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Tumour necrosis factor-α suppresses the hypoxic response by NF-κB-dependent induction of inhibitory PAS domain protein in PC12 cells

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Inflammation is often accompanied by hypoxia. However, crosstalk between signalling pathways activated by inflammation and signalling events that control adaptive response to hypoxia is not fully understood. Here we show that exposure to tumour necrosis factor-α (TNF-α) activates expression of the inhibitory PAS domain protein (IPAS) to suppress the hypoxic response caused by hypoxia-inducible factor (HIF)-1 and HIF-2 in rat pheochromocytoma PC12 cells but not in human hepatoma Hep3B cells. This induction of IPAS was dependent on the nuclear factor-κB (NF-κB) pathway and attenuated hypoxic induction of HIF-1 target genes such as tyrosine hydroxylase (TH) and vascular endothelial growth factor (VEGF). HIF-dependent reporter activity in hypoxia was also decreased following TNF-α treatment. Knockdown of IPAS mRNA by small interfering RNA (siRNA) restored the TNF-α-suppressed hypoxic response. These results indicate that TNF-α is a cell-type specific suppressor of HIFs and suggest a novel crosstalk between stimulation by inflammatory mediators and HIF-dependent hypoxic response.

Keywords: HIF-1/hypoxic response/IPAS/NF-κB/TNF-α.

Abbreviations: $CoCl_2$, cobalt chloride; HIF, hypoxia-inducible factor; HRE, hypoxia response element; IPAS, inhibitory PAS domain protein; NF-κB, nuclear factor-κB; NF-κB-RE, NF-κB response element; ROS, reactive oxygen species; siRNA, small interfering RNA; TH, tyrosine hydroxylase; TNF-α, tumour necrosis factor-α; TNF-R, TNF-receptor; VEGF, vascular endothelial growth factor.

There are several lines of evidence demonstrating that inflamed tissues are hypoxic due to their elevated metabolic activity and metabolically active infiltrating inflammatory cells (*1*, *2*). Blood vessel stenosis and microthrombosis caused by inflammation also decrease oxygen supply resulting in hypoxia (*3*). In the microenvironment of hypoxia, hypoxia-inducible factors (HIFs) act as master regulators of adaptation to hypoxia, leading to transcriptional activation of various genes that participate in angiogenesis, glucose metabolism, cell proliferation and cell survival (*4*). HIFs are composed of two subunits, the oxygen-dependent HIF-α subunit and the constitutively expressed aryl hydrocarbon receptor nuclear translocator (Arnt, HIF-1β) subunit (*5*). There are three HIF-α subunits, HIF-1α, HIF-2α (HLF, EPAS-1) and HIF-3α (*5–8*). HIF-1α and HIF-2α are particularly critical for the hypoxic response. On the other hand, HIF-3α has lower transcriptional activity than HIF-1α and HIF-2α due to lack of the C-terminal activation domain. Therefore HIF-3α is considered to be a negative regulator of the hypoxic response (*9*). Inhibitory PAS domain protein (IPAS) is a splicing variant of HIF-3α and directly binds to HIF-1α and HIF-2α to abrogate their binding activity to hypoxia-response element (HRE), resulting in suppression of HIF activity (*10*, *11*). IPAS is induced in response to hypoxia via HIF-1 activation in some cell-types through its unique promoter containing an HRE, which is recognized by HIF-1 and HIF-2 (*11*, *12*). Recently we reported transcriptional activation of the IPAS gene via the nuclear factor-κB (NF-κB) pathway stimulated by cobalt chloride ($CoCl_2$)-dependent reactive oxygen species (ROS) and ROS-evoked calcium influx (*13*).

Tumour necrosis factor-α (TNF-α) is a major mediator of inflammation, and sustained activation of TNF-α signalling has been implicated in the pathogenesis of a wide spectrum of diseases (*14*). TNF-α signals through two distinct cell surface receptors, TNF-receptor 1 (TNF-R1) and TNF-receptor 2 (TNF-R2). TNF-R1 is widely expressed on all cells of the body, whereas TNF-R2 exhibits more restricted expression on certain subpopulations of immune cells and a few other cell types (*15*, *16*). TNF-α binding to TNF-R activates the transcription factor NF-κB via the canonical pathway. NF-κB targets many genes that facilitate inflammation, cellular immortality, cell survival, angiogenesis, proliferation, tumour promotion and metastasis (*17*). Recent studies have shown that inflammatory mediators including TNF-α activate HIF-1α under normoxic conditions at the transcriptional and/or translational levels in a cell type-specific manner (*2*, *18*).

In this article, we report that NF-κB activation in PC12 cells upregulates IPAS gene expression without activating HIF-1α and HIF-2α. This

TNF-α-stimulated IPAS expression attenuates cellular responses to hypoxia and chemical hypoxia caused by $CoCl_2$.

Materials and Methods

Cytokines and chemicals

Rat and human TNF-α were purchased from Wako Pure Chemical Industries (Osaka, Japan), and used for PC12 cells and Hep3B cells, respectively. TNF-α was dissolved in 0.1% bovine serum albumin. BMS-345541 was obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was from Dojindo Laboratories (Kumamoto, Japan).

Cell culture

Rat pheochromocytoma PC12 cells and human hepatoma Hep3B cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, and maintained as described (*13*).

Construction of plasmids

Construction of pG5ELuc and pGL3-HRE4SV40Luc was described previously (*19*, *20*). pGL4-E1bLuc2 was constructed by inserting the blunt-ended SalI–NcoI fragment of pG5ELuc into the blunt-ended BglII and NcoI sites of pGL4.10[Luc2] (Promega, Madison, WI, USA). pGL4-NF-κB-RE5E1bLuc2 was constructed by inserting the synthesized oligonucleotides with five copies of the NF-κB response element sequence, 5′-CTAGC GGGAA TTTCC GGGGA C TTTC CGGGA ATTTC CGGGG ACTTT CCGGG AATTT CCA-3′ and 5′-GATCT GGAAA TTCCC GGAAA GTCCC CG GAA ATTCC CGGAA AGTCC CCGGA AATTC CCG-3′, into the NheI and BglII sites of pGL4-ElbLuc2. pGL4-SV40Luc2 and pGL4-HRESV40Luc2 were constructed by inserting the XhoI–HindIII fragments of pGL3-promoter and pGL3-HRE4SV40Luc, respectively, into the XhoI and HindIII sites of pGL4.10[Luc2]. All constructions were validated by sequence analysis.

DNA transfection and luciferase assay

PC12 cells (1×10^5) were seeded in polyethleneimine (PEI)-coated 24-well plates (*21*) and Hep3B cells (7.5×10^4) were plated in 24-well plates. These cells were cultured 1 day prior to transfection. The cells were transfected with 0.05 μg of luciferase reporter plasmid and 0.05 μg of a β-galactosidase expression plasmid, pBOS-LacZ, with the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Four hours after transfection, cells were treated with TNF-α for 6 or 12 h. Exposure to $CoCl_2$ or hypoxia (1% O_2) was performed 1 h after TNF-α treatment. Cell lysates were prepared using Passive Lysis Buffer (Promega), and luciferase activity was determined with the Luciferase Assay System (Promega), and normalized to β-galactosidase activity as described previously (*20*).

RNA isolation and RT-PCR

PC12 cells (1×10^6) were seeded in PEI-coated 6-well plates and Hep3B cells (4×10^5) were plated in 60-mm dishes. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) 1 day before experiments. After TNF-α treatment, total RNA was isolated using RNAiso plus (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. Reverse transcription and PCR were performed as described previously (*13*). PCR cycles were chosen within the linear range of amplification. The PCR procedure consisted of 16 cycles of reaction for 18 S rRNA, 19 cycles for TH, 24 cycles for HIF-2α and vascular endothelial growth factor (VEGF), 27 cycles for HIF-1α, 28 cycles for TNF-R1, 30 cycles for pVHL, 33 cycles for HIF-3α and human IPAS, 35 cycles for rat IPAS and TNF-R2 of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The primers of 18 S rRNA, rat IPAS, HIF-1α, HIF-2α, HIF-3α, pVHL, tyrosine hydroxylase (TH) and VEGF were previously described (*13*, *22*). The other primers were as follows: human IPAS, 5′-GATGG TGCTA CTCTT GGTCT C-3′ (forward) and 5′-TAGCC CAGCA CAATT CCCTC-3′ (reverse); TNF-R1, 5′-TGCGA GGTGT GTGAT AAAGG CAC-3′ (forward) and 5′-TCCCT ACAAA TGATG GAGTA GACC-3′ (reverse); TNF-R2, 5′-GATGA CAAAT CCCAG GATGC-3′ (forward) and 5′- GCTAC AGACG TTCAC GATGC A-3′ (reverse). The PCR products were electrophoresed on 1.5–2.0% agarose gels and bands were quantified by using the Scion Image software. For rat and human IPAS primers, the rat and human IPAS putative exon 16 sequences were found by homology search in the NCBI database.

Western blot analysis

Cytosolic and nuclear extracts were prepared as described previously (*20*). The extracts (5 μg protein) were resolved on 7.5% SDS–PAGE, and proteins were transferred to the Immobilon-P Transfer Membrane (Millipore Corporation, Bedfold, MA, USA). A mouse monoclonal HIF-1α antibody (Novus Biologicals, Littleton, CO, USA) diluted 1 : 1000, a rabbit polyclonal HIF-2α antibody (Novus Biologicals) diluted 1 : 1000, a rabbit polyclonal p65 antibody (Millipore) diluted 1 : 2000, a rabbit polyclonal GAPDH antibody (Trevigen, Gaithersburg, MD, USA) diluted 1 : 200 and a goat polyclonal Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 200 were used as the primary antibodies, and an HRP-linked mouse, rabbit or goat antibody (GE Healthcare, Milwaukee, WI, USA) diluted 1 : 10000 was used as the secondary antibody. Signals were detected using the ECL plus detection kit (GE Healthcare).

RNA interference

Small interfering RNA (siRNA) sequences targeting for green fluorescent protein (GFP) and IPAS/HIF-3α were previously described (*13*). The cells were treated with annealing siRNAs using the Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's protocol. 48 h after transfection, luciferase assay and RT–PCR were carried out.

MTT assay

Cell viability was determined using the MTT assay as described (*23*). PC12 cells (1×10^5) were seeded in PEI-coated 24-well plates and used for assay. Treatment of cells with TNF-α, $CoCl_2$ and hypoxia were performed similarly as described in the luciferase assay procedure.

Statistical analysis

The data are expressed as mean ± standard deviation (SD) from at least three independent experiments. The statistical significance was performed using Student's *t*-test and $P < 0.05$ were considered significant.

Results

Induction of IPAS expression following TNF-α treatment in PC12 cells

In a previous work, we found that ROS-evoked NF-κB activation upregulates IPAS gene expression (*13*). To investigate whether IPAS is induced by TNF-α that activates NF-κB, IPAS mRNA levels were examined by RT-PCR in two different cell types, a pheochromocytoma cell line PC12 cells and a hepatoma cell line Hep3B cells. There was an ~2.5-fold increase in IPAS mRNA levels in PC12 cells when treated with 100 ng/ml TNF-α (Fig. 1A). On the other hand, IPAS mRNA levels were weakly decreased in Hep3B cells by TNF-α treatment (Fig. 1B). The maximal expression of IPAS mRNA in PC12 cells occurred 6 h after TNF-α treatment (Fig. 1C). These results indicate that IPAS is induced by TNF-α in a dose- and time-dependent manner in PC12 cells, and the expression of IPAS mRNA may be regulated in a cell type-specific manner.

In order to confirm the activation of NF-κB by TNF-α, we introduced a reporter plasmid containing NF-κB response elements in the promoter region (Fig. 2A) into PC12 cells, and treated with TNF-α. An ~5-fold induction of NF-κB-dependent reporter

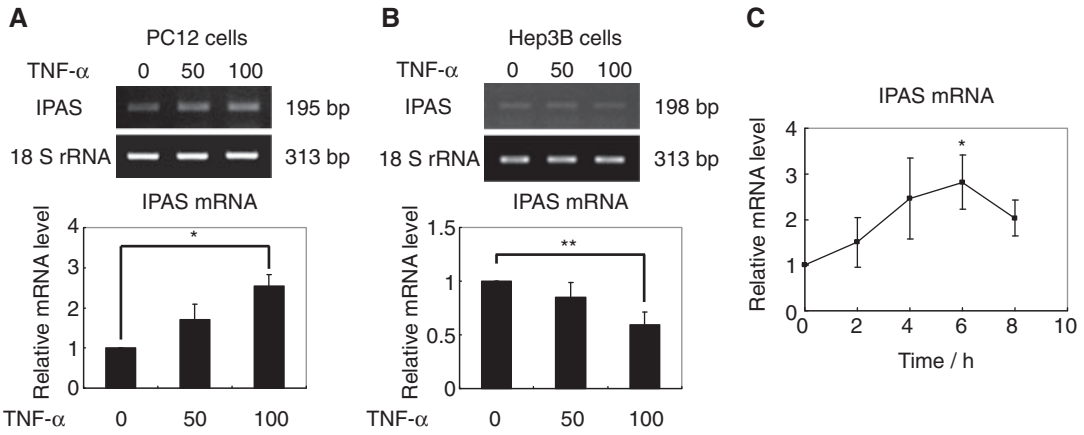

Fig. 1 Induction of IPAS expression by TNF-α treatment in PC12 cells. (A and B) Induction of IPAS mRNA caused by TNF-α. PC12 cells (A) and Hep3B cells (B) were treated with TNF-α at a concentration of 0, 50 and 100 ng/ml for 6 h. PCR products were analysed on agarose gels and quantified. The data were normalized to 18 S rRNA. (C) Time course of induction of IPAS mRNA. PC12 cells were exposed to 100 ng/ml TNF-α for the indicated times. IPAS mRNA levels were determined by RT–PCR and normalized to 18 S rRNA. $*P<0.05$ for indicated comparison. $**P<0.01$ for indicated comparison. Data shown are means ± SD of at least three independent experiments.

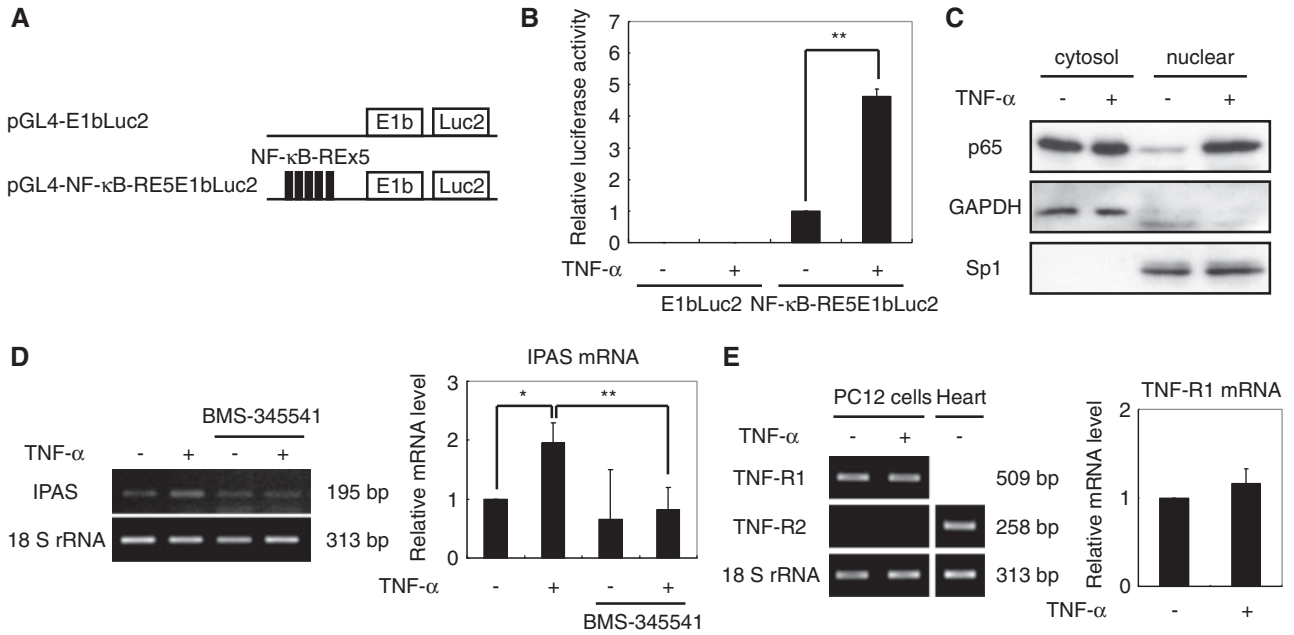

Fig. 2 Activation of NF-κB by TNF-α in PC12 cells. (A) Schematic representation of NF-κB-RE-dependent reporter plasmids. (B) Induction of NF-κB-dependent reporter activity by TNF-α. Cells were treated with 100 ng/ml TNF-α for 12 h after the transfection, and cell lysates were prepared for assay of luciferase activity. (C) Nuclear translocation of p65. Cells were exposed to 100 ng/ml TNF-α for 6 h, and nuclear extracts were prepared. p65 was detected by western blot analysis. GAPDH and Sp1 were used as controls of cytosolic and nuclear fractions, respectively. (D) Effects of BMS-345541 on the TNF-α-induced expression of IPAS mRNA. Cells were pretreated with 1.5 μM BMS-345541 for 30 min and exposed to 100 ng/ml TNF-α for 6 h. (E) Expression of TNF-R1 and TNF-R2 mRNAs in PC12 cells. TNF-R1 and TNF-R2 mRNA levels were quantified by RT–PCR. The data were normalized to 18 S rRNA. $*P<0.05$ for indicated comparison. $**P<0.01$ for indicated comparison. Data shown are means ± SD of three independent experiments.

activity was found in response to TNF-α (Fig. 2B). Since TNF-α predominantly activates the p65/p50 dimer of the NF-κB family via the canonical pathway (*17*), we analysed nuclear translocation of the p65 subunit. Translocation of p65 was clearly observed following TNF-α treatment (Fig. 2C). Furthermore, BMS-345541, a specific inhibitor of IKKβ and a weak inhibitor of IKKα, blocked the induction of IPAS mRNA by TNF-α (Fig. 2D). These results indicate that TNF-α activates the canonical NF-κB pathway to induce IPAS in PC12 cells.

NF-κB activation by TNF-α is mediated by two distinct cell surface receptors, TNF-R1 and TNF-R2 (*15*, *16*). To examine expression of cell-type specific TNF-R2 in PC12 cells, we determined the relative mRNA levels of TNF-R1 and TNF-R2. Expression of TNF-R1 mRNA was detectable and remained unchanged by the treatment of TNF-α (Fig. 2E). On the other hand, TNF-R2 mRNA, which was detectable in the heart, could not be detected (Fig. 2E). These results suggest that activation of NF-κB by TNF-α occurs through TNF-R1 in PC12 cells.

TNF-α-induced suppression of the hypoxic response in PC12 cells

To investigate whether TNF-α-induced IPAS expression suppresses the hypoxic response in PC12 cells, we analysed the effect of TNF-α on HIF-dependent reporter activity in the cells treated with $CoCl_2$ or hypoxia (1% O_2). In the previous study, $CoCl_2$ and hypoxia cause HIF-activation via different mechanisms in PC12 cells (*13*). Therefore we tested suppression by TNF-α of the $CoCl_2$- and hypoxia-induced cellular response in PC12 cells. HIF-dependent reporter activities that were increased by $CoCl_2$ or hypoxia were decreased by ~30% or 35%, respectively, in the cells treated with TNF-α for 12 h (Figs 3B and 4A). A similar result was also obtained following TNF-α treatment for 6 h (Supplementary Fig. S1A and B). Treatment of cells with $CoCl_2$ or hypoxia had little effect on NF-κB induction by TNF-α (Figs 3C and 4B). TNF-α treatment did not substantially affect nuclear accumulation of HIF-1α and HIF-2α proteins (Figs 3D and 4C). Since cell viability was unchanged by TNF-α treatment (Figs 3E and 4D), the decrease in reporter activity is not due to cell death by TNF-α.

Next, we analysed effect of TNF-α treatment on HIF-target gene expression using RT–PCR. Induced expression of TH mRNA by $CoCl_2$ was weakly but reproducibly reduced by ~15% in the cells treated with TNF-α (Fig. 3F), and hypoxia-induced TH mRNA was also suppressed to a similar extent (Fig. 4E). Elevated VEGF mRNA levels by $CoCl_2$ or hypoxia were largely reduced by ~60% or 30%,

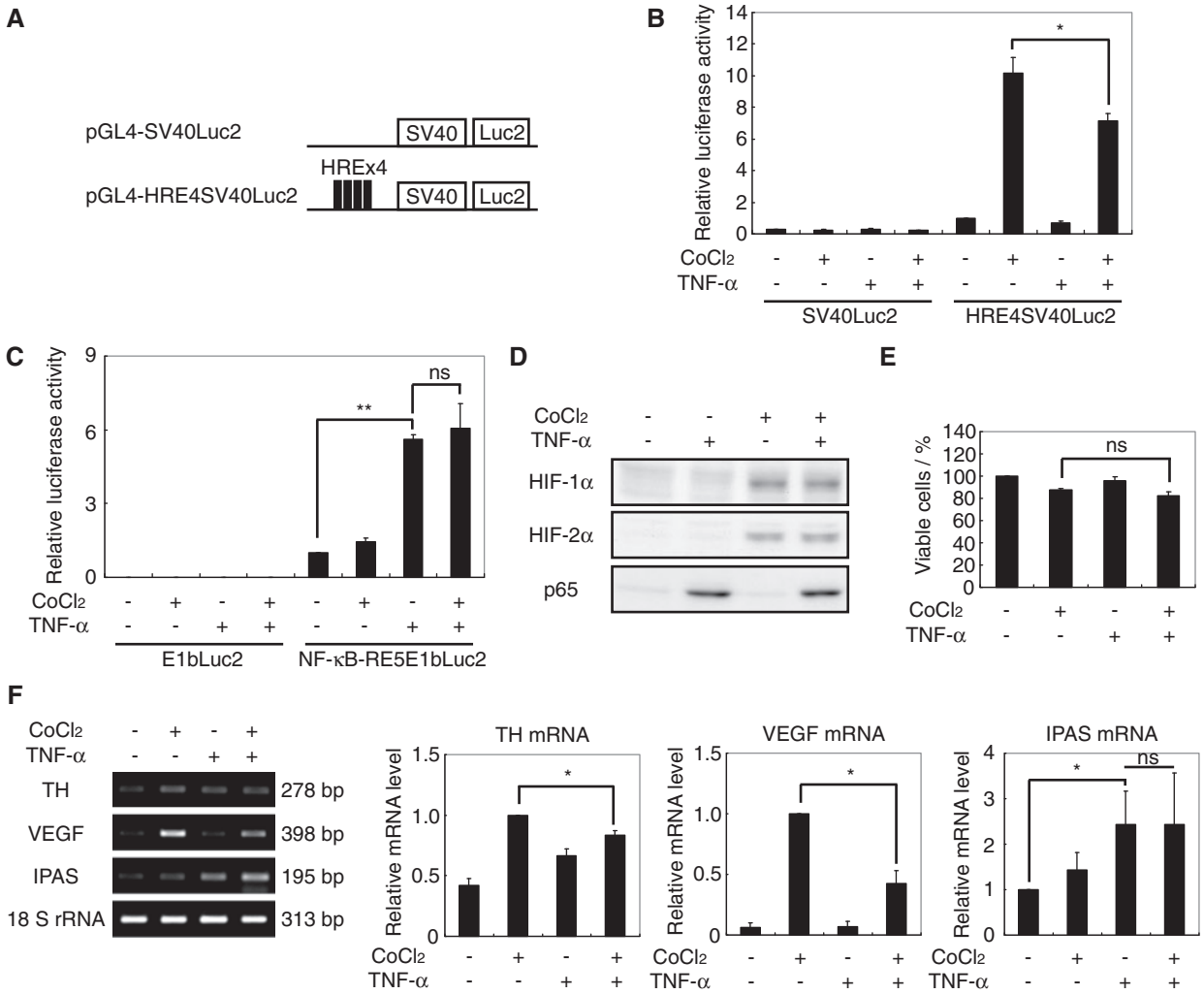

Fig. 3 Suppression of the $CoCl_2$-induced hypoxic response by TNF-α in PC12 cells. (A) Schematic representation of HRE-dependent reporter plasmids. (B) Suppression of HRE-dependent reporter activity by TNF-α. PC12 cells were pretreated with 100 ng/ml TNF-α for 1 h and exposed to 100 μM $CoCl_2$ for 12 h. (C) Effect of $CoCl_2$ treatment on the TNF-α-induced NF-κB-RE-dependent reporter activity. Cells were pretreated with 100 ng/ml TNF-α for 1 h and then exposed to 100 μM $CoCl_2$ for 12 h. (D) Effect of TNF-α on activation of HIF-α. Nuclear extracts were prepared from cells treated with $CoCl_2$ in presence or absence TNF-α and used for western blot analysis. Cells were pretreated with 100 ng/ml TNF-α for 1 h and then exposed to 100 μM $CoCl_2$ for 5 h. p65 was used as a positive control for TNF-α-dependent nuclear accumulation. (E) Viability of $CoCl_2$-treated PC12 cells in the presence or absence of TNF-α was determined using MTT assay. Cells were treated with 100 ng/ml TNF-α for 12 h. One hour after TNF-α treatment, 100 μM $CoCl_2$ were added for MTT assay. (F) Suppression of TH and VEGF gene expression by TNF-α. PC12 cells were pretreated with 100 ng/ml TNF-α for 1 h and then exposed to 100 μM $CoCl_2$ for 5 h. TH, VEGF and IPAS mRNA levels were determined by RT–PCR. The data were normalized to 18 S rRNA. $*P<0.05$ for indicated comparison. $**P<0.01$ for indicated comparison. ns, no significance. Data shown are means ± SD of three independent experiments.

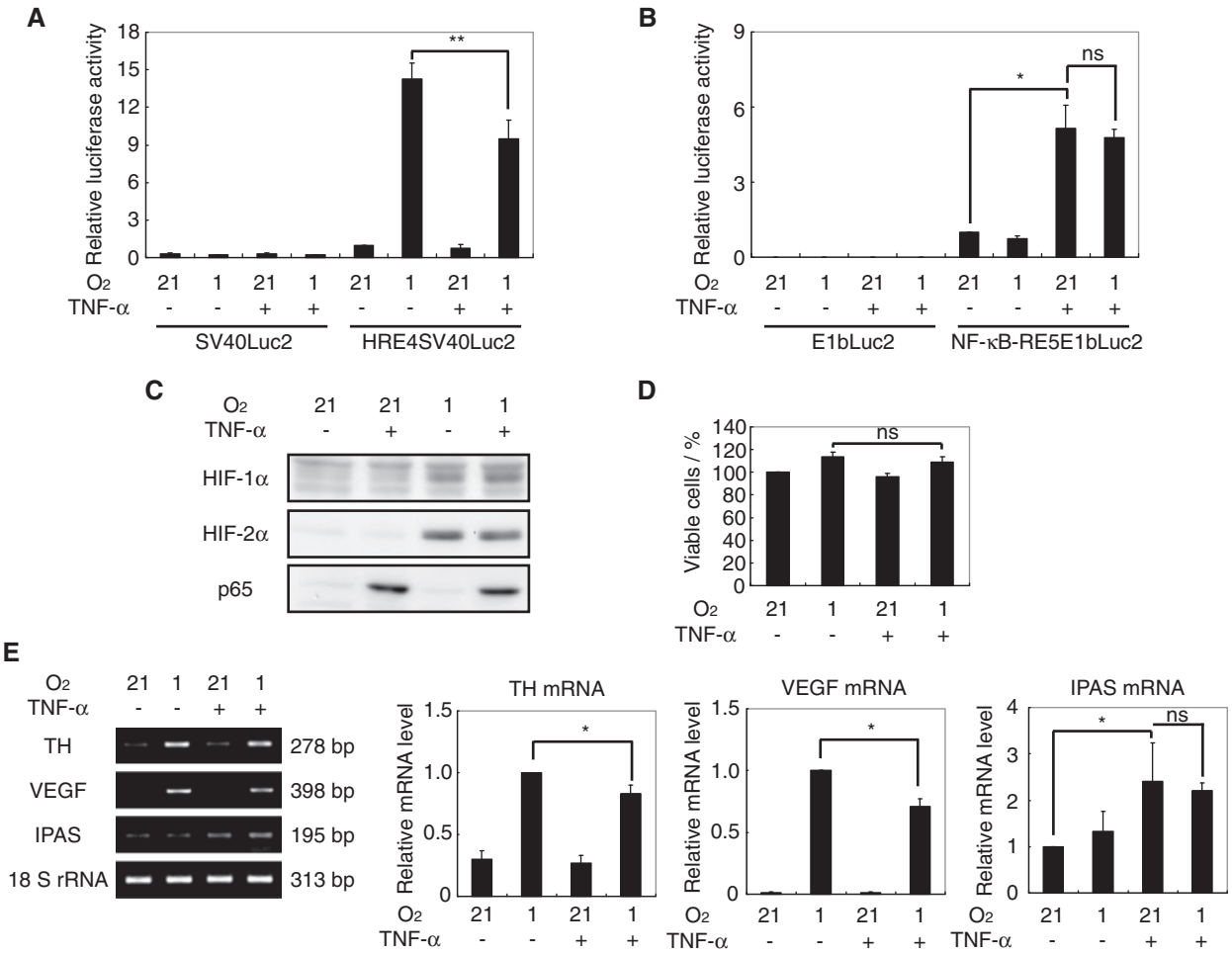

Fig. 4 Suppression of the low oxygen-induced hypoxic response by TNF-α in PC12 cells. (A) Suppression of HRE-dependent reporter activity by TNF-α. PC12 cells were pretreated with 100 ng/ml TNF-α for 1 h and exposed to 1% O_2 for 12 h. (B) Effect of hypoxia treatment on the TNF-α-induced NF-κB-RE-dependent reporter activity. Cells were pretreated with 100 ng/ml TNF-α for 1 h and then exposed to 1% O_2 for 12 h. (C) Effect of TNF-α on activation of HIF-α. Nuclear extracts were prepared from cells treated with hypoxia in presence or absence TNF-α and used for western blot analysis. Cells were pretreated with 100 ng/ml TNF-α for 1 h and then exposed to 1% O_2 for 5 h. p65 was used as a positive control for TNF-α-dependent nuclear accumulation. (D) Viability of 1% O_2-treated PC12 cells in the presence or absence of TNF-α was determined using MTT assay. Cells were treated with 100 ng/ml TNF-α for 12 h. One hour after TNF-α treatment, cells were exposed to 1% O_2 for MTT assay. (E) Suppression of TH and VEGF gene expression by TNF-α. PC12 cells were pretreated with 100 ng/ml TNF-α for 1 h and then exposed to 1% O_2 for 5 h. TH, VEGF and IPAS mRNA levels were determined by RT–PCR. The data were normalized to 18 S rRNA. $^*P < 0.05$ for indicated comparison. $^{**}P < 0.01$ for indicated comparison. ns, no significance. Data shown are means ± SD of three independent experiments.

respectively, following TNF-α treatment (Figs 3F and 4E). IPAS mRNA levels were similarly induced by TNF-α regardless of the treatment with $CoCl_2$ or hypoxia (Figs 3F and 4E).

We introduced IPAS siRNA to PC12 cells and analysed the effect of TNF-α on the $CoCl_2$-induced hypoxic response. TNF-α-induced IPAS mRNA was largely decreased by the treatment with IPAS/HIF-3α siRNA (Fig. 5A). In accordance with the reduction, expression levels of TH mRNA and VEGF mRNA suppressed by TNF-α were recovered by the treatment (Fig. 5A). HIF-dependent reporter activity suppressed by TNF-α was also significantly restored by the siRNA treatment (Fig. 5B). Taken together, these results strongly suggest that TNF-α-induced IPAS gene expression suppresses the HIF-1-dependent hypoxic response caused by $CoCl_2$ or hypoxia.

Discussion

Lipopolysaccaride and inflammatory cytokines are a stimulus for HIF-1α activation in myeloid cells under normoxic conditions as well as hypoxic conditions (*2*, *18*). Similar observations were made in tumour cells, epithelial cells and vascular smooth muscle cells (*2*, *18*, *24*). Mechanistical studies revealed that NF-κB, a central regulator in innate immunity, activated by canonical IKK pathways activates HIF-1α gene expression by direct binding to its gene control region (*25*, *26*). In addition, TNF-α activates HIF-1α via the translational regulation of the PI3K-AKT-mTOR signal pathway (*27*). On the other hand, HIF-1 was shown to mediate NF-κB activation in neutrophils under anoxic conditions (*28*). Hypoxia itself can stimulate NF-κB activation by inhibiting prolyl hydroxylases that negatively modulate IKKβ catalytic

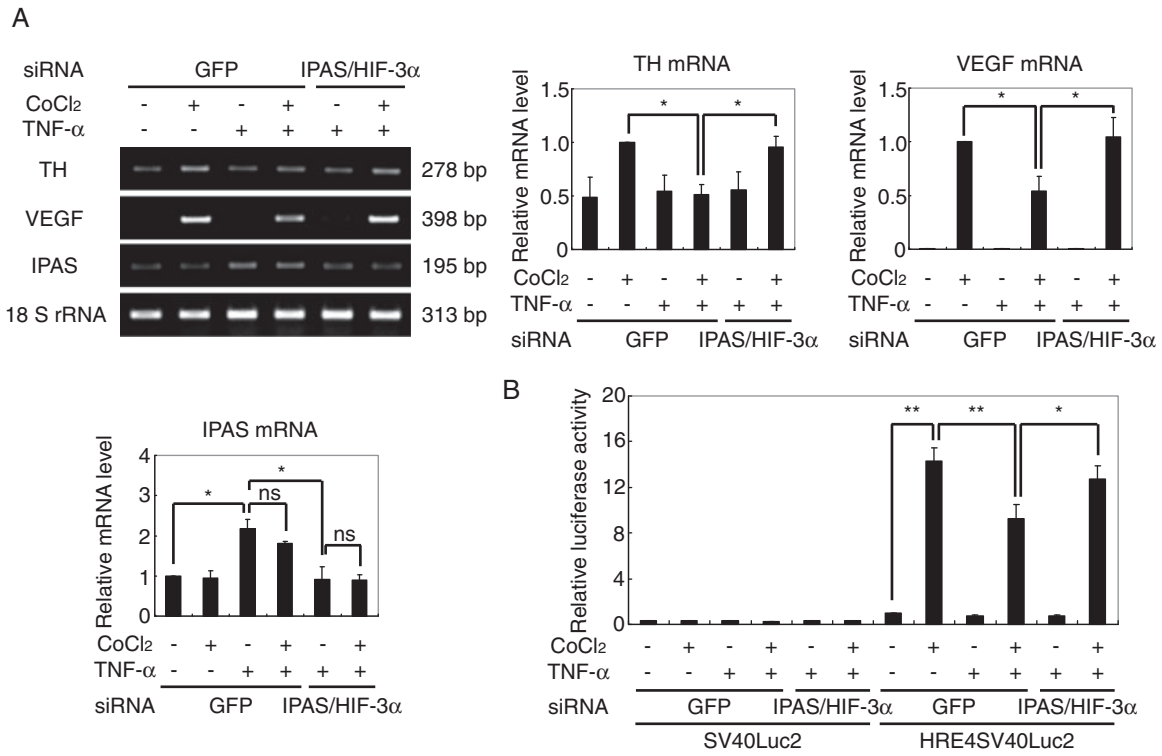

Fig. 5 Effect of IPAS siRNA treatment on suppression of the hypoxic response by TNF-α in PC12 cells. (A) Recovery of TH and VEGF mRNA expression by IPAS/HIF-3α siRNA treatment. Cells were transfected with IPAS/HIF-3α siRNA for 48 h, treated with 100 ng/ml TNF-α for 1 h and exposed to 100 μM $CoCl_2$ for 5 h. GFP siRNA was used as a control. mRNA levels were determined by RT–PCR and normalized to 18 S rRNA. (B) Recovery of $CoCl_2$-induced reporter activity by IPAS/HIF-3α siRNA treatment. PC12 cells were treated with IPAS/HIF-3α siRNA for 48 h. After siRNA treatment, cells were transfected with the HIF-dependent reporter plasmid. 4 h after transfection, cells were treated with 100 ng/ml TNF-α for 1 h and exposed to 100 μM $CoCl_2$ for 12 h. $^*P<0.05$ for indicated comparison. $^{**}P<0.01$ for indicated comparison. ns, no significance. Data shown are means ± SD of three independent experiments.

activity (*29*). These findings demonstrate that NF-κB and HIF-1 form a positive loop activated under hypoxic and inflamic conditions in neutrophils and some other cells (*30*). When transcriptional activation of the HIF-α genes following TNF-α treatment was analysed in PC12 cells, mRNA levels of HIF-1α, HIF-2α and HIF-3α were unchanged (Supplementary Fig. S2A). HIF-1α and HIF-2α proteins were hardly detected in the nucleus of cells treated with TNF-α as shown in Fig. 3D. We also examined the effect of TNF-α on HIF-dependent reporter activity by introducing a reporter plasmid containing HREs. HIF-dependent reporter activity was not increased by the treatment with TNF-α, thus ruling out the possibility that a subtle activation of HIF-1 was caused by TNF-α in PC12 cells (Supplementary Fig. S2B). Taken together, these results indicate that TNF-α was unable to activate HIF expression in PC12 cells and strongly suggest that increase in IPAS gene expression by TNF-α is independent of activity of HIF-1α and its family members.

A few reports have shown the presence of negative effects of NF-κB on HIF-1 activity. A negative feedback loop mediated at the microRNA level was reported between the NF-κB and HIF pathways; miR155 induced by lipopolysaccaride downregulates HIF-1α in hematopoietic stem cells (*31*). We previously found that a simultaneous activation of the PI3K-AKT-mTOR pathway and calcium signalling by cobalt-induced ROS leads to activation of NF-κB to enhance IPAS gene expression, leading to repression of HIF-1 activity in PC12 cells (*13*). All these reports analysing positive and negative regulation in hypoxic responses by inflammation demonstrate that the intracellular signalling pathways that affect HIF-1 activity are highly dependent upon the cell type and stimulus.

Presently it is unclear why induction of IPAS mRNA by TNF-α was not observed in Hep3B cells. PC12 cell-specific transcription factors acting cooperatively with NF-κB may be involved in the induction. Present study clearly demonstrates that upregulation of the IPAS gene by TNF-α is independent of HIF activation and can be induced even in normoxia. This finding suggests that IPAS may have an additional function indifferent to suppression of the hypoxic response in inflammation under the control of NF-κB. In relation to this issue, NF-κB in neural cells has a function totally different from that in immune cells and cancer cells. In neural cells, exogenous stimulation that activates NF-κB contributes to cell death (*32*). Recently, we found that IPAS has a pro-apoptotic activity mediated through its C-terminal region (*33*). TNF-α induced IPAS activity may play a role in TNF-α induced cell death in PC12 cells, although induced expression of IPAS did not induce cell death in the experimental conditions used in this study. In summary, we showed that TNF-α

induced IPAS gene expression in PC12 cells via activation of NF-κB. Furthermore, TNF-α-induced IPAS was shown to suppress the $CoCl_2$- or hypoxia-induced response of the cells. These results demonstrate that a novel crosstalk is present between hypoxic responses and inflammatory mediators.

SUPPLEMENTARY DATA

Supplementary Data are available at *JB* online.

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Conflict of interest
None declared.

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