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ARTICLE

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High glucose and hydrogen peroxide increase c-Myc and haeme-oxygenase 1 mRNA levels in rat pancreatic islets without activating NFκB

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Abstract *Aims/hypothesis:* Hyperglycaemia and the pro-inflammatory cytokine IL-1β induce similar alterations of beta cell gene expression, including up-regulation of *c-Myc* and *haeme-oxygenase 1*. These effects of hyperglycaemia may result from nuclear factor-kappa B (NFκB) activation by oxidative stress. To test this hypothesis, we compared the effects of IL-1β, high glucose, and hydrogen peroxide, on NFκB DNA binding activity and target gene mRNA levels in cultured rat islets. *Methods:* Rat islets were pre-cultured for 1 week in serum-free RPMI medium ontaining 10 mmol/l glucose, and further cultured in glucose concentrations of 5–30 mmol/l plus various test substances. Islet NFκB activity was measured by ELISA and gene mRNA expression was measured by RT-PCR. *Results:* IL-1β consistently increased islet NFκB activity and c-Myc, haeme-oxygenase 1, inducible nitric oxide synthase (iNOS), Fas, and inhibitor of NFκB alpha (IκBα) mRNA levels. In comparison, 1- to 7-day culture in 30 mmol/l instead of 10 mmol/l glucose stimulated islet c-Myc and haeme-oxygenase 1 expression without affecting NFκB activity or iNOS and IκBα mRNA levels. Fas mRNA levels only increased after 1 week in 30 mmol/l glucose. Overnight exposure to hydrogen peroxide mimicked the effects of 30 mmol/l glucose on haeme-oxygenase 1 and c-Myc mRNA levels without activating NFκB. On the other hand, the antioxidant *N*-acetyl-L-cysteine inhibited the stimulation of haeme-oxygenase 1 and c-Myc expression by 30 mmol/l glucose and/or hydrogen peroxide. *Conclusions/interpretation:* In contrast to IL-1β, high glucose and hydrogen peroxide do not activate NFκB in cultured rat islets. It is suggested that the stimulation of islet c-Myc and haeme-oxygenase 1 expression by 30 mmol/l glucose results from activation of a distinct, probably oxidative-stress-dependent signalling pathway.

Keywords Antioxidant · Glucose toxicity · NFκB · Oxidative stress · Pancreatic beta cell

Abbreviations GSIS: Glucose-stimulated insulin secretion · HO: Haeme oxygenase · H_2O_2: Hydrogen peroxide · IκBα: Inhibitor of κB alpha · iNOS: inducible nitric oxide synthase · NAC: *N*-acetyl-L-cysteine · NFκB: Nuclear factor of kappa B chain · PKCβ$_2$: Protein kinase C β$_2$ · TBP: TATA-box binding protein

Introduction

Type 2 diabetes results from the combination of insulin resistance and defective glucose-stimulated insulin secretion (GSIS) [1, 2]. This beta cell dysfunction seems to result from a moderate reduction in beta cell mass [3, 4] and profound functional alterations of the remaining beta cells [5, 6]. In contrast, type 1 diabetes is characterised by selective immune destruction of beta cells and subsequent lack of insulin secretion [7].

Chronic hyperglycaemia induces phenotypic alterations of rodent beta cells, including GSIS abnormalities, reduced expression of specific genes (loss of beta cell differentiation), hypertrophy, slight increase in apoptosis, and increased expression of genes normally repressed in fully differentiated beta cells [6, 8–14]. These phenotypic alterations may contribute to the progressive worsening of GSIS in type 2 diabetes [15, 16]. In comparison, in vitro exposure to inflammatory cytokines involved in the pathogenesis of type 1 diabetes, e.g. IL-1β, induces similar alterations of the beta cell phenotype [7, 17, 18]. These effects of IL-1β largely result from receptor-mediated activation of the transcription factor nuclear factor of kappa B chain (NFκB) [19, 20].

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Among the genes induced by IL-1β and supraphysiological glucose concentrations, the transcription factor c-Myc stimulates apoptosis more than proliferation in rodent beta cells [21–23], whereas the antioxidant enzyme haeme-oxygenase 1 (HO1) improves beta cell survival under various stressful conditions [17, 24–26]. In vitro, overnight culture of rat islets in the presence of 30 mmol/l glucose (G30) instead of 10 mmol/l glucose (G10) markedly stimulated expression of HO1 and c-Myc at the mRNA and protein levels in a Ca^{2+}- and cyclic AMP-dependent manner without affecting c-Myc or HO1 mRNA stability [27, 28]. Of note, culture in glucose concentrations lower than G10 (G2–G5), a stressful condition for rat beta cells, also stimulated islet HO1 and c-Myc expression. These V-shaped glucose concentration response curves for changes in islet c-Myc and HO1 expression with a minimum at G10 are similar to that of beta cell death [29]. Whereas the stimulation of beta cell apoptosis by prolonged culture in low glucose may be due to activation of AMP-dependent protein kinase and stimulation of c-Myc expression under these conditions [23, 30], it has been suggested that the deleterious effects of supraphysiological glucose concentrations on the beta cell phenotype result from NFκB activation by oxidative stress [12, 31–34] or by induction of production and autocrine actions of IL-1β [35].

The aim of this study was to evaluate the role of NFκB activation in the changes of beta cell gene expression by high glucose concentrations. Therefore, we tested the effects of high glucose on both NFκB DNA binding activity and the mRNA levels of HO1, c-Myc and other NFκB target genes in cultured rat islets. These effects were compared to those of IL-1β, a positive control for NFκB activation and stimulation of NFκB target gene expression, and to those of hydrogen peroxide (H_2O_2), a positive control for oxidative stress. The effects of glucose and H_2O_2 were tested with and without addition of the antioxidant *N*-acetyl-L-cysteine (NAC).

Materials and methods

Materials Human IL-1β was kindly provided by Dr. R.E. Aurigemma (National Cancer Institute Biological Resources Branch Preclinical Repository, Rockville, MD, USA), and diluted, aliquoted and stored as recommended. NAC, H_2O_2 and aminoguanidine hydrochloride were purchased from Merck (Darmstadt, Germany), Acros Organics (Geel, Belgium) and Sigma (St. Louis, MO, USA) respectively. The inhibitors of p38MAPK (SB203580) and of ERK activation (PD98059) were purchased from Campro scientific (Veenendaal, The Netherlands) and Research Biochemical International (Natick, MA, USA) respectively.

Islet isolation and culture Male Wistar rats (180–200 g) were obtained from the animal facility of the Faculty of Medicine at the University of Louvain (Brussels, Belgium). Their pancreatic islets were isolated by collagenase digestion of the gland, separated from the digest by density gradient centrifugation, and handpicked under a stereomicroscope [28]. They were then pre-cultured for 1 week at 37°C in serum-free RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing G10 and 5 g/l BSA (fraction V, Roche, Basel, Switzerland), 100 IU/ml penicillin, and 100 µg/ml streptomycin in the presence of 5% CO_2. Large islets (>150- to 200-µm diameter) that frequently develop central necrosis were discarded. After preculture, the islets were further cultured in the same medium containing 5 mmol/l glucose (G5), G10 or G30 and various test substances. This test period lasted from 15 min up to 8 days, so that the total duration of culture never exceeded 15 days. In all cases, the medium was renewed after 1 day, then every other day. At the end of the culture, a portion of the medium was withdrawn for insulin secretion measurement, and the islets were processed for measurement of NFκB DNA binding activity or mRNA levels.

All experiments were conducted in accord with accepted standards of humane animal care and were approved by the Institutional Committee on Animal Experimentation from the Faculty of Medicine of the University of Louvain.

Measurement of islet NFκB DNA binding activity After culture, batches of 100 islets were washed three times in ice-cold PBS (10 mmol/l phosphate buffer, pH 7.5, 150 mmol/l NaCl), followed by rapid centrifugation for 4 min at 1,000 g. The TransAM NFκB ELISA kit (Active Motif, Rixensart, Belgium) was then used for whole islet cell extract preparation and measurement of NFκB (p65) DNA-binding activity according to the manufacturer's instructions. A 30-min exposure to 50 IU/ml IL-1β in the presence of G10 was used as a positive control condition for islet NFκB activation. The TransAM NFκB ELISA kit was validated by comparing its results with those obtained by electrophoretic mobility shift assay using an NFκB consensus oligonucleotide [36]. Comparable results were obtained with both methods in insulin-producing RINm5 cells exposed to IL-1β for 30 min. Moreover, the signal intensity of NFκB activation detected by the ELISA kit was proportional to the number of IL-1β-treated cells in RINm5 cells, INS-1 cells and primary beta cells (25, 50 and 100×10^3 cells) (data not shown).

RNA extraction and cDNA synthesis Islet total RNA was extracted and reverse transcribed into cDNA, as previously described [27, 28], using random hexamers and 200 units of M-MLV Reverse Transcriptase RNase H^- Point Mutant (Promega, Madison, WI, USA).

Duplex radioactive PCR TATA-box binding protein (*TBP*) was used as a housekeeping gene. The islet *HO1* and *c-Myc:TBP* mRNA ratios were determined by simultaneous amplification of *c-Myc* or *HO1* plus *TBP* islet cDNAs using a validated radioactive hot-start PCR protocol. The primer sequences, reaction conditions and PCR validation experiments have been reported previously [27, 28]. The PCR products were separated on a 6% PAGE and the amount of [α-^{32}P] dCTP incorporated in each amplimer was quantified with a Cyclone Storage Phosphor System (Packard, Meriden, CT, USA). The ratio "gene/TBP" was calculated

for each test condition, and normalised to the same ratio in control islets cultured in G10 within the same experimental series. Relative to total RNA, TBP mRNA levels remained constant under our different experimental conditions, unless otherwise specified.

Real-time fluorescent PCR Real-time PCR was performed with the iCycler iQ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA), using the fluorescent dye SYBR green I (Molecular Probe, Leiden, The Netherlands) [37]. cDNA amplifications were performed in duplicates in a 25-µl reaction volume containing 12.5-µl iQ Supermix (Bio-Rad), cDNA (0.25–16 ng total RNA equivalents) or water, 300 nmol/l sense and antisense primers, SYBR Green I at a 10^{-5} dilution, and 10 nmol/l fluorescein for initial well-to-well fluorescence normalisation. The thermal cycle profile was a 3-min denaturing step at 95°C to release DNA polymerase activity followed by 40 cycles of amplification, each composed of a 15-s denaturation step at 95°C, a 45- to 60-s annealing step at 60–62°C, and an eventual 15-s step at 82–83°C (Table 1). After amplification, the specificity of PCR products was verified by melting curve analysis [38], and the threshold cycle was determined using iCycler iQ software 3.0a (Bio-Rad). Under these conditions, PCR efficiencies ranged from 0.95 to 1.0. Within each PCR, "gene" mRNA levels were determined by comparison with a standard curve prepared by serial 4-fold dilutions of an appropriate control cDNA (Table 1), and expressed relative to the mRNA levels in control islets cultured in G10.

Primers Except for Fas primers [12], pairs of primers were designed using Hybsimulator 4.0 software (Advanced Gene Computing Technologies, Irvine, CA, USA). The specificity of sense and antisense primers (Table 1) was checked by BLAST search on the Genbank database. For each gene studied, the size of the amplicon corresponded to the expected one, based on published sequences.

Measurement of insulin secretion Insulin concentrations in the culture media were assayed by radioimmunoassay using rat insulin as a standard [39].

Data analysis Results are means±SEM for the indicated number of independent experiments. Statistical analysis was performed using unpaired Student's *t*-test when only two groups were compared, one-way ANOVA followed by a Newman–Keuls test, or two-way ANOVA as indicated. Differences were considered significant at a p value of less than 0.05.

Results

Time-dependent effect of high glucose on islet HO1 and c-Myc mRNA levels After 1-week preculture in the presence of G10, further culture in G30 instead of G10 induced a time-dependent increase in islet HO1 and c-Myc/TBP mRNA ratios that persisted for at least up to 3 days (Fig. 1 and Table 2, series 1). These increases were also observed in freshly isolated islets cultured for 1 week in G30 instead of G10 (Table 2, series 2). Significant increases in islet HO1 and c-Myc/TBP mRNA ratios were also induced by a 6-h exposure to IL-1β in the presence of G10 (Table 2).

Effect of high glucose on NFκB (p65) DNA binding activity After 1-week preculture in G10, islet NFκB (p65) DNA binding activity was not significantly affected by further culture in G5, G10 or G30 for 15, 30 or 60 min (data not shown, n=3), 1–24 h, and 1–8 days (Fig. 2a and b). Under these conditions, glucose markedly stimulated insulin secretion in a concentration-dependent manner (Fig. 2c and d; two-way ANOVA, $p<0.001$ or less for the glucose effect, $p>0.2$ for the time effect). Contrasting with the absence of a glucose effect, a 30-min exposure to IL-1β in the presence of G10 strongly and consistently activated NFκB DNA binding activity in these islets ($p<0.01$ or less

Table 1 Sequences of oligonucleotide primers and reaction conditions for real-time PCR, and characteristics of PCR products

Gene	5′-Sense primer-3′	5′-Antisense primer-3′	Input[a]	Standard curve cDNA[b]	Annealing (°C–s)	Extension (°C–s)	Size (bp)	Tm (°C)
Fas	TGA.GCC.TTG.CAC.ACG.AAC	CAC.AAC.AGC.CCC.CAA.GAT	16	Control islets	62–45	82–15	322	85.0
iNOS	TGA.CCT.GAA.AGA.GGA.AAA.GGA	CAC.GTG.AGG.ATC.AAA.AAC.TGG	16	IL-1β treated islets	60–45	83–15	200	88.0
IκBα	TGG.TCT.CGC.TCC.TGT.TGA.AG	GTA.AGC.TGG.TAG.GGG.GAG.TAG	4	IL-1β treated islets	62–45	82–15	76	85.0
ProIL-1β	GAT.GAT.GAC.GAC.CTG.CTA.GTG.T	GTT.GGC.TTA.TGT.TCT.GTC.CAT.TGA.G	16	LPS-rat liver	62–60	none	142	84.5
TBP	ACC.CTT.CAC.CAA.TGA.CTC.CTA.TG	ACT.TCG.TGC.CAG.AAA.TGC.TGA	4–16	variable	variable	variable	157	85.5

Tm melting temperature
[a]Islet sample cDNA input per tube (ng total RNA equivalent)
[b]The standard curve cDNA was either prepared from control precultured islets eventually treated for 6 h with 50 IU/ml IL-1β, or prepared from the liver of a rat infected with lipopolysaccharide (LPS-rat) [58]

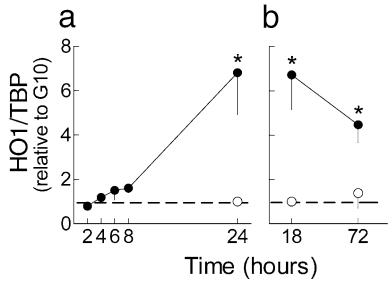

Fig. 1 Time-dependent effect of 30 mmol/l glucose on islet HO1 mRNA levels. After 1-week preculture in 10 mmol/l glucose (G10), rat islets were cultured for 2–24 h (**a**) or 18 and 72 h (**b**) in G10 or 30 mmol/l glucose (G30) (*open or closed circles* respectively). At the end of culture, the islet HO1/TBP mRNA ratio was determined and expressed relative to the ratio in islets cultured for 24 h (**a**) or 18 h (**b**) in G10 (*dotted line*). Data are means±SEM for four independent experiments. *p<0.05 or less vs islets cultured in G10

at each time point, n=3). Similar results were obtained using RPMI medium containing 10% heat-inactivated calf serum instead of BSA for preculture and culture of the islets (data not shown).

Effects of high glucose on islet mRNA levels of various NFκB-dependent genes In the experiments in which islet c-Myc and HO1/TBP mRNA ratios were significantly increased by G30 and IL-1β (Table 2), TBP, iNOS and IκBα mRNA levels were not significantly affected by glucose, whereas Fas mRNA levels were slightly decreased after overnight culture in G30, and significantly increased after 1 week under these conditions (Table 3). In comparison, a 6-h exposure to IL-1β in the presence of G10 consistently increased islet Fas, iNOS and IκBα without affecting TBP mRNA levels.

Effects of antioxidants on islet HO1 and c-Myc mRNA induction by G30 After 1-week preculture in G10 and further culture for 18 h in G10 or G30, the addition of a combination of 10 mmol/l NAC and 1 mmol/l aminoguanidine to the medium decreased the islet HO1/TBP mRNA ratio in G10, and completely abrogated its increase by G30. These effects were reproduced by NAC alone, whereas aminoguanidine reduced the islet HO1/TBP mRNA ratio in both G10 and G30 by about 40%, without affecting its relative increase by G30 (Fig. 3a). In another series of experiments, in which rat islets were cultured overnight in the presence of increasing NAC concentrations, the increase in the islet HO1/TBP mRNA ratio produced by G30 was unaffected by 0.01–0.1 mmol/l NAC, but was maximally inhibited by 1–10 mmol/l NAC (n=3, p<0.05 vs G30 alone, data not shown). In a subsequent series of experiments, 1 mmol/l NAC abrogated and reduced by about 50% the stimulatory effect of G30 on islet HO1 and c-Myc/TBP mRNA ratios respectively (Fig. 3b and c).

Effects of hydrogen peroxide on islet HO1 and c-Myc mRNA levels After 1-week preculture in G10 and further culture for 18 h in G10 plus increasing concentrations of H_2O_2, the islet HO1 and c-Myc/TBP mRNA ratios were unaffected by 0.2–1 μmol/l H_2O_2, but were increased by 5 μmol/l H_2O_2 (n=3, p<0.01 vs G10 alone, data not shown). Higher concentrations of H_2O_2 (10–25 μmol/l) induced macroscopical alterations of the islets, reduced the amount of total RNA recovered per islet, and decreased the mRNA expression of TBP relative to the input of cDNA (data not shown). These alterations prevented reliable determination of changes in islet HO1 and c-Myc mRNA levels after islet treatment with a high concentration of H_2O_2. In a subsequent series of experiments, the stimulatory effect of 5 μmol/l H_2O_2 on islet HO1 and c-Myc mRNA levels was synergistic with that of G30, and was abrogated by 1 mmol/l NAC (Fig. 3b and c).

Effects of H_2O_2 on islet NFκB (p65) DNA binding activity and mRNA levels of various NFκB-dependent genes After 1-week preculture in G10, further culture for 15 min to 18 h in the presence of G10 and 5 μmol/l H_2O_2 did not stimulate islet NFκB (p65) DNA binding activity, in contrast to a 30-min exposure to 50 IU/ml IL-1β (Fig. 4). Accordingly, islet Fas and IκBα mRNA levels were not, or only slightly, increased by culture for 18 h in the presence of 5 μmol/l H_2O_2. Under these conditions, islet iNOS mRNA levels were increased to a much lesser extent by H_2O_2 than by IL-1β (~3-fold vs ~700-fold increase respectively) (Table 3, series 1).

Table 2 Comparison of the effects of 30 mmol/l glucose and IL-1β on islet HO1 and c-Myc/TBP mRNA ratios (relative to 10 mmol/l glucose)

Gene	Experimental series 1			Experimental series 2	
	G10 (18 h), n=8	G30 (18 h), n=8	G10+IL-1β (6 h), n=3	G10 (1 week), n=6–7	G30 (1 week), n=6–7
HO1/TBP	1.00±0.16	5.10±1.16[a]	2.16±0.46[a]	1.00±0.27	3.55±0.59[a]
c-Myc/TBP	1.00±0.22	3.99±0.39[a]	5.11±0.62[a]	1.00±0.19	2.52±0.61[a]

In series 1, rat islets were first precultured in G10 as described above, before being further cultured for 18 h in the presence of G10 or G30, or treated with 50 IU/ml IL-1β for the last 6 h of culture in G10 (G10+IL-1β). In series 2, immediately after isolation, rat islets were cultured for 1 week in the presence of G10 or G30. At the end of culture, islet c-Myc and HO1/TBP mRNA ratios were determined by duplex radioactive RT-PCR and expressed relative to the ratio measured in islets cultured in G10. Data are means±SEM for the indicated number of independent observations

G10 10 mmol/l glucose, *G30* 30 mmol/l glucose

[a] p<0.05 or less vs islets cultured in G10 by unpaired t-test

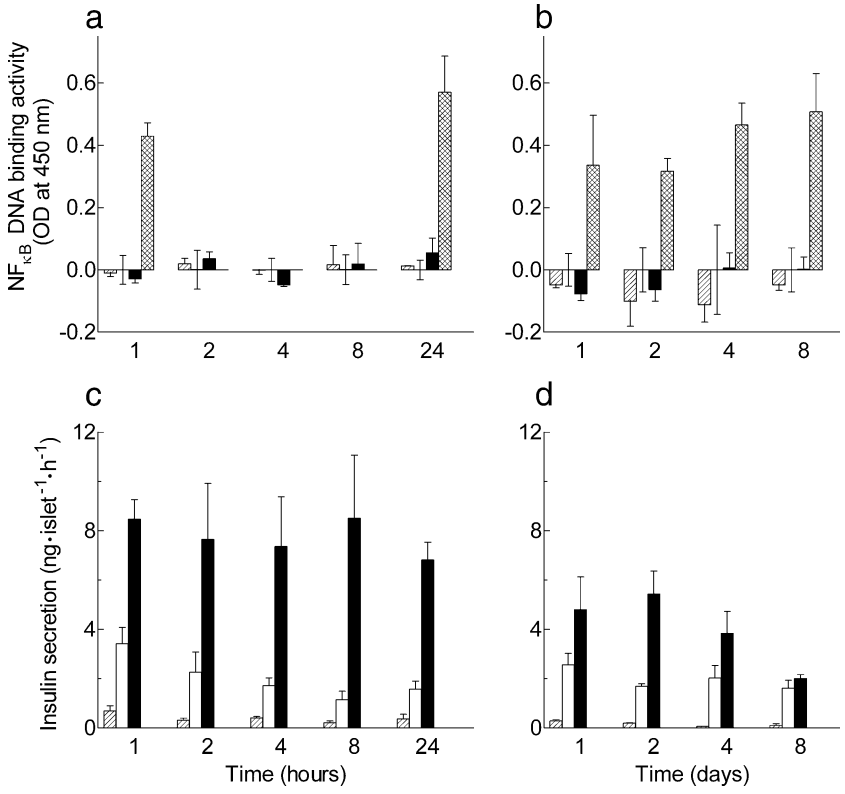

Fig. 2 Time-dependent effect of glucose on NFκB (p65) DNA binding activity and insulin secretion in cultured rat islets. After preculture in 10 mmol/l glucose, rat islets were cultured for 1–24 h (**a**, **c**) or 1–8 days (**b**, **d**) in the presence of 5 mmol/l glucose (G5, *hatched columns*), 10 mmol/l glucose (G10, *open columns*) or 30 mmol/l glucose (G30, *filled columns*). At several time points, islets cultured in G10 were treated for the last 30 min with 50 IU/ml IL-1β (G10+IL-1β, *cross-hatched columns*). Cellular extracts (**a**, **b**) were prepared, and the DNA binding activity of the p65 subunit of NFκB was measured by an ELISA as described above. Within each experiment, the absorbance value obtained for islets cultured in G10 was subtracted from the absorbance value for other groups of islets; results from islets cultured in G10 are shown as 0±SEM (*second column at each time point*). At the end of culture, insulin concentration (**c**) was measured in the medium by RIA and the rate of secretion per hour of culture was calculated. **d**. Hourly rate of insulin secretion over the last 2 days of culture. Results are means±SEM for three independent experiments

Table 3 Comparison of the effects of 30 mmol/l glucose, IL-1β and hydrogen peroxide on islet mRNA levels of TBP and various NFκB target genes (relative to 10 mmol/l glucose)

Gene	Experimental series 1			Experimental series 2
	G30/G10 (18 h), n=8[a]	G10+IL-1β/G10 (6 h), n=3	G10+H_2O_2 /G10 (18 h), n=4	G30/G10 (1 week), n=6–7
TBP	1.13±0.04	1.1±0.2	0.98±0.07	1.00±0.07
Fas	0.81±0.11[b]	7.7±1.2[b,c]	1.16±0.20	2.66±0.35[b]
iNOS[d]	1.00±0.36	718±112[b,c]	2.86±0.77[b]	1.36±0.34
IκBα	1.14±0.12	13.9±9.7 [c]	1.48±0.10[b]	0.97±0.22
proIL-1β	0.68±0.21[b]	7.2±2.5[c]	0.74±0.32	1.02±0.30

In series 1, rat islets were first precultured in G10 as described above, before being further cultured for 18 h in the presence of G10, G30 or G10+5 μmol/l H_2O_2 (G10+H_2O_2), or treated with 50 IU/ml IL-1β for the last 6 h of culture in G10 (G10+IL-1β). In series 2, immediately after isolation, rat islets were cultured for 1 week in the presence of G10 or G30. Islet TBP, Fas, iNOS, IκBα and proIL-1β mRNA levels were determined by real-time RT-PCR and expressed relative to the mRNA levels in islets cultured in G10 for 18 h (three left columns) or 1 week (right column). Data are means±SEM for the indicated number of independent observations

G10 10 mmol/l glucose, *G30* 30 mmol/l glucose, H_2O_2 hydrogen peroxide

[a] n=6 for iNOS that was below the detection limit in two out of eight cDNAs from islets cultured in G30

[b] Relative change in gene mRNA levels significantly different from that in TBP mRNA levels in the same samples, $p<0.05$ or less by unpaired t-test

[c] Significant effect of IL-1β vs G30 and H_2O_2 on the relative change in gene mRNA levels, $p<0.05$ or less by one-way ANOVA+Newman–Keuls test

[d] iNOS mRNA expression was very low in islets cultured for 18 h in G10 or G30, with mean±SE threshold cycle values of 29.1±0.6 and 30.4 ±0.5 respectively

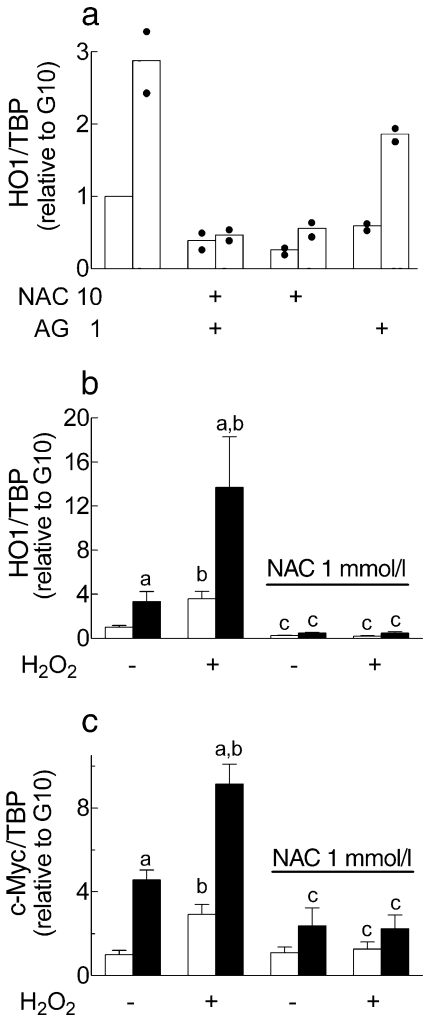

Fig. 3 Effect of antioxidants and hydrogen peroxide on the stimulation of islet HO1 and c-Myc mRNA expression by 30 mmol/l glucose. **a** After preculture in 10 mmol/l glucose (G10), rat islets were cultured for 18 h in the presence of G10 (*open columns*) or 30 mmol/l glucose (G30, *grey columns*) with or without 10 mmol/l *N*-acetyl-L-cysteine (NAC) and 1 mmol/l aminoguanidine (AG) as indicated. **b, c** The islets were cultured for 18 h in G10 (*open columns*) or G30 (*filled columns*) with or without 5 µmol/l hydrogen peroxide (H_2O_2) in the presence or absence of 1 mmol/l NAC. At the end of culture, islet total RNA was extracted and the HO1 and c-Myc/TBP mRNA ratios, determined by duplex RT-PCR, were expressed relative to the ratio in islets cultured in G10. Data are means for two experiments plus individual data points (**a**) or means±SEM for three independent experiments (**b**, **c**). [a]Significant effect of G30 ($p \leq 0.05$); [b]significant effect of H_2O_2 ($p \leq 0.05$); [c]significant effect of NAC ($p \leq 0.05$)

Effect of high glucose, H_2O_2 and IL-1β on proIL-1β mRNA expression After 1-week preculture, islet proIL-1β mRNA levels were not increased by further culture in G30 or G10+5 µmol/l H_2O_2. In contrast, a 30-min exposure to 50 IU/ml IL-1β markedly increased islet proIL-1β mRNA levels (Table 3).

Effect of p38MAPK and ERK pathway inhibition on islet HO1 and c-Myc mRNA expression After 1-week preculture, the addition of an inhibitor of p38MAPK (SB203580) or of ERK activation (PD98059) to the culture medium did not affect glucose stimulation of insulin secretion during culture, but reduced by about 50% the stimulation of islet HO1 (and to a lesser extent c-Myc) mRNA expression by G30 as compared to G10 (Fig. 5).

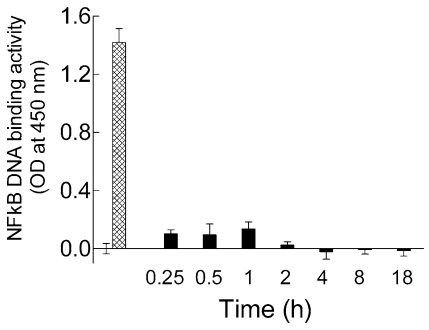

Fig. 4 Effect of hydrogen peroxide on islet NFκB (p65) DNA binding activity. After preculture, rat islets were cultured for 15 min up to 18 h in the presence of 10 mmol/l glucose (G10) and 5 µmol/l hydrogen peroxide (*filled columns*). Control islets cultured in G10 were treated with 50 IU/ml IL-1β for the last 30 min of culture (G10 +IL-1β, *cross-hatched column*). Cellular extracts were prepared, and the DNA binding activity of the p65 subunit of NFκB was measured as described above. Within each experiment, the absorbance value obtained for islets cultured in G10 was subtracted from the absorbance value for other groups of islets; results from islets cultured in G10 are shown as 0±SEM (on the *left* of G10+IL-1β data). Data are means±SEM for four independent experiments

Discussion

This study was performed with rat islets precultured for 1 week in serum-free RPMI medium containing G10. This preculture is routinely used in our lab because it preserves glucose stimulus-secretion coupling events and islet cell morphology [40] while allowing islet recovery from the stress of isolation [27, 28]. As such, it is an important condition to detect an effect of high glucose on islet expression of stress-induced genes such as *c-Myc* and *HO1*, two genes that are early markers of alterations induced by high glucose in beta cell gene expression [11, 12, 27, 28].

Our results demonstrate that the in vitro stimulation of rat islet HO1 and c-Myc mRNA expression by supraphysiological glucose occurs without activation of the p65 subunit of NFκB, which has been previously shown to be the crucial component of the activated NFκB complex in pancreatic beta cells [41, 42]. Accordingly, islet mRNA expression of NFκB target genes such as *iNOS* and *IκBα* were not affected by overnight and 1-week culture in G30 as compared to G10, whereas Fas was increased after 1 week only. These results make it unlikely that other NFκB subunits (p50, c-Rel) migrate to the nucleus and compensate for the lack of p65 activation under these culture conditions. In contrast with G30, IL-1β consistently and markedly increased islet NFκB DNA binding activity and mRNA expression of all NFκB target genes tested under our culture conditions, in agreement with studies on the effects of IL-1β in purified rat beta cells [17, 19, 20]. Therefore, our results do not support our previous hypoth-

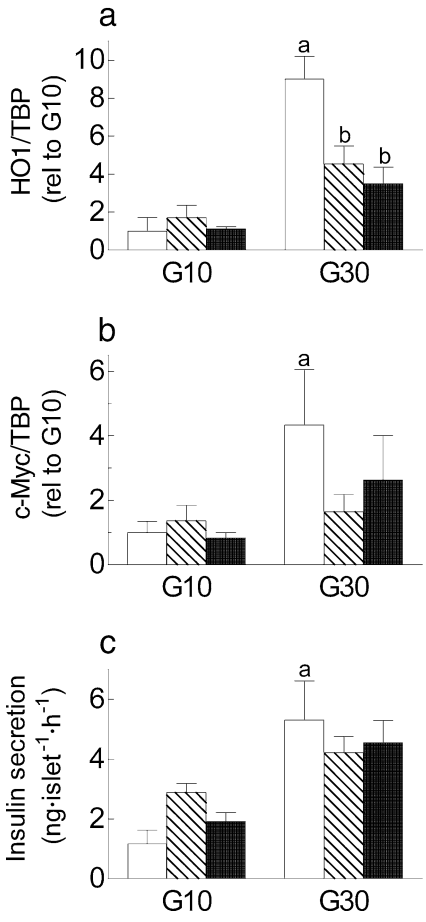

Fig. 5 Effect of inhibitors of p38MAPK and ERK activation on the stimulation of islet HO1 and c-Myc mRNA expression by 30 mmol/l glucose. After preculture, rat islets were cultured for 18 h in the presence of 10 mmol/l glucose (G10) and 30 mmol/l glucose (G30) alone (*open columns*), G10 and G30 with 10 μmol/l SB203580 (*hatched columns*), or G10 and G30 with 50 μmol/l PD98059 (*filled columns*). At the end of culture, islet total RNA was extracted and the HO1 (**a**) and c-Myc/TBP (**b**) mRNA ratios, determined by duplex RT-PCR, were expressed relative to the ratio in islets cultured in G10. At the end of culture, insulin concentration (**c**) was measured in the medium by RIA and the rate of secretion per hour of culture was calculated. Data are means±SEM for three independent experiments. [a]Significant effect of G30 ($p<0.05$); [b]significant effect of test agent in G30 ($p<0.05$)

esis that the stimulation of islet HO1 and c-Myc expression by high glucose might result from NFκB activation [28].

It could be argued that we failed to detect an effect of glucose on rat islet NFκB DNA binding activity because this activation is transient. However, islet HO1 and c-Myc expression were maximally induced after 18–24 h and remained elevated after several days of culture in G30, whereas islet NFκB activity was not increased after 15 min up to 8 days of culture in high glucose. It could also be argued that the sensitivity of the assay was too low. However, glucose did not affect islet mRNA levels of iNOS and IκBα, two NFκB target genes induced about 700- and 15-fold by IL-1β. It is therefore unlikely that high glucose would activate islet NFκB without inducing a detectable increase in iNOS and IκBα mRNA levels. Finally, the lack of glucose effect on islet NFκB DNA binding activity was confirmed in rat islets exposed to G30 instead of G10 for 1–4 days in serum-containing RPMI, and in FACS-purified rat beta cells (attached to poly-lysine-coated dishes) exposed to high glucose for 12–24 h, thereby excluding the possibility that our results are due to the absence of serum or lack of islet cell attachment during culture. In the latter type of experiments, NFκB activation was evaluated by immunofluorescent determination of the cytoplasmic and nuclear localisation of NFκB, as previously described [43]. Thus, after 12–24 h of culture in G28 vs G5–G10, NFκB remained localised in the cytoplasm, whereas it was mainly localised in the nucleus after IL-1β treatment (cytoplasmic localisation in 99.6±0.4 and 99.2±0.5% of the cells cultured for 24 h in G10 and G30 respectively, $n=3$, $p>0.05$; nuclear localisation in 95.6±2.2 vs 0.4±0.4% of the cells exposed to IL-1β vs controls, $n=3$, $p<0.001$).

Interestingly, hydrogen peroxide reproduced the effect of G30 on islet HO1 and c-Myc mRNA expression without activating NFκB, while the antioxidant NAC reduced or abrogated these effects of G30 and/or hydrogen peroxide. Although an increase in production of reactive oxygen species was not directly demonstrated under our particular culture conditions, these data nevertheless suggest that oxidative stress is involved in the stimulation of islet HO1 and c-Myc mRNA expression by hyperglycaemia, but through the activation of transcription factor(s) other than NFκB. Although it is usually accepted that oxidative stress activates NFκB, such a causal link has been recently challenged [44]. Besides NFκB DNA binding sites, rat *hmox1* and *c-Myc* gene promoters contain consensus sequences for binding of other transcription factors activated by oxidative stress, i.e. AP1, CREB, ATF4, HIF, SRE, etc. [45, 46]. In this regard, while the stimulation of c-Myc expression by high glucose could result from the activation of PKCβ$_2$ [47], our preliminary results indicate that the stimulation of islet HO1 expression may involve glucose activation of both p38MAPK and ERK [48].

A role of oxidative stress in the stimulation of islet HO1 and c-Myc expression by G30 is compatible with a recent study showing that culture in high glucose increases reactive oxygen species production in rat pancreatic islets [33]. It is also compatible with earlier reports on the beneficial effect of antioxidants, including NAC, on beta cell glucose toxicity in models of type 2 diabetes [32, 33, 49, 50]. It should be noted, however, that very high NAC or cysteine concentrations can be detrimental (pro-oxidant?) to pancreatic beta cells [51].

It has recently been reported that NFκB activation and subsequent stimulation of beta cell apoptosis in isolated human islets cultured for a few days in high glucose result from an increase in beta cell production of IL-1β with autocrine effects [35]. These observations, together with similarities between the effects of cytokines and high glucose on beta cell gene expression, have led to the proposal that type 2, like type 1 diabetes, is a chronic inflammatory disease [52]. Our finding that NFκB activity (using the same ELISA kit as in [35]) is not affected by

glucose in cultured rat islets markedly contrasts with these results. Another difference with regard to the observations in human islets is that proIL-1β mRNA expression by rat islets was unaffected by G30 or hydrogen peroxide although it was induced by IL-1β. The stimulatory effect of IL-1β on its own expression was not reproduced in FACS-purified rat beta cells [53] (Liu and Eizirik, unpublished data), and could thus occur in non-beta cells of the islet. Whatever the cell type involved in this response, these results indicate that, under our experimental conditions, G30 did not stimulate IL-1β release by rat islets.

The reasons for the discrepancy between our and the above-mentioned studies are unclear. We consider the species difference (rat vs human) an unlikely explanation, because prolonged culture in G30 instead of G10 induces similar alterations of glucose-stimulated-insulin secretion in rat and human islets [9, 10, 40]. Moreover, in three well characterised human islet preparations containing more than 50% insulin-positive beta cells, culture in 28 instead of 5.6 mmol/l glucose for 2 or 7 days failed to induce mRNA expression of the NFκB target genes *Fas* and *IL-1β*, indicating that NFκB was not activated in human islets under these culture conditions (Welsh et al., preliminary observations). Of note, Fas and IL-1β mRNAs were detected at 30- and 100-fold higher levels respectively in stimulated human dendritic cells. Regarding the differences in culture conditions, we have shown that glucose does not activate rat islet NFκB in various culture systems (with or without serum, floating or attached whole islets, dispersed cells attached to poly-lysine coated dishes). Our current hypothesis is that culturing human islets spread on an extracellular matrix [35] permits long-term survival/proliferation of non-endocrine cells that inevitably contaminate human islet preparations [54]. It is possible that hyperglycaemia activates the p65 subunit of NFκB, in a manner dependent on oxidative stress, in duct cells, endothelial cells, or fibroblasts [55–57]. In contrast, our culture of floating islets in serum-free medium allows optimal survival of endocrine cells in the absence of contaminating cell types, as judged by optical and electron microscopy of islet sections [40]. The conserved islet structure (floating round islets with central beta cells and peripheral non-beta cells) should also preserve beta cell interactions with their neighbouring beta cells and with components of the extracellular matrix [40]. Further studies are definitely required before one can conclude that one culture system is more appropriate than the other for the in vitro study of islet cell (patho)physiology.

In conclusion, supraphysiological glucose concentrations stimulate c-Myc and HO1 expression without activating NFκB in cultured rat islets. This strongly suggests that IL-1β receptor-mediated activation of NFκB is not involved in the functional alterations of rat and human beta cells cultured under similar conditions [9, 10, 40]. Data from our present and previous studies [27, 28] on the stimulation of rat islet c-Myc and HO1 expression by G30 are summarised in Fig. 6. Altogether, these results suggest that the stimulation of islet c-Myc and HO1 mRNA expression by high glucose results from the activation of a signalling pathway that is dependent on Ca^{2+}, cyclic AMP and oxidative stress, and independent of NFκB activation. The respective role and potential overlap of these pathways in high-glucose-induced alterations of the beta cell phenotype remain to be clarified.

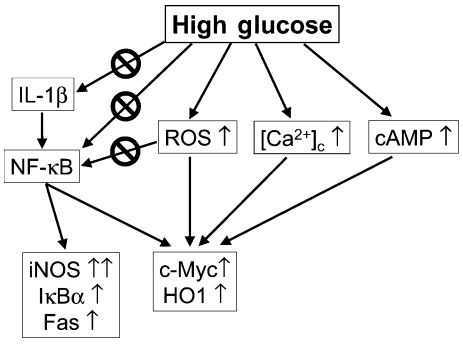

Fig. 6 Pathways involved in the stimulation of rat islet HO1 and c-Myc expression by high glucose concentrations. The stimulation of rat islet c-Myc and HO1 expression by 30 mmol/l glucose is inhibited by substances that reduce Ca^{2+} influx (diazoxide and nimodipine), and by the α_2-adrenergic agonist clonidine, which inhibits adenylate cyclase in beta cells [27, 28]. It is also inhibited by the antioxidant *N*-acetyl-L-cysteine (this study). The increase in c-Myc and HO1 expression, which can be reproduced by membrane permeant dibutyryl cyclic AMP and by low concentrations of hydrogen peroxide, occurs in the absence of IL-1β production or NFκB activation. In contrast, IL-1β markedly activates rat islet NFκB and strongly increases islet mRNA levels of iNOS, IκBα and Fas. Protein kinases (PKCβ_2, p38MAPK, ERK, etc.) potentially involved in these pathways are not depicted on this scheme. ROS, reactive oxygen species. $[Ca^{2+}]_c$ Cytosolic Ca^{2+} concentration, *cAMP* cyclic AMP

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