Reinvestigation of the effect of carbenoxolone on the induction of heat shock proteins

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Received: 20 May 2008 / Revised: 30 January 2009 / Accepted: 5 February 2009 / Published online: 31 March 2009
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Abstract Carbenoxolone (CBX) is a semisynthetic derivative of the licorice root substance glycyrrhizinic acid and has been previously reported to induce only heat shock protein 70 [Hsp70, HSPA1A (the systematic name of heat shock protein is given in the parenthesis after each HSP, according to the recent nomenclature guidelines, Kampinga et al., *Cell Stress Chaperones*, 14:105–111, 2008) but not other heat shock proteins (HSPs) (Nagayama et al., *Life Sci.* 69:2867–2873, 2001). In this study, we reinvestigated the effect of CBX on the induction of HSPs in HeLa and human neuroblastoma (A-172) cells. CBX clearly induced not only Hsp70 but also Hsp90 (HSPC1), Hsp40 (DNAJB1), and Hsp27 (HSPB1) at concentrations of 10 to 800 μ M for 16 h incubation. At higher concentrations (more than 400 μ M), however, CBX appeared to be toxic. Treatment of cells with CBX resulted in enhanced phosphorylation and acquisition of DNA-binding ability of heat shock transcription factor 1 (HSF1). Furthermore, characteristic HSF1 granules were formed in the nucleus, suggesting that the induction of HSPs by CBX is mediated by the activation of HSF1. Furthermore, thermotolerance was induced by CBX treatment, as determined by clonogenic survival. Although the precise target of CBX is not known at present, these results indicate that CBX is one of the molecular chaperone inducers and suggest that some pharmacological activities of CBX might be ascribable in part to its molecular chaperone-inducing property.

Keywords Carbenoxolone (CBX) · Heat shock proteins · Molecular chaperones · HSF1 activation · Thermotolerance

Introduction

Molecular chaperones, which are mostly heat- or stress-induced proteins (HSPs), are known to regulate various cellular functions. These include (1) assistance in folding of nascent polypeptides or in refolding of partially denatured proteins, (2) protein transport across intracellular membranes, (3) regulation of apoptosis, (4) regulation of cytoskeletal organization, (5) inhibition of protein denaturation, (6) suppression of aggregate formation by disabled proteins, and (7) targeting of severely damaged proteins to the degradation system or to the formation of large aggregates for sequestration (Parsell and Lindquist 1993; Hartl and Hayer-Hartl 2002; Morimoto 2002). Therefore, molecular chaperones have been envisioned to be endogenous cytoprotective factors, lifeguards, or guardians of the proteome (Morimoto and Santoro 1998; Jaattela 1999; Ohtsuka and Hata 2000). Recent studies indicate that moderate overexpression of molecular chaperones could confer on cells and tissues stress tolerance and provide beneficial effects on various pathological states associated with protein misfolding and protein aggregation (Muchowski and Wacker 2005). In addition, molecular chaperones have been reported to be involved in the process of antigen presentation, as well as in the stimulation of innate and adaptive immune responses by working outside of cells as danger signals or immunoactivators (Srivastava 2002; Calderwood et al. 2006; Ohtsuka et al. 2007). Mild heat shock, preconditioning stresses, transfection of HSP genes, and some chemical compounds are usually used to elicit the overexpression of molecular chaperones.

Recently, many types of low molecular weight chemical inducers and coinducers of HSPs have been reported, including nonsteroidal anti-inflammatory drugs, Hsp90 inhibitors, arachidonic acid and prostaglandins, proteasome

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inhibitors, anti-ulcer drugs, and major constituents of some herbal medicines, which are summarized in several reviews (Westerheide and Morimoto 2005; Soti et al. 2005; Ohtsuka et al. 2005). Among these inducers, some chemicals have been reported to induce a specific HSP(s) but not the others. For example, herbimycin-A stimulated only Hsp70 synthesis and protected cells against a subsequent, normally lethal heat stress, but it could not induce other HSPs such as Hsp90, Hsp60, and Hsp27 in cultured cardiomyocytes (Morris et al. 1996). Monophosphoryl lipid A, an endotoxin derivative, which has potent anti-ischemic activity, enhanced only Hsp70 expression but did not provoke the synthesis of Hsp27, Hsp32, and Hsp90 in adult rat cardiac myocytes (Nayeem et al. 1997). Selective induction of Hsp27 and alpha B-crystallin, but not Hsp70 and other HSPs, was shown by several chemical compounds such as anisomycin (Kato et al. 1999), sphingosine 1-phosphate (Kozawa et al. 1999), hydroxyurea (Eskenazi et al. 1998), short-chain fatty acids (Ren et al. 2001), and thrombin (Hirade et al. 2002). Furthermore, carbenoxolone (CBX) was reported to induce only Hsp70 but not Hsp90 or Hsp40 (Nagayama et al. 2001). In most of these reports, however, the authors did not investigate the activation of heat shock factor 1 (HSF1), a key transcription factor for the induction of HSPs. The principal concept of heat shock or stress response is that the induction of HSPs is usually mediated by the activation of HSF1 (Morimoto 1998). Since most of the HSP genes have a consensus nucleotide sequence $(-NGAAN-)_n$ called the heat shock element (HSE) at the promoter region of each HSP gene to which the activated HSF1 binds, it is very difficult to consider that a specific HSP(s), but not the others, would be induced by some stresses or chemicals. Therefore, we decided to reinvestigate the effects of the abovementioned chemicals on the induction of HSPs and chose CBX because a previous report showed that in spite of the activation of HSF1, which was demonstrated as a band shift by Western blot, only Hsp70 was induced by CBX but not Hsp90 or Hsp40 (Nagayama et al. 2001). In this paper, we report the induction of Hsp70 as well as Hsp90, Hsp40, and Hsp27 through the activation of HSF1 by the treatment with CBX in HeLa and human neuroblastoma A-172 cells.

Materials and methods

Cells, cell culture, and chemicals

HeLa cells and human neuroblastoma A-172 cells were used in this study. Cells were cultured in Dulbecco's modified Eagle's minimal essential medium (D-MEM, GIBCO, Grand Island, NE, USA) supplemented with 10%

fetal bovine serum (GIBCO) and 1% penicillin–streptomycin (GIBCO) and incubated in a CO₂ incubator with 5% CO₂ and 95% air at 37°C. Serum-free D-MEM was also used as indicated. A stock solution of CBX (10 mM in phosphate-buffered saline [PBS]; Sigma, St. Louis, MO, USA) was added to the culture medium at the indicated final concentrations. When cells were heated, the culture dishes were sealed with parafilm and immersed in a water bath, the temperature of which was controlled within $\pm 0.1^\circ\text{C}$. Other reagents and chemicals were of the highest purity available from Wako Pure Chemicals (Osaka, Japan) and Nakalai Tesque (Kyoto, Japan).

Immunological methods

For Western blotting, cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (2.3% SDS, 62.5 mM Tris–HCl [pH 6.8], 5% β -mercaptoethanol, 10% glycerol) and boiled for 5 min. The protein concentration of each cell lysate was determined by a Pierce protein assay kit (Pierce, Rockford, IL, USA). Proteins in a 10- μg sample were separated by standard SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schnell, Dassel, Germany). The blotted membranes were probed with the first antibody (1/1,000 to 1/2,000 dilution). The first antibodies used in this study were rabbit polyclonal anti-HSF1 (SPA-901, StressGen, Victoria, Canada), rabbit polyclonal anti-Hsp90 (H114, Santa Cruz Biotechnol. Inc., USA), mouse monoclonal anti-Hsp70 (SRB-810, MBL, Nagoya, Japan), rabbit polyclonal anti-Hsp40 (Hattori et al. 1992), and rabbit polyclonal anti-Hsp27 (SPA-800, StressGen) antibodies. The membranes were then probed with the horseradish peroxidase-conjugated corresponding immunoglobulin G (IgG; Zymed, San Francisco, USA, 1/1,000 dilution). They were then treated with enhanced chemiluminescence reagent (Amersham, Piscataway, NJ, USA), and signals were detected by exposure of the membranes on X-ray film (Kodak, Rochester, NY, USA). The relative signal intensity was quantified by densitometry with NIH Image software (Scicon Image) on a personal computer.

To determine the intracellular localization of HSF1, cells grown on a cover slip were fixed in 100% methanol at -20°C for 10 min and then treated with 10% normal goat serum to inhibit nonspecific binding. They were then processed for immunofluorescence staining by using rabbit polyclonal anti-HSF1 antibody (SPA-901, StressGen, 1/200 dilution) as the first antibody, and fluorescein-isothiocyanate-conjugated anti-rabbit IgG (Zymed, 1/100 dilution) as the second antibody. The cells were observed through a Fluorophoto microscope (Nikon, Tokyo, Japan).

Gel mobility shift assay

To detect the complex of activated HSF1 with the HSE, a gel mobility shift assay was performed by LightShift Chemiluminescent EMSA kit (Pierce) with a biotin-streptavidin non-R1 system as described previously (Yan et al. 2004; Saito et al. 2005). The oligonucleotide sequence of the HSE probe was as follows:

HSE of *Drosophila* HSP70 (+): 5'-gcctcgaatgttcgc-gaagtttcg-3'

HSE of *Drosophila* HSP70 (-): 5'-cgaaacttcgcgaacattc-gaggc-3'

To prepare the cell extract, HeLa cells were lysed with NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Then, the cell extracts (5 µg of total protein) were incubated with 50 fmol of biotinylated HSE oligonucleotide probe in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 500 mM KCl, 100 mM NaCl, 5% glycerol, and 10 mM dithiothreitol in a final volume of 20 µl for 30 min at 23°C. After incubation, the assay mixtures were applied to 5% polyacrylamide non-denaturing gels and electrophoresed. Gels were then transferred to nylon membranes. HSE-HSF1 complexes on each blot were visualized by the chemiluminescent reaction with streptavidin/horseradish peroxidase on X-ray film (Kodak).

Transfection and reporter assay

To test whether or not the induction of HSPs by CBX treatment was dependent on the HSE consensus sequence, we performed a reporter assay with the promoter region of the human Hsp40 gene (Hata and Ohtsuka 1998). The Hsp40 gene promoter region was introduced into the plasmid PGVG carrying the luciferase reporter gene (Picagene, Toyo Ink, Tokyo, Japan). A plasmid pGV-Nr (-277) has the HSE as well as other general promoter sequences. Plasmid DNA was purified by Qiagen Plasmid Kit (Qiagen, Valencia, USA). For the transfection experiment, HeLa cells were seeded at 1×10^6 cells/35 mm diameter dish and grown for 20 h. Then, 2 µg of each plasmid was transfected into HeLa cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After a 24-h incubation, the medium was replaced with fresh medium, and cells were treated with CBX at indicated concentrations for 24 h or heated at 42°C for 1 h and then recovered for 5 h at 37°C. The cells were then washed with ice-cold PBS and lysed with 120 µl of lysis buffer of Luciferase Reporter Assay System (Promega, Madison, WI, USA). The protein concentration of each lysate was measured by protein assay kit (Pierce) and normalized. Luciferase activities were measured by the

Luciferase Reporter Assay System (Promega), and light emission was detected with a Wallac 1420 multilabel counter (Perkin Elmer, Boston, MA, USA).

Cell survival assay

A clonogenic cell survival assay was performed according to the standard method. In brief, exponentially growing HeLa cells were trypsinized; the number of cells were counted with a hemocytometer and diluted in culture medium. An appropriate number of cells to yield 50 to 200 colonies per flask were inoculated into flasks of 60 mm in diameter. The flasks were incubated at 37°C for 5 h to allow cells to attach to the substrate of the flasks. The cells were then subjected to prior heating at 42°C for 2 h and then recovered for 3 h at 37°C or subjected to prior treatment with 400 µM CBX for 16 h. After the prior treatment, the cells were heated at 45°C for 15 min for test heating, after which they were cultured again at 37°C for 12 days for colony formation. The cells were fixed and stained with hematoxylin–eosin, and colonies of 50 or more cells were scored.

Results

Induction of HSPs by the treatment of CBX

We first examined the effect of CBX on the induction of HSPs in cultured HeLa and human neuroblastoma A-172 cells by Western blotting. CBX clearly induced not only Hsp70 but also Hsp90, Hsp40, and Hsp27 at concentrations of 10 to 800 µM in HeLa cells (Fig. 1a) and of 100 to 800 µM in A-172 cells (Fig. 2a) in roughly a dose-dependent manner. The effect of CBX on the HSP induction appears to be dependent on cell lines used because HSP induction in A-172 cells at concentration of 10 to 100 µM CBX was not obvious as compared with that in HeLa cells. Expression levels of each HSP increased at 2 h or later after the addition of CBX at 400 µM (Figs. 1c and 2c).

CBX is known to bind to plasma proteins more than 99.95% (Parke and Lindup 1973; Davidson et al. 1986). We therefore investigated the induction of HSPs by CBX in serum-free medium. As shown in Fig. 3, CBX could induce HSPs in HeLa cells at concentrations as low as 1 to 10 µM.

The toxic effect of CBX was estimated by the standard trypan blue dye-exclusion test and cell morphology. When HeLa cells were treated with CBX at 400 µM, cell viability gradually decreased, and cells became rounded and detached from the substrate of the culture dish at 16 h or later after CBX addition (data not shown). However, when the

Fig. 1 Induction of HSPs by carbenoxolone (CBX) in human HeLa cells. **a** Cells were treated with CBX at concentrations of 10 to 800 μ M for 16 h. **c** Cells were treated with 400 μ M CBX for the indicated periods. Each HSP was detected with a specific antibody by Western blotting, and the relative density of each band in **a** and **c** was measured by densitometry and shown in **b** and **d**, respectively. The relative amount of each HSP in nontreated control cells was taken as 1.0

culture medium was replaced with fresh medium after a 16- to 20-h treatment with a 400 μ M or higher concentration of CBX, cell growth recovered. Therefore, long-term treatment of cells by CBX at higher concentrations (more than 400 μ M) seems to be toxic.

Activation of HSF1 by CBX

Induction of HSPs is known to be usually mediated by the activation of heat shock transcription factor 1 (HSF1) in a multistep process that includes phosphorylation, oligomer-

Fig. 2 Induction of HSPs by CBX in human neuroblastoma A-172 cells. **a** Cells were treated with CBX at concentrations of 100 to 800 μ M for 16 h. **c** Cells were treated with 400 μ M CBX for the indicated periods. Each HSP was detected with a specific antibody by Western blotting, and the relative density of each band in **a** and **c** was measured by densitometry and shown in **b** and **d**, respectively. The relative amount of each HSP in nontreated control cells was taken as 1.0

Fig. 3 Induction of HSPs in HeLa cells by CBX in serum-free culture medium. Cells were treated with CBX at concentrations of 1 to 200 μ M for 24 h in serum-free D-MEM. Detection of HSPs and densitometric analysis were done as described for Figs. 1 and 2

ization, relocation into the nucleus, acquisition of DNA-binding competent state, and the formation of characteristic HSF1 granules in the nucleus (Sarge et al. 1993; Morimoto 1998). Treatment of HeLa cells with 400 μ M CBX for 2 to 8 h (Fig. 4a, lanes 3–6) or 800 μ M CBX for 0.5 to 2 h (Fig. 4b, lanes 3–6) resulted in up-shift of the HSF1 band in Western blotting, indicating the phosphorylation of HSF1.

Fig. 4 Phosphorylation of heat shock factor 1 (HSF1) by the treatment of CBX in HeLa cells. **a**, **b** Cells were treated with 400 and 800 μ M CBX, respectively, for the indicated period, and HSF1 was detected by Western blotting. Treatment of cells with CBX resulted in a clear mobility shift of the HSF1 signal as compared with that of nontreated control cells at a concentration of 400 μ M for 2 to 8 h (**a**, lanes 3–6) and 800 μ M for 0.5 to 2 h (**b**, lanes 3–6)

As shown in Fig. 4a, the HSF1 band shifted to an intermediate position between control and heated samples at 400 μ M CBX. However, at 800 μ M CBX, the band shifted to the same mobility as heat. These data suggested different modification, perhaps different amount of phosphorylation, at the different CBX concentrations. With continuous treatment of cells with 400 μ M CBX for 16 to 24 h, the phosphorylated form of HSF1 disappeared (Fig. 4a, lanes 8–10), indicating dephosphorylation of HSF1.

Intracellular localization of HSF1 was examined by indirect immunofluorescence staining. HSF1 was localized throughout in the nucleus at normal temperature (Fig. 5b). Upon heat shock (42°C for 2 h), typical HSF1 stress granules were formed in the nucleus (arrows in Fig. 5d). These granules were clearly different from the phase dense nucleolus. When HeLa cells were treated with CBX at 400 μ M for 2 h or at 800 μ M for 1 h, obvious HSF1 stress granules were formed (arrows in Figs. 5f and 5 h, respectively).

The acquisition of the DNA-binding ability of HSF1 by CBX treatment was demonstrated by a gel mobility shift assay with the HSE oligonucleotide. Specific protein–DNA complexes were detected in cells treated with 400 μ M CBX for 2 to 6 h (Fig. 6a, lanes 3–5), as well as heat shocked cells (42°C for 2 h, Fig. 6a, lane 2). The formation of these complexes was inhibited in the presence of excess unlabelled HSE oligonucleotide (Fig. 6b), which suggests that the protein bound to HSE was HSF1. Even in the continuous presence of CBX, specific DNA–protein complex was remarkably decreased at 8 h of treatment (Fig. 6a, lane 6), which roughly correspond to the time course of dephosphorylation of HSF1 (Fig. 4a), suggesting the attenuation process of HSF1 activity (Sarge et al. 1993; Yan et al. 2004). These results clearly showed that induction of HSPs by CBX is mediated by the activation of HSF1.

Furthermore, induction of HSPs by CBX treatment was dependent on the HSE consensus sequence at the promoter region of Hsp40 gene as demonstrated by a reporter assay (Fig. 7). Although the fold induction of luciferase was not so high (1.2- to 1.6-fold at concentrations of 10 to 100 μ M CBX), a positive result was consistently obtained. In a previous report, the fold induction of luciferase in reporter assay was also two to threefolds at concentrations of 10 to 100 μ M CBX (Nagayama et al. 2001). Curiously, luciferase enzyme activity could not be obviously detected in a reporter assay at concentration of 400 to 800 μ M CBX under our experimental conditions, in spite of clear induction of HSPs (Figs. 1 and 2) and apparent activation of HSF1 (Figs. 4, 5, and 6) at these high concentrations. We also tried to detect luciferase protein in a reporter assay with Hsp70 promoter at 400 to 800 μ M CBX by Western blotting, but induction of the protein was again not evident. Of course, luciferase protein was clearly induced in heated

Fig. 5 Formation of typical HSF1 stress granules in the nucleus by the CBX treatment in HeLa cells. **a, b** Nontreated control cells; **c, d** cells heated at 42°C for 2 h; **e, f** cells treated with 400 μM CBX for 2 h; **g, h** cells treated with 800 μM CBX for 1 h. The phase-contrast micrographs are shown in **a, c, e, and g**; the HSF1-specific

fluorescence micrographs of each corresponding field are shown in **b, d, f, and h**. Note that typical HSF1 granules were formed in the nucleus in CBX-treated cells (arrows in **f** and **h**), similar granules were also observed in heated cells (arrows in **d**)

sample (approximately 15-fold induction, data not shown). These contradictory data might be explained that HSE at the promoter region in an exogenous plasmid used in our reporter assay could not interact properly with CBX-activated HSF1. Alternatively, CBX could induce HSPs in an HSF1-independent mechanism. The precise molecular mechanism of HSP induction by CBX is remained to be elucidated.

Induction of thermotolerance by CBX

When HSPs are expressed at an elevated level, thermotolerance is always induced (Ohtsuka and Hata 2000). To test

whether CBX could induce thermotolerance or not, a clonogenic survival assay was performed. As shown in Fig. 8, treatment of cells with 400 μM CBX for 16 h could clearly induce thermotolerance as comparable with the prior treatment at 42°C for 2 h or 42°C for 2 h and then recovered for 3 h at 37°C.

Discussion

In a previous report of Nagayama et al. (2001), they showed that only Hsp70 but not Hsp90 or Hsp40 was induced by CBX in HeLa cells as demonstrated by Western

Fig. 6 Acquisition of DNA-binding ability of HSF1 by the CBX treatment in HeLa cells. **a** Cell extracts were prepared from cells treated under various conditions and subjected to the gel mobility shift assay. *Lane 1* Nontreated control cells; *lane 2*, cells heated at 42°C for 2 h; *lanes 3–6* cells treated with 400 μM CBX for 2, 4, 6, and 8 h, respectively. The specific HSF1-DNA (HSE) complex is indicated by

the arrowhead. **b** Inhibition of the HSF1-HSE complex with excess unlabelled oligonucleotide competitor. Specific HSF1-HSE complexes detected in extracts from cells heated at 42°C for 2 h (*lane 1*) or treated with 400 μM CBX for 4 h (*lane 3*) disappeared in the presence of excess unlabelled oligonucleotide (*lanes 2 and 4*)

Fig. 7 A reporter assay using promoter region of human Hsp40 gene. The plasmid pGV-Nr (-277) has complete HSE sequence (Hata and Ohtsuka 1998). *Lane 1* Nontreated control cells; *lane 2* cells heated at 42°C for 2 h then recovered at 37°C for 3 h; *lanes 3–5* cells treated with CBX at concentration of 10 to 100 μM, respectively, for 16 h. Luciferase activity of nontreated control cells was taken as 1.0

blotting. The reason why other HSPs except Hsp70 were not induced by CBX in a previous report is not known in spite of the activation of HSF1. In the present report, we clearly demonstrated the induction of Hsp70 as well as Hsp90, Hsp40, and Hsp27 by the treatment with CBX in

Fig. 8 Development of thermotolerance in HeLa cells treated with CBX. Cells were heated at 42°C for 2 h (triangle), heated at 42°C for 2 h and then recovered for 3 h at 37°C (square) or treated with 400 μM CBX for 16 h (ex symbol) and then heated at 45°C for 15 min. Clonogenic survival of cells without prior treatment (circle) was compared with those of prior treatment (triangle, square, ex symbol). The numbers in the figure indicate percent survival, taking survival of nontreat control cells as 100% (diamond). Data points represent the means of three independent experiments

HeLa and A-172 neuroblastoma cells, and this process might be mediated by the activation of HSF1. The induction level of HSPs by CBX seems to be dependent on its concentration and cell lines used. Although the direct target of CBX is not known at present, one possibility is that the induction of oxidative stress in mitochondria by CBX (Salvi et al. 2005) might lead to the enhanced expression of HSPs.

CBX [3-(3-carboxy-1-oxopropoxy)-11-oxo-olean-12-en-29-oic acid] is a semisynthetic succinyl ester of glycyrrhizinic acid (licorice root substance) and has been used as an effective treatment for peptic ulceration since the 1960s (Pinder et al. 1976; Parke 1983). Its medical use, however, is limited by side effects of a mineralcorticoid aldosterone-like property with hypokalemia, weight gain, hypertension, and retention of sodium, chloride, and water (Baron 1983; Zimmerman 1984). It was later proved that mineralcorticoid-like activity of CBX is due to its inhibitory effect on 11β-hydroxysteroid dehydrogenase, which is responsible for the interconversion of cortisol to cortisone (Stewart et al. 1987; Stewart et al. 1990). Furthermore, CBX is a blocker of gap junction intercellular communication by binding to connexins (Davidson et al. 1986; Davidson and Baumgarten 1988) and has been reported to have anticonvulsant and neuroprotective effects in experimental animals (Hosseinzadeh and Nassiri Asl 2003; Gareri et al. 2004; de Pina-Benabou et al. 2005).

It has been reported that CBX has a cytoprotective effect on the gastric mucosa against a variety of irritant agents and stress-induced gastric damage in various experimental systems. CBX is known to potentiate the activity of glycosyl transferase and stimulate gastric mucus production, inhibit peptic activity, and inhibit prostaglandin degradation, which in turn increase endogenous prostaglandins level in gastric juice (Pinder et al. 1976; Parke 1983). The molecular mechanism of the cytoprotective effect of CBX, however, is not fully understood at present.

HSPs have molecular chaperone activity and are known to be endogenous cytoprotective factors and lifeguards. Indeed, the induction of HSPs by hyperthermia (Nakamura et al. 1991), geranylgeranylacetone (Hirakawa et al. 1996), and aspirin (Jin et al. 1999) could protect gastric mucosa against necrotizing stimuli in experimental animals. Whether or not CBX can induce HSPs in gastric mucosa has not been examined yet, the cytoprotective effect of CBX might be partly due to its chaperone inducing activity.

Acknowledgments This work was supported by a Grant-in-Aid for the High-Tech Research Center Establishment Project (Chubu University, 2002-2006) from the Japanese Ministry of Education, Culture, Sports, Science and Technology. We thank Mr. H. Kimura and T. Kurachi, Miss A. Komanoya, and N. Mase for their excellent technical assistance.

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