Angiotensin II causes beta cell dysfunction through an ER stress induced pro-inflammatory response

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Abstract

The metabolic syndrome is associated with an increase in the activation of renin angiotensin system (RAS) and inhibition of RAS reduces the incidence of new onset diabetes. Importantly, angiotensin II (AngII), independently of its vasoconstrictor action causes beta-cell inflammation and dysfunction, which may be an early step in the development of type-2 diabetes. The aim of this study was to determine how AngII causes beta cell dysfunction. Islets of Langerhans were isolated from C57BL/6J mice that had been infused with AngII in the presence or absence of taurine-conjugated ursodeoxycholic acid (TUDCA) and effects on ER stress, inflammation and beta cell function determined. The mechanism of action of AngII was further investigated using isolated murine islets and clonal beta cells.

We show that AngII triggers ER stress, an increase in the mRNA expression of pro-inflammatory cytokines, and beta cell dysfunction in murine islets of Langerhans both *in vivo* and *ex vivo*. These effects were significantly attenuated by TUDCA, an inhibitor of ER stress. We also show that AngII-induced ER stress, is required for the increased expression of pro-inflammatory cytokines and, is caused by ROS and IP3 receptor activation.

These data reveal that the induction of ER stress is critical for AngII-induced beta cell dysfunction and indicates how therapies that promote ER homeostasis may be beneficial in the prevention of type-2 diabetes.

Abbreviations: AngII (angiotensin II), 2APB (2-aminoethoxydiphenyl borate), AT1R (angiotensin type 1 receptor), ATF4 (activating transcription factor 4), CHOP (C/EBP Homologous Protein), ER (endoplasmic reticulum), GK (glucokinase), IRE1 (inositol requiring enzyme 1), PERK (PKR-like ER kinase), RAS (renin angiotensin system), TUDCA (taurine-conjugated ursodeoxycholic acid), UPR (unfolded protein response),

Introduction

Elevated blood pressure due to increased activation of the renin angiotensin system (RAS) is an important feature of the metabolic syndrome. This constitutes a series of metabolic disorders that increase the risk of developing type-2 diabetes and cardiovascular disease. Importantly, the pharmacological inhibition of RAS reduces the incidence of new onset type-2 diabetes in high risk populations (1–3) and RAS blockade in several animal models of diabetes improves pancreatic beta cell function (4–8). Conversely, the infusion of AngII into mice causes beta cell dysfunction (9–11). As the components of the RAS system have been detected in islets and this 'local' RAS plays an important role in regulating islet mass and function (7,9), the effects of RAS blockade *in vivo* is likely mediated by inhibiting locally produced AngII.

The detrimental effects of AngII on beta cell function were largely attributed to vasoconstriction resulting in decreased delivery of glucose to pancreatic beta cells (12–14). However, it has recently been shown that AngII infusion in mice causes beta cell dysfunction independently of AngII's effect on blood pressure (11). In support of this, the treatment of either isolated human or rodent islets with AngII also causes beta cell dysfunction (9–11). The damaging effects of AngII on beta cell function, both *in vivo* and *in vitro,* have been ascribed to an increase in the expression of pro-inflammatory cytokines, in particular IL-1β (11), and there is a growing body of evidence indicating that inflammation is important in the development of beta cell dysfunction in type-2 diabetes (15–17). Endoplasmic reticulum (ER) stress is also associated with the loss of beta cell function and viability in type-2 diabetes (18–21). This stress is sensed by the ER transmembrane proteins: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1α (IRE1) that activate an adaptive response called the unfolded protein response (UPR) (21–23). If the UPR is unable

76 to alleviate ER stress beta cell dysfunction and death can occur through the chronic activation

77 of a UPR, which activates a number of pro-apoptotic and pro-inflammatory signaling pathways

78 (16,23,24). Given that AngII also increases the expression of pro-inflammatory cytokines and

79 causes beta cell dysfunction (11), we hypothesised that ER stress may play an important role

80 in AngII-mediated beta cell inflammation and dysfunction.

81

82

83 **Materials and Methods**

84 *Cell culture.* Mouse insulinoma 6 (MIN6) cells (25) were used between passages 25 and 35 at

85 ~80% confluence and cultured as previously described (26).

86

87 *Islet isolation.* Pancreatic islets were isolated from adult C57BL/6J mice (Animal Resources

88 Centre, Perth, Australia)). Briefly the pancreas was inflated by injecting 3 ml of RPMI 1640

89 (Invitrogen) containing 1mg/ml collagenase (Sigma-Aldrich, Australia) through the common

90 pancreatic duct and the pancreas excised. Islets were then isolated as previously described

91 (26).

92

93 *Animal experimentation.* All experiments were approved by the Animal Ethics Committee of

94 RMIT University (#1504). Male C57BL/6J mice (10 weeks of age) obtained from the Animal

95 Resources Centre (Perth, Australia) were kept at $22\pm1^\circ$C on a 12-h light/dark cycle. All mice

96 were fed standard mouse chow and water ad libitum. After 1 week of acclimatization, the mice

97 were randomly assigned to 3 groups: 1) sham (control mice infused with PBS, n=8); AngII

98 (mice infused with human AngII, dissolved in sterile 1XPBS, at 416ng.kg^{-1}.min^{-1} using

99 subcutaneous ALZET® mini-osmotic pumps (USA) for 2 weeks, n=8); or AT (mice infused

100 with AngII with daily intra-peritoneal injection of TUDCA at 150 mg.kg^{-1}.day^{-1} for 2 weeks,

101   n=8). Human Ang II (≥93%HPLC) was purchased from Sigma. The body weight and plasma

102   glucose levels were recorded three times weekly during the experiment. Systolic blood pressure

103   (SBP) was measured using the CODA tail-cuff blood pressure system (ADInstruments Pty

104   Ltd., Australia).

105   For Glucose tolerance tests (GTT; 2.5 g glucose/kg BW, ip) mice were fasted for 5 h

106   prior to blood samples being collected via the tail vein and blood glucose concentration

107   determined using a glucometer (AccuCheck Proforma Nano; Roche, Victoria, Australia). For

108   insulin measurements blood samples were centrifuged (2000 rpm, 2 min at 4°C) and the plasma

109   insulin concentrations measured by ELISA (Linco Research, St. Louis, MO) in collaboration

110   with the Department of Physiology, Monash University, Melbourne. Mice were euthanized

111   with CO_2. The disposition index, a composite measure of beta cell function (27), was also

112   calculated using the following formula: $\Delta I_{0-30}/\Delta G_{0-30} \times 1/\text{fasting insulin}$.

113

114   *Western-blotting and Immunohistochemistry.* SDS-PAGE and western blotting were

115   performed as previously described (28) using antibodies against: BiP (BD Transduction

116   Laboratories, USA), phospho-IRE1α (Ser 724) (Abcam, USA), phospho-PERK (Thr 980),

117   phospho-eIF2α (Ser 51), CHOP, ATF4, and GAPDH (Cell Signaling Technology, USA).

118   Immunohistochemistry was performed on fixed and paraffin embedded pancreatic sections

119   using anti-CHOP, and Alexa Fluor 488 conjugated antibodies. All antibodies were used as per

120   manufacturer's instructions.

121

122   *Transfection, RNA isolation and qPCR analyses.* Silencer® Select siRNAs against *Ire* (cat. no.

123   S95857) and *Xbp* (cat. no. S76114) were purchased from from Ambion®oligos (Thermo

124   Scientific, USA).siRNA oligos were tranfected using Lipofectamine® RNAiMAX (Thermo

125   Scientific, USA) according to the manufacturer's protocol. Total RNA was isolated using the

126 ReliaPrep™ RNA Cell Miniprep System (Promega, USA). Reverse transcription was carried

127 out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK).

128 Quantitative PCR was carried out using the SYBR® Green PCR Master Mix (Applied

129 Biosystems, UK) using primers described in ESM Table 1. The gene expression from each

130 sample was analysed in duplicate and normalized against the housekeeper 18S. All reactions

131 were performed on the Rotor-Gene Q (Qiagen, USA). The results are expressed as relative

132 gene expression using the ΔCt method (29).

133

134 *Glucose stimulated insulin secretion (GSIS).* Isolated islets were cultured overnight in RPMI

135 1640 medium supplemented with L-glutamine (20 mmol/l), and FBS (5%). GSIS was

136 performed as previously described (28). Insulin ELISA was performed as described above.

137

138 *Quantification of superoxide levels* Superoxide levels were measured using L-012 (Tocris

139 Bioscience, USA) enhanced chemiluminescence as previously described (30). MIN6 cells were

140 plated on a 96-well Optiplate (PerkinElmer, Melbourne, Australia), incubated with 100 µmol/L

141 L-012, and luminescence measured using a BMG Clariostar plate reader (BMG Labtech,

142 Melbourne, Australia).

143

144 *Statistical analysis* Data are expressed as mean ± SE, unless otherwise stated. Data were

145 analysed by one-way ANOVA followed by Tukey's post-hoc test for multiple comparison

146 between means using Prism 6 (GraphPad Software, USA). Differences were considered

147 statistically significant at $p < 0.05$.

148

149 **Results**

Angiotensin II induces ER stress and impairs beta cell function in mouse islets of Langerhans.

To investigate the role of ER stress on AngII-dependent beta cell dysfunction, mouse islets of Langerhans were treated with AngII for 96 h in the presence or absence of TUDCA, a chemical chaperone that inhibits ER stress (31). AngII caused beta cell dysfunction as demonstrated by a marked increase of basal insulin secretion and significant decrease in the stimulatory index compared to control islets reflecting the loss of the glucose-stimulated insulin secretion (GSIS) (Fig. 1a and b). This correlated with a decrease in the mRNA expression of glucokinase *(Gk)* (Fig. 1c), whereas the mRNA expression of glucose transporter-2 (*Glut-2*) and pancreatic and duodenal homeobox 1 (*Pdx1*) were increased (Fig 1c). AngII treatment also significantly increased the expression of the pro-inflammatory cytokines interleukin 1β (*Il-1β*) and tumor necrosis factor α (*Tnf-α*) (Fig. 1d). However, no significant changes in monocyte chemotactic protein 1 (*Mcp-1*) were detected. Importantly, AngII caused a marked increase in the expression of markers of ER stress (Fig. 1e), including immunoglobulin binding protein (*BiP*/*Grp78*), the spliced form of X-box binding protein 1 (*Xbp1s*), endoplasmic reticulum oxidoreductin 1 (*Ero1l*), peptidyl-prolyl cis-trans isomerase (*Fkbp11*), ER degradation enhancing α-mannosidase-like protein (*Edem*), activating transcription factor 4 (*Atf4*) and the pro-apoptotic transcription factor C/EBP Homologous Protein (*Chop* also known as *Gadd153*) (32,33). The co-administration of TUDCA with AngII significantly restored beta cell function, as demonstrated by a reduction in basal insulin secretion (Fig. 1a), an improved stimulatory index (Fig. 1b) and the restoration of *glucokinase* expression to control levels (Fig 1c). TUDCA also significantly decreased the expression of the pro-inflammatory cytokines *Il-1β* and *Tnf-α* (Fig 1d) and all the markers of ER stress investigated (Fig. 1e). Thus AngII causes beta cell dysfunction, ER stress and inflammation in mouse islets of Langerhans and these effects occur independently of AngII's systemic vasoconstrictive effects. Moreover, as TUDCA counteracts

174　the effects of AngII it is likely that the deleterious effects of AngII treatment observed here are

175　mediated through ER stress.

176

177　*ER stress precedes the induction of pro-inflammatory cytokines in AngII treated MIN6 cells*

178　*and Islets of Langerhans.* We initially investigated the efficacy of using the pancreatic beta

179　cell line MIN6 as a model to further investigate AngII-induced-ER stress and inflammation.

180　MIN6 cells were treated with AngII or thapsigargin, a pharmacological inducer of ER stress,

181　in the presence or absence of TUDCA (ESM Fig 1). As observed in islets, AngII treatment

182　caused ER stress and an increase in the expression of *Il-1β,* which was inhibited by TUDCA.

183　Thapsigargin also evoked a UPR and increased the expression of *Il-1β*.

184　To investigate the temporal relationship between AngII-induced ER stress and the expression

185　of pro-inflammatory cytokines, MIN6 cells were treated with AngII for up to 96 h and the

186　induction of ER stress and the expression of *Il-1β, Tnf-α* and *Mcp-1* were monitored. AngII

187　rapidly induced ER stress (within 2 h) as determined by the phosphorylation status of: IRE1α,

188　PERK, PERK′s substrate eIF2α, and an increase in the expression of BiP, ATF4 and CHOP

189　and *Xbp1s,* (Fig. 2a and b). AngII increased the expression of thioredoxin interacting protein

190　(TXNIP) (16) (Fig. 2b) at 6h and the expression of the pro-inflammatory cytokine *Il-1β* and

191　*Tnf-α* mRNA by 6 h and 48 h respectively (Fig. 2c). No changes in the expression of *Mcp1*

192　were detected (Fig. 2c). AngII treatment of mouse islets caused an increased in the expression

193　of *Xbp1s, Atf4* and *Chop* which preceded an increase in the expression of *Il-1β* and *Tnfα* (Fig

194　2d).

195　Therefore, the occurrence of ER stress precedes an increase in the expression of the pro-

196　inflammatory cytokines providing evidence that AngII-induced ER stress may promote

197　inflammation in beta cells.

198

The role of PERK and IRE1α in AngII-induced expression of the pro-inflammatory cytokines.
To determine how ER stress increases the expression of the pro-inflammatory cytokines, we investigated the effect of a selective inhibitor of PERK (GSK2606414 (PERKi)) and siRNA mediated knock-down of *Ire1α* or *Xbp1* on AngII-induced *Il-1β* expression. GSK2606414 inhibited AngII-induced phosphorylation of eIF2α (Fig. 3a) and expression of ATF4, CHOP (Fig. 3a), *Txnip* (Fig. 3b) and importantly *Il-1β* (Fig. 3b). As anticipated siRNAs directed towards *Ire1α* or *Xbp1* significantly reduced *Xbp1s* basal expression and AngII induced increases in *Xbp1s* expression (Fig. 3c). Importantly, siRNA-mediated knock-down of *Ire1α* or *Xbp1* also inhibited AngII evoked increases in *Txnip and Il-1β* expression (Fig. 3d). These results provide evidence that both PERK and IRE1α are required for Ang II to induce a pro-inflammatory response.

210

Angiotensin-II induced ER stress is dependent upon both IP3R and NOX activation. AngII-induced ER stress and inflammation is dependent on AT1R activation as irbesartan (IRB), an angiotensin 1 receptor (AT1R) antagonist, attenuated both AngII-induced ER stress and *Il-1β* expression (ESM Fig. 2). The AT1R classically couples to Gq/11 and activates NADPH oxidase (NOX) and phospholipase-C (PLC) resulting in an increase in IP3 and ROS (34,35). IP3 stimulates ER calcium release (34) and ROS has been shown to sensitise the IP3 receptor (IP3R) (36). As a decrease in ER calcium can induce ER stress (26) we investigated whether the effects of AngII were mediated by an IP3R-dependent mechanism. MIN6 cells were treated with AngII in the presence or absence of selective IP3 receptor antagonists, 2-aminoethoxydiphenyl borate (2APB) and xestospongin-C (XestC). 2APB and Xest-C inhibited AngII-induced ER stress as determined by a significant decrease in eIF2α phosphorylation, as

222   well as the expression of ATF4 and CHOP (Fig. 4a). Therefore, AngII-induced ER stress

223   requires IP3R activation indicating that AngII-induced ER stress is likely to be mediated by a

224   decrease in ER calcium. However, for reasons which are unclear, at 6h treatment of cells with

225   AngII in the presence of xestospongin-C potentiated AngII-induced eIF2α phosphorylation

226   (Fig. 4a).

227   To investigate the role of ROS, changes in superoxide production in MIN6 cells in response to

228   AngII in the presence or absence of the AT1R antagonist irbesartan, and two selective

229   inhibitors of NOX, apocyin and diphenyleneiodonium (DPI) was determined (Fig. 4b). As

230   anticipated AngII increased superoxide levels and this was inhibited by apocynin (at 10 and

231   300 μM), DPI and irbesartan (Fig. 4b). Importantly, apocynin or DPI also inhibited AngII-

232   induced ER stress as determined by the phosphorylation of eIF2α and the expression of ATF4

233   and CHOP (Fig. 4c). These data provide evidence that ROS is required for AngII-induced ER

234   stress. Given that IP3R activation is also required, it is possible that ROS promotes ER stress

235   by sensitizing the IP3R (36).

236   *High glucose potentiates AngII-induced ER stress.* To investigated the effect of glucose

237   concentration on AngII-induced ER stress. MIN6 cells were incubated at either low (5.5mM)

238   glucose or high (25mM) glucose and the effect of AngII on ER stress determined. AngII

239   treatment of MIN6 cells incubated at low glucose caused a significant increase in the

240   expression of CHOP and ATF4 which marks the presence of ER stress. Interesting, incubation

241   at high glucose (25mM) potentiated the effect of AngII on ER stress (ESM Fig 3a). These

242   experiments were repeated using isolated murine islets and similar results were obtained (ESM

243   Fig 3b). Thus, high glucose potentiates the effects of AngII on ER stress.

244

Angiotensin II infusion of mice causes ER stress and an increase in the expression of pro-inflammatory cytokines in pancreatic islets. To assess whether a chronic elevation in AngII caused ER stress in islets *in vivo* and whether this was important in the induction of pro-inflammatory cytokines, mice were infused with AngII for 2 weeks with or without the co-administration of TUDCA. Following AngII infusion, mice displayed a marked impairment of glucose tolerance (Fig. 5a, b) together with elevated levels of fasting plasma insulin (Fig. 5c), likely due to the known detrimental effects of AngII on insulin sensitivity (37). Interestingly, there was also evidence of beta cell dysfunction as determined by a decrease in the disposition index (Fig. 5d). The co-administration of TUDCA improved glucose tolerance, reduced plasma insulin levels (Fig. 5a and c) and rescued beta cell function (Fig. 5d). All these effects occurred independently of changes in body weight, adiposity, or a sustained increase in systolic blood pressure (SBP;ESM Fig 4). Importantly, islets isolated from these AngII infused animals showed signs of ER stress as evidenced by an increase in the expression of *Xbp1s*, *Fkbp11, Ero1l, Edem, Atf4* and *Chop* and *Txnip* (Fig. 5e). Although no change in the expression of *BiP*, an adaptive marker of the UPR, was detected (Fig. 5e). Importantly, *Il-1β* and *Tnf-α,* expression were also augmented by AngII infusion (Fig. 5f). Interestingly, as observed in isolated islets, AngII caused an increase in *Pdx1* and *Glut2* expression but a decrease in *glucokinase* expression (Fig. 5g). The co-administration of TUDCA inhibited AngII-stimulated increase in the expression of markers of ER stress (Fig. 5e), the pro-inflammatory cytokines (Fig. 5f) and the markers of beta-cell function (Fig. 5g). Taken together, these data provide evidence that *in vivo* AngII causes ER stress and that this increases the production of pro-inflammatory cytokines in islets.

To determine whether macrophages were present in islets isolated from AngII treated mice we looked for the presence of *F4/80,* a macrophage specific marker, by qPCR (ESM Fig. 5).

11

269   Although *F4/80 mRNA was detected in islets* its expression was unchanged by AngII indicating

270   the macrophages are unlikely to be the source of the pro-inflammatory cytokines.

271

272   **Discussion**

273   This study provides strong evidence that AngII causes ER stress to beta cells/islets both *in vitro*

274   and *in vivo* and that this results in a pro-inflammatory phenotype and beta cell dysfunction. In

275   addition, we provide a novel insight into how AngII causes ER stress (Fig. 6). We show that at

276   high glucose AngII, via AT1R activation, promotes IP3R activation and an increase in ROS.

277   This, likely via a decrease in ER calcium, results in ER stress and an increase in the expression

278   of pro-inflammatory cytokines mediated by the activation of PERK and IRE1α.

279   Although inflammation and the UPR are protective responses, chronic inflammation and/or

280   UPR activation is associated with the pathogenesis of many diseases including type-2 diabetes

281   (23). Indeed chronic inflammation has been implicated in beta cell dysfunction in type-2

282   diabetes and treatment with either anakinra, an IL-1 receptor antagonist, or anti IL-1β

283   antibodies improves beta cell function, improves glycemic control and reduces inflammation

284   (38–40). Moreover, IL-1β antagonism protects against the deleterious effects of AngII on islet

285   function in HFD fed mice (11). Thus inflammation is a key mediator of AngII-induced beta

286   cell dysfunction (11). A role for ER stress in AngII-mediated inflammation has recently been

287   shown in other cell types/tissues (41–43), yet the mechanism by which this occurs had not been

288   fully explored.

289   In this study we demonstrate that AngII-stimulated increase in pro-inflammatory cytokine

290   expression in islets and beta cells is caused by ER stress as: 1) *Il-1β* and *Tnf-α* expression is

291   inhibited by TUDCA; 2) *Il-1β* expression is inhibited by inhibition/reduced expression of

292　PERK and IRE1α/XBP1 ; 3) ER stress precedes an increase in the expression of *Il-1β* and *Tnf-*

293　*α* and; 4) thapsigargin increases the expression of *Il-1β* and *Tnf-α*. This ER stress-induced

294　increase in cytokine expression may be mediated by TXNIP promoted by the activation of

295　PERK and IRE1α as: 1) pharmacologically induced ER stress increases *Il-1β* mRNA

296　expression through an increase in TXNIP expression, stimulated by the activation of PERK

297　and IRE1 (16,44); 2)　AngII increases *Txnip* expression via a PERK- and IRE1α- dependent

298　mechanism (Fig. 3) and; 3) *Txnip* precedes the expression of *Tnf-α* and *Il-1β* and is inhibited

299　by TUDCA AngII induced ER stress is prevented by IP3R inhibition (Fig. 4). Intriguingly,

300　inhibitors of NOX or the IP3R inhibit AngII-induced ER stress.As ER calcium depletion is

301　known to cause ER stress (26,45–48) and ROS can potentiate IP3-dependent calcium release

302　(36), ER calcium depletion is the likely cause of AngII-induced ER stress.

303　We show that chronic AngII treatment causes a decrease in beta cell function both *in vitro* and

304　*in vivo* as evaluated by a loss of GSIS caused by an increase in the release of insulin at a lower

305　threshold of glucose (Fig. 1 and 5). Similar results have been observed in rodent and human

306　islets chronically treated with AngII and in HFD fed mice infused with AngII (11).

307　Interestingly, the loss of GSIS observed in this study was associated with decreased

308　*glucokinase* expression (Fig. 1 and 6), a protein which sets the threshold for GSIS (49,50). As

309　the expression of glucokinase and GLUT2 are positively related to the state of differentiation

310　of the beta cells and their expression is stimulated by PDX1 (51–53), it is surprising that AngII

311　increases *Pdx1* and *Glut2* expression both *in vitro* and *in vivo* (Fig. 1 and 6). However, we

312　detected no change in PDX1 protein expression (results not shown). Thus the significance of

313　these changes in mRNA expression are unclear.

314　The components of the RAS system are expressed in islets and these are up-regulated in animal

315　models of diabetes. Thus locally generated AngII rather than systemic AngII likely play an

important role in islet inflammation and ultimately beta cell dysfunction in type-2 diabetes (7,9,54). Interestingly, the AT1R antagonist losartan reduces high glucose induced ER stress and decreased beta cell function in human islets (55). Moreover, the administration of losartan to *db/db* mice improves islet function and mass, delays the onset of diabetes yet, has no effect on insulin sensitivity (7). Thus high glucose induced ER stress and beta cell dysfunction is, at least in part, mediated by AngII acting via the AT1R. Interestingly, we found that high glucose potentiated the effects of AngII on ER stress in MIN6 cells and murine islets. In addition, AngII exacerbates palmitate-induced ER stress in MIN6 cells (unpublished results). Therefore hyperglyceamia and/or obesity may potentiate the deleterious effect of increased local AngII on beta cell function by exacerbating ER stress.

Together, these findings provide evidence that ER stress is a critical link between AngII and the induction of pro-inflammatory cytokines and that this may represent an initiating and/or early step in the development of beta cell dysfunction in type-2 diabetes.

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506

507 **Figure legends**

508 **Figure 1. Angiotensin II evokes ER stress and impairs beta cell function in mouse islets**

509 **of Langerhans**. Mouse islets of Langerhans were treated with AngII (1μmol/l) (AngII) in the

510 absence or presence of TUDCA (500 μg/ml) (AngII plus TUDCA = A+T) for 4 days prior to:

511 (**a**) performing a GSIS assay to determine (**b**) the stimulatory index or (**c-e**) qPCR analyses of:

512 (**c**) markers of beta cell function (*glucokinase (Gk); Glut2 and Pdx1*); (**d**) pro-inflammatory

513 cytokines (*Il-1β, Tnf-α,* and *Mcp-1*);(**e**) markers of ER stress (*BiP, Xbp1s, Ero1l, Fkbp11,*

514 *Edem, Atf4, Chop,*); The results are expressed as the mean +/- S.E.M of three independent

515 experiments. * *p*<0.05, ** *p*<0.01 vs control; †† *p*<0.01 for the compared groups.

516

517 **Figure 2**. **ER stress precedes the expression of pro-inflammatory cytokines.** MIN6 cells

518 were treated for up to 96 h with 1μmol/l AngII. **(a)** Western-blot analysis of BiP, ATF4, CHOP

519 and the phosphorylated form of IRE1α (p-IRE1), PERK (p-PERK) and eIF2α (p-eIF2α).

520 GAPDH was used as a loading control. Densitometric analyses of the results are presented

521 below. qPCR analyses of: **(b)** markers of ER stress (*Xbp1s, Chop, Tnxip*) and; **(c)** pro-

522 inflammatory cytokines (*Il-1β, Tnf-α* and *Mcp-1*). (**d**) Mouse islets were treated for up to 42 h

523 with 1μmol/l AngII. qPCR analyses of markers of ER stress (*Xbp1s, Atf4, Chop, Tnxip*) and

524 pro-inflammatory cytokines (*Il-1β* and *Tnf-α*). The results are expressed as mean +/- S.E.M of

525 four independent experiments. * $p<0.05$, ** $p<0.01$ vs control.

526

527 **Figure 3. Role of PERK and IRE1 in Angiotensin II induced expression of *Il-1β*.** MIN6

528 cells were treated with 1μmol/l AngII for 6 h in the presence or absence of vehicle (DMSO),

529 0.5 μmol/l GSK2606414 (PERKi) **(a)** Western-blot analysis of BiP, ATF4, CHOP phospho-

530 IRE1α (p-IRE1α), phospho-PERK (p-PERK) and phospho-eIF2α (p-eIF2α). GAPDH was

531 used as a loading control. Densitometric analyses of the results are presented below. **(b)** qPCR

532 analyses of *Txnip, Il-1β* and *Xbp1s*. The results are expressed as mean +/- S.E.M of three

533 independent experiments. * $p<0.05$, ** $p<0.01$ vs their control; ♦♦ $p<0.01$ vs the control group

534 of siCon. MIN6 cells were transfected with control siRNA or siRNA against *Ire* or *Xbp*. 96 h

535 post transfection cells were treated with 1μmol/l AngII for 6 h prior to qPCR analyses of **(c)**

536 *Xbp1s, Ire1α and* **(d)** *Txnip,* and *Il-1β.*

537

538 **Figure 4. IP3R and NOX activation is required for angiotensin II induced ER stress**. **(a)**

539 MIN6 cells were treated with 1μmol/l AngII for 2 ~~or 6 h~~ in the presence of 2-

540 aminoethoxydiphenyl borate (2APB) or xestospongin-C (XestC) (10μmol/l). Western-blot

541 analysis of BiP, ATF4, CHOP, phospho-IRE1α (p-IRE1α) and phospho-eIF2α (p-eIF2α).

542 GAPDH was used as a loading control. Densitometric analyses of the results are presented

543 below. The results are expressed as mean +/- S.E.M of three independent experiments. *

544       $p<0.05$, ** $p<0.01$ vs control; # $p<0.05$ for the compared groups. **(b)** MIN6 were treated with

545   1μmol/l AngII for 6 h in the presence or absence of: 100 nmol/l irbesartan (IRB); 1 (+), 10 (++)

546   or 300 μmol/l (+++) apocycin (Apo) or; 10 μmol/l diphenyleneiodonium (DPI). Superoxide

547   levels were measured using L-012 enhanced chemiluminescence. The results are expressed as

548   mean +/- S.E.M (NS (not significant)), ** $p<0.01$, * $p<0.05$; vs AngII treated and †† $p<0.01$

549   vs control). **(c)** Representitive western-blot of BiP, ATF4, CHOP, phospho-IRE1and phospho-

550   eIF2α. GAPDH was used as a loading control. DPI and Apo used was 10 μmol/l.

551

552   **Figure 5. Angiotensin II infusion in mice causes beta cell dysfunction, ER stress and and**

553   **increase in the expression of pro-inflammatory cytokines**. C57BL/6J male mice were

554   infused with AngII (at 416ng.kg^{-1}.min^{-1}) for 2 weeks. Where indicated TUDCA (150 mg.kg^{-1}

555   .day^{-1}) was also administered (Sham (–●–), AngII (–■–), AngII plus TUDCA (A+T) / (–□–)).**(a)**

556   A GTT was conducted after 2 weeks of infusion and **(b)** the incremental area under the curve

557   (iAUC) and **(c)** plasma insulin levels determined. **(d)** Disposition index is expressed as median

558   +/- interquartile range. The results are expressed as mean +/- S.E.M. with eight mice per group

559   unless otherwise stated. * $p<0.05$, ** $p<0.01$ vs sham; †† $p<0.01$ vs AngII. Pancreatic islets

560   were then isolated and mRNA expression of:**(e)** markers of ER stress; **(f)** pro-inflammatory

561   cytokines and; **(g)** genes related to beta cell function were determined by qPCR analysis. The

562   results are expressed as mean +/- S.E.M. with four mice per group. * $p<0.05$, ** $p<0.01$ vs

563   control; † $p<0.05$, †† $p<0.01$ for the compared groups.

564

565   **Figure 7. Schematic showing how angiotensin II causes inflammation in beta cells.** AngII

566   binds to the AT1R resulting in the activation of Gαq/PLCβ and NOX. This increases the

567   production of ROS and IP3. ROS possibly sensitizes the IP3R for subsequent IP3-dependent

568 calcium release from the ER. This causes ER stress and the activation of the UPR, which

569 promotes an increase in the expression of pro-inflammatory cytokines.
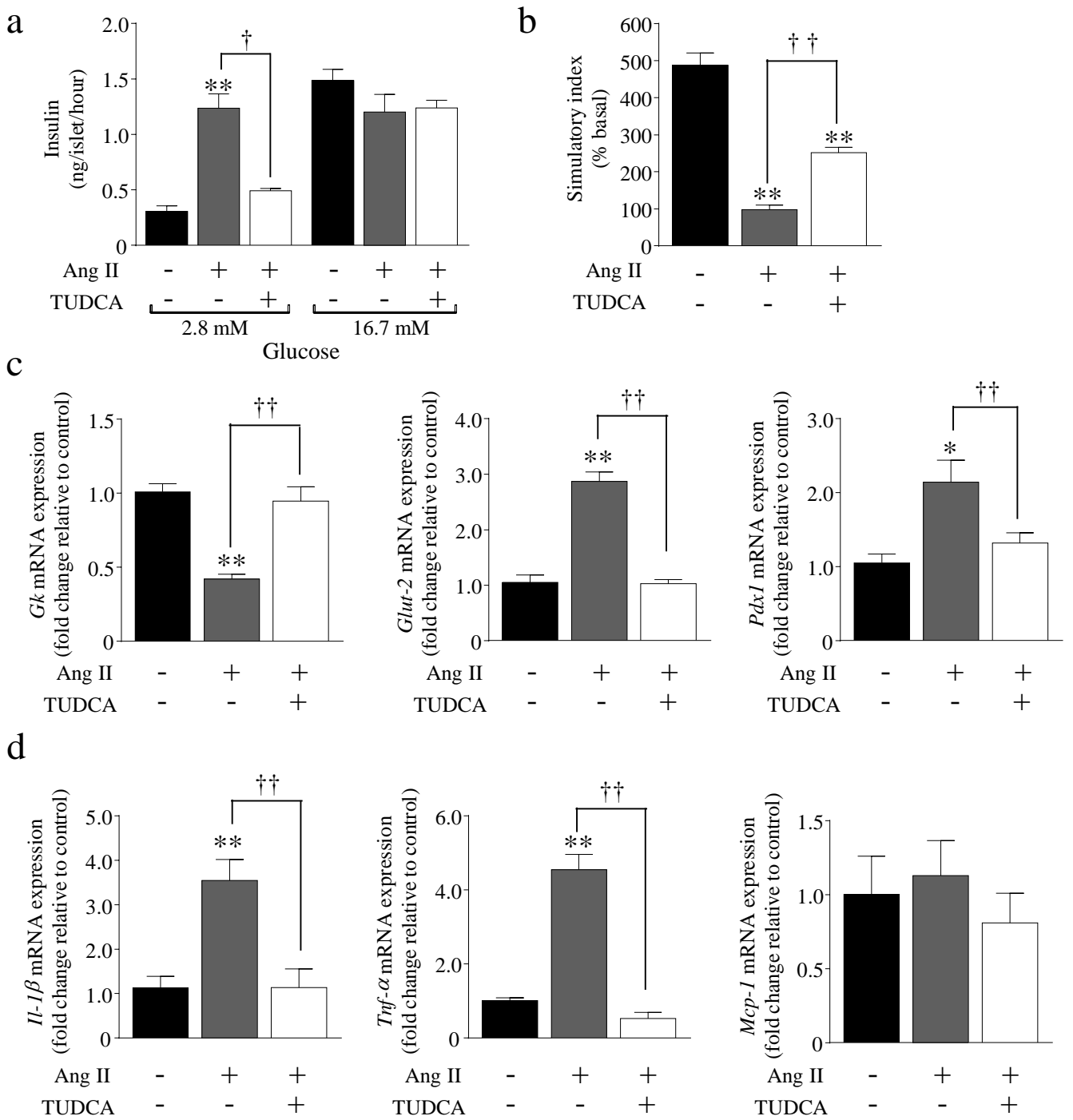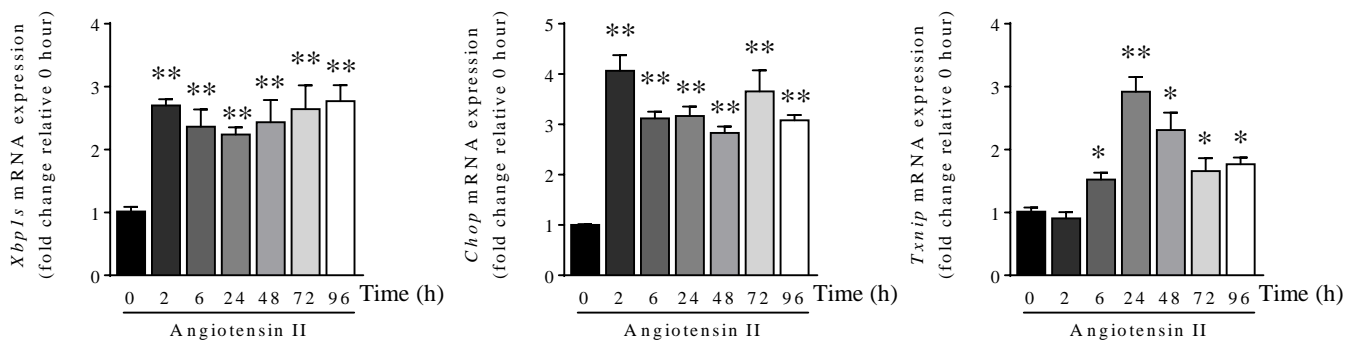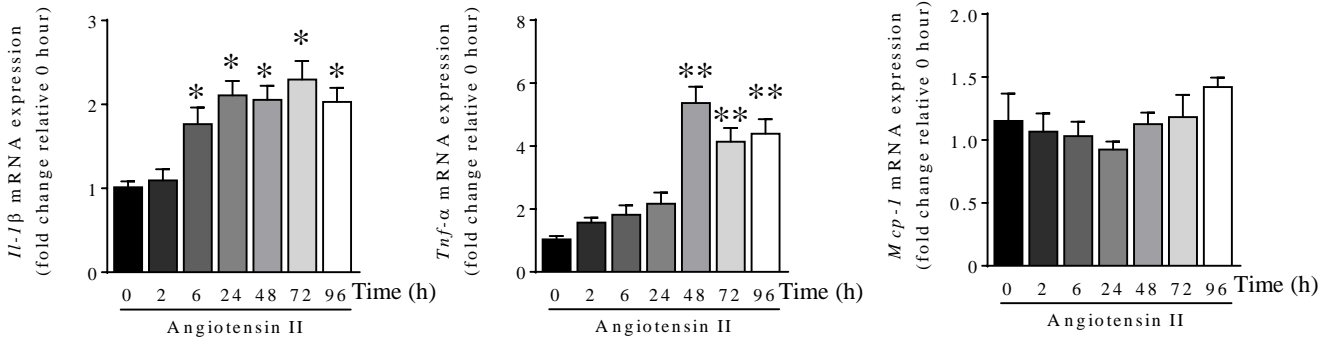
Figure 1

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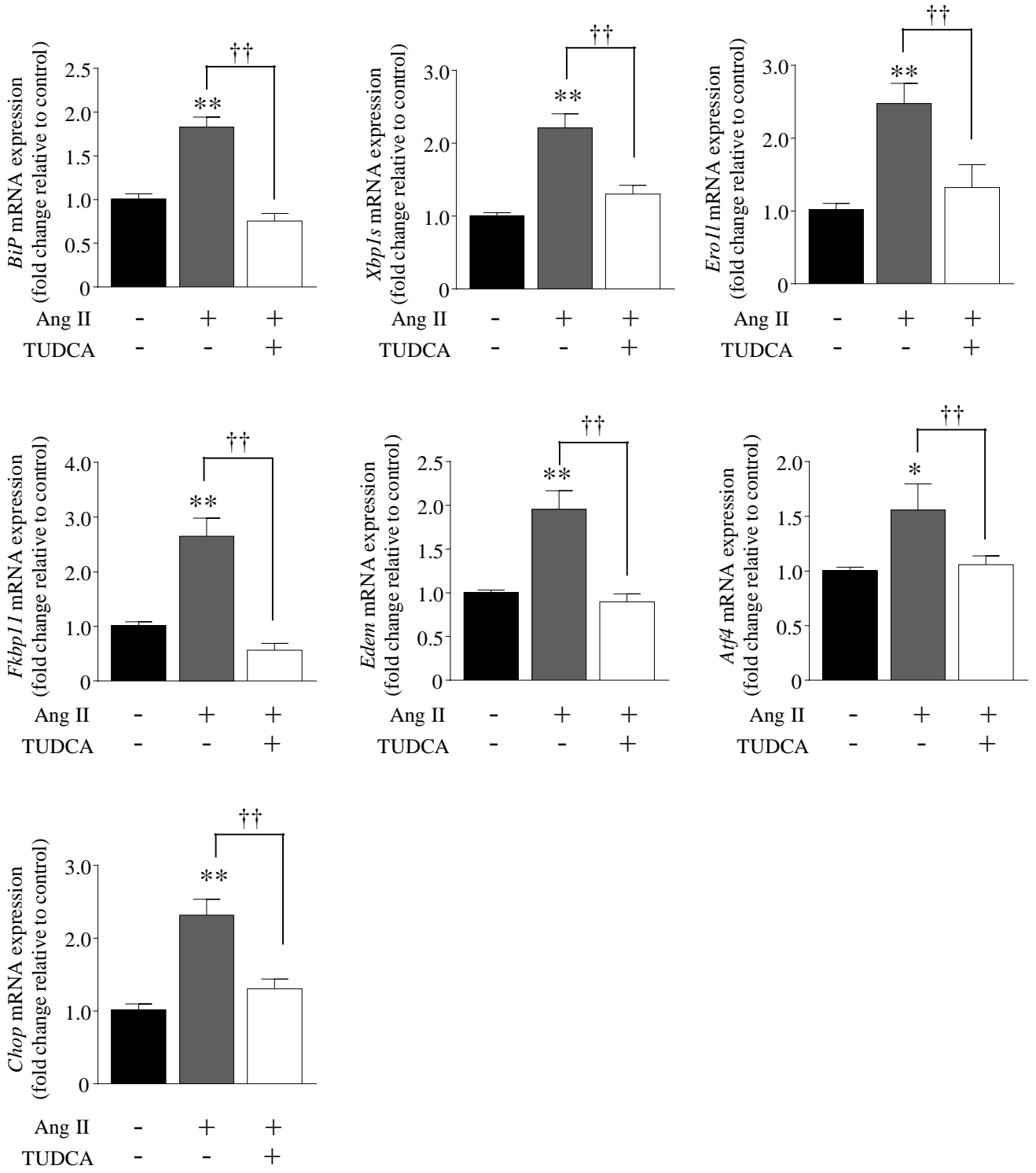
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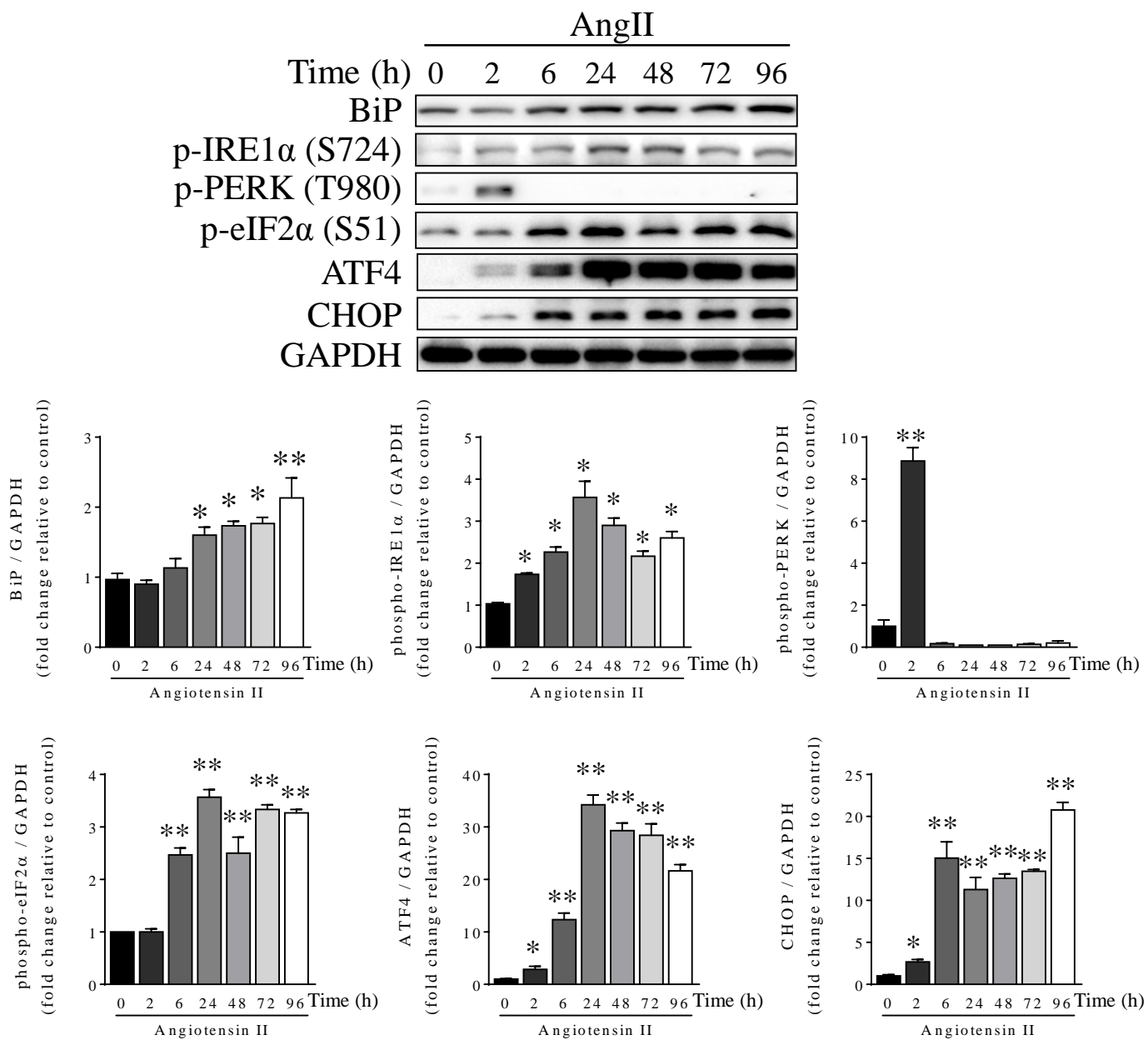
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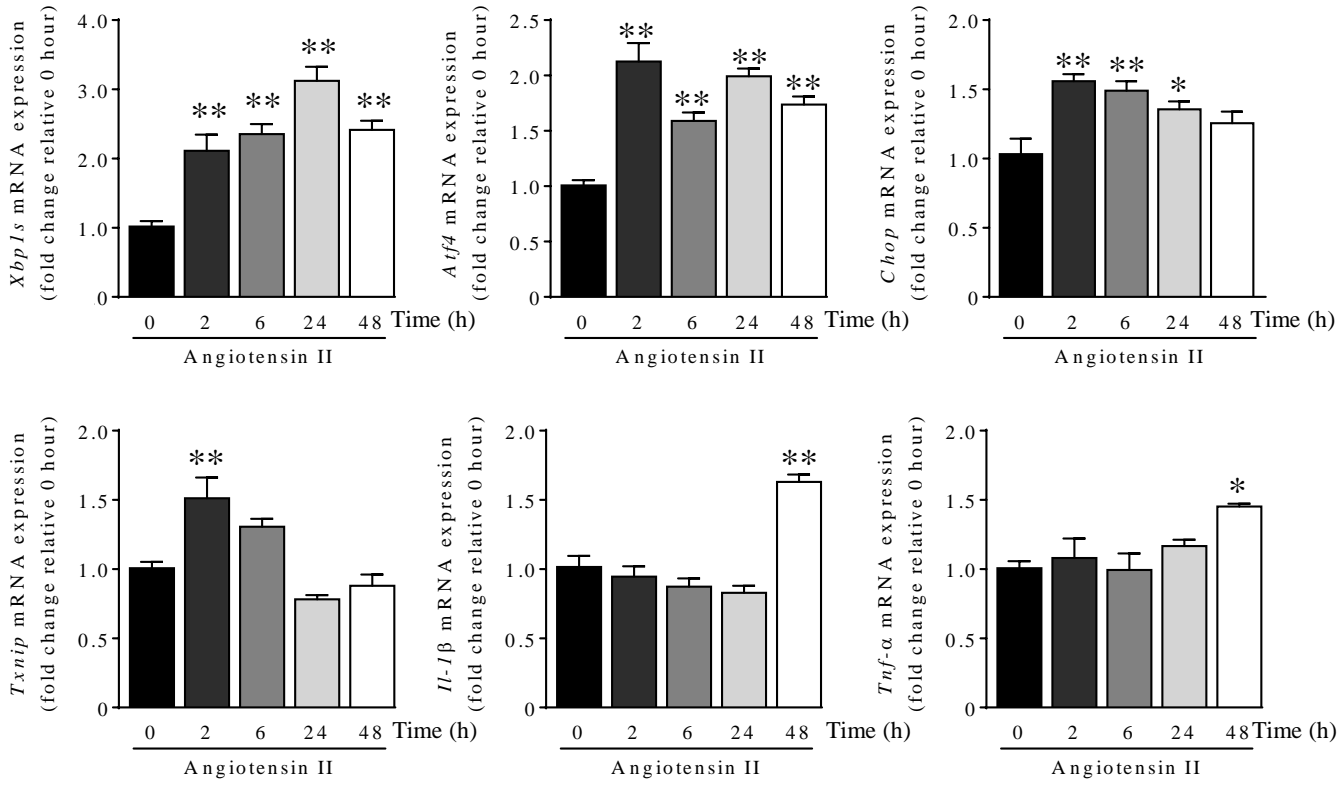
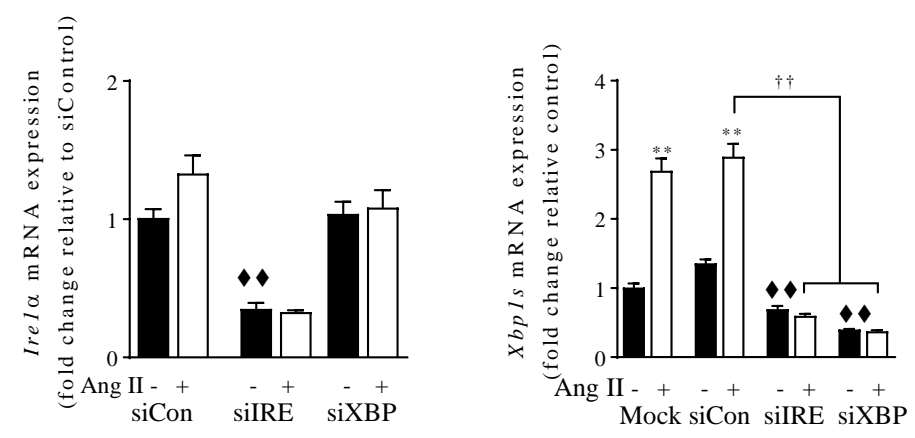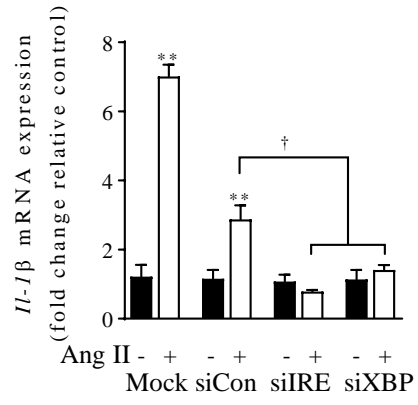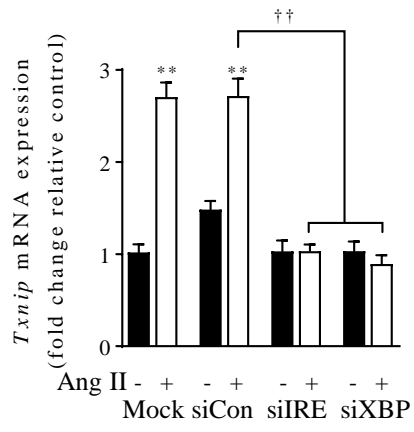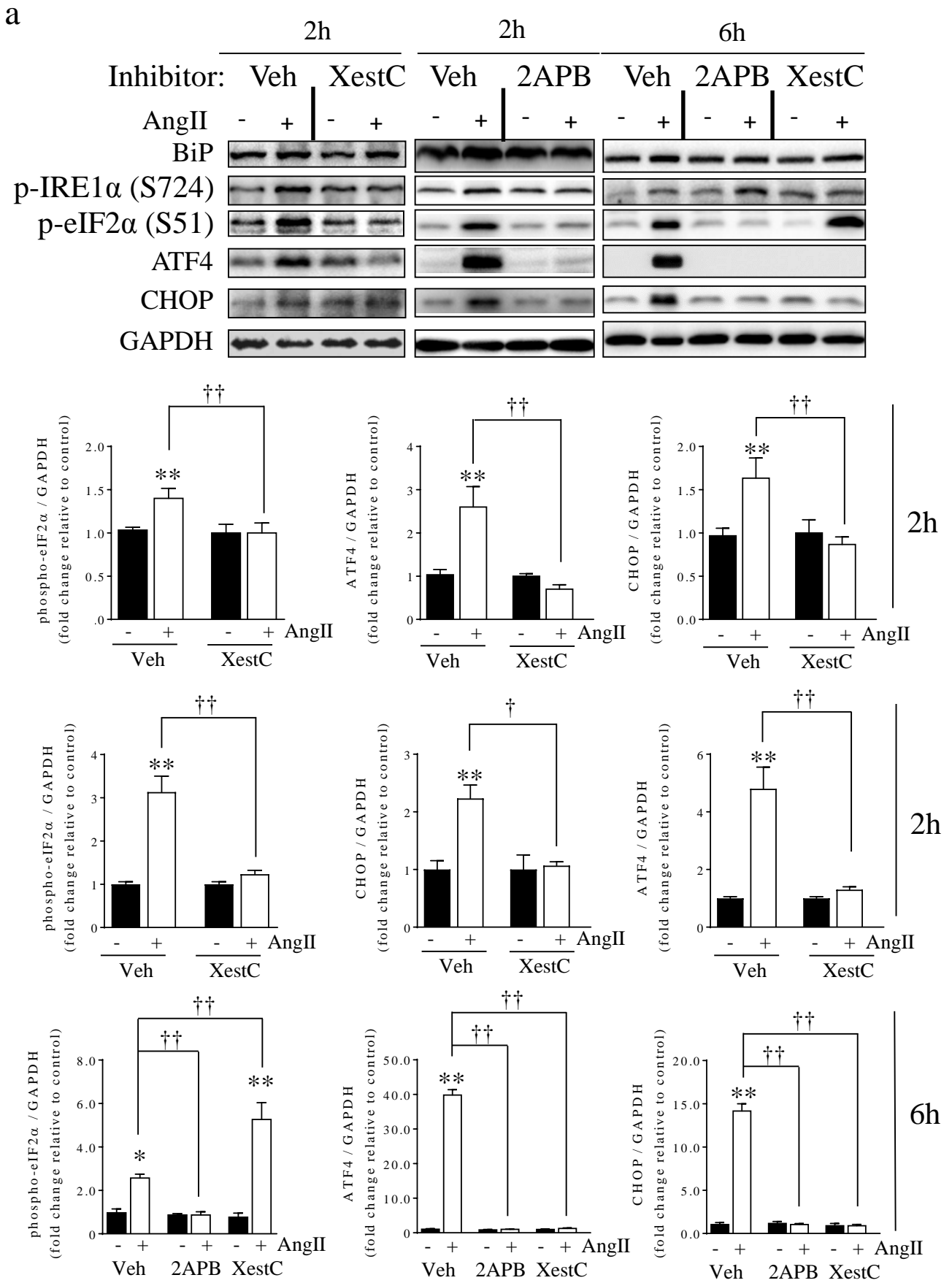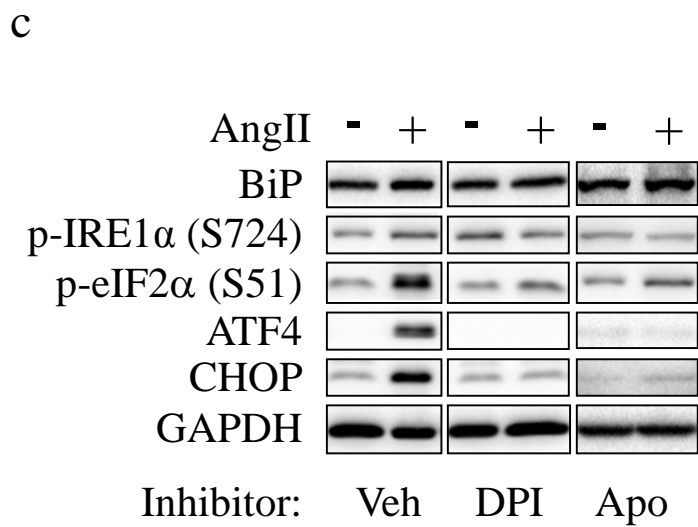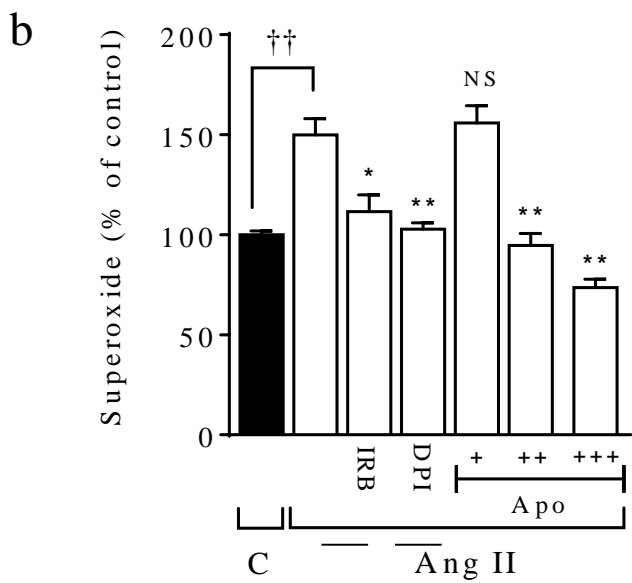Figure 2 continued
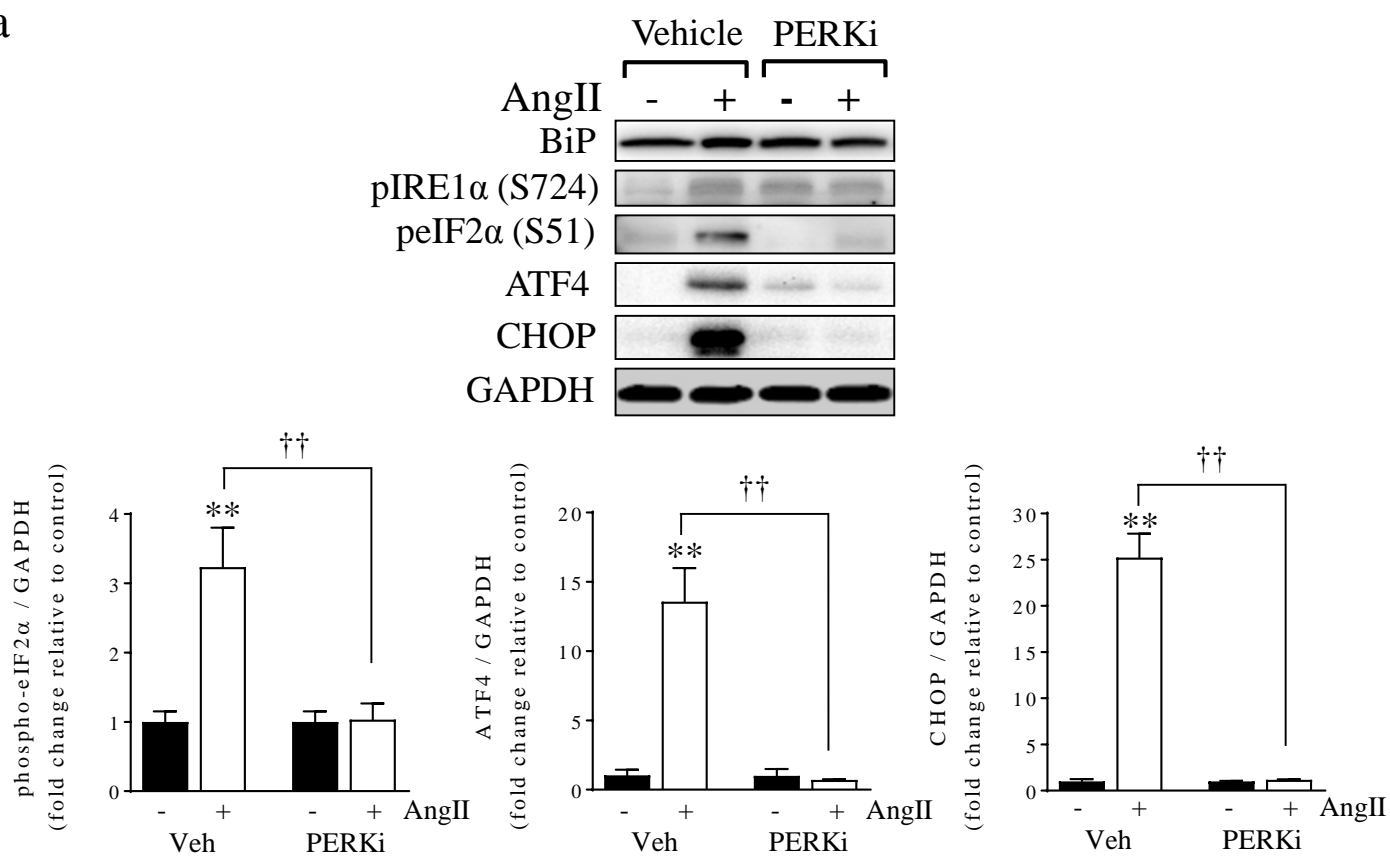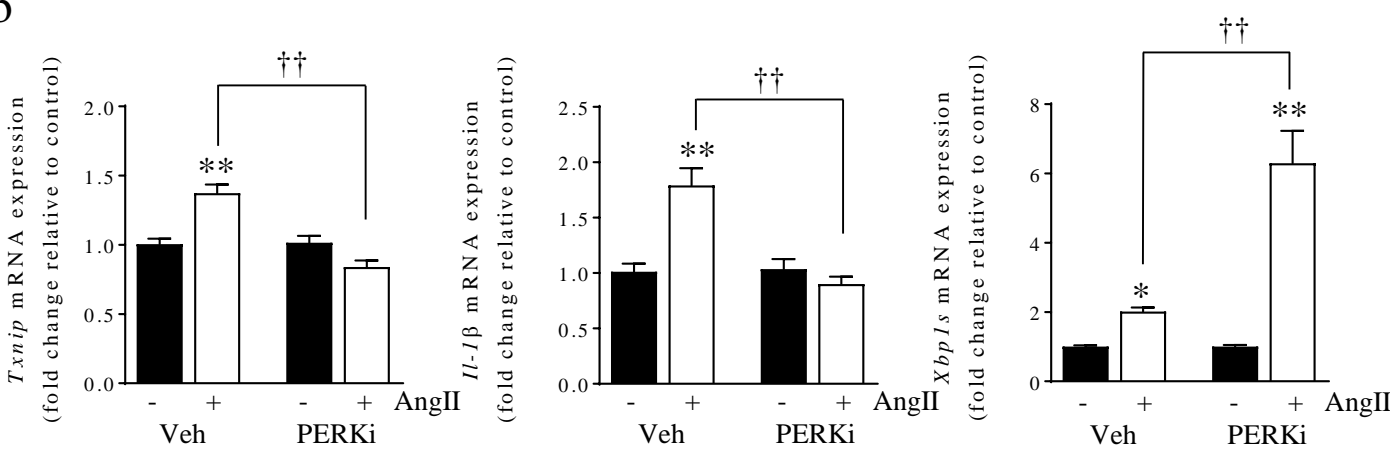
Figure 3

d

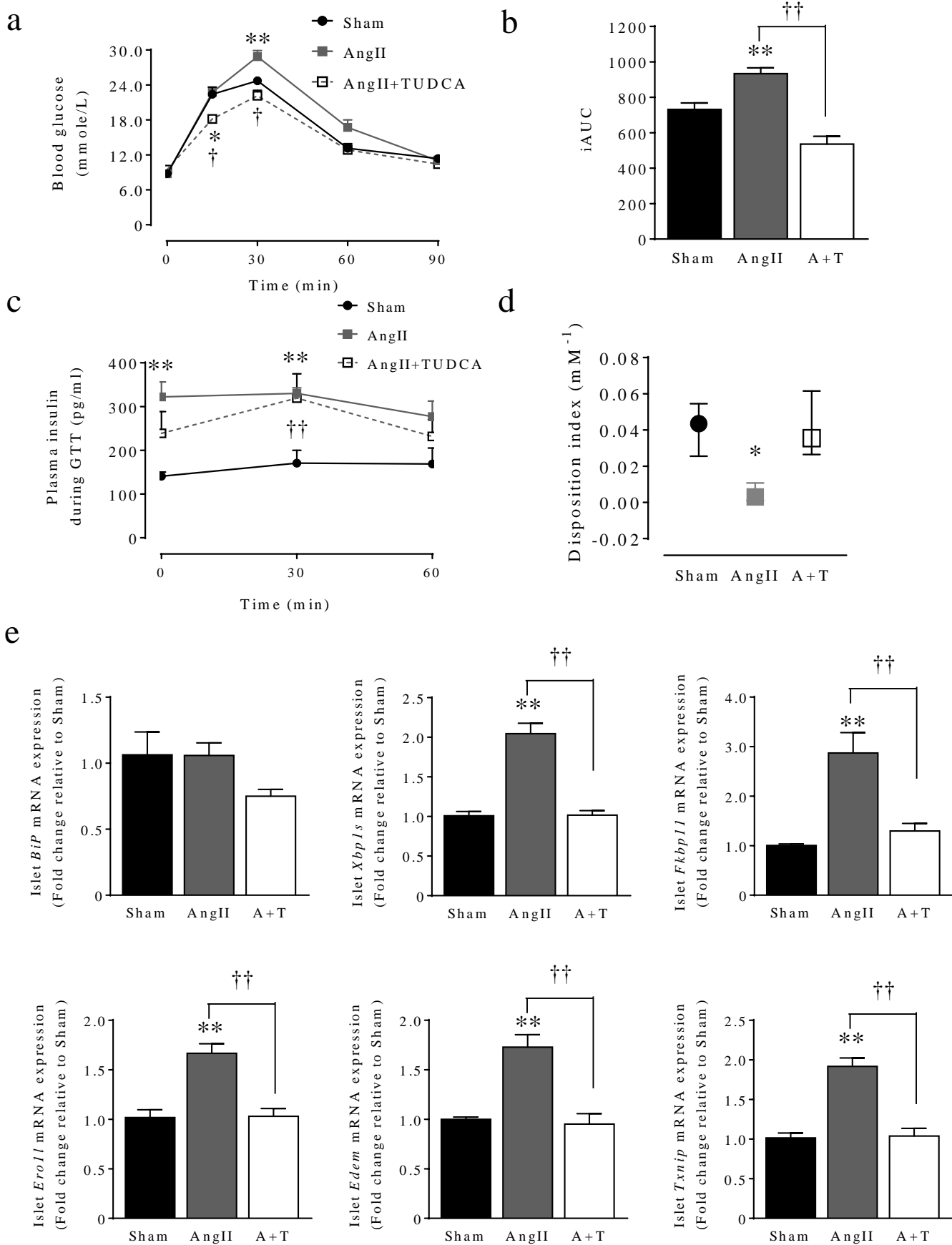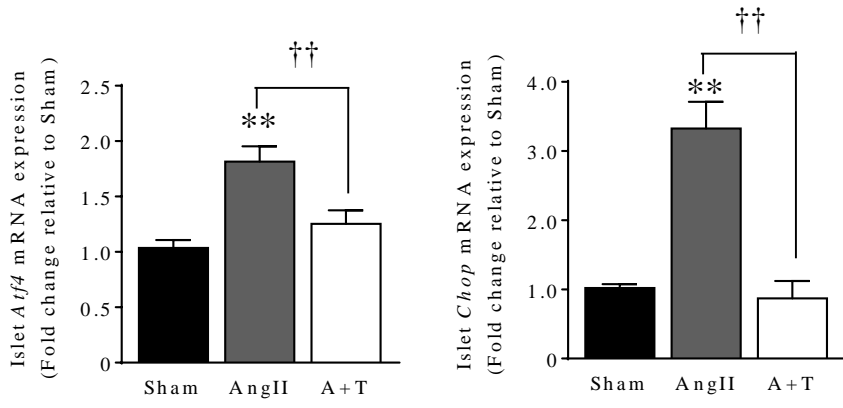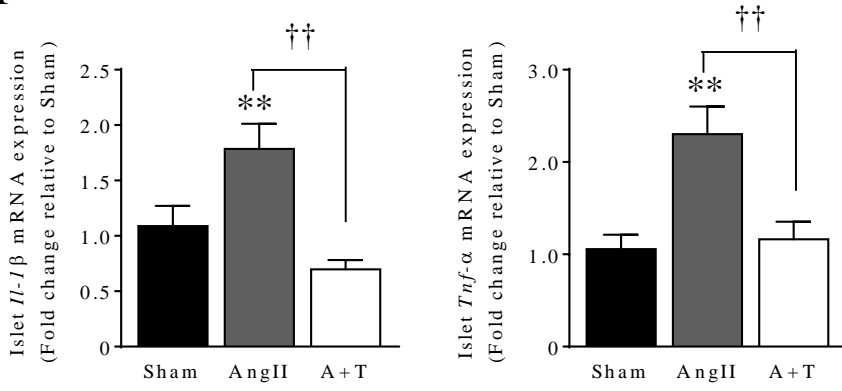

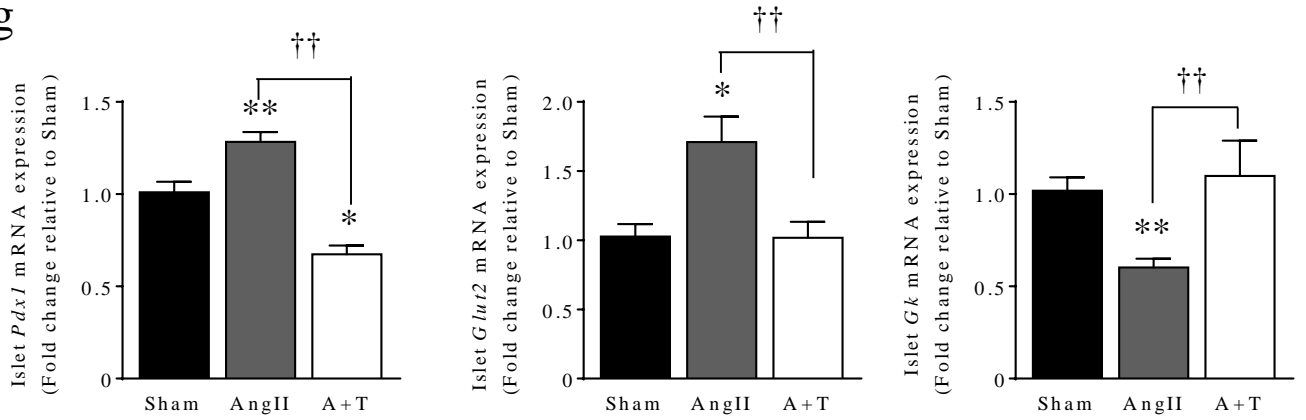Figure 3 continued

Figure 4

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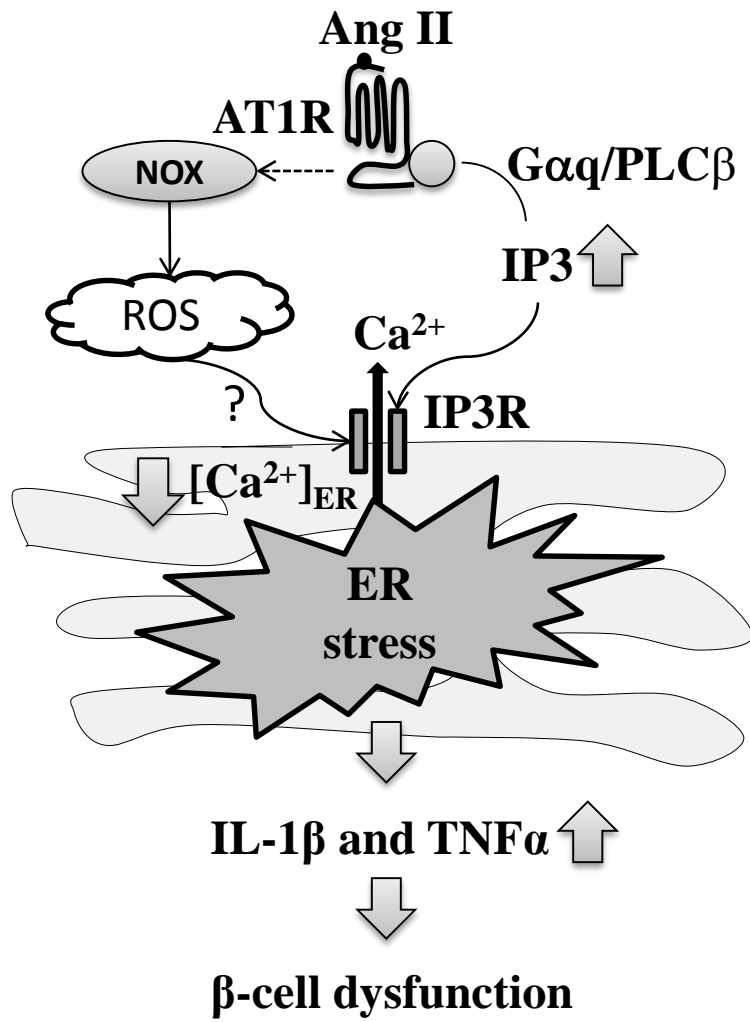
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Figure 5

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Figure 5 continued

Figure 6