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Exogenous nucleosides modulate expression and activity of transcription factors in Caco-2 cells☆,☆☆

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Abstract

Dietary nucleotides (NTs) have an important role in cellular and humoral immunity, intestinal growth, differentiation and recovery from tissue damage. Nucleosides (NSs) are the best-absorbed chemical form of NTs in the intestinal epithelium. The aim of this study was to evaluate the effects of NSs on the activity and expression of multiple transcription factors (TFs) in Caco-2 cells, as a possible molecular mechanism by which NSs modulate gene expression in human intestinal cells. The effects of NS-supplemented media on human Caco-2 cell proliferation, viability, protein and RNA concentration were determined, and the activity and expression profiles of multiple TFs were analyzed by using an array-based technology. Exogenous NSs did not affect Caco-2 cell proliferation or viability but increased the protein content in cytoplasm and nucleus and the nuclear protein/RNA ratio. The addition of NSs to the media increased the expression and activity of the TFs *CCAAT displacement protein* (CUX1), *v-ets avian erythroblastosis virus E26 oncogene homolog 1* (ETS1) and *SMAD family member 2*. In contrast, NS addition decreased the expression and activity of the general *upstream stimulatory factor 1* (USF1), *glucocorticoid receptor* (NR3C1), NFKB and *tumor protein p53*. In conclusion, our results suggest that exogenous NSs affect the expression and activity of several TFs involved in cell growth, differentiation, apoptosis, immune response and inflammation.

Keywords: Exogenous nucleosides; Transcription factors; Caco-2 cells; Intestine; Gene expression

1. Introduction

Nucleosides (NSs) and nucleotides (NTs) are naturally present in all foods as individual monomers and nucleic acids and they are absorbed by intestinal epithelium mainly in the form of NSs [1]. They participate in nearly all biochemical processes of the organism. Supplementation of diets with NTs or NSs is common in infant formulas and enteral nutrition because of their positive effects in conditions of rapid growth, such as the neonatal period and tissue repair after surgery or injury [2–5]. They serve as building blocks for RNA and DNA synthesis and have important effects on the growth and differentiation of cells, especially those with a rapid turnover, such as the immune system and the gastrointestinal tract [6–11]. Moreover, intestinal homeostasis may depend on exogenous supplies of NTs because the *de novo* NT synthesis seems to be limited. Therefore, NTs can be considered as "semi-essential" nutrients, especially during periods of starvation, injury or stress [12–16].

Dietary NTs have been shown to modulate many genes, including intestinal cytokines and genes involved in NT metabolism and apoptosis [17–20]. However, little is known about the molecular mechanisms by which dietary NTs modulate gene expression. It has been postulated that dietary NTs may influence protein biosynthesis

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by regulating the intracellular NT pool [21]. In addition, signal transduction mediated by the interaction of exogenous NSs and their cell membrane receptors may contribute to modulating gene expression [3].

Eukaryotic gene expression is mainly controlled by transcription factors (TFs) through their interaction with specific regulatory DNA elements; TFs modulate the frequency and efficiency of transcriptional initiation and/or RNA elongation. The expression and activity of TFs are often regulated in a specific cell type in relation to the cell cycle. Regulation of TFs may also be mediated by interactions with other molecules, including NTs. However, the role of NTs in TF expression and activity has not been systematically investigated.

The objective of the present study was to evaluate the effects of exogenous NSs on the activity and expression of multiple TFs. For this purpose, cultured human colon adenocarcinoma cells (Caco-2) were grown for 3 days in NS-supplemented medium (+NS) and compared with cells grown in medium not supplemented with NSs (−NS). An array-based technology was used to simultaneously profile TF activities and expression.

2. Materials and methods

2.1. Materials

All chemicals (molecular biology grade) were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise noted. Labware and cell culture components were purchased from Corning (Madrid, Spain) and Invitrogen (Life Technologies, Barcelona, Spain), respectively. The TranSignal Human TF cDNA and Protein/DNA Arrays kits were purchased from Panomics, Inc. (Redwood City, CA, USA).

2.2. Cell line and culture conditions

The human colorectal adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (ATCC HTB-37, Manassas, VA). Cells were maintained at 37°C in an atmosphere containing 5% CO_2, in DMEM with Earle's salts adjusted to contain 1.5 g/L sodium bicarbonate, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum (FBS). The culture medium was changed twice weekly during maintenance.

2.3. NS treatment and experimental groups

A mixture containing equimolecular concentrations of thymidine, uridine, cytidine, guanosine and adenosine was added to the culture medium at a final concentration of 50 μM of each NS, which is within the range of physiological values, as judged by the NT content provided by foods such as human milk [22] and the capacity of absorption in the intestine. NSs are absorbed mainly in the proximal intestine; nevertheless, we used colonic Caco-2 cells as a model. It is known that Caco-2 cells express equilibrative NT transporters but not the concentrative transporters CNT1 and CNT2 [23] and it is unclear if they express CNT3 [24]. CNT3 is present in the intestine, with maximal expression in duodenum and to a low extent in all sections of colon [25]. Thus, under physiological conditions, the concentrative capacity of CNT3 would probably allow transient intracellular NS concentrations similar to those used in this study.

Confluent dishes of Caco-2 cells were trypsinized, and 1×10^5 cells/cm^2 were plated in six-well tissue culture plates with the above-described medium. Twenty-four hours later, the medium was replaced with FBS-free medium and NSs were added (+NS). Control dishes were identical cultures that did not contain NSs (−NS). Media were replaced every 24 h. Cells with NSs and control cells were maintained in culture for 72 h and reached ~70% confluency in all cases. After treatments, the supernatant from each well was collected and centrifuged at 3000×*g*. Cell layers were harvested by trypsinization, centrifuged at 3000×*g* and washed with PBS.

2.4. Cell proliferation, viability and cytotoxicity assays

Cell proliferation and viability were determined by counting all cells and trypan blue-excluding cells, respectively. Cells in solution were incubated with 0.4% trypan blue (1:1) and counted using a hemocytometer. The cytotoxic effect was determined by measuring lactate dehydrogenase (LDH) activity, an index of cell injury, in the supernatant from each well. The assay was performed in an optically clear 96-well flat-bottomed microtiter plate; 30 μl of centrifuged medium was incubated with 80 μl of β-NADH (1.45 mM) for 5 min at 37°C. The kinetic determination of LDH was initiated by adding 20 μl of sodium pyruvate (25 mM). The initial oxidation rate of NADH is proportional to the LDH catalytic activity. LDH activity in the sample was calculated by measuring the per time absorbance decrease at 340 nm.

2.5. Nuclear and cytoplasmic protein extracts

Cells were harvested and washed with PBS followed by washing in 5 cellular volumes of hypotonic sample buffer composed of 10 mM Hepes, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol. Pellets were resuspended in 3 cellular volumes of the hypotonic sample buffer, homogenized by pipetting and kept on ice for 10 min before centrifugation at 3000×*g* for 15 min. Supernatants corresponded to the cytoplasmic extract fractions, whereas pellets corresponded to the nuclear fractions. Nuclear protein extracts were obtained by osmotic shock of the nuclear fractions, which were resuspended in 0.5 nuclear volumes of low-salt sample buffer (20 mM Hepes, pH 7.6, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) followed by addition of an equal volume of high-salt sample buffer (low-salt sample buffer supplemented with 1.2 M KCl). Samples were centrifuged at 14,000×*g* for 30 min, and the supernatants, corresponding to the nuclear extract fractions, were dialyzed in 20 mM Hepes, pH 7.6, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol. The Bicinchoninic Acid Protein Assay Kit was used to measure total protein from culture supernatants and nuclear and cellular protein extracts.

2.6. RNA isolation

Cells were harvested and washed with PBS followed by lysis and homogenization in the presence of a highly denaturing guanidine thiocyanate buffer, according to the manufacturer's protocol. Ethanol was added to the lysate to provide ideal binding conditions. The sample was then applied to an RNeasy Mini spin column (Qiagen, Hilden, Germany), and contaminants were washed away. RNA was eluted in 30 μl water.

2.7. Expression pattern of multiple TFs

The Panomics TranSignal Human TF cDNA Array was used to profile the TF expression according to the manufacturer's instructions. This array is spotted with DNA encoding 119 different TFs. Each TF is represented twice in the array. Briefly, RNA isolated from the samples was reverse-transcribed using the TranSignal probe mix to create biotin-labeled cDNA probes. Then, these biotin-labeled probes were hybridized to TranSignal array membranes, and results were visualized using chemiluminiscent detection. The density of each spot was analyzed with ImageJ software (version 1.41, NIH, USA). The arrays were normalized with respect to the mean value of the housekeeping genes β-actin, GAPDH and/or ubiquitin spotted on the array, which served as the baseline. After normalizing each array, spot intensities on separate arrays were compared (see Section 2.9).

2.8. Functional analysis of TFs

To profile the activities of multiple TFs simultaneously, we used a Panomics TranSignal Protein/DNA Arrays kit, following the manufacturer's instructions. This array is spotted with 54 different consensus binding sequences, present in duplicates. Briefly, the nuclear extract obtained from cells cultured in the presence or absence of NSs was pre-incubated with a set of biotin-labeled DNA oligonucleotides corresponding to TF binding sites in order to allow the formation of DNA/protein complexes. The protein/DNA complexes were separated from the free probes. Then, the probes in the complexes were extracted and hybridized to the TranSignal Array. Results were visualized using chemiluminiscent detection. The density of each spot was analyzed using ImageJ software (version 1.41, NIH, USA). After normalizing each array, spot intensities on separate arrays were compared (see Section 2.9).

2.9. Statistical analysis

Measurements of protein and RNA concentrations were done at least in triplicate in three independent experiments. We compared two groups of arrays, one from samples with NS-supplemented media (+NS) and the other from control samples (−NS). For each group of arrays, we measured three arrays, corresponding to three independent experiments. All values were expressed as median and range (min–max values) except for cell proliferation (mean±S.D.).

Array statistical analysis to compare the effects of NS supplementation on expression and activities of TFs was performed by ANOVA models and *a posteriori* corrections by multiple false discovery rate (FDR) using the Partek Genomics Suite version 6.09.10 software. Normalization of cDNA TF arrays data by geometric averaging of multiple internal control genes (GADPH, β-actin and ubiquitin) was done according to Vandesompele et al. [26]. Normalization of the TF protein arrays was done using the appropriate standard dot/spots given by the array supplier. The use of these housekeeping genes for normalization was based on the fact that no significant differences were observed between their expression in the presence or absence of NTs (P=.607, P=.565 and P=.459, respectively).

The change in expression or activity level for a TF was expressed as fold change (Fc). Fc results represent the ratio of the data from NS-supplemented cells to those from control cells. Generally, a twofold or more increase or decrease in spot intensity was associated with a statistically significant difference.

Other study parameters were compared between NS-supplemented and control cultured cells using the non-parametric Mann–Whitney test. $P<.05$ was considered significant in all tests. SPSS software (version 15.0, SPSS Inc. Chicago, IL) was used for those statistical analyses.

3. Results

3.1. Cell proliferation, viability and cytotoxicity

The effect of NSs on TF expression and activity was determined by culturing human Caco-2 cells in the presence or absence of NSs for 3 days. The mean±S.D. cell count from triplicates of three independent experiments was 3.83±0.6 ($\times 10^5$) cells/ml in the NS-treated cells (+NS) versus 3.39±0.4 ($\times 10^5$) cells/ml in the control cells (−NS). Similar trypan blue exclusion (cell viability) values were obtained for the −NS and +NS treatments (data not shown). Results for LDH release (cell destruction marker), expressed in U/L as median and range (min–max) of triplicates from three independent experiments, were 8.36 (7.66–8.71) (+NS) and 9.41 (8.71–9.54) (−NS), with no significant differences between the groups.

3.2. Protein and RNA concentration

Fig. 1A shows the protein concentration of nuclear and cytoplasmic extracts (NE and CE, respectively) and the extracellular protein concentration in the supernatant (SP) of Caco-2 cells grown in the presence or absence of the NS mixture. NS addition increased the concentrations of nuclear and cytoplasmic proteins ($P<.05$) but did not affect extracellular protein concentrations. Results for protein concentration expressed in micrograms per microliter as median and range (min–max) were 5.99 (5.76–6.11) (+NS) and 5.17 (4.60–5.67) (−NS) for NE, 7.07 (7.00–7.13) (+NS) and 5.37 (5.19–6.83) (−NS) for CE and 4.64 (4.54–5.05) (+NS) and 5.09 (5.03–5.30) (−NS) for SP. The RNA concentration expressed as median and range (min–max) from triplicates of three independent experiments was significantly lower in the NS-supplemented group [686.29 (649.04–717.82) μg/mL for +NS cells vs. 769.29 (725.56–806.12) μg/mL for −NS cells, $P<.05$] (Fig. 1B) but the nuclear protein/RNA ratio was significantly higher ($P<.05$). The median value and range from triplicates of three independent experiments was 8.72 (8.51–8.87) μg nuclear protein/μg RNA for +NS cells versus 7.12 (5.71–7.37) μg nuclear protein/μg RNA for −NS cells ($P<.05$).

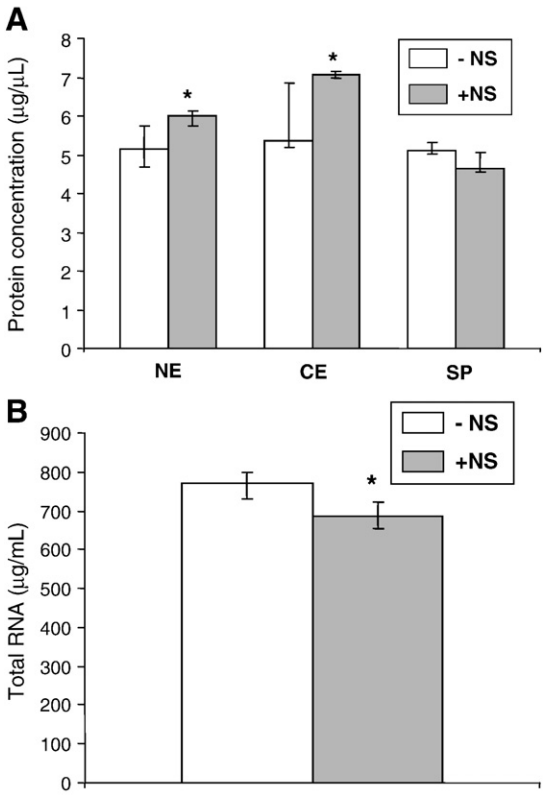

Fig. 1. Effect of exogenous NSs on protein concentration (A) in nuclear extract (NE), cytoplasmic extract (CE) and supernatant (SP) and total RNA concentration (B) obtained from human Caco-2 cells grown in media with (gray bar) or without (control, white bar) NS mixture for 3 days. Values are expressed in median±range (n=triplicates of three independent experiments). *Significant difference versus control, $P<.05$.

3.3. TF expression

Analysis of the arrays revealed that 58 out of 119 TF cDNAs spotted on the arrays were detected in our experiment (Table 1). Fig. 2 shows the hierarchical bidimensional clustering of TF transcripts differentially expressed up to $P<.1$. Array statistical analysis was performed after accurate normalization of array data by geometric averaging of multiple internal control genes (GADPH, β-actin and ubiquitin) (see Section 2.9). Most of the affected TFs were general TFs and TFs regulating cell growth, differentiation, apoptosis, immune response and inflammation. Real-time PCR was performed for two TFs that showed increased expression in the array analysis upon addition of NSs (*CUX1* and *STAT5*) and for one TF that was down-regulated (*PBX2*). These experiments confirmed the array observations in that *CUX1* and *STAT5* mRNAs were more abundant, whereas *PBX2* mRNA was less abundant (data not shown).

3.4. TF protein activity

TF activity was determined by purification of the TFs that were in an active DNA-binding conformation. Table 2 shows the effect of NS supplementation on the activity of the 24 TFs detected out of the 54 TFs spotted on the arrays. Fig. 3 shows the hierarchical bidimensional clustering of TF differential activities up to $P<.1$. Array statistical analysis was performed after accurate normalization of arrays using the appropriate standard spots given by the array supplier (see Section 2.9). As in TF gene expression, the most affected TFs were general TFs and TFs that regulate cell growth, differentiation, apoptosis, immune response and inflammation.

3.5. Activity and expression of specific TFs in Caco-2 cells in the presence of exogenous NSs

Fig. 4 compares the expression and activity of the 20 TFs detected in both types of array (mRNA and protein activity). Fig. 4A depicts the TFs whose activity and expression changes were in the same direction. In +NS cells, we found increases in activity and expression of the TFs *CCAAT displacement protein* (also known as CUX1), a potential candidate as a general repressor of developmentally regulated genes; *v-ets avian erythroblastosis virus E26 oncogene homolog 1* (*ETS1*), involved in stem cell development, cell senescence and death and tumorigenesis; and *SMAD2*, a member of the SMAD family involved in TGF signaling for cell growth and differentiation. In contrast, the expression of *SMAD4* and *SMAD5* was lower in the presence of exogenous NSs, which had no significant effect on TGFB1 activity (Table 1). On the contrary, in +NS cells, we found decreases in activity and expression of *glucocorticoid receptor NR3C1*, the general TF *upstream stimulatory factor* (USF) *USF1* and the *tumor protein p53* TF. TP53 responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair or changes in metabolism and activates *nuclear factor of kappa*

Table 1
TF expression in Caco-2 cells grown in NS supplemented media (+NS) versus non-supplemented media (−NS)

	−NS		+NS		FDR *P* value	Fc
	Median	Range	Median	Range		
Up-regulated TFs						
General TFs						
Activating transcription factor 1; ATF1	1893	320	3741	697	.006710	2.07
Activating transcription factor 2; ATF2 (CREB2)	3683	115	7410	1496	.005841	1.85
Cut-like 1 (CCAAT displacement protein); CUX1 (CDP)	124	94	1523	654	.005841	12.01
Transcription factor Sp2; SP2	3385	83	7037	674	.001052	2.00
Transcription factor Sp3; SP3	1168	237	2285	494	.012073	2.01
Cell growth and differentiation TFs						
E2F transcription factor 5, p130-binding; E2F5	291	146	1326	1020	.013494	4.73
Early growth response 1; EGR1	1099	300	4419	1211	.002784	3.95
ets variant gene 5 (ets-related molecule); ETV5	300	141	1263	81	.004879	3.95
Mothers against decapentaplegic, Drosophila, homolog of, 2 (SMAD family member 2); SMAD2	3229	1218	6143	1721	.038165	2.00
Peroxisome proliferator-activated receptor-gamma, coactivator 1 alpha; PPARGC1A (PPARγ_coact)	1360	555	3070	766	.032507	1.93
Signal transducer and activator of transcription 4; STAT4	6073	483	13,162	1449	.001120	2.05
Signal transducer and activator of transcription 5A; STAT5A	6777	201	17,371	1648	.000763	2.50
Signal transducer and activator of transcription 6 (IL-4 induced); STAT6	16,202	2300	38,335	4773	.001120	2.41
v-ets avian erythroblastosis virus E26 oncogene homolog 1; ETS1	291	12	1132	284	.002525	3.51
v-jun avian sarcoma virus 17 oncogene homolog (oncogene Jun; AP1, included); JUN (c-jun)	587	230	3040	1132	.007862	4.83
Down-regulated/repressed TFs						
General TFs						
TAF4 RNA polymerase II, TATA box-binding protein (TBP)-associated factor, 135 kDa; TAF4 (TAFII135)	40,057	4440	21,736	7608	.020537	−1.99
Upstream stimulatory factor 1; USF1	3972	1040	2172	626	.026142	−1.98
Upstream stimulatory factor 2, c-fos interacting; USF2	15,210	3091	7829	502	.010641	−1.90
Cell growth and differentiation TFs						
GATA-binding protein 1 (globin transcription factor 1); GATA1	3287	1467	1701	564	.032627	−1.98
Glucocorticoid receptor (nuclear receptor subfamily 3, group C, member 1); NR3C1 (GCCR)	3749	935	1625	108	.004071	−2.31
Mothers against decapentaplegic, Drosophila, homolog of, 4 (SMAD family member 4); SMAD4	10,114	5187	5074	1712	.040411	−2.12
Mothers against decapentaplegic, Drosophila, homolog of, 5 (SMAD family member 5); SMAD5	4714	572	2458	463	.006561	−1.96
Myogenic differentiation antigen 1 (myogenic factor D); MYOD1 (MYOD)	43,288	3238	24,377	983	.000320	−1.88
Nuclear factor erythroid 2-like 1; NFE2L1	4179	1319	2154	497	.014971	−2.02
Nuclear factor erythroid 2-like 3; NFE2L3	5915	2163	2867	729	.032507	−1.99
POU domain, class 1, transcription factor 1 (growth hormone factor 1); POU1F1 (PIT1)	10,778	4091	4494	1359	.014947	−2.56
POU domain, class 2, transcription factor 1 (octamer-binding transcription factor 1); POU2F1 (OCT1)	3676	1264	1776	616	.047252	−1.92
Pre-B-cell leukemia transcription factor 2; PBX2 (HOX12)	6940	1172	1917	658	.002784	−3.38
Pre-B-cell leukemia transcription factor interacting protein 1 (pre-b-cell leukemia interacting protein 1); PBXP1 (HPIP)	5563	3429	1614	1019	.042304	−3.32
Retinoid X receptor, alpha; RXRA	44,464	12,705	23,932	7881	.035563	−2.08
Signal transducer and activator of transcription 1, 91 kDa; STAT1	2867	908	1525	785	.047252	−2.13
Immune response and inflammation TFs						
Interferon-stimulated transcription factor 3, gamma (interferon regulatory factor 9); ISGF3 (IRF9)	29,759	3563	14,342	4116	.011958	−1.98
Nuclear factor of kappa light chain gene enhancer in B cells 2 (p52/p100); NFKB2	57,816	10,312	29,590	9076	.020537	−2.03
v-rel avian reticuloendotheliosis viral oncogene homolog A; RELA (p65 subunit)	116,224	15,117	57,390	12,032	.005841	−2.15
v-rel avian reticuloendotheliosis viral oncogene homolog B; RELB	30,156	8311	14,333	6248	.032507	−2.06
Apoptosis TF						
Tumor protein p53; TP53	15,155	3098	7189	1150	.005841	−2.09
Unaltered TFs						
General TFs						
CCAAT/enhancer binding-protein, alpha (C/EBP-alpha); CEBPA	1164	1117	500	1178	.371591	−1.96
Transcription factor AP2-alpha (activating enhancer-binding protein 2-alpha); TFAP2A (AP-2α)	8673	9615	22,020	4341	.095217*	1.98
Transcription factor Sp1; SP1	6182	1103	6406	160	.450112	1.05
Cell growth and differentiation TFs						
cAMP response element-binding protein 1; CREB1 (CREB)	4503	1325	2147	1436	.080258*	−1.85
E2F transcription factor 1; E2F1	36,207	8514	35,671	3930	.980439	1.01
Early growth response 2 (Krox-20 homolog, Drosophila); EGR2	36,894	16,310	35,152	11,413	.632192	−1.14
Early growth response 3; EGR3	4695	1931	3911	2773	.359605	−1.79
Endothelial differentiation-related factor 1 (multiprotein bridging factor 1); EDF1 (MBF1)	50,989	8574	51,105	1307	.417866	−1.04
Forkhead box H1; FOXH1 (FAST1)	3694	1462	2229	889	.056956*	−1.91
Forkhead box I1; FOXI1 (HNF-3)	10,679	5640	3496	1708	.071331*	−2.72
Nuclear factor erythroid 2, p45 subunit; NFE2	4777	2721	1111	649	.055868*	−3.14
Nuclear factor erythroid 2-like 2; NFE2L2	2564	1020	1182	1101	.166539	−2.17
Oncogene jun-B; JUNB	42,172	25,764	95,105	48,455	.173905	2.39

Table 1 (*continued*)

	−NS		+NS		FDR *P* value	Fc
	Median	Range	Median	Range		
Peroxisome proliferator-activated receptor gamma; PPARG	11,888	9380	12,162	9143	.980439	1.01
POU domain, class 4, transcription factor 1; POU4F1 (BRN3A)	49,443	44,533	80,144	73,732	.325500	1.97
Retinoic acid receptor, alpha; RARA	113,069	9390	107,805	12,653	.534782	−1.04
Signal transducer and activator of transcription 3 (acute-phase response factor); STAT3	3960	465	4344	103	.335699	1.07
Thyroid hormone receptor, alpha-1 (v-erb-a avian erythroblastic leukemia viral oncogene homolog 1); THRA (TR)	6499	846	7004	1324	.962020	1.02
v-fos FBJ murine osteosarcoma viral oncogene homolog B; FOSB	1175	436	1056	435	.976315	−1.03
v-myb avian myeloblastosis viral oncogene homolog; MYB (c-myb)	63,224	6940	58,778	11,975	.920799	−1.02
v-myc avian myelocytomatosis viral oncogene homolog (c-myc responsive region); MYC (c-Myc)	4948	1200	2286	1356	.051951*	−1.91
Immune response and inflammation TFs						
Interferon regulatory factor 2; IRF2	4003	1557	4156	1545	.980439	−1.01

Values are medians and range of arbitrary units relative to the internal control GAPDH (n=3 independent experiments), determined by image analysis of the spots. Array statistical analysis to compare the effects of NS supplementation and samples was performed by ANOVA models and *a posteriori* corrections by multiple FDR. Accurate normalization of array data by geometric averaging of multiple internal control genes (GADPH, β-actin and ubiquitin) was previously done. Relative over- or underexpression of the TFs from Caco-2 cells incubated in supplemented NS media (+NS) in comparison with control cells (−NS) is expressed as fold change (Fc). Fc results represent the ratio of the data from NS-supplemented cells to those from control cells after normalization with the combination of the three internal control genes and averaging of different scores. Significant differences with respect to control (−NS) are represented by *P* values corrected by FDR <.05. * Statistical trend with respect to control (−NS) cells (P<.1).

light chain gene enhancer in B cells (NFKB), a TF linked to inflammatory events that can protect from or contribute to apoptosis [27], whose activity and expression were decreased in +NS cells.

Fig. 4B shows the TFs unaffected by NSs and the TFs whose changes in expression and activity were not in the same direction. Thus, NS addition had no effect on the activity or expression of *E2F transcription factor 1* (*E2F1*), while *E2F5*, which contributes to the regulation of early G1 events [28], was overexpressed (Table 1). The expression and activity of the enhancer of basal transcriptional activity *transcription factor AP2-alpha* (*TFAP2A*) were not affected in +NS cells. Addition of NSs affected activity but not expression of *cAMP response element-binding protein*, *CREB1* (originally called CREB), which mediates diverse transcriptional regulatory effects; the *v-myb avian myeloblastosis viral oncogene homolog* (MYB), which is critical for controlling the proliferation and differentiation of hematopoietic stem and progenitor cells via interaction with p300 [29]; *thyroid hormone receptor* (THR); RAR (*retinoic acid receptor*); and *nuclear factor erythroid 2, p45 subunit* (*NFE2*), which plays a role in all aspects of hemoglobin production, including heme and globin synthesis and iron procurement. Similar to *CREB1*, although above the statistical threshold P<.05, the expression of *NFE2* showed a statistical trend (P<.1). The expression of different *nuclear factor erythroid 2-like* TFs was either unmodified (*NFE2L2*) or lower (*NFE2L1*, *NFE2L3*) in +NS cells (Table 1). TFs whose changes in expression and activity

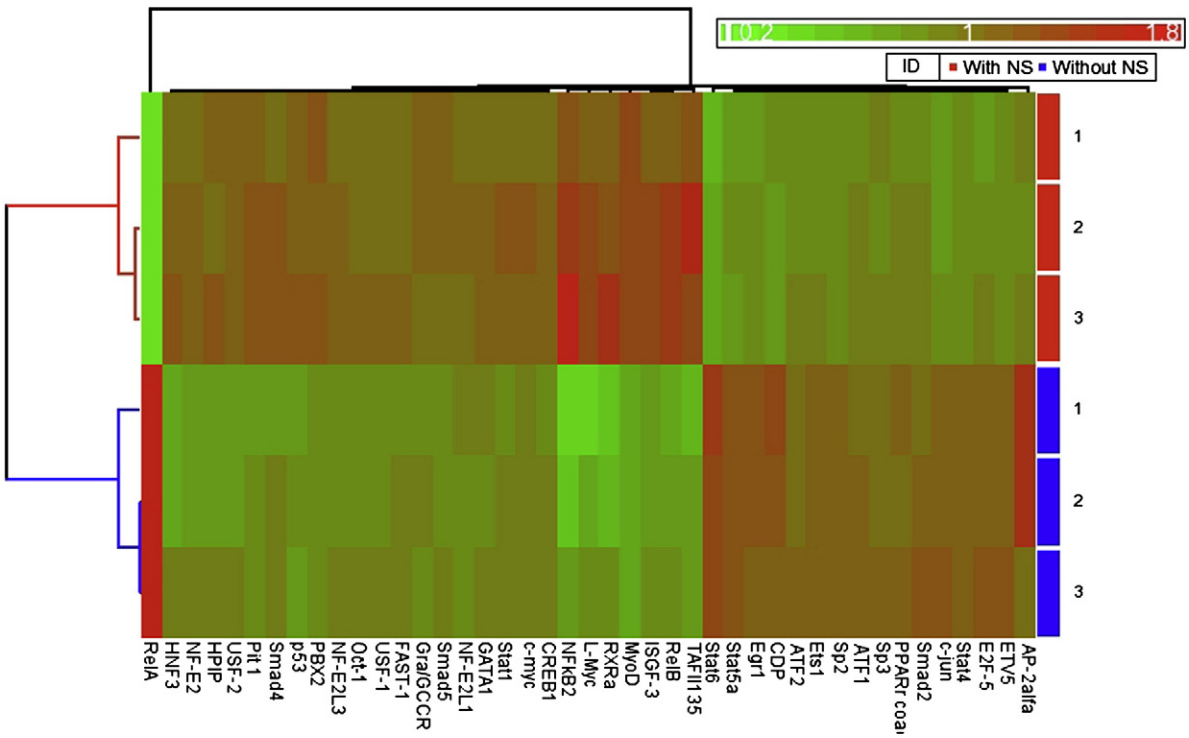

Fig. 2. Hierarchical bidimensional clustering of TF transcripts differentially expressed in Caco-2 cultured cells in the presence and absence of NSs (P<.1). Green and red indicate down- and up-regulated genes, respectively. The magnitude of the changes is proportional to the darkness of the color. Each row represents a separate sample (1 to 3) and each column represents a transcript. Each cell in the matrix represents the log2 of the abundance of a transcript for a particular sample.

Table 2
TF protein activity in Caco-2 cells grown in NS supplemented media (+NS) versus non-supplemented media (−NS)

	−NS		+NS		FDR *P* value	Fc
	Median	Range	Median	Range		
Activated TFs						
General TFs						
Cut-like 1 (CCAAT displacement protein); CUX1 (CDP)	293	150	3817	1469	.000543	12.38
TATA box-binding protein; TBP (TFIID)	10,840	1123	31,525	9093	.000543	3.21
Cell growth and differentiation TFs						
MAX protein (myc-associated factor X); MAX	460	131	2861	845	.000543	6.52
Mothers against decapentaplegic, *Drosophila*, homolog of, 2 (SMAD family member 2); SMAD2	3205	644	14,976	5214	.000543	5.15
Retinoic acid receptor; RAR	195	68	606	125	.001092	2.97
Retinoid-X receptor; RXR	6104	126	13,359	2798	.000837	2.10
v-ets avian erythroblastosis virus E26 oncogene homolog 1; ETS1	356	82	1182	213	.000543	3.21
v-myb avian myeloblastosis viral oncogene homolog; MYB (c-Myb)	17,696	4886	73,912	22,519	.000763	4.04
Inhibited TFs						
General TFs						
Transcription factor Sp1; SP1	45,984	7246	4935	1229	.000403	−9.40
Upstream stimulatory factor 1; USF1	10,345	1816	3186	624	.000543	−3.17
Cell growth and differentiation TFs						
cAMP response element-binding protein 1; CREB1 (CREB)	93,302	7128	26,751	25,622	.011774	−3.12
Glucocorticoid receptor (nuclear receptor subfamily 3, group C, member 1); NR3C1 (GCCR)	8765	1126	2166	851	.000915	−4.64
Nuclear factor erythroid 2, p45 subunit; NFE2	26,767	5409	5908	3543	.001730	−4.29
Signal transducer and activator of transcription 4; STAT4	26,451	1329	14,496	2234	.000591	−1.91
Thyroid hormone receptor; THR (TR)	108,920	25,471	52,580	1948	.000969	−1.99
Vitamin D receptor (1,25-dihydroxyvitamin D3 receptor); VDR	44,205	9650	24,341	2178	.000969	−1.86
Immune response and inflammation TF						
Nuclear factor of kappa light chain gene enhancer in B cells 1; NFKB1	23,055	3725	5869	3977	.006511	−4.73
Apoptosis TF						
Tumor protein p53; TP53	14,560	4439	4808	1379	.000969	−3.10
Non-modified TFs						
General TFs						
Heat-shock transcription factor; HSF	1250	475	1121	705	.740965	−1.09
Transcription factor AP2-alpha (activating enhancer-binding protein 2-alpha); TFAP2A (AP-2α)	6544	11,604	19,056	5205	.100987	2.31
Cell growth and differentiation TFs						
E2F transcription factor 1; E2F1	60,269	23,549	58,416	15,260	.830693	−1.03
Early growth response; EGR	80,204	2959	80,447	17,523	.552149	1.05
Paired box gene 5 (B-cell lineage-specific activator protein); PAX5	37,438	7544	41,402	12,004	.552149	1.08
Transforming growth factor, beta-1; TGFB1	15,356	8090	20,905	8958	.830693	1.06

Values are medians and range of arbitrary units relative to control spots (n=3 independent experiments), determined by image analysis of the spots. Array statistical analysis to compare the effects of NS supplementation and samples was performed by ANOVA models and *a posteriori* corrections by multiple FDR. Relative over- or underactivation of the TFs from Caco-2 cells incubated in supplemented NS media (+NS) in comparison with control cells (−NS) is expressed as fold change (Fc). Fc results represent the ratio of the data from NS-supplemented cells to those from control cells after normalization and averaging of triplicate scores. Significant differences with respect to control (−NS) are represented by *P* values corrected by FDR <.05.

were not in the same direction were *STAT4* (*signal transducer and activator of transcription 4*) and RXR (*retinoid X receptor*).

Addition of exogenous NSs did not affect the activity of *early growth response* TFs (EGR). However, the expression of *EGR1* was higher in +NS cells, while expression of *EGR2* and *EGR3*, involved in promoting a T-cell-receptor-induced negative regulatory genetic program [30], was not affected (Table 1).

The TFs SP1, SP2 and SP3 are general DNA-binding proteins that interact with a variety of gene promoters containing GC-box elements. In our experiments, the expression of *SP2* and *SP3* was higher in +NS cells (Table 1), while *SP1* expression was unchanged and its activity was markedly lower (Fig. 4B).

4. Discussion

The main finding of this study was that exogenous NSs affect the expression and activity of several TFs involved in cell growth, differentiation, apoptosis, immune response and inflammation.

Our results indicate an increase in the protein content of the Caco-2 cells, consistent with other studies in which exogenous NSs modulate protein synthesis not only in the small intestine but also in the liver as a result of tissue-specific nucleic acid changes [8]. No data on the effect of exogenous NSs on the total amount of RNA in human intestine are available, but the increased nuclear protein/RNA ratio in our experiments indicates a higher translational activity and/or reduced degradation of protein. The effect of NSs on Caco-2 cell protein content was observed most clearly in the cytoplasm and in the nucleus, suggesting that NSs may affect TF synthesis and therefore the expression of specific genes. This is in agreement with other studies that reported that dietary NTs and NSs influence gene expression in the intestine and in intestinal cell lines [18,31] and in rat liver, modulating extracellular matrix genes in primary hepatocytes and in liver stellate cells, both when cultured separately and together [21].

There is evidence that the effects of purine NTs may be mediated through intestinal gene transcription and, more specifically, through the action of TFs [32]. Of particular interest is the fact that NTs modulate the transcription of the *hypoxanthine-guanine phosphoribosyltransferase* (*HGPRT*), which is a key enzyme in the *de novo* synthesis of NTs and the way to incorporate exogenous NTs. In vivo footprinting assays on the promoter region of *HGPRT* mapped a potential AP2-binding site (TFAP2A) and multiple Spl-binding sites [33]. TFAP2A acts as an enhancer of basal transcriptional activity, and its expression is increased by a cis-acting element that contains a critical binding site for ETS1, whose overexpression induces TFAP2A promoter activity [34]. In our study, *TFAP2A* showed a trend to be overexpressed, and the expression of *ETS1* was significantly higher in the presence of exogenous NSs, adding further support to the concept of an increased utilization of purines by intestinal cells when exposed to exogenous NTs and NSs. These data also provide additional evidence for a mechanism by which the intestinal epithelium maintains and regulates the salvage of purines and NTs necessary for its high cell turnover.

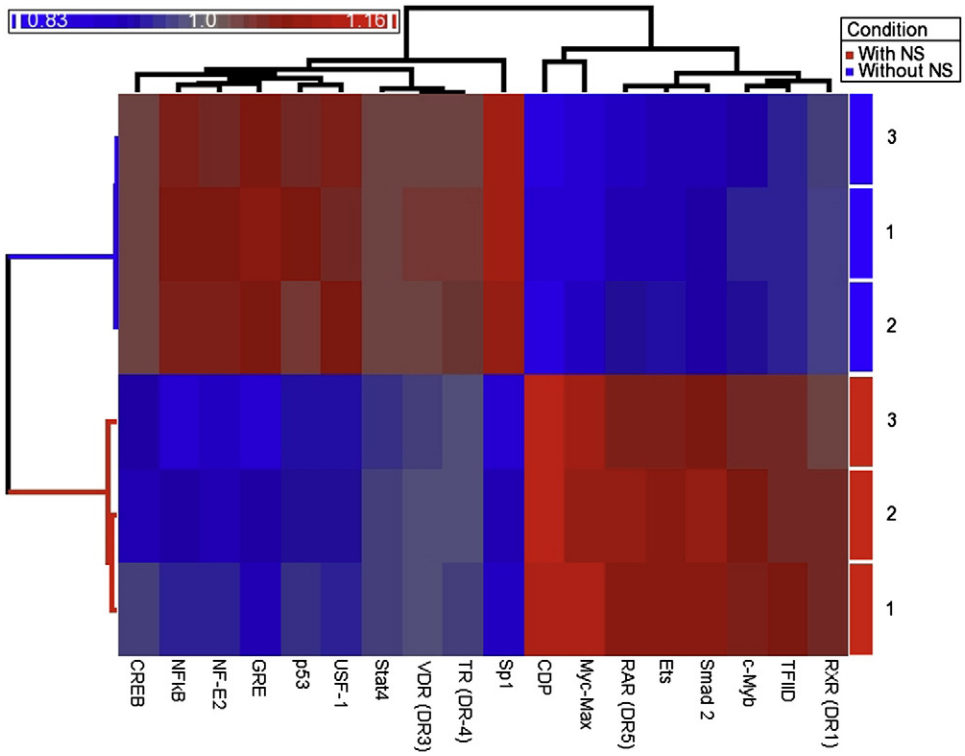

Fig. 3. Hierarchical bidimensional clustering of TF differential activities in Caco-2 cultured cells in the presence and absence of NSs ($P<.1$). Red and blue indicate increased and decreased TF activities, respectively. The magnitude of the changes in activity is proportional to the darkness of the color. Each row represents a separate sample (1 to 3) and each column represents a TF. Each cell in the matrix represents the log2 of the protein content for a TF and a particular sample.

IEC-6 cell proliferation is higher with a mixture of NSs that contains thymidine but lower with a mixture containing >75 μM of uridine [35]. In the present study, no effects on proliferation (cell count) or LDH release (cell destruction marker) were observed. This finding is in agreement with a previous report demonstrating that the proliferation and differentiation of Caco-2 cells under normal culture conditions were not affected by an NT supplement containing physiological levels of each NT [36].

The ubiquitously expressed USF is involved in the transcription of a wide variety of genes and consists of two related polypeptides: USF1, which belongs to the *v-myc avian myelocytomatosis viral oncogene homolog* (MYC) family, and USF2. In our study, the activity and expression of *USF1* and the expression of *USF2* were lower in +NS cells, and *MYC* showed a trend to be underexpressed, whereas there was a higher activity of the *myc-associated factor* (MAX), which is part of a heteromeric complex associated with *MYC* and whose mRNA is reported to be relatively stable and not modulated by proliferation or differentiation [37]. MYC regulates the expression and transcription of numerous target genes that control cell growth, proliferation and cell cycle progression, such as the *E2F* genes. Alterations in *MYC* and *MYB*, which are both classified as oncogenes, are found in more than one third of human solid tumors, and some alterations and allelic deletions of MYB have been correlated with the progression and metastasis of carcinomas and sarcomas [38]. Previous experiments found that overexpression of *MYC* or *E2F1* causes apoptosis under certain conditions [39]. However, E2F1 may function as a critical antiapoptotic factor in human and rodent liver cancer through its ability to counteract MYC-driven apoptosis via activation of MYB pathway [40]. Moreover, *E2F5* transcription is highest in mid-G1 phase, before *E2F1* expression is detectable, contributing to the regulation of early G1 events, including G0/G1 transition. The increased MYB activity and *E2F5* expression together with the potentially reduced *MYC* and unmodified *E2F1* expressions found in our study may support the concept of counter-interactions that would leave the proliferation of Caco-2 cells unchanged in the presence of NSs.

EGR1 is another putative G0/G1 switch regulatory gene whose expression was higher in +NS cells. Liu et al. [41] observed that the *EGR1* gene product directly controls *TGFB1* gene expression, and they found that EGR1-dependent expression of *TGFB1* inhibited human cancer cell growth in model cells. *TGFB1* gene expression regulates proliferation, differentiation and other functions in many cell types, and dysregulation of TGFB activation and signaling may result in apoptosis. Responses to TGFB family members may be mediated by a DNA-binding complex formed by FAST1 (*forkhead box H1*), SMAD2 and SMAD4 [42]. In our study, the activity of TGFB1 was not modified, although NSs had differential effects on other TFs implicated in regulating cell growth and differentiation, increasing the activity and expression of some (*SMAD2*) and decreasing the expression of others (*SMAD4* and *SMAD5*).

Regarding immunity, which is one of the most important processes affected by dietary NTs, we have observed a higher expression and slightly lower activity of *STAT4*, a higher expression of *STAT5A* and *STAT6* and a lower expression of *STAT1* versus control cells (Table 1). Moreover, *STAT3* showed a trend for higher expression. STAT proteins have, as their name implies, a dual function of signal transduction and transcription activation [43]. STAT4 is phosphorylated in response to interleukin (IL)-12 and is essential for IL-12 signal transduction. Cell-mediated immunity is dependent on IL-12 production by macrophages and dendritic cells, and Lovato et al. [44] found that intestinal T cells from Crohn's disease patients showed constitutive activation of STAT3 and STAT4. Moreover, STAT3, STAT5 and STAT6 but not STAT1, STAT2 or STAT4 are involved in signaling from the leptin receptor and in the physiologic action of leptin [45,46].

CREB1 is phosphorylated and activated by T-cell activation and is required for induction of TF AP1 (c-Jun) and subsequent IL-2

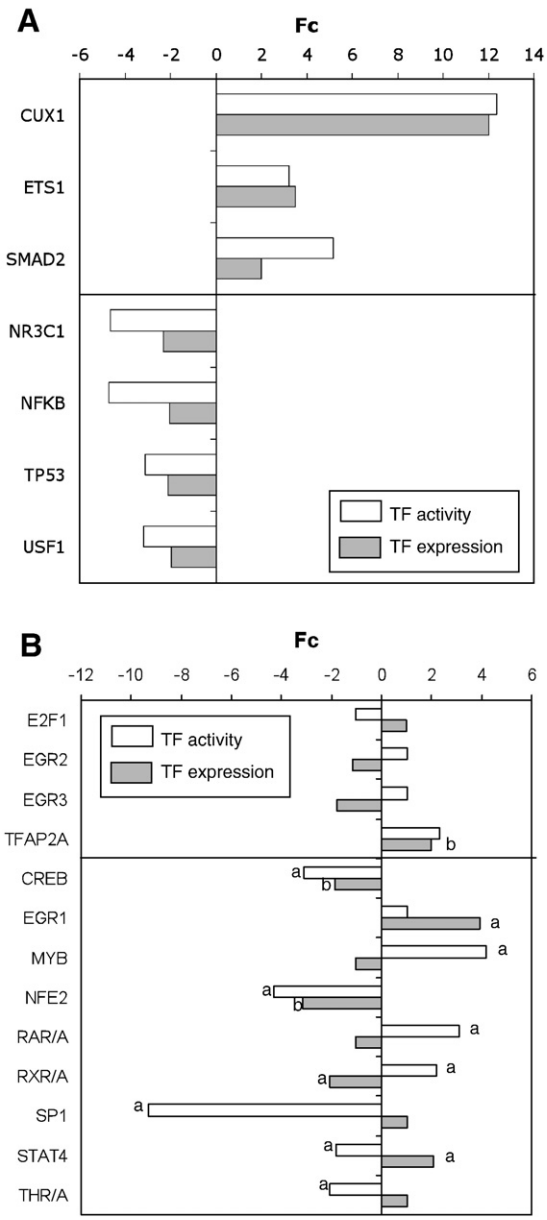

Fig. 4. Comparison of the activity and expression of specific TFs in Caco-2 cells in the presence of exogenous NSs. Results were expressed as fold change (Fc) of the activity values (white bars) and the expression values (gray bars) from cells grown in NS-supplemented media in comparison with non-supplemented media. Fc represents the ratio of the data from NS-supplemented cells to those from control cells after normalization and averaging of triplicate scores (n=3 independent experiments). Panel A shows the TFs that are statistically different versus control (P<.05) and simultaneously activated and up-regulated (top) or inhibited and down-regulated (bottom). Panel B shows the non-modified (top) or differently activated and expressed (bottom) TFs. (a) Significant differences versus control cells (P<.05). (b) Trend with respect to control cells (P<.1).

production and cell cycle progression [47]. In the present study, *CREB* showed a trend to be underexpressed and the activity was lower in +NS cells. Other CRE-binding proteins are the *activating transcription factors ATF1* and *ATF2*, which bind to cAMP response elements (CREs), form a homodimer or heterodimer with c-Jun and stimulate CRE-dependent transcription. AP1 is constituted by a group of related dimeric basic region-leucine zipper proteins. JUN (*Jun N terminal kinase*) is the most potent transcriptional activator in this group, and its transcriptional activity is attenuated and sometimes antagonized by JUNB. In our experiments, the expression of *ATF1*, *ATF2* and *JUN* but not *JUNB* was higher in the presence of NSs.

Regarding cell growth and differentiation, our data on TFs also give additional insight. Thus, in our study, both the expression and activity of CDP were higher in +NS cells. The general activity of CDP is thought to act by preventing binding of positively acting CCAAT factors to promoters, and its postulated mechanism of action makes it a potential candidate as a general repressor of developmentally regulated genes. In our study, the expression of *PPAR-gamma coactivator 1 alpha* (*PPARGC1A*) and activity of RAR and RXR were higher in +NS cells but the expression of *PPARG* (*peroxisome proliferator-activated receptor PPAR-gamma*, member of the nuclear hormone receptor subfamily of TFs), *RAR* or *RXR* was unchanged or slightly lower. Retinoid acid is also implicated in many aspects of vertebrate development and homeostasis. Retinoid signaling is transduced by the families of nuclear receptors RAR and RXR, which form RXR/RAR heterodimers. The PPARs are among the TFs that form heterodimers with RXRs and regulate transcription of various genes. PPAR-gamma is believed to be involved in adipocyte differentiation, while PPARGC1A has a low inherent transcriptional activity when not bound to a TF. According to Puigserver et al. [48], the docking of PPARGC1 to PPARG stimulates an apparent conformational change in PPARGC1 that permits binding to different TFs, resulting in an increase in transcriptional activity. Thus, TF docking can switch on the activity of a coactivator protein. Our results showed that the activity of *NFE2* was lower in +NS cells. Peters et al. [49] demonstrated *Nfe2* expression in the mouse small intestine and NFE2 binding activity in nuclear extracts of Caco-2. Caco-2 cells possess properties of the small intestine, including the ability to transport iron.

Regarding apoptosis, expression of Bax, an apoptosis-inducer gene, is higher with addition of AMP [19], suggesting that the induction of apoptosis may be channeled through the *tumor protein p53* (TP53) pathway; however, this effect was not seen for CMP. TP53 inhibits cell growth through activation of cell cycle arrest and inhibits apoptosis via nontranscriptional cytoplasmic processes. In our array analyses, both the expression and activity of TP53 were significantly lower in +NS cells. On the other hand, the apoptotic pathways induced by SP1 overexpression are cell type specific and require the SP1 DNA-binding domain [50]. In our experiments, although there was no significant effect on *SP1* expression, +NS cells showed a markedly lower SP1 activity and a higher expression of *SP3*, an inhibitory member of the SP gene family [51]. These results, together with those for TP53, suggest that NSs may have a preventive effect against apoptosis. Moreover, a direct coactivator target for SP1 is the general TF TAF4, which was underexpressed in the presence of NSs in our experiments.

Dietary NTs have been reported to affect the maturation and differentiation of intestinal lymphocytes, which are closer to enterocytes, exerting a selective effect on different lymphocyte populations. In general, they appear to promote the development of T-helper lymphocytes and consequently the maturation and differentiation of B cells [52]. Thus, NSs would affect the differentiation of the whole gut-associated lymphoid tissue, including enterocytes and the cross-talk between these cells and lymphocytes, through modification of the gene expression of TFs related to cytokine synthesis. This would in turn affect the immunological response and inflammation. Inappropriate activation of NFKB complex (NFKB1, NFKB2, REL, RELA or RELB among other members) has been linked to inflammatory events, while complete and persistent NFKB inhibition has been directly linked to apoptosis, inappropriate immune cell development and delayed cell growth. Our results indicate that addition of NSs to the medium decreases the expression of the *v-rel avian reticuloendotheliosis viral oncogene homolog A* or *B* (*RELA* or *RELB*) (Table 1)

and *NFKB* and the activity of NFKB, suggesting that NSs have an anti-inflammatory effect. Although inflammatory responses in many cell types are coordinated by the opposing actions of NFKB and the *glucocorticoid receptor* (NR3C1), crucial to gene expression, both the activity and expression of *NR3C1* were lower in +NSs cells in the present study.

In conclusion, NSs affect differential gene expression in a number of TFs involved in the regulation of cell growth, differentiation and apoptosis. The expression and activity of the general TF *CCAAT displacement protein* were higher and the expression and activity of *upstream stimulatory factor 1* were lower in +NS cells. Among the TFs involved in the regulation of cell growth and differentiation, the expression and activity of *ETS1* and *SMAD2* were higher but the expression and activity of *glucocorticoid receptor NR3C1* were lower in +NS cells. The activity and expression of *NFKB* and *tumor protein p53*, TFs involved in immune response, inflammation and apoptosis, were lower in +NS cells. Furthermore, the expression and activity of some TFs were not regulated in the same direction, which may be due to binding of cofactors, change in protein turnover or limiting cofactors, among others. Further studies are required to specifically address the role of NTs in TF-mediated events.

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