Synergistic effect of standardized extract of *Asparagus officinalis* stem and heat shock on progesterone synthesis with lipid droplets and mitochondrial function in bovine granulosa cells

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Abstract

Progesterone (P4) is a well-known steroid hormone that plays a key role in oocyte growth and the maintenance of pregnancy in mammals, including cattle. Heat stress (HS) has an adverse effect on P4 synthesis through an imbalance in the cellular redox status. We have recently revealed that a standardized extract of *Asparagus officinalis* stem (EAS) increases P4 through non-HS induction of heat shock protein 70 (HSP70) and a synergistic increase of HSP70 by enhancing the intracellular redox balance, which was adversely affected by HS in bovine granulosa cells (GCs). Bovine GCs collected from bovine ovarian follicles were cultured at 38.5°C and 41°C for 12 h with or without 5 mg/mL EAS. After treatment, cells and culture suppernatant were collected for the analysis. Enzyme-linked immunosorbent assay (ELISA) was performed to detect in P4 level. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was used to detect expression of steroidogenesis related genes. Fluorescence staining was used to detect mitochondrial activity and lipid droplet.

P4 level was increased by EAS treatment in association with increase in steroidogenic acute regulatory protein (STAR), 3β-hydroxysteroid dehydrogenase (3β-HSD), mitochondrial membrane activity and lipid droplet both under non-HS and HS conditions. Notably, synergistic effect of EAS with HS co-treatment was observed to show a greater increase in P4 synthesis when comparison with EAS treatment under non-HS condition. Furthermore, inhibition of HSP70 significantly reduced EAS-induced P4 synthesis, mitochondrial activity and synthesis of lipid droplets. These results suggest that P4 synthesis by EAS is mediated

by the steroidogenesis pathway via HSP70-regulated activation of *STAR* and *3β-HSD*, together with improved mitochondrial activity and lipid metabolism in bovine GCs. Moreover, effect of EAS has a synergistic effect of with HSP70-regulated steroidogenesis pathway.

Keywords: Bovine granulosa cells, EAS, HSP70, Progesterone, synergistic effect

1. Introduction

Progesterone (P4) is an important steroid hormone that plays a pivotal role in the establishment of uterine receptivity, oocyte maturation, and the maintenance of pregnancy [1,2]. In mammals, granulosa cells (GCs) play a fundamental role in the maturation and acquisition of developmental competence in bovine oocytes [3]. GCs differentiate into luteal cells that are responsible for P4 production [4]. Therefore, GCs are good models for inducing steroidogenesis under heat stress (HS) conditions to evaluate the importance of maintaining reproductive functions.

P4 synthesis in GCs is regulated by cholesterol transport into the mitochondria by steroidogenesis enzymes, such as STAR, cytochrome P450 family 11 subfamily a member 1 (CYP11A1), and 3β-HSD [5,6]. Importantly, mitochondria play a central role in steroidogenesis and are abundant in GCs [6,7]. Moreover, P4 synthesis in bovine GCs is associated with lipid metabolism [8]. In addition, cholesterol from lipid droplets is one of the available sources for P4 biosynthesis [7]. One of the main transcription factors that control lipid metabolism by regulating enzymes required for cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis is *SREBP-1* [9]. Thus, P4 synthesis in bovine GCs is regulated by steroidogenesis, mitochondrial function, and lipid metabolism. However, a critical issue that affects dairy farming is the reduction of reproductive performance in high-yielding dairy cows [10]. The reproductive performance of dairy cows is decreased by increased body

66 temperature [11,12] which decreases ovarian function, follicular growth, and corpus luteum
67 function in summer.
68 High ambient temperature produces reactive oxygen species (ROS) with reduced P4
69 production in bovine GCs [13]. In addition, a negative effect of ROS on P4 levels has been
70 reported in human follicular fluid [14]. Follicular fluid contains soluble factors secreted from
71 GCs that maintain the intrafollicular redox status [15,16]. ROS induces redox status
72 imbalance, which leads to cellular damage, alteration of cellular function, and physiological
73 processes [17,18]. Thus, HS has an adverse impact on P4 synthesis owing to HS-induced
74 toxic factors that disturb the redox imbalance in GCs.
75 Under stress conditions, cellular conditions are restored by the induction of heat shock
76 proteins (HSPs), which act as molecular chaperones by folding and unfolding proteins while
77 maintaining homeostasis of the cells [19]. Therefore, strengthening chaperone systems by
78 inducing HSPs is one of the keys to regulating their functions in various biological pathways
79 in ovarian functions, including P4 synthesis. However, as described above, HS is
80 accompanied by the induction of ROS with harmful effects.
81 Recently, a standardized extract of *Asparagus officinalis* stem (EAS) was shown to induce
82 HS-independent HSP70 in human promyelocytic leukemia cells [20]. In addition, we have
83 revealed that EAS has a beneficial effect in inducing HSP70 under non-HS conditions and
84 maintaining redox balance through ROS reduction and glutathione (GSH) synthesis in bovine
85 GCs [21]. Moreover, EAS showed a synergistic increase in HSP70 expression and an

improvement in the intracellular redox status of bovine GCs, which was adversely affected by HS [21]. The abundance regulation of EAS-induced HSP70 which affects redox balance under both HS and non-HS conditions may compromise P4 synthesis in bovine GCs. In addition, the effects of EAS on P4 biosynthesis under HS and non-HS conditions in bovine GCs cells have not yet been investigated. In the present study, we investigated the effects of EAS on P4 levels, steroidogenesis genes, mitochondrial function, and lipid metabolism under non-HS and HS conditions.

2. Materials and Methods

Extract of *Asparagus officinalis* stem (EAS), which was extracted from asparagus (*A. officinalis* L.), was produced following a previously described method [22] and commercially available as a supplement (ETAS®).

2.1 Collection and culture of bovine GCs

Bovine ovaries were obtained from a local abattoir and were transported within 4 hours to the laboratory at 20°C. The ovaries were washed several times with a sterile saline solution. Oocytes were collected from follicles (2-8 mm in diameter) without signs of atresia, using a disposable 18-gauge needle attached to a 10-mL syringe. After cumulus-oocyte complexes were picked up, the remaining follicular fluid with floating GCs cells was cultured in

106 Dulbecco's modified Eagle's medium (high glucose) (DMEM) (Wako, Osaka, Japan)
107 containing 5% fetal bovine serum (FBS), 0.06 g/L penicillin G potassium (Nacalai Tesque,
108 Kyoto, Japan), and 0.1 g/L streptomycin sulfate (Nacalai Tesque, Kyoto, Japan) at 38.5°C
109 under 5% CO_2 in air.
110 After overnight culture, the theca cell cluster layer was gently removed from the bottom of
111 the cell culture dishes. The cells remaining at the bottom of the dish were bovine GCs, which
112 were then washed with calcium- and magnesium-free phosphate-buffered saline (PBS) (–),
113 and cultured in 5% FBS in DMEM at 38.5°C under 5% CO_2 in air. When the cells reached
114 confluence, they were washed with PBS (–) and dissociated from the substratum with PBS
115 (–) containing 0.05% trypsin and 0.53 mM Ethylenediaminetetraacetic acid (EDTA) for 2 min
116 at 38.5°C in a CO_2 incubator. After the addition of 5% FBS in DMEM to inactivate trypsin
117 activity, the separated cell suspension was centrifuged at 1,200 × *g* for 3 min. To culture
118 these bovine GCs, 1×10^5 cells/mL viable cells were seeded in each well of a 4-well dish
119 (Thermo Fisher Scientific) or 8-well slide chamber (Thermo Fisher Scientific) and cultured at
120 38.5°C under 5% CO_2 in air. After the cells reached 70% confluence, the medium was
121 replaced with 0.9 mL of DMEM in 5% FBS together with 0.1 mL of EAS solution in PBS (–),
122 and 0.1 mL of PBS (–) was added to the control group. The cells were then cultured at 38.5°C
123 or 41°C under 5% CO_2 atmosphere.
124
125 *2.2 Experimental design*

126 To investigate the effect of EAS on P4 production, the cells were cultured at 38.5°C for 12
127 h with 0.5, 1, 5, and 10 mg/mL of EAS. In addition to evaluating the effects of EAS on P4
128 synthesis under non-HS and HS conditions, cells were cultured at 38.5°C and 41°C for 12 h
129 with or without 5 mg/mL EAS. To confirm the role of EAS-induced HSP70 in P4 synthesis,
130 EAS-treated cells were exposed to 10 μM pifithrin-μ (2-phenylethynesulfonamide:PES)
131 (StressMarq Biosciences Inc., Victoria, Canada) under non-HS and HS conditions. After
132 culture, cells and culture medium were used for mRNA expression analysis, P4
133 measurement, and detection of lipid droplets and mitochondria.
134
135 *2.3 P4 measurement*
136 The concentrations of P4 in culture supernatants of bovine CGs were measured by enzyme-
137 linked immunosorbent assay (ELISA) using P4 measurement kits (ADI- 900– 011, Enzo Life
138 Sciences, USA). The intra- and inter-assay coefficients of variation for P4 were less than
139 10%. The aforementioned experiments were performed in duplicate.
140
141 *2.4 RNA extraction and quantitative reverse-transcription polymerase chain reaction (RT-*
142 *qPCR)*
143 Total RNA was extracted from the cells using ISOGEN II (Nippon Gene, Toyama, Japan)
144 according to the manufacturer's instructions. The RNA concentration was measured using
145 spectrophotometry (NanoDrop ND-2000; Thermo Fisher Scientific). All RNA samples were

146 stored at −80°C until use. Total RNA was reverse transcribed using ReverTra Ace qPCR RT
147 Master Mix with gDNA remover (TOYOBO Life Science, Osaka, Japan) with a thermal cycler
148 (Astec GeneAtlas Type G Thermal Cycler; ASTEC, Fukuoka, Japan). All cDNA samples were
149 stored at − 30°C until use for Quantitative PCR. Specific primers (Table S1) were designed
150 using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qPCR was performed
151 using THUNDERBIRD SYBR qPCR Mix (Toyobo Life Science) at a final primer concentration
152 of 0.5 μM. To quantify mRNA expression levels, PCR was performed under the following
153 conditions with the LightCycler Nano (Roche Diagnostics, Basel, Switzerland): 1 cycle at
154 95°C for 30 s (denaturation), 45 cycles at 95°C for 10 s (denaturation), 55°C for 15 s (primer
155 annealing), and 72°C for 30 s (extension). Relative mRNA abundance was calculated using
156 the ΔΔCt method with H2AFZ as a reference gene.
157
158 *2.5 Immunodetection of HSP70*
159 HSP70 immunofluorescence analysis was performed as previously described [21]. After
160 GCs were cultured in 8-well slide chambers, they were fixed with 4% paraformaldehyde
161 diluted with PBS (–), permeabilized with 0.2% Triton X-100 in PBS (–), blocked with 2% (w/v)
162 BSA (Sigma-Aldrich) in PBS (–), and incubated at 4°C overnight with rabbit anti-human
163 HSP70 polyclonal primary antibody (SPC-103; StressMarq Biosciences Inc., Victoria,
164 Canada). After washing three times with PBS, the cells were incubated for 30 min with the
165 secondary antibody Alexa Fluor 488 donkey anti-rabbit IgG (A21206; Thermo Fisher

166 Scientific). The 8-well slide chambers were then mounted with mounting solution (Fluoro-
167 KEEPER Anti fade Reagent, Non-Hardening Type with DAPI, Nacalai Tesque) and observed
168 under a fluorescence microscope (Leica Camera AG, Wetzlar, Germany).
169
170 *2.6 Detection of Lipid droplet and mitochondrial activity*
171 For the detection of lipid droplets, cells were washed with PBS (–) after culture and fixed
172 with 4% PFA in PBS (–) for 10 min at room temperature. After washing the cells three times
173 with PBS (–) for 5 min, they were treated with 1 μg/mL Nile Red (Wako, Osaka, Japan) for
174 30 min at 38.5°C in a 5% CO_2 atmosphere.
175 To detect mitochondrial activity, cells were washed with PBS (–) after culture and treated
176 with 200 nM MitoTracker™ Green FM (Thermo Fischer Scientific) for 30 min at 38.5°C in a
177 5% CO_2 atmosphere.
178 Cells from both detections were then washed with PBS (–), and 10 μL of mounting solution
179 (Nacalai Tesque) was added to the cells and covered with a cover glass. Fluorescence
180 images were obtained using a fluorescence microscope (Leica, Wetzlar, Germany).
181 Fluorescence intensity was quantified using ImageJ software v1.52A (National Institutes of
182 Health; http://imagej.nih.gov/ij/). Corrected total cell fluorescence (CTCF) was carried out
183 following a previously described formula [23]:
184 1. CTCF = integrated density (total area of selected cells × mean fluorescence of
185 background readings).

2. CTCF per cell = CTCF/ Ncells

where “integrated density” is the integrated intensity of pixels for all cells in the image, total cell area is the number of pixels of all cells, background fluorescence is the average mean gray value of nearby regions containing no cells, and Ncells is the number of cells that was measured by counting fluorescently labeled nuclei from images.

Images were deconvolved using Huygens Professional software (Hilversum, Netherlands)

2.7 Statistical analysis

Experimental data are shown as mean ± standard error of the mean (S.E.M.). Analysis of variance (ANOVA) and Tukey’s test were performed using R software (version 3.5.3; https://www.r-project.org/). Probabilities of less than 5% ($P < 0.05$) indicate statistically significant differences.

3. Results

3.1 Effects of EAS on P4 production

We investigated the effects of various doses of EAS on P4 secretion in bovine GCs. As shown in Figure 1, we found that EAS significantly ($p < 0.001$) promoted P4 levels peaking at 5 mg/mL. Collectively, EAS showed a dose-dependent induction of P4 production.

205 *3.2 Effects of EAS on P4 production and steroidogenic and lipid metabolism gene expression*
206 *in bovine GCs under non-HS and HS conditions*
207 In our previous study, we showed that EAS induces HSP70 and improves redox balance in
208 both non-HS and HS conditions [21]. In the present study, we investigated whether EAS
209 affects P4 synthesis in both environments, together with the expression of steroidogenesis-
210 related genes in EAS-treated bovine GCs, under non-HS and HS conditions. As shown in
211 Figure 2A, P4 levels were significantly ($p < 0.001$) increased by EAS compared with the
212 untreated control. However, P4 levels in HS-treated cells tended to be lower than that for the
213 control cells ($p < 0.1$) (Figure 2A). Interestingly, P4 levels were significantly increased ($p <$
214 0.001) by EAS under HS conditions (Figure 2A).
215 Furthermore, *STAR* and *3β-HSD* mRNA expression levels were similar to P4 levels in the
216 EAS and EAS+HS groups ($p < 0.001$) (Figure 2B, 2C). In contrast, EAS did not affect the
217 expression of *CYP11A1, progesterone receptor*, or *estrogen receptor* under either non-HS
218 or HS conditions ($p > 0.05$) (Figure 2D and Figure S1).
219 Moreover, SREBP-1 expression was significantly increased in both the EAS and EAS+HS
220 groups, similar to the pattern of P4 levels and expression of steroidogenic genes ($p < 0.05$)
221 (Figure 2E).
222 These results confirmed that EAS-induced P4 production is affected by the enhancement *of*
223 *STAR*, *3β-HSD*, and *SREBP-1,* and it has the greatest effect on P4 synthesis in bovine GCs

under HS conditions. Notably, HS treatment had a synergistic effect with EAS on the increase in *STAR, 3β-HSD,* and *SREBP-1*.

3.3 Effects of EAS on lipid metabolism and mitochondrial activity in bovine GCs under non-HS and HS conditions

To clarify the effects of EAS on P4 levels, steroidogenesis, and lipid metabolism gene expression, we examined lipid synthesis and mitochondrial function in EAS-treated cells under non-HS and HS conditions. As shown in Figure 3A, EAS increased lipid droplets and mitochondrial activity under non-HS and HS conditions. Remarkably, the EAS-induced HSP70 protein was expressed in both the nucleus and cytoplasm, but HS-induced HSP70 was localized mainly in the nucleus (Figure 3A).

Next, we deconvoluted the acquired lipid metabolism and mitochondrial activity images. Deconvolution of the images efficiently removes background noise pixels in lipid droplets and mitochondrial images of African green monkey kidney fibroblast cells [24]. After deconvolution, the lipid droplets and mitochondrial structure appeared to be assembled from multiple particles (Figure 3A). Notably, the localization of lipid droplets and mitochondrial function were mainly observed in the cytosol around the nucleus (Figure 3A). The results show that EAS increased lipid droplets and mitochondrial particles under non-HS and HS conditions.

243 Similar to P4 synthesis, the fluorescence levels of lipid droplets and mitochondria in the
244 EAS+HS group were significantly ($p < 0.001$) higher than those in the other treatment groups
245 (Figure 3B, C). HS significantly ($p < 0.05$) increased the number of lipid droplets but
246 significantly ($p < 0.01$) reduced mitochondrial activity (Figure 3B, C). These results show that
247 EAS and its synergistic effect with HS increased lipid metabolism and mitochondrial activity,
248 and these results may be related to EAS-induced HSP70.
249
250 *3.4 Inhibition of HSP70 attenuates P4 synthesis, lipid metabolism, and mitochondrial activity*
251 *in EAS-treated bovine GCs under non-HS and HS conditions*
252 We hypothesized that EAS-induced HSP70 may be involved in the effects of EAS on P4
253 synthesis, lipid droplet formation, and mitochondrial function in bovine GCs under non-HS
254 and HS conditions. To test this hypothesis, we evaluated the effects of HSP70 inhibition on
255 P4 synthesis, steroidogenic gene expression, lipid synthesis, and mitochondrial activity in
256 EAS-treated cells under both non-HS and HS conditions. As expected, PES significantly
257 decreased EAS-induced P4 synthesis related to reduced P4, *STAR, 3β-HSD, and SREBP-*
258 *1* levels under both non-HS and HS conditions ($p < 0.05$) (Figure 4A, B, C, E). However, an
259 HSP70 inhibitor did not affect *CYP11A1* expression ($p > 0.05$) (Figure 4D).
260 In addition, HSP70 blockage inhibited the EAS-mediated upregulation of fluorescent levels
261 of lipid droplets and mitochondria, whereas HSP70 expression was unchanged (Figure 5).
262 Consistently, PES suppressed lipid synthesis and mitochondrial activity in EAS-treated

bovine GCs under non-HS and HS conditions ($p < 0.05$) (Figure 6). Taken together, these data suggest that EAS-induced P4 synthesis is regulated by EAS-induced HSP70.

4. Discussion

In the present study, we confirmed that EAS induces HSP70-mediated P4 synthesis through steroidogenic genes associated with the induction of lipid metabolism and mitochondrial activity in bovine GCs under both non-HS and HS conditions. Notably, EAS treatment under HS treatment synergistically increased P4 synthesis with increased expression of *STAR*, *3β-HSD,* and *SREBP-1*, lipid metabolism, and mitochondrial activity.

The present results show that P4 levels in bovine GCs were highest when treated with 5 mg/mL EAS. This result was similar to that of our previous study in which bovine GCs were treated with or without 5 mg/mL EAS [21]. EAS exerts beneficial effects during stress and improves sleep quality in humans [25]. Other studies have revealed beneficial effects of EAS on brain cell function [26]. Recent evidence has suggested that the central nervous system can synthesize steroid hormones from cholesterol [27]. P4 synthesis is initiated by cholesterol import into the mitochondria by STAR, after which CYP11A1 transforms cholesterol to pregnenolone, which is converted into P4 by 3β-HSD [28]. In GCs, P4 binds to the progesterone receptor and facilitates its activity in gene transcription and cellular responses [29]. Under *in vitro* conditions, bovine GCs are good models for P4 studies because of luteinization of this cell line, which involves the switch from the production of

283 estrogens to P4 [30]. In the present study, EAS strongly increased P4 levels and the
284 expression of *STAR* and *3β-HSD*, but did not affect the expression of *CYP11A1,*
285 *progesterone receptor,* and *estrogen receptor*. These results suggest that EAS regulates P4
286 synthesis through *STAR* and *3β-HSD* pathways in the mitochondria of bovine GCs. In
287 addition, EAS enhanced P4 levels but did not affect P4 related signal pathway of GCs through
288 the progesterone receptor in an autocrine or paracrine manner. Furthermore, HS can
289 synergistically promote this pathway with EAS treatment, resulting in promotion of P4
290 synthesis. Importantly, mitochondria play a central role in steroidogenesis enzymatic activity
291 [6,7]. Surprisingly, in our results, EAS increased mitochondrial activity in response to HS,
292 despite the fact that HS itself decreased mitochondrial activity.
293 Generally, HS in the summer season is known to cause a decrease in ovarian function, with
294 reduced P4 levels and corpus luteum size [31–33]. In mammalian cells, lipid droplets are the
295 cholesterol preservation sites for steroid hormones [34]. In the fat tissue of Japanese black
296 cattle, SREBP-1 affects lipid metabolism [35]. Our present results show that lipid droplets
297 and *SREBP-1* were induced by both EAS and HS at the same level, and the highest
298 expression levels were observed after co-treatment with EAS and HS. Collectively, EAS-
299 induced P4 synthesis through enhancement of STAR, 3β-HSD enzyme activity,
300 mitochondrial activity, and lipid metabolism in bovine GCs. In addition, significant effect of
301 EAS and HS co-treatment on P4 synthesis was also found in the present study.

In our previous study, EAS was shown to enhance redox balance through the reduction of ROS levels under ordinary non-HS conditions, and, surprisingly, EAS had a significant effect on HSP70 induction and ROS reduction in bovine GCs even under HS conditions compared with the increased ROS and decreased GSH under HS conditions without EAS [21].

In a previous study, STAR, a key enzyme in P4 synthesis, was shown to be inhibited by ROS in rat Leydig cells [36]. ROS also blocks P4 synthesis through CYP11A1 and 3β-HSD in human granulosa luteal cells [37]. In bovine GCs, HS-induced ROS reduce *STAR* and *CYP11A1* gene expression [13]. Hydrogen peroxide, the most popular ROS, decreases P4 levels, *STAR*, and *3β-HSD* gene expression in bovine GCs [38]. In addition, high concentrations of ROS negatively affect mitochondria in mouse GCs cells [39]. Therefore, redox balance might also be important in regulating steroidogenesis-related genes and mitochondrial activity in bovine GCs.

In addition, lipid droplets are markers of cellular stress, and they play an important role in maintaining redox status under stress conditions [40]. Under stress conditions, lipid droplets sequester toxic lipids and delay the release of lipids, and this lipid droplet biosynthesis maintains redox balance [40]. Thus, EAS-treated GCs in this study, while maintaining redox balance, possibly caused the stabilization of lipid droplets as a source of cholesterol compared to non-EAS-treated cells. Collectively, the enhancement of P4 synthesis by EAS treatment while maintaining redox status depends on the ROS levels in bovine GCs.

In the present study, HS induced lipid droplet formation and reduced mitochondrial activity in bovine GCs. Our previous study suggested that ROS and ROS-induced DNA damage are detected mainly in the nuclei of bovine GCs under HS conditions [21]. In bovine somatic cell nuclear transfer embryos, mitochondrial and DNA damage are induced by an increase in ROS [41]. Thus, impairment of DNA damage in the nucleus reduces mitochondrial function in HS bovine GCs and affects gene expression. Previous studies have shown that lipid droplets have dynamic functions in cellular metabolism [42]. In mice, mitochondrial damage is caused by hypoxia-induced formation of lipid droplets [43]. Thus, the high levels of lipid droplets in bovine GCs induced by HS can be explained by mitochondrial dysfunction. Collectively, in our results, HS-induced ROS damage in the nucleus impaired mitochondrial function, resulting in the accumulation of lipid droplets in bovine GCs. EAS and EAS plus HS treatment also induced lipid droplet formation, and these results are related with expression of HSP70 that will be discussed in more details below. In our previous study, overall data confirmed that the beneficial effects of EAS-induced HSP70 are mediated by regulating redox balance and protecting cells from HS-related harmful effects in bovine GCs [21].

To show the relationship between HSP70, the steroidogenesis pathway, and mitochondrial function, we inhibited HSP70 activity. Inhibition of HSP70 by PES did not affect the expression of HSP70 protein; however, HSP70 inhibition clearly reduced mitochondrial activity and lipid droplet synthesis. Previous studies have shown that PES disrupts the co-chaperone and substrate proteins of HSP70 without affecting HSP70 expression; thus, PES

341 inhibits the function of HSP70 protein in multiple cell signaling pathways [44]. In the present
342 study, PES strongly inhibited P4 synthesis, expression of *STAR*, *3β-HSD*, and *SREBP-1*,
343 mitochondrial activity, and lipid droplet synthesis in bovine GCs, even in the presence of EAS,
344 under both non-HS and HS conditions. Therefore, these data strongly support the hypothesis
345 that an increase in EAS-induced HSP70 promotes P4 synthesis. In addition, HSP70
346 localization was detected in both the nucleus and cytoplasm of EAS-treated cells, particularly
347 under HS conditions. In mammalian cells, HSP70 has chaperone functions in both the
348 nucleus and cytoplasm [45,46].
349 In our previous study, EAS-induced HSP70 reduces DNA damage by reducing ROS, which
350 are mainly generated in the nucleus in bovine GCs [21]. Modulation of redox balance-
351 regulated mitochondrial activity and lipid droplets reduces ROS damage in the nuclei of
352 bovine GCs. In addition, the transcription of steroidogenesis enzymes mainly occurs in the
353 nucleus [47]. Therefore, EAS-induced HSP70 balances the redox status to improve
354 steroidogenesis enzyme activity, mitochondrial function, and lipid synthesis in bovine GCs.
355 On the other hand, synthetic chemical chaperones increase 3β-HSD metabolic activity in the
356 inner mitochondrial membrane by promoting the folding speed of 3β-HSD from the native to
357 the active state [48]. In rat adipocyte lipid droplets, HSP70 stimulation is involved in stabilizing
358 the droplet monolayer, protein folding, and mobilization of nascent proteins to the lipid
359 droplets [49]. The size of lipid droplets and fat accumulation are increased by HSP70 through
360 the enhancement of *SREBP-1* in mouse liver cells [50]. Therefore, EAS-induced HSP70

361 enhances the accumulation of lipid droplets and 3β-HSD activity in the mitochondria in the
362 cytoplasm with the chaperone function of bovine GCs. In the present study, promotion effect
363 of EAS on P4 synthesis via steroidogenesis pathway, mitochondrial functions, lipid synthesis
364 with synergistic augmentation with HS. These results suggest the EAS is a potential tool for
365 improving the reproductive functions by regulating of intracellular redox and molecular
366 chaperone. Recent study has revealed that the HSP inducer in the EAS was purified and
367 identified as a mixture of Asparagus-Derived Proline-Containing 3-Alkyldiketopiperazines
368 (Asparaprolines) [20]. However, evaluation of such components on the detailed cellular
369 functions is not clarified. Further study will clarify the beneficial effect on cellular functions
370 and regulation of reproductive performance.
371
372 **5.Conclusion**
373 P4 synthesis by EAS is mediated by the steroidogenesis pathway via HSP70-regulated
374 activation of STAR and 3β-HSD, together with improved mitochondrial activity and lipid
375 metabolism in bovine GCs. Moreover, EAS has synergistic effect of with HSP70-regulated
376 steroidogenesis pathway. These findings provide a basis for the application of EAS to
377 improve reproductive functions in mammals, including cattle, in combination with HS
378 treatment.
379
380 **Supplementary materials:**

Table S1: Primer sequences used for qRT-PCR

Figure S1: Effects of EAS on progesterone receptor and estrogen receptor in bovine GCs cells under non-HS and HS conditions

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Author contributions

Masashi Takahashi: Conceptualization, Supervision, Formal analysis, Writing-review & editing, Project administration. **Khoi Thieu Ho**: Conceptualization, Validation, Formal analysis, Investigation, Visualization, Writing-original draft, Writing-review & editing. **Hanako Bai**: Methodology, Writing-review & editing. **Manabu Kawahara**: Methodology, Writing-review & editing. **Kohei Homma**: Resources, Writing-review & editing. **Jun Takanari**: Resources, Writing-review & editing. **Ahmed Zaky Balboula**: Formal analysis, Writing-review & editing. **Khang Thi Kim Nguyen**: Formal analysis, Writing-review & editing.

Figure legends

Figure 1. Effect of various concentrations of EAS on P4 production in bovine GCs.

Cells were subjected to various concentrations of EAS (0.5, 1, 5, and 10 mg/mL) for 12 h at 38.5°C (n = 5). Detection of P4 secretion was performed with ELISA in cell supernatants. Data are shown as the mean ± S.E.M, a vs. b ($p < 0.001$), a vs. c ($p < 0.05$), and b vs. c ($p < 0.05$).

Figure 2. Effect of EAS on P4 synthesis and expression of steroidogenic genes in bovine GCs under non-HS and HS conditions.

Cells were treated with or without 5 mg/mL of EAS at 38.5°C (control, EAS) or 41°C (HS, HS + EAS) for 12 h. Detection of P4 secretion was performed with ELISA in cell supernatants (A, n = 3). The mRNA expression levels of STAR, 3β-HSD, CYP11A1, and SREBP-1 (B–E, respectively, n = 5) are presented relative to H2AFZ as a reference gene. Data are shown as the mean ± S.E.M, (A) a vs. b ($p < 0.001$), a vs. c ($p < 0.001$), b vs. c ($p < 0.05$), (B) a vs. b ($p < 0.05$), a vs. c ($p < 0.001$), b vs. c ($p < 0.05$), (C) a vs. b ($p < 0.01$), a vs. c ($p < 0.001$), and b vs. c ($p < 0.001$), and (E) a vs. b ($p < 0.05$), a vs. c ($p < 0.001$), b vs. c ($p < 0.05$).

Figure 3. Effect of EAS on the synthesis of lipid droplets and mitochondrial activity in bovine GCs under non-HS and HS conditions.

611 Cells were treated with or without 5 mg/mL of EAS at 38.5°C (control, EAS) or at 41°C (HS,
612 HS + EAS) for 12 h. Fluorescence staining and enlarged image of mitochondria, lipid droplets,
613 and HSP70 are shown (A). Scale bar = 50 μm; original magnification = 63×. The fluorescence
614 intensity of mitochondria and lipid droplets was analyzed using CTCF (B and C, respectively,
615 n = 5). Data are shown as the mean ± S.E.M, (B) a vs. b ($p < 0.05$), a vs. c ($p < 0.001$), b vs.
616 c ($p < 0.001$), (C) a vs. b ($p < 0.01$), a vs. c ($p < 0.01$), a vs. d ($p < 0.001$), b vs. c ($p < 0.001$),
617 b vs. d ($p < 0.001$), c vs. d ($p < 0.001$).
618
619 **Figure 4. Effect of HSP70 inhibition on EAS-induced P4 synthesis and expression of**
620 **steroidogenic genes in bovine GCs under non-HS and HS conditions.**
621 Cells were treated with or without 10 μM PES to 38.5°C (control, EAS, EAS + PES) or at
622 41°C (HS, HS + EAS, HS + EAS + PES) for 12 h. Detection of P4 secretion was performed
623 with ELISA in cell supernatants (A, n = 3). The mRNA expression levels of STAR, 3β-HSD,
624 CYP11A1, and SREBP-1 (B–E, respectively, n = 5) are presented relative to H2AFZ as a
625 reference gene. Data are shown as the mean ± S.E.M, (A) a vs. b ($p < 0.05$), a vs. c
626 ($p < 0.001$), and b vs. c ($p < 0.01$), (B) a vs. b ($p < 0.001$), a vs. c ($p < 0.001$), and b vs. c
627 ($p < 0.05$); (C) a vs. b ($p < 0.05$); a vs. c ($p < 0.001$), b vs. c ($p < 0.001$), and (E) a vs. b (p
628 < 0.001), a vs. c ($p < 0.001$), b vs. c ($p < 0.01$).
629

Figure 5. HSP70 inhibitor reversed EAS-induced lipid metabolism and mitochondrial activity in bovine GCs under non-HS and HS conditions.

Cells were treated with or without 10 μM PES at 38.5°C (control, EAS, EAS + PES) or at 41°C (HS, HS + EAS, HS + EAS + PES) for 12 h. Fluorescence staining images of mitochondria, lipid droplets, and HSP70 are shown. Scale bar = 50 μm; original magnification = 63×.

Figure 6. Fluorescence intensity of lipid droplets and mitochondrial activity in bovine GCs treated with EAS under non-HS and HS conditions.

The fluorescence intensity of lipid droplets and mitochondrial activity in cells treated with EAS under non-HS and HS conditions was analyzed by CTCF (A and B, respectively, n = 5). Data are shown as the mean ± S.E.M, (A) a vs. b ($p < 0.05$), a vs. c ($p < 0.001$), and b vs. c ($p < 0.05$), (B) a vs. b ($p < 0.05$), a vs. c ($p < 0.05$), a vs. d ($p < 0.001$), b vs. c ($p < 0.001$), b vs. d ($p < 0.05$), c vs. d ($p < 0.001$).

Highlights

- EAS increases progesterone synthesis by increasing of steroidogenesis-related genes
- EAS increases lipid synthesis and mitochondrial activity
- Synnergistic effect of EAS and heat shock on progesterone synthesis
- HSP70 inhibition decreases EAS-stimulated progesterone synthesis pathway

Graphical abstract

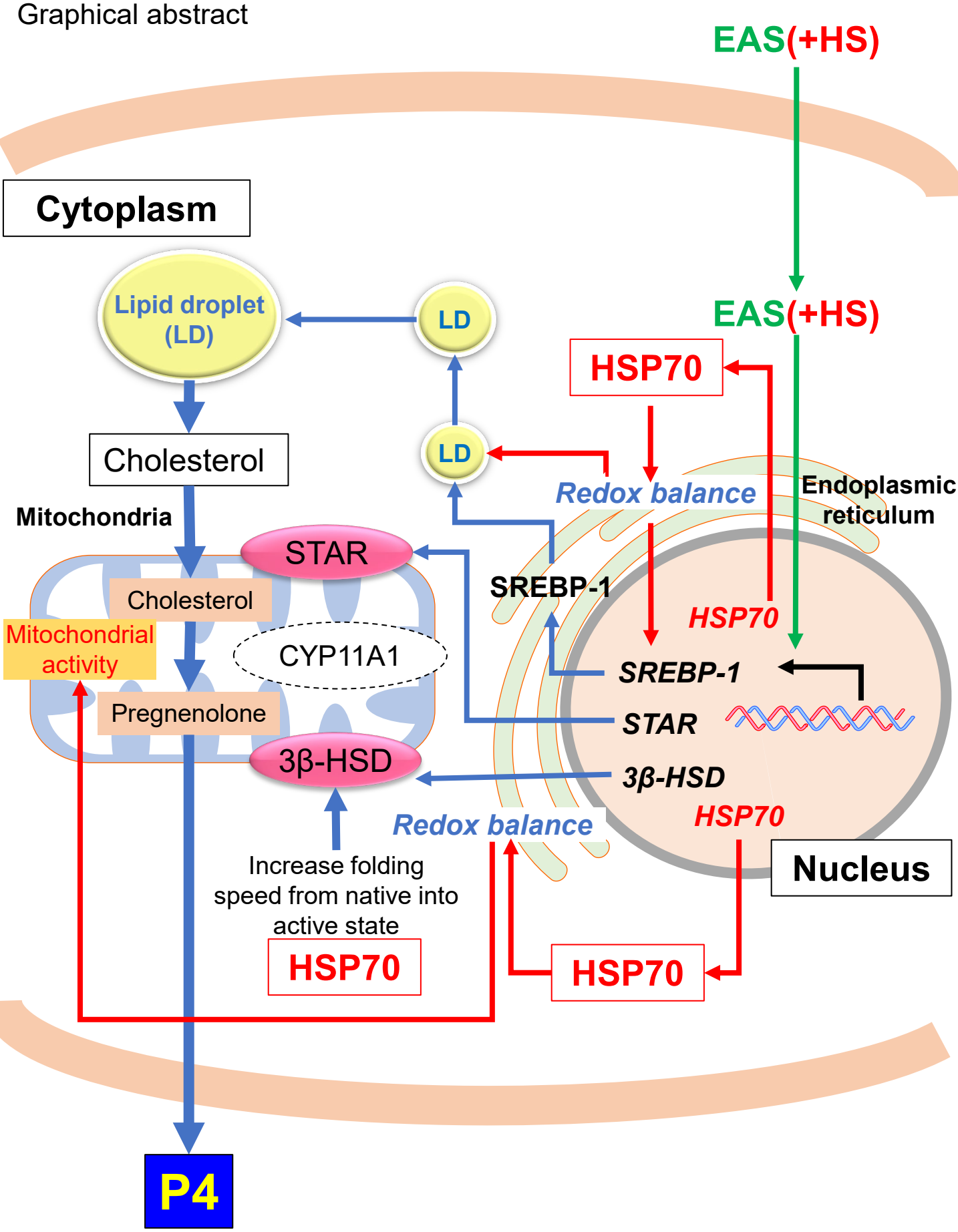


Figure 1

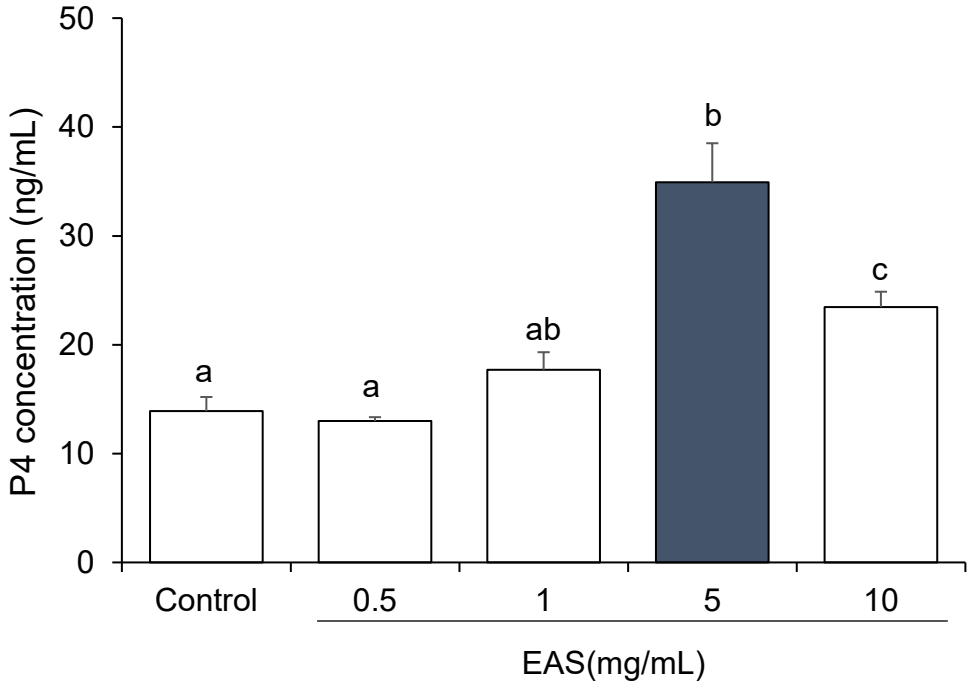

50
40
30
20
10
0
P4 concentration (ng/mL)
a
a
ab
b
c
Control
0.5
1
5
10
EAS(mg/mL)

Figure 2

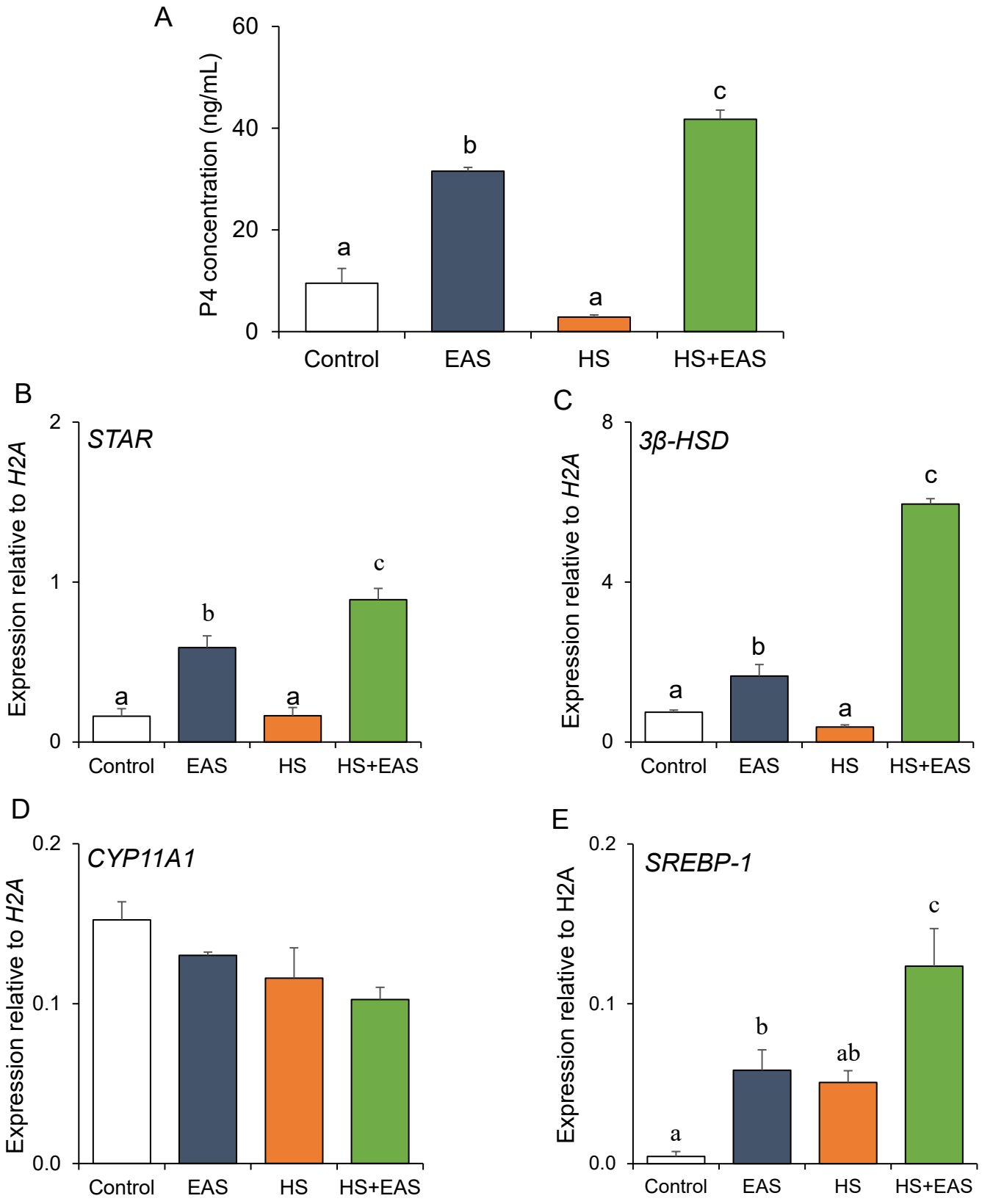
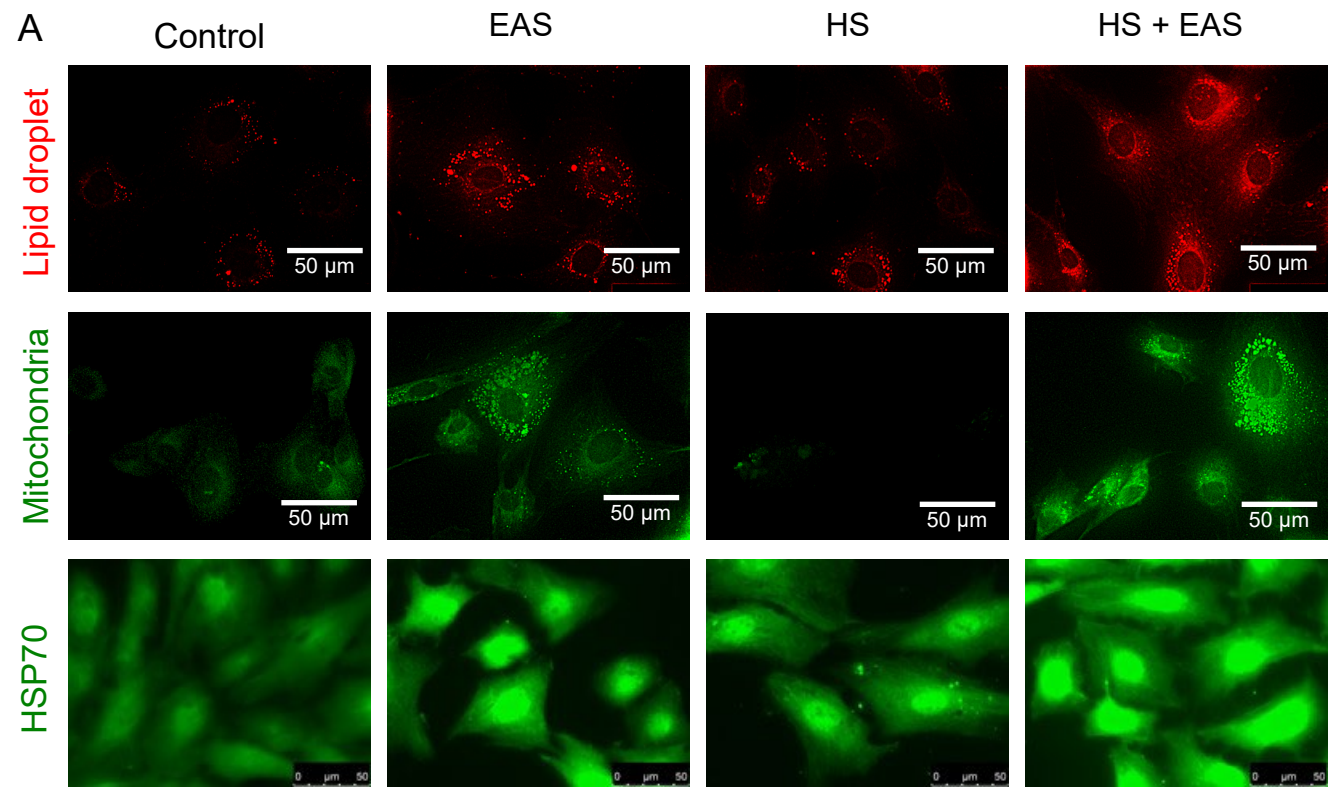
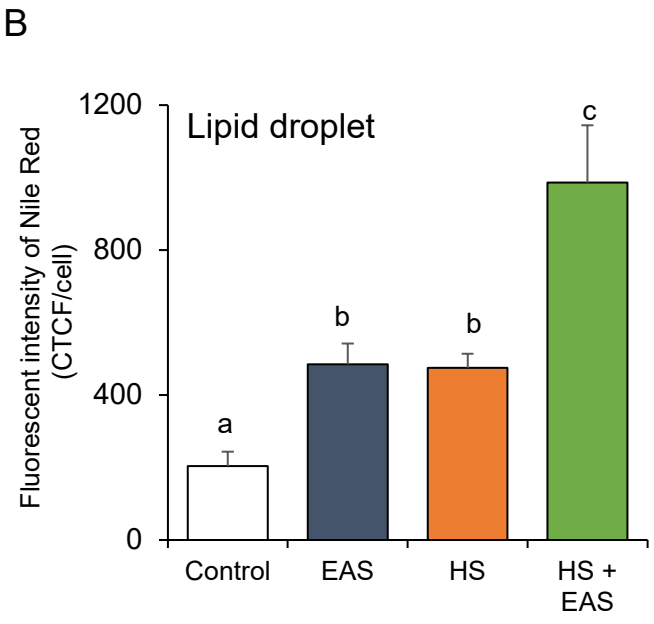

A
P4 concentration (ng/mL)
60
40
20
0
a
b
a
c
Control
EAS
HS
HS+EAS
B
STAR
Expression relative to H2A
2
1
0
a
b
a
c
Control
EAS
HS
HS+EAS
C
3β-HSD
Expression relative to H2A
8
4
0
a
b
a
c
Control
EAS
HS
HS+EAS
D
CYP11A1
Expression relative to H2A
0.2
0.1
0.0
Control
EAS
HS
HS+EAS
E
SREBP-1
Expression relative to H2A
0.2
0.1
0.0
a
b
ab
c
Control
EAS
HS
HS+EAS

Figure 3


A
Control
EAS
HS
HS + EAS
Lipid droplet
Mitochondria
HSP70
50 μm


B
Lipid droplet
Fluorescent intensity of Nile Red (CTCF/cell)
1200
800
400
0
a
b
b
c
Control
EAS
HS
HS + EAS

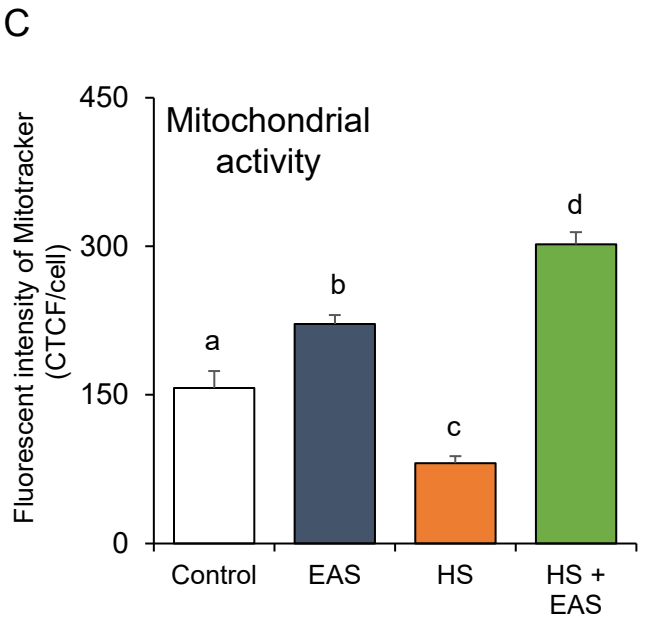

C
Mitochondrial activity
Fluorescent intensity of Mitotracker (CTCF/cell)
450
300
150
0
a
b
c
d
Control
EAS
HS
HS + EAS

Figure 4

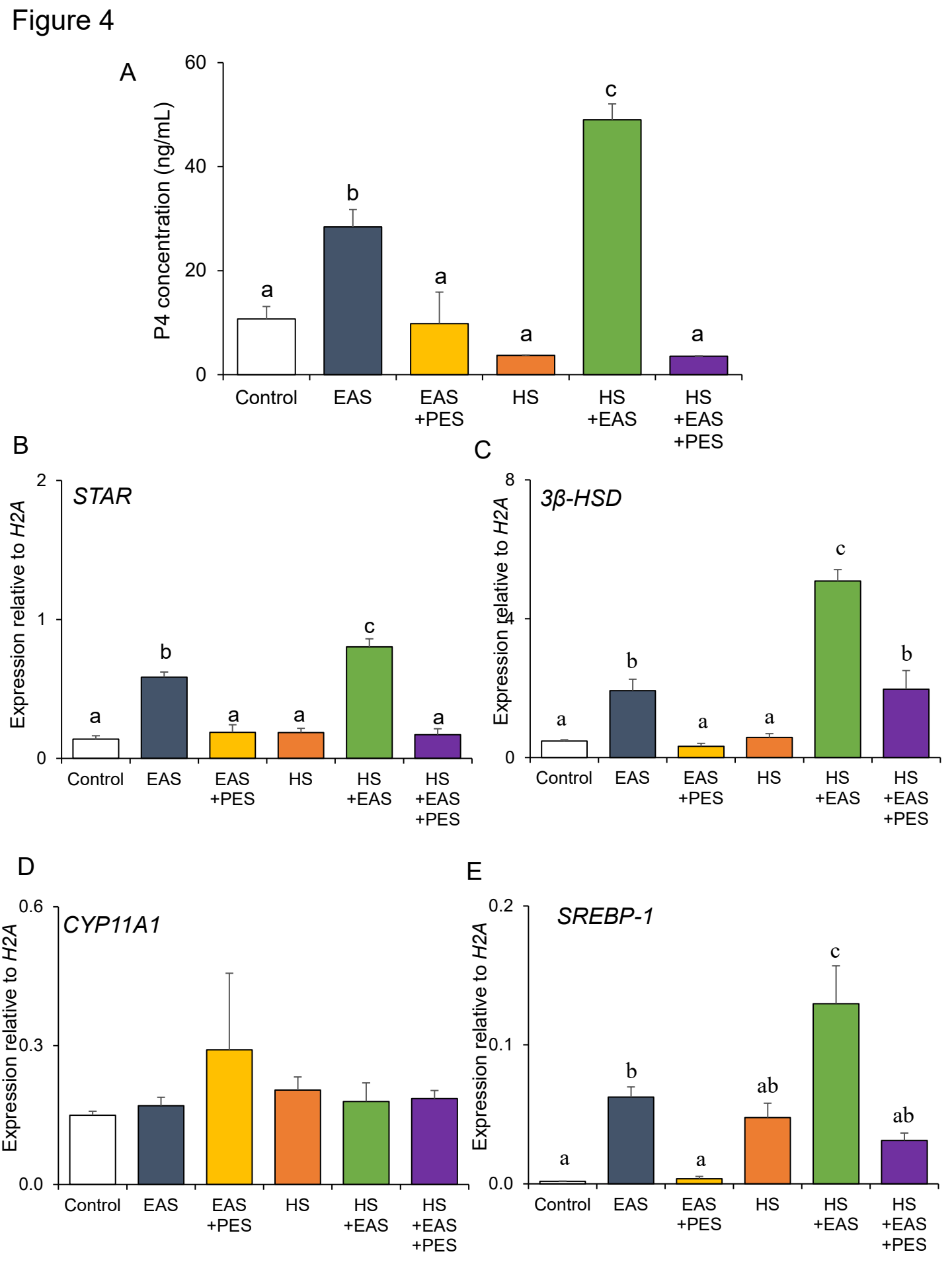

A
P4 concentration (ng/mL)
60
40
20
0
a
b
a
a
c
a
Control
EAS
EAS +PES
HS
HS +EAS
HS +EAS +PES
B
STAR
Expression relative to H2A
2
1
0
a
b
a
a
c
a
C
3β-HSD
Expression relative to H2A
8
4
0
a
b
a
a
c
b
D
CYP11A1
Expression relative to H2A
0.6
0.3
0.0
E
SREBP-1
Expression relative to H2A
0.2
0.1
0.0
a
b
a
ab
c
ab

Figure 5

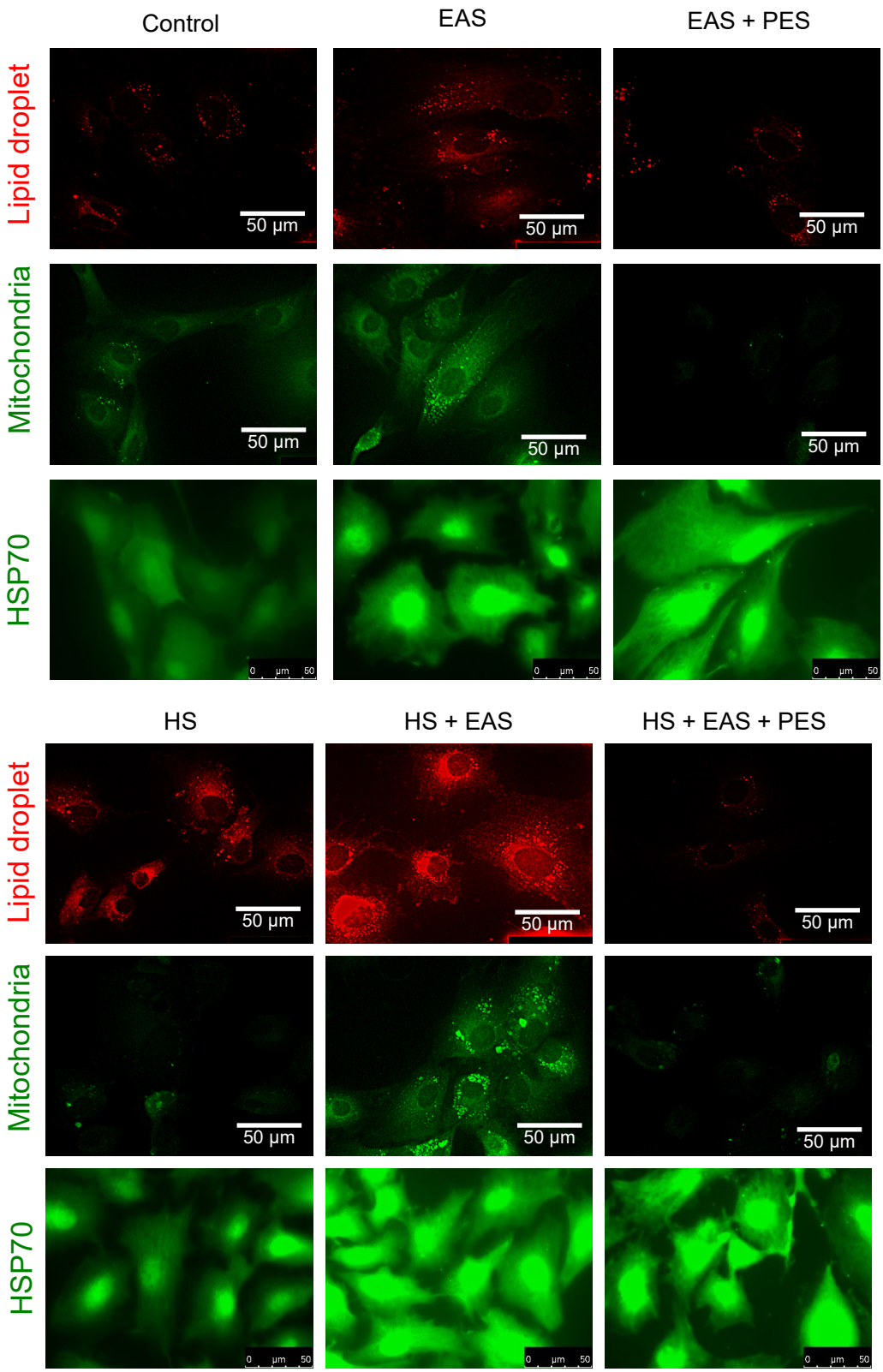
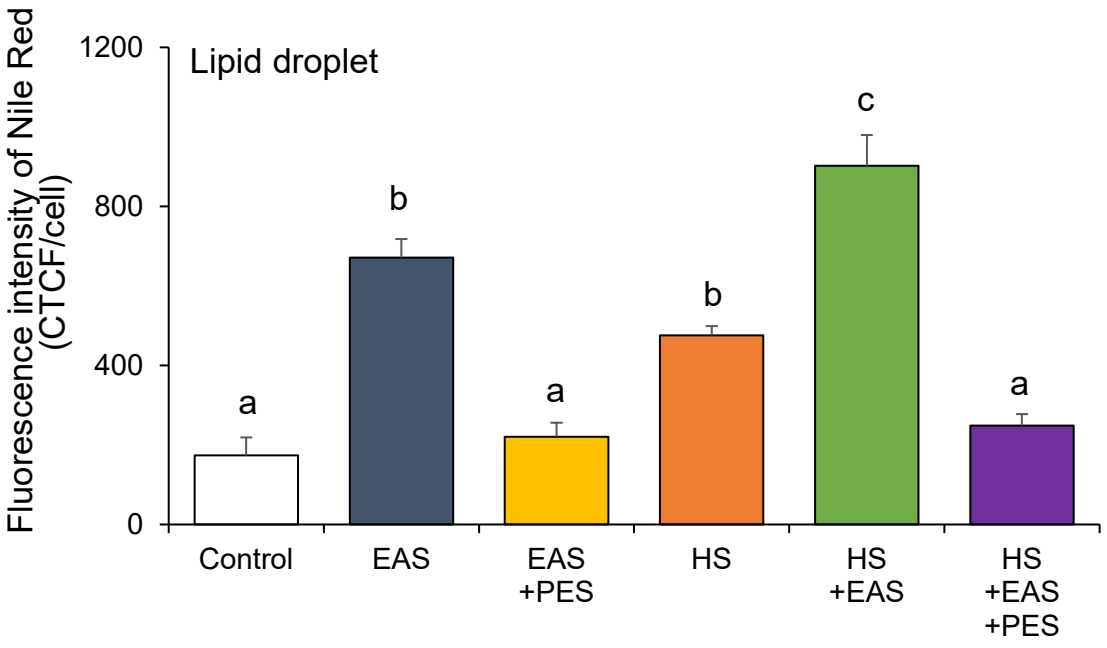
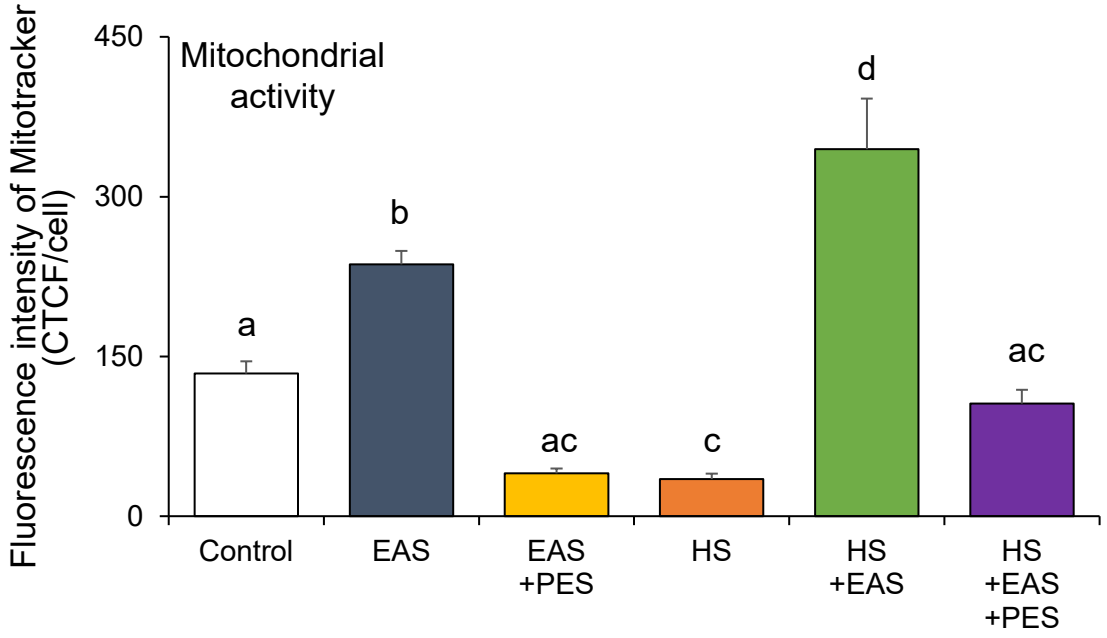

Control
EAS
EAS + PES
Lipid droplet
Mitochondria
HSP70
50 μm
HS
HS + EAS
HS + EAS + PES
Lipid droplet
Mitochondria
HSP70
50 μm

Figure 6


Lipid droplet
Fluorescence intensity of Nile Red (CTCF/cell)
1200
800
400
0
a
b
a
b
c
a
Control
EAS
EAS +PES
HS
HS +EAS
HS +EAS +PES


Mitochondrial activity
Fluorescence intensity of Mitotracker (CTCF/cell)
450
300
150
0
a
b
ac
c
d
ac
Control
EAS
EAS +PES
HS
HS +EAS
HS +EAS +PES