

Analysis of mitogen-activated protein kinase pathways used by interleukin 1 in tissues *in vivo*: activation of hepatic c-Jun N-terminal kinases 1 and 2, and mitogen-activated protein kinase kinases 4 and 7

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The effects of interleukin 1 (IL-1) are mediated by the activation of protein kinase signalling pathways, which have been well characterized in cultured cells. We have investigated the activation of these pathways in rabbit liver and other tissues after the systemic administration of IL-1 α . In liver there was 30–40-fold activation of c-Jun N-terminal kinase (JNK) and 5-fold activation of both JNK kinases, mitogen-activated protein kinase (MAPK) kinase (MKK)4 and MKK7. IL-1 α also caused 2–3-fold activation of p38 MAPK and degradation of the inhibitor of nuclear factor κ B ('I κ B'), although no activation of extracellular signal-regulated protein kinase (ERK) (p42/44 MAPK) was observed. The use of antibodies against specific JNK isoforms showed that, in liver, short (p46) JNK1 and long (p54) JNK2 are the predominant forms activated, with smaller amounts of long JNK1 and short JNK2. No active JNK3 was detected. A similar

pattern of JNK activation was seen in lung, spleen, skeletal muscle and kidney. Significant JNK3 activity was detectable only in the brain, although little activation of the JNK pathway in response to IL-1 α was observed in this tissue. This distribution of active JNK isoforms probably results from a different expression of JNKs within the tissues, rather than from a selective activation of isoforms. We conclude that IL-1 α might activate a more restricted set of signalling pathways in tissues *in vivo* than it does in cultured cells, where ERK and JNK3 activation are often observed. Cultured cells might represent a 'repair' phenotype that undergoes a broader set of responses to the cytokine.

Key words: liver, nuclear factor κ B, stress-activated protein kinase.

INTRODUCTION

Interleukin 1 (IL-1) is a potent pro-inflammatory cytokine produced by activated monocytes and macrophages that causes pleiotropic effects in many different cell types. It is made at sites of injury or infection and, by up-regulating adhesion molecules and chemotaxins, causes leucocytes to move from blood vessels into tissues. Local production of IL-1 also causes the resorption of connective tissue matrices by resident cells. In addition, IL-1 exerts systemic effects; most notably it causes fever and the production of acute-phase proteins by the liver. Excessive production of IL-1 and the biologically similar cytokine tumour necrosis factor (TNF) is strongly implicated in causing tissue damage in chronic inflammatory diseases such as rheumatoid arthritis.

The signalling pathways activated by IL-1 have been widely studied and elucidated in cultured cells. However, little has been reported of the pathways activated by the cytokine in tissues *in vivo*. IL-1 signals by binding to the type 1 IL-1 receptor (IL-1R1). On ligand binding, IL-1R1 recruits its co-receptor, the IL-1-receptor accessory protein ('IRAcP') [1] and a complex is formed that includes the IL-1-receptor-activated kinase ('IRAK') [2], and the death-domain-containing protein Myd88 [3]. This complex, via an association with TNF-receptor-associated factor 6 ('TRAF 6') [4], activates downstream pathways. In fibroblasts,

for example, IL-1 activates five downstream protein kinase pathways. These include the three mitogen-activated-protein kinase (MAPK) pathways, which are the extracellular signal-regulated kinase (ERK) or p42/p44 MAPK pathway [5], the p38 MAPK pathway [6] and the c-Jun N-terminal kinase (JNK) pathway [7]. IL-1 also activates the inhibitor of nuclear factor κ B (I κ B) kinase pathway [8,9], which leads to the activation of nuclear factor κ B (NF- κ B). Lastly, the TNF- and IL-1-activated protein kinase (TIPK) is also activated by IL-1 in some cells; this enzyme, which phosphorylates a QSL (Gln-Ser-Leu) motif in β casein *in vitro* [10], is less well characterized and has not been cloned. These pathways regulate the expression of target genes by controlling transcription factor activity and also post-transcriptional events such as mRNA stability.

The MAPKs are a family of proline-directed serine/threonine protein kinases that are activated in response to many extracellular stimuli. This results from their phosphorylation on threonine and tyrosine residues in a Thr-Xaa-Tyr motif, in which the intervening amino acid varies between family members. Phosphorylation of both hydroxyamino acids is catalysed by a family of dual-specificity kinases [MAPK kinases (MKKs)] and is required for maximal MAPK activity.

The ERK pathway is strongly activated by growth factors such as platelet-derived growth factor ('PDGF'), as well as many other stimuli. In contrast, p38 MAPK and JNK are at best

Abbreviations used: CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; ERK, extracellular signal-regulated protein kinase; GST, glutathione S-transferase; HA, haemagglutinin; I κ B, inhibitor of nuclear factor κ B; IL-1, interleukin 1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated-protein kinase; MAPKAPK-2, MAPK-activated protein kinase 2; MKK, MAPK kinase; NF- κ B, nuclear factor κ B; TIPK, TNF- and IL-1-activated protein kinase; TNF, tumour necrosis factor.

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weakly activated by growth factors but are strongly activated by cellular stresses and by pro-inflammatory agents such as endotoxin and the cytokines IL-1 and TNF [6,11–14]. Each type of MAPK is specifically regulated by MKKs: ERK by MKK1 and MKK2, p38 by MKK3 and MKK6, and JNK by MKK4 and MKK7. The nature of the kinases upstream of MKKs in IL-1 signalling is not entirely clear. Many MKK kinases have been cloned that can cause the activation of p38 MAPK and JNK: the TGF β -activated kinase (TAK1), which is activated by IL-1 and (indirectly) activates p38 and JNK, is the best candidate for an IL-1-activated MKK kinase [15].

We previously purified two forms of activated JNK2 from liver of rabbits injected with IL-1 [7] and subsequently showed that an IL-1-stimulated activator of JNK purified from the tissue corresponded to MKK7 [16]. There are three closely related JNK genes (1, 2 and 3) that give rise to different splice variants resulting in ten potential isoforms [17]. The physiological reason for the existence of so many isoforms is not known and the nature of the JNK proteins expressed by different tissues has not been explored. We have made antisera against specific JNK forms and used them to investigate the nature of the IL-1-activated JNKs in liver and other tissues. We have also further investigated the MKKs involved in JNK activation and also the effect of IL-1 α *in vivo* on the other signalling pathways potentially used by the cytokine. Our results suggest that the signalling pathways activated by IL-1 α in tissues *in vivo* might be more restricted than those commonly seen in cultured cells.

MATERIALS AND METHODS

Materials

All reagents were from Sigma (Poole, Dorset, U.K.) or Merck Ltd (Poole, Dorset, U.K.) unless stated otherwise. Human IL-1 α was made as described [12]. [γ -³²P]ATP (Reactiv) was from Amersham International (Little Chalfont, Bucks., U.K.). Protein G–Sephacryl was from Amersham Pharmacia Biotech. cDNA for histidine-tagged murine MAPK-activated protein kinase 2 (MAPKAPK-2) was a gift from M. Gaestel (Max Delbrück Center for Molecular Medicine, Berlin, Germany). cDNA for JNK2 α 2 (K55R) and JNK1 α 2 were gifts from M. Kracht (Hannover Medical School, Hannover, Germany). cDNA species for rat GST–SAPK β (rat homologue of JNK3 α 2) and human glutathione S-transferase-conjugated Jun (GST–Jun) (residues 1–135) were gifts from J. R. Woodgett (Ontario Cancer Institute, Toronto, Ontario, Canada). Recombinant proteins were expressed in *Escherichia coli* and the proteins were purified by standard methods. Rat anti-haemagglutinin (anti-HA) antibody was from Boehringer Mannheim (Mannheim, Germany). Murine anti-JNK1 monoclonal antibody (raised against the short form of JNK1) was from PharMingen (San Diego, CA, U.S.A.). Rabbit antibodies against ERK-2, MKK4 and I κ B were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit antisera against synthetic peptides were all raised at the Babraham Institute (Cambridge, U.K.). Their specificities were as follows: anti-(short JNK 2) was against the peptide KNGVVKDQPSAQMQQ (single-letter amino acid codes) (human, C-terminal 15 residues), anti-(long JNK 2) was against DSSLDASTGPLEGCR (human, C-terminal 15 residues), anti-(short JNK 3) was against GVVKGQPSAQQVQQ (human, C-terminal 15 residues), anti-(p38 MAPK) was against ISFVPPPLDQEEMES (rat p38 α , C-terminal 15 residues) and anti-MKK7 was against CRTSGVLSQHHLPPFR (murine, C-terminal 15 residues).

Plasmids

The HA-tag expression vector pCMV3RHA (in which CMV stands for cytomegalovirus) was constructed by the insertion of an oligonucleotide (5'-CTAGCATGTACCCATACGATGTTCTGACTATGCGGAATTCTCGAGATATCTAGA-3') at the *Xba*I site of pCMV3R [18]. The 5' *Xba*I site was destroyed and the remaining *Xho*I and *Xba*I sites (shown in bold) were used for insertion of JNK cDNA species. JNKs were amplified by PCR with the following primers (restriction sites are in bold; initiating methionine residues were altered): 5' for JNK1 α 1 (short JNK1), 5'-GCGCCTCGAGGAGCAGAAGCAAGCGT-GACAACAAT-3'; 3' for JNK1 α 1, 5'-GCGCCTCGAGTCACTGCTGCACCTGTGCTAAAGGAGAGGGCTGCC-C-3'; 5' for JNK1 α 2 (long JNK1), 5'-GCGCACTAGTGAG-CAGAAGCAAGCGTGACAACAAT-3'; 3' for JNK1 α 2, 5'-GCGCACTAGTTCATCTACAGCAGCCAGAGG-3'; 5' for JNK2 α 1 (short JNK2), 5'-GCGCCTCGAGGAGCGACAGT-AAATGTGACAGTC-3'; 3' for JNK2 α 1, 5'-GCGCCTCGAGTTACTGCTGCATCTGTGCTGAAGGCTGATCTTTTAC-AAC-3'; 5' for JNK2 α 2 (long JNK2), 5'-GCGCACTAGTGA-GCCACAGTAAATGTGACAGTC-3'; 3' for JNK2 α 2, 5'-GCGCACTAGTCTATCATCGACAGCCTTCAAGGGG-3'; 5' for JNK3 α 1 and JNK3 α 2 (short JNK3 and long JNK3), 5'-GCGCCTCGAGGAGCCTCCATTTCTTATACTACTGCA-G-3'; 3' for JNK3 α 1, 5'-GCGCCTCGAGTCACTGCTGCAC-CTGTGCTGAAGGAGAAGGCTGTC-3'; 3' for JNK3 α 2, 5'-GCGCCTCGAGTCACTGCAACAACCCAGGG-3'.

Expression of recombinant JNKs in Cos-7 cells

Each cDNA (10 μ g) was mixed with 50 μ l of Superfect transfection reagent (Quiagen, Crawley, West Sussex, U.K.) and 500 μ l of Dulbecco's modified Eagle's medium (DMEM). After 15 min, 3 ml of DMEM/10% (v/v) fetal calf serum was added and this mixture was added to a 10 cm dish of Cos-7 cells (approx. 75% confluence). After 3 h, a further 10 ml of DMEM/10% (v/v) fetal calf serum was added to each dish and the cells were left for 40 h. The medium was then removed and cells were lysed in 2 ml of lysis buffer [20 mM Hepes (pH 7.4)/20 mM β -glycerophosphate/10 mM NaF/0.5 mM EDTA/0.5 mM EGTA/0.2 M NaCl/2 mM dithiothreitol/1 mM PMSF/10 μ g/ml aprotinin/10 μ M *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64)/1% (v/v) Triton X-100] for 10 min on ice. Lysates were clarified by centrifugation in a Microfuge (16000 *g* for 15 min at 4 $^{\circ}$ C).

Immunoprecipitation of recombinant JNKs

Expression of recombinant HA-JNKs was assessed by Western blotting on PVDF membrane with anti-HA antibody at 1:2000 dilution. All blocking and antibody incubations were in Tris-buffered saline/0.1% (v/v) Tween 20/5% (v/v) non-fat milk. Blots were developed by enhanced chemifluorescence (Amersham International) and antigen was quantified with a FLA-2000 PhosphorImager (Fuji, Raytek Scientific Ltd, Sheffield, South Yorks., U.K.). Lysates were then adjusted by adding lysate of untransfected Cos-7 cells to give approximately the same levels of HA-JNKs and total protein. Adjusted lysates were split into five for the preparation of immunoprecipitates, which were made with non-immune serum, the anti-JNK1 monoclonal antibody or the rabbit antisera against short JNK2, long JNK2 and short JNK3. The adjusted lysates were mixed with an equal volume of 2 \times immunoprecipitation buffer [1 \times immunoprecipitation buffer consists of 20 mM Hepes, pH 7.4, 20 mM β -glycerophosphate,

10 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA, 0.2 M NaCl, 2 mM dithiothreitol, 1% (v/v) Nonidet P40, 0.5% sodium deoxycholate and 0.1% SDS], 30 μ l of Protein A-agarose and 5 μ l of a non-immune serum or relevant antiserum or antibody. Samples were rotated for 3 h at 4 °C. Protein A-agarose pellets were then washed three times in 1 ml of immunoprecipitation buffer and twice in 1 ml of kinase wash buffer [20 mM Hepes (pH 7.4)/20 mM β -glycerophosphate/10 mM NaF/0.5 mM EDTA/0.5 mM EGTA/2 mM dithiothreitol/10 mM MgCl₂]. SDS/PAGE loading buffer (50 μ l) was added to the Protein A-agarose beads and samples were subjected to SDS/PAGE. The immunoprecipitation of HA-JNKs was assessed by Western blotting with the anti-HA antibody as described above.

Injection of animals with IL-1 α

Female Dutch rabbits were sedated with Hypnorm [Janssen Pharmaceuticals (Wantage, Oxon., U.K.)] and injected via an ear vein with IL-1 α (5 μ g/kg) or vehicle. After 4 min a rapidly lethal anaesthetic [Sagatal (pentobarbitone sodium urethane) from Rhone Merieux (Harlow, Essex, U.K.)] was administered and the tissues were rapidly removed and placed on ice before processing or storage at -70 °C. The estimated times from the injection of IL-1 to placing tissues on ice were as follows: liver, 6 min; spleen, 7 min; lung, 8 min; skeletal muscle, 9 min; brain, 11 min; kidney, 12 min.

Preparation of tissue extracts

For immunoprecipitation or Western blotting, 250 mg of tissue (fresh or frozen) was homogenized in a Polytron (Kinematica, Littan, Switzerland) in 5 ml of lysis buffer lacking Triton X-100. For MonoQ chromatography, homogenates were prepared in chromatography buffer: this was the lysis buffer but lacking both Triton X-100 and NaCl and in which 20 mM Tris/HCl, pH 8.5, was substituted for Hepes. Homogenates were clarified by centrifugation in a Microfuge (16000 *g* for 15 min at 4 °C).

Western blotting for I κ B

Western blots were performed essentially as described for anti-HA blots; anti-I κ B was used at 1:1000 dilution, the blots were developed with enhanced chemiluminescence rather than enhanced chemifluorescence and proteins were detected on X-ray film.

MonoQ chromatography

A MonoQ column [1 ml (HR 5/5); Pharmacia Biotech, Uppsala, Sweden] was equilibrated in chromatography buffer; 20 mg of liver sample was applied to the column. A 20 ml gradient from 0 to 0.5 M NaCl in chromatography buffer was used to elute proteins; 20 fractions (1 ml) were collected and JNK activity in the fractions was measured directly in the kinase assay or after immunoprecipitation with JNK antibodies. An alternative protocol was used to generate fractions for in-gel kinase assays. Here, homogenate from IL-1-treated rabbit liver (generated as above) containing 1 mg of total protein was applied to a pre-equilibrated MonoQ PC 1.6/5 column; proteins were eluted as 20 fractions (0.2 ml) with a linear 0–0.3 M NaCl gradient.

Immunoprecipitation of chromatography fractions and tissue extracts

The protein concentration of tissue extracts was adjusted to 2 mg/ml (as judged by Bradford protein assay). Tissue extract (500 μ l) or chromatography fraction aliquot (300 μ l) was mixed

with an equal volume of 2 \times immunoprecipitation buffer (for the assay of MKK4 and MKK7, sodium deoxycholate was omitted from the immunoprecipitation buffer at this stage and in subsequent washes) containing 5 μ l of diluted antibody or antiserum, and 30 μ l of Protein A-agarose slurry. Samples were then treated as described for the immunoprecipitation of recombinant JNKs; the kinases immunosorbed to Protein A-agarose were assayed for activity. Immunoprecipitations for analysis by in-gel kinase assay were prepared with 180 μ l of chromatography fraction, 180 μ l of 2 \times immunoprecipitation buffer, 5 μ l of anti-JNK1 antibody and 30 μ l of Protein G-Sepharose slurry. Immunoprecipitations of JNKs from cultured cells (MRC-5 or HeLa) were performed similarly, after treatment of cells with 20 ng/ml IL-1 α (or vehicle) for 15 min.

Protein kinase assays

Samples of chromatography fractions, or protein A beads containing immunosorbed protein kinase, were assayed for protein kinase activity as follows. To each pellet was added 10 μ l of kinase wash buffer, 10 μ l of stock solution of substrate at 100 μ g/ml [His-MAPKAPK-2 for p38 MAPK; GST-Jun (1–135) for JNK; myelin basic protein for p42 MAPK; GST-JNK2 α 2 (K55R) for MKK4 and MKK7] and 10 μ l of kinase assay buffer [150 mM Tris/HCl (pH 7.4)/30 mM MgCl₂/60 μ M ATP/0.4 μ Ci/ μ l [γ -³²P]ATP/1 mM PMSF/10 μ g/ml aprotinin/10 μ M E-64]. Samples were shaken for 20 min at room temperature and, after the addition of 20 μ l of SDS sample buffer, were run on SDS/PAGE. Gels were stained and dried and the incorporation of ³²P into the substrate was quantified with a PhosphorImager.

In-gel kinase assays

Samples (10 μ l of monoQ chromatography fractions or entire pellets from immunoprecipitations) were boiled in sample buffer and separated on SDS/10% polyacrylamide mini-gels containing 1.2 mg/ml GST-Jun (1–135). Gels were rinsed three times at room temperature in 20% (v/v) propan-2-ol/50 mM Tris/HCl (pH 8.0) (20 min for each rinse) to remove SDS, and three times (20 min each) in 50 mM Tris/HCl (pH 8.0)/5 mM 2-mercaptoethanol. Proteins were denatured at room temperature with two washes (30 min each) in 7 M guanidinium chloride/50 mM Tris/HCl (pH 8.0)/5 mM 2-mercaptoethanol and renatured at 4 °C with five washes (one for 30 min, two for 1 h, one for 14 h and one for 30 min) in 50 mM Tris/HCl (pH 8.0)/5 mM 2-mercaptoethanol/0.04% (v/v) Tween 40. Gels were re-equilibrated to room temperature with two 30 min washes in 40 mM Hepes (pH 8.0)/2 mM dithiothreitol/10 mM MgCl₂. Phosphorylation of in-gel substrate was then performed for 3 h at room temperature in 40 mM Hepes (pH 8.0)/2 mM dithiothreitol/0.5 mM EGTA/10 mM MgCl₂/50 μ M ATP containing 0.6 mCi/ml [γ -³²P]ATP. Gels were then washed extensively with 5% (w/v) trichloroacetic acid/1% (w/v) disodium pyrophosphate until no further radioactivity was being stripped from the gels. Incorporation of labelled phosphate was quantified by PhosphorImager.

RESULTS

Characterization of anti-JNK antibodies

The terminology of the JNK isoforms is as follows [17]: JNK1, JNK2 and JNK3 represent three highly similar genes. JNK1 and JNK2 occur as α or β variants depending on the presence of short alternative sequences in subdomains IX and X. The antisera used here did not distinguish between these. All three JNKs occur in alternatively spliced forms, giving rise to distinct

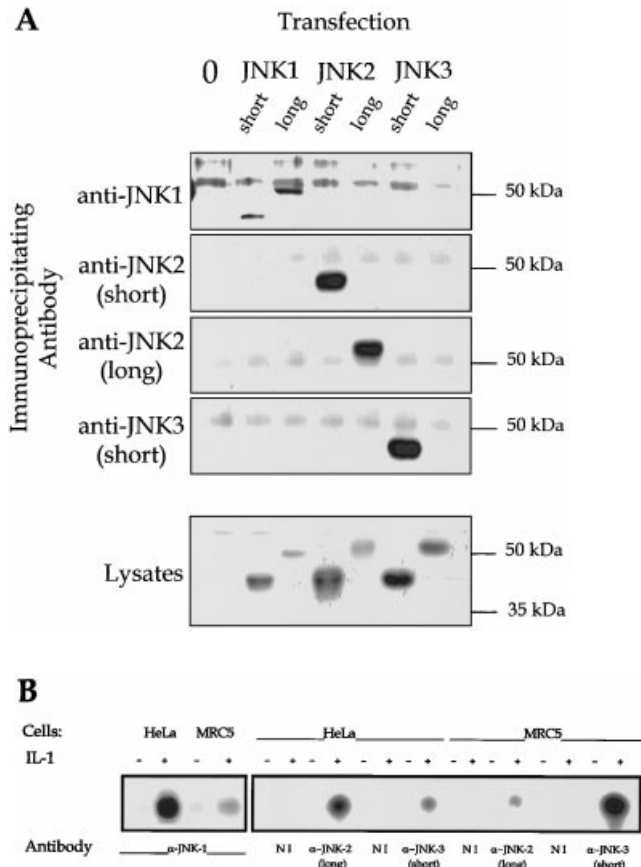


Figure 1 Specificity of anti-JNK antibodies

(A) Cos-7 cells were left untransfected (0) or were transfected with HA-tagged JNKs. Cell lysates were adjusted to give similar HA-JNK and protein concentrations. Western blotting of the lysates with anti-HA antibody is shown in the bottom panel (lysates). Immunoprecipitates were prepared from the adjusted lysates by use of the following: the anti-JNK1 antibody and the anti-(short JNK2), anti-(long JNK2) and anti-(short JNK3) antisera. Precipitated HA-JNKs were detected by Western blotting with the anti-HA antibody and enhanced chemiluminescence. See the Materials and methods section for details. Some cross-reactivity of the (anti-rat) secondary antibody with (murine) heavy chain is observed in the top panel. (B) Immunoprecipitates from lysates (corrected for protein concentration) of cells stimulated with IL-1 or vehicle were prepared with the antibodies against JNK1, long JNK2, short JNK3 or non-immune rabbit serum (NI) as indicated, and assayed for activity against GST-Jun (1–135). The result shown is typical of three experiments.

C-termini. Thus each JNK has a short or a long form: the short forms designated JNK1 α 1, JNK1 β 1, JNK2 α 1 and JNK2 β 1 are detected as 46 kDa proteins; JNK3 α 1 is 48 kDa. The long forms JNK1 α 2, JNK1 β 2, JNK2 α 2 and JNK2 β 2 correspond to 54 kDa proteins and JNK3 α 2 is 57 kDa [17]. We made rabbit antisera against the C-terminal 15 residues of the long and short forms of JNK2 and the short form of JNK3. We refer to these as anti-(short JNK2), anti-(long JNK2) and anti-(short JNK3). A monoclonal antibody raised against the short form of JNK1 was obtained commercially.

The specificity of the rabbit antisera for immunoprecipitation of different JNK isoforms was determined as follows. cDNA species were used as templates for the PCR-based generation of short and long α forms of each JNK. These six constructs were cloned into a mammalian expression vector (pCMVRHA) and expressed with an N-terminal HA tag in COS-7 cells. Lysates were prepared and the levels of expression of recombinant JNKs

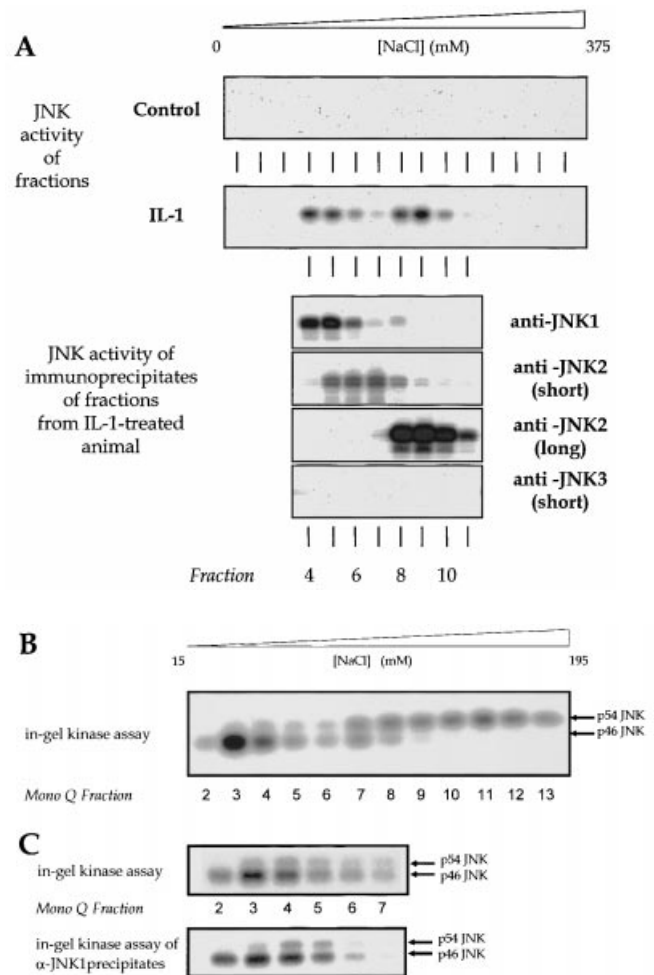


Figure 2 IL-1 activates JNK1 and JNK2 in rabbit liver

(A) MonoQ chromatography was performed on extracts of liver from rabbits injected with vehicle (control) or IL-1. JNK activity of the fractions was detected by assaying them on GST-Jun (1–135) substrate (top two panels). Immunoprecipitates were prepared from equal volumes of the fractions by using the four anti-JNK antibodies; precipitated JNK activity was detected in the kinase assay as described above (bottom four panels). See the Materials and methods section for details. (B) MonoQ chromatography fractions (see the Materials and methods section for details of the shallower NaCl gradient used) were assayed for activity of long (p54) JNK and short (p46) JNK activated by IL-1 in rabbit liver by in-gel kinase assay against GST-Jun (1–135). (C) Immunoprecipitation of early JNK (p54 and p46) peaks by anti-JNK1 antibody, shown on in-gel assay.

(HA-JNKs) were examined by Western blotting with an anti-HA antibody. Development of the blot by enhanced chemiluminescence allowed the quantification of HA-JNK expression with the use of a PhosphorImager. Lysates were then adjusted by dilution with lysates of untransfected Cos-7 cells to approximately equal HA-JNK concentrations (Figure 1A, bottom panel). Immunoprecipitates were prepared from these adjusted lysates by the addition of the four anti-JNK antisera; levels of precipitated HA-JNKs were assessed by Western blotting with the anti-HA antibody (Figure 1A, top four panels). The antibody against JNK1 precipitated both short and long forms but did not react with JNK2 or JNK3. The antisera against short JNK2, against long JNK2 and against short JNK3 all specifically immunoprecipitated their respective antigens.

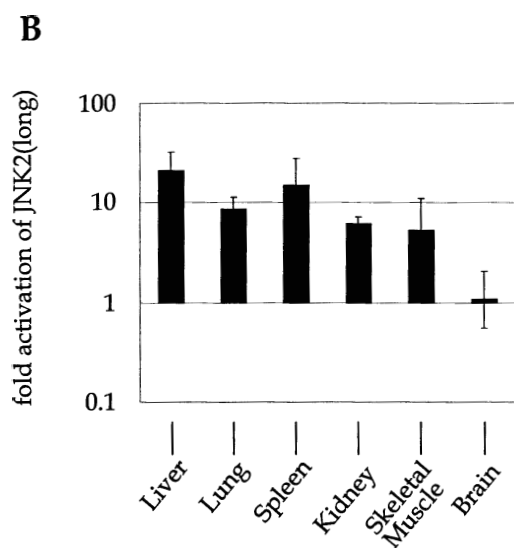
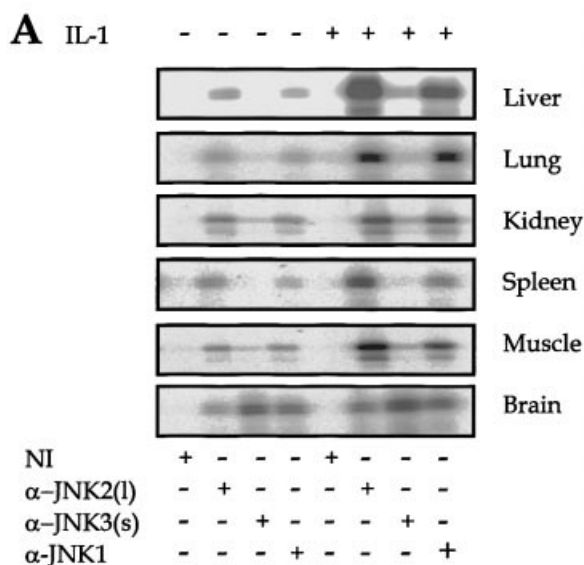


Figure 3 Activation of JNKs by IL-1 in rabbit tissues

(A) Rabbit tissues were analysed in pairs, one unstimulated and one IL-1-stimulated. Immunoprecipitates were prepared from tissue lysates with the anti-JNK1, the anti-(long JNK2) and the anti-(short JNK3) antibodies. A non-immune serum (NI) was included as a control. The activity of the precipitated JNKs was measured on GST-Jun (1–135) substrate. Phosphorylated substrate was detected by autoradiography. (B) Activation of long JNK2 by IL-1 in rabbit tissues, measured as described above. A PhosphorImager was used to quantify substrate phosphorylation. The fold activation of long JNK2 in each tissue on stimulation by IL-1 was determined in three pairs of rabbits. Results are geometric means \pm S.E.M. for these triplicates.

The ability of the antibodies against JNK1, long JNK2 and short JNK3 to precipitate active kinase from cultured cells was assessed by kinase assay *in vitro* with GST-Jun (1–135) as substrate. Treatment of both MRC-5 fibroblasts and HeLa cells with IL-1 α resulted in activation of the JNK isoforms precipitated by all three antibodies (Figure 1B). No significant activity was precipitated from IL-1-treated cells by non-immune rabbit serum or by the specific antibodies from unstimulated cells. Stimulation by IL-1 α resulted in strong activation of JNK1 in HeLa cells, with less JNK2 and even less JNK3 detected. In contrast, JNK3 was the predominant JNK detected in IL-1 α -stimulated fibroblasts.

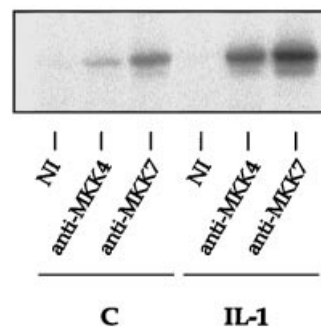


Figure 4 Activation of MKK4 and MKK7 by IL-1 in rabbit liver

Immunoprecipitates were prepared from lysates of unstimulated (C) or IL-1-stimulated rabbit liver by using antibodies against MKK4 and MKK7. Kinase assays were performed *in vitro* on the precipitates with GST-JNK2 (K55R). Phosphorylated substrate was revealed by SDS/PAGE and autoradiography. The result shown is typical of three experiments.

Identification of IL-1-activated JNK isoforms in rabbit liver

We previously showed by purification and peptide sequencing that systemic administration of IL-1 α caused a rapid activation of short and long forms of JNK2 in rabbit liver [7]. We used the various JNK antibodies to characterize further the IL-1 α -activated forms of JNK in rabbit liver. Liver homogenates were prepared from IL-1 α -injected animals and were chromatographed on a MonoQ column. Aliquots of column fractions were immunoprecipitated with each of the four antibodies. JNK activity in the fractions (Figure 2A, top two panels) and in the immunoprecipitates (Figure 2A, bottom four panels) was measured. Two main peaks of JNK activity were eluted by the gradient. Activity from the first peak was precipitated by the antibody against JNK1, and from the second by the antiserum against long JNK2. The antiserum against short JNK2 precipitated activity between the two peaks. The antiserum against JNK3 precipitated no detectable activity. The fact that there was no distinct peak of JNK activity corresponding to short JNK2 suggested that it was a relatively minor component compared with long JNK2 and JNK1.

Activated hepatic JNK1 is predominantly the p46 form

In-gel kinase assay of fractions from MonoQ chromatography of IL-1 α -stimulated rabbit liver (Figure 2B) revealed two main peaks of Jun phosphorylation: a p46 JNK activity that was eluted as an early, sharp peak (fractions 3 and 4; 30–60 mM NaCl), and a p54 JNK activity that was eluted as a later, broad peak (fractions 7–13; 90–195 mM NaCl). In addition, a minor peak of p46 JNK was eluted between the two main peaks (fractions 10 and 11; 90–120 mM NaCl) and a minor peak of p54 JNK activity was approximately co-eluted with the main p46 peak. The main peak of long JNK corresponds to the activity precipitated by the anti-(long JNK2) antiserum, and the minor peak of short JNK to that precipitated by the anti-(short JNK2) antiserum; the in-gel assay thus confirmed that activated hepatic JNK2 is primarily the long form. Both the early peaks detected by the in-gel assay (the minor long peak and the main short peak) were precipitated by the anti-JNK1 monoclonal antibody (Figure 2C). Thus the activated hepatic JNK1 (in contrast with JNK2) is predominantly in the short form, with a small amount of the long form (identifiable by in-gel kinase assay).

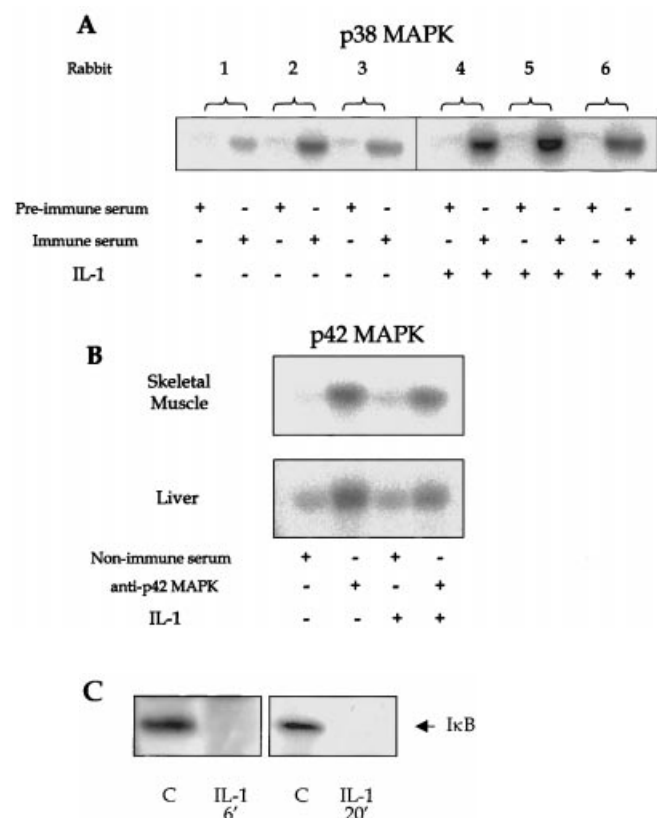


Figure 5 IL-1 activates p38 MAPK and I κ B degradation but not ERK in rabbit liver

(A) Immunoprecipitates were prepared from extracts of livers from six animals (three control and three injected with IL-1 as before) with the anti-(p38 MAPK) antiserum. Immunoprecipitated p38 MAPK activity was measured on His₆-MAPKAPK-2 substrate. See the Materials and methods section for details. (B) Anti-ERK-2 immunoprecipitates were prepared from extracts of liver and skeletal muscle from vehicle-injected or IL-1-injected rabbits; ERK-2 activity was measured against myelin basic protein substrate. The result shown is typical of three experiments. (C) Liver extracts were prepared as before; 1 mg of tissue protein was analysed by SDS/PAGE, transferred to PVDF membrane and I κ B was detected by blotting with anti-I κ B antibody at 1:1000 dilution. See the Materials and methods section for details. The result shown is typical of three experiments.

IL-1 α activates JNK1 and JNK2 but not JNK3 in other tissues

The anti-JNK antibodies were then used to examine whether IL-1 α caused a similar profile of JNK activation in other tissues. Rabbits were injected intravenously with IL-1, left for 4 min and killed as before. Tissues were rapidly removed and placed on ice before the preparation of extracts at 4 °C. Immunoprecipitation assays were performed with the various antibodies. Figure 3(A) shows a representative experiment. IL-1 α caused the activation of long JNK2 and JNK1 in lung, spleen, kidney and skeletal muscle as well as in liver. The mean levels of activation varied from 30-fold in liver to 7-fold in skeletal muscle. No significant JNK3 activity was detected in any tissue apart from brain. Little activation of the JNK isoforms was detected in brain (1–2-fold in several experiments) (Figures 3A and 3B).

IL-1 α activates both MKK4 and MKK7 in rabbit liver

Two JNK activators are known: MKK4 and MKK7. It has been reported that in some cell lines IL-1 activates MKK7 and not MKK4, whereas both JNK kinases are activated in response to

stressful stimuli [19–21]. We measured the activity of both of these kinases by immunoprecipitating them from liver extracts and assaying their ability to phosphorylate a kinase-dead JNK substrate. Figure 4 shows a representative experiment in which IL-1 α caused approx. 6-fold activation of both enzymes.

IL-1 α activates p38 MAPK and I κ B degradation, but not ERK, in rabbit liver

We next investigated the activation in liver of other signalling pathways known to be stimulated by IL-1 in cultured cells. The activity of p38 MAPK was measured by immunoprecipitation assay of extracts prepared as before. Three pairs of livers (control and IL-1) were used. IL-1 α caused a 2–3-fold activation (Figure 5A). Significant variation in the levels of p38 activity between extracts from the three unstimulated livers was observed.

IL-1 α did not cause an activation of ERK-2 in liver or skeletal muscle, as judged by an immunoprecipitation assay on myelin basic protein (Figure 5B). This contrasts with a strong activation of ERK by IL-1 previously observed in many cell lines [5,22].

Lastly we checked whether IL-1 α caused the degradation of I κ B. Tissue extracts were prepared 6 and 20 min after the injection of IL-1 α and then Western blotted. The hepatic I κ B had been degraded by 6 min, which was consistent with expected activation of the NF- κ B pathway (Figure 5C).

DISCUSSION

The physiological reasons for the existence of three JNK genes and at least ten potential isoforms remain unclear. Characterization of the various JNK antibodies described here showed that they were specific for the form used as antigen. For this reason we used them to characterize further the JNK forms activated by IL-1 α *in vivo* in the liver, which is an important target tissue. Most of the JNK activated by IL-1 α comprised the short form of JNK1 and the long form of JNK2. Long JNK1 and short JNK2 seemed to be minor components. No JNK3 was detected, which was surprising because the antibody against short JNK3 strongly precipitates IL-1 α activated JNK from a variety of cultured cells, including fibroblasts and HeLa cells (Figure 1B), and vascular endothelial cells [22]. A relatively large amount of JNK3 activity was found in the brain but there was little regulation by IL-1 α compared with that seen in other tissues. This pattern was consistent with the reported distribution of JNK3 mRNA in mouse tissues, in which significant expression was restricted to brain and testis [17]. This suggests that the relative activities of JNK isoforms detected in tissues is likely to reflect the relative expression of the three JNK genes; certainly we have found no evidence for the differential activation of isoforms by different stimuli in cultured cells (W. Davis and J. Saklatvala, unpublished work).

The activation of hepatic JNKs after injection of IL-1 α is presumably by MKK4 as well as MKK7, because both these upstream kinases were activated. This finding contrasts with some reports in cell lines in which IL-1 has been found to activate only MKK4, although both JNK kinases were reported to be activated by stressful stimuli [19–21]. We have also found the regulation of MKK4 (as well as MKK7) by IL-1 α , with the assay described, in many cell lines including pig aortic endothelial cells and KB cells (W. Davis and J. Saklatvala, unpublished work).

We previously purified an IL-1-induced activator, identified as MKK7, from rabbit liver [16]. In that work JNK activation was measured by its ability to phosphorylate c-Jun; MKK4 was not detected. It is interesting to consider the finding of strong activation of both kinases *in vivo* in the light of the findings of

Lawler et al. [21]. They showed that, *in vitro*, MKK4 exhibited a strong preference for the tyrosine residue in the JNK phosphorylation motif (TPY) and did not phosphorylate the threonine residue. MKK7, in contrast, phosphorylated the threonine residue preferentially but was also able to phosphorylate the tyrosine residue, although to a much smaller extent. To be active, MAPKs need both hydroxyamino acids to be phosphorylated. Consequently MKK4 acting alone might not be a significant activator but might synergize strongly with MKK7. We found that both MKKs were activated *in vivo* by IL-1 α approx. 6-fold: this would result in a synergistic activation, which is consistent with the level of activation that we observe (approx. 30-fold).

Maximal JNK activation therefore might require two activators. This contrasts with ERK and p38 MAPKs, whose MKKs show a more equal preference for the tyrosine and threonine residues of their respective activation motifs. The need for two activators raises the possibility that they could be regulated separately and might be activated by different upstream kinases. Generally, MKK4 has been thought to be activated by MEK1 [23,24], whereas MKK7 might be activated by mixed-lineage kinase 3 ('MLK3') because of the association of MKK7 and MLK3 with the JNK-interacting protein ('JIP') [25].

Besides investigating the JNK pathway components activated by IL-1 α in a physiological system *in vivo*, we also examined the other pathways known to be activated by IL-1 in cell culture. Both p38 MAPK and I κ B degradation were also stimulated in liver, although a lower activation of p38 than that caused by IL-1 α in many cell lines [26,27] was observed. This suggests that the p38 pathway *in vivo* is less responsive to IL-1 α than is the JNK pathway, in which comparable activation to that observed in cell lines is elicited by injection of the cytokine. In our previous study we were unable to show convincing activation of p38 [16]. This might have been because there was variation in basal activity between different liver samples. In these experiments we used three separate pairs of animals. However, we were still unable to find evidence of activation of ERK by IL-1, although this is widely found in cells in culture. We have also been unable to detect activation of TIPK in tissues of IL-1-injected rabbits (A. Finch and J. Saklatvala, unpublished work). It might be that differentiated non-dividing cells respond to IL-1 in a limited manner *in vivo*, whereas dividing 'repair' cells such as fibroblasts undergo a wider range of changes in gene expression in response to the cytokine. Many cultured cells might resemble this 'repair' phenotype more closely than that of differentiated, resting tissue, and might therefore respond to IL-1 by activating more intracellular signalling pathways. The expression of significant levels of JNK3 in these cells, as well as the responsiveness to IL-1 of the ERK and TIPK pathways, might be a reflection of this difference.

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