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Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria

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Abstract

The genus *Propionibacterium* is composed of dairy and cutaneous bacteria which produce short-chain fatty acids (SCFA), mainly propionate and acetate, by fermentation. Here, we show that *P. acidipropionici* and *freudenreichii*, two species which can survive in the human intestine, can kill two human colorectal carcinoma cell lines by apoptosis. Propionate and acetate were identified as the major cytotoxic components secreted by the bacteria. Bacterial culture supernatants as well as pure SCFA induced typical signs of apoptosis including a loss of mitochondrial transmembrane potential, the generation of reactive oxygen species, caspase-3 processing, and nuclear chromatin condensation. The oncoprotein Bcl-2, which is known to prevent apoptosis via mitochondrial effects, and the cytomegalovirus-encoded protein vMIA, which inhibits apoptosis and interacts with the mitochondrial adenine nucleotide translocator (ANT), both inhibited cell death induced by propionibacterial SCFA, suggesting that mitochondria and ANT are involved in the cell death pathway. Accordingly, propionate and acetate induced mitochondrial swelling when added to purified mitochondria *in vitro*. Moreover, they specifically permeabi-lize proteoliposomes containing ANT, indicating that ANT can be a critical target in SCFA-induced apoptosis. We suggest that propionibacteria could constitute probiotics efficient in digestive cancer prophylaxis via their ability to produce apoptosis-inducing SCFA.

Abbreviations: ANT, adenine nucleotide translocator; Atr., atractyloside; SN, bacterial DMEM supernatant; Co., control; 4- MUP, 4-methylumbelliferyl phosphate; 4-MU, 4-methylumbellifer-one; $\Delta\Psi m$, mitochondrial transmembrane potential; csA, cyclosporin A; $DiOC_{(6)}3$, 3-3′-dihexyloxacarbocyanineiodide; HE, hydroethidine; PTPC, permeability transition pore complex; PBR, peripheral benzodiazepin receptor; RT, room temperature; ROS, reactive oxygen species; SCFA, short-chain fatty acids; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VDAC, voltage-dependent anion channel.

Introduction

Oncogenesis is determined by a combination of genetic factors and environmental causes including radiation, chemical carcinogens and diet. Indeed, the role of diet in cancer development is strongly supported by epidemiological studies, in particular in the case of cancers of the digestive tract.[1] The major impact of eating habits on the prevalence of colon cancers has triggered efforts to design an optimal diet and/or to create food supplements specifically reducing the risk of cancer. Probiotics are non-pathogenic micro-organisms that, when ingested, exert a positive influence on the health or physiology of the host.[2] They can influence intestinal physiology either directly or indirectly through regulation of the endogenous microflora. Among different dietary bacteria, the propionibacteria form a genus, which is found in specific dairy products such as Swiss-type cheese. The metabolism of propionibacteria relies on the anaerobic conversion of carbohydrates and lactic acid to short-chain fatty acids (SCFA), in particular propionate and acetate.[3] It has been previously shown that the SCFA butyrate (which is generated by endogenous bacteria not belonging to the genus *Propionibacterium*),[4,5] induces apoptosis in colon cancer cells but not in normal cells.[5–8] Thus, with the aim to define a new cancer prophylaxis based on dietary probiotics supplementation, we investigated the cytotoxic potential of propionibacteria on human colon cancer cells.

In the course of apoptosis, mitochondrial alterations consist primarily in an increase in mitochondrial membrane permeability, due at least in part, to the opening of the permeability transition pore complex (PTPC). The PTPC is a protein complex located at the contact site between the two mitochondrial membranes.[9] It is composed of several proteins including hexokinase (cytosol), porin, also called voltage-dependent anion channel (VDAC, a major protein in the outer membrane), peripheral benzodiazepin receptor (PBR, outer membrane), ANT (a major protein in the inner membrane) and cyclophilin D (matrix). Recently, it has been shown that PTPC is involved in the apoptotic process induced by a variety of pro-apoptotic signals such as pro-apoptotic Bcl-2-family members,[10,11] viral proteins,[12,13] chemotherapeutic agents[14,15,16] and lipids such as palmitate[17] and ganglioside GD3.[18] Upon apoptosis induction,

the PTPC protein ANT may form a large non-specific pore, allowing for the diffusion of molecules up to 1500 Da on the inner mitochondrial membrane. This leads to the dissipation of the inner transmembrane potential ($\Delta\Psi$m), enhanced generation of reactive oxygen species (ROS), colloidosmotic swelling of the mitochondrial matrix, and permeabilization of the outer membrane with consecutive release of apoptogenic proteins from the intermembrane space to the cytoplasm.[19,20] All these events are prevented by oncoproteins from Bcl-2 family, which have been shown to interact with PTPC components, in particular ANT[10,11,21] and VDAC.[22]

Of note, most anti-cancer agents, albeit cytotoxic, have no direct effects on mitochondria. Rather, they elicit signal transduction pathways which indirectly affect mitochondria. Prominent examples include the p53-induced upregulation of proteins acting on mitochondria (e.g. the Bcl-2 antagonist Bax, p53-Ap1, proline oxidase)[23] as well as the generation of second messengers including Ca^{2+}, ganglioside GD3, NO, and ROS.[15] Recently, it has been discovered that a

number of experimental chemotherapeutic agents directly affect mitochondria. This applies to betulinic acid,[24,25] lonidamine,[26] arsenite,[27] 6[3-adamantyl-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437),[27] 2-chloro-2′deoxyadenosine, 2-chloro-2′-arafluorodeoxyadenosine,[29] verteporfin[16] and MT-21.[30]

The aim of the present study was to determine whether propionibacteria could kill colon cancer cells. For this purpose, we chose three propionibacteria strains selected for their tolerance toward digestive stresses,[31,32] namely *Propionibacterium acidipropionici* strain CNRZ80, *P. freudenreichii* subsp. *freudenreichii* strain ITG18, and *P. freudenreichii* subsp. *shermanii* strain SI41, SI41 being a commercially available probiotic. Our data demonstrate that these strains kill human cancer cell lines, such as HeLa, HT29 and Caco2 cells, apparently via SCFA. We have investigated the mechanism whereby SCFA induce apoptosis and provide evidence that they directly act on mitochondria to permeabilize their membranes.
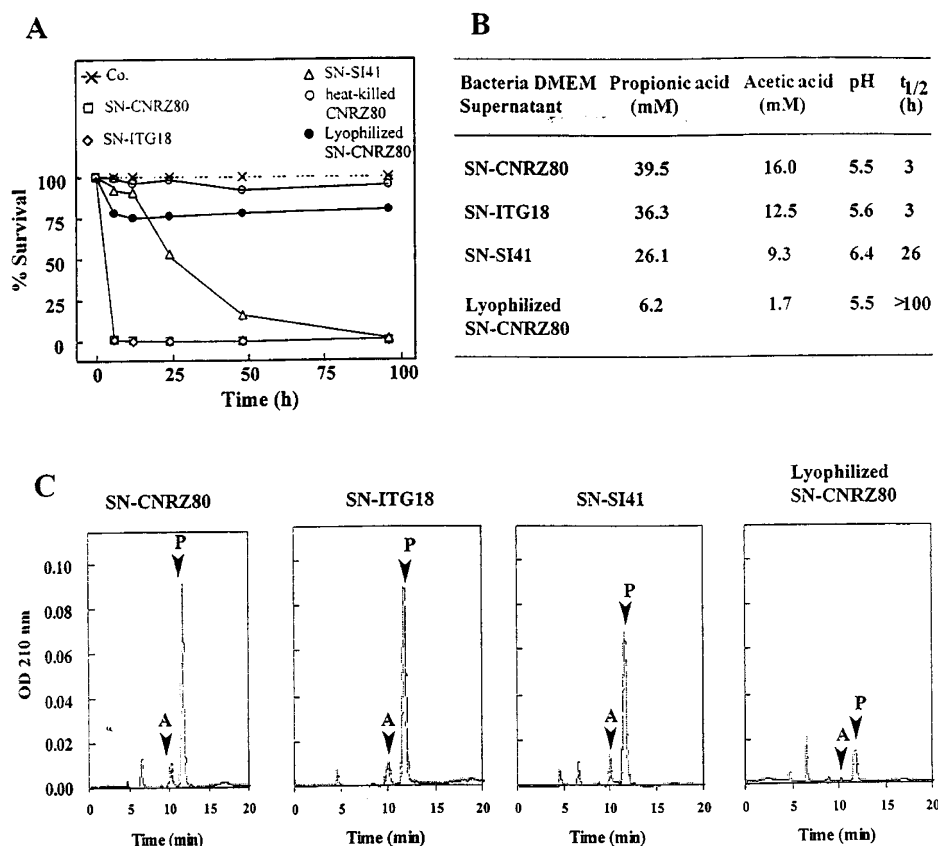

Bacteria DMEM Supernatant	Propionic acid (mM)	Acetic acid (mM)	pH	$t_{1/2}$ (h)
SN-CNRZ80	39.5	16.0	5.5	3
SN-ITG18	36.3	12.5	5.6	3
SN-SI41	26.1	9.3	6.4	26
Lyophilized SN-CNRZ80	6.2	1.7	5.5	>100

Figure 1 SCFA mediate propionibacteria-induced cell death. (**A**) Effects of propionibacteria on HT29 colon cancer cells. 1.10^6 HT29 cells were co-cultured with supernatants of different strains of propionibacteria, namely *P. acidipropionici* CNRZ80, *P. freudenreichii* subsp. *freudenreichii* ITG18, or *P. freudenreichii* subsp.*shermanii* SI41. As controls, cell cultures were inoculated with heat-killed CNRZ80 strain or with a lyophilized supernatant of strain CNRZ80. Cell viability was determined after different periods of culture. This experiment has been reproduced three times. (**B**) Chromatographic analysis of SCFA in DMEM culture supernatants (SN). DMEM was inoculated with CNRZ80, ITG18 or SI41 strain. After growth, supernatants were prepared by centrifugation prior to chromatographic quantification of acetate (A) and propionate (P) in comparison with molecular standards, as described in Material and Methods. As a control, SN-CNRZ80 was lyophilized extensively and reconstituted before analysis in the same conditions. (**C**) Composition and activity of propionibacterial supernatants. The concentration of short-chain fatty acids was analysed by HPLC chromatography as in B, and the time course leading to 50% of HT29 cell death ($t_{1/2}$) was determined as in A

Results

Propionibacteria kill colorectal cancer cell lines

To investigate the cytotoxic potential of different strains and species of diary propionibacteria, we co-cultured HT29 colorectal carcinoma cells with *Propionibacterium acidipropionici* strain CNRZ80, *P. freudenreichii* subsp. *freudenreichii* strain ITG18, and *P. freudenreichii* subsp. *shermanii* strain SI41. All these strains caused cell killing (not shown). The cytocidal effect of propionibacteria could not be attributed to bacterial invasion of host cells (not shown) nor to surface interactions, since heat-inactivation abolished cell killing. Rather, the supernatants (SN) of propionibacteria sufficed to kill HT29 colon carcinoma cells (Figure 1A), Caco 2 cells (not shown) and Jurkat lymphoma cells (not shown) suggesting that cancer cell killing was due to the presence of soluble factors contained in the medium. As a control of bacterial genus specificity, the *Escherichia coli* SN effect was also evaluated. Accordingly, the *E.coli* SN did not induce HT29 cell killing. The cytotoxic effects were fast : half-maximal killing by SN derived from CNRZ80 or ITG18 occurred within 3 h and that of SN derived from SI41 occurred within 24 h (Figure 1A). Lyophilization abolished the cytotoxic effect of SN from CNRZ80 (Figure 1A), suggesting that the lethal

compound contained in the SN was volatile. We performed HPLC analyses of the SN to detect the presence of candidate volatile molecules. Acetate and propionate were identified as the major SCFA in SN from CNRZ80, ITG18 and SI41 (Figure 1C). Both fatty acids were partially eliminated by lyophilization of SN CNRZ80 (Figure 1C).
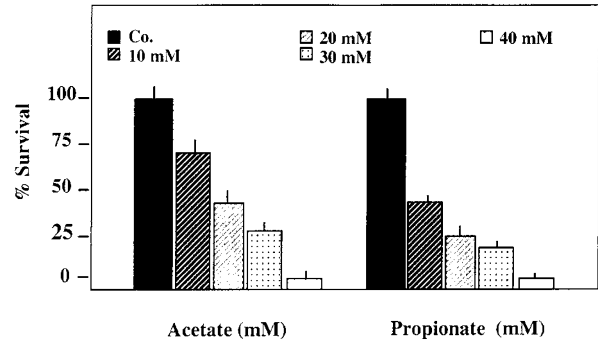

Figure 2 Effects of pure acetate and propionate on the viability of HT29 cells. 1.10^6 cells were left untreated (Co.) or were treated with various concentrations of propionate or acetate. The percentage of surviving cells was determined after 48 h of culture by Trypan blue exclusion. Results are mean values of three independent experiments $\pm$ S.E.M.
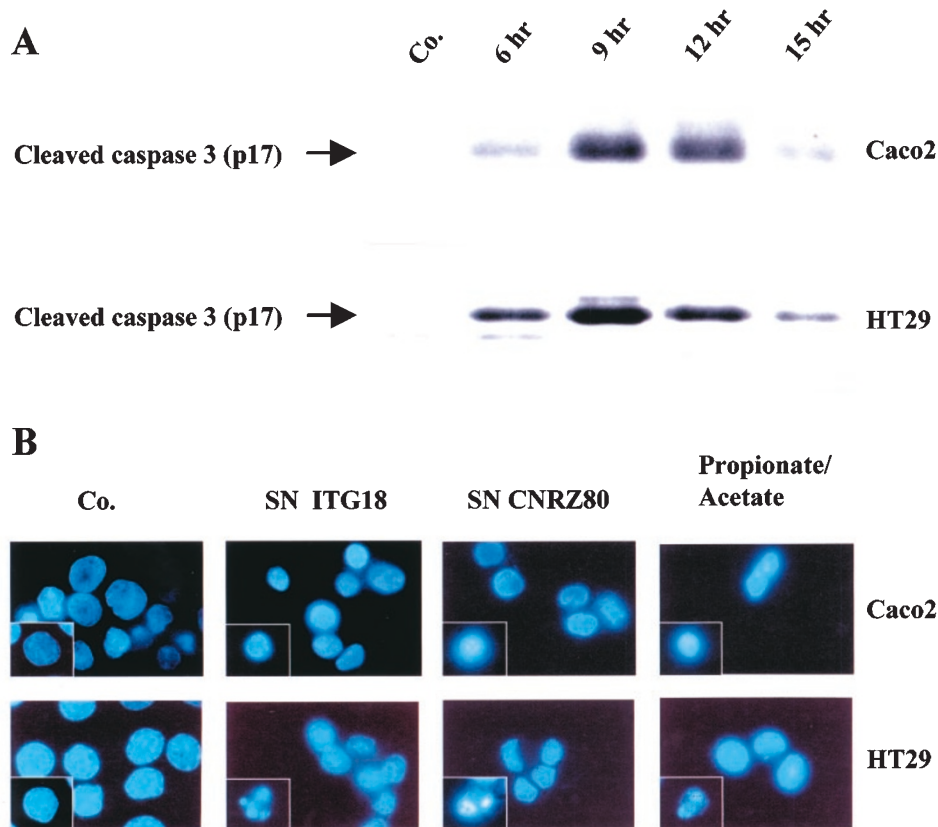

Figure 3 SCFA produced by propionibacteria induce apoptosis in HT29 and Caco2 cells. (**A**) SCFA-induced caspase 3 processing. Cells were treated by SCFA in DMEM. At different times, 5×10^6 cells were subjected to SDS–PAGE, and the cleaved caspase 3 (p17) subunit p20 was detected by immunoblot. (**B**) SCFA induced nuclear condensation. Cells were cultured (Co.) or treated for 24 or 48 h (inserts) in the presence of SN-CNRZ80, SN-ITG18 or sodium propionate and acetate, followed by Hoechst 33324 staining and fluorescence microscopy
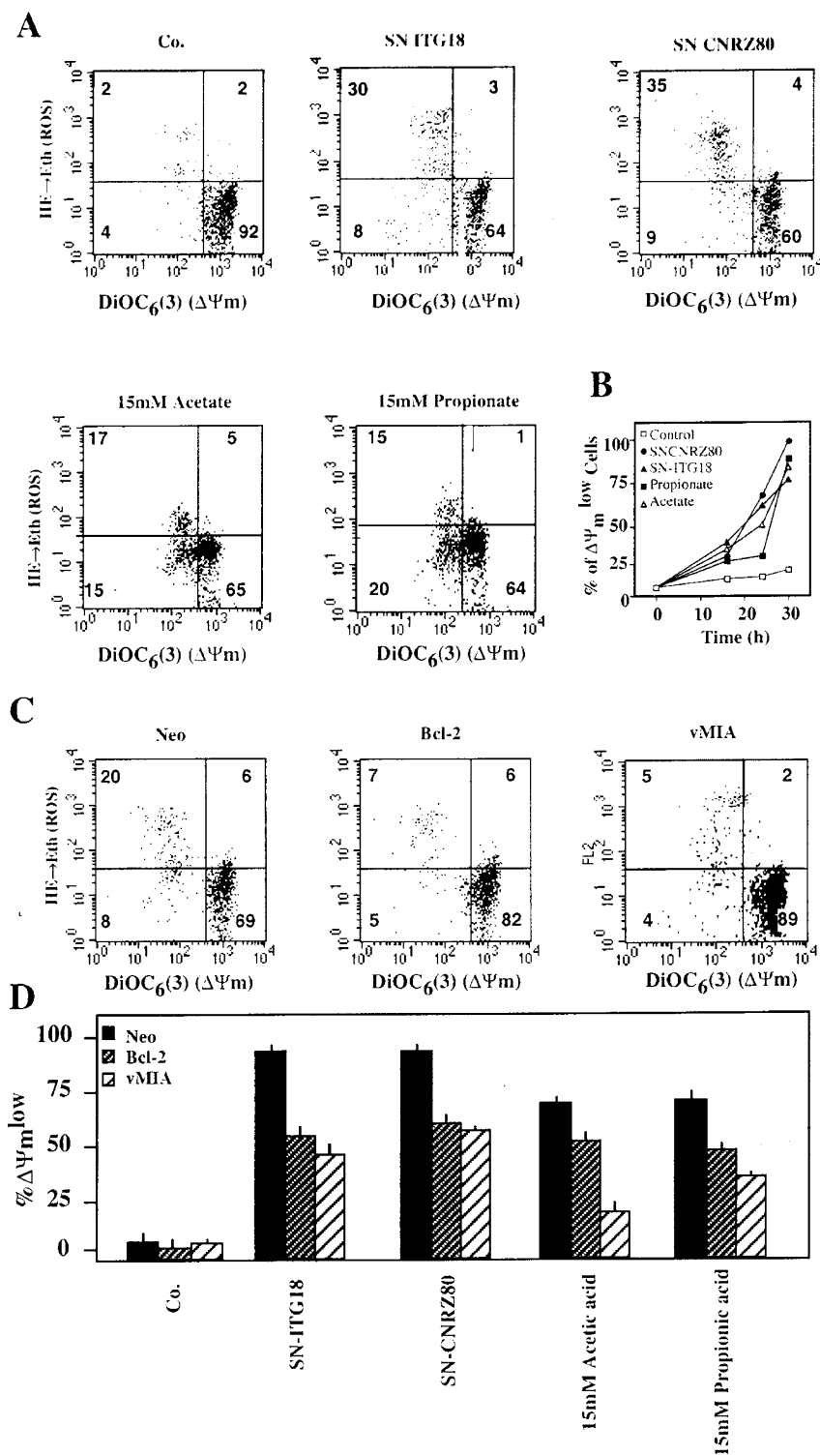
Figure 4 Effect of propionibacterial supernatants and SCFA on apoptosis-associated mitochondrial parameters. (**A**) Determination of the dissipation of the inner membrane potential ($\Delta\Psi m$) and the generation of reactive oxygen species (ROS). HT29 cells were cultured for 16 h with SN-CNRZ80, SN-ITG18, propionate or acetate, followed by staining with DiOC$_{(6)}$3 and HE and cytofluorimetric analysis. Numbers indicate the percentage of cells found in each quadrant. (**B**) Kinetic analysis of the $\Delta\Psi m$ dissipation. $\Delta\Psi m$ was measured at 0, 16, 24 and 36 h of cell treatment by DiOC$_{(6)}$3 staining as in **A**. (**C**) Inhibition of apoptosis by Bcl-2 and vMIA. HeLa cells transfected with the vector only (Neo), the human Bcl-2 gene or the cytomegalovirus-derived vMIA gene, were treated with 15 mM acetate for 16 h, followed by DiOC$_{(6)}$3/HE staining and cytofluorometric analysis as in **A**. (**D**) Comparison of the effects of propionibacterial supernatants and pure SCFA on HeLa cell expressing the Neo resistance gene only, Bcl-2 or vMIA. Data are representative of two experiments

No butyrate formation could be detected under these conditions. The ratios propionate/acetate ranged between 2.5 and 2.9, as expected for propionibacteria, which generally produce more propionate than acetate.[3] The SN-CNRZ80 contained more acetate (16 mM) and propionate (39.5 mM) than the SN-ITG18 (12.5 and 36.3 mM respectively) and also more than the SN-SI41 (9.3 and 26.1 mM) (Figure 1B), correlating with the cytotoxic potential of the supernatants (Figure 1C). We then tested the cytotoxicity of propionate and acetate and found that these compounds killed HT-29 cells with an ED_{50} of 11 and 20 mM respectively (Figure 2). Taken together, these data indicate that propionibacteria efficiently kill colon carcinoma cells, at least in part, due to their specific property to produce two SCFA, propionate and acetate.

Propionibacterial SCFA induce apoptosis of colon carcinoma cells

Apoptosis proceeds via a series of biochemical events different from those occurring during necrosis. For instance, apoptosis but not necrosis usually involves the activation of caspases and nuclear alterations such as chromatin condensation. To characterize the propionibacterial SCFA-induced cell death, the processing of caspase 3 was analyzed by SDS–PAGE and immunoblotting of SN and SCFA-treated HT29 and Caco2 cells (Figure 3A). The bacterial SN-ITG18 and -CNRZ80 (data not shown), as well as the mixture of propionate and acetate in a [2 : 1] molar ratio, induced the cleavage of pro-caspase 3 and the generation of the active form of caspase 3, the subunit p20 (Figure 3A). In addition, SN-treated HT29 and Caco2 cells were labeled with the Hoechst 33324 dye and analyzed for chromatin condensation by fluorescence microscopy (Figure 3B). In comparison to untreated cells, the nuclei of SN-CNRZ80, SN-ITG18, propionate and acetate-treated cells shrank, chromatin condensed, and finally nuclei fragmented into apoptotic bodies (Figure 3B). In conclusion, the mode of death triggered by propionibacteria is apoptosis rather than necrosis.

Propionibacterial SCFA-induced apoptosis involves mitochondrial changes which are antagonized by Bcl-2 and an ANT-targeted viral protein

With the goal of determining whether the mechanisms of propionibacterial SCFA-induced cell death involve mitochondria, we measured two critical mitochondrial parameters, the inner membrane potential, $\Delta\Psi m$, and the generation of ROS, using a combination of two fluorescent probes, $DiOC_{(6)}3$ (which is $\Delta\Psi m$-sensitive) and HE (which detects ROS). As shown in Figure 4A, untreated cells (Co.) exhibited a high $\Delta\Psi m$ and a low HE fluorescence. SN from ITG18 and CNRZ80, as well as acetate or propionate, induced a reduction in the $DiOC_{(6)}3$-dependent fluorescence and an increase in the HE-dependent fluorescence, indicating an increase in the mitochondrial inner membrane permeability and an enhanced generation of ROS (Figure

4A). The kinetics of the $\Delta\Psi m$ dissipation elicited by propionibacterial SN, acetate or propionate were comparable (Figure 4B). Recently, vMIA, a protein encoded by human cytomegalovirus, has been demonstrated to prevent apoptosis, presumably via an interaction with ANT.[33] HeLa cell lines stably transfected with a control vector only (Neo), Bcl-2, or vMIA were treated with acetate, followed by determination of the $\Delta\Psi m$ and ROS generation. Bcl-2 and vMIA conferred a significant protection against the mitochondrial effects of acetate (Figure 4C), propionate, and the SN or ITG18 and CNRZ80 (Figure 4D). This indicates that the mitochondrial effects of propionibacterial SCFA are regulated by Bcl-2 and vMIA.

Propionibacterial SCFA act on purified mitochondria, as well as on ANT reconstituted into proteoliposomes

When isolated mouse liver mitochondria were treated with 100 μM Ca2+, they underwent large amplitude swelling due to the opening of the PTPC pore, a process which could be inhibited by pre-treatment with cyclosporin A (CsA), a ligand of the mitochondrial matrix protein cyclophilin D, one of the PTPC constituents (Figure 5). Similarly, the SN of ITG18 or CNRZ80, as well as SCFA, induced the swelling of mitochondria in a dose-dependent manner (Figure 5). These effects were largely inhibited by CsA, suggesting that opening of the PTPC rather than non-specific membrane destabilization by fatty acids accounts for membrane permeabilization. To identify the SCFA target within the PTPC, we investigated the effects of SN and SCFA on phosphatidylcholine/cardiolipin liposomes containing ANT or not (plain liposomes). Briefly, ANT was purified from rat heart mitochondria and reconstituted in liposomes as detailed in Material and Methods. Then, as an indication of ANT pore opening (Belzacq *et al.*, 2001),[16] we measured the release of a fluorescent probe, 4-MUP, from the liposomal lumen. *Propionibacterium*-produced SCFA were devoid of any permeabilizing effect on plain liposomes (not shown), yet had a potent 4-MUP-releasing effect on ANT-proteoliposomes (Figure 6). The SCFA effects were inhibited by the two natural ligands of ANT, ATP and ADP. In conclusion, ANT could be the target, or one of the targets, of SCFA-mediated mitochondrial membrane permeabilization.

Discussion

In this study, we report that different strains and species of propionibacteria kill cancer cells via the metabolic production of two SCFA, propionate and acetate. This notion is based on the evidence that (i) SN of propionibacteria were as cytotoxic as live strains (Figure 1A); (ii) the cytotoxic effect of various propionibacterial strains correlated with the amount of SCFA they produce (Figure 1B,C), (iii) removal of propionate and acetate by lyophilization from the SN abolished their cytotoxic activity (Figure 1B,C); and (iv) all subcellular and molecular effects mediated by SN of propionibacteria could be mimicked by propionate and acetate alone or in combination (Figures
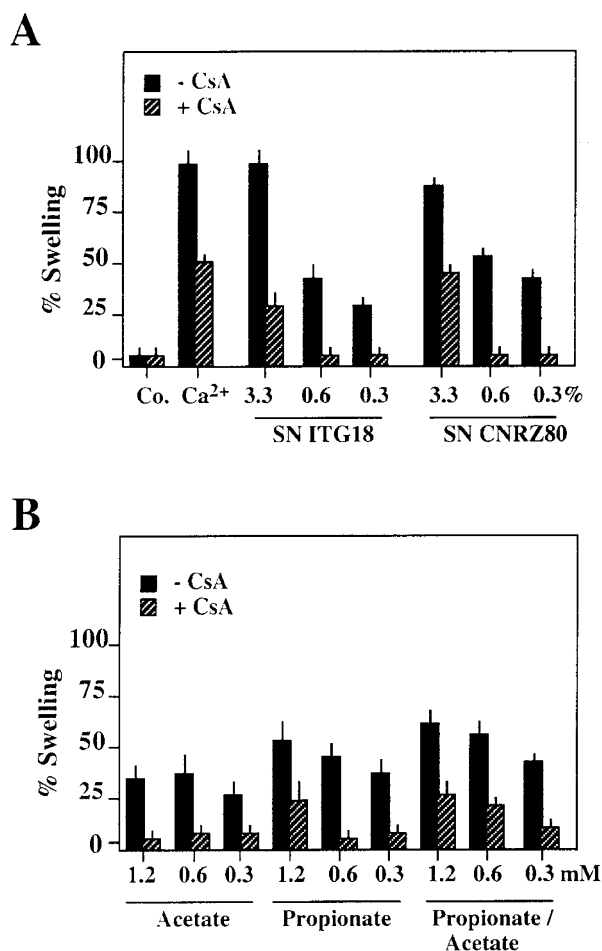
A

B

Figure 5 Propionibacterial SCFA trigger large amplitude swelling in isolated mitochondria (**A**) Swelling induced by SN of ITG18 or CNRZ80. Mouse liver mitochondria were isolated on a Percoll® gradient, and aliquots (110 μg proteins/ml) were treated with SN of ITG18, CNRZ80 or 100 μM calcium. Absorbance at 540 mm was recorded for 20 min. 100% swelling represents the maximal swelling obtained with 100 μM calcium. Optionally, CsA was added 5 min before the indicated agents. (**B**) Swelling of mitochondria induced by SCFA. The same procedure as described in (A) was applied to various doses of acetate, propionate, or a mixture of both acids in a [2 : 1] molar ratio. Values represent mean percentages ± S.E.M. from three independent experiments

Figure 6 SCFA effects on ANT-containing liposomes. 4-MUP was encapsulated into ANT liposomes. Then, ANT liposomes were incubated with 1 mM ADP, 1 mM ATP or liposome buffer only (Co.) for 30 min. Atractyloside or the indicated SCFA were added for another hour at RT. The percentage of 4-MUP release (indicative of membrane permeabilization) was determined as described in Material and Methods. Values represent mean percentages ± S.E.M. from three independent experiments

2–6). The ED$_{50}$ of propionate and acetate were found to be relatively high, around 15 mM. Nonetheless, these two SCFA do not kill cells in a non-specific fashion, based on the finding that they induce apoptosis rather than necrosis (Figure 3), and that their cytotoxic effects were antagonized by overexpression of two apoptosis inhibitors, Bcl-2 and vMIA (Figure 4C).

What is then the mechanism of apoptosis induction by propionibacterial SCFA? In many models, apoptosis can be divided in three phases, an initiation phase (pre-mitochondrial phase), a decision phase (mitochondrial phase) and a degradation phase (cytoplasmic, nuclear and membranous alterations.[19,20] As for butyrate[7,34] and the long-chain fatty acid, palmitate,[17] propionate and acetate-induced apoptosis occurred via the stereotyped biochemical events that belong to the three phases model, including mitochondrial alterations, caspase activation and nuclear degradation.
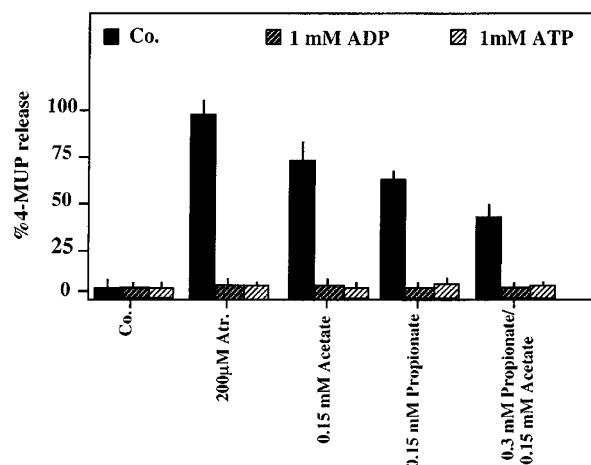
More precisely, the subcellular events in Caco2 and HT29 cells treated with propionibacterial SCFA were, at least, ΔΨm dissipation, ROS generation, caspase 3 processing, chromatin condensation and nuclear fragmentation (Figures 3,4). Our observations that mitochondrial inhibitors of apoptosis Bcl-2[1,11] and vMIA[33] prevent partially propionibacterial SCFA-induced cell killing suggest that mitochondria play a critical role in the death process (Figure 4C). However, additional time course experiments would be necessary to determine if mitochondria support an initiator role or not in SCFA-induced apoptosis.

Surprisingly, we found that propionibacterial SCFA induced CsA-inhibitable swelling of isolated mitochondria (Figure 5). Since CsA reportedly inhibits the PTPC, this argues for the ability of SCFA to act directly on mitochondria and, more precisely on the PTPC. Stimulated by the fact that vMIA selectively interacts with ANT,[33] one of the PTPC components, and that vMIA inhibits propionibacterial SCFA-triggered apoptosis (Figure 4C), we investigated the possibility that SCFA might directly act on ANT. Propionibacterial SCFA permeabilized ANT liposomes but failed to act on protein-free liposomes (Figure 6). Apparently, this effect involves a conformational or functional change of ANT, because it is inhibited by ATP and ADP (Figure 6). The functional SCFA–ANT interaction is reminiscent of that observed for palmitate and similar saturated long-chain fatty acids, which have previously been reported to bind to ANT[35,36] and to induce PTPC opening.[37,38] Of note, the SCFA effects obtained on purified mitochondria and ANT liposomes were observed at much lower doses, around 150–300 μM (Figures 5,6), than the doses required for cell killing. This may be due to the fact that the plasma membrane is not permeable to the anionic form of propionibacterial SCFA[39] and that the anionic form predominates at neutral pH.

Having elucidated the pro-apoptotic mode of action of some strains of propionibacteria, it is tempting to speculate on the medical and therapeutic applications of propionibacterial SCFA. *P. acnes*, a human skin pathogen, has been previously shown to induce apoptosis of various cells indirectly via the stimulation of TNFα synthesis by immune cells.[40] In sharp contrast, we observed that three dairy strains of *Propionibacterium* induced apoptosis directly, in HT29, Caco2, or HeLa cells, without the need of accessory cells (Figure 1,3,4), apparently via SCFA. In humans, after supplementation, dairy propionibacteria survive to the digestive stress (mediated by acidic pH, bile salts or hydrolases) at concentrations compatible with potential probiotic activities (fecal concentration $> 10^6$ CFU/g[34]). In particular, the *P. freudenreichii* strain SI41, has been shown to survive the transit through the human digestive tract,[41] which is linked to highly efficient adaptive response to digestive stresses.[31,32] Indeed, in a recent study,[32] propionibacteria (strain SI41) were ingested by seven healthy volunteers at doses ranging from 5.10^9 (equivalent to 10 g of cheese) to 5.10^{10} (equivalent to 100 g of cheese). In the optimal protocol, propionibacteria were recovered in the human faeces at concentrations between 10^6 and 10^7 CFU per g of faeces, while they were undetectable before treatment. The maximal faecal concentrations of acetate and propionate were 45.7 mM and 11.3 mM, respectively, after this treatment, *versus* 18.3 mM and 4.9 mM before the treatment. Here, acetate concentration in DMEM culture SN ranged from 9 to 16 mM and propionate concentration from 26 to 40 mM, depending on the propionibacterial strain used. In parallel, propionibacterial population reached 15.10^6 to 75.10^6 CFU.ml (data not shown). Thus, propionibacterial population and SCFAs concentrations were in the same range in both *in vitro* and *in vivo* experiments. Based on SCFA concentrations *in vitro* and *in vivo*, it is not unlikely that propionibacteria can favor apoptosis induction of tumor cells via the local production of SCFA. Moreover, *in vivo*, carbohydrates that have not been resorbed in the small intestine, are delivered to the colon where they are fermented by the anaerobic microflora to SCFA.[6] Thus, the concentration of total SCFA (acetate+butyrate+propionate) has been reported to reach values of 100 mM in the lumen of the hind gut.[6] Butyrate, the most studied SCFA, has been found to stimulate proliferation of normal crypt cells, yet apoptosis of colorectal cancer cells.[6] Moreover, butyrate has been reported to induce colorectal carcinoma cell apoptosis via a process that involves cell cycle arrest in the G0-G1 and G2-M phases,[7] inhibition of a histone deactylase, activation of a DEVD-caspase, cleavage of the cyclin-dependent kinase inhibitor p21[Waf1/Cip1,34] Bcl-2 protection[42,43] and DNA fragmentation.[5] This led to the general proposal that SCFA could play a role in digestive cancer prevention. Indeed, high fiber diet which favors butyrate production, reduces the expression of Bcl-2, decreases the rate of aberrant crypt foci in rats, and also reduces the incidence of human colorectal cancer,[44,45,46] Thus, our results extend the hypothesis that SCFA might have a prophylactic action on colon cancer[8] to propionate and acetate. Considering that dairy propionibacteria are known to adhere to human ileal

glycoproteins,[47,48] to Caco-2 cells *in vitro* (our unpublished data), as well as to the intestinal mucus,[49] they might serve for the local delivery of SCFA at the colonocyte level. Dairy propionibacteria, including *P. acidipropioninici* and *P. freudenreichii* are generally recognized as safe bacteria and have already been used as human probiotics, mainly due to their bifidogenic effect.[41] Future clinical and/or epidemiological studies will determine whether these probiotics will also reduce the incidence of colon cancer.

Material and Methods

Chemicals

When not specified, chemicals were purchased from SIGMA, and protocols were those indicated by suppliers.

Cell lines and culture conditions

HT29 cells (a gift from Zweibaum A., INSERM U178, Villejuif, France), Caco2 cells (a gift from Kaeffer B., INRA UR1026, Nantes, France), HeLa cell clones stably transfected with the pcDNA3 vector only, Bcl-2, or vMIA[33] (a gift from Goldmacher V., ImmunoGen, Inc., Cambridge, MA) were cultured in DMEM medium supplemented with 10% heat inactivated-fetal calf serum and antibiotics at 37°C under 5% CO_2.

Bacterial cultures and supernatants preparation

Propionibacterium acidipropionici strain CNRZ80 (Centre National de Recherches Zootechniques, Jouy-en-Josas, France), *P. freudenreichii* subsp. *freudenreichii* strain ITG18 (Institut Technique du Gruyère, Rennes, France) and *P. freudenreichii* subsp. *shermanii* strain SI41 (Standa-Industrie, Caen, France) were routinely cultivated on a modified yeast extract-lactate (YEL) medium and stored at −80°C in the same medium supplemented with 15% glycerol. Such cultures were used to inoculate DMEM medium supplemented with 10% heat inactivated-fetal calf serum, but without antibiotics. After growth, bacterial DMEM supernatants (SN) were prepared by centrifugation (12 000 *g*, 4°C, 15 min) and 0.22 μm-filter-sterilized. All bacterial cultures were less than four passages from the collection stock.

Lyophilization of bacterial supernatant SN-CNRZ80

To lower the amount of SCFA in SN, lyophilization was performed as follows. A CNRZ80 DMEM culture supernatant was brought to pH 2.5 using 2N HCl in order to protonate SCFA before freezing at −80°C. The resulting product was lyophilized and kept 3 days under vacuum (0.05 mbar, 10°C) to favor evaporation of volatile compounds. Bidistilled water was then added up to the initial volume and 2N NaOH used to adjust the pH to its initial value (pH 5.5). The reconstituted supernatant was 0.22 μm-filter sterilized, and its SCFA content was analyzed by chromatography.

Chromatographic analysis

Organic acids were analyzed by high-performance liquid chromatography (HPLC, Gold, Beckman, USA) using UV detection at 210 nm. The anion exchange column (6×300 mm, Aminex A_6) was operated at RT with H_2SO_4 0.01 N (0.5 ml/min) as eluent. Standard solutions of lactic acid, acetic acid, propionic acid and butyric acid of known concentrations were used for column calibration.

Cell treatment and viability monitoring

HT29 and Caco2 cells were plated onto flat-bottom 24-well culture plates at 1.10^5 cells/well. When cells reached 75% confluence, the medium was replaced with fresh medium containing SN-CNRZ80, SN-ITG18, acetate and propionate. For co-cultures, the medium was replaced with DMEM inoculated with each of the three propionibacteria tested. Heat-inactivated bacteria (80°C, 15 min) were also inoculated as a comparison. Three wells per plate were kept as controls and received unmodified DMEM. After 0, 6, 12, 24, 28 and 96 h of contact, cells were trypsinized, dissociated with DMEM and diluted in a 0.4% buffered saline trypan blue solution. Live cells were counted under a Zeiss microscope using a Malassez counting chamber, and counts were expressed as a percentage of the mean obtained for the three untreated wells.

Fluorescence microscopy

$0.5.10^6$ treated and untreated HT29 and Caco2 cells were trypsinized and resuspended in fresh DMEM supplemented with 2 μM of the DNA-intercalating Hoechst H33342 as previously described.[12] After a 10-min incubation at 37°C, cells were examined with a fluorescence microscope (Leica, DMRB type, filter, 340–380 nm; 430 nm).

Western blotting

5.10^6 HT29 and Caco2 cells were trypsinized and lyzed in SDS-sample buffer by sonication, followed by boiling for 5 min. The protein extracts were separated on 13% polyacrylamide gels. Proteins were then transferred onto nitrocellulose membranes using a semi-dry system. Caspase-3 subunit was detected using a cleaved caspase-3 rabbit monoclonal antibody (D175, Cell Signaling Technology, MA, USA) and alkaline phosphatase-conjugated goat IgGs directed against rabbit IgGs as primary and secondary antibodies, respectively. The substrate used for detection was 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium.

Cytofluorimetric analysis of apoptosis

$0.3.10^6$ HT29, HeLa-Neo, HeLa-Bcl-2, and HeLa-Bcl-2 cells cultured overnight at 37°C with the indicated doses of bacterial SN, propionate and/or acetate. Trypsinized cells were labeled with 40 nM 3-3′-dihexyloxacarbocyanineiodide (DiOC$_{(6)}$3; Molecular Probes, Eugene, OR, USA) and 2 μM dihydroethidine (HE; Molecular Probes, Eugene, OR, USA) for 10 min at 37°C. Cells were then analyzed by cytofluorimetry (FACSVantage, Becton Dickinson) as previously described.[49]

Spectrophotometric determination of mitochondrial matrix swelling

Mouse liver mitochondria were purified and resuspended in a buffer containing 200 mM sucrose, 10 mM Tris-MOPS (pH 7.4), 5 mM Tris-succinate, 1 mM Tris-phosphate, 2 μM rotenone, and 10 μM EGTA. Large amplitude mitochondrial swelling was measured spectroscopically by the loss of absorbance at 540 nm, as described.[50] When indicated, 10 μM cyclosporin A were added to the mitochondria (110 μg proteins/ml) prior to the addition of DMEM containing 10% fetal calf serum as a negative control, 100 μM calcium as a 100% control, or the indicated dose of bacterial supernatants, propionate or acetate, and absorbance was recorded for 20 min.

ANT liposomes

ANT was purified from rat heart mitochondria[11] and was reconstituted in phosphatidylcholine/cardiolipin (45:1, w:w) liposomes by a surfactant dialysis method.[11,21] ANT-proteoliposomes were sonicated in the presence of 1 mM 4-MUP and 10 mM KCl (50 W, 22 sec, Branson sonifier 250) on ice as previously described.[16] Then, liposomes were separated on Sephadex G-25 columns (PD-10, Pharmacia) from unencapsulated products. 25 μl-aliquots of liposomes were mixed with 25 μl of various concentrations of the pro-apoptotic inducer atractyloside (200 μM) or SCFA dissolved in 10 mM Hepes, 125 mM saccharose (pH 7.4), diluted to 3 ml and incubated for 1 h at RT. ATP and ADP were added to the liposomes 30 min prior to liposome treatment. After addition of 10 μl-alkaline phosphatase (5 U/ml, Boehringer Mannheim) diluted in liposomes buffer+0.5 mM MgCl$_2$, samples were incubated for 15 min at 37°C under constant agitation and the enzymatic conversion of 4-MUP in 4-MU was stopped by addition of 150 μl Stop buffer (10 mM Hepes–NaOH, 200 mM EDTA, pH 10). The 4-MU fluorescence was quantified using a Perkin Elmer spectrofluorimeter. Atractyloside, a pro-apoptotic permeability transition inducer, was used in each experiment as a standard to determine the 100% response. The percentage of 4-MUP release induced by SCFA was calculated as following: [(fluorescence of liposomes treated by SCFA – fluorescence of untreated liposomes) /(fluorescence of liposomes treated by atractyloside – fluorescence of untreated liposomes)] $\times$ 100.

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