1 Ice plant (*Mesembryanthemum crystallinum*) extract promotes lipolysis in mouse

2 3T3-L1 adipocytes via extracellular signal-regulated kinase activation

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23 ***Keywords****: ATGL, ERK, HSL, MTT, PPARγ, perilipin*

24

25 ***Running title:*** *Lipolysis is induced by Ice plant extract*

26 **ABSTRACT**

27 The anti-obesity effect of ice plant (IP; *Mesembryanthemum crystallinum*), a
28 salt-resistant African plant, has recently attracted increased attention. IP is rich in
29 pinitol, which lowers blood sugar, and myo-inositol, which prevents fatty liver disease.
30 Furthermore, IP can potentially prevent or reduce the symptoms of metabolic syndrome.
31 However, the details of the physiological mechanisms and mechanisms of action of IP
32 are unclear. A previous study by our group demonstrated the capability of IP extract to
33 prevent adipogenesis in 3T3-L1 preadipocytes. Here, we analyzed the physiological
34 function of IP extract on lipolysis in 3T3-L1 cells and the underlying mechanisms of
35 this process. We found that the release of glycerol from cells treated with IP extract
36 increased in an IP dose-dependent manner. IP extract exhibited cytotoxic activity at
37 concentrations above 4 mg/ml. Real-time PCR and western blotting showed that IP
38 extract downregulated peroxisome proliferator activated receptor γ (*PPARγ*), hormone
39 sensitive lipase (*HSL*) and adipose triglyceride lipase (*ATGL*) in a
40 concentration-dependent manner, but did not affect HSL-Ser563, HSL-Ser660, or
41 perilipin phosphorylation. Although the cAMP-dependent protein kinase A
42 (PKA)-specific inhibitor H89 did not affect IP extract-induced lipolysis, the
43 extracellular signal regulated kinase (ERK1/2) inhibitor U0126 significantly abrogated

44 IP extract-activated glycerol release. Furthermore, IP extract strongly enhanced ERK1/2

45 phosphorylation at the concentrations used in the study. These results suggest that IP

46 extract augments lipolysis by enhancing ERK phosphorylation.

47 # 1. INTRODUCTION

48 Lipolysis is an important lipid metabolic function in white adipose tissue (WAT).
49 Under conditions of energy deficiency, accumulated triacylglycerol (TAG) in adipose
50 tissue is immediately hydrolyzed by lipases, which releases glycerol and fatty acids for
51 use as sources of energy.[1] Lipolysis is strictly regulated by the hormones insulin and
52 catecholamines, and among the lipases, hormone-sensitive lipase (HSL) is a particularly
53 important rate-limiting enzyme.[2] The activation of HSL is a multi-step process: (1)
54 catecholamine binds to the G-protein conjugate receptor to activate adenyl cyclase
55 (AC), (2) the activated AC increases the intracellular concentration of cAMP, which
56 activates cAMP-dependent protein kinase A (PKA), and (3) PKA phosphorylates
57 residues Ser563, Ser659, and Ser660 of HSL.[3] The phosphorylated HSL then forms a
58 complex with PKA-phosphorylated perilipin, which induces the hydrolysis of cytosolic
59 TAG- and diacylglycerol (DAG)-containing lipid droplets.[4-6] Perilipin normally inhibits
60 HSL from hydrolyzing lipid droplets and enhances the action of HSL when it is
61 phosphorylated in response to catecholamine stimulation. Recently, increasing evidence
62 has revealed the importance of the extracellular signal-regulated kinase (ERK1/2)
63 pathway for lipolysis activation.[7,8] ERK1/2 can regulate adipocyte lipolysis by
64 phosphorylating HSL on residue Ser 600 and increasing its activity.[9]

65 In recent years, plant-derived physiologically active substances (phytochemicals)
66 have been associated with the reversal and prevention of obesity. The efficacy and
67 mechanisms of action of phytochemicals vary enormously, and each phytochemical can
68 exert a range of physiological actions at various sites of action; caffeine and ephedrine
69 enhance energy consumption, capsaicin and berberine inhibit lipid formation, and
70 flavonoids and conjugated linoleic acid promote lipolysis.[10]

71 In this study, we focused on the anti-obesity effect of ice plant (IP;
72 *Mesembryanthemum crystallinum*). IP, which is native to Africa, is a salt-resistant plant
73 that produces substances useful for controlling the damage by salt and ultraviolet light.
74 IP contains numerous functional substances, including myo-inositol, which acts to
75 prevent fatty liver disease, and pinitol, which lowers blood sugar and increases insulin
76 sensitivity as well as the antioxidants β-cyanins, proline, pantothenic acid, β-carotene,
77 and other flavonoids.[11-16] Previous studies have shown that crude extracts of IP inhibit
78 the differentiation of mouse 3T3-L1 preadipocytes.[17]

79 This study aimed to determine the lipolysis-related physiological functions of IP
80 extract and to elucidate the mechanisms of action of IP by using 3T3-L1 adipocytes.

81

82 # 2. MATERIALS AND METHODS

83 **2.1. Materials**

84 Dried powder made from IP grown in indoor planters (Tsuburina, Nihon Advanced
85 Agri Co., Shiga, Japan) was used in the experiments. IP powder was dissolved in
86 distilled water and passed through a filter paper. The supernatant was filter sterilized,
87 and the IP samples thus obtained were stored at -80 ºC. The concentrations of IP are
88 expressed as the weight of IP dissolved in 1 mL of culture medium. H89 and U0126
89 were purchased from Cayman Chemical (Ann Arbor, MI, USA), dissolved in DMSO,
90 and stored at -80ºC.

91 **2.2. Cell culture**

92 Mouse 3T3-L1 preadipocytes (Institute of Physical and Chemical Research Cell
93 Bank, Tsukuba, Ibaraki, Japan) were cultured in DMEM-high glucose (Dulbecco's
94 modified minimum essential; 4500 mg/L glucose [pH 7.3]) (Sigma-Aldrich, St. Louis,
95 MO, USA) containing 10% fetal bovine serum (Sanko Junyaku, Tokyo, Japan), 100
96 U/mL penicillin (Wako, Osaka, Japan), and 100 μg/mL streptomycin (Meiji Seika,
97 Tokyo, Japan). Cells were cultured in either 24-well plates at 5×10^4 cells/well or 60 mm
98 dishes at 3×10^5 cells/dish and maintained in a 5% CO_2 incubator at 37 °C (NAPCO,
99 New York, USA). After the cells reached confluence, they were cultured for 2 days in
100 DMEM-high glucose culture medium containing DMI (10 μg/mL insulin (Wako), 1 μM

101 dexamethasone (Sigma-Aldrich), and 500 μM 3-isobutyl-1-methylxanthine

102 (Sigma-Aldrich)). After incubation with DMI for 2 days, the cells were cultured for an

103 additional 2 days in DMEM-high glucose containing 5 μg/mL insulin. The culture

104 medium was then exchanged for DMEM-high glucose with no additives. 3T3-L1

105 adipocytes were used at day 10 for further experiments.

106 **2.3. Glycerol release assay**

107 The amount of glycerol released from adipocytes into the culture medium was

108 analyzed using an E-test Wako kit (Wako). Absorbance at 600 nm was measured using a

109 Spectra Max microplate reader (Spectra Max190; Molecular Devices Corporation) and

110 converted to the amount of glycerol. Total protein was measured using a BCA Protein

111 Assay Kit (Reducing Agent Compatible; Wako).

112 **Cell viability assay**

113 Thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) was used to measure the

114 cytotoxicity of IP.[18] Briefly, 10 day-old 3T3-L1 adipocytes, cultured in 24-well plates,

115 were incubated with IP extract (0-5 mg/mL) for 48 h. MTT (5 mg/mL) was added to the

116 culture medium at a ratio of 1:10, and the cells were cultured for an additional 3 h in the

117 CO_2 incubator. The culture medium was then removed, formazan was dissolved in 0.04

118 mol/L HCl/isopropanol, and the absorbance was measured at 570 nm.

119 **2.5. Real-time PCR analysis**

120 RNA extraction was performed according to the acid

121 guanidinium-phenol-chloroform method.[19] After treatment with DNase I (Takara Bio,

122 Otsu, Shiga, Japan), M-MLV reverse transcriptase (Takara) was used to synthesize

123 cDNA. Real-time PCR was performed using the KAPA SYBR FAST qPCR Kit (Kapa

124 Biosystems, Boston, MA, USA). cDNA was amplified using 40 cycles of 3 min at 95°C,

125 3 s at 95°C, and 20 s at 60°C. The following primers were used: β-actin

126 (5′-AGATGTGGATCAGCAAGCAGG-3′ and

127 5′-AACGCAGCTCAGTAACAGTCC-3′), peroxisome proliferator activated receptor *γ*

128 (*PPARγ*) (5′-AAACTCTGGGAGATTCTCCT-3′ and

129 5′-TGGCATCTCTGTGTCAAC-3′), *HSL* (5′-GCTGTCTGAAGGCTCTGAGTTGC-3′

130 and 5′-AAGACCACATCGCCCACAGC-3′), and adipose triglyceride lipase (*ATGL*)

131 (5′-TGCTGGAGGCCTGTGTGGAA-3′ and

132 5′-TCAGGGACATCAGGCAGCCACT-3′). β-actin was used as an internal control.

133 **2.6. Western blot analysis**

134 3T3-L1 adipocytes were harvested in RIPA buffer (50 mmol/L Tris-HCl (pH 7.4),

135 150 mmol/L NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% NP40, 1 mmol/L EDTA, 2

136 mmol/L NaF, and 1 mmol/L Na_3VO_4). After protein transfer, the membranes were

137 blocked for 1 h in TBS-T buffer containing 2% BSA. Then, primary antibodies
138 (anti-HSL563, anti-HSL660, anti-HSL, anti-ATGL, anti-perilipin, anti-pERK,
139 anti-ERK, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell
140 Signaling Technology), dissolved in TBS-T buffer, were added for overnight incubation.
141 The LuminoGLO reagent (Cell Signaling Technology) was used for protein detection.
142 The protein bands were analyzed using chemiluminescence (EZ-Capture MG, ATTO,
143 Tokyo, Japan). The band intensity was analyzed using ImageJ analysis software.

144 **2.7. Statistical analysis**

145 Results are expressed as means ± S.D. Statistical analysis was performed using one
146 way or two way analysis of variance (ANOVA) followed by the Neuman–Keuls post
147 hoc test, or the student unpaired *t*-test. P values below 0.05 were considered significant.

148

149 # 3. RESULTS

150 **3.1. Lipolytic effect of IP extract**

151 The physiological effects of IP extract on lipid storage in differentiated adipocytes
152 were analyzed. Ten days after differentiation was induced, 3T3-L1 adipocytes were
153 cultured in the presence of IP extract (0–1.5 mg/mL). After incubation with IP for 12 or
154 24 h, the amount of glycerol released into the culture medium was measured (Figure 1).

155 The addition of IP extract increased the glycerol content of the culture medium in a
156 time- and concentration-dependent manner. Thus, IP extract promotes lipolysis in
157 differentiated adipocytes. Next, we investigated the effect of IP extract on cell viability.
158 Adipocytes differentiated for 10 days were treated with IP extract (0–5 mg/mL) for 48
159 h, and cell viability was measured using an MTT assay (Figure 1). Cell viability
160 decreased significantly at IP extract concentrations at or above 4 mg/mL. However, cell
161 viability was largely unaffected at IP extract concentrations at or below 3 mg/mL.
162 Therefore, subsequent experiments were performed using IP extract concentrations
163 below 3 mg/mL.

164 **3.2. IP extract induced changes in *PPARγ*, *ATGL* and *HSL* gene expression**

165 PPARγ is involved not only in lipid formation but also in regulating the expression
166 of the *HSL* and *ATGL* genes, which are responsible for lipolysis.[20] Figure 2 shows the
167 mRNA expression levels of *PPARγ*, *ATGL* and *HSL*, which were dramatically lower in
168 growth media with added IP extract. Moreover, western blot analysis showed lower
169 ATGL protein expression (Figure 3A).

170 **3.3. IP extract has no effect on HSL and perilipin phosphorylation**

171 HSL activity is strictly controlled by phosphorylation. cAMP-activated PKA
172 phosphorylates Ser563, Ser659, and Ser660 of HSL, whereupon HSL hydrolyzes TAG

173 and DAG.[21,3] Figure 3 shows the western blot analysis of perilipin phosphorylation and
174 HSL phosphorylation (Ser563 and Ser660 residues). IP extract did not enhance the
175 phosphorylation of perilipin (Figure 3A). Additionally, no significant difference was
176 observed in HSL phosphorylation at the Ser563 and Ser660 residues after IP extract
177 addition (Figure 3B). In contrast, isoproterenol, which was used as a positive control,
178 clearly increased the amount of the perilipin isoform at 67 kDa, reflecting an increase in
179 phosphorylation and activation, and enhanced the phosphorylation of HSL at both sites
180 (Ser563 and Ser660).

181 **3.4. Suppression of IP lipolysis by the ERK1/2 inhibitor U0126**

182 HSL enzyme activity is regulated by phosphorylation; HSL can be activated by
183 phosphorylation via the PKA and ERK pathways. Therefore, we investigated the effect
184 of PKA and ERK1/2 pathway inhibitors (H89 and U126, respectively) on the lipolytic
185 effect of IP extract. H89 is a selective inhibitor of PKA, while U0126 selectively
186 inhibits MAPK/ERK1/2 kinase (MEK), which activates ERK1/2. Differentiated
187 adipocytes were cultured for 1 h in the presence of either H89 (10 μM) or U0126 (50
188 μM). IP extract (0-1.5 mg/mL) was then added to the adipocytes. After incubation for
189 12 and 24 h, the glycerol content of the culture medium was measured. The
190 lipolysis-enhancing effect of IP extract was not reduced by H89 (Figure 4A). However,

191 U0126 significantly inhibited IP extract-induced lipolysis. Consistent with the inhibitor
192 assay, western blot analysis indicated that IP extract strongly induced the
193 phosphorylation of ERK1/2 kinase in a concentration-dependent manner (Figure 4B).
194 This IP extract-induced phosphorylation was effectively attenuated by the U0126
195 inhibitor. These data indicate that the ERK1/2 phosphorylation pathway is involved in
196 IP extract-enhanced lipolysis.
197

198 ## 4. DISCUSSION

199 In this study, we showed that IP extract enhances lipolysis in mature adipocytes, and
200 is not cytotoxic (Figure 1). We also showed that IP extract is involved in lipolysis by
201 enhancing ERK1/2 kinase phosphorylation (Figure 4).
202 *ATGL* expression is increased by PPAR agonists and glucocorticoids, whereas
203 insulin decreases its expression. Recent findings showed that mTOR complex 1
204 (mTORC1)-dependent signaling reduces *ATGL* expression.[22] Inversely, activation of
205 FoxO1 by SIRT1-induced deacetylation activates lipolysis by increasing *ATGL*
206 expression.[23] However, the abundance of *ATGL* and *HSL* expression does not always
207 correlate with cellular lipolytic activity.[24] Thus, cellular lipase expressions are
208 inadequate as indicators of enzyme activities. In fact, IP extract used at 1.5 mg/mL

209 decreases the expression of *PPARγ*, *ATGL* and *HSL* expressions.

210 HSL is normally present in the cytoplasm.[27] In this study, HSL was phosphorylated
211 by PKA at three residues (Ser563, Ser659, and Ser660) or by ERK1/2 at one residue
212 (Ser600), after which it localizes to lipid droplets.[9,25] After localization to lipid droplets,
213 the hydrolytic activity of HSL on TAG and DAG increases.[2] ERK1/2 is a
214 serine/threonine kinase that is stimulated by cytokines such as insulin and tumor
215 necrosis factor α (TNFα), growth factors, oxidative stress, and other factors, and it is
216 activated via MEK.[26] H89, which was used in this study, selectively suppresses PKA,
217 while U0126 directly inhibits MEK activity. Our results show that although the IP
218 extract has a negative effect on the expression of *HSL*, it has no effect on the
219 phosphorylation and activation of perilipin and HSL at Ser563 and Ser660. In
220 agreement with this finding, H89 did not attenuate IP extract-induced lipolysis. Between
221 the inhibitors used, only U0126 had the capability to abolish IP extract-enhanced
222 lipolysis; IP extract enhanced the phosphorylation of ERK1/2 in a
223 concentration-dependent manner, and this phosphorylation was significantly attenuated
224 by U0126.

225 HSL activity can also be affected by inhibitory pathways. The Ser656 residue of
226 HSL can be phosphorylated by AMP-activated protein kinase (AMPK), which inhibits

227 HSL activity.[27] Previous studies have demonstrated the existence of cytokines, such as
228 TNFα, resistin, interleukin 6, leptin, and ciliary neurotrophic factor, that inhibit as well
229 as activate AMPK, and the regulation of lipolysis by these cytokines was the focus of a
230 previous study.[28] AMPK is also an important factor in the regulation of lipolysis, and it
231 would be interesting to determine whether IP is involved in regulating AMPK activity.

232 Interestingly, a recent report has demonstrated that mTORC1 is also associated with
233 lipolysis.[22] Ras homolog enriched in brain (Rheb) acts on mTOR as a positive
234 regulatory factor. 3T3-L1 cells that have undergone transformation to enable the stable
235 expression of Rheb release smaller amounts of glycerol. The mTOR inhibitor rapamycin
236 enhances TAG hydrolysis and fatty acid release in 3T3-L1 cells.[29] Another study
237 demonstrated the positive effect of Trans-10, cis-12 CLA on lipolysis via convergent
238 increase of mTOR and ERK1/2 activation in human adipocytes.[30] Recently, it was
239 reported that the control of ERK1/2 phosphorylation is closely related to the
240 Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII), and inhibition of CaMKII
241 blunted ERK1/2 phosphorylation and glycerol release.[31] It is possible that IP extract,
242 which acts to suppress adipocyte differentiation and enhance lipolysis, is involved in the
243 mTOR signaling pathway or in CaMKII phosphorylation. We are currently investigating
244 the mechanism of action of IP extract on the mTOR and CaMKII signaling pathways.

In this study, we showed that IP extract acts on differentiated mature adipocytes to enhance the hydrolysis of lipids. As mentioned above, IP has been found to contain a large amount of pinitol, myo-inositol as well as antioxidant compounds. Two independent studies related to lipid metabolism demonstrated that pinitol has no effect on adipogenesis in 3T3-L1 preadipocytes, whereas, myo-inositol increased lipid accumulation and reduced basal lipolysis in the same cell line.[33,34] For this reason, future studies are needed to isolate and identify the functional substances endowed with anti-adipogeneic and lipolytic effects of IP extract, as well as in vivo analysis of their bioavailability. It is possible to expand on the results of this study and examine the use of IP as an effective functional food to prevent and treat metabolic syndrome.

ACKNOWLEDGMENTS

This work was supported by a grant for Science and Education for the University of Tsukuba.

AUTHOR DISCLOSURE STATEMENT

The authors have no conflicts of interest to declare.

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361
362

363 **FIGURE LEGENDS**

364 **Figure 1. Effect of IP extract on adipocyte lipolysis and viability**

365 3T3-L1 preadipocytes were cultured for 10 days after induction with DMI. IP extract
366 (0–1.5 mg/mL) was added, and after treatment for 12 or 24 h, absorption at 600 nm was
367 measured to quantify the glycerol content of the culture media. The glycerol content
368 was standardized according to the total protein content. The graph shows values relative
369 to the control. Results are representative of 3 independent experiments with n = 3 for
370 each. The error bars indicate SD. For statistical analysis, two ways ANOVA, followed
371 by the Newman-Keuls post hoc test was used. *a, b,* and *c* indicate significant difference
372 at P < 0.05 between different treatments at 12 h; *d, e,* and *f* indicate significant
373 difference at P < 0.05 between different treatments at 24 h. * shows significant
374 difference at $P < 0.05$ among treatment at same concentration (A). IP extract (0–5.0
375 mg/mL) was added, and after treatment for 48 h, the MTT assay was used to measure
376 cell viability. The graph shows percentages relative to the control. Results are
377 representative of 3 independent experiments with n = 3 for each. The error bars indicate
378 SD. *$P < 0.05$, vs. the control was regarded as statistically significant by using Student
379 *t*-test (B).

380

381 **Figure 2. Effect of IP extract on gene expression**

382 3T3-L1 cells were differentiated for 10 days. Then, IP extract (0–1.5 mg/mL) was
383 added, and the cells were incubated for 24 h before gene expression was investigated
384 using real-time PCR. The graph shows the values relative to the control. Results are
385 representative of 3 independent experiments. The error bars indicate SD. *$P < 0.05$ vs.
386 the control was regarded as statistically significant (Student *t*-test).

387

388 **Figure 3. Effect of IP extract on ATGL expression, perilipin phosphorylation, and**
389 **HSL phosphorylation**

390 3T3-L1 cells were cultured for 10 days after induction with DMI. Then, IP extract (0–
391 1.5 mg/mL) or isoproterenol (30 nmol/L) was added, and the cells were incubated for
392 12 or 24 h. ATGL expression, perilipin phosphorylation (A), and HSL phosphorylation
393 (B) were investigated using western blot analysis. The intensity of the detected bands
394 was converted to numerical values using ImageJ. The error bars indicate the SD of 3
395 independent experiments. *$P < 0.05$, **$P < 0.01$, §$P < 0.05$, and §§$P < 0.01$ vs. the
396 control of each group were regarded as statistically significant (Student *t*-test).

397

398 **Figure 4. Effect of PKA inhibitor (H89) and ERK inhibitor (U0126) on IP**

399 **extract-stimulated lipolysis (A) and ERK phosphorylation (B)**

400 3T3-L1 preadipocytes were cultured for 10 days after induction with DMI. H89 or
401 U0126 was added to the culture medium, and the cells were cultured for 1 h. Then, IP
402 extract (1.5 mg/mL) was added to the culture medium, and the cells were incubated for
403 12 or 24 h before the amount of glycerol released into the culture medium was
404 measured. The graph shows values relative to the control. Results are representative of 3
405 independent experiments with n = 3 for each. The error bars indicate SD. For statistical
406 analysis, two ways ANOVA, followed by the Newman-Keuls post hoc test was used. *
407 $P < 0.05$, vs. the IP (0.75 mg/mL) treated cells at same time; and $^{\#}P < 0.05$, vs. the IP
408 (1.5mg/mL) treated cells at the same time, were regarded as statistically significant (A).
409 ERK phosphorylation was investigated using western blot analysis. Results are
410 representative of 3 independent experiments. The error bars indicate SD. *$P < 0.05$, **P
411 < 0.01, §$P < 0.05$, and §§$P < 0.01$ vs. the control of each group were regarded as
412 statistically significant. ¤¤$P < 0.01$, Ħ$P < 0.01$ vs. the IP extract (1.5 mg/mL) treatment
413 in each group was regarded as statistically significant (Student *t*-test) (B).