Vitamin C inhibits leptin secretion and some glucose/lipid metabolic pathways in primary rat adipocytes

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Abstract

Antioxidant-based treatments are emerging as an interesting approach to possibly counteract obesity fat accumulation complications, since this is accompanied by an increased systemic oxidative stress. The aim of this study was to analyze specific metabolic effects of vitamin C (VC) on epididymal primary rat adipocytes. Cells were isolated and incubated for 72 h in culture medium, in the absence or presence of 1·6 nM insulin, within a range of VC concentrations (5–1000 μM). Glucose- and lipid-related variables as well as the secretion/expression patterns of several obesity-related genes were assessed. It was observed that VC dose dependently inhibited glucose uptake and lactate production, and also reduced glycerol release in both control and insulin-treated cells. Also, VC caused a dramatic concentration-dependent fall in leptin secretion especially in insulin-stimulated cells. In addition, VC (200 μM) induced *Cdkn1a* and *Casp8*, partially inhibited *Irs3*, and together with insulin drastically reduced *Gpdh* (listed as *Gpd1* in the MGI database) gene expressions. Finally, VC and insulin down-regulatory effects were observed on extracellular and intracellular reactive oxygen species production respectively. In summary, this experimental assay describes a specific effect of VC in isolated rat adipocytes on glucose and fat metabolism, and on the secretion/expression of important obesity-related proteins.

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Introduction

Worldwide, obesity is emerging as one of the major health threats (Powers *et al.* 2007). Indeed, it is well known that an excessive body fat accumulation, which defines this disease, could lead to several associated clinical manifestations such as type 2 diabetes, metabolic syndrome features, cardiovascular events, and arthritis (Bray 2004). These effects are related to a white adipose tissue (WAT) overgrowth and also to an impaired production and secretion of endogenous products by the enlarged adipocytes or the macrophages coexisting in the tissue (Bray 2004), which often have pro-inflammatory properties (Fantuzzi 2005). Actually, it has been reported that several inflammatory products derived from this tissue, such as TNF-α, IL6, MCP-1 (listed as CCL2 in the MGI database), and iNOS (NOS2), correlate with increased body adiposity (Ferrante 2007). In addition, it has been reported that inflammatory-related pathways are activated in obesity and insulin resistance states (Yuan *et al.* 2001, Cai *et al.* 2005). Besides the secretion of these pro-inflammatory cytokines, the adipose tissue produces other substances that also have important local and systemic effects (Fantuzzi 2005). Among these molecules, leptin, which is related to the control of food intake and energy expenditure (Zhang *et al.* 1994), and adiponectin, which is related to significant insulin sensitivity improvements (Kim *et al.* 2007), as well as visfatin, which has been reported to have controversial associations with insulin resistance and obesity, are metabolically relevant (Fukuhara *et al.* 2005, Haider *et al.* 2006, Varma *et al.* 2007).

On the other hand, in obesity, a mitochondrial dysfunction and reactive oxygen species (ROS) overproduction as well as an association between oxidative stress and insulin resistance have been observed (Martinez 2006). In this sense, in obese patients (Vincent & Taylor 2006) and in overweight animal models (Furukawa *et al.* 2004, Milagro *et al.* 2006), an elevated oxidative stress has been documented. Furthermore, depletion of the antioxidant defenses has also been described in obesity (Mutlu-Turkoglu *et al.* 2003). This oxidative stress unbalanced status is related to chronic inflammation and hyperleptinemia (Vincent & Taylor 2006). Thus, induced monocyte migration to the adipose tissue and high secretion levels of some WAT-secreted adipokines are directly implicated in the obesity-associated ROS overproduction. This fact links the pathogenic secretion pattern of WAT with an enhanced oxidative stress status (Moreno-Aliaga *et al.* 2005).

The abilities of antioxidant vitamins include free radical depletion, nitric oxide synthesis or release control, ROS production inhibition, and the induction of antioxidant enzymes (Flora 2007). In this context, it

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has been observed that the dietary antioxidant ascorbic acid or vitamin C (VC) is negatively associated with the presence of several conditions such as hypertension, gallbladder disease, stroke, cancers, and atherosclerosis (Bsoul & Terezhalmy 2004), and also with the occurrence of obesity (Canoy *et al.* 2005, Johnston 2005). Among the beneficial effects of ascorbic acid on obesity-related mechanism, the modulation of adipocyte lipolysis (Misekova *et al.* 1993, Hasegawa *et al.* 2002, Senen *et al.* 2002, Garcia-Diaz *et al.* 2009), glucocorticoid release from adrenal glands (Doulas *et al.* 1987), hyperglycemia improvement and glycosylation decrease in obese diabetic mice (Abdel-Wahab *et al.* 2002), and an inhibition of the inflammatory response (Carcamo *et al.* 2002) have been described.

Taking all these findings into account, in this study, an attempt to identify the potential relationships between gene expression and secretion of WAT adipokines as affected by VC treatment in rat primary culture adipocytes was made. Moreover, this work aimed to analyze whether this antioxidant treatment has implications concerning the glucose and lipid metabolism and in the oxidative stress status of rat fat cells.

Materials and methods

Materials

DMEM, 100× minimal essential medium (MEM) non-essential amino acids, penicillin/streptomycin, heat-inactivated fetal bovine serum (FBS), 10× MEM, nystatin, Trizol Reagent, and M-MLV reverse transcriptase were obtained from Invitrogen. BSA, HEPES, insulin, and 2′,7′-dichlorofluorescein (DCFH) were all obtained from Sigma–Aldrich Company. Collagen (Purecol) was purchased from Nutacon (Leimuiden, The Netherlands). Type I collagenase was supplied by Worthington Biochemical Corporation (Lakewood, NJ, USA), and VC by Panreac Quimica (Barcelona, Spain). Glycerol-3-phosphate dehydrogenase (GPDH, listed as GPD1 in the MGI database), glycerol kinase (GK, GYK), ATP, and NAD were obtained from Roche Diagnostics.

Animals

Eight-week-old male Wistar rats (±250 g weight) that were supplied by the Center for Applied Pharmacobiology Research (CIFA, Pamplona, Spain) were housed in temperature-controlled rooms at 21–23 °C under a 12-h light cycle (lights were switched off at 2000 h). The rats ($n=7$) were killed, and their epididymal WAT was removed for adipocyte isolation. All the procedures were performed according to the European National and Institutional Guidelines of the Animal Care and Use Committee at the University of Navarra.

Adipocyte isolation and culture

Primary cultures were performed according to protocols described elsewhere (Lorente-Cebrian *et al.* 2009). Epididymal WAT (3–4 g) of each rat ($n=7$) was minced using scissors in HEPES–phosphate buffer (pH 7·4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2·2 mM $CaCl_2{\cdot}2H_2O$, 1·25 mM $MgSO_4{\cdot}7H_2O$, 0·45 mM KH_2PO_4, 2·17 mM Na_2HPO_4, and 10 mM HEPES). WAT fragments were digested in the same buffer with type I collagenase (1·25 mg/ml per 0·5 g tissue) at 37 °C with gentle shaking for 30 min. The resulting cell suspension was diluted in the buffer, and then the adipocytes were isolated from the undigested tissue by filtration through a 400-μm nylon mesh and washed three times with alternate centrifugations at 500 ***g***. for 6 min. Isolated adipocytes were then resuspended in DMEM supplemented with 1% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, 100 U/ml nystatin, and 1× MEM non-essential amino acids, followed by incubation for 40 min at 37 °C in 5% CO_2. The resulting isolated adipocytes (150 μl of a 2:1 mix of packed cells/culture medium) were then plated on 500 μl of a collagen matrix (pH 7, 7 parts collagen: 1 part 10× MEM) in six-well culture plates. After 40–50 min of incubation at 37 °C in 5% CO_2, the culture medium containing 0 or 1·6 nM insulin and different concentrations (0, 5, 10, 50, 200, and 1000 μM) of VC was added. Cells were maintained in an incubator at 37 °C in 5% CO_2 up to 72 h. VC was freshly added every day. Aliquots of the culture medium were collected at the end of the experimental trial, and conserved at −80 °C for further assays.

Culture medium determinations

All the assays were performed on 72-h treatment samples. Cell viability ($n=5$) was measured using the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit according to the manufacturer's indications (Cayman Chemical Company, Ann Arbor, MI, USA). LDH activity (μU/ml) in the culture medium was used as an indicator of cell membrane integrity, and thus as a measurement of cell necrosis/apoptosis. Cell viability was assayed at the end of the experimental period in control and insulin/VC (200 μM)-treated cells. Glucose and lactate concentrations in the medium were measured using the HK-CP kit obtained from ABX Diagnostic (Montpellier, France) and the L-lactate kit obtained from Randox Laboratories (Crumlin, UK) respectively using an automatized COBAS MIRA equipment (Roche). The amount of released carbon as lactate per amount of carbon taken up as glucose over this time (glucose to lactate %) was calculated as (lactate concentration/glucose concentration)×100. Glycerol concentration in the medium, as a measure

of the adipocyte lipolytic response, was determined by a colorimetric method (Campion & Martinez 2004). Briefly, culture medium of each sample was incubated with 25 μg/ml GPDH and 250 mU/ml GK in the presence of 43·6 mM $MgCl_2$, 200 mM glycine, 5·2% hydrazine, 1·24 mM ATP, and 573 μM NAD for 40 min. Finally, sample absorbance was measured at 340 nm. Leptin secretion was determined using the Rat Leptin ELISA kit obtained from Linco Research (St Charles, MO, USA), adiponectin using the Mouse/Rat Adiponectin ELISA kit obtained from B-Bridge International (Mountain View, CA, USA), and visfatin using the Visfatin EIA kit obtained from ALPCO Diagnostics (Salem, NH, USA).

Gene expression assays

Total RNA was isolated from isolated epididymal adipocytes incubated with or without 1·6 nM insulin and treated or not treated with 200 μM VC using Trizol (Invitrogen) according to the manufacturer's protocol. Purified total RNA from adipocytes was then treated with DNAse (DNAfree kit; Ambion Inc., Austin, TX, USA), and used to generate cDNA with M-MLV reverse transcriptase (Invitrogen). Relative real-time PCR was performed on an ABI PRISM 7000 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Taqman probes for rat leptin, adiponectin, visfatin, *Gpdh*, insulin receptor substrate 3 (*Irs3*), cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), caspase 8 (*Casp8*), *Gapdh*, and *18S* rRNA were also supplied by Applied Biosystems. All the expression levels of the target genes studied were normalized by the two selected internal controls, *Gapdh* and *18S*, applying the GeNorm software (http://medgen.ugent.be/~jvdesomp/genorm/; Vandesompele *et al.* 2002).

ROS determination

DCFH was used for intracellular and extracellular ROS concentration measurements with a protocol described elsewhere (Fu *et al.* 2008). Once inside the cell, this molecule is cleaved by endogenous esterases and can no longer pass out of the cell membrane. The de-esterified product becomes a fluorescent compound after oxidation by ROS (Brandt & Keston 1965). Briefly, cells were incubated with 10 μM DCFH for 40 min at 37 °C in 5% CO_2, frozen for at least 1 h at −80 °C, and then lysed using 1000 μl lysis buffer (150 mM NaCl, 0·1% Triton, and 10 mM Tris). Finally, 200 μl of each lysate were plated on a 96-well black plate (Labsystems, Barcelona, Spain). For extracellular ROS determinations, 300 μl of culture medium of each sample (from 1000 μl of total incubation volume) after the 72-h treatment were also incubated with 10 μM DCFH for 40 min at 37 °C in 5% CO_2, frozen for at least 1 h at −80 °C, and then 200 μl from this incubation mix were loaded on a 96-well black plate. Finally, fluorescence intensity was measured using a POLARstar spectrofluorometer plate reader (BMG Labtechnologies, Offenburg, Germany) at an excitation of 485 nm wavelength and at an emission of 530 nm wavelength.

Statistical analysis

Due to reduced sample size in some of the experimental groups, non-parametric analyses were performed. Thus, Kruskal–Wallis test was used followed by Mann–Whitney *U* tests for further comparisons. Results are shown using box and whisker plots expressing median and interquartile range. For ROS determinations, parametric analyses (two-way ANOVA) were performed due to their normal distribution, which was determined by the Shapiro–Wilk test, and equal sample number among experimental groups. These results were expressed by mean ± S.E.M. A probability of $P<0·05$ was set for determining significant differences. All the analyses were performed using the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA) and the SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Glucose and lipid metabolism

Initially, LDH cytotoxicity assay presented no statistical differences among the experimental groups, indicating that neither insulin nor VC induced necrosis/apoptosis in rat epididymal adipocytes after the 72-h treatment at the assayed doses (data not shown). Adipocytes treated with VC exhibited lower glucose uptake than controls (Fig. 1A). On the other hand, insulin induced a marginally significant ($P=0·053$) inducing effect on the glucose uptake of the adipocytes. Specifically, among the adipocytes without insulin treatment, 200 μM VC induced a significant decrease in the glucose uptake compared with the controls ($P<0·05$). Moreover, in adipocytes that were incubated with insulin, the VC treatment induced a substantial and concentration-dependent decrease in the glucose uptake, with this reduction being statistically significant with concentrations over 50 μM versus adipocytes treated with insulin alone ($P<0·05$ for 50, and $P<0·01$ for 200 and 1000 μM).

On the other hand, the lactate production of the adipocytes was not significantly affected by the insulin treatment (Fig. 1B), but increasing concentrations of VC induced a lower production of this anaerobic metabolite, which was statistically significant over

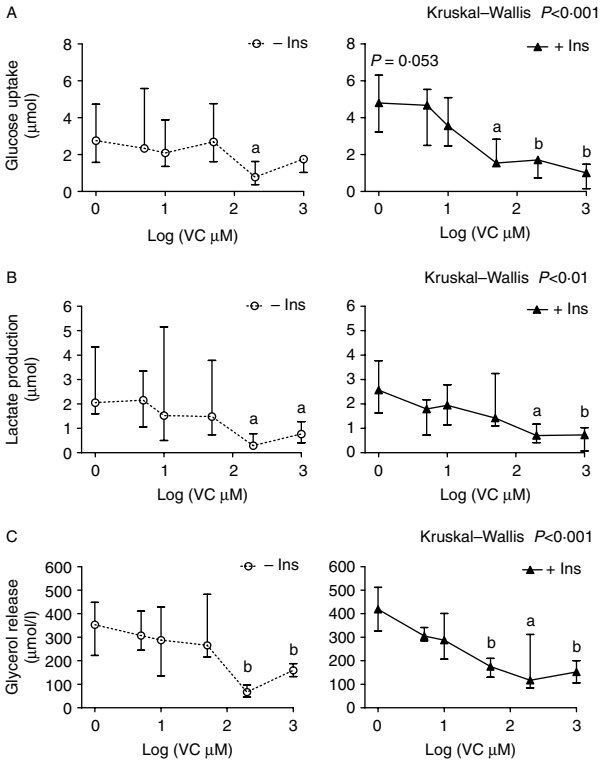

Figure 1 Effects of vitamin C (log (5–1000 μM)) in the absence (white circles) or presence (black triangles) of 1·6 nM insulin on (A) glucose uptake, (B) lactate production, and (C) glycerol release in isolated rat adipocytes over 72 h in culture. Data ($n \geq 4$, median and interquartile range). $^{a}P<0{\cdot}05$ and $^{b}P<0{\cdot}01$ versus control cells (with or without insulin treatment). $P=0{\cdot}053$, control versus control + insulin cells. VC, vitamin C; Ins, insulin.

200 μM VC for both untreated ($P<0{\cdot}05$ for 200 and 1000 μM versus control cells) and insulin-treated cells ($P<0{\cdot}05$ for 200 and $P<0{\cdot}01$ for 1000 μM versus cells treated with insulin alone). The percentage of glucose converted to lactate was not affected by either VC or insulin treatment (data not shown).

Finally, the amount of glycerol released (Fig. 1C), as a lipolysis marker, showed a VC-reduced pattern that was nearly the same as that shown by lactate that was produced, presenting significant differences in non-insulin-treated cells at VC concentrations of 200 and 1000 μM ($P<0{\cdot}01$ for both versus control cells) and in insulin-treated cells at 50 ($P<0{\cdot}01$), 200 ($P<0{\cdot}05$), and 1000 μM ($P<0{\cdot}01$) compared with the cells treated with insulin alone.

Adipokine secretion

The role of VC in the secretion of different adipokines by the adipocytes was also evaluated (Fig. 2). In first instance, leptin secretion into the culture medium after 72 h was detected (Fig. 2A), and significant effects of insulin and VC treatment on its secretion were observed ($P<0{\cdot}001$). Increasing concentrations of VC induced a dose-dependent inhibition on both basal ($P<0{\cdot}05$ for 200 and 1000 μM versus control cells) and insulin-stimulated leptin secretion ($P<0{\cdot}01$ for 200 and 1000 μM versus insulin-treated cells). Insulin treatment effectively induced leptin secretion with respect to the control cells ($P<0{\cdot}05$). In contrast, in the case of adiponectin, despite the fact that a significant global effect was detected ($P<0{\cdot}05$), no differences among experimental groups were observed (Fig. 2B). Finally, concerning visfatin secretion into the culture medium, no significant differences were detected in the VC-treated cells (Fig. 2C).

Furthermore, the leptin secretion into the culture medium presented positive and significant correlations with the glucose uptake of the VC-treated adipocytes in both untreated ($r=0{\cdot}566$, $P<0{\cdot}01$) and insulin-stimulated cells ($r=0{\cdot}797$, $P<0{\cdot}001$; Fig. 3A and B respectively), and also with glycerol release in both untreated ($r=0{\cdot}787$, $P<0{\cdot}001$) and insulin-stimulated cells ($r=0{\cdot}791$, $P<0{\cdot}001$; Fig. 3C and D respectively).

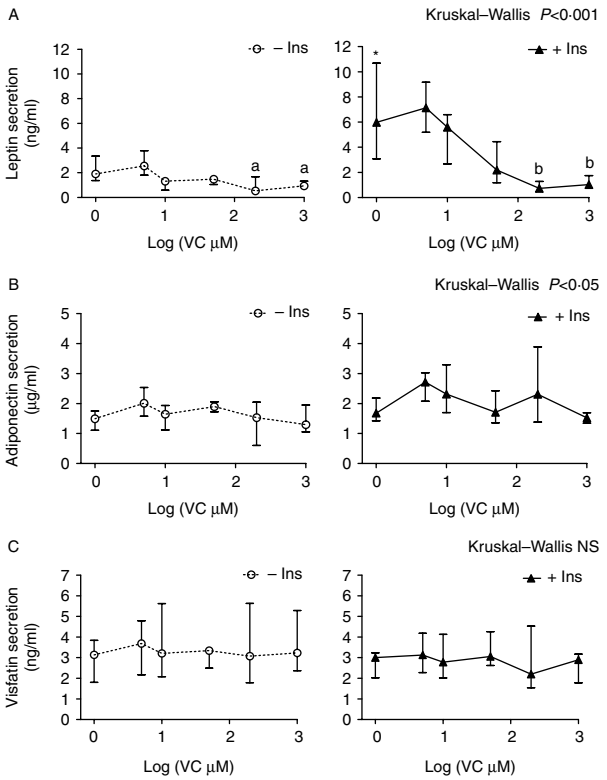

Figure 2 Effects of vitamin C (log (5–1000 μM)) in the absence (white circles) or presence (black triangles) of 1·6 nM insulin on (A) leptin, (B) adiponectin, and (C) visfatin secretion in isolated rat adipocytes over 72 h in culture. Data ($n \geq 4$, median and interquartile range). $^{a}P<0{\cdot}05$ and $^{b}P<0{\cdot}01$ versus control cells (with or without insulin treatment). $^{*}P<0{\cdot}05$, control versus control + insulin cells. VC, vitamin C; Ins, insulin; NS, not significant.

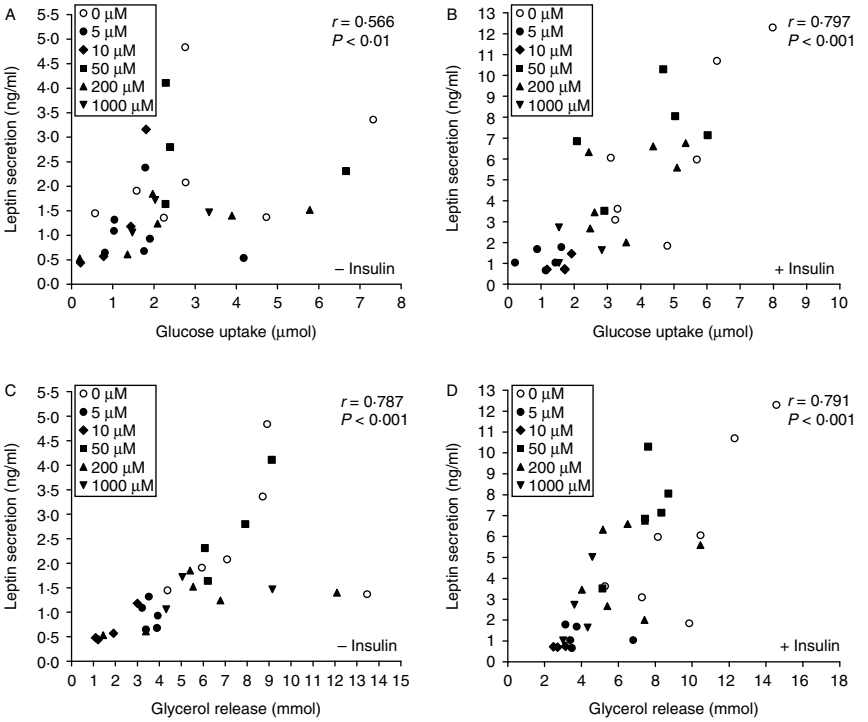

Figure 3 Analysis of the association between leptin secretion and (A) basal glucose uptake, (B) insulin-stimulated glucose uptake, and the amount of glycerol released in the (C) absence and (D) presence of insulin. All the VC concentrations (0–1000 μM) are included. Graph symbols represent: open circles, 0 μM; closed circles, 5 μM; diamonds, 10 μM; squares, 50 μM; triangles, 200 μM; and inverted triangles, 1000 μM VC concentrations. *r*, Spearman's correlation coefficient.

mRNA expression of specific adipokines and obesity-related genes

For these analyses, 200 μM VC and control samples were chosen for total mRNA extraction followed by gene expression of the three selected adipokines. This concentration was considered physiological according to Levine *et al.* (1996). Leptin mRNA expression presented no significant differences in insulin- or VC-treated cells (Fig. 4A), but a correlation with the glucose uptake in insulin-stimulated cells ($r=0{\cdot}810$, $P<0{\cdot}05$) was detected. Regarding the other assayed adipokines, no statistically significant differences were observed in adiponectin mRNA expression (Fig. 4B), but a slightly significant effect of the treatments on visfatin gene expression was detected (Fig. 4C), probably induced by the elevated mRNA expression found in VC+insulin-treated adipocytes.

Gene expression of several other genes involved in obesity-related mechanism was also examined. Important and significant modifications were observed in the *Gpdh* mRNA expression ($P<0{\cdot}05$), induced mainly by a drastic inhibition observed in the VC+ insulin-treated adipocytes (Fig. 5A). The *Irs3* gene expression (Fig. 5B) was not significantly modified by insulin or VC treatment. Finally, the *Cdkn1a* and *Casp8* mRNA expressions presented significant modifications ($P<0{\cdot}05$ for both), mainly due to the observed inducing effects of the VC treatment (Fig. 5D and E).

ROS production

Intracellular and extracellular ROS concentrations were measured after the 72-h treatment. Marked effects ($P<0{\cdot}05$) were detected on the intracellular ROS concentration of the isolated adipocytes, mainly due to the inhibition observed in insulin-treated cells (Fig. 6A). On the other hand, the extracellular ROS levels also presented reductions ($P<0{\cdot}05$) induced by VC incubation in both insulin-untreated and insulin-treated cells (Fig. 6B).

Discussion

Available data show inconclusive evidences regarding possible relationships between antioxidant treatments and obesity prevention or treatment mechanisms (Valdecantos *et al.* 2009). Research with antioxidants,

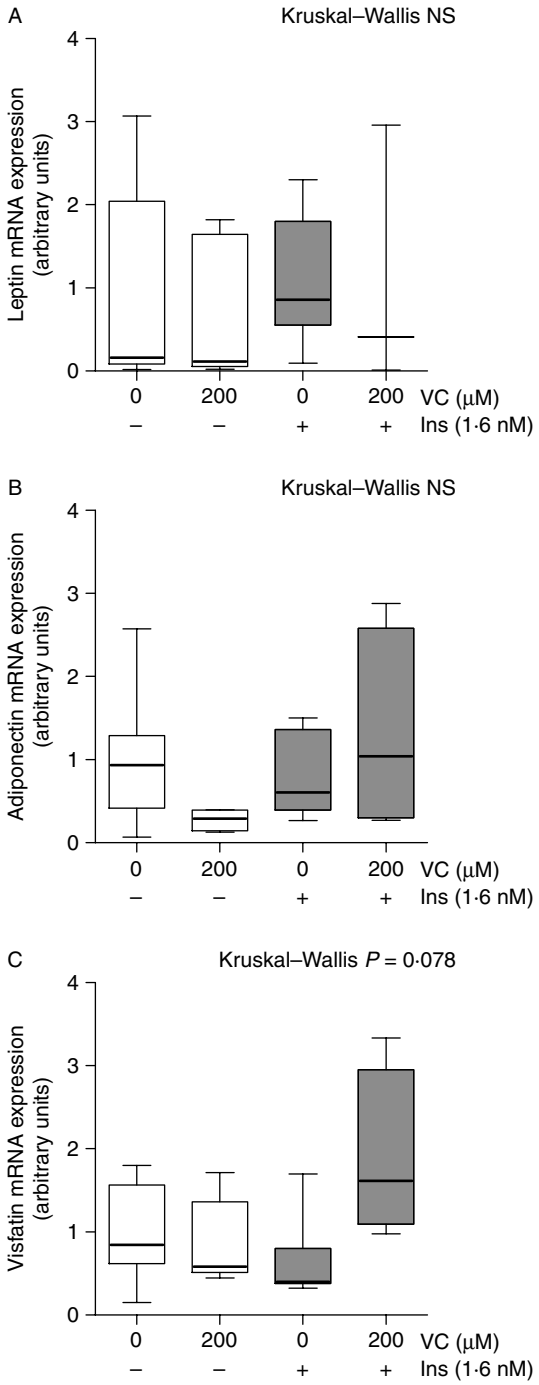

Figure 4 Effects of vitamin C (200 μM) in the absence (white boxes) or presence (grey boxes) of 1·6 nM insulin on (A) leptin, (B) adiponectin, and (C) visfatin mRNA expressions in isolated rat adipocytes over 72 h in culture. Data ($n \geq 4$, median and interquartile range). VC, vitamin C; Ins, insulin; NS, not significant.

which are labile products, could have discrepant outcomes concerning metabolic changes due to experimental conditions. However, in humans, inverse associations between VC plasma levels and body mass index (Johnston 2005), waist–hip ratio (Canoy *et al.* 2005), and cardiovascular disease risk were observed (Kurl *et al.* 2002). Moreover, a VC supplementation induced some beneficial effects on glucose and lipid metabolism in type 2 diabetic patients (Paolisso *et al.* 1995).

In animal studies, it was observed that VC supplementation induced insulin resistance improvement in hyperglycemic *ob/ob* mice (Abdel-Wahab *et al.* 2002), and decreased body weight gain in guinea pigs (Sorensen *et al.* 1974). Studies by our research group have found a high-fat diet-induced adiposity reduction by VC supplementation in rats (Campion *et al.* 2006, Garcia-Diaz *et al.* 2007) and modifications in adipocyte catecholamine-induced lipolysis (Garcia-Diaz *et al.* 2009). *In vitro* studies have reported controversial VC effects on adipocyte differentiation (Ono *et al.* 1990, Krieger-Brauer & Kather 1995, Galinier *et al.* 2006).

In the present study, *in vitro* effects of VC incubation on epididymal rat adipocyte metabolism and secretory functions were evaluated. The glucose uptake inhibition observed in adipocytes without insulin treatment, and especially in adipocytes under insulin treatment, could be partially explained by the fact that dehydroascorbic acid (DHA; the oxidized form of VC that is transported inside cells) possibly competes with glucose for GLUT1 (SLC2A1) and GLUT3 (SLC2A3; Arrigoni & De Tullio 2002), and for GLUT4 (SLC2A4; Vera *et al.* 1993) transporters respectively. However, it was reported previously that in primary cultures of rat hippocampal neurons, VC accumulation inhibited the glucose transport inside the cytoplasm independently of this competition (Patel *et al.* 2001). The lactate production inhibition by VC is in agreement with a study that described a lactic acid plasma concentration decrease in rats with streptozotocin-induced diabetes by a VC/E treatment (Ruperez *et al.* 2008). On the other hand, no statistical effects of VC were observed on the percentage of glucose carbon released as lactate, suggesting that VC did not participate in the aerobic/anaerobic metabolism of rat adipocytes over a 72-h culture treatment. Finally, the results showed no glycerol modulation release by insulin. Although the antilipolytic properties of this molecule are well known (Elks & Manganiello 1985), the present data are in agreement with the data reported by Perez-Matute *et al.* (2007), indicating the lack of lipolysis modulation by insulin in this experimental model. Despite this, the lower glycerol release induced by VC may indicate inhibited fat utilization in both insulin-treated and non-treated adipocytes. It has been described that rats fed with high-fat diet with VC supplementation presented a decreased isoproterenol-induced lipolysis compared with the rats fed with high-fat diet alone (Garcia-Diaz *et al.* 2009).

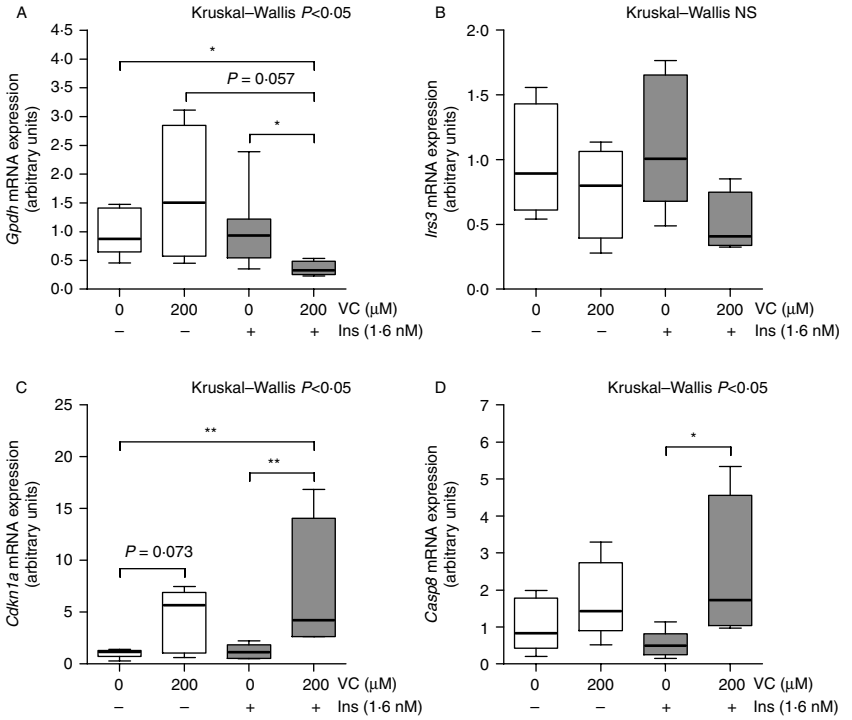

Figure 5 Effects of vitamin C (200 μM) in the absence (white boxes) or presence (grey boxes) of 1·6 nM insulin on mRNA expression of several genes involved in (A) adipocyte differentiation (*Gpdh*), (B) insulin signaling (*Irs3*), (C) cell cycle (*Cdkn1a*), and (D) apoptosis (*Casp8*) in isolated rat adipocytes over 72 h in culture. Data ($n \geq 4$, median and interquartile range). VC, vitamin C; Ins, insulin; *$P<0·05$; **$P<0·01$; NS, not significant.

Regarding the secretion and expression of some adipokines, this study has demonstrated an important inhibitory effect of VC on leptin secretion, which is in agreement with the previously reported reducing effects on leptin circulatory levels of a diet with VC supplementation given to high-fat diet-fed rats for 56 days (Garcia-Diaz *et al.* 2007). In that work, the observed reduction in leptin secretion was accompanied by a decrease in body weight and adiposity. However, the results of the present study suggest that the leptin secretion inhibition was mainly due to specific effects of the VC treatment over the adipocytes and not due to mass-reducing effects on the leptin secretor tissue. In another study, it was reported that the leptin expression and secretion in cultured rat adipocytes were decreased by glucose uptake inhibition (Mueller *et al.* 1998). In the present study, the positive correlation between leptin secretion and glucose uptake observed in both insulin-treated and non-treated cells suggests at first glance that the glucose uptake inhibition by VC itself could be determining the leptin secretion. In this context, previous studies indicated that glucose utilization stimulates leptin production by driving the glucose metabolism to oxidation or lipogenesis, rather than anaerobic lactate production (Mueller *et al.* 2000). Moreover, leptin secretion presented significant positive associations with glycerol release. This could be explained by reports describing that leptin directly inhibits lipogenesis (Ramsay 2003) and stimulates lipolysis in adipocytes (Fruhbeck *et al.* 1997). This feature could also in part explain the lower glycerol release occurring in the VC-incubated adipocytes. The fact that insulin induced no higher leptin gene expression could be explained by possible mRNA expression fluctuations during culture. Therefore, the elevated leptin secretion observed in insulin-treated cells could be due to an induced gene expression that decreased time-dependently, which was undetected at the end of the treatment. It was described that insulin-treated primary culture epididymal adipocytes produced more leptin during the first 2 h of *in vitro* incubations (Barr *et al.* 1997). Also, an acute significant increase in leptin secretion after a 2-h 100 nM insulin treatment, without changes in its gene expression (Bradley & Cheatham 1999), was observed. Moreover, it was described that biosynthetic release rates of leptin correlate with tissue leptin content and adiposity, but not with leptin mRNA levels in humans (Lee *et al.* 2007*a*). Finally, the lack of effects observed on the leptin mRNA expression by VC could be due to the well-known ascorbic acid instability (Feng *et al.* 1977), and also could be due to the lack of insulin effects, since

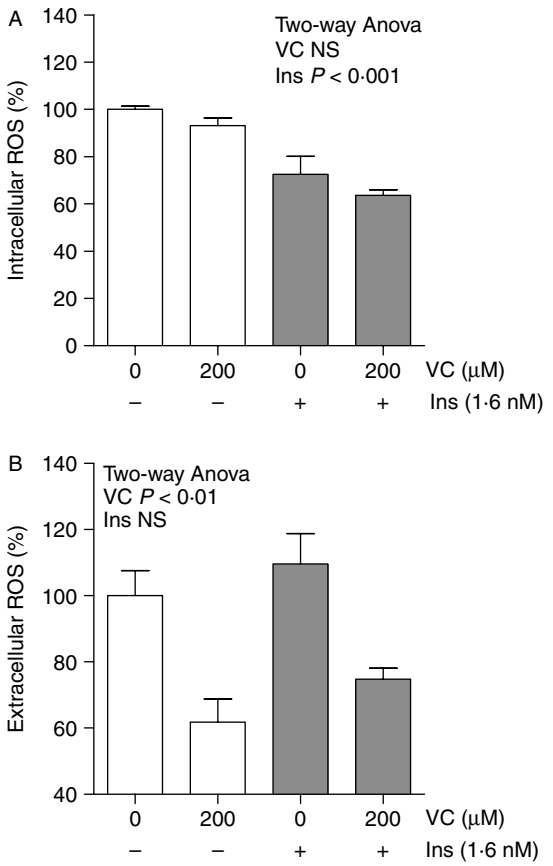

Figure 6 Effects of vitamin C (200 μM) in the absence or presence of 1·6 nM insulin on intracellular (A) and extracellular (B) ROS formation in isolated rat adipocytes over 72 h in culture. Data are expressed in percentage ($n=3$, mean ± S.E.M). VC, vitamin C; Ins, insulin.

the most dramatic inhibitory effects of VC were observed on insulin-induced leptin secretion. Furthermore, no data evidencing a possible intracellular leptin accumulation by VC have been reported previously. In any case, mRNA levels do not always correlate with the given protein product, perhaps by post-transcriptional regulation mechanisms (Lackner & Bahler 2008). In fact, it is known that both feeding and insulin treatments increase leptin translation (Lee *et al.* 2007*b*).

Regarding the mRNA expression analysis of other obesity-related genes, the data suggest that VC treatment could possibly modify some mechanisms related to adipocyte differentiation, insulin resistance, inflammation, and apoptosis. In the present study, a lowering effect of VC+insulin co-treatment on *Gpdh* mRNA expression was observed. The available literature reported a reduced GPDH activity in mature adipocytes exposed to VC treatment (Hasegawa *et al.* 2002) and differentiation inhibition, and consequently, reduced GPDH activity in 3T3-L1 cells (Krieger-Brauer & Kather 1995). On the other hand, it is known that insulin is necessary for triglyceride accumulation in preadipocytes (Ntambi & Young-Cheul 2000). In fact, the utilization of insulin for differentiation induction of different preadipocytes cell lines is common (Ailhaud 1997). However, the treated adipocytes in this study were mature cells isolated directly from WAT that did not present important differentiation machinery activity. This contradicts the lipolytic properties described for VC (Hasegawa *et al.* 2002), but this could be explained by an extra down-regulation of the lipogenic pathways by VC treatment.

It has been observed that VC induced a slight decrease in *Irs3* mRNA expression in subcutaneous and retroperitoneal fat pads of high-fat VC-supplemented diet-fed rats with respect to the high-fat diet-alone-fed obese rats (Garcia-Diaz *et al.* 2007). This slight mRNA inhibition was also observed in the current study, especially in cells under insulin treatment, suggesting a direct relationship between VC and insulin resistance/sensitivity pathways. It has been described earlier that *Irs3* overexpression induces higher translocation of GLUT4 proteins to the membranes of rat adipose cells, and also that mutant and non-functional IRS3 inhibits insulin action (Zhou *et al.* 1999). Consequently, down-regulation of this gene could have contributed to the observed glucose uptake VC-mediated inhibition. However, some insulin sensitivity improvement effects of a VC supplementation in high-fat diet-fed rats have been described previously (Garcia-Diaz *et al.* 2009). Therefore, these systemic insulin-sensitizing effects of VC could be driven through other mechanisms rather than through IRS3 modulation in adipocytes.

The *Cdkn1a* and *Casp8* mRNA overexpression observed in VC-treated adipocytes could indicate an induced cell apoptosis (Hershenson 2004, Baumgartner *et al.* 2007). These results are in agreement with the data reported by Senen *et al.* (2002), who described how VC injections in WAT deposits of rats decrease adipocyte cell number. Despite this evidence, no effect of 200 μM VC treatment on cell integrity/viability has been observed, suggesting that the expression of these genes was not directly related to cell death. Furthermore, in a study by our research group (Boque *et al.* 2009), a reduced mRNA expression of *Cdkn1a* and *p57* in subcutaneous WAT of rats fed with a high-fat diet with respect to the controls was observed, suggesting that this process could lead to higher adipose cell proliferation. It was also observed that a VC dietary supplementation induced slightly, but not significantly, the *Cdkn1a* and *p57* gene expressions with respect to a high-fat-alone-fed group, which is in agreement with the data reported in the present work.

It has been described that oxidative stress in excessively accumulated adipose tissue is an important trigger for the onset of obesity-related metabolic syndrome features (Furukawa *et al.* 2004). Some studies

described an important ROS-scavenging effect of this antioxidant vitamin (Perticone *et al.* 2001, Arrigoni & De Tullio 2002). Besides, it has been observed that an induced oxidative stress decreased GLUT4 expression by impairing the protein binding to its promoter, reducing glucose uptake (Pessler *et al.* 2001). On the other hand, another study described that a ROS production pathway stimulation is related to higher GLUT1 transcription (Kao & Fong 2008). It is known that the oxidized form of VC (DHA) is preferentially transported by GLUT1 (Vera *et al.* 1993), suggesting that VC could also induce the glucose uptake inhibition observed due to its ROS-scavenging properties. Therefore, the present results suggest that VC treatment could be involved in ROS formation, indicating that this inhibition could be implicated in some of the metabolic effects described earlier.

Taking all these into account, even though leptin is a recognized insulin-sensitizing agent (Dyck 2009), the inhibited secretion of this adipokine induced by VC counteracts the possible insulin improvement properties of this molecule. However, other previously observed effects of this vitamin, such as inhibition of the lipolytic rate (Garcia-Diaz *et al.* 2009) and oxidative stress inhibition (Vincent *et al.* 2009), could be associated with this possible beneficial effect, both of which are in agreement with the present results.

In summary, VC inhibited some glucose and lipid metabolism indicators, and also reduced the secretion of leptin and modified the expression of some important obesity-related proteins in primary culture rat adipocytes. The glucose uptake decrease could be due to VC–glucose transport competition, a fact that possibly leads to leptin secretion inhibition, which in turn could drive the observed lipolysis inhibition. Also, the effects of VC on ROS modulations could be involved in glucose and lipid metabolism regulation. The possible insulin-sensitizing properties of VC could also be attributed to other systemic effects rather than to direct inhibitory effects on leptin secretion in isolated adipocytes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Abdel-Wahab YH, O'Harte FP, Mooney MH, Barnett CR & Flatt PR 2002 Vitamin C supplementation decreases insulin glycation and improves glucose homeostasis in obese hyperglycemic (ob/ob) mice. *Metabolism: Clinical and Experimental* **51** 514–517.

Ailhaud G 1997 Molecular mechanisms of adipocyte differentiation. *Journal of Endocrinology* **155** 201–202.

Arrigoni O & De Tullio MC 2002 Ascorbic acid: much more than just an antioxidant. *Biochimica et Biophysica Acta* **1569** 1–9.

Barr VA, Malide D, Zarnowski MJ, Taylor SI & Cushman SW 1997 Insulin stimulates both leptin secretion and production by rat white adipose tissue. *Endocrinology* **138** 4463–4472.

Baumgartner HK, Gerasimenko JV, Thorne C, Ashurst LH, Barrow SL, Chvanov MA, Gillies S, Criddle DN, Tepikin AV, Petersen OH *et al.* 2007 Caspase-8-mediated apoptosis induced by oxidative stress is independent of the intrinsic pathway and dependent on cathepsins. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **293** G296–G307.

Boque N, Campion J, Milagro FI, Moreno-Aliaga MJ & Martinez JA 2009 Some cyclin-dependent kinase inhibitors-related genes are regulated by vitamin C in a model of diet-induced obesity. *Biological and Pharmaceutical Bulletin* **32** 1462–1468.

Bradley RL & Cheatham B 1999 Regulation of ob gene expression and leptin secretion by insulin and dexamethasone in rat adipocytes. *Diabetes* **48** 272–278.

Brandt R & Keston AS 1965 Synthesis of diacetyldichlorofluorescin: a stable reagent for fluorometric analysis. *Analytical Biochemistry* **11** 6–9.

Bray GA 2004 Medical consequences of obesity. *Journal of Clinical Endocrinology and Metabolism* **89** 2583–2589.

Bsoul SA & Terezhalmy GT 2004 Vitamin C in health and disease. *Journal of Contemporary Dental Practice* **5** 1–13.

Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J & Shoelson SE 2005 Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nature Medicine* **11** 183–190.

Campion J & Martinez J 2004 Ketoconazole, an antifungal agent, protects against adiposity induced by a cafeteria diet. *Hormone and Metabolic Research* **36** 485–491.

Campion J, Milagro FI, Fernandez D & Martinez JA 2006 Diferential gene expression and adiposity reduction induced by ascorbic acid supplementation in a cafeteria model of obesity. *Journal of Physiology and Biochemistry* **62** 71–80.

Canoy D, Wareham N, Welch A, Bingham S, Luben R, Day N & Khaw KT 2005 Plasma ascorbic acid concentrations and fat distribution in 19,068 British men and women in the European Prospective Investigation into Cancer and Nutrition Norfolk cohort study. *American Journal of Clinical Nutrition* **82** 1203–1209.

Carcamo JM, Pedraza A, Borquez-Ojeda O & Golde DW 2002 Vitamin C suppresses TNF alpha-induced NF kappa B activation by inhibiting I kappa B alpha phosphorylation. *Biochemistry* **41** 12995–13002.

Doulas NL, Constantopoulos A & Litsios B 1987 Effect of ascorbic acid on guinea pig adrenal adenylate cyclase activity and plasma cortisol. *Journal of Nutrition* **117** 1108–1114.

Dyck DJ 2009 Adipokines as regulators of muscle metabolism and insulin sensitivity. *Applied Physiology, Nutrition, and Metabolism* **34** 396–402.

Elks ML & Manganiello VC 1985 Antilipolytic action of insulin: role of cAMP phosphodiesterase activation. *Endocrinology* **116** 2119–2121.

Fantuzzi G 2005 Adipose tissue, adipokines, and inflammation. *Journal of Allergy and Clinical Immunology* **115** 911–919.

Feng J, Melcher AH, Brunette DM & Moe HK 1977 Determination of L-ascorbic acid levels in culture medium: concentrations in commercial media and maintenance of levels under conditions of organ culture. *In Vitro* **13** 91–99.

Ferrante AW 2007 Obesity-induced inflammation: a metabolic dialogue in the language of inflammation. *Journal of Internal Medicine* **262** 408–414.

Flora SJ 2007 Role of free radicals and antioxidants in health and disease. *Cellular and Molecular Biology* **53** 1–2.

Fruhbeck G, Aguado M & Martinez JA 1997 *In vitro* lipolytic effect of leptin on mouse adipocytes: evidence for a possible autocrine/paracrine role of leptin. *Biochemical and Biophysical Research Communications* **240** 590–594.

Fu J, Liang X, Chen Y, Tang L, Zhang QH & Dong Q 2008 Oxidative stress as a component of chromium-induced cytotoxicity in rat calvarial osteoblasts. *Cell Biology and Toxicology* **24** 201–212.

Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H *et al.* 2005 Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* **307** 426–430.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M & Shimomura I 2004 Increased oxidative stress in obesity and its impact on metabolic syndrome. *Journal of Clinical Investigation* **114** 1752–1761.

Galinier A, Carriere A, Fernandez Y, Carpene C, Andre M, Caspar-Bauguil S, Thouvenot JP, Periquet B, Penicaud L & Casteilla L 2006 Adipose tissue proadipogenic redox changes in obesity. *Journal of Biological Chemistry* **281** 12682–12687.

Garcia-Diaz D, Campion J, Milagro FI & Martinez JA 2007 Adiposity dependent apelin gene expression: relationships with oxidative and inflammation markers. *Molecular and Cellular Biochemistry* **305** 87–94.

Garcia-Diaz DF, Campion J, Milagro FI, Paternain L, Solomon A & Martinez JA 2009 Ascorbic acid oral treatment modifies lipolytic response and behavioural activity but not glucocorticoid metabolism in cafeteria diet fed rats. *Acta Physiologica* **195** 449–457.

Haider DG, Schindler K, Schaller G, Prager G, Wolzt M & Ludvik B 2006 Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding. *Journal of Clinical Endocrinology and Metabolism* **91** 1578–1581.

Hasegawa N, Niimi N & Odani F 2002 Vitamin C is one of the lipolytic substances in green tea. *Phytotherapy Research* **16** 91–92.

Hershenson MB 2004 p21Waf1/Cip1 and the prevention of oxidative stress. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **286** L502–L505.

Johnston CS 2005 Strategies for healthy weight loss: from vitamin C to the glycemic response. *Journal of the American College of Nutrition* **24** 158–165.

Kao YS & Fong JC 2008 Endothelin-1 induces glut1 transcription through enhanced interaction between Sp1 and NF-kappaB transcription factors. *Cellular Signalling* **20** 771–778.

Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, Durand JL, Li H, Li G *et al.* 2007 Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *Journal of Clinical Investigation* **117** 2621–2637.

Krieger-Brauer HI & Kather H 1995 Antagonistic effects of different members of the fibroblast and platelet-derived growth factor families on adipose conversion and NADPH-dependent H_2O_2 generation in 3T3 L1-cells. *Biochemical Journal* **307** 549–556.

Kurl S, Tuomainen TP, Laukkanen JA, Nyyssonen K, Lakka T, Sivenius J & Salonen JT 2002 Plasma vitamin C modifies the association between hypertension and risk of stroke. *Stroke* **33** 1568–1573.

Lackner DH & Bahler J 2008 Translational control of gene expression from transcripts to transcriptomes. *International Review of Cell and Molecular Biology* **271** 199–251.

Lee MJ, Wang Y, Ricci MR, Sullivan S, Russell CD & Fried SK 2007*a* Acute and chronic regulation of leptin synthesis, storage, and secretion by insulin and dexamethasone in human adipose tissue. *American Journal of Physiology. Endocrinology and Metabolism* **292** E858–E864.

Lee MJ, Yang RZ, Gong DW & Fried SK 2007*b* Feeding and insulin increase leptin translation. Importance of the leptin mRNA untranslated regions. *Journal of Biological Chemistry* **282** 72–80.

Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J *et al.* 1996 Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *PNAS* **93** 3704–3709.

Lorente-Cebrian S, Bustos M, Marti A, Martinez JA & Moreno-Aliaga MJ 2009 Eicosapentaenoic acid stimulates AMP-activated protein kinase and increases visfatin secretion in cultured murine adipocytes. *Clinical Science* **117** 243–249.

Martinez JA 2006 Mitochondrial oxidative stress and inflammation: an slalom to obesity and insulin resistance. *Journal of Physiology and Biochemistry* **62** 303–306.

Milagro FI, Campion J & Martinez JA 2006 Weight gain induced by high-fat feeding involves increased liver oxidative stress. *Obesity* **14** 1118–1123.

Misekova D, Lincova D & Hynie S 1993 The effect of ascorbic acid on adrenergic lipolysis. *Sborník Lékarský* **94** 55–62.

Moreno-Aliaga M, Campion J, Milagro FI, Berjon A & Martinez JA 2005 Adiposity and proinflammatory state: the chicken or the egg. *Adipocytes* **1** 1–16.

Mueller WM, Gregoire FM, Stanhope KL, Mobbs CV, Mizuno TM, Warden CH, Stern JS & Havel PJ 1998 Evidence that glucose metabolism regulates leptin secretion from cultured rat adipocytes. *Endocrinology* **139** 551–558.

Mueller WM, Stanhope KL, Gregoire F, Evans JL & Havel PJ 2000 Effects of metformin and vanadium on leptin secretion from cultured rat adipocytes. *Obesity Research* **8** 530–539.

Mutlu-Turkoglu U, Oztezcan S, Telci A, Orhan Y, Aykac-Toker G, Sivas A & Uysal M 2003 An increase in lipoprotein oxidation and endogenous lipid peroxides in serum of obese women. *Clinical and Experimental Medicine* **2** 171–174.

Ntambi JM & Young-Cheul K 2000 Adipocyte differentiation and gene expression. *Journal of Nutrition* **130** 3122S–3126S.

Ono M, Aratani Y, Kitagawa I & Kitagawa Y 1990 Ascorbic acid phosphate stimulates type IV collagen synthesis and accelerates adipose conversion of 3T3-L1 cells. *Experimental Cell Research* **187** 309–314.

Paolisso G, Balbi V, Volpe C, Varricchio G, Gambardella A, Saccomanno F, Ammendola S, Varricchio M & D'Onofrio F 1995 Metabolic benefits deriving from chronic vitamin C supplementation in aged non-insulin dependent diabetics. *Journal of the American College of Nutrition* **14** 387–392.

Patel M, McIntosh L, Bliss T, Ho D & Sapolsky R 2001 Interactions among ascorbate, dehydroascorbate and glucose transport in cultured hippocampal neurons and glia. *Brain Research* **916** 127–135.

Perez-Matute P, Marti A, Martinez JA, Fernandez-Otero MP, Stanhope KL, Havel PJ & Moreno-Aliaga MJ 2007 Conjugated linoleic acid inhibits glucose metabolism, leptin and adiponectin secretion in primary cultured rat adipocytes. *Molecular and Cellular Endocrinology* **268** 50–58.

Perticone F, Ceravolo R, Candigliota M, Ventura G, Iacopino S, Sinopoli F & Mattioli PL 2001 Obesity and body fat distribution induce endothelial dysfunction by oxidative stress: protective effect of vitamin C. *Diabetes* **50** 159–165.

Pessler D, Rudich A & Bashan N 2001 Oxidative stress impairs nuclear proteins binding to the insulin responsive element in the GLUT4 promoter. *Diabetologia* **44** 2156–2164.

Powers KA, Rehrig ST & Jones DB 2007 Financial impact of obesity and bariatric surgery. *Medical Clinics of North America* **91** 321–338.

Ramsay TG 2003 Porcine leptin inhibits lipogenesis in porcine adipocytes. *Journal of Animal Science* **81** 3008–3017.

Ruperez FJ, Garcia-Martinez D, Baena B, Maeso N, Cifuentes A, Barbas C & Herrera E 2008 Evolution of oxidative stress parameters and response to oral vitamins E and C in streptozotocin-induced diabetic rats. *Journal of Pharmacy and Pharmacology* **60** 871–878.

Senen D, Adanali G, Ayhan M, Gorgu M & Erdogan B 2002 Contribution of vitamin C administration for increasing lipolysis. *Aesthetic Plastic Surgery* **26** 123–125.

Sorensen DI, Devine MM & Rivers JM 1974 Catabolism and tissue levels of ascorbic acid following long-term massive doses in the guinea pig. *Journal of Nutrition* **104** 1041–1048.

Valdecantos MP, Perez-Matute P & Martinez JA 2009 Obesity and oxidative stress: role of antioxidant supplementation. *Revista de Investigación Clínica* **61** 127–139.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** research0034.1–research0034.11.

Varma V, Yao-Borengasser A, Rasouli N, Bodles AM, Phanavanh B, Lee MJ, Starks T, Kern LM, Spencer HJ, McGehee RE *et al.* 2007 Human visfatin expression: relationship to insulin sensitivity, intramyocellular lipids, and inflammation. *Journal of Clinical Endocrinology and Metabolism* **92** 666–672.

Vera JC, Rivas CI, Fischbarg J & Golde DW 1993 Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. *Nature* **364** 79–82.

Vincent HK & Taylor AG 2006 Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *International Journal of Obesity* **30** 400–418.

Vincent HK, Bourguignon CM, Weltman AL, Vincent KR, Barrett E, Innes KE & Taylor AG 2009 Effects of antioxidant supplementation on insulin sensitivity, endothelial adhesion molecules, and oxidative stress in normal-weight and overweight young adults. *Metabolism: Clinical and Experimental* **58** 254–262.

Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M & Shoelson SE 2001 Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* **293** 1673–1677.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature* **372** 425–432.

Zhou L, Chen H, Xu P, Cong LN, Sciacchitano S, Li Y, Graham D, Jacobs AR, Taylor SI & Quon MJ 1999 Action of insulin receptor substrate-3 (IRS-3) and IRS-4 to stimulate translocation of GLUT4 in rat adipose cells. *Molecular Endocrinology* **13** 505–514.

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