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ARTICLE

TNF-α acutely upregulates amylin expression in murine pancreatic beta cells

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Abstract
Aims/hypothesis Amylin, a secretory protein mainly produced by pancreatic beta cells, is elevated in the circulation of patients with diseases related to acute and chronic inflammation, including acute pancreatitis, pancreas graft rejection, obesity and insulin resistance. TNF-α is involved in these disorders. We investigated the effect of TNF-α on amylin levels and the underlying mechanisms, using murine pancreatic beta cell line MIN6 and pancreatic islets.
Methods Amylin, proinsulin and prohormone convertase 1/3, 2 (*Pc1/3, Pc2* [also known as *Pcsk1/3* and *Pcsk2*, respectively]) mRNA levels, and amylin promoter and nuclear factor κB (NF-κB) activation were examined by real-time PCR and luciferase reporter assay, respectively. Amylin protein level and mitogen-activated protein kinase phosphorylation were detected by western blot. Activator protein 1 (AP1) activation was examined by electrophoretic mobility shift assay (EMSA).
Results TNF-α acutely induced amylin expression at the transcriptional level and increased proamylin and the intermediate form of amylin in MIN6 cells and islets. However, it had no effect on proinsulin, *Pc1/3* and *Pc2* expression. Studies with (1) MIN6 cells treated with inhibitors of MEK1/2, c-Jun-N-terminal kinase (JNK) or protein kinase Cζ (PKC_ζ), (2) MIN6 cells expressing a *c-Jun*-dominant negative construct and (3) islets from *Fos* knockout mice demonstrated that TNF-α induced amylin expression through the PKC_ζ−extracellular signal-regulated kinase (ERK)/JNK pathways. EMSA showed that PKC_ζ, JNK and ERK1/2 were involved in TNF-α-induced AP1 activation, suggesting that TNF-α induces murine amylin expression through the $PKC_\zeta - ERK1/2 - AP1$ and $PKC_\zeta -$ JNK − AP1 pathways. Further studies showed that TNF-α also induced murine amylin expression through the phosphatidylinositol 3 kinase–NF-κB signalling pathway and enhanced human amylin promoter activation through NF-κB and AP1.
Conclusions/interpretation TNF-α acutely induces amylin gene expression in beta cells through multiple signalling pathways, possibly contributing to amylin elevation in acute inflammation-related pancreatic disorders.

Keywords Amylin · Beta cell · Gene expression · Islet amyloid polypeptide · Pancreatic islet · Signalling transduction · TNF-α

Abbreviations

AP1	Activator protein 1
EMSA	Electrophoretic mobility shift assay
Nfkbia-DN	*Nfkbia* dominant-negative construct
JNK	c-Jun-N-terminal kinase
c-Jun DN	*c-Jun* dominant-negative construct
MAPK	Mitogen-activated protein kinase

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MEK	Mitogen-activated protein kinase kinase
Myr−PKC$_\zeta$	Protein kinase C$_\zeta$ pseudosubstrate
NF-κB	Nuclear factor κB
NF-κB-Luc	NF-κB luciferase reporter construct
PC	Prohormone convertase
PDX-1	Pancreatic and duodenal homeobox 1
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C

Introduction

Amylin, also known as islet amyloid polypeptide (IAPP), is a normal secretory protein mainly expressed by pancreatic beta cells. It plays important roles in energy homeostasis, bone growth and brain development [1–3]. Amylin was discovered in 1987 as a major component of amyloid deposits in the pancreas of type 2 diabetes patients [4, 5]. Elevated circulating levels of amylin have been detected in patients with severe acute pancreatitis [6], pancreas transplantation [7], obesity and insulin resistance [8–10]. Pancreatic amylin mRNA and plasma amylin levels are also elevated in genetically obese, insulin-resistant rats [11]. However, the mechanisms underlying amylin expression are not completely understood.

TNF-α is a critical player in the inflammatory response in acute pancreatitis [12] and has been reported to be involved in pancreas graft dysfunction [13]. Circulating TNF-α is increased in obesity [14] and has been implicated as a causative factor in obesity-associated insulin resistance and the pathogenesis of type 2 diabetes [15, 16]. In this study, we used the cultured murine pancreatic beta cell line MIN6 and pancreatic islets, as well as human amylin promoter luciferase reporter constructs, to examine the effect of TNF-α on amylin expression and further explore the mechanisms involved.

Methods

Materials TNF-α was purchased from Peprotech (Rocky Hill, NJ, USA). PD98059, SP600125, protein kinase Cζ (PKC$_\zeta$) pseudosubstrate (Myr-PKC$_\zeta$), wortmannin, sulfasalazine and pyrrolidine dithiocarbamate were from Calbiochem (La Jolla, CA, USA). Ficoll 400 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). DMEM was from Gibco BRL (Burlington, ON, Canada). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). Unless otherwise stated, all other reagents were purchased from Sigma Aldrich (St Louis, MO, USA).

Pancreatic islet preparation and cell culture Pancreatic islets were isolated from C57/B6 mice (Shanghai SLAC Laboratory Animal Company, Shanghai, China) or *Fos* knockout mice [17] by type V collagenase digestion followed by Ficoll 400 gradient separation, as described previously [18]. Islets were cultured in DMEM containing 5.6 mmol/l glucose, 10% FBS (vol./vol.), 100 U/ml penicillin and 100 μg/ml streptomycin (Bio Basis, Markham, ON, Canada). All experiments using animals were in accordance with the 'Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) and were approved by the Biological Research Ethics Committee, Institute for Nutritional Sciences, Chinese Academy of Sciences. MIN6 cells were cultured at 37°C in DMEM containing 5.6 mmol/l glucose, 10% FBS and antibiotics in a humidified atmosphere with 5% CO_2.

RNA extraction and real-time PCR Total RNA was extracted from MIN6 cells or mouse pancreatic islets using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and depleted of contaminating DNA with RNase-free DNase (TAKARA Biotechnology, Otsu, Shiga, Japan). cDNA was synthesised from 2 μg RNA with M-MuLV reverse transcriptase (Fermentas, Burlington, ON, Canada) and random hexamer. Reverse-transcribed cDNA in triplicate samples was checked for amylin, proinsulin 1, proinsulin 2, prohormone convertase 1/3 (*Pc1/3* [also known as *Pcsk1/3*]) or *Pc2* (also known as *Pcsk2*) mRNA expression by quantitative real-time PCR with Power SYBR Green PCR master Mix (Applied Biosystems, Warrington, UK) on a sequence detector (ABI Prism 7500; Applied Biosystems, Foster City, CA, USA). The primers for real-time PCR are listed in Electronic supplementary material (ESM) Table 1. Amplification of the target cDNA was normalised to β-actin expression. Relative levels of target mRNA expression were calculated using the $2^{-\Delta\Delta C_t}$ method.

Western blotting MIN6 cells were stabilised in KRB buffer for 2 h followed by stimulation with TNF-α for 5 min. Phosphorylation of ERK1/2 or c-Jun-N-terminal kinase (JNK) was examined by western blot as previously described [19]. Levels of ERK1/2 or JNK were also examined to ensure equal loading. Amylin protein levels in murine islets were examined by western blot with anti-rat amylin antiserum (T-4145; Peninsula Laboratory, Belmont, CA, USA) as previously described [20].

Plasmid construction and luciferase reporter assay The human amylin promoter fragments between −222 and 450, and −391 and 450 of the transcriptional start site of amylin gene [21] were amplified by PCR and cloned into the pGL3-basic luciferase reporter plasmid (Promega, Madison, WI, USA) between the MIuI and XhoI sites. The constructs were named −222/450 and −391/450, respectively, and

confirmed by restriction enzyme digestion as well as sequencing. Amylin promoter luciferase plasmid was co-transfected with the pRL-TK Renilla plasmid into MIN6 cells with a 40:1 ratio using Lipofectamine 2000. At 36 h after the transfection, cells were cultured for another 8 h in DMEM containing 0.5 mmol/l glucose and 2% FBS (vol./vol.), with or without 2.8 nmol/l TNF-α. Luciferase activities of the promoter construct and the pRL-TK construct were measured sequentially using a reporter system (Dual-Luciferase Reporter Assay; Promega). Variation in transfection efficiency was normalised by dividing the promoter construct activity by the respective co-transfected pRL-TK luciferase activity.

For nuclear factor κB (NF-κB)-dependent luciferase reporter assay, 5× NF-κB luciferase reporter construct (*NF-κB*-Luc) was co-transfected with pRL-TK Renilla plasmid into the MIN6 cells using Lipofectamine 2000 as described above. At 36 h after the transfection, cells were treated for 1 h with or without various inhibitors, followed by stimulation with TNF-α for an additional 6 h. Luciferase activities were detected and normalised as above.

Electrophoretic mobility shift assay MIN6 cells were cultured for 12 h in medium without FBS, then treated for 1 h with or without various inhibitors, followed by treatment with 2.8 nmol/l TNF-α for another 2 h. The nuclear extracts were prepared and electrophoretic mobility shift assay (EMSA) was performed with a kit (LightShift Chemiluminescent EMSA kit; Pierce Chemical, Rockford, IL, USA) as previously described [22]. The oligonucleotide sequences of activator protein 1 (AP1) probes were: amylin AP1 probe: 5′-AAGAGCTTGAGTCACACAAGA-3′; consensus AP1 probe: 5′-CGCTTGATGACTCAGCCGGAA-3′.

Statistical analysis Results are expressed as means ± SD. Statistical analysis was performed using ANOVA for time course and dose-response, and Student's *t* test for other data.

Results

TNF-α induces murine amylin expression To determine the effect of TNF-α on amylin gene expression, MIN6 cells were challenged with different concentrations of TNF-α for different lengths of time and the mRNA expression of amylin were detected by quantitative real-time PCR. As shown in Fig. 1a, b, MIN6 cells cultured in DMEM containing 5.6 mmol/l glucose expressed transcripts for amylin. This expression was significantly enhanced by TNF-α in time- and dose-dependent manners. The minimal concentration of TNF-α needed to significantly induce amylin gene expression was 0.57 nmol/l. Consistent with the results obtained from MIN6 cells, mRNA expression of amylin in murine pancreatic islets was significantly enhanced by TNF-α after 9 h of stimulation (Fig. 1c). Interestingly, the inductive effect of TNF-α on amylin mRNA was more potent in islets than in MIN6 cells, suggesting that amylin expression in response to TNF-α is more sensitive in islets than in transformed beta cells. As amylin and insulin are co-localised in beta cells and co-secreted in response to glucose [23], we then examined the effect of TNF-α on proinsulin expression in MIN6 cells. While TNF-α upregulated amylin mRNA levels in MIN6 cells and murine primary islets in a time-dependent manner (Fig. 1a, ESM Fig. 1), it had no effect on proinsulin 1 and proinsulin 2 mRNA expressions in MIN6 cells (Fig. 1d) and islets (ESM Fig. 1), suggesting that TNF-α specifically induces amylin expression in beta cells. We checked the effect of TNF-α on cytokine expression in MIN6 cells and found that 3 to 15 h of TNF-α treatment at 2.87 nmol/l had no effect on *Il1β* (also known as *Il1b*), *Il6* or *Mcp-1* (also known as *Ccl2*) mRNA expression (data not shown). These results suggest that the inductive effect of TNF-α on amylin expression was not indirectly mediated by cytokines induced by TNF-α.

To determine the effect of TNF-α on amylin mRNA stability, MIN6 cells pretreated for 9 h with or without 2.87 nmol/l TNF-α were cultured with 5 μg/ml actinomycin D for 2, 5 and 7 h, after which amylin mRNA levels were examined by real-time PCR. Although TNF-α markedly increased amylin mRNA expression, there was no significant difference in the curves of mRNA decay between TNF-α-treated and control groups (Fig. 1e), suggesting that TNF-α increased amylin expression at the transcriptional level.

We next examined the effect of TNF-α on amylin protein production. Western blot assay showed that in the resting state, mature amylin is the main form of amylin in murine pancreatic islets. Stimulation of murine islets with 2.87 nmol/l TNF-α or 16 mmol/l glucose for 24 h significantly increased levels of proamylin (~8 kDa) and the intermediate form of amylin (~6 kDa) (Fig. 2a, b). TNF-α had no significant effect on the expression of *Pc1/3* or *Pc2* (Fig. 2c), which are responsible for proamylin processing [24]. These results might explain the increase of proamylin and the intermediate form of amylin by TNF-α stimulation.

The $PKC\zeta-ERK1/2-AP1$ and $PKC\zeta-JNK-AP1$ pathways are involved in TNF-α-induced amylin gene expression It has been reported that the biological effects of TNF-α are mediated through mitogen-activated protein kinase (MAPK) pathways [25]. We observed that pretreatment of MIN6 cells with PD98059, a MEK 1/2 inhibitor, or with SP600125, an inhibitor of JNK, inhibited TNF-α-

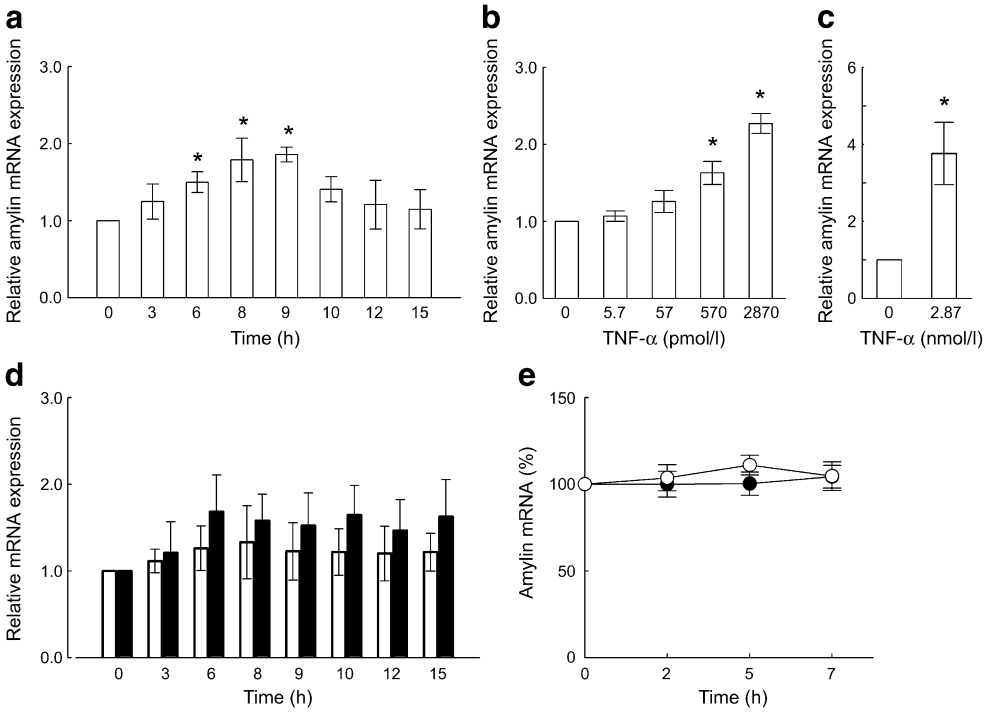

Fig. 1 TNF-α upregulates amylin gene expression. MIN6 cells were incubated with 2.87 nmol/l TNF-α for different time periods (**a**, **d**) or (**b**) with different concentrations of TNF-α for 9 h. Total RNA was then extracted and examined for amylin (**a**, **b**) or proinsulin (**d**) mRNA expression by real-time PCR. White bars, proinsulin 1; black bars, proinsulin 2. **c** Mouse pancreatic islets were treated with 2.87 nmol/l TNF-α for 9 h and amylin mRNA expression examined by real-time PCR. **e** MIN6 cells pretreated with (white circles) or without (control, black circles) 2.87 nmol/l TNF-α for 9 h were cultured with 5 μg/ml actinomycin D for the indicated time intervals. Amylin mRNA expression was then examined by real-time PCR. All data are shown as mean ± SD of three independent experiments. *$p<0.05$ vs MIN6 cells or islets cultured with control medium

induced amylin gene expression (Fig. 3a). PD98059 and SP600125 at the tested concentrations had no effect on cell viability as examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown). These results indicate that TNF-α might upregulate amylin gene expression through activation of signalling pathways related to ERK 1/2 and JNK. As PD98059 and SP600125 have also been reported to inhibit other protein kinases [26], it is possible that protein kinases other than ERK1/2 and JNK may also be involved in amylin upregulation by TNF-α.

Protein kinase C_ζ, a member of the atypical PKC family, acts as a signalling molecule of TNF-α and has been shown to induce MAPK activation [27]. We examined

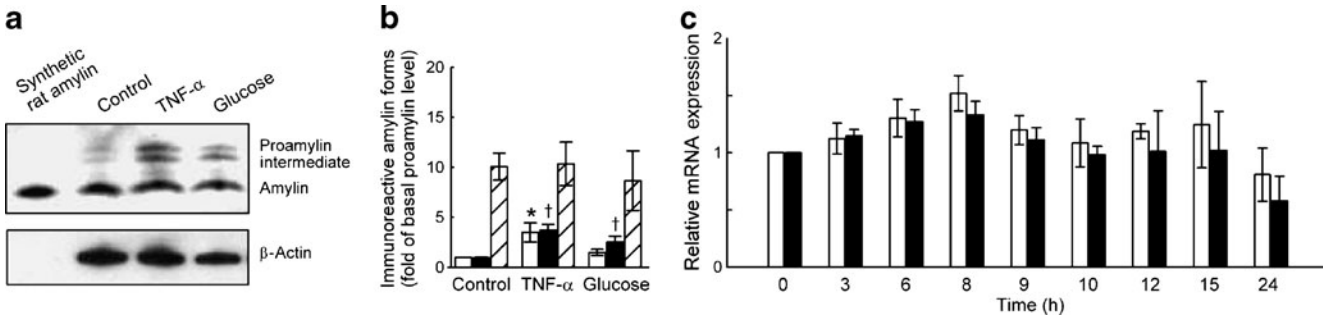

Fig. 2 TNF-α induces amylin protein production. **a** Murine pancreatic islets were cultured overnight in control medium containing 2.8 mmol/l glucose, followed by treatment with 2.87 nmol/l TNF-α or 16 mmol/l glucose for 24 h. Amylin protein levels were examined by western blot assay. A representative gel is shown, in which synthetic rat amylin was used as amylin positive control. **b** Quantification of blot (**a**). White bars, proamylin; black bars, intermediate form of amylin; hatched bars, amylin. *$p<0.05$ vs proamylin in islets cultured with control medium; †$p<0.05$ vs intermediate form of amylin in islets cultured with control medium. **c** MIN6 cells were incubated with 2.87 nmol/l TNF-α for different time periods, then total RNA was extracted and examined for prohormone convertase 1/3 (white bars) and 2 (black bars) mRNA by real-time PCR. All data are shown as mean ± SD of three independent experiments

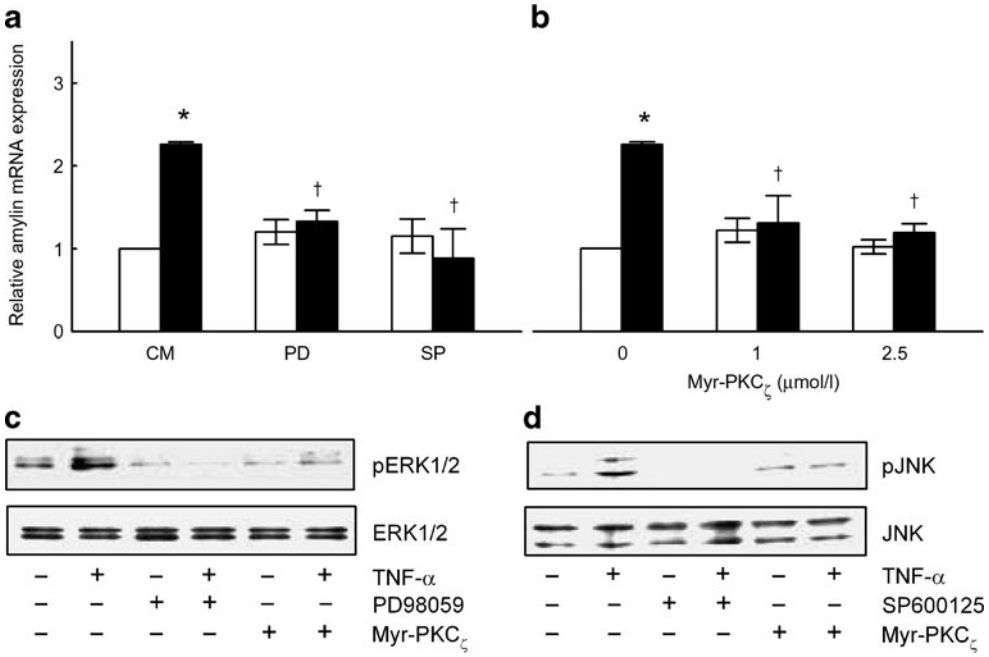

Fig. 3 PKCζ–ERK1/2 and PKCζ–JNK signalling pathways are involved in TNF-α-induced amylin gene expression. **a** MIN6 cells were incubated for 1 h with control medium (CM), 30 μmol/l PD98059 (PD), 50 μmol/l SP600125 (SP) or (**b**) different concentrations of Myr–PKCζ, then stimulated with 2.87 nmol/l TNF-α (black bars) for 9 h and examined for amylin expression by real-time PCR. White bars, control. Values are mean ± SD of three independent experiments; *$p<0.05$ vs cells cultured with CM; †$p<0.05$ compared with cells treated with TNF-α alone. **c, d** MIN6 cells pretreated for 2 h with 2.5 μmol/l Myr–PKCζ, 30 μmol/l PD or 50 μmol/l SP were stimulated with 2.87 nmol/l TNF-α for 5 min. ERK1/2 (**c**) or JNK (**d**) phosphorylation was examined by western blot. The experiments were performed at least three times and representative results are shown

whether PKCζ was involved in TNF-α-induced amylin gene expression. Pretreatment of MIN6 cells with Myr-PKCζ, an inhibitor of PKCζ, significantly inhibited 2.87 nmol/l TNF-α-induced amylin mRNA expression (Fig. 3b). As Myr-PKCζ at the tested concentrations had no effect on cell viability (data not shown), these results suggest that PKCζ is also involved in TNF-α-induced amylin gene expression. Then we examined the relationship between PKCζ, ERK 1/2 and JNK activation by TNF-α. As shown in Fig. 3c, d, treatment of MIN6 cells with 2.87 nmol/l TNF-α stimulated rapid phosphorylation of ERK1/2 and JNK. Pretreatment of MIN6 cells with Myr-PKCζ significantly inhibited this phosphorylation. As positive controls, MEK 1/2 inhibitor PD98059 and JNK inhibitor SP600125 inhibited TNF-α-induced ERK 1/2 and JNK phosphorylation, respectively. These results indicate that TNF-α induces amylin expression in MIN6 cells through activation of the PKCζ–ERK1/2 and PKCζ–JNK pathways.

AP1 is a crucial transcription factor in TNF-α signalling pathway and has been reported to act as downstream molecule of MAPK [25]. AP1 is formed by dimerisation of Jun proteins (c-Jun, JunB and JunD) or heterodimerisation of a Jun protein with a Fos protein (c-Fos, FosB, Fra-1 and Fra-2) [28]. Analysis with AliBaba version 2.1 software (http://wwwiti.cs.uni-magdeburg.de/grabe/alibaba2) revealed the existence of a few AP1 binding sites in the promoter region of murine amylin gene. To examine whether AP1 is involved in TNF-α-induced amylin gene expression, we transfected MIN6 cells with a control vector pcDNA 3.1 or a *c-Jun* dominant-negative construct (*c-Jun* DN) [29] and found that overexpression of *c-Jun* DN significantly attenuated TNF-α-induced amylin gene expression in MIN6 cells (Fig. 4a). Transfection of murine primary pancreatic islets with *c-Jun* DN also inhibited the inductive effect of TNF-α on amylin gene expression (Fig. 4b). Further studies showed that TNF-α failed to induce amylin expression in islets isolated from *Fos* knockout mice (Fig. 4c). These results suggest that AP1, which is composed of c-Jun and c-Fos, plays an essential role in induction of amylin gene expression by TNF-α.

We next tested whether AP1 is a transcription factor downstream of PKCζ–ERK1/2 and PKCζ–JNK signalling, which mediates amylin upregulation by TNF-α. EMSA assay with a consensus AP1 probe or an amylin AP1 probe that contains the AP1 binding sequence at the promoter region (−1574/−1568) of murine amylin gene showed that TNF-α significantly increased the binding activity of AP1 in MIN6 cells (Fig. 5a, b). In TNF-α-stimulated cells, excess unlabelled consensus AP1 probe and amylin AP1 probe both competed for consensus AP1 binding, while excess amylin AP1 probe competed for amylin AP1 binding (Fig. 5a), supporting the notion that

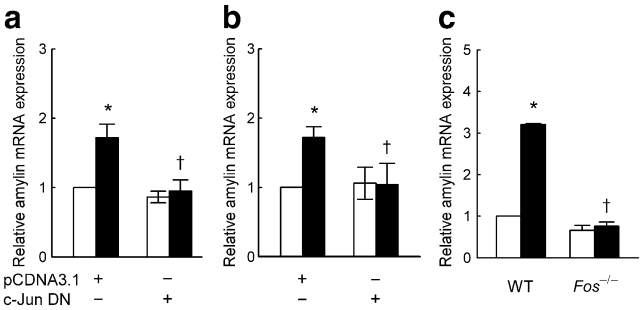

Fig. 4 TNF-α stimulates amylin gene expression through activation of AP1. MIN6 cells (**a**) or mouse pancreatic islets (**b**) were transfected for 36 h with *c-Jun* DN or control vector pCDNA 3.1, then treated with (black bars) or without (control, white bars) 2.87 nmol/l TNF-α for another 9 h. Amylin mRNA expression was examined by real-time PCR. **c** Mouse pancreatic islets isolated from wild type (WT) or *Fos*$^{-/-}$ mice were treated with 2.87 nmol/l TNF-α for 9 h and amylin mRNA expression examined by real-time PCR. All data are shown as mean ± SD of three independent experiments. *$p<0.05$ vs pcDNA3.1-transfected MIN6 cells or islets from WT mice cultured with control medium; †$p<0.05$ vs pcDNA3.1-transfected MIN6 cells or islets from WT mice in response to TNF-α

the amyln AP1 probe is specific; moreover, TNF-α induced AP1 binding to the promoter region of amylin gene. The increased amylin AP1 activity induced by TNF-α was significantly inhibited by pretreatment of MIN6 cells with Myr-PKC$_\zeta$, SP600125 or PD98059 (Fig. 5b,c). Taken together, these results suggest that TNF-α induced amylin gene expression in beta cells through the PKC$_\zeta$−ERK1/2−AP1 and PKC$_\zeta$−JNK−AP1 pathways.

TNF-α induces amylin gene expression through pathways related to that of phosphatidylinositol 3- kinase–NF-κB Transcription factor NF-κB is a key component of the TNF-α signal transduction pathway [25]. Phosphatidylinositol 3 kinase (PI3K) has been reported to be an upstream molecule of NF-κB [30]. Thus we checked whether PI3K and NF-κB were involved in TNF-α-induced amylin gene expression in pancreatic beta cells. Pretreatment of MIN6 cells with wortmannin (PI3K inhibitor) or sulfasalazine (NF-κB inhibitor) [31] significantly inhibited TNF-α-induced amylin gene expression (Fig. 6a). Wortmannin and sulfasalazine at the tested concentrations had no effect on cell viability (data not shown). These results suggest that PI3K and NF-κB are involved in amylin upregulation by TNF-α. To further confirm that NF-κB activation is involved in the induction of amylin expression by TNF-α, we transfected MIN6 cells or mouse pancreatic islets with an *Nfkbia* dominant-negative construct (*Nfkbia*-DN) [32] or control vector flag-zeo. Overexpression of *Nfkbia*-DN in MIN6 cells (Fig. 6b) and islets (Fig. 6c) both significantly attenuated TNF-α-induced amylin gene expression, suggesting an essential role of NF-κB in the upregulation of amylin expression by TNF-α.

We next examined whether PI3K is an upstream signalling molecule in NF-κB activation by TNF-α. Compared with MIN6 cells transfected with pGL3-basic plasmid, cells transfected with *NF-κB*-Luc responded to TNF-α with a 5.6-fold increase of luciferase activity. Pretreatment of *NF-κB*-Luc transfected cells with wortmannin and pyrrolidine dithiocarbamate (NF-κB inhibitor) both significantly inhibited TNF-α-induced NF-κB activation (Fig. 6d), indicating that PI3K mediates NF-κB activation by TNF-α in beta cells. These results suggest that pathways related to that of PI3K–NF-κB are involved in TNF-α-induced amylin gene expression in mouse beta cells.

TNF-α induces human amylin promoter activation through AP1 and NF-κB It has been reported that transcription of

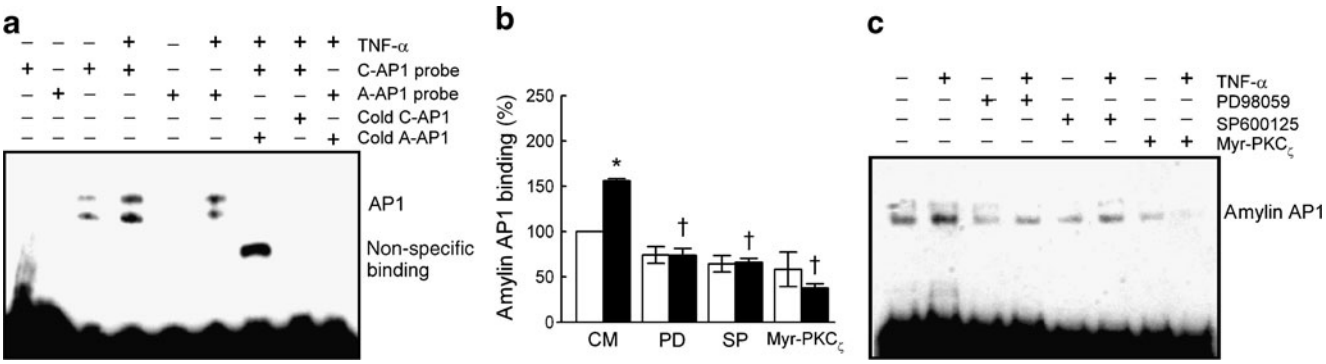

Fig. 5 TNF-α activates AP1 through ERK1/2, JNK and PKC$_\zeta$. **a** MIN6 cells were treated for 2 h with or without 2.87 nmol/l TNF-α, and the nuclear protein was extracted and applied for EMSA using biotin-labelled amylin AP1 probe (A-AP1 probe) or consensus AP1 probe (C-AP1 probe). As competitor we used 100-fold of unlabelled consensus AP1 probe (cold-C-AP1) or amylin AP1 probe (cold-A-AP1). The experiments were performed at least three times and representative results are shown. **b** MIN6 cells pretreated for 1 h with control medium (CM), 2.5 μmol/l Myr−PKC$_\zeta$, 50 μmol/l SP600125 (SP) or 30 μmol/l PD98059 (PD) were stimulated with (black bars) or without (white bars) 2.87 nmol/l TNF-α for another 2 h. The nuclear protein was extracted and applied for EMSA using biotin-labelled amylin AP1 probe. Values are means ± SD of three independent experiments; *$p<0.05$ vs cells cultured with CM; †$p<0.05$ compared with cells treated with 2.87 nmol/l TNF-α alone. **c** A representative gel for experiment shown above (**b**)

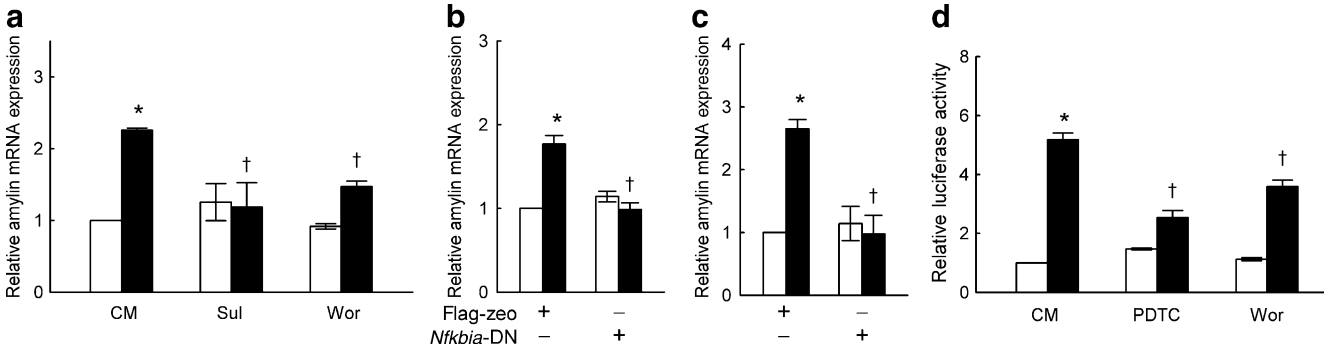

Fig. 6 Involvement of PI3K–NF-κB signalling pathway in TNF-α-induced amylin gene expression in MIN6 cells and pancreatic islets. **a** MIN6 cells pretreated for 1 h with control medium (CM), 50 μmol/l sulfasalazine (Sul) or 100 nmol/l wortmannin (Wor) were stimulated with (black bars) or without (white bars) 2.87 nmol/l TNF-α for 9 h, then amylin mRNA expression was examined by real-time PCR. *$p<0.05$ vs cells cultured with control medium; †$p<0.05$ compared with cells treated with TNF-α alone. **b** MIN6 cells or mouse pancreatic islets (**c**) transiently transfected with *Nfkbia*-DN or control vector flag-zeo for 36 h were treated with control medium (white bars) or 2.87 nmol/l TNF-α (black bars) for another 9 h and amylin mRNA expression was examined by real-time PCR. *$p<0.05$ vs flag-zeo-transfected cells cultured with control medium; †$p<0.05$ vs flag-zeo-transfected cells in response to TNF-α. **d** The pGL3-basic or NF-κB-Luc construct was co-transfected with the pRL-TK renilla luciferase reporter plasmid into MIN6 cells. After 36 h, cells were treated with control medium (CM) or medium containing various inhibitors (20 μmol/l pyrrolidine dithiocarbamate [PDTC], 100 nmol/l wortmannin) for 1 h followed by 2.87 nmol/l TNF-α (black bars; white bars, control) for additional 6 h.. Luciferase activities were measured, normalised to renilla expression of the pRL-TK plasmid and are shown as fold over the activity of the pGL3-basic construct in control medium. *$p<0.05$ vs cells cultured in control medium; †$p<0.05$ vs cells treated with TNF-α alone. All data are shown as mean ± SD of three independent experiments

human amylin gene is controlled by a promoter region spanning from −2,798 to 450 relative to the transcriptional start site. The −222/450 and −391/450 sequences have approximately the same transcription activity as the whole promoter region of human amylin [21]. We therefore constructed luciferase reporter plasmids containing these two sequences to test the effect of TNF-α on human amylin gene transcription. Compared with MIN6 cells transfected with pGL-3-basic plasmid, cells transfected with human amylin promoter constructs −391/450 or −222/450 both responded to TNF-α (Fig. 7a, b), demonstrating that human amylin gene expression is also enhanced by TNF-α.

We analysed transcription factor binding sites in the human amylin promoter region with AliBaba2 software and found that five AP1 and two NF-κB binding sites were located in the region from −222 to 450 (Table 1). Then we further tested whether AP1 and NF-κB were also involved in TNF-α-induced human amylin expression. Pretreatment of −222/450 construct-transfected MIN6 cells with curcumin (AP1 inhibitor) [33] or sulfasalazine (NF-κB inhibitor) both significantly inhibited TNF-α-induced human amylin promoter activation (Fig. 7c), suggesting that AP1 and NF-κB also mediate TNF-α-induced human amylin gene expression.

Discussion

In the current study, we investigated the effect of TNF-α on amylin gene expression in the mouse pancreatic beta cell line MIN6 and in pancreatic islets. We found that TNF-α acutely induced amylin expression at mRNA and protein levels, but had no effect on proinsulin expression. We further demonstrated that TNF-α had no effect on amylin mRNA stability and that it enhanced amylin gene expression through pathways related to the PKC_{ζ}–ERK/JNK–AP1 and PI3K–NF-κB signalling pathways (Fig. 8). Human amylin gene is also upregulated by TNF-α through activation of AP1 and NF-κB.

Shepherd et al. [34] reported that treatment with TNF-α (0.57 nmol/l, 48 h) had no effect on human amylin gene promoter activity and decreased amylin mRNA level in rat islets. The inconsistent results of human amylin promoter activation by TNF-α in their and our study may be due to different human amylin promoter regions and/or different TNF-α concentrations/treatment times used in the experiments. Our studies on amylin mRNA and protein level as well as amylin mRNA stability all demonstrated that TNF-α could upregulate amylin expression. We also found that when MIN6 cells were stimulated with 0.57 nmol/l TNF-α for up to 48 h, amylin mRNA level was robustly increased after 9 h stimulation followed by a gradual recovery to basal level (ESM Fig. 2). All these results show that TNF-α acutely induces murine amylin gene expression and promotes human amylin transcription.

Increased circulating levels of TNF-α and amylin have been detected in obese and insulin-resistant human patients [8–10, 14, 35]. Plasma level of amylin or TNF-α is also elevated in genetically obese, insulin-resistant animal models [11, 35]. Although we didn't observe inductive effects of TNF-α on amylin expression after prolonged treatment of MIN6 cells with TNF-α for up to 48 h, TNF-α

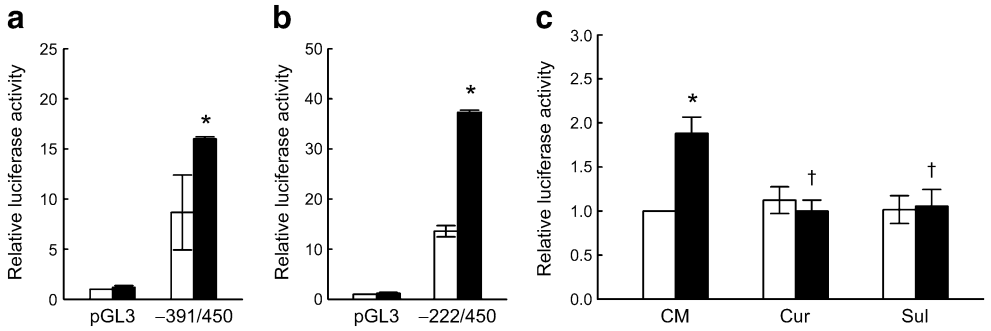

Fig. 7 TNF-α activates human amylin promoter through AP1 and NF-κB. **a**, The pGL3-basic vector (pGL3) or human amylin promoter luciferase constructs, −391/450 and (**b**) −222/450, was co-transfected with the pRL-TK renilla luciferase reporter plasmid into MIN6 cells. After 36 h, the cells were stimulated with 2.87 nmol/l TNF-α (black bars; control, white bars) and luciferase activities were measured 8 h later. **c** MIN6 cells transfected with −222/450 construct were incubated for 1 h with control medium (CM), 10 μmol/l curcumin (Cur) or 500 μmol/l sulfalasazine (Sul), then stimulated for 8 h with 2.87 nmol/l TNF-α (black bars; control, white bars) and examined for luciferase activities. All data are shown as mean ± SD of three independent experiments with each condition tested in triplicate. *$p<0.05$ for amylin promoter luciferase construct-transfected cells in response to TNF-α vs control medium; $^{\dagger}p<0.05$ compared with cells treated with 2.87 nmol/l TNF-α alone

may nevertheless induce amylin expression under acute inflammation-related conditions. Elevation of plasma amylin concentration was observed in severe acute pancreatitis [6]. Although amylin may be increased as a consequence of islet cell necrosis, it may also be induced by TNF-α, which plays an important role in the inflammatory response in pancreatitis. In islet transplantation, TNF-α has been shown to be produced locally by macrophages from the recipient and the donor [36, 37]. The elevation of amylin in the circulation of patients with islet transplantation may result from amylin upregulation by TNF-α, but needs further investigation.

Using a series of biochemical and molecular biology methods with specific inhibitors of PKCζ, MAPK, PI3K and NF-κB, with dominant-negative constructs for *c-Jun* and *Nfkbia*, and with islets from *Fos* knockout mice, we demonstrated that TNF-α-induced amylin expression in murine pancreatic beta cells through the PKCζ−JNK−AP1, PKCζ−ERK1/2−AP1 and PI3K–NF-κB pathways. There was no difference in AP1 activation by TNF-α between *Nfkbia*-DN plasmid-transfected cells and control vector-transfected cells (ESM Fig. 3), suggesting that PI3K–NF-κB and PKCζ−JNK/ERK1/2−AP1 are two independent pathways involved in murine amylin upregulation by TNF-α.

Our results demonstrated that AP1 and NF-κB not only mediated amylin upregulation by TNF-α in mouse pancreatic islets, but also participated in TNF-α-induced human amylin promoter activation. While analysis with AliBaba2 revealed the existence of AP1 binding sites in the promoter regions of human and mouse amylin genes, NF-κB binding sites only exist in the promoter region of human amylin gene. Therefore, NF-κB activation may play an indirect role in murine amylin upregulation by TNF-α. AP1 and NF-κB are also the downstream signalling molecules of other proinflammatory cytokines. Our studies revealed that TNF-α-induced murine amylin expression is independent of IL1β, IL6 or monocyte chemoattractant protein 1,

Table 1 Putative transcriptional factor binding sites in the promoter region (−222/+450) of human amylin gene predicted with AliBaba2

Position per transcription factor	Sequence
AP1	
−214 to +208	TGACACA
−141 to +135	TGACCCA
−83 to +77	TGACAGA
−59 to +53	GATGTCA
+85 to +91	TGACATT
NF-κB	
+123 to +133	CTGGGAGAGTT
+246 to +256	TTGGGAAAGTA

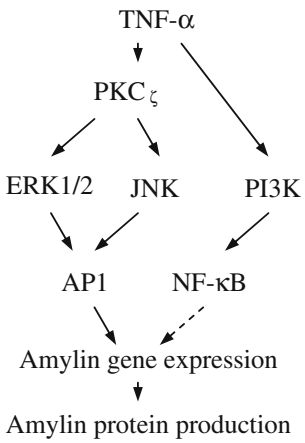

Fig. 8 Proposed pathways for TNF-α-induced amylin expression and production in mouse pancreatic beta cells. This overview is based on the present findings and does not exclude participation of other signalling pathways or complementary signals within the diagram presented

suggesting that TNF-α signalling directly activates AP1 and NF-κB to promote amylin expression. Ca^{2+} and pancreatic and duodenal homeobox 1 (PDX-1) have been reported to be involved in the upregulation of amylin gene expression by other stimuli, such as glucose and NEFA [20, 38]. Pretreatment of MIN6 cells with EGTA (an extracellular calcium chelator) or 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) (an intracellular calcium chelator), or transfection of MIN6 cells with dominant-negative PDX-1 expression plasmid had no influence on amylin upregulation by TNF-α (data not shown), suggesting that Ca^{2+} and PDX-1 are not involved in TNF-α-induced amylin expression.

Immunoreactivity for the NH_2-terminal flanking region of proamylin has been found in islet amyloid deposits in pancreas from type 2 diabetic patients [39]. It has been proposed that impaired processing of proamylin may lead to hypersecretion of unprocessed or partially processed forms of proamylin that may have a higher tendency to aggregate than mature amylin [40]. It has been reported that chronic exposure to high concentrations of glucose upregulates *Pc1/3* and *Pc2* mRNA levels and increases cellular content of amylin precursors in murine beta cells [41, 42]. However, acute treatment with glucose induces prohormone convertase (PC)1/3 and PC2 production in MIN6 cells at the translational level, but not the transcriptional level [43]. Our results reveal that acute treatment with TNF-α also had no acute effect on *Pc1/3* and *Pc2* mRNA expressions in MIN6 cells. We found that glucose and TNF-α increased levels of proamylin and the intermediate form of amylin in murine islets. Although glucose acutely upregulated PC1/3 and PC2 at the translational level, the activity of these enzymes might not have increased accordingly, leading to the increase of amylin precursors. The disproportional increase between PC1/3 and PC2 activity and proamylin expression may also have applied to TNF-α-induced increase of amylin precursors, but needs further investigation. Widespread amyloid depositions have been found in transplanted islets in type 1 diabetes patients and mouse models of type 1 diabetes [44, 45]. Increased plasma amylin has been reported as a sign of impaired beta cell function in type 1 diabetic patients after kidney and pancreas transplantation [7]. The state of systemic inflammation and the inflammatory mediators have been proposed as causal roles in islet transplantation failure [46]. Amylin upregulation by proinflammatory cytokine TNF-α might contribute to plasma amylin elevation and amyloid deposition. But the effect of TNF-α on human proamylin processing and amylin aggregation/deposition needs to be examined with murine islets transfected with human proamylin or transgenic mice expressing human proamylin in beta cells [47].

Taken together, our studies demonstrate that TNF-α acutely induces amylin gene expression in murine beta cells through pathways related to the PKC_ζ−JNK−AP1, PKC_ζ−ERK1/2−AP1 and PI3K-NF-κB signalling pathways. AP1 and NF-κB are also involved in upregulation of human amylin transcription. Our results and the available evidence suggest that in addition to direct involvement in inflammatory disorders and insulin resistance, TNF-α may play an important role in overexpression of amylin under acute inflammatory conditions.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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