Stable isotope-based dynamic metabolic profiling is applied in this paper to elucidate the mechanism by which butyrate induces cell differentiation in HT29 cells. We utilized butyrate-sensitive (HT29) cells incubated with [1,2-13C2]glucose or [1,2-13C2]butyrate as single tracers to observe the changes in metabolic fluxes in these cells. In HT29 cells, increasing concentrations of butyrate inhibited glucose uptake, glucose oxidation, and nucleic acid ribose synthesis in a dose-dependent fashion. Glucose carbon utilization for de novo fatty acid synthesis and tricarboxylic acid cycle flux was replaced by butyrate. We also demonstrated that these changes are not present in butyrate-resistant pancreatic adenocarcinoma MIA cells. The results suggest that the mechanism by which colon carcinoma cells acquire a differentiated phenotype is through a replacement of glucose for butyrate as the main carbon source for macromolecule biosynthesis and energy production. This provides a better understanding of cell differentiation through metabolic adaptive changes in response to butyrate in HT29 cells, demonstrating that variations in metabolic pathway substrate flow are powerful regulators of tumor cell proliferation and differentiation.

Butyrate is a four-carbon short chain fatty acid produced by fermentation of fiber polysaccharides by the intestinal microflora of the human colon (1). Butyrate is utilized primarily by colon epithelial cells as a substrate for energy production (2). Previous studies demonstrated that deficiency in the availability or utilization of butyrate causes colitis and may be involved in colon carcinogenesis (3).

Butyrate has been shown to induce dose dependent differentiation of various malignant cell lines (4–6). Studies performed in recent years in colorectal cancer have shown that there is a cell cycle arrest in G1 phase due to the activation of cyclin D3 and p21Waf1/Cip1 after incubation with butyrate (7). These studies implicate many known cell signaling events in mediating the cell-differentiating effect of butyrate, including cyclin-dependent kinase inhibitors (8), mitogen-activated phosphorylase kinases (9), down-regulation of c-myc (10), and the proinflammatory transcription factor NF-κB (11, 12). Among the genes studied using gene array technology, the most significantly affected were those of transcription factors related to cell growth, apoptosis, and oxidative metabolism (13, 14). How butyrate induces these specific molecular changes is mostly unknown. To assess how known genetic modifications can be translated into metabolic changes characteristic of differentiated cells, techniques allowing analysis of the levels of low molecular weight compounds are required. Stable isotope-based dynamic metabolic profiling using gas chromatography/mass spectrometry (GC/MS)1 is a new tool with a largely untapped potential in the field of functional genomics. In this paper we demonstrate the usefulness of this technique to elucidate the metabolic mechanism underlying butyrate induced cell differentiation.

The task will be performed using metabolomic and fluxomic analysis to investigate whether butyrate-induced differentiation in HT29 (butyrate-sensitive) colon and MIA (butyrate-resistant) pancreatic adenocarcinoma cells involves the reversion of metabolic reactions characteristic of undifferentiated cells. Such results would strongly support the importance of gene-nutrient interactions on the platform of cellular metabolic processes and the level of their intermediates as key metabolic signals/ligands to the transcriptional regulation of mammalian cell growth and differentiation.

MATERIALS AND METHODS

Cell Lines and Culture—HT29 human colon adenocarcinoma cells and MIA pancreatic adenocarcinoma cells (obtained from the American Type Culture Collection) were grown in minimum essential medium in the presence of 10% fetal bovine serum at 37 °C in 95% air, 5% CO2 for 3 days before the start of the experiments. Just prior to butyrate treatment and stable isotope labeling, the medium was removed and fresh Dulbecco’s modified Eagle’s medium (with l-glutamine, without and sodium pyruvate; Invitrogen) was added with 10% fetal bovine serum, 10 mM of glucose and increasing doses of butyrate (0, 0.1, 1, and 5 or 10 mM), for 72 h. Two separate experiments were performed in each cell line: 1) to study the effect of butyrate on glucose metabolism, cells were incubated with [1,2-13C2]glucose (50% isotope enrichment) and increasing doses of butyrate; and 2) when the metabolism of butyrate itself was studied, the cultured cells were incubated with unlabeled glucose but increasing doses of [1,2-13C2]butyrate (50% isotope enrichment). Stable [1,2-13C2]l-glucose and [1,2-13C2]l-butyrata

1 The abbreviations used are: GC/MS, gas chromatography/mass spectrometry; IRMS, isotope ratio mass spectrometer.

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isotopes were purchased with >99% purity and 99% isotope enrichment for each position (Isotec Inc., Miamisburg, OH).

Cell cultures were started with the same cell number \((2 \times 10^6)\), which was achieved using standard cell counting techniques. At the end of the experiment, the final cell numbers were also measured with these standard procedures. HT29 and MIA cells were selected for the study because HT29 differentiate with butyrate, whereas MIA cells do not. The metabolic differences of these cell lines are reported in order to reveal differentiation-specific carbon flow and substrate redistribution changes in tumor cells.

**Alkaline Phosphatase Activity**—This enzyme activity was measured as a marker of degree of cell differentiation after butyrate treatment according to the published procedures (15). Alkaline phosphatase was expressed as nmol of substrate converted per min and per mg of protein. Protein determination was done using the BCA protein assay (Pierce).

**Glucose and Lactate**—The levels of glucose and lactate in media were measured using a Cobas Mira chemistry analyzer (Roche Applied Science). Glucose and butyrate oxidation by the cells was determined by media \(^{13}C/^{12}C\) ratios in released \(CO_2\) by a Finnegan Delta-S isotope ratio mass spectrometer (IRMS) (16). To obtain the \(CO_2\) from the medium, 200 \(\mu\)l of the cell medium were added to a hermetically closed vial, and then 50 \(\mu\)l of 0.1 \(M\) NaHCO\(_3\) and 50 \(\mu\)l of 0.12 \(M\) HCl were added to react with the medium and liberate the \(CO_2\), which was injected directly into the IRMS. The value obtained from the spectrometer is \(\delta_{\text{PDB}}\), which is equal to the ratio between \(^{13}C\) and \(^{12}C\) from the sample minus the \(^{13}C/^{12}C\) from the reference (air) divided from the ratio of the reference. Release of \(^{13}CO_2\) was measured to estimate glucose or butyrate carbon utilization through oxidation by the cell lines and was expressed as the difference between the amount released by each treatment and the amount released by the control.

Lactate from the cell culture medium was extracted by ethyl acetate after acidification with HCl. Lactate was derivatized to its propylamide-heptafluorobutyric form and the \(m/z\) 256 (carbons 1–5 of lactate, chemical ionization) was monitored for the detection of \(m/z\) 256 with one \(^{13}C\) label and \(m/z\) 258 with two \(^{13}C\) labels. \(m/z\) 256 originates from glucose metabolized by direct oxidation via the oxidative steps of the pentose phosphate pathways and then recycled to glycolysis via the non-oxidative pentose cycle. From these results, we can predict pentose cycle flux relative to the glycolytic flux, and it is calculated by the \(m_1/m_2\) ratio in lactate (17).

**RNA Ribose**—RNA ribose was isolated by acid hydrolysis of cellular RNA after Trizol purification of cell extracts. Ribose isolated from RNA was derivatized to its aldonitrile acetate form using hydroxyl-amine in pyridine and acetic anhydride. We monitored the ion cluster around the \(m/z\) 256 (carbons 1–5 of ribose, chemical ionization) to find the molar enrichment and positional distribution of \(^{13}C\) labels in ribose (17).

**Glutamate**—Glutamate was separated from the cell medium using ion-exchange chromatography (18). Glutamate was converted to its \(n\)-trifluoroacetyl-\(n\)-butyl derivative and the ion clusters \(m/z\) 198 (carbons 2–5 of glutamate, electron impact ionization) and \(m/z\) 152 (carbons 4–5 of glutamate, electron impact ionization) were monitored. The different isotomers of glutamate allowed us to determine the parameter \(Y\), which is the anaplerotic flux (pyruvate carboxylase) related to the tricarboxylic acid cycle flux (expressed as the fraction of oxaloacetate entering and completing a full turn of the tricarboxylic acid cycle) (19).

![Effect of butyrate on cell differentiation after 72 h of incubation](http://www.jbc.org/)

**FIG. 1. Effect of butyrate on cell differentiation after 72 h of incubation.** Alkaline phosphatase activity, measured as a marker of cell differentiation, is expressed as nmol·min\(^{-1}\)·mg\(^{-1}\) of protein in the cell extract. *, \(p < 0.05\); **, \(p < 0.01\).

![Effect of butyrate on glucose consumption and on lactate production](http://www.jbc.org/)

**FIG. 2. Effect of butyrate on glucose consumption and on lactate production.** The results of each isotope experiment were combined to show the changes in medium glucose or lactate concentration after a 72-h incubation. Triplicates were performed for each butyrate concentration with a total \(n = 12\). ***, \(p < 0.01\).
Fatty Acids—Fatty acids were extracted by saponification of the Trizol cell extract after removal of the RNA-containing supernatant. Cell debris was treated with 30% KOH and 100% ethanol overnight, and the extraction was performed using petroleum ether. Fatty acids were converted to their methylated derivative using 0.5N methanolic HCl. Palmitate was monitored at $m/z$ 270 and stearate at $m/z$ 298.

The enrichment of acetyl units in HT29 and MIA cells in response to butyrate treatment was determined using the mass isotopomer distribution analysis approach of different isotopomers of palmitate, an abundant cell membrane lipid readily recovered by biological mass spectrometry from cell pellets (20). Lipid synthesis is also dependent on glucose carbons, as they are the primary source of acetyl-CoA, which is then incorporated into fatty acids through de novo synthesis (C16, palmitate). Acetyl-CoA enrichment was calculated from the $m_4/m_2$ ratio using the formula $m_4/m_2 = (1 - r)/r$, where $r = [(m_1/m_2)/(1 + 0.5(m_1/m_2))]$ and is the anaplerotic flux relative to the tricarboxylic acid cycle flux. $^*, p < 0.05; ^**, p < 0.01$. In the case of labeled butyrate, $m_2$ (C-2–C-3) is obtained because of the high acetyl-CoA enrichment coming from butyrate that is recycled by the tricarboxylic acid cycle; this labeled glutamate is not related to the pyruvate carboxylase activity and hence is marked not applicable (N/A).

Gas Chromatography/Mass Spectrometry—Mass spectral data were obtained on the HP5973 mass selective detector connected to an HP6890 gas chromatograph. The settings are as follows: GC inlet 230 °C, transfer line 280 °C, MS source 230 °C, MS quad 150 °C. An HP-5 capillary column (30-m length, 250-μm diameter, 0.25-μm film thickness) was used for analysis of glucose, ribose, glutamate, and lactate. A Bpx70 column (25-m length, 220-μm diameter, 0.25-μm film thickness; SGE Incorporated, Austin, TX) was used for fatty acid analysis with specific temperature programming for each compound studied.

Data Analysis and Statistical Methods—In vitro experiments were carried out using three cultures each time for each treatment regimen and then repeated twice. Mass spectral analyses were carried out by three independent automatic injections of 1 μl of each sample by the automatic sampler and were accepted only if the standard sample deviation was less than 1% of the normalized peak intensity. Statistical analyses were performed using the parametric unpaired, two-tailed independent sample $t$ test with 99% confidence intervals. $^*, p < 0.05; ^**, p < 0.01$. In the case of labeled butyrate, $m_2$ (C-2–C-3) is obtained because of the high acetyl-CoA enrichment coming from butyrate that is recycled by the tricarboxylic acid cycle; this labeled glutamate is not related to the pyruvate carboxylase activity and hence is marked not applicable (N/A).

FIG. 3. Butyrate metabolism and its effect on tricarboxylic acid cycle as determined by mass isotopomers of glutamate. Pyruvate dehydrogenase activity relative to β-oxidation ($m_2$ C-4–C-5) is calculated by subtracting $m_2$ of $m_1$ 152 from $m_2$ of $m_1$ 198 fragments. The enrichment depends of how much butyrate is β-oxidized instead of glucose. Pyruvate carboxylase activity ($m_2$ C-2–C-3) is the $m_2$ from the $m_1$ 152 fragment of glutamate. $Y$ is calculated using the formula $Y = (1 - r)/r$, where $r = [(m_1/m_2)/(1 + 0.5(m_1/m_2))]$ and is the anaplerotic flux relative to the tricarboxylic acid cycle flux. $*, p < 0.05; **, p < 0.01$. In the case of labeled butyrate, $m_2$ (C-2–C-3) is obtained because of the high acetyl-CoA enrichment coming from butyrate that is recycled by the tricarboxylic acid cycle; this labeled glutamate is not related to the pyruvate carboxylase activity and hence is marked not applicable (N/A).

RESULTS

Effect of Butyrate on Cell Differentiation—The anti-proliferative/cell differentiating effect of sodium butyrate was evident in HT29 cells by an increase in cell differentiation, as assessed by the increase in the activity of the differentiation marker enzyme alkaline phosphatase (Fig. 1) as well as by a visible change in cell morphology. Glucose consumption decreased in a dose-dependent manner in HT29 cells after 0.1, 1 and 5 mM of butyrate, whereas MIA cells did not show any decrease in

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glucose consumption or cell differentiation (Fig. 2). Lactate production was also decreased in a dose-dependent manner in HT29 cells after 0.1, 1, 5, and 10 mM butyrate treatment, whereas MIA cells continued high lactate production (see Fig. 2). These results indicate that glycolysis substrate flow readily responds to butyrate only in differentiating HT29, but not in MIA cells.

Butyrate Metabolism in HT29 and MIA Cells—β-Oxidation of butyrate produces acetyl-CoA, which can either be oxidized in the mitochondria via the tricarboxylic acid cycle or reutilized for de novo lipogenesis. Thus, oxidation of butyrate generates acetyl-CoA and malonyl-CoA, both being potent metabolic regulators of glucose and fatty acid oxidation. β-Oxidation of $[1,2^{-13}C_2]$butyrate generates $[1,2^{-13}C_2]$acetyl-CoA, which can be incorporated into carbon 4 and 5 of glutamate, which is in equilibrium with α-ketoglutarate. The measurement of enrichment in C-4–C-5 of glutamate reflects the utilization of butyrate in energy production. In experiments with $[1,2^{-13}C_2]$glucose, oxidation of butyrate produces acetyl-CoA, which can either be oxidized in the mitochondria via the tricarboxylic acid cycle or reutilized for de novo lipogenesis. Thus, oxidation of butyrate generates acetyl-CoA and malonyl-CoA, both being potent metabolic regulators of glucose and fatty acid oxidation. β-Oxidation of $[1,2^{-13}C_2]$butyrate generates $[1,2^{-13}C_2]$acetyl-CoA, which can be incorporated into carbon 4 and 5 of glutamate, which is in equilibrium with α-ketoglutarate. The measurement of enrichment in C-4–C-5 of glutamate reflects the utilization of butyrate in energy production. In experiments with $[1,2^{-13}C_2]$glucose.
and unlabeled butyrate, the unlabeled acetyl-CoA from butyrate dilutes the enrichment of C-4–C-5 of glutamate. These results are shown in Fig. 3. When [1,2-13C2]butyrate was used as the tracer, the enrichment of m2 C-4–C-5 of glutamate steadily increased in both cell lines, reflecting the increasing butyrate oxidation. The HT29 cells were able to metabolize butyrate better than the MIA cells, as demonstrated by the higher m2 enrichment in glutamate. The high acetyl-CoA enrichment in HT29 cells resulted in the combination of 13C in carbons 3 and 4 generating m2 C-2–C-3 in /H9251-ketoglutarate, as the first labeled molecule continues through the tricarboxylic acid cycle as oxalacetate. When [1,2-13C2]glucose was used as the tracer, increasing butyrate oxidation and suppression of pyruvate dehydrogenase activity contributed to the decreasing enrichment in m2 C-4–C-5 of glutamate in both cell lines. [1,2-13C2]Glucose can also label carbons 2 and 3 of glutamate via pyruvate carboxylase. The decrease in the label of the upper part of the glutamate molecule (m2 C-2–C-3) indicates a decrease in the pyruvate carboxylase activity. Because anaplerosis relative to the tricarboxylic acid cycle (∆Y) remained relatively unchanged or was increased, unlabeled substrates must have entered the tricarboxylic acid cycle to make up for the decrease in pyruvate carboxylase activity.

β-Oxidation of butyrate also contributes to the precursor pool of malonyl-CoA for de novo lipogenesis. Incubation with labeled butyrate showed a dose response increase in the incorporation of labeled acetyl-CoA in palmitate synthesis, indicating a switch between the utilization of glucose and butyrate for fatty acid synthesis. This increase was higher in the differentiated HT29 cells than in MIA cells (Fig. 4). For the same reason, the decreased label incorporation into the fatty acids of MIA cells from [1,2-13C2]glucose due to dilution by unlabeled acetyl-CoA from butyrate was less prominent than in HT29 cells.

Effect of Butyrate on Flux Distribution between Glycolysis and the Pentose Cycle—Lactate labeling from [1,2-13C2]glucose and [1,2-13C2]butyrate was measured using GC/MS in separate experiments. Lactate 13C labeling was observed only when glucose tracer was used and was absent when the butyrate tracer was used. These findings indicate that 13C in butyrate is not incorporated into pyruvate through gluconeogenesis in HT29 or MIA cells. The results of lactate isotopomer production from [1,2-13C2]glucose are shown in Fig. 6. In addition to m2 lactate ([2,3-13C2]lactate), a significant fraction of singly labeled lactate was detected. Because recycling of label via the tricarboxylic acid cycle has been ruled out by the lack of labeling from butyrate, the slight increase in m1 in HT29 cells indicates an increase of the oxidative pentose phosphate path-
way. The relative amount of glucose that is converted indirectly to lactate through the pentose cycle as a percentage of the glycolytic flux can be calculated from the m1/m2 ratio. The pentose cycle in HT29 cells was about twice that of the MIA cells. A dose-dependent increase in the pentose cycle was observed in HT29 cells treated with butyrate. No such effect was observed in MIA cells.

Butyrate treatment resulted in a decrease in 13C incorporation into ribose of nucleic acid from glucose in a dose-dependent manner in HT29 cells (see Fig. 7). The average number of 13C atoms/molecule was reduced by 40% after 5 mM butyrate treatment. The reduction in ribose synthesis was much more modest (a reduction of about 15%) in MIA cells. Because butyrate treatment stimulates the oxidative pentose phosphate pathway (lactate isotopomer data in Fig. 6), the reduction of ribose synthesis in HT29 cells was caused by reduced substrate flux through the non-oxidative steps of the pentose cycle. The reduced transketolase activity is evident in the decreased m2 ribose fraction in the HT29 cells. In contrast, the effect of butyrate treatment on MIA cells ribose synthesis was much less than that observed in HT29 cells.

**DISCUSSION**

The results of this study demonstrate that HT29 colon adenocarcinoma cells exhibit profound metabolic adaptive changes in connection with phenotype modification and differentiation in response to butyrate. Colon HT29 cells responded to increasing doses of butyrate by expressing alkaline phosphatase, a potent cell differentiation marker. This is in good agreement with the increasing expression of several differentiation markers in HT29 cells reported in the literature (21). As determined from the metabolic profile, increasing concentrations of butyrate inhibited glucose uptake, glucose oxidation, and nucleic acid ribose synthesis in HT29 cells. In contradiction to these changes, there was a dose-dependent increase in de novo fatty acid synthesis utilizing butyrate carbons, whereas the utilization of glucose carbons for this purpose was diminished. There was also a significant increase in pentose cycle activity, affecting primarily the oxidative branch, which indicates an increase of NADPH production that is necessary for the increased fatty acid synthesis profile reported herein (Fig. 8). MIA cells, on the other hand, did not differentiate in response to butyrate, and the metabolic profile of MIA cells remained essentially unaffected. In contrast to HT29 cells, butyrate did not reduce glucose utilization, lactate production, or ribose synthesis in MIA cells.

The ability of HT29 cells and the inability of MIA cells to respond to butyrate present an interesting paradigm of nutrient gene interactions. HT29 cells originate from colon epithelial cells, which have the physiological role of metabolizing butyrate to prevent it from reaching toxic levels in the circulation. The transformation of colon epithelia into malignant HT29 cells requires prompt adaptation to a different nutrient environment in the absence of butyrate. The metabolic profile under such nutrient condition is similar to that of cultured MIA cells. However, when HT29 cells are exposed to butyrate, these cells re-adapt to a high butyrate nutrient condition, altering the expression of many genes of the glucose and lipid metabolic pathways. As a result, HT29 cells exhibit a different metabolic profile when treated with butyrate, whereas MIA cells are incapable of adapting to the high butyrate nutrient environment. This is demonstrated by the fact that labeled butyrate was poorly utilized by MIA cells for mitochondrial oxidation and lipogenesis.

The present study also offers an example of how cells sense...
changes in the nutrient environment and how they respond to these changes. The metabolism of butyrate significantly alters glucose metabolic pathways and the rate of energy production in responsive cells. The inhibition of glucose metabolic pathways and excess butyrate oxidation potentially changes the intracellular concentrations of many glucose and fatty acid.

**Fig. 7.** Effect of butyrate on RNA ribose synthesis. Ribose isotopomers obtained from the experiment with glucose label are shown. The m2 isotopomers of ribose are indicative of the non-oxidative pentose phosphate pathway flux, whereas the m1 isotopomers indicate the oxidative combined with the non-oxidative pentose phosphate pathway flux producing ribose. $\Sigma mn$ represents the molar enrichment of $^{13}$C in ribose for each condition and each cell line and is indicative of the ribose that is synthesized. *, $p < 0.05$; **, $p < 0.01$.

**Fig. 8.** Metabolic profile changes associated with butyrate-induced differentiation. Colon cells normally utilize butyrate as the major fuel substrate. In malignant transformation, these cells acquire the ability to utilize glucose as the major substrate. When HT29 cells, a colon cancer cell line, are exposed to high concentration of butyrate, butyrate induces HT29 cells to differentiate back to their butyrate-utilizing phenotype. These metabolic profile changes in HT29 cells between these two metabolic phenotypes are shown. In the figure, the intensity of the arrows represents the magnitude of substrate flux. In the gray box, we have listed some of the major genetic changes accompanying butyrate utilization by these cells (13, 14). Metabolic profiling provides a roadmap by which regulation of cell cycle as well as gene expression by metabolic intermediates may be investigated.
metabolic intermediates, which serve as signals for transcriptional, translational, and post-translational events that alter the metabolic phenotype of responsive cells.

In summary, HT29 cells assume two distinct metabolic phenotypes in response to changes in the nutrient environment. In the presence of butyrate, HT29 cells decrease the rate of glucose utilization and readily substitute glucose with butyrate. This change in metabolic phenotype is accompanied by molecular and morphological changes. The metabolic intermediates of butyrate may play a role not only as substrates for macromolecule synthesis and energy production but also as nuclear receptor-signaling ligands. Depending on the genetic background, changes in substrate availability result in the transformation of cells that exhibit a glucose-metabolizing phenotype, which is highly implicated in the proliferative cellular phenotype in cancer (22). This study demonstrates that metabolic enzymes and their substrates may serve as high efficiency, non-genetic novel targets and agents, respectively, for future cancer therapies (23).

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