The scaffold protein IB1/JIP-1 controls the activation of JNK in rat stressed urothelium

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Summary

The c-Jun N-terminal kinase (JNK) is critical for cell survival, differentiation, apoptosis and tumorigenesis. This signalling pathway requires the presence of the scaffold protein Islet-Brain1/c-Jun N-terminal kinase interacting protein-1 (IB1/JIP-1). Immunolabeling and in situ hybridisation of bladder sections showed that IB1/JIP-1 is expressed in urothelial cells. The functional role of IB1/JIP-1 in the urothelium was therefore studied in vivo in a model of complete rat bladder outlet obstruction. This parietal stress, which is due to urine retention, reduced the content of IB1/JIP-1 in urothelial cells and consequently induced a drastic increase in JNK activity and AP-1 binding activity. Using a viral gene transfer approach, the stress-induced activation of JNK was prevented by overexpressing IB1/JIP-1. Conversely, the JNK activity was increased in urothelial cells where the IB1/JIP-1 content was experimentally reduced using an antisense RNA strategy. Furthermore, JNK activation was found to be increased in non-stressed urothelial cells of heterozygous mice carrying a selective disruption of the *IB1/JIP-1* gene. These data established that mechanical stress in urothelial cells in vivo induces a robust JNK activation as a consequence of regulated expression of the scaffold protein IB1/JIP-1. This result highlights a critical role for that scaffold protein in the homeostasis of the urothelium and unravels a new potential target to regulate the JNK pathway in this tissue.

Key words: bladder, urothelium, islet-Brain1, JIP-1, JNK, c-Jun, adenovirus

Introduction

Various physical stresses such as osmolarity, heat, pH, redox state and radiation have profound effects in mammalian cells. As a consequence of these stimuli, cells may undergo either proliferation, differentiation, apoptosis or tumorigenesis (Davis, 2000). Mitogen-activated protein kinases (MAPK) are enzymes involved in regulating the response to these stimuli. In mammals, three major groups of MAPKs have been identified (Schaeffer and Weber, 1999). The c-Jun N-terminal kinases (JNK) are activated by stimuli, including proinflammatory cytokines, DNA damaging agents (cisplatinum and alkylating agents) and mechanical stress. Once activated, the JNK phosphorylates c-Jun which, in turn, mediates various cellular effects by modulating the transcriptional activity of AP-1 (Pulverer et al., 1991; Smeal et al., 1991).

The control of JNK activity is therefore a critical component of the cell's response to stress (Davis, 2000). Recently, the JNK activity was shown to be controlled in part by the mammalian scaffold protein Islet-Brain1/c-Jun N-terminal kinase interacting protein-1 (IB1/JIP-1). This protein binds to three kinases, MLK3 (a MAPKKK), MKK7 (a MAPKK) and JNK (a MAPK) in a single transduction complex (Whitmarsh et al., 1998; Yasuda et al., 1999). These kinases together constitute an ordered unit of sequential signalling molecules, transducing a variety of stress signals (Davis, 2000).

IB1/JIP1 was first studied in the brain and in the endocrine pancreas, where the protein was found to be highly expressed. In pancreatic β cells the high content of IB1/JIP-1 limits the output of JNK signalling toward apoptosis. Furthermore, a single missense mutation (S/N) mutation in the *IB1/JIP-1* gene was recently shown to be responsible for accelerated cell death and secondary diabetes in humans (Bonny et al., 1998; Bonny et al., 2000; Waeber et al., 2000). In the brain, IB1/JIP-1 is localised in neurons (Pellet et al., 2000) and interacts functionally with p190 RhoGEF (Meyer et al., 1999), the ApoE receptor 2 (ApoER2) (Stockinger et al., 2000) and the low-density-lipoprotein (LDL)-related-receptor protein, megalin (Gotthardt et al., 2000). Recently IB1/JIP-1 and its different isoforms have been demonstrated to directly interact with kinesins, a family of motor proteins required for axonal transport in neuronal cells. These data revealed that the scaffold IB1/JIP1 protein family are cargos for kinesins and could participate in the formation of multiprotein complexes linking motor proteins to their membranous cargo (Verhey et al., 2001).

Bladder carcinoma originating from transitional cells is a highly prevalent disease, which has steadily increased in incidence over the past years (Jung and Messing, 2000). The functional role of JNK signal transduction in the bladder and in the urothelial cells is mostly unexplored. In neoplasic non-urothelial cells, cell transformation induced by Ras required the activation of the JNK pathway (Pulverer et al., 1991; Smeal et al., 1991). Furthermore, Ras-induced tumorigenicity is suppressed by mutation of the JNK phosphorylation sites on c-Jun (Behrens et al., 2000). Lastly, an increase in c-Jun

expression was shown to be correlated with rapid tumor proliferation and aggressive cell growth in transitional cell carcinoma (TCC) (Skopelitou et al., 1997).

Here, we have evaluated the regulation of the JNK in urothelial cells in vivo. We have demonstrated that this signalling pathway is activated by parietal stress and is dependent upon the scaffold protein IB1/JIP-1. We prevented JNK activation by overexpressing IB1/JIP-1 and, conversely, a decreased IB1/JIP-1 content is associated with an increase in JNK activity. Moreover, JNK was overactivated in mice carrying a selective disruption of the *IB1/JIP-1* gene as heterozygous carriers. Taken together, these data unravel a critical role for IB1/JIP-1 in the control of the JNK activity in urothelial cells.

Materials and Methods

Bladder outlet obstruction and recombinant adenoviral infection

Our institutional review committee for animal experimentation approved the procedures we used for animal care, surgery and euthanasia. Male Wistar rats weighing between 220-260 g were used. For surgical procedures, all rats were anaesthetised by intra-peritoneal injection 5.5 mg/100 g body weight of sodium pentobarbital following by repeated injection of 2 mg/100 g every hour. A cutaneous transversal incision was made cranially to the preputial orifice to expose the corpus penis. Urethral muscle were separated from the connective tissue and the penis was double ligated, resulting in a caudal urethral obstruction. After seven hours, this surgery resulted in a 10-fold increase ($P<0.01$) in the volume of urine retained within the bladder. Seven hours later, the animals were killed with an overdose of sodium pentobarbital, the bladder was separated from the ventral prostatic lobes, ligated at its hilum and removed. Empty bladders were rapidly frozen in liquid nitrogen. Controls rats were exposed to the same surgical manipulations, except for the urethral ligature. For gene transfer (Engler et al., 1999; Morris et al., 1994), we exteriorised the bladder, and urine was evacuated by aspiration with a 0.3×20 mm needle. The bladder was washed with 1 ml of PBS and then instilled with recombinant adenovirus (20×109 pfu) diluted in 300 ml of PBS containing 15% ethanol, 3% sucrose and 2 mM $MgCl_2$. The animals were kept anaesthetised for one hour to prevent voiding, thereby insuring an adequate incubation period for the virus with superficial bladder cells. After a period of 48 hours, bladder outlet obstruction was performed.

In situ hybridisation

For in situ hybridisation, after sacrifice, rats were infused with 30 ml diethyl pyrocarbonate (DEPC, Sigma)/PBS (phosphate-buffered saline). The bladder was rapidly excised, frozen in 2-methylbutane precooled in liquid nitrogen, then frozen in OCT medium (Miles Inc., IN, USA) and cryostat sectioned at 12 mm thickness. Sections were fixed in 4% paraformaldehyde in PBS for 10 minutes, rinsed in DEPC/PBS and analysed by in situ hybridisation as previously described (Braissant et al., 1996; Haefliger et al., 1999b). Briefly, hybridisation with two non-overlapping rat IB1/JIP-1 digoxygenin-labeled antisense riboprobes (550 bp EcoR1-Apa1 and 377 bp Apa1-Xho of the rat IB1/JIP-1 cDNA) was carried out for 40 hours at 58°C in 5× standard saline citrate (SSC) and 50% formamide. Sections were washed for 30 minutes at room temperature in 2×SSC, then for one hour at 65°C in 2×SSC, one hour at 65°C in 0.1×SSC and then stained for alkaline phosphatase. Stained sections were dehydrated and mounted with Eukitt (O. Kindler Co., Germany). The specificity of hybridisation was ascertained using two sense IB1/JIP-1 RNA of the same length, GC content and specific activity as the corresponding antisense probe.

Immunofluorescence

For light microscopy studies, rats were sacrificed and then perfused through the left ventricle with 30 ml of PBS. The bladder was rapidly excised and quickly frozen in 2-methylbutane precooled in liquid nitrogen. The bladders were frozen in OCT medium (Miles Inc., IN) and cryo-sectioned at about 5-8 μm thickness. Sections were rinsed in PBS, incubated for 45 minutes in a buffer containing 3% BSA (Bovin Serum Albumin) and 0.1% triton and then exposed for 20 hours to antibodies against IB1/JIP-1 diluted in PBS. Primary antibodies were detected using fluorescein-isothiocyanate-labeled anti-rabbit immunoglobulins (Biosystem, France). Sections were then rinsed in PBS, stained with Evans' blue, viewed with a Leica microscope (Leica, West Germany) and photographed on Kodak Tmax 400 films.

RNA isolation and northern blot analysis

The bladder was homogenised in a 4 M guanidine hydrothiocyanate buffer containing 25 mM sodium citrate and 100 mM β-mercaptoethanol using a Kinametic polytron blender (Kinametica, Switzerland). Total RNA was extracted by the acid guanidium isothiocyanate method, and yields were evaluated by absorbance at 260 nm. 10-15 mg total RNA were fractionated by size on 1% agarose gels containing 8% formaldehyde (Fluka, Switzerland) and 1×MOPS buffer (3-[N-Morpholino]propanesulfonic acid, Fluka, Switzerland). Capillary transfer in the presence of 10×SSC transferred RNAs overnight to gene screen membrane (Du Pont de Nemours GmbH, NEN Division, Germany). Membranes were UV cross-linked and vacuum-baked for two hours at 80°C. After prehybridisation, total mRNA levels were determined by hybridisation with randomly primed (Boehringer-Mannheim, Germany) cDNA that were specific for IB1 (Bonny et al., 1998), and glyceradheyde-3-phosphate dehydrogenase (GAPDH) (Haefliger et al., 1999a) was labeled with [α-^{32}P]dCTP (Amersham, Switzerland).

Antibodies

Polyclonal IB1/JIP-1 antiserum was described previously (Bonny et al., 1998). These antibodies were affinity purified from crude serum using a Hitrap NHS-activated affinity sepharose column (Amersham Pharmacia Biotech AB Switzerand), which was coupled to the immunogenic protein. Polyclonal antibodies (JNK, p-JNK, p38, p-p38, ERK, p-ERK, c-Jun) were purchased from New England Biolabs (Bioconcept, Switzerland).

Western blotting

Immediately after sacrifice, rats were infused through the heart with 30 ml PBS, the bladders were excised and rapidly frozen in liquid nitrogen, or urothelial cells were scraped from freshly removed bladder using a scalpel, and frozen. The bladders were homogenised with a polytron in the passive lysis buffer (Promega, Switzerland). The urothelial cells were lysed in Mono Q buffer (1% Triton X-100, 0.5% Nonidet P-40,20 mM Tris-HCl, pH 7.5, 0.2 mM sodium vanadate, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 5 mM PMSF). Western blots of nuclear extract were performed using the same samples prepared for the EMSA. The DC protein assay reagent kit (Bio-Rad Laboratories, Switzerland) determined protein content. Aliquots of bladder or urothelial cells (150 mg for IB1/JIP-1, 50 mg for MAPK, 30 mg for c-Jun) were fractionated by electrophoresis in a 10% polyacrylamide gel and immunoblotted onto Immobilon PVDF membranes (Millipore Co, MA) overnight at a constant voltage of 30 V. Membranes were incubated for one hour at room temperature in PBS containing 5% milk and 0.1% Tween 20 (blocking buffer) and then incubated for two hours at room temperature with antibodies directed against IB1/JIP-1. Antibodies against JNK, p-JNK, p38, p-p38, ERK, p-ERK and c-Jun were incubated overnight at 4°C in

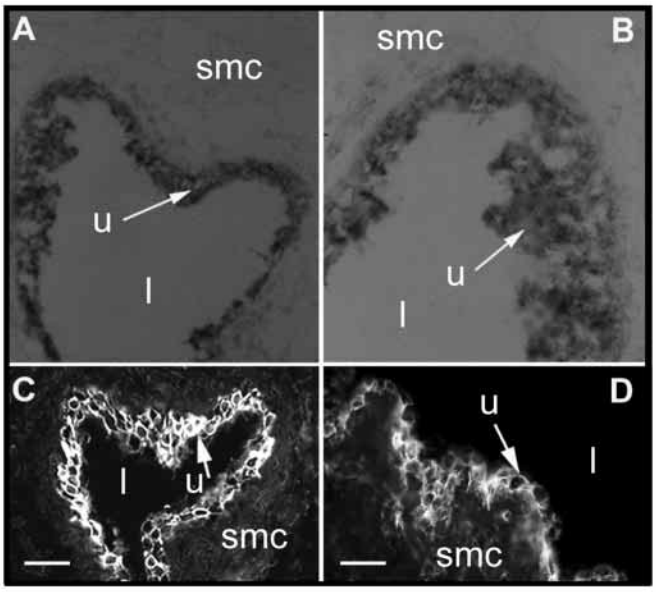


Fig. 1. The scaffold protein IB1/JIP-1 is present in urothelial cells of rat bladder. In situ hybridisation using two non-overlapping cDNA probes specific for IB1/JIP-1 (A,B) demonstrated the presence of IB1/JIP-1 transcripts in the urothelium of rat bladder. These transcripts are translated into immunodetectable protein in non-dilated and dilated bladder respectively (C,D). Smooth muscle cells (smc); Urothelium (u); Lumen (l). Bar represents 15 μm in A and C and 25 μm in B and D.

blocking buffer. Specific antigen-antibody complexes were detected with the HRP western blot detection system (Amersham, Switzerland). Each lane shows a sample from a different rat.

Protein kinase assays

JNK activity was measured using glutathione S-transferase-c-Jun(1-79) bound to glutathione-Sepharose 4B (Bonny et al., 2000). Urothelial cells scraped from freshly removed bladder were lysed in 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 7.6, 0.25 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1mM dithiothreitol, 1mM PMSF (phenylmethylsulfonyl fluoride), 2 mM sodium vanadate, 20 μg/ml aprotinin and 5 μg/m leupeptin. Lysates were centrifuged at 14000 ***g*** for 10 minutes to remove any nuclei, and supernatants (50 μg of protein) were mixed with 19 μl of glutathione S-transferase-c-Jun. The mixture was rotated at 4°C for one hour, washed twice in lysis buffer and twice in kinase buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 10 mM p-nitrophenylphosphate, 1 mM dithiothreitol and 50 μm sodium vanadate). Beads were suspended in 40 μl of kinase buffer with 10 μCi of [γ-^{32}P]ATP and incubated at 30°C for 25 minutes. Samples were boiled in loading buffer, and phosphorylated proteins were resolved on 10% SDS polyacrylamide gels. To verify the selectivity of the JNK assay, cell lysates were fractionated by Mono Q ion exchange chromatography, and each fraction assayed as described above. Fractions were immunoblotted with rabbit antiserum recognising JNK. Only fractions containing immunoreactive JNK phosphorylated the glutathione S-transferase-c-Jun(1-79) protein.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of freshly scraped urothelial cells from control or dilated bladders were prepared as previously described (Andres et al., 1999). The protein concentration was measured using a Bio-Rad protein measurement kit. For EMSA, the double-stranded DNA probe corresponding to the AP-1 consensus sequence was labeled with [α-^{32}P]dCTP by the Klenow enzymes. The labeled probe was incubated with 5 mg of the nuclear protein and 1 mg of poly d(I-C) in a total

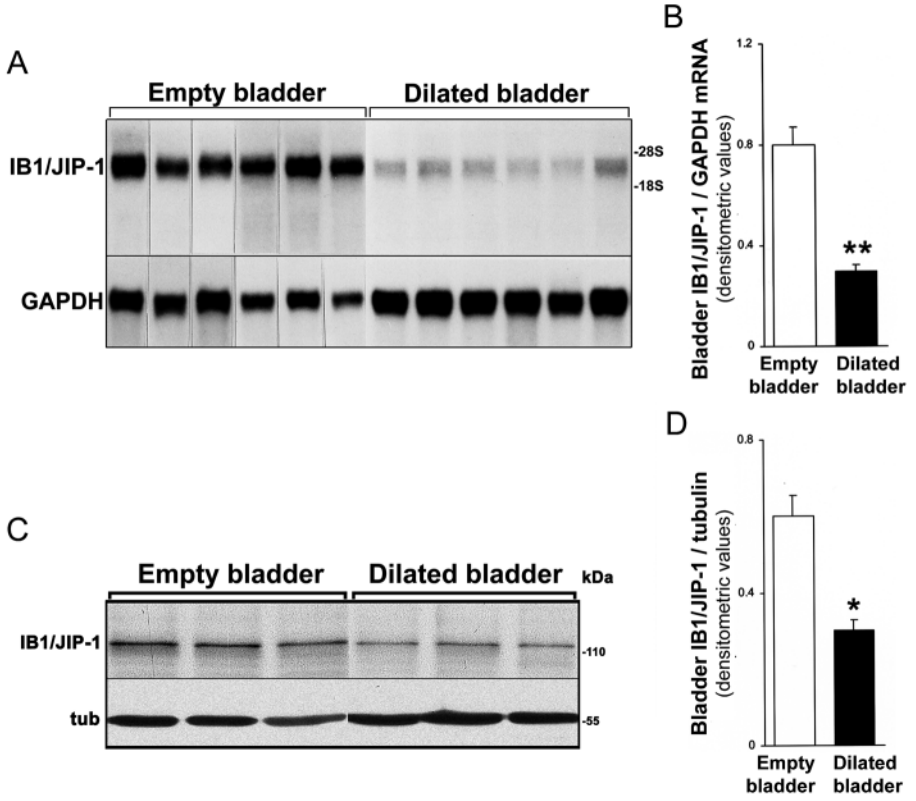


Fig. 2. Regulated expression of the scaffold protein IB1/JIP-1. (A) Northern blot analysis of bladders revealed that the level of IB1/JIP-1 transcripts is drastically decreased in dilated bladder after seven hours of dilation. In the same samples, levels of GAPDH mRNA were slightly increased (1.5 fold) in the dilated bladder. Each lane is from a different animal and was loaded with 10 μg mRNA. (B) Quantitative assessment of sixteen experiments (one per rat) confirmed that the levels of IB1/JIP-1 mRNA were decreased by three fold compared with control values. Values represent ratios of densitometric measurements of IB1/JIP-1 and GAPDH mRNAs and are expressed as mean±s.e.m. (n=16± s.e.m.; *P<0.01). (C) IB1/JIP-1 content was reduced in dilated bladder after seven hours of dilation as assessed by western blot analysis. Equal protein loading was evaluated by measuring the level of tubulin. (D) Quantitative evaluation of four immunoblots revealed that the levels of IB1/JIP-1 were reduced by 50% in samples of dilated bladder (seven hours after ligation) compared with their controls. Values represent ratios of densitometric measurements of IB1/JIP-1 and tubulin. (mean±s.e.m.; n=5; P<0.05).

volume of 20 ml containing 10% glycerol, 15 mM Hepes-NaOH, pH 7.8, 5 mM $MgCl_2$, 5 mM dithiothreitol and 50 mM KCl. After incubation at 4°C for 30 minutes, the reaction mixtures were resolved on a 4% non-denaturing polyacrylamide gel electrophoresis in 0.5×TBE buffer (1×TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0), and the products were detected by autoradiography. Antiserum against c-Jun (H-79) was purchased from Santa Cruz biotechnology, Inc. (CA). For the competition experiments, each unlabeled oligonucleotide was added to the reaction mixture prior the addition of the labeled probe. For the antibody experiments, antiserum was added to the extracts and the mixture was incubated for two hours on ice prior to the addition of the labeled oligonucleotides.

Generation of recombinant adenoviruses

To overexpress IB1 or inhibit its expression, we have constructed recombinant adenoviruses comprising the complete cDNA of rat IB1/JIP-1 (Bonny et al., 1998) in the sense orientation or in the antisense orientation. Adenoviruses expressing the green fluorescent protein (GFP) alone were used as a control. To generate the adenoviruses, the cDNAs were inserted into the plasmid pXC15 (Schaack et al., 1995), and adenoviruses were then generated by homologous recombination in 293 cells following cotransfection by the calcium phosphate procedure of the plasmid pJM17 (Hitt et al., 1998) and pXC15 constructs. Viruses were further plaque-purified three times on HR911 cells (IntroGene, Leiden, The Netherlands). Two rounds of CsCl centrifugation purified large stock viruses. After the second centrifugation, the virus band (1.5 ml) was collected and dialysed at 4°C against three changes (at least 200 volumes each) of 10 mM Hepes pH 8.0, 150 mM NaCl in a Slide-A-Lyzer (0.5-3.0 ml capacity), gamma irradiated 10K dialysis cassette (Pierce, Switzerland). The recombinant adenoviruses allowed expression of IB1/JIP-1, GFP and/or the IB1/JIP-1 antisense RNA under the control of the strong immediate early cytomegalovirus (CMV) promoter.

Statistical analysis

Densitometric analysis of signals (autoradiograms) were performed using a Molecular Dynamics scanner (Sunnyvale, USA), which integrates areas and corrects for background. Data were expressed as mean ±s.e.m. and were compared using the Student's *t* test. Statistical significance was defined at a value $P<0.05$ (*), $P<0.01$ (**) and, $P<0.001$ (***).

Results

The scaffold protein IB1/JIP-1 is present in urothelial cells

IB1/JIP-1 was previously reported to be highly expressed in the brain (Pellet et al., 2000) and in the endocrine pancreas (Bonny et al., 1998). We investigated whether IB1/JIP-1 is expressed in the urothelium in rat bladder by in situ hybridisation and immunofluorescence. Using two non-overlapping rat cDNA probes, we demonstrated the presence of IB1/JIP-1 mRNA in urothelial (Fig. 1A,B) but not smooth muscle (smc) or stromal cells of the bladder. No signal was detected using a sense probe (data not shown). Furthermore IB1/JIP-1 was immunodetectable in these cells (Fig. 1C,D).

Regulated expression of the scaffold protein IB1/JIP-1

We then evaluated whether IB1/JIP-1 expression in urothelium is regulated in vivo by mechanical stress. Northern blot analysis of RNA isolated from the complete rat bladder revealed that IB1/JIP-1 mRNA was decreased three-fold in the dilated compared to the empty bladder (Fig. 2A,B). This modulation correlated with a decrease in immunoreactive IB1/JIP-1, as evaluated by western blot analysis of protein isolated from stressed or non stressed bladders (Fig. 2C). A two-fold decrease in IB1/JIP-1 content was observed and quantitatively assessed by densitometric measurements of IB1/JIP-1 and tubulin (Fig. 2D).

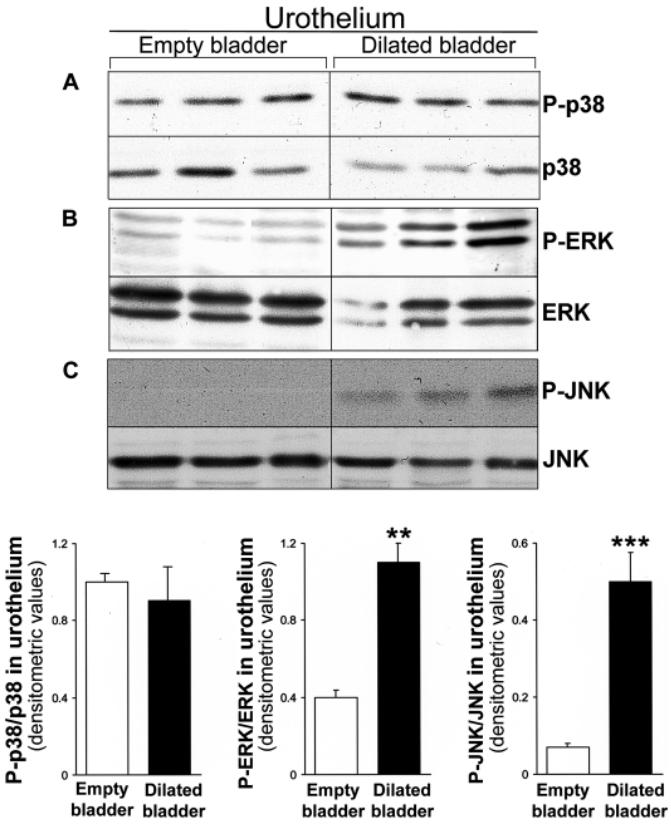


Fig. 3. Parietal stressed is associated with a selective increase phosphorylated MAPK ERK and JNK but not p38 in urothelial cells. Western blot analysis of freshly scraped urothelial cells using specific antibodies against the phosphorylated and the non phosphorylated form of the MAPKs p38 (A), ERK (B), JNK (C) demonstrates an increase in the phosphorylated form of the MAPKs ERK and JNK but not in p38 in urothelium of dilated bladder after seven hours of obstruction, compared with empty bladder. Each lane is from a different animal and quantitative assessment of five experiments (one per rat) confirmed that the levels of P-JNK and P-ERK were increased six-fold and four-fold respectively over control values. Values represent ratios of densitometric measurements of either P-JNK or JNK, P-ERK and ERK or P-p38 and p38 and are expressed as mean±s.e.m.

MAPKs are regulated by parietal stress in urothelial cells

Since mechanical stress was shown to be associated in mammalian cells with MAPK activation (Davis, 2000), we evaluated whether p38, ERK and JNK signalling transduction kinases were detected in freshly scraped urothelial cells. As observed in Fig. 3A,B,C, the three kinases were detected by western blot analysis in non-stressed bladder. The putative activation of the kinases was evaluated by measuring the specific phospho-kinases. The mechanical stress was associated

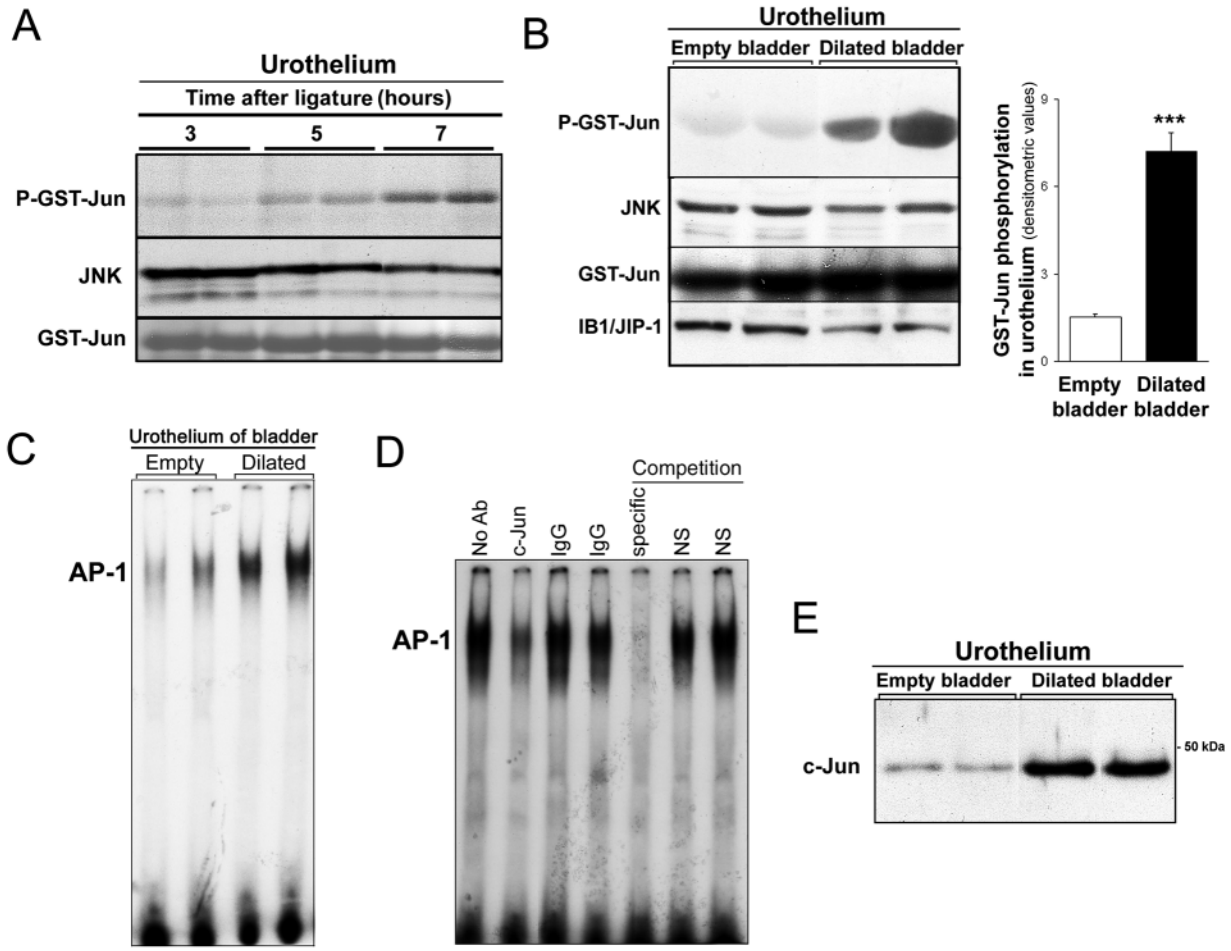


Fig. 4. Phosphorylated JNK activity is functionally active and associated with an increase in AP-1 binding activity. (A) JNK activity was evaluated in urothelial cells. Endogenous JNK was first purified using GST-Jun, and then a solid phase kinase assay was performed. [γ-^{33}P]-phosphorylated substrates (P-GST-Jun) were separated on a polyacrylamide gel. Western blotting monitored JNK expression in the same samples. The gel was stained with Coomassie blue to evaluate the loading of substrate (GST-Jun). P-GST-Jun showed a progressive increase during the development of the dilated bladder. Maximum expression occurred seven hours after ligature. All lanes were loaded with 50 µg of protein. (B) JNK activity in urothelium was monitored seven hours after ligature. Quantitative assessment demonstrated that urothelial cells of dilated bladder increased c-Jun phosphorylation about five fold compared to the empty bladder. On the same samples, IB1/JIP-1 content was decreased in freshly scraped urothelial cells from the dilated bladder. Values represent densitometric measurements of six independent experiments and are expressed as mean±s.e.m. ***=P<0.001 level. (C) Electrophoretic mobility shift assay (EMSA) showed that AP-1 binding activity is increased in stressed urothelium seven hours after ligature. Each lane shows a sample from a different rat. All lanes were loaded with 7 µg. (D) Specificity of the AP-1 shift. Nuclear extracts of freshly scraped urothelial cells were pre-incubated with specific c-Jun antibody (c-Jun) or two different irrelevant antibodies (IgG). AP-1 shift was competed with the incubation of a cold-specific competitor (specific) but not with non-specific oligonucleotides (NS). (E) Western blot analysis of c-Jun expression on the same nuclear extract of freshly scraped urothelial cells used for EMSA experiment seven hours after ligature demonstrated a 400% increase of c-Jun expression in dilated bladder compared with the control.

in urothelium, seven hours after ligature, with a selective increased in phosphorylated forms of ERK and JNK but not p38. Quantitative assessment showed that, compared to control values, the levels of p-JNK and p-ERK were significantly increased in urothelial cells of dilated bladder (Fig. 3).

The phosphorylated JNK is functionally active and associated with an increase in c-Jun expression and AP-1 binding activity

IB1/JIP-1 is implicated in JNK-mediated signal transduction. Solid phase assays using GST-Jun as the substrate were used to evaluate JNK activity. A progressive increase in JNK activation in stressed urothelial cells was observed (Fig. 4A). After seven hours of ligation, a five-fold increase in JNK activation was detected (Fig. 4B), which correlates well with the increased phosphorylated state of JNK in urothelial cells of dilated bladder. On the very same samples, western blot analysis demonstrated that IB1/JIP-1 was decreased as previously observed in Fig. 2B. Gene transcription is induced by the phosphorylation of the trans-acting domains of various transcription factors including c-Jun (Ip and Davis, 1998), which is one of the major components of the AP-1 complex. AP-1 binding activity evaluated by EMSA was strongly induced in stress urothelial cells (Fig. 4C). The specificity of the urothelial AP-1 shift was demonstrated by specific and unrelated competitions (Fig. 4D). To demonstrate the presence of c-Jun in the urothelial AP-1 complex, we incubated nuclear

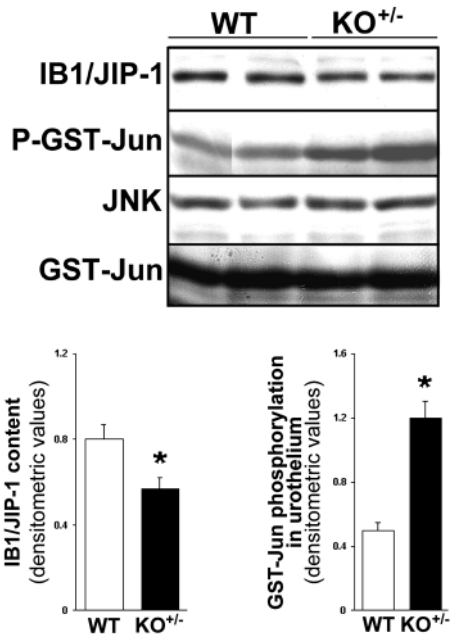


Fig. 5. The IB1/JIP-1 content is critical for mediating JNK activity. Western blot analysis and a solid phase kinase assay using GST-Jun as the substrate revealed a decrease in IB1/JIP-1 expression associated and an increase of JNK activity (P-GST-Jun) in empty bladders of mice heterozygous ($KO^{+/-}$) compared with wild-type mice (WT). The JNK content was evaluated by western blotting. Equal loading of substrate was confirmed by Coomassie blue staining of the gel (GST-jun). Quantitative assessments of IB1/JIP-1 and c-Jun phosphorylation in urothelium of mice, which had had the *IB1/JIP-1* gene selectively disrupted, demonstrated an inverse relationship between the levels of IB1/JIP-1 content and the JNK activity compared to basal level in wild-type mice.

extracts with either a specific c-Jun antibody or an irrelevant antibodies (Fig. 4D). Moreover, on the same nuclear extracts, modulation of AP-1 activity was associated with an increase of c-Jun expression in stress-induced urothelial cells compared to their controls, as observed by western blot analysis (Fig. 4E).

The IB1/JIP-1 content is critical for the control of JNK activity

To evaluate the potential role of IB1/JIP-1 in the control of urothelial JNK activation, we measured the JNK activity in the urothelium of mice in which the *IB1/JIP-1* gene has been disrupted. Since homozygous IB1/JIP-$1^{-/-}$ mice are associated with embryonic lethality (Thompson et al., 2001), we studied heterozygous IB1/JIP-$1^{+/-}$ mice. These mice have a normal phenotype but the IB1/JIP-1 content is decrease by 40% in the urothelium compared to their wild-type littermates ($^{+/+}$) (Thompson et al., 2001). Concomitantly, JNK activity is increased by two fold (Fig. 5). These data indicate that the JNK activity is controlled, in non-stressed conditions, by the IB1/JIP-1 content.

Gene transfer of IB1/JIP-1 prevents the JNK activation

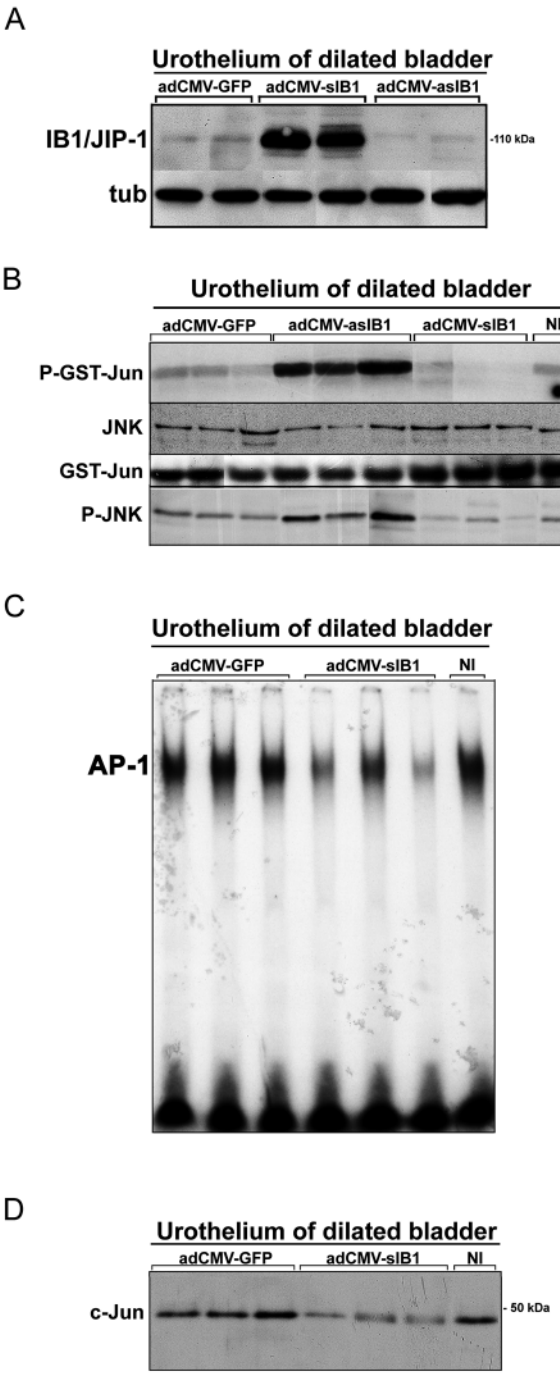
IB1/JIP-1 content was experimentally modulated in urothelium using a recombinant adenovirus gene transfer approach. We used recombinant adenoviruses containing the complete IB1/JIP-1 cDNA inserted in the sense or antisense orientation (adCMV-sIB1 or adCMV-asIB1). An adenovirus expressing only GFP was used as control. To study the efficiency of adenoviral-mediated transfer of IB1/JIP-1, western blot analysis of stressed urothelial cells infected with adCMV-GFP, adCMV-sIB1 or adCMV-asIB1/JIP-1 was used and showed that the cells infected with the adCMV-sIB1 express high amounts of IB1/JIP-1 compared with urothelial extract infected with adCMV-GFP. In contrast, the IB1/JIP-1 content was decreased with adCMV-asIB1 (Fig. 6A). On the same samples infected with the adCMV-asIB1, JNK activity was increased in stressed urothelial cells (Fig. 6B) and was associated with an increase in P-JNK as evaluated by western blot analysis. On the other hand, when the IB1/JIP-1 content was experimentally increased by adCMV-sIB1/JIP-1 (Fig. 6B), JNK activity was decreased compared to the controls (adCMV-GFP or non infected urothelial cells (NI)). Quantitative assessment demonstrated a three-fold increase in c-Jun phosphorylation in stressed urothelial cells infected with the adCMV-asIB1 and inversely a two-fold decrease in stressed urothelial cells infected with the adCMV-sIB1 compared to controls (adCMV-GFP, NI) (Fig. 6B).

By EMSA, the AP-1 binding activity was also found to be decreased in nuclear extracts obtained from urothelial cells overexpressing IB1/JIP-1 (Fig. 6C). Western blot analysis (Fig. 6D) done with the same nuclear extracts revealed a decrease of c-Jun expression in urothelium of dilated bladder infected with the overexpressing IB1/JIP-1 recombinant adenovirus (adCMV-sIB1/JIP-1).

Discussion

IB1/JIP-1 has been previously shown to be expressed in the brain (Pellet et al., 2000) and in the endocrine pancreas (Bonny et al., 1998). Here, we show that both in situ hybridisation and immunofluorescence concurred to show that this protein is expressed not only within neuronal and pancreatic β cells but also within urothelial cells of the rat bladder. This scaffold protein was recognised to contribute to the cell-signalling pathway by binding to the three kinases MLK3, MKK7 and JNK, which constitute a signalling module leading to the activation of JNK.

We investigated a rat model of complete bladder outlet obstruction (Tammela et al., 1993) to study in vivo the activity of MAPKs, as mechanical stress is known to activate the JNK pathway in many systems (Komuro et al., 1996; Li et al., 1999; Matsuda et al., 1998; Ishida et al., 1999). In this model, ligation of the urethra induces an increased voiding pressure, which enhances bladder wall stress. In these conditions, we observed a drastic increase in the activated state of JNK associated with an increased of AP-1 binding activity. Furthermore, this urothelial AP-1 complex is constructed by c-Jun, the physiological substrate of JNK. These data suggested that the active JNK could potentiate, through c-Jun phosphorylation, the ability of AP-1 to activate transcription, which, in turn, would increase the c-Jun expression, as observed here in mechanically stressed urothelium. This increased c-Jun synthesis could result in the increased AP-1 binding activity observed (Karin, 1995). Mechanical stress reduced the IB1/JIP-1 content in the urothelium, and using an adenovirus



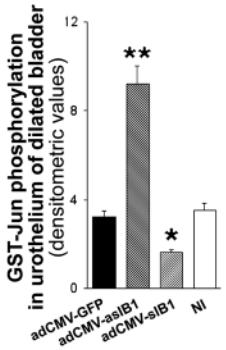


Fig. 6. Gene transfer of IB1/JIP-1 can prevent the JNK activity. (A) Western blot analysis of scraped urothelial cells infected with different adenovirus constructs showed that IB1/JIP-1 content is largely increased in cells overexpressing IB1/JIP-1 (adCMV-sIB1), whereas a decreased expression was observed when cells were infected with the adCMV-asIB1 compared to control adCMV-GFP. Protein loading was evaluated by tubulin. **(**B) JNK activity analysis in urothelial cells of dilated bladder revealed that cells infected with adCMV-asIB1 increased their JNK activity; in contrast, cells infected with adCMV-sIB1 decreased their JNK activity. Quantitative assessment demonstrated that the JNK activity is increased about three-fold in urothelial cells of dilated bladder infected with adCMV-asIB1 compared with non infected bladder (NI) or infected with control adenovirus (adCMV-GFP). In contrast, a 50% reduction in JNK activity is observed in cells infected with adCMV-sIB1. In the same samples, an increase in P-JNK was detected by immunoblot in urothelial cells infected with adCMV-asIB1; in contrast, a decrease in P-JNK content was observed in cells infected with adCMV-sIB1 compared to controls (NI, adCMV-GFP). **(**C) Electrophoretic mobility shift assay (EMSA) revealed a decrease in AP-1 binding activity in stress urothelium transduced with adCMV-sIB1/JIP-1 compared to non-infected dilated urothelium (NI) or cells transduced with the control adenovirus. **(**D) Western blot analysis of c-Jun expression on the same nuclear extract of dilated urothelial cells demonstrated a decrease in c-Jun expression in bladder transduced with adCMV-sIB1 compared to non infected bladder (NI) or infected with the control adenovirus.

gene transfer approach, we could demonstrate that IB1/JIP-1 is a critical regulator of JNK activity. Moreover, we showed that increased levels of IB1/JIP-1 induced by viral gene transfer could prevent the stress-induced activation of JNK and regulate c-Jun expression and AP-1 activity. Conversely, JNK activity was increased in urothelial cells where the IB1/JIP-1 content was experimentally reduced using an antisense RNA strategy. Moreover, reduced IB1/JIP-1 content in urothelial cells of heterozygous mice carrying a disruption of the *IB1/JIP-1* gene leads to a basal increase in JNK activity. Therefore, IB1/JIP-1 plays a crucial role in the dilated urothelium by controlling the effects of JNK signalling under mechanical stress. Taken together, these data unravel for the first time a critical role for IB1/JIP-1 in the control of the JNK signalling pathway in vivo. The stoichiometry of the interaction between scaffold proteins and protein kinases may be critical for the regulation of protein kinase activity; high levels of IB1/JIP1 would be predicted to inhibit JNK activation by titrating the protein kinase components into separate IB1/JIP1 complexes, whereas lower levels are likely to form functional complexes leading to JNK activation (Yasuda et al., 1999). It is important to emphasize that the in vitro protein kinase assay may not be a true reflection of JNK activity measured in the cells. JNK interacts tightly with IB1/JIP-1 and with less affinity with its substrate c-Jun. This means that in cells from dilated bladder where there is reduced IB1/JIP1 expression, more JNK is likely to be pulled down, resulting in increased c-Jun phosphorylation, which may not reflect the situation in vivo.

We previously reported that IB1/JIP-1 regulates cytokine-induced apoptosis in insulin-producing cells (Bonny et al., 2000). In sympathetic neurons, the NGF withdrawal induced apoptosis, which could be prevented by the overexpression of the JBD (JNK binding domain) of IB1/JIP-1 (Harding et al., 2000). These data indicated that IB1/JIP-1 plays an anti-apoptotic function in β cells and neurons by controlling the JNK signalling pathway. In the bladder, the most prominent response to acute bladder distention is a urothelial cells proliferation (Tammela et al., 1993) associated with an increase in smooth muscle cells volume (hypertrophy) (Levin et al., 1990) as well as lamina propria and connective tissue proliferation (Tong et al., 1992). In the same model it was

demonstrated that Cox-2 is upregulated and may participate in cell proliferation (Park et al., 1999). By TUNEL and Hoechst/propidium iodide staining, we did not observe an increase apoptotic rate (data not shown), but it remains technically difficult to evaluate this apoptotic rate in vivo. Further studies will be required to evaluate, in vitro, if a decrease of IB1 content, in this specialised epithelium, increased cell death.

Although JNK is implicated in cancer, the potential contribution of the JNK signalling is still unclear. Recent investigations concurred that JNK is required for stress-induced apoptosis in primary cells (Garay et al., 2000; Ho et al., 2000). In contrast, antisense JNK oligonucleotides inhibit the growth of tumor cells and can induce apoptosis (Bost et al., 1999; Potapova et al., 2000). It is possible that proteins that inhibit JNK activity could act as an oncogene by supressing the regulated apoptosis induced by the JNK pathway activation (Adler et al., 1999; Kurokawa et al., 2000), but this speculative role remains to be evaluated in further studies in vitro. All these data revealed a potential role for IB1/JIP-1 in bladder oncogenesis; IB1/JIP-1 may therefore represent an alternative target for gene therapy of cell proliferation or tumorogenesis in the bladder as a new regulator of the JNK signalling pathway.

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