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Antibiotics induce mitonuclear protein imbalance but fail to inhibit respiration and nutrient activation in pancreatic β-cells

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ABSTRACT

Chloramphenicol and several other antibiotics targeting bacterial ribosomes inhibit mitochondrial protein translation. Inhibition of mitochondrial protein synthesis leads to mitonuclear protein imbalance and reduced respiratory rates as confirmed here in HeLa and PC12 cells. Unexpectedly, respiration in INS-1E insulinoma cells and primary human islets was unaltered in the presence of chloramphenicol. Resting respiratory rates and glucose stimulated acceleration of respiration were also not lowered when a range of antibiotics including, thiamphenicol, streptomycin, gentamycin and doxycycline known to interfere with bacterial protein synthesis were tested. However, chloramphenicol efficiently reduced mitochondrial protein synthesis in INS-1E cells, lowering expression of the mtDNA encoded COX1 subunit of the respiratory chain but not the nuclear encoded ATP-synthase subunit ATP5A. Despite a marked reduction of the essential respiratory chain subunit COX1, normal respiratory rates were maintained in INS-1E cells. ATP-synthase dependent respiration was even elevated in chloramphenicol treated INS-1E cells. Consistent with these findings, glucose-dependent calcium signaling reflecting metabolism-secretion coupling in beta-cells, was augmented. We conclude that antibiotics targeting mitochondria are able to cause mitonuclear protein imbalance in insulin secreting cells. We hypothesize that in contrast to other cell types, compensatory mechanisms are sufficiently strong to maintain normal respiratory rates and surprisingly even result in augmented ATP-synthase dependent respiration and calcium signaling following glucose stimulation. The result suggests that in insulin secreting cells only lowering COX1 below a threshold level may result in a measurable impairment of respiration. When focusing on mitochondrial function, care should be taken when including antibiotics targeting translation for long-term cell culture as depending on the sensitivity of the cell type analyzed, respiration, mitonuclear protein imbalance or down-stream signaling may be altered.

1. Introduction

Mitochondria carry their own small genome encoding the mitochondrial 16S and 12S rRNA, 22 tRNAs and 13 proteins of the respiratory chain [24,4]. The large majority of the more than 1200 mitochondrial proteins are therefore transcribed in the nucleus and the proteins imported into the organelle [18]. The respiratory chain complexes are composed of a total of about 90 proteins. The 13 proteins expressed inside mitochondria are essential subunits of complex I, III, IV and V [24,4]. For example complex IV is built from 13 proteins 3 of which COX1, COX2 and COX3 are mtDNA encoded [18,27]. COX1 and COX2 form the catalytic core of complex IV directly involved in the reduction of oxygen the final electron acceptor of the respiratory chain [27].

In order to express the small set of mitochondrial proteins, the organelle has maintained the molecular machineries required for DNA replication, transcription and protein translation [18]. To form the mitochondrial ribosome alone, 78 ribosomal proteins must be imported from the cytosol into the mitochondrial matrix [20]. The mitochondrial ribosome (55S) is structurally quite distinct from eukaryotic cytoplasmic (80S) or bacterial (70S) ribosomes [20,26]. For instance, human mitochondrial ribosomes lack several RNA stem structures of bacterial

Abbreviations: AA, antimycin A; DMSO, dimethyl sulfoxide; FCS, fetal calf serum (heat-inactivated); INS-1E, rat insulinoma cell line clone 1E; KRBH, Krebs-Ringer bicarbonate HEPES buffer; OCR, oxygen consumption rate; Rot, rotenone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; YC3.6, fluorescent protein yellow cameleon version 3.6

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rRNA but rely on a larger number of ribosomal proteins to form the translation machinery. These differences are surprising as mitochondria are thought to be derived from alpha-proteobacteria [1,26] and their ribosomes are sensitive to several classes of antibiotics targeting the bacterial ribosome at different steps during the peptide elongation cycle [10,12,14,17,2,21,31]. Mitochondrial and bacterial ribosomes may be sensitive to the same molecular entities as the general molecular mechanisms of protein biosynthesis are shared and therefore key nucleotides in the rRNA and core amino acid sequences of ribosomal proteins are conserved [20,3,31,9].

Tetracyclines such as doxycycline for example bind to the 30S bacterial ribosomal subunit and prevent accommodation of amino acid-tRNAs to the acceptor site on the bacterial ribosome thereby blocking peptide elongation [5]. Tetracycline also slow mitochondrial protein synthesis dramatically, lowering the expression of mitochondrial but not nuclear encoded proteins [10,12,15,17]. This inhibition of mitochondrial translation lowers respiratory rates and slows proliferation in mammalian cells [12,19]. Reduced expression of mtDNA encoded proteins with normal unchanged expression of nuclear encoded respiratory chain complex subunits has been termed mitonuclear protein imbalance [10]. The stoichiometric imbalance between mitochondrial and nuclear encoded respiratory chain subunits leads to the mitochondrial unfolded protein response [13]. This processes affects cell function beyond respiration. An impressive example is the extension of lifespan in C. elegans, which is observed when the mitochondrial unfolded protein response is induced during development [10].

Chloramphenicol uses a distinct mechanism blocking the nascent peptide chain by interfering with peptide bond formation on the bacterial ribosome [31]. Chloramphenicol also specifically lowers mitochondrial translation and reduces respiratory rates without affecting the expression of nuclear encoded imported mitochondrial proteins [10,17,21].

Streptomycin and gentamicin are aminoglycosides members of another commonly used class of antibiotics. They bind near the acceptor site on ribosomes, where they cause codon misreading and inhibition of tRNA mRNA complex translocation [16,9]. The gentamicin binding site on the rRNA is homologous between bacteria and mitochondria. This region on the mitochondrial rRNA is susceptible to gentamicin albeit at higher concentrations of the antibiotic [9]. As a consequence gentamycin is able to lower mitochondrial translation and proliferation in mammalian cells [12,2].

Given their effect on mitochondrial translation antibiotics targeting the ribosome can have side-effects in human. One such well-described side-effect is a measurable sign of hearing loss in 20% of patients treated with aminoglycosides [11,9]. A link with mitochondria has been firmly established as specific mitochondrial rRNA mutations in human have been shown to dramatically increase the sensitivity to aminoglycosides [9].

Another cell type strongly dependent on mitochondria is the pancreatic β-cell [30]. This is illustrated for example by maternally inherited diabetes and deafness caused by a heteroplasmic mtDNA mutation in the gene encoding the leucine tRNA [28]. The diabetes phenotypes in these patients is due to a progressive loss of β-cell function and viability.

The main function of the pancreatic β-cell is to secrete the blood glucose lowering hormone insulin [22]. For proper regulation of hormone secretion, the β-cell senses a large number of signals reflecting the nutritional status. Mitochondria are essential in β-cells, linking nutrient sensing and metabolism to insulin granule exocytosis [30]. Mitochondria are required for oxidative metabolism of glucose and amino acids and are generating metabolic signals triggering and amplifying insulin exocytosis. Mitochondrially generated ATP is the main signal of the triggering pathway as it induces closure of plasma membrane K_{ATP} channels. The associated depolarization of the plasmamembrane opens voltage-dependent calcium channels leading to Ca^{2+} rises that trigger insulin granule exocytosis [22,29,6]. Based on this strong reliance of β-cells on mitochondria for energy homeostasis and signal generation for insulin secretion, we speculated that antibiotics by impairing mitochondrial translation of respiratory chain complex subunits should reduce mitochondrial activation of respiration by nutrients also blocking metabolism-secretion coupling. Here we study antibiotics targeting mitochondrial protein synthesis and their ability to affect respiration in INS-1E cells and primary β-cells in the context of the human islet.

2. Materials and methods

2.1. Reagents

Chemicals were from Sigma-Aldrich (Switzerland), Invitrogen (Switzerland), Thermo Fisher Scientific (Waltham, MA, USA), VWR (Switzerland) or Tocris (Switzerland) unless otherwise indicated. The $YC3.6_{cyto}$ pcDNA3 construct (22) was kindly provided by Prof. A. Miyawaki (Riken Brain Science Institute, Wako, Japan).

2.2. INS-1E cell culture

INS-1E cells were obtained from Prof. C. Wollheim and Prof. P. Maechler (University of Geneva). INS-1E cells were cultured at 37 °C in a humidified atmosphere (5% CO_2) in RPMI-1640 medium (GIBCO #21875-034) containing 11 mM glucose, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Brunschwig AG, Switzerland), 10 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol. For regular culture of INS-1E cells the antibiotics penicillin (50 μg/ml) and streptomycin (100 μg/ml) were included. To study the effects of antibiotics on the expression of mtDNA encoded proteins or respiration of INS-1E cells the antibiotics were removed 2 days before initiation of the experiments.

2.3. HeLa cell culture

HeLa cells are from the European Collection of Authenticated Cell Cultures and cultured at 37 °C in a humidified atmosphere (5% CO2) in DMEM medium containing 25 mM glucose, L-glutamine without pyruvate (GIBCO #41966-029). The medium was complemented with 10% (v/v) FCS (Chemie Brunschwig, Switzerland). Depending on the conditions used penicillin (50 μg/ml) and streptomycin (100 μg/ml) were added to the medium.

2.4. PC12 cell culture

PC12 rat pheocromocytoma cells were obtained from the European Collection of Authenticated Cell Cultures and cultured at 37 °C in a humidified atmosphere (5% CO_2) in RPMI-1640 medium (GIBCO #21875-034) containing 11 mM glucose and supplemented with 7.5% fetal bovine serum, 7.5% horse serum, penicillin (50 μg/ml) and streptomycin (100 μg/ml).

2.5. Human islet

Human islets were from non-diabetic deceased donors were purchased from Tebu-bio (Le Perray-en-Yvelines, France). Donors had consented to donate organs for medical research and the local independent ethics committee of the Canton of Vaud (Switzerland) had approved the use of these human samples. Islets were cultured at 37 °C in a humidified atmosphere (5% CO_2) in CMRL 1066 medium 5.6 mM glucose without glutamine (GIBCO #21530027). The medium was supplemented with 10% (v/v) heat-inactivated FBS (Chemie Brunschwig AG, Switzerland) and 2 mM L-glutamine. For human islet culture the antibiotics penicillin (50 μg/ml) and streptomycin 100 μg/ml were included in the medium. Upon receipt the islets were allowed to recover for 24 h in complete CMRL 1066 medium before plating them on 804G matrix coated plastic wells (Seahorse XF96 V3 PS Cell

Culture Microplates #101085-004). The islets were then kept in complete CMRL 1066 for 48 h before initiation of the respiration experiments.

To generate the matrix for human islets, 804G cells were cultured in 75 cm^2 bottles containing DMEM (GIBCO #11966-025) with L-Glutamine, supplemented with 10% FBS, 5.6 mM glucose, 110 μg/ml sodium pyruvate, penicillin (50 μg/ml) and streptomycin (100 μg/ml). When reaching confluency the cells were trypsinized and divided into six 175 cm^2 flasks and grown until they were 50% confluent. The medium was then replaced with 50 ml fresh medium without FBS and further incubated for 72 h. The medium was then collected and centrifuged at 1000 rpm for 5 min. The cleared supernatants were frozen at −20 °C and stored until use.

2.6. Oxygen consumption measurements

Oxygen consumption was measured using a XF96 instrument (Seahorse Biosciences, MA, USA). INS-1E cells were seeded into Seahorse tissue culture plates (Seahorse XF96 V3 PS Cell Culture Microplates #101085-004) at a density of 40000 cells per well. Similar experiments were performed with HeLa (plated at 40000 cells per well) or PC12 cells (40000 cells per well). One day after plating the cells antibiotics were removed from the medium. Two days before the experiment, chloramphenicol (CA) or other antibiotics as specified in the figures were added at increasing concentrations to the medium. During this treatment, control cells were cultured under identical conditions without antibiotics. For respiration analysis, the cells were washed twice and incubated in basal KRBH buffer containing 2.5 mM glucose, 140 mm NaCl, 3.6 mm KCl, 0.5 mm NaH_2PO_4, 0.5 mm $MgSO_4$, 1.5 mm $CaCl_2$, 10 mm HEPES, and 5 mm $NaHCO_3$ (pH 7.4). The cells were kept for 30 min at 37 °C inside the Seahorse instrument. Respiration rates were determined every 6 min at 37 °C. Following each addition the instrument 3 min of mixing were followed by 3 min of oxygen consumption measurements. 5 measurements were performed for each condition. Respiratory chain inhibitors were added as indicated in the figures at the following concentrations: oligomycin (2.5 μg/ml) or rotenone (1 μM) plus antimycin A (1 μg/ml).

Human islets were cultured and plated as described in Section 2.5. Per 96-well 50 human islets were seeded. The islets were kept in the continuous presence of penicillin (50 μg/ml) and streptomycin 100 μg/ml. In addition, the islets were treated for 48 h without (control) or with chloramphenicol (20 μg/ml). The wells at the rim of the 96-well plates were not used for these measurements to avoid edge effects. The outer wells on the 96-well plates were prone to show different respiration rates compared to the neighbouring wells more centrally localized. KRBH was the same as for the cell lines except that 1 mM glucose was used as the basal buffer before stimulation with a final concentration of 16.7 mM glucose.

2.7. Western-blots

Protein analysis was performed on cells grown initially to 50% confluency. At this point, the medium was switched to the corresponding medium lacking standard antibiotics. Unless indicated otherwise the cells were cultured 2 more days before protein extraction. During this time, the cells were cultured in the presence or absence of test antibiotics. Information on the duration of the chloramphenicol treatments, the concentrations used and alternative antibiotics tested can be found in the figure legends. Whole cells were lysed for 15 min on ice in RIPA buffer complemented with cOmplete (Mini, EDTA-free Protease Inhibitor Cocktail; Roche 11836170001 provided by Sigma-Aldrich (Switzerland)). The lysate was centrifuged at 11000×*g* for 5 min at 4 °C, and the protein content of the supernatant was determined using the Pierce® BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The protein lysate (20 μg protein/lane) was loaded on Mini-PROTEAN TGX Precast Gels 4–15% (Bio-Rad 4561083, 4561085, 4561093, 4561095) SDS-PAGE gels. The Invitrogen iBlot 7-Minute Blotting System (Invitrogen; Switzerland) was used to transfer the proteins to nitrocellulose membranes (Invitrogen iBlot® Transfer Stack, nitrocellulose, regular size #IB301001). Uniform protein transfer was assessed with Ponceau Red (Sigma-Aldrich; Switzerland) staining. Tris buffered saline containing Tween20 (0.1%) and milk powder (5% w/v) was used as blocking and antibody solution. Antibodies recognizing COXI (abcam ab14715) ATP5A (abcam ab14748) and Hsp60 (Cell Signaling 12165) were used at 1/1000. Detection of TOM20 (Santa Cruz Biotechnology sc-11415) was performed as a loading control. As secondary antibodies, horseradish peroxidase–conjugated antibodies were used. Western bands were revealed using chemiluminescence detection solutions (Amersham Biosciences, Pittsburgh, PA, USA). Luminescence images were taken on a Fusion FX Vilber Lourmat instrument.

2.8. Single cell Ca^{2+} imaging

Cytosolic Ca^{2+} was measured with the genetically encoded sensor $YC3.6_{cyto}$. INS-1E cells were plated on polyornithin-coated 35-mm-diameter glass bottom dishes (MatTek, MA, USA) and transfected with a pcDNA3 vector carrying the $YC3.6_{cyto}$ insert expressed under the CMV promoter. Transfection of INS-1E cells was achieved using PolyPlus (Polyplus-Transfection SA, Illkirch-Graffenstaden, France) using the Jetprime transfection reagent according to the manufacturer's protocol. The medium was changed after 4 h and the cells were cultured for 48 h in the presence of chloramphenicol or without antibiotics in the medium. Two days after transfection cells were washed in KRBH. The glass bottom dishes were inserted in a thermostatic chamber (Life Imaging Services, Switzerland) and maintained at 37 °C on the microscope. Cells were imaged on a DMI6000 B inverted fluorescence microscope, using a HCX PL APO 63x/1.40-0.60 NA oil immersion objective (Leica Microsystems, Germany) and an Evolve 512 back illuminated CCD with 16×16 pixels camera (Photometrics, AZ, USA). Cells were excited at 430 nm through a BP436/20 filter. The two emission images were acquired with BP480/40 and BP535/30 emission filters. Fluorescence ratios were calculated in MetaFluor 7.0 (Meta Imaging Series, Molecular Devices, CA, USA) and analyzed in Excel (Microsoft, WA, USA) and GraphPad Prism 5 (GraphPad, Software, CA, USA). Images were taken every 2 s.

2.9. Statistical analysis

Values are given as mean ± standard error of the mean (s.e.m.). N is the number of independent experiments. n is the number of replicates in an individual experiment. *p*-values were obtained by Student's *t*-test.

3. Results

3.1. Inhibition of mitochondrial translation by chloramphenicol impairs HeLa cell respiratory function

Impaired mitochondrial translation slows expression of the 13 proteins encoded by mtDNA with a consequential loss of respiratory chain complex function [10,21]. Like several other antibiotics affecting bacterial protein biosynthesis, chloramphenicol inhibits mitochondrial translation [17]. Consistent with these earlier findings, chloramphenicol added to the culture medium of HeLa (human epithelial carcinoma) cells caused a pronounced reduction of respiration (Fig. 1A). After 48 h in the presence of chloramphenicol (20 μg/ml) respiratory rates were reduced by more than 2-fold. Strongest inhibition was observed at 100 μg/ml chloramphenicol (Fig. 1B). Consistent with its effect on mitochondrial translation, chloramphenicol (20 μg/ml) lowered COX1 expression in HeLa cells (Fig. 1C). COX1 is a core catalytic subunit of the cytochrome c oxidase complex of the respiratory chain and is encoded by the mtDNA.

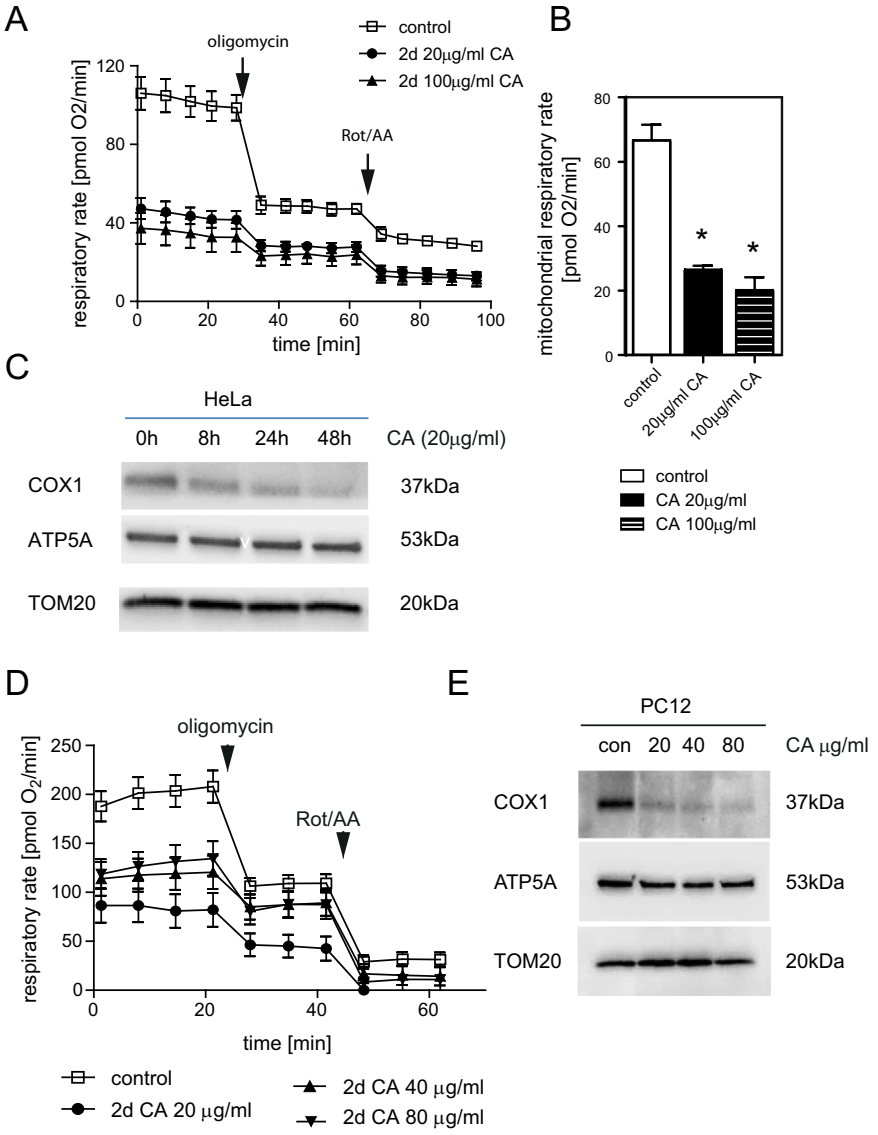

Fig. 1. The mitochondrial translation inhibitor chloramphenicol impairs respiratory chain function in HeLa and PC12 cells. HeLa cells were cultured for 2 days in DMEM 25 mM glucose containing standard antibiotics (streptomycin 100 μg/ml) penicillin (20 μg/ml) plus varying concentrations of chloramphenicol. (A) Respiratory rates were measured over time in KRBH medium (2.5 mM glucose). Respiratory rates were measured every 6 min in HeLa cells cultured previously under control conditions (empty squares), the presence of chloramphenicol (20 μg/ml; black circles) or a high concentration of chloramphenicol (100 μg/ml; black triangles). The respiratory chain inhibitors oligomycin (2.5 μg/ml) and rotenone (1 μM) plus antimycin A (1 μg/ml) were added as indicated (arrows). Shown is a single experiment performed in sextuplicate. The experiment was performed twice. (B) Quantification of mitochondrial respiratory rates (basal respiration before oligomycin addition minus non-mitochondrial respiration after rotenone plus antimycin A). Shown is mean +/-s.e.m. (n=6); *p < 0.001. (C) Analysis of protein lysates (20 μg/lane) obtained from HeLa cells maintained in DMEM culture medium containing chloramphenicol (20 μg/ml) for increasing amounts of time (0–48 h). COX1 (upper panel), ATP5A (middle panel) and TOM20 (lower panel) were detected using specific antibodies. Shown is one of two independent experiments. (D) PC12 cells were cultured for 2 days in RPMI-1640 11 mM glucose in the absence of antibiotics (control; empty squares) or increasing concentrations of chloramphenicol (20 μg/ml; filled circles/40 μg/ml; filled triangles/80 μg/ml; inverted filled triangles). Respiratory rates were determined every 6 min in KRBH (2.5 mM glucose) medium. Oligomycin (2.5 μg/ml) was added to inhibit ATP-synthase dependent respiration. Rotenone (1 μM) plus antimycin A (1 μg/ml) were added to block mitochondrial respiration. Data is the average of 16 measurements +/-s.e.m. Shown is one of two independent experiments showing qualitatively similar results. (E) Protein bands from PC12 protein lysates (20 μg/lane) were detected using antibodies recognizing COX1 (upper panel), ATP-synthase 5A (ATP5A; middle panel) and TOM20 (lower panel). Cells were cultures for 48 h in the absence (con) or increasing concentrations of chloramphenicol as indicated. Qualitatively similar results were obtained in 2 independent experiments.

As such this protein is translated inside mitochondria and its activity is directly linked to oxygen consumption. Reduced COX1 levels were already observed after 8 h of chloramphenicol in the culture medium. The COX1 protein continued to decrease thereafter and was only weakly expressed at 48 h. This effect of chloramphenicol was specific to mtDNA encoded respiratory chain subunits. Expression of ATP-synthase subunit α a subunit of complex V of the respiratory chain encoded in the nucleus was unaffected by chloramphenicol treatment over the same time course (Fig. 1C). TOM20 a protein of the mitochondrial protein import machinery was also unaltered by antibiotic treatment. The results demonstrate that chloramphenicol specifically lowers the expression of mitochondrial encoded proteins without reducing mitochondrial number or expression of nuclear encoded proteins of the respiratory chain. As the respiratory chain is assembled from stoichiometric protein complexes with both mitochondrial and nuclear encoded subunits, specific inhibition of mitochondrial translation leads to mitonuclear protein imbalance [10].

3.2. Respiration in PC12 cells is sensitive to the mitochondrial translation inhibitor chloramphenicol

Similar results were obtained in PC12 (rat adrenal phaeochromocytoma) cells. Culture for 48 h in the presence of 20 μg/ml chloram-

phenicol caused a marked reduction in the respiratory rates compared to control PC12 cells grown in medium without antibiotics (Fig. 1D). Increasing the chloramphenicol concentration to 40 and 80 μg/ml did not further reduce respiration. COX1 expression was strongly reduced in PC12 cells after 48 h treatment with chloramphenicol (Fig. 1E). Expression of the nuclear encoded mitochondrial proteins ATP-synthase subunit α and TOM20 were not changed by chloramphenicol demonstrating specific inhibition of mitochondrial encoded proteins also in PC12 cells.

3.3. Chloramphenicol does not reduce respiration in INS-1E insulin secreting cells

INS-1E (rat insulinoma) cells, like primary pancreatic β-cells, strongly depend on mitochondrial function to link glucose metabolism to the stimulation of insulin granule exocytosis [30]. Respiratory rates in INS-1E cells are enhanced in response to stimulatory glucose concentrations [6]. Surprisingly, basal respiratory rates and glucose-induced respiration were not significantly different between control INS-1E cells or when grown for 2 days in the presence of 20 μg/ml chloramphenicol (Fig. 2A). Standard culture medium for INS-1E cells contains the antibiotics penicillin (50 μg/ml) and streptomycin (100 μg/ml). These antibiotics were not added to the medium during these experiments to specifically study the effects of chloramphenicol. We were unable to inhibit respiration in INS-1E cells when using chloramphenicol even after increasing the concentration to 40 and 80 μg/ml (Fig. 2A). Mitochondrial respiration at basal 2.5 mM glucose (Fig. 2B) and glucose-induced (16.7 mM) respiration (Fig. 2C) were not significantly altered when treating the INS-1E cells for 48 h with 20–80 μg/ml chloramphenicol. Further elevating antibiotic concentrations to 160 and 320 μg/ml caused cell death in a large fraction of the cells. Cells treated with such high concentrations were not further studied. INS-1E cells were also assessed for cell growth determining total protein content after 48 h culture in the presence of increasing chloramphenicol concentrations. A small but significant reduction of protein content was measured in lysates of INS-1E cells grown in chloramphenicol (Fig. 2D). This decrease suggests the antibiotic slightly reduces cell proliferation or induces some cell death.

Respiratory rates in insulin secreting cells measured under basal and glucose stimulated conditions may be below the maximal respiratory capacity. This would explain why respiratory rates in INS-1E can be maintained even when antibiotics partially inhibit translation of mtDNA. To study close to maximal respiration we uncoupled mitochondria using FCCP (1 μM) in the presence of the ATP-synthase blocker oligomycin. The uncoupler caused an immediate transient acceleration of respiration, which was indistinguishable between control and chloramphenicol treated INS-1E cells (Fig. 2E). Increasing the concentrations of FCCP to 2 or 5 μM did not further enhance respiratory rates whether or not the antibiotic was added to the culture medium (data not shown). We conclude that unlike HeLa, PC12 (Fig. 1) and other cell types described in the literature [12,21], respiration in INS-1E cells (Fig. 2) is unusually resistant to the mitochondrial translation inhibitor chloramphenicol.

3.4. Chloramphenicol does neither lower basal respiration nor the respiratory response to glucose in human primary β-cells

The observed maintained respiration after treatment with chloramphenicol may be a peculiarity of the INS-1E cells. We therefore also tested the impact of chloramphenicol on human islets. Human islets are composed of about 50% pancreatic β-cells. Other endocrine cells types constitute most of the remaining islet mass and therefore contribute to basal respiration of human islets. The glucose-induced respiratory response should be mainly due to β-cells given the responsiveness of β-cell mitochondria to a raise in glucose [25,6].

Following culture of human islets in medium containing chloramphenicol (20 μg/ml) neither basal (1 mM glucose) respiration nor the glucose-induced (16.7 mM) elevation of respiration were changed compared to control islets (Fig. 2F). We conclude that respiration of primary pancreatic β-cells and possibly other main cell types in the human islet micro-organ are largely insensitive to the antibiotic chloramphenicol.

3.5. Respiration in INS-1E cells is insensitive to several antibiotics targeting mitochondrial protein synthesis

The relative insensitivity of INS-1E cells may be specific to chloramaphenicol. We therefore also studied several other antibiotics. Thiamphenicol is a structural analogue of chloramaphenicol [33]. Thiamphenicol is an inhibitor of ribosomal function and inhibits mitochondrial translation with a potency similar to chloramphenicol [33,7]. Over a concentration range of thiamphenicol (mol. weight: 356.2 g/mol) similar to the concentrations tested for chloramphenicol (mol. weight: 323.1 g/mol) INS-1E cell respiration was not reduced (Fig. 3A). The modest increase of respiratory function observed at higher concentrations of thiamphenicol tested was not significant.

For regular culture, INS-1E cells are maintained in standard medium containing the two antibiotics penicillin (50 μg/ml) and streptomycin (100 μg/ml). Penicillin disrupts the bacterial cell wall and a member of this family of antibiotics was shown to have no impact on the translation of mtDNA encoded proteins or respiration in several mammalian cell types tested [19]. On the other hand, aminoglycosides such as streptomycin or gentamicin are known to act through the inhibition of ribosomes and can cause toxic effects in humans likely through their inhibition of the mitochondrial ribosome [11,9]. Here we tested whether the antibiotics included in the INS-1E culture medium to prevent bacterial growth had any effect on respiration. Culture of INS-1E cells for 2 days in the presence or absence of penicillin and streptomycin had no significant effect on basal or glucose induced respiratory rates (Fig. 3B).

Another relevant antibiotic is gentamicin. It is used as an antibiotic in culture medium of human islets at 0.05 mg/ml [32,6]. This concentration of gentamicin as well as concentrations up to 2 mg/ml (40× higher) had no effect on basal or glucose-induced respiration in INS-1E cells (Fig. 3C). For comparison, oxidative metabolism in cochlear outer hair cells was rapidly slowed by gentamicin when used at a concentration of 0.3 mg/ml [11]. Further elevating the concentration of gentamicin to 4 or 8 mg/ml caused progressively cell death and therefore partial respectively complete loss of respiration in INS-1E cells (Fig. 3D).

Respiratory rates in INS-1E insulin secreting cells were also unaffected by doxycycline when tested at concentrations ranging from 1 to 10 μg/ml (Fig. 3E, F). In other cell types doxycycline (1 μg/ml and below) has been shown to have inhibitory effects [12,19]. Chloramphenicol, gentamicin and doxycycline target ribosomes and thereby protein synthesis through different molecular mechanism. We therefore conclude that respiration in INS-1E insulin secreting cells is surprisingly resistant to different antibiotics affecting mitochondrial translation.

3.6. Chloramphenicol inhibits synthesis of the mtDNA encoded respiratory chain subunit COX1 in INS-1E cells

A possible interpretation of the data is that insulin secreting cells continue to respire at normal rates as they may be unusually insensitive to inhibitors of mitochondrial protein synthesis. Therefore, similar to our experiments in HeLa (Fig. 1C) and PC12 (Fig. 1E) cells, we followed COX1 expression as a read-out for mitochondrial protein synthesis. INS-1E cells were cultured for increasing durations in medium containing chloramphenicol (20 μg/ml). At this concentration, which has no effect on respiratory rates, COX1 levels were reduced gradually in the presence of the antibiotic with a notable reduction after

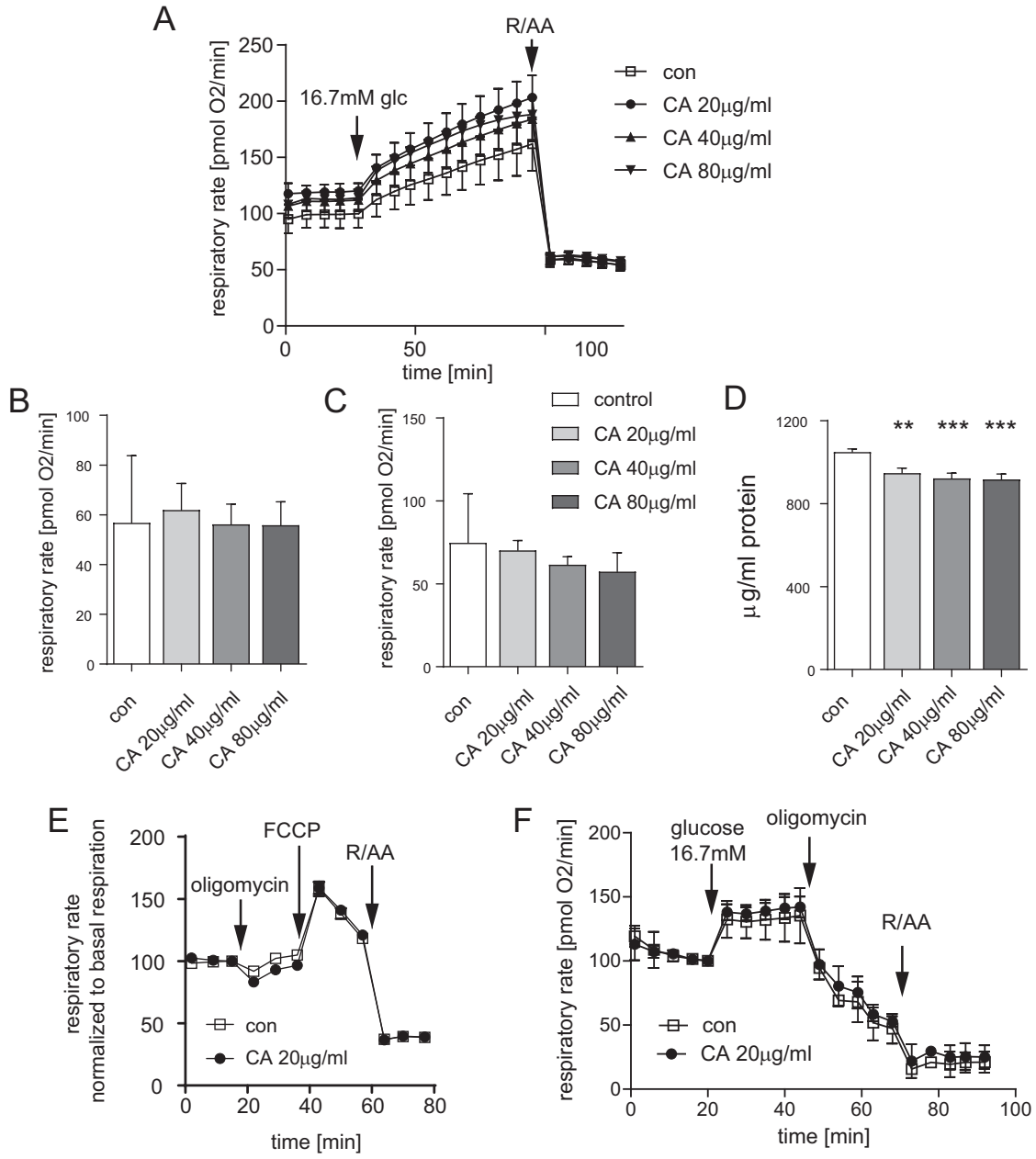

Fig. 2. Respiration in INS-1E cells and human islets is relatively insensitive to the mitochondrial translation inhibitor chloramphenicol. INS-1E cells were cultured for 2 days in RPMI (11 mM glucose) containing varying concentrations of chloramphenicol. For these experiments no streptomycin or penicillin was added to the culture medium. Respiration was measured every 6 min in KRBH medium (2.5 mM glucose). The cells were stimulated with glucose (16.7 mM; arrow) before final inhibition of mitochondrial respiration (second arrow) with rotenone (1 μM) plus antimycin A (1 μg/ml). Control INS-1E cells (empty squares) were directly compared to cells grown in increasing concentrations of chloramphenicol: 20 μg/ml (circles), 40 μg/ml (triangles) or 80 μg/ml (inverted triangles). A representative experiment performed in sextuplicate is shown. Data is the average +/-s.e.m. (B, C) Results from 4 independent experiments as shown in A were analyzed (+/-s.e.m.). Respiratory rates of cells kept under basal (2.5 mM glucose; B) and after glucose stimulation (16.7 mM; C) were quantified. The data is the mean +/- s.e.m. (D) Quantification of protein content in INS-1E cells grown for 48 h in medium containing increasing concentrations of chloramphenicol. INS-1E cells grown in 24-well plates were extracted in 100μl of lysis buffer. A significant reduction of protein content compared to control was observed. $^{**}p < 0.01$, $^{***}p < 0.001$. (E) Uncoupled respiration was analyzed in INS-1E cells grown in control medium (empty squares) and medium containing chloramphenicol (black circles; CA 20 μg/ml). Oligomycin (2.5 μg/ml), FCCP (1 μM) and rotenone (1 μM) plus antimycin A (1 μg/ml) were added (arrows). (F) Human islets were bound to 804 G matrix coated assay plates. The islets were cultured in CMRL medium (5.6 mM glucose) in the presence (CA 20 μg/ml; black circles) or absence (control empty squares) of chloramphenicol. For these experiments 50 μg/ml penicillin and 100 μg/ml streptomycin were added during islet culture. During the measurement, the islets were initially kept in KRBH 1 mM glucose before raising the glucose concentration to 16.7 mM as indicated. Respiration was inhibited stepwise using the ATP-synthase inhibitor oligomycin (2.5 μg/ml) followed by the complex I and III inhibitors rotenone (1 μM) plus antimycin A (1 μg/ml). Qualitatively similar results were obtained with islets from two different donors. Shown are the results mean +/- s.e.m. from one donor performed in triplicate.

24 h and a very marked reduction of COX1 when compared to control after 48 h (Fig. 4A). Expression of the nuclear encoded respiratory chain subunit ATP-synthase subunit α and the mitochondrial import protein TOM20 were not altered by the chloramphenicol treatment (Fig. 4A). INS-1E cells may express a large excess of mitochondrially encoded proteins and as a result maintain normal oxygen consumption even when mitochondrial translation is inhibited by antibiotics. Comparison of protein levels of TOM20 and COX1 in INS-1E and PC12 cells show however that neither the mitochondrial content nor expression of mtDNA encoded proteins is different between the two rat cell lines (Fig. 4B). Furthermore, we directly compared the sensitivity of COX1 expression to chloramphenicol in INS-1E (Fig. 4C, D) and

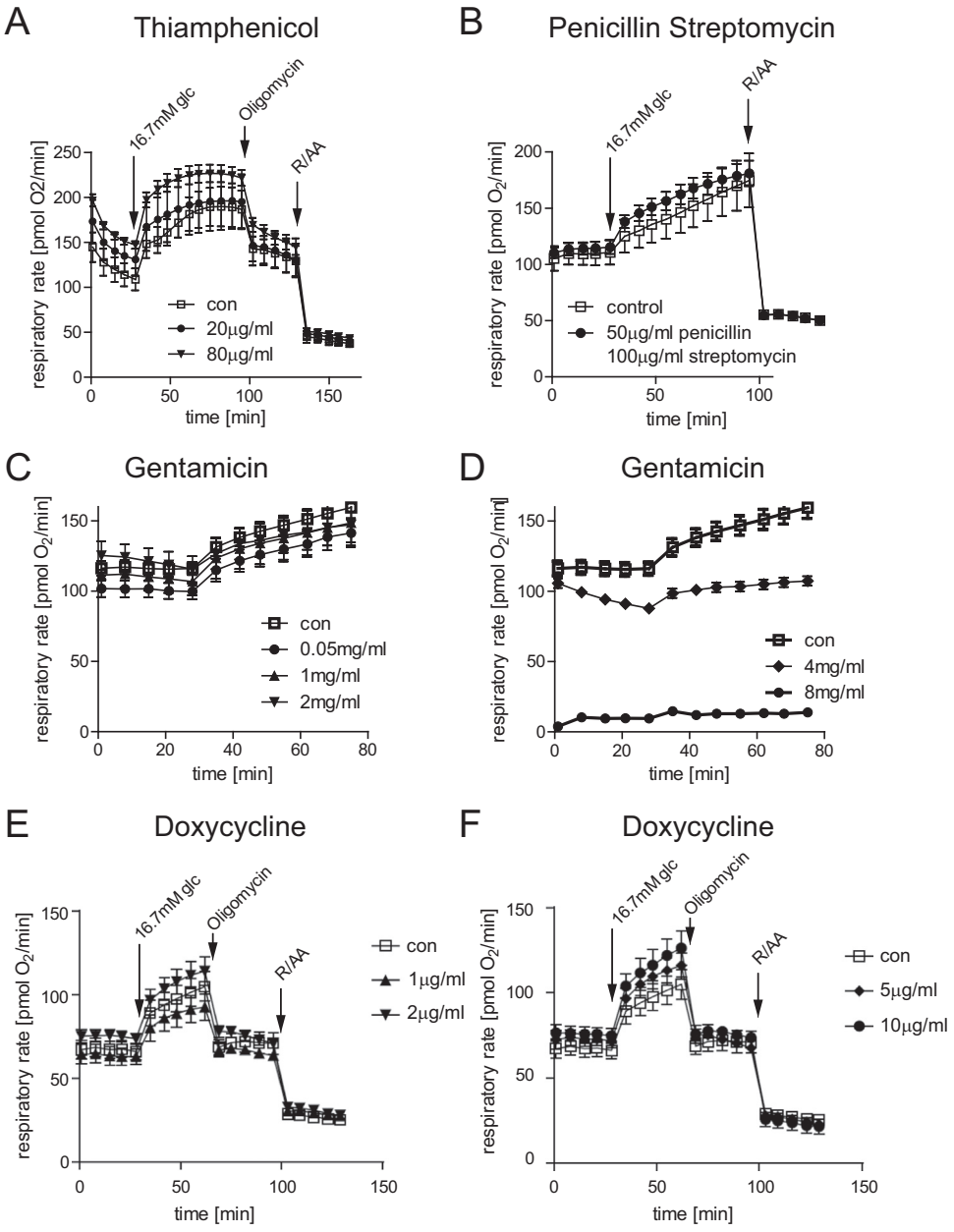

Fig. 3. Failure of several antibiotics targeting mitochondrial translation to lower respiration in INS-1E insulin secreting cells. INS-1E cells were cultures and analyzed as described in Fig. 2. The cells were treated with different antibiotics in INS-1E medium for 2 days prior to the experiment. (A) Thiamphenicol was added at 20 µg/ml (black circles) or 80 µg/ml (inverted triangles) and compared to cells maintained in INS-1E medium (con; empty squares). Shown is the mean+/- s.e.m. from 8 wells. Qualitatively similar results were obtained in 3 independent experiments. (B) INS-1E cells were grown in control INS-medium containing the antibiotics penicillin (50 µg/ml) and streptomycin (100 µg/ml) or control INS-medium lacking antibiotics. Respiratory rates of cells grown in the absence of antibiotics (control; empty squares) or in regular INS-medium with penicillin and streptomycin (black circles) are compared. Mean +/- s.e.m. (n=5) is shown. Similar results were obtained in the two independent experiments performed. (C, D) INS-1E cells were grown in INS-1E medium lacking antibiotics (con; empty squares) or containing increasing concentration of the mitochondrial translation inhibitor gentamycin. (C) Respiratory responses to glucose were studied in cells maintained for 2 days in the absence (empty squares) or presence of gentamicin 0.05 mg/ml (black circles), 1 mg/ml (black triangles), 2 mg/ml (inverted black triangles) or (D) 4 mg/ml (black diamonds) or 8 mg/ml (black circles). For each panel the mean (+/-s.e.m.) of 8 replicates for each experiment and condition are shown. Qualitative similar results were obtained in 2 independent experiments (8 measurements each). (E, F) Effect of doxycycline in the culture medium on INS-1E cell respiration. Respiratory rates in basal and glucose stimulated control cells (empty squares) or medium containing increasing concentrations of doxycycline (E) 1 µg/ml (black triangles), (E) 2 µg/ml (inverted triangles), (F) 5 µg/ml (black diamonds) or (F) 10 µg/ml (black circles) were measured. Results are the average of 5 measurements +/-s.e.m. Respiratory chain inhibitors were use as describes in Fig. 2.

PC12 cells (Fig. E, F). Quantification of Western band intensities shows that the COX1/ATP5A protein ratio in INS-1E cells is lowered at all chloramphenicol concentrations tested (20–80 µg/ml; Fig. 4C, D). COX1 expression was slightly less sensitive to chloramphenicol in PC12 cells (Fig. 4E, F). The results demonstrate that like in other cell types, chloramphenicol is able to inhibit expression of mtDNA encoded proteins in INS-1E cells. Such specific inhibition of mitochondrial protein expression can lead to mitonuclear protein imbalance and an associated mitochondrial unfolded protein response. One of the marker proteins induced by the mitochondrial unfolded protein response is Hsp60. Indeed, induction of Hsp60 was observed in both INS-1E and PC12 cells (Fig. 4C, E) following chloramphenicol treatment. In INS-1E cells like in other cell types, chloramphenicol impairs the balance between mitochondrial and nuclear expression of respiratory chain subunits and as a consequence induces the mitochondrial unfolded protein response.

The resistance of INS-1E cell respiration to antibiotics targeting translation cannot be explained by high initial rates of mitochondrial

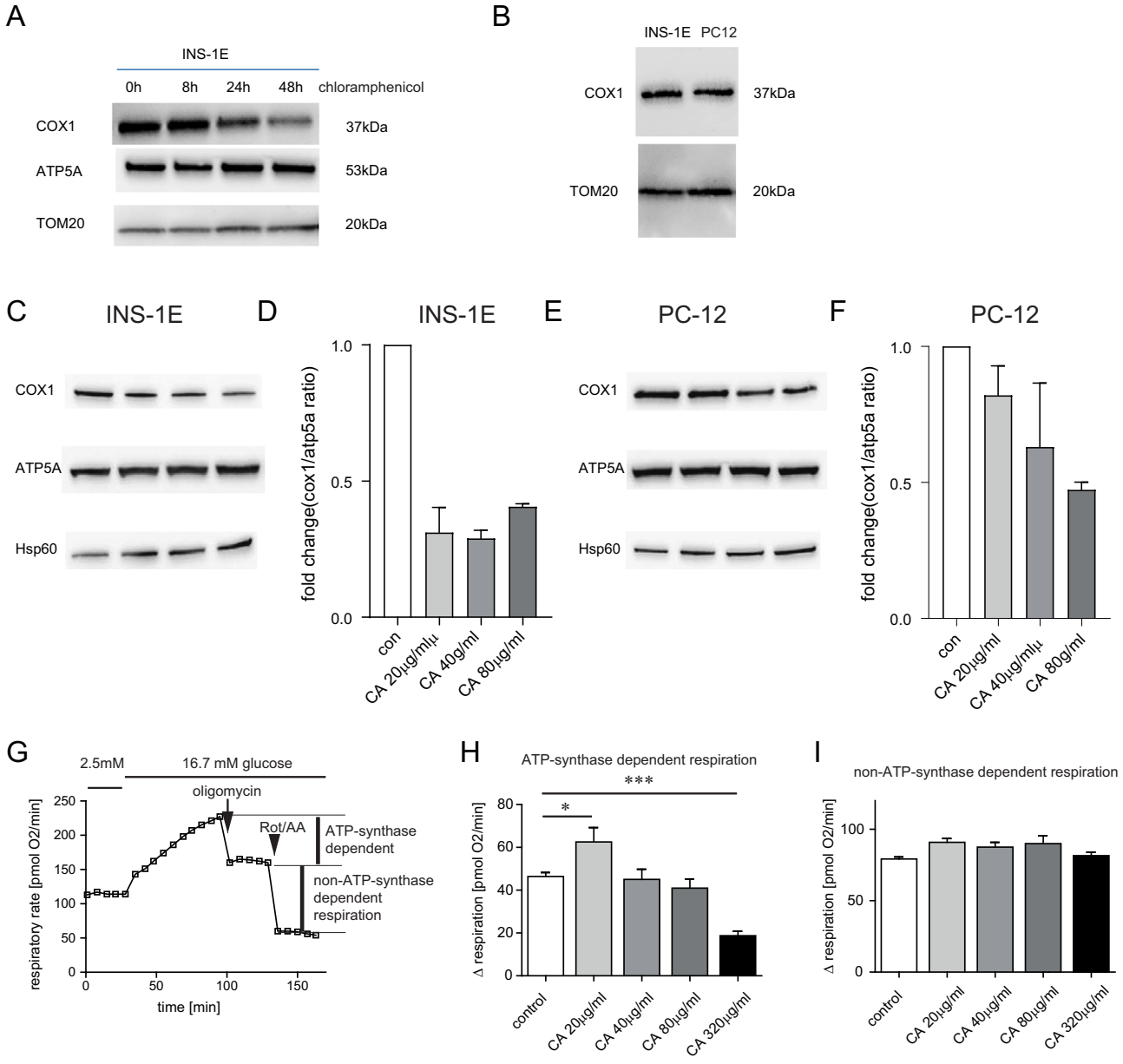

Fig. 4. Chloramphenicol inhibits expression of the mtDNA encoded respiratory chain subunit COX1 without impairing ATP-synthase dependent respiration. (A) INS-1E cells were cultured for increasing amounts of time in medium containing chloramphenicol (20 µg/ml) as indicated in the figure. INS-1E cell lysates (20 µg/lane) were analyzed by Western blotting for the expression of COX1 (upper panel), ATP5A (middle panel) and TOM20 (lower panel). An example of three independent experiments is shown. (B) Comparison of COX1 (upper panel) and TOM20 (lower panel) expressed in the two rat cell lines INS-1E and PC12. Equal amounts of protein were loaded (20 µg/lane). Shown is one of three independent experiments. (C-F) Expression of COX1 (upper panel) was followed in INS-1E (C) and PC12 cells (E) following treatment with chloramphenicol as indicated. For control ATP5A expression was detected (middle panel). Induction of Hsp60 expression in response to the antibiotic (lower panel). (D, F) Expression of COX1 was quantified and divided by the ATP5A signal and expressed as fold change compared to the control condition (no chloramphenicol; white bar). The quantification is the average +/-s.e.m. from 3 independent experiments. (G) INS-1E respiratory rates were studied after glucose stimulation. ATP-synthase dependent respiration and other mitochondrial respiration were calculated as illustrated. Acute reduction of the respiratory rate after oligomycin (2.5 µg/ml) addition was taken as a read-out of ATP-synthase dependent respiration. The remaining mitochondrial respiration was calculated as the difference in respiration caused by rotenone (1 µM) plus antimycin A (1 µg/ml) subsequent to oligomycin inhibition. (H) ATP-synthase dependent respiration and (I) non-ATP-synthase dependent mitochondrial respiration was calculated from 16 measurements from 2 independent experiments. Cells cultured in control INS-medium (white bars) were compared to INS-1E cells grown for 2 days in INS-medium containing increasing concentrations of chloramphenicol (20–320 µg/ml, grey bars). *$p < 0.05$, ***$p < 0.0001$.

translation or a failure of the antibiotic to lower mitochondrial protein expression in insulin secreting cells.

3.7. Mitonuclear protein imbalance does not lower mitochondrial ATP synthesis dependent respiration in INS-1E cells

At the concentration and time point where chloramphenicol causes mitonuclear imbalance, we tested in more depth whether the antibiotic was altering energy metabolism. As a read-out of mitochondrial ATP-synthesis, we followed oligomycin dependent respiration (Fig. 4G, H). Oligomycin is a specific inhibitor of the ATP-synthase and therefore the fraction of respiration inhibited by oligomycin is proportional to mitochondrial ATP synthesis. The remainder mitochondrial respiration can be measured by determining the changes in respiration by subsequent addition of a combination of the complex I and III blockers rotenone and antimycin A (Fig. 1). The two parameters were calculated from experiments such as illustrated in Fig. 4G. Culture for 2 days in medium containing chloramphenicol ranging from 20 to 80 µg/ml did neither lower ATP-synthase dependent respiration nor the fraction of respiration blocked by rotenone plus antimycin A (Figs. 4H, 4I). Only

after 2 days at 320 μg/ml, the highest concentration tested, did we observe a strong reduction of ATP-synthase dependent respiration (Fig. 4H). Interestingly, even under these conditions where ATP-synthase dependent respiration was reduced non-ATP-synthase dependent respiration was preserved (Fig. 4I). Of note, chloramphenicol at 20 μg/ml did not only fail to lower but actually slightly elevated ATP-synthase dependent respiration (Fig. 4H) suggesting that mild mitonuclear protein imbalance may augment glucose-dependent ATP synthesis. Taken together, we conclude that chloramphenicol at concentrations inhibiting mitochondrial translation does not interfere with nutrient-induced ATP production in insulin secreting cells.

3.8. Chloramphenicol does not impair Ca^{2+} signaling associated with metabolism secretion coupling

Mitochondria are essential to link glucose metabolism to plasma membrane electrical activity and cytosolic Ca^{2+} transients resulting in insulin secretion. Cytosolic Ca^{2+} signals are a sensitive read-out of metabolism secretion coupling but also mitochondrial function in insulin secreting cells [6]. To assess whether inhibition of mitochondrial translation by chloramphenicol and the associated mitonuclear protein imbalance affects β-cell function, we expressed the cytosolic Ca^{2+} sensor YC3.6 in INS-1E cells. Raising glucose from 2.5 to 16.7 mM induced a control Ca^{2+} response (Fig. 5A). Ca^{2+} transients were initiated in response to glucose with a delay of 1–2 min and persisted for 15 min in the continued presence of high glucose. After 2 days culture of INS-1E cells in chloramphenicol (20 μg/ml) containing media, the glucose-stimulated Ca^{2+} response was fully preserved (Fig. 5B). Calculation of the area under curve showed that glucose-induced Ca^{2+} signals were in fact elevated compared to the control cells (Fig. 5C). The Ca^{2+} response to plasma membrane depolarization using KCl (30 mM) was unchanged in chloramphenicol treated cells compared to control (Fig. 5D), which demonstrates that overall Ca^{2+} homeostasis is not strongly altered after antibiotic treatment. Our results show that Ca^{2+} signaling specifically linked to metabolism-secretion coupling is slightly enhanced in INS-1E cells when mitochondrial protein translation is inhibited. This phenotype is consistent with the observed elevated ATP-synthase dependent respiration in the presence of chloramphenicol (20 μg/ml; Fig. 4H).

4. Discussion

Several antibiotics targeting bacterial protein synthesis have been shown to inhibit mitochondrial protein synthesis without affecting

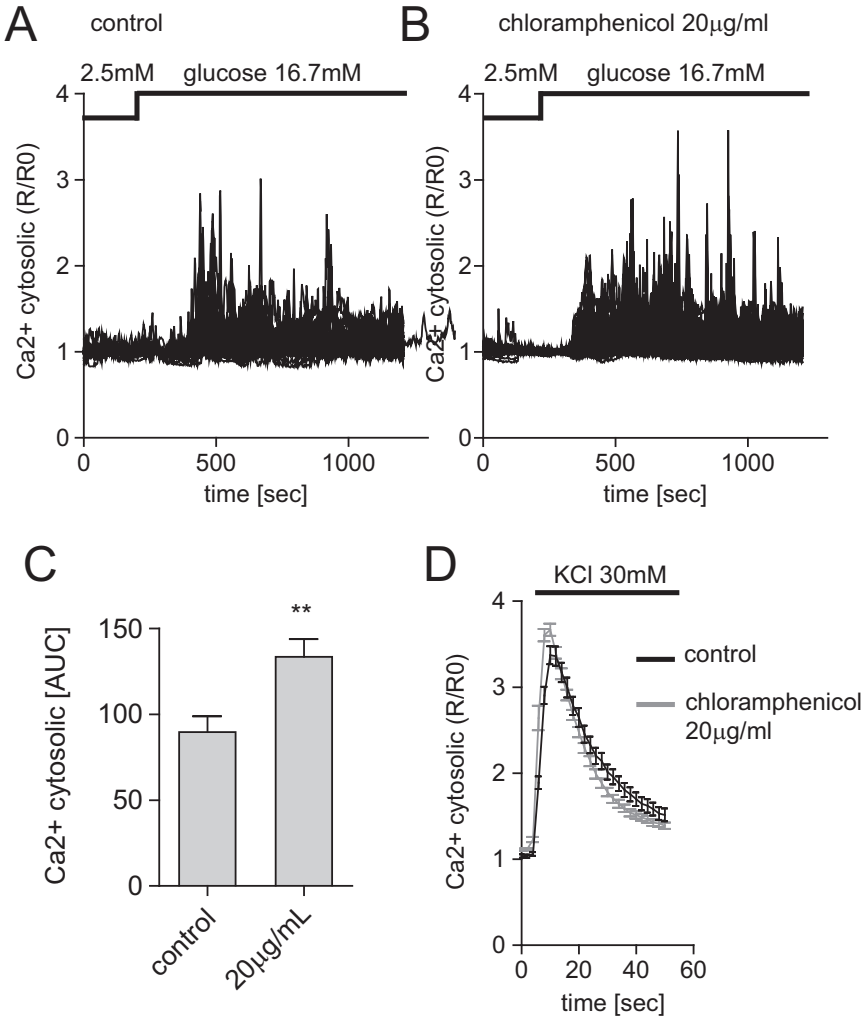

Fig. 5. Glucose dependent Ca^{2+}signaling is elevated after inhibition of mitochondrial protein synthesis with chloramphenicol. INS-1E cells expressing the cytosolic Ca^{2+} probe YC3.6 were analyzed in KRBH 2.5 mM glucose and stimulated with glucose (16.7 mM). Prior to the experiment the cells were cultured in RPMI 11 mM glucose in the absence (A) or presence (B) of chloramphenicol (20 μg/ml). Ca^{2+} responses from 50 cells are overlaid in each panel. (C) Glucose-dependent Ca^{2+} rises were quantified as area under the curve over the basal FRET ratio R/R0. The results are the mean +/- s.e.m. from 3 independent experiments. Per condition 50 cells were included in the analysis. (D) Cells were cultured and analyzed as in A and B. Average (+/-s.e.m.; N=3) Ca^{2+} response from control INS-1E cells (black trace) or cultured in medium containing chloramphenicol (20 μg/ml; grey trace) depolarized with 30 mM KCl are shown. A total of 50 cells were analyzed per condition. Mean +/- s.e.m.

cytosolic ribosomes. Therefore antibiotics such as chloramphenicol, doxycycline, streptomycin or gentamicin lower expression of mtDNA encoded essential subunits of the respiratory chain resulting in partial loss of respiratory capacity in a large number of cell types and organisms [12,19]. Here we have confirmed such earlier findings in HeLa cells (derived from human cervical cancer cells) and PC12 cells (derived from rat adrenal gland phaeochromocytoma) using chloramphenicol. In these cell types, chloramphenicol reduced the expression of COX1 without affecting the expression of nuclear encoded mitochondrial proteins (ATP5A and TOM20). In HeLa cells and PC12 cells, we measured a corresponding 2.5-fold respectively 2-fold reduction of the mitochondrial respiratory rates when chloramphenicol (20 μg/ml) was added to the cell culture medium 2 days prior to the experiment. In contrast, respiration in INS-1E cells is quite resistant to chloramphenicol. This was also true for primary β-cells in the context of intact human islets. At concentrations strongly reducing respiration in the other cell types, respiratory rates were normal in insulin secreting cells. Both basal respiration in 2.5 mM glucose (mostly uncoupled respiration) and glucose (16.7 mM) induced respiratory rates (primarily ATP-synthase dependent) were not lowered by chloramphenicol. In INS-1E cells, even further increasing the chloramphenicol concentration was not sufficient to inhibit respiration.

Interestingly, chloramphenicol retained its ability to inhibit mitochondrial translation in INS-1E cells. Chloramphenicol (20 μg/ml) caused a strong reduction of COX1 protein levels in INS-1E cell mitochondria. This finding suggests the antibiotic is able to inhibit the mitochondrial translation machinery even in insulin secreting cells. COX1 is an essential subunit of complex IV of the respiratory chain. Its direct involvement in the reduction of oxygen suggests changes in COX1 and therefore complex IV activity should cause a proportional reduction in respiratory rates. This was not the case for insulin secreting cells. A possible interpretation of our results is that some cell types such as β-cells express an excess of respiratory chain complexes or specifically an excess of mtDNA encoded proteins. This explanation however seems highly unlikely as COX1 expression levels were similar in the two rat cell lines INS-1E and PC12 despite the fact that their respiration is resistant respectively sensitive to chloramphenicol.

In several experiments, we observed that at very high concentration of the antibiotics respiration in general was reduced (Fig. 3D; data not shown). We did not further analyze these experiments as they are likely confounded by the impact of antibiotics on cell proliferation and cell death via mitochondrial dysfunction. The effects on respiration and cell viability are hard to dissect as loss of respiratory chain activity below a threshold level is likely harmful for β-cells as glycolytic flux and mitochondrial energy metabolism are tightly linked [25]. Severely impaired mitochondrial function in this cell type also slows glycolysis causing energy crisis and cell death.

Taken together, the sensitivity of mitochondrial respiration to antibiotics is cell type specific with β-cells being surprisingly insensitive compared to other cell types. A number of examples in the literature show that also for other cell types there may be no perfect correlation between the expression levels of mtDNA encoded proteins and respiratory rates. Respiration seems to be at least partially resistant even when the level of COX1 or other subunits of the respiratory chain were very markedly reduced. These observations suggest that a certain excess of respiratory chain complexes are expressed in different cell types. A modest reduction of the mitochondrially encoded subunits may leads to mitochondrial protein imbalance without yet affecting respiratory rates. Only once mitochondrial protein translation is suppressed below a threshold will the respiratory rates be inhibited. Our data in β-cells shows that there is a large discrepancy between the amount of chloramphenicol required to inhibit mitochondrial translation and the effective concentration to lower respiration.

In a recent study by Moullan et al. [19], the authors warn from the use of the tetracycline antibiotic doxycycline for the control of transgenes expression using the Tet-on and Tet-off system [19]. Indeed, they show that doxycycline at concentrations used to control transgene expression (0.5–1 μg/ml) lowers mitochondrial translation and respiration. Such impairment of mitochondrial function by doxycycline was demonstrated in a large number of cell types and organisms [12,19]. Respiratory rates in INS-1E insulin secreting cells on the other hand were unaffected by doxycycline when tested at or above the concentrations having inhibitory effects in other cell types.

Caution in the use of antibiotics in biological experiments also applies to other antibiotics developed for the inhibition of bacterial translation as they may have side-effects on mitochondria. This is true for chloramphenicol as shown here for HeLa and PC12 cells and by earlier studies [21]. Many transformed cell lines are poor users of mitochondria relying mostly on glycolysis for energy metabolism. A shift away from mitochondrial energy metabolism may be worsened due to the continuous culture in media containing antibiotics, which inhibit mitochondrial translation.

We tested several antibiotics for their ability to alter respiration in INS-1E cells. Regular INS-medium contains a combination of the antibiotics penicillin (50 μg/ml) and streptomycin (100 μg/ml) and similar antibiotic combinations have been used for human islet long-term culture or preparation for transplantation [23,8]. Presence of these antibiotics in the medium did not significantly alter the respiratory rates of INS-1E cells. Gentamicin (0.05 mg/ml) has also been used to prevent bacterial growth in human islet cultures [32,6]. Gentamicin neither altered basal nor glucose-induced respiration in INS-1E cells. Antibiotics used for the culture of insulin secreting cells therefore do not affect their respiratory function.

Insulin secreting cells, as demonstrated in this study, seem less affected as mitochondrial respiration is to a large extent resistant to mitochondrial translation inhibitors. It should be kept in mind that lowering mtDNA translation even without altering respiration may affect cell function. Auwerx and colleagues have shown that relative changes in the expression of mitochondrially and nuclear encoded respiratory chain subunits results in a mitochondrial unfolded protein response [10,19]. The stoichiometric imbalance of nuclear and mitochondrial respiratory complex subunits will result in the accumulation of unassembled respiratory chain complex subunits targeted for degradation. The stoichiometric imbalance leading to the mitochondrial unfolded protein response also influence nuclear gene expression and therefore cell function beyond mitochondria. Interestingly, this mitochondrial unfolded protein response has also been shown to increase life-span in mice and worms [10].

In INS-1E cells, inhibition of COX1 expression was associated with elevated Hsp60 expression likely due to the induction of the mitochondrial unfolded protein response. At optimal concentrations of chloramphenicol the mitochondrial unfolded protein response may actually have beneficial effects on energy metabolism and metabolism-secretion coupling. We noticed for instance that chloramphenicol enhanced the responsiveness of INS-1E cells to glucose as measured following cytosolic Ca^{2+} signals. Cytosolic Ca^{2+} rises are a sensitive read-out of metabolism-secretion coupling in β-cells. INS-1E cells cultured in the presence of chloramphenicol (20 μg/ml) for 2 days displayed larger glucose-induced cytosolic Ca^{2+} rises than the control INS-1E cells (Fig. 5A, B, C). The here observed elevated Ca^{2+} signals could indicate improved mitochondrial function possibly as a result of the mitochondrial unfolded protein response. Consistent with this interpretation, we also observed a small increase in ATP-synthase dependent respiration when chloramphenicol (20 μg/μl) was present in the culture medium (Fig. 4H). Depending on concentration of chloramphenicol employed, we may study opposing effects on mitochondrial energy metabolism. On the one hand, inhibition of translation and at higher concentrations of the antibiotics respiratory impairment. On the other hand, partial inhibition of mitochondrial translation with an associated mitochondrial unfolded protein response that improves the function of the existing mitochondrial pool. Possible improvement of mitochondrial

function by antibiotics and its relation to the mitochondrial unfolded protein response will require further studies.

Authors contribution

A.W. has written the manuscript. J. S-D and I. C. have designed and executed the experiments. C. B. has optimized Western detection protocols for this study. S.L. has contributed to protein and Western analysis.

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