学位論文

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# Effect of TNF-α on the expression of ABCA1 in pancreatic β-cells

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16 **Abstract**

17 ATP-binding cassette transporter A1 (ABCA1), a 254-kD membrane protein, is a
18 key regulator of lipid efflux from cells to apolipoproteins. ABCA1 in pancreatic β-cells
19 influences insulin secretion and cholesterol homeostasis. Tumor necrosis factor (TNF)-α
20 is a pleiotropic cytokine that elicits a wide spectrum of physiological events, including
21 cell proliferation, differentiation, and apoptosis, and is also known to decrease glucose-
22 dependent insulin secretion in pancreatic islets. In the present study, we examined the role
23 of TNF-α on ABCA1 expression in rat pancreatic islets and INS-1 cells. ABCA1 protein
24 levels decreased in response to rising concentrations of TNF-α in pancreatic islets. Real-
25 time polymerase chain reaction analysis showed a significant decrease in ABCA1 mRNA
26 expression. In parallel with its effect on endogenous ABCA1 mRNA levels, TNF-α
27 suppressed the activity of a reporter construct containing the ABCA1 promoter. This
28 effect was abrogated by BIRB796, but not by SB203580 or LY-294002. The constitutively
29 active form of p38 mitogen-activated protein kinase (MAPK) γ suppressed ABCA1
30 promoter activity but not p38-MAPK (α, β), while a dominant-negative mutant of p38-
31 MAPK γ blocked the effect of TNF-α on ABCA1 promoter activity. BIRB796 inhibited

the increased cholesterol ester content induced by TNF-α. BIRB796 cancelled the decreased insulin content nor ABCA1 suppression caused by TNF-α in INS-1 cells. We checked the influence of TNF-α of insulin secretion and glucose-stimulated insulin secretion in rat pancreatic islet and INS-1 cell. TNF-α suppressed the insulin secretion and glucose-stimulated insulin secretion in both rat pancreatic islet and INS-1 cell. In summary, TNF-α suppressed the expression of endogenous ABCA1 and suppres the insulin secretion in pancreatic islets and INS-1 cells. These findings raise the possibility that TNF-α may affect insulin secretion by controlling ABCA1 expression.

**Key words**

tumor necrosis factor-α, ATP-binding cassette transporter A1, p38MAPK, pancreatic β cells, cholesterol accumulation

45 **Introduction**

46 Tumor necrosis factor (TNF)-α is a multifunctional pro-inflammatory cytokine that
47 is associated with some pathological processes such as apoptosis, proliferation,
48 inflammation, and immunoregulation. A previous report indicated that TNF-α played a
49 role in impaired secretion of insulin, which frequently occurs in type 2 diabetes (Donath
50 *et al.* 2003). In pancreatic β-cells, TNF-α alone inhibited glucose-stimulated insulin
51 secretion (GSIS) (Zhang & Kim 1995; Dunger *et al.* 1996; Tsiotra *et al.* 2001). In the rat
52 pancreatic β-cell line, INS-1, most molecules involved in all steps of insulin secretion,
53 from glucose sensing to insulin exocytosis, were not quantitatively altered by TNF-α
54 treatment, nor was the expression of insulin and PDX-1 decreased. There was little
55 change in the glucose-stimulated ATP level between TNF-α-treated and -untreated INS-1
56 cells, demonstrating that TNF-α does not affect glucose metabolism (Tsiotra *et al.* 2001;
57 Kim *et al.* 2008).

58 A previous report indicated that TNF-α inhibited GSIS by reducing glucose-
59 stimulated Ca2+ influx, possibly through the activation of c-Jun N-terminal kinase (JNK)
60 and p38 mitogen-activated protein kinase (MAPK), and NF-κB inflammatory signals.

61 Thus, our findings suggest that the activation of stress and inflammatory signals can
62 contribute to the inhibition of GSIS during the development of diabetes (Kim *et al.* 2008).
63 However, the detailed mechanisms have not been clarified.
64 Type 2 diabetes is characterized by progressive β-cell dysfunction and loss of β-cell
65 mass. The reasons for β-cell dysfunction in type 2 diabetes are incompletely understood.
66 Recently, abnormalities in cholesterol metabolism have emerged as a potential
67 contributor to β-cell dysfunction (Brunham *et al.* 2008; Kruit *et al.* 2010). In pancreatic
68 β-cells, lipotoxicity induced by cholesterol accumulation results in apoptosis and
69 impaired insulin secretion (Unger 1995; Zhou & Grill 1995). ATP-binding cassette
70 transporter A1 (ABCA1), a 254-kDa membrane protein, is a pivotal regulator of lipid
71 efflux from cells to apolipoproteins and plays an important role in reverse cholesterol
72 transport (Fielding & Fielding 1995). Previously, it was reported that specific inactivation
73 of the ABCA1 gene in β-cells induced impaired glucose tolerance and defective insulin
74 secretion in mice, but normal insulin sensitivity was retained (Brunham *et al.* 2007). The
75 absence of ABCA1 in pancreatic islets altered cholesterol homeostasis and impaired
76 insulin secretion in vitro, indicating that cholesterol accumulation may contribute to β-

77 cell dysfunction in type 2 diabetes (Brunham *et al.* 2008).

78 In this study, we hypothesized that TNF-α might increase cholesterol accumulation

79 via the ABCA1 transporter to induce lipotoxicity in pancreatic β-cells, thus attenuating

80 insulin secretion.

81

82 **Materials and Method**

83 Cell culture

84 The INS-1 cells originated from a rat insulinoma cell line developed and propagated

85 at the Division of Biochimie Cliniqe (courtesy of C. B. Wollheim, Geneva, Switzerland).

86 The present experiments were performed using cell passages 9~35, the cells being

87 trypsinized every 7 days. These cells were cultured in RPMI1640 media (SIGAMA,

88 Tokyo, Japan) containing 11.2 mmol/l glucose and supplemented with 10% heat-

89 inactivated fetal bovine serum (Dainippon Pharmaceutical Co., Ltd. Tokyo, Japan), 50

90 μmol/l 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells

91 were incubated in humidified 5% CO2 at 37°C. When 80% confluent, the cells were

92 washed twice and incubated with 0.5% fetal bovine serum RPMI 1640 media for 6 h.

93 Then the cells were treated with varying doses of angiotensin II for 24 h before harvesting

94 as described previously (Yu *et al.* 2004).

95

96 Western blot analysis

97 The 15-30 μg proteins were separated by a 7.5% SDS-PAGE and transferred to

98 polyvinylidene difluoride membrane for immunoblotting. After blocking with 7.5% skim

99 milk in PBS (pH 7.2) overnight at 4°C, the membrane was incubated with 0.1% Tween

100 20 in PBS (PBS-T) containing the anti-ABCA1 antibody, anti-insulin (Santa Cruz

101 Biotechnology Inc; diluted to 1:200) overnight at 4°C or anti-GAPDH antibody (Biomol

102 Research, Plymouth Meeting, PA; diluted to 1:5000) for 1 h at room temperature as

103 described previously (Nishiuchi *et al.* 2010). Membrane was washed three times with

104 PBS-T, 10 min each, and then incubated for 1h at room temperature with the appropriate

105 horseradish peroxidase-linked secondary antibody (DakoCytomation; diluted to 1:2000).

106 Membranes were again washed three times, 10 min each, and antigen-antibody

107 complexes were visualized by ECL (GE Healthcare).

108

109 Real-time PCR

110 PCR was performed with a final volume of 20 µl in LightCycler (Roche, Mannheim,

111 Germany) glass capillaries. The sequences of the forward and reverse ABCA1 primers

112 were 5'-CCCGGCGGAGTAGAAAGG-3' and 5'-AGGGCGATGCAAACAAAGAC-3',

113 respectively. Each set of PCR reactions included water as a negative control and 5

114 dilutions of the standard. Known amounts of DNA were then diluted to make the

115 standards, and a regression curve of crossing points versus concentration was generated

116 with LightCycler as described previously (Miyai *et al.* 2011). GAPDH was used as the

117 housekeeping standard.

118

119 Transfection of INS-1 cells and luciferase reporter gene assay

120 We used a construct (pABCA1-LUC) containing the human ABCA1 promoter

121 obtained using PCR and the luciferase reporter gene as previously described (Nagao *et al.*

122 2006). Purified promoter plasmid was transfected into INS-1 using Lipofectamine (Life

123 Technologies, Gaithersburg, MD). Transfected cells were maintained in medium

124 containing 20ng/ml TNF for 24 h with or without pre-treatment with LY (LY294002,

125 10μmol/l), PD (PD98095, 10μmol/l), SB (SB203580, 1μmol/l), H-89 (1μmol/l) or

126 STO609 (1μg/ml) to separately inhibit the PI3K, MEK, p38MAPK, PKA, or CaMKK

127 signaling pathway for 30 min (Yu *et al.* 2010). Then transfected cells were harvested, and

128 the promoter activity of ABCA1 was measured in an aliquot of the cytoplasmic

129 preparation. 40μL aliquots were taken for the luciferase assay, which was performed

130 according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

131

132 Plasmid preparation.

133 An expression vector encoding a constitu- tively active p38 MAPK (α, β, γ) and a

134 dominant-negative mutant of p38 MAPK (p38-DN) were kindly provided by Dr. Z. Wu

135 (Hong Kong University of Science & Technology) as described previously (Murao *et al.*

136 2008).

137

138 Cholesterol content assay

139 To measure cellular cholesterol content concentration, we used a colorimetric assay

140 that utilizes reagents widely used for the measurement of cholesterol in conjunction with

141 a random-access chemistry analyzer ARCHITECT c8000 as described previously (Lyu *et*
142 *al.* 2016).
143
144 Glucose-stimulated insulin secretion (GSIS)
145 Pancreatic islets or INS-1 cells treated with varying treatments were starved in
146 Krebs-Ringer bicarbonate (KRB) buffer containing 120 mM NaCl, 5 mM KCl, 1.1 mM
147 MgCl2, 2.5 mM CaCl2, 25 mM NaHCO3 and 0.1% bovine serum albumin (pH 7.4) for
148 1 h. Following, cells were incubated in KRB buffer containing 3.3 mM glucose for 1 h
149 and then the medium was replaced by KRB buffer supplemented with varying glucose
150 concentration (basal 3.3 mM;stimulatory 16.7 mM) together with other test regents. After
151 1 h incubation, the supernatant was harvested and used for insulin measurement by
152 ELISA kit (Shibayagi, Japan). All the incubation was performed in 5% CO2 incubator at
153 37°C.
154
155 Animals
156 All procedures involving animals were in accordance with Japanese laws and

157 approved by the Animal Care Committee of Kagawa University. 7-week-old Sprague

158 Dawley （SD） rats were purchased from UNIMEDIA and housed at controlled

159 temperature (25°C) and lighting (12 h light/dark cycles) in compliance with the Guide for

160 Experimental Animal Research. After 1-week adaptation, animals were sacrificed, and

161 pancreas was extracted.Pancreatic islets isolationIsolation of pancreatic islets from rat

162 was performed as described previously(Hellman *et al.* 2012). Briefly, the pancreas was

163 digested by collagenase type XI at 37°C for 15 min and then islets were purified and

164 collected by Histopaque1077. Finally, islets (100-200 μm) were picked and placed

165 RPMI1640 media supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml

166 penicillin, and 0.1 mg/ml streptomycin in 5% CO2 incubator at 37°C.

167

168 Statistical analysis

169 Data was expressed as mean ± SEM. Results were analyzed by one-way ANOVA

170 and Student's t test. A value of $P < 0.05$ was considered statistically being significant.

171 All experiments were performed at least three times.

172

173 **Results**

174 Glucose-stimulated insulin secretion was reduced by TNF-α

175 Previous report suggested that accumulation of cholesterol in pancreatic beta cells

176 resulted in impaired insulin secretion. Thus, we checked the abundance of insulin

177 secretion in INS-1 cells treated with TNF-α. As shown in Fig. 1A, TNF-α significantly

178 decreased the amount of insulin. Following, glucose-stimulated insulin secretion (GSIS)

179 assay showed that high glucose (16.7 mM) increased insulin secretion to compared with

180 basal level (3.3 mM, Fig. 1B). INS-1 cells treated with TNF-α were not able to release

181 insulin response to high glucose stimulation (Fig. 1B). These data demonstrated that TNF-

182 α decreased glucose-stimulated insulin secretion in pancreatic beta cells. So, we measured

183 the insulin secretion in rat pancreatic islet treated with TNF-α. TNF-α significantly

184 decreased the amount of insulin in rat pancreatic islet (Fig 1C). It also suppressed the

185 GSIS in rat pancreatic islet treated with TNF-α (Fig 1D).

186

187 TNF-α decreased the expression of ABCA1 in rat pancreatic islets

188 We examined the effect of TNF-α on the expression of ABCA1 in pancreatic islets

189 from rats. Both the protein and mRNA expression of ABCA1 was significantly decreased

190 by treatment with TNF-α (Fig. 2).

191 Concentration-response and time course of TNF-α-mediated suppression of ABCA1 in

192 the rat pancreatic β-cell line, INS-1

193 Total protein was isolated from INS-1 cells treated with varying concentrations

194 (0~20 ng/mL) or varying incubation periods (0~72 h) of TNF-α. Protein samples were

195 subjected to western blotting to detect the expression of ABCA1. These analyses (Fig.

196 3A) showed a decreased abundance of ABCA1 protein in response to rising

197 concentrations of TNF-α, with maximum induction observed at 20 ng/mL. The time

198 course of changes in ABCA1 protein levels were also analyzed up to 72 h after treatment

199 with TNF-α by western blot analysis. In pancreatic islets, there was a decrease in ABCA1

200 expression after 48 h with levels reaching 50% of control after 72 h (Fig. 3B).

201 Furthermore, the abundance of ABCA1 mRNA also decreased after treatment with TNF-

202 α (Fig. 3C).

203

204 TNF-α decreased the transcription of ABCA1 via the MEK/ERK signaling pathway in

205 INS-1 cells

206 Because TNF-α lowered the abundance of both ABCA1 protein and mRNA in INS-

207 1 cells, we speculated that TNF-α regulated the activity of the ABCA1 promoter in INS-

208 1 cells. For these studies, luciferase activity was measured in INS-1 cells transfected with

209 pABCA1-LUC and exposed to 10 ng/mL TNF-α (Fig. 4A). In agreement with the protein

210 and mRNA levels, TNF-α treatment also inhibited the activity of the promoter. Together,

211 these results clearly show that TNF-α suppresses the activity of the ABCA1 gene in INS-

212 1 cells.

213 Next, we used LY294002 (10μM), SB203580 (1μM), or BIRB796 (1μM) to

214 separately inhibit the PI3K, p38-MAPK (α, β), or p38-MAPK (α, β, γ) signaling pathways.

215 The results showed that only inhibition of the p38MAPK γ signaling pathway blocked

216 the effect of TNF-α on ABCA1 promoter activity. This suggests that TNF-α may regulate

217 ABCA1 expression via this pathway (Fig. 4B).

218

219 p38-MAPK regulated ABCA1 promoter activity

220 Because TNF-α suppressed ABCA1 promoter activity, the use of constitutively

221 active forms of p38-MAPK should mimic the effects of TNF-α. To test this hypothesis,

222 we expressed constitutively active forms of p38-MAPK (α, β, γ) in INS-1 cells carrying

223 a reporter gene containing the ABCA1 promoter. The results showed that the

224 constitutively active form of p38-MAPK γ, but not α or β, suppressed ABCA1 promoter

225 activity in INS-1 cells (Fig. 5A). The converse of this study is using a dominant-negative

226 mutant of p38-MAPK γ (p38-DN) to block the effect of TNF-α on ABCA1 promoter

227 activity (Fig. 5B). Consistent with the above results, TNF-α treatment inhibited ABCA1

228 promoter activity while the expression of p38-DN masked the ability of TNF-α to

229 suppress this activity. Together, these findings support the idea that the p38-MAPK γ

230 pathway is required for TNF-α suppression of ABCA1 promoter activity in INS-1 cells.

231

232 Effect of TNF-α on cholesterol content in INS-1 cells

233 ABCA1 is a cholesterol transporter that facilitates lipid efflux from cells. Thus, we

234 investigated the effect of TNF-α on cholesterol ester content in INS-1 cells. Figure 6A

235 shows that INS-1 cells treated with TNF-α have larger and more Oil Red O-stained lipid

236 droplets than untreated control cells. The p38-MAPK inhibitor, BIRB796, blocked the

237 effect of TNF-α on lipid accumulation of INS-1 cells.

238 We also measured the intracellular cholesterol ester concentration in INS-1 cells
239 quantitatively. TNF-α increased cholesterol content, and the cholesterol ester
240 concentration in cells increased about 25% compared to untreated cells (Fig. 6B). As
241 predicted, the p38-MAPK inhibitor, BIRB796, inhibited the increment of cholesterol
242 ester content by TNF-α (Fig. 6B) but had no significant effect on basal cholesterol ester
243 levels in the absence of TNF-α.

244

245 Effect of TNF-α on the insulin content in INS-1 cells

246 A previous study reported that the pancreatic knockout of ABCA1 resulted in
247 cholesterol accumulation and, subsequently, impaired insulin secretion (Brunham *et al.*
248 2007). To test this possibility in our system, we measured the insulin content in INS-1
249 cells treated with TNF-α. These cells showed a decreased insulin content in parallel with
250 ABCA1 suppression (Fig. 7). However, BIRB796 prevented neither the decreased insulin
251 content nor ABCA1 suppression caused by TNF-α.

252

253 **Discussion**

254 In this study, we found that TNF-α inhibited the expression of ABCA1 in the
255 pancreatic β-cell line, INS-1, and isolated rat islets. TNF-α alone, or in combination with
256 other cytokines, is associated with type I and type II diabetes mellitus (Argilés *et al.* 1994).
257 While destruction of β-cells by cytokines may be responsible for type I diabetes,
258 cytokines appear to play a multifaceted role in type II diabetes.

259 Previous reports suggested that the cytokines, interleukin-1, interferon-γ, and TNF-
260 α, alone or combination, decreased GSIS (Zhang & Kim 1995). While the insulin content
261 in cytoplasm was not changed by treatment with TNF-α (Zhang & Kim 1995), the
262 reduction of glucose utilization via decreased GLUT2 or glucokinase was suggested to
263 be a cause for TNF-α-induced inhibition of GSIS (Park *et al.* 1999). However, Kim et al.
264 demonstrated that a defect in glucose metabolism was not the main cause of TNF-α-
265 induced GSIS inhibition. Furthermore, they showed that most molecules involved in the
266 steps from glucose sensing to insulin exocytosis were not quantitatively altered by TNF-
267 α treatment, and that the expression of insulin and PDX-1 was not decreased. The minor
268 change in the glucose-stimulated ATP level between TNF-α-treated and -untreated INS-1

269 cells demonstrates that TNF-α does not affect glucose metabolism (Kim *et al.* 2008).

270 Elevated cholesterol levels in pancreatic islet cells, either in ob/ob mice that lack
271 ApoE and have diabetogenic obesity, or in transformed β-cell lines directly overloaded
272 with cholesterol, reduce GSIS (LENZEN & GUumlNTER 1978). This correlation is
273 consistent with that between the reduction in insulin secretion and the elevation of
274 pancreatic islet cell cholesterol levels in mice lacking β-cell ABCA1 (Brunham *et al.* 2007,
275 2008). This suggests that cholesterol has a direct effect on reducing β-cell functions.

276 In the current study, we showed that treatment with TNF-α increased the cholesterol
277 ester level and decreased insulin secretion. The important role of β-cell ABCA1 in glucose
278 homeostasis was further underscored by the finding that rosiglitazone increased β-cell
279 ABCA1 expression (Brunham *et al.* 2007). Furthermore, we previously found that
280 exendin-4, a glucagon-like peptide-1 analog, and insulin-like growth factor-I, have
281 stimulatory effects on ABCA-1 expression at the transcriptional level, thereby influencing
282 insulin secretion in pancreatic β-cells (Li *et al.* 2010; Lyu *et al.* 2016).

283 One goal of the present study was to examine in more detail the signaling pathways
284 activated by TNF-α that affect ABCA1 gene expression. TNF-α-induced responses are

285 mediated through the TNF-α receptor (Locksley *et al.* 2001). The levels of phospho-JNK,

286 phospho-p38, and NF-κB are enhanced by TNF-α treatment, and inhibitors of these

287 MAPK and inflammatory signals reduce TNF-α-induced GSIS inhibition (Kim *et al.*

288 2008). MAPKs contribute to direct cellular responses to various stimuli. p38/MAPK, a

289 member of the MAPK superfamily that transduces extracellular responses, is known for

290 its regulatory roles in apoptosis, cytokine production, transcriptional regulation, and

291 cytoskeletal reorganization (Zarubin & Han 2005). p38, a class of serine/threonine

292 MAPKs, is composed of four isoforms (α, β, γ, and δ) with more than 60% overall

293 sequence homology and over 90% identity within the kinase domains (Cuenda &

294 Rousseau 2007).

295 The use of BIRB796 decreases the activity of the four p38-MAPK subunits by

296 almost 100%, while SB203582 fails to exert an obvious effect on the activities of the γ

297 and δ isoforms (Kuma *et al.* 2005). Furthermore, the amounts of p38-MAPK β and p38-

298 MAPK δ are only 10.6 and 0.08% of that of p38-MAPK α, respectively (Dingar *et al.*

299 2010). Previous studies, as well as the present study, have focused on the α and γ subunits

300 of p38-MAPK. The present study found that BIRB796 treatment inhibited the effects of

301 TNF-α, including the enhancement of glucose uptake, increasing the cholesterol content,
302 and decreasing ABCA1 expression, while SB203580 treatment did not exert any
303 inhibitory effects. Because the distribution of the β and δ subunits is significantly lower
304 than that of the α subunit, while γ is similar to α (LU *et al.* 2015), it is assumed that the
305 functional effect of TNF-α in cells is mainly mediated via the p38-MAPK γ subunit. These
306 results clearly demonstrate that the p38-MAPK signaling pathway, particularly p38-
307 MAPK γ, may have an important role in TNF-α-mediated down-regulation of ABCA1
308 expression. This was confirmed using CA-p38-MAPK γ or DN-p38-MAPK γ (Fig. 5).
309 In type 2 diabetes, p38/MAPK can be activated by high concentrations of glucose
310 and advanced glycation end products, and may play important roles in its pathogenesis
311 (Chen *et al.* 1994). We previously examined glucose-mediated regulation of ABCA1 gene
312 expression in vascular smooth muscle cells (Yu *et al.* 2010). The results showed that the
313 expression of ABCA1 mRNA and protein decreased after the cells were treated with 22.4
314 mM glucose for 48 h, and aslo high glucose-induced ABCA1 suppression was sensitive
315 to p38-MAPK inhibitors. On the other hand, glucose-dependent insulinotropic
316 polypeptide potentiated GSIS, insulin biosynthesis, and β-cell proliferation and survival.

317 Importantly, a previous report discovered that glucose-dependent insulinotropic
318 polypeptide suppressed p38-MAPK and JNK via Akt-mediated changes in the
319 phosphorylation state of apoptosis signal-regulating kinase 1 in INS-1 cells and human
320 islets, resulting in inhibition of its activity (Widenmaier *et al.* 2009).

321 Tangier disease (TD) is a rare autosomal recessive disease caused by mutations in
322 the ABCA1 gene. Patients with TD exhibit high density lipoprotein deficiency, resulting
323 in the accumulation of cholesteryl esters in many tissues, such as liver, tonsils, spleen,
324 gastrointestinal mucosa, lymph nodes, and peripheral nerves (Mott *et al.* 2000).
325 Furthermore, ABCA1 is expressed in pancreatic β-cells (Brunham *et al.* 2007), suggesting
326 that ABCA1 deficiency, along with abnormal lipid rafts, might lead to the dysfunction of
327 pancreatic β-cells in TD patients. Koseki et al. reported that all patients with TD suffered
328 from type 2 diabetes. A characteristic of diabetes in TD patients might be the extremely
329 low insulinogenic index value, suggesting that ABCA1 plays a critical functional role in
330 insulin secretion from β-cells (Koseki *et al.* 2009). In the current study, INS-1 cells treated
331 with TNF-α suppressed ABCA1-decreased insulin secretion in response to changes in the
332 glucose concentration.

333 fIn summary, our studies show that TNF-α suppressed the expression of endogenous
334 ABCA1 in pancreatic islets and INS-1 cells. These findings raise the possibility that TNF-
335 α may affect insulin secretion by controlling ABCA1 expression in pancreatic β-cells,
336 which may be of therapeutic value in the treatment of diabetes mellitus.

337

338 **Declaration of interest**

339 Conflict of Interest: The authors declare that they have no conflict of interest.

340

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345 **Author contributions**

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356

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478

**Figure legends**

Figure 1. Effect of tumor necrosis factor (TNF)-α on insulin secretion and glucose stimulated insulin secretion in INS-1 cells and pancreatic islet.

(A) INS-1 cells were incubated with 10 ng/mL TNF-α for 24 h. Fold of insulin secretion has significantly decreased in treated with TNF-α ($P < 0.05$). (B) To compared with 3.3 mM glucose group, 16.7mM glucose group has significantly increased of insulin secretion. But treated with TNF-α, the increased level of insulin secretion in 16.7mM glucose has canceled. (C) Rat pancreatic islet were incubated with 10 ng/mL TNF-α for 24 h. Fold of insulin secretion has significantly decreased in treated with TNF-α ($P < 0.05$). (D) To compared with 3.3 mM glucose group, 16.7mM glucose group has significantly increased of insulin secretion. But treated with TNF-α, the increased level of insulin secretion in 16.7mM glucose has canceled.

Figure 2. Effect of tumor necrosis factor (TNF)-α on the expression of ATP-binding cassette transporter A1 (ABCA1) in rat pancreatic islets.

The effect of TNF-α on the expression of ABCA1 protein (A) and mRNA (B) in rat pancreatic islets. The ABCA1/GAPDH ratios are shown as percentages of control. Each data point shows the mean ± SEM of three separate experiments for each treatment group.

496 *Significantly different compared to the control ($P < 0.05$).

497

498 Figure 3. Effect of tumor necrosis factor (TNF)-α on the expression of ATP-binding

499 cassette transporter A1 (ABCA1) in INS-1 cells.

500 (A) Dose-dependent suppression of ABCA1 protein by TNF-α. INS-1 cells were

501 seeded in six-well plates and exposed to the indicated amounts of TNF-α for 24 h. ABCA1

502 in the total cell lysate was detected by western blot analysis. GAPDH served as the control

503 and is shown at the bottom of each lane. The ABCA1/GAPDH ratios are shown as

504 percentages of control in the lower panel. Each data point shows the mean ± SE of three

505 separate experiments. *Significantly different from control ($P < 0.05$). (B) Time-

506 dependent suppression of ABCA1 protein by TNF-α. INS-1 cells were incubated for the

507 indicated periods of time in the presence of 10 ng/mL TNF-α. GAPDH served as the

508 control and is shown at the bottom of each lane. The ABCA1/GAPDH ratios are shown

509 as percentages of control in the lower panel. Each data point shows the mean ± SE of

510 three separate experiments. *Significantly different from control ($P < 0.05$). (C)

511 Abundance of ABCA1 mRNA in INS-1 cells treated with 10 ng/mL TNF-α. The

512 ABCA1/GAPDH ratios are shown as percentages of control. Each data point shows the

513 mean ± SEM of three separate experiments for each treatment group. *Significantly

514 different from control ($P < 0.05$).

515

516 Figure 4. Effect of tumor necrosis factor (TNF)-α on ATP-binding cassette transporter A1

517 (ABCA1) promoter activity in INS-1 cells.

518 (A) TNF-α decreases ABCA1 gene transcription. INS-1 cells were transfected with

519 1 μg of pABCA1-LUC and treated with TNF-α for 24 h prior to harvesting. All assays

520 were corrected for β-galactosidase activity. The total amount of protein in every reaction

521 was identical. The results are expressed as relative luciferase activity compared with

522 control cells arbitrarily set at 100. Each data point shows the mean ± SE of three separate

523 transfections that were performed. *Significantly different from control ($P < 0.05$). (B)

524 Effects of the phosphatidylinositol 3-kinase inhibitor, LY-294002 (LY), and the p38-

525 mitogen-activated protein kinase (MAPK) inhibitors, SB-203580 (SB) and BIRB796

526 (BIRB), on ABCA1 transcriptional activity in INS-1 cells treated with TNF-α. The p38-

527 MAPK inhibitors block the actions of TNF-α. Vehicle was 0.1% dimethyl sulfoxide. Each

528 data point shows the mean ± SE of three independent transfections that were performed .

529 *Significantly different from only TNF-α ($P < 0.05$).

530

531 Figure 5. Role of the p38-mitogen-activated protein kinase (MAPK) signal transduction

532 pathway on ABCA1 promoter activity inhibited by tumor necrosis factor (TNF)-α.

533 (A) Effects of p38-MAPK isoforms on ABCA1 promoter activity. INS-1 cells were

534 transfected with pABCA1-LUC and either an empty vector (control) or expression

535 vectors of constitutively active forms of p38-MAPK (α, -β, -γ) for 24 h prior to cell

536 harvesting. All assays were corrected for β-galactosidase activity. The total amount of

537 protein in each reaction was identical. The results are expressed as relative luciferase

538 activity compared with control cells arbitrarily set at 100. Each data point shows the mean

539 ± SE of four separate transfections that were performed on different days. *Significantly

540 different from control (*$P < 0.05$). N.S., no significant difference. (B) Dominant-negative

541 p38-MAPKγ (p38γ-DN) blocks the TNF-α-induced inhibition of ABCA1 transcription.

542 INS-1 cells were transfected with pABCA1-LUC and either the empty vector or p38γ-

543 DN, then treated with TNF-α for 24 h prior to cell harvesting. The results are expressed

544 as relative luciferase activity compared with control cells arbitrarily set at 100. Each data

545 point shows the mean ± SE of four separate transfections that were performed on different

546 days. *Significantly different from control ($P < 0.05$). N.S., no significant difference.

547

548 Figure 6. Effect of tumor necrosis factor (TNF)-α on cholesterol accumulation in INS-1

549 cells.

550 INS-1 cells were pretreated with dimethyl sulfoxide or BIRB796 (BIRB) for 30 min,

551 then incubated with 10 ng/mL TNF-α for 24 h. Cells were then stained with Oil Red O

552 (A) or lysed to measure intracellular cholesterol content (B). Percentages of cholesterol

553 content per cell relative to control are shown as means ± SEM of three separate

554 experiments. *Significantly different compared to control ($P < 0.05$). #Significantly

555 different compared to dimethyl sulfoxide plus TNF-α ($P < 0.05$).

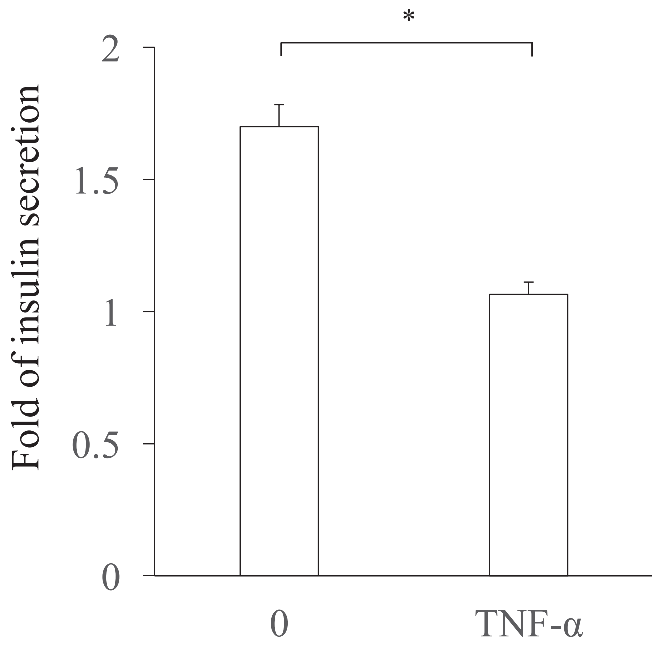
556

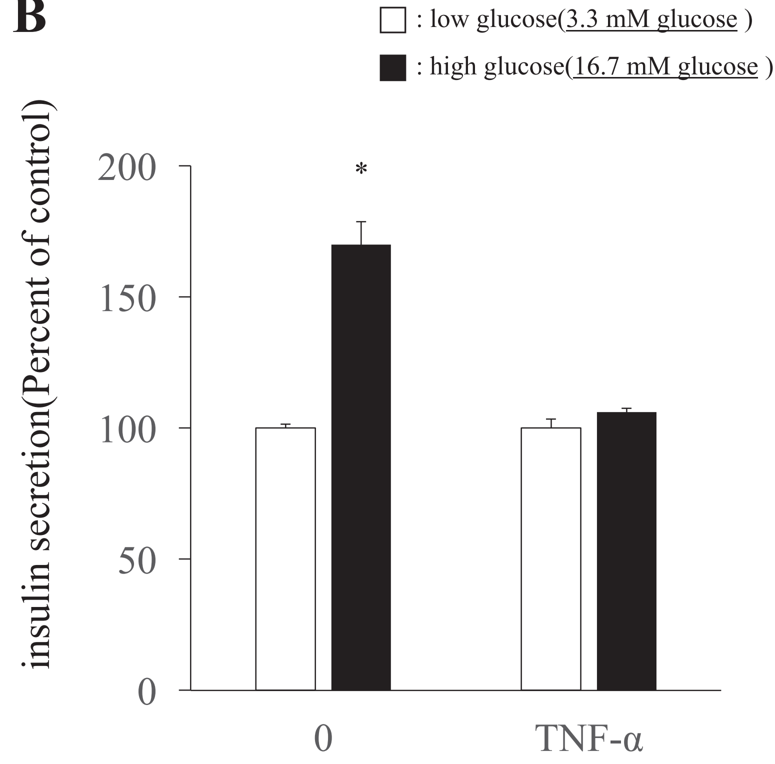
557

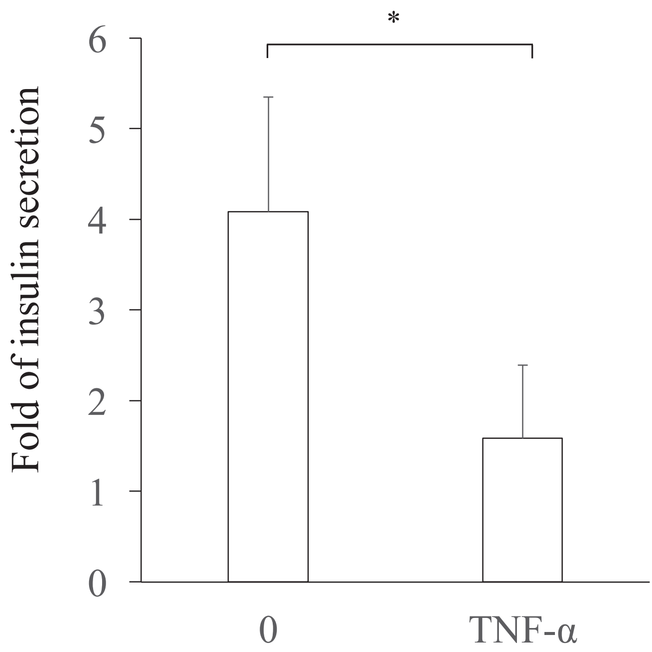
558 Figure 7. Effect of tumor necrosis factor (TNF)-α on ABCA1 expression and insulin

559 synthesis in INS-1 cells.

(A) INS-1 cells were pretreated with dimethyl sulfoxide or BIRB796 (BIRB) for 30 min and then incubated with 10 ng/mL TNF-α for 24 h. Protein extracts were subjected to western blot analysis to determine ABCA1 expression. The ABCA1/GAPDH ratios are shown as mean ± SEM percentages of control from three separate experiments for each treatment group. *Significantly different compared to DMSO ($P < 0.05$). #Significantly different compared to DMSO + TNF-α ($P < 0.05$). (B) Abundance of insulin in INS-1 cells treated with TNF-α. INS-1 cells were pretreated with dimethyl sulfoxide or BIRB for 30 min, then incubated with 10 ng/mL TNF-α for 24 h. Protein extracts were subjected to western blot analysis to determine insulin expression. The insulin/GAPDH ratios are shown as mean ± SEM percentages of control from three separate experiments for each treatment group. *Significantly different compared to DMSO ($P < 0.05$). #Significantly different compared to DMSO + TNF-α ($P < 0.05$).


A
*
Fold of insulin secretion
2
1.5
1
0.5
0
0
TNF-α


B
: low glucose(3.3 mM glucose )
: high glucose(16.7 mM glucose )
insulin secretion(Percent of control)
*
200
150
100
50
0
0
TNF-α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*
Fold of insulin secretion
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2
1
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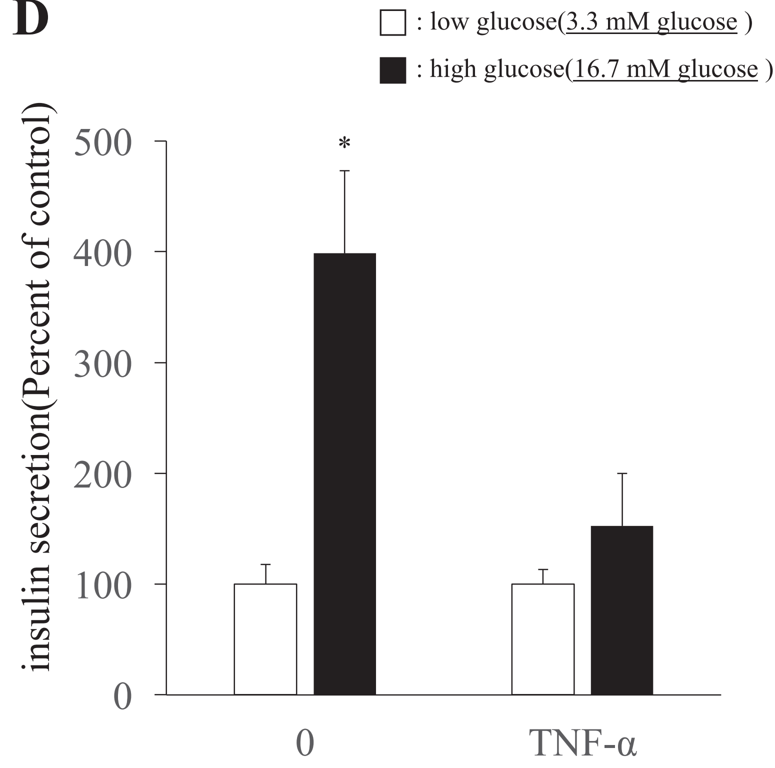
D
: low glucose(3.3 mM glucose )
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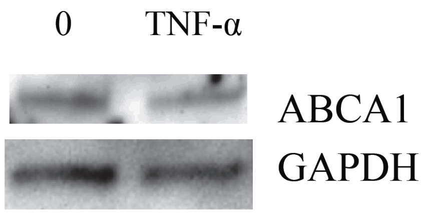
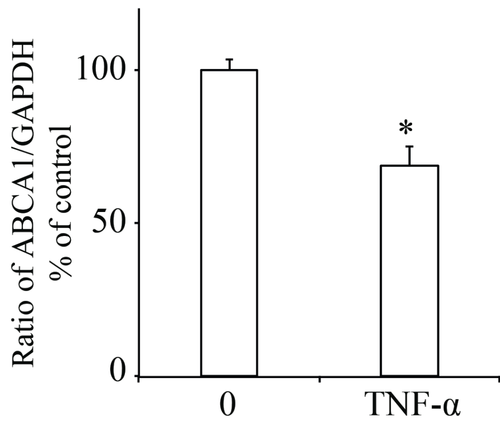
insulin secretion(Percent of control)
*
500
400
300
200
100
0
0
TNF-α

A

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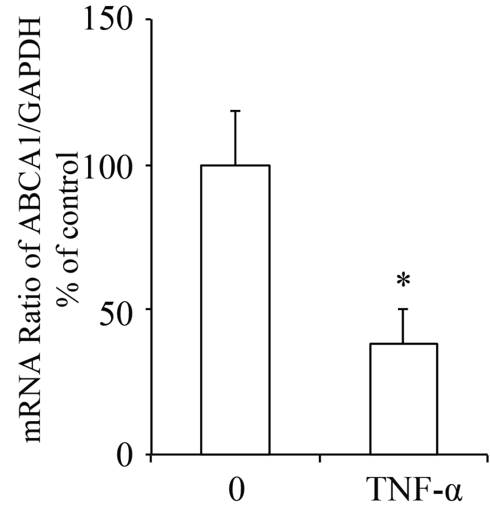

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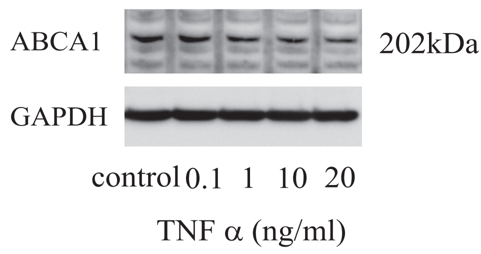


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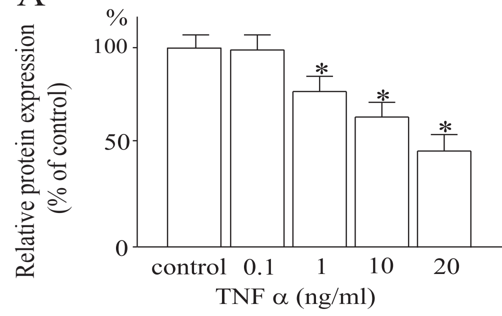


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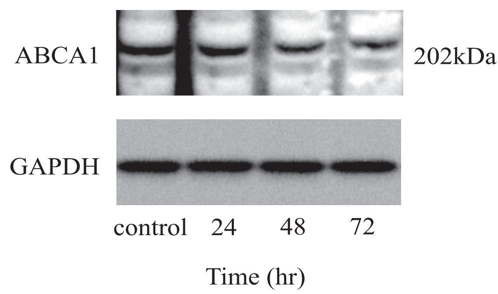


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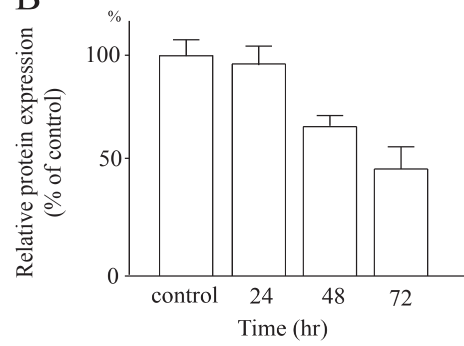


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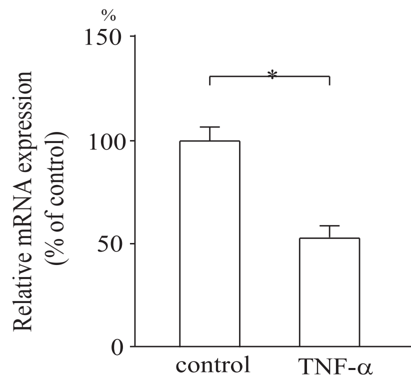
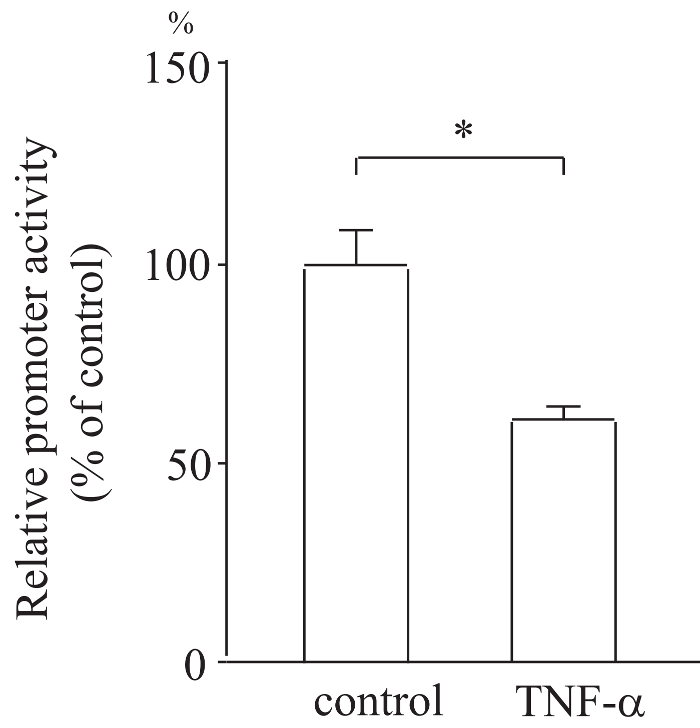
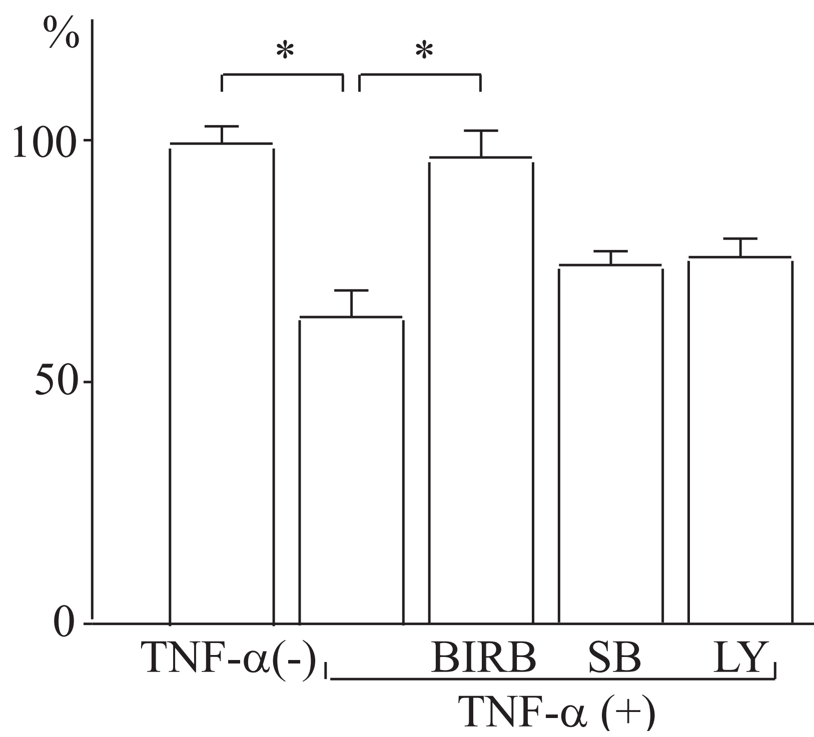
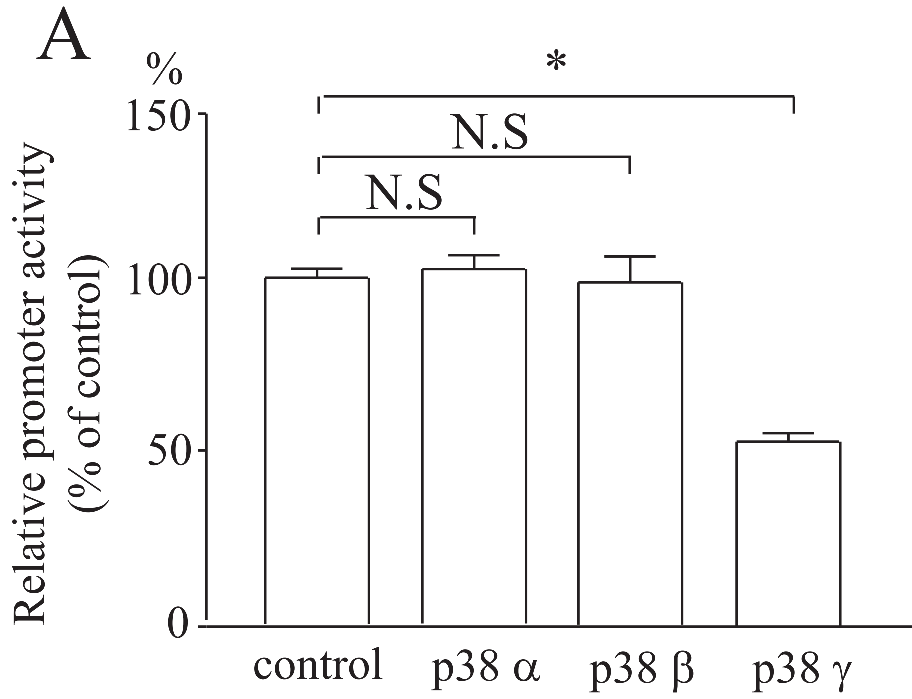
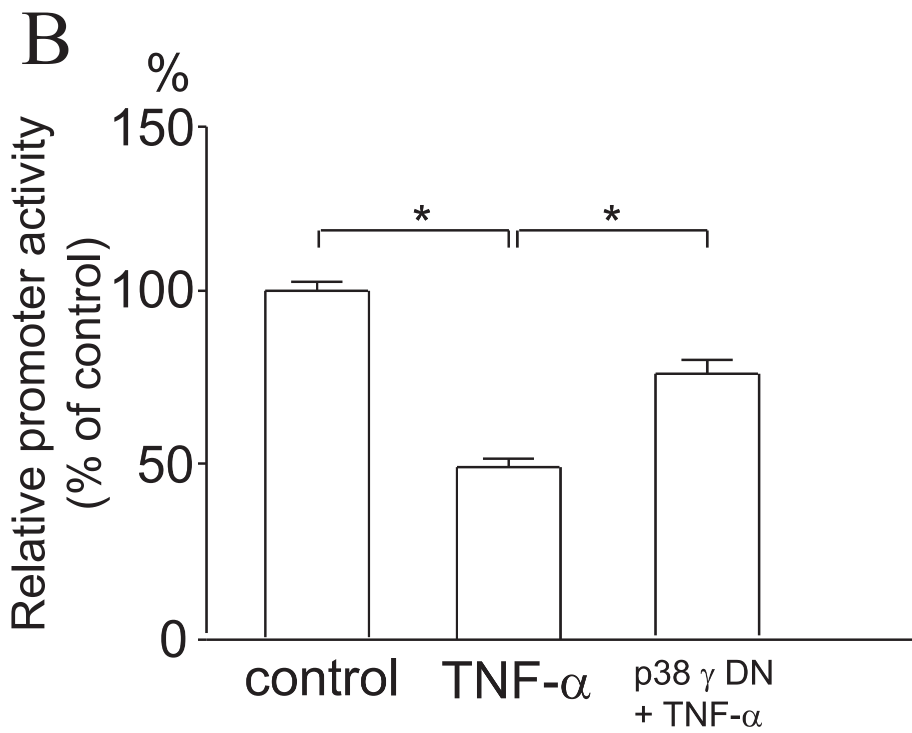
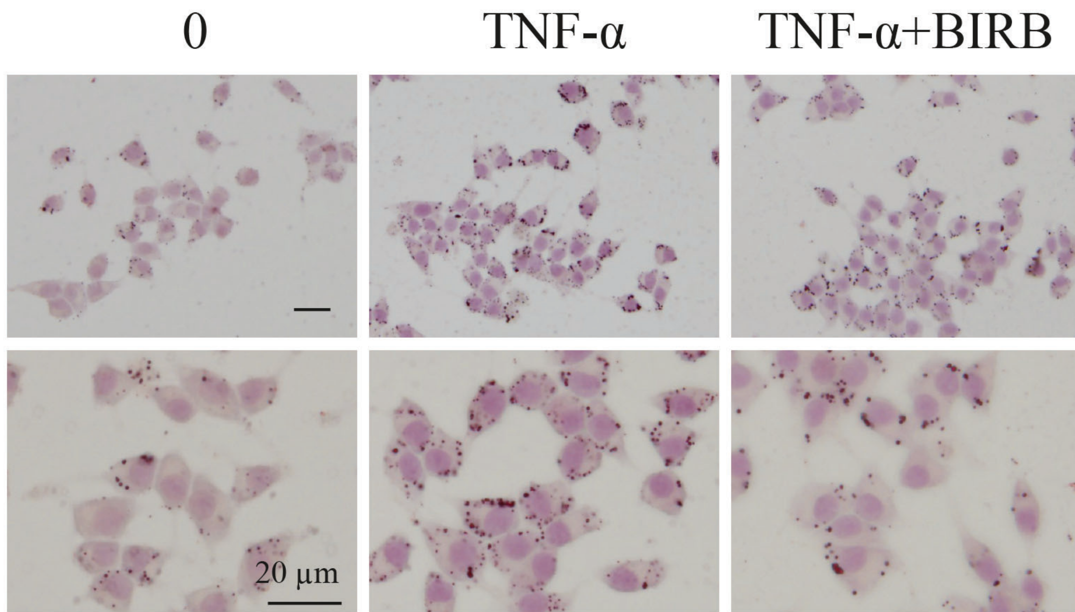


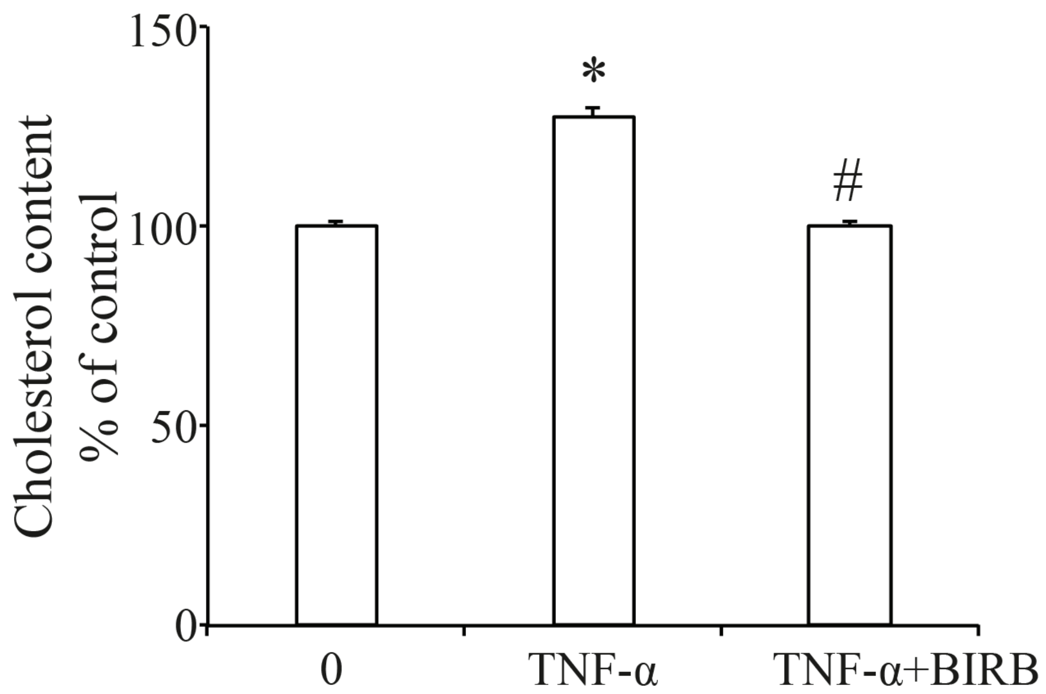

A
%
150
100
50
0
*
Relative promoter activity
(% of control)
control
TNF-α


B
%
100
50
0
*
*
Relative promoter activity
(% of control)
TNF-α(-)
BIRB
SB
LY
TNF-α (+)

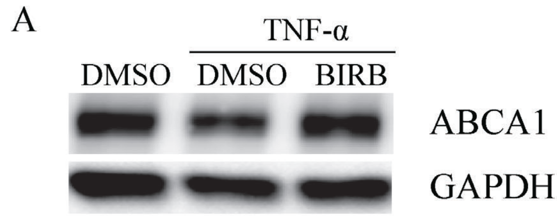

A
%
*
150
N.S
N.S
100
50
0
Relative promoter activity
(% of control)
control
p38 α
p38 β
p38 γ

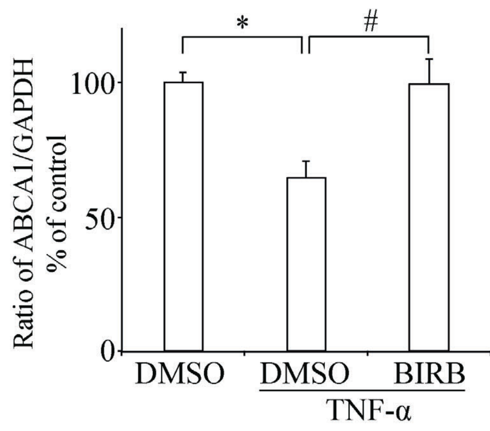

B
%
150
*
*
100
50
0
Relative promoter activity
(% of control)
control
TNF-α
p38 γ DN
+ TNF-α


A
0
TNF-α
TNF-α+BIRB
20 μm


B
Cholesterol content
% of control
150
100
50
0
*
#
0
TNF-α
TNF-α+BIRB

A

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