Some Cyclin-Dependent Kinase Inhibitors-Related Genes Are Regulated by Vitamin C in a Model of Diet-Induced Obesity

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Received March 31, 2009; accepted May 22, 2009; published online May 28, 2009

The aim of this research was to investigate differential gene expression of cyclin-dependent kinase inhibitors (CKIs) in white adipose tissue (WAT) and liver from high-fat fed male Wistar rats with or without vitamin C (VC) supplementation (750 mg/kg of body weight). After 56 d of experimentation, animals fed on a cafeteria diet increased significantly body weights and total body fat. Reverse transcription-polymerase chain reaction (RT-PCR) studies showed that cafeteria diet decreased p21 and p57 mRNA expression in subcutaneous WAT and increased p21 mRNA in liver. Overall, these data provide new information about the role of high fat intake on mRNA levels of several CKIs with implications in adipogenesis, cell metabolism and weight homeostasis. Interestingly, VC supplementation partially prevented diet-induced adiposity and increased p27 mRNA in liver without any changes in the other tissues and genes analyzed. Thus, hepatic mRNA changes induced by ascorbic acid indicate a possible role of these genes in diet-induced oxidative stress processes.

Key words cyclin-dependent kinase inhibitor; cafeteria diet; ascorbic acid; adipose tissue; fatty liver

Cell cycle progression implies the up and down-regulation of a number of genes. The key genes of this pathway are p21 (Cdkn1a), p27 (Cdkn1b) and p57 (Cdkn1c), which belong to the Cip/Kip family of cyclin-dependent kinases inhibitors (CKIs). They are regulated by the F-box protein Skp2 and in the case of p21 it is also under the control of the p53 tumor suppressor protein, which is activated when DNA is damaged. During the last years, many investigations have focused on CKIs research due to their relationship with cancer. Promising results have prompted that several scientific groups devote their efforts to investigate the relation of these molecules with other biological processes like adipogenesis or β-cell function among other functions. In this context, it has been recently reported a role of the Cip/Kip family in adipogenesis by regulating adipocyte proliferation and differentiation. Specifically, it has been described that p21 and p27 are major regulators of adipocyte number in vivo and knockout animals lacking one or both of these proteins provide models for adipocyte hyperplasia studies. Moreover, Okada et al. (2007) have observed that abdominal irradiation was able to decrease the body weight gain of ob/ob mice and induce p21 gene expression in adipose tissue. They have also reported that this reduction of body weight was accompanied by decreased adipose tissue weight without changes in adipocyte size. On the other hand, it has been described that mice fed with a high-fat diet during 25 weeks develop adipocyte hyperplasia and hypertrophy, which was accompanied by a diminished p27 expression and an up-regulation of Skp2 protein in white adipose tissue (WAT). In this way, Skp2 knockout mice had a decrease in both subcutaneous and visceral fat pad mass and adipocyte number. Furthermore, p53 has also been related to obesity, since it has been demonstrated that this gene is highly induced in adipocytes from ob/ob mice in a fed state and that it plays an important role in the pathogenesis of fatty liver.

In this context, cafeteria diet fed animals is an accepted model of obesity induced by high-fat feeding that shares common Western diet features and drives to metabolic syndrome conditions. Moreover, an enhanced oxidative stress status arising from an excessive release of reactive oxygen species has been documented in obese patients and overweight animals. In this context, adiposity and hyperinsulinemia may enhance liver oxidative stress in cafeteria in comparison to control rats. Moreover, pharmacological doses of vitamin C (VC), which have not been related with toxicity, have a protective role in the development of adiposity induced by a high fat diet, and p21 and p57 may play a possible role in this effect. In this sense, CKIs and cell cycle progression are regulated by oxidative stress and reactive oxygen species (ROS), which allow to hypothesize a potential role of these genes in the development of diet-induced obesity.

The aim of this study was to analyze potential variations in mRNA levels of some key genes involved in cell cycle regulation in a rat model of obesity, and to determine a possible protective role of VC in preventing diet-induced adiposity through the regulation of CKIs.

MATERIALS AND METHODS

Animals Briefly, experiments were performed with twenty-five male Wistar rats from CIFA (Centre of Pharmaceutical Applied Investigation) of the University of Navarra with an initial weight of 250 g. Animals were kept in an isolated room with a constantly regulated temperature between 21 and 23 °C, and controlled (50±10%) humidity in a 12 h artificial light cycle. They were randomized in three groups: Control group (n=8), Cafeteria group (n=8) and Cafeteria+ VC (CafVitC) group (n=9). Control rats were fed with a standard pelleted chow diet from Harlan Ibérica (Barcelona, Spain) while Cafeteria and CafVitC rats were fed with a high-fat diet (cafeteria diet: 50% fat, 39% carbohydrates and 11% protein) to generate a diet-induced obesity model. Moreover, CafVitC group included a daily dose of VC (Pancreac Quimica SA, Barcelona, Spain) of 750 mg/kg rat, adequately distributed with the cafeteria diet. The different
groups of rats had ad libitum water and food access, while body weight and food intake were daily recorded. At the sacrifice day (56 d), every animal was weighted for being anesthetized in a fasted state with Ketamine (50 mg/kg intraperitoneally (i.p.), Parke-Davis, Madrid, Spain) and Medetomidine (0.025 mg/kg i.p., Pfizer SA, Madrid, Spain). Body composition was measured using an EM-SCAN (Springfield, IL, U.S.A.) as described elsewhere.17,26) After the sacrifice by decapitation, blood was collected and serum was stored immediately at −80 °C. All the procedures performed agreed with the national and institutional guidelines of the Animal Care and Use Committee at the University of Navarra.

**Serum and Tissue Measurements** Serum triglycerides were determined with the RANDOX kit for the in vitro diagnostic of triglycerides (Randox LTD. Laboratories, Ardmore Road, U.K.), glucose was measured using the HK-CP kit (ABX diagnostics, Montpellier, France) and total protein with total protein 250 kit (ABX-Diagnostic, Geneve, Switzerland) adapted for a COBAS MIRA (Roche, Basel, Switzerland) equipment. Hepatic malondialdehyde (MDA) was determined by a colorimetric assay for lipid peroxidation (Bioxytech, Portland, Geneve, OR, U.S.A.) and leptin and insulin quantification was performed by radioimmunoassay (RIA) following the protocol described by the manufacturer (Linco Research, MI, U.S.A.). Finally, the homeostatic model assessment (HOMA), as an insulin resistance index, was calculated using the following formula: (fasting plasma insulin×plasma glucose)/22.5.

**Determination of DNA Content** Total DNA content from liver and from subcutaneous and retroperitoneal adipose tissues was analyzed using a fluorimetric technique (CyQUANT Cell Proliferation Assay Kit C-7026). Briefly, 250 mg of tissue was homogenized in a lysis buffer (NaCl 150 mM, Tris 10 mM pH 8, Triton-X-100 0.1%). Then, 0.5 μl of the dye was added to a 200 μl of the diluted sample and DNA was quantified using a bacteriophage λDNA standard curve in a Polarstar fluorometer with an excitation wavelength of 480 nm and an emission wavelength of 520 nm as described elsewhere.27)

**Gene Expression Analysis** From frozen rat tissues (−80 °C), total RNA was isolated by Trizol method (Invitrogen, CA, U.S.A.). Purified RNA was treated with DNase (Ambion, TX, U.S.A.) and used to generate cDNA with reverse transcriptase Maloney Murine Leukemia reverse transcriptase (M-MLV). Next, quantitative real-time polymerase chain reaction (PCR) was performed on an ABI PRISM 7000 HT Sequence Detection System (Applied Biosystems, CA, U.S.A.). Specific Taqman probes for rat (Applied Biosystems) p21 (Rn00589996_m1), p57 (Rn00711097_m1), p27 (Rn00582195_m1), Skp2 (Rn01402565_g1), p53 (Rn00755717_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Rn99999916_s1) and 18S (Hs9999991_s1) were used. Changes in gene expression between Control, Cafeteria and CafVitC groups were calculated using the 2−ΔΔCt method. In order to normalize expression values, Genorm software was applied for liver and retroperitoneal adipose tissue, whereas GAPDH was the housekeeping for subcutaneous adipose tissue.

**Statistics** All results are expressed as the average mean±standard error. Means comparisons were tested by Kruskal–Wallis test and groups were separated using the U Mann–Whitney test. Association analyses were performed using the Spearman correlation test. A level of probability up at p<0.05 was set up as statistically significant and p<0.01 as very statistically significant. For the statistical tests, SPSS software for Windows 15.0 was used (Chicago, IL, U.S.A.).

**RESULTS**

Animals fed on cafeteria diet during 56 d increased significantly body weight (p=0.003), adiposity as total body fat (p=0.001), and energy food intake (p=0.001) in comparison to control chow fed group (Table 1). Moreover, high-fat diet induced a marked hyperleptinemia (p=0.002), hyperglycemia, hyperinsulinemia and increased HOMA-index. In addition, liver weight and the levels of hepatic malondialdehyde, a parameter of tissue oxidative stress, were significantly affected by this diet (p=0.014 and p=0.012, respectively). On the other hand, when was compared to cafeteria group (Table 1), VC supplementation on high fat diet (CafVitC group) reduced significantly body weight gain (p=0.024), as well as total body fat and leptinemia (p=0.009 and p=0.034), protecting thus against cafeteria-induced overweightness. Interestingly, this action on weight gain was independent of energy intake (Table 1) as it is indicated by the significant reduction of efficiency in CafVitC group compared to cafeteria group (p=0.036).

Regarding the main goal of this work, gene expression control within proliferative pathway by high fat diet and VC supplementation in rats, RT-PCR analyses showed tissue and fat pad-specific regulation (Figs. 1—3). Thus, in the liver (Fig. 1) cafeteria diet induced a significant increase (two folds, p=0.021) of p21 mRNA when was compared to chow fed rats. The analysis of the remaining genes concerning this

**Table 1. Body Composition, Energy Intake, Serum Leptin and Hepatic MDA in the Experimental Dietary Groups (Control, Cafeteria and CafVitC)**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>Cafeteria (n=8)</th>
<th>CafVitC (n=9)</th>
<th>C vs. Caf</th>
<th>p</th>
<th>C vs. CafVitC</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Final body weight (g)</td>
<td>409.38±11.68</td>
<td>503.25±22.63</td>
<td>447.44±10.90</td>
<td>**</td>
<td>*</td>
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<tr>
<td>Total body fat content (g/100 g)</td>
<td>14.21±0.94</td>
<td>21.87±1.36</td>
<td>17.55±0.74</td>
<td>**</td>
<td>**</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Energy food intake (kcal)</td>
<td>83.76±1.30</td>
<td>154.76±10.91</td>
<td>150.70±4.27</td>
<td>**</td>
<td>n.s.</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Efficiency (g/100 kcal)</td>
<td>3.07±0.13</td>
<td>2.73±0.21</td>
<td>2.14±0.09</td>
<td>n.s.</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Plama leptin (ng/ml)</td>
<td>3.35±1.01</td>
<td>14.26±2.01</td>
<td>8.32±1.34</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>8.96±8.96</td>
<td>12.21±1.21</td>
<td>10.37±0.68</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>MDA/protein ratio (μmol/g)</td>
<td>0.30±0.03</td>
<td>0.54±0.06</td>
<td>0.47±0.09</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
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</table>

All the results are expressed as the mean value±standard error. Statistical analyses were performed using Kruskal–Wallis test and groups were separated using the U Mann–Whitney test. n.s., not significant; * p<0.05; ** p<0.01; C: Control; Caf: Cafeteria; CafVitC, Cafeteria + VC.
metabolic pathway analyzed in liver did not show statistical changes in gene expression by high fat intake, despite of a tendency to increase p57 and p53 mRNA levels. When the effect of VC supplementation was studied in liver, the only gene significantly overexpressed was the key-protein p27 mRNA (about five folds, \(p < 0.002\)). Finally, total DNA amount was similar between the three groups indicating that there does not apparently exist a diet or VC induced hepatocyte proliferation (C: 11.01 ± 0.12, Caf: 10.07 ± 0.97, CafVitC: 10.39 ± 0.71 mg of DNA per total weight depot).

Rats fed on cafeteria diet showed a significant decrease of p21 and p57 mRNA expression levels (both almost two folds, \(p = 0.019\) and \(p = 0.040\)) in subcutaneous adipose tissue in comparison to the Control group (Fig. 2). Both p27 and p53 mRNAs showed a tendency to a decreased expression, without reaching statistical significance. Interestingly, concerning the hypothesis of a possible VC-controlled adipocyte hyperplasia, this antioxidant administration did not affect significantly any of the investigated genes when CafVitC group was compared against Cafeteria group. In the case of the retroperitoneal fat depot, cell cycle controlling mRNAs expression was not seemingly affected (Fig. 3) by either high fat intake nor VC supplementation, indicating a depot-specific gene expression regulation depending on WAT body localization. In order to clarify proliferation diet induced proliferation, total DNA content was analyzed in the two fat pads. Thus, DNA amount in Cafeteria group increased more than 300% (\(p < 0.003\)) and CafVitC group 2 fold (\(p = 0.008\)) in comparison to control group (C: 1.00 ± 0.08, Caf: 3.23 ± 0.04, CafVitC: 1.92 ± 0.21 mg of DNA per weight depot). In the case of retroperitoneal adipose tissue, despite of showing significant differences (\(p = 0.012\)), total DNA

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**Fig. 1. Gene Expression Analysis Detected by RT-PCR in Liver of Control, Cafeteria and CafVitC Groups**

All results are expressed as fold of change respect to controls (control set at unity), and showed as mean ± S.E.M. (dotted bars, Control group; white bars, Cafeteria group; grey bars, CafVitC group; *p < 0.05; **p < 0.01).

**Fig. 2. Gene Expression Analysis Detected by RT-PCR in Subcutaneous WAT of Control, Cafeteria and CafVitC Groups**

All results are expressed as fold of change respect to controls (control set at unity), and showed as mean ± S.E.M. (dotted bars, Control group; white bars, Cafeteria group; grey bars, CafVitC group; *p < 0.05).
from high-fat group was less increased (about 50%) in comparison to controls rats (C: 0.56±0.02, Caf: 0.78±0.06, CatVitC: 1.02±0.13 mg of DNA per total weight depot).

Finally, in order to characterize the role of these genes as possible biomarkers of high-fat induced obesity or a VC protective effect, we analyzed the correlation of gene expression data with some of the biological key-variables affected by the experimental diets (Fig. 4). In relation to WAT, and despite the lack of significant changes in p53 mRNA levels by cafeteria diet, association analyses for Control and Cafeteria groups revealed that subcutaneous p53 mRNA levels may be a very relevant marker of adiposity (Fig. 4A), associated to total body fat (Spearman = −0.678, p=0.005). Moreover, and specially for subcutaneous adipose depot, a very significant and negative association (Spearman = −0.581, p=0.007) was detected after analyzing p21 mRNA gene expression and total DNA content from this depot, showing again a potential role of this protein in cell cycle regulation by high-fat diet. On the other hand, association analysis for Cafeteria and CatVitC groups indicated p27 mRNA as the cell-cycle key gene in relation to the protective effect of VC in liver. Thus, statistical studies showed significant correlations between p27 and almost all the analyzed variables, presenting stronger negative associations (Fig. 4B) between this gene and total body fat (Spearman = −0.875, p<0.001), serum leptin levels (Spearman = −0.703, p=0.002) and MDA (Spearman = −0.778, p<0.001).

DISCUSSION

The main goal of this research was to investigate possible changes in the regulation of several cell-cycle controlling genes as affected by a diet with high fat content and the role of VC supplementation in two WAT depots and liver. The current study revealed a differential tissue and fat pad-specific regulation of the analyzed genes, which was evidenced...
in three genes of the subcutaneous adipose tissue (p21, p57 and p53) and one in liver (p21). On the other hand, this study found that the p27 gene was upregulated by vitamin C in liver. Some of those genes were selected after a microarray assay from a previous experiment, revealing a myriad of changes at the transcriptional level.23)

It is widely recognized that high fat intake induces both adipocyte hyperplasia and hypertrophy,28,29 generating an increased adiposity, chronic inflammation and obesity-related features such as insulin resistance and hyperleptinemia.48) Several publications have recently confirmed that some of the genes of CKIs family may be related to the process of cell proliferation and differentiation within the adipose tissue,30,31) which could involve hyperplasia mechanisms concerning obesity onset.

Adipocyte hyperplasia begins with a number of cell divisions (mitotic clonal expansion), followed by a growth arrest that induces gene expression for fat metabolism, thereby accumulating large cytosolic fat droplets.7) Cell division occurs when D and E type cyclins bind to their CDK partners and allows the cell cycle progression from G1 to S phase. This progression can be inhibited by cyclin-dependent kinase inhibitors, which bind and inactivate cyclin/CDK complexes and so they play a regulatory role in differentiation, apoptosis, tumor suppression and cell cycle progression.39) In this sense, some studies have revealed that expression of p21 and p27 inhibitors in 3T3-L1 is almost undetectable in subconfluent cells. Thus, it has been proposed that p53 might influence cellular turnover processes and might shorten the life span of adipocytes.15)

Curiously, it seems that Skp2 gene expression is not apparently involved in the regulation of the studied genes,13,43) although it has been reported a role for the F-box protein Skp2 in the degradation of p27,30,31 p57,44) p2145) and E2F-1.46) Thus, Skp2 mediates polyubiquitylation, targeting modified substrates for degradation by the 26S proteasome.47) Some authors have reported that Skp2 expression increases when preadipocytes are induced to differentiate, and that inhibition of Skp2 led to accumulation of p27 suggesting that the absence of Skp2 may have effects on mitotic clonal expansion.14)

The profiling pattern induced by the cafeteria diet feeding on the set of analyzed genes in the liver seems to be completely different to the adipose tissue outcome. The diet caused a significant increase of p21 mRNA expression and a tendency to increase the expression of p57 mRNA levels. These results are in some accordance with those obtained from other authors,48) who demonstrated an overexpression of p21 gene in the non-alcoholic fatty liver of rodents. Thus, p21 action consists in the inhibition of hepatocyte cell cycle, since other authors have observed that steatotic livers have lower phosphorylation of retinoblastoma proteins, avoiding

Regarding fat-depot differences between CKIs expression in the subcutaneous and the retroperitoneal adipose tissue, it could be due to the different proliferating capacity of these two depots. Thus, DiGirolamo et al.40) described regional adipose growth differences, with a high proliferating rate of the subcutaneous adipose tissue compared with the visceral one, which has been confirmed also in humans.41) Moreover, the results of DNA experiments indicated that hyperplasia was higher in the subcutaneous than in the retroperitoneal fat pad. So, the dietary management induced changes in body weight and adiposity which could be due to an increase of adipose tissue cellularity in the subcutaneous adipose tissue, marked by the decreased expression of CKIs, with minor effects on the retroperitoneal depot.

Otherwise, p53 could be involved in the reduction of p21 mRNA in subcutaneous adipocytes induced by the diet, since it has been described a p53 response element in the human p21 promoter,15,42) but not in the diminished expression of p57. So, the increased accumulation of intracellular lipids due to the high-fat diet intake, may be a cellular stress associated to large adipocytes that could lead to the activation of the p53/p21 pathway, required for survival of hypertrophic adipocytes. Thus, it has been proposed that p53 might influence cellular turnover processes and might shorten the life span of adipocytes.15)
the liberation of E2F transcription factors and so resulting in the arrest of fatty hepatocytes in G1 phase of the cell cycle. This situation may inhibit the hepatic tissue capacity of regeneration and lead to the fatty liver pathogenesis, since the overexpression of p21 in the liver was followed by hepatic lipid accumulation and increased hepatic triglycerides. In fact, high fat diet is known to induce liver oxidative stress in the animals and contribute to hepatic disorders aggravating the cafeteria-induced metabolic syndrome. Thus, increased hepatic ROS and lipid peroxidation causes inflammation and non-alcoholic fatty liver disease, a clinical relevant issue, that is a major cause of liver-related morbidity, which has been related to human obesity in epidemiological studies.

In spite of the lack of effect of VC supplementation in adipose tissue gene expression profiling, this antioxidant induced a clear and promising effect specifically on p27 mRNA gene expression in liver. Moreover, correlation studies indicate a strong and very significant statistical association between hepatic MDA and p27 mRNA levels, when adipose tissue gene expression is considered. It is noted that the liberation of E2F transcription factors and so resulting in the arrest of fatty hepatocytes in G1 phase of the cell cycle. This situation may inhibit the hepatic tissue capacity of regeneration and lead to the fatty liver pathogenesis, since the overexpression of p21 in the liver was followed by hepatic lipid accumulation and increased hepatic triglycerides. In fact, high fat diet is known to induce liver oxidative stress in the animals and contribute to hepatic disorders aggravating the cafeteria-induced metabolic syndrome. Thus, increased hepatic ROS and lipid peroxidation causes inflammation and non-alcoholic fatty liver disease, a clinical relevant issue, that is a major cause of liver-related morbidity, which has been related to human obesity in epidemiological studies.

In conclusion, this study confirms a tissue-specific regulation of some cell cycle key genes as affected by cafeteria diet and adipose tissue environment. The results of this experiment open a promising field to modify the expression of Cip/Kip family genes in adipose tissue, taking into account the role of several epigenetic mechanisms involved in their regulation. Finally, this research may be useful in order to investigate the possible administration of pharmacological agents, or functional ingredients such as saponins, for inhibiting some of the damaging effects of the westernized diets involving a high fat consumption and a low antioxidant intake.

Acknowledgments The authors wish to thank to Linea Especial (LE’97) from the University of Navarra, MEC grant (AGL2006-04716/ALI), as well as Department of Education of the Government of Navarra for financial support.

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