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Uncoupling Protein-2 Participates in Cellular Defense against Oxidative Stress in Clonal β-Cells

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The role of uncoupling protein-2 (UCP-2) in β-cells is presently unclear. We have tested the notion that UCP-2 participates in β-cell defense against oxidants. Expression of the UCP-2 gene in clonal β-cells (INS-1) was decreased by 45% after 48 h of culture with vitamin E and selenite. When INS-1 cells were exposed to 200 μM H_2O_2 for 5 min, the cell viability (MTT assay) decreased to 85 ± 1, 61 ± 1, 40 ± 2, and 39 ± 2% of control when measured respectively 30 min, 2 h, 6 h, and 16 h after H_2O_2 exposure. At corresponding time points UCP-2 mRNA levels were 1.01 ± 0.09, 1.53 ± 0.15 ($P < 0.05$), 1.44 ± 0.18 ($P = 0.06$), and 1.12 ± 0.09 fold of control, i.e., transiently increased. We next tested whether overexpression of UCP-2 could enhance resistance of β-cells toward H_2O_2 toxicity. A cotransfection method using EGFP as a suitable marker and a human cDNA UCP-2 construct was used for transient overexpression of UCP-2. Transfected cells expressed the gene about 30-fold more than normal cells. After exposure to H_2O_2 (200 μm, 5 min), the survival of UCP-2 overexpressing cells was measured 30–45 min later by flow cytometry. Survival was 13 ± 0.05% higher than control (EGFP only) cells, $P < 0.004$ for difference. The results indicate that oxidative stress induces UCP-2 expression in β-cells, and that UCP-2 serves a role in β-cell defense against oxidative stress. © 2001 Academic Press

Key Words: **uncoupling protein-2; reactive oxygen species; β-cells; diabetes mellitus; INS-1 cells; vitamin E; H_2O_2.**

The physiological role of uncoupling protein 2 (UCP-2) is presently unclear. Uncoupling of mitochondrial oxidation leads to dissipation of energy as heat. Predictably, thermogenesis is the main function of the earlier discovered uncoupling protein-1 (UCP-1) (1). However, although uncoupling activity has been documented for UCP-2 both *in vitro* (2, 3) and *in vivo* (4), increased thermogenesis seems not to be the major physiological function of UCP-2 (1). It was recently suggested that the uncoupling activity of UCP-2 is physiologically important in modulating the generation of reactive oxygen species (ROS) from mitochondria of certain cell types. For example, it was reported that LPS treatment increased UCP-2 mRNA in lung and stomach (5). In hepatocytes, fatty infiltration, which presumably increases the production of ROS, also greatly increases expression of UCP-2 (6). In macrophages, the production of ROS appears regulated by UCP-2 in conjunction with physiological function. Thus, disruption of the UCP-2 gene increases ROS production from macrophages and, in parallel protects against lethal infections by Toxoplasma Gondi. (7). Presumably, this effect is due to macrophages having acquired a higher than normal killing potential.

Pancreatic β-cells are destroyed by autoimmune, probably cytokine-mediated, assaults (8), leading to diabetes. The mechanisms of defense, or lack of defense of β-cells against toxicity are therefore the subject of intense investigation. It is clear that β-cells display low expression and activity of many of the enzymes involved in antioxidant defense (9, 10). At the same time, there is evidence for a high level of expression of UCP-2 in β-cells (11). Hypothetically, this constellation could signify that a putative antioxidative effect of UCP-2 is of special importance in β-cells. Altogether, these findings warrant studies on the role of UCP-2 for antioxidant defense in β-cells.

We initially screened for an antioxidant role of UCP-2 in β-cells by testing the effect of antioxidants (vitamin E together with selenite) in a clonal β-cell line. Upon finding a reducing effect on UCP-2 expression, we then tested for effects by H_2O_2 on UCP-2 expression. Next, we directly assessed the effect of

Abbreviations used: EGFP, enhanced green fluorescence protein; FCS, fetal calf serum; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide. ROS, reactive oxygen species; UCP, uncoupling protein.

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UCP-2 for β-cell antioxidant defense by testing whether overexpression of UCP-2 protects against H_2O_2-induced toxicity.

MATERIALS AND METHODS

Cell culture and incubations. INS-1 cells (Passage number between 30–60) were obtained from Dr. Claes Wollheim, Geneva, Switzerland. Cells were grown in monolayer cultures as described previously (12) in RPMI-1640 medium containing 11 mmol/1 glucose supplemented with 10 mmol/1 HEPES, 10% heat-inactivated fetal calf serum, 2 mmol/1 L-glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified (5% CO_2, 95% air) atmosphere. Cells were seeded 7 days before use in 25-cm^2 flasks at a density of 1.2×10^6 cells per flask. After 6–7 days (at 60–80% confluence), cells were employed in appropriate test protocols.

MTT assay. As described previously (13), a stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was prepared in phosphate buffered saline (PBS), diluted in RPMI medium and added to cell-containing wells at a concentration of 0.5 mg/ml, 100 μl per well, after first removing additives and medium. The plates were then incubated for 3 h at 37°C in 5% CO_2. Most of the liquid was carefully aspirated, leaving a total volume of 50 μl in each well. One hundred microliters of 2-propanol supplemented with HCl (3.3 ml/l) was added to solubilize the MTT formazan. The plates were then placed on a mechanical shaker for 20 to 60 min at room temperature for complete solubilization. Absorbance was measured on a multi scan plus reader with a 588-nm wavelength filter. All experiments were performed at least twice, each with 16 parallels.

Preparation of pUCP-2 expression vector and transient transfection. A full-length human UCP-2 cDNA in the pCR 2.1 vector (Invitrogen) (a gift of Dr. C. Warden, Rowe Genetics, University of California, Davis, CA) was digested with *Eco*RI to excise the UCP-2 open reading frame fragment. The UCP-2 open reading frame fragment (1060 bp) was then cloned into the *Eco*RI sites of pEGFP-N1 vector (Clontech, CA) to make the recombinant expression plasmid pUCP-2. After first having removed the enhanced green fluorescence protein (EGFP) gene by digesting the pEGFP-N1 vector with *Eco*RI, the correct sequence and orientation of the UCP-2 insert was verified by DNA sequencing. Twenty-four hours before gene transfection, INS-1 cells were replated in six-well tissue culture wells. Three μg recombinant vector pUCP-2, together with 1 μg EGFP vector, was used to co-transfect INS-1 cells using FuGene-6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to recommendations from the manufacturer. Control cells were transfected with 4 μg of EGFP vector. After transfection, cells were cultured for an additional 48 h. High level expression of the UCP-2 cDNA was confirmed by Northern blot analysis. Efficiency of transfection was monitored by co-transfection of the pUCP-2 vector with pEGFP-N1 expressing EGFP protein.

Assay of UCP-2 mRNA. INS-1 cells were seeded 7 days before use in 25-cm^2 flasks at a density of 1.2×10^6 cells per flask. After 6–7 days, (60–80% confluence) cells were incubated with or without 200 μM H_2O_2 for 5 min. Cells were then washed with phosphate-buffered saline (PBS) and subsequently cultured for 30 minutes, 2 h, 6 h or 16 h in RPMI medium containing 11 mM glucose. After culture, cells were washed again with ice-cold PBS, and total RNA isolated using a high pure RNA isolation kit (Roche). Total RNA was electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, blotted onto Hybond N^+ membrane (Amersham, Aylesbury, United Kingdom) by capillary transfer, and crosslinked to the membrane by baking (20 min, at 120°C). Hybridization was carried out overnight in ExpressHyb hybridization solution (Clontech) to the UCP-2 cDNA probe labeled with [^{32}P]dCTP, using a rediprime DNA labeling system kit (Amersham). Membranes were washed five times for 20 min in 2× SSC, 1% SDS at 60°C and twice for 20 min in 0.1× SSC, 0.5% SDS at 50°C. Hybridized blots were placed in a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA) for overnight exposure, and analyzed using a Molecular Dynamics ImageQuant software. Prior to rehybridization, the membranes were stripped of probe by boiling in 0.5% SDS for 10 min followed by an additional 10 min in the hot solution after removing it from heat. Membranes were rehybridized with a human β-actin cDNA probe to control for the amount of RNA present in each lane.

Flow cytometry. Cells subjected to UCP-2 and EGFP transfection procedure as well as cells subjected only to EGFP transfections were washed twice with PBS. Cells were detached by treating with 0.05% EDTA, which was dissolved in PBS, and re-suspended (5.0×10^5/ml) in culture medium containing 11 mM glucose followed by exposure to H_2O_2 (200 μM) at 37°C for 5 min. After aspiration of medium and washing, cells were placed on ice and incubated with 1 μg/ml propidium iodide (PI) for 10 min. The total time elapsed from the exposure to H_2O_2 until the start of measurements varied between 30 and 45 min. Green fluorescence of cells and transfection efficiency were monitored on a Coulter Epics Elike ESP flow cytometer (Coulter Beckman, Hialeah, FL) using a 15-mW argon laser (488 nm), 550-nm dichronic longpass filter and 525 ± 15-nm bandpass filter. Total cell counts for all samples were 10,000. Survival of EGFP overexpressing cells with or without exposure to H_2O_2 were analyzed by flow cytometry using 640-nm longpass filter and 675 ± 15-nm bandpass filter, measuring the fluorescence for PI. Each sample was measured in duplicate. According to the values of forward scatter, side scatter and red fluorescence (PI), debris, aggregates and dead cells could be recognized and were gated out of analysis.

Presentation of results. All results is expressed as the mean ± SE. Significance testing was performed using Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Effects of Vitamin E Together with Selenite on UCP-2 Expression

The expression of UCP-2 in INS-1 cells was decreased by 45 ± 9.1% ($n = 4$, $P < 0.05$) after a 48 h culture with 50 μM vitamin E together with 250 nM sodium selenite.

Effects of H_2O_2 on Cell Viability

Viability was assayed by MTT assay (Fig. 1B). Cell viability decreased by 15, 39, 60, and 61%, respectively, when registered after 30 min, 2 h, 6 h, and 16 h of H_2O_2 exposure.

Effects of H_2O_2 on UCP-2 mRNA Expression

As shown in Fig. 1A, no induction of UCP-2 mRNA was seen 30 min after the exposure to H_2O_2. However, 2 h after exposure, UCP-2 mRNA was increased (1.53 ± 0.15, $P < 0.05$) as well as after 6 h (1.44 ± 0.18, $P < 0.06$) compared to untreated cells. The level of UCP-2 mRNA was only marginally increased 16 h after H_2O_2 exposure (1.12 ± 0.09, NS) compared to cells which had not been exposed to H_2O_2.

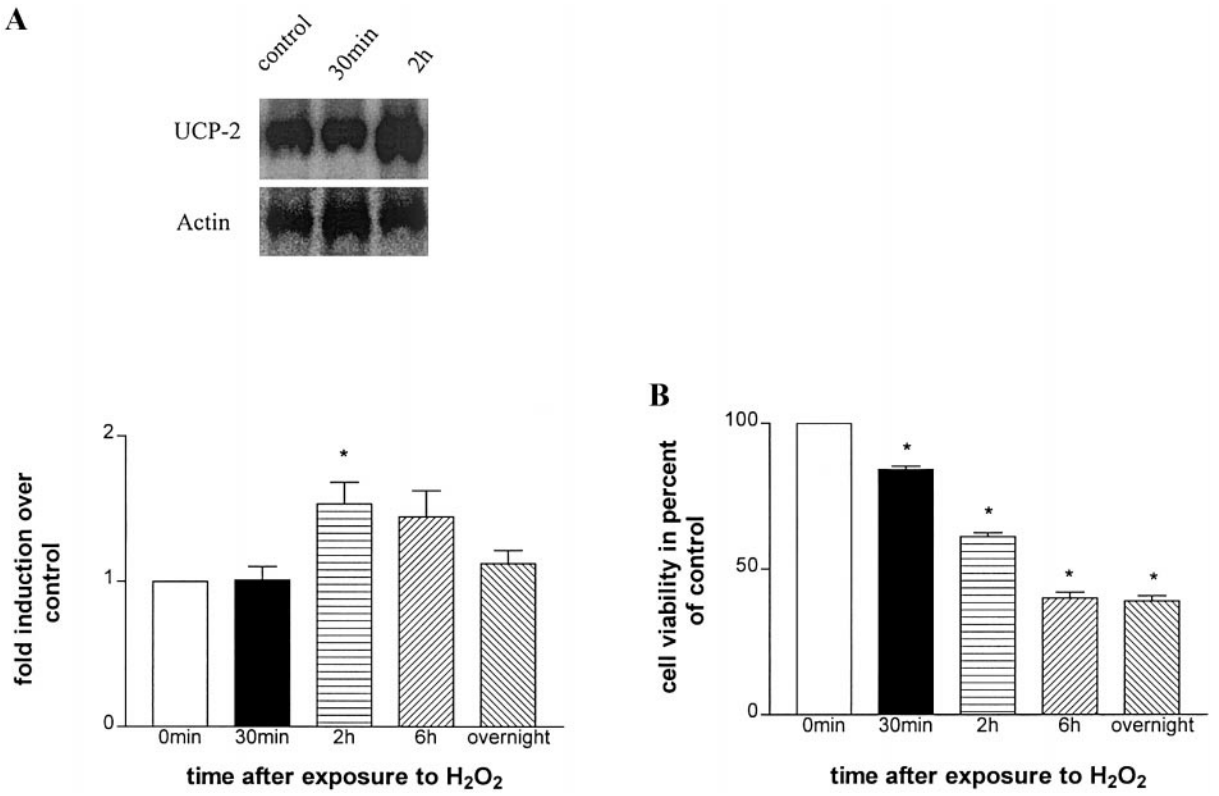

FIG. 1. Effect of H_2O_2 exposure on cell survival and UCP-2 mRNA levels. INS-1 cells were exposed to H_2O_2 (200 μm) for 5 min. After removal of H_2O_2 by washing, the cells were cultured for the time periods indicated in the figure. UCP-2 mRNA transcript was measured by Northern blot hybridization (A) and cells viability was then assayed by MTT assay (B). Ratios of UCP-2/β-actin mRNA band intensity were determined with a phosphorimager. Results are expressed relative to control conditions (*$P < 0.05$ vs control).

Effects of Overexpression of UCP-2 in INS-1 Cells on Toxic Effects of H_2O_2

Cells doubly transfected with EGFP and UCP-2 markedly over-expressed the UCP-2 gene (Fig. 2). With a contribution of 10% transfected cells to the UCP-2 mRNA level of the total number of cells assayed, the degree of overexpression was estimated to be about 30-fold.

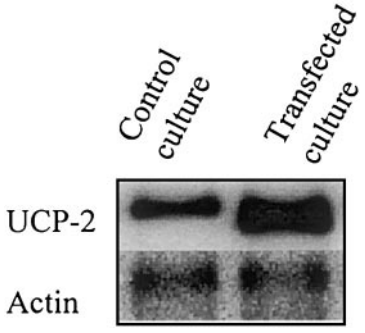

FIG. 2. Overexpression of UCP-2 in INS-1 cells. INS-1 cells were transiently transfected with *pUCP-2* expression vector using Fugene-6. The methods of transfection were described under Materials and Methods. Successful transfection was confirmed by Northern blot analysis.

Overexpression of UCP-2 was accompanied by increased cell survival after H_2O_2 exposure when compared with cells transfected only with EGFP. The protective effect was moderate (13 $\pm$ 0.05%) but statistically highly significant (Table 1).

DISCUSSION

Three findings of the present study support the notion that UCP-2 plays a role in β-cell antioxidant defense. First, exposure to antioxidants (vitamin E, together with selenite) was found to lower UCP-2 expression. Second, exposure to H_2O_2 causes an evanescent but significant rise in UCP-2 mRNA levels. Third, overexpression of UCP-2 is accompanied by protection against H_2O_2-induced toxicity.

The inhibitory effect of antioxidants on UCP-2 expression suggests the presence of tonic oxidative stress in INS-1 cells. Such a notion could explain the high level of expression of UCP-2 that we observe in these cells, a level higher than in pancreatic islets (our unpublished observations). Further studies with antioxidants are however needed to characterize the mechanisms behind the presently recorded effects with Vitamin E together with selenite.

TABLE 1

Effect of UCP-2 Overexpression on Toxicity of H_2O_2

	EGFP overexpressing cells (control)	UCP-2 overexpressing cells	Difference (%)
Percent of survival†	80.25 ± 3.98	93.25 ± 3.68	13.0 ± 0.05*

* $P < 0.005$ vs control.
† Expressed as percentage of cells not exposed to H_2O_2.

Exposure to H_2O_2 was chosen as a model of oxidative stress providing oxygen radicals in the form of H_2O_2 itself and the superoxide anion O_2^-. Exposure to H_2O_2 has recognized toxicity towards β-cells (14, 15). Also, agents which chemically induce diabetes like alloxan and streptozotocin may do so by H_2O_2 production (16).

A lag period of more than 6 h for induction of UCP-2 mRNA was observed in a previous study (6) in which UCP-2 mRNA was induced by recombinant tumor necrosis factor in cultures of primary hepatocytes and in hepatocytes of LPS-treated rats. The difference in time course vis-à-vis our results could be explained by the indirect rather than direct generation of oxygen radicals in the previous study, as well as the continued presence of the toxic agent. Indeed, the evanescent effect on UCP-2 expression that we observe may be secondary to the washout of H_2O_2 that was performed after 5 min of exposure to H_2O_2.

Our study shows, to our knowledge for the first time, that overexpression of UCP-2 is associated with protection from ROS-induced toxicity. The effect observed was moderate but statistically highly significant. Many other antioxidant defense systems are operative in cells, including pancreatic β-cells, (although, as previously mentioned, such systems are probably be less efficient in β-cells than in other cell types). A moderate rather than a dramatic effect on protection by UCP-2 overexpression was therefore to be expected.

One important limitation of the present study, as well as other studies in the field, is the lack of reliable measurements of UCP-2 protein. As recently reviewed (5), the presently available antibodies against the protein are not specific.

A protective effect of UCP-2 against oxidative stress could be of special significance in insulin producing β-cells. Previous work in bioengineering β-cells indicates that protection increases when several antioxidant factors are combined (17). Adding UCP-2 to these factors could, at least theoretically, further enhance the survival of engineered β-cells also under *in vivo* conditions.

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