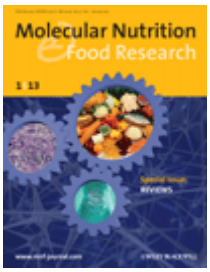


Aspalathin protects insulin-producing β cells against glucotoxicity and oxidative stress-induced cell death

Journal:	*Molecular Nutrition and Food Research*
Manuscript ID	mnfr.201901009.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Moens, Céline; Vrije Universiteit Brussel - Campus Jette, Cell Differentiation Lab -DIFF Bensellam, Mohammed; Université catholique de Louvain, : Institut de recherche expérimentale et clinique, Pôle d'endocrinologie, diabète et nutrition Himpe, Eddy; Vrije Universiteit Brussel - Campus Jette, Cell Differentiation Lab -DIFF Muller, Christo; South African Medical Research Council, Biomedical Research and Innovation Platform Jonas, Jean-Christophe; Université catholique de Louvain, Institut de recherche expérimentale et clinique, Pôle d'endocrinologie, diabète et nutrition Bouwens, Luc; Vrije Universiteit Brussel - Campus Jette, Cell Differentiation Lab -DIFF
Keywords:	Aspalathin, Apoptosis, Diabetes, Glucotoxicity, Oxidative stress

Aspalathin protects insulin-producing β cells against glucotoxicity and oxidative stress-induced cell death

Céline Moens[1*], Mohammed Bensellam[2], Eddy Himpe[1], Christo J.F. Muller[3], Jean-Christophe Jonas[2], Luc Bouwens[1]

[1]Cell Differentiation Lab, Vrije Universiteit Brussel, Brussels, Belgium

[2]Université Catholique de Louvain, Institut de Recherche Expérimentale et Clinique, Pôle d'Endocrinologie, Diabète et Nutrition, Brussels, Belgium

[3]Biomedical Research and Innovation Platform, South African Medical Research Council, Tygerberg, South Africa

*Correspondence: Céline Moens, Cell Differentiation Lab, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium. Fax: +32-2477-4458; E-mail: celine.moens@vub.be

Keywords: Aspalathin, Apoptosis, Diabetes, Glucotoxicity, Oxidative stress

Abbreviations

ANOVA	One-way analysis of variance
ARE	Antioxidant responsive element
CYCA	Cyclophilin A
C_t	Cycle threshold
DDIT3	DNA Damage Inducible Transcript 3
ECL	Enhanced Chemiluminescence
FBS	Fetal bovine serum
HMW	High molecular weight

HMOX1	Heme oxygenase 1
HSPA5	Heat Shock Protein Family A (Hsp70) Member 5
INS1E	Insulinoma 1E
KEAP1	Kelch-like ECH-associated protein 1
NIH	National Institutes of Health
NOD	Non-obese diabetic
NQO-1	NAD(P)H Quinone Dehydrogenase 1
NRF2	Nuclear factor erythroid 2-related factor 2
P62	Sequestosome-1
PVDF	Polyvinylidene difluoride
RIPA	Radio immunoprecipitation assay
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1
STZ	Streptozotocin
T2D	Type 2 diabetes
TBS	Tris buffered saline
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TXNIP	Thioredoxin interacting protein

Abstract

Scope

Aspalathin, the main polyphenolic phytochemical of rooibos (*Aspalathus linearis*), has been attributed with health promoting properties, including a glucose lowering effect that could prove interesting for application as nutraceutical or therapeutic in (pre-) diabetics. Preservation

of β cell mass in the pancreas is considered a key issue for diabetes prevention or treatment, therefore we aimed to investigate whether aspalathin also has β cell cytoprotective potential.

Methods and results

Rat pancreatic islets and the β cell line INS1E were studied *in vitro* after exposure to various cytotoxic agents, namely streptozotocin, hydrogen peroxide or chronic high glucose. The effect of aspalathin on cell survival and apoptosis was studied. Expression of relevant cytoprotective genes was analyzed by qRT-PCR and proteins by Western blot. Aspalathin was found to protect β cells against cytotoxicity and apoptosis. This was associated with increased translocation of nuclear factor erythroid 2-related factor 2 (NRF2) and expression of its antioxidant target genes heme oxygenase 1 (*Hmox1*), NAD(P)H quinone dehydrogenase 1 (*Nqo-1*) and superoxide dismutase 1 (*Sod1*).

Conclusion

It is proposed that aspalathin protects β cells against glucotoxicity and oxidative stress by increasing the expression of NRF2-regulated antioxidant enzymes. This indicates that aspalathin is an interesting β cell cytoprotectant.

1 Introduction

There is an unmet need in finding new medicines to treat or prevent type 2 diabetes (T2D). T2D is a common chronic and progressive metabolic disease marked by hyperglycemia. It originates from the failure of pancreatic β cells to compensate for increased insulin needs due to insulin resistance of the body. The resulting high glucose levels, together with high dietary lipid levels, exert deleterious effects on β cell function and survival which is denoted as glucolipotoxicity. Glucolipotoxicity thus leads to the progressive decline of functional β cell mass, worsening the disease over time. The reduction in β cell mass is now considered a major contributor to T2D pathogenesis and as one of the most urgent priorities for diabetes research.[1, 2] It is now

generally agreed that more effective antidiabetic therapies are needed that not only lower blood glucose concentrations but that also slow down or halt progressive loss of β cell mass and function.[3, 4]

High circulating levels of both glucose and free fatty acids are known to induce oxidative stress in β cells.[5, 6] β cells are particularly sensitive to oxidative stress due to low expression levels of antioxidant enzymes.[7] This suggests that therapy with antioxidants may represent a useful pharmacological approach for the management of T2D.[8]

In addition to therapeutic drugs, nutraceuticals with glucose-lowering and β cell cytoprotective effects may have a prophylactic potential and can be considered an important area for further research.[9] Diets rich in polyphenols, including flavonoids, have been associated with lower risk of T2D[10, 11] and were shown to improve glucose metabolism in people with increased risk for diabetes.[12] Several studies have reported a beneficial role of rooibos extract or its components in diabetes.[13] Rooibos tea is a popular caffeine-free beverage and a rich source of polyphenols, with as major component the dihydrochalcone C-glucoside aspalathin, which is unique to the rooibos plant *Aspalathus linearis*.[14] Aspalathin has been attributed anti-hyperglycemic and cardioprotective effects and is considered a potent anti-oxidant.[15] It was found to suppress the rise in blood glycemia and to improve glucose tolerance in db/db mice,[16] ob/ob mice[17] and streptozotocin (STZ)-treated rats.[18] *In vitro*, it increased the glucose uptake by myocytes,[16–18] it increased insulin secretion by RIN-5F β cells[16] and suppressed the formation of reactive oxygen species (ROS) in these cells.[17] In cardiomyocytes exposed to high glucose, aspalathin was shown recently to stimulate the transcription of antioxidant and phase II detoxification genes in a NRF2-dependent manner.[19]

A protective effect of aspalathin against β cell death has not been documented so far. The present study was initiated to verify whether aspalathin protects β cells in culture against

oxidative stress-induced cell death and glucotoxicity and whether it alters the cellular antioxidant response.

2 Experimental Section

2.1 Culture of INS1E β cells and primary rat islets

The INS1E β cell line originates from a rat insulinoma and is characterized by the production of insulin. The INS1E β cell line was kindly provided by Prof. Claes B. Wollheim at the Centre Médical Universitaire de Genève, Geneva, Switzerland. Passages between 61 and 79 were taken for experiments. The cells were cultivated in RPMI medium 1640 (1X) with 2mM GlutaMAX™-I and L-glutamine. The medium was supplemented with 5% fetal bovine serum (FBS), 100µg/mL streptomycin, 100units/mL penicillin, 1mM sodium pyruvate, 10mM HEPES and 50µM β-mercaptoethanol (all ThermoFisher Scientific, Massachusetts, United States). The glucose concentration of the standard medium was 11mM (control), unless stated otherwise.

Primary rat islets were isolated from the pancreas of ~200g male Wistar rats by collagenase digestion, separated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St Louis, MI, USA) and handpicked under a stereomicroscope as previously described.[20] Before treatment, islets were precultured for 1 week in RPMI medium 1640 (1X) containing 10mM glucose and supplemented with 5g/L BSA, 2mM L-glutamine, 100U/mL penicillin and 100µg/mL streptomycin. All experiments were approved by the Institutional Committee on Animal Experimentation of the Health Sciences Sector at UCLouvain (Project 2017/UCL/MD/014).

2.2 Aspalathin

3-(3,4-Dihydroxyphenyl)-1-(3-β-D-glucopyranosyl-2,4,6-trihydroxyphenyl)-1-propanone (Aspalathin), a water-soluble dihydrochalcone C-glucoside, was synthesized by High Force Research Ltd (Durham, UK) with a purity of ca. 98% based on HPLC (batch SZ1-356-54).[21] It was kindly provided by Prof. Christo Müller of the South African Medical Research Council (Cape Town, South Africa).

2.3 Dosage Information/ Dosage Regimen

INS1E cells were treated with various concentrations (0, 5, 10, 30 or 60µM) aspalathin. The biggest protective effect was seen with 30 and 60µM aspalathin. Thus 30 and 60µM were selected as the concentrations for further experiments. Additionally, the cell toxicity of aspalathin was investigated at 100µM. 100µM aspalathin did not induce cell toxicity.

2.4 *In vitro* cytotoxicity treatments

Cells for cytotoxicity experiments were seeded in 96-well plates with a density of 25.000 cells per well. They were incubated 48h to guarantee full attachment of the cells to the bottom. Aspalathin was added to the RPMI culture medium 24h prior to the insult at concentrations of 0, 5, 10, 30 or 60µM. After 24h, fresh aspalathin-containing medium was added to the wells and as a toxic insult STZ or hydrogen peroxide (H_2O_2) was added. STZ (Sigma-Aldrich) was dissolved in citrate buffer and used at a concentration of 1mM during a 1h incubation. H_2O_2 (VWR, Pennsylvania, United States) was used at a concentration of 100µM and cells were incubated 30 min. After the insult, the medium was removed and new medium with respective aspalathin concentrations was added. Standard medium containing 11mM glucose (NaCl 0.9% from Baxter, Illinois, United States) was used as control. For high glucose conditions, standard medium supplemented with 22mM glucose, to reach a final concentration of 33mM glucose, (D-(+)-Glucose solution from Sigma-Aldrich) was used. 18h after insult, cells were used for

analysis. For glucotoxicity tests, INS1E β cells were seeded in 24-well plates and incubated 48h as described above. Wells then received normal or high glucose for 72h. After 24h, 0 or 30µM aspalathin was added for the last 48h before analysis (see next section).

Batches of 100 to 150 primary rat islets were cultured for 1 week in RPMI medium containing 10mM glucose (control condition) or supplemented with 20mM glucose to reach a final concentration of 30mM glucose (glucotoxicity) in the absence or presence of 30µM aspalathin. The culture medium was renewed every day.

2.5 Assessment of apoptotic and necrotic β cell death by Hoechst-Propidium Iodide staining

Apoptotic INS1E β cells were counted using the DNA binding dyes Hoechst 33342 (10µg/mL, Sigma-Aldrich) and propidium iodide (PI, 5µg/mL, Sigma-Aldrich). Cells were incubated with these dyes for 1h at 37°C, washed and fixed with 4% paraformaldehyde for 15 min. Apoptotic and necrotic cells were counted under the Nikon TE2000 Eclipse fluorescence microscope (Nikon, Tokyo, Japan). Quantification was performed using NIS AR2.30 imaging software (Nikon). Microscopic images (20x magnification) were taken from different regions in the wells to ensure a representation of the full well. Cells which were Hoechst positive and PI negative were counted as early apoptotic cells, Hoechst positive and PI positive cells with the occurrence of picnotic nuclei, were counted as late apoptotic cells and cells that were only PI positive were counted as necrotic cells. Total cell death was defined by adding the percentage necrotic cells to the percentage apoptotic cells. Correct counting of the apoptotic cells was confirmed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (see below).

2.6 Assessment of apoptotic β cell death by TUNEL staining

TUNEL staining (version 18, Roche, Basel, Switzerland) was performed to ensure correct counting of the apoptotic cells with Hoechst-PI staining. TUNEL staining detects DNA fragmentation by labeling the 3'- hydroxyl end of DNA breaks which occurs during apoptosis. After culture, islets were collected and washed in ice-cold PBS, fixed in 4% (wt/vol) paraformaldehyde for 4h and embedded in paraffin. The percentage of apoptotic β cells was determined on 5µm-thick islet sections using the *In Situ* Cell Death Detection Kit-POD (Roche Diagnostics) following manufacturer's instructions. These sections were then immunoprobed for insulin using a rabbit monoclonal anti-insulin antibody (3014, Cell Signaling Technology, Danvers, MA, USA) and an AlexaFluor 594-conjugated anti-rabbit secondary antibody (Thermofisher Scientific). Islet $TUNEL^+$/$insulin^+$/$DAPI^+$ cells were counted on digital images acquired using an Axio Imager Z1 fluorescence microscope (Carl Zeiss,Oberkochen, Germany).

2.7 MTS cytotoxicity assay

Cell viability/cytotoxicity was also determined using the CellTiter 96 Aq_{ueous} one solution cell proliferation assay (Promega, Madison, USA). This is a colometric method to determine the number of viable cells. When tetrazolium compound is added to metabolically active cells, this substance is reduced by NADPH or NADH into a colored formazan product. An amount of 10µL of tetrazolium compound was added to each well of the plate and then incubated for 2h at 37°C. The optical density (OD) was read at 490nm using an iMark microplate reader (Bio-rad, California, United States). The cell viability percentage was calculated by the formula: OD test sample/ OD control sample x 100. Untreated wells were taken as control samples. The reading was a mean of at least three wells for each experimental condition.

2.8 RNA extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

For RNA-analysis by qRT-PCR, INS1E β cells were seeded in 6-well plates at a density of $1x10^6$ cells per well and subsequently incubated for 48h as described above. Wells received 24h treatment in normal glucose conditions with 0, 30 or 60µM aspalathin and were then harvested for mRNA extraction. Total RNA isolation of the INS1E β cells was performed using the GeneElute™ Mammalian Total RNA Miniprep Kit RTN70 (Sigma-Aldrich). The RNA concentration was quantified by Nanodrop 2000 (ThermoFisher Scientific). All RNA samples were subjected to DNase treatment using the DNase I kit (ThermoFisher Scientific; EN0521). The GoScript Reverse Transcription System (Promega, A5000) was used to prepare the cDNA. PCR analysis was performed using the 7900HT Fast Real Time PCR system (Applied Biosystems, California, United States) and PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, A25742). Predesigned SYBRGreen® Kicqstart® primers (Millipore Sigma-Aldrich; primers *Sod1* gene) and self-designed DNA oligo primers (Integrated DNA Technologies, Iowa, United States; all the other primers) were used. Genes that were analyzed are: *β-actin* (GeneID: 81822), *Nqo-1* (GeneID: 24314), *Hmox1* (GeneID: 24451) and *Sod1* (GeneID: 24786, pair 1). The primer sequences are listed in Table S1, Supporting Information. 3µL primer mix (1.5µM per primer) was mixed with 10µL of PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific), 5µL of DEPC water and 2.5ng RNA was added in each well. Dissociation curves and lengths of amplified products with agarose gel electrophoresis were investigated to ensure specific amplification of the target gene. Relative expression levels of the genes of interest were calculated to the expression level of β-actin as an endogenous reference gene using the delta cycle threshold (C_t).

Islet total RNA was isolated and reverse transcribed as previously reported.[20] Real-time RT-PCR was performed using the SYBR Green method and a CFX96 optical cycler detection

system (Bio-Rad). Genes that were analysed are: cyclophilin A (*Cyca*; GeneID: 25518), *Hmox1* (GeneID: 24451), *Txnip* (GeneID: 117514), *Ddit3* (GeneID: 29467) and *Hspa5* (GeneID: 25617). The value obtained for a gene product was normalised to the control gene *Cyca* and expressed as fold change of the value in the control condition.

2.9 Protein extraction and Western blot

For determining the effect of aspalathin on protein expression, INS1E β cells were seeded as described in caption 2.7. Wells received 24h treatment with 0, 30 or 60µM aspalathin in normal glucose conditions. Each passage represented an independent experiment. The cells were harvested, washed with ice-cold PBS and lysed for 10 min in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing 1% protease/phosphatase inhibitor (Cell Signaling Technology) on ice. Unbroken cells were spun down for 5 min at 16000g. For the translocation experiments of NRF2 to the nucleus, the nuclear and cytoplasmic extraction kit (ThermoFisher Scientific) was used following the manufacturer protocol. Protein concentration of the different fractions were measured with the protein assay from Bio-rad. Equal amounts of protein (25µg) mixed with an equal amount of 2x Laemmli sample buffer (Bio-rad) were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-rad). A maximum of two replicates for each condition were loaded on the same gel. After saturation (1h) of non-specific binding sites using blocking buffer (tris buffered saline (TBS) containing 5% non-fat milk (Cell Signaling Technology) and 0.1% Tween 20), blots were probed with primary antibodies (1/1000 TBS containing 5% BSA (Merck, Darmstadt, Germany) and 0.1% Tween 20) overnight at 4°C. The blots were washed three times for 5 min with TBS 0.1% Tween 20 and incubated with species specific HRP-labelled IgG (1/5000 in blocking buffer, GE healthcare, Chicago, United states) for 1h. Antibodies against sequestosome 1 (P62; 23214), β-actin (4970) and histone H3 (9715) were from Cell Signaling Technology. Antibody against

NRF2 (ab137550) was from Abcam (Cambridge, United Kingdom). Blots were washed three times with TBS 0.1% Tween 20 for 5 min and protein signal was visualized using western lighting plus enhanced chemiluminescence (ECL; Perkin Elmer, Waltham, United states) as recommended by the manufacturer. Chemiluminescence was captured with the Odyssey Fc Imaging System from LI-COR bioscience (Lincoln, United States). Densitometric analysis was performed using ImageJ 1.46r National Institutes of Health (NIH; Bethesda, United States). Control groups of independent experiments were normalized against each other. Experimental conditions were then compared to the control group.

2.10 Statistics

GraphPad Prism 6.0 software was used to perform statistical analysis (GraphPad Software, San Diego, CA, USA). Differences among groups in cell viability, Hoechst-PI staining, TUNEL staining, MTS assay and Western blotting were determined using a one- or two-way analysis of variance (ANOVA) with Tukey's post-hoc test. qRT-PCR data was analyzed by a paired one-way ANOVA with Tukey's post hoc test or a two-way ANOVA with Sidak's or Holm-Sidak's post hoc test. Normality test indicated Gaussian distribution. Independent repeats are indicated as n. All data are expressed as mean ± SEM (Standard Error of the Mean) and all tests were performed at the 0.05 level of significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ###$p < 0.001$ and ####$p < 0.0001$.

3 Results

3.1 Aspalathin protects INS1E β cells against streptozotocin and H_2O_2 induced apoptotic cell death

To induce oxidative stress in β cells, we first used STZ which is a β cell-selective toxin that acutely acts as a donor of multiple forms of ROS, against which β cells have little defense.[7] In

the presence of 33mM glucose, short STZ exposure induces mainly apoptotic β cell death[22] that can be demonstrated by the Hoechst-PI or TUNEL method. We found an excellent correlation between the results of both methods (data not shown). With the Hoechst-PI method (Fig. 1A), 30μM aspalathin showed a strong anti-apoptotic, cytoprotective effect (~80%) on INS1E β cells. Lower concentrations of aspalathin showed less but significant cytoprotective effect against STZ-induced apoptosis, indicating a dose-dependent effect (Fig. 1A). The β-cytoprotective effect of aspalathin could also be demonstrated by the MTS method, a tetrazolium-based assay that measures dehydrogenases in metabolically active cells. This assay proved to be less sensitive than the Hoechst-PI (or TUNEL) method, with no significant effect of aspalathin in the concentration range 5-10μM (data not shown), although a significant cytoprotective effect was noted at 30 and 60μM (Fig. 1B).

Aspalathin also had a cytoprotective effect on INS1E β cells exposed to 100μM of H_2O_2, another inducer of oxidative stress. This was shown by Hoechst-PI staining (Fig. 2A) and MTS assay (Fig. 2B).

3.2 Aspalathin protects against glucotoxic cell death in pancreatic islet β cells

Having established that aspalathin offers protection to oxidative stressors in the previous section, we examined whether it would also protect the INS1E β cells from glucotoxicity which is thought to operate largely via oxidative stress in β cells and is a physiologically more relevant stressor.

Aspalathin (30μM) was found to significantly decrease the frequency of apoptotic INS1E β cells when cell death was induced by a prolonged exposure to elevated glucose concentrations (Fig. 3A). Although the INS1E β cell line represents a valuable model which has been successfully used in functional screens for drug discovery,[3] cultured pancreatic islets may more closely reproduce *in vivo* conditions. Therefore, we cultured islets of Langerhans isolated

from rat pancreas for 7 days at normal or high glucose concentrations, in the presence or absence of aspalathin. Chronic high glucose induced apoptotic β cell death in the islets as demonstrated by TUNEL staining and this cytotoxicity was completely abolished in the presence of 30μM aspalathin (Fig. 3B and 3C).

3.3 Aspalathin stimulates the expression of oxidative stress response genes

To try to understand the mechanism by which aspalathin offers protection to glucotoxicity and oxidative stress, we analyzed the islet cells by qRT-PCR for the expression of *Hmox1*, which is known as an important antioxidant and prosurvival gene involved in β cells.[8] Aspalathin was found to significantly increase the expression of *Hmox1* both in normal and high glucose conditions (Fig. 4A). We also found a significant repression by aspalathin of the high oxidative stress-induced proapoptotic gene thioredoxin interacting protein (*Txnip*)[8] (Fig. 4B). Aspalathin treatment also markedly downregulated the mRNA levels of DNA damage-inducible transcript 3 (*Ddit3* or *Chop*) which is a known proapoptotic gene induced under various types of stress including ER- and oxidative stress (Fig. 4C). In addition, aspalathin treatment significantly reduced the mRNA levels of heat-shock protein family A member 5 (*Hspa5* or *Bip*), a marker of ER-stress (Fig. 4D).

These observations suggest that aspalathin treatment protects β cells against high glucose-induced oxidative stress and ER stress which may explain the observed protection against high glucose-induced apoptosis.

In INS1E β cells, exposure to aspalathin in normal glucose conditions leads to an increased transcription of *Hmox1* (Fig. 5A), *Nqo1* (Fig. 5B) and also of *Sod1* (Fig. 5C) which all are oxidative stress response genes. All genes encode antioxidant enzymes and their expression is regulated by the NRF2- Kelch-like ECH-associated protein 1 (KEAP1)- antioxidant responsive element (ARE) pathway.[23] KEAP1 protein acts as a redox sensor which normally targets

NRF2 for degradation. In conditions of oxidative stress, NRF2 may detach from KEAP1 and translocate to the nucleus where it accumulates and binds to the ARE. This initiates expression of cytoprotective genes like *Hmox1* and *Nqo1*. To find out whether aspalathin treatment induces NRF2 accumulation in the nucleus, Western blot analysis of nuclear extracts from INS1E β cells exposed to aspalathin for 24h was performed. The results confirm accumulation of NRF2 in the nucleus in a dose dependent fashion, which is in line with the ARE-activation (Fig. 6A and B).

We also analyzed the expression of P62 protein, which is a stress-sensitive protein and redox sensor that can interact with the NRF2-KEAP1-ARE pathway[24, 25]. In INS1E β cells exposed to aspalathin in normal culture conditions, the expression of the P62 protein (~60kDa) was increased within 24h as seen by Western blot (Fig. 7A and B). Moreover, we noted a significant increase of an altered form of P62 with reduced electrophoretic mobility in denaturing SDS-PAGE conditions. This high molecular weight (HMW) P62 band did not accumulate at 60kDa, but rather between approximately 120 and 170kDa (Fig. 7A and C) and was already noted after 30-60 min of aspalathin exposure (data not shown). This observation may indicate a direct interaction between aspalathin and P62 protein and contribute to activation of NRF2 (see Discussion).

4 Discussion

In this study, it was found that the polyphenolic flavonoid aspalathin, which is enriched in unfermented extracts from the rooibos plant (*Aspalathus linearis*), can protect pancreatic islet β cells from the cytotoxic effects of different stressors, namely STZ, H_2O_2 and chronic high glucose (glucotoxicity). Exposure of β cells to H_2O_2 and prolonged high glucose are known to induce oxidative stress and β cell death.[8] STZ also induces oxidative stress and apoptosis in β

cells.[22] In diabetes, oxidative stress is complex and can be induced by multiple mechanisms involving different types of ROS. These are superoxide anion, hydroxyl radical, singlet oxygen, H_2O_2 and peroxynitrite, all of which can lead to cell death. STZ induces the formation of all these kinds of ROS and therefore is an appropriate inducer of multiple levels of oxidative stress for diabetes studies.[26, 27]

The β-cytoprotective effect of aspalathin against glucotoxicity and oxidative stress-induced apoptosis was found in the INS1E β cell line and primary rat islet β cells. In these cells, aspalathin was found to repress the expression of proapoptotic genes, like *Txnip* and *Ddit3*, and to activate the expression of protective antioxidant response genes, like *Hmox1*.

Increased transcription of the *Hmox1* gene is the primary mechanism for increased HMOX1 antioxidant activity and HMOX1-upregulating agents have been considered as potential therapeutic agents for β cell protection.[28] HMOX1 has also anti-inflammatory and immunomodulatory properties besides its antioxidant and cytoprotective effects, which makes it more interesting in diabetes. It is reported that pharmacological enhancement of the HMOX1 pathway exerts protective effects against the development of diabetes via several mechanisms.[29] Upregulation of *Hmox1* was shown to protect pancreatic β cells from oxidative injury induced by high glucose concentrations, resulting in preservation of insulin secretion *in vitro*.[30] In non-obese diabetic (NOD) mice, a well-established model of type 1 diabetes, administration of a *Hmox1* inducer decreased pancreatic superoxide contents, which in return resulted in suppression of β cell loss.[31] Similar results were found in transgenic mice that overexpress *Hmox1* in β cells.[32] In our study, aspalathin also increased the expression of *Nqo-1* and *Sod1* in INS1E β cells. NQO-1 is an antioxidative enzyme that plays a role in protection against the toxicity and carcinogenicity of electrophiles and oxidants.[23] SOD1 is also an important antioxidative enzyme that catalyzes the dismutation of superoxide anion into oxygen

and H_2O_2.[33] It is well documented that HMOX1, NQO-1 and SOD1 are positively regulated by NRF2, a transcription factor and master regulator of antioxidants and anti-apoptotic proteins.[34] Moreover, it has been reported that nuclear HMOX1 interacts with NRF2 and stabilizes it from ubiquitin-proteasomal degradation, thereby prolonging its accumulation in the nucleus.[35] Our Western blot results confirm that aspalathin induces nuclear accumulation of NRF2 protein. This is interesting as the NRF2 system is considered as a crucial defense pathway to prevent reactive oxygen damage in pancreatic β cells and protect the cells in physiological and pathological conditions.[36]

The KEAP1-NRF2 pathway is considered as the major regulator of cytoprotective responses to oxidative stress.[23, 34] NRF2 is normally bound to the E3 ubiquitin ligase adaptor protein KEAP1, which causes NRF2 ubiquitination and proteasomal degradation. In the canonical pathway, the KEAP1 protein contains critical cysteines that can be modified by nitric oxide, electrophiles and ROS leading to conformational changes and its inactivation. Consequently, NRF2 is stabilized and released by KEAP1. As a result, NRF2 can translocate to the nucleus and activate the ARE-mediated transcription of cytoprotective genes.

In the noncanonical pathway, P62 protein accumulates, e.g. when autophagic flux is compromised, and KEAP1 is sequestered and captured by P62 through interaction with the NRF2 binding site. KEAP1 can no longer bind NRF2, leading to increased NRF2 signaling.[23, 34, 37, 38] In our study, aspalathin was found to increase the level of P62 protein in INS1E β cells, and it also induced increased levels of HMW P62 aggregates (within just a short exposure time). The formation of the P62 aggregates is an interesting observation that others have denoted as oxidative crosslinking of P62 oligomers.[37, 39] KEAP1 is a very cysteine-rich protein, making it a redox sensor in the cell.[34] The same is also true for P62 which contains oxidation-sensitive cysteine residues allowing P62 to also sense (like KEAP1) ROS under oxidative stress

conditions.[37] The induction of P62 oligomerization by aspalathin thus suggests a direct interaction with P62 and activation of NRF2 and antioxidant enzymes via the noncanonical pathway. It remains possible that aspalathin also can directly interact with the cysteine residues of the KEAP1 protein, resulting in the canonical activation of NRF2. Interaction with cysteine-rich proteins has been observed with other polyphenolic flavonoids that induced the NRF2 pathway.[40]

Since oxidative stress is considered a central driving feature of β cell dysfunction and cell death, antioxidant therapies are predicted to be of great value to complement existing therapeutic strategies. However, early clinical trials with traditional antioxidants such as ascorbic acid or tocopherol have provided disappointing outcomes.[8] It has been argued, however, that these antioxidant drugs may be insufficient to combat the high levels of oxidative stress to which β cells are exposed in (pre-) diabetics. A better therapeutic approach would be to target cellular programs driving antioxidant function within the β cell.[6] The goal therefore should be to find mechanism-based antioxidants, i.e. small molecule activators of endogenous antioxidant mechanisms that could have clinical benefit by protecting β cells from oxidative stress.

We show that aspalathin acts as a mechanism-based antioxidant that protects β cells against oxidative stress and glucotoxicity-induced cell death. As mechanism we propose a direct interaction of aspalathin with P62 driving the noncanonical activation of the KEAP1-NRF2 system which induces the expression of antioxidant enzymes like HMOX1 that results in buffering ROS and preventing β cell apoptosis (Fig. 8). Further studies are needed to fully unravel the involved molecular mechanisms.

In addition to the presently reported β-cytoprotective effect of aspalathin *in vitro*, previous studies have reported hypoglycemic[16–18] and cardioprotective[19] effects of aspalathin

treatment *in vivo*. These findings warrant further studies of aspalathin as a potential candidate for prevention or treatment of diabetes as a next generation medicine or nutraceutical.

Author Contributions

L.B., E.H., M.B. and C.M. designed the study. E.H., M.B. and C.M. performed the experiments and obtained the data. L.B., E.D., M.B and C.M. performed the analysis and interpretation of the data. L.B. and C.M. drafted the manuscript, and L.B., JC.J., M.B., E.H. and C.J.F.M. revised the manuscript. All authors approved the final version of the manuscript to be published.

Acknowledgements

This work has been supported by a BRIDGE Strategic Platform Grant from Innoviris-Brussels, "Personalized medicine in diabetes" (DiaType). M.B. was supported by a MOVE-in Louvain/EC Marie-Curie incoming postdoctoral fellowship and is actually supported by a fellowship from the Clinical Research Fund, Cliniques Universitaires Saint-Luc at UCLouvain. JC.J. is Research Director of the Fonds de la Recherche Scientifique-FNRS, Belgium. The authors wish to thank Johan Louw and C.J.F.M. from the South African Medical Research Council for stimulating discussions. We also wish to thank Emmy De Blay for immunofluorescent stainings and Jonathan Baldan and Isabelle Houbracken for scientific discussion.

Conflict of interest

The authors declare no competing interests linked to this manuscript.

5 References

[1] G.C. Weir, S. Bonner-Weir, *Ann N Y Acad Sci* **2013**, *1281*, 92.

[2] J.J. Meier, R.C. Bonadonna, *Diabetes Care* **2013**, *36 Suppl 2*, S113.

[3] A. Vetere, A. Choudhary, S.M. Burns, B.K. Wagner, *Nat Rev Drug Discov* **2014**, *13*, 278.

[4] S.E. Kahn, M.E. Cooper, S. Del Prato, *Lancet* **2014**, *383*, 1068.

[5] R.P. Robertson, *J Biol Chem* **2004**, *279*, 42351.

[6] S.Z. Hasnain, J.B. Prins, M.A. McGuckin, *J Mol Endocrinol* **2016**, *56*, R33.

[7] S. Lenzen, J. Drinkgern, M. Tiedge, *Free Radic. Biol. Med.* **1996**, *20*, 463.

[8] M. Bensellam, D.R. Laybutt, J.C. Jonas, *Mol Cell Endocrinol* **2012**, *364*, 1.

[9] E.J. Verspohl, *Pharmacol Rev* **2012**, *64*, 188.

[10] B.A. Graf, P.E. Milbury, J.B. Blumberg, *J Med Food* **2005**, *8*, 281.

[11] N.M. Wedick, A. Pan, A. Cassidy, E.B. Rimm, L. Sampson, B. Rosner, W. Willett, F.B. Hu, Q. Sun, R.M. van Dam, *Am J Clin Nutr* **2012**, *95*, 925.

[12] L. Bozzetto, G. Annuzzi, G. Pacini, G. Costabile, C. Vetrani, M. Vitale, E. Griffo, A. Giacco, C. De Natale, S. Cocozza, G. Della Pepa, A. Tura, G. Riccardi, A.A. Rivellese, *Diabetologia* **2015**, *58*, 1551.

[13] M. Sasaki, N. Nishida, M. Shimada, *Molecules* **2018**, *23*.

[14] B.H. Koeppen, D.G. Roux, *Biochem J* **1966**, *99*, 604.

[15] A. von Gadow, E. Joubert, C. F. Hansmann, *J. Agric. Food Chem.* **1997**, *45*, 632.

[16] A. Kawano, H. Nakamura, S. Hata, M. Minakawa, Y. Miura, K. Yagasaki, *Phytomedicine* **2009**, *16*, 437.

[17] M.J. Son, M. Minakawa, Y. Miura, K. Yagasaki, *Eur J Nutr* **2013**, *52*, 1607.

[18] C.J. Muller, E. Joubert, D. de Beer, M. Sanderson, C.J. Malherbe, S.J. Fey, J. Louw, *Phytomedicine* **2012**, *20*, 32.

[19] P. V Dludla, C.J. Muller, E. Joubert, J. Louw, M.F. Essop, K.B. Gabuza, S. Ghoor, B. Huisamen, R. Johnson, *Molecules* **2017**, *22*.

[20] M. Bensellam, L. Van Lommel, L. Overbergh, F.C. Schuit, J.C. Jonas, *Diabetologia* **2009**, *52*, 463.

[21] Z. Han, M.C. Achilonu, P.S. Kendrekar, E. Joubert, D. Ferreira, S.L. Bonnet, J.H. van der Westhuizen, *J Nat Prod* **2014**, *77*, 583.

[22] E. Himpe, D.A. Cunha, I. Song, M. Bugliani, P. Marchetti, M. Cnop, L. Bouwens, *PLoS One* **2016**, *11*, e0157604.

[23] A. Loboda, M. Damulewicz, E. Pyza, A. Jozkowicz, J. Dulak, *Cell Mol Life Sci* **2016**, *73*, 3221.

[24] A. Lau, X.-J. Wang, F. Zhao, N.F. Villeneuve, T. Wu, T. Jiang, Z. Sun, E. White, D.D. Zhang, *Mol. Cell. Biol.* **2010**, *30*, 3275.

[25] M. Komatsu, H. Kurokawa, S. Waguri, K. Taguchi, A. Kobayashi, Y. Ichimura, Y.-S. Sou, I. Ueno, A. Sakamoto, K.I. Tong, M. Kim, Y. Nishito, S. Iemura, T. Natsume, T. Ueno, E. Kominami, H. Motohashi, K. Tanaka, M. Yamamoto, *Nat. Cell Biol.* **2010**, *12*, 213.

[26] T. Szkudelski, *Physiol Res* **2001**, *50*, 537.

[27] N. Auberval, S. Dal, W. Bietiger, E. Seyfritz, J. Peluso, C. Muller, M. Zhao, E. Marchioni, M. Pinget, N. Jeandidier, E. Maillard, V. Schini-Kerth, S. Sigrist, *Evid Based Complement Altern. Med* **2015**, *2015*, 859048.

[28] Y. Shan, R.W. Lambrecht, S.E. Donohue, H.L. Bonkovsky, *Faseb J* **2006**, *20*, 2651.

[29] N.G. Abraham, A. Kappas, *Pharmacol Rev* **2008**, *60*, 79.

[30] K.C. Won, J.S. Moon, M.J. Eun, J.S. Yoon, K.A. Chun, I.H. Cho, Y.W. Kim, H.W. Lee, *J Korean Med Sci* **2006**, *21*, 418.

[31] M. Li, S. Peterson, D. Husney, M. Inaba, K. Guo, A. Kappas, S. Ikehara, N.G. Abraham, *Cell Cycle* **2007**, *6*, 567.

[32] S.H. Huang, C.H. Chu, J.C. Yu, W.C. Chuang, G.J. Lin, P.L. Chen, F.C. Chou, L.Y.

Chau, H.K. Sytwu, *Diabetologia* **2010**, *53*, 2389.

[33] S. Dal, S. Sigrist, *Dis. (Basel, Switzerland)* **2016**, *4*.

[34] E. Kansanen, S.M. Kuosmanen, H. Leinonen, A.L. Levonen, *Redox Biol* **2013**, *1*, 45.

[35] C. Biswas, N. Shah, M. Muthu, P. La, A.P. Fernando, S. Sengupta, G. Yang, P.A. Dennery, *J Biol Chem* **2014**, *289*, 26882.

[36] Y. Yagishita, T. Fukutomi, A. Sugawara, H. Kawamura, T. Takahashi, J. Pi, A. Uruno, M. Yamamoto, *Diabetes* **2014**, *63*, 605.

[37] B. Carroll, E.G. Otten, D. Manni, R. Stefanatos, F.M. Menzies, G.R. Smith, D. Jurk, N. Kenneth, S. Wilkinson, J.F. Passos, J. Attems, E.A. Veal, E. Teyssou, D. Seilhean, S. Millecamps, E.L. Eskelinen, A.K. Bronowska, D.C. Rubinsztein, A. Sanz, V.I. Korolchuk, *Nat Commun* **2018**, *9*, 256.

[38] Y. Ichimura, S. Waguri, Y.-S. Sou, S. Kageyama, J. Hasegawa, R. Ishimura, T. Saito, Y. Yang, T. Kouno, T. Fukutomi, T. Hoshii, A. Hirao, K. Takagi, T. Mizushima, H. Motohashi, M.-S. Lee, T. Yoshimori, K. Tanaka, M. Yamamoto, M. Komatsu, *Mol. Cell* **2013**, *51*, 618.

[39] E. Donohue, A.D. Balgi, M. Komatsu, M. Roberge, *PLoS One* **2014**, *9*, e114964.

[40] S. Magesh, Y. Chen, L. Hu, *Med Res Rev* **2012**, *32*, 687.

Figure captions

Figure 1. INS1E β cells were cultivated in 96-well plates. Cells received aspalathin at indicated concentrations 24h prior to STZ insult and at the time of STZ insult (1mM STZ for 1 hour). STZ-controls received citrate buffer (CB). After STZ or CB insult, the medium was replaced with fresh medium containing aspalathin treatment and 33mM glucose. 18h after insult, cells were analyzed for cell viability by MTS assay or by Hoechst-PI staining. STZ = streptozotocin and ASPA = aspalathin. **(A)** Total apoptotic cells (%) counted with Hoechst-PI staining **(B)**

MTS assay for cell viability (% of control). Average of three independent plates ± SEM. Differences between were analyzed by one-way ANOVA with Tukey's post hoc test. * above bars indicate comparison versus control. # above bars indicate comparison versus STZ. Lines indicate comparison between two treatments. ** $p < 0.01$, *** $p < 0.001$,**** $p < 0.0001$, ### $p < 0.001$ and #### $p < 0.0001$.

Figure 2. INS1E β cells were cultivated in 96-well plates. Wells received 0 or 30µM aspalathin treatment 24h prior to H_2O_2 insult and at the time of H_2O_2 insult (100µM H_2O_2 or control medium for 30 min). Post-insult, the medium was replaced and 0 or 30µM aspalathin was added for another 18h before analysis. Cells were analyzed for cell viability by MTS assay or stained with Hoechst-PI staining. ASPA = 30µM aspalathin, H_2O_2 = 100µM H_2O_2, and H_2O_2+ASPA = 100µM H_2O_2 + 30µM aspalathin. **(A)** Total apoptotic cells (%) counted with Hoechst-PI staining **(B)** MTS assay for cell viability (% of control). Mean ± SEM (n=3-5). Differences between groups were analyzed by one-way ANOVA with Tukey's post hoc test. * above bars indicate comparison versus control. Lines indicate comparison between two groups. ** $p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Figure 3. (A) INS1E β cells were cultivated in a 24-well plate. Wells received for 72h normal (10-11mM) or high (30-33mM) glucose treatment with or without 30µM of aspalathin treatment. After 72h, cells were used for analysis by Hoechst-PI staining. ASPA = 30µM aspalathin. Mean total apoptotic cells (%) ± SEM (n=3). **(B)** Rat islets were cultured for 1 week in the presence of 10 or 30mM glucose with or without 30µM aspalathin. The percentage of apoptotic β-cells was assessed by TUNEL staining. Data are means ± SEM for 3 experiments. **(C)** Representative TUNEL/insulin immunostaining images. Scale bar, 50µm. Differences between groups in A and B were analyzed by two-way ANOVA with Tukey's post hoc test. #

for the effect of aspalathin and * for the effect of high glucose. ## $p < 0.01$ and #### $p < 0.0001$. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 4. Effects of aspalathin treatment on the expression of islet stress response genes. Rat islets were cultured for 1 week in the presence of 10 or 30mM glucose with or without 30µM aspalathin. Changes in the mRNA levels of **(A)** the antioxidant gene *Hmox1*, **(B)** the proapoptotic oxidative stress-responsive gene *Txnip* and **(C and D)** the ER stress-responsive genes *Ddit3* and *Hspa5*. Data are means ± SEM for 3 experiments. Differences between groups were analyzed by two-way ANOVA with Sidak's or Holm-Sidak's post hoc test. # for the effect of aspalathin and * for the effect of high glucose. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 5. For mRNA expression analysis by qRT-PCR, INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment in normal glucose conditions with 0, 30 or 60µM aspalathin and were then harvested for mRNA extraction. **(A-C)** Relative fold change (vs. β-actin) of *Hmox1*, *Nqo1* and *Sod1*. Ordinary one-way ANOVA with Tukey's post-hoc test, mean ± SEM (n=3). * above bars indicate comparison versus control. Lines indicate comparison between two treatments. * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$.

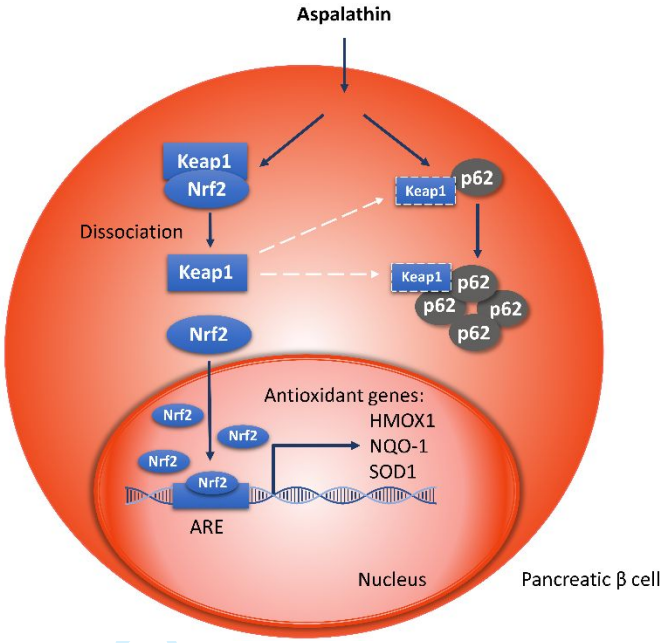
Figure 6. For the NRF2 translocation experiments analyzed by Western blotting, INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment with 0, 30 or 60µM aspalathin in the presence of normal glucose conditions and the nuclear protein fraction was isolated. **(A)** Three Western blots are representative of seven independent experiments (n=7). Entire blots of these abbreviated pieces can be found in Figure S2, Supporting Information. **(B)** The histogram

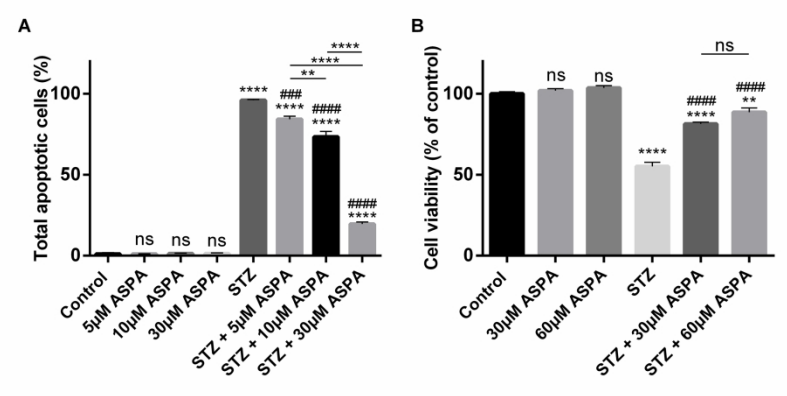
represents the percentage of increase of nuclear NRF2 normalized with nuclear marker histone H3. Values are mean ± SEM from seven independent experiments (n=7). Ordinary one-way ANOVA with Tukey's post-hoc test, mean ± SEM. * above bars indicate comparison versus control. Lines indicate comparison between two treatments. ** $p < 0.01$.

Figure 7. For the P62 experiments analyzed by Western blotting, INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment with 0, 30 or 60μM aspalathin in the presence of normal glucose conditions and total protein fraction was isolated. **(A)** Three Western blots are representative of four independent experiments (n=4). Entire blots of these abbreviated pieces can be found in Figure S3, Supporting Information. **(B)** The histogram represents the percentage of increase of P62 and **(C)** HMW P62 aggregates normalized with β-actin. Values are mean ± SEM from four independent experiments (n=4). Ordinary one-way ANOVA with Tukey's post-hoc test, mean ± SEM. * above bars indicate comparison versus control. Lines indicate comparison between two treatments. * $p < 0.05$ and ** $p < 0.01$.

Figure 8. Proposed working mechanism for aspalathin-induced expression of antioxidant enzymes. Aspalathin is proposed to interact with cysteine residues in KEAP1 and P62 proteins. In the case of KEAP1, this liberates NRF2 which then can accumulate in the nucleus to activate transcription of ARE target genes like *Hmox1*, *Nqo1* and *Sod1*. In the case of P62, this induces the formation of HMW P62 aggregates.

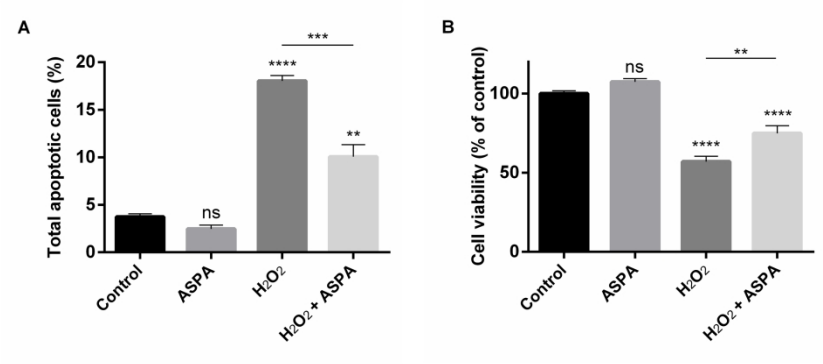
Aspalathin, the major antioxidative component of rooibos tea, has antidiabetic potential. The present study shows that aspalathin protects β cells *in vitro* against oxidative damage. It proposes the interaction of aspalathin with KEAP1 and P62 proteins leading to accumulation of NRF2 in the nucleus and activation of the transcription of anti-oxidant protective genes like *Hmox1*, *Nqo1* and *Sod1*.





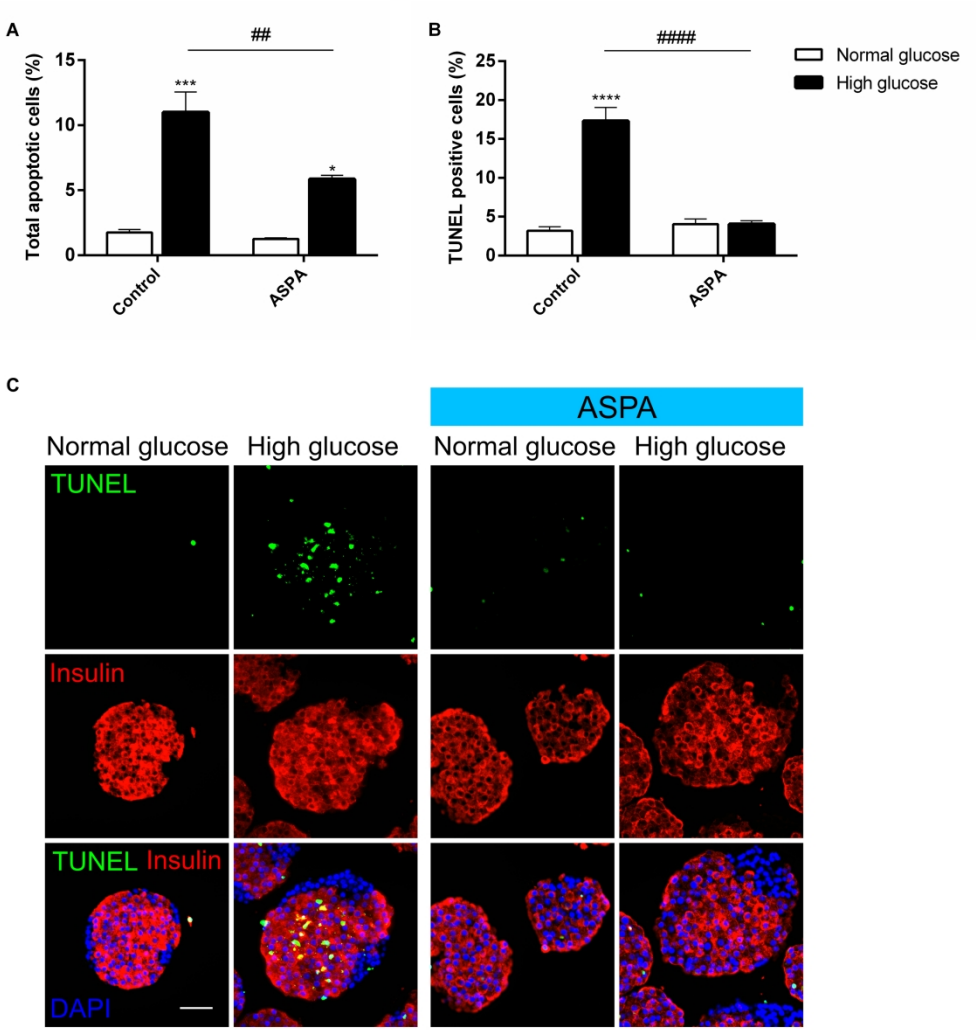
INS1E β cells were cultivated in 96-well plates. Cells received aspalathin at indicated concentrations 24h prior to STZ insult and at the time of STZ insult (1mM STZ for 1 hour). STZ-controls received citrate buffer (CB). After STZ or CB insult, the medium was replaced with fresh medium containing aspalathin treatment and 33mM glucose. 18h after insult, cells were analyzed for cell viability by MTS assay or by Hoechst-PI staining. STZ = streptozotocin and ASPA = aspalathin. (A) Total apoptotic cells (%) counted with Hoechst-PI staining (B) MTS assay for cell viability (% of control). Average of three independent plates ± SEM. Differences between were analyzed by one-way ANOVA with Tukey's post hoc test. * above bars indicate comparison versus control. # above bars indicate comparison versus STZ. Lines indicate comparison between two treatments. ** $p < 0.01$, *** $p < 0.001$,**** $p < 0.0001$, ### $p < 0.001$ and #### $p < 0.0001$.

149x59mm (600 x 600 DPI)



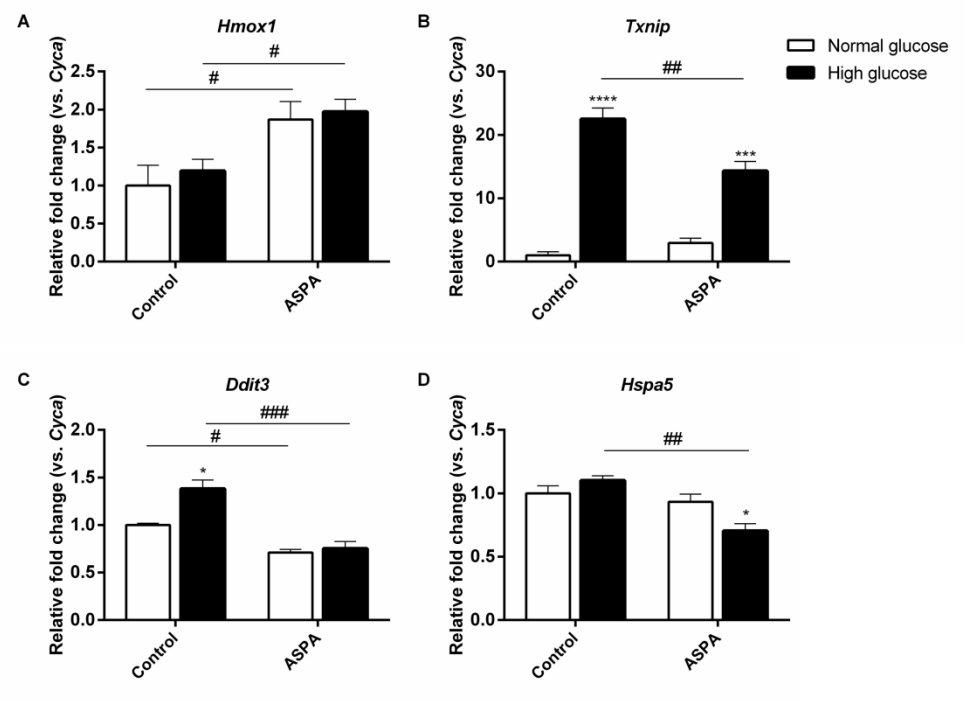
INS1E β cells were cultivated in 96-well plates. Wells received 0 or 30μM aspalathin treatment 24h prior to H2O2 insult and at the time of H2O2 insult (100μM H2O2 or control medium for 30 min). Post-insult, the medium was replaced and 0 or 30μM aspalathin was added for another 18h before analysis. Cells were analyzed for cell viability by MTS assay or stained with Hoechst-PI staining. ASPA = 30μM aspalathin, H2O2 = 100μM H2O2, and H2O2+ASPA = 100μM H2O2 + 30μM aspalathin. (A) Total apoptotic cells (%) counted with Hoechst-PI staining (B) MTS assay for cell viability (% of control). Mean ± SEM (n=3-5). Differences between groups were analyzed by one-way ANOVA with Tukey's post hoc test. * above bars indicate comparison versus control. Lines indicate comparison between two groups. ** $p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

149x59mm (600 x 600 DPI)



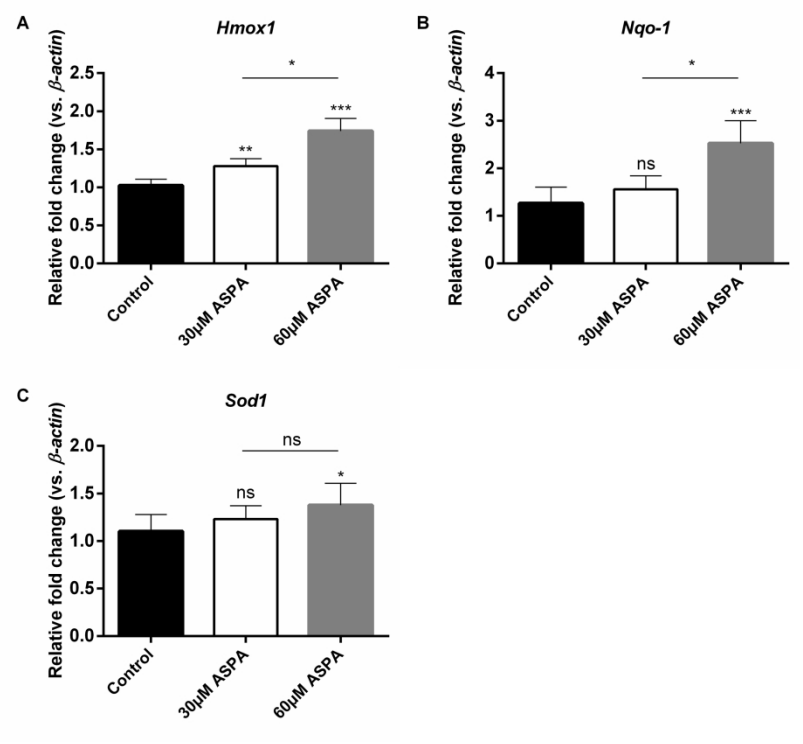
(A) INS1E β cells were cultivated in a 24-well plate. Wells received for 72h normal (10-11mM) or high (30-33mM) glucose treatment with or without 30µM of aspalathin treatment. After 72h, cells were used for analysis by Hoechst-PI staining. ASPA = 30µM aspalathin. Mean total apoptotic cells (%) ± SEM (n=3). (B) Rat islets were cultured for 1 week in the presence of 10 or 30mM glucose with or without 30µM aspalathin. The percentage of apoptotic □-cells was assessed by TUNEL staining. Data are means ± SEM for 3 experiments. (C) Representative TUNEL/insulin immunostaining images. Scale bar, 50µm. Differences between groups in A and B were analyzed by two-way ANOVA with Tukey's post hoc test. # for the effect of aspalathin and * for the effect of high glucose. ## $p < 0.01$ and #### $p < 0.0001$. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$.

149x159mm (600 x 600 DPI)



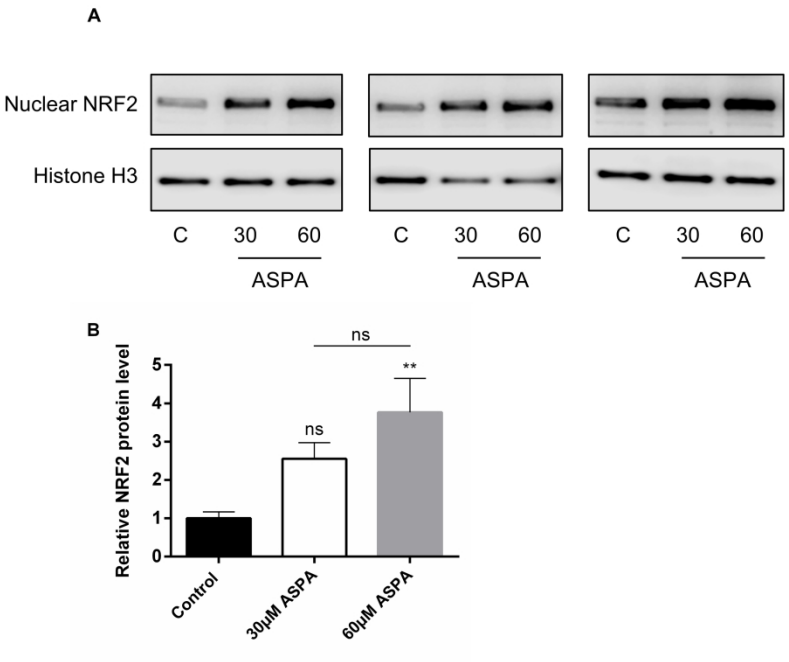
Effects of aspalathin treatment on the expression of islet stress response genes. Rat islets were cultured for 1 week in the presence of 10 or 30mM glucose with or without 30μM aspalathin. Changes in the mRNA levels of (A) the antioxidant gene Hmox1, (B) the proapoptotic oxidative stress-responsive gene Txnip and (C and D) the ER stress-responsive genes Ddit3 and Hspa5. Data are means ± SEM for 3 experiments. Differences between groups were analyzed by two-way ANOVA with Sidak's or Holm-Sidak's post hoc test. # for the effect of aspalathin and * for the effect of high glucose. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$.

149x119mm (600 x 600 DPI)



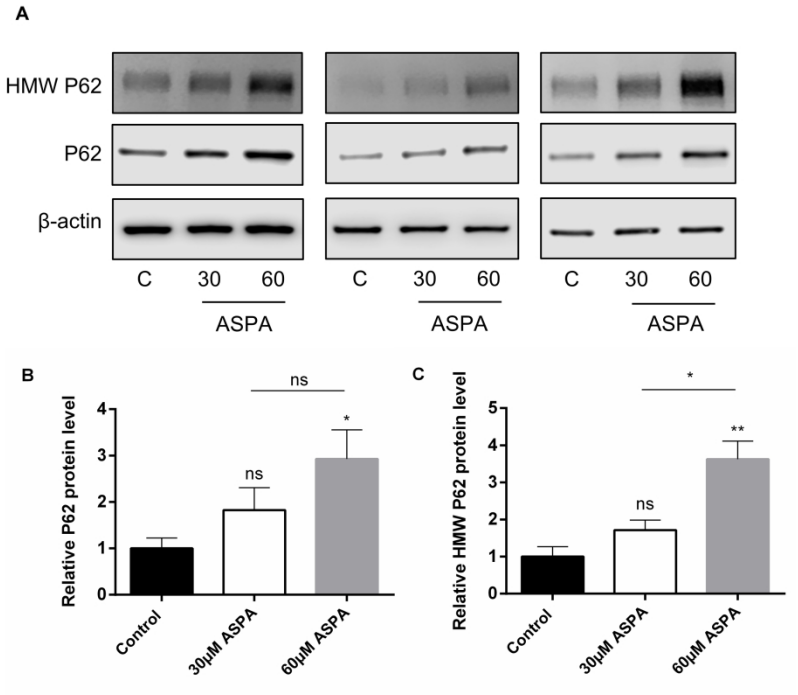
For mRNA expression analysis by qRT-PCR, INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment in normal glucose conditions with 0, 30 or 60μM aspalathin and were then harvested for mRNA extraction. (A-C) Relative fold change (vs. β-actin) of Hmox1, Nqo1 and Sod1. Ordinary one-way ANOVA with Tukey's post-hoc test, mean ± SEM (n=3). * above bars indicate comparison versus control. Lines indicate comparison between two treatments. * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$.

149x119mm (600 x 600 DPI)



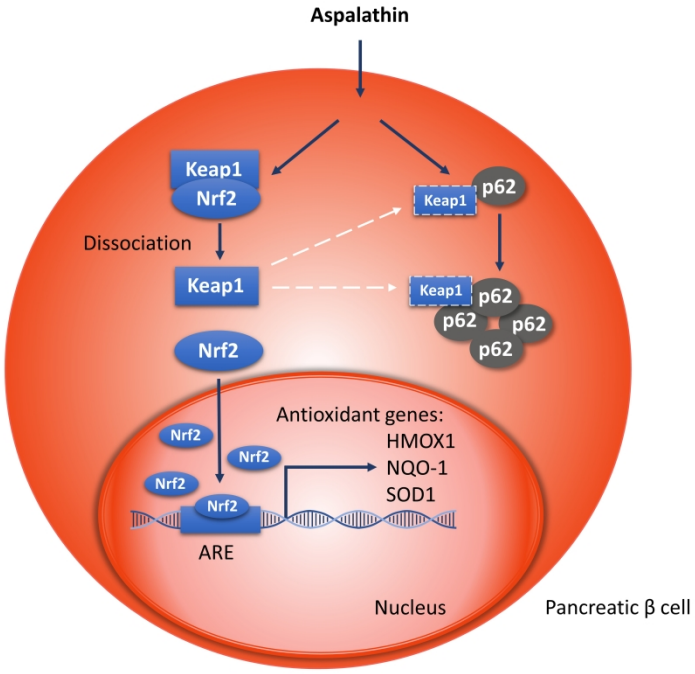
For the NRF2 translocation experiments analyzed by Western blotting, INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment with 0, 30 or 60µM aspalathin in the presence of normal glucose conditions and the nuclear protein fraction was isolated. (A) Three Western blots are representative of seven independent experiments (n=7). Entire blots of these abbreviated pieces can be found in Figure S2, Supporting Information. (B) The histogram represents the percentage of increase of nuclear NRF2 normalized with nuclear marker histone H3. Values are mean ± SEM from seven independent experiments (n=7). Ordinary one-way ANOVA with Tukey's post-hoc test, mean ± SEM. * above bars indicate comparison versus control. Lines indicate comparison between two treatments. ** $p < 0.01$.

149x119mm (600 x 600 DPI)



For the P62 experiments analyzed by Western blotting, INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment with 0, 30 or 60μM aspalathin in the presence of normal glucose conditions and total protein fraction was isolated. (A) Three Western blots are representative of four independent experiments (n=4). Entire blots of these abbreviated pieces can be found in Figure S3, Supporting Information. (B) The histogram represents the percentage of increase of P62 and (C) HMW P62 aggregates normalized with β-actin. Values are mean ± SEM from four independent experiments (n=4). Ordinary one-way ANOVA with Tukey's post-hoc test, mean ± SEM. * above bars indicate comparison versus control. Lines indicate comparison between two treatments. * $p < 0.05$ and ** $p < 0.01$.

149x119mm (600 x 600 DPI)



Proposed working mechanism for aspalathin-induced expression of antioxidant enzymes. Aspalathin is proposed to interact with cysteine residues in KEAP1 and P62 proteins. In the case of KEAP1, this liberates NRF2 which then can accumulate in the nucleus to activate transcription of ARE target genes like Hmox1, Nqo1 and Sod1. In the case of P62, this induces the formation of HMW P62 aggregates.

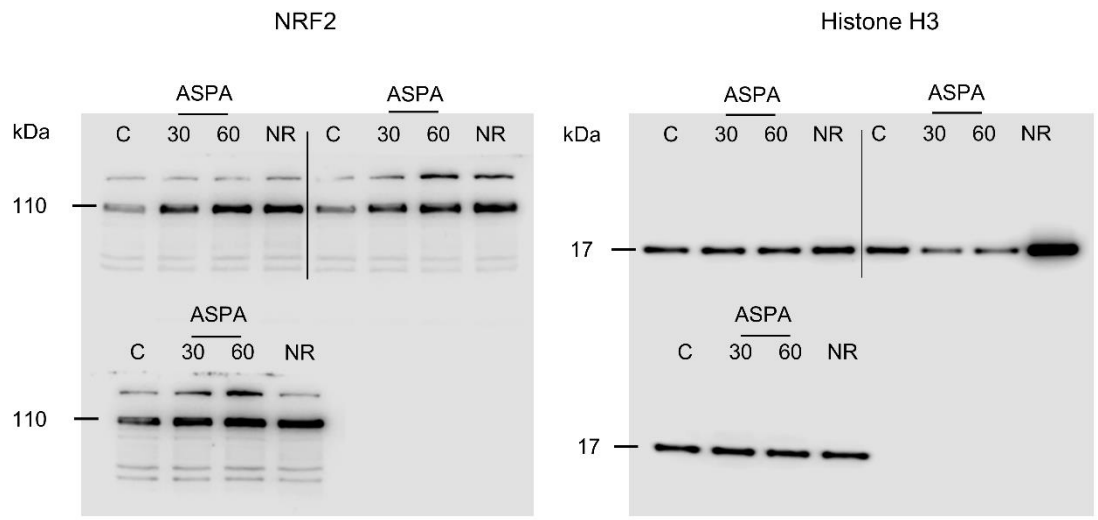
254x190mm (600 x 600 DPI)

Supporting Information Table S1. Sequences of primers for qRT-PCR.

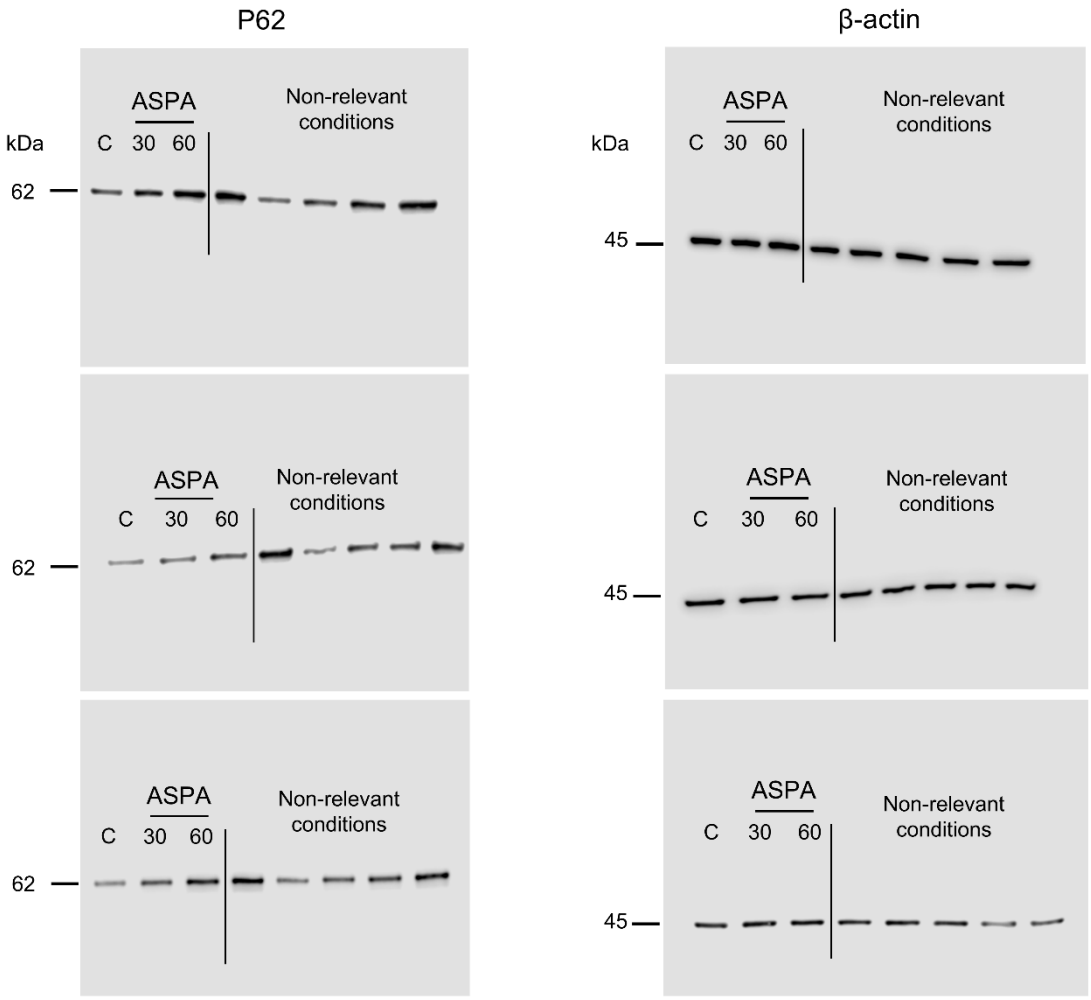
Primers for INS1E β cells qRT-PCR		
	Forward	**Reverse**
β-actin	CGCGAGTACAACCTTCTTGC	CGTCATCCATGGCGAACTGG
Hmox1 (Ho1)	GTCCCAGGATTTGTCCGAGG	GGAGGCCATCACCAGCTTAAA
Nqo-1	CAGAAACGACATCACAGGGGA	AGCACTCTCTCAAACCAGCC
Sod1	AATGTGTCCATTGAAGATCG	CACATAGGGAATGTTTATTGGG

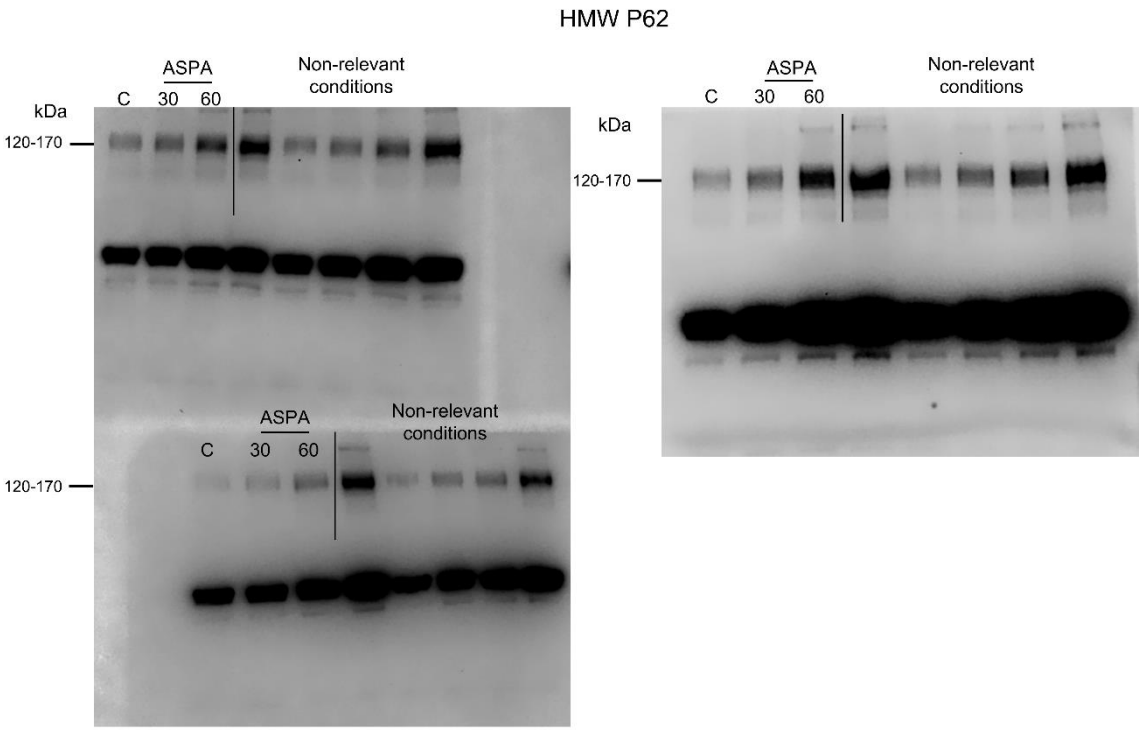
Primers for primary rat islets qRT-PCR		
	Forward	**Reverse**
***Ddit3* (*Chop*)**	GTCACAAGCACCTCCCAAAG	CCACTCTGTTTCCGTTTCCTAG
***Txnip* (*Vdup1*)**	GTGTGAAGTTACCCGAGTCAAAG	CACCATCTCGTTCTCACCTGTA
***Hspa5* (*Bip*)**	TCAGCCCACCGTAACAATCA	AAGGTGACTTCAATCTGGGGTA
***Hmox1* (*Ho1*)**	ACAGCATGTCCCAGGATTTGT	AAGGAGGCCATCACCAGCTT
***Cyca* (*Ppia*)**	AACCCCACCGTGTTCTTC	TGCCTTCTTTCACCTTCCC

Aliases of gene symbols given in parentheses



Supporting Information Figure S2. Entire Western blots of the abbreviated pieces shown in Figure 6A for NRF2 (110kDa) and Histone H3 (17kDa). INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment with 0, 30 or 60µM aspalathin in the presence of normal glucose conditions and the nuclear protein fraction was isolated. NR = non-relevant conditions, not relevant for this study.


P62
β-actin
ASPA
Non-relevant conditions
C 30 60
kDa
62
45


HMW P62
ASPA
Non-relevant conditions
C 30 60
kDa
120-170

Supporting Information Figure S3. Entire Western blots of the abbreviated pieces shown in Figure 7A for P62 (62kDa), HMW P62 (120-170kDa) and β-actin (45kDa). INS1E β cells were cultivated in 6-well plates. Cells received 24h treatment with 0, 30 or 60µM aspalathin in the presence of normal glucose conditions and total protein fraction was isolated. Non-relevant conditions = not relevant for this study.