Vitamin C modulates the interaction between adipocytes and macrophages

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Abbreviations:

LDH, lactate dehydrogenase; NO, nitric oxide; VC, vitamin C.

Keywords:

Ascorbic acid, cell culture, coculture, inflammation, obesity

Abstract

Scope: Increased adiposity is related with monocyte infiltration into the adipose tissue which accentuates inflammation. Antioxidant treatments emerge as approaches to counteract this phenomenon. Methods and results: Cocultures of differentiated 3T3-L1 adipocytes and RAW264.7 macrophages were incubated for 24-72 h with/without 100 nM insulin and/or 200 μM vitamin C (VC). Nitric oxide (NO) secretion (24 h) was measured. Also, expression (24 h) and secretion (72 h) of MCP-1, leptin and apelin were analyzed. NO secretion was significantly inhibited by insulin and VC only in cocultures. MCP-1 expression/secretion was enhanced in cocultures. Insulin incubation reduced MCP-1 expression in both cultures and VC only in controls. Both treatments inhibited MCP-1 secretion in cocultures. Apelin gene expression was induced in cocultures. Insulin induced apelin mRNA expression, but VC inhibited its expression in cocultures under insulin treatment. Apelin secretion was notably induced by insulin and inhibited by VC in cocultures. Leptin expression was decreased in coculture, while presented no effects by VC. Conclusion: VC importantly modulates the established pro-inflammatory state in the interaction between adipocytes and macrophages.

1 Introduction

Obesity is often accompanied by a low-grade inflammation condition in the adipose tissue [1]. Indeed, adipokines, cytokines, and other factors produced by this tissue could be responsible for this process [2]. This situation could lead to macrophage infiltration, consequently aggravating the obesity state [3]. The cross-talk between inflammatory macrophages and adipocytes may influence insulin resistance, since macrophages contribute to the development of insulin resistance in obese patients, while weight loss reduces macrophage infiltration and the expression of inflammation-related factors in adipose tissue [4, 5]. Thus, several inflammatory products derived from this tissue, such as TNF-α, IL-6, MCP-1, and nitric oxide (NO) correlates with increased body adiposity [6], and appear to participate in the induction and maintenance of the chronic inflammatory state associated with obesity [7]. Therefore, a reduction in the inflammatory status based on antioxidant/anti-inflammatory agents could constitute a potential treatment to improve insulin-sensitivity and to avoid adverse obesity-associated consequences [8]. Among these agents, vitamin C (VC) has been claimed as one of the most important natural antioxidants, due to its well-known ROS scavenging properties [9, 10]. Furthermore, it has been described that this molecule induced a significant reduction in the inflammatory response mediated by NFκB [11]. Experiments of our research group have found that a VC treatment was correlated with a decrease body weight gain, leptin plasma levels, and apelin gene expression in rat adipose tissue [12], with modulations in lipolysis [13] and with an inhibition in the glucose metabolism and leptin secretion of isolated adipocytes also from rats [14]. In this context, the use of a dual cellular system mimicking adipose tissue physiology

could be an excellent model to get in-depth into the mechanisms involved in the possible antioxidant protective effect of VC.

2 Materials and methods

Cell culture and treatment

3T3-L1 mouse preadipocytes (ATCC, Rockville, USA) and RAW264.7 mouse macrophages (obtained from the Centre for Applied Medical Research of the University of Navarra) cell lines were maintained at 37 ºC in a humidified atmosphere containing 5% CO_2. 3T3-L1 cells were cultured in twelve-well plates in DMEM containing 4.5 g/L glucose and 10% calf serum. Two days after full confluence, cells were differentiated by incubation with 0.5 mM isobutylmethylxanthine, 1 μm dexamethasone, 10 μg/ml insulin, in 4.5 g/L mM glucose DMEM supplemented with 10% fetal bovine serum for 2 days and then for the next 2 days with 10 μg/ml insulin, in 4.5 g/L mM glucose DMEM supplemented with 10% fetal bovine serum. Thereafter, cells were maintained and re-fed every 2 or 3 days with media without any hormones until 14 days after differentiation induction, when between 80 and 90% of the cells exhibited the mature adipocyte phenotype.

Adipocytes and macrophages coculture

Coculture of adipocytes and macrophages was performed in a contact system according to a protocol previously described [15]. Serum-starved 14 days-differentiated 3T3-L1 cells were cultured in twelve-well plates, and RAW264.7 cells (67,000 cells/cm^2), were plated onto 3T3-L1. The cells were cultured in contact to each other, in the presence or absence of 100 nM insulin and 200 μM VC, and harvested at 24 h. Also a group of cells was maintained until 72 h for adipokine/cytokine production measurements. As control, equal number of adipocytes and macrophages in relation to those in the contact system, were separately cultured and mixed after harvest. Culture media were collected and

stored at -80 ºC until further measurements. For all analyses, comparisons between control and coculture meant finding differences between mixtures of both cells types when were in contact against when were separated. For secretion analyses, the amount detected of each molecule of interest that was secreted by adipocytes alone was added to the amount released by macrophages alone. This sum was then compared with the secretion of both cells in the coculture. The VC dose utilized was previously used by our research group and it is considered to be within physiological levels [14, 16].

Cell viability assay

Cell viability was measured with the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit at 24 h according to manufacturer`s instructions (sensitivity = $>10^3$ cells) (Cayman Chemical Company, Ann Arbor, USA).

Nitric oxide release measurement

The amount of nitrite in cell-free culture supernatants was measured, in 24 h samples, using Griess reagent according to manufacturer`s protocol (working range = 0.43-65 µM Nitrite) (Sigma-Aldrich Company, St. Louis, USA). Briefly, 50 µl of supernatant was mixed with an equivalent volume of Griess reagent in a 96-well flat bottom plate. After a 10 min incubation in darkness, absorbance at 540 nm was measured, and the amount of nitrite was calculated from a $NaNO_2$ standard curve. The results were corrected by cell viability [17].

Gene expression assays

Total RNA was isolated from 24 h samples using Trizol (Invitrogen, Paisley, UK), according to supplier`s protocol. Purified total RNA (2 µg) from adipocytes and

macrophages was then treated with DNAse (DNAfree kit; Ambion Inc., Austin, USA) and used to generate first strand cDNA with M-MLV reverse transcriptase (Invitrogen, Paisley, UK), utilizing random hexamers (Invitrogen, Paisley, UK), and dNTP mix (Bioline, London, UK), according to manufacturer`s protocol. The resultant cDNA was amplified with primers specific for mouse MCP-1 (Mm99999056_m1), leptin (Mm00434759_m1), and apelin (Mm00443562_m1) in a total volume of 10 μl. Real-time PCR was performed in an ABI PRISM 7000 HT Sequence Detection System following manufacturer's recommendations (Applied Biosystems, California, USA). The real-time PCR program utilized was: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s, and 60 ºC for 1 min. PCR products were analyzed with the SDS 2.3 and the RQ Manager 1.2 software (Applied Biosystems, California, USA). All the expression levels of the target genes studied were normalized by the expression of cyclophilin (Mm02342430_g1) as the selected internal control, probe that was also supplied by Applied Biosystems (California, USA). Fold change between groups was calculated using the $2^{-\Delta\Delta Ct}$ method. All procedures were performed according to a previously described protocol [14].

Secretion analyses

MCP-1 secretion to culture media was measured using the MCP-1 Mouse ELISA Kit (sensitivity = >9 pg/ml; intra-assay precision = 3.7-5.4 %CV; inter-assay precision 5.4-7.7 %CV) (Invitrogen, Paisley, UK), apelin secretion using the Apelin-12 EIA Kit (sensitivity = >0.07 ng/ml, according to manufacturer; intra-assay precision = 5 %CV; inter-assay precision 14 %CV, according to [18]) (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany), and leptin secretion using the Rat Leptin ELISA kit obtained from Linco Research (sensitivity = >0.04 ng/ml; intra-assay precision = 1.88-2.49

%CV; inter-assay precision 2.95-3.93 %CV) (St Charles, MO, USA) using an automatized Triturus equipment (Grifols International, Barcelona, Spain). All secretion measurements were performed in 72 h samples. The results were corrected by cell viability.

Statistical analyses

All results are expressed as mean ± standard deviation (SD). All data were evaluated using a factorial two-way ANOVA. When interaction was detected, Student t tests were performed for group comparisons. Association analyses were performed using the Pearson correlation coefficient. A probability of $p<0.05$ was set up for determining statistically significant differences. All the statistical analyses were performed using the SPSS 15.0 for Windows software (SPSS Inc., Chicago, USA).

3 Results

Cell viability

Cell integrity was decreased (p<0.001) in coculture (Figure 1). However, both VC and insulin increased cell viability (p<0.001 and p<0.01, respectively), and only an interaction between coculture and VC was observed (p<0.05), due to the significant improving effects of VC especially in the coculture system. When data were analyzed by culture type, VC protected cells from toxicity in both cultures (p<0.05 in controls and p<0.001 in coculture), but insulin only increased significantly cell integrity in cocultures (p<0.05).

NO concentrations

The NO secretion was not induced by the coculture (Figure 3). However, a statistically significant interaction (p<0.05) between insulin and VC incubations was observed, possibly due to inhibitions in NO production induced separately by VC and insulin incubations in the coculture system.

Gene expression

MCP-1 mRNA expression was induced (p<0.001) in the coculture system as compared to controls. Insulin incubation inhibited (p<0.01) the expression of this cytokine in both cultures types. VC incubation presented lowering effects only in control culture (p<0.05) (Figure 4A). Apelin gene expression was induced (p<0.001) in the coculture system respect to controls. Insulin also induced apelin mRNA expression (p<0.001), and a significant interaction between VC and the culture system was observed (p<0.05), due to a VC-induced inhibition in coculture under insulin treatment (Figure 4B). Leptin

mRNA expression was decreased (p<0.05) in coculture system respect to controls, and no effects by VC or insulin were observed (Figure 4C).

Protein secretion

MCP-1 secretion to culture media was induced (p<0.001) in the coculture system respect to control. On the other hand, VC and insulin induced significant lowering effects (p<0.01 and p<0.05, respectively). Furthermore, significant interactions between the effects of the coculture and the effects of both treatments (insulin, p<0.05; VC, p<0.01) were observed, probably due to the inhibitory effects of both molecules on the coculture-induced MCP-1 secretion. When analyzed separately, VC incubation presented a lowering effect only on the coculture (p<0.01). Same tendency was observed for insulin incubation (p<0.05) (Figure 5A). Apelin secretion was inhibited in the coculture respect to controls (p<0.01). On the other hand, the secretion of this adipokine was significantly induced by insulin (p<0.01) and inhibited by VC treatments (p<0.001) (Figure 5B). Interactions between the effects of the coculture and the effects of insulin (p=0.056) and VC (p<0.001), and also between both treatments (p<0.01), were observed. These interactions could be due to the important inducing effects of insulin (p<0.01), and inhibiting effects of VC (p<0.001) only in cocultures, which was also observed when analyzed separately. Leptin secretion to culture media was not affected by the coculture, but was induced significantly by insulin (p<0.001) (Figure 5C). When the analysis was separated regarding culture type, stimulatory effects of insulin on leptin secretion were observed in both control (p<0.001) and coculture (p<0.01) systems.

Association studies

Statistically significant negative correlations were observed between apelin and MCP-1 gene expressions, in control (r = -0.713, p<0.01) and in coculture (r = -0.809, p<0.01). Also, a significant positive correlation was detected between NO production and MCP-1 mRNA expression (r = 0.464, p<0.05) and secretion (r = 0.597, p<0.01). Finally, negative correlations were observed between leptin mRNA expression and MCP-1 gene expression (r = -0.457, p<0.05) and secretion (r = -0.449, p<0.05), and between cell viability and MCP-1 gene expression (r = -0.864; p<0.001) and secretion (r = -0.777, p<0.001).

4 Discussion

In the obese state, adipose tissue is characterized by an increased systemic oxidative stress and a chronic low grade inflammation [19]. This enhanced inflammatory state is accompanied by an increased macrophage infiltration in the fat tissue [2]. The macrophages interact with the adipocytes driving to insulin-resistance [3]. In the current study, an antioxidant treatment (VC) was applied into the adipocyte-macrophage interaction in order to try to counteract this vicious cycle. Inflammatory state was achieved in the coculture system, as suggested by the increased MCP-1 mRNA expression and protein secretion [15]. On the other hand, VC seems to inhibit the NO production only in cocultures. However, this outcome is contradictory since it has been described that VC induced the NO formation in activated macrophages [20]. In this sense, it was observed that an iNOS activity inhibition leads to increased lipolysis in adipose tissue [21]. This finding agrees with previous research that described a lower lipolytic response of adipocytes under VC incubations [13, 14]. Therefore, VC could drive the NO inhibition observed by anti-lipolytic processes.

Also, it is known that insulin induces NO production, and that insulin-resistance leads to a decreased nitric oxide production [22]. In the present results, insulin seemed to fail to induce NO synthesis. Nevertheless, it has been described that insulin impairs the iNOS-mediated NO production, but on skeletal muscle [23]. However, when insulin and VC were both present in coculture NO production seemed to increase, suggesting that the previously described inducing-inflammatory effects of these molecules regarding NO concentration appears only when these two molecules were occurring in coculture. Though, this is the first time that a VC incubation is evaluated in a coculture system of adipocytes and macrophages.

Regarding cell viability, it was observed an elevated LDH activity in culture media of the coculture system respect to controls, indicating increased cell death. As is stated in the introduction, it has been established that the cross-talk between inflammatory macrophages and adipocytes may influence insulin resistance [4, 5]. However, in order to this association is established, macrophages population inside the adipose tissue must first increase. Adipose tissue in enlargement, as in obesity development, presents a downregulation of MKP-1 which leads to an increased secretion of MCP-1 [24]. This phenomenon, is accompanied by a hypoxia-induced endoplasmatic reticulum stress in obese adipose tissue [25], and by an increase in ROS production [19], both factors involved in adipokine secretion dysregulation. In this scenario, MCP-1 induces massive infiltration of macrophages into the adipose tissue, which participates in inflammatory pathways. Together with MCP-1, FFA are secreted by adipocytes and induce the activation of pro-inflammatory cascades in macrophages, resulting in TNF-α release, which in turn stimulate pro-inflammatory cytokine production and lipolysis in adipocyte through NFκB-dependent and –independent mechanisms [15], establishing a vicious cycle [26]. Furthermore, it has been observed that TNF-α treatment can induce apoptosis in human adipocytes [27], and that adipocyte cell death has been related to macrophage presence in the adipose tissue [28]. These facts agree with the observed negative correlations between cell viability and MCP-1 expression/secretion. Therefore, VC could protect from this cell death by its inhibitory properties over NFκB-mediated inflammatory response [11]. On the other hand, it has been described that VC incubations reduced oxLDL-induced macrophages death [29, 30].

Furthermore, insulin inhibits MCP-1 expression, which is consistent with a previous research report [31]. Also, in the present study, it has been described that the VC incubation reduced MCP-1 expression and secretion especially in cocultures. MCP-1

plays a crucial role in the inflammatory-response increment in obesity, by enhancing monocyte migration and activation of macrophages [32]. NF-κB is one of the mayor inductors of MCP-1 [33] and iNOS [34], and it has been reported that VC inhibits the activation of NF-κB induced by TNF-α in the endothelial cell line ECV304, in primary HUVECs, and in humans cell lines such as HeLa, monocytic U937, myeloid leukemia HL-60, and breast MCF7 [11, 35].

On the other hand, apelin gene expression was significantly increased in coculture, and an interaction between VC and the coculture system in the apelin gene expression was observed, due to a VC-induced inhibition in the adipocyte-macrophage system under insulin treatment. A significant modulation of apelin was observed only in cocultures. This result agrees with a previous report of our group describing a lowering effect of VC over apelin in a pathologic obesity model [12]. However, this inhibitory effect, in cocultures, was only observed at the protein secretion level but not in the mRNA expression. This suggests that the apelin secretion modulation by VC could imply post-translational mechanisms [36]. In fact, in the present work, no significant correlation between apelin gene expression and secretion was observed. On the other hand, it has been described that apelin prevents macrophage-induced inflammation [37]. This fact is supported in the present work by the negative correlations observed between apelin and MCP-1 expression in control culture and in coculture. This finding suggests a beneficial role of this adipokine in obesity-induced inflammation [38]. VC decreased insulin-induced apelin expression only in coculture, which possibly leads to higher MCP-1 expression under both hormones treatment in this system. Furthermore, a significant correlation between NO production and MCP-1 expression was observed, showing a reduction in both variables in the control cultures, and with incubations of insulin and VC. However, the protein secretion of apelin and MCP-1 did not presented significant

negative correlations, suggesting that maybe in coculture, apelin expression/secretion are not dependent on MCP-1 expression/secretion and vice versa.

Finally, regarding leptin expression, the lower leptin expression in the coculture system contradicts the fact that a pro-inflammatory state leads to higher leptin secretion [39]. Furthermore, a significant negative correlation were observed between leptin mRNA expression and MCP-1 gene expression, suggesting a relationship of leptin with a decreased inflammatory state [40, 41]. One explanation could be that the 24h coculture probably not mimics a fully established inflammatory state as in pathogenic adipose tissue. In fact, in this system no hypertrophied adipocytes are present, as occur in obesity which leads to increased leptin levels [42]. Moreover, the leptin secretion was not modified by the coculture. Nevertheless, the slight inhibitory effect of VC observed in the contact system is supported by previous observations [12, 14].

Summing up, a VC treatment leads to improvements in the adipocyte-macrophage inflammatory vicious cycle, including inhibition of NO and ROS production and a decrease in insulin-induced MCP-1 and apelin expression/secretion. In some of our own previous experiments [12-14], marginally beneficial effects on the insulin-resistance status by VC treatments have been observed, but the mechanisms underlying these events have not been elucidated. In the present article, the fact that the VC treatment effectively reduce the inflammation in co-coculture, suggest that this could be a pathway on which vitamin C exert its anti-insulin-resistance features. Finally, these experiments suggest that the use of a dual cell system mimicking adipose tissue inflammation during obesity can be used in screening strategies that search novel bioactive compounds with potential antiobesity and antidiabetic properties.

Acknowledgments

Conflict of interest statement

The authors declare that they have no conflict of interest regarding the contents of this manuscript.

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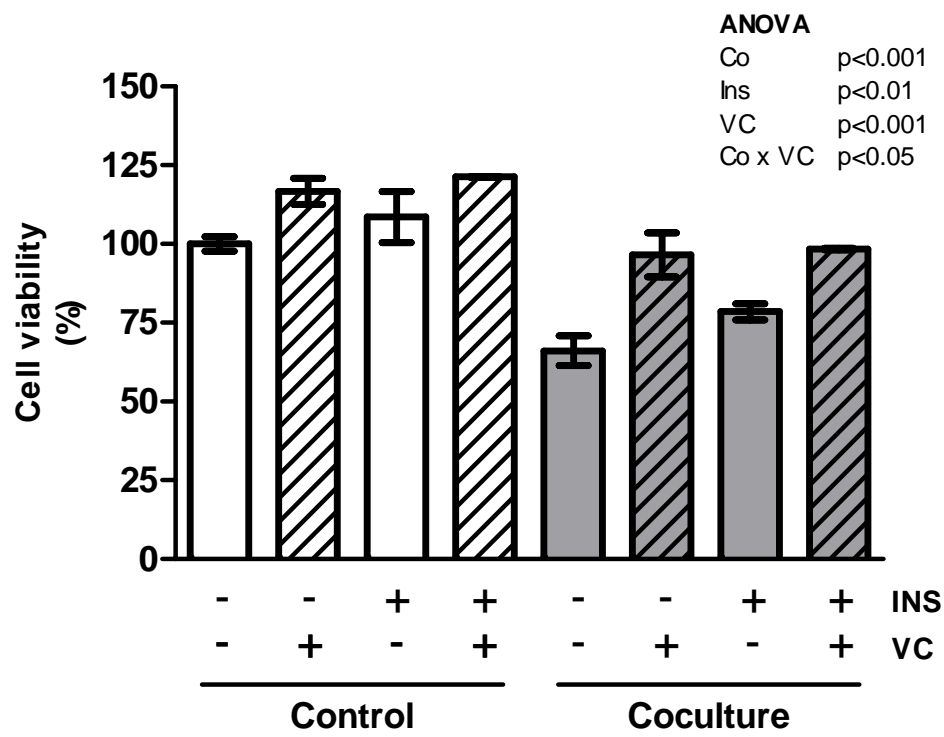
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Figure 1. Cell viability in the coculture system. Cell integrity in control cultures and the coculture system was determined after 24 h of treatment with insulin and/or vitamin C. Data (n=3) are expressed as mean ± SD. Two-way ANOVA was performed to identify statistical effects. Co, coculture; INS, insulin; VC, vitamin C.

Figure 2. NO concentration in the coculture system. NO production (corrected by cell viability) in control cultures and the coculture system was determined after 24 h of treatment with insulin and/or vitamin C. Data (n=3) are expressed as mean ± SD. Two-way ANOVA was performed to identify statistical effects. Co, coculture; INS, insulin; VC, vitamin C.

Figure 3. Gene expression analysis of an inflammatory marker and two adipokines in the coculture system. mRNA expression of MCP-1 (A), apelin (B), and leptin (C) in control cultures and the coculture system was determined after 24 h of treatment with insulin and/or vitamin C. Data (n=3) are expressed as mean ± SD. Two-way ANOVA was performed to identify statistical effects. Co, coculture; INS, insulin; VC, vitamin C.
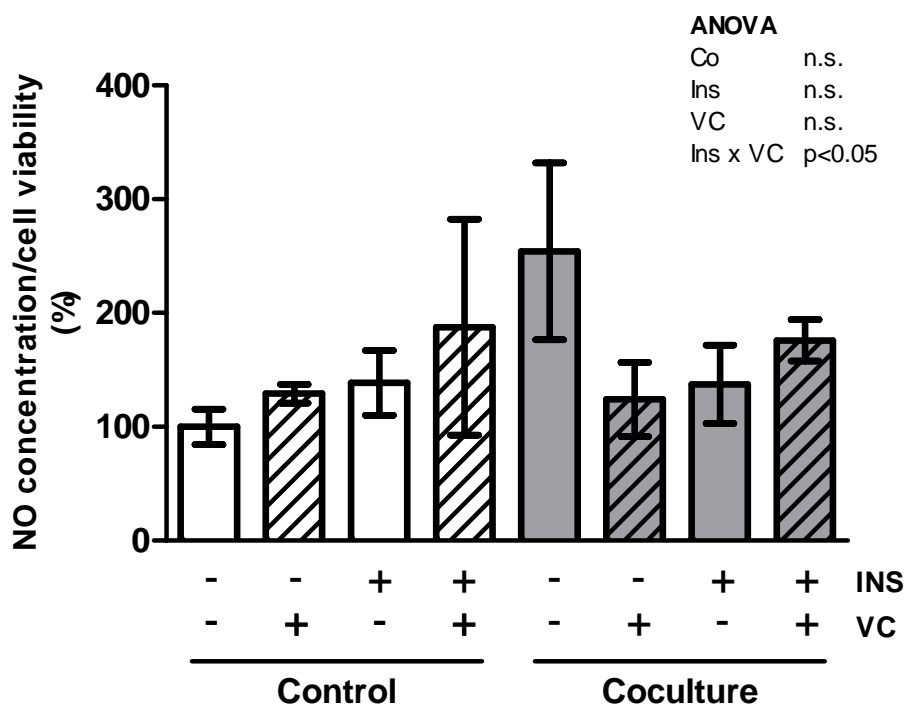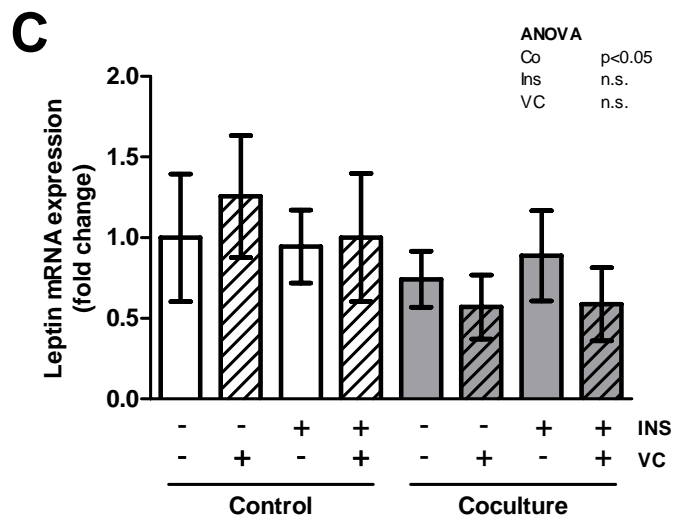
Figure 4. Secretion analysis of an inflammatory marker and two adipokines in the coculture system (corrected by cell viability). Concentration in culture media of MCP-1 (A), apelin (B), and leptin (C) in control cultures and the coculture system was determined after 72 h of treatment with insulin and/or vitamin C. Data (n=3) are expressed as mean ± SD. Two-way ANOVA was performed to identify statistical effects. Co, coculture; INS, insulin; VC, vitamin C.
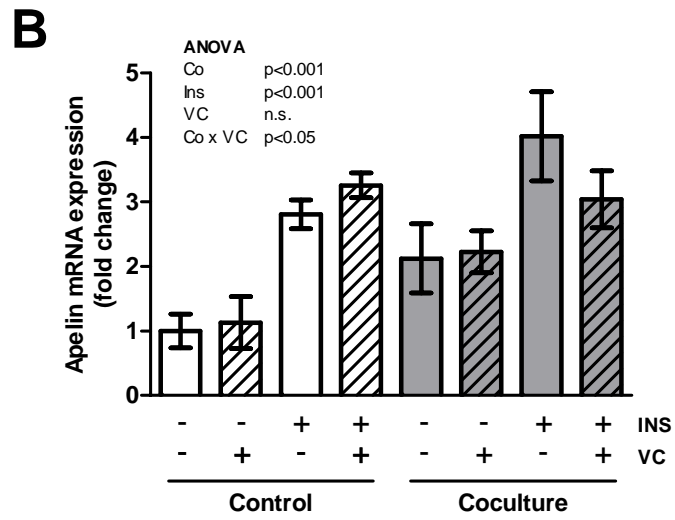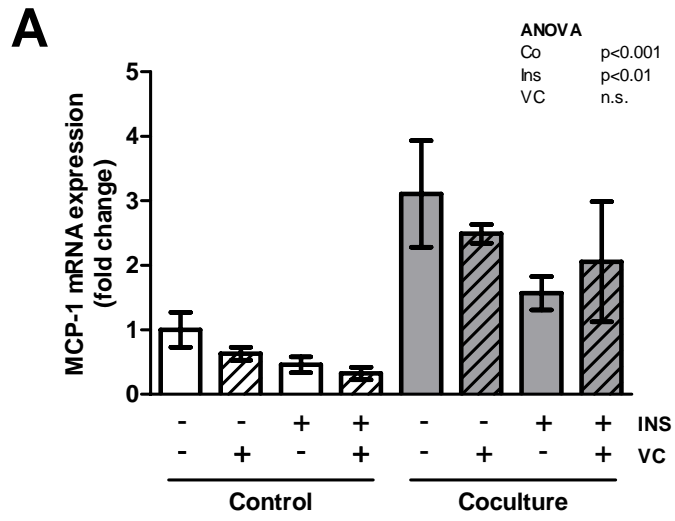
Figure 1

Figure 2

Figure 3

Figure 4