Haeme-oxygenase 1 expression in rat pancreatic beta cells is stimulated by supraphysiological glucose concentrations and by cyclic AMP

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Abstract

Aim/hypothesis. Increased expression of haeme-oxygenase 1 (HO1) and other antioxidant enzymes could improve pancreatic beta-cell survival under stressful conditions, including hyperglycaemia. However, how hyperglycaemia increases islet HO1 expression is not known.

Methods. Rat islets were pre-cultured for 1 week in RPMI medium containing 10 mmol·l⁻¹ glucose (G10), and further cultured overnight in G5-G30 plus various test substances. Islet HO1 mRNA and protein expression was measured by semiquantitative RT-PCR, western blot, and immunohistochemistry.

Results. Islet HO1 mRNA expression was minimal after overnight culture in G10, slightly increased in G5, and increased by five- to ten-fold in G30 in parallel with a heterogeneous increase in beta-cell HO1 protein expression. The effect of G30 was fully inhibited by agents decreasing cytosolic Ca²⁺ (diazoxide, nimodipine), but was only slightly reproduced by agents raising Ca²⁺ (tolbutamide, 30 mmol·l⁻¹ potassium). It was

also suppressed by the α_2 -adrenoceptor agonist clonidine, whereas dibutyryl-cyclic-AMP largely increased beta-cell HO1 expression. The induction of HO1 mRNA expression by G30 was independent from changes in medium insulin concentration, but was completely inhibited by a cocktail of antioxidants. In contrast to HO1, islet mRNA expression of glutathione peroxidase and constitutive haeme-oxygenase 2 were not affected by G30, nor by dibutyryl-cyclic-AMP.

Conclusion/interpretation. High glucose and dibutyryl-cyclic-AMP stimulate expression of HO1 in rat pancreatic beta cells. The inhibition of HO1 expression in G30 by nimodipine, clonidine, and antioxidants, suggests that Ca²⁺ influx and cyclic-AMP are necessary for the generation of oxidative stress by G30, or for the stimulation of beta-cell HO1 expression by increased oxidative stress. [Diabetologia (2003) 46:1234–1244]

Keywords Antioxidant, cytosolic calcium concentration, gene expression, islet, oxidative stress.

In rodent models of chronic hyperglycaemia, alterations of beta-cell function are associated with beta-cell

Received: 24 January 2003 / Revised: 7 May 2003

Published online: 24 July 2003

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Abbreviations: HO, Heme oxygenase; GPx, glutathione peroxidase; TBP, TATA-box binding protein; Gn, n mmol·l⁻¹ glucose; [Ca²⁺]_i, intracellular Ca²⁺ concentration; cDNA, complementary DNA; mRNA, messenger ribonucleic acid; NF- κ B, nuclear factor of κ B.

hypertrophy and decreased expression of genes involved in beta-cell development and glucose-stimulated insulin secretion (transcription factors, metabolic enzymes, ion channels, insulin) [1, 2, 3, 4]. On the other hand, several genes that are not, or only slightly, expressed in normal beta cells, are induced in islets from hyperglycaemic animals. Some of them, like glucose-6-phosphatase, lactate dehydrogenase A and monocarboxylate transporters, could affect beta-cell function [3, 4]. Others, like the early response gene c-Myc, might contribute to changes in beta-cell differentiation, growth, and survival [3, 5, 6, 7].

In islets from rats submitted to a 90% pancreatectomy, hyperglycaemia also stimulates the expression of

antioxidant enzymes, such as glutathione-peroxidase (GPx) and the inducible isoform of haeme-oxygenase (HO1) [8]. As in other cell types [9, 10], this increase in HO1 and GPx expression could result from an increase in oxidative stress and serve to protect beta cells from this stress. Indeed, overexpression of HO1, GPx, or other antioxidant enzymes in pancreatic beta cells improves their survival and function under various stressful conditions [11, 12, 13, 14, 15, 16, 17, 18]. In view of the important protective role that these enzymes might play in beta cells exposed to chronic hyperglycaemia, we have investigated whether and how glucose affects beta-cell HO1 and GPx expression in cultured rat pancreatic islets.

Materials and methods

Materials. The following drugs were used: diazoxide (kindly provided by Schering-Plough Avondale, Rathdrum, Co. Wicklow, Ireland); nimodipine (Bayer A.G, Leverkusen, Germany); clonidine hydrochloride (Boehringer, Ingelheim, Germany); tolbutamide, dibutyl- γ -cyclic-AMP (2'-O-dibutyladenosine 3':5'-cyclic monophosphate), and aminoguanidine hydrochloride (Sigma, St. Louis, Mo., USA); N-acetyl-L-cysteine (Merck, Darmstadt, Germany), L(+)-ascorbic acid (Janssen Chimica, Beerse, Belgium), and human insulin (Actrapid 100 UI·ml⁻¹, Novo Nordisk, Bagsvaerd, Denmark). Stock solutions of diazoxide (50 mmol·l⁻¹) and tolbutamide (50 mmol·l⁻¹) were prepared in 0.1 mol·l⁻¹ NaOH; nimodipine was dissolved in dimethylsulfoxide at a concentration of 1 mmol·l⁻¹ and diluted 10⁻³ in the culture medium.

Islet isolation. Male Wistar rats were anesthetized by intraperitoneal injection of 100 mg·kg⁻¹ pentobarbital (Nembutal, Abbott Laboratories) and their pancreatic islets were isolated by collagenase digestion of the gland as previously described [5]. They were then separated from the digest by density gradient centrifugation using Histopaque 1077, microdissected, and hand-picked under a stereomicroscope to ensure high purity of the preparation. Experimental procedures were approved by the ethic committee for animal experimentation of the School of Medicine, University of Louvain.

Islet culture. Islets were cultured at 37°C with 5% CO₂ in RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) containing 100 IU·ml⁻¹ penicillin and 100 µg·ml⁻¹ streptomycin, and supplemented with 5 g·l⁻¹ bovine serum albumin (BSA) instead of 10%

fetal calf serum. In most cases, the islets from one pancreas (~250–500) were precultured in 9 ml of medium containing 10 mmol·l⁻¹ glucose (G10) for 1 week, during which the medium was renewed after 1 day, then every other day. Large islets (>200 µm diameter) with signs of central necrosis were discarded. After this week of preculture, the islets were cultured 18 h in batches of 50 to 80 in 1.5 ml of RPMI medium containing G2-G30 and various test substances. At the end of this 18 h culture, a portion of the medium was withdrawn for insulin secretion measurement, and the islets were processed for measurement of gene expression by RT-PCR, Western blot, or immunohistochemistry.

Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]_i). [Ca²⁺]_i was measured by microspectrofluorimetry using Quantal cell 700 m (Visitech, Sunderland, UK), as previously described [19]. At the end of culture, the islets were loaded for 2 to 3 h with 2 µmol·l⁻¹ FuraPE3 acetoxymethylester (Teflabs, Austin, Tex., USA) under the same culture conditions. The islets were then perfused at a flow rate of about 1 ml·min⁻¹ with RPMI medium containing 1 g·l⁻¹ BSA and continuously gassed with O₂:CO₂ (95:5). Fura-PE3 was alternately excited at 340 and 380 nm, and the 510 nm fluorescence emitted from the islets was recorded. The data were automatically corrected for background fluorescence. The [Ca²⁺]_i was calculated by comparing the ratio of the fluorescence at each pixel to a calibration curve based on the equation of Grynkiewicz [20]. The mean [Ca²⁺]_i in the islet was then calculated by averaging the [Ca²⁺]_i at all pixels of the islet.

Islet cDNA synthesis. Some of the PCR were carried out with islet cDNA samples from a previous study on the stimulation of islet c-Myc expression by high glucose concentrations [5]. In this case, at least two additional experiments have been done, resulting in insulin secretion values slightly different from those published previously. Islet total RNA extraction, quantification and reverse-transcription in cDNA were carried out exactly as described [5].

HO1 mRNA half-life determination. After 1 week preculture in RPMI-G10 medium, islets were cultured 18 h in the presence of G10 or G30. Each group was then divided in batches of 45 to 60 islets, further incubated for 0, 40, 80 and 120 min in the same medium supplemented with 5 µg/ml actinomycin D, and processed for RT-PCR measurement of HO1 and TBP mRNA.

Primers. Pairs of primers were designed using HybSimulator 4.0 software (Advanced Gene Computing Technologies, Irvine, Calif., USA). The specificity of sense and antisense primers (Table 1) was checked by BLAST search on Genbank database. For each gene studied, the size of amplicon corresponded to the expected one, based on published sequences.

Table 1. Oligonucleotide primers (sequences and concentrations), and cycling conditions for duplex radioactive PCR amplification of rat islet gene cDNAs

Gene name	Size (bp)	Sense primer	Antisense primer	ratio of gene/TBP primer concentrations (nmol·l ⁻¹)	Annealing (°C)	Cycle number
HO1	408	cac.gca.tat.acc.cgc.tac.ctg	cgg.tgt.ctg.gga.tga.act.agt	400/80	60	25
HO2	378	ttt.act.tca.cat.act.cag.ccc.ttg.a	gct.tga.att.gct.ggg.cat.tgt.c	200/200	62	25
GPx	353	agt.tcg.gac.atc.agg.aga.atg.g	gga.cag.cag.ggc.ttc.tat.atc.g	600/100	60	27
TBP ^a	190	acc.ctt.cac.caa.tga.ctc.cta.tg	atg.atg.act.gca.gca.aat.cgc		60–62	25–27

^a Oligonucleotide primer sequences from [56]

Fig. 1A, B. Duplex radioactive PCR amplification of HO1/TBP islet cDNA. (A) PCR were stopped after 22–30 cycles of amplification, and the products were separated by PAGE. (B) the logarithm of band intensities was plotted against the number of cycles. PCR for experimental samples were stopped within the exponential phase of cDNA amplification (cf Table 1)

Duplex radioactive. PCR Polymerization reactions were done with a Perkin Elmer 9700 Thermocycler in a 25 μ l reaction volume containing 1.5 μ l cDNA (10 ng total RNA equivalents), 80 μ mol·l⁻¹ cold dNTPs, 46 kBq [α -³²P]dCTP (110 TBq·mmol⁻¹, Amersham Pharmacia Biotech, Buckinghamshire, UK), GeneAmp Gold PCR buffer and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, Calif., USA). To allow optimal quantitation of relative islet gene expression, PCR were stopped in the exponential phase of amplification of each product (Fig. 1), as described previously [3, 5]. The thermal cycle profile was a 10-min denaturing step at 94°C to release DNA polymerase activity, followed by 25 to 27 cycles of amplification (1 min denaturation at 94°C, 1 min annealing at 60 to 62°C, 1 min extension at 72°C), and a final extension step of 10 min at 72°C (Table 1). In each set, the gene product of interest was amplified with the TATA-binding protein (TBP) to correct for experimental variations between samples (TBP mRNA expression remained constant under the different experimental conditions). The amplimers were then separated on a 6% PAGE in Tris borate EDTA buffer, the gel was dried and the amount of [α -³²P]dCTP incorporated in each amplimer was quantitated with a Cyclone Storage Phosphor System (Packard, Meriden, Conn., USA). The ratio “gene/TBP” was calculated for each sample, and normalized, within each experiment, to the ratio measured in islets cultured in G10.

Western blot. Equal batches of islets (~300–400) were cultured 18 h under various conditions, washed three times with ice-cold phosphate-buffered saline and precipitated with trichloroacetic acid (100 g·l⁻¹). After double ether extraction, the pellet was dried and solubilized in modified Laemmli buffer (20 g·l⁻¹ SDS, 1/10 (vol/vol) glycerol, 100 mmol·l⁻¹ dithiothreitol, 60 mmol·l⁻¹ Tris (pH 6.8) and 100 mg·l⁻¹ bromophenol blue). Total islet proteins were then separated on a 12 or 15% SDS-PAGE and electrophoretically transferred to PVDF membranes (Dupont NEN, Boston, Mass., USA). On several occasions, se-

rial four-fold dilutions of recombinant HO1 protein with a Mr of 30 (Stressgen, Victoria, Canada) were electrophoresed in parallel with experimental samples. The amount of protein present in each lane was checked by Ponceau S staining, and membranes with unequal protein loading or transfer efficiency were discarded. The membrane was blocked for 2 h at room temperature with 3% non-fat dry milk in Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 (NFDM-TBST). It was then incubated overnight at 4°C with mouse monoclonal anti-HO1 antibody diluted 1/20 000 in NFDM-TBST (Stressgen), washed with TBST and incubated 1 h at room temperature with HRP-conjugated anti-mouse IgG (Fc specific) antibody diluted 1/50 000 in NFDM-TBST. After six washes, detection of bound antibodies was carried out with the Supersignal West Femto kit (Pierce, Rockford, Ill., USA) using Kodak X-AR films. Band intensities were quantitated by scanning densitometry using Image Master Total Lab (Amersham Pharmacia Biotech).

Immunostaining. At the end of culture, the islets were washed, fixed in 1% paraformaldehyde for 4 h at room temperature and routinely embedded in paraffin, then 5- μ m-thick sections were deparaffinized, rehydrated and washed with 0.05 mol·l⁻¹ Tris-HCl-buffered solution (TBS, pH 7.4). After inactivation of endogenous peroxidases by a 30-min incubation in 0.3% H₂O₂, the islet sections were treated with microwaves (citrate buffer pH 5.6, 5×5 min, 700 W), and incubated with 10% normal goat serum (NGS, ICN, USA) in TBS for 30 min. Thereafter, the sections were incubated overnight at 4°C with mouse monoclonal anti-HO1 antibody diluted 1/100. After washes, this antibody was detected by EnVision⁺ peroxidase anti-mouse system (Dako A/S, Glostrup, Denmark). Finally, the peroxidase activity was revealed by immersing the sections in TBS containing 0.05% diaminobenzidine (DAB; Fluka, Germany; brown deposit) and 0.01% H₂O₂ for 10 min. When the primary antibody was omitted or replaced by an unrelated antibody with similar characteristics (monoclonal anti-Digoxigenin antibody, Roche, Germany), no staining was detected on islet sections.

The HO1 DAB-staining was followed by the detection of the non-beta-cells by a fluorescent immunodetection system. After an elution step (0.1 M HCl, 30 min), the sections were incubated with a cocktail of anti-glucagon (Novocastra) and anti-somatostatin (gift from W. Gepts, Brussels, Belgium) rabbit polyclonal antibodies, respectively diluted at 1/100 and 1/200 (overnight at 4°C). These antibodies were detected with a swine anti-rabbit antibody conjugated with fluorescein isothiocyanate (green fluorescent colour; Dako) diluted at 1/20 (2 h at room temperature). After washes, the slides were covered with a fluorescent mounting medium (Vector, Burlingame, Calif., USA), observed with an Axioplan Photomicroscope (Zeiss, Hallbergmoos, Germany). All images were captured with a digital Axiocam camera (Zeiss). The microscope devices and camera settings, related to DAB or fluorescence staining, have been maintained similar through all the experiment.

Insulin determinations. Insulin concentrations in the culture media were determined by radioimmunoassay using rat insulin as a standard.

Data analysis. Shown are means $\pm$ S.E.M. for the indicated number of batches of islets, or representative experiments for two to three independent observations. Statistical significance of differences between groups was assessed by one-way ANOVA followed by a test of Newman-Keuls, or by two-tailed unpaired Student's *t* test when only two groups were com-

pared. Differences were considered significant when $p < 0.05$. For mRNA half-life determination, mathematical analysis of the data was done using Prism 3.02 software (GraphPad Software, San Diego, Calif., USA).

Results

Changes in islet HO1 mRNA expression induced by isolation and culture. The islet HO1/TBP mRNA ratio was low in fresh islets (0.4 ± 0.1 , $n=4$). It was, however, increased by 15- to 30-fold after overnight culture in RPMI medium containing 5, 10, 20 or 30 mmol·l⁻¹ glucose (G5-G30) (6.9 ± 1.0 , 8.5 ± 1.5 , 10.4 ± 1.1 and 11.6 ± 0.9 respectively, $n=4$). After 1 week of culture in the presence of G10, the HO1/TBP mRNA ratio was low again, only slightly higher than in fresh islets (1.06 ± 0.12 , $n=4$, $p < 0.01$). All subsequent experiments were carried out with islets initially cultured for 1 week in RPMI medium containing G10.

Fig. 2A, B. Effect of glucose on islet HO1/TBP mRNA ratio. The islets were precultured for 1 week in RPMI medium (supplemented with 5 g·l⁻¹ BSA instead of 10% FCS) in the presence of G10. Batches of 40–60 islets were either directly processed for total RNA extraction (P), or cultured 18 h in RPMI medium containing 5, 10, 20 or 30 mmol·l⁻¹ glucose, as indicated. At the end of culture, insulin secretion (*open circles*) was measured and islet total RNA was extracted. The HO1/TBP mRNA ratios (*closed circles*) were determined by RT-PCR and expressed relative to the ratio in islets cultured in G10. Shown are representative PCR results (A), and means $\pm$ S.E.M. for seven experiments (B). * $p < 0.01$ or less vs minimal value

Fig. 3A, B. Effect of glucose on islet HO1 mRNA half-life. After 1 week of preculture in G10, the islets were cultured 18 h in RPMI medium containing G10 or G30. Batches of 40–60 islets were then cultured in the same medium supplemented with 5 μ g·ml⁻¹ actinomycin D for the indicated time. Their total RNA was extracted and the decrease in HO1 and TBP mRNAs was determined by RT-PCR. At time 0, HO1/TBP mRNA ratio was increased ~10-fold in G30 vs G10. As islet cyclophilin mRNA expression does not change within 2 h of actinomycin D treatment [5], HO1 and TBP band intensities were divided by cyclophilin intensity at each time point. Each ratio was then expressed in percent of the ratio at time 0. The data were fitted to a one phase exponential decay equation ($Y = \text{Span} \cdot e^{(-K \cdot X)} + \text{Plateau}$ where Span and plateau are fixed at 100 and 0% respectively and half life = $0.69/K$). Data are means $\pm$ S.E.M. for three experiments (B) or representative PCR results (A)

Fig. 4A, B. Effect of glucose and cyclic-AMP on islet HO1 protein expression. After preculture for one week in G10, equal batches of ~300 islets were cultured 18 h in RPMI medium containing G10 or G30 and various test substances, as indicated (1 μ mol·l⁻¹ nimodipine, 1 μ mol·l⁻¹ clonidine or 1 mmol·l⁻¹ dibutyryl-cyclic-AMP). Islet HO1 protein expression was measured by Western blot analysis on a 12% SDS-PAGE, in parallel with 1.25, 5 and 20 ng of recombinant HO1 protein (truncated form with a Mr of 30). Equal protein loading and transfer across lanes were assessed by Ponceau S staining of the membrane (not shown). Blots are representative for two (nimodipine and clonidine effects) or at least three (G30 and cyclic-AMP effects) experiments

Fig. 5A–I. Heterogeneity of HO1 induction by G30 and dibutyl- γ -cyclic-AMP in pancreatic islet cells. Immunohistochemical detection of HO1 (A,D,G) and the non-beta-cell islet hormones glucagon and somatostatin (B,E,H) (both images are superimposed in C,F,I) on sections of islets pre-cultured for 1 week in G10 and further cultured overnight in RPMI medium containing G10 (A–C), G30 (D–F) or G10+1 mmol·l⁻¹ dibutyl- γ -cyclic-AMP (G–I). For each type of staining, islet pictures were taken at the same magnification, using the same exposure parameters. Results are representative for two separate experiments

Effect of glucose on islet HO1 mRNA expression. After preculture of the islets for 1 week in G10, the HO1/TBP mRNA ratio was not affected by a further overnight culture in G10, was slightly, but not significantly ($p>0.05$), increased in G5, and was strongly increased in G20–G30 (Fig. 2A,B). Under these conditions, the islet HO1 mRNA half-life was short and similar after overnight culture in G10 or G30 (85 min [72–105] and 87 min [83–91] respectively, mean and [95% confidence interval], $n=3$) (Fig. 3). As expected,

glucose dose-dependently stimulated insulin secretion during this additional culture period (Fig. 2B; 23 ± 4 -fold increase from G5 to G30).

The validity of duplex radioactive PCR for measuring changes in islet HO1/TBP mRNA ratio was confirmed a posteriori by real-time PCR. In a subset of four experiments comparing three glucose concentrations, the relative changes in islet HO1/TBP mRNA ratio were found to be similar using either real-time PCR or duplex radioactive PCR (data not shown).

Effect of G30 on islet cell HO1 protein expression. After 1-week preculture in G10, a further overnight culture in G30 strongly increased islet HO1 protein expression [Fig. 4A; 8.0 ± 1.4 -fold increase in HO1 band intensity by G30 ($n=3$, $p<0.05$)]. This increase occurred in the cytosol of beta cells located both at the periphery and in the core of the islets, and was highly variable between cells (Fig. 5A–F). Groups of peripheral glucagon- or somatostatin-positive cells (non-beta cells) were not stained by the anti-HO1 antibody under these conditions.

Fig. 6A–F. Effects of $[Ca^{2+}]_i$ - and cyclic-AMP-increasing and -decreasing agents on islet HO1/TBP mRNA ratio. After pre-culture for 1 week in G10, batches of 40–60 islets were cultured 18 h in RPMI medium containing G10 or G30 and various test substances, as indicated [50 $\mu\text{mol}\cdot\text{l}^{-1}$ diazoxide, 100 $\mu\text{mol}\cdot\text{l}^{-1}$ tolbutamide; 1 $\mu\text{mol}\cdot\text{l}^{-1}$ nimodipine, 1 $\mu\text{mol}\cdot\text{l}^{-1}$ clonidine, 1 $\text{mmol}\cdot\text{l}^{-1}$ dibutyl-AMP and 30 $\text{mmol}\cdot\text{l}^{-1}$ KCl + 100 $\mu\text{mol}\cdot\text{l}^{-1}$ diazoxide (K30Dz)]. At the end of culture, insulin secretion was measured and islet total RNA was extracted. The HO1/TBP mRNA ratios are expressed relative to the ratio in islets cultured in G10 alone. Results are means $\pm$ S.E.M. for six–nine experiments. * significant effect of test substance at the same glucose concentration, $p < 0.05$ or less; # significant effect of test substance vs K30Dz, $p < 0.01$

Role of cytosolic Ca^{2+} in the induction of islet HO1 expression by high glucose. Inhibition of glucose-induced Ca^{2+} influx and insulin secretion with either diazoxide or nimodipine fully inhibited the stimulation of HO1 expression by G30 (Fig. 4A, Fig. 6A–D). However, stimulation of Ca^{2+} influx and insulin secretion with tolbutamide or high K^+ only slightly increased the islet HO1/TBP mRNA ratio (Fig. 6A–D).

Effect of clonidine and cyclic-AMP on islet HO1 expression. The stimulation of HO1 expression and insulin secretion by G30 were both suppressed by the α_2 -adrenergic receptor agonist clonidine (Fig. 6E,F, Fig. 4A). These inhibitory effects of clonidine were also observed in the presence of high K^+ (Fig. 6C,D). In contrast, the membrane-permeant dibutyl-AMP strongly increased the islet HO1/TBP mRNA ratio in both G10 and G30, in parallel with insulin secretion (Fig. 6E,F). This effect of dibutyl-AMP, which was not inhibited by clonidine, nor by nimodipine, resulted in a large increase in HO1 protein expression (Fig. 4B). In the presence of G10, dibutyl-AMP increased the HO1 protein band intensity by 13.4 ± 4.6 fold ($n=3$, $p < 0.01$). Again, this

increase mainly occurred in the cytosol of beta cells, and was highly variable from cell to cell (Fig. 5G–I).

Effects of clonidine on islet $[Ca^{2+}]_i$. We next evaluated the possibility that clonidine inhibits islet HO1 expression in the presence of G30 by reducing $[Ca^{2+}]_i$ under these conditions. During perfusion with RPMI medium containing G30, $[Ca^{2+}]_i$ was high (187 ± 3 $\text{nmol}\cdot\text{l}^{-1}$, $n=21$). Under these conditions, clonidine only slightly and transiently reduced the mean islet $[Ca^{2+}]_i$ (Fig. 7A). During perfusion with RPMI medium containing G10 and 100 $\mu\text{mol}\cdot\text{l}^{-1}$ diazoxide, $[Ca^{2+}]_i$ was low (116 ± 3 $\text{nmol}\cdot\text{l}^{-1}$, $n=5$). As expected, membrane depolarization with 30 $\text{mmol}\cdot\text{l}^{-1}$ K^+ triggered a rapid and sustained increase in $[Ca^{2+}]_i$ which was not affected by addition of clonidine (Fig. 7B). After overnight culture and perfusion with 1 $\mu\text{mol}\cdot\text{l}^{-1}$ clonidine, mean islet $[Ca^{2+}]_i$ was similar to that in control islets, during stimulation with either G30 (189 ± 3 and 198 ± 4 $\text{nmol}\cdot\text{l}^{-1}$ with or without clonidine respectively, $n=6$) or high K^+ + diazoxide (174 ± 4 and 177 ± 3 $\text{nmol}\cdot\text{l}^{-1}$ with or without clonidine respectively, $n=12$).

Effect of exogenous insulin on islet HO1 mRNA expression. Addition of 1 $\mu\text{mol}\cdot\text{l}^{-1}$ exogenous insulin to the culture medium did not affect islet HO1 expression in G10, nor its induction by G30 (Fig. 8A,B). Conversely, when G30-induced HO1 expression and insulin secretion were inhibited by nimodipine, the HO1/TBP mRNA ratio was not affected by addition of increasing concentrations of exogenous insulin to the culture medium (Fig. 8C,D).

Effect of antioxidants on islet HO1 mRNA expression. We also tested the effect of a cocktail of antioxidant substances (N-acetyl-L-cystein, aminoguanidine and ascorbic acid) on islet HO1 mRNA expression in the presence of various glucose concentrations ranging from unphysiologically low (G2) to pathologically high (G30). As previously observed (Fig. 2), the islet

Fig. 7A, B. Acute effects of clonidine on islet $[Ca^{2+}]_i$. After 1 week preculture in G10, furaPE3-loaded islets were perfused with RPMI medium containing 1 g.l⁻¹ BSA and **A:** G30 or **B:** G10, 100 $\mu\text{mol.l}^{-1}$ diazoxide and 2.7 (G10Dz) or 30 mmol.l⁻¹ KCl (K30). Clonidine (1 $\mu\text{mol.l}^{-1}$) was then added to the medium for 20–25 min. Traces are means for 21 islets from three different cultures (**A**) or five islets from a single culture (**B**)

Fig. 8A–D. Effect of exogenous insulin on the stimulation of islet HO1 mRNA expression by high glucose and its inhibition by nimodipine. After preculture for 1 week in G10, batches of 50–70 islets were cultured 18 h in RPMI medium containing G10 or G30 with or without 1 $\mu\text{mol.l}^{-1}$ insulin (**A,B**), or in G30+1 $\mu\text{mol.l}^{-1}$ nimodipine and increasing concentrations of insulin (**C,D**). Islet total RNA was extracted for RT-PCR determination of islet HO1/TBP mRNA ratio, and the medium was saved for insulin concentration determination by RIA. Results are means $\pm$ S.E.M. for three–five experiments. * significant effect ($p < 0.05$ or less) of G30 by two-tailed unpaired t-test (**A,B**); * $p < 0.01$ or less vs G10 alone by ANOVA + test of Newmann-Keuls (**C,D**)

Fig. 9. Effect of a cocktail of antioxidants on the stimulation of islet HO1 mRNA expression by low and high glucose. After preculture for 1 week in G10, batches of 70–120 islets were cultured 18 h in RPMI medium containing G2–G30 without (open circles) or with (closed circles) 1 mmol.l⁻¹ ascorbic acid +10 mmol.l⁻¹ N-acetyl-L-cysteine +1 mmol.l⁻¹ aminoguanidine. Islet total RNA was extracted for RT-PCR determination of islet HO1/TBP mRNA ratio. Results are means $\pm$ S.E.M. for three experiments. * $p < 0.01$ or less vs G10

HO1/TBP mRNA ratio was minimal after culture in G10, slightly increased in G5, and significantly increased in G30 ($p < 0.01$). Interestingly, in islets cultured in the presence of G2, the HO1/TBP mRNA ratio was even larger than in islet cultured in G30. In the presence of antioxidants, the stimulation of islet HO1 expression by overnight culture in G30 was completely abrogated, whereas that induced by G2 remained unaffected (Fig. 9).

Lack of effect of glucose and dibutyl-cyclic-AMP on islet heme oxygenase 2 (HO2) and GPx mRNA expression. After one-week preculture in G10, one more night culture in G30 instead of G10 did not affect the islet HO2/TBP mRNA ratio (1.40 ± 0.15 vs 1.37 ± 0.16 in G30 and G10 respectively, $n=5$), nor their GPx/TBP mRNA ratio (0.35 ± 0.1 and 0.32 ± 0.06 in G30 and G10 respectively, $n=5$). Similarly, addition of 1 mmol.l⁻¹ dibutyl-cyclic-AMP in the presence of G10 did not affect the islet HO2/TBP mRNA ratio (1.91 ± 0.23 and 1.73 ± 0.16 with and without dibutyl-cyclic-AMP respectively, $n=4$), nor their GPx/TBP mRNA ratio (0.28 ± 0.01 and 0.23 ± 0.02 with and without dibutyl-cyclic-AMP respectively, $n=4$).

Discussion

HO is the rate-limiting enzyme for the NADPH-dependent degradation of haeme in biliverdin, Fe²⁺ and CO [9]. Biliverdin is further reduced by biliverdin reductase in bilirubin, whereas Fe²⁺ is used for ferritin

synthesis. The resulting decrease in heme and Fe^{2+} concentration, and increase in bilirubin and CO production, could contribute to cellular protection against oxidative or inflammatory stresses [9, 21].

In vivo and in freshly isolated islets, beta cells only express the constitutive HO2 isoform which has been proposed to play a role in the regulation of islet hormone secretion [22, 23]. The expression of HO2 in islets is usually stable [13, 22, 24], with the possible exception of an increase after exposure to lipopolysaccharides [16]. It is therefore not surprising that the islet HO2/TBP mRNA ratio was unaffected by culture in the presence of high glucose concentrations or dibutyl-cyclic-AMP. In contrast, the inducible HO1 isoform is only expressed in beta cells submitted to various stressful stimuli, including islet isolation and culture [13], exposure to cytokines [11, 13, 15, 25], endotoxins [16], hydrogen peroxide [24], and high glucose concentrations [8]. As in other cell types [10], HO1 expression is thought to reflect an increase in beta-cell oxidative stress under these conditions [8, 11, 13, 14, 15, 26].

High glucose and cyclic-AMP stimulate beta-cell HO1 expression. The present study shows that, in the presence of stable concentrations of other nutrients (lipids, amino acids), raising glucose from G10 to G20-G30 rapidly (≤ 18 h) induces rat beta-cell HO1 expression without affecting islet HO1 mRNA half-life. This effect of G30 therefore likely results from transcriptional activation of the *ho1* gene. The islet HO activity was not measured, but is known to correlate well with HO1 protein expression [9, 21]. The results also show that the GPx/TBP mRNA ratio was not affected by G30. A similar dissociation between changes in islet HO1 and GPx expression has been observed in beta cells exposed to cytokines and various agents that stimulate oxidative stress [12, 25], in contrast with the parallel induction of both gene mRNAs in islets from rats submitted to a 90% pancreatectomy [8]. A possible explanation for the different effect of in vivo and in vitro hyperglycaemia on islet GPx mRNA expression might be that fresh islets contain a large number of endothelial cells that do express GPx when exposed to hyperglycaemia [27], whereas cultured islets lose these cells within a few days [28].

Our results also add dibutyl-cyclic-AMP to the long list of substances that increase beta-cell HO1 expression in vitro. A similar effect of cyclic-AMP raising agents has been reported in other cell types, and can likely be ascribed to the presence of a cyclic-AMP-responsive element in the promoter of the *ho1* gene [10]. Interestingly, if this type of agents also stimulate HO1 expression in human beta cells, they could be used, as an alternative to in vitro treatment with hemin or protoporphyrins, to induce HO1 expression and improve beta-cell survival in islet transplantation programs [11, 14]. In this context, it is tempting

to speculate that the anti-apoptotic effect of glucagon-like-peptide-1 and its analog exendin-4 in pancreatic beta cells [29, 30, 31] could be mediated, at least in part, by an increase in beta-cell HO1 expression.

Role of Ca^{2+} influx and exocytosis in the stimulation of islet HO1 expression by G30. Upon glucose stimulation, acceleration of beta-cell metabolism leads to plasma membrane depolarisation, Ca^{2+} influx, and increase in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) that is the triggering signal for insulin secretion [32]. The ability of both diazoxide and nimodipine to abolish the stimulation of HO1 expression by G30 shows the absolute dependence of the effect of G30 on Ca^{2+} influx. However, increasing $[\text{Ca}^{2+}]_i$ [33] and insulin secretion by tolbutamide and high K^+ only weakly increased the islet HO1/TBP mRNA ratio. This shows that neither the stimulation of Ca^{2+} influx nor the associated exocytosis is sufficient to trigger islet HO1 expression. Together with the lack of effect of exogenous insulin on the islet HO1/TBP mRNA ratio, these results also rule out an autocrine effect of beta-cell secretory products on HO1 expression. We conclude that Ca^{2+} influx only plays a permissive role in the stimulation of islet HO1 expression by G30, in contrast with its regulatory role in insulin secretion. Such a permissive role is further supported by the fact that, in rat islets, $[\text{Ca}^{2+}]_i$ is increased upon stimulation from G5 to G10, but little affected between G10 and G30 [34], whereas islet HO1 expression is only stimulated by supraphysiological (above G10) glucose concentrations.

Role of cyclic-AMP in the stimulation of islet HO1 expression by G30. There is no doubt that changes in islet cyclic-AMP content affect islet HO1 expression in a Ca^{2+} -independent manner. Thus, the stimulation of islet HO1 expression by dibutyl-cyclic-AMP persisted in the presence of nimodipine. Conversely, the α_2 -adrenergic receptor agonist clonidine fully prevented islet HO1 induction by G30, even in the presence of high K^+ , conditions under which clonidine did not decrease islet $[\text{Ca}^{2+}]_i$. This suggests that the inhibition of HO1 expression by clonidine does not result from a decrease in $[\text{Ca}^{2+}]_i$ [23], but is rather due to a decrease in cyclic-AMP content [37]. This conclusion is supported by the observation that the inhibitory effect of clonidine was completely abrogated in the presence of dibutyl-cyclic-AMP.

Glucose is known to increase beta-cell cyclic-AMP content by a ill-defined mechanism requiring Ca^{2+} [38]. It is thus possible that the stimulation of islet HO1 expression by G30 results from such an increase in islet cyclic-AMP content. This hypothesis is compatible with the inhibitory effect of both $[\text{Ca}^{2+}]_i$ - and cyclic-AMP-decreasing agents, and with the poor stimulatory effect of $[\text{Ca}^{2+}]_i$ -raising agents alone, on islet HO1 expression.

Possible role of oxidative stress in the stimulation of islet HO1 expression by G30. An increase in HO1 expression is often considered to be a marker of oxidative stress [9, 10, 39]. Inhibition of this increase by a cocktail of antioxidants suggests that this is also the case in islets cultured overnight in G30. Interestingly, these antioxidants also effectively reduced the severity of various alterations of the beta-cell phenotype induced by prolonged exposure to high glucose or D-ribose concentrations [17, 40, 41, 42, 43]. Our results now suggest that the generation of oxidative stress by G30 is a rapid event (≤ 18 h), and that the increase in islet HO1 mRNA expression is one of the earliest and most sensitive, though not specific, marker of beta-cell oxidative stress. In that respect, the heterogeneity in beta-cell HO1 staining might reflect an heterogeneity of rat beta-cell susceptibility to G30-induced oxidative stress. Since the induction of HO1 by G30 was abrogated by diazoxide, nimodipine, clonidine, or antioxidants, it seems that Ca^{2+} and cyclic-AMP are involved either in the generation of oxidative stress by supraphysiological glucose concentrations, or in the stimulation of beta-cell *ho1* gene transcription by oxidative stress.

Possible transcription factors involved in HO1 induction by G30. The HO1 promoter contains consensus binding sites for a large number of transcription factors, e.g. NF- κ B, AP-1, CREB, hypoxia-inducible factor 1, Myc/Max heterodimers, Sp1... [44], some of which could be activated by high glucose and/or oxidative stress [5, 45, 46, 47, 48]. Among them, the nuclear factor of κ B (NF- κ B) is an obvious candidate for mediating the effect of G30 on HO1 expression for several reasons. Firstly, its activation by beta cell exposure to interleukin-1 β induces both HO1 and c-Myc expression [49]. Secondly, the stimulation of islet HO1 expression by hyperglycaemia in islets from rats submitted to a 90% pancreatectomy is accompanied by an increase in NF- κ B binding to its consensus DNA binding site [8]. Thirdly, high glucose concentrations activate NF- κ B in human islets cultured on extracellular matrix, by a mechanism that could involve interleukin-1 β production by, and autocrine effect on, beta cells [50].

Possible significance of the V-shaped glucose-concentration response curve for changes in islet HO1 mRNA expression. The islet HO1/TBP mRNA ratio was minimal after culture in the presence of G10, the optimal glucose concentration for rat beta-cell survival in culture [51, 52]. In contrast, overnight culture in the presence of either very low (G2) or supraphysiological (G20-G30) glucose concentrations induced a large increase in islet HO1 mRNA expression. Such a V-shaped glucose-concentration response curve has previously been observed under similar culture conditions for changes in islet c-Myc expression [5, 53],

and after a few days of culture, for changes in beta-cell death [52]. This raises the possibility of causal relationships between these three phenomena at both ends of the curve. However, it is important to note that the low and high glucose concentrations stimulate beta-cell apoptosis, c-Myc and HO1 expression, by distinct mechanisms. The increase in these three parameters after culture in high glucose concentrations is dependent on an increase in beta-cell $[\text{Ca}^{2+}]_i$, whereas $[\text{Ca}^{2+}]_i$ is known to be low in the presence of low glucose concentrations. Another difference is that antioxidants prevented the effect of high glucose on HO1 induction without influencing that of low glucose. In this respect, the mechanism leading to HO1 induction by low glucose in beta cells is different from that previously reported in human hepatoma HepG2 cells [54], in which stimulation of HO1 expression by glucose deprivation likely results from an increased production of reactive oxygen species by the mitochondrial electron transport chain [55].

In view of the unusual V-shaped glucose-concentration response curve for changes in the expression of several beta-cell gene mRNAs, we strongly suggest that studies addressing the effect of high glucose on beta-cell gene expression include the intermediate glucose concentration of 10 mmol·l⁻¹ in addition to the basal (G3-G5) and high glucose concentrations (G20-G30) usually tested.

In conclusion, in the presence of stable concentrations of other nutrients, supraphysiological glucose concentrations (20–30 mmol·l⁻¹) and dibutyl-AMP increase beta-cell HO1 expression in cultured rat islets. The complete inhibition of G30-induced HO1 expression by either diazoxide, nimodipine, clonidine, or a cocktail of antioxidants, strongly suggests that the generation of oxidative stress by G30, or the effect of oxidative stress on beta-cell *ho1* gene transcription, depends on an increase in $[\text{Ca}^{2+}]_i$ and cyclic-AMP.

Acknowledgements. We thank A. Fiévet and F. Knockaert for expert technical help. This work was supported by Grants 1.5133.00, 3.4552.98 and 3.4594.99 from the Fonds National de la Recherche Scientifique (Brussels), the Interuniversity Poles of Attraction Program (P5/17)-Belgian State, and Grant 00/05-260 from the General Direction of Scientific Research of the French Community of Belgium. J.C.J. is Research Associate of the National Fund for Scientific Research, Brussels, Belgium.

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