Screening of metabolism-disrupting chemicals on pancreatic α-cells using in vitro methods

Reinaldo Sousa Dos Santos[1,2], Ignacio Babiloni-Chust[1,2], Laura Marroqui[1,2], and Angel Nadal[1,2*]

[1]*Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE), Universidad Miguel Hernández de Elche, Alicante, Spain.*

[2]*CIBER de Diabetes y Enfermedades Metabólicas Asociadas, Instituto de Salud Carlos III.*

*** Corresponding author:**

Angel Nadal

Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche, IDiBE, Universidad Miguel Hernández de Elche, Ed. Torregaitán, Av. de la Universidad s/n 03202 Elche, Spain.

Tel. +34 965222002

Email: nadal@umh.es

Disclosure Statement The authors have nothing to disclose.

Abstract

Metabolism-disrupting chemicals (MDCs) are endocrine disruptors with obesogenic and/or diabetogenic action. There is mounting evidence linking exposure to MDCs to increased susceptibility to diabetes. Despite the important role of glucagon in glucose homeostasis, there is little information on the effects of MDCs on α-cells. Furthermore, there are no methods to identify and test MDCs with the potential to alter α-cell viability and function. Here we used the mouse α-cell line αTC1-9 to evaluate the effects of MDCs on cell viability and glucagon secretion. We tested six chemicals at concentrations within human exposure (from 0.1 pM to 1 μM): bisphenol-A (BPA), tributyltin (TBT), perfluorooctanoic acid (PFOA), triphenylphosphate (TPP), triclosan (TCS), and dichlorodiphenyldichloroethylene (DDE). Using two different approaches, MTT assay and DNA-binding dyes, we observed that BPA and TBT decreased α-cell viability via a mechanism that depends on the activation of estrogen receptors and PPARγ, respectively. These two chemicals induced ROS production, but barely altered the expression of endoplasmic reticulum (ER) stress markers. Although PFOA, TPP, TCS, and DDE did not alter cell viability nor induced ROS generation or ER stress, all four compounds negatively affected glucagon secretion. Our findings suggest that αTC1-9 cells seem an appropriate model to test chemicals with metabolism-disrupting activity and that the improvement of the test methods proposed herein could be incorporated into protocols for the screening of diabetogenic MDCs.

Keywords: Apoptosis, Diabetes, Endocrine disruptors, Glucagon secretion, Metabolism-disrupting chemicals, Pancreatic α-cells, Test methods

1. Introduction

Diabetes has been considered one of the most serious metabolic diseases and its global prevalence has been rising at an alarming rate [1]. One of the emerging risks factors that promote diabetes development is our extensive exposure to environmental chemicals that act as endocrine disruptors [2,3].

Despite their differences in etiology and pathogenesis, both type 1 (T1D) and type 2 diabetes (T2D) present dysfunctional glucagon-producing α-cells and impaired glucagon secretion [4–7]. Glucagon is considered a critical regulator of glycemia because it counteracts the glucose-lowering effects of insulin by stimulating hepatic glycogenolysis and gluconeogenesis [8,9]. In the context of diabetes, dysregulated glucagon secretion may contribute to the observed insulin-induced hypoglycemia in T1D and hyperglycemia in early stages of T2D [10,11].

Accumulating data suggest that metabolism-disrupting chemicals (MDCs), a class of endocrine disruptors that alter susceptibility to metabolic disorders [12,13], impact β-cell function and survival by different molecular initiated events (MIE). For instance, bisphenol A (BPA), a well-studied MDC, can alter insulin secretion in primary mouse islets/β-cells by activating estrogen receptors and altering ion channel expression and activity [14–17]. Moreover, BPA induces β-cell apoptosis in an estrogen receptor-dependent manner, where BPA increases reactive oxygen species (ROS) generation and disrupts the formation of ERα/ERβ heterodimers [18–20]. Tributyltin (TBT), another well-known MDC, induces β-cell dysfunction and demise as well as ROS production by a mechanism involving peroxisome proliferator-activated receptor γ (PPARγ) [20–22]. Despite the mounting body of evidence that has been gathered about how MDCs affect β-cells, much less is known regarding the effects of these chemicals on pancreatic α-cells. The fact that α-cell dysfunction poses a risk for both T1D and T2D individuals reinforces the importance of identifying MDCs that might impact α-cell survival and/or function.

As part of GOLIATH, a European Union Horizon 2020-funded project focused on designing MDC testing approaches [23], we have recently described test methods to identify MDCs that might affect β-cells [20]. In our previous study, BPA and TBT were considered as positive controls, while other four chemicals, namely perfluorooctanoic acid (PFOA), triphenylphosphate (TPP), triclosan (TCS) and dichlorodiphenyldichloroethylene (DDE), were used as "unknown" chemicals due to the lack of data about their effects on β-cells. In the present work we tested these chemicals

79 in α-cells following an adverse outcome pathway framework, where the MIE was studied
80 by pharmacology and two key events, namely ROS production and expression of
81 endoplasmic reticulum (ER) stress markers. As adverse effects, we assessed α-cell
82 viability and glucagon secretion.
83
84 **2. Results**
85 *2.1. MDCs affect α-cell survival*
86 We have previously shown that the effects of MDCs on β-cell survival could be evaluated
87 by two different approaches, namely the MTT assay and staining with the DNA-binding
88 dyes Hoechst 33342 and propidium iodide (HO/PI) [20]. Here, we used these same
89 methods to evaluate whether different MDCs could affect α-cell viability. For this
90 purpose, the mouse α-cell line αTC1-9 was exposed to a range of concentrations of each
91 MDC. First, we measured cell viability by MTT assay following treatment for 48 h and
92 72 h (Figure 1A-F and Supplementary Figure 1). Considering the range of concentrations
93 established based on biomonitoring studies, BPA and TBT decreased α-cell viability in a
94 dose-dependent manner (Figure 1A,B, Supplementary Figure 1E,F), whereas the other
95 four MDCs tested did not change cell viability (Figure 1C-F, Supplementary Figure 1G-
96 J). At their highest concentrations, i.e., 1 μM for BPA and 200 nM for TBT, BPA and
97 TBT decreased viability by around 10% and 30%, respectively, after treatment for 48 h.
98 Exposure to higher concentrations of PFOA, TPP, TCN, and DDE showed that 1 mM of
99 these chemicals had a robust effect on αTC1-9 cell viability, which was reduced by nearly
100 80-90% upon treatment (Supplementary Figure 1A-D).
101 Next, we assessed αTC1-9 viability using the DNA-binding dyes HO/PI upon 24 h
102 treatment (Figure 1G-I). The high sensitivity of this method allowed us to detect that very
103 low concentrations of either BPA (Figure 1G) or TBT (Figure 1H) induced α-cell
104 apoptosis. While 1 μM BPA promoted a 2-fold increase in apoptosis, a 3-fold induction
105 was seen in 200 nM TBT-treated cells. As shown by MTT, treatment with concentrations
106 of PFOA, TPP, TCN, and DDE within the range observed in biomonitoring studies did
107 not induce apoptosis (Figure 1I).
108 As in our previous work [20], a mix of the proinflammatory cytokines interleukin-1β (IL-
109 1β) and interferon-γ (IFNγ) was used as positive control. As expected, IL-1β + IFNγ
110 decreased viability or induced apoptosis in our model (Figure 1,2, Supplementary Figure
111 1E-J).

112

113 *2.2. Estrogen receptors and PPARγ are involved in BPA- and TBT-induced α-cell*
114 *apoptosis*

115 BPA is a xenoestrogen that, through the activation of the estrogen receptors ERα, ERβ,
116 and G protein-coupled estrogen receptor (GPER) can affect β-cell function and survival
117 [14,19,20,24]. We observed that BPA-induced apoptosis was totally blunted by
118 concomitant treatment with the pure estrogen receptor antagonist ICI 182,780 (Figure
119 2A). As TBT is an agonist of both the retinoid X receptors (RXRs) and the PPARγ
120 [25,26], we used the PPARγ antagonist T0070907 to investigate if PPARγ activation
121 would be involved in TBT-induced apoptosis. As shown in Figure 2B, TBT-induced
122 apoptosis was mostly blocked by T0070907. These findings suggest that estrogen
123 receptors and PPARγ are involved in the MIE whereby BPA and TBT, respectively,
124 induce α-cell death.

125

126 *2.3. BPA and TBT promote ROS production*

127 Previous data show that exposure to BPA and TBT, but not to PFOA, stimulate ROS
128 production in β-cells [18–22]. Hence, we measured ROS generation in α-cells upon
129 exposure to all six MDCs. In αTC1-9 cells, a 30-40% increase in ROS levels was
130 observed upon treatment with BPA or TBT, whereas PFOA, TPP, TCN, and DDE did not
131 change ROS production in these cells (Figure 3A-F). Of note, menadione was used as a
132 positive control.

133

134 *2.4. Exposure to different MDCs does not induce ER stress*

135 Accumulating evidence suggests that some endocrine disruptors induce ER stress, which
136 might lead to apoptosis in a variety of models [27]. We measured the protein expression
137 of two ER stress markers, namely immunoglobulin heavy chain-binding protein (BiP)
138 and the phosphorylated form of the eukaryotic initiation factor-2α (p-eIF2α), in αTC1-9
139 cells treated with each of the six chemicals for 24 h. Exposure to 1 μM BPA reduced BiP
140 expression by 45%, while no changes were observed in p-eIF2α levels (Figure 4A-C).
141 Treatment with TBT slightly augmented BiP levels without changing p-eIF2α expression
142 (Figure 4D-F). Expression of BiP and p-eIF2α was not modified by exposure to PFOA,
143 TPP, TCN, and DDE (Figure 4G-I).

144

145 *2.5. α-cell function is perturbed by different MDCs*

146 As a test method to assess α-cell function, we measured static glucagon secretion under
147 stimulatory and inhibitory conditions (0.5 mM and 11 mM glucose, respectively) upon
148 exposure to different doses of each MDC for 48 h (Figure 5). BPA (Figure 5A) and TBT
149 (Figure 5B) did not change glucose-regulated glucagon secretion, despite a trend in cells
150 treated with higher concentrations of TBT. The greatest changes were observed in cells
151 exposed to PFOA; at a stimulatory glucose concentration (i.e., 0.5 mM), PFOA induced
152 a decrease in glucagon secretion in a dose-dependent manner, reaching a reduction of
153 nearly 25% at 1 μM compared with vehicle-treated cells. At high glucose, 10 pM PFOA
154 augmented glucagon secretion (Figure 5C). Exposure to TPP also diminished glucagon
155 release at low glucose, albeit only 100 pM TPP was statistically significant (Figure 5D).
156 Curiously, we observed that TCS and DDE modulated glucagon secretion in a non-
157 monotonic dose response-dependent manner, in which exposure to 10 nM TCS and 100
158 pM DDE significantly reduced glucose-regulated glucagon secretion at a stimulatory
159 glucose concentration, while exposure to higher doses of each MDC did not change
160 secretion (Figure 5E and 5F). Of note, 1 μM TCS also increased glucagon release at high
161 glucose (Figure 5E).

162

163 **3. Discussion**

164 Aiming to detect environmental pollutants that could have metabolism disrupting activity
165 in α-cells, here we propose different in vitro test methods to assess α-cell viability (MTT
166 assay and staining with DNA-binding dyes) and function (glucagon secretion).

167 Up to now, there are no human α-cell lines available for research use as they are difficult
168 to generate, mainly due to the paucity of information about the glucagon promoter and its
169 lack of specificity for α-cells [28]. Among the limited α-cell lines existing for rodents, we
170 used the αTC1 Clone 9 (or αTC1-9), which is more differentiated than the parental αTC1
171 cell line and produces only glucagon (but not insulin or preproinsulin) [29]. Since its
172 cloning from the parental αTC1 cells, studies by us and other have confirmed that αTC1-
173 9 cells are a useful model to investigate α-cell physiology. This cell line allows us to
174 assess several cellular parameters, including Ca^{2+} signaling [30,31], electrical activity
175 [31], and glucagon secretion [30,32–35]. Furthermore, αTC1-9 response to different
176 stimuli, such as proinflammatory cytokines and leptin, is similar to their primary cell
177 counterparts [29,31,36,37].

178

179 *3.1. α-cell viability tests*

180 Using two previously established approaches to determine cell viability, namely the MTT
181 assay and HO/PI DNA-binding dyes [20], we showed that low doses of BPA and TBT
182 induced α-cell apoptosis, while only extremely high doses (i.e., >100 μM) of PFOA, TPP,
183 TCS and DDE negatively affected α-cell viability.

184 Although recent evidence shows that BPA affects β-cell survival [18–20,38,39], BPA
185 effects on α-cells are still unclear. To our knowledge, there is only a recently published
186 study that analyzed pancreatic sections from rats given 4.5 μg/L BPA in drinking water
187 for 45 days [40]. In this study, α-cells from BPA-treated rats presented signs of apoptosis,
188 such as cell shrinkage and small dense nuclei, as well as higher levels of caspase 3
189 reactivity compared to vehicle-treated animals. Glucagon immunostaining confirmed that
190 BPA-treated rats had less α-cells than control rats [40]. Despite the different approaches,
191 these data agree with our results showing that BPA induces apoptosis in vitro.

192 BPA is a known xenoestrogen that induces β-cell apoptosis via activation of the estrogen
193 receptors ERα, ERβ, and GPER [19,20]. As all three estrogen receptors are expressed in
194 α-cells [41], we investigated whether these receptors were involved in BPA-induced
195 apoptosis in αTC1-9 cells. In line with our previous findings in β-cells, the pure estrogen
196 receptor antagonist ICI 182,780 prevented BPA-induced apoptosis, suggesting that the
197 activation of estrogen receptors is part of the MIE underlying BPA effects in α-cells.

198 It has been reported that in vivo and in vitro exposure to TBT results in β-cell death
199 [21,22,42,43]. Here we show for the first time that TBT also induces α-cell apoptosis at
200 doses as low as 1 nM. Curiously, at the highest dose, TBT effect on viability was stronger
201 than the mix of proinflammatory cytokines used as positive control. This effect on
202 viability was mostly abrogated by a PPARγ antagonist, which suggests that this receptor
203 is involved in TBT-induced apoptosis, as we have previously described in β-cells [20].

204 PFOA, TPP, TCN, and DDE only reduced cell viability when αTC1-9 cells were exposed
205 to concentrations higher than 100 μM, which agree with prior studies in β-cells. We
206 showed that concentrations of PFOA above 20 μM induced apoptosis in the human
207 EndoC-βH1 cell line and the rat INS-1E cells [20]. In the rat RIN-m5F β-cell line, doses
208 of PFOA higher than 100 μM induced apoptosis after 48 h, whereas 1 μM PFOA did not
209 affect cell survival [44]. In MIN6, a mouse β-cell line, as well as in primary mouse islets,
210 up to 300 μM PFOA did not change viability; doses above 500 μM, however, induced
211 apoptosis upon 24 h treatment [45]. Up to 1 μM TPP did not induce apoptosis in EndoC-
212 βH1 and INS-1E cells as well as in dispersed mouse islets, even after 72 h exposure [20].

213 Exposure to 17 and 35 μM TCS increased necrosis in MIN6 cells, while lower doses (up
214 to 7 μM) did not change viability in MIN6, EndoC-βH1, and INS-1E cells [20,46]. Lastly,
215 DDE treatment ranging from 10 fM to 50 μM had no effect on the viability of different
216 β-cell lines, namely EndoC-βH1, INS-1E, and MIN6, or in dispersed mouse islets, when
217 cells were treated for up to 48 h [20,47,48]. On the other hand, longer DDE exposure
218 (from 4 to 8 days) diminished viability even at the lowest dose in INS-1E cells [48]. Taken
219 together, our findings suggest that αTC1-9 cells are a reliable model for the identification
220 of MDCs that could affect α-cell survival. Moreover, we confirm that the test methods
221 used herein are suitable for the assessment of cell viability/apoptosis in response to
222 MDCs.
223 Following an adverse outcome pathway framework, we next looked at two key events
224 that could be involved in cell death and dysfunction, namely oxidative stress and ER stress
225 [49,50]. For this purpose, we measured ROS production and the expression of two ER
226 stress markers, namely BiP and p-eIF2α. Like it has been previously reported in β-cells
227 [18–22,38], BPA and TBT induced ROS production in αTC1-9 cells; of note, exposure
228 to the other four MDCs did not change ROS production at the concentrations tested. As
229 ROS levels were increased only by chemicals that induced apoptosis, it is reasonable to
230 state that generation of ROS is a key event in the pathway to MDC-induced α-cell
231 apoptosis. Regarding the ER stress markers, expression of the chaperone BiP was reduced
232 by 1 μM BPA and slightly increased by 200 nM TBT. PFOA, TPP, TCS, and DDE did
233 not alter BiP expression. None of the six chemicals tested changed the expression of the
234 activated form of eIF2α (i.e., p-eIF2α) at the doses tested. At first glance, this lack of
235 major changes in the expression of ER stress markers may be unexpected for BPA and
236 TBT, as these MDCs, which induce α-cell apoptosis, activate the ER stress response in
237 other cell types [27,51–55]. However, it is important to keep in mind that the expression
238 of ER stress markers may depend on the amount and time of exposure to a given stimulus.
239 Moreover, it is possible that activation of the ER stress response is not part of the
240 mechanism whereby BPA and TBT induce apoptosis in α-cells. Finally, contrary to β-
241 cells, where ER stress is a key component contributing to β-cell loss, α-cells are more
242 resistant to ER stress-mediated apoptosis [56]. Overall, these data suggest that MDC-
243 induced ROS production is a key event that might be used as a substitute for measurement
244 of cell viability, as it properly predicted α-cell apoptosis.
245
246 *3.2. α-cell function tests*

247 As glucagon is a hormone responsible for opposing the hypoglycemic effects of insulin,
248 maintenance of adequate α-cell function is crucial for the regulation of glucose
249 homeostasis.
250 Even though the effects of different MDCs on insulin release have been largely explored
251 [12,57,58], whether exposure to these chemicals affects α-cell function remains largely
252 unknown. The only study available to date showed that acute treatment with 1 nM BPA,
253 suppressed low glucose-induced intracellular Ca^{2+} oscillations in primary mouse α-cells
254 within intact pancreatic islets [59]. As intracellular Ca^{2+} signaling is intimately linked to
255 glucagon secretion (see below), one might speculate that BPA-induced suppression of
256 Ca^{2+} oscillations could ultimately inhibit glucagon release at low glucose concentrations.
257 Unfortunately, glucagon secretion was not measured in Alonso-Magdalena et al study. In
258 the present work we observed that glucose-regulated glucagon secretion remained
259 unchanged upon BPA exposure for 48 h. It would be interesting to investigate if Ca^{2+}
260 signals are altered in the αTC1-9 cell line.
261 Contrary to β-cells, where TBT increases glucose-stimulated insulin secretion in a variety
262 of models [20–22,43], here we did not observe a clear effect on glucagon secretion.
263 However, as a clear trend is seen at both glucose concentrations, it may be that higher
264 TBT doses result in augmented glucagon release.
265 The four MDCs that did not affect α-cell viability, PFOA, TPP, TCS, and DDE, disturbed
266 α-cell function in some way. Among them, PFOA was the one that induced the most
267 changes in glucagon secretion, where doses ranging from 100 pM to 1 μM PFOA
268 impaired glucagon secretion at glucose-stimulating levels. Our in vitro findings seem to
269 contradict a recent in vivo study where oral administration of PFOA to adult male mice
270 (1.25 mg/kg PFOA for 28 days) resulted in high blood glucagon levels, increased fasting
271 glycemia, and augmented hepatic gluconeogenesis [60]. These apparently opposing
272 outcomes might be due to different PFOA doses and exposure time between studies. The
273 concentrations of PFOA used herein are within the range observed in biomonitoring
274 studies, which varied between 650 pM and 34 nM [61]. Zheng et al., on the other hand,
275 observed that the serum levels of PFOA at the end of exposure were around 134 μM [60].
276 Furthermore, in the present study we treated αTC1-9 cells for only 48 h, while Zheng and
277 collaborators exposed mice to PFOA for 28 days, which may be long enough to allow α-
278 cells to adapt and start secreting more glucagon. Unfortunately, as we have not tested
279 longer time points, we cannot directly compare our findings with the aforementioned in
280 vivo study. Along with prior studies showing that PFOA exposure also impairs β-cell

281 function [20,45,62], our findings in α-cells strongly suggest that PFOA acts as a
282 diabetogenic MDC.
283 TCS and DDE affected glucagon secretion at a stimulatory glucose concentration in a
284 non-monotonic dose response-dependent manner. Non-monotonic dose response curves
285 have been demonstrated for endocrine-disrupting chemicals in several biological systems,
286 such as cell lines, organ cultures, and animal models, and [63,64]. As these findings may
287 have important implications for risk assessment toxicology, it will be interesting to further
288 explore the mechanisms underlying TCS and DDE non-monotonic effect on α-cell
289 function.
290 Contrary to β-cells, where the stimulus-secretion coupling model is well-established, the
291 exact mechanisms regulating glucose-modulated glucagon secretion are still under
292 debate. Glucagon secretion can be regulated by intrinsic (exerted within the α-cell itself)
293 and paracrine (involvement of factors released from neighboring β- and δ-cells)
294 mechanisms. A generally accepted intrinsic model suggests that, at low glucose
295 concentrations, there is less glucose entering the cells via the glucose transporters, which
296 will result in decreased intracellular ATP/ADP ratio. This lower ATP/ADP ratio leads to
297 moderate activity of K_{ATP} channels, which causes membrane depolarization and
298 subsequent opening of voltage-dependent Na^+ and Ca^{2+} channels. The resulting increase
299 in intracellular Ca^{2+} triggers exocytosis of glucagon secretory granules. Activation of
300 store-operated Ca^{2+} entry due to ER Ca^{2+} depletion and cAMP-induced potentiation of
301 Ca^{2+}-induced Ca^{2+} release from the ER also contribute to exocytosis of glucagon-
302 containing vesicles [8,9,65,66]. Our findings suggest that MDCs affecting α-cell function
303 could be disrupting any step of this complex secretory pathway. In fact, considering our
304 experimental approach, i.e., direct treatment of a cell line, it is likely that PFOA, TPP,
305 TCS, and DDE might be interfering with intrinsic mechanisms controlling glucagon
306 release, such as glucose transport/metabolism, activity/expression of ion channels, and/or
307 exocytosis. Additionally, as we used an α-cell line instead of whole pancreatic islets, we
308 cannot discard the possibility that any of the MDCs tested in our study may affect the
309 paracrine regulation of glucagon secretion. This option should be investigated in future
310 studies. Either way, our study shows that MDC-induced α-cell dysregulation may
311 contribute to the diabetogenic actions of these chemical pollutants.
312 In conclusion, our present findings suggest that αTC1-9 cells represent a valid model for
313 the identification of MDCs with potential diabetogenic activity. We also validated in α-
314 cells the test methods for assessment of cell viability presented in our previous study.

315 Generation of ROS, but not expression of ER stress markers, could be a suitable surrogate
316 for the evaluation of cell viability. Lastly, measurement of glucagon secretion seems a
317 valuable test method to explore whether a given MDC affects α-cell function.
318
319 **4. Materials and Methods**
320 *4.1. Chemicals*
321 Chemicals used in this work were acquired as follows: BPA (Cat. No. 239658), TBT (Cat.
322 No. T50202), PFOA (Cat. No. 77262), TPP (Cat. No. 241288), TCS (Cat. No. PHR1338),
323 and DDE (Cat. No. 123897) were obtained from Sigma-Aldrich (Barcelona, Spain). MDC
324 stock solutions were weekly prepared by dissolution in 100% cell-culture grade, sterile-
325 filtered DMSO (Sigma-Aldrich; Cat No D2650) and stored at -20 °C between uses.
326 Recombinant human IL-1β was obtained from R&D Systems (Abingdon, UK; Cat. No.
327 201-LB/CF) and recombinant murine IFNγ from PeproTech (Rocky Hill, NJ, USA; Cat.
328 No. 315-05). ICI 182,780 (Cat. No. 1047) and T0070907 (Cat. No. HY-13202) were
329 obtained from Tocris Cookson Ltd (Avonmouth, UK) and MedChem Express (New
330 Jersey, USA), respectively. T0070907 was redosed every 8-10 h due to its short half-life.
331
332 *4.2. Culture of αTC1-9 cells*
333 Mouse glucagon-releasing cell line αTC1-9 (RRID: CVCL_0150, ATCC, Manassas, VA,
334 USA) was cultured in DMEM (Invitrogen, Barcelona, Spain) without phenol red
335 supplemented with 2 mM l-glutamine, 19 mM $NaHCO_3$, 15 mM HEPES, 10% inactivated
336 FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mM non-essential amino acids,
337 and a final glucose concentration of 16 mM [35]. Cells were kept at 37 °C in a humidified
338 atmosphere of 95% O_2 and 5% CO_2.
339
340 *4.3. Assessment of cell viability by MTT assay*
341 The MTT assay was performed as previously described [20]. Briefly, MTT was added to
342 each well (final concentration: 0.5 mg/mL) and incubated for 3 h at 37°C. Following
343 incubation, the supernatant was removed by aspiration and formazan crystals were
344 dissolved by addition of 100 µL DMSO. The absorbance was measured at 595 nm using
345 an iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA) and the percentage
346 of cell viability was calculated.
347
348 *4.4. Assessment of cell viability by DNA-binding dyes*

349 Percentage of living, apoptotic and necrotic cells was assessed upon staining with DNA-
350 binding dyes Hoechst 33342 and propidium iodide [67,68]. A minimum of 500 cells per
351 experimental condition was counted by two different observers, one of them being
352 unaware of sample identity to avoid bias (agreement between results from both observers
353 was >90%). Results are expressed as percentage of apoptosis.
354
355 *4.5. DCF assay*
356 Generation of ROS was evaluated using the fluorescent probe 2',7'-dichlorofluorescein
357 di-acetate (DCF; Sigma-Aldrich) as described [20]. DCF fluorescence was quantified in
358 a POLASTAR plate reader (BMG Labtech, Germany). Data are represented as DCF
359 fluorescence corrected by total protein. Menadione (15 μM for 90 minutes) was employed
360 as a positive control.
361
362 *4.6. Western blotting*
363 Cells were washed with cold PBS and lysed in Laemmli buffer. Cells extracts were
364 resolved on 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad).
365 Immunoblot analysis was performed by overnight incubation with antibodies against BiP,
366 p-eIF2α, and α-Tubulin as described [19]. The antibodies used herein are listed in
367 Supplementary Table 1.
368
369 *4.7. Glucagon secretion*
370 Cells were washed and then preincubated in a modified Krebs-Ringer buffer (120 mM
371 NaCl, 5.4 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.4 mM $CaCl_2$, 20 mM HEPES
372 pH 7.4, and 0.1% BSA) added of 5.6 mM glucose for 2 h before stimulation. At the end
373 of this incubation, cells were sequentially stimulated with low (0.5 mM) and then high
374 glucose (11 mM) for 30 min (each stimulation). After each stimulatory period, the
375 incubation medium was collected, placed onto ice, and centrifuged at 1400 *g*, 5 min at 4
376 °C. The supernatant was transferred into a fresh tube containing aprotinin (20 mg/L) and
377 stored at -80 °C until glucagon measurements. Glucagon levels were assessed using a
378 mouse glucagon ELISA kit (Mercodia, Uppsala, Sweden). Data are normalized to
379 glucagon secretion at low glucose in vehicle-treated cells.
380
381 *4.8. Data analysis*

The GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. Data are presented as the mean ± SEM. Statistical analyses were performed using Student's t-test, one-way ANOVA or two-way ANOVA as stated in the figure legends. One- and two-way ANOVA were followed by Dunnett's test as post hoc analysis. p values ≤ 0.05 were considered statistically significant.

Funding sources

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement GOLIATH No. 825489 (AN), Ministerio de Ciencia e Innovación, Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER) grant PID2020-117294RB-I00 (AN) and PROMETEO II/2020/006 (AN) supported by Generalitat Valenciana, Spain. The authors' laboratories are also financed by the grant PID2020-117569RA-I00 from Ministerio de Ciencia e Innovación, Agencia Estatal de Investigación (AEI) (LM). This research was supported by CIBER-Consorcio Centro de Investigación Biomédica en Red (CB07/08/0002), Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación.

Acknowledgements

The authors thank Beatriz Bonmati Botella, Maria Luisa Navarro, and Salomé Ramon for their excellent technical assistance.

Author contributions

R.S.d.S.: Conceptualization, Supervision, Visualization, Investigation, Formal analysis, Writing – original draft & editing; I.B.-C.: Investigation, Formal analysis, Writing – review & editing; L.M.: Investigation, Formal analysis, Writing – review & editing; A.N.: Conceptualization, Supervision, Visualization, Resources, Funding acquisition, Writing – original draft & editing, Project administration. All authors have read and given approval to the final version of the manuscript.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Spanish Royal Decree 1201/2005 and the European Community Council directive 2010/63/EU.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Sun, H.; Saeedi, P.; Karuranga, S.; Pinkepank, M.; Ogurtsova, K.; Duncan, B.B.; Stein, C.; Basit, A.; Chan, J.C.N.; Mbanya, J.C.; et al. IDF Diabetes Atlas: Global, Regional and Country-Level Diabetes Prevalence Estimates for 2021 and Projections for 2045. *Diabetes Res. Clin. Pract.* **2022**, *183*, 109119, doi:10.1016/j.diabres.2021.109119.
2. Gore, A.C.; Chappell, V.A.; Fenton, S.E.; Flaws, J.A.; Nadal, A.; Prins, G.S.; Toppari, J.; Zoeller, R.T. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr. Rev.* **2015**, *36*, doi:10.1210/er.2015-1010.
3. Sargis, R.M.; Simmons, R.A. Environmental Neglect: Endocrine Disruptors as Underappreciated but Potentially Modifiable Diabetes Risk Factors. *Diabetologia* **2019**, *62*, 1811–1822, doi:10.1007/s00125-019-4940-z.
4. Marroquí, L.; Alonso-Magdalena, P.; Merino, B.; Fuentes, E.; Nadal, A.; Quesada, I. Nutrient Regulation of Glucagon Secretion: Involvement in Metabolism and Diabetes. *Nutr. Res. Rev.* **2014**, *27*, 48–62, doi:10.1017/S0954422414000031.
5. Brissova, M.; Haliyur, R.; Saunders, D.; Shrestha, S.; Dai, C.; Blodgett, D.M.; Bottino, R.; Campbell-Thompson, M.; Aramandla, R.; Poffenberger, G.; et al. α Cell Function and Gene Expression Are Compromised in Type 1 Diabetes. *Cell Rep.* **2018**, *22*, 2667–2676, doi:10.1016/j.celrep.2018.02.032.
6. Gromada, J.; Chabosseau, P.; Rutter, G.A. The α-Cell in Diabetes Mellitus. *Nat. Rev. Endocrinol.* **2018**, *14*, 694–704, doi:10.1038/s41574-018-0097-y.
7. Dai, X.-Q.; Camunas-Soler, J.; Briant, L.J.B.; dos Santos, T.; Spigelman, A.F.; Walker, E.M.; Arrojo e Drigo, R.; Bautista, A.; Jones, R.C.; Avrahami, D.; et al. Heterogenous Impairment of α Cell Function in Type 2 Diabetes Is Linked to Cell Maturation State. *Cell Metab.* **2022**, *34*, 256-268.e5, doi:10.1016/j.cmet.2021.12.021.
8. Quesada, I.; Tudurí, E.; Ripoll, C.; Nadal, Á. Physiology of the Pancreatic α-Cell and Glucagon Secretion: Role in Glucose Homeostasis and Diabetes. *J. Endocrinol.* **2008**, *199*, 5–19, doi:10.1677/JOE-08-0290.
9. Wendt, A.; Eliasson, L. Pancreatic α-Cells – The Unsung Heroes in Islet Function. *Semin. Cell Dev. Biol.* **2020**, *103*, 41–50, doi:10.1016/j.semcdb.2020.01.006.
10. Dunning, B.E.; Gerich, J.E. The Role of α-Cell Dysregulation in Fasting and Postprandial Hyperglycemia in Type 2 Diabetes and Therapeutic Implications. *Endocr. Rev.* **2007**, *28*, 253–283, doi:10.1210/er.2006-0026.
11. Cryer, P.E. Minireview: Glucagon in the Pathogenesis of Hypoglycemia and Hyperglycemia in Diabetes. *Endocrinology* **2012**, *153*, 1039–1048, doi:10.1210/en.2011-1499.
12. Heindel, J.J.; Blumberg, B.; Cave, M.; Machtinger, R.; Mantovani, A.; Mendez, M.A.; Nadal, A.; Palanza, P.; Panzica, G.; Sargis, R.; et al. Metabolism Disrupting Chemicals and Metabolic Disorders. *Reprod. Toxicol.* **2017**, *68*, 3–33, doi:10.1016/j.reprotox.2016.10.001.
13. Nadal, A.; Quesada, I.; Tudurí, E.; Nogueiras, R.; Alonso-Magdalena, P. Endocrine-Disrupting Chemicals and the Regulation of Energy Balance. *Nat. Rev. Endocrinol.* **2017**, *13*, doi:10.1038/nrendo.2017.51.

14. Alonso-Magdalena, P.; Ropero, A.B.; Carrera, M.P.; Cederroth, C.R.; Baquié, M.; Gauthier, B.R.; Nef, S.; Stefani, E.; Nadal, A. Pancreatic Insulin Content Regulation by the Estrogen Receptor ERα. *PLoS ONE* **2008**, *3*, doi:10.1371/journal.pone.0002069.
15. Villar-Pazos, S.; Martinez-Pinna, J.; Castellano-Muñoz, M.; Alonso-Magdalena, P.; Marroqui, L.; Quesada, I.; Gustafsson, J.A.; Nadal, A. Molecular Mechanisms Involved in the Non-Monotonic Effect of Bisphenol-A on Ca2+ Entry in Mouse Pancreatic β-Cells. *Sci. Rep.* **2017**, *7*, 11770, doi:10.1038/s41598-017-11995-3.
16. Martinez-Pinna, J.; Marroqui, L.; Hmadcha, A.; Lopez-Beas, J.; Soriano, S.; Villar-Pazos, S.; Alonso-Magdalena, P.; Dos Santos, R.S.; Quesada, I.; Martin, F.; et al. Oestrogen Receptor β Mediates the Actions of Bisphenol-A on Ion Channel Expression in Mouse Pancreatic Beta Cells. *Diabetologia* **2019**, *62*, 1667–1680, doi:10.1007/s00125-019-4925-y.
17. Marroqui, L.; Martinez-Pinna, J.; Castellano-Muñoz, M.; dos Santos, R.S.; Medina-Gali, R.M.; Soriano, S.; Quesada, I.; Gustafsson, J.A.; Encinar, J.A.; Nadal, A. Bisphenol-S and Bisphenol-F Alter Mouse Pancreatic β-Cell Ion Channel Expression and Activity and Insulin Release through an Estrogen Receptor ERβ Mediated Pathway. *Chemosphere* **2021**, *265*, doi:10.1016/j.chemosphere.2020.129051.
18. Carchia, E.; Porreca, I.; Almeida, P.J.; D'Angelo, F.; Cuomo, D.; Ceccarelli, M.; De Felice, M.; Mallardo, M.; Ambrosino, C. Evaluation of Low Doses BPA-Induced Perturbation of Glycemia by Toxicogenomics Points to a Primary Role of Pancreatic Islets and to the Mechanism of Toxicity. *Cell Death Dis.* **2015**, *6*, e1959–e1959, doi:10.1038/cddis.2015.319.
19. Babiloni-Chust, I.; dos Santos, R.S.; Medina-Gali, R.M.; Perez-Serna, A.A.; Encinar, J.-A.; Martinez-Pinna, J.; Gustafsson, J.-A.; Marroqui, L.; Nadal, A. G Protein-Coupled Estrogen Receptor Activation by Bisphenol-A Disrupts the Protection from Apoptosis Conferred by the Estrogen Receptors ERα and ERβ in Pancreatic Beta Cells. *Environ. Int.* **2022**, *164*, 107250, doi:10.1016/j.envint.2022.107250.
20. Dos Santos, R.S.; Medina-Gali, R.M.; Babiloni-Chust, I.; Marroqui, L.; Nadal, A. In Vitro Assays to Identify Metabolism-Disrupting Chemicals with Diabetogenic Activity in a Human Pancreatic β-Cell Model. *Int. J. Mol. Sci.* **2022**, *23*, 5040, doi:10.3390/ijms23095040.
21. Chen, Y.W.; Lan, K.C.; Tsai, J.R.; Weng, T.I.; Yang, C.Y.; Liu, S.H. Tributyltin Exposure at Noncytotoxic Doses Dysregulates Pancreatic β-Cell Function in Vitro and in Vivo. *Arch. Toxicol.* **2017**, *91*, 3135–3144, doi:10.1007/s00204-017-1940-y.
22. Huang, C.F.; Yang, C.Y.; Tsai, J.R.; Wu, C.T.; Liu, S.H.; Lan, K.C. Low-Dose Tributyltin Exposure Induces an Oxidative Stress-Triggered JNK-Related Pancreatic β-Cell Apoptosis and a Reversible Hypoinsulinemic Hyperglycemia in Mice. *Sci. Rep.* **2018**, *8*, doi:10.1038/s41598-018-24076-w.
23. Legler, J.; Zalko, D.; Jourdan, F.; Jacobs, M.; Fromenty, B.; Balaguer, P.; Bourguet, W.; Kos, V.M.; Nadal, A.; Beausoleil, C.; et al. The GOLIATH Project: Towards an Internationally Harmonised Approach for Testing Metabolism Disrupting Compounds. *Int. J. Mol. Sci.* **2020**, *21*, 3480, doi:10.3390/ijms21103480.
24. Soriano, S.; Alonso-Magdalena, P.; García-Arévalo, M.; Novials, A.; Muhammed, S.J.; Salehi, A.; Gustafsson, J.A.; Quesada, I.; Nadal, A. Rapid Insulinotropic Action of Low Doses of Bisphenol-A on Mouse and Human Islets of Langerhans: Role of Estrogen Receptor β. *PLoS ONE* **2012**, *7*, e31109, doi:10.1371/journal.pone.0031109.

25. Grün, F.; Blumberg, B. Environmental Obesogens: Organotins and Endocrine Disruption via Nuclear Receptor Signaling. *Endocrinology* **2006**, *147*, s50–s55, doi:10.1210/en.2005-1129.
26. le Maire, A.; Grimaldi, M.; Roecklin, D.; Dagnino, S.; Vivat-Hannah, V.; Balaguer, P.; Bourguet, W. Activation of RXR–PPAR Heterodimers by Organotin Environmental Endocrine Disruptors. *EMBO Rep.* **2009**, *10*, 367–373, doi:10.1038/embor.2009.8.
27. Kim, K.; Kwon, J.-S.; Ahn, C.; Jeung, E.-B. Endocrine-Disrupting Chemicals and Their Adverse Effects on the Endoplasmic Reticulum. *Int. J. Mol. Sci.* **2022**, *23*, 1581, doi:10.3390/ijms23031581.
28. Scharfmann, R.; Staels, W.; Albagli, O. The Supply Chain of Human Pancreatic β Cell Lines. *J. Clin. Invest.* **2019**, *129*, 3511–3520, doi:10.1172/JCI129484.
29. Hamaguchi, K.; Leiter, E.H. Comparison of Cytokine Effects on Mouse Pancreatic α-Cell and β-Cell Lines Viability, Secretory Function, and MHC Antigen Expression. *Diabetes* **1990**, *39*, 415–425, doi:10.2337/diab.39.4.415.
30. Ravier, M.A.; Rutter, G.A. Glucose or Insulin, but Not Zinc Ions, Inhibit Glucagon Secretion From Mouse Pancreatic α-Cells. *Diabetes* **2005**, *54*, 1789–1797, doi:10.2337/diabetes.54.6.1789.
31. Tuduri, E.; Marroqui, L.; Soriano, S.; Ropero, A.B.; Batista, T.M.; Piquer, S.; Lopez-Boado, M.A.; Carneiro, E.M.; Gomis, R.; Nadal, A.; et al. Inhibitory Effects of Leptin on Pancreatic α-Cell Function. *Diabetes* **2009**, *58*, 1616–1624, doi:10.2337/db08-1787.
32. McKinnon, C.M.; Ravier, M.A.; Rutter, G.A. FoxO1 Is Required for the Regulation of Preproglucagon Gene Expression by Insulin in Pancreatic ATC1-9 Cells*. *J. Biol. Chem.* **2006**, *281*, 39358–39369, doi:10.1074/jbc.M605022200.
33. Leibiger, B.; Moede, T.; Muhandiramlage, T.P.; Kaiser, D.; Sanchez, P.V.; Leibiger, I.B.; Berggren, P.-O. Glucagon Regulates Its Own Synthesis by Autocrine Signaling. *Proc. Natl. Acad. Sci.* **2012**, *109*, 20925–20930, doi:10.1073/pnas.1212870110.
34. Vieira, E.; Marroquí, L.; Figueroa, A.C.; Merino, B.; Fernandez-Ruiz, R.; Nadal, A.; Burris, T.P.; Gomis, R.; Quesada, I. Involvement of the Clock Gene Rev-Erb Alpha in the Regulation of Glucagon Secretion in Pancreatic Alpha-Cells. *PLoS ONE* **2013**, *8*, doi:10.1371/journal.pone.0069939.
35. Quesada-Candela, C.; Tudurí, E.; Marroquí, L.; Alonso-Magdalena, P.; Quesada, I.; Nadal, Á. Morphological and Functional Adaptations of Pancreatic Alpha-Cells during Late Pregnancy in the Mouse. *Metabolism* **2020**, *102*, 153963, doi:10.1016/j.metabol.2019.153963.
36. Marroquí, L.; Vieira, E.; Gonzalez, A.; Nadal, A.; Quesada, I. Leptin Downregulates Expression of the Gene Encoding Glucagon in AlphaTC1-9 Cells and Mouse Islets. *Diabetologia* **2011**, *54*, 843–851, doi:10.1007/s00125-010-2024-1.
37. Marroqui, L.; Lopes, M.; dos Santos, R.S.; Grieco, F.A.; Roivainen, M.; Richardson, S.J.; Morgan, N.G.; de Beeck, A.O.; Eizirik, D.L. Differential Cell Autonomous Responses Determine the Outcome of Coxsackievirus Infections in Murine Pancreatic α and β Cells. *eLife* **2015**, *4*, 1–23, doi:10.7554/eLife.06990.
38. Lin, Y.; Sun, X.; Qiu, L.; Wei, J.; Huang, Q.; Fang, C.; Ye, T.; Kang, M.; Shen, H.; Dong, S. Exposure to Bisphenol A Induces Dysfunction of Insulin Secretion and Apoptosis through the Damage of Mitochondria in Rat Insulinoma (INS-1) Cells. *Cell Death Dis.* **2013**, *4*, e460–e460, doi:10.1038/cddis.2012.206.
39. Bansal, A.; Rashid, C.; Xin, F.; Li, C.; Polyak, E.; Duemler, A.; van der Meer, T.; Stefaniak, M.; Wajid, S.; Doliba, N.; et al. Sex- and Dose-Specific Effects of

Maternal Bisphenol A Exposure on Pancreatic Islets of First- and Second-Generation Adult Mice Offspring. *Environ. Health Perspect.* **2017**, *125*, doi:10.1289/EHP1674.

40. Morsi, A.A.; Mersal, E.A.; Alsabih, A.O.; Abdelmoneim, A.M.; Sakr, E.M.; Alakabawy, S.; Elfawal, R.G.; Naji, M.; Aljanfawe, H.J.; Alshateb, F.H.; et al. Apoptotic Susceptibility of Pancreatic Alpha Cells to Environmentally Relevant Dose Levels of Bisphenol-A versus Dibutyl Phthalate Is Mediated by HSP60/Caspase-3 Expression in Male Albino Rats. *Cell Biol. Int.* **2022**, *n/a*, 1–14, doi:10.1002/cbin.11909.
41. Handgraaf, S.; Dusaulcy, R.; Visentin, F.; Philippe, J.; Gosmain, Y. 17-β Estradiol Regulates Proglucagon-Derived Peptide Secretion in Mouse and Human α- and L Cells. *JCI Insight* **2018**, *3*, doi:10.1172/jci.insight.98569.
42. Zuo, Z.; Wu, T.; Lin, M.; Zhang, S.; Yan, F.; Yang, Z.; Wang, Y.; Wang, C. Chronic Exposure to Tributyltin Chloride Induces Pancreatic Islet Cell Apoptosis and Disrupts Glucose Homeostasis in Male Mice. *Environ. Sci. Technol.* **2014**, *48*, 5179–5186, doi:10.1021/es404729p.
43. Ghaemmaleki, F.; Mohammadi, P.; Baeeri, M.; Navaei-Nigjeh, M.; Abdollahi, M.; Mostafalou, S. Estrogens Counteract Tributyltin-Induced Toxicity in the Rat Islets of Langerhans. *Heliyon* **2020**, *6*, e03562, doi:10.1016/j.heliyon.2020.e03562.
44. Suh, K.S.; Choi, E.M.; Kim, Y.J.; Hong, S.M.; Park, S.Y.; Rhee, S.Y.; Oh, S.; Kim, S.W.; Pak, Y.K.; Choe, W.; et al. Perfluorooctanoic Acid Induces Oxidative Damage and Mitochondrial Dysfunction in Pancreatic β-Cells. *Mol. Med. Rep.* **2017**, *15*, 3871–3878, doi:10.3892/mmr.2017.6452.
45. He, X.; Wu, D.; Xu, Y.; Zhang, Y.; Sun, Y.; Chang, X.; Zhu, Y.; Tang, W. Perfluorooctanoic Acid Promotes Pancreatic β Cell Dysfunction and Apoptosis through ER Stress and the ATF4/CHOP/TRIB3 Pathway. *Environ. Sci. Pollut. Res.* **2022**, doi:10.1007/s11356-022-21188-9.
46. Ajao, C.; Andersson, M.A.; Teplova, V.V.; Nagy, S.; Gahmberg, C.G.; Andersson, L.C.; Hautaniemi, M.; Kakasi, B.; Roivainen, M.; Salkinoja-Salonen, M. Mitochondrial Toxicity of Triclosan on Mammalian Cells. *Toxicol. Rep.* **2015**, *2*, 624–637, doi:10.1016/j.toxrep.2015.03.012.
47. Al-Abdulla, R.; Ferrero, H.; Soriano, S.; Boronat-Belda, T.; Alonso-Magdalena, P. Screening of Relevant Metabolism-Disrupting Chemicals on Pancreatic β-Cells: Evaluation of Murine and Human In Vitro Models. *Int. J. Mol. Sci.* **2022**, *23*, 4182, doi:10.3390/ijms23084182.
48. Bresson, S.E.; Isom, S.; Jensen, E.T.; Huber, S.; Oulhote, Y.; Rigdon, J.; Lovato, J.; Liese, A.D.; Pihoker, C.; Dabelea, D.; et al. Associations between Persistent Organic Pollutants and Type 1 Diabetes in Youth. *Environ. Int.* **2022**, *163*, 107175, doi:10.1016/j.envint.2022.107175.
49. Cao, S.S.; Kaufman, R.J. Endoplasmic Reticulum Stress and Oxidative Stress in Cell Fate Decision and Human Disease. *Antioxid. Redox Signal.* **2014**, *21*, 396–413, doi:10.1089/ars.2014.5851.
50. Hasnain, S.Z.; Prins, J.B.; McGuckin, M.A. Oxidative and Endoplasmic Reticulum Stress in β-Cell Dysfunction in Diabetes. *J. Mol. Endocrinol.* **2016**, *56*, R33–R54, doi:10.1530/JME-15-0232.
51. Asahi, J.; Kamo, H.; Baba, R.; Doi, Y.; Yamashita, A.; Murakami, D.; Hanada, A.; Hirano, T. Bisphenol A Induces Endoplasmic Reticulum Stress-Associated Apoptosis in Mouse Non-Parenchymal Hepatocytes. *Life Sci.* **2010**, *87*, 431–438, doi:10.1016/j.lfs.2010.08.007.

52. Isomura, M.; Kotake, Y.; Masuda, K.; Miyara, M.; Okuda, K.; Samizo, S.; Sanoh, S.; Hosoi, T.; Ozawa, K.; Ohta, S. Tributyltin-Induced Endoplasmic Reticulum Stress and Its Ca2+-Mediated Mechanism. *Toxicol. Appl. Pharmacol.* **2013**, *272*, 137–146, doi:10.1016/j.taap.2013.05.026.
53. Moreno-Gómez-Toledano, R.; Sánchez-Esteban, S.; Cook, A.; Mínguez-Moratinos, M.; Ramírez-Carracedo, R.; Reventún, P.; Delgado-Marín, M.; Bosch, R.J.; Saura, M. Bisphenol A Induces Accelerated Cell Aging in Murine Endothelium. *Biomolecules* **2021**, *11*, 1429, doi:10.3390/biom11101429.
54. Ferreira, R.; Amaral, C.; Correia-da-Silva, G.; Almada, M.; Borges, M.; Cunha, S.C.; Fernandes, J.O.; Teixeira, N. Bisphenols A, F, S and AF Trigger Apoptosis and/or Endoplasmic Reticulum Stress in Human Endometrial Stromal Cells. *Toxicology* **2022**, *478*, 153282, doi:10.1016/j.tox.2022.153282.
55. Liu, J.; Chen, T.; Wang, S.; Wu, H.; Xu, S. BPA Exposure Aggravates Necroptosis of Myocardial Tissue in Selenium Deficient Broilers through NO-Dependent Endoplasmic Reticulum Stress. *Toxicology* **2022**, *472*, 153190, doi:10.1016/j.tox.2022.153190.
56. Marroqui, L.; Masini, M.; Merino, B.; Grieco, F.A.; Millard, I.; Dubois, C.; Quesada, I.; Marchetti, P.; Cnop, M.; Eizirik, D.L. Pancreatic α Cells Are Resistant to Metabolic Stress-Induced Apoptosis in Type 2 Diabetes. *EBioMedicine* **2015**, *2*, 378–385, doi:10.1016/j.ebiom.2015.03.012.
57. Alonso-Magdalena, P.; Quesada, I.; Nadal, A. Endocrine Disruptors in the Etiology of Type 2 Diabetes Mellitus. *Nat. Rev. Endocrinol.* **2011**, *7*, 346–353, doi:10.1038/nrendo.2011.56.
58. Mimoto, M.S.; Nadal, A.; Sargis, R.M. Polluted Pathways: Mechanisms of Metabolic Disruption by Endocrine Disrupting Chemicals. *Curr. Environ. Health Rep.* **2017**, *4*, 208–222, doi:10.1007/s40572-017-0137-0.
59. Alonso-Magdalena, P.; Laribi, O.; Ropero, A.B.; Fuentes, E.; Ripoll, C.; Soria, B.; Nadal, A. Low Doses of Bisphenol A and Diethylstilbestrol Impair Ca2+ Signals in Pancreatic α-Cells through a Nonclassical Membrane Estrogen Receptor within Intact Islets of Langerhans. *Environ. Health Perspect.* **2005**, *113*, 969–977, doi:10.1289/ehp.8002.
60. Zheng, F.; Sheng, N.; Zhang, H.; Yan, S.; Zhang, J.; Wang, J. Perfluorooctanoic Acid Exposure Disturbs Glucose Metabolism in Mouse Liver. *Toxicol. Appl. Pharmacol.* **2017**, *335*, doi:10.1016/j.taap.2017.09.019.
61. Göckener, B.; Weber, T.; Rüdel, H.; Bücking, M.; Kolossa-Gehring, M. Human Biomonitoring of Per- and Polyfluoroalkyl Substances in German Blood Plasma Samples from 1982 to 2019. *Environ. Int.* **2020**, *145*, 106123, doi:10.1016/j.envint.2020.106123.
62. Qin, W.P.; Cao, L.Y.; Li, C.H.; Guo, L.H.; Colbourne, J.; Ren, X.M. Perfluoroalkyl Substances Stimulate Insulin Secretion by Islet β Cells via G Protein-Coupled Receptor 40. *Environ. Sci. Technol.* **2020**, *54*, 3428–3436, doi:10.1021/acs.est.9b07295.
63. Vandenberg, L.N.; Colborn, T.; Hayes, T.B.; Heindel, J.J.; Jacobs, D.R., Jr.; Lee, D.-H.; Shioda, T.; Soto, A.M.; vom Saal, F.S.; Welshons, W.V.; et al. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. *Endocr. Rev.* **2012**, *33*, 378–455, doi:10.1210/er.2011-1050.
64. Vandenberg, L.N. Non-Monotonic Dose Responses in Studies of Endocrine Disrupting Chemicals: Bisphenol a as a Case Study. *Dose-Response* **2014**, *12*, dose-response.13-020.Vandenberg, doi:10.2203/dose-response.13-020.Vandenberg.

65. Gilon, P. The Role of α-Cells in Islet Function and Glucose Homeostasis in Health and Type 2 Diabetes. *J. Mol. Biol.* **2020**, *432*, 1367–1394, doi:10.1016/j.jmb.2020.01.004.
66. Acreman, S.; Zhang, Q. Regulation of α-Cell Glucagon Secretion: The Role of Second Messengers. *Chronic Dis. Transl. Med.* **2022**, *8*, 7–18, doi:10.1016/j.cdtm.2021.06.001.
67. Santin, I.; Dos Santos, R.S.; Eizirik, D.L. Pancreatic Beta Cell Survival and Signaling Pathways: Effects of Type 1 Diabetes-Associated Genetic Variants. In *Methods in Molecular Biology*; Humana Press: New York, NY, USA, 2016; Vol. 1433, pp. 21–54.
68. Dos Santos, R.S.; Daures, M.; Philippi, A.; Romero, S.; Marselli, L.; Marchetti, P.; Senée, V.; Bacq, D.; Besse, C.; Baz, B.; et al. DUTPase (DUT) Is Mutated in a Novel Monogenic Syndrome with Diabetes and Bone Marrow Failure. *Diabetes* **2017**, *66*, 1086–1093, doi:10.2337/db16-0839.

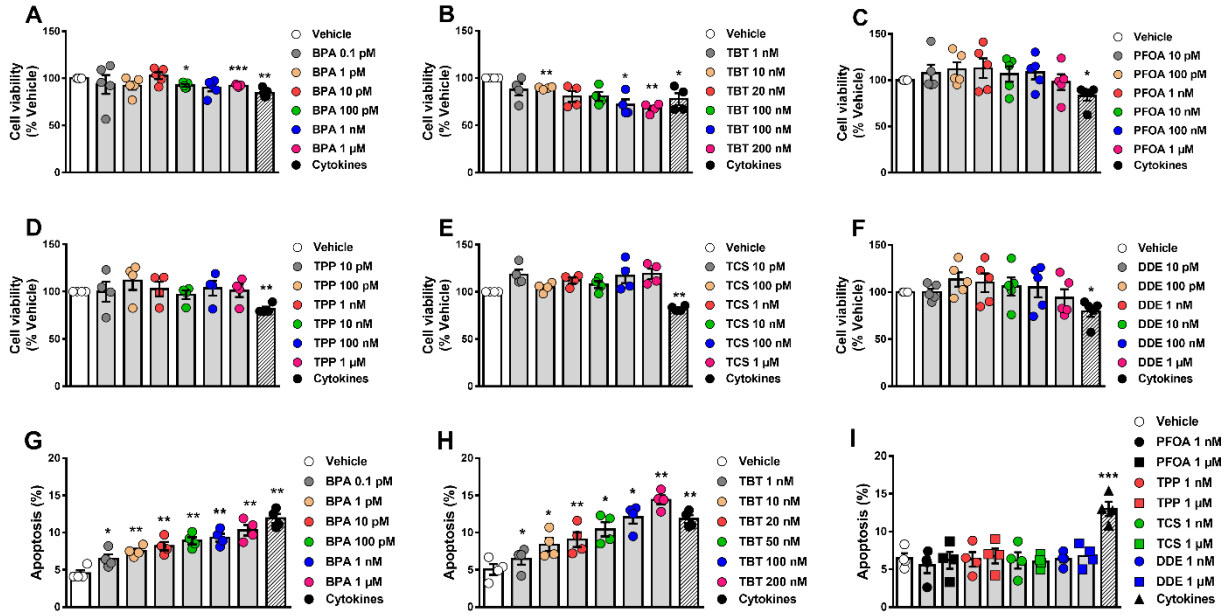
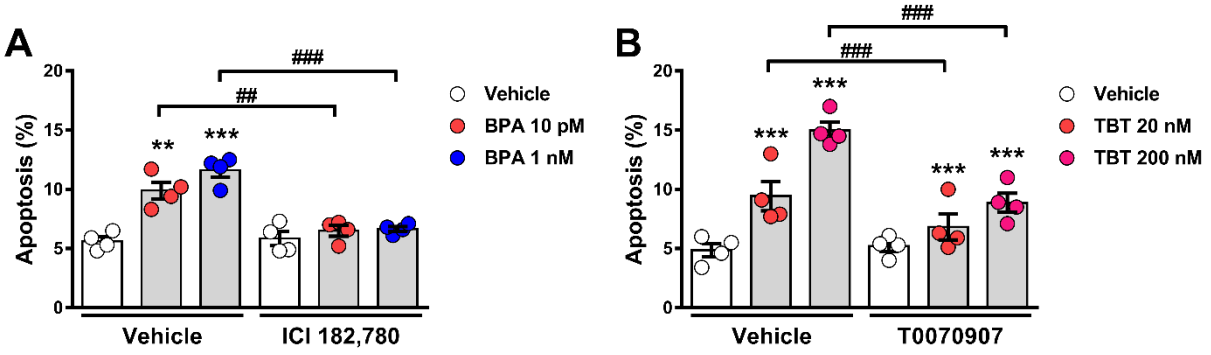


Figure 1. α-cell viability upon MDC exposure. (A-F) αTC1-9 cells were treated with vehicle (DMSO) or different doses of BPA (A), TBT (B), PFOA (C), TPP (D), TCS (E), or DDE (F) for 48 h. A cocktail of the cytokines IL-1β + IFNγ (50 and 1000 U/ml, respectively) was used as a positive control. Cell viability was evaluated by MTT assay. (G-I) αTC1-9 cells were treated with vehicle (DMSO) or different doses of BPA (G), TBT (H), PFOA, TPP, TCS, or DDE (I) for 24 h. Apoptosis was evaluated using HO and PI staining. Data are shown as means ± SEM (n = 4–5 independent experiments, where each dot represents an independent experiment). * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. Vehicle. MDCs vs. Vehicle by one-way ANOVA; Cytokines vs. Vehicle by two-tailed Student's t test.



686 **Figure 2. Estrogen receptors and PPARγ are implicated in BPA- and TBT-induced**
687 **α-cell apoptosis, respectively.** (A) αTC1-9 cells were treated with vehicle (DMSO) or
688 BPA (10 pM or 1 nM) in the absence or presence of 1 μM ICI 182,780 for 24 h. (B)
689 αTC1-9 cells were treated with vehicle (DMSO) or TBT (20 nM or 200 nM) in the
690 absence or presence of 100 nM T0070907 for 24 h. Apoptosis was evaluated using HO
691 and PI staining. Data are shown as means ± SEM (n = 4 independent experiments, where
692 each dot represents an independent experiment). ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. its
693 respective Vehicle; $^{\#\#}$ $p \leq 0.01$ and $^{\#\#\#}$ $p \leq 0.001$ as indicated by bars. Two-way ANOVA.

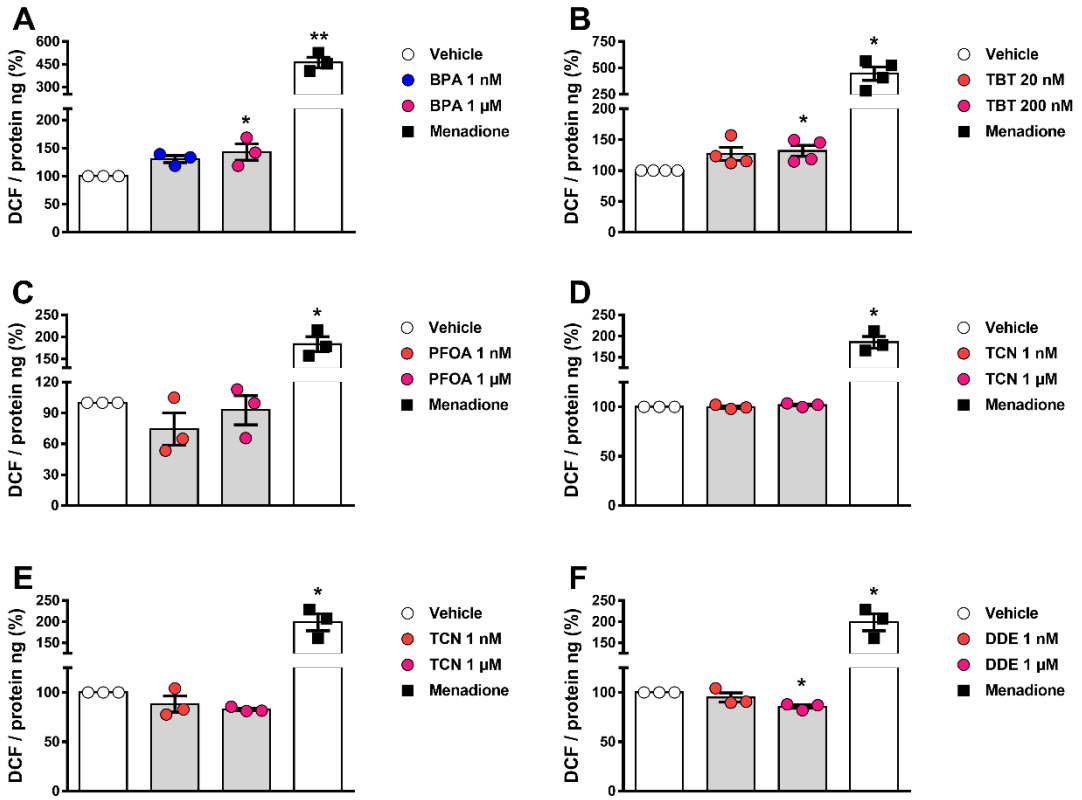


Figure 3. ROS production upon MDC exposure. αTC1-9 cells were treated with vehicle (DMSO) or different doses of BPA (A), TBT (B), PFOA (C), TPP (D), TCS (E), or DDE (F) for 24 h. Menadione (15 µM for 90 minutes) was used as a positive control. Generation of ROS was assessed by oxidation of the fluorescent probe DCF and normalized by total protein. Data are shown as means ± SEM (n = 3–4 independent experiments, where each dot represents an independent experiment). * $p \leq 0.05$ and ** $p \leq 0.01$ vs. Vehicle. MDCs vs. Vehicle by one-way ANOVA; Menadione vs. Vehicle by two-tailed Student's t test.

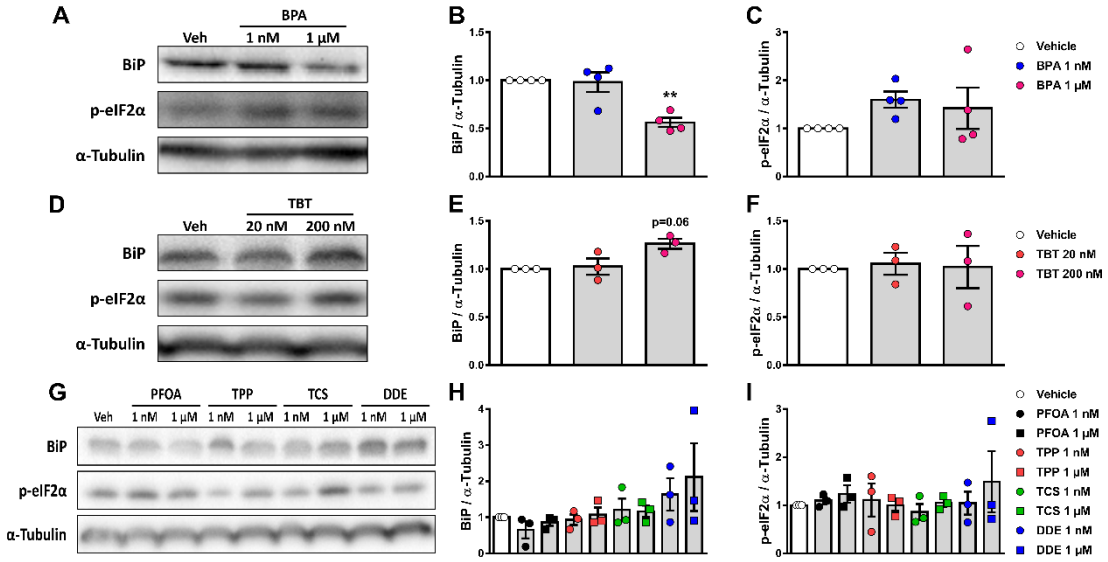


Figure 4. Expression of ER stress markers upon MDC exposure. αTC1-9 cells were treated with vehicle (DMSO) or different doses of BPA (A-C), TBT (D-F), PFOA, TPP, TCS, or DDE (G-I) for 24 h. Protein expression was measured by western blot. Representative images of three independent experiments are shown (A,D,G) and densitometry results are presented for BiP (B,E,H) and p-eIF2α (C,F,I). Data are shown as means ± SEM (n = 3-4 independent experiments, where each dot represents an independent experiment). ** $p \leq 0.01$ vs. Vehicle. MDCs vs. Vehicle by one-way ANOVA.

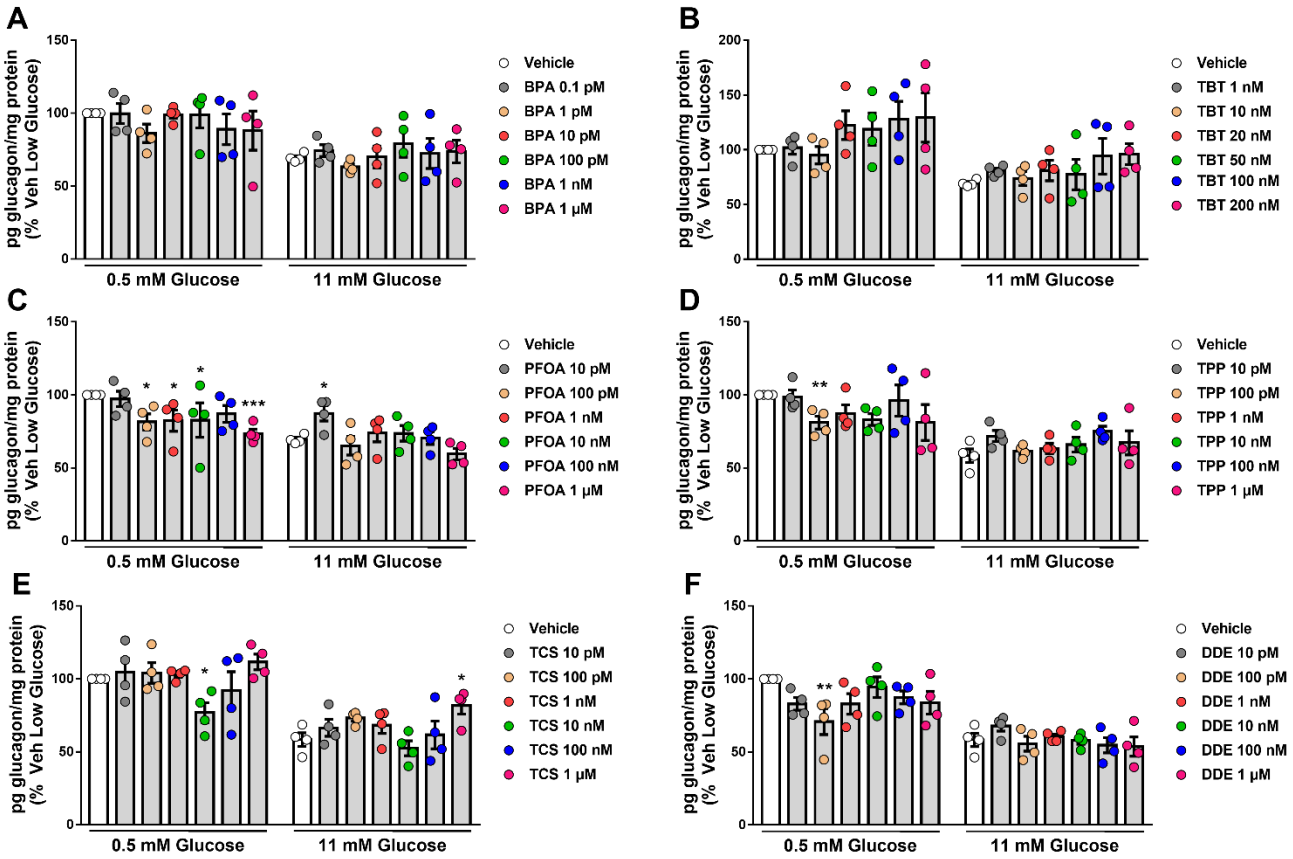


Figure 5. Glucagon secretion upon MDC exposure. αTC1-9 cells were treated with vehicle (DMSO) or different doses of BPA (A), TBT (B), PFOA (C), TPP (D), TCS (E), or DDE (F) for 48 h. Glucagon secretion was measured at 0.5 and 11 mM glucose, and glucagon released into the medium was measured by ELISA. Data are normalized to glucagon secretion at low glucose (i.e., 0.5 mM) in vehicle-treated cells. Data are shown as means ± SEM (n = 4 independent experiments, where each dot represents an independent experiment). * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. its respective Vehicle. Two-way ANOVA.