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Xanthohumol Influences Preadipocyte Differentiation: Implication of Antiproliferative and Apoptotic Effects

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There is interest in the research of natural compounds that may interfere with the adipocyte life cycle, due to the growing prevalence of obesity and related complications. We aimed at studying the effect of xanthohumol (XN), a *Humulus lupulus* L. prenylflavonoid, on adipocytes measuring differentiation, proliferation, and apoptosis in 3T3-L1 cells. XN reduced differentiation, as revealed by decreased lipid content and peroxisome proliferator-activated receptor γ expression, an effect more pronounced when cells were treated before or during differentiation induction. XN also decreased proliferation, as measured by sulforhodamine staining (IC_{50} between 26 and 12 μM for 24, 48, and 72 h), and preadipocyte Ki67 expression. Apoptosis was increased in preadipocytes and adipocytes. NF-κB activity was stimulated by XN in preadipocytes. Results suggest that XN may reduce adipocyte number, contributing to adipocyte hypertrophy. Taking into consideration the consequences of adipocyte hypertrophy, XN does not seem to improve the metabolic profile associated with obesity.

KEYWORDS: Adipocyte; adipogenesis; apoptosis; NF-κB; obesity; proliferation; xanthohumol

INTRODUCTION

Obesity is considered an epidemic of modern society, and its prevalence keeps increasing (*1*). Life style is pointed as the main cause, mainly involving the ingestion of high energy foods and low physical activity, as energy excess is stored in the form of triacylglycerols (TAG) in the adipose tissue (AT) (*2*). However, since the discovery of leptin and its production in AT, this tissue is gaining the status of an endocrine organ. Many other hormones, peptides, and cytokines produced in the AT have been described so far, collectively termed adipokines (*3*), many of which are implicated in the inflammatory status associated with obesity (*4, 5*).

AT growth results from an increase in adipocyte size (hypertrophy), adipocyte number (hyperplastic AT), or both (*6, 7*). For many years it was believed that adipocyte number was determined during childhood, and that the increase of AT was exclusively due to hypertrophy of adipose cells. However, this idea has been set apart with the demonstration that AT cells are able to incorporate tritiated thymidine reflecting the synthesis of nucleic acids during proliferation (*8*). Consequently, preadipocyte proliferation throughout adulthood is an important factor in the determination of adipocyte number in adults. Although adipocyte size may reflect the balance between lipolysis and lipogenesis, it is also influenced by the number of differentiated adipocytes since the same amount of TAG can be stored in many small adipocytes or in fewer larger cells.

Hypertrophic obesity is more common in adults and is correlated with metabolic complications of obesity, including insulin resistance, type 2 diabetes, hypertension, and coronary heart disease (*6*). Some authors have been attributing responsibility for obesity-related outcomes to inflammation generated in the AT, but lipid excess deposition in other organs is also likely to contribute. For this reason, the perspective of reducing differentiation of preadipocytes into mature adipocytes as a strategy to prevent obesity is beginning to lose strength toward a new hypothesis: that decreasing adipocyte size may overcome metabolic stress (*6, 9, 10*).

There is currently intense research in the field of functional foods to treat obesity and its complications. Specifically, some polyphenols, including epigallocatechin-3-gallate, procyanidins, genistein, and resveratrol have been demonstrated to modulate adipose tissue metabolism and adipocyte cell cycle (*11*−*14*). Xanthohumol (XN) is a prenylated flavonoid derived from hops (*Humulus lupulus* L.) (**Figure 1**), that may be present in low concentrations in beer (*15*). Hop components have been described to inhibit the metabolic activation of xenobiotics (*16*−*18*), prostaglandin, and NO production (*19, 20*) and to exert antitumor activity in hypoxic tumor cells (*21*). Additionally, it was demonstrated that XN exerts antiproliferative, pro-apoptotic,

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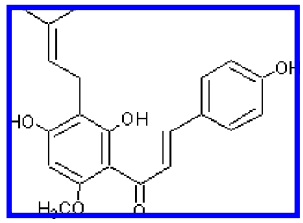

Figure 1. Chemical structure of xanthohumol.

and antiangiogenic effects in several tumor cell lines or xenografts (*22*−*24*). Recent reports also relate this compound to effects on energy metabolism (*25*−*27*).

Despite the current knowledge on XN properties, the interference of this flavonoid with adipose tissue biology is still unclear. The aim of the present work is to analyze the interaction of XN with adipose tissue development by investigating the effects of the flavonoid on the murine preadipocyte cell line, 3T3-L1. The influence of XN on distinct phases of the adipogenic process, including lipid accumulation, cell proliferation, apoptosis, and NF-κB activity have been assessed.

MATERIALS AND METHODS

Materials. Antibiotic-antimycotic solution, bovine serum albumin (BSA), oil red O, sulforhodamine B (SRB), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and trypsin-EDTA solution were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Gelatine, Triton X-100, and *p*-formaldehyde were obtained from Merck (Darmstadt, Germany). DAPI, terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) kit, and Tripure isolation reagent were obtained from Roche Applied Science (Basel, Switzerland). Goat polyclonal immunoglobulin G anti-Ki67 and donkey antigoat immunoglobulin G-FITC were obtained from Santa Cruz Biotechnologies (CA). Nuclear factor-kappa B (NF-κB) TransAM Flexi NFκB Family kit was from Active Motif (Belgium). Primers for RT-PCR analysis were purchased from Metabion International (Martinsried, Germany). XN (purity of 90%) (*28*) was kindly supplied by Hopsteiner (Mainburg, Germany) through Instituto de Bebidas e Saúde. XN was dissolved in ethanol (100 mM) and stored at −80 °C until use. To test the effect, XN or the solvent were dissolved in incubation media. The concentration of ethanol in incubation media was less than 0.1%. Determination of XN stability (5 and 20 μM) when dissolved in culture medium and incubated at 37 °C for 24, 48, and 72 h was determined by HPLC-DAD as described previously (*28*).

Cell Culture. 3T3-L1 preadipocytes were obtained from the American type Culture Collection, through LGC Promochem (Barcelona, Spain) and were maintained in a humidified atmosphere of 5% CO_2−95% air. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with phenol red and supplemented with 2.5 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L $NaHCO_3$, 10% heat-inactivated fetal bovine serum (45 °C, 30 min), 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 μg/mL amphotericin B. At 80% confluence, culture was split 1:3 by incubating the cells with trypsin solution (37 °C, 5 min) and subcultured in 22.1 cm^2 polystyrene culture plates. For studies in adipocytes, differentiation was induced 2 days after cells reached full confluence (day 0) with DMEM supplemented as described above and containing 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), dexamethasone (0.25 μM), and insulin (10 μg/ml). After 2 days, the differentiation cocktail was removed and the culture medium changed to original DMEM, being renewed every 2−3 days. Adipocytes were used at days 7−8 when approximately 80% of control cells were differentiated.

Cell Differentiation. Preadipocytes were seeded (4×10^4 cells/well, 1.91 cm^2 culture plates) in wells precoated with 0.2% gelatine. Cells were treated with XN (from 0.01 to 20 μM) in different periods during cellular differentiation (**Figure 2A**). Seven days after the beginning of differentiation, the culture medium was removed, and cells were washed with PBS and fixed with *p*-formaldehyde (4% in PBS) for 1 h at room temperature. The cells were stained with 0.3% oil red O solution in isopropanol:distilled water (3:2). The culture plate was washed 4 times with distilled water and air-dried. Oil red O in the cells was dissolved in 200 μL of isopropanol, and the absorbance measured at 492 nm with reference at 650 nm. For visualization at the microscope, the cells were cultured on glass coverslips precoated with 0.2% gelatine in 24-well culture plates, and the same treatment and staining procedure were performed. Coverslips were removed from the culture plates, mounted on glass slides, and observed under the microscope (Nikon 50i, Nikon, Japan) at 400× magnification.

RNA and Protein Extraction. Adipocytes were treated with XN (treatment scheme A) or vehicle dissolved in the culture medium. In the case of preadipocytes, 24 h after seeding, cells were treated for another 24 h with the compound. Total RNA and protein were extracted using Tripure isolation reagent, according to the manufacturer's instructions. Extracted RNA was dissolved in RNase-free water, proteins were dissolved in 0.1% sodium dodecylsulfate solution, and samples were stored at −80 °C.

RT-PCR. Five micrograms of RNA were used as template for cDNA production through the incubation with reverse transcriptase (Reverase, Bioron GmbH) for 1 h at 45 °C, in 10 μM random hexamers, 0.375 mM per dNTP, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol, and 40 units RNase inhibitor (RNaseOUT™, Gibco BRL), followed by 10 min at 95 °C to inactivate the enzyme. Samples were incubated for 30 min at 37 °C with 0.1 mg/mL RNase A (Sigma). PCR amplification was performed in the presence of 1.2 mM $MgCl_2$, 0.5 mM each primer, 0.2 mM dNTPs and 2 U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron GmbH), and 2 μL of RT product, in a final volume of 25 μL. Simultaneous amplification of the target gene with the invariant housekeeping gene GAPDH was performed. Primer sequences for PPARγ were TGG GGA TGT CTC ACA ATG CCA (sense) and TTC CTG TCA AGA TCG CCC TCG (antisense) and for GAPDH primer sequences were AGT ATG ATG ACA TCA AGA AGG (sense) and ATG GTA TTC AAG AGA GTA GGG (antisense). Expected sizes of PCR products were 200 and 421 base pairs, respectively. Amplification started with denaturation at 94 °C for 1 min and 30 cycles of denaturation at 92 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 °C for 50 s, and a final elongation at 72 °C for 5 min. PCR products were visualized on a 1.6% agarose gel with ethidium bromide staining. The expression of each transcript was normalized to the expression of GAPDH of the same sample and compared using Gel Pro Analyzer software.

Cell Proliferation. *Meaurement of Whole Culture Protein.* Cells were seeded (8×10^3 cells/well) in 96-well plates, and 24 h later XN was added to the culture in different concentrations (from 0.1 to 50 μM) for 24, 48, and 72 h. At the end of each experiment, the cells were fixed by addition of 25 μL of ice-cold 50% (w/v) trichloroacetic acid and incubated for 1 h at 4 °C. Cells were then gently washed 4 times with distilled water, and plates were air-dried before staining for 15 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid as described (*29*). SRB was removed, and cultures were quickly rinsed 5 times with 1% acetic acid to remove unbound dye. After drying, the bound dye was solubilized with 150 μL of Tris-HCl (10 mM, pH 10.5), and the absorbance was determined at 550 nm with reference at 650 nm on a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

KI67 Immunocytochemistry. Preadipocytes were seeded (4×10^4 cells/well; 1.91 cm^2 culture plates) on glass coverslips precoated with 0.2% gelatine. After 24 h incubation, cells were treated with XN for 24 h. After treatment, cells were fixed with 4% (w/v) *p*-formaldehyde solution and blocked with 4% (w/v) BSA in phosphate buffered solution (PBS, pH 7.4). Cells were then incubated with goat polyclonal immunoglobulin G anti-Ki67 (diluted 1:50 in BSA in PBS) followed by donkey antigoat immunoglobulin G-FITC (diluted 1:200 in BSA in PBS). Cell nuclei were counterstained with DAPI (1 μg/ml in methanol). Coverslips were mounted on glass slides and blindly observed at the fluorescence microscope (Nikon 50i, Nikon, Japan) at 400× magnification. Proliferating cells were counted in five randomly chosen optical fields. Proliferation was expressed as the percentage of Ki67-labeled cells (proliferating) over total cell number (nuclei with DAPI labeling).

TUNEL Assay. Preadipocytes were seeded on glass coverslips precoated with 0.2% gelatine. To evaluate apoptosis in preadipocytes,

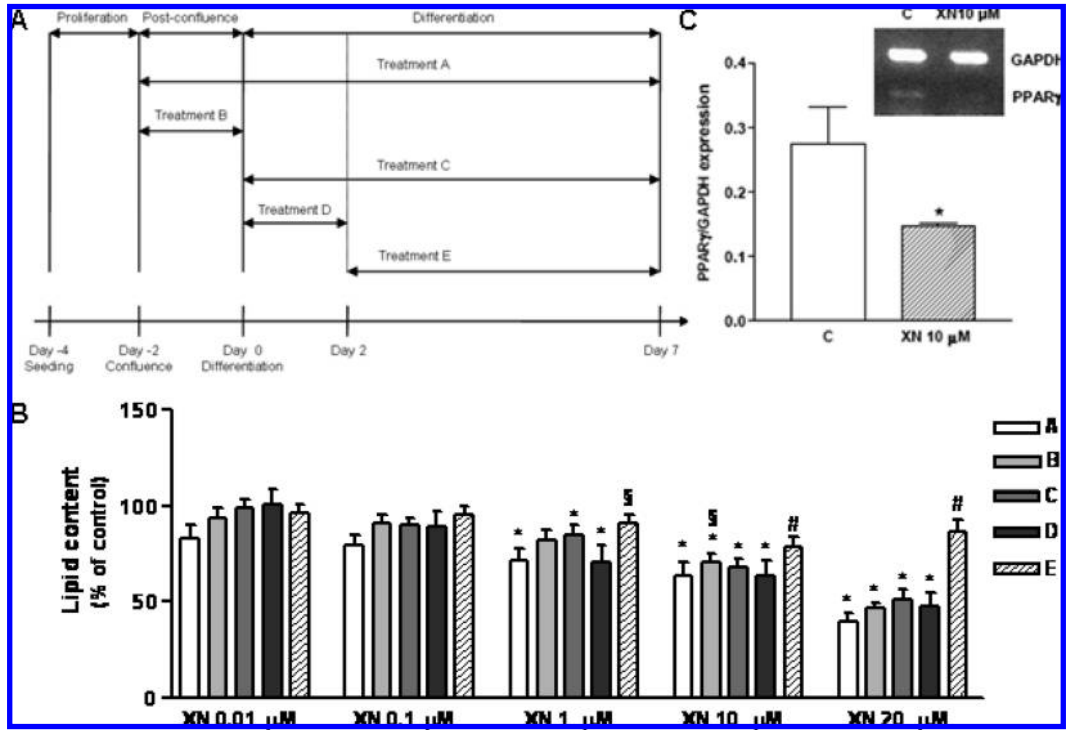

Figure 2. (**A**) Schemes of 3T3-L1 cell treatment with xanthohumol (XN). Treatment A: from confluence until seven days after the beginning of the differentiation protocol. Treatment B: from confluence until the beginning of differentiation protocol. Treatment C: from the beginning of differentiation protocol until day 7. Treatment D: only during the addition of differentiation cocktail. Treatment E: from after the addition of differentiation cocktail until day 7. (**B**) Effect of XN in different stages of preadipocyte differentiation. Preadipocytes were confluent at day −2, and at day 0 the differentiation cocktail (0.5 mM IBMX, 0.25 μM dexamethasone, and 10 μg/mL of insulin) was added. Two days later, cells were placed with fresh medium, and seven days after the beginning of differentiation cells were fixed and lipid content assessed after oil red O staining. Results are presented as mean ± SEM of at least three independent experiments performed in triplicate. *$p < 0.05$ vs respective control; §$p < 0.05$ vs treatment A and D; #$p < 0.05$ vs treatment A−D. (**C**) PPARγ expression in adipocytes treated with XN or vehicle. Expression was determined in differentiated cells after treatment and the optical density of bands estimated using Gel Pro Analyzer software. Results are presented as mean ± SEM of triplicate determinations. *$p < 0.05$ vs control.

after 24 h of culture, cells were treated with XN (5 μM) or vehicle (ethanol 0.1%) for 24 h. For studies with adipocytes, preadipocytes were differentiated as described above and at day 7 of differentiation were treated for 24 h with XN or vehicle. Apoptosis was determined by TUNEL according to the instructions of the producer. Briefly, cells were fixed with 4% *p*-formaldehyde solution in PBS for 30 min, permeabilized, and incubated with fluorescein isothiocyanate-conjugated dUTP for 1 h. DAPI was used to stain total nuclei. Coverslips were mounted on glass slides and visualized under a fluorescence microscope (Nikon 50i, Nikon, Japan). Fluorescein-labeled and DAPI-labeled cells were counted in five randomly chosen optical fields per slide, and the number of apoptotic cells is presented as a percentage of total cells.

NF-κB Activation. NF-κB activation was measured in total proteins extracted from preadipocytes or adipocytes treated as described above, and measurement of NF-κB was performed following the instructions of the manufacturer. In brief, 20 μg of proteins was incubated with a biotinylated oligonuceotide containing the NF-κB consensus site and immobilized on a streptavidin-coated 96-well plate with immobilized site. Sample wells were incubated with NF-κB primary antibody (p50 subunit), followed by incubation with HRP-conjugated secondary antibody. Quantification was performed at 450 nm with reference at 650 nm. Determinations were carried out in duplicate in three separate experiments.

Statistical Analysis. Results are arithmetic means and standard error of mean (SEM). IC_{50}s are geometric means with respective confidence intervals (CI) at 95%. Differences between two groups were evaluated using Student's *t* test whereas to determine statistical differences between more than two groups one way analysis of variance was used followed by Bonferroni's test. When the variances of the means differed significantly, nonparametric tests were applied. Differences between means were considered significant when $p < 0.05$.

RESULTS

XN Stability. Two different culture media were prepared, one with 5 μM and another with 20 μM of XN. These media were incubated at 37 °C for 24, 48, and 72 h, and XN concentration in the media was determined after incubation. There was no significant difference between XN concentration before and after incubation. After 24, 48, and 72 h of incubation, the concentration of XN found in culture medium was 88.6 ± 2.7%, 90.7 ± 1.9%, and 107.3 ± 3.9%, respectively, of that of culture medium with 5 μM XN without incubation. For the concentration of 20 μM, XN stability was 94.9 ± 1.9%, 98.1 ± 1.6%, and 100.1 ± 1.5%, respectively, of that of the culture medium containing the same concentration of XN but without being subject to incubation at 37 °C.

XN Decreased Differentiation. The effect of XN treatment on the lipid content of adipocytes, treated during different differentiation stages, is displayed in **Figure 2**. A reduction in lipid content of cells was found for all treatment schemes, except for scheme E, when cells were treated with XN only after the induction of differentiation and removal of the differentiation cocktail (**Figure 2B**). For the remaining treatments, the effect of XN on lipid accumulation was concentration-dependent. The reduction of lipid accumulation was most evident for treatments that consisted of incubating the 3T3-L1 cells from confluence (day −2) until day 2 of differentiation or later. For treatment A, there was a marked difference from control or treatment E, incubation with 20 μM of XN having reduced lipid accumulation to 40% of control. In treatment B (from day −2 to day 0) and treatment D (from day 0 to day 2 of differentiation) the lipid

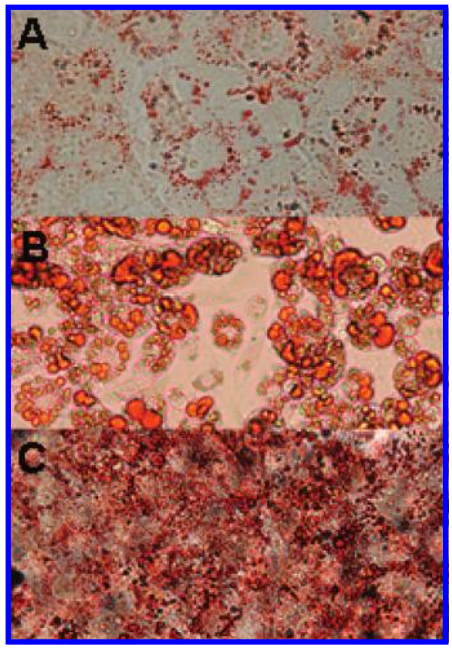

Figure 3. Lipid content of adipocytes evidenced by oil red O staining. (**A**) Control cells at day 0, (**B**) control cells at day 7 of differentiation, and (**C**) adipocytes treated (treatment A) with xanthohumol (XN, 20 μM) at day 7 of differentiation (400× magnification).

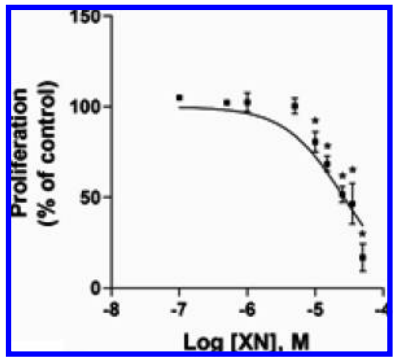

Figure 4. Effect of xantohumol (XN) in preadipocyte proliferation. Proliferation was estimated from the measurement of cell culture proteins with sulforhodamine B, after the treatment with different concentrations of XN (0.1−50 μM) for 24 h. Results are expressed as mean ± SEM of at least three independent experiments carried out in triplicate. *$p < 0.05$ vs control.

content was significantly decreased to approximately 47% of control. The effect of XN upon differentiation was confirmed by the finding of decreased PPARγ expression after treatment (scheme A) with XN (**Figure 2C**). Morphological observation of differentiated cells with or without XN treatment did also reveal a clear decrease in lipid droplet size (**Figure 3**A−C).

XN Reduced Preadipocyte Proliferation. As shown in **Figure 4**, XN reduced whole culture protein content, an indirect indicator of cell proliferation. This was evident for all incubation times with XN, and the effect was concentration-dependent. From 10 μM onward, the difference in proliferation rates of cells was significant when compared with control. At 50 μM, XN treatment for 48 and 72 h of incubation, cytotoxicity was observed. IC_{50}s for the effect of XN on proliferation were 26 μM, 12 μM, and 17 μM after 24, 48, and 72 h of incubation respectively. Evaluation of the effect of XN on cell proliferation by Ki67 immunocytochemisty showed that, after 24 h of treatment with 5 μM of XN, the percentage of Ki67-expressing cells decreased. Whereas in vehicle-treated cells 8.59 ± 0.71% of all cells expressed Ki67, in cells treated with 5 μM XN only 3.11 ± 0.02% of all cells were expressing this marker (**Figure 5**). No significant difference was observed in the percentage of Ki67 immunostained cells after incubation with 1 μM XN in comparison to control.

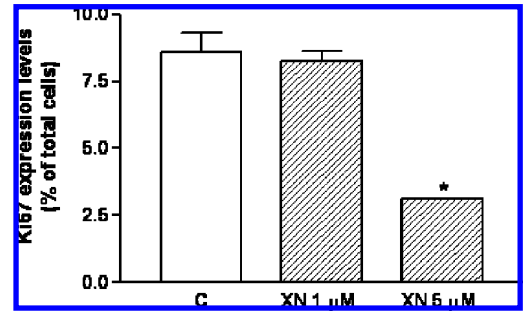

Figure 5. Effect of xanthohumol (XN) on Ki67 expression in preadipocytes. Cells were treated for 24 h with XN or the vehicle (C, 0.1% ethanol). Results are presented as mean ± SEM of at least three independent experiments performed in triplicate. *$p < 0.05$ vs control.

XN Increased Apoptosis. After treatment with 5 μM XN, apoptosis was increased both in preadipocytes and adipocytes (**Figure 6**). In preadipocytes (**Figure 6A**), apoptosis was increased about 2 times in comparison to control cells (3.77 ± 0.51 compared to 1.35 ± 0.22 in controls). In differentiated cells treated for 24 h with 5 μM of XN (**Figure 6B**), apoptosis was more markedly increased, being about 5 times higher than in control cells (23.2 ± 2.35 compared to 3.72 ± 1.05 in controls). Apart from fluorescein labeling of dUTP, apoptotic cells did also show signs of chromatin condensation (**Figure 6C**).

NF-κB Activation in XN-Treated Preadipocytes. XN treatment (10 μM) of preadipocytes resulted in a significant increase in NF-κB p50 subunit activity (to 113.9 ± 3.6% of control) (**Figure 7A**). In the case of adipocytes, no effect on the transcription factor activity was detected (**Figure 7B**).

DISCUSSION

In this work, we describe the modulation of 3T3-L1 preadipocyte differentiation by the prenylflavonoid from hop, xanthohumol. Although a similar observation has been reported while this manuscript was in preparation (*26*), our approach allowed us to determine in which phase of the adipogenic differentiation the effect of XN was most relevant, which may be important for the clarification of its mechanism of action. We have shown that XN reduced lipid accumulation most strongly when cells were incubated with the compound 24 h after plating, until full differentiation (treatment A), but on the contrary, it had little or no effect when added to the culture medium after the adipogenic challenge (treatment E). However, the presence of xanthohumol only during the time of treatment with the differentiation medium (treatment D) did affect cell lipid content significantly, explaining part of the effect observed with treatment A. Another hint in favor of the effect of XN occurring early in the differentiation process is the evidence that by incubating postconfluent cells with the compound (treatment B), a marked reduction of adipogenesis also occurs.

The effects on differentiation after incubation in these two different stages reveal a multilevel action of XN. After confluence, preadipocytes undergo cell-to-cell contact inhibition of growth which is considered the first step of preadipocytes toward differentiation (*7*). The transition of preadipocytes to adipocytes requires the combined action of cell-cycle and differentiation

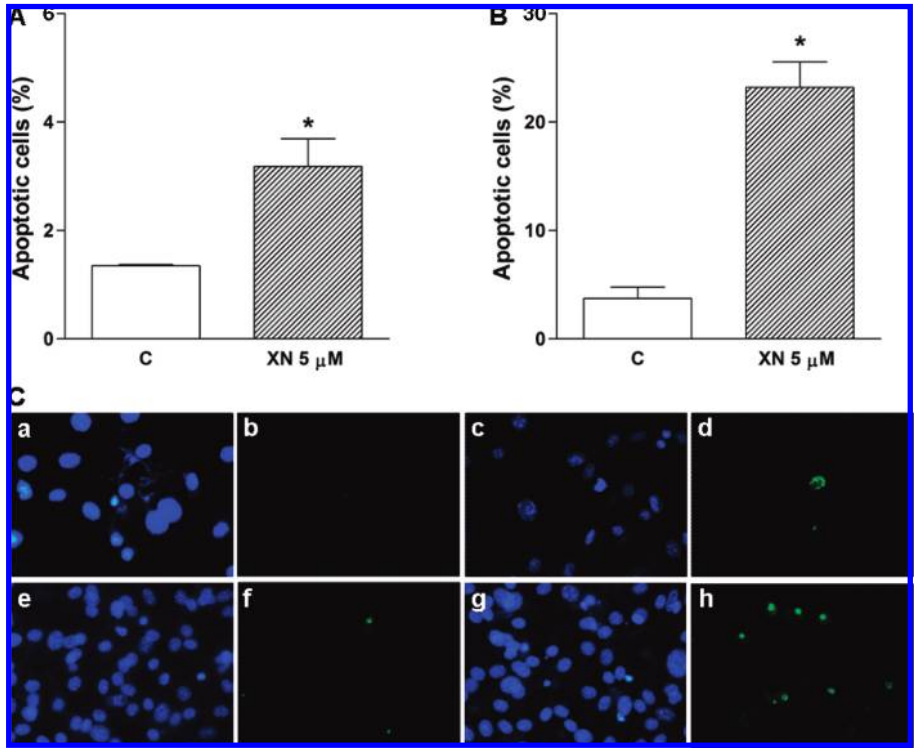

Figure 6. Effect of xanthohumol (XN) in preadipocyte (**A**) and adipocyte (**B**) apoptosis. Preadipocytes were treated 24 h after seeding, and adipocytes were treated on day 7 with XN or vehicle (C, 0.1% ethanol) for 24 h. Results are presented as mean ± SEM of at least three independent experiments performed in triplicate. *$p < 0.05$ vs respective control. (**C**) Fluorescence micrographs of apoptotic cells. Control (b) and XN (5 μM)-treated (d) apoptotic preadipocytes. Control (f) and XN (5 μM)-treated (h) apoptotic adipocytes. The correspondent total cell nuclei stained with DAPI appear in blue (a, c, e, g). All photographs were obtained under a 400× magnification.

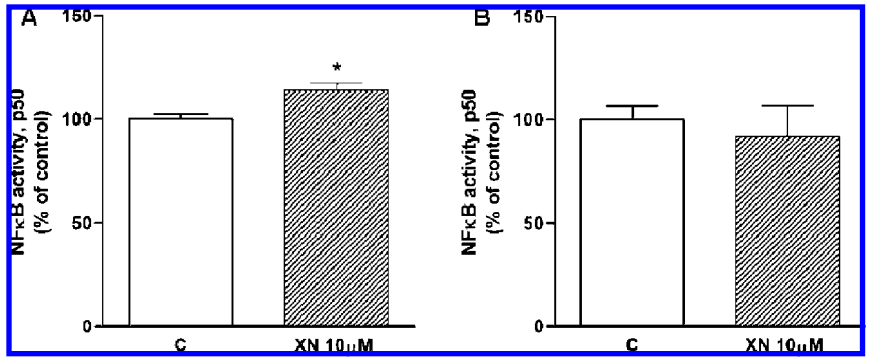

Figure 7. Effect of xanthohumol (XN) in preadipocyte (**A**) and adipocyte (**B**) NF-κB activity. Preadipocytes were treated 24 h after seeding, and adipocytes were treated on day 7 with XN or vehicle (C, 0.1% ethanol) for 24 h. Determinations of subunit p50 transcriptional activity were made in total protein extracts. Results are presented as mean ± SEM of three independent experiments performed in duplicate. *$p < 0.05$ vs respective control.

factors which are triggered synchronously and accelerated by the addition of the adipogenic medium to the cells (*7*). Among the best studied regulators of the adipogenic process is PPARγ, a nuclear receptor expressed at very low levels in preadipocytes that is activated during the adipogenic process and coordinates the expression of a multitude of proteins related to the functional properties of mature adipocytes (*3, 30*). PPARγ transcriptional activity has been demonstrated to be an essential factor for adipogenesis both in vivo and in vitro (*31*). In line with the results obtained for cell lipid content, we have shown that treatment of adipocytes with 10 μM XN reduced by half the expression of PPARγ. This is in agreement with previous results (*26*) where a decrease in PPARγ expression after treatment with 25 μM XN is also shown, along with the inhibition of C/EBPα and decreased differentiation markers diacylglycerol transferase and adipocyte protein 2.

Although PPARγ modulation is an important mechanism contributing to the decrease in cell lipid content and differentiation, it should not be the sole mechanism operated by XN, as the compound begins to act before the addition of differentiation agents. Adipogenesis involves several cell divisions: when cells enter the differentiation program postconfluent mitosis with DNA replication and clonal expansion take place, resulting in cell doubling (*7*). Cell cycle regulating properties of XN have been often described in different experimental cell lines, with the involvement of many different proposed mechanisms (*19, 24, 29*). In the present work, we show that, in 3T3-L1 cells, this modulation of proliferation occurs as well. This is shown in incubations with XN for 24, 48, and 72 h in exponential cell growth conditions, i.e., in subconfluence, to avoid the contact inhibition effect. The IC_{50}s calculated for XN are between 26 and 12 μM, within the same range of concentrations which

produced significant effects in adipocyte differentiation. Adding to this measurement of whole cell culture protein, we have also found that the expression of the proliferation-specific nuclear protein Ki67 is decreased by the treatment with 5 μM XN for 24 h. Although at this concentration no significant effect was observed with SRB measurements, it is likely that the expression of Ki67 precedes the decrease in cell culture protein. Inhibition of DNA polymerase activity by xanthohumol has been previously proposed as a mechanism for its cell cycle-regulating actions (*19*). In fact, in previous studies it has been shown that XN treatment significantly decreases [^{3}H]-thymidine incorporation into DNA in several cancer cells lines (*23, 24, 29*). This is in good agreement with DNA replication inhibition, and might well explain an impairment of postconfluent mitosis in 3T3-L1, with repercussion on adipogenic differentiation.

Comparison of the cellular content of the culture before and after treatment with XN also allowed determining that for 48 and 72 h of treatment the protein content is lower when compared to the beginning of treatment denoting not only inhibition of growth but also cytotoxicity. Given the described apoptotic properties of XN (*23, 24, 32*), we hypothesized that this could also be occurring in this cell line. TUNEL revealed that preadipocytes, after 24 h of treatment with XN, displayed signs of apoptosis. Apoptotic effects of XN have also been found in mature adipocytes, and the cellular pathways have been further explored elsewhere (*26*). These involve the increase of cytoplasmic cytochrome *c* and cleaved poly(ADP-ribose) polymerase and activation of the effector caspase-3/7. Thus, apoptosis is implicated both in the decrease in cell culture protein and in the decrease of lipid accumulation. Although the increase in apoptosis and inhibition of proliferation would suffice to reduce the number of adipocytes through the decrease in cell precursors, interference with the adipogenic process becomes clear by the downregulation of PPARγ expression by XN.

NFκB is a nuclear transcription factor involved in the regulation of transcription of a large number of molecules. It has been shown to participate in numerous cell events like proliferation, apoptosis, inflammation, and stress (*33*). Lately, the involvement of NFκB activity in the regulation of PPARγ expression has been shown. Upon NFκB activation by TNFα, 3T3-L1 cells express lower levels of PPARγ and differentiation is inhibited (*34*). Our results show an ability of XN to activate NFκB, and this might contribute to the effect observed in PPARγ expression and differentiation. The activation of NFκB by XN also suggests the implication of this nuclear transcription factor as an effector in the modulation of proliferation and apoptosis herein observed. However, it is necessary to bear in mind that, given the small extension of NFκB activity increase in preadipocytes and absence of effect in adipocytes, it is very likely that other mechanisms are involved in the regulation of differentiation, proliferation, and apoptosis.

XN shares similar biological effects on preadipocytes and adipocytes with other polyphenols, such as epigallocatechin-3-gallate, genistein, procyanidins, and resveratrol, which have also been shown to decrease preadipocyte proliferation, clonal expansion of adipocytes, increase apoptosis in mature adipocytes and precursors, and inhibit differentiation. Mechanisms for their actions were in many cases closely related to their antioxidant potential, resulting in the modulation of mitogen-activated protein kinases, involved in redox signaling and control of cell cycle and cell death (*14*). Therefore, this is yet another possible mechanism underlying the actions of XN observed in the present study.

Altogether, our results demonstrate that XN is able to influence the number of fat accumulating cells, through the reduction of proliferation, increase of apoptosis, and impairment of differentiation. The reduction of white adipose tissue weight by XN has been previously shown in KK-Ay mice treated with an XN-supplemented diet (*25*). Apart from alterations in AT cellularity, the modulation of lipid metabolism may also be involved. In preliminary experiments, we found that XN did not alter lipogenesis, measured by glycerol-3-phosphate dehydrogenase activity and lipolysis stimulated by isoproterenol. On the other hand, it was able to reduce basal glycerol release, a measure of lipolysis, in 3T3-L1 cells (results not shown).

Our in vitro results cannot be extrapolated to in vivo situations of administration of the compound. It has been described that after oral administration XN is extensively metabolized in the intestine and liver to form, among others, XN conjugates (*35*−*38*). However, although the concentrations and time of contact with the cells used in this work may not mimic what is achievable after ingestion of XN-rich food sources, there are commercially available hop extracts that supply high concentrations of XN and the food industry is making an effort to fortify foods in XN, contributing to higher ingested amounts (*15*). Furthermore, the lipophilicity of XN raises the possibility that this compound be accumulated in the adipose tissue where it may reach higher concentration and exert local effects.

It is worth emphasizing that a reduction of adipocyte number may not be favorable in a setting of positive energy balance, as AT capacity to buffer excess energy intake may be easily overcome, resulting in lipotoxicity from lipid overload accumulation in organs such as the pancreas, the liver, and the muscle (*10*). In consequence of reduced adipocyte number, the scarcity of competent cells to accumulate fat may lead to adipocyte hypertrophy. The deleterious consequences of adipocyte hypertrophy have been shown in type 2 diabetes and obesity-related inflammation, as large adipocyte size is correlated to insulin resistance (*6*), higher circulating pro-inflammatory factors, and lower circulating adiponectin levels. Futhermore, adipocyte oversizing and liability to rupture (*9*) have been implicated in the presence of macrophage infiltration in the adipose tissue (*39*). Therefore, adipose tissue plasticity to accommodate surplus energy seems a key feature to avoid metabolic disturbances (*10*). Thus, the meaning of the effects here described for XN requires further elucidation. In vivo exploration of the effects of XN on the adipose tissue, namely on an energy excess setting, would help clarify this point.

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