'Archivio M, et al. Oxidised LDL modulate adipogenesis in 3T3-L1 preadipocytes by affecting the balance between cell proliferation and differentiation. FEBS Lett 2006;580:2421–2429
9. D'Archivio M, Scazzocchio B, Filesi C, et al. Oxidised LDL up-regulate CD36 expression by the Nrf2 pathway in 3T3-L1 preadipocytes. FEBS Lett 2008;582:2291–2298
10. Scazzocchio B, Varì R, D'Archivio M, et al. Oxidized LDL impair adipocyte response to insulin by activating serine/threonine kinases. J Lipid Res 2009;50:832–845
11. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ. Diabetes 2006;55:1537–1545
12. Yamauchi T, Kamon J, Waki H, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med 2001;7:941–946
13. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol Metab 2002;13:84–89
14. Lindsay RS, Funahashi T, Hanson RL, et al. Adiponectin and development of type 2 diabetes in the Pima Indian population. Lancet 2002;360:57–58
15. Iwaki M, Matsuda M, Maeda N, et al. Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 2003;52:1655–1663
16. Spiegelman BM. PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. Diabetes 1998;47:507–514
17. Badman MK, Flier JS. The adipocyte as an active participant in energy balance and metabolism. Gastroenterology 2007;132:2103–2115
18. Miyazaki Y, Mahankali A, Wajcberg E, Bajaj M, Mandarino LJ, DeFronzo RA. Effect of pioglitazone on circulating adipocytokine levels and insulin sensitivity in type 2 diabetic patients. J Clin Endocrinol Metab 2004;89:4312–4319
19. Biesalski HK. Diabetes preventive components in the Mediterranean diet. Eur J Nutr 2004;43(Suppl. 1):I/26–30
20. Lindström J, Ilanne-Parikka P, Peltonen M, et al.; Finnish Diabetes Prevention Study Group. Sustained reduction in the incidence of type 2 diabetes by lifestyle intervention: follow-up of the Finnish Diabetes Prevention Study. Lancet 2006;368:1673–1679
21. Tuomilehto J, Lindström J, Eriksson JG, et al.; Finnish Diabetes Prevention Study Group. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 2001;344: 1343–1350
22. Saura-Calixto F, Goñi I. Definition of the Mediterranean diet based on bioactive compounds. Crit Rev Food Sci Nutr 2009;49:145–152
23. Hertog MG, Hollman PC, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. Nutr Cancer 1993;20:21–29
24. Kay CD, Mazza G, Holub BJ, Wang J. Anthocyanin metabolites in human urine and serum. Br J Nutr 2004;91:933–942
25. Kay CD, Mazza GJ, Holub BJ. Anthocyanins exist in the circulation primarily as metabolites in adult men. J Nutr 2005;135:2582–2588
26. Mazza G, Kay CD. Bioactivity, absorption, and metabolism of anthocyanins. In *Recent Advances in Polyphenols Research*. Vol. 1. Lattanzio V, Daayf F, Eds. Oxford, Blackwell, 2008, p. 228–262
27. Tsuda T, Horio F, Osawa T. Absorption and metabolism of cyanidin 3-*O*-beta-D-glucoside in rats. FEBS Lett 1999;449:179–182
28. Vitaglione P, Donnarumma G, Napolitano A, et al. Protocatechuic acid is the major human metabolite of cyanidin-glucosides. J Nutr 2007;137:2043–2048
29. Cao G, Muccitelli HU, Sánchez-Moreno C, Prior RL. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. Am J Clin Nutr 2001;73:920–926
30. Galvano F, La Fauci L, Lazzarino G, et al. Cyanidins: metabolism and biological properties. J Nutr Biochem 2004;15:2–11
31. Serraino I, Dugo L, Dugo P, et al. Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. Life Sci 2003;73:1097–1114
32. Tsuda T, Horio F, Uchida K, Aoki H, Osawa T. Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. J Nutr 2003;133:2125–2130

33. Seymour EM, Lewis SK, Urcuyo-Llanes DE, et al. Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. J Med Food 2009;12:935–942
34. Xia M, Hou M, Zhu H, et al. Anthocyanins induce cholesterol efflux from mouse peritoneal macrophages: the role of the peroxisome proliferator-activated receptor gamma-liver X receptor alpha-ABCA1 pathway. J Biol Chem 2005;280:36792–36801
35. Masella R, Varì R, D'Archivio M, et al. Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. J Nutr 2004;134:785–791
36. Kristensen K, Pedersen SB, Richelsen B. Regulation of leptin by steroid hormones in rat adipose tissue. Biochem Biophys Res Commun 1999;259:624–630
37. Tanti JF, Cormont M, Grémeaux T, Le Marchand-Brustel Y. Assays of glucose entry, glucose transporter amount, and translocation. Methods Mol Biol 2001;155:157–165
38. Piper RC, Hess LJ, James DE. Differential sorting of two glucose transporters expressed in insulin-sensitive cells. Am J Physiol 1991;260:C570–C580
39. McKeel DW, Jarett L. Preparation and characterization of a plasma membrane fraction from isolated fat cells. J Cell Biol 1970;44:417–432
40. Arai Y, Kojima T, Takayama M, Hirose N. The metabolic syndrome, IGF-1, and insulin action. Mol Cell Endocrinol 2009;299:124–128
41. Cusi K, Maezono K, Osman A, et al. Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. J Clin Invest 2000;105:311–320
42. Folli F, Saad MJ, Backer JM, Kahn CR. Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. J Clin Invest 1993;92:1787–1794
43. Adisakwattana S, Charoenlertkul P, Yibchok-Anun S. alpha-Glucosidase inhibitory activity of cyanidin-3-galactoside and synergistic effect with acarbose. J Enzyme Inhib Med Chem 2009;24:65–69
44. Takikawa M, Inoue S, Horio F, Tsuda T. Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. J Nutr 2010; 140: 527–533
45. Nizamutdinova IT, Jin YC, Chung JI, et al. The anti-diabetic effect of anthocyanins in streptozotocin-induced diabetic rats through glucose transporter 4 regulation and prevention of insulin resistance and pancreatic apoptosis. Mol Nutr Food Res 2009;53:1419–1429
46. Mazza G, Kay CD, Cottrell T, Holub BJ. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J Agric Food Chem 2002;50:7731–7737
47. Koli R, Erlund I, Jula A, Marniemi J, Mattila P, Alfthan G. Bioavailability of various polyphenols from a diet containing moderate amounts of berries. J Agric Food Chem 2010;58:3927–3932
48. Liang KW, Lee WJ, Lee WL, Ting CT, Sheu WH. Decreased ratio of high-molecular-weight to total adiponectin is associated with angiographic coronary atherosclerosis severity but not restenosis. Clin Chim Acta 2009; 405:114–118
49. Tsuda T, Ueno Y, Aoki H, et al. Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes. Biochem Biophys Res Commun 2004;316:149–157
50. Tsuda T, Ueno Y, Yoshikawa T, Kojo H, Osawa T. Microarray profiling of gene expression in human adipocytes in response to anthocyanins. Biochem Pharmacol 2006;71:1184–1197