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Original Contribution

# The deleterious metabolic and genotoxic effects of the bacterial metabolite *p*-cresol on colonic epithelial cells

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ABSTRACT

*p*-Cresol that is produced by the intestinal microbiota from the amino acid tyrosine is found at millimolar concentrations in the human feces. The effects of this metabolite on colonic epithelial cells were tested in this study. Using the human colonic epithelial HT-29 $Glc^{-/+}$ cell line, we found that 0.8 mM *p*-cresol inhibits cell proliferation, an effect concomitant with an accumulation of the cells in the S phase and with a slight increase of cell detachment without necrotic effect. At this concentration, *p*-cresol inhibited oxygen consumption in HT-29 $Glc^{-/+}$ cells. In rat normal colonocytes, *p*-cresol also inhibited respiration. Pretreatment of HT-29 $Glc^{-/+}$ cells with 0.8 mM *p*-cresol for 1 day resulted in an increase of the state 3 oxygen consumption and of the cell maximal respiratory capacity with concomitant increased anion superoxide production. At higher concentrations (1.6 and 3.2 mM), *p*-cresol showed similar effects but additionally increased after 1 day the proton leak through the inner mitochondrial membrane, decreasing the mitochondrial bioenergetic activity. At these concentrations, *p*-cresol was found to be genotoxic toward HT-29 $Glc^{-/+}$ and also LS-174T intestinal cells. Lastly, a decreased ATP intracellular content was observed after 3 days treatment. *p*-Cresol at 0.8 mM concentration inhibits colonocyte respiration and proliferation. In response, cells can mobilize their "respiratory reserve." At higher concentrations, *p*-cresol pretreatment uncouples cell respiration and ATP synthesis, increases DNA damage, and finally decreases the ATP cell content. Thus, we have identified *p*-cresol as a metabolic troublemaker and as a genotoxic agent toward colonocytes.



## 1. Introduction

The degradation of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) by the intestinal microbiota produces phenolic and indolic metabolic end products [12]. Among these bacterial metabolites, the phenolic compound *p*-cresol is produced from L-tyrosine in the large intestine lumen [46], notably by the anaerobic flora of the left colon [4]. Phenolic compound concentrations are high in the distal part of the large intestine where protein fermentation is intense [31]. Indeed, in humans, a 6.2 mM concentration of phenolic compounds has been measured in the distal colon while a 1.4 mM concentration was found in the proximal colon [45]. Phenol and *p*-cresol account for 70% of all luminal products of dissimilatory aromatic amino acid metabolism in the distal gut. The *p*-cresol concentration in the human feces averages approximately 0.4 mM [19,27]. These compounds are absorbed from the intestinal luminal content to the bloodstream by colonocytes, metabolized in the liver, and finally excreted by the kidneys with more than 90% of urinary phenolic compounds being recovered as *p*-cresol [21,31,41]. An increase of the protein intake raises the urinary [18] and fecal [49] concentrations of *p*-cresol. This is related to the fact that undigested or partially digested alimentary, endogenous proteins and peptides are transferred from the small intestine to the large intestine where they are degraded by the microbiota proteases and peptidases which release free amino acids (including L-tyrosine) used for bacterial metabolism [3]. Conversely, the fecal excretion and concentration of *p*-cresol are diminished by a high resistant starch diet [2]. Thus, the amount and the nature of alimentary compounds can

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modify the luminal concentration of *p*-cresol. Interestingly, distinct urinary metabolic footprints were described in colorectal cancer (CRC) patients when compared with healthy counterparts with altered levels of metabolites derived from gut microbial-host co-metabolism. Among this panel of bacterial metabolites, *p*-cresol was selected as able to discriminate CRC subjects from their healthy counterparts [11].

Previous studies evaluating the genotoxicity of *p*-cresol reported divergent results depending on the cell types studied [10,17]. In addition, decreased cell proliferation together with increased reactive oxygen species production has been observed in endothelial and mononuclear cells treated with *p*-cresol [9].

*p*-Cresol is also well known to be a potential uremic toxin [23,39]. Indeed, in patients with chronic kidney disease (CKD), the accumulation of *p*-cresol and its metabolite *p*-cresyl-sulfate contributes to CKD-associated disorders [13,42]. Surprisingly there is a paucity of data on the effect of *p*-cresol on the colon epithelium even though this metabolite is present at high concentrations in the luminal content and is in vicinity to colonic epithelial cells [54].

In that context, the aim of the present study was to document the effects of *p*-cresol on colonic epithelial cells using the human colonic adenocarcinoma cells HT-29 $Glc^{-/+}$ which have retained major metabolic characteristics of normal colonocytes [28]. We examined both the acute and longer-term effects of *p*-cresol on colonocytes, with emphasis on mitochondrial bioenergetics, cell proliferation, and genotoxic effects. In addition, we discussed the likely causal links between these different cellular parameters.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals including *p*-cresol (99% pure) were obtained from Sigma (St. Louis, MO).

### 2.2. Cell culture and cell proliferation

The human adenocarcinoma cell line HT-29 $Glc^{-/+}$ was established in permanent culture in 1975. HT29-$Glc^{-/+}$ cells used in this study were selected by Zweibaum et al. from parental cells by growing them in a glucose-free medium for 36 passages, then leaving them to grow at 37 °C under 5% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum and containing 4 mM glutamine, 25 mM D-glucose. HT29-$Glc^{-/+}$ cells were used between passages 37 and 65 (one passage every week) and were seeded at density of $2 \times 10^4$ cells/$cm^2$ on day 0. The cells were cultured in six well plates or in T75 flasks without or with *p*-cresol freshly dissolved in the culture medium. The culture medium was changed every day.

For cell proliferation, cells were pretreated with or without *p*-cresol 0.2, 0.4, or 0.8 mM from day 3 to day 4 or from day 3 to day 7 after seeding. Then, the culture medium was recovered to count floating cells. Adherent cells were isolated using a phosphate-buffered saline (PBS) containing 1 g $L^{-1}$ EDTA and 0.25 g $L^{-1}$ trypsin and counted with a hematocytometer.

LS-174T human epithelial colorectal adenocarcinoma cells (ATCC No. CL-188) were grown in αMEM medium supplemented with 10% FBS.

### 2.3. Isolation of rat colonocytes

Colonic epithelial cells (colonocytes) were isolated as described [35] from Wistar Han 200–240 g rat colon (Harlan Laboratories Gannat, France). Briefly, the colon was removed and flushed cleaned with NaCl 9 g $L^{-1}$ and then the colonocytes were isolated using the perfusion method with EDTA followed by hyaluronidase treatment. Colonocytes were maintained in DMEM medium up to oxygen consumption measurements.

### 2.4. Measurement of membrane integrity

The *in vitro* TOX7 toxicology assay kit (Sigma, St. Louis, MO) was used. After cell culture with or without *p*-cresol, the culture medium was recovered and centrifuged. Soluble LDH was measured in the supernatant (corresponding to LDH released from necrotic cells) and in the floating and adhering cells. Relative LDH activity was measured as percentages of total LDH activity.

### 2.5. Cell cycle distribution

Floating cells recovered in the culture medium and adherent cells recovered by trypsinization following treatment with *p*-cresol or butyrate (as positive control) were washed and incubated overnight with 70% ice-cold ethanol in PBS prior to staining with a propidium iodide solution (40 μg $mL^{-1}$ PI) in PBS containing 50 μg $mL^{-1}$ RNase A (30 min, 37 °C, in dark). The immunofluorescent staining of incorporated PI was measured on $20 \times 10^3$ cells sorted with a FACSCalibur flow cytometer and analysis was performed using Cell Quest software [26]. FlowJo software was used to generate DNA content frequency histograms and to quantify the amount of cells in the individual cell cycle phases including sub-G0/G1 population.

### 2.6. Oxygen cell consumption

Oxygen consumption was measured by polarography (Oxygraph Hansatech Inst., Norfolk, UK) at 37 °C using a Clark-type electrode as described [1]. Control and cells pretreated with *p*-cresol were isolated and $5 \times 10^6$ cells were resuspended in 1.5 mL air-saturated incubation mixture (20 mM Hepes buffer containing 200 mM mannitol, 5 mM $KH_2PO_4$, 2.5 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.4, enriched with 0.1% bovine serum albumin) and placed in the oxygraph chamber for oxygen consumption measurement. Basal oxygen consumption (state 3) was measured in the absence of any exogenous agent. Uncoupled rate was determined after addition of the proton ionophore carbonyl cyanide 4-trifluoromethoxy phenyl hydrazone (FCCP 1.5 μg $mL^{-1}$). The oxygen consumption due to the proton leak (state 4) was measured after the addition of the Fo/F1 ATPase inhibitor oligomycine (0.5 μg $mL^{-1}$). For evaluating the acute effects of *p*-cresol on oxygen consumption, the compound was added as a single shot in the chamber of an oxygraph containing untreated cells.

### 2.7. Anion superoxide measurement

HT-29 $Glc^{-/+}$ cells were seeded at a density of $2 \times 10^4$ cells/$cm^2$ density on day 0. At day 3, cells were cultured without or with *p*-cresol. Then cells were isolated with trypsin, and floating and adhering cells were labeled using 5 μM dihydroethidium (DHE, Molecular Probes, Eugene, OR) for 20 min at 37 °C in the dark to detect superoxide anion ($O_2^{\cdot-}$) production. After washes with PBS, fluorescence emission of oxidized DHE was measured by flow cytometry [26]. Menadione (250 μM, 20 min) was used as positive control. Superposition of control and menadione histograms allowed determination of the percentages of cells producing $O_2^{\cdot-}$.

### 2.8. Genotoxicity measurement

For genotoxicity measurement, the γH2AX (phosphorylated form of H2AX histone) In Cell Western (ICW) technique was performed as previously described [24]. Briefly, HT-29 $Glc^{-/+}$ or

LS-174T colon epithelial cells were distributed in cell culture plate ($36 \times 10^3$ cells, 200 μL/well). Sixteen hours later, the cells were treated in serum-free medium containing *p*-cresol. For each plate, etoposide (Eto, 1 μM) was used as a positive control. After 24 h of treatment, the cells were fixed in 4% paraformaldehyde. Paraformaldehyde was neutralized and cells were permeabilized with 0.2% Triton X-100 in PBS. Cells were blocked with MAXblock Blocking medium supplemented with phosphatase inhibitor PHOSTOP and RNase A, followed by 2 h incubation with rabbit monoclonal anti γH2AX (Clone 20E3, Cell Signaling) in PST buffer. Secondary detection was carried out using an infrared fluorescent dye conjugated to goat antibody with an absorption peak at 770 nm (CF770, Biotium). For DNA labeling, RedDot 2 (Biotium) was used in conjugation with the secondary antibody. After 1 h incubation, DNA and γH2AX were simultaneously visualized using an Odyssey infrared imaging scanner (LiCorScienceTec, Les Ulis, France). The relative fluorescence units from the scanning were used for quantitative analysis. For the determination of genotoxicity, relative fluorescent units for γH2AX per cell (as determined by γH2AX divided by DNA content) were divided by the respective controls (vehicle only) to determine the change in phosphorylation of H2AX levels compared with control. To determine relative cell count (RCC), the DNA content recorded in the different experiments was compared to the DNA content in control cells. Genotoxicity was considered positive when a compound produced a level of cytotoxicity below 50% and led to at least 1.3-fold γH2AX induction compared to the control.

### 2.9. Mitochondrial NADPH/NADH dehydrogenase activities

MTS is a MTT-like assay that is highly dependent on mitochondrial activity. The method is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. This conversion is carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. MTS reduction is analyzed by a colorimetric assay to study mitochondrial dehydrogenase activity as a test of cytotoxicity. Cells were plated at density of $2 \times 10^4$ cells/cm$^2$ on day 0. At day 4, cells were cultured with *p*-cresol and then washed, and their ability to reduce MTS was evaluated by using the CellTiter 96 AQueous assay according to the manufacturer's instructions (Promega, WI, USA). The absorbance at λ 490 nm was measured using a Multi-Mode microplate reader (Synergy HT, BioTek, and Sunrise, Tecan; respectively).

### 2.10. ATP cell content

The intracellular ATP content was measured using the CellTiter-Glo kit, which generates a luminescent signal proportional to the amount of ATP present in the assay [8]. Cells were plated at a density of $2 \times 10^4$ cells/cm$^2$ on day 0. At day 4, cells were incubated with *p*-cresol and ATP was assayed according to the manufacturer's instructions (Promega, WI, USA) with luminescence being measured using a Multi-Mode microplate reader. The ATP levels were normalized by the protein contents.

### 2.11. Data analysis

The results are expressed as mean value (± SEM). Statistical analysis was performed using the Student *t* test, ANOVA, and Tukey multiple-comparisons test when appropriate. Differences with $P$ value $< 0.05$ were considered as statistically significant.

## 3. Results

### 3.1. Effect of p-cresol on HT-29 Glc$^{-/+}$ cell proliferation

As indicated in Fig. 1A, when cells were cultured with increasing concentrations of *p*-cresol, the 0.8 mM concentration markedly and significantly decreased the number of adherent cells both after 1 and 4 days treatment when compared with the control experiment (without *p*-cresol). This *p*-cresol concentration was in addition able to modestly increase the amount of floating cells in the incubation medium after 4 days treatment but not after 1 day treatment (Fig. 1B). When comparing the respective number of adhering vs floating cells (Figs. 1A and B), it appears that the marked reduction of cell growth due to 0.8 mM *p*-cresol is only very partly due to cell detachment.

### 3.2. Effect of p-cresol on HT-29 Glc$^{-/+}$ cell viability

As shown in Fig. 2, when the cells were cultured for 2 days in the presence of 0.8 mM *p*-cresol, and in good accordance with the results obtained in the experiments related to cell proliferation, this treatment slightly but significantly decreases the number of adherent cells and accordingly increased the number of floating cells in the incubation medium. However, the percentage of free LDH activity in the incubation medium, which is an indicator of cell necrosis, was not modified by *p*-cresol, whatever the concentration used (Fig. 2). When a higher *p*-cresol concentration (1.6 mM) was used, the results were superimposable with the results obtained using 0.8 mM *p*-cresol (Fig. 2). The highest concentration tested (3.2 mM) did not provoked cell necrosis even though it slightly increased the number of floating cells in the culture medium (Fig. 2).

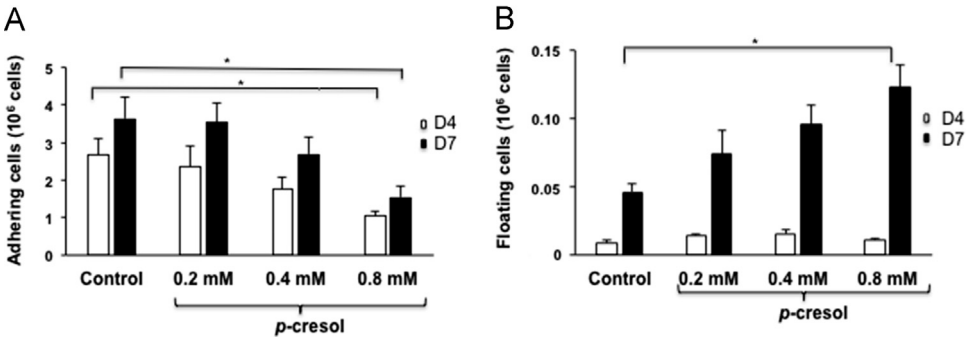


**Fig. 1.** Effects of *p*-cresol on HT-29 Glc$^{-}$/+ cell proliferation and adhesion. Three days after cell seeding, the cells were cultured without (control) or with increasing concentrations of *p*-cresol for 1 and 4 days with the culture medium changed every day. Adhering (A) and floating cells (B) were measured at day 4 (1 day treatment) and at day 7 (4 days treatment). The results (mean ± SEM) were obtained from 5 independent experiments. * Indicates significantly different values ($P < 0.05$).

### 3.3. Effect of p-cresol on HT-29 Glc$^{-/+}$ cell cycle

As indicated in Fig. 3, when *p*-cresol was added at increasing doses in the incubation medium, the 0.8 mM concentration was able after 1 and 2 days of treatment to provoke a marked accumulation of the HT-29 Glc$^{-/+}$ cells in the S-cell cycle phase and a decrease of the relative number of HT-29 Glc$^{-/+}$ cells in the G0/G1 phase. The relative amount of cells in the subdiploid G1 peak, characteristic of cells undergoing nuclear fragmentation, was not modified by the treatment with 0.8 mM *p*-cresol (Fig. 3). When 10 mM butyrate was used as a positive control, the amount of cells in the S phase decreased, as expected, after 1 day treatment while after 2 days treatment, butyrate increased the number of apoptotic cells (Fig. 3).

### 3.4. Acute effect of p-cresol on HT-29 Glc$^{-/+}$ cell oxygen consumption

As shown in Fig. 4A, when *p*-cresol was added at 0.8 mM concentration to the HT-29 Glc$^{-/+}$ cells isolated 4 days after seeding, it decreased the state 3 basal cell oxygen consumption.

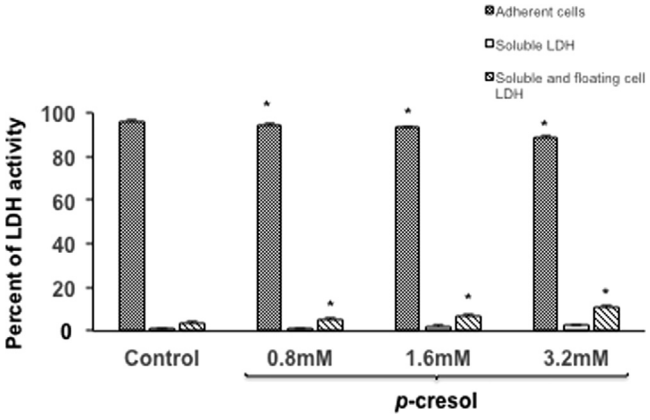


**Fig. 2.** Effects of *p*-cresol on HT-29 Glc$^{-}$/+ cell viability. Three days after cell seeding, the cells were cultured without (control) or with increasing concentration of *p*-cresol for 2 days with the culture medium changed every day. The LDH activity was measured in adherent cells, in the culture medium, and in the floating cells. The results (mean ± SEM) were obtained from 6 independent experiments. * indicates significantly different values between controls and matched groups ($P < 0.05$).

No reversion of oxygen consumption back to the basal value was observed up to the longest time tested (10 min, data not shown). When *p*-cresol was used at 1.6 and 3.2 mM, the inhibition of oxygen consumption was not vastly different when compared with the effect recorded at 0.8 mM *p*-cresol (Fig. 4A). When the same experiments were performed using HT-29 Glc$^{-/+}$ cells isolated 7 days after seeding, the inhibitory effect of *p*-cresol was similar to the effect recorded with cells isolated at day 4 (Fig. 4B), indicating that the acute *p*-cresol effect on HT-29 Glc$^{-/+}$ cells is not dependent on the duration of the cell culture after seeding.

When the cells were isolated 7 days after seeding and cell oxygen consumption was measured either in the sole presence of the Fo/F1 ATPase inhibitor oligomycin or in the presence of 1.6 mM *p*-cresol and oligomycin, if the cell oxygen consumption is expressed in percentages of the basal state 3 oxygen consumption (no agent added), we found no significant difference between the percentage calculated in the sole presence of oligomycin (44.4% ± 3.1 of the state 3, $n = 10$) and the percentage calculated in the presence of *p*-cresol and oligomycin (56.6% ± 7.3 of the state 3, $n = 10$, data not shown) indicating that *p*-cresol had no immediate uncoupling effect in acute experiments. Likewise, when the cell oxygen consumption was measured either in the sole presence of the uncoupler FCCP or in the presence of 1.6 mM *p*-cresol and FCCP, and expressed in percentages of the basal state 3 oxygen consumption, we found no significant difference between the percentage calculated in the sole presence of FCCP (300.6% ± 10.7 of the state 3, $n = 10$) and the percentage calculated in the presence of *p*-cresol and FCCP (263.6% ± 30.4 of the state 3, $n = 10$, data not shown), indicating that *p*-cresol did not increase the maximal respiratory rate in acute experiments.

When *p*-cresol was added on isolated rat normal colonocytes at the intermediate concentration (1.6 mM), it also decreased cellular state 3 oxygen consumption (Fig. 4C), confirming the results obtained with the HT-29 Glc$^{-/+}$ cell model.

### 3.5. Effect of p-cresol pretreatment on HT-29 Glc$^{-/+}$ cell oxygen consumption

As indicated in Fig. 5A, when HT-29 Glc$^{-/+}$ cells were pretreated for 1 day in the presence of 0.8 mM *p*-cresol, and then isolated for measurement of oxygen consumption under different experimental conditions, the state 3 basal oxygen consumption was significantly increased. When the same experiment was done in the presence of

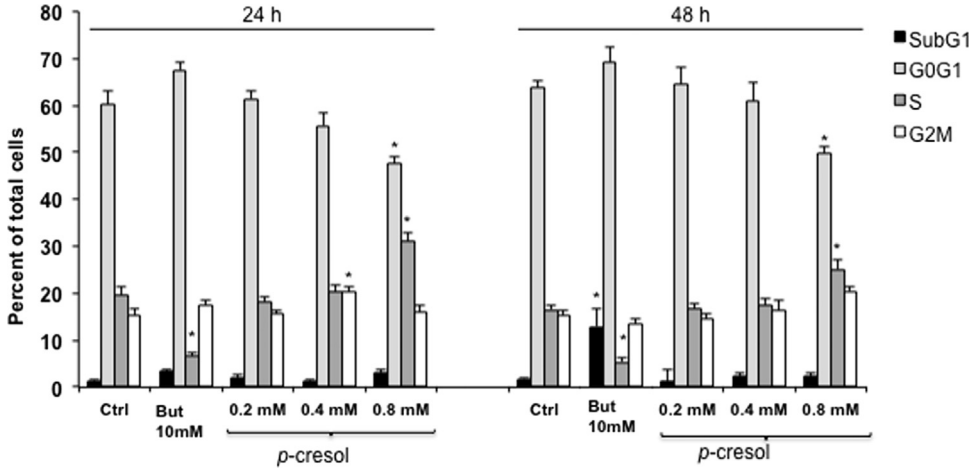


**Fig. 3.** Effects of *p*-cresol on HT-29 Glc$^{-}$/+ cell distribution in the cell cycle phases. Three days after cell seeding, cells were cultured without (control) or with increasing concentrations of *p*-cresol added to the culture medium for 1 (24 h) or 2 (48 h) days, with the culture media changed every day. Adherent and floating cells were pooled together and stained with PI before cell cycle analysis by flow cytometry. A positive control was done in the presence of 10 mM butyrate. The results (mean ± SEM) were obtained from 6 independent experiments. * indicates significantly different values compared to control group ($P < 0.05$).

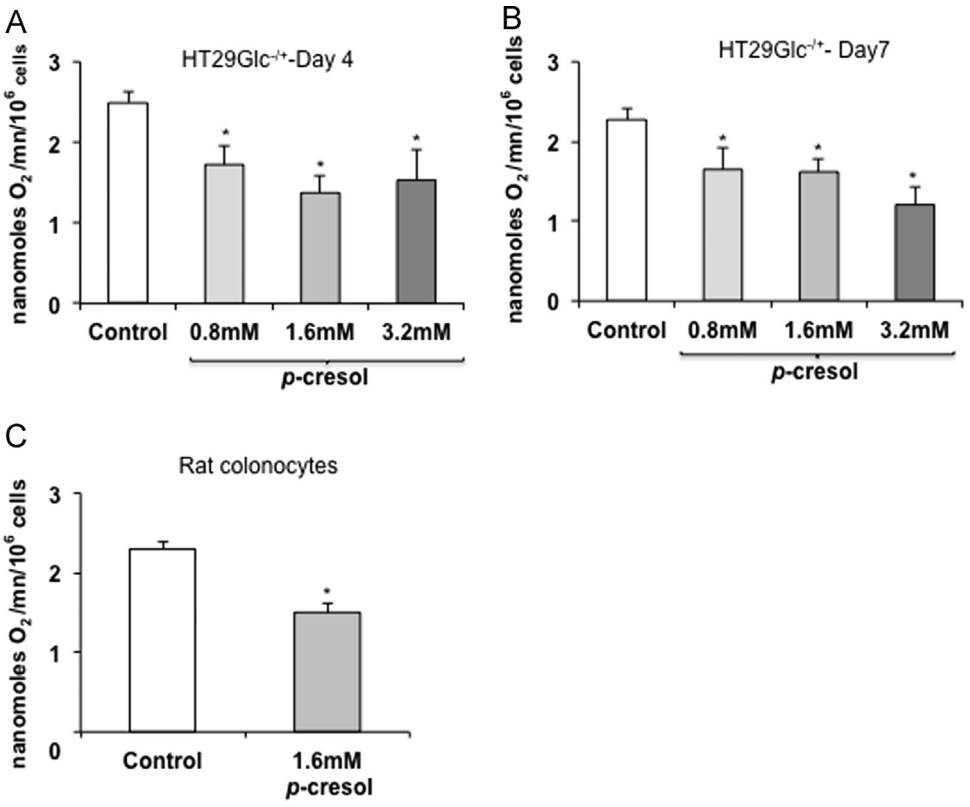


**Fig. 4.** Effects of acute treatment with *p*-cresol on HT-29 $Glc^{-/+}$ cell oxygen consumption. Four days (A) or 7 days (B) after seeding, cells were isolated and used for $O_2$ consumption measurements. Rate of oxygen consumption was measured first without any exogenous compound (state 3) which corresponds to the basal oxygen consumption (control). Then a single shot of increasing concentration of *p*-cresol was added and oxygen consumption rate was measured after respiration stabilization. Rat colonocytes were isolated and incubated in the presence of the intermediate concentration of *p*-cresol 1.6 mM (C). The results (mean ± SEM) were obtained from at least 7 independent experiments. * indicates significantly different values between control and matched groups ($P < 0.05$).

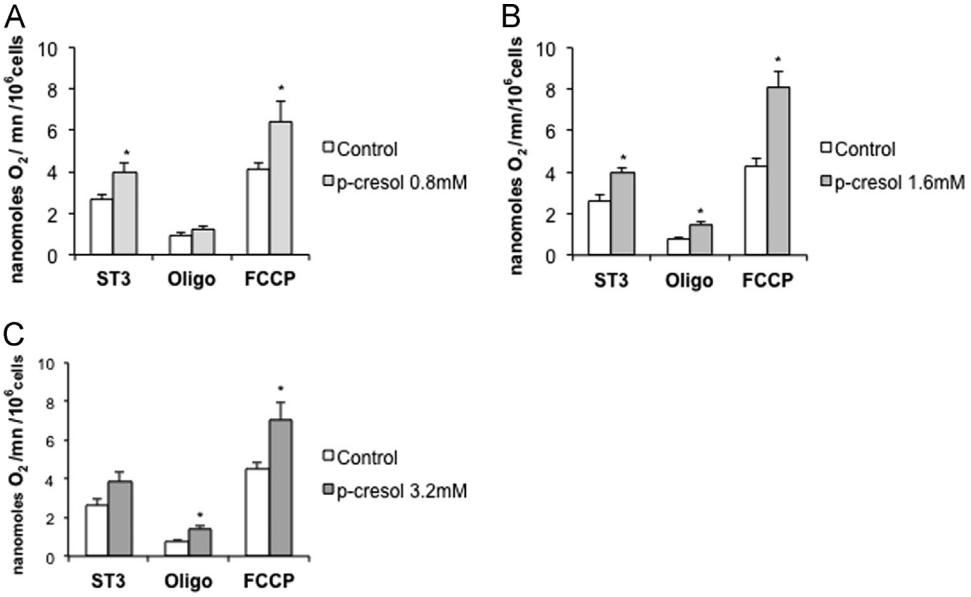


**Fig. 5.** Effects of *p*-cresol pretreatment on HT-29 $Glc^{-}/+$ cell oxygen consumption. Three days after cell seeding, cells were cultured for 1 day without (control) or with 0.8 mM (A), 1.6 mM (B), and 3.2 mM *p*-cresol (C) and the cells were then isolated for oxygen consumption measurement first without any exogenous agent (state 3 (ST3)) and after addition of the Fo/F1 ATPase inhibitor oligomycin (Oligo) or the uncoupler FCCP. The results (mean ± SEM) were obtained from at least 7 independent experiments. * indicates significantly different values between control and matched groups ($P < 0.05$).

the uncoupling agent FCCP, a significant increase of the oxygen consumption was also observed, indicating an increase of the maximal oxygen consumption capacity in the mitochondrial respiratory chain. In the presence of the inhibitor of Fo/F1 ATPase oligomycin, the oxygen consumption was not different between the cells treated with *p*-cresol and the control untreated cells, indicating a similar proton leak in both experimental situations.

When the HT-29 $Glc^{-/+}$ cells were pretreated for 1 day with a higher *p*-cresol concentration (1.6 mM, see Fig. 5B), as for the 0.8 mM concentration, we observed an increase of oxygen

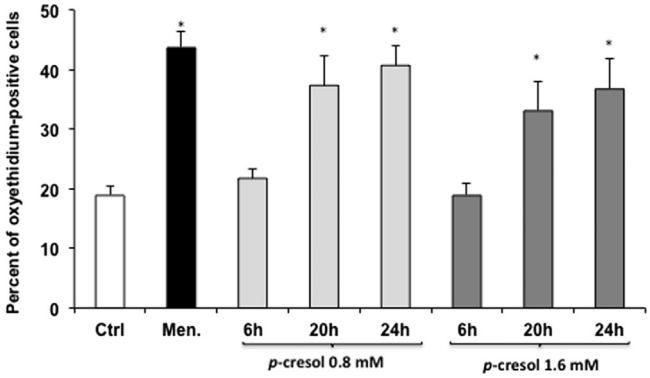


**Fig. 6.** Effects of *p*-cresol pretreatment on HT-29 Glc$^{-}$/+ cell anion superoxide production. Three days after cell seeding, cells were cultured for 6, 20, and 24 h without (control) or with *p*-cresol, and cells were then isolated and labeled with dihydroethidium for the measurement of oxyethidium fluorescence emission by flow cytometry. The prooxidant Menadione (Men., 250 μM, 20 min) was used as positive control. The results (mean ± SEM) were obtained from 6 independent experiments. * indicates significantly different values ($P < 0.05$) compared to control group.

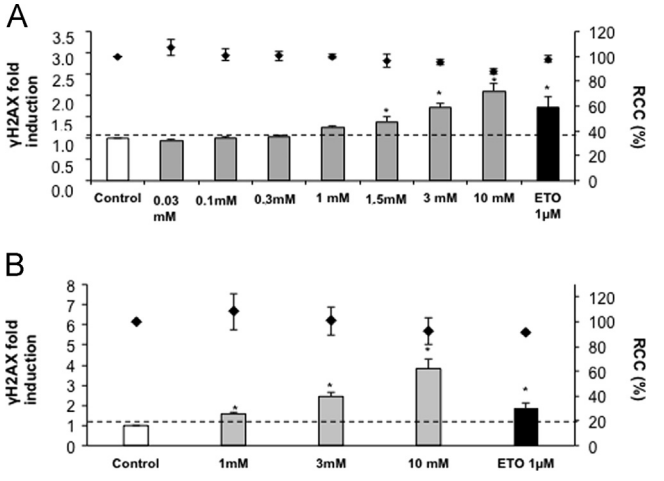


**Fig. 7.** Genotoxic effects of *p*-cresol on colon epithelial cell lines. HT-29 Glc$^{-/+}$ cells (A) or LS-174T (B) were cultured for 24 h with vehicle only (control), etoposide (Eto, positive control), or with increasing concentrations of *p*-cresol. The γH2AX ICW assay was used to quantify DNA damage (left *Y* axis) and cytotoxicity (right *Y* axis). The results are expressed as fold change for H2AX phosphorylation compared to negative control (histogram) and relative cell count (RCC, individual points) expressed as % of control cell DNA content. The results (mean ± SEM) were obtained from at least 3 independent experiments in duplicate. * indicates values significantly different compared to negative control ($P < 0.05$).

consumption both in the basal (state 3) condition and in the uncoupled situation. Moreover, we observed a significant increase of the oxygen consumption measured in the presence of oligomycin. This indicates that the mitochondria of the HT-29 Glc$^{-/+}$ cells pretreated with 1.6 mM *p*-cresol are markedly less coupled compared to mitochondria from untreated cells. When the cells were treated for 1 day with the 3.2 mM concentration, the results were almost identical to the results obtained using the 1.6 mM concentration (Fig. 5C).

### 3.6. Effects of p-cresol pretreatment on HT-29 Glc$^{-/+}$ cell anion superoxide production

When the cells were incubated with 0.8 mM *p*-cresol, the production of anion superoxide significantly increased after 20 and 24 h of treatment but not after 6 h (Fig. 6). Using a higher concentration of *p*-cresol (1.6 mM) did not further increase the anion superoxide production.

### 3.7. Genotoxic effects of p-cresol on colon epithelial cell lines

Because anion superoxide production may act as genotoxic compounds, we measured with the γH2AX ICW assay, the genotoxic potential of *p*-cresol on HT-29 Glc$^{-/+}$ cells. We found that 24 h *p*-cresol treatment dose dependently induced the phosphorylation of the H2AX histone, indicating that *p*-cresol induced DNA damage (Fig. 7A). This effect was statistically significant from 1.5 mM concentration of *p*-cresol and above on HT-29 Glc$^{-/+}$ cells (Fig. 7A). Similar results were obtained using another human colon epithelial cell line, that is, LS-174T cells (Fig. 7B). The genotoxic effect of *p*-cresol was not related to any cytotoxic effect of this compound (Figs. 7A and B).

### 3.8. Effects of p-cresol pretreatment on mitochondrial NADPH/NADH deshydrogenase activities

By using the MTS assay, we found that the treatment of cells for 1 and 2 days with 3.2 mM *p*-cresol decreased the mitochondrial NADPH/NADH dehydrogenase activities when compared with control experiments (no *p*-cresol, see Figs. 8A and B). A lower concentration of *p*-cresol (1.6 mM) has an effect on this parameter only after 2 days of treatment.

### 3.9. Effects of p-cresol pretreatment on HT-29 Glc$^{-/+}$ cell ATP content

As indicated on Figs. 9A to C, although 0.8, 1.6, and 3.2 mM *p*-cresol had no effect on the intracellular concentration of ATP after 1 (A) and 2 day treatment (B), a decrease was clearly visible after 3 days of treatment (C).

## 4. Discussion

Our work with the human colonocytes HT-29 Glc$^{-/+}$ cell shows that a single shot of *p*-cresol at a moderate concentration (0.8 mM) decreased within minutes the cell oxygen consumption. Using rat isolated colonocytes which can be maintained viable for 1 h, we found that normal colonic epithelial cells are also sensitive to *p*-cresol for their respiration, thus validating the HT-29 Glc$^{-/+}$ cell model for such study.

In response to the pretreatment with 0.8 mM *p*-cresol, the HT-29 Glc$^{-/+}$ cells were found to increase their state 3 basal oxygen consumption and their maximal respiratory rate within 1 day. The maximal rate of electron transfer through the mitochondrial respiratory chain is revealed by the maximal oxygen consumption observed in the presence of the respiratory uncoupler FCCP. The increase of the basal and maximal respiratory rate likely corresponds to an attempt of the cells to counteract the effect of *p*-cresol on the cell respiration [33]. This most likely corresponds to a cell adaptation to the pretreatment with *p*-cresol since *p*-cresol did not increase the maximal respiratory rate in acute experiments.

However, despite this latter attempt, we measured in the meantime a decrease of HT-29 Glc$^{-/+}$ colonocyte proliferation, detectable within 1 day, which coincided with an accumulation of cells in the S-phase cell cycle and with some minor cell detachment. Reduced proliferation rate may be considered as an adaptative response to the impaired ATP synthesis in case of partial inhibition of respiration, allowing maintenance of cell integrity [29]. Indeed, a decrease in ATP cell content is associated and used as an indicator of cell necrosis [52]. Buttgereit and Brand documented that in mammalian cells, macromolecule biosynthesis is very sensitive to energy supply with a rate of protein and

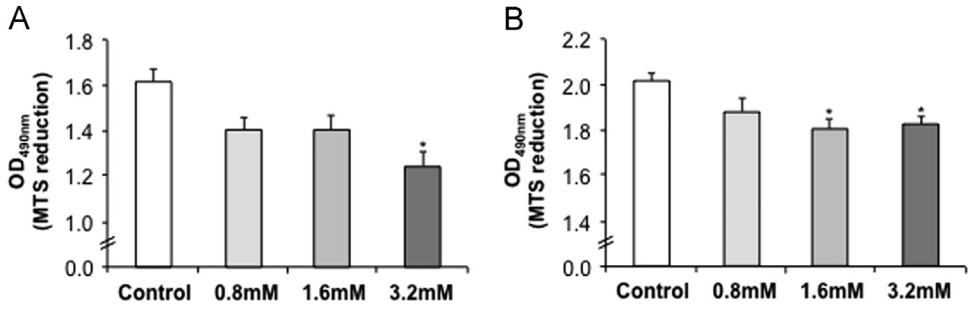


**Fig. 8.** Effects of *p*-cresol pretreatment on mitochondrial NADPH/NADH deshydrogenase activities. Three days after cell seeding, HT-29 $Glc^{-}/+$ cells were cultured for 1 (A) and 2 days (B) without (control) or with increasing concentrations of *p*-cresol with the culture medium changed every day. Then the cells were tested for their mitochondrial NADPH/NADH deshydrogenase activities by using the MTS test. The results (mean ± SEM) were obtained from 3 independent experiments in quadruplicates. * indicates significantly different values ($P < 0.05$).

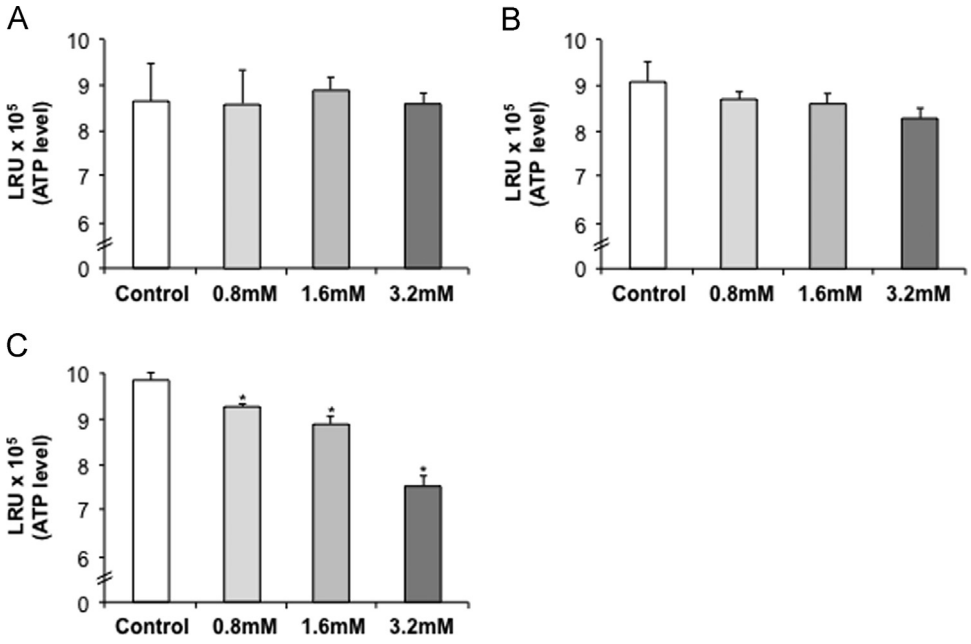


**Fig. 9.** Effects of *p*-cresol pretreatment on the ATP HT-29 $Glc^{-}/+$ cell content. Three days after cell seeding, cells were cultured for 1 (A), 2 (B), and 3 days (C) without (control) or with increasing concentrations of *p*-cresol with the culture medium changed every day. Then the cells were tested for their intracellular ATP content. The results (mean ± SEM) were obtained from 3 independent experiments in quadruplicates. * indicates significantly different values ($P < 0.05$).

polynucleotide synthesis decreasing to 40% when the respiration is inhibited by 30% [6]. As a matter of fact, HT-29 $Glc^{-/+}$ cells maintained up to 3 days of treatment with *p*-cresol a constant ATP intracellular content and showed no sign of cell necrosis nor apoptosis.

Following pretreatment with 0.8 mM *p*-cresol, an increased production of anion superoxide by the HT-29 $Glc^{-/+}$ cells was measured after 20 h but not after 6 h treatment with *p*-cresol, indicating a lag period for cellular response to the treatment. In most eukaryotic cell types, anion superoxide are produced mainly (but not exclusively) at the mitochondrial complex I and III level in the course of oxidative phosphorylation, particularly in situations of mitochondrial complex activity inhibition [7,15,16,22,38,50,51]. The rate of mitochondrial anion superoxide production is dependent on the metabolic state of the mitochondria. Higher production can be explained by an increased proportion of the respiratory chain components in their reduced state and by a longer life duration of some intermediates involved in proton transfer like ubisemiquinone [16]. Then, we propose that the effect of *p*-cresol on the production of anion superoxide corresponds, at least partly, to an effect of this compound on the redox state and/or life duration of some intermediates of the mitochondrial respiratory chain. In other words, the acute effect of *p*-cresol toward colonocytes is an inhibition of the respiration while the longer-term effect of this bacterial metabolite may involve the modified redox state of respiratory chain element(s). Interestingly, it has been shown [25,36] that *p*-cresol affects NAD- and succinate-linked respiration in liver mitochondria, and the inhibitory effect of *p*-cresol on the NAD-linked respiration was found to be stronger than those on the succinate-linked respiration, with little effect on the P/O ratio in liver mitochondria using glutamate or succinate as oxidative substrates. These results indicate that liver mitochondria are likely one of the targets for the hepatotoxic actions of *p*-cresol. Further experiments outside of the scope of this study are necessary to further decipher the mechanisms involved in the acute and longer-term effects of *p*-cresol, notably on the different mitochondrial complexes in colonic epithelial cells.

When higher concentrations of *p*-cresol were used (1.6 and 3.2 mM), interestingly we also observed after 1 day pretreatment, in addition to the effects documented with the 0.8 mM concentration, an increased cell oxygen consumption in the presence of the Fo/F1 ATPase inhibitor oligomycin. This result demonstrates that these *p*-cresol concentrations lead to an increase of the mitochondrial proton leak, an increase which favors mitochondrial uncoupling between oxygen consumption and ATP synthesis. Once again, this most likely corresponds to a cell adaptation to the

presence of *p*-cresol for 1 day since *p*-cresol did not increase the proton leak in acute experiments.

A mild uncoupling is considered as a way to speed up the respiratory chain and then to reduce the production of superoxide anion [34,43,47]. However, this mild uncoupling observed after pretreatment with 1.6 mM *p*-cresol did not coincide with any marked reduction of anion superoxide production when compared with the production measured with a lower concentration (0.8 mM) with no uncoupling effect, thus questioning the efficiency of uncoupling for such a purpose. In addition, decreased cellular bioenergetic performance is known to represent a price to be paid for uncoupling. Indeed, in that case, the protons are transferred from the mitochondrial intermembrane space to the matrix without ATP synthesis through the Fo/F1 ATPase.

Respiratory coupling in cells restricts the cellular respiration to a rate matching the ATP turnover. The consequence of the inhibition of cell respiration and of mitochondrial uncoupling, in terms of ATP intracellular content, was not immediate but was clearly visible after 3 days treatment at a time when presumably, despite decreased proliferation rate, the ATP synthesis does not meet anymore the ATP consumption by the colonic epithelial cells.

Regarding the important point of the genotoxic effect of *p*-cresol on colonocytes, our data show that after 1 day treatment, there was a dose-related induction of DNA damage by *p*-cresol in colonocytes as recorded by the γH2AX ICW assay on HT-29 $Glc^{-/+}$ cells and also on LS-174T human colonic epithelial cells. Increased reactive oxygen species production is often considered to be associated with genomic DNA damage [32,48,53]. However, our data showing that the dose-dependent genotoxic effect of *p*-cresol can be dissociated from the effect on anion superoxide production suggest that the damaging effect of *p*-cresol on the colonocyte DNA may involve other unknown pathways than oxidative stress, maybe, for instance, following *p*-cresol bioactivation in colonocytes. Importantly, our study is the first to evaluate the genotoxicity of this bacterial metabolite toward colonocytes. As those cells are directly facing the large intestine lumen where *p*-cresol is produced, they are probably the most exposed cells of the body to this bacterial metabolite. Importantly, positive correlation between DNA damage in colonocytes and *p*-cresol concentration in the large intestine content has been described in rats fed with varying levels of protein [49,54].

From our data, it appears that *p*-cresol can be considered both as a metabolic troublemaker and as a genotoxic luminal bacterial metabolite toward colonic epithelial cells. Limitations of this study are related to (i) the imprecision regarding the true luminal concentration of the unbound form of *p*-cresol in the immediate vicinity of the colonic epithelial cells (notably near the pluripotent stem cells located at the bottom of the crypt [37], a parameter difficult to assess for technical reasons; and (ii) to the unknown capacity of *p*-cresol to diffuse, according to its luminal concentration, across the mucous layer covering the epithelial cells [37]. Regarding this latter point, the fact that *p*-cresol is largely absorbed through the large intestine epithelium and excreted in the urine indicates that the passage of this compound across the mucous layer is efficient.

With these reservations in mind, the present study and previous ones indicate that the colon epithelial layer is facing a luminal content characterized by the presence of deleterious compounds, including *p*-cresol, when present in excess [20]. Indeed, we previously reported that other amino acid-derived bacterial metabolites, including hydrogen sulfide [5] and ammonia [1], act at excessive luminal concentrations as inhibitors of colonocyte respiration. In a context of a mean protein consumption largely above the recommended daily amount in industrialized countries [14,40,44] and taking into account that the increased protein consumption increases the *p*-cresol fecal concentration [49] our study showing that *p*-cresol is both a genotoxic agent and a metabolic troublemaker in human colonocytes calls for further studies regarding the potential impact of long-term increased luminal concentration of *p*-cresol in the course of colorectal carcinogenesis [30]. Indeed, the identification of luminal compounds with deleterious effects on the process of colonic epithelium renewal associated with genotoxic effects, and finally loss of epithelial homeostasis, appears to represent an important step in the understanding of the relationship between the modifications of the colonocyte luminal environment and the preneoplasic and neoplasic processes.

In conclusion, our study represents a new contribution on the effects of bacterial metabolites on colonocytes. We show that *p*-cresol acts in acute exposure as a metabolic troublemaker inhibiting cell respiration. At 0.8 mM concentration, *p*-cresol pretreatment, despite a mobilization of the "respiratory reserve," decreases cell proliferation but maintains the ATP intracellular content and the cell viability. At a higher concentration, *p*-cresol pretreatment increases proton leak through the mitochondrial inner membrane, resulting in decreased bioenergetics performance and finally decreased ATP intracellular content. At these concentrations, *p*-cresol is genotoxic for colonocytes reinforcing the view that *p*-cresol at excessive concentration is deleterious for these cells.

## Author disclosure statement

No competing financial interest exists.

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