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1 **Effects of parabens on apoptosis induced by serum-free medium**

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18
19 **ABSTRACT**

20
21 Alkyl esters of *p*-hydroxybenzoic acids (parabens), an endocrine disrupter,
22 are used as preservatives in cosmetics and foods. In this study, to

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understand the relationship between parabens and differentiation in infants, the effects of parabens on apoptosis induced by serum deprivation in PC12 cells were investigated. In addition, apoptosis-related factors were assayed. As results, a tendency toward enhancement of apoptosis was observed in the cells cultured in the serum-free medium with methylparaben, and this tendency was suggested to be related to the contents of Bad. Butylparaben did not show any tendency to enhance apoptosis.

Keywords: PC12 cell, endocrine disrupter, food additives, apoptotic factors, methylparaben, butylparaben

INTRODUCTION

Parabens, alkyl esters of *p*-hydroxybenzoic acid, are most commonly used as preservatives in foods, cosmetics, toiletries and pharmaceuticals because of their relatively low toxicity in humans and their effective antimicrobial activity.

p-Hydroxy substitution on the aromatic ring has been recognized as an

important requirement for the estrogenic activity of some alkylphenols.^[1]

Parabens are structurally similar to alkylphenols. These compounds exert weak estrogenic activity as described by Lemini et al.^[2], Routledge et al.^[3], Hossaini et al.^[4] and Byford et al.^[5] The magnitude of the estrogenic response increases with the alkyl group size, as shown by the fact that ethylparaben, propylparaben, and butylparaben are approximately 150,000-, 30,000- and 10,000-fold less potent than 17 β -estradiol.^[6]

Some studies have examined the effects of early exposure to environmental toxicants using embryos.^[7-8] They reported that exposure to some chemicals increased mortality, induced morphologic deformations, increased apoptosis, and accelerated embryogenesis in the embryo stage. White^[9] reported growth effects of methylparaben, propylparaben and EDTA on a femur weight in organ cultures of embryonic chicks. The methylparaben and propylparaben concentrations found to stimulate growth were 0.25 to 2.5 μ g/mL and 0.025 to 2.5 μ g/mL, respectively. These results suggested that parabens might have some effects on infants and embryos. From this viewpoint, there were some interesting reports that endocrine disrupters affected apoptosis in cells.^[10-13]

66 Apoptosis is a morphological and biochemical description of a
67 physiological cell death mechanism that is commonly associated with
68 programmed events necessary for the differentiation and development of
69 individuals and organs.^[14-15] Maroto and Perez-Polo ^[15] wrote that
70 although apoptosis is often referred to as programmed cell death, the two
71 terms are not synonymous. Apoptotic events are not programmed but
72 rather occur in response to environmental cellular signals in many
73 instances.^[16] The activation of apoptosis by the signals and its role in
74 organisms need to be studied. Apoptotic cell death is characterized by
75 chromatin condensation, DNA fragmentation and membrane blebbing,
76 resulting in the formation of apoptotic bodies.^[14] Apoptosis is regulated
77 and executed by two major protein families, the Bcl-2 and caspase
78 families, which are highly conserved from worms to humans.^[17-18] Each
79 caspase is synthesized as an inactive precursor, which is activated only
80 after a cell has received a death signal.^[19] The Bcl-2 family, which is
81 involved in the regulation of caspase activity, is subdivided into
82 pro-apoptotic (for example, Bax and Bad) and anti-apoptotic (for example,
83 Bcl-2) members, which share one or more similar regions.^[20-21] However,
84 the influence of parabens on apoptosis in cultured cells has not been

investigated.

In rat pheochromocytoma PC12 cells, apoptosis is induced when the cells are cultured in serum-free medium. Apoptotic PC12 cells exhibit DNA fragmentation.^[22-23] Thus, these cells have proven to be a useful model for studying the mechanism of induction and inhibition of apoptosis.^[15, 24]

In this study, to determine the effects of parabens in differentiation of embryos and infants, their effects on apoptosis induced by serum deprivation were investigated using the PC12 cell system. In addition, apoptotic factors such as caspase-3 and the Bcl-2 family in PC12 cells exposed to parabens were measured by enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase (RT)-PCR.

MATERIALS AND METHODS

Materials

PC12, a rat pheochromocytoma cell line, was purchased from the American Type Culture Collection (USA and Canada). Dulbecco's

modified Eagle's medium (DMEM), streptavidin-conjugated peroxidase,
phosphate-citrate buffer with sodium perborate capsules and
o-phenyldiamine dihydrochloride (OPD) were from Sigma (St. Louis,
MO, USA). Fetal bovine serum (FBS) was from HyClone (Rockville, MD,
USA). The caspase assay kit, SV total RNA isolation kit, RT-PCR kit and
access RT-PCR introductory kit were from Promega (Madison, WI, USA).
Methyl p-hydroxybenzoate (methylparaben) and butyl p-hydroxybenzoate
(butylparaben) were obtained from Nacalai Tesque (Kyoto, Japan). Biotin
16-dUTP, proteinase K and the blocking reagent were from Roche
Diagnostics (Mannheim, Germany). Polyclonal antibodies against Bax,
Bad and Bcl-2 were from Oncogene Research Products (Boston, MA,
USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The
secondary antibody, biotinylated donkey anti-rabbit immunoglobulin, was
from Amersham Pharmacia Biotech (Buckinghamshire, UK). The DNA
7500 assay kit and RNA 6000 Nano Assay kit were from Agilent
Technologies (Waldbronn, Germany). Other chemicals were of analytical
reagent grade. Methylparaben and butylparaben were dissolved in ethanol
and used as the vehicle.

Cell Culture and Exposure Conditions

PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO₂. Inactivation of FBS was carried out at 56°C for 30 min. The cells were preincubated in 25 cm² flasks (Nunc, USA) for two or three days, and then the medium was replaced with serum/serum-free DMEM with or without methylparaben or butylparaben as follows.

Cells were incubated in serum-free DMEM, 0, 0.001, 0.01, 0.1 and 10 mg/mL (final concentration) of methylparaben or butylparaben for one, two and three days. Five µL of a 1000-fold concentration of methylparaben or butylparaben in ethanol was added to the 5 mL cell medium. Five microliters of ethanol was added to the serum-free DMEM and DMEM supplemented with 10% FBS as positive and negative control of apoptosis, respectively. When the medium was changed to serum-deprived medium, cells in the flask were washed twice with serum-free DMEM.

Oberdoerster et al. ^[25] reported that 150mM ethanol-induced enhancement of cell death by serum deprivation increased the extent of apoptosis in

PC12 cells. Furthermore, Neuman et al. ^[26] observed that 100mM ethanol induced apoptosis in cultured skin cells, through 40 mM ethanol rarely affected apoptosis in the cells. Based on these reports, it was presumed that using ethanol at a concentration of 1 μ L/mL (approximately 17mM) in this study did not affect apoptosis in PC12 cells.

Nuclear DNA Isolation

After that the PC12 cells were treated with 0 to 10 μ g/mL methylparaben and butylparaben, and 1 μ L/mL ethanol as a control for three days. The cells were then harvested using a scraper, and centrifuged at 1,500 rpm for 10 min. Then the supernatants were removed and the cells were washed twice at 1,500 rpm with 10 mM Tris-5 mM HCl containing 150 mM NaCl for 5 min. The obtained cells were incubated with 10 mM Tris-5 mM HCl, 0.1 M EDTA, 0.5% SDS and 20 μ g/mL pancreatic RNAase for 1 hr at 37°C. Proteinase K was added to the suspension up to 100 μ g/mL, and the enzyme reaction was carried out for 3 hr at 50°C. After cooling, the nuclear DNA was serially extracted with phenol and chloroform.

160

161 **Quantification of DNA Fragmentation in PC12 Cells by the**
162 **TdT-mediated dUTP-biotin Nick End Labeling (TUNEL) Method**

163

164 The DNA obtained from PC12 cells treated with methylparaben and
165 butylparaben was resuspended in 100 mM Tris-HCl, pH 8.0, containing 1
166 mM EDTA, and equal amounts of DNA were put into 96-well plates.

167 Quantification of DNA fragmentation in PC12 cells was done by the
168 method of Kurasaki et al.^[27]

169

170 **ELISA of Apoptotic Factors in PC12 Cells**

171

172 The PC12 cells were incubated with 0 to 10 µg/mL methylparaben or
173 butylparaben, or 1 µL/mL ethanol as a control for one, two, or three days.

174 After the cultivation, the cells were harvested using a scraper. The

175 harvested cells were washed twice at 1,500 rpm with 10 mM Tris-5 mM

176 HCl buffer containing 150 mM NaCl for 5 min. The cells were then

177 resuspended in 10 mM Tris-5 mM HCl buffer containing 150 mM NaCl

178 and disrupted by sonication for 30 sec with a Sonifier 250 (Branson). The

cell debris and unbroken cells were removed by centrifugation. The contents of Bad, Bax and Bcl-2 in the cells were measured by the method of Yamanoshita et al.^[12] The cell extracts after incubation with parabens were adjusted to equal protein concentrations with 40 mM Tris-5, mM HCl containing 150 mM NaCl for ELISA. The primary antibody for Bad, Bax or Bcl-2 (diluted 1:250 with 1% [v/v] blocking reagent in the same buffer) was reacted with antigens for 1 hr. The contents of the three factors were calculated from the absorbance measured at 405 nm with a Microplate Reader model 450 (BIO-RAD).

RNA Isolation

The total RNA of PC-12 cells treated with methylparaben or butylparaben was extracted using an SV total RNA isolation kit according to the instruction manual after the cells were treated with parabens. The details are described in the legend of Figure 5. The total RNA obtained was verified using a RNA 6000 Nano Assay kit with an Agilent 2100 Bio Analyzer. Total RNA was stored at -20°C until use for one or two days.

Gene Expression by RT-PCR

Expression of bad, bax and bcl-2 mRNAs, in the PC12 cells exposed to parabens was detected using the Access RT-PCR kit and Access RT-PCR introductory kit according to the manufacturer's instructions. The PCR primers of bad, bax, bcl-2 and β -actin were designed from their DNA sequences.^[28] The samples were heated at 48°C for 45 min and subsequently at 94°C for 2 min for enzyme reaction. The time course of one cycle was 94°C for 30 s for denaturation, 60°C for β -actin and bax, 55°C for bad and 47°C for bcl-2 for 1 min for annealing, and 68°C for 2 min for extension. The number of cycles performed was 30 or 40. β -Actin was chosen as an internal control. PCR products were verified using a DNA 7500 Assay kit with a 2100 Bio Analyzer (Agilent Technologies, USA) according to the manufacturer's instructions.

Measurement of Caspase-3 Like Activity

PC12 cells were incubated in the medium containing a caspase inhibitor, Z-VAD or Z-VAD-free medium with 0 to 10 μ g/mL methylparaben or 1

217 $\mu\text{L/mL}$ ethanol as a control for two days. The activity of caspase-3-like
218 protease was detected using the Caspase Assay System kit. The activity of
219 caspase-3 like protease in the PC12 cells was indirectly measured as
220 absorbance at 405 nm depending on the pNA released from the cleaved
221 substrate. After overnight incubation, absorbance at 405 nm was
222 measured as the caspase-3 like activity

223

224 **Statistical Analysis**

225

226 Each value is expressed as mean $\pm$ SEM. Analysis of variance (ANOVA)
227 followed by the Bonferroni/Dunn multicomparison test was employed.
228 Statistical analyses were performed using StatView (HULINKS, Tokyo,
229 Japan).

230

231 **RESULTS**

232

233 **Effects of Methylparaben and Butylparaben on Apoptosis**

234

235 To study the effects of methylparaben and butylparaben on apoptosis,

TUNEL signals from nuclear DNA in PC12 cells cultured in serum-free medium and treated with 0.001, 0.01, 0.1, 1 and 10 µg/mL methylparaben or butylparaben for three days were measured (Fig. 1).

The TUNEL signals in the cells cultured in the serum-deprived medium were significantly increased as compared with those in the control cells.

The signals in the DNA of the cells treated with methylparaben showed a tendency to increase (Fig. 1a). On the other hand, TUNEL signals in the cells treated with butylparaben at various concentrations showed no significant differences (Fig. 1b).

These changes did not show the dose-response effect. However these kinds of irregular responses were showed in similar experiments.^[13, 29]

Contents of Apoptotic Factors Measured by ELISA

To understand the reason for the different tendencies of TUNEL signals between the PC12 cells treated with methylparaben and butylparaben, the contents of the apoptotic factors Bad, Bax and Bcl-2 in the PC12 cells cultured in serum-free medium containing 0.001, 0.01, 0.1, 1 or 10 µg/mL methylparaben or butylparaben for one, two or three days, were measured

255 by ELISA (Figs. 2-4).

256 After one-day exposure, the contents of Bad increased according to the
257 increase in the concentration of methylparaben as expected (Fig. 2a). Bad
258 contents in the cells exposed to 0.1 and 10 µg/mL methylparaben for one
259 day increased significantly ($P<0.05$). After two days of exposure, the
260 contents of Bad increased slightly in the cells treated with methylparaben
261 (Fig. 2b). Bad contents increased significantly ($P<0.05$) in cells treated
262 with 1 µg/mL methylparaben for two days. Unexpectedly, however, there
263 was no significant difference among Bad contents in cells treated with the
264 various concentrations of methylparaben for three days (Fig. 2c).

265 There was no significant difference among Bad contents in the cells
266 treated with butylparaben for one, two or three days at the various
267 concentrations as expected (Figs. 2d-f).

268 In addition, there was no significant difference among Bax contents in the
269 cells treated with methylparaben or butylparaben for one, two or three
270 days at the various concentrations (Fig. 3).

271 Bcl-2 contents in the cells exposed to 0.1 µg/mL methylparaben for one
272 day increased significantly ($P<0.05$; Fig. 4a). However, there was no
273 significant difference among Bcl-2 contents in the cells treated with

274 methylparaben for two and three days at the various concentrations (Figs.
275 4b and c).

276 There was no significant difference among Bcl-2 contents in the cells
277 treated with butylparaben for one, two or three days at the various
278 concentrations (Figs. 4d-f).

279

280 **Expression of Apoptotic Factor mRNAs Detected by RT-PCR**

281

282 To verify changes of apoptotic factors in PC12 cells treated with
283 methylparaben or butylparaben, the expression of bad, bax and bcl-2
284 mRNAs in PC12 cells cultured in the serum-free medium with 0.001, 0.01,
285 0.1, 1 or 10 µg/mL methylparaben or butylparaben added for one, two or
286 three days, was measured by RT-PCR.

287 There was a tendency for the expression of bad mRNA to increase in the
288 cells treated with methylparaben after one day similar to the changes of
289 Bad contents detected by ELISA in the cells treated with methylparaben
290 for one and two days (Figs. 5a and b). There was no significant difference
291 of bad mRNA expression among cells treated with methylparaben for two
292 and three days at the various concentrations (Fig. 5c), the same as seen in

Figure 2C. After one- and two-day exposures, there was a tendency for the bad mRNA in the cells treated with butylparaben to increase (Figs. 5d and e). There was no significant difference of bad mRNA expression among the cells treated with butylparaben for three days at 0.001 to 1 $\mu\text{g/mL}$ (Fig. 5f). However the cells died as a result of treatment with 10 $\mu\text{g/mL}$ butylparaben for two and three days (Figs. 5e and f). There was no significant difference among bax and bcl-2 mRNAs in the cells treated with methylparaben or butylparaben for one, two or three days at the various concentrations (data not shown).

Measurement of Caspase-3 Like Activity

To investigate whether apoptosis-related factors under the conditions of apoptosis induced by serum deprivation were changed by addition of methylparaben, caspase-3 activity was measured in PC12 cells cultured in serum-free medium containing methylparaben (Fig. 6). The caspase-3 like activity in the cells increased with increasing concentrations of methylparaben as expected. On the other hand the activity in the cells did not change with buthylparaben (data not shown).

DISCUSSION

The aim of this experiment was to study effects of parabens on apoptosis induced in advance. PC12 cells are well known to be a useful model for studying the mechanisms of induction and inhibition of apoptosis under conditions with and without serum, because apoptosis is induced by serum deprivation in the cells. In our laboratory the influence of endocrine disrupters on apoptosis induced by serum deprivation has been investigated using PC12 cells.^[12-13, 30] When serum is present, apoptosis was not induced by addition of parabens (data not shown). This means that parabens cannot induce apoptosis itself. On the other hand, under serum deprivation, a tendency for apoptosis to be induced occurred after addition of methylparaben in the *in vitro* model system described above (Fig. 1a). This means that there is a possibility for enhancement of apoptosis by addition of methylparaben. In this study serum was used as a regulatory factor for apoptosis. The any tendency for apoptosis to be induced by serum deprivation did not show in the cells treated with various concentrations of butylparaben (Fig. 1b).

It was reported that the binding of parabens to the estrogen α -receptor (ER α) of MCF7, human breast cancer cells, increased with increasing alkyl chain length.^[5] Moreover, the estrogenic activity of parabens has been demonstrated to increase with increasing length of the linear alkyl chain from methylparaben to butylparaben.^[1] In addition, 4×10^{-4} M methylparaben and 3×10^{-6} M butylparaben showed 10% of the activity of 10^{-7} M 17- β estradiol. These concentrations of parabens are more than ten mg/mL. However, in the present study, the TUNEL signals showed no relationship between alkyl group size and effects on apoptosis in PC12 cells treated with parabens (Figs. 1a and b). From the results, it was indicated that the effects of parabens on apoptosis in the cells were independent of the function of ER α , because PC12 is an ER α -negative cell line.

To study whether enhancement of apoptosis by treatment with methylparaben or butylparaben was regulated by the apoptotic factors Bax, Bad and Bcl-2, we measured the relative contents of these apoptotic factors in PC12 cells treated with methylparaben or butylparaben under serum-free conditions using ELISA and RT-PCR. The content of Bad, a pro-apoptotic protein, was slightly increased with increasing

350 concentrations of methylparaben for one day (Fig. 2a). However, no
351 significant effects were seen on the expression of bad mRNA, although
352 there was a tendency toward an increase after one-day exposure. No
353 changes were observed in the contents of Bax and Bcl-2 under the same
354 conditions. These results could indicate that the enhancement of
355 apoptosis in the PC12 cells cultured in serum-free medium containing
356 methylparaben could be associated with an increase of Bad content.
357 Moreover, caspase-3 activity was increased in the cells treated with 0.001
358 to 10 µg/mL methylparaben (Fig. 6). It was considered that the
359 enhancement of apoptosis in the PC12 cells treated with methylparaben
360 was mediated by the caspase-3 pathway.

361 Paraben-induced cytotoxicity has been speculated to depend not only on
362 the dose of paraben, but also on the length of the exposure period.
363 However, the contents of the apoptotic factors in the cells treated with
364 parabens differed markedly at one day as compared with those for two and
365 three days. Maroto and Peres-Polo ^[15] reported that expression of Bad and
366 Bax was decreased by serum deprivation for 3hr and 24hr in PC12 cells.
367 However, in this study, the tendency for Bad contents to increase in cells
368 treated with methylpareben was only observed on day one, although the

369 contents of Bax and Bcl-2 showed no changes under the same conditions
370 for one to three days.

371 In this study, PC12 cells died after the treatment with butylparaben at 10
372 $\mu\text{g/mL}$ (5.15×10^{-5} M) (Figs. 5e-f). Darbre et al. ^[31] reported that
373 benzylparaben was cytotoxic at a concentration of 10^{-4} M for
374 ER α -negative MDA-MB-231 human breast cells. On the other hand,
375 methylparaben showed cytotoxicity at about 400 $\mu\text{g/mL}$ in human THP-1
376 cells.^[32] With the experimental concentration (maximum 10 $\mu\text{g/mL}$) in
377 this study, methylparaben did not show any cytotoxicity.

378 Janjua et al. ^[33] reported that butylparaben peaked in serum after a few
379 hours, reaching 81 $\mu\text{g/L}$ after daily whole-body topical application of a 2
380 mg/cm^2 basic cream formulation (2% [w/w]) for two weeks. In addition,
381 five days after the treatment about 20 $\mu\text{g/L}$ butylpareben remained in the
382 serum. Gelb et al. ^[34] investigated effects of intravenously administered
383 methylparaben on cerebral blood flow in the human brain. They reported
384 that the concentration of methylparaben in serum was more than 30 $\mu\text{g/L}$.
385 On the basis of these reports, the concentrations of methylparaben and
386 butylparaben used in this study were in the expected range in human
387 blood after uptake of the materials containing parabens.

In this study, to investigate the effects of parabens on differentiation and development, the relationship between apoptosis induced by serum deprivation in PC12 cells and parabens was studied. Apoptosis is a physiological cell death mechanism that is commonly associated with programmed events necessary for the differentiation and development of individuals and organs.^[14-15] We found that there was no significant effect of parabens on induced apoptosis in PC12 cells. However, methylparaben showed a tendency to enhance of apoptosis (Fig. 1). In addition, methylparaben slightly increased the contents of Bad (Fig. 2) and caspase-3 activity (Fig. 6), thereby possibly further enhancing the apoptosis induced by the serum deprivation. As parabens are hydrophobic compounds, they can be transferred from the maternal body to infants.^[7-8]

CONCLUSION

From the results, a tendency toward enhancement of apoptosis was observed in the cells cultured in the serum-free medium with methylparaben, and this tendency was suggested to be related to the contents of Bad. Butylparaben did not show any tendency to enhance

407 apoptosis. Exposure to trace levels of some chemicals increases
408 morphologic deformations and caused adverse effects on embryogenesis
409 in the embryo stage. The human population is chronically exposed to
410 parabens through many thousands of foods, drugs and cosmetic products.
411 From this viewpoint, to clarify the detailed effects of long-term exposure
412 to parabens, further investigation is needed.

413

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415

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FIGURE CAPTIONS

Figure 1. Effects on DNA fragmentation in PC12 cells treated with methylparaben (a; n=6) or butylparaben (b; n=5) for three days. The TUNEL signals of DNA fragmentation are presented as relative percentages against those of cells cultured in serum-free medium containing 1 μ L/mL ethanol. Asterisks denote values significantly different (**P<0.05) or having a tendency to be different (*P<0.1) from cells incubated with serum-free medium without parabens. (mean $\pm$ SEM)

Figure 2. Contents of Bad in PC12 cells treated with methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f) days. The contents of Bad are presented as relative percentages against those of cells cultured in serum-free medium containing 1 μ L/mL ethanol for one day. The mean $\pm$ SEM was calculated four (a, b, c) and five (d, e, f) independent experiments. Asterisks denote values significantly different (**P<0.05) from these of cells incubated in serum-free medium without paraben.

Figure 3. Contents of Bax in PC12 cells treated with methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f) days. The contents of Bax are presented as relative percentages against those of cells cultured in serum-free medium containing 1 μ L/mL ethanol for one day. The mean $\pm$ SEM was calculated four (a, b, c) and five (d, e, f) independent experiments.

Figure 4. Contents of Bcl-2 in PC12 cells treated with methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f)

days. The contents of Bcl-2 are presented as a relative percentage against those of cells cultured in serum-free medium containing 1 μ L/mL of ethanol for one day. The mean $\pm$ SEM was calculated for four (a, b, c) and five (d, e, f) independent experiments. Asterisk denotes a value significantly different (**P<0.05) from cells incubated in serum-free medium without parabens.

Figure 5. Contents of bad mRNA in PC12 cells treated with 0 to 10 μ g/mL methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f) days. The contents of bad mRNA are presented as relative percentages against those of cells cultured in medium containing serum and 1 μ L/mL ethanol. The mean $\pm$ SEM was calculated from three (one day) and five (two and three days) independent experiments.

Figure 6. The specific activity of caspase-3 in PC12 cells treated with 0.001 to 10 μ g/mL methylparaben and serum deprivation for two days. The specific activity of caspase-3 is presented as a relative value against that of the cells cultured in medium containing serum and 1 μ L/mL ethanol as a control. The values are means from two representative experiments.

Fig.1

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Fig.2

■ serum + ethanol
□ serum-free + ethanol (Control)
■ serum-free + parabens

a

Relative contents of Bad (%)

b

c

serum + parabens

d

e

f

0 0.001 0.01 0.1 1 10 ($\mu\text{g/mL}$)

Chart	Condition	Relative contents of Bad (%)	Significance
a	serum + ethanol	~90	
	serum-free + ethanol (Control)	~100	
	serum-free + parabens (0.001)	~105	
	serum-free + parabens (0.01)	~115	
	serum-free + parabens (0.1)	~130	**
	serum-free + parabens (1)	~115	
	serum-free + parabens (10)	~135	**
b	serum + ethanol	~120	
	serum-free + ethanol (Control)	~95	
	serum-free + parabens (0.001)	~105	
	serum-free + parabens (0.01)	~90	
	serum-free + parabens (0.1)	~100	
	serum-free + parabens (1)	~130	**
	serum-free + parabens (10)	~105	
c	serum + ethanol	~130	
	serum-free + ethanol (Control)	~105	
	serum-free + parabens (0.001)	~100	
	serum-free + parabens (0.01)	~95	
	serum-free + parabens (0.1)	~90	
	serum-free + parabens (1)	~100	
	serum-free + parabens (10)	~95	
d	serum + ethanol	~115	
	serum-free + ethanol (Control)	~100	
	serum-free + parabens (0.001)	~95	
	serum-free + parabens (0.01)	~95	
	serum-free + parabens (0.1)	~100	
	serum-free + parabens (1)	~105	
	serum-free + parabens (10)	~95	
e	serum + ethanol	~110	
	serum-free + ethanol (Control)	~75	
	serum-free + parabens (0.001)	~75	
	serum-free + parabens (0.01)	~70	
	serum-free + parabens (0.1)	~75	
	serum-free + parabens (1)	~80	
	serum-free + parabens (10)	~75	
f	serum + ethanol	~105	
	serum-free + ethanol (Control)	~75	
	serum-free + parabens (0.001)	~75	
	serum-free + parabens (0.01)	~65	
	serum-free + parabens (0.1)	~75	
	serum-free + parabens (1)	~70	
	serum-free + parabens (10)	~70	

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Fig.3

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Fig.4

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Fig.5

Fig.6

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