Transient oxidative stress damages mitochondrial machinery inducing persistent ß-cell dysfunction

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SUPPORTING INFORMATION

Figure S1. Dose-response of $\Delta\Psi_m$ in INS-1E cells 3 days after transient oxidative stress. INS-1E cells seeded in 24-well plates were further cultured for 3 days following 10 min transient oxidative stress (200 µM H_2O_2 Stressed or 500 µM H_2O_2 Stressed). Mitochondrial membrane potential was monitored as Rhodamine-123 fluorescence and hyperpolarization was induced by raising glucose from basal 2.5 mM to stimulatory 15 mM (see arrow, Glucose). Complete depolarization of the mitochondrial membrane was evoked by addition of 1 µM of the uncoupler FCCP (see arrow, FCCP). Traces are means ± S.D. of 1 representative out of 5 independent experiments, each performed in quadruplicate.

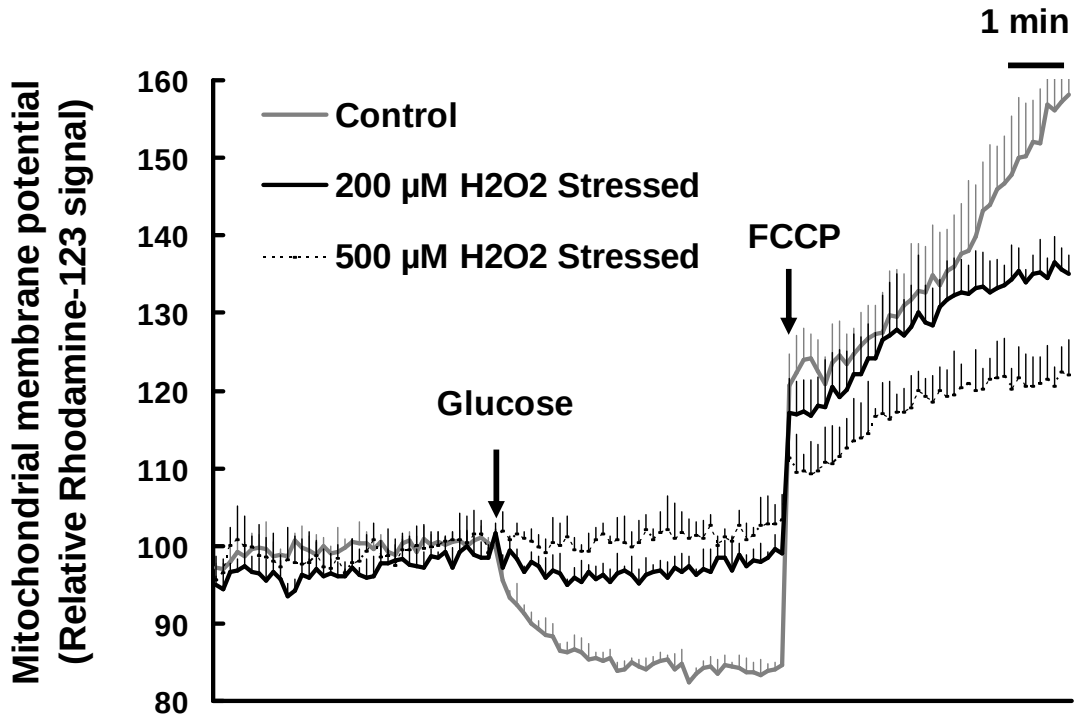
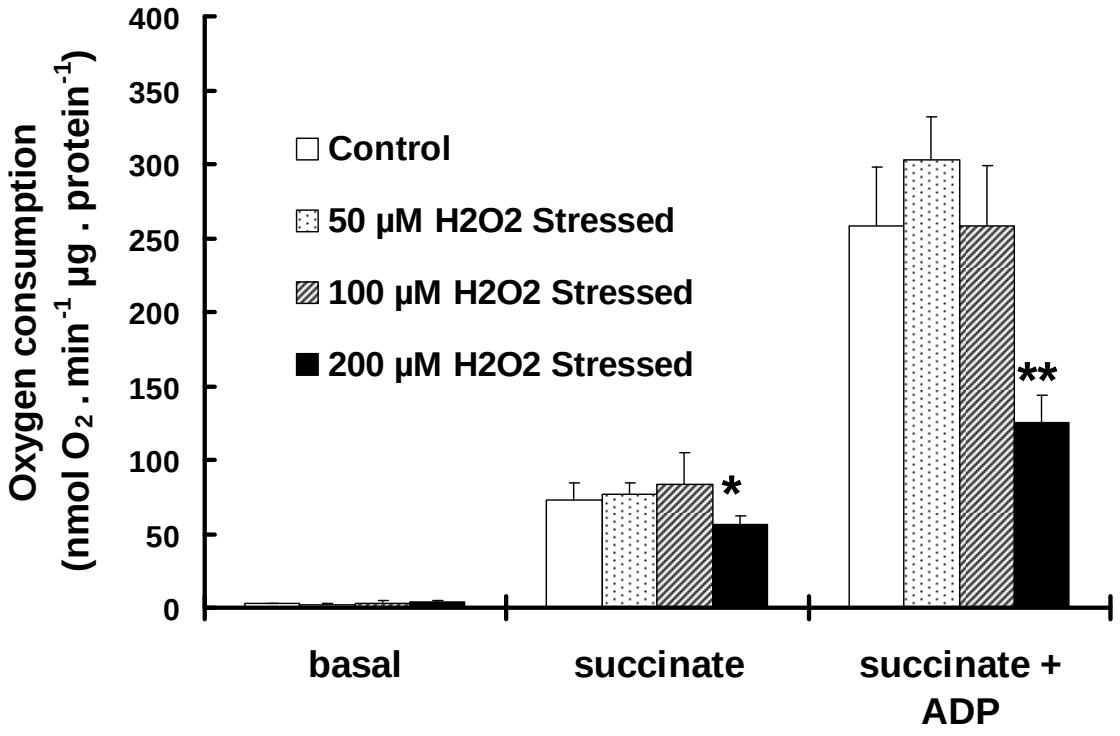


Figure S2. Dose-response of O_2 consumption in INS-1E mitochondria 3 days after transient oxidative stress. INS-1E cells were cultured for 3 days following 10 min transient oxidative stress (50 µM H_2O_2 Stressed, 100 µM H_2O_2 Stressed or 200 µM H_2O_2 Stressed) before mitochondria isolation. Isolated mitochondria were placed into an oxymeter chamber and stabilized for at least 10 min without substrates (basal). Then, complex II dependent O_2 consumption was stimulated by adding 5 mM succinate (succinate), followed by further 150 µM ADP addition (succinate + ADP). Columns represent means ± S.E.M. of 4 independent experiments. **P*<0.01, ***P*<0.005 *versus* corresponding controls.



Immunohistochemistry, cell proliferation and TUNEL assays. At the end of the culture period, INS-1E cells were washed with PBS and fixed in 4% paraformaldehyde/PBS for 20 min at room temperature. Immunochemical detection of insulin was performed as previously described (1). Nuclei were stained with DAPI (10 µg/ml; Sigma). Cover slips were mounted using DAKO fluorescent mounting medium and visualized using a Zeiss Axiophot I. For proliferation, control or stressed INS-1E cells were labelled with 10 µM BrdU for 8 hours. Proliferation was quantified using an immunohistochemical assay kit as described by the manufacturer (BrdU labelling and detection Kit, Roche Diagnostics, Switzerland). Cell death was measured by the TUNEL assay (In Situ Cell Death Detection Kit, Roche). Results are expressed as a percentage of BrdU or TMR-fluorescein-labeled nuclei (TUNEL-positive cells) over the total amount of INS-1E cells (nuclei staining by DAPI).

Figure S3. Cell death and proliferation rates of INS-1E cells at different time points after oxidative stress. INS-1E cells not subjected the absence of oxidative stress (T=0) or following transient oxidative stress (200 µM H_2O_2 for 10 min, Stressed) were cultured for the indicated time points. (A) Apoptosis and (B) cell proliferation were measured by the TUNEL assay and by BrdU incorporation, respectively. For proliferation, cells were cultured in the presence of BrdU (10 µM) for 8 hrs. Representative immunofluorescent composite image of INS-1E cells positive for (A) TUNEL or (B) immunostained for BrdU (green), insulin (red) and DAPI (nuclei, blue) are shown. Quantifications of results are depicted as percentage of positive cells over the total amount of insulin positive cells, shown in Fig. 1A and B.

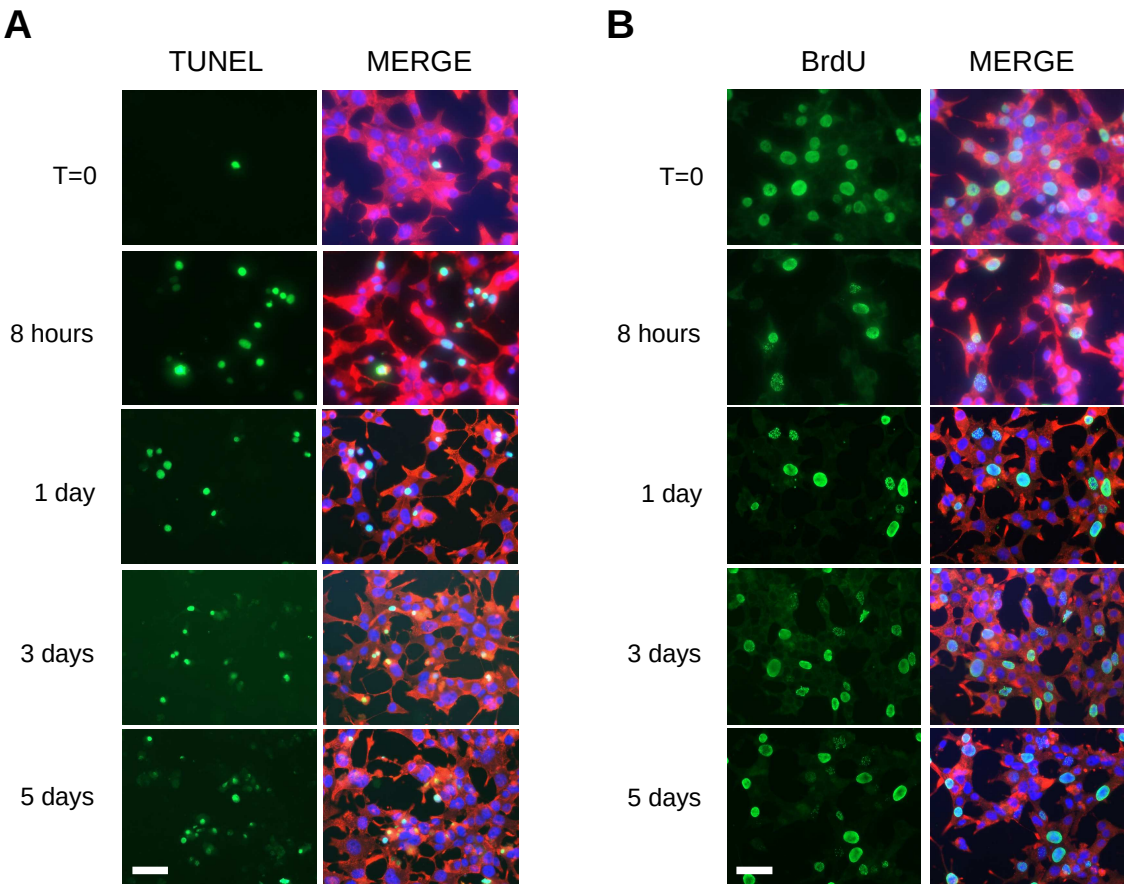

A
TUNEL
MERGE
T=0
8 hours
1 day
3 days
5 days
B
BrdU
MERGE
T=0
8 hours
1 day
3 days
5 days

Figure S4. Cellular calcium changes in INS-1E cells 3 days post-stress. Cytosolic calcium changes were monitored in cells preincubated for 90 min with 2 µM Fura-2AM (Teflab, Austin, TX) in KRBH at 37°C and then washed before experiment. Ratiometric measurements of Fura-2 fluorescence were performed in a plate-reader fluorimeter (Fluostar Optima) with filters set at 510 nm for excitation and 340/380 nm for emission. Calcium rises were induced by addition of 30 mM KCl (arrow) at basal 2.5 mM glucose. Values are means ± S.D. of 4 wells in 1 out of 3 independent experiments.

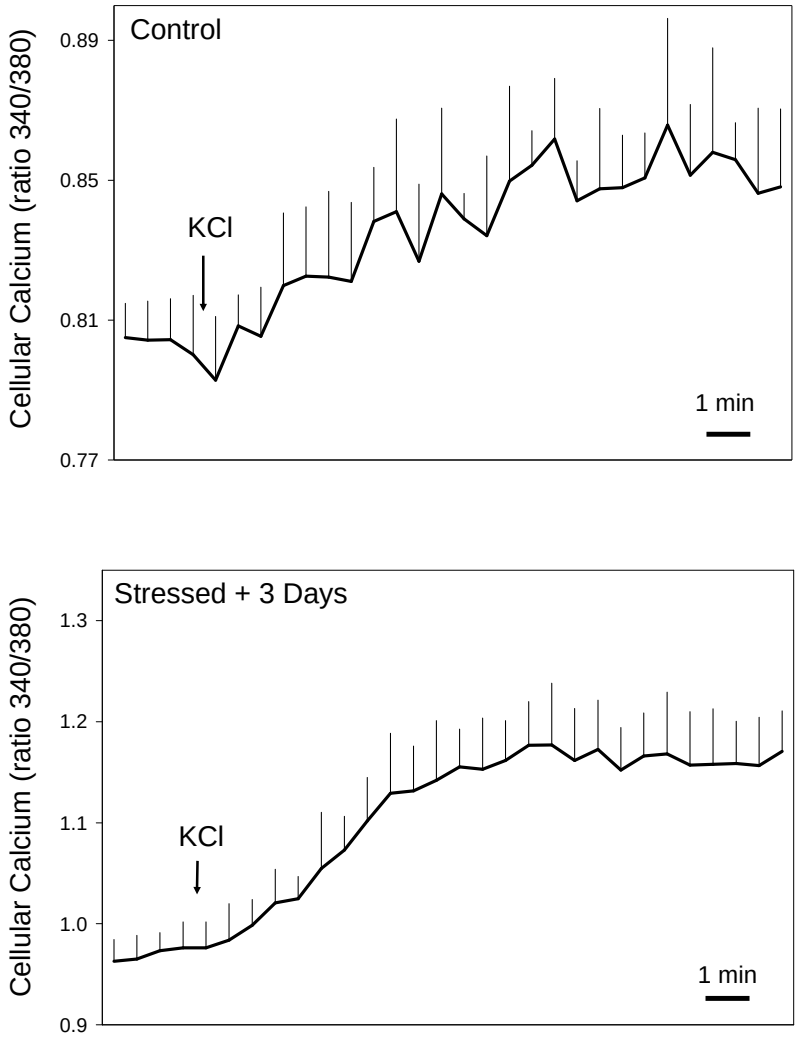


Table S1. Primer sequences for quantitative real-time PCR.

Genes	Sense (5'-3')	Antisense (5'-3')
ND6	TGG GTG GGA TGT TGG TTG TAT	TCA AGT TTC CGG ATA TTC CTC AGT
COX I	AGC TGG CTT CGT CCA CTG ATT	GGG CTT TTG CTC ATG TGT CAT
TFAM	GGG AAG AGC AAA TGG CTG AA	TCA CAC TGC GAC GGA TGA GA
PGC-1α	TGA CAC AAC GCG GAC AGA AC	CCG CAG ATT TAC GGT GCA TT
SOD1	TTC CAT CAT TGG CCG TA	AAG CGG CTT CCA GCA TTTC
SOD2	TGG TGT GAG CTG CTC TTG ATT G	GCC CCA ACA CAG AGA TGG AAT A
CAT	CAT GAA TGG CTA TGG CTC ACA	CAA GTT TTT GAT GCC CTG GTC
GPx	AGC CGG CTA CAA TGT CAG GTT	GGC ATC GTC CCC ATT TAC AC
UCP2	CTT CTG CAC CAC CGT CAT TG	TAC TGG CCC AAG GCA GAG TT
RPS29	GCT GAA CAT GTG CCG ACA GT	GGT CGC TTA GTC CAA CTT AAT GAA G

Measurement of ER stress markers in INS-1E cells.

Changes in cellular redox status may also secondarily affect the ER. Accordingly, we evaluated possible endoplasmic reticulum (ER) stress, 6 h and 12 h after 10 min of 200 µM H_2O_2 stress. Total RNA from INS-1E cells was extracted and then converted to cDNA. Primer sequences for the ER stress markers C/EBP homologous protein (*CHOP*, also known as *GADD153* or *DDIT3*), Ig heavy chain binding protein (*BiP*, also known as *GRP78* or *HSPA5*) and spliced X-box binding protein-1 (*XBP1S*) were reported before (2,3). Gene expression was measured and corrected for the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as described (4).

None of the markers of ER stress studied (*CHOP, BiP* and *XBP1S*) were modified 6 hours and 12 hours after a 10 min exposure to 200 µM H_2O_2 (Supplementary Table S2), suggesting that ER stress is not involved in the long term deleterious effects of an acute oxidative stress in INS-1E cells and that the observed mitochondrial dysfunction is specific to this organelle. To further examine whether ER stress can be triggered by oxidative stress, an additional experimental model was tested of longer exposure to lower concentrations of H_2O_2. In these experiments INS-1E cells were exposed for 1 h to 40 µM H_2O_2 and then studied after 2, 6 or 16 hours. Again there were no changes in the expression of *CHOP, BiP* and *XBP1S* (see Supplementary Table S3). Similar findings were obtained when INS-1E cells were exposed for 1 h to 15-30 µM H_2O_2 and studied after 2-16 h (data not shown). These observations

suggest that H_2O_2-induced oxidative stress, under the present experimental conditions, does not lead to ER stress in ß-cells.

Table S2. Measurement of ß-cell ER stress markers in INS-1E cells. Six hours and 12 hours after acute (10 min) oxidant exposure (200 μM H_2O_2), ER stress markers were detected by real time RT-PCR and corrected for GAPDH expression. Results are means ± S.E. of 3 independent experiments.

Genes	Control (6 hrs)	Stressed (6 hrs)	Control (12 hrs)	Stressed (12 hrs)
CHOP	53.60 ± 47.23	85.32 ± 60.42	63.99 ± 49.80	56.50 ± 11.33
BiP	4601.53 ± 2742.81	4933.05 ± 2581.49	7252.79 ± 3819.39	3170.4 ± 1308.57
XBP-1S	124.92 ± 87.53	84.16 ± 45.04	104.51 ± 39.31	102.95 ± 48.97

Table S3. Measurement of ß-cell ER stress markers in INS-1E cells 2, 6 or 16 hours after a 1 hour exposure to 40 μM H_2O_2.

After 2, 6 or 16 hours following 1 hour exposure to 40 μM H_2O_2, ER stress markers were detected by real time RT-PCR and corrected per GAPDH. Results are means ± S.E. of 5 independent experiments.

Genes	Control (2 hrs)	Stressed (2 hrs)	Control (6 hrs)	Stressed (6 hrs)	Control (16 hrs)	Stressed (16 hrs)
CHOP	58.60 ± 21.52	65.58 ± 23.99	62.61 ± 19.53	68.71 ± 12.44	62.52 ± 19.63	46.37 ± 16.41
BiP	11971.21 ± 1821.53	10430.58 ± 1929.56	9732.91 ± 1270.31	5783.06 ± 1192.35	10339.03 ± 1309.18	11145.21 ± 2104.40
XBP1S	582.31 ± 142.60	371.87 ± 78.38	555.05 ± 110.42	333.00 ± 14.48	583.77 ± 110.44	623.16 ± 139.15

1. Brun, T., Duhamel, D. L., Hu He, K. H., Wollheim, C. B., and Gauthier, B. R. (2007) *Oncogene* **26**, 4261-4271
2. Kharroubi, I., Ladriere, L., Cardozo, A. K., Dogusan, Z., Cnop, M., and Eizirik, D. L. (2004) *Endocrinology* **145**, 5087-5096
3. Pirot, P., Naamane, N., Libert, F., Magnusson, N. E., Orntoft, T. F., Cardozo, A. K., and Eizirik, D. L. (2007) *Diabetologia* **50**, 1006-1014
4. Cnop, M., Ladriere, L., Hekerman, P., Ortis, F., Cardozo, A. K., Dogusan, Z., Flamez, D., Boyce, M., Yuan, J., and Eizirik, D. L. (2007) *J Biol Chem* **282**, 3989-3997