



Title	Impact of capsaicin, an active component of chili pepper, on pathogenic chlamydial growth (<i>Chlamydia trachomatis</i> and <i>Chlamydia pneumoniae</i>) in immortal human epithelial HeLa cells
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**Impact of capsaicin, an active component of chili pepper, on pathogenic
chlamydial growth (*Chlamydia trachomatis* and *Chlamydia
pneumoniae*) in immortal human epithelial HeLa cells**

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25 **Abstract**

26 *Chlamydia trachomatis* is the leading cause of sexually transmitted infections
27 worldwide. Capsaicin, a component of chili pepper, which can stimulate actin
28 remodeling via capsaicin receptor TRPV1 (transient receptor potential vanilloid 1) and
29 anti-inflammatory effects via PPAR γ (peroxisome proliferator-activated receptor- γ) and
30 LXR α (liver X receptor α), is a potential candidate to control chlamydial growth in host
31 cells. We examined whether capsaicin could inhibit *C. trachomatis* growth in immortal
32 human epithelial HeLa cells. Inclusion forming unit and quantitative PCR assays
33 showed that capsaicin significantly inhibited bacterial growth in cells in a
34 dose-dependent manner, even in the presence of cycloheximide, a eukaryotic protein
35 synthesis inhibitor. Confocal microscopic and transmission electron microscopic
36 observations revealed an obvious decrease in bacterial numbers to inclusions bodies
37 formed in the cells. Although capsaicin can stimulate the apoptosis of cells, no increase
38 in cleaved PARP (poly (ADP-ribose) polymerase), an apoptotic indicator, was observed
39 at a working concentration. All of the drugs tested (capsazepine, a TRPV1 antagonist;
40 5CPPSS-50, an LXR α inhibitor; and T0070907, a PPAR γ inhibitor) had no effect on
41 chlamydial inhibition in the presence of capsaicin. In addition, we also confirmed that
42 capsaicin inhibited *Chlamydia pneumoniae* growth, indicating a phenomena not specific
43 to *C. trachomatis*. Thus, we conclude that capsaicin can block chlamydial growth
44 without the requirement of host cell protein synthesis, but by another, yet to be defined,
45 mechanism.

46

47 **Keywords:** *Chlamydia trachomatis*; capsaicin; HeLa cells; TRPV1; PPAR γ ; LXR α

48

49 **Introduction**

50 *Chlamydia trachomatis* is an obligate intracellular bacterium with a unique
51 developmental cycle, consisting of elementary (EB) and reticulate body (RB) forms.
52 Differentiation from EB to RB (or re-differentiation from RB to EB) forms occurs in
53 inclusion bodies [1]. The maturation process from EB to RB forms in infected host cells
54 has been investigated and requires actin remodeling [2-4], lipid metabolism [5-7] and
55 inflammatory responses [8-10], although it remains to be fully understood.

56 *Chlamydia* are the leading cause of bacterial sexually transmitted diseases
57 worldwide, with an estimated 100 million chlamydial infections detected annually [11].
58 Such infections are often asymptomatic in women [12-14] and can be left untreated,
59 resulting in serious outcomes with ductal obstruction, pelvic inflammatory disease,
60 tubal occlusion and extrauterine pregnancy [15-17]. The prevalence of *C. trachomatis*
61 following prophylactic countermeasures therefore needs to be investigated.

62 The use of natural compounds that are easily and safely ingested within a daily
63 diet is a new approach to prophylactically overcome a variety of infectious risks. For
64 example, chili peppers contain capsaicin (8-methyl-N-vanillyl-6-nonenamide), a
65 phenolic compound produced by all plants [18], that has been well investigated [19-22].
66 Capsaicin can inhibit the growth of human pathogenic bacteria such as cell-invasive
67 group A streptococci [20], *Vibrio cholera* [21], *Escherichia coli* O157:H7 [22] and
68 *Salmonella enterica* serovar Typhimurium [22], indicating its potential application
69 against a broad range of bacterial infectious diseases.

70 Capsaicin can also modulate cellular dynamics by actin remodeling via the
71 capsaicin receptor TRPV1 (transient receptor potential vanilloid 1), which is a channel
72 expressed at a subset of sensory neurons involved in pain sensation and other

73 non-neuronal sites in mammalian cells [18, 23], and by exerting anti-inflammatory
74 effects via PPAR γ (peroxisome proliferator-activated receptor-gamma) [18, 24] and
75 LXR α (liver X receptor α) [18, 24]. Specifically, TRPV1 activation altered F-actin
76 organization in an ERK1/2- and MLC2-dependent manner [23], and the inhibitory
77 action of capsaicin on lipopolysaccharide (LPS)-induced IL-1 β , IL-6 and TNF- α
78 production occurred depending on the activation of both PPAR γ and LXR α [24].

79 Thus, interestingly, it appears that the activity of capsaicin is similar to that of
80 chlamydia, raising the idea that bacteria and capsaicin may compete with resulting
81 effects on actin remodeling and inflammatory activity, and ultimately chlamydial
82 growth inhibition. We therefore examined whether capsaicin can inhibit *C. trachomatis*
83 growth in immortal human epithelial HeLa cells. Here, for the first time, we showed
84 that capsaicin can inhibit chlamydial growth with minimal requirement for host
85 responses.

86

87

88 **Materials and methods**

89 *Drugs*

90 Capsaicin (Tokyo Chemical Industry Co., Ltd.) was resolved in 95% ethanol at a
91 stock concentration, 50 mg/ml. Capsazepine (Wako) (antagonist for TRPV1),
92 5CPPSS-50 (Wako) (inhibitor for LXR α), and T0070907 (CAY) (inhibitor for PPAR γ)
93 were resolved in DMSO at 50, 15 and 50 mM, respectively. Cycloheximide
94 (eukaryote-specific protein synthesis inhibitor) purchased from Sigma was resolved in
95 DMSO at 20 mg/ml. These drugs were stored at -80 °C until use.

96

97 *Immortal human cell lines*

98 Immortal human epithelial HeLa and HEp-2 cells were purchased from the
99 American Type Culture Collection and the Riken Cell Bank (Tsukuba, Japan),
100 respectively. Both of the cell lines were cultured at 37 °C in 5% CO₂ in Dulbecco's
101 Modified Eagle's Medium (Sigma) containing 10% heat-inactivated fetal calf serum and
102 antibiotics [gentamicin (10 µg/ml) (Sigma), vancomycin (10 µg/ml) (Sigma) and
103 amphotericin B (0.5 µg/ml) (Sigma)] [25, 26].

104

105 *Bacteria and inclusion forming unit (IFU) assay*

106 *C. trachomatis* strain UW-3/CX (*ompA* genotype: D) was purchased from ATCC.
107 *Chlamydia pneumoniae* strain TW183 was kindly provided by G. Byrne, University of
108 Tennessee (Memphis, TN, USA). *C. trachomatis* and *C. pneumoniae* were propagated
109 in HeLa and HEp-2 cells, respectively, as described previously [25, 26]. In brief, the
110 infected cells were harvested on day 2 (for *C. trachomatis*) or day 3 (for *C.*
111 *pneumoniae*), and disrupted by freezing-thawing. After centrifugation to remove cell
112 debris, bacteria were collected by high-speed centrifugation. Bacterial pellets were
113 resuspended in sucrose–phosphate–glutamic acid buffer (0.2 M sucrose, 3.8 mM
114 KH₂PO₄, 6.7 mM Na₂HPO₄, 5 mM L-glutamic acid, pH 7.4) and stored at -80 °C until
115 use. The numbers of infectious progeny were determined as IFUs by counting
116 chlamydial inclusions formed in HEp-2 cells, using a fluorescein isothiocyanate
117 (FITC)-conjugated monoclonal antibody specific to *Chlamydia* LPS (with Evans Blue)
118 (Denka Seiken) [25].

119

120 *Infection of cells*

121 HeLa and HEp-2 cells, adjusted to a concentration of 2×10^5 cells/well, were infected
122 with bacteria at a MOI of 1 (*C. trachomatis*) or 3 (*C. pneumoniae*), respectively,
123 followed by centrifugation (800×g, 30 min) at room temperature. After washing to
124 remove redundant bacteria with Hank's balanced salt solution (Sigma), infected cells at
125 a concentration of 2×10^5 cells/well were seeded into 24-well plates, and then incubated
126 for up to 48 h (*C. trachomatis*) or 72 h (*C. pneumoniae*) in the presence or absence of
127 various drugs (working concentration: 1–100 µg/ml capsaicin, 10 µM capsazepine, 15
128 µM 5CPPSS-50, 10 µM T0070907, 2 µg/ml cycloheximide). Cells were collected in a
129 time-dependent manner for assessing IFU numbers, chlamydial 16S rDNA quantities,
130 inclusion formation, and bacterial morphological changes. In addition, using a cell
131 counting kit-8 (Dojindo), it was confirmed that these drugs, used at the working
132 concentrations, exerted minimal cytotoxic effects on the cells over the culture period.

133

134 *Assessment of inclusion formation*

135 Cells were collected to determine the morphology of chlamydial inclusions, as
136 described previously [25]. In brief, after the cells were fixed onto a slide with ethanol,
137 they were stained with FITC-conjugated monoclonal anti-*Chlamydia* antibody specific
138 to *Chlamydia* LPS (with Evans Blue) (Denka Seiken). Cells stained with specific
139 antibodies were observed using a conventional [IX71 (Olympus)] or a confocal laser
140 microscope [LSM510 (Carl Zeiss Japan Group)].

141

142 *Transmission electron microscopy (TEM)*

143 TEM was performed in accordance with the following method. In brief, cells

144 were immersed in a fixative containing 3% glutaraldehyde in 0.1 M PBS pH 7.4, for 24
145 h at 4 °C. After briefly washing with PBS, cells were processed for alcohol dehydration
146 and embedded in Epon 812. Ultra-thin sections of cells were stained with lead citrate
147 and uranium acetate before viewing by TEM (Hitachi H7600; Hitachi). The Hanaichi
148 Ultrastructure Research Institute prepared the ultra-thin sections and carried out
149 observations by TEM.

150

151 *Quantitative (q) PCR*

152 DNA was extracted from the cultured cells using the LaboPass™ Tissue Mini kit
153 (Hokkaido System Science). PCR was performed using primer sets specific to
154 chlamydial 16S rDNA (which recognize a wide range of chlamydiae) (sense primer,
155 5'-GGA CCT TAG CTG GAC TTG ACA TGT-3'; antisense primer, 5'-CCA TGC AGC
156 ACC TGT GTA TCT G-3') [27] and glyceraldehyde 3-phosphate dehydrogenase
157 (*gapdh*) (sense primer: 5'-AAC GGG AAG CTC ACT GGC ATG-3', antisense primer:
158 5'-TCC ACC AAC CTG TTG CTG TAG-3') [28]. The number of bacteria per culture
159 was expressed as the ratio of chlamydial 16S rDNA:*gapdh*.

160

161 *Assessment of cell death*

162 Cell death was estimated by changes in nuclear morphology and the appearance of
163 condensed chromatin using DAPI staining.

164

165 *Assessment of mitochondrial membrane integrity*

166 HeLa cells were incubated with capsaicin (0–200 µg/ml) or staurosporine (10 µM) for
167 48 h, and then incubated with 100 nM MitoTracker Red CMXRos (Invitrogen) for 30

168 min at 37 °C according to the manufacturer's instructions. After fixing in 4%
169 paraformaldehyde, the cells were observed under a fluorescence microscope.

170

171 *Western blot analysis*

172 Cells were collected and then boiled for 5 min at 100 °C in a reducing sample buffer
173 containing 2-mercaptoethanol. Then, the samples were separated by 10% (w/v)
174 SDS-PAGE, and the separated proteins were transferred to polyvinylidene difluoride
175 membrane. Membranes were blocked with 5% (w/v) skim milk in Tris-buffered saline
176 and then incubated with an anti-PARP (poly (ADP-ribose) polymerase) antibody (Roche
177 Diagnostics), TRPV1 rabbit polyclonal antibody, α -tubulin monoclonal antibody
178 (Sigma) or rabbit IgG as a control (Sigma) for 1 h at room temperature, followed by a
179 HRP-conjugated goat anti-rabbit IgG (KLP) or goat anti-mouse IgG and IgM antibody
180 (Jackson ImmunoResearch) for 1 h at room temperature. Labeled proteins were
181 visualized with Pierce ECL western blotting substrate, according to the manufacturer's
182 protocol (Thermo Fisher Scientific).

183

184 *Statistical analysis*

185 Comparisons among groups (more than three) were performed by a multiple
186 comparison test for parametric analysis using the Bonferroni/Dunn method. Comparison
187 between two groups was also performed by the Student's *t* test. A *p*-value of less than
188 0.05 was considered significant. Calculations were performed in Excel for Mac (2011)
189 with Statcel3C.

190

191

192 **Results**

193 *Chlamydial growth in HeLa cells in the presence of capsaicin*

194 We first compared the growth of *C. trachomatis* in HeLa cells in the presence or
195 absence of capsaicin. Conventional fluorescent microscopic observations revealed that
196 compared with the control, capsaicin exposure drastically restricted the expansion of
197 chlamydial inclusion size, although there was no difference of the number of infected
198 cells in the presence or absence of capsaicin (Fig. 1A, Green spots). An IFU assay
199 confirmed that the number of infectious progeny (EB) significantly decreased following
200 capsaicin exposure in a dose-dependent manner (Fig. 1B, Black bar); capsaicin
201 exposure similarly inhibited the growth of *C. pneumoniae* in HeLa cells, indicating that
202 this phenomenon is not specific to the *C. trachomatis* strain used in this study (Fig. 1B,
203 White bar). Furthermore, qPCR data revealed that, consistent with the inclusion
204 morphology and IFU data, the amount of chlamydial 16S rDNA significantly decreased
205 following capsaicin exposure in a dose-dependent manner, indicating that no persistent
206 infection occurred (Fig. 1C). These data suggested that capsaicin exposure suppressed *C.*
207 *trachomatis* infection in HeLa cells as well as *C. pneumoniae* infection.

208 To confirm this, morphological changes in *C. trachomatis* in the presence of
209 capsaicin were assessed by confocal laser microscopy and TEM. Consistent with the
210 results of conventional microscopy, confocal laser microscopic observations showed
211 that chlamydial inclusion size diminished in a dose-dependent manner and no typical
212 inclusion bodies were seen particularly after high-dose exposure of capsaicin (100
213 $\mu\text{g/ml}$) (Fig. 2). TEM observations also revealed that, compared with the control (Fig.
214 3A), the number of bacteria in inclusions formed in the infected cells was limited in the
215 presence of capsaicin and few re-differentiated EB in inclusions were seen (Fig. 3B);

216 EB (relatively small size and high density) and RB (large size and pale density) were
217 observed with distinct morphological traits. Furthermore, detached bacterial cell walls
218 (Fig. 3C) and disrupted bacteria in inclusion bodies (Fig. 3D) were also observed. Thus,
219 the chlamydial cell structure in the infected cells was morphologically impaired by
220 treatment with capsaicin.

221 Taken together, these results indicated that capsaicin could inhibit chlamydial
222 growth, through blocking the maturation of the bacteria following bacterial disruption.

223

224 *Mechanism by which capsaicin inhibits chlamydial growth*

225 It has previously been reported that capsaicin can induce apoptotic cell death via
226 mitochondria dysfunction and caspase-3 activation in immortal cell lines such as
227 MCF-7 or HeLa cells [29, 30]. We therefore assessed whether apoptosis induced by
228 capsaicin exposure was involved in the inhibition of chlamydial growth. When
229 compared with the control using staurosporine as a general apoptosis inducer, the
230 increase in the number of cells with condensed chromatin visualized by DAPI staining
231 was minimal at a working concentration of capsaicin after 48 h incubation, although a
232 slight increase was seen at a higher working concentration (125 $\mu\text{g/ml}$) (Fig. 4A). Thus,
233 at the usual working concentration of capsaicin, cell death was minimal. To confirm this,
234 we determined the minimal concentration of capsaicin inducing apoptosis of HeLa cells
235 using a more sensitive western blot-based approach to detect PARP cleavage, a critical
236 step in the induction of apoptosis [31]. No PARP cleavage was detected in the presence
237 of less than 100 $\mu\text{g/ml}$ capsaicin, which was the working concentration used in this
238 study (Fig. 4B). In addition, MitoTracker staining revealed that following exposure to
239 capsaicin (100 $\mu\text{g/ml}$) mitochondrial membrane integrity was maintained, supporting

240 the findings from western blot analysis above (Online Resource 1). Collectively, these
241 results indicated that the effect of apoptosis induction following capsaicin exposure on
242 the inhibition of chlamydial growth was minimal.

243 Recent studies have revealed that capsaicin modulates cellular dynamics by
244 actin remodeling via capsaicin receptor TRPV1 [18, 23] and exerts anti-inflammatory
245 effects via PPAR γ [18, 24] and LXR α [18, 24]. Specifically, TRPV1 activation altered
246 F-actin organization in an ERK1/2- and MLC2-dependent manner [23], and the
247 inhibitory action of capsaicin on LPS-induced IL-1 β , IL-6 and TNF- α production was
248 dependent on the activation of both PPAR γ and LXR α [24]. We therefore assessed,
249 using drugs that block the signal transduction of capsaicin [10 μ M capsazepine
250 (antagonist for TRPV1), 15 μ M 5CPPSS-50 (inhibitor for LXR α) and 10 μ M T0070907
251 (inhibitor for PPAR γ)], whether the effects on cellular dynamics by actin remodeling
252 and the anti-inflammatory effects induced by capsaicin exposure were involved in
253 chlamydial growth inhibition. None of the drugs were able to completely restore
254 chlamydial growth following capsaicin exposure, although a slight improvement in
255 growth was detected (Fig. 5). In addition, we confirmed TRPV1 expression on the HeLa
256 cells used in this study by western blotting with TRPV1 rabbit polyclonal antibody
257 (Online Resource 2). Since none of the drugs had an effect on chlamydial inhibition in
258 the presence of capsaicin, capsaicin may inhibit chlamydial growth, presumably with
259 minimal requirement for host responses. To confirm this, we examined whether
260 chlamydial growth inhibition induced by capsaicin exposure could be recovered by
261 stimulation with cycloheximide (2 μ g/ml), a eukaryote-specific protein synthesis
262 inhibitor. As expected, regardless of the presence of the drug, capsaicin exposure

263 similarly inhibited chlamydial growth (Fig. 6).

264 Taken together, we concluded that capsaicin inhibited chlamydial growth by
265 blocking bacterial maturation following bacterial disruption, but with minimal
266 requirement for host responses.

267

268

269 **Discussion**

270 The use of natural compounds that can be easily and safely ingested as part of a
271 daily diet is a new approach to prophylactically overcome a variety of infectious risks.
272 One such compound that has been well investigated is capsaicin
273 (8-methyl-N-vanillyl-6-nonenamide: MW 305.41), a small phenolic compound with
274 hydrophobicity that is produced by all plants including chili peppers [18-22]. Several
275 studies have reported that capsaicin can inhibit the growth of human pathogenic bacteria
276 such as cell-invasive group A streptococci [20], *V. cholera* [21], *E. coli* O157:H7 [22]
277 and *S. enterica* serovar Typhimurium [22]. Here, for the first time, we showed that
278 capsaicin directly inhibited the growth of *C. trachomatis*, an obligate intracellular
279 pathogen, in human immortal epithelial HeLa cells, with minimal requirement for host
280 responses.

281 An IFU assay revealed that capsaicin dramatically diminished the number of
282 chlamydial infectious progeny, and morphological observations by fluorescence
283 microscopy and TEM revealed that, compared with the control, capsaicin exposure
284 drastically restricted the expansion of chlamydial inclusions. However, there was no
285 difference in the number of infected cells in the presence or absence of capsaicin (see
286 Fig. 1A). Furthermore, pretreatment of the bacteria with capsaicin had no effect on

287 chlamydial growth inhibition (data not shown). Thus, the results indicated that capsaicin
288 worked against the bacteria in HeLa cells rather than by blocking bacterial attachment
289 to host cells. As mentioned above, it appears that, due to its hydrophobic nature [18],
290 capsaicin can easily penetrate bacteria across first the cellular membrane and then the
291 inclusion membrane. Interestingly, capsaicin exposure also inhibited the growth of *C.*
292 *pneumoniae* in HeLa cells, indicating that this phenomenon is not specific to the *C.*
293 *trachomatis* strain used in this study. Meanwhile, the effects of capsaicin on the
294 inhibition of *C. pneumoniae* growth into the IFU assessment were weak with only one
295 log reduction compared with those on *C. trachomatis*; however, the reason for this
296 remains to be clarified. It is possible that because of the slow growth rate of *C.*
297 *pneumoniae* capsaicin may target the metabolic active site of the bacteria that is
298 required for growth. Furthermore, although further studies should be required for
299 clarifying clinical aspect of capsaicin against chlamydial infection, it implies that
300 capsaicin also has a potential to component to treat *C. pneumoniae*, causing pneumonia
301 with chronic disorder such as asthma [32].

302 Interestingly, TEM observations revealed that capsaicin not only affected
303 bacterial inclusion size by blocking bacterial maturation, but also caused bacterial
304 morphological changes involving cell wall detachment and bacterial disruption. Our
305 previous studies similarly showed that treatment of HEp-2 cells with a steroid receptor
306 antagonist RU486 (mifepristone), which is a small hydrophobic molecule, significantly
307 inhibited the growth of *C. pneumoniae* in a dose-dependent manner with disruption of
308 the bacteria due to direct binding of the drug to the bacteria [33]. Furthermore, we found
309 that 2-amino-phenoxazine-3-one (phenoxazine derivate, Phx-3: a small hydrophobic
310 molecule) induced the destruction of *C. pneumoniae* in inclusion bodies in immortal

311 epithelial HEp-2 cells, resulting in bacterial growth inhibition [34]. Interestingly, since
312 chlamydial maturation in inclusion bodies is required for manipulation of infected host
313 cells through several effector molecules secreted by type III machinery [35], the
314 dysfunction of type III secretion machinery by the binding of small hydrophobic
315 molecules impaired chlamydia growth in host cells [36]. Although more studies are
316 needed to clarify the mechanisms by which capsaicin causes bacterial morphological
317 changes, it may be that the interaction of small hydrophobic molecules with unknown
318 receptors on the bacterial cell wall is responsible for the dysfunction in bacterial
319 maturation.

320 It is well established that capsaicin evokes several host responses including
321 apoptosis [29, 30], actin remodeling [18, 23] and anti-inflammatory effects [18, 24].
322 However, neither cleaved PARP, mitochondrial dysfunction nor recovery of chlamydial
323 growth inhibition in the presence of inhibitors, capsazepine, 5CPPSS-50 and T0070907,
324 was observed with a working concentration of capsaicin. Furthermore, regardless of the
325 presence or absence of cycloheximide, capsaicin similarly inhibited chlamydial growth.
326 Thus, although at present the mechanism by which bacterial growth is inhibited in the
327 presence of capsaicin remains unknown, we concluded that the influence of host
328 responses stimulated by capsaicin on growth inhibition was minimal. In addition, the
329 presence of the inhibitors along with capsaicin boosted chlamydial growth inhibition,
330 although at present the reason for this remains to be clarified. Because no effect was
331 seen with either inhibitor alone (data not shown), these inhibitors may help capsaicin to
332 access to the bacteria by crossing the membrane.

333 It is well recognized that capsaicin, responsible for the "hot" taste of chili
334 peppers and chili pepper extracts, is a valuable pharmacological agent with therapeutic

335 applications in controlling pain and inflammation [18-22]. However, although orally
336 administered capsaicin is absorbed into the intestinal and stomach tissues [37], the
337 majority of adsorbed capsaicin is rapidly metabolized in the liver with
338 16-hydroxycapsaicin, 17-hydroxycapsaicin or 16,17-dehydrocapsaicin, and no intact
339 capsaicin was detected in the tissues or bloodstream four days after oral administration
340 [38]. Drug delivery strategies to improve the bioavailability and therapeutic effects of
341 capsaicin have been investigated and have shown that nanoparticles or liposomes can be
342 used to efficiently deliver intact capsaicin to the bloodstream [39, 40]. Thus, while
343 capsaicin is a promising component to treat *C. trachomatis* infection (presumably also
344 against *C. pneumoniae* infection), further studies are needed to clarify whether dietary
345 ingestion of capsaicin using an appropriate delivery system could target chlamydia
346 infection of the vaginal tissue and/or other sites such as respiratory tract.

347 In conclusion, we found that capsaicin, a component of chili pepper, can block
348 chlamydial growth (*C. trachomatis* and *C. pneumoniae*) without the requirement of host
349 cell protein synthesis by an, as yet, undetermined mechanism.

350

351

352 **Conflict of interest**

353 None.

354

355

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360

361

362 **Appendix A. Supplementary data**

363 Online Resource 1: MitoTracker staining showing mitochondrial membrane integrity

364 after exposure to capsaicin at 100 $\mu\text{g/ml}$. Normal mitochondria showed strong red

365 staining compared with abnormal mitochondria (10 μM staurosporine). Magnification,

366 $\times 600$.

367 Online Resource 2: Western blotting showing TRPV1 expression on HeLa cells.

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485

486 **Figure legends**

487

488 **Fig. 1.** Representative conventional fluorescence microscopic images showing inclusion
489 formation and growth of chlamydiae in HeLa cells in the presence or absence of
490 capsaicin at 48 h after infection. **A:** *C. trachomatis*-infected cells in the presence or
491 absence of capsaicin (0–100 µg/ml) were stained with FITC-conjugated anti-*Chlamydia*
492 antibody. The images were captured at 48 h after infection. Green spots, inclusion
493 bodies. Control, diluted DMSO. Magnification, ×400. **B:** Number of chlamydial
494 infectious progeny were estimated at 48 h (*C. trachomatis*) or 72 h (*C. pneumoniae*)
495 after infection by an IFU assay. Control, diluted DMSO. Ct, *C. trachomatis*. Cp, *C.*
496 *pneumoniae*. Data show the mean + SD. * $p < 0.05$ versus the value of each of the
497 controls. **C:** Amount of *C. trachomatis* 16S rDNA was estimated by qPCR. Data are
498 expressed as the ratio of 16S rDNA:*gapdh*. See the Materials and methods. Data show
499 the mean + SD. * $p < 0.05$ versus the value of the control.

500

501 **Fig. 2.** Representative confocal laser fluorescence microscopic images showing
502 inclusion formation and growth for *C. trachomatis* in HeLa cells in the presence or
503 absence of capsaicin at 48 h after infection. *C. trachomatis*-infected cells in the presence
504 or absence of capsaicin (0–100 µg/ml) were stained with FITC-conjugated
505 anti-*Chlamydia* antibody. Blue, DAPI. Green spots, inclusion bodies. Control, diluted
506 DMSO.

507

508 **Fig. 3.** Representative TEM image showing the bacterial morphological traits in

509 inclusion bodies of *C. trachomatis* formed in HeLa cells at 48 h after infection. Arrows
510 show inclusion bodies. **A:** Control, diluted DMSO. **B–D:** + capsaicin, infected cells
511 were incubated with 100 µg/ml capsaicin. Arrows show representative EBs. Stars show
512 representative RBs.

513

514 **Fig. 4.** Influence of apoptosis induced by capsaicin exposure on the growth inhibition of
515 *C. trachomatis*. Cells were analyzed at 48 h after infection according to the following
516 traits. **A:** DAPI-stained cells were analyzed for morphological changes including
517 condensed chromatin. Staurosporine, which induces apoptosis, was included as a
518 positive control. Data show the mean + SD (%). * $p < 0.05$ versus the value for the
519 staurosporine control. **B:** Representative western blot images showing the induction of
520 apoptosis by capsaicin exposure. Open arrow shows PARP cleaved by apoptosis
521 induction. Staurosporine treatment was included as a positive control.

522

523 **Fig. 5.** Influence of inhibitors against capsaicin on chlamydial growth inhibition
524 following capsaicin exposure. HeLa cells with or without *C. trachomatis* infection were
525 incubated for up to 48 h in the presence or absence of inhibitors [10 µM capsazepine
526 (antagonist for TRPV1), 15 µM 5CPPSS-50 (inhibitor for LXR α), 10 µM T0070907
527 (inhibitor for PPAR γ)] with or without capsaicin (50 µg/ml). The number of infectious
528 progeny was estimated by an IFU assay at 48 h after infection. Control, diluted DMSO.
529 Data show the mean + SD. * $p < 0.05$ versus the value of the control.

530

531 **Fig. 6.** Influence of cycloheximide (eukaryote-specific protein synthesis inhibitor) on

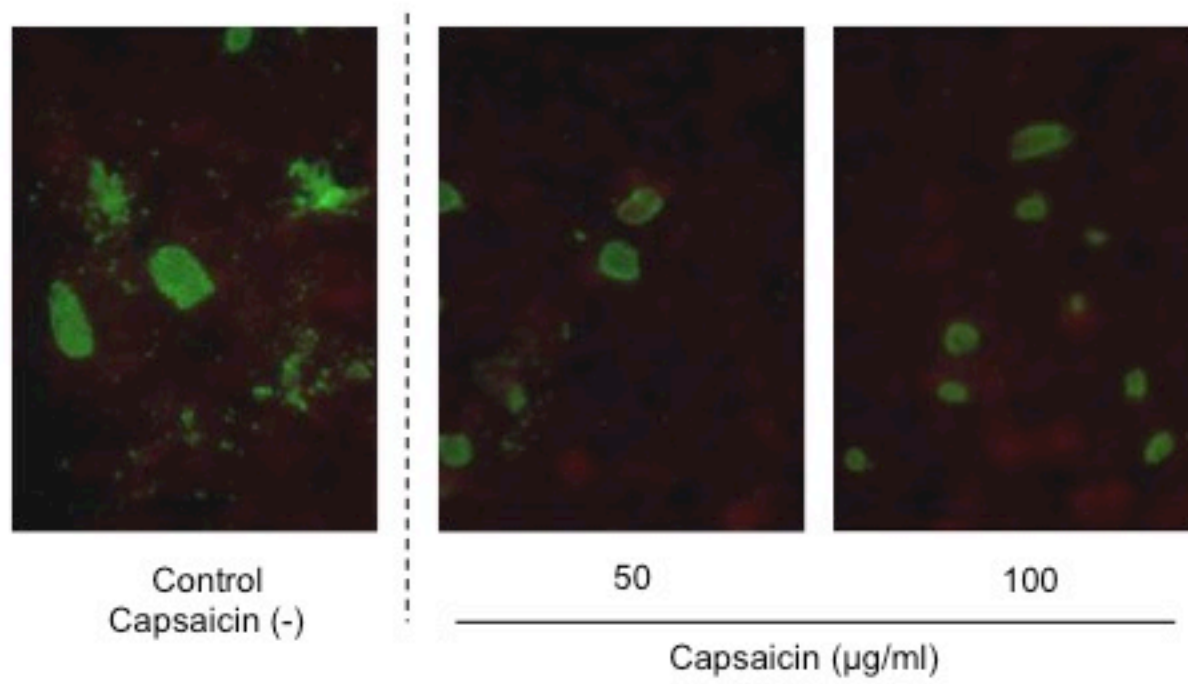
532 chlamydia growth inhibition following capsaicin exposure. *C. trachomatis*-infected
533 cells were incubated in the presence or absence of cycloheximide (2 µg/ml) with or
534 without capsaicin (50 µg/ml). After incubation, the number of infectious progeny was
535 estimated by an IFU assay at 48 h after infection. Data show the mean + SD. * $p < 0.05$
536 versus the value of each of the controls “Capsaicin (-)”.

537

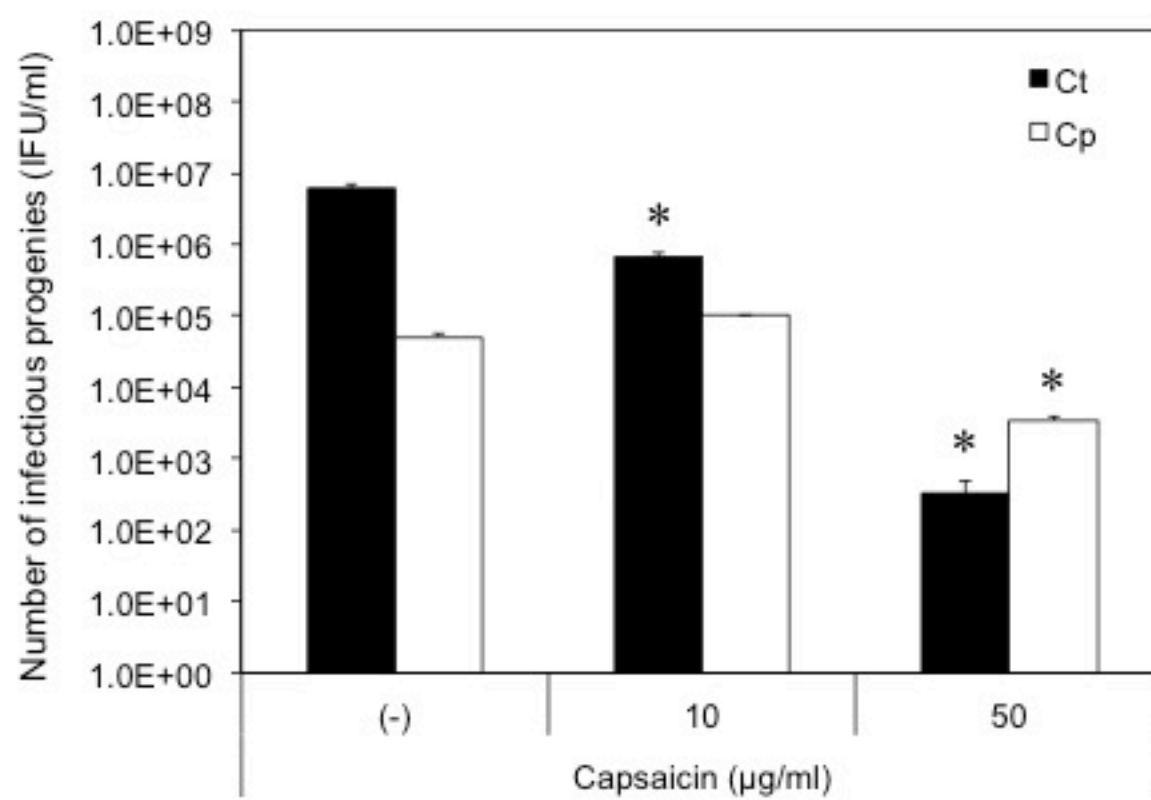
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Fig. 1

A



B



C

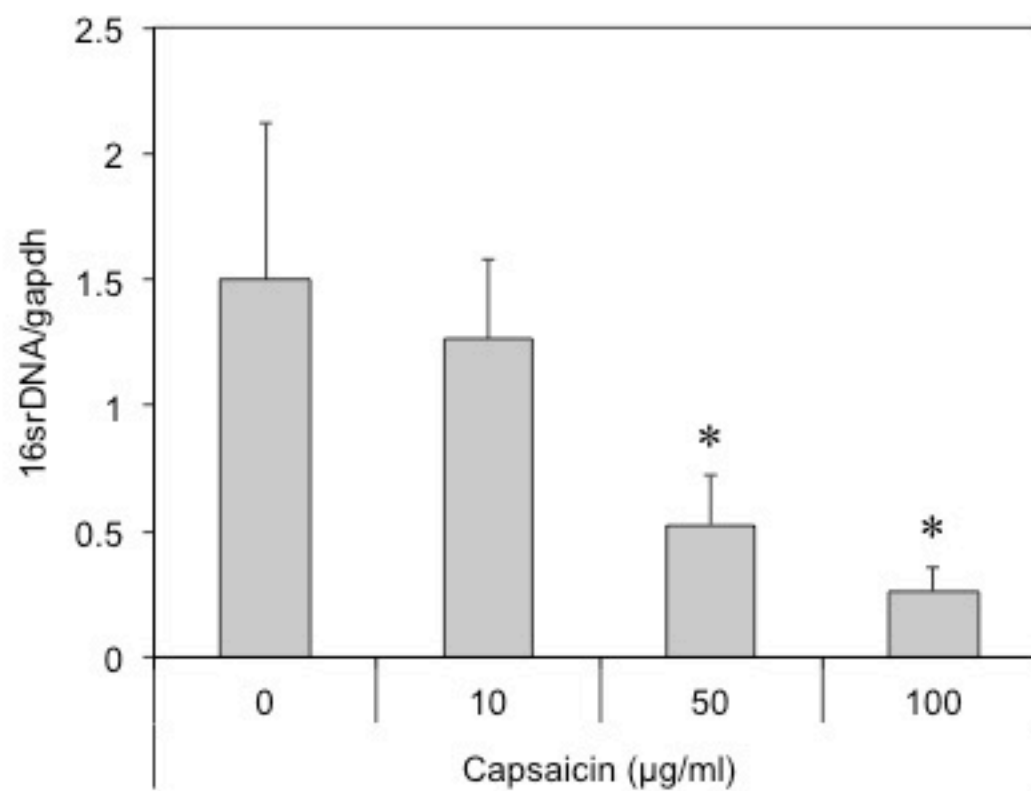


Fig. 2

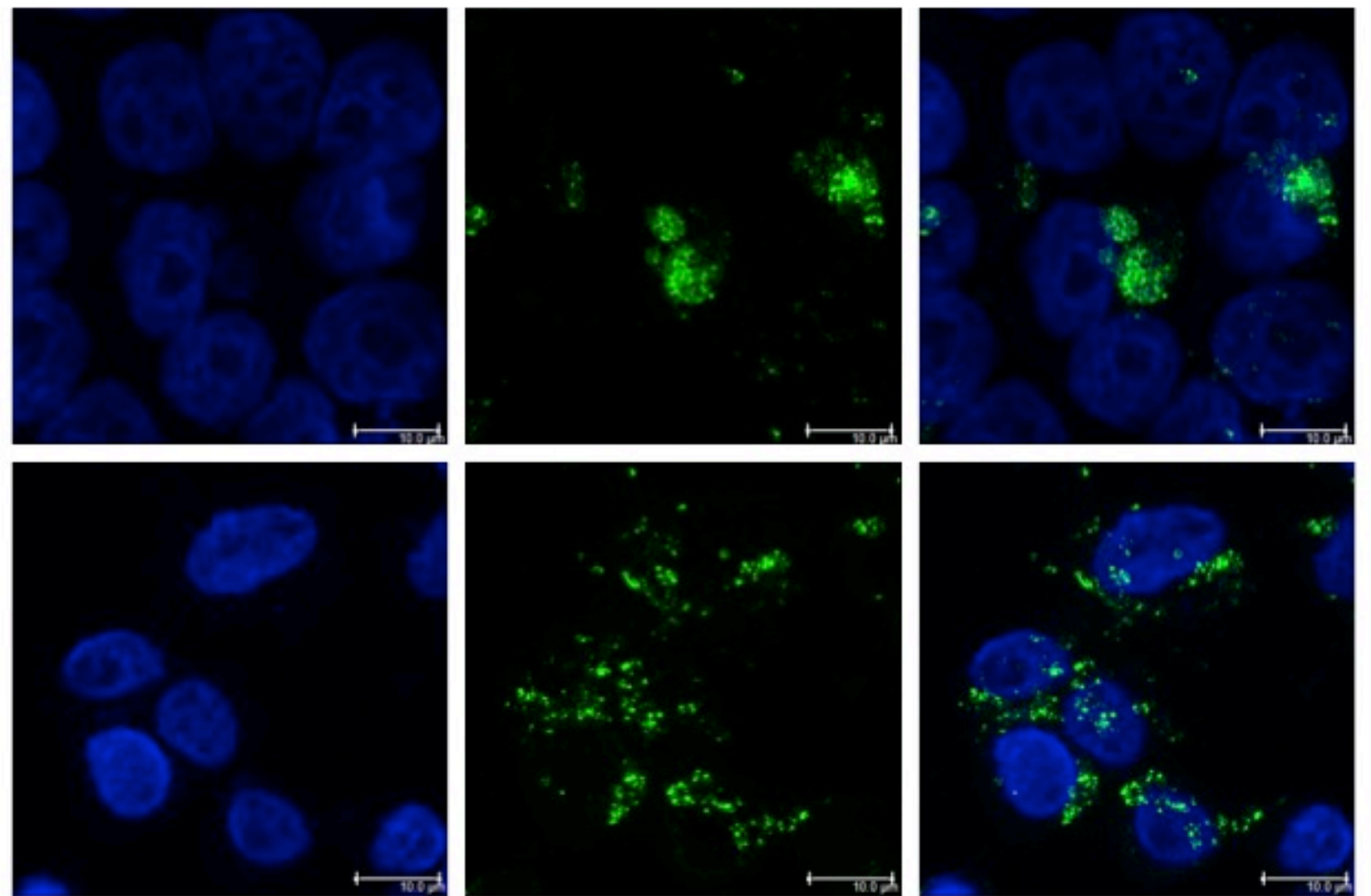
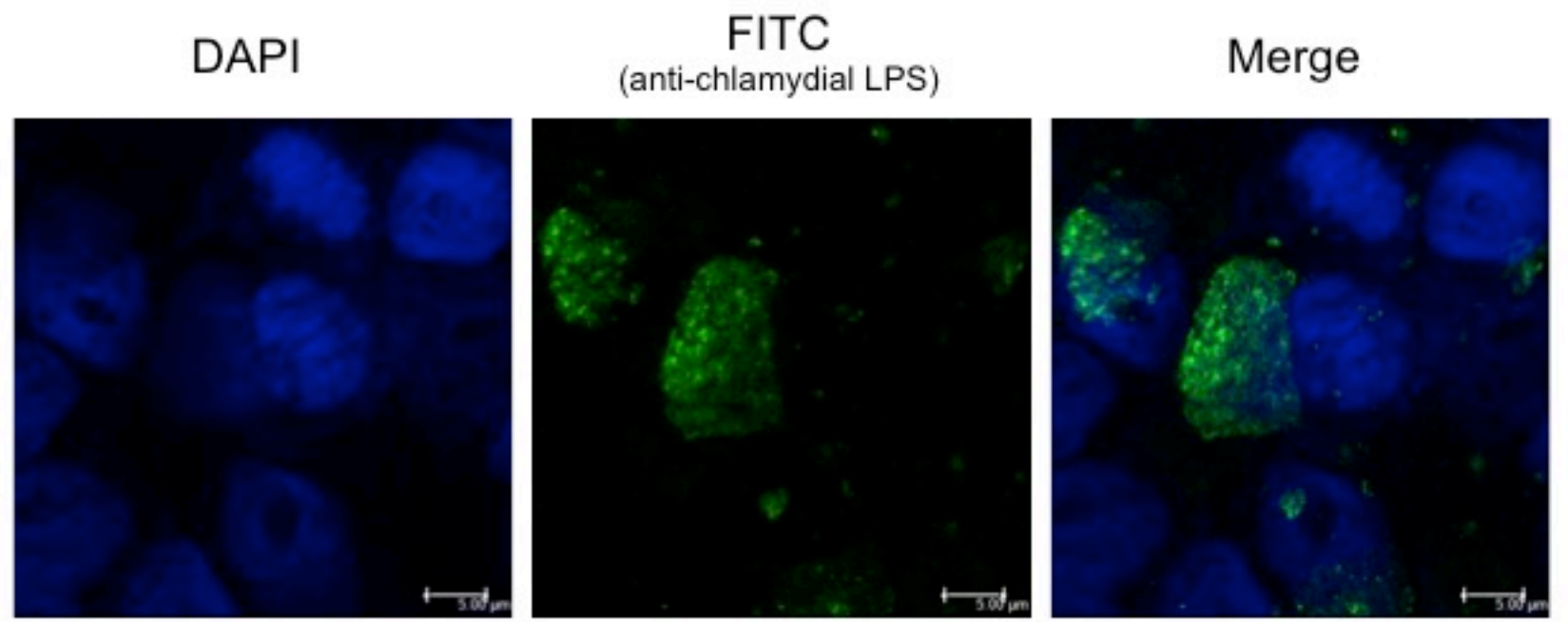


Fig. 3

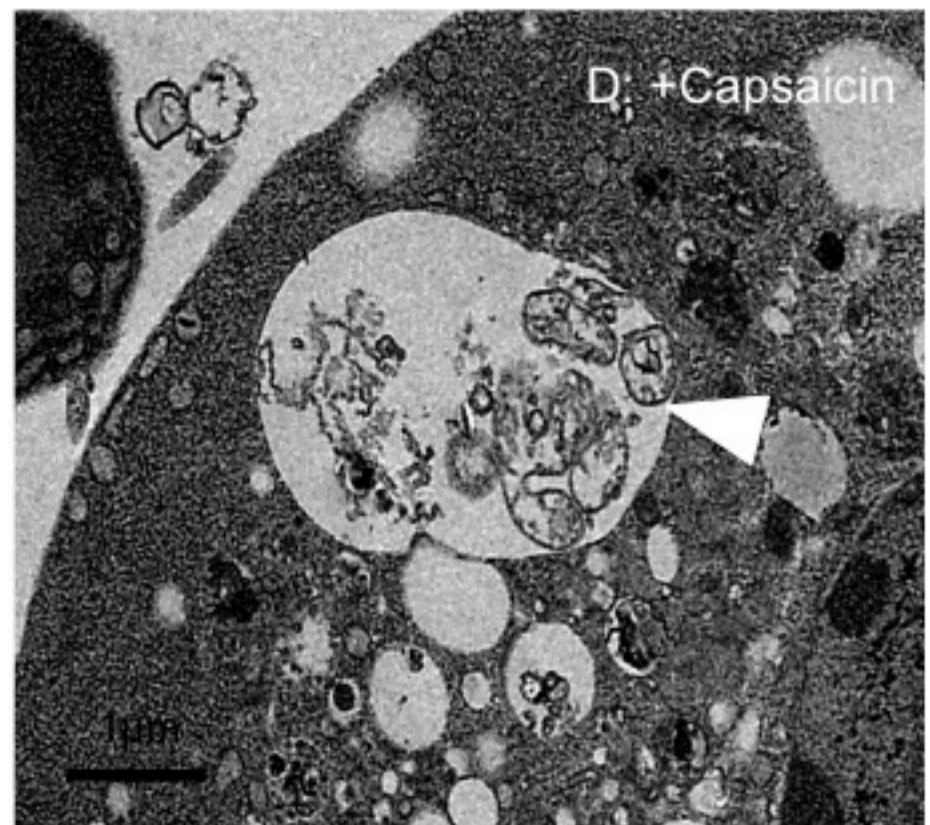
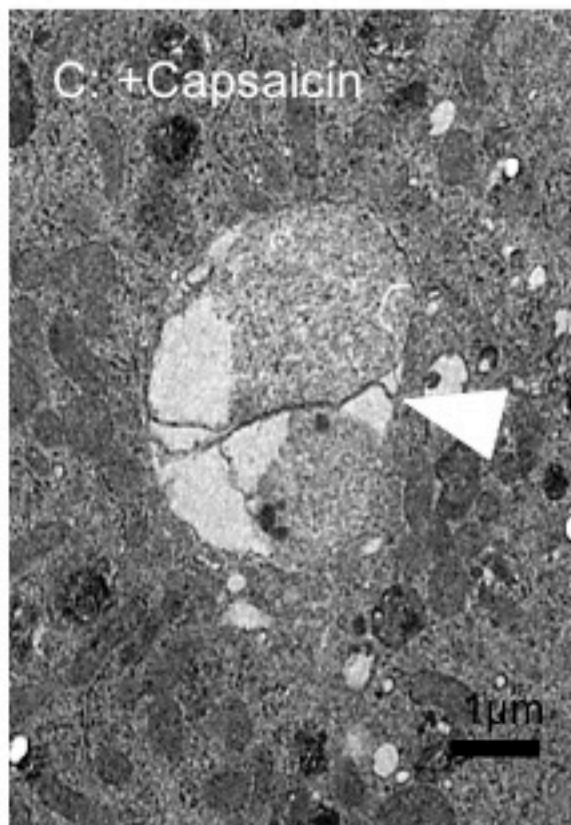
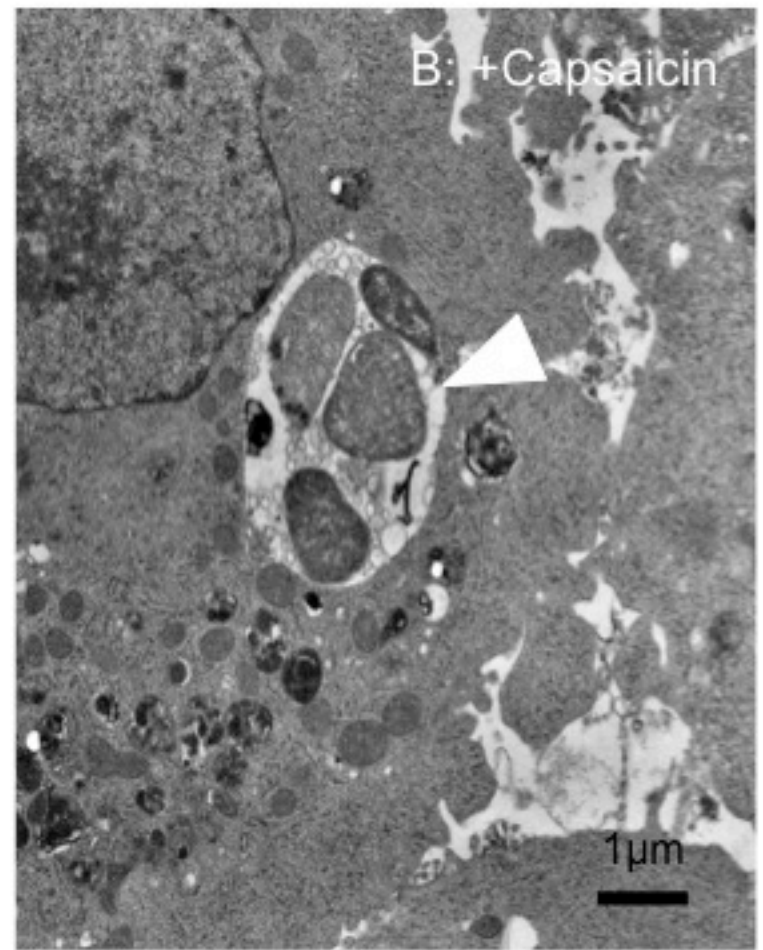
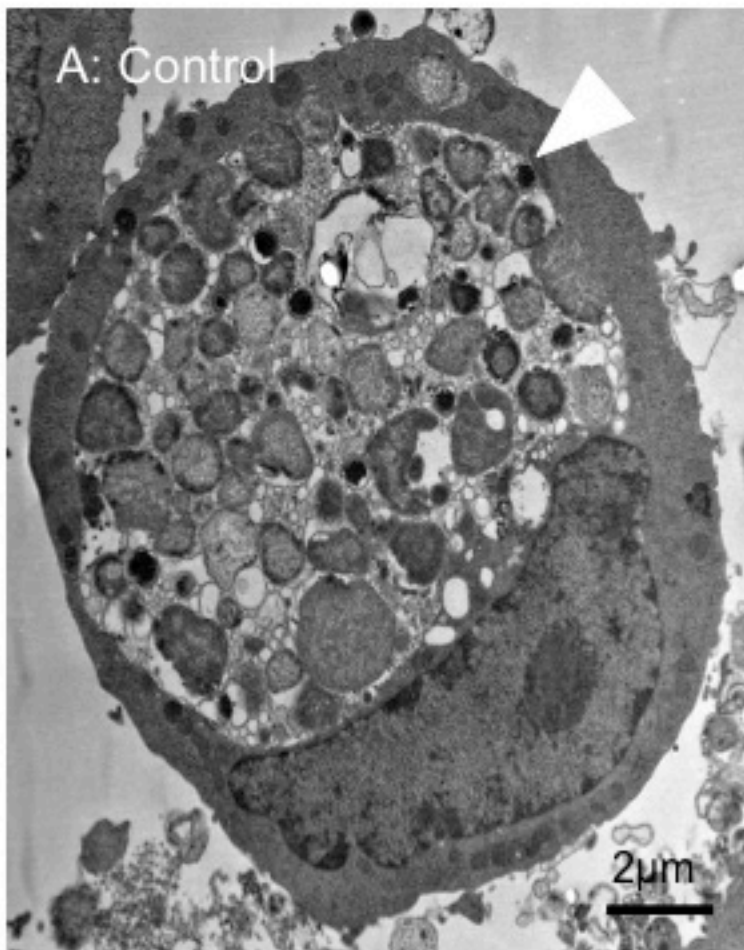
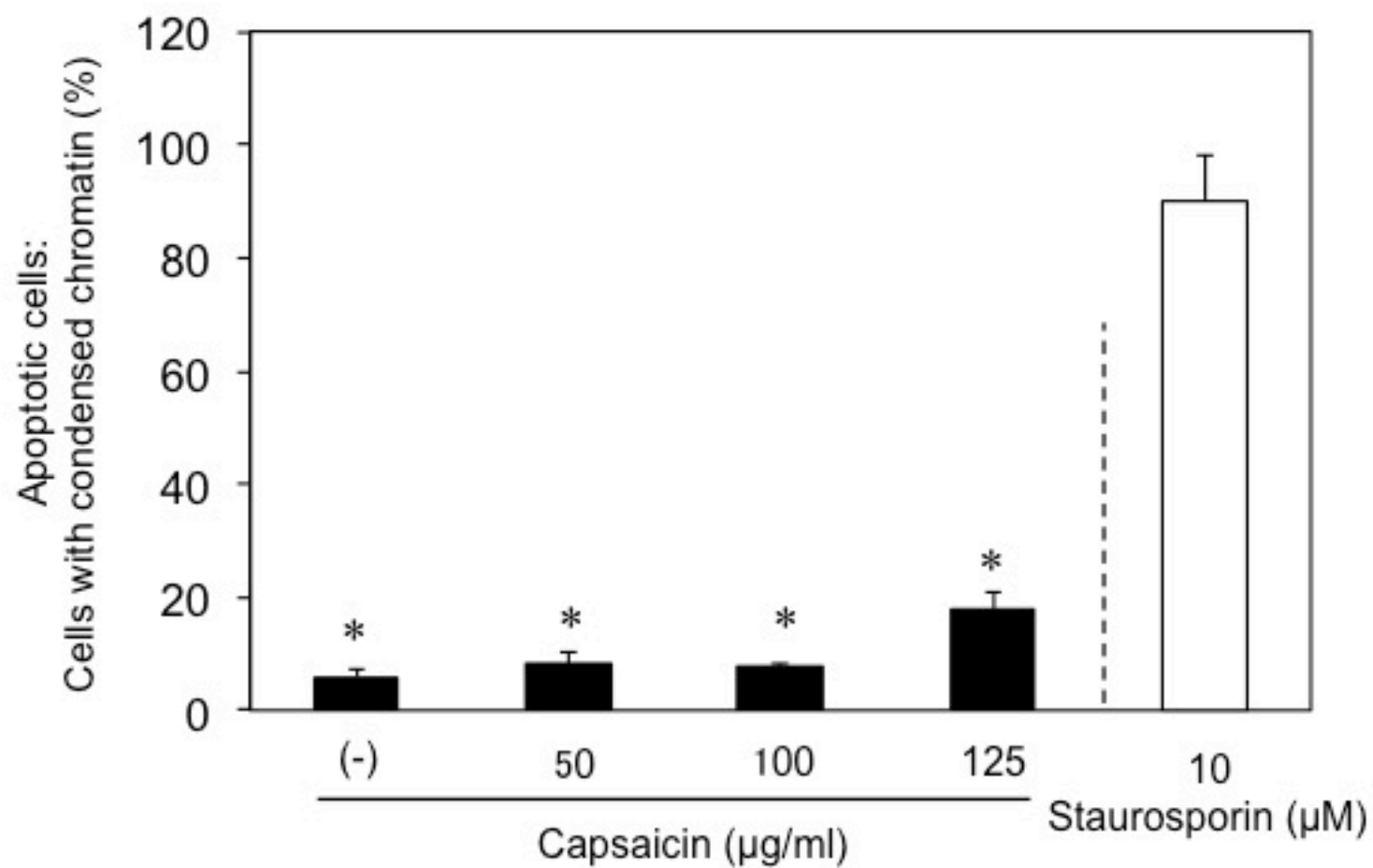


Fig. 4

A



B

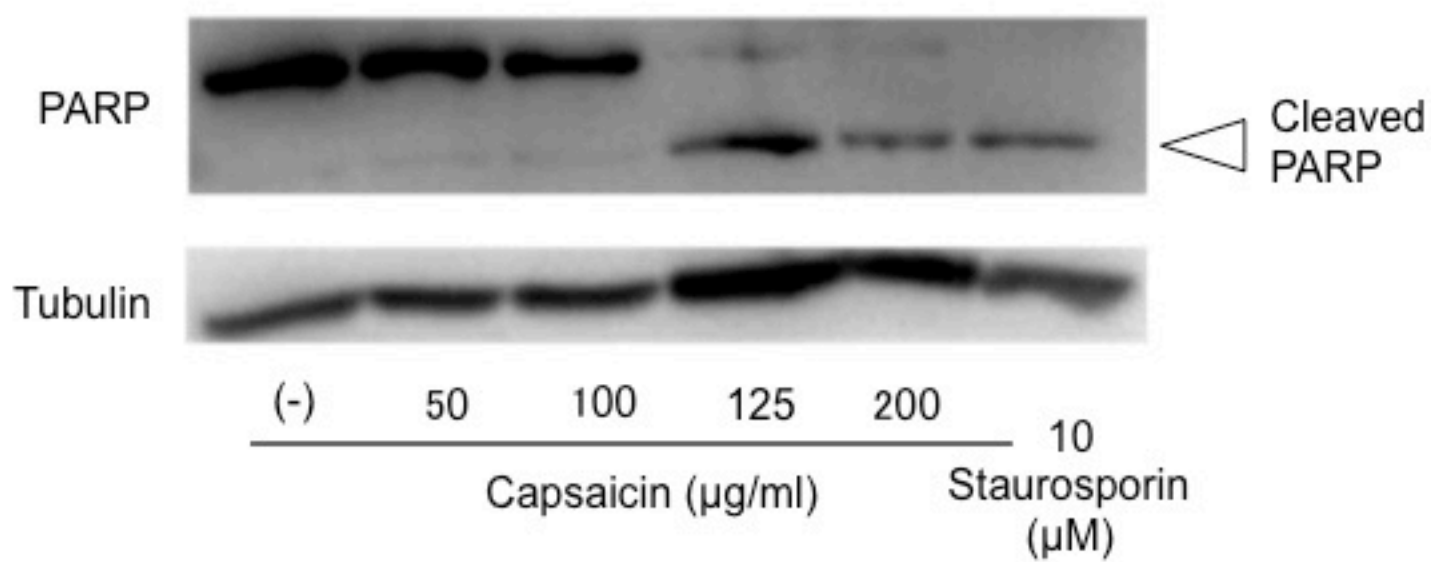
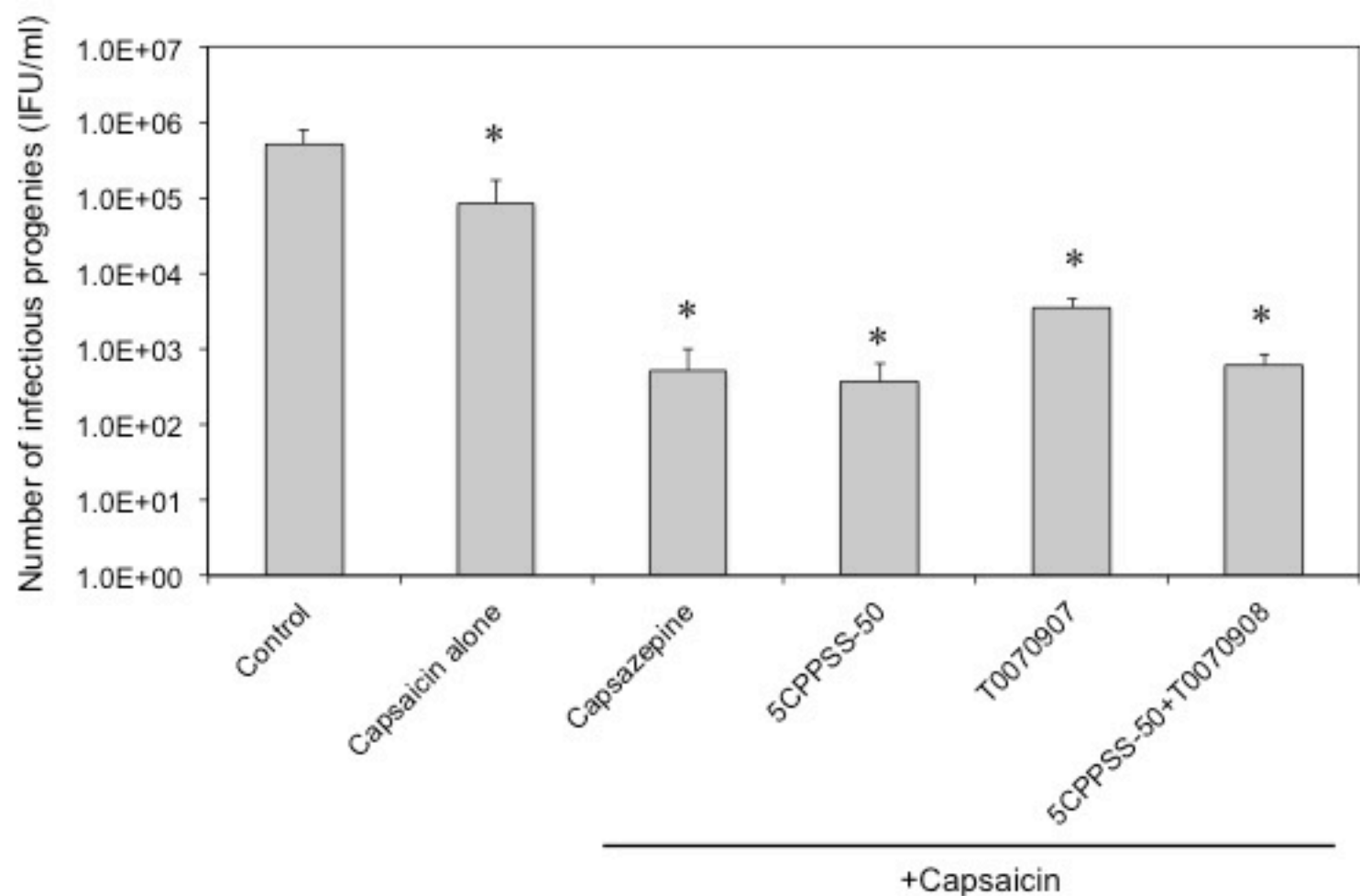
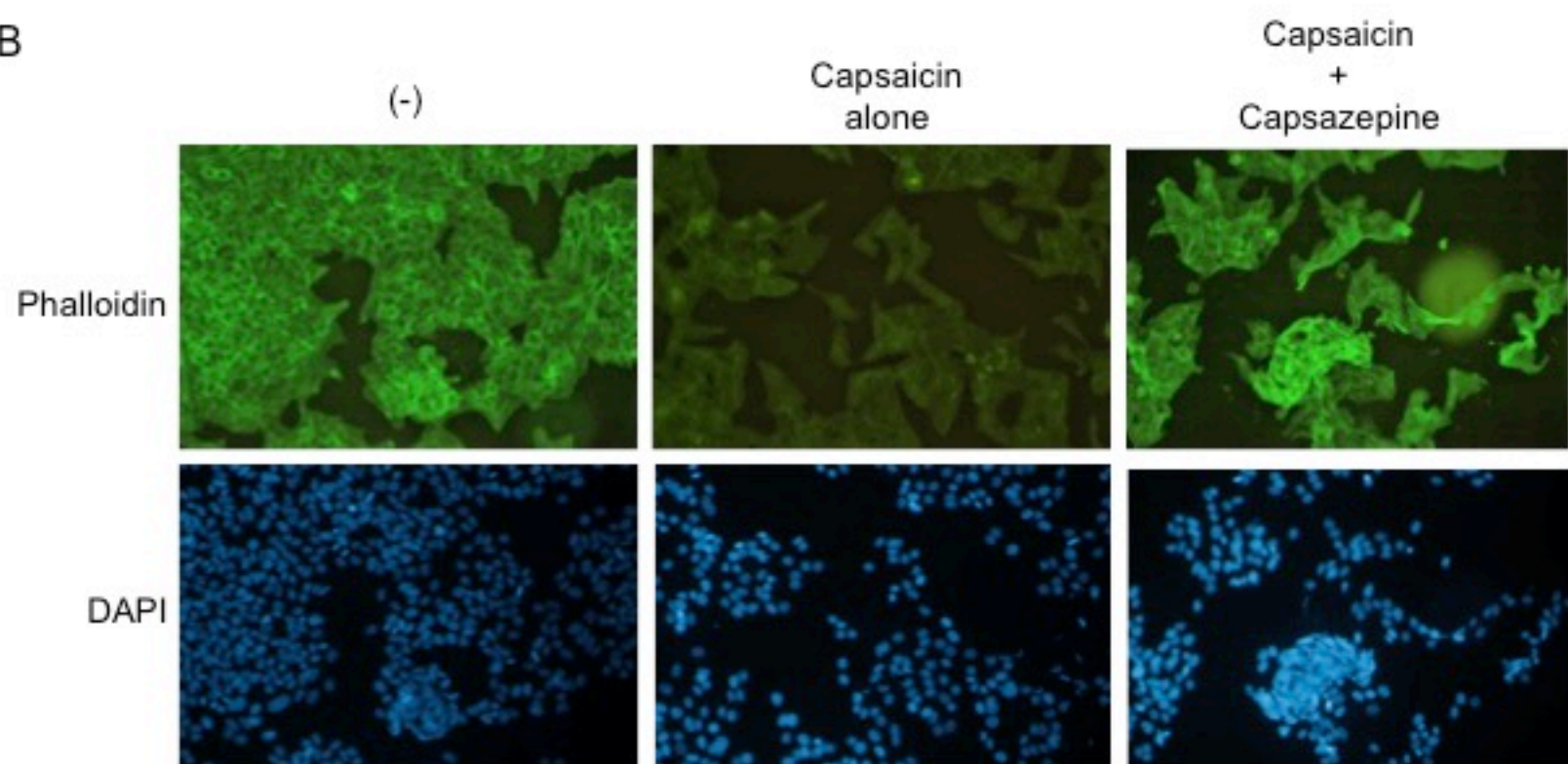


Fig. 5

A



B



C

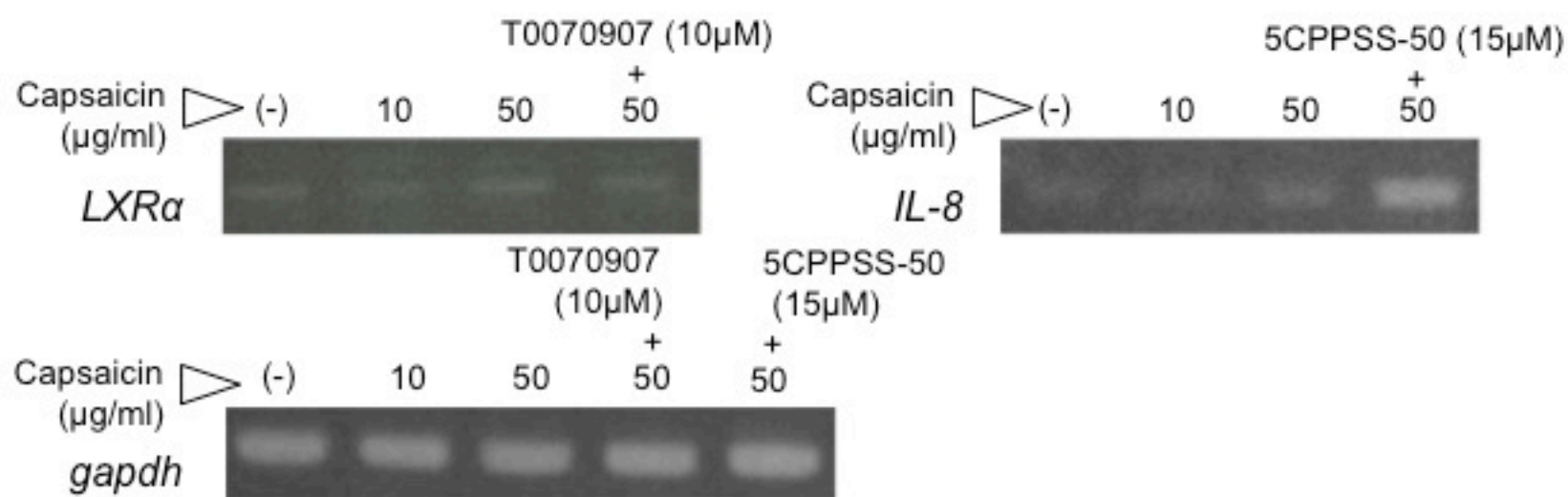


Fig. 6

