

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 interleukin-1 β (IL-1 β) induce similar alterations of beta-cell gene expression, including up-regulation of c-Myc and haeme-oxygenase 1 (HO1). These effects of hyperglycaemia may result from nuclear factor-kappa B (NF κ B) activation by oxidative stress. To test this hypothesis, we compared the effects of IL-1 β , high glucose, and hydrogen peroxide (H₂O₂), on NF κ B 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 containing 10 mmol/l glucose (G10), and further cultured in G5-G30 plus various test substances. Islet NF κ B activity was measured by ELISA and gene mRNA expression was measured by RT-PCR.

Results. IL-1 β consistently increased islet NF κ B activity and c-Myc, HO1, inducible NO synthase (iNOS), Fas, and inhibitor of NF κ B alpha (I κ B α) mRNA levels. In comparison, 1-7 day culture in G30 instead of G10 stimulated islet c-Myc and HO1 expression without affecting NF κ B activity or iNOS and I κ B α mRNA levels. Fas mRNA levels only increased after 1 week in G30. Overnight exposure to H₂O₂ mimicked G30 effects on HO1 and c-Myc mRNA levels without activating NF κ B. On the other hand, the antioxidant N-acetyl-L-cysteine inhibited the stimulation of HO1 and c-Myc expression by G30 and/or H₂O₂.

Conclusions/interpretation. In contrast with IL-1 β , high glucose and H₂O₂ do not activate NF κ B in cultured rat islets. It is suggested that the stimulation of islet c-Myc and HO1 expression by G30 results from activation of a distinct, likely oxidative stress-dependent, signaling pathway.

Keywords: antioxidant, glucose toxicity, NF κ B, oxidative stress, pancreatic beta-cell.

Abbreviations: IL-1 β , interleukin-1 beta ; HO, haeme oxygenase; NF κ B, nuclear factor of kappa B chain; I κ B α , Inhibitor of κ B alpha; Gn, n mmol/l glucose; TBP, TATA-box binding protein; ROS, reactive oxygen species; [Ca²⁺]_c, cytosolic Ca²⁺ concentration; PKC β ₂, protein kinase C β ₂.

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 characterized 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, i.e. interleukin-1 β (IL-1 β), induces similar alterations of the beta-cell phenotype [7,17,18]. These effects of IL-1 β largely result from receptor-mediated activation of the transcription factor NF κ B [19,20].

Among the genes induced by both IL-1 β 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 stimuli [17,24-26]. *In vitro*, overnight culture of rat islets in the presence of 30 mmol/l glucose (G30) instead of G10 markedly stimulated expression of HO1 and c-Myc at the mRNA and protein levels in a Ca²⁺- 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 those of hydrogen peroxide (H₂O₂), a positive control for oxidative stress. The effects of glucose and H₂O₂ were tested with and without addition of the antioxidant N-acetyl-L-cysteine (NAC).

MATERIALS AND METHODS

Materials : The following drugs were used : human interleukin-1 beta (IL-1 β), kindly provided by Dr. R. E. Aurigemma (National Cancer Institute Biological Resources Branch Preclinical Repository, Rockville, MD, USA), was diluted, aliquoted and stored as recommended. N-Acetyl-L-Cysteine, hydrogen peroxide 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 hand-picked under a stereomicroscope [28]. They were then pre-cultured for one week at 37°C in serum-free RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) containing 10 mmol/l glucose (G10) and 5 g/l bovine serum albumin (BSA fraction V, Roche, Basel, Switzerland), 100 IU.ml⁻¹ penicillin, and 100 μ g.ml⁻¹ streptomycin in the presence of 5% CO₂. Large islets (>150-200 μ m diameter) that frequently develop central necrosis were discarded. After preculture, the islets were further cultured in the same medium containing 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 NF κ B 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 thrice in ice-cold Phosphate-Buffered-Saline (10 mmol/l phosphate buffer, pH 7.5, 150 mmol/l NaCl), followed by rapid centrifugation for 4 min at 1000 x 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 following manufacturer instructions. A 30 min exposure to 50 U.l⁻¹ 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 x 10³ 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 Corporation).

Duplex radioactive PCR. TATA-box binding protein (TBP) was used as a house keeping gene. The islet HO1 and c-Myc to 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-³²P] dCTP incorporated in each amplicon was quantified with a Cyclone Storage Phosphor System (Packard, Meriden, CT). The ratio “gene/TBP” was calculated for each test condition, and normalized 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 were performed with the iCycler iQ Real Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.), using the fluorescent dye SYBR green I (Molecular Probe, Leiden, The Netherlands) [37]. cDNA amplifications were performed in duplicates in a 25 μ l reaction volume containing 12.5 μ l 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 normalization. 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 sec denaturation step at 95°C, a 45-60 sec annealing step at 60-62°C, and an eventual 15 sec 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). The specificity of sense and antisense primers (Table 1) was checked by BLAST search on 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 $\pm$ S.E.M. 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 when $P < 0.05$.

RESULTS

Time-dependent effect of high glucose on islet HO1 and c-Myc mRNA levels. After one 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 series 1 in Table 2). These increases were also observed in freshly isolated islets cultured for 1 week in G30 instead of G10 (series 2 in Table 2). Significant increases in islet HO1 and c-Myc/TBP mRNA ratios were also induced by a 6 h exposure to IL-1 β in the presence of G10 (Table 2).

Effect of high glucose on NF κ B (p65) DNA binding activity. After one week preculture in G10, the islet NF κ B (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 to 24 h, and 1 to 8 days (Fig. 2A,B). Under these conditions, glucose markedly stimulated insulin secretion in a concentration-dependent manner (Fig. 2C,D)(Two-way ANOVA: $p < 0.001$ or less for the glucose effect, $p > 0.2$ for the time effect). In contrast with the absence of glucose effect, a 30 min exposure to IL-1 β in the presence of G10 strongly and consistently activated NF κ B DNA-binding activity in these islets ($P < 0.01$ or less 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 NF κ B-dependent genes. In the experiments in which islet c-Myc and HO1/TBP mRNA ratios were significantly increased by G30 and IL-1 β (Table 2), TBP, iNOS and I κ B α mRNA levels were not significantly affected by glucose, whereas Fas mRNA levels were slightly decreased after overnight culture in G30, and significantly increased after one week under these conditions (Table 3). In comparison, a 6 h exposure to IL-1 β in the presence of G10 consistently increased islet Fas, iNOS and I κ B α without affecting TBP mRNA levels.

Effects of antioxidants on islet HO1 and c-Myc mRNA induction by G30. After one week preculture in G10 and further culture for 18 h in G10 or G30, addition of a combination of 10

mmol/l N-acetyl-L-cysteine (NAC) and 1 mmol/l amino guanidine (AG) 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 AG reduced by ~40% the islet HO1/TBP mRNA ratio in both G10 and G30 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 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 respectively abrogated and reduced by ~50% the stimulatory effect of G30 on islet HO1 and c-Myc/TBP mRNA ratios (Fig. 3B,C).

Effects of hydrogen peroxide (H₂O₂) on islet HO1 and c-Myc mRNA levels. After one week preculture in G10 and further culture for 18 h in G10 plus increasing concentrations of H₂O₂, the islet HO1 and c-Myc/TBP mRNA ratios were unaffected by 0.2-1 μ mol/l H₂O₂, but were significantly increased by 5 μ mol/l H₂O₂ ($n=3$, $P<0.01$ vs G10 alone, data not shown). Higher concentrations of H₂O₂ (10-25 μ mol/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 H₂O₂. In a subsequent series of experiments, the stimulatory effect of 5 μ mol/l H₂O₂ on islet HO1 and c-Myc mRNA levels was synergistic with that of G30, and was abrogated by 1 mmol/l NAC (Fig. 3B,C).

Effects of H₂O₂ on islet NF κ B (p65) DNA binding activity and mRNA levels of various NF κ B-dependent genes. After one week preculture in G10, further culture for 15 min to 18 h in the presence of G10 and 5 μ mol/l H₂O₂ did not stimulate islet NF κ B (p65) DNA binding activity, in contrast to a 30 min exposure to 50 U/ml IL-1 β (Fig. 4). Accordingly, islet Fas and I κ B α mRNA levels were not, or only slightly, increased by culture for 18 h in the presence of 5 μ mol/l H₂O₂. Under these conditions, islet iNOS mRNA levels were increased

to a much lesser extent by H₂O₂ than by IL-1 β (~3-fold vs ~700-fold increase respectively) (series 1 in Table 3).

Effect of high glucose, H₂O₂ and IL-1 β on proIL-1 β mRNA expression. After one week preculture, islet proIL-1 β mRNA levels were not increased by further culture in G30 or G10 + 5 μ mol/l H₂O₂. In contrast, a 30 min exposure to 50 UI.ml⁻¹ IL-1 β markedly increased islet proIL-1 β mRNA levels (Table 3).

Effect of p38MAPK and ERK pathway inhibition on islet HO1 and c-Myc mRNA expression. After one week preculture, addition of an inhibitor of p38MAPK (SB203580) or ERK-activation (PD 98059) to the culture medium did not affect glucose stimulation of insulin secretion during culture, but reduced by ~50% the stimulation of islet HO1 (and to a lesser extent c-Myc) mRNA expression by G30 (Fig. 5).

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 high glucose-induced alterations 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 one week culture in G30 as compared to G10, whereas Fas was increased after one 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 hypothesis 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 18-24h 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 I κ B α , two NF κ B target genes induced ~700- and ~15-fold by IL-1 β . It is therefore unlikely that high glucose would activate islet NF κ B without inducing a detectable increase in iNOS and I κ B α mRNA levels. Finally, the lack of glucose effect on islet NF κ B DNA binding activity was confirmed in rat islets exposed to G30 vs 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, NF κ B activation was evaluated by immunofluorescent determination of the cytoplasmic and nuclear localization of NF κ B, as previously described [43]. Thus, after 12-24 h of culture in 28 vs 5 and 10 mmol/l glucose, NF κ B remained localized in the cytoplasm, whereas it was mainly (>95%) localized in the nucleus after IL-1 β treatment (cytoplasmic localization in 99.6 ± 0.4 and 99.2 ± 0.5 % of the cells cultured for 24h in G10 and G30 respectively, $n=3$, $P>0.05$; nuclear localization in 95.6 ± 2.2 vs. 0.4 ± 0.4 % of the cells exposed to IL-1 β 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 NF κ B, while the antioxidant NAC reduced or abrogated these effects of G30 and/or hydrogen peroxide. Although an increase in ROS production 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 NF κ B. Although it is usually accepted that oxidative stress activates NF κ B, such a causal link has been recently challenged [44]. Besides NF κ B 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 PKC β_2 [47], our preliminary results indicate that the stimulation of islet HO1 expression may involve glucose activation of both p38 MAPK 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], and 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 reference [35]) is not affected by glucose in cultured rat islets markedly contrasts with these results. Another difference with 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 D and Eizirik DL, 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 of 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]. More conclusively, in 3 well characterized 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 (N. Welsh, I. Kharroubi, M. Cnop, D.L. Eizirik, preliminary observations). Of note, Fas and IL-1 β mRNAs were detected at respectively 30 and 100-fold higher levels in stimulated human dendritic cells. Regarding the differences in culture conditions, we have shown that glucose does not activate rat islet NF κ B 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 might be in duct cells, endothelial cells, or fibroblasts that hyperglycaemia activates the p65 subunit of NF κ B in an oxidative stress-dependent manner [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 neighboring beta-cells and with components of the extracellular matrix [40]. Further studies are definitely required before one could conclude that one or the other culture system is more appropriate for the in vitro study of islet cell (patho)physiology.

In conclusion, supraphysiological glucose concentrations stimulates c-Myc and HO1 expression without activating NF κ B in cultured rat islets. This strongly suggests that IL-1 β receptor-mediated activation of NF κ B 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 summarized 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 Ca²⁺, cyclic AMP and oxidative stress-dependent signaling pathway independent from NF κ B activation. The respective role and potential overlap of these pathways in high-glucose induced alterations of the beta-cell phenotype remains to be clarified.

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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-sec)	Extension (°C-sec)	Size (bp)	T _m (°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-1 β treated islets	60-45	83-15	200	88.0
I κ B α	TGG.TCT.CGC.TCC.TGT.TGA.AG	GTA.AGC.TGG.TAG.GGG.GAG.TAG	4	IL-1 β treated islets	62-45	82-15	76	85.0
ProIL-1 β	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

^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 6h with 50 UI/ml IL-1 β , or prepared from the liver of a lipopolysaccharide-injected rat (LPS-rat)[58].

Table 2 : Comparison of the effects of G30 and IL-1 β on islet HO1 and c-Myc to TBP mRNA ratios.

<i>gene to TBP mRNA ratio (relative to G10)</i>					
gene	Experimental series 1			Experimental series 2	
	G10	G30	G10 + IL-1 β	G10	G30
	(18 h)	(18 h)	(6 h)	(1 week)	(1 week)
	n=8	n=8	n=3	n=6-7	n=6-7
HO1/TBP	1.00 $\pm$ 0.16	5.10 $\pm$ 1.16*	2.16 $\pm$ 0.46'	1.00 $\pm$ 0.27	3.55 $\pm$ 0.59*
c-Myc/TBP	1.00 $\pm$ 0.22	3.99 $\pm$ 0.39*	5.11 $\pm$ 0.62'	1.00 $\pm$ 0.19	2.52 $\pm$ 0.61*

In series 1, rat islets were first precultured in G10 as described under “Methods”, before being further cultured for 18h in the presence of G10 or G30, or treated with 50 UI/ml IL-1 β for the last 6 h of culture in G10 (G10 + IL-1 β). 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 $\pm$ S.E.M. for the indicated number of independent observations. *, $P < 0.05$ or less vs islets cultured in G10 by unpaired t-test.

Table 3 : Comparison of the effects of G30, IL-1 β and H₂O₂ on islet mRNA levels of TBP and various NF κ B target genes.

gene	<i>mRNA levels (relative to G10)</i>			
	Experimental series 1			Experimental series 2
	G30/G10	G10 + IL-1 β /G10	G10 + H ₂ O ₂ /G10	G30/G10
	(18h) n=8 ^a	(6h) n=3	(18 h) n=4	(1 week) n=6-7
TBP	1.13 $\pm$ 0.04	1.1 $\pm$ 0.2	0.98 $\pm$ 0.07	1.00 $\pm$ 0.07
Fas	0.81 $\pm$ 0.11 ^b	7.7 $\pm$ 1.2 ^{b,c}	1.16 $\pm$ 0.20	2.66 $\pm$ 0.35 ^b
iNOS ^d	1.00 $\pm$ 0.36	718 $\pm$ 112 ^{b,c}	2.86 $\pm$ 0.77 ^b	1.36 $\pm$ 0.34
I κ B α	1.14 $\pm$ 0.12	13.9 $\pm$ 9.7 ^c	1.48 $\pm$ 0.10 ^b	0.97 $\pm$ 0.22
proIL-1 β	0.68 $\pm$ 0.21 ^b	7.2 $\pm$ 2.5 ^c	0.74 $\pm$ 0.32	1.02 $\pm$ 0.30

In series 1, rat islets were first precultured in G10 as described under “Methods”, before being further cultured for 18h in the presence of G10, G30 or G10 + 5 μ mol/l H₂O₂ (G10 + H₂O₂), or treated with 50 UI/ml IL-1 β for the last 6 h of culture in G10 (G10 + IL-1 β). In series 2, immediately after isolation, rat islets were cultured for 1 week in the presence of G10 or G30. Islet TBP, Fas, iNOS, I κ B α and proIL-1 β mRNA levels were determined by real-time RT-PCR and expressed relative to the mRNA levels in islets cultured in G10 for 18 h (3 left columns) or 1 week (right column). Data are means $\pm$ S.E.M. for the indicated number of independent observations; ^a, n=6 for iNOS that was below detection limit in 2 out of 8 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-1 β vs G30 and H₂O₂ on the relative change in gene mRNA levels, $P < 0.05$ or less by one way ANOVA + test of Newman-Keuls. ^d, iNOS mRNA expression was very low in islets cultured for 18h in G10 or G30, with mean $\pm$ S.E. Ct values of 29.1 $\pm$ 0.6 and 30.4 $\pm$ 0.5 respectively.

LEGENDS TO FIGURES

Figure 1 : Time-dependent effect of G30 on islet HO1 mRNA levels. After 1 week preculture in G10, rat islets were cultured for 2 to 24h (panel a) or 18 and 72 h (panel b) in 10 or 30 mmol/l glucose (G10 or G30, open or closed circles respectively). At the end of culture, islet HO1/TBP mRNA ratio was determined and expressed relative to the ratio in islets cultured for 24 h (panel a) or 18 h (panel b) in G10 (dotted line). Data are means $\pm$ S.E.M. for 4 independent experiments. *, $P < 0.05$ or less vs islets cultured in G10.

Figure 2 : Time-dependent effect of glucose on NF κ B (p65) DNA binding activity (A, B) and insulin secretion (C, D) in cultured rat islets. After preculture in G10, rat islets were cultured for 1 to 24 h (panels a and c) or 1 to 8 days (panels b and d) in the presence of G5 (hatched columns), G10 (open columns) or G30 (filled columns). At several time points, islets cultured in G10 were treated for the last 30 min with 50 IU/ml IL-1 β (G10 + IL-1 β , double hatched columns). Panels a and b, cellular extracts were prepared, and the DNA binding activity of the p65 subunit of NF κ B was measured by an ELISA as described under "Methods". 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 $0 \pm$ S.E.M (second column at each time point). Panel c, at the end of culture, insulin concentration was measured in the medium by RIA and the rate of secretion per hour of culture was calculated. Panel d, hourly rate of insulin secretion over the last two days of culture. Results are means $\pm$ S.E.M. for 3 independent experiments.

Figure 3: Effect of antioxidants and hydrogen peroxide (H₂O₂) on the stimulation of islet HO1 and c-Myc mRNA expression by G30. Panel a, after preculture in G10, rat islets were cultured for 18 h in the presence of G10 (open columns) or G30 (gray columns) with or without 10 mmol/l N-acetyl-L-cysteine (NAC) and 1 mmol/l aminoguanidine (AG) as indicated. Panels b and c, the islets were cultured for 18h in G10 (open columns) or G30

(filled columns) with or without 5 $\mu\text{mol/l}$ 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 2 experiments plus individual data points (panel a) or means $\pm$ S.E.M. for 3 independent experiments (panels b and c). ^a, significant effect of G30; ^b, significant effect of H_2O_2 ; ^c, significant effect of NAC.

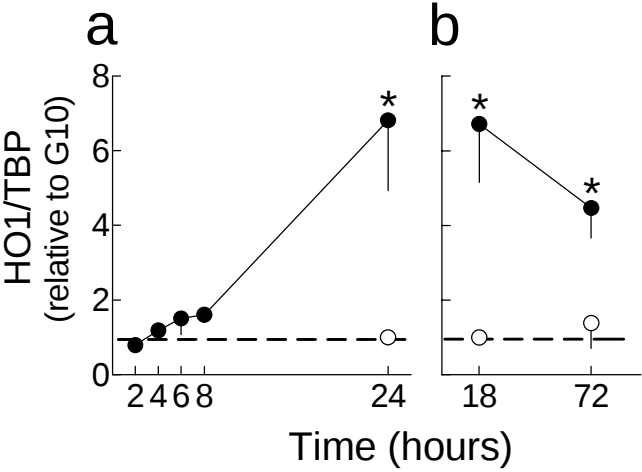
Figure 4 : Effect of hydrogen peroxide (H_2O_2) on islet NF κ B (p65) DNA binding activity.

After preculture, rat islets were cultured for 15 min up to 18 h in the presence of G10 and 5 $\mu\text{mol/l}$ H_2O_2 (filled columns). Control islets cultured in G10 were treated with 50 IU/ml IL-1 β for the last 30 min of culture (G10 + IL-1 β , double hatched column). Cellular extracts were prepared, and the DNA binding activity of the p65 subunit of NF κ B was measured as described under “Methods”. 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 $0 \pm$ S.E.M (on the left of G10 + IL-1 β data). Data are means $\pm$ S.E.M. for 4 independent experiments

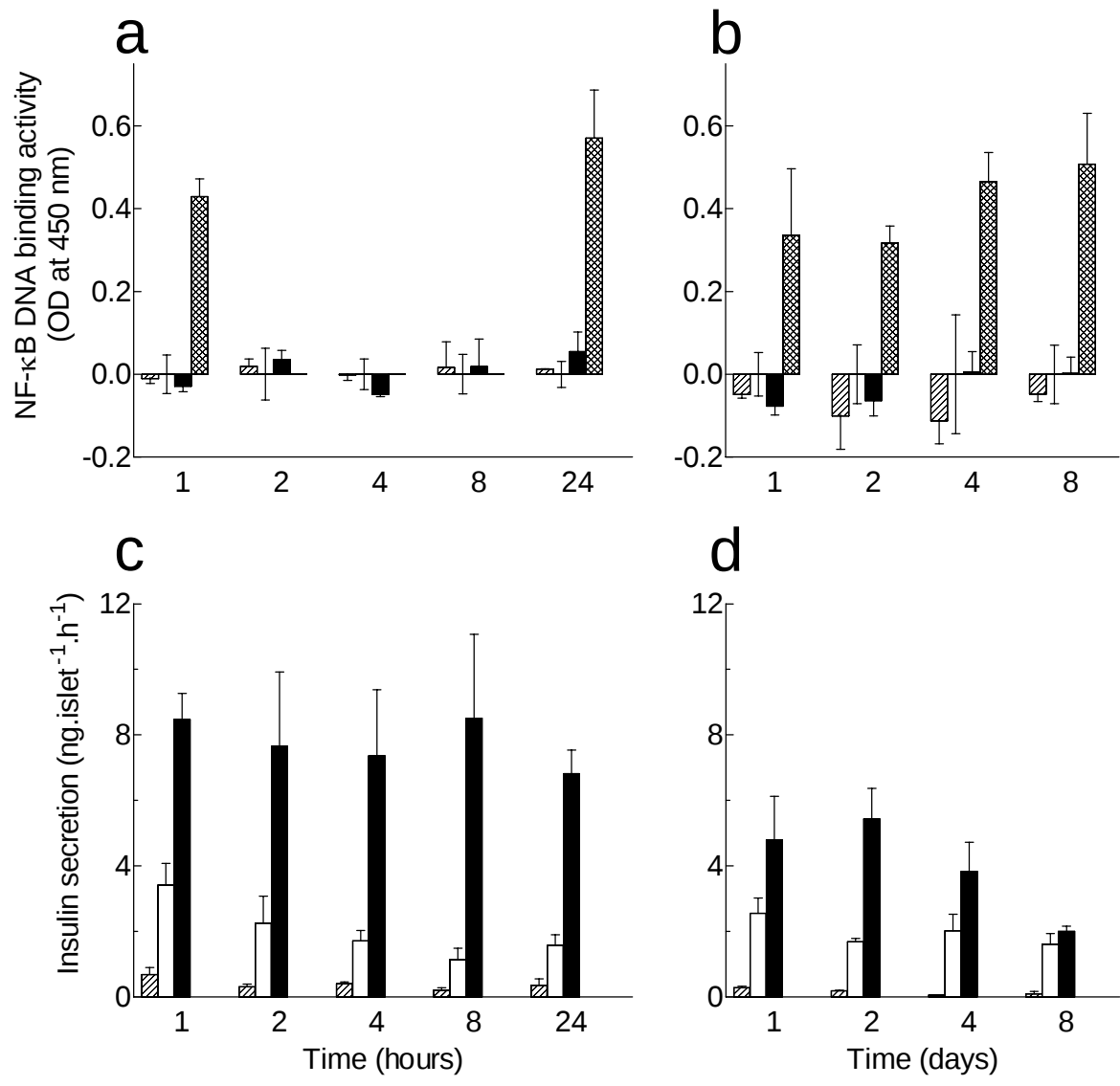
Figure 5 : Effect of inhibitors of p38MAPK and ERK activation on the stimulation of islet HO1 and c-Myc mRNA expression by G30. After preculture, rat islets were cultured for 18h in the presence of G10 and G30 alone (open columns), G10 and G30 with 10 $\mu\text{mol/l}$ SB203580 (hatched columns), or G10 and G30 with 50 $\mu\text{mol/l}$ PD98059 (filled columns). At the end of culture, islet total RNA was extracted and the HO1 (panel a) and c-Myc/TBP (panel 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 (panel c) was measured in the medium by RIA and the rate of secretion per hour of culture was calculated. Data are means $\pm$ S.E.M for 3 independent experiments. ^a, significant effect of G30; ^b, significant effect of test agent in G30.

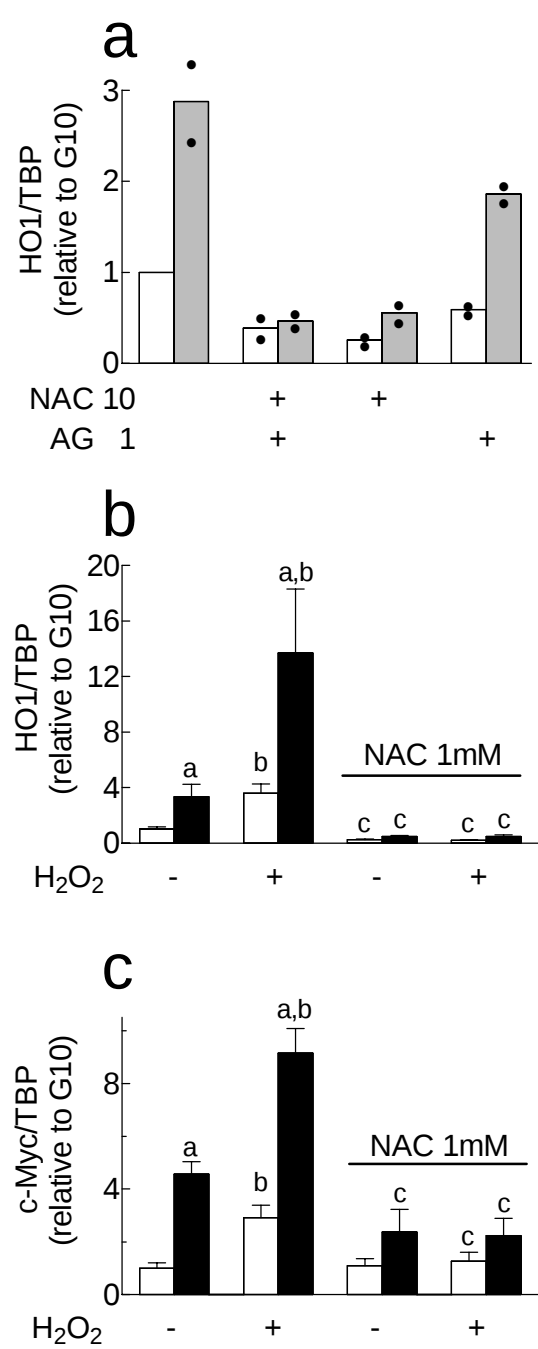
Figure 6 : Pathways involved in the stimulation of rat islet HO1 and c-Myc expression by high glucose concentrations. The stimulation of rat islet c-Myc and HO1 expression by G30 is inhibited by substances that reduce Ca^{2+} influx (diazoxide and nimodipine), and by the α_2 -adrenergic agonist clonidine which inhibits adenylate cyclase in beta-cells [27,28]. It is also inhibited by the antioxidant NAC (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-1 β production or NF κ B activation. In contrast, IL-1 β markedly activates rat islet NF κ B and strongly increases islet mRNA levels of iNOS, I κ B α and Fas. Protein kinases (PKC β_2 , p38MAPK, ERK...) potentially involved in these pathways are not depicted on this scheme. ROS, reactive oxygen species; $[\text{Ca}^{2+}]_c$, cytosolic Ca^{2+} concentration; cAMP, cyclic AMP.

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Figure 1

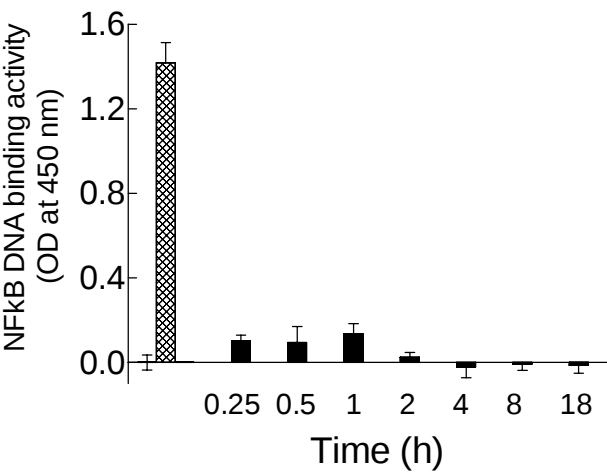


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Figure 2

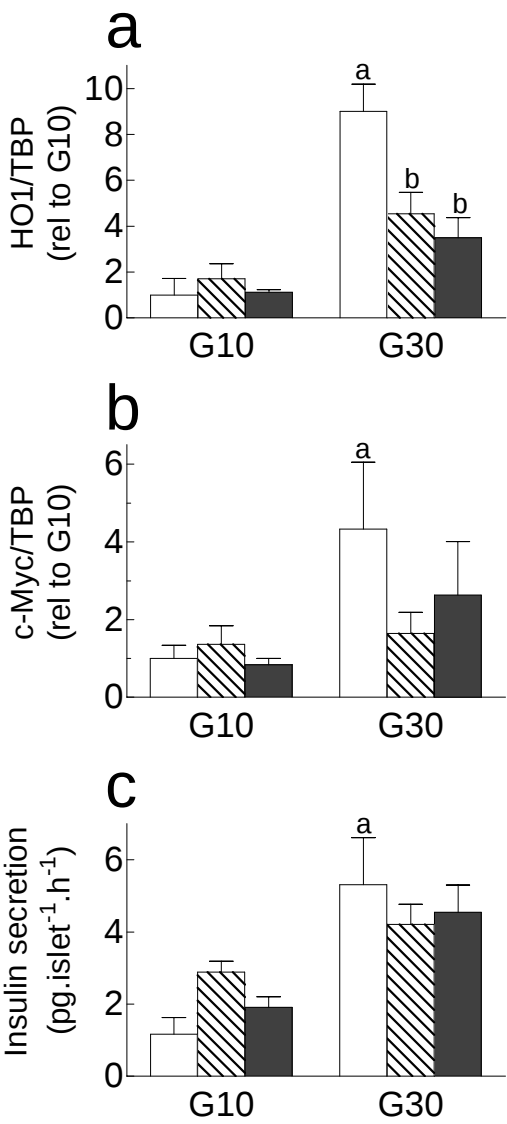




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Figure 4



Elouil et al.
Figure 5



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Figure 6

