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Marked over expression of uncoupling protein-2 in beta cells exerts minor effects on mitochondrial metabolism

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ABSTRACT

Evidence is conflicting as to the impact of elevated levels of uncoupling protein-2 (UCP-2) on insulin-producing beta cells. Here we investigated effects of a fourfold induction of UCP-2 protein primarily on mitochondrial parameters and tested for replication of positive findings at a lower level of induction.

We transfected INS-1 cells to obtain a tet-on inducible cell line. A 48 h exposure to 1 μg/ml of doxycycline (dox) induced UCP-2 fourfold (424 ± 113%, mean ± SEM) and 0.1 μg/ml twofold (178 ± 29%, $n = 3$).

Fourfold induced cells displayed normal viability (MTT, apoptosis), normal cellular insulin contents and, glucose-induced insulin secretion (+27 ± 11%) as well as D-[U-^{14}C]-glucose oxidation (+5 ± 9% at 11 mM glucose). Oxidation of [1-^{14}C]-oleate was increased from 4088 to 5797 fmol/μg prot/2 h at 3.3 mM glucose, $p < 0.03$. Oxidation of L-[^{14}C(U)]-glutamine was unaffected. Induction of UCP-2 did not significantly affect measures of mitochondrial membrane potential (Rhodamine 123) or mitochondrial mass (Mitotracker Green) and did not affect ATP levels. Oligomycin-inhibited oxygen consumption (a measure of mitochondrial uncoupling) was marginally increased, the effect being significant in comparison with dox-only treated cells, $p < 0.05$. Oxygen radicals, assessed by dichlorofluorescin diacetate, were decreased by 30%, $p < 0.025$. Testing for the lower level of UCP-2 induction did not reproduce any of the positive findings. A fourfold induction of UCP-2 was required to exert minor metabolic effects. These findings question an impact of moderately elevated UCP-2 levels in beta cells as seen in diabetes.

1. Introduction

Over expression of uncoupling protein-2 (UCP-2) has been proposed to be diabetogenic because of adverse effects on beta cell function. Evidence supporting this notion includes the demonstration of better insulin secretion from pancreatic islets of ucp −/− vs. wild-type mice [1], the coupling of hyperglycemia to increased UCP-2 expression [2,3], and the amelioration of diabetes by transferring a knock-out mutation to the ob/ob/ mouse, an animal model of type 2 diabetes [1]. However, recent in vivo studies did not replicate the latter effects and in fact displayed opposite findings [4]. Likewise there are discrepancies as to the functional effects of over-expression of UCP-2 in vitro. Inhibitory effects have been reported [5] as well as stimulatory ones [6] or no effects [7]. Because of conflicting results no agreement on the impact of elevated UCP-2 has been reached.

Different degrees of elevated UCP-2 between studies could be one reason for conflicting results. We undertook the present study to compare effects of two levels of over expression. Because of the mitochondrial localization of UCP-2 we primarily tested parameters related to mitochondrial metabolism.

2. Materials and methods

2.1. Materials

FuGENE-6™ from Roche Diagnostics (Mannheim, Germany) and BD Tet-ON™ gene expression systems were from BD Bioscience Clonetech (Mountain View Calif). Dual-Luciferase® Reporter Assay System was from Promega (Madison, Wisconsin), D-[U-^{14}C] glucose, [1-^{14}C]-oleic acid and L-[^{14}C(U)]-glutamine, Hybond N$^+$ membranes and ECL Western blotting detection reagents were from Amersham Pharmacia Biotech (Buckinghamshire, UK) and Perkin Elmer Inc. (Waltham, Massachusetts). Other materials were from Sigma–Aldrich Chemical Co. (St. Louis, MO) or from sources specified below.

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2.2. Cell culture and transfections

INS-1 cells were a gift from Claes Wollheim, Geneva, Switzerland. INS-1 cells were grown in monolayer cultures in RPMI-1640 medium containing 11 mM glucose and supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM-glutamine, 1 mM sodium pyruvate, 50 μM mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified (5% CO_2, 95% air) atmosphere at 37 °C. The cells were sub cultured once a week after detachment with 0.01% trypsin in 0.02% EDTA. The medium was changed every 3–4 days of culture.

To generate a cell line which over expresses UCP-2 in the presence of doxycycline (dox), the INS-1 cell line was doubly stable transfected, using the BD Tet-ON™ gene expression system. INS-1 cells were plated in 5 cm culture plates containing 10 ml complete RPMI medium at a density of 5×10^6 cells/plate. After 2 days of incubation pTet-On regulator plasmid (2 μg) was transfected into the INS-1 cells by using FuGENE 6 as transfection reagent, the transfection being performed as recommended by the manufacturer. The cells were then incubated for 2 days before initiating drug selection by geneticin (150 μg/ml). The medium was replaced with complete RPMI medium containing geneticin every 3–4 days. Transformed colonies were clearly visible after 5 weeks. Eleven colonies were clonally expanded and further analyzed. The Tet-On clones were screened for low background and highest dox-dependent induction by a dual-luciferase assay. Clones with the highest fold-induction were stably transfected with the pTRE2-hyg-UCP-2 vector (2 μg) by the same procedure described above. The plasmid used for the second transfection was constructed by subcloning a full-length human UCP-2 cDNA (a gift from Dr. C. Warden, Row Genetics, University of California, Davis, CA) into the expression vector pTRE2-hyg. The correct sequence and orientation of the UCP-2 insert was verified by DNA sequencing. Doubly stable transfected Tet-On UCP-2 clones were selected in presence of Hygromycine (100 μg/ml). Six clones were obtained and screened for a dox-induced dose-dependent increase in UCP-2 protein as detected by Western Blots. One of these clones was used in the present study (named UCP-2 cells). One of the clones containing only the regulator-plasmid (named Tet-on cells) was used for appropriate control experiments.

Exposure to dox was performed 1–2 days after seeding. Cells were cultured in RPMI medium (which contained glutamine 2 mM, added by Sigma, and an additional 2 mM added by us) with or without dox for 48 h. Subsequent to a dose-response study (Fig. 1), we used 1.0 μg/ml of dox for all further experiments and 0.1 μg/ml when testing for reproduction of positive findings.

2.3. Insulin secretion and insulin contents

Cells were seeded (3×10^5 per well, 3–4 wells per condition) in a 24 well plate and incubated over night before ± dox exposure. Cells were then pre-incubated with modified Krebs-Ringer bicarbonate-HEPES buffer (KRBH) medium for 4 h. The KRBH medium was supplemented with 10 mM HEPES and 0.1% BSA.

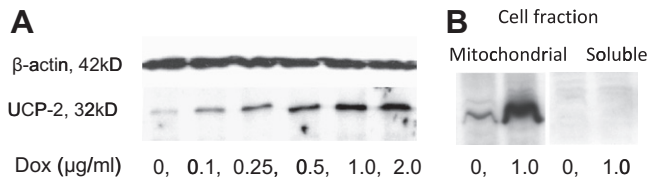

Fig. 1. Effects of a 48 h exposure to 0.1–2.0 μg/ml of dox on UCP-2 and β-actin. (A) A representative curve is shown (Western blotting, separate gels for UCP-2 and β-actin). (B) Signals from a mitochondrial and cytosolic fraction are compared.

Pre incubation media did not contain glucose. Final batch type incubations were carried out in 1 ml of KRBH for 60 min with 3.3, 11 or 27 mM glucose. Aliquots of media were secured. Insulin contents of cells were extracted by 200 μl of acid-ethanol (0.18 mol/l HCl in 95% ethanol). Immunoreactive insulin was assayed by RIA [8].

2.4. Glucose, oleate and glutamine oxidation

Production of $^{14}CO_2$ from D-[U-^{14}C]-glucose was measured basically as described [9]. Cells were incubated in 1 ml glass vials containing 100 μl of KRBH medium together with 0.2–0.5 μCi of D-[U-^{14}C]-glucose plus non-radioactive glucose to achieve a final concentration of 3.3, 11 or 27 mM. The glass vials were placed in 20 ml scintillation tubes that were gassed with O_2/CO_2 (95:5) and capped airtight with rubber membranes. The tubes were shaken continuously for 120 min at 37 °C in a water bath. Metabolism was stopped by an injection of 100 μl of 0.1 M HCL into the glass vials followed by injection of 250 μl of hyamine hydroxide into the outer vials (= scintillation tubes). The sealed scintillation bottles were left overnight at room temperature to absorb $^{14}CO_2$ into the hyamine. Blank incubations were treated identically.

Production of [l-$^{14}CO_2$] from [l-^{14}C]-oleate and $^{14}CO_2$ from L-[^{14}C(U)]-glutamine was assessed as for glucose oxidation, except that 0.16 μCi of [l-^{14}C]-oleate or 0.2 μCi of L-[^{14}C(U)]-glutamine was added during 120 min incubations.

2.5. Oxygen consumption

The oxygen consumption (of intact cells) was measured by Clark-type polarographic oxygen sensors and high-resolution respirometry (Oxygraph-2 k, OROBOROS, Innsbruck, Austria). Samples of 10^6 cells/cm^3 suspended in cell culture medium were added to a chamber with magnetic stirring and allowed to equilibrate with air for 2 minutes before closing the chamber and recording oxygen uptake at basal respiration. Uncoupled respiration was induced by adding the ATP synthase inhibitor oligomycin (2 μg/ml). After that, the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was titrated (up to 5 μM) to achieve a state of maximum respiratory capacity. Rotenone (0.5 μM) and antimycin (2.5 μM), inhibitors of complex I and III, were used to obtain the inhibited state and the corresponding residual oxygen consumption (ROX) values. Oxygen consumption rates were calculated as the negative time derivate of the oxygen concentration present in the chamber (pmol/s/mill cells). All values were corrected for ROX.

2.6. Western blots

The procedure in general has been described [10] Fifteen to twenty μg protein, equal amounts for each lane, were applied to 12% (for UCP-2) or 8% (for beta actin) sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE) and run for 1 h at 150 V, then transferred to nitrocellulose for 1 h at 250 mA. The membranes were blocked 2 h at room temperature with 5% fat-free milk and 0.1% Tween-20 in TBS. Membranes were incubated with UCP-2 antibody (polyclonal goat IgG1 from Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:300) or beta-actin (monoclonal mouse IgG1 from Sigma–Aldrich, 1:10.000) overnight at 4 °C, followed by incubation with horseradish-peroxidase-linked second antibody (rabbit anti goat or sheep anti mouse, 1:5000) for 1 h at room temperature. Immuno-reactive bands were visualized by chemiluminescence (ECL, Amersham).

Mitochondrial protein was isolated by the MS853 Mitochondrial Isolation Kit for Cultured Cells (Mito Sciences Inc., Oregon). For immunoblotting we used an antibody "cocktail" (Mito

Sciences) containing mouse monoclonal antibodies against complexes (C) *I–V* subunits, these being for CI the NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8), for CII succinate dehydrogenase subunit B (SDHB), for CIII ubiquinol cytochrome C reductase core protein 2 (UQCRC2), for CIV mitochondrial encoded cytochrome C oxidase subunit 1 (MTCO1) and for CV ATP synthase alpha subunit (ATP5A). The Micro BCA Protein Assay Kit (Thermo scientific, Rockford, IL) was used for protein quantification.

2.7. ATP

The ATP Bioluminescence assay Kit HS II (Roche Diagnostics, Mannheim, Germany) was used. Triplicates of each sample/standard were measured and averaged.

2.8. DNA

The Fluorescent DNA Quantification kit (Bio-Rad, Hercules, CA) was used for quantification in oxygen consumption experiments.

2.9. Mitochondrial membrane potential (MMP)

MMP was assessed by double staining with the fluorescent dyes Rhodamine 123 and propidium iodide (Sigma–Aldrich). After induction by 1 μg/ml dox the medium was aspirated and the cells washed. They were then detached by treating with 0.05% EDTA (in PBS) and re-suspended (4×10^5/ml) in Hank's solution. Cells were then incubated with 10 μg/ml Rhodamine 123 at 37 °C for 10 min, and subsequently with 1 μg/ml propidium iodide for 10 min on ice. The MMP was assessed with duplicates using a 15 mW argon laser (488 nm), a 550 nm dichronic long pass filter and a 525 ± 15 nm band pass filter detecting Rhodamine 123 and 675 ± 10 nm band pass filter detecting propidium iodide. According to the values of forward scatter, side scatter and red fluorescence (propidium iodide), debris, aggregates and dead cells could be identified and were gated out of analysis. Fluorescence was measured on a logarithmic scale. Results are expressed as the ratio for mean fluorescence intensity of the potential-sensitive probe Rhodamine 123 in samples measured with or without prior exposure to FCCP.

2.10. Mitochondrial mass

Cells were loaded with 20 and 50 nM Mitotracker Green (Molecular Probes) for 30 min at 37 °C. The probe is taken up into mitochondria, producing a fluorescence signal which is not affected by the prevailing MMP, thereby providing a measure of mitochondrial mass.

2.11. Apoptosis

Cells were detached by treating with 0.05% EDTA in PBS and collected by centrifugation. Apoptotic and necrotic cells were dual stained with Annexin V and propidium iodide using an Apoptosis Assay Kit# (V13241, Molecular Probes). Flow cytometric analysis was performed using a 15 mW argon laser (488 nm), 550 nm diachronic long-pass filter and 550 ± 20 nm band-pass filter detecting Annexin V and a 675 ± 15 nm band-pass filter detecting propidium iodide.

2.12. Oxidative stress

The probe 2′7′-dichlorofluorescin diacetate (Molecular Probes) was used to assess ROS generation. Oxidation by ROS produces the fluorescent compound 2′7′-dichlorofluorescein (DCF). Cells were loaded with 5 μM of the probe for 20 min at 37 °C. The production of ROS was assessed by measuring the fluorescent signal evoked by DCF in the setting of a 488 nm argon laser and 550 ± 20 band-pass filters.

2.13. MTT assay

A measure of cell viability was obtained by the 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [11].

2.14. Cell counting and trypan blue

A sample of the cell suspension was mixed (1:1) with trypan blue stain (0,4%) to separate/count viable and dead cells in a Countess automatic cell counter (Invitrogen, Carlsbad, California).

2.15. Carnithine palmityol transferase I (CPT1)

Activity of CPT1was measured basically as described [12].

2.16. Statistics

Results were calculated as means ± SE. Significance testing was carried out using Students' t-test (paired or unpaired differences as appropriate). A p-value <0.05 (two-sided) was considered significant.

3. Results

3.1. Dox induces dose-dependent increases in UCP-2 protein

A 48 h induction period with concentrations of dox ranging from 0.1 to 2 μg/ml produced dose-related increases in UCP-2 protein (Fig. 1). In three separate experiments the increase was, for 0.1 μg/ml of dox 178 ± 29% and for 1 μg/ml 424 ± 113%. Beta actin was not affected (Fig. 1A). Over-expressed UCP-2 was localized to mitochondria (Fig. 1B).

3.2. UCP-2 induction fails to affect cellular insulin contents or secretion

A 48 h induction period with 0.1–2 μg/ml of dox did not affect insulin contents of cells (results not shown). A fourfold induction of UCP-2 (by 1 μg/ml of dox) failed to affect glucose (11 mM)-induced insulin secretion (27 ± 11%, n = 5, Supplemental Table S1).

3.3. Cell characteristics

There were no significant effects by fourfold UCP-2 induction on viability as assessed by MTT, by trypan blue, or by annexin V (results not shown). Nor were any effects on growth rates observed. Hence, the number of UCP-2 cells after induction was 106 ± 4% (n = 6) of non-induced cells. Corresponding number for dox treatment of Tet-on cells was 96 ± 4% (n = 3).

3.4. ATP contents

ATP contents of non-induced UCP-2 cells was 139 ± 28%, (n = 4) compared to fourfold induced unexposed cells.

3.5. UCP-2 induction enhances oleate but not glucose or glutamine oxidation

Oxidation of oleate was expectedly [13] markedly dependent on the prevailing glucose concentration: it was >40-fold greater at 3.3 compared with 27 mM glucose in induced and non-induced cells (Fig. 2A). At 3.3 mM glucose, a fourfold induction of UCP-2

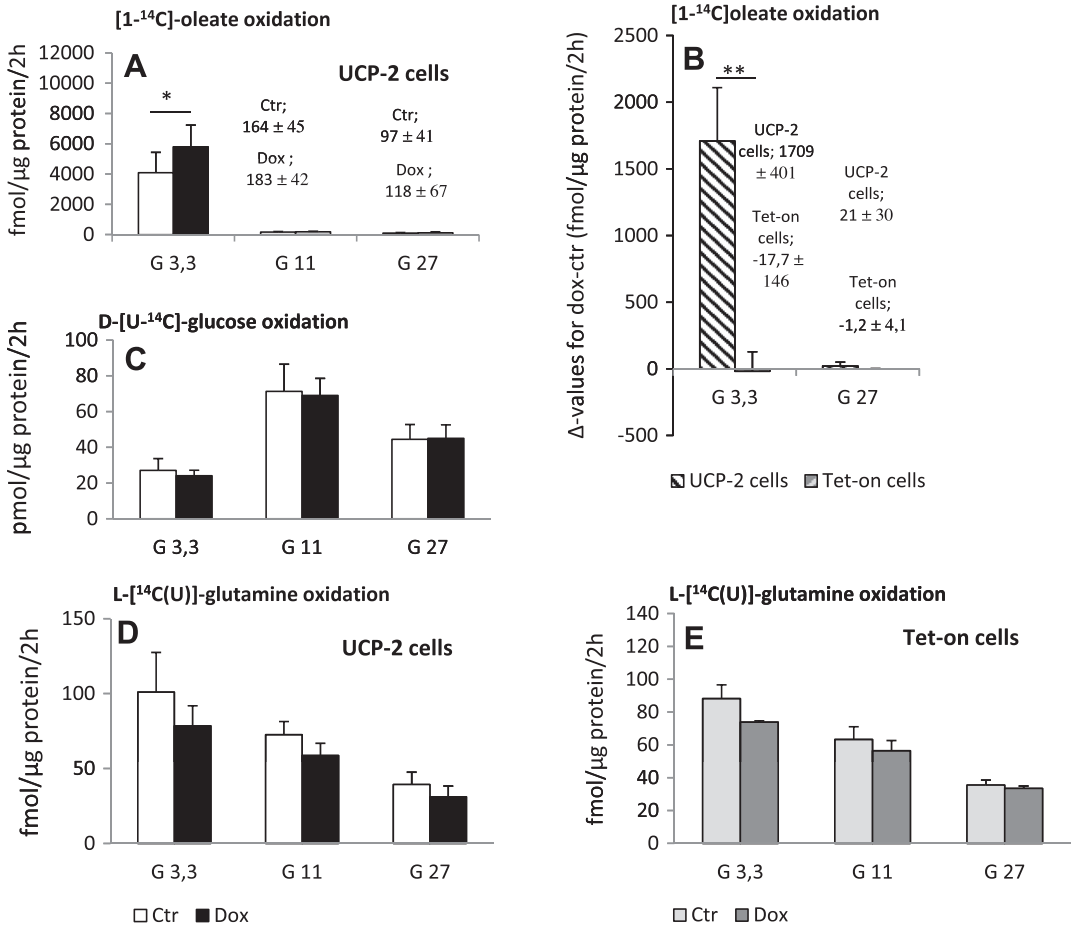

Fig. 2. Effects of UCP-2 induction and dox per se on ^{14}C-nutrient oxidation. Controls (Ctr) are cells not exposed to dox. Glucose (G) concentrations during oxidation were 3.3, 11 and 27 mM. For bars that are barely visible, numeric values are indicated in the graphs. Data are mean ± SEM of 3–12 experiments, each consisting of 3–5 parallel measurements per glucose concentration. $^{*}p < 0.03$, $^{**}p < 0.02$.

increased the [$^{14}CO_2$]-production from [l-^{14}C]-oleate by 1709 ± 401 fmol/µg protein/2 h (Fig. 2A and B). No effect was seen by the lower level of induction (results not shown). There was no discernible effect by dox in the cells that lacked coupling to the UCP-2 gene (Tet-on cells, Fig. 2B).

UCP-2 induction failed to affect the oxidation of D-[U-^{14}C]-glucose, whether tested at 3.3, 11 or 27 mM of glucose (Fig. 2C). The oxidation of glutamine was inhibited by exposure to dox in the UCP-2 cells. However, a similar inhibition was seen after exposing Tet-on cells to dox (Fig. 2D and E).

3.6. CPT1

We tested for effects on the activity of carnithine palmityol transferase I (CPT1), CPT1 being a rate-limiting enzyme for fatty acid oxidation. Enzyme activity was responsive (depressed) by 24 h of elevated glucose (27 mM), results not shown. There was however no discernible effect on the enzyme activity by UCP-2 induction (38.0 vs 37.5 fmol/10 min/10 µg protein, mean of 6 experiments).

3.7. MMP and mitochondrial mass

We assessed MMP by rhodamine 123. By this method, a decreased ratio of fluorescence of FCCP in relation to basal fluorescence (FCCP:basal) would signify depolarization. A non-significant tendency for such an effect was observed (Table 1).

Table 1
Mitochondrial membrane potential (MMP) and mitochondrial mass in UCP-2 cells.

	MMP (n = 6)		Mitochondrial mass (n = 3)	
	Fluorescence intensity level			
	Rhodamine 123		Mitotracker Green (MTG)	
	FCCP (100 µM)	FCCP (200 µM)	MTG (20 nM)	MTG (50 nM)
	FCCP/no FCCP			
No dox	1.3 ± 0.02	1.72 ± 0.05	749 ± 79	1502 ± 107
Dox	1.31 ± 3.3	1.62 ± 0.11	774 ± 34	1526 ± 115

Mitochondrial mass (by the Mitotracker Green probe) was not affected (Table 1).

3.8. UCP-2 induction affects mitochondrial complex I and IV

Subunits of complex 1 and complex IV were reduced by UCP-2 induction (Table 2). Dox exposure also reduced these proteins in Tet-on cells. Taking into account the latter influence the effect of UCP-2 induction was still significant.

3.9. Oxygen consumption

Effects on oxygen consumption were evaluated in the context of "dox" effects per se in the Tet-on cells. A fourfold induction of UCP-2 did not affect basal respiration in UCP-2 cells but reduced

Table 2
Effect of UCP-2 up-regulation on mitochondrial complex protein levels.

Complex (C) subunit	Amount of protein in dox-induced cells (as% of no dox)				
	CI-NDUFB8	CII-SDHB	CIII-UQCR2	CIV-MTCO1	CV-ATP5A
Subunit (kDa)	20	30	47	39	53
UCP-2 cell	67 ± 5[a,b]	110 ± 5	101 ± 6	68 ± 3[a,b]	102 ± 10
Tet-on cell	87 ± 9	101 ± 8	95 ± 7	92 ± 9	103 ± 7

a: $p < 0.003$ for effect of dox, b: $p < 0.04$ for comparing dox-effect in UCP-2 cells ($n = 5$) vs. Tet-on cells ($n = 3$).

respiration in Tet-on cells. Hence, basal respiration was slightly increased in UCP-2 cells when the Tet-on influence was taken into account. UCP-2 induction failed to affect FFCP-induced oxygen consumption taking into account a similar effect of dox in Tet-on cells (Fig. 3A and B).

UCP-2 induction significantly increased the oxygen consumption remaining after oligomycin (by 8.2% vs −8.5% for dox in Tet-on cells, Fig. 3C). Induction was associated with a small increase in uncoupling (Fig. 3D and E).

Exposure to oleate, (100 μM), did not affect oxygen consumption either with or without induction of UCP-2 (results not shown). Higher concentrations of oleate (200 and 400 μM) increased oxygen consumption, however with no difference between induced and non-induced cells (results not shown).

3.10. UCP-2 induction and ROS

Decrease was variable and only significant after performing a large number of experiments (20.9 ± 3.9 without vs.14.7 ± 2.1 with prior dox induction, $n = 18$, $p < 0.025$).

4. Discussion

Our results show that a fourfold over-expression of UCP-2 in INS-1 beta cells mildly affects mitochondrial metabolism, the two salient effects being an increased fraction of oxygen consumption independent of ATP synthesis and an increase in fatty acid oxidation. The effect on oxygen consumption is compatible with increased uncoupling; the effect was small but compatible with the

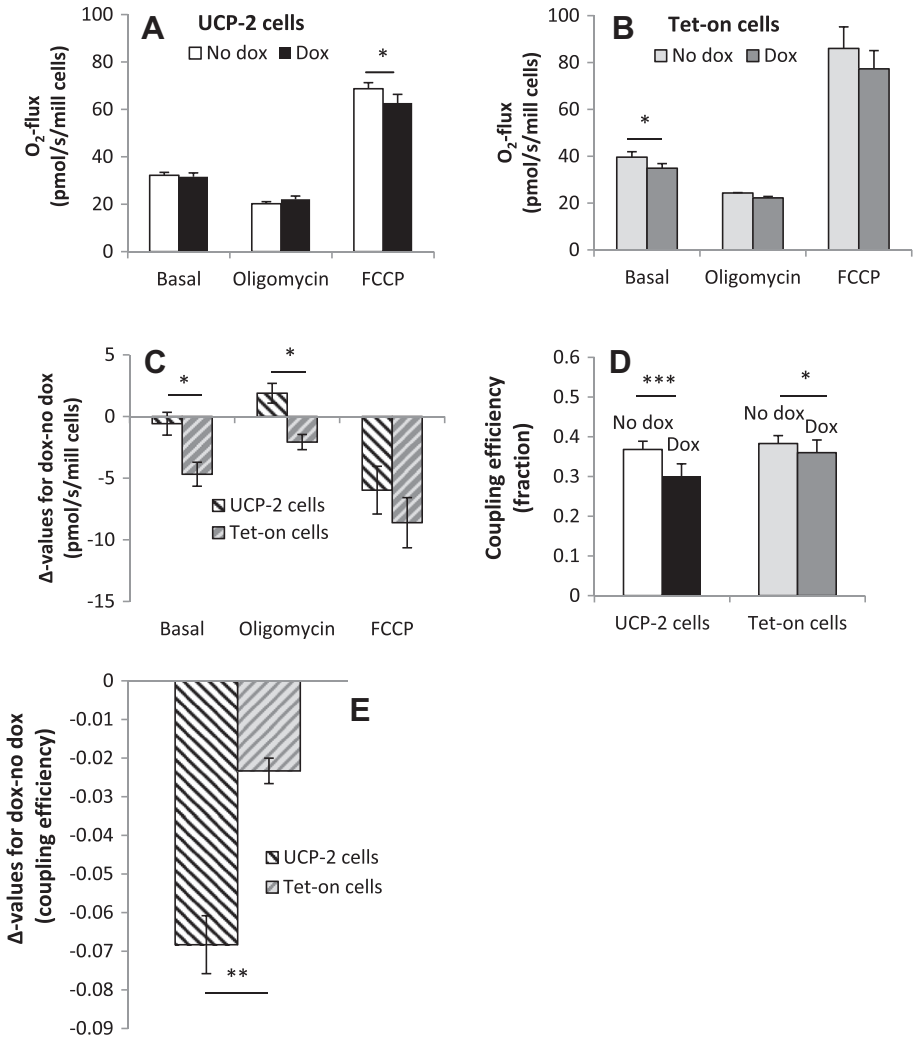

Fig. 3. Effects of UCP-2 induction and dox per se on respiration. (A and B) O_2-flux in UCP-2 and Tet-on cells at basal conditions, in the presence of oligomycin and subsequently FCCP. (C) Dox-effect as Δ O_2 flux in UCP-2 cells vs. Tet-on cells. (D) Coupling efficiency was estimated as the fraction of basal respiration that was inhibited by oligomycin. (E) Dox-effect as Δ coupling efficiency in UCP-2 cells vs. Tet-on cells. Data are mean ± SEM of 6 (UCP-2 cells) or 3 (Tet-on cells) separate experiments, each consisting of 2–4 parallel measurements. $^{*}p < 0.05$, $^{**}p < 0.004$, $^{***}p < 0.0003$.

opposite effect being recorded after UCP-2 reduction by siRNA [14]. Fatty acids stimulate UCP-2 [13], therefore reciprocity was not unexpected. The increased oxidation of oleate that we report was also seen in INS-1 cells in which UCP-2 was over-expressed by adenovirus infection [5]. These findings disagree with the increased oxidation that was reported in islets from UCP2 −/− islets [15]. The discrepancy could possibly relate to influences by non-beta cells which constitute a significant minority of islet cells, see further below.

We followed up our findings on oleate oxidation by measuring the activity of the CPT1 enzyme and by testing for enhanced uncoupling activity of oleate as a result of UCP-2 induction. However, we could not register any influence on the activity of this enzyme by UCP-2 over expression. Nor did we find a synergizing effect on fatty-acid induced uncoupling. Other possible mechanisms remain to be explored.

The lack of effects on glucose oxidation that we find concurs with previous reports [16,7]. A novel observation is the lack of a positive effect on the oxidation of glutamine, a feature which was hypothesized to be coupled to the action of UCP-2 in immune cells [17].

The use of clonal beta cells rather than pancreatic islets could be viewed as a limitation of this study, since studies in isolated islets are closer to the in vivo situation. Islets however contain approximately thirty percent non-beta cells, the contribution of which could obscure the regulation and effects of UCP-2 in beta cells. Indeed, high levels of UCP-2 have been reported in glucagon-secreting alpha cells [18], which make up 15–20% of islet mass. Hence, the use of isolated beta cells rather than islets for this study appears to have merit.

Induction by dox in appropriately manipulated cells has the advantage of allowing tests for effects at a defined time of exposure and level of over-expression. However, a "dox" effect per se constitutes a possible pitfall in the interpretation of effects [19]. We did not find any "dox" effects on viability or growth parameters, hence excluding overt toxicity. However, we find "dox" effects on some of the functional parameters studied. It became clear that results in UCP-2 cells had to be "corrected" for "dox" effects. We are aware that such the correction (which has rarely been recognized as necessary in the past) introduces some variability and may affect significance testing of effects.

The effects of UCP-2 induction that we observe (including confirmation of a decrease in ROS, proposed to be important for antioxidative defense [7,13,20]) were apparent only after a fourfold elevation of UCP-2. This raises doubt on the functional – and clinical – importance in vivo on beta cells by moderate elevations of UCP2 in diabetes. UCP-2 may exert a more important permissive role on beta cell function as suggested by the findings of Affourtit and Brand [14].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.101.

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