Stress- and cell death-related signals in nerve growth factor-treated PC12 cells are differentially affected by varying butyric acid amounts

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Contents

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Abstract

Periodontal disease painlessly progresses; however, its underlying mechanisms are still unknown. Butyric acid (BA), a periodontopathic metabolite, suppresses β-nerve growth factor (NGF)-induced neurite outgrowth of rat pheochromocytoma PC12 cells, and may contribute to the painlessness of the disease. Additionally, increased intracellular calcium concentrations and ER stress induced in response to cell death and oxidative stress are involved in neurite retraction. In the present study, NGF-treated PC12 cells were exposed to varying BA concentrations, and the resulting stress-related signals (H_2O_2, glutathione reductase, calcium, plasma membrane Ca^{2+} ATPase, and GADD153/CHOP) were determined. Similarly, this study also investigated cell death-related signals (FasL, TNF-α, TWEAK, TRAIL, Caspases, and NF-κB).

Overall, results suggest that BA-related oxidative stress-induced neurodegeneration occurs via an increased accumulation of intracellular calcium and ER stress. Moreover, different BA concentrations may trigger different cell death-related signals. TWEAK levels decreased in a BA dose-dependent manner, whereas TRAIL levels increased, and FasL and TNF-α levels only increased in response to high BA concentrations. Further, Caspase-3, Caspase-8, and Caspase-9 activities increased, whereas, NF-κB activity remained unaffected. These results suggest that stress- and cell death-signaling in NGF-treated

PC12 cells are differently affected depending on BA concentration.

The following events are likely to occur in periodontal disease scenarios: (1) during early stages, low BA concentration result in stress- and cell death-related signals favoring neurite nonproliferation; and (2) during later stages, the accumulation of BA results in higher concentrations, leading to stress- and cell death-related signals favoring neurodegeneration. More importantly, these results suggest that the absence of neuropathic pain at any periodontal disease progression stage can be attributed to the accumulation of BA regardless of the concentration.

Introduction

Butyric acid (BA) accumulation has been linked to periodontal disease progression while neuropathic pain is absent in the early stages of periodontal disease, likely related to decreased neurite outgrowth (1,2). Consequentially, this is detrimental for the early detection and treatment of the disease. In an earlier work, it was previously proposed that neuropathic pain absence is associated with BA-induced neurite retraction attributable to oxidative stress and calcium (3). Neurite retraction is related to cell death while cells utilize several signals for cell death execution (4,5). Surprisingly, BA effects on the various cell death signals have not been fully understood. A better understanding of the influence of BA-induced cell death signaling in neuronal cells could shed light on why neuropathic pain is absent at the onset of periodontal disease and, equally important, this may lead to novel therapeutic strategies allowing for the early detection and treatment of periodontal disease.

In this study, β-nerve growth factor (NGF)-treated rat pheochromocytoma PC12 cells were utilized to determine the effects of varying BA amounts on selected stress-related signals. Subsequently, the effects of low and high BA amounts on extrinsic and intrinsic cell death-related signaling were established. Similarly, correlating neuropathic pain

absence at the different stages of periodontal disease progression to the various cell death signals activated by differing BA amounts was likewise attempted.

Materials and Methods

PC12 treatment and processing

PC12 cells [RIKEN Cell Bank, Tsukuba, Japan. Mature PC12 cells (1×10^6 cells mL^{-1})] were routinely maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich Co., Missouri, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, Illinois, USA), 50 U mL^{-1} penicillin (Life Technologies, California, USA), and 50 μg mL^{-1} streptomycin (Life Technologies). PC12 cells were grown with 100 ng mL^{-1} NGF (R&D Systems, Minneapolis, USA) and used for downstream biochemical analyses. Cell cultures were treated with 0 (control), 0.5, 1, and 5 mM BA amounts using sodium butyrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h. For this study, 5 mM was considered as the periodontal disease level-BA (PDL-BA) amount (2,3). Cell cultures samples were centrifuged at 10,000×g for 10 min at 4°C and both the supernatant (culture medium) and cell precipitates (cell extracts) were collected for further analyses. N-PER™ Neuronal Protein Extraction Reagent (Thermo Scientific) was used for sample processing while BCA™ Protein Assay Kit (Thermo Scientific) was used for standardization prior to downstream analyses. Pierce® Detergent Removal Spin Columns

(Thermo Scientific) was used to remove detergents from all samples for further downstream analyses.

Determination of stress-related signals

Neuronal cells are regularly exposed to oxidative and ER stresses (6). This study focused on selected stress-related signals or molecules that may influence neuronal oxidative and ER stresses. Neuronal hydrogen peroxide (H_2O_2), glutathione reductase (GR), and calcium concentrations were measured using the Red Hydrogen Peroxide Assay Kit (Enzo Life Science Inc., Pennsylvania, USA), Glutathione Reductase Activity Colorimetric Assay Kit (BioVision Inc., California, USA) and Calcium Colorimetric Assay Kit (BioVision), respectively. All kits were performed following manufacturer's recommendations. For both H_2O_2 and GR measurements, cell extracts were used and both cell extracts and cell medium were utilized for calcium concentration measurements.

Western blotting was performed to semi-quantify neuronal plasma membrane calcium ATPase (PMCA) levels. Briefly and in chronological order, neuronal proteins were separated through SDS-PAGE, wet transfer was performed on Hybond-C nitrocellulose membrane (Amersham Biosciences Co., New Jersey, USA), membranes were blocked with DifcoTM Skim Milk (Becton Dickinson Co., New Jersey, USA), probing was

performed with the corresponding primary and secondary antibodies, and finally, immunoreactive proteins were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific). Anti-PMCA (Thermo Scientific) was used to detect neuronal PMCA while Anti-GAPDH (GeneTex Inc., California, USA) was used to detect glyceraldehydes-3-phosphate (GAPDH) which serves as control. ImageJ software was used to quantify the protein bands (7).

Neuronal growth arrest and DNA damage-inducible gene (GADD153) levels were quantified thorough the pre-optimized ELISA method. Briefly and in chronological order, antigen coating (at 1 μg mL^{-1} concentration) on polystyrene plate was done using sodium bicarbonate-sodium carbonate buffer (Polysciences, Inc., Pennsylvania, USA), blocking was performed using PBS with 1% BSA blocking buffer (GeneTex) for 24 h, HRP conjugated GADD153/CHOP antibody (Novus Biologicals LLC, Colorado, USA) was utilized to detect neuronal GADD153, and peroxidase detection was performed using SIGMA*FAST*™ OPD tablets (Sigma). PBS/Tween® Solution (PanReac AppliChem, Darmstadt, Germany) was used for all washing steps and hydrochloric acid (1.0 M) was used as the ELISA stop solution. All ELISA measurements were done in Abs 450 nm. GADD153 protein (GeneTex) was used to establish a protein standard to estimate neuronal GADD153 amounts.

Cell death visualization and confirmation

NGF-treated PC12 cells were washed with PBS, fixed with 3% formaldehyde for 20 min, and treated with 0.1% Triton X-100 for 5 min. All steps were made in room temperature. Subsequently, cells were stained with 2.5 μM SYTOX orange solution (Life Technologies) for 10 min at room temperature and viewed with a confocal-fluorescence microscope.

Quantification of extrinsic cell death-associated signals

Extrinsic cell death signals studied mainly refer to Fas ligand (FasL), TNF-related weak inducer of apoptosis (TWEAK), tumor necrosis factor alpha (TNF-α), and TNF-related apoptosis-inducing ligand (TRAIL). For signal detection, cell culture medium (supernatant) was used. Rat Fas Ligand ELISA Kit (RayBio®, Georgia, USA), Rat TWEAK R ELISA Kit (RayBio®), Rat TNF-α ELISA Kit (AssayPro LLC., Missouri, USA) were used to measure FasL, TWEAK, and TNF-α concentrations, respectively. All kits were performed following manufacturers' recommendations. TRAIL levels were quantified through the pre-optimized ELISA method as it was described earlier in "Material and methods". HRP-conjugated anti-TRAIL antibody (Bioss Antibodies Inc., Massachusetts, USA) was used.

Measurement of intrinsic cell death associated signals

Intrinsic cell death signals studied mainly focus on Caspase-3, Caspase-8, Caspase-9, Caspase-10, Cytochrome C (CytC), NF-κB, and BH3 interacting-domain death agonist (Bid). For signal detection, cell precipitates (extract) were used. Investigated caspases (Caspase-3, Caspase-8, Caspase-9, Caspase-10) were measured using Caspase-3/CPP32 Colorimetric Assay Kit (Biovision), FLICE/Caspase-8 Colorimetric Assay Kit (Biovision), Caspase-9 Colorimetric Assay Kit (Biovision), and Caspase-10 Colorimetric Assay Kit (GeneTex), respectively. NF-κB/p65 ELISA Kit (Novus Biological) was used to measure NF-κB levels. All kits were performed following manufacturer's recommendations. Western blotting was performed using anti-CytC (Signalway Antibody LLC, Maryland, USA), anti-Bid (Acris Antibodies, Inc., California, USA), and anti-GADPH (Gene-Tex) to detect neuronal CytC, Bid, and GAPDH proteins, respectively. Western blotting protocol performed followed what was earlier described.

Statistical analysis

Statistical analyses were performed throughout the study. All data obtained were first checked with the Andersen-Darling normality test and, if passed ($p > 0.05$), the statistical significance of differences between control (0 mM BA), and varying BA concentrations

studied (0.5, 1, and 5 mM BA) were determined by Student's *t* test (two-tailed). A significance level of 95% ($p < 0.05$) was considered statistically significant.

Results

High BA amounts induce neuronal oxidative stress

To establish the oxidative stress-related signals associated with the varying BA levels, H_2O_2 and GR amounts were quantified. As seen in Fig. 1a, it was found that at low BA (0.5 mM), H_2O_2 levels were increased while GR (an antioxidant) levels (Fig. 1b) remained unchanged and, in contrast, it was observed that at high BA (1 and 5 mM), H_2O_2 amounts were increased while GR amounts decreased. Pro-oxidants, like H_2O_2, have dual function and may either serve as a signaling or stress-related molecule (8). Moreover, stress-related increase in pro-oxidant amounts is accompanied by either an increase or decrease in anti-oxidant levels (3,9-11). In this regard, it is believed that the increase in H_2O_2 levels at low BA is related to signaling, whereas, high BA is associated with oxidative stress induction (12). However, at PDL-BA level, H_2O_2 amounts were lowered compared to 1 mM BA treatment. It was suspected that this was attributable to neuronal cell death. Additional discussion would be made later.

Interestingly and in contrast with the previous report (3) wherein catalase concentration increased with respect to increased H_2O_2 levels, GR amounts decreased. This could be attributable to decreased glutathione levels. Glutathione serves several

roles which include an anti-apoptotic function while GR helps maintain the glutathione homeostasis in order to prevent oxidative stress induction (13). Decreased GR amounts would suggest that glutathione homeostasis was altered thereby inducing oxidative stress consistent with the results and, similarly, this could putatively imply that glutathione levels were reduced which hypothetically may lead to cell death (in this case neurodegeneration). Moreover, oxidative stress is related to neuronal Ca^{2+} levels (3) while Ca^{2+} is involved in ether extracellular or intracellular signaling (14).

BA only affects intracellular Ca^{2+} levels

To elucidate both the extracellular and intracellular Ca^{2+} levels at varying BA amounts, Ca^{2+} amounts in the cell culture medium (extracellular) and cell lysate (intracellular) were measured. As seen in Fig. 1c, it is observed that intracellular Ca^{2+} levels were only increased in high BA amounts while no difference was observed in both low BA amount and extracellular Ca^{2+} levels. Ca^{2+} signaling plays a significant part in several extracellular and intracellular activities (14) which would insinuate that low BA amount has no significant effect on the intracellular Ca^{2+} environment and BA (regardless of concentration) does not promote Ca^{2+} extrusion to the extracellular environment. This would insinuate that only high BA amounts (possibly ascribable to

oxidative stress induction) could affect neuronal Ca^{2+} homeostasis, particularly in the intracellular environment. Considering extracellular Ca^{2+} amounts were unchanged, it is suspected that neuronal Na/Ca exchanger was not affected by BA presence. However, it is possible that an increase in Ca^{2+} levels would affect neuronal PMCA since intracellular Ca^{2+} homeostasis was altered.

To determine the effect of varying BA amounts on neuronal PMCA level, Western blotting was performed and the relative density was determined. It was found that the relative PMCA amounts were higher at 0.5 and 1 mM BA concentrations compared to the control and PDL-BA concentration (Fig. 1d). In addition, it was observed that the relative PMCA amount among PC12 cells treated with PDL-BA concentration is lower compared to PC12 cells treated with 1 mM BA concentration. PMCA has been associated with several vital neuronal activities and among which is neurodegeneration (15). This would suggest that BA-related increase in PMCA amounts (0.5 and 1 mM) is possibly related to neuronal activities such as neuronal maturation while BA-related decrease in PMCA amount (PDL-BA) is putatively related neurodegeneration. Additional discussion with regards to the relative decrease in PMCA amounts at PDL-BA level will be made later. Oxidative stress induction and Ca^{2+} accumulation have been associated with ER stress stimulation (16) and one major component often associated with ER

stress is GADD153.

GADD153 is differentially affected by varying BA amounts

To confirm how GADD153 levels were affected by varying BA amounts, GADD153 levels were measured. It was observed that GADD153 levels were higher in both 0.5 and 1 mM BA amounts while GADD153 levels in the control and PDL-BA amount are similar (Fig. 1e). GADD153 has a dual function serving as a pro-apoptotic molecule that functions as a negative inhibitor of CCAAT/enhancer binding proteins (C/EBPs) and, likewise, as an ER stress marker (16). Moreover, GADD153 helps sensitize cells to agents that may disrupt ER function and activates other genes by inhibiting C/EBPs (16,17). This implies that increased GADD153 levels can either be detrimental (ER stress induction) or beneficial (maintaining ER function) to the cell. Considering that oxidative stress and Ca^{2+} accumulation only occurred in high BA amounts, it was postulate that PC12 cells exposed to low BA amount would mediate an increase in neuronal GADD153 levels that is beneficial, whereas, increase in neuronal GADD153 levels associated with high BA amounts in detrimental.

Interestingly, in PDL-BA-treated cells, GADD153 levels were lower compared to 1 mM BA amount (Fig. 1e). This is consistent with the observations in neuronal H_2O_2

(Fig. 1a), GR (Fig. 1b), and PMCA (Fig. 1d) levels. Thus, it is probable that the reversal of these measurements at PDL-BA amount could be due to neurodegeneration whereby the neuronal cell reverts back to an immature state (18). This would imply two things: [1] oxidative stress and Ca^{2+} levels elicited an increase in GADD153 levels but is not enough to trigger neurodegeneration at 1 mM BA amount; and [2] oxidative stress and Ca^{2+} levels reached the threshold at 1 mM BA, while GADD153 levels remained high after 1 mM BA (Fig. 1e). Presumably, this triggered GADD153 levels to revert back to its original state (similar to control) and may consequentially result to neurodegeneration which may explain why there is no apparent dose-dependent effect on the varying neuronal factors (H_2O_2, GR, PMCA, and GADD153) studied at increasing BA concentration.

Chromatin condensation occurs at PDL-BA amount

To verify whether decreased GADD153 levels in cells treated with PDL-BA amount was attributable to neurodegeneration, chromatin condensation between control and PDL-BA-treated PC12 cells were compared. As shown in Fig. 1f, chromatin condensation occurred only in PDL-BA treated cells. C/EBP inhibition has long been correlated to chromatin condensation (19). Moreover, concurrent oxidative stress

induction, Ca^{2+} accumulation, and GADD153 reversion among PDL-BA-related cells are consistent signs of neurodegeneration (16,18). Similarly, this would also suggest that PC12 cells treated with 1 mM BA amount are not fully committed to neurodegeneration (or at the early stages) as compared to cells treated with PDL-BA amount where neurodegeneration is fully committed. At this point, a possible speculation is that varying BA amounts may trigger differing cell death-related signals.

TWEAK was dose-dependently decreased and TRAIL was increased

To determine the effect of BA on both TWEAK and TRAIL, extracellular TWEAK and TRAIL amounts and, subsequently, intracellular Caspase-10 and Caspase-8 levels (both associated to TWEAK and TRAIL) were measured. Interestingly, results show that TWEAK was decreased by BA dose-dependently (Fig. 2a) and TRAIL was increased in the presence of BA regardless of amount (Fig. 2b) which suggests that both signals are affected by BA and, similarly, BA effects are dependent on its amount. Interestingly, no significant differences in Caspase-10 activity was detected (Fig. 1c), whereas, high BA amount resulted to an increase in Caspase-8 activity (Fig. 1d). Caspase-8 is a key mediator of cell death signals and activated upon receptor binding of the respective death ligands (20) while Caspase-10 also works as initiator of death receptor-mediated

apoptosis (21). These results would imply that BA-related effects in TWEAK and TRAIL does not affect Caspase-10 activity. Similarly, BA-related does-dependent decrease in TWEAK amounts is inconsistent with increased Caspase-8 activity which suggests that even though TWEAK was dose-dependently affected by varying BA amounts, it is not involved in BA-induced cell death signaling. In contrast and considering TRAIL levels were increased similar to Caspase-8 activity at high BA amounts, it is possible that TRAIL is utilized by BA for cell death signaling, specifically, at high BA amounts. Moreover, at low BA amount, TRAIL was increased while Caspase-8 was unaffected which lead us to suspect that increased TRAIL levels at low BA amount are unrelated to cell death signaling. Interestingly, Caspase-8 level at PDL-BA amount was lower compared to 1 mM BA amount. This is possibly attributed to neurodegeneration as a consequence of reverting back to immature state (18). Nevertheless, results here would insinuate that Caspase-8 may play a role in inducing BA-related neurodegeneration. However, considering Caspase-8 is also involved in TNF-α signaling, BA was similarly checked if it was utilized for cell death signaling related to TNF-α signaling pathway.

High BA amounts increase TNF-α levels putatively via TNF-R1

To determine BA effects on TNF-α, TNF-α concentration was quantified. We observed that TNF-α levels were increased only in high amounts (Fig. 3a) which would imply that TNF-α level were affected by high BA amounts, whereas, low BA amount had no effect. It is possible that high BA activates TNF-α-related receptors while low BA has no effect on TNF-α related receptors. TNF-α stimulation is induce by H_2O_2 accumulation (22) consistent with an earlier result (Fig. 1a) and previous work (10). Moreover, TNF is a marker of disease activity and is also crucial for the induction of local prospective immune response against bacterial infection (23). This would suggest that TNF is activated at periodontal infection sites.

It is worth mentioning that NF-κB (particularly p65) levels were not affected regardless of BA concentration (Fig. 3b). Initially, this would imply that neither TNF-R1 nor TNF-R2 receptors (both recognized by TNF-α) were utilized, however, considering Caspase-8 was increased (Fig. 1d) then this suggests that the TNF-R1 receptor was putatively utilized. Coincidentally, TNF-R1 signaling is activated during bacterial infection (24) and, in a BA-related periodontal disease scenario, it is hypothesized that BA-related TNF-α activation utilizes the TNF-R1 receptor. It is worth mentioning that asides from TWEAK, TRAIL, and TNF-α cell death signaling, Caspase-8 is also

utilized in FasL signaling (20).

FasL amounts were increased only in PDL-BA amount

To determine BA effects on FasL, I quantified FasL levels. It was observed that FasL amounts were increased only in PDL-BA amount (Fig. 4a). This would suggest that FasL is not affected by 0.5 and 1.0 mM BA amounts but rather is only affected by PDL-BA amount. CytC is one biomolecule associated to FasL and is released from the mitochondria of intrinsic pathway during apoptosis (5). In addition, Bid, an apoptosis-related gene belonging to the Bcl-2 family, is activated upon receiving an apoptotic signal from Caspase-8. Once activated, Bid migrates to the mitochondria and binds with Bcl-2 homologous antagonist/killer (BAK) to induce CytC release (25). To determine the effect of BA on CytC and Bid, Western blotting analyses were performed. The CytC protein band only decreased at PDL-BA amount (Fig. 4b). Conversely, the estimated protein concentration in the Bid band was similar at all BA concentrations.

Moreover, it was found that FasL was increased while CytC was decreased at PDL-BA amount which presume is ascribable to Caspase-9 (26). To determine BA effects on Caspase-9, Caspase-9 levels were measured. It was found that Caspase-9 activity was increased in high BA amount (Fig. 4c). Caspase-9 has always been linked

to Caspase-8 activation (5) which would explain why both Caspase-9 and Caspase-8 are increased at high BA amounts (Figs. 4c, 2d). Moreover, Caspase-9 signals Caspase-3 activation (26) and to determine BA effects on Caspase-3, Caspase-3 amounts were quantified. As seen in Fig. 4d, it was observed that Caspase-3 activities were increased in high BA amounts (Fig. 4d). This would imply that both Caspase-9 and Caspase-3 are not affected by lower BA amounts but rather are only affected in high BA levels. Similarly, it was also observed that Caspase-9 and Caspase-3 levels at PDL-BA were lower compared to 1 mM BA amount possibly ascribable to reverting back to an immature state consistent with neurodegeneration (18).

Discussion

Periodontal disease progression is classified into three stages: mild, moderate, and severe (in reference to periodontitis) while BA accumulation is a pre-requisite for periodontal disease initiation and, subsequently, upon reaching PDL-BA amount would result full-blown periodontal disease (2,27). One hallmark characteristic during periodontal disease progression is neuropathic pain absence (27). Pain signaling requires neurite proliferation and any process that would inhibit signaling between neurites would result to neuropathic pain absence (28). In an earlier work (3), it was previously shown that high BA amounts cause neurite retraction and, in this regard, it is possible that BA-induced neurite retraction may contribute to neuropathic pain absence during periodontal disease progression.

Neurite retraction is a common feature during neurodegeneration, whereas, neurodegeneration has been associated with stress-related signals (altered calcium homeostasis and induction of both oxidative and ER stresses) (6,29). Subsequently, stress-related signals are commonly associated with cell death-related signals (29) which in-turn can affect neurites, in particular: [1] TWEAK is involved in neurite proliferation, whereas, decreased TWEAK amounts result in non-proliferation (30)

which would suggest that BA presence (regardless of amount) inhibit neurite proliferation; [2] TRAIL plays a role in neuronal cell death (31) which would imply that BA-induced increase in TRAIL levels are linked to cell death wherein low BA amount inhibit neurite proliferation, whereas, high BA amounts induce neurite retraction; [3] TNF-α induce cell death (32) which would insinuate that increase in TNF-α levels associated to only high BA amounts are attributable to cell death induction consistent with neurite retraction (3,4). FasL signaling in neuron cells lead to neurite retraction ascribable to cell death (3,33) consistent with these findings and earlier work with regards to PDL-BA-induced neurite retraction (3).

Taken together, it is assumed that BA has the potential to contribute to neuropathic pain absence during periodontal disease progression. In particular, it is probable that at high BA amounts both BA-stimulated stress- and cell death-related signals contribute to neurodegeneration, whereas, at low BA amount both BA-stimulated stress- and cell death-related signals are not associated with neurodegeneration but rather to neurite non-proliferation consistent with the presence and absence of BA-induced neurite retraction, respectively (3). Moreover, it is proposed that the following possible association between the BA-affected extracellular cell death signals and the stage of periodontal disease in view of neuropathic pain absence: [1] decreased TWEAK levels

are associated to mild periodontal disease, wherein, pain absence is correlated to neurite non-proliferation; [2] increased TRAIL levels (at low BA amount) are associated to mild periodontal disease, wherein, pain absence is correlated to neurite non-proliferation, whereas, increased TRAIL levels (at high BA amounts) are related moderate periodontal disease, wherein, pain absence is ascribable to neurite retraction; [3] increased TNF-α levels are related to moderate periodontal disease, wherein, pain absence is ascribable to neurite retraction; and [4] increased FasL levels are linked to severe periodontal disease, wherein, pain absence is also attributed to neurite retraction and, additionally, can cause bone loss (34).

Conclusions

This study aimed at elucidating the influence of varying BA amounts on stress- and cell death-related signals in PC12 cells. The conclusions of the study are as follows:

(1) BA-related oxidative stress-induced neurodegeneration occurs via an increased accumulation of intracellular calcium and ER stress.

(2) Cell death-signaling in NGF-treated PC12 cells are affected differently by different BA concentrations.

(3) The absence of neuropathic pain at any periodontal disease progression stage may be due to BA accumulation, regardless of the concentration.

From these results, it was suggested that stress- and cell death-signaling in NGF-treated PC12 cells are differently affected depending on BA concentration.

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Figures

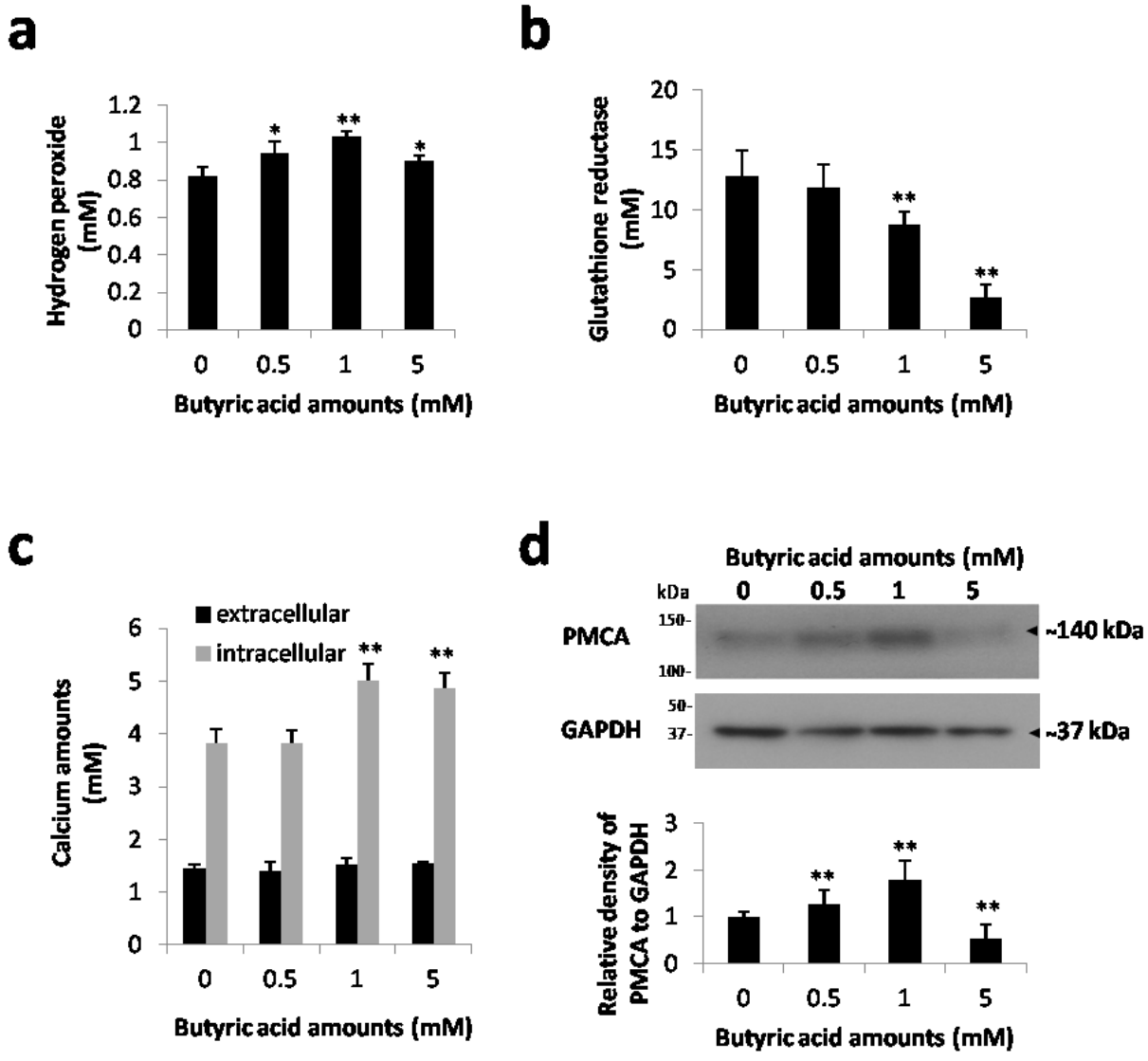
a
Hydrogen peroxide (mM)
1.2
1
0.8
0.6
0.4
0.2
0
*
**
*
0
0.5
1
5
Butyric acid amounts (mM)
b
Glutathione reductase (mM)
20
15
10
5
0
**
**
0
0.5
1
5
Butyric acid amounts (mM)
c
extracellular
intracellular
Calcium amounts (mM)
6
5
4
3
2
1
0
**
**
0
0.5
1
5
Butyric acid amounts (mM)
d
Butyric acid amounts (mM)
kDa
0
0.5
1
5
PMCA
150-
100-
~140 kDa
GAPDH
50-
37-
~37 kDa
Relative density of PMCA to GAPDH
3
2
1
0
**
**
**
0
0.5
1
5
Butyric acid amounts (mM)
e
GADD153/CHOP amounts (ng mL-1)
8
7
6
5
4
3
2
1
0
**
**
0
0.5
1
5
Butyric acid amounts (mM)
f
Control
BA-treated
2.5 µm
2.5 µm

Fig. 1. Activation of stress-related signals is dependent on BA amounts. **(a)** Hydrogen peroxide, and **(b)** glutathione reductase assays shown are representative signals for oxidative stress. **(c)** Calcium levels and **(d)** plasma membrane calcium ATPase (PMCA) protein bands presented are representative signals for calcium homeostasis. Antibodies used to detect glyceraldehydes-3-phosphate (GAPDH) and PMCA are indicated (*upper panel*). Relative density of PMCA to GAPDH is shown (*lower panel*). **(e)** GADD153/CHOP levels shown is a representative signal for ER stress. **(f)** Chromatin condensation between control (untreated PC12 cells) and BA-treated (5 mM) PC12 cells are displayed to confirm neuronal cell death. Varying BA amounts (0, 0.5, 1, 5 mM) are indicated. Assay and relative density results shown are mean ± SE, $n = 3$ independent samples. Statistical significance of differences between the varying BA concentrations in all assay performed was determined using Student's *t* test (*$p < 0.05$, **$p < 0.01$)

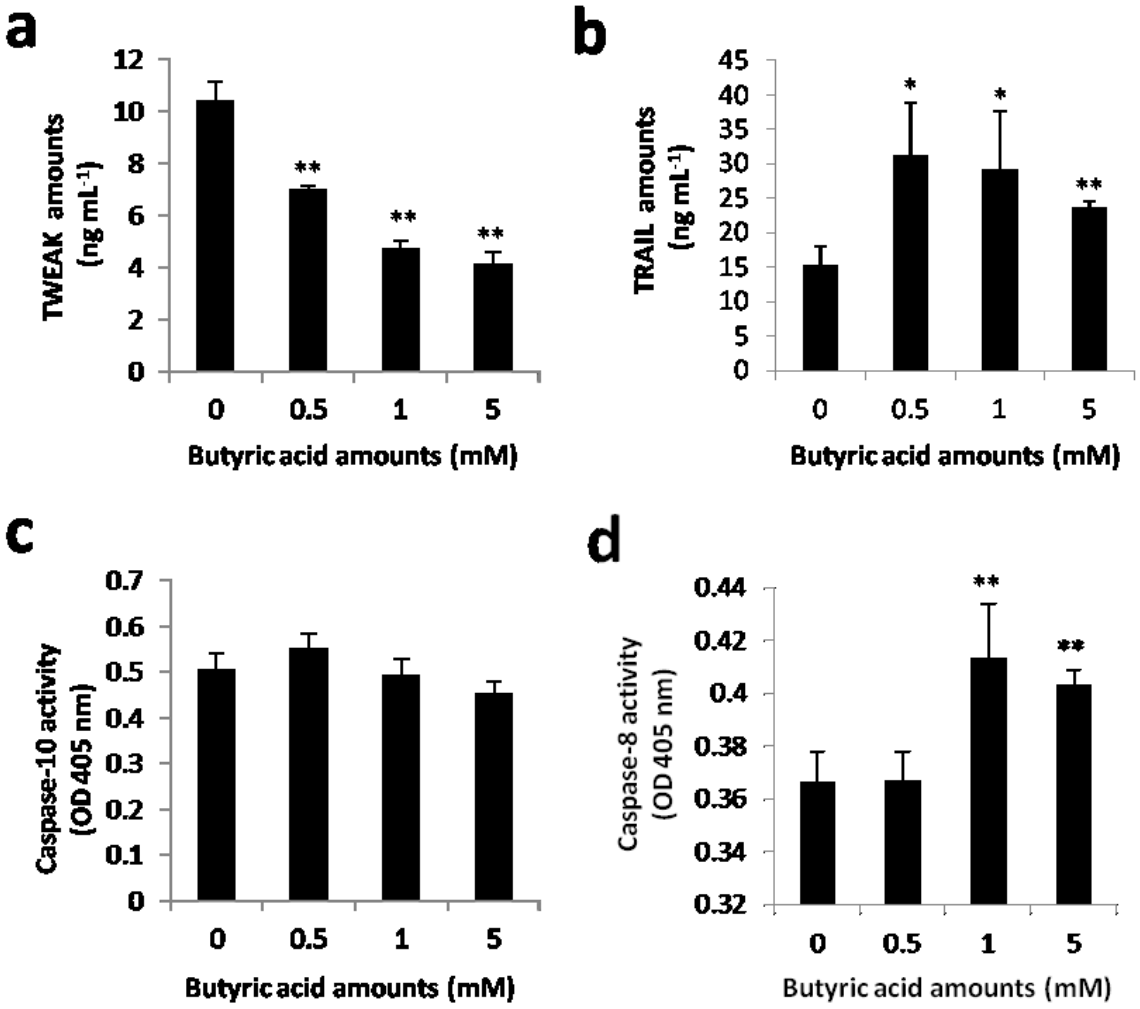

Fig. 2. Decreased TWEAK and increased TRAIL levels in NGF-treated PC12 cells are attributable to BA presence. **(a)** TWEAK and **(b)** TRAIL amounts in the NGF-treated PC12 cell culture medium. **(c)** Caspase-10 and **(d)** Caspase-8 activities in the NGF-treated PC12 cell. Varying BA amounts (0, 0.5, 1, 5 mM) are indicated. Results shown are mean ± SE, $n = 3$ independent samples. Statistical significance of differences between the varying BA concentrations in all assay performed was determined using Student's t test (*$p < 0.05$, **$p < 0.01$)

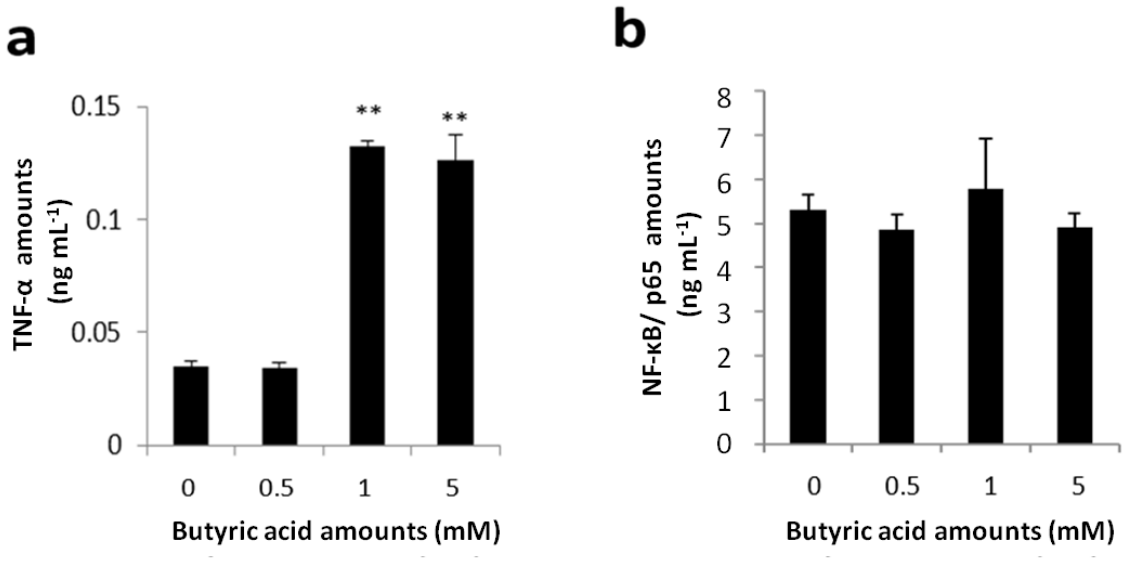

Fig. 3. High BA amounts increase TNF-α levels in NGF-treated PC12 cells. **(a)** TNF-α amounts in the NGF-treated PC12 cell culture medium. **(b)** NF-κB/p65 amounts in the NGF-treated PC12 cell. Varying BA amounts (0, 0.5, 1, 5 mM) are indicated. Results shown are mean ± SE, $n = 3$ independent samples. Statistical significance of differences between the varying BA concentrations in all assay performed was determined using Student's t test (**$p < 0.01$)

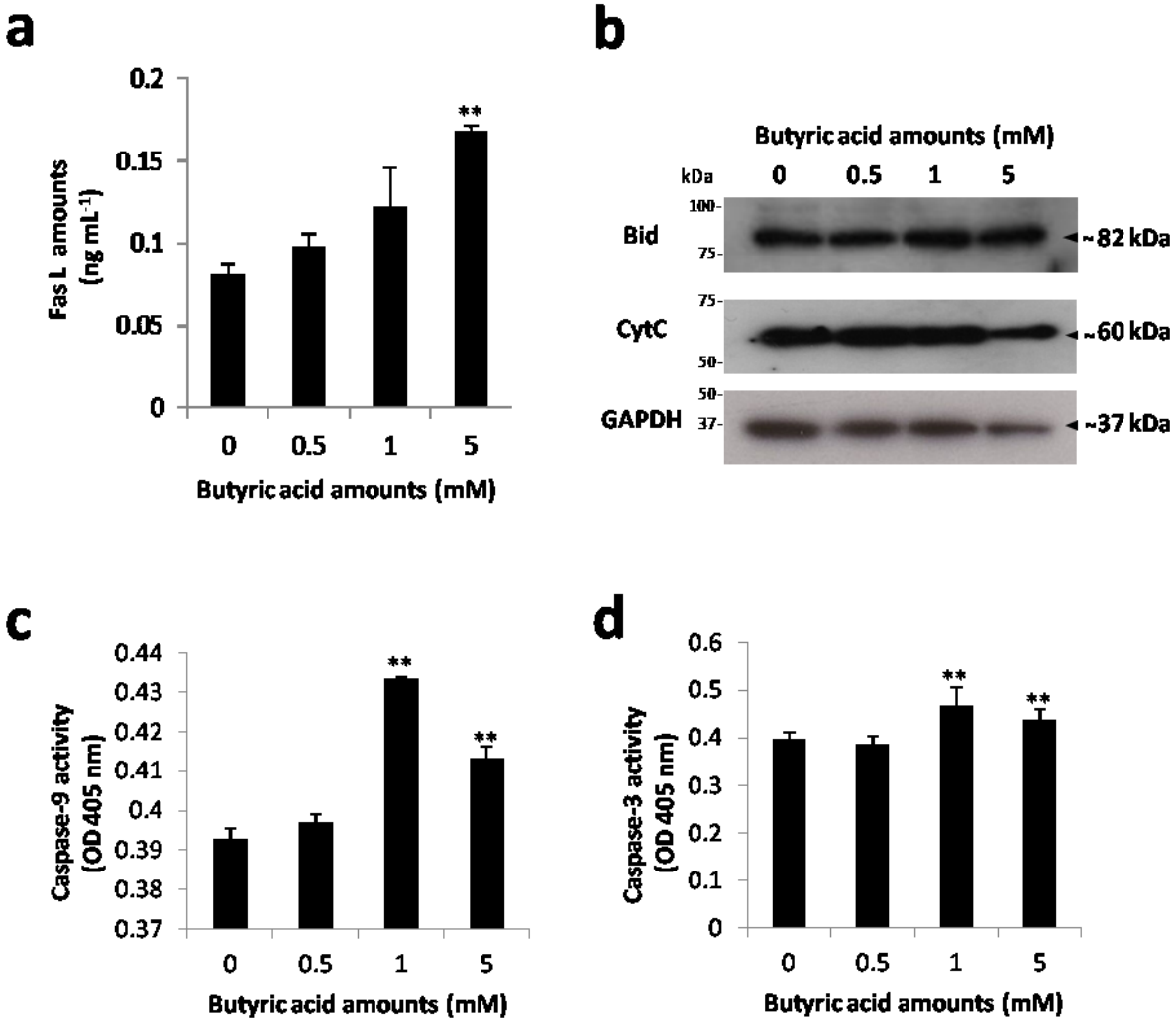

Fig. 4. FasL amounts in NGF-treated PC12 cells are increased only in PDL-BA level. **(a)** FasL amounts in the NGF-treated PC12 cell culture medium. **(b)** Western blotting analyses. Antibodies used to detect glyceraldehydes-3-phosphate (GAPDH), BH3 interacting-domain death agonist (Bid), and cytochrome c (CytC) are shown. **(c)** Caspase-9 and **(d)** Caspase-3 activities in the NGF-treated PC12 cell. Varying BA amounts (0, 0.5, 1, 5 mM) are indicated. Assay and Relative density results shown are mean ± SE, $n = 3$ independent samples. The statistical significance of differences between the varying BA concentrations in all assay performed was determined using Student's t test (**$p < 0.01$)