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Butyrate-induced differentiation of PC12 cells to chromaffin cells involves cell adhesion and induction of extracellular proteins and cell adhesion proteins

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PC12 cells were differentiated into the cells of chromaffin phenotype by butyrate treatment. Cells were aggregated and formed tight cell adhesion. To investigate the molecular change in this differentiation, we examined expression levels of cell adhesion proteins and extracellular proteins during butyrate induced-differentiation of PC12 cells. Integrin β1, integrin α7, E cadherin, VCAM, collagen-I, fibronectin, desmoglein and connexin were increased during differentiation. The levels of clusterin and secreted clusterin were also increased. These increased levels of cell adhesion proteins and extracellular proteins appear to induce cell aggregation and tight cell adhesion. The levels of p21, p27 and p16 were increased probably because of differentiation-related growth arrest during differentiation. Prolonged incubation of butyrate up to 1 day was required for differentiation. Signal transduction pathways for this differentiatiom could not be identified since various inhibitors had no effect. The results showed that butyrate-induced differentiation of PC12 cells to chromaffin cells involves tight cell adhesion and induction of extracellular proteins and cell adhesion proteins.

Keywords: butyrate; PC12; differentiation; chromaffin cells; cell adhesion proteins

Introduction

PC12 pheochromocytoma cells have been widely used as a model for the study of neuronal differentiation (Fujita et al. 1989). PC12 cells were differentiated into sympathetic-like neurons involving neurite outgrowth by treatment of NGF (Greene and Tischler 1976). PC12 cells were also reported to be differentiated to chromaffin cells by butyrate (Byrd and Alho 1987). While NGF-induced differentiation of PC12 cells into sympathetic-like neurons has been well characterized, butyrate-induced differentiation of PC12 cells into chromaffin cells has not been well investigated at the molecular level.

Butyrate is a histone deacetylase inhibitor that arrests cell proliferation, induces differentiation and regulates gene expression (Kruh 1982; Davie 2003) through changing histone acetylation. Nucleosomal histone undergoes reversible acetylation of the amino group at the specific lysine residues located in their N-terminal tail on the histone core (Allfrey et al. 1964). Increased acetylation is generally correlated with active transcription and increases the accessibility of transcription factors to their binding site on DNA (Lee et al. 1993; Vettese-Dadey et al. 1996). DNA synthesis and cellular proliferation were inhibited during butyrate-induced differentiation of PC12 cells. In response to butyrate, PC12 cells increase cell-cell adhesion and expression of neuron-specific enolase (NSE) (Byrd and Alho 1987), proenkephalin gene products (Byrd et al. 1987) and transglutaminase (Byrd and Lichti 1987). However, the detailed molecular changes during butyrate-induced differentiation of PC12 cells have not been thoroughly characterized.

Here we show that expression levels of cell adhesion proteins, extracellular proteins and cyclin-dependent kinase inhibitors were increased and thereby cell adhesion is induced during butyrate-induced differentiation of PC12 cells.

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Materials and methods

Materials

Sodium butyrate, PMSF, leupeptin, aprotinin and pepstatin A were purchased from Sigma. The ECL system was obtained from Amersham Biosciences. Trizol reagent was obtained from Life technologies. Anti-collagen-I, anti-collagen-III, anti-C cadherin, anti-E cadherin, anti-fibronectin, anti-clusterin, anti-connexin and anti-desmoglein were purchased from BD Transduction Laboratories. Anti-p21 antibody was obtained from Pharmingen and anti-p27, anti-p16, anti-VCAM, anti-NCAM, anti-integrin β1, anti-integrin β3 and anti-integrin α7 were obtained from Santacruz. Anti-neuron-specific enolase (NSE) antibody was purchased from Nouvus.

Cell culture

PC12 cells were cultured in RPMI medium containing 10% horse serum and 5% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Differentiation of PC12 cells was induced by addition of sodium butyrate (5 mM).

Western blot analysis

Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% NP-40, 0.1 mM PMSF, 10 μg/ml each leupeptin, aprotinin and pepstatin A). 50 μg of cell lysate was separated on a SDS-polyacrylamide gel and transferred to Immun-Blot PVDF membrane. The membranes were incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibody. The resulting bands were visualized using the ECL system. Primary antibodies used in this work were anti-collagen-I, anti-collagen-III, anti-C cadherin, anti-E cadherin, anti-fibronectin, anti-clusterin, anti-connexin and anti-desmoglein, anti-p21, anti-p27 and anti-p16, anti-VCAM, anti-NCAM, anti-integrin β1, anti-integrin β3 and anti-integrin α7.

Northern blot analysis

Total RNA was prepared from cells with Trizol reagent. Total RNA was separated in a formaldehyde gel by electrophoresis and transferred to nylon membranes. Northern blot analysis was performed using a clusterin probe that was prepared by in vitro transcription of a clusterin cDNA T7 clone in the presence of α-^{32}P CTP.

Immunostaining

Cells were treated with sodium butyrate and indicated inhibitors for 24 or 48 h and then fixed in 4% formaldehyde. The cells were permeabilized in a 0.2% Triton X-100 in TBS (Tris-buffered saline). After washing with washing buffer (0.1% Triton X-100 in TBS), the cells were incubated with 2% BSA in TBS for blocking. The cells were incubated with anti-neuron-specific enolase (NSE) antibody for 2 h. The cells were washed with washing buffer and incubated with FITC-conjugated anti-rabbit IgG. Images were observed under the fluorescence microscope.

Results

Butyrate-induced differentiation of PC12 cells to chromaffin cells involves cell aggregation and tight cell adhesion

To observe the morphological changes during butyrate-induced differentiation of PC 12 cells, cells were treated with 5 mM sodium butyrate for 24 and 48 h. PC 12 cells were aggregated at first and then showed tight adhesion among cells. Boundaries of cells became unclear although nuclei of cells were clearly distinguished. This change was apparent in 24 h and was completed in 48 h (Figure 1).

Expression levels of cell adhesion proteins and extracellular proteins increased during butyrate-induced differentiation

Since differentiation accompanies aggregation and cell adhesion, increase in expression of cell adhesion molecules (CAMs) including gap junction proteins and extracellular proteins appears to be involved. Therefore, the expression levels of cell adhesion proteins and extracellular proteins were examined. Integrin β1, an adhesion protein, increased up to 18 h but decreased at 24 h. Integrin α7 was increased during entire differentiation while integrin β3 was decreased (Figure 2A). Cadherin C and E were increased at 6 h and peaked at 24 h (Figure 2B). Vascular cell adhesion molecule (V-CAM) began to increase at 6 h and peaked at 24 h. However, the protein level of neuronal cell adhesion molecule (N-CAM) was not changed (Figure 2C). Gap junction-related proteins, connexin and desmoglein were increased at 6 h and maintained up to 48 h (Figure 2D). To observe the involvement of actin and extracellular matrix, actin, collagen and fibronectin were examined during butyrate-induced differentiation of PC12 cells. Collagen-I was increased at 12 h and kept increasing throughout entire differentiation period. However, the level of collagen-III protein was not changed (Figure 2E). Fibronectin

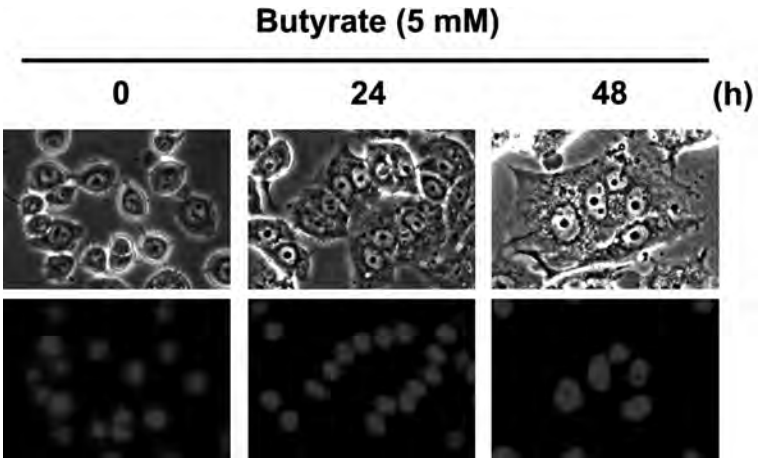

Figure 1. Morphological changes during butyrate-induced differentiation of PC12 cells to chromaffin cells. PC12 cells were treated with 5 mM sodium butyrate for 0, 24 and 48 h as indicated. Nuclei were stained using DAPI. Morphological change was observed under the fluorescence microscope with $\times 400$ magnification.

and actin increased during differentiation (Figure 2F). These results showed that cell interaction proteins and gap junction-related proteins were increased during butyrate-induced differentiation of PC12 cells. Expression of clusterin protein and mRNA were also examined. The protein level of clusterin increased up to 48 h little by little. We also tested secreted clusterin in culture media because clusterin was known to be secreted to outside the cell. Secreted clusterin began to increase at 18 h and kept increasing up to 48 h (Figure 2G). The clusterin mRNA level also increased from 18 h to 48 h (Figure 2H). The result indicated that the levels of various cell adhesion

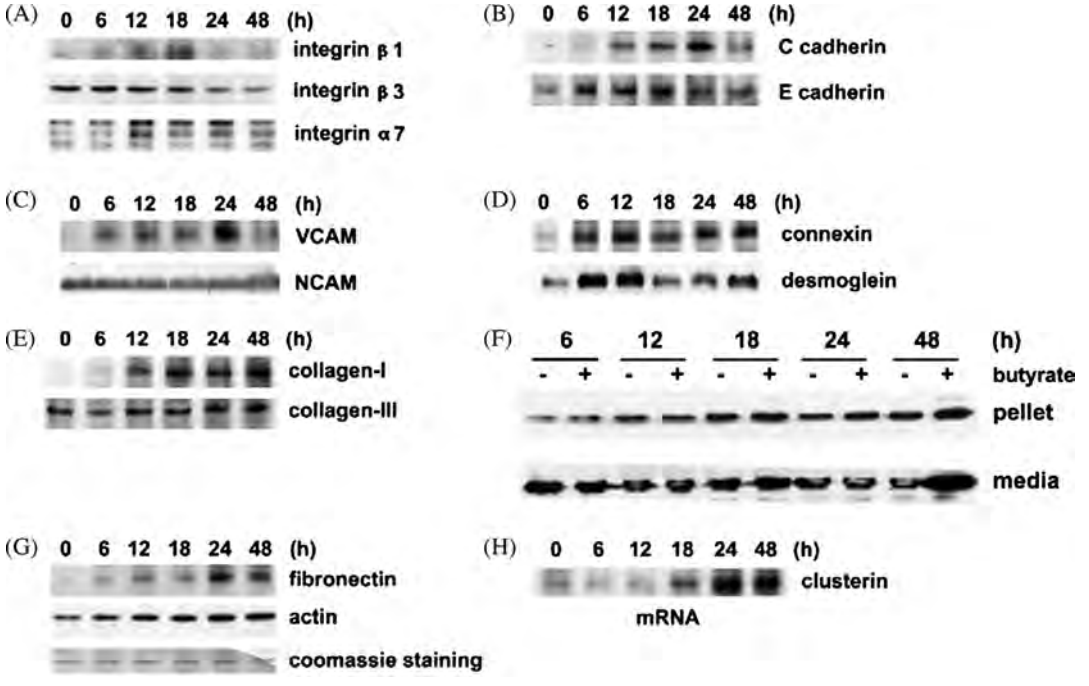

Figure 2. Change in expression levels of the cell adhesion-related proteins and extracellular proteins during butyrate-induced differentiation of PC12 cells. (A–F) PC12 cells were treated with 5 mM sodium butyrate. Cells were harvested at 6, 12, 18, 24 and 48 h. Cell lysates were prepared and Western blot analysis was performed by using antibodies against integrin β1, β2 and β3 (A), C cadherin and E cadherin (B), V-CAM and N-CAM (C), connexin and desmoglein (D) and collagen I and III (E) as described in Materials and methods. (G) PC12 cells were treated with or without 5 mM sodium butyrate for 6, 12, 18, 24 and 48 h. Protein level (pellet), secreted protein level (media) and RNA level (H) of clusterin were measured by Western blot using anti-clusterin antibody or Northern blot analysis, respectively. The experiments were repeated three times.

proteins including gap junction proteins and extracellular proteins increased in company with cell adhesion during butyrate-induced differentiation of PC12 cells.

Expression levels of cyclin-dependent kinase inhibitors (CDKI) increased during butyrate-induced differentiation of PC12 cells

Since it has been reported that p21 was transcriptionally up-regulated by butyrate (Richon et al. 2000), we examined the expression levels of CDKI proteins during butyrate-induced differentiation of PC12 cells. The level of p21 increased at 6 h and kept continuously increasing up to 48 h. The pattern of expression of p16 and p27 was similar to that of p21 to 24 h (Figure 3). These results show that CDKI proteins were induced for growth arrest as a prerequisite for butyrate-induced differentiation of PC12 cells.

Butyrate-induced differentiation of PC12 cells requires a prolonged incubation with butyrate

To examine the incubation time required to induce differentiation of PC12 cells, cells were incubated with media containing butyrate for 1, 2 and 4 h. Then cells were washed with butyrate-free media twice prior to cultivating in the regular media. Cells were not differentiated by butyrate treatment for $1 \sim 4$ h (Figure 4). The results suggest that butyrate-induced differentiation of PC12 cells requires a prolonged incubation of butyrate of up to 1 day. To determine whether a specific signal transduction pathway was involved in butyrate-induced differentiation of PC12 cells, various inhibitors of signal transduction pathways were tested. When PD98059, an inhibitor of MEK1, SB203580, an inhibitor of p38 MAPK, and wortmannin, an inhibitor of PI3K, were co-treated with butyrate, butyrate-induced differentiation of PC12 cells was not affected. The level of neuron-specific enolase (NSE) was also examined by immunostaining as a control differentiation-specific protein since its level is known to be increased during differentiation of

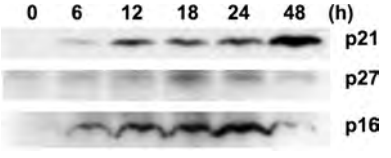

Figure 3. Change in expression levels of CDKIs during butyrate-induced differentiation of PC12 cells. PC12 cells were treated with 5 mM sodium butyrate. Cells were harvested 6, 12, 18, 24 and 48 h after treatment. Cell lysates were prepared and Western blot analysis was performed using anti-CDKI antibodies. The experiments were repeated three times.

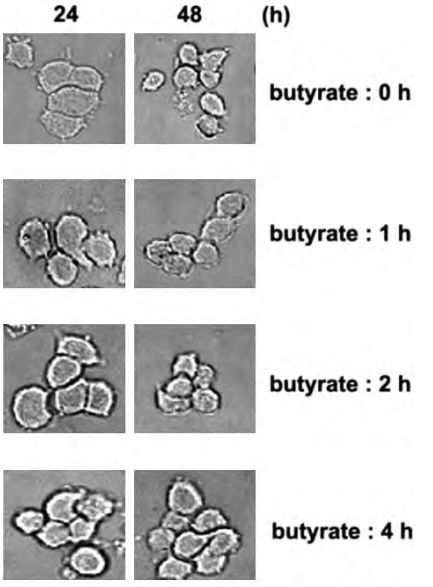

Figure 4. Butyrate-induced differentiation of PC12 requires prolonged treatment with butyrate. PC12 cells were treated with 5 mM sodium butyrate for 0, 1, 2 and 4 h as indicated and washed with butyrate-free media. Cells were then maintained in regular media for 24 and 48 h. Morphological change was observed under the microscope with $\times 400$ magnification.

PC12 cells by sodium butyrate (Byrd and Alho 1987). NSE immunoreactivity was increased by sodium butyrate as expected (green color) and various inhibitors did not affect the increase of immunoreactivity. Genistein, an inhibitor of several tyrosine kinases, also could not change the differentiation (data not shown). Therefore, we do not currently know the signal pathway that is involved in butyrate-induced differentiation of PC12 cells.

Discussion

Even though PC12 cells are known to differentiate to the cells of chromaffin phenotype by treatment with histone deacetylase inhibitors, the molecular mechanism for differentiation and the detailed changes of gene expression during differentiation have not been thoroughly elucidated. The present study showed that PC12 cells underwent aggregation and cell adhesion in response to butyrate. Levels of many extracellular proteins and cell-cell interaction related proteins were increased. Integrins are cell surface receptors that transduce outside-in signaling and inside-out signaling (Paschos et al. 2009). Integrins are regulated during development (Meighan and Schwarzbauer 2008). During butyrate-induced differentiation of PC12 cells to chromaffin phenotype, integrin beta 1 and alpha 7 were increased (Figure 2A). Fibronectin

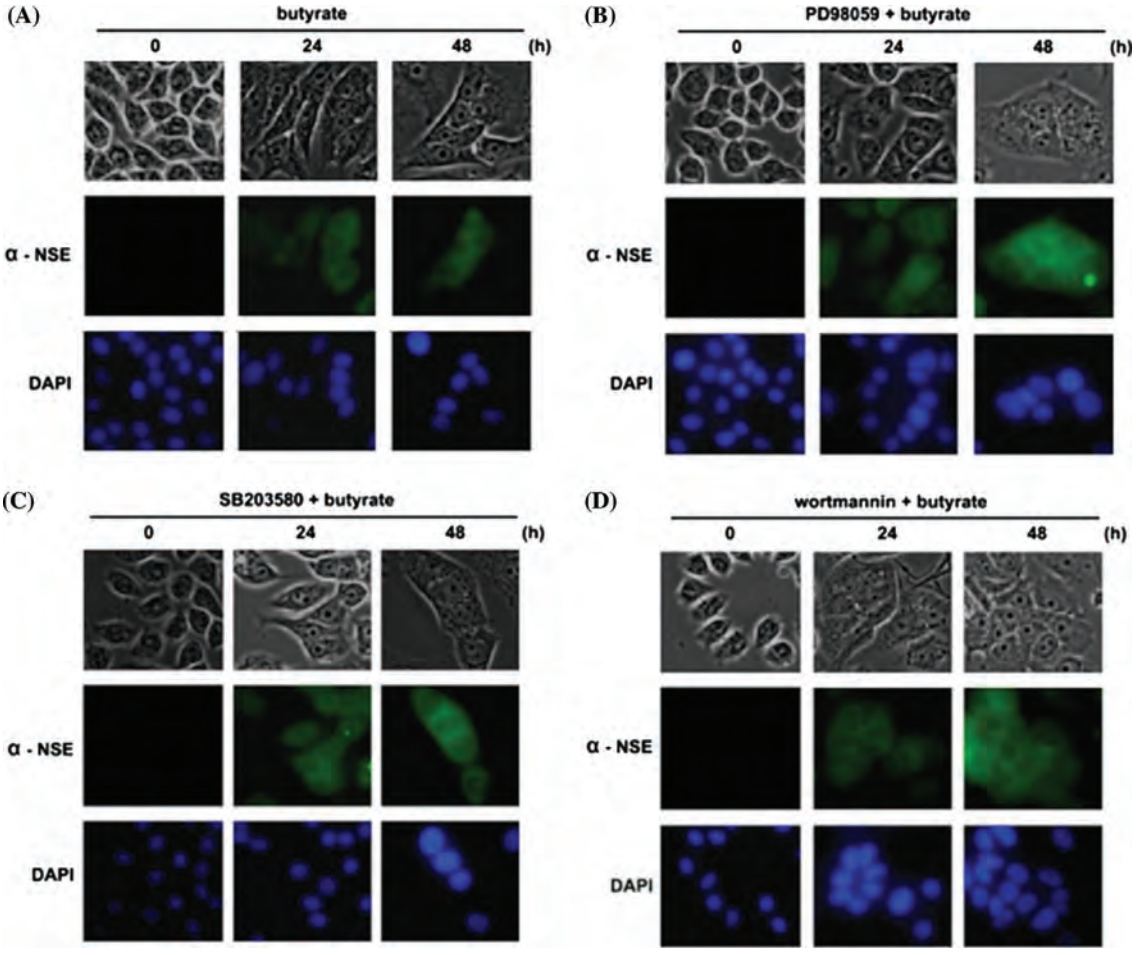

Figure 5. Butyrate-induce differentiation of PC12 cells was not affected by various signal transduction inhibitors. PC12 cells were treated with sodium butyrate (A) and at the same time with PD98059 (B), SB203580 (C) and wortmannin (D) for 12, 24 and 48 h. Cells were permeabilized and incubated with anti-neuron-specific enolase (α-NSE) antibody as described in Materials and methods. Nuclei were stained using DAPI. Morphological change was observed under the fluorescence microscope with $\times 400$ magnification.

and collagen, which are ligands of integrin, were increased (Figure 2E and F). Cadherins are transmembrane proteins that play an important role in cell adhesion (Gumbiner 1996; Makrilia et al. 2009). Their superfamily includes cadherins, desmogleins and protocadherins. E-cadherins are well known cadherin members that are found in epithelial tissues. During differentiation of PC12 cells, E-cadherin and desmogleins were increased. Desmoglein is one of the cadherin superfamily (Figure 2B and D). Vascular cell adhesion molecule 1 (VCAM-1) is expressed on blood vessels and activated endothelial cells. VCAM-1 is up-regulated in response to cytokines in endotherial cells. Neural cell adhesion molecule (NCAM) is mainly expressed on the surface of neurons and plays a role in cell-cell adhesion and neurite outgrowth. During differentiation of PC12 cells, VCAM-1 was increased but not NCAM (Figure 2C). Clusterin is a chaperone molecule and disulfide-linked heterodimeric protein which is implicated in many physiological processes such as programmed cell death, cell-cell adhesion, sperm maturation and tissue remodeling (Jones and Jomary 2002; Nuutinen et al. 2009). It was reported that histone deacetylase inhibitors and 5-aza-2-deoxycytidine increase the expression of clusterin and secretion of clusterin proteins in neural cells (Nuutinen et al. 2005). During differentiation of PC12 cells, levels of clusterin and secretion were increased (Figure 2G). The mRNA level of clusterin also increased, indicating that its transcription was increased (Figure 2H). Overall, increased levels of cell adhesion proteins and extracellular proteins may mediate cell aggregation and cell adhesion during differentiation although the detailed mechanism for cell adhesion is not currently available.

As seen in Figure 4, butyrate-induced differentiation of PC12 cells requires a prolonged incubation of butyrate, as does NGF-induced differentiation of PC12 cells to sympathetic-like neurons. When PD98059, an inhibitor of MEK1, SB203580, an inhibitor of p38 MAPK, and wortmannin, an inhibitor of PI3K, were co-treated with butyrate, PC12 cells were differentiated by butyrate regardless of these inhibitors (Figure 5). Genistein, an inhibitor of several tyrosine kinases, also did not change the differentiation (data not shown). Therefore, an unidentified signal transduction pathway may be involved in butyrate-induced differentiation of PC12 cells; or activation of specific genes through histone deacetylation may be directly involved. The activation of specific genes appears be specific to PC12 cells since butyrate treatment did not change other types of cells. Further investigation of the molecular mechanism of this differentiation will provide a better understanding of this phenomenon.

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