High glucose activates pituitary proopiomelanocortin gene expression: possible role of free radical-sensitive transcription factors

Koichi Asaba[1] Yasumasa Iwasaki[1*] Masato Asai[2] Masanori Yoshida[2] Takeshi Nigawara[2] Machiko Kambayashi[1] Kozo Hashimoto[1]

[1]*Department of Endocrinology, Metabolism, and Nephrology, Kochi Medical School, Kochi University, Nankoku 783-8505, Japan*

[2]*Department of Medicine, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan*

Running title: Hyperglycemia activates POMC expression

*Correspondence to: Yasumasa Iwasaki, M.D., Ph.D., Department of Endocrinology, Metabolism, and Nephrology, Kochi Medical School, Kochi University, Nankoku 783-8505, Japan

Tel: +81-88-880-2343; Fax: +81-88-880-2344

E-mail: iwasaki@med.kochi-u.ac.jp

Abstract

Background Hyperglycemia is recognized as a metabolic stress, and indeed it is known to stimulate hypothalamo-pituitary-adrenal (HPA) axis, a representative anti-stress system, in patients with diabetes mellitus or in animal models of hyperglycemia. Thus, we tried to clarify the molecular mechanism of glucose-induced HPA axis activation.

Methods We studied the effect of high glucose on the transcriptional regulation of proopiomelanocortin (POMC) gene which encodes adrenocorticotropic hormone, a central mediator of HPA axis using AtT20 corticotroph cell line *in vitro*.

Results We found that high glucose concentration (24 mM) significantly stimulated the 5'-promoter activity of POMC gene. The effect was promoter-specific, and was mimicked by nuclear factor kappa-B (NF-κB)- or AP1-responsive promoters but not by cAMP-responsive element- or serum response element-containing promoters. Furthermore, the stimulatory effect of high glucose on POMC gene was eliminated by NF-κB and AP1 inhibitors, suggesting the involvement of the transcriptional factors. The POMC 5'-promoter has the canonical NF-κB consensus sequence, and gel shift assay showed the binding of NF-κB to the element. Finally, the effect of high glucose was completely abolished by treatment with a radical quencher TEMPOL.

Conclusions Our data suggest that hyperglycemia activates POMC gene expression, at least partly, via NF-κB/AP1, and that high glucose-induced free radical generation may mediate the activation of these transcription factors, which in turn stimulates the transcription of POMC gene.

Key words hyperglycemia, diabetes mellitus, proopiomelanocortin, adrenocorticoropin, nuclear factor-kappaB

Introduction

Hypothalamo-pituitary-adrenal (HPA) axis is recognized as a major anti-stress system in higher organisms, and pituitary adrenocorticotropin (ACTH) plays a pivotal role in such a way that it regulates the secretion of adrenal glucocorticoid hormone under the control of hypothalamic releasing hormones, paracrine factors, or systemic factors. A variety of stressors is known to activate HPA axis, including mental, physical, or chemical stresses such as hypoxia or hypoglycemia.

Interestingly, it has also been shown that HPA axis is activated in diabetes mellitus. Indeed, plasma ACTH and cortisol levels are elevated in patients with untreated diabetes mellitus, suggesting that hyperglycemia is a metabolic stress [1-3]. Animal experiments using a diabetic rat model support the observation as well [4-6]. However, the molecular mechanism(s) regarding the level of glucose concentration which stimulates the activity of the HPA axis is not known. Recently, Nishikawa et al. have reported that high glucose facilitates the generation of mitochondrial oxygen radical, which is responsible for a variety of cellular responses in diabetes [7]. Thus, one possible explanation is that high glucose-induced oxidative stress is involved in the regulation of HPA axis during hyperglycemia. If that is the case, oxidative stress may be one of the stresses which directly activates the HPA axis.

To test the hypothesis, in this study, we examined the effect of elevated glucose concentration on the expression of proopiomelanocortin (POMC) gene which encodes ACTH in pituitary corticotroph cells. Our *in vitro* data support the hypothesis, showing that high glucose potently stimulates the 5'-promoter activity of the POMC gene. The effect was almost completely eliminated by antioxidant treatment, suggesting the involvement of reactive oxygen species (ROS). Furthermore, the radical-sensitive transcription factor nuclear factor kappa-B (NF-κB) and AP1 are suggested to mediate, at least in part, the effect of ROS-induced activation of POMC gene transcription.

Experimental procedures

Materials

Pyrrolidine dithiocarbamate (PDTC), curcumin, L-glucose, and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were obtained from Sigma (St. Louis, MO). H89, Ro-32-0432, PD98059, SB203580, and wortmannin were from Calbiochem (San Diego, CA), and K252a was from Alomone Labs. (Jerusalem, Israel). pCRE (cAMP-response element)-Luc, pAP1-Luc, pSRE (serum-response element)-Luc, and pNF-κB-Luc were purchased from Stratagene (La Jolla, CA).

Transfection and cell culture

Establishment of the AtT20PL cell line used in this study was described elsewhere [8]. Briefly, the pituitary corticotroph cell line AtT20/D16v was transfected stably with a plasmid (pA3Luc) containing an ≈0.7 kb fragment of the rat POMC gene 5'-promoter (-708 to +64; +1 indicates the transcription start site), and a transformed cell line, designated as AtT20PL, was used for subsequent experiments.

The cells were maintained in a T_{75} culture flask with DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Invitrogen) under a 5% CO_2 / 95 % atmosphere at 37 °C. Culture medium was changed twice a week, and the cells were subcultured once a week.

Experiments

AtT20PL cells were plated in 24-well plates with approximately 50% confluency and cultured with DMEM (high glucose) supplemented with 10% FBS . Two days after plating, the medium was once changed to DMEM containing 3 mM glucose supplemented with 0.5% FBS and insulin (10 nM). Then the cells were cultured under different concentrations of glucose (3-24 mM) for the defined time interval based on the protocol of each experiment.

At the end of incubation, the culture medium was removed, and the cells were harvested for the luciferase assay, as described previously [8]. In some experiments, AtT20D16v cells were transfected transiently with the test plasmids using a lipofection method. The experimental procedure otherwise was the same as that for AtT20PL cells.

RT-PCR

RNA was isolated from the AtT20PL cells using RNeasy kit (Qiagen, Hilden, Germany), and 5 μg of the total RNA was used for the reverse transcription reaction with SuperScript II reverse transcriptase (Invitrogen). The expression of NF-κB-related proteins (p65, p50, IkappaB) were then analyzed by PCR with Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) using the primer sets as follows: sense, 5'- TCAATGGCTACACAGGACCA-3' and antisense, 5'- CACTGTCACCTGGAAGCAGA-3' for mouse p65; sense, 5'- CACCTAGCTGCCAAAGAAGG-3' and antisense, 5'- AGGCTCAAAGTTCTCCACCA-3' for mouse p50; sense, 5'- GCCGACGCAGATGAATGGTG-3' and antisense, 5'- TGTTCATTAGACAGGCCTTGGT-3' for mouse IκBβ. All the primer sets were designed to span at least one intron to avoid the genomic DNA-derived amplification.

Western blot analysis

Cellular protein was extracted by a NE-PAR nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL), and cytoplasmic extracts were separated onto 7.5% SDS-PAGE, followed by electroblotting to nitrocellulose membrane. Then the membranes were incubated with primary antibodies against NF-κB-p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at room temperature. After washing, the secondary antibody (horseradish peroxidase-coupled rabbit IgG) was added and incubation was continued at room temperature. Membranes were then washed as above, followed by a last wash in TBS, and immunoreactive proteins were detected with the ECL-Western blot system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Electromobility shift assay (EMSA)

Nuclear extract was prepared using a NE-PAR nuclear and cytoplasmic extraction kit (Pierce). The extract was then incubated for 6 h with the double-stranded, 3'-end-biotinylated oligonucleotide probe (50 fmol) encompassing the consensus NF-κB binding sequence (sense, 5'-CGACCGGGAAGCCCCCCCTC-3'biotin, antisense, 5'-GAGGGGGGGTTCCCGGTCG-3'biotin), or the NF-κB binding sequence in the 5'-promoter of POMC gene (-151 to -141), and the mixture was subjected to 4% nondenaturing polyacrylamide gel (160V for 4 h). Finally, the biotinylated DNA was transferred to a nylon membrane using capillary transfer and cross-linked, after which the biotin-labeled DNA was detected with digital imaging apparatus (ATTO, Japan).

Data analysis

Samples in each group of the experiments were in triplicate or quadruplicate. All data were expressed as mean ± SEM. When the statistical analyses were performed, data were compared by one way analysis of variance with Fisher's multiple range test, and p values below 0.05 were considered significant.

Results

Effect of high glucose on the POMC 5'-promoter activity

We first examined the effect of high glucose on POMC gene expression in AtT20PL cells. As shown in Figure 1, the rise in the concentration of glucose from 3 to 24 mM significantly stimulated the POMC gene transcription. The maximal effect was obtained 6 h after the treatment, and more than a 2-fold increase was observed.

Effects of high glucose on canonical responsive elements-mediated transcription

To clarify which signal transduction pathway(s) is involved in the high glucose-induced effect observed above, we then carried out a transient transfection experiment in AtT20 cells using a

variety of reporter constructs containing a canonical responsive element-luciferase fusion gene. Among the reporter plasmids tested, similar positive effects were observed on the NF-κB- and AP-1-mediated transcription (Figure 2). In contrast, high glucose did not influence the CRE- or SRE-mediated transcription. These results suggest that the high glucose-induced effect is not a non-specific effect but rather a promoter-specific one, and that both of these representative stress-sensitive transcription factors are activated by high glucose.

Effect of L-glucose on the POMC 5'-promoter activity

To further confirm the specificity of the high glucose-induced effect, we compared the effect of D-glucose and metabolically inactive L-glucose in AtT20PL cells. As shown in Figure 3, the rise in the concentration of L-glucose from 3 to 24 mM caused only an ≈30% increase in the POMC gene expression, whereas D-glucose stimulated it to more than 2-fold. These data indicate that the effect of D-glucose is not caused by a non-specific osmogenic effect but by a D-glucose-specific (probably metabolic) effect. Nonetheless, the minimal but significant increase by L-glucose suggests that increased osmolality may activate some signaling pathway(s) possibly through an osmotic stress-mediated effect.

Effects of signal transduction inhibitors on high-glucose mediated POMC gene transcription

To clarify the signal transduction pathway(s) involved in the observed effect more precisely, we tested the effect of a variety of signal transduction inhibitors on high glucose-mediated increase in the POMC gene 5'-promoter activity. As shown in Figure 4, treatment of the cells with H89, Ro-32-0432, K252a, PD98059, and SB203580 for protein kinase A, protein kinase C, calmodulin kinases, p42/44 and p38 MAP kinase inhibitors, respectively, did not interrupt high glucose-induced POMC gene expression. In contrast, the NF-κB inhibitor PDTC and the AP1 inhibitor curcumin completely eliminated the glucose-mediated increase, indicating that the transcription factors play central roles in the glucose-induced effect. In addition, wortmannin, a phosphatidylinositol 3 kinase (PI_3K) inhibitor, significantly blunted

the effect of high glucose, suggesting a partial involvement of the PI_3K-mediated signaling pathway.

Expression of NF-κB transcription factors in AtT20PL cells

Since the putative NF-κB binding sites are located in the 5'-promoter region of the POMC gene (Figure 5A), we analyzed the expression of trans-acting factors, p50 (Rel) and p65 (RelA), of the NF-κB components as well as IkappaB in AtT20PL cells by. As shown in Figure 5B, bands corresponding to mRNA of all the protein were strongly amplified, suggesting that NF-κB is indeed expressed in these cells. The expression of p50 and p65 proteins were also confirmed by Western blotting (Figure 5C).

Effect of high glucose on DNA binding of NF-κB

To see if high glucose enhances the DNA binding of NF-κB, EMSA analysis was carried out using canonical or POMC 5'-promoter-derived NF-κB binding sequences as probes. The results showed that high glucose treatment (24 mM for 2 h) clearly enhanced the protein binding to the canonical probe (Figure 6, left). The effect on the POMC probe was less obvious than that on the canonical probe, but significant increase was still obtained [852 ± 25 and 1056 ± 36 in low and high glucose groups, respectively (arbitrary unit, n=2 in each group, $p<0.05$), estimated by a densitometric analysis]. A representative photograph is shown in Figure 6 (right).

Effect of an antioxidant TEMPOL on high-glucose mediated POMC gene transcription

As shown above, the transcription factors NF-κB and AP-1 were activated by high glucose (Figure 2) in AtT20PL cells, and they are known to be both radical-sensitive [9, 10]. Accordingly, we finally examined whether glucose-induced overproduction of free radical is responsible for the activation of the POMC gene transcription. As shown in Figure 7, pre-treatment of the cells with TEMPOL (2 mM), a potent free radical scavenger, completely abolished the positive effect of glucose. Thus, our data strongly suggest that high

glucose-induced activation of the POMC gene transcription is, in most part, mediated by oxidative stress.

Discussion

In this study, we found that high glucose directly increases the expression of POMC gene which encodes ACTH and other stress-related peptides. Our data also showed that the same treatment activates the NF-κB- and AP1-dependent transcription as well. The effect of high glucose on the POMC gene was completely eliminated by NF-κB inhibitor or antioxidant, further suggesting the involvement of ROS and ROS-sensitive NF-κB/AP1 transcription factors. Altogether, the present findings indicate that hyperglycemia-induced oxidative stress activates HPA axis, which may account for the reported elevation of plasma ACTH and cortisol in patients or animal models with uncontrolled diabetes mellitus.

It is well known that ACTH is a key factor for regulating HPA axis, a representative stress-coping system in humans and mammals. ACTH-producing pituitary corticotroph cells receive a variety of stress-related signals such as hypothalamic hormones (CRH, vasopressin), paracrine hormones, and cytokines, and after integration of these inputs, they secrete ACTH which specifically stimulate adrenal glucocorticoid hormone, cortisol or corticosterone, a representative anti-stress hormone. Thus, synthesis and secretion of ACTH is a key step for regulating HPA axis in both physiological and pathological conditions. Indeed, expression of ACTH is known to be elevated in a variety of stress paradigms. The most typical stress is a physiological stress (pain or trauma), but emotional or even chemical stress (hypoxia or hypoglycemia) are known to activate HPA axis as well.

In addition, it has been recognized for a long time that HPA axis is also activated in patients with uncontrolled diabetes mellitus. Roy et al. reported the increase in plasma ACTH and plasma and urinary cortisol in diabetic patients [1-3]. *In vivo* experiments using the type 2 diabetic rat model showed similar effects [4-6], and Schwartz et al. found a decrease in hypothalamic CRH expression despite the increase in ACTH and cortisol,

suggesting the existence of unknown factor(s) stimulating the HPA axis at the pituitary level during high blood glucose [5]. Our *in vitro* findings in the present study strongly suggest that hyperglycemia *per se* is a stressor, and stimulates the synthesis of ACTH in the pituitary cells. The effect seems not to be a non-specific metabolic effect but rather a promoter-specific event, because similar effects were observed in some (NF-κB or AP-1-luciferase) but not in other (CRE- or SRE-luciferase) constructs. Furthermore, an equimolar concentration of metabolically inactive L-glucose only partially mimicked the effect of D-glucose, indicating that the event is not caused by hyperosmolality itself. Interestingly, however, L-glucose also had a mild but significant stimulatory effect, possibly due to the hyperosmolality-induced free radical generation [11, 12] (see below).

It is important to clarify which signal transduction pathway(s) mediates the high glucose-induced upregulation of POMC gene. Our data show that high glucose had similar positive effects on transcription caused by NF-κB and AP-1, both of which are known to be oxygen radical-sensitive transcription factors. Recent reports indicate that high glucose concentration elicits enhanced mitochondrial glucose oxidation, and subsequent overproduction of ROS, especially oxygen radicals, is responsible for a variety of pathological events observed in diabetic patients [7]. Based on this paradigm, it is plausible to speculate that high glucose-induced ROS activates NF-κB and AP-1 [10], which in turn exerts a positive effect on POMC gene. The involvement of ROS is also supported by our finding showing that radical quencher TEMPOL completely abolished the effect of high glucose. Furthermore, curcumin and PDTC, well-known antioxidants and inhibitors of AP1 and NF-κB, respectively, also eliminated the effect, suggesting the importance of the transcription factors. The binding sites of both transcription factors are located in the 5'-promoter region of the POMC gene. Regarding the role of NF-κB, EMSA analysis showed the actual binding of the protein, whereas the effect of high glucose was minimal. This might partly be because basal activity of NF-κB-dependent transcription is already high in AtT20 cells, and the glucose mediated effect on transcriptional activity is relatively low (≈2-fold). Furthermore, recent studies suggest that the transactivation capacity of NF-κB is

determined not only by nuclear translocation (quantitative change) but also by phosphorylation of p65 (RelA) (qualitative change) via a variety of signaling pathways [13], which is not detected by EMSA. The role of another ROS-sensitive factor AP1 in the transcriptional regulation of POMC gene has been well characterized previously [14]. In addition, our data suggest the possible involvement of the PI_3K signaling pathway in high glucose-mediated effect, because wortmannin, a specific inhibitor of PI_3K partly eliminated the glucose-stimulated POMC gene expression.

Considering the fact that the high glucose-induced ROS overproduction is a systemic phenomena, it may influence glucocorticoid action as well as production *in vivo*. Recent data clearly show that NF-κB is activated in a variety of tissues throughout the body, probably through the generation of high glucose-induced ROS and/or advanced glycation endproduct (AGE) [15, 16]. Since NF-κB antagonizes glucocorticoid receptor function [17], high glucose is supposed to cause decreased glucocorticoid sensitivity. Furthermore, increased ROS is reported to impair glucocorticoid receptor function [18, 19]. These data altogether suggest that high glucose with increased ROS activates HPA axis and glucocorticoid secretion but simultaneously impairs the sensitivity to glucocorticoid hormone in the peripheral organ. This may explain why the activated HPA axis does not necessarily induce clinically overt Cushing's syndrome in patients with uncontrolled diabetes. Nevertheless, the continuous activation of HPA axis may contribute, to some extent, to the development of central obesity and/or the metabolic syndrome frequently observed in diabetic patients [20].

In conclusion, our data show a new paradigm suggesting strongly that extracellular high glucose is a stress which activates HPA axis. More specifically, glucose-induced oxidative stress with resultant activation of radical-sensitive transcription factors such as NF-κB may, at least partly, account for the molecular mechanism underlying the hyperglycemia-induced activation of HPA axis observed in patients with uncontrolled diabetes mellitus.

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References

1. Roy M, Collier B, Roy A. Hypothalamic-pituitary-adrenal axis dysregulation among diabetic outpatients. *Psychiatry Res* 1990; **31**: 31-37.
2. Roy MS, Roy A, Gallucci WT, *et al*. The ovine corticotropin-releasing hormone-stimulation test in type I diabetic patients and controls: suggestion of mild chronic hypercortisolism. *Metabolism* 1993; **42**: 696-700.
3. Roy MS, Roy A, Brown S. Increased urinary-free cortisol outputs in diabetic patients. *J Diabetes Complications* 1998; **12**: 24-27.
4. Tojo C, Takao T, Nishioka T, Numata Y, Suemaru S, Hashimoto K. Hypothalamic-pituitary-adrenal axis in WBN/Kob rats with non-insulin dependent diabetes mellitus. *Endocr J* 1996; **43**: 233-239.
5. Schwartz MW, Strack AM, Dallman MF. Evidence that elevated plasma corticosterone levels are the cause of reduced hypothalamic corticotrophin-releasing hormone gene expression in diabetes. *Regul Pept* 1997; **72**: 105-112.
6. Takao T, Tojo C, Nishioka T, Hashimoto K. Increased adrenocorticotropin responses to acute stress in Otsuka Long-Evans Tokushima Fatty (type 2 diabetic) rats. *Brain Res* 2000; **852**: 110-115.
7. Nishikawa T, Edelstein D, Du XL, *et al*. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000; **404**: 787-790.
8. Aoki Y, Iwasaki Y, Katahira M, Oiso Y, Saito H. Regulation of the rat proopio-melanocortin gene expression in AtT-20 cells. I: Effects of the common secretagogues. *Endocrinology* 1997; **138**: 1923-1929.
9. Lavrovsky Y, Chatterjee B, Clark RA, Roy AK. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp Gerontol* 2000; **35**: 521-532.
10. Fan H, Sun B, Gu Q, Lafond-Walker A, Cao S, Becker LC. Oxygen radicals trigger activation of NF-kappaB and AP-1 and upregulation of ICAM-1 in reperfused canine

heart. *Am J Physiol Heart Circ Physiol* 2002; **282**: H1778-H1786.

11. Loitsch SM, von Mallinckrodt C, Kippenberger S, Steinhilber D, Wagner TO, Bargon J. Reactive oxygen intermediates are involved in IL-8 production induced by hyperosmotic stress in human bronchial epithelial cells. *Biochem Biophys Res Commun* 2000; **276**: 571-578.
12. Takeda K, Kato M, Wu J, *et al*. Osmotic stress-mediated activation of RET kinases involves intracellular disulfide-bonded dimer formation. *Antioxid Redox Signal* 2001; **3**: 473-482.
13. Vermeulen L, De Wilde G, Notebaert S, Vanden Berghe W, Haegeman G. Regulation of the transcriptional activity of the nuclear factor-κB p65 subunit. *Biochem Pharmacol* 2002; **64**: 963-970.
14. Becquet D, Guillaumond F, Bosler O, Francois-Bellan AM. Long-term variations of AP-1 composition after CRH stimulation: consequence on POMC gene regulation. *Mol Cell Endocrinol* 2001; **175**: 93-100.
15. Yerneni KK, Bai W, Khan BV, Medford RM, Natarajan R. Hyperglycemia-induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells. *Diabetes* 1999; **48**: 855-864.
16. Piepr GM, Riazul H. Activation of nuclear factor kappaB in cultured endothelial cells by increased glucose concentration: prevention by calphostin C. *J Cardiovasc Pharamacol* 1997; **30**: 528-532.
17. McKay LI, Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev* 1999; **20**: 435-459.
18. Makino Y, Okamoto K, Yoshikawa N, *et al*. Thioredoxin: a redox-regulating cellular cofactor for glucocorticoid hormone action. Cross talk between endocrine control of stress response and cellular antioxidant defense system. *J Clin Invest* 1996; **98**:2469-2477.
19. Okamoto K, Tanaka H, Ogawa H, *et al*. Redox-dependent regulation of nuclear import of the glucocorticoid receptor. *J Biol Chem* 1999; **274**: 10363-10371.

20. Rosmond R. Stress induced disturbances of the HPA axis: a pathway to Type 2 diabetes? *Med Sci Monit* 2003; **9**: RA35-RA39.

Figure legends

Figure 1. Effect of high glucose on the POMC 5'-promoter activity in AtT20PL cells. Cells were treated with DMEM containing 3 mM (control; open bar) or 24 mM glucose (closed bars) for the defined time interval (h). Each value is shown as a percentage of the control value. *p<0.05 vs. control (value at time 0).

Figure 2. Effect of high glucose on the CRE-, AP1-, SRE-, NF-κB-mediated transcriptional activity in AtT20 cells. Cells were transfected transiently with pCRE-, pAP1-, pSRE- and pNF-κB-Luc reporter plasmids. On the next day, cells were incubated for 18 h with DMEM containing 3 mM glucose, and then treated with DMEM containing 3 mM (open bars) or 24 mM (closed bars) glucose for 6 h. Each value is shown as a percentage of the value of the 3 mM group. *p<0.05 vs. value of 3 mM group.

Figure 3. Effects of D- or L-glucose (24 mM) on the POMC 5'-promoter activity in AtT20PL cells. Cells were treated with DMEM containing 3 (open bars) or 24 mM (closed bars) of D-glucose (left), or 3 or 24 mM of L-glucose (right) for 6 h. Each value is shown as a percentage of the value of 3 mM group. *p<0.05 vs. value of 3 mM group.

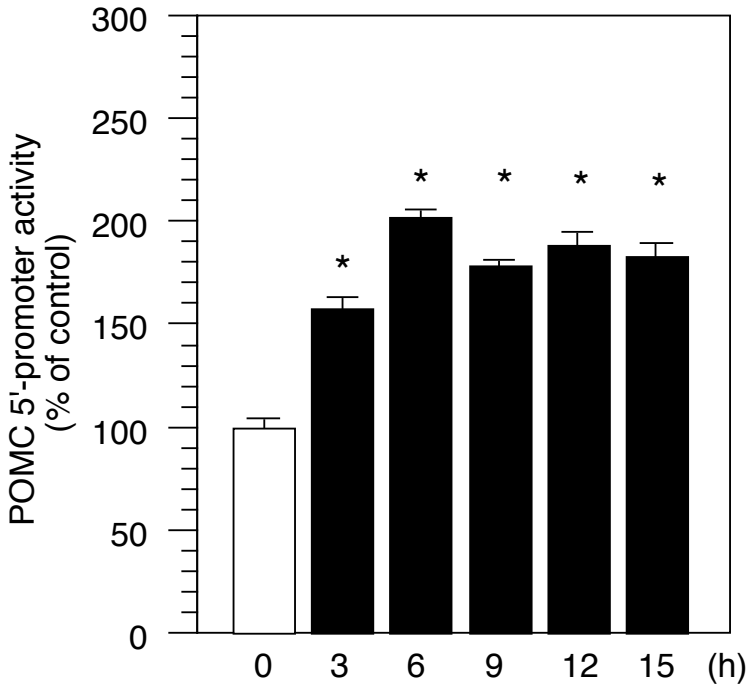
Figure 4. Effects of a variety of protein kinase inhibitors on high glucose-mediated POMC 5'-promoter activity in AtT20PL cells. Cells were incubated with DMEM containing 3 mM glucose for 18 h, and then treated with DMEM containing 3 (open bars) or 24 mM (closed bars) glucose for 6 h. One hour prior to the change in glucose concentration, cells were treated with vehicle (control), wortmannin (Wort; 1 μM), H89 (30 μM), Ro-32-0432 (Ro; 5 μM), K252a (K252;1 μM), curcumin (Curc; 10 μM), PDTC (100 μM), PD98059 (PD; 20 μM), and SB203580 (SB; 10 μM) until the end of experiment. Each value is shown as a percentage of the value of the corresponding 3 mM group. *p<0.05 vs. value of 3 mM group.

Figure 5. A. The nucleotide sequence of the 5'-promoter region of the rat POMC gene used in this study. Defined and/or putative binding sites for representative transcription factors including NF-κB are underlined. B. RT-PCR analysis of each subunit of NF-κB mRNA expression in AtT20PL cells. Bands corresponding to p50, p65 and IκBβ were amplified. M represents molecular size marker (100 bp ladder). C. Western blot analysis of each subunit of NF-κB protein in AtT20PL cells. Bands corresponding to p50 and p65 of NF-κB components were observed. Open triangles represent molecular size marker (48 kDa).

Figure 6. EMSA analysis. AtT20PL cells were incubated with DMEM containing 3 mM glucose for 18 h, and then treated with DMEM containing either 3 or 24 mM glucose for 2 h, and the nuclear extracts was applied for EMSA. Either canonical or POMC 5'-promoter-derived NF-κB binding sequence was used as probes. LG, low glucose (3 mM); HG, high glucose (24 mM).

Figure 7. Effect of radical quencher TEMPOL on high glucose-mediated POMC 5'-promoter activity in AtT20PL cells. Cells were incubated with DMEM containing 3 mM glucose for 18 h, and then treated with DMEM containing either 3 mM (open bars) or 24 mM (closed bars) glucose for 6 h. One hour prior to the change in glucose concentration, cells were treated with vehicle (control) or TEMPOL (2 mM) until the end of experiment. Each value is shown as a percentage of the value of the corresponding 3 mM group. *$p<0.05$ vs. value of 3 mM group.

Figure 1


POMC 5'-promoter activity
(% of control)
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6
9
12
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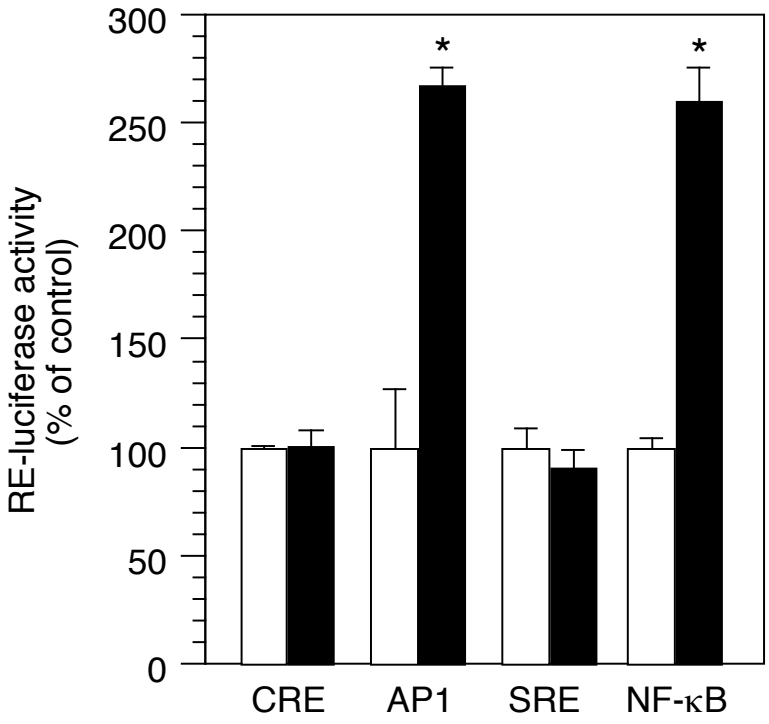
(h)

Figure 2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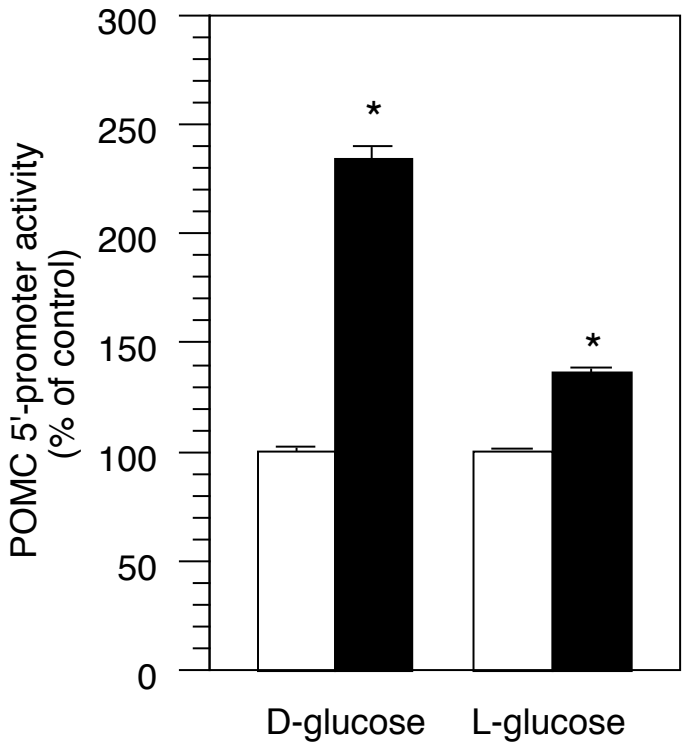
CRE
AP1
SRE
NF-κB

Figure 3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(% of control)
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D-glucose
L-glucose

Figure 4

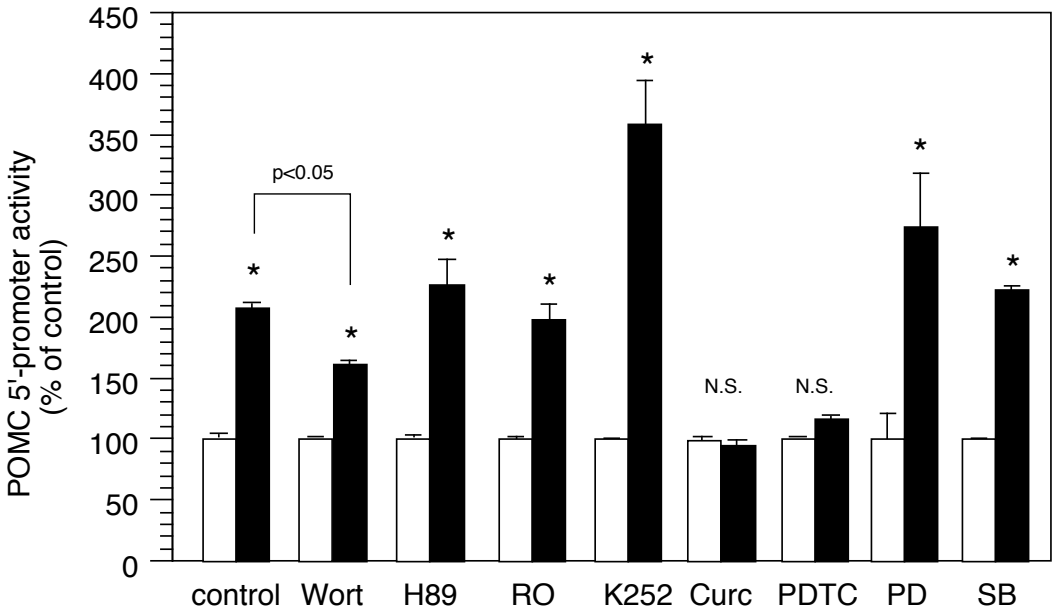
POMC 5'-promoter activity
(% of control)
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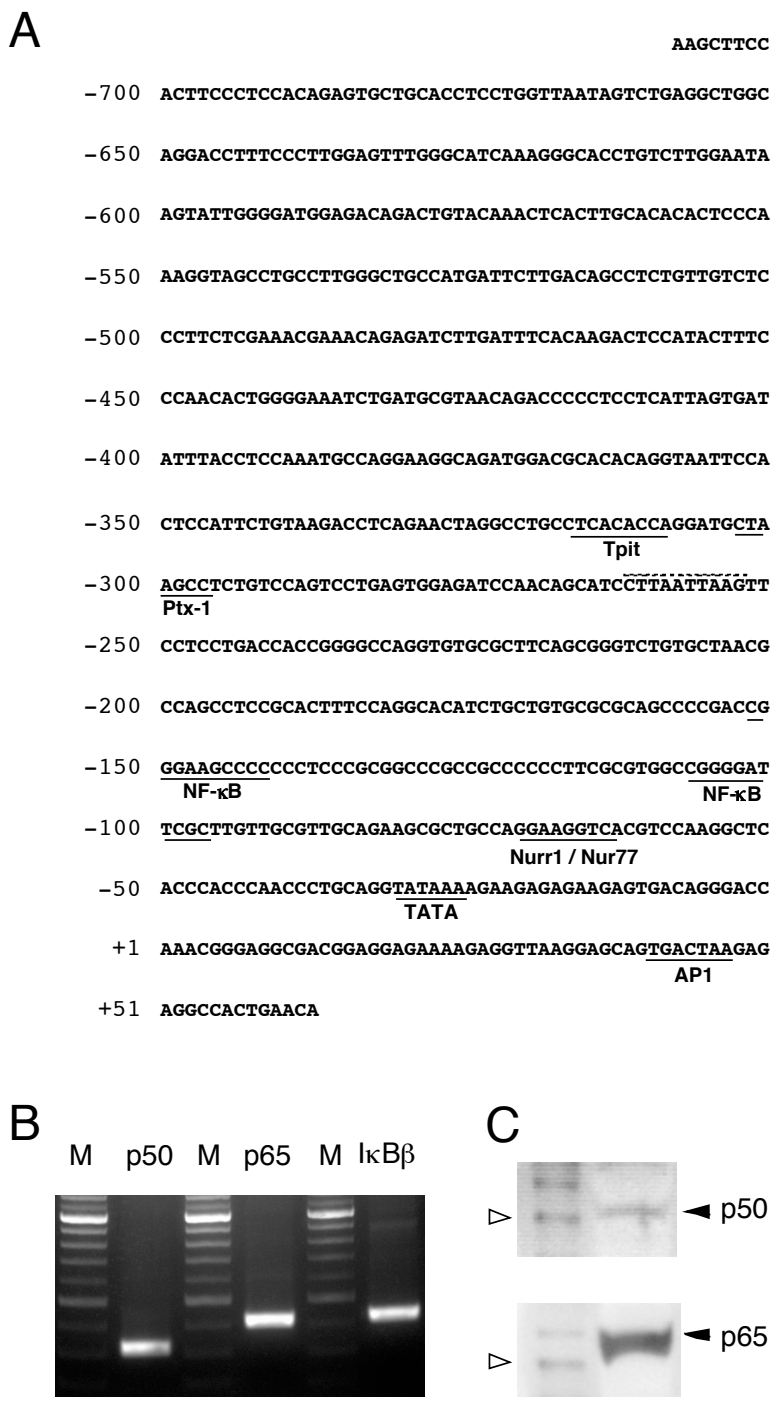
Figure 5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NF-κB
NF-κB
-100 TCGCTTGTTGCGTTGCAGAAGCGCTGCCAGGAAGGTCACGTCCAAGGCTC
Nurr1 / Nur77
-50 ACCCACCCAACCCTGCAGGTATAAAAGAAGAGAGAAGAGTGACAGGGACC
TATA
+1 AAACGGGAGGCGACGGAGGAGAAAAGAGGTTAAGGAGCAGTGACTAAGAG
AP1
+51 AGGCCACTGAACA
B
M p50 M p65 M IκBβ
C
p50
p65

Figure 6

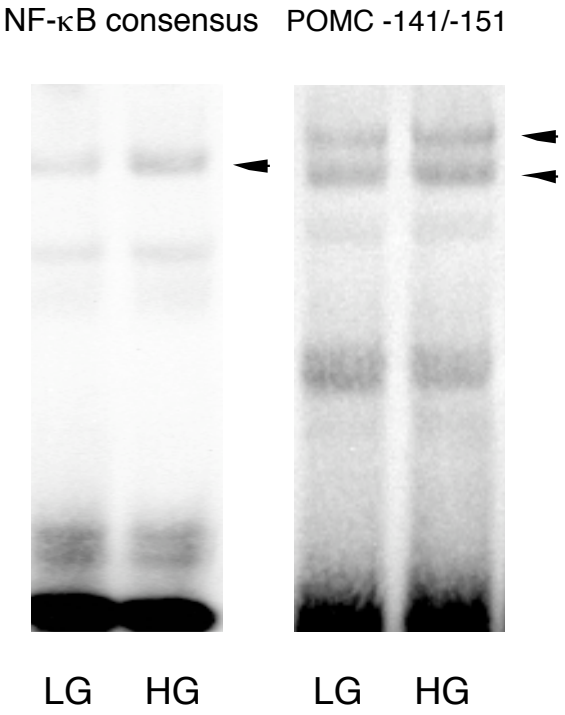
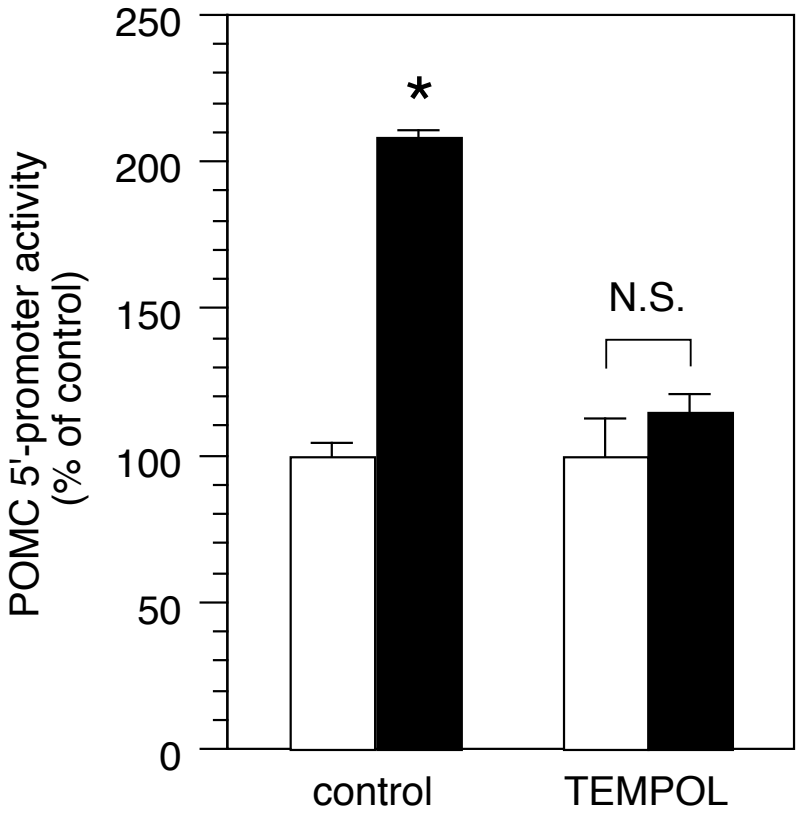


Figure 7


250
200
150
100
50
0
POMC 5'-promoter activity
(% of control)
*
N.S.
control
TEMPOL