

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 μ M). 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 μ M) 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

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 & Terezhalmay 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 CaCl₂·2H₂O, 1.25 mM MgSO₄·7H₂O, 0.45 mM KH₂PO₄, 2.17 mM Na₂HPO₄, 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- μ m 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 μ g/ml streptomycin, 100 U/ml nystatin, and 1× MEM non-essential amino acids, followed by incubation for 40 min at 37 °C in 5% CO₂. The resulting isolated adipocytes (150 μ l of a 2:1 mix of packed cells/culture medium) were then plated on 500 μ l 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% CO₂, the culture medium containing 0 or 1.6 nM insulin and different concentrations (0, 5, 10, 50, 200, and 1000 μ M) of VC was added. Cells were maintained in an incubator at 37 °C in 5% CO₂ 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 (μ U/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 μ M)-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 MgCl₂, 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% CO₂, 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% CO₂, 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

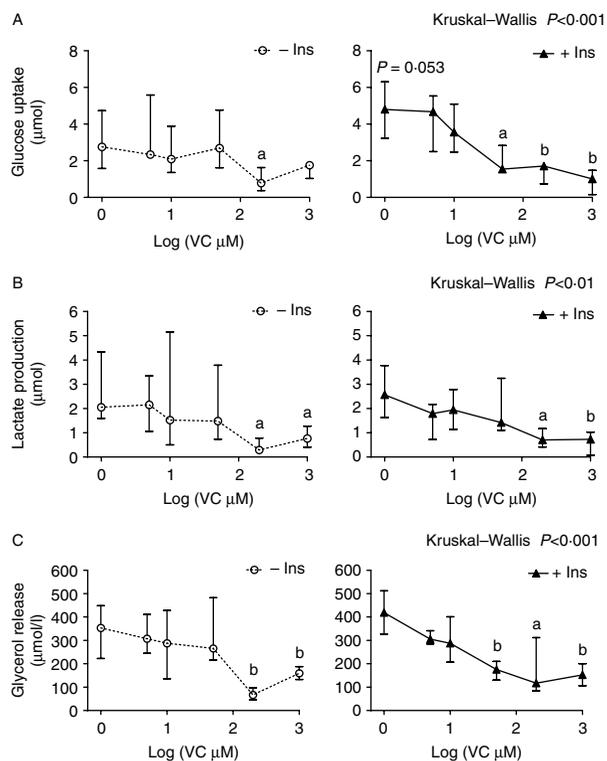


Figure 1 Effects of vitamin C (log (5–1000 μM)) in the absence (white circles) or presence (black triangles) of 1.6 nM insulin on (A) glucose uptake, (B) lactate production, and (C) glycerol release in isolated rat adipocytes over 72 h in culture. Data ($n \geq 4$, median and interquartile range). ^a $P < 0.05$ and ^b $P < 0.01$ versus control cells (with or without insulin treatment). $P = 0.053$, control versus control+insulin cells. VC, vitamin C; Ins, insulin.

200 μM VC for both untreated ($P < 0.05$ for 200 and 1000 μM versus control cells) and insulin-treated cells ($P < 0.05$ for 200 and $P < 0.01$ 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 ($P < 0.01$ for both versus control cells) and in insulin-treated cells at 50 ($P < 0.01$), 200 ($P < 0.05$), and 1000 μM ($P < 0.01$) 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 ($P < 0.001$). Increasing concentrations of VC induced a dose-dependent inhibition on both basal ($P < 0.05$ for 200 and 1000 μM versus control cells) and insulin-stimulated leptin secretion ($P < 0.01$ for 200 and 1000 μM versus insulin-treated cells). Insulin treatment effectively induced leptin secretion with respect to the control cells ($P < 0.05$). In contrast, in the case of adiponectin, despite the fact that a significant global effect was detected ($P < 0.05$), 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 ($r = 0.566$, $P < 0.01$) and insulin-stimulated cells ($r = 0.797$, $P < 0.001$; Fig. 3A and B respectively), and also with glycerol release in both untreated ($r = 0.787$, $P < 0.001$) and insulin-stimulated cells ($r = 0.791$, $P < 0.001$; Fig. 3C and D respectively).

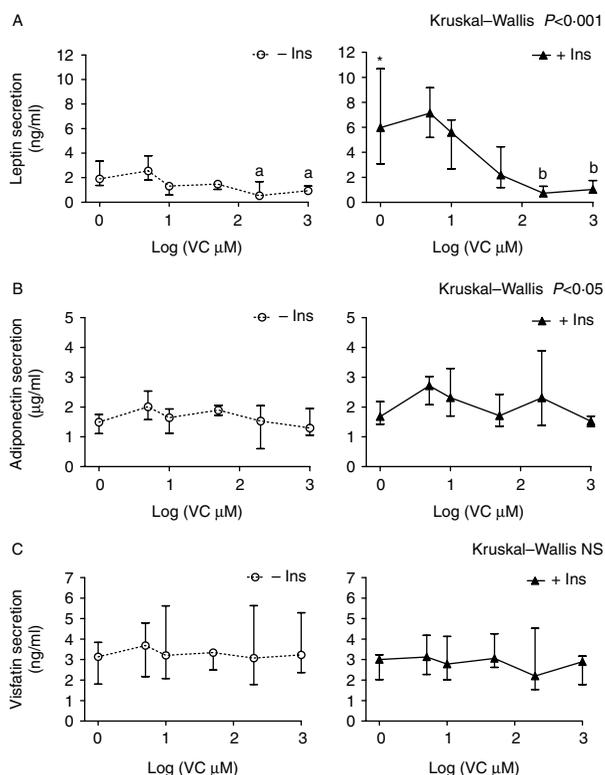


Figure 2 Effects of vitamin C (log (5–1000 μM)) in the absence (white circles) or presence (black triangles) of 1.6 nM insulin on (A) leptin, (B) adiponectin, and (C) visfatin secretion in isolated rat adipocytes over 72 h in culture. Data ($n \geq 4$, median and interquartile range). ^a $P < 0.05$ and ^b $P < 0.01$ versus control cells (with or without insulin treatment). * $P < 0.05$, control versus control+insulin cells. VC, vitamin C; Ins, insulin; NS, not significant.

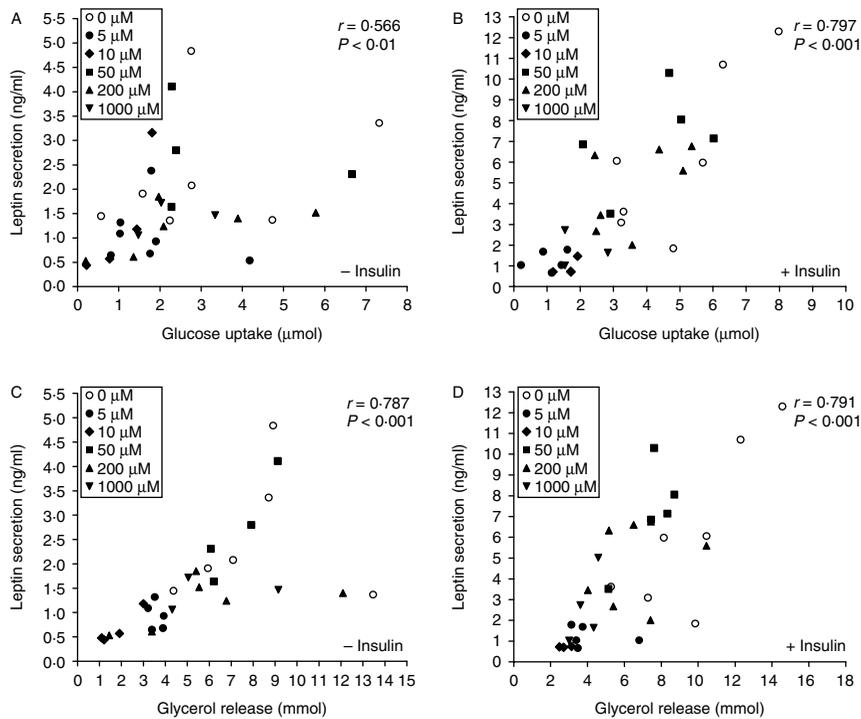


Figure 3 Analysis of the association between leptin secretion and (A) basal glucose uptake, (B) insulin-stimulated glucose uptake, and the amount of glycerol released in the (C) absence and (D) presence of insulin. All the VC concentrations (0–1000 μM) are included. Graph symbols represent: open circles, 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 ($r=0.810$, $P<0.05$) 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 ($P<0.05$), 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 ($P<0.05$ 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 ($P<0.05$) 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 ($P<0.05$) 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,

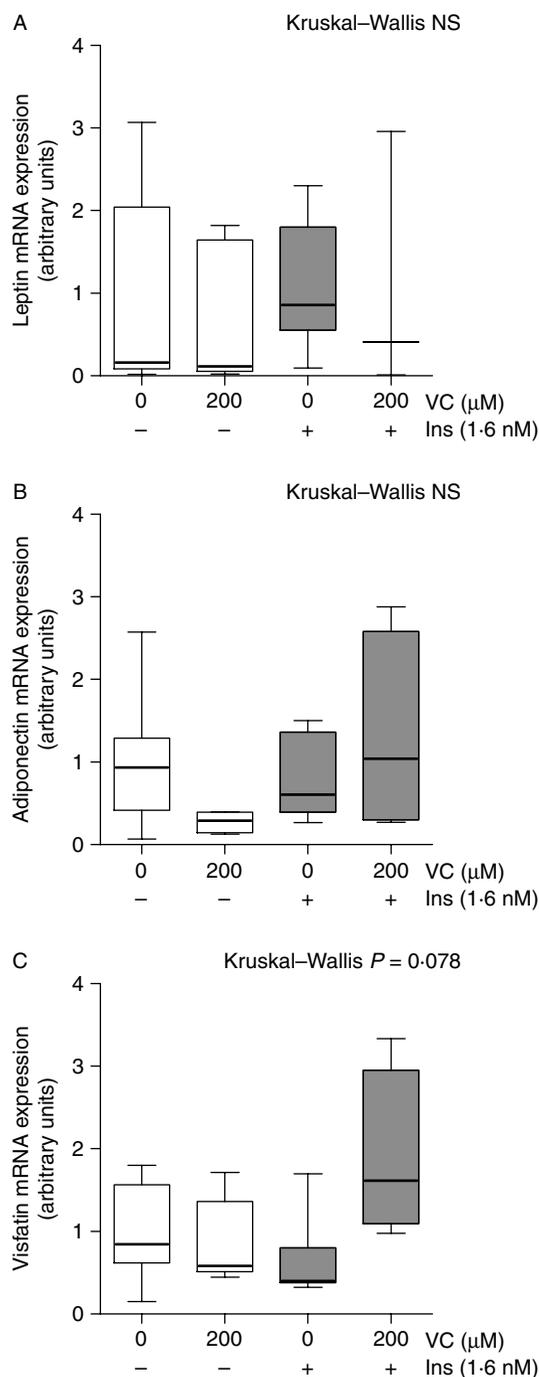


Figure 4 Effects of vitamin C (200 μM) in the absence (white boxes) or presence (grey boxes) of 1.6 nM insulin on (A) leptin, (B) adiponectin, and (C) visfatin mRNA expressions in isolated rat adipocytes over 72 h in culture. Data ($n \geq 4$, median and interquartile range). VC, vitamin C; Ins, insulin; 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).

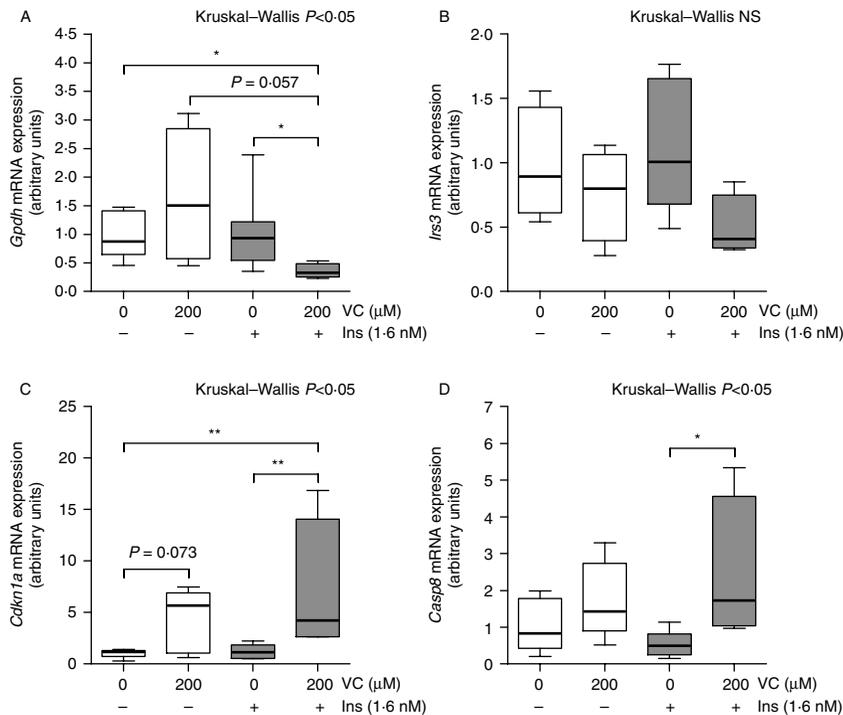


Figure 5 Effects of vitamin C (200 μ M) in the absence (white boxes) or presence (grey boxes) of 1.6 nM insulin on mRNA expression of several genes involved in (A) adipocyte differentiation (*Gpdh*), (B) insulin signaling (*Irs3*), (C) cell cycle (*Cdkn1a*), and (D) apoptosis (*Casp8*) in isolated rat 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.* 2007a). 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

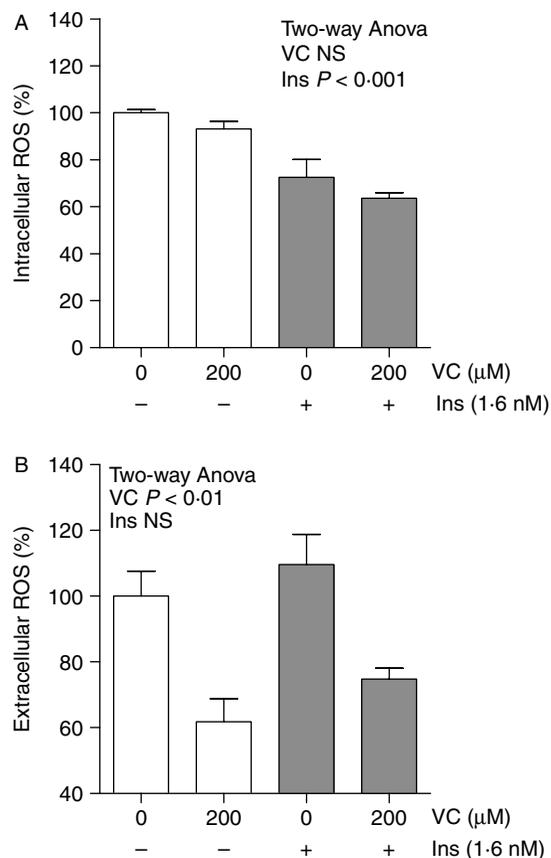


Figure 6 Effects of vitamin C (200 µM) in the absence or presence of 1.6 nM insulin on intracellular (A) and extracellular (B) ROS formation in isolated rat adipocytes over 72 h in culture. Data are expressed in percentage ($n=3$, mean \pm S.E.M.). VC, vitamin C; Ins, insulin.

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.* 2007b).

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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