Exogenous Nucleosides Modulate Proliferation of Rat Intestinal Epithelial IEC-6 Cells¹,²

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Abstract

Exogenous nucleotides are considered semiessential nutritional components that play an important role in intestinal development, maintenance, and recovery from tissue damage. Nucleosides (NS) are the best-absorbed chemical form of nucleotides in the intestinal epithelium. The aim of this work was to clarify, at the cellular level, the effects described in vivo. Under conditions of high intracellular availability of NS, we studied the effects of 2 NS mixtures on the NS uptake and intracellular distribution and on the proliferation, morphology, viability, and cell-cycle phase distribution of rat intestinal epithelial cell line 6. Purine and pyrimidine NS showed a similar uptake profile, but the intracellular incorporation of guanosine was greater than that of uridine, without differences in intracellular distribution. Proliferation assays demonstrated that IEC-6 cell proliferation is increased by a mixture containing thymidine but decreased by one containing uridine. In fact, the antiproliferative effect started at 75 µmol/L, which indicated that it may not be correct to consider concentrations of uridine >75 µmol/L as physiological. Interestingly, these effects were not related to increased cell necrosis or apoptosis or to changed cell morphology but rather to a reduced S-phase and increased G0/G1 phase of the cell cycle. In summary, our results suggest that NS molecules are well-absorbed by rat intestinal epithelial cell line 6 cells, whose proliferation can be promoted or inhibited (according to the NS mixtures used) by a mechanism that is not dependent on the toxicity of the mixtures. J. Nutr. 137: 879–884, 2007.

Introduction

Nucleotides (NT)⁸ participate in almost all biochemical processes of the organism in different ways (1–3). They can be synthesized de novo from simple molecules, with considerable consumption of ATP, or synthesized by the salvage pathway, in which new NT are created by preformed bases and nucleosides (NS) derived from diet, from liver via blood, or from intracellular nucleic acid degradation (4).

Because of the existence of the de novo synthesis pathway, NT are not considered essential nutrients nor have there been reports of diseases related to a deficit in the exogenous contribution of NT (5,6). Nevertheless, numerous studies have indicated that exogenous NT are important for normal gastro-intestinal development and maintenance (7–11), leading to their description as semiessential or conditionally essential nutrients for tissues with a rapid cell turnover rate and limited capacity for de novo synthesis (6,12).

A small amount of NT is absorbed through cell membranes and it is mainly NS, which appear after release of phosphate groups, that cross into cells (13–15). This process occurs through an active sodium cotransport mechanism, localized in the apical cellular membrane in intestinal epithelium, and through passive transport by facilitated diffusion, localized in basolateral membrane (16,17). The differential localization of transporters supports the hypothesis that coordinated action permits the trans-epithelial transport of NS (18).

The objective of this study was to evaluate effects of exogenous NS, the best-absorbed chemical form of NT, on the intestinal epithelium, to clarify, at the cellular level, the effects described in vivo. For this purpose, we studied the intracellular incorporation capacity of exogenous NS and their influence on the growth, morphology, cell cycle phase distribution, and cell viability of rat intestinal epithelial cell line 6 (IEC-6). These cells were isolated from rat jejunum (19). It is important to consider that these cells come from intestinal crypts, where the effective concentrations of NS may not be the same as that in the luminal epithelium.
Materials and Methods

Cell line and culture conditions. IEC-6 cell line was obtained from the Technical Service Center (ATCC: CRL-1592) of the University of Granada. Cells were maintained at 37°C in an atmosphere containing 5% CO2, with DMEM supplemented with 5% heat-inactivated fetal bovine serum, 100 IU/L bovine insulin, 2% l-glutamine, 2.7% sodium bicarbonate, 1% Hepes buffer, 40 mg/L gentamicin, and 500 mg/L ampicillin. In assays with NS and tritium, the serum was replaced by 0.5% heat-inactivated diazylated fetal bovine serum and 1 mL/L of MTO (serum replacement, Becton Dickinson Labware) to avoid possible input of NS or NT from normal serum.

Nucleoside treatment and experimental groups. NS frequently found in plasma were used in the assays. To differentiate between DNA and RNA, 2 mixtures were used that differed solely in the presence of uridine or thymidine, respectively (20). One group was treated with an NS mixture composed of thymidine, cytidine, guanosine, and inosine (T-CGI). Another group was treated with an NS mixture composed of uridine, cytidine, guanosine, and inosine (U-CGI). A Control group received no NS supplementation. Unless otherwise stated, the final concentration of each NS in the mixtures was 100 μmol/L, and the medium was changed every 12 h in all experiments. All NS were purchased from Sigma Chemical. Starting cell density for all experiments was 5 × 103 cells/cm² (low density) and, after the conclusion of the respective assays, in no case was a confluence >70% reached.

Tritium assays. Uridine and guanosine were selected for studies of NS incorporation, intracellular distribution, and differences between pyrimidine and purine chemical groups in IEC-6 cells. The following culture media were prepared: T-CGI/tritium, composed of DMEM supplemented with thymidine, cytidine, and inosine (100 μmol/L each) plus 200 μCi/mL tritiated guanosine (Moravek Biochemicals) brought to 100 μmol/L with nontritiated guanosine; U-CGI/tritium, similar to the previous medium but with guanosine, cytidine, and inosine (100 μmol/L each) plus 200 μCi/mL tritiated uridine (Moravek Biochemicals) brought to 100 μmol/L with nontritiated uridine; and control medium containing no NS or tritium.

Nucleoside clearance from culture medium. Cells seeded in Transwells (Corning) were incubated with T-CGI/tritium, U-CGI/tritium, or control culture medium. After incubation in the apical chamber for 0, 0.5, 1, 2, 3, 4, 6, 8, 10, or 12 h, the medium was collected, diluted 1:3000 (v/v) with scintillation support medium (Ecolumen, ICN Biomedicals), and total radioactive amount was then determined by liquid scintillation counting (Beckman, LS6000).

Intracellular nucleoside incorporation by electron microscopy autoradiography. Monolayers from the above Transwell cultures at 0.5, 1, or 4 h were washed 3 times in cold PBS at 4°C, prefixed for 60 min at 4°C with 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L, pH 7.4), fixed with 1% osmium tetroxide in cacodylate buffer for 60 min at 4°C (Merck), dehydrated in ethanol, and embedded in Embed 812 (Electron Microscopy Science). Ultrathin sections (90–120 nm) were collected in Formvar-coated copper grid, double-stained with 3% uranyl acetate for 15 min and 6% lead citrate for 1 min, air dried, and coated with a 5 nm-thick carbon layer (21).

Next, the grids were coated with Ilfodd L4 nuclear emulsion (Ilfodd Imaging) by the “wire loop method” (22) and kept in the dark for 80 d at 4°C. Subsequently, grids were developed with phenidone-ascorbic acid mixture for 2 min, washed with distilled water, and fixed with 30% sodium thiosulfate for 5 min. The developing mixture was composed of 1.5% ascorbic acid, 0.25% photographic grade phenidone, 0.6% potassium bromide, 1.3% potassium carbonate, and 6% potassium thiocyanate (Sigma Chemical).

Grids were studied under transmission electron microscope (Zeiss 902, Carl Zeiss). Images were scanned, and silver grains were counted. Three variables related to radioactive density were determined, calculating the incorporation index (marks per area unit × 10,000, with each real μm2 on electron micrographs equivalent to 2997.4 units of digital area) for the whole cell (total incorporation index, TII), the nucleus (nuclear incorporation index, NII), and the cytoplasm (cytoplasmic incorporation index, CII).

Proliferation assays. Cells were incubated with T-CGI, U-CGI, or control culture media. On d 1–7, four samples per treatment were fixed with 10% cold trichloroacetic and stained with 0.4% B sulforhodamine in 1% acetic acid, and the dye that was bound to cells was subsequently resuspended with 10 mmol/L Tris-base buffer (pH 10.5). Optical density was then determined using a Titerrek multiscan colorimeter at 492 nm (23).

Effects of increasing concentrations of the T-CGI and U-CGI NS mixtures were characterized by seeding cells and exposing them to concentrations of 10, 25, 50, 75, or 100 μmol/L. On d 7, cells were fixed and processed for B sulforhodamine staining, as described above.

Protein content. To verify differences in proliferation among experimental groups, total protein amount was measured after 7 d of incubation. Cell layers were harvested with PBS-EDTA (0.02%) solution and washed with PBS, and pellets were resuspended in sample buffer composed of 62.5 mmol/L Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodeyl sulfate (Sigma Chemical), 5% 2-mercaptoethanol, 0.5% bromophenol blue, and 100 mmol/L dithiorthiol (Bio-Rad). Then, samples were homogenized, boiled, and centrifuged at 14000 × g for 10 min. Total protein amount of the supernatant was measured by the Lowry method (24).

Morphology. The study of cell morphology was carried out using a Nikon TM Phase Contrast-2 ELWD inverted optical microscope.

Cell viability and apoptotic assays. Effects of NS mixtures on cell viability, necrosis, and apoptosis were evaluated by monitoring the binding of FITC-labeled annexin V and the entry of propidium iodide (PI) into cell nuclei by means of the TACS Annexin V-FITC apoptosis detection kit (R & D Systems). Cells were incubated with or without NS mixtures. After 7 d of culture, cells were processed according to manufacturer’s instructions and analyzed in a Vantage flow cytometer (Becton Dickinson).

Cell cycle phase distribution analysis. Cells were incubated with T-CGI, U-CGI, or control culture media. After 7 d of culture, the medium was aspirated and cells were harvested with a PBS-EDTA (0.02%) solution, washed twice with sample buffer (1 g/L glucose in PBS without Ca++ or Mg++) and fixed overnight in 70% cold ethanol. Next, cells were pelleted, washed once with sample buffer, and resuspended in 50 mg/ml PI staining solution for 30 min in the dark. Samples were then analyzed using a Vantage flow cytometer (Becton Dickinson) with CellFIT cell cycle analysis software.

Statistical analysis. All values are means ± SD. Statistical analysis was carried out using Statgraphics, version 5.1 (Statistical Graphics, 2000). The analyses performed were simple regression, 1- or 2-way ANOVA, and multiple mean comparisons using Fisher’s least significant differences test. Significant differences were determined at P < 0.05. Graphics of the collected data were made using Microsoft Office Excel 2003 software (Microsoft Corporation).

Results

Nucleoside clearance from culture medium. The clearance profiles of guanosine and uridine were similar, with no significant differences between their values at any incubation time. The highest clearance rate occurred from 0 to 4 h. At 0.5 h, 36.86% of the uridine and 30.76% of the guanosine were cleared. Culture medium contained 25.89% and 21.07% of initial concentrations of uridine and guanosine at 4 h, and 9.69% and 8.01%, respectively, at 12 h (data not shown). Resulting expressions from the experimental data regression were adjusted to an inverse function of the independent variable
with \( P < 0.01 \) in both cases: percentage of remaining uridine = 
\[
\frac{1}{0.011332 + 0.00763866 \times \text{incubation time}},
\]
and percentage of remaining guanosine = 
\[
\frac{1}{0.0093928 + 0.00961567 \times \text{incubation time}}.
\]

**Intracellular nucleoside incorporation by electron microscopy autoradiography.** On the electron micrographs, marks were observed in specific organelles (e.g., rough endoplasmic reticulum and mitochondrion) independent of incubation time (Fig. 1). Large amounts of silver grains were frequently observed in heterochromatic regions and nucleoli. A predominance of marks in the nucleus was clearly noted. However, an ordered and defined route of NS incorporation through intracellular compartments was not observed.

Both time of incubation (30 min, 1 h, and 4 h) and the presence of tritiated guanosine or uridine affected the 3 indexes of incorporation \( (P < 0.05) \). The total incorporation index initially was higher for uridine than for guanosine. At the end of the assay, however, the TII \( (P < 0.01) \) and CII indexes \( (P < 0.05) \) were higher for guanosine than for uridine, and the NII index did not differ between them. There was greater mark density for both NS in the nucleus than in the cytoplasm (1.81-fold for guanosine and 2.49-fold for uridine at 4 h) (Fig. 2).

**Proliferation assays.** Growth curves showed that cells initially grew at the same rate regardless of the treatment (control, T-CGI, or U-CGI). From d 4, the proliferation rate was significantly higher in the T-CGI group than in the other 2 groups \( (P < 0.01) \), and this difference persisted throughout the assay. From that day, the rate was lower in the U-CGI group than in the control group, and this difference was significant from d 6 (Fig. 3).

These differences in proliferation rates were corroborated by quantification of total protein production on d 7 of treatment (data not shown), which showed a 1.66-fold and 0.65-fold in cells incubated with mixtures T-CGI and U-CGI, respectively, compared with controls.

Cultivation of cells at increasing concentrations for 7 d showed an increased growth in the T-CGI group than in the other 2 groups from a concentration of 25 \( \mu \text{mol/L} \), whereas growth in the U-CGI group did not differ from that of the control group until a concentration of 75 \( \mu \text{mol/L} \) was reached (Fig. 4).

**Morphology.** Direct observation showed a homogeneous monolayer of cells comprised of strongly joined colonies of
The absorption capacity is lower in fetal rat hepatocytes than in adult rat hepatocytes or IEC-6 cells. In the former, Sáez-Lara et al. (20) found that 40–50% of guanosine and 70% of uridine remained in culture medium after 48 h of incubation. These authors also reported the interesting result that NS uptake was minimally influenced by the mixture used (T-CGI or U-CGI).

**Intracellular nucleoside incorporation.** Absorbed NS augments the intracellular nucleotide pool (27,28) that can be used for nucleic acid synthesis (29,30). Indeed, the preferential localization of radioactive grains in the cell nucleus, as found in the present study, suggests that the IEC-6 cells mainly used the NS for this purpose, at least during the incubation period assayed.

An analysis of the distribution of radioactive marks showed some differences between guanosine and uridine. At 4 h, the TII index was higher for guanosine than for uridine, although this difference was not observed in the absorption curve, which may be due to a differential use of exogenous NS by IEC-6 cells.

In 1994, Sanderson et al. (31) studied differentiated human colon adenocarcinoma cells (Caco-2) and observed transepithelial transport from the apical to the basolateral membrane and vice-versa; the former was faster and showed greater capacity than the latter, and guanosine was one of the most rapidly transported NS. Therefore, there may have been variations in levels of transepithelial transport in our assays, leaving differentially tritiated NS in the lower compartment of transwells, which may explain the differences found between guanosine and uridine.

**Proliferation.** Ohyanagi et al. (32) incubated primary cultures of hepatocytes with inosine, GMP, cytidine, uridine, and thymidine. They found that NS produced an inhibition of proliferation within 24 h, which was more marked for U-CGI (20).

The total NS concentration of mixtures was 400 μmol/L (T-CGI or U-CGI) in the present study. These combinations and concentrations of NS were recently used by other authors in hepatocytes (20,27,33) and were considered closer to physiological values because they were <1 mmol/L and no toxicity was found in a 24-h viability assay (20).

Ohyanagi et al. (32) did not find that uridine concentration <230 μmol/L reduced proliferation after examining the first 24 h of incubation, whereas significant differences in proliferation emerged after d 6 of culture in the present study. Moreover, uridine was present in body fluids at concentrations <75 μmol/L (34), which was the lowest concentration that produced inhibition in cultures of the present study. The above data suggest that uridine levels >75 μmol/L should not be considered physiological values in IEC-6 cells.

In adult rat hepatocyte cultures, NS mixtures did not modify cell proliferation or viability (27). However, an incubation period of only 24 h was studied, and effects on enterocytes were observed after longer incubation in the present study. In fetal rat hepatocytes, however, both mixtures produced an increase of proliferation within 24 h, which was more marked for U-CGI (20).

NT in human milk (AMP, CMP, GMP, IMP, and UMP) did not affect the proliferation rate of Caco-2 tumor cells under normal nutritional conditions, but they increased the growth rate in cells when nutritional stress conditions were simulated by glutamine removal. IEC-6 cells were affected under both nutritional conditions and showed an increase in cell proliferation (29,35).

**Discussion**

The role of exogenous NS as a semiessential nutritional component is becoming more widely accepted after the publication of investigations into their gastrointestinal, immunological, and hepatic effects (4,6,12,25,26). Nevertheless, the mechanisms by which these effects are exerted by NS have not yet been clarified.

**Nucleoside clearance.** Observations of NS uptake in IEC-6 cells showed that both types of molecules, purine (guanosine) and pyrimidine (uridine), were rapidly cleared from culture mediums after 48 h of incubation. These differences might be due to a differential use of exogenous NS by IEC-6 cells.

In the flow cytometry study (Supplemental Fig. 2), control cultures contained 60.73% of cells in G0/G1, 15.55% in G2/M, and 23.73% in S-phase; T-CGI group showed a reduction in S-phase (9.06%) and a corresponding accumulation in G0/G1 (69.38%) and G2/M (21.56%); and U-CGI group showed an intermediate picture, with a significant reduction vs. controls in S (20.10%) that was solely due to an increase in G0/G1 (66.69%), with no significant difference in the G2/M-phase percentage (13.22%).

**Cell-cycle phase distribution analysis.** In the flow cytometry study (Supplemental Fig. 1), control cultures contained 60.73% of cells in G0/G1, 15.55% in G2/M, and 23.73% in S-phase; T-CGI group showed a reduction in S-phase (9.06%) and a corresponding accumulation in G0/G1 (69.38%) and G2/M (21.56%); and U-CGI group showed an intermediate picture, with a significant reduction vs. controls in S (20.10%) that was solely due to an increase in G0/G1 (66.69%), with no significant difference in the G2/M-phase percentage (13.22%).

**Cell viability and apoptotic assays.** Similar values were obtained among the control (93.34% of viable cells, 4.52% of necrotic cells, and 2.15% of apoptotic cells), T-CGI (95.35%, 4.17, and 1.27%, respectively) and U-CGI (93.29, 4.76, and 1.95%, respectively) groups, with no significant differences in respective percentages among them (Supplemental Fig. 1).
In the present assays, significant differences in the proliferation of IEC-6 cells were found, but not due to a glutamine restriction. Our study differs importantly from others insofar as others have used NT as the chemical form of purines and pyrimidines, whereas we used NS, which are better absorbed (13–15). In this context, Holen et al. (36) found that proliferation rate did not differ when IEC-6 cells were incubated with complex mixtures containing DNA sodium salt extracted from fish spawn or yeast RNA, except under nutritional stress conditions without glutamine and with a 50% reduction in fetal bovine serum. In our opinion, there is lower cellular bioavailability of NS from these complex molecules than from mixtures of isolated NS, because it depends on extracellular degradation of DNA and RNA by different catabolic enzymes (37), some of which are related to the differentiation status of cells (38).

We initially speculated that the inhibition of proliferation by the U-CGI mixture in our assays might be due either to a possible toxicity of the mixture, which would affect cell viability, or to its stimulation of cell differentiation by cell cycle blockade, with cells kept in nonproliferative state and acquiring differentiated characteristics.

**Cell viability and apoptosis.** Epithelial homeostasis is maintained by the balance between cell division and apoptosis (39). Our IEC-6 cells showed similar apoptosis and necrosis levels to those reported by other authors in control cultures of IEC-6 cells (40,41). Moreover, because these data did not differ among experimental groups, the differences in proliferation profile cannot be explained by variations in cellular viability, necrosis, or apoptosis.

Holen et al. (36) also found an inhibition of Caco-2 cell proliferation under optimal nutritional conditions after treatment with DNA sodium salt extracted from fish spawn and yeast RNA. They did not consider the mixture to be toxic, because growth was promoted under conditions of nutritional stress (see above). They ascribed the antiproliferative effect to the stimulation of PI2Y receptors by exogenous NT, inducing apoptosis and cell growth inhibition (42).

**Cell cycle phase distribution.** NS, especially the T-CGI mixture, reduced the percentage of cells in S-phase. This appears to be due to a reduction by the NS in the time needed for cells to complete S-phase, thereby increasing G2/M phase accumulation, as observed in the T-CGI group. However, the U-CGI group showed no difference with controls in G2/M, and there was a lower proliferation rate in both groups. On the other hand, the NS produced an increased percentage of cells in G0-G1 phase, considered necessary to begin the differentiation process (43). Cell cycle phase distribution in the U-CGI group showed intermediate behavior between those in the T-CGI and control groups. However, it was not possible to detect variations that explained the differences in antiproliferative response.

In summary, exogenous NS are molecules that are well-absorbed by intestinal epithelial cells and that can promote or inhibit their proliferation according to the mixture used. Our results also indicate that >75 μM/L of uridine should not be considered a physiological value for IEC-6, and lower NS concentrations should therefore be applied in future assays. Finally, the antiproliferative effect of the U-CGI mixture was not associated with a reduction in cell viability. Further studies are warranted on the effects exerted by T-CGI and U-CGI mixtures on the differentiation of IEC-6 cells.

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