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Phenyl *N*-tert-butylnitrone Down-regulates IL-1β-stimulated Matrix Metalloproteinase-13 Gene Expression In Human Chondrocytes: Suppression of c-Jun NH_2-terminal Kinase, p38-MAP Kinase And Activating Protein-1.

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Abstract.

Cytokine mediated induction and overexpression of matrix metalloproteinases (MMPs) is recognized as an important factor in the pathogenesis of arthritis. Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is known to superinduce the expression and production of MMP-13 in many cell types. Phenyl *N*-tert-butylnitrone (PBN), a spin trap agent, inhibited the IL-1β-induced expression of MMP-13 in human osteoarthritis chondrocytes (OA chondrocytes). Down-regulation of MMP-13 expression correlated with the inhibition of mitogen activated protein kinase (MAPK) sub-groups c-Jun NH_2-terminal Kinase (JNK) and p38-MAPK activation, accumulation of phospho-c-jun and the DNA binding activity of AP-1. Results of *in vitro* kinase assays showed that exogenously added PBN completely blocked the c-Jun phosphorylating activity of JNK. Interestingly, using *in* vitro kinase assay we also found that chondrocyte p38-MAPK phosphorylate c-Jun and that PBN was not very effective in inhibiting c-Jun phosphorylating activity of p38-MAPK. In addition, PBN did not block the ATF-2 phosphorylating activity of p38-MAPK and Elk-1 phosphorylating activity of ERK p44/p42 *in vitro* indicating that PBN may act selectively to inhibit the phosphorylation of c-Jun in OA chondrocytes. Taken together our results for the first time demonstrate that PBN suppress the IL-1β-stimulated expression of MMP-13 in OA chondrocytes and that this was achieved by inhibiting the activation of JNK and AP-1. These results suggest that use of PBN or compounds derived from it may be of potential benefit in inhibiting signaling events associated with cartilage degradation in arthritis.

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that elicits the induction of several mediators of cartilage degradation and therefore plays a pivotal role in the pathogenesis of arthritis (Mengshol et al, 2000; Kraan and van den Berg, 2000). Involvement of IL-1β in cartilage degradation was established from studies showing that intra-articular administration of IL-1β in rabbit and mouse joints results in loss of proteoglycans from the cartilage that persists for a long period of time (Kraan and van den Berg, 2000). These inflammatory effects of IL-1β, including the induction of mediators of cartilage degradation, are mediated via activation of the mitogen activated protein kinases (MAPKs) family of serine/threonine kinases. MAPKs participate in different intracellular signaling pathways controlling a wide spectrum of cellular processes including growth, differentiation, transformation, apoptosis and stress responses (Chang and Karin, 2001). The three ubiquitously expressed subgroups of MAPK family, namely extracellular regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38-MAPK are structurally related and play a key role in transmitting the signal from the cell surface to the nucleus. The JNK and p38-MAPK subgroups strongly respond to stress and inflammatory signals and are known to regulate the induction of molecules associated with cartilage degradation in arthritis (Vincenti and Brinckerhoff, 2001). In the promoter region of most of the genes implicated in inflammation binding sites for the transcription factor activating protein-1 (AP-1) are present. A constituent of AP-1 homo or heterodimers is the c-Jun which is preferentially phosphorylated by the JNK at specific sites (serine 63, serine 73) on the amino-terminal transactivation domain resulting in enhanced transcriptional activity of AP-1 (Karin, 1995).

Matrix metalloproteinases (MMPs) are a large group of enzymes that play a crucial role in tissue remodeling as well as in the destruction of cartilage and bone in an arthritic joint.

Production and release of MMPs is microenvironmental and is induced by several factors including the pro-inflammatory cytokine IL-1β (van den Berg, 2000). MMPs are capable of degrading a wide variety of extracellular matrix components (reviewed in Mengshol et al., 2002). Among the various MMPs, MMP-13 is of particular importance as it is found elevated in joint disorders (Mitchell et al., 1996) and can cleave type-II collagen (CII), the major component of the cartilage matrix, more efficiently. Studies have documented that in arthritic joints degradation of CII is excessive due to increased cleavage of CII by MMPs (Billinghurst et al., 2000). Other studies have shown that excessive activity of MMP-13 can produce the type of pathology seen in OA joints (Neuhold et al., 2001). Expression of MMP-13 is also increased in majority of rheumatoid arthritis (RA) patients and can be correlated with systemic markers of inflammation (Westhoff, et al 1999). Pro-inflammatory cytokine-induced expression of MMP-13 in human chondrocytes and in animal models of arthritis is dependent on the activation of the MAPK sub-group JNK and the transcription factor AP-1 (Han et al., 2001; Liacini, et al 2002).

Phenyl *N*-tert-butylnitrone (PBN), a synthetic pharmacological spin trap agent produce a diverse array of pharmacological effects in animal models of oxidative tissue injury (Ho et al., 2000; Kotake, 1999; Endoh et al., 2001). Several studies have attributed the inhibition of oxidative stress by PBN to its properties of free radical (peroxy, hydroxyl, NO) scavenging and down regulation of pro-inflammatory cytokine expression and up-regulation of anti-inflammatory cytokine IL-10 (reviewed in Kotake, 1999; also see Sang, et al., 1999). PBN has also been shown to decrease basal protein phosphorylation with concomitantly increased phosphatase activity and also inhibited the IL-1β-induced activation of p38 MAPK and H_2O_2 biosynthesis in rat glial cells (Robinson et al., 1999). Other studies have shown that PBN inhibits stress-sensitive signaling pathways in gerbil hippocampus and the induction of neurite outgrowth

in PC12 cells via activation of Ras-ERK1/2 pathway and activation of protein kinase C (Tsuji et al., 2000, 2001). However, studies on the modulation of IL-1β-induced stimuli in OA chondrocytes by PBN have not yet been reported.

In the present study we show for the first time that PBN is a potent inhibitor of IL-1β-stimulated induction and expression of MMP-13 in OA chondrocytes *in vitro*. We also demonstrate that PBN-mediated inhibition of MMP-13 expression correlated with the inhibition of JNK activation, reduction in the levels of phosphorylated c-Jun, and inhibition of the DNA binding activity of the transcription factor AP-1. Furthermore, in *in vitro* kinase assays PBN completely blocked the c-Jun phosphorylating activity of JNK but not of p38-MAPK indicating that c-Jun may also be a substrate for p38-MAPK in OA chondrocytes. In addition, PBN had no significant inhibitory effect on the ATF-2 phosphorylating activity of p38-MAPK and Elk-1 phosphorylating activity of ERK p44/p42 in *in vitro* kinase assays. These results suggest that use of PBN or compounds derived from it may selectively target the IL-1β-induced activation of JNK and AP-1 thereby inhibiting the production of MMPs in arthritic joints.

MATERIALS AND METHODS

Reagents. Cell culture medium was from either Cellgro (Mediatech Inc., Herndon, VA) or GIBCO-BRL (Bethesda, MD). PBN was purchased from Alexis Biochemicals (San Diego, CA) and recombinant human IL-1β was purchased from R & D Systems (St. Paul, MN). N-acetyl cysteine (NAC), MTT, NP-40, protease inhibitors (leupeptin, pepstatin A, PMSF, DTT) and phosphatase inhibitors β−glycerophosphate and sodium orthovanadate (Na_3VO_4) were purchased from Sigma Chemical Co., (St. Louis, MO).

Culture of OA chondrocytes and treatments. Human OA cartilage samples from the hip were procured through the Cooperative Human Tissue Network and with prior approval of the Institutional Review Board of University Hospitals of Cleveland. In all of the experiments described in this report, primary chondrocytes prepared by the enzymatic digestion of cartilage (Singh et al., 2002; Islam et al., 2001) were used. OA chondrocytes were plated (1×10^6/ml) in 35mm culture dishes (Becton-Dickinson, Franklin Lakes, NJ) in DMEM:F-12 (1:1) with 10% FCS (GIBCO BRL) and allowed to adhere for 72 hours at 37°C and 5% CO_2 in a tissue culture incubator. A stock solution of PBN (200 mM) was prepared in PBS and filter sterilized. OA chondrocytes were serum starved overnight and then treated with IL-1β (5 ng/ml) and PBN in serum free DMEM:F-12 for time period indicated under each figure. The dose of PBN (10mM) used in the present study was based on the dose used in earlier published studies (Endoh et al., 2001).

IL-1β bioassay. To determine whether PBN inhibits the bioactivity of IL-1β *in vitro*, IL-1β-induced proliferation of mouse thymocytes in the presence and absence of PBN was measured essentially as described (Mossman, 1983). Thymocytes proliferation was measured using the MTT based cell proliferation assay kit commercially available (R & D Systems) according to the

instructions of the manufacturer. Color intensity was read at 570 nm using the Bio-Tek ELISA Reader (Bio-Tek Instruments, Winooski, VT).

Western immunoblot analysis. OA Chondrocytes were treated as described above and 1 ml of culture medium was precipitated with 10% trichloroacetic acid (TCA) at 4°C. TCA-precipitated proteins were collected and resolved by SDS/PAGE and transferred to nitrocellulose membranes. Western blots were probed with a goat anti-human MMP-13 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the reacting proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia, Pistacaway, NJ). For the analysis of MAPKs, OA chondrocytes were washed with cold PBS and lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 and 1% SDS, supplemented with protease inhibitors (1μg/ml leupeptin, 1μg/ml pepstatin A, 1mM PMSF and 1mM DTT) and phosphatase inhibitors (1 mM β–glycerophosphate and 1 mM Na_3VO_4). Equal amount (25 μg) of protein was resolved by SDS-PAGE on a 4-20% gradient gel and transferred to nitrocellulose membranes and the Western blots were probed with phosphorylation and non-phosphorylation state-specific polyclonal rabbit anti-human p38-MAPK (Cell Signaling Technologies, Beverly, MA), anti-ERK p44/p42, anti-JNKp46/p54, and anti-c-Jun antibodies (all from Santa Cruz).

MMP-13 activity assay. MMP-13 activity was determined in the culture supernatants using a commercially available MMP-13 activity assay kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and was expressed as $\delta Absorbance_{405}/h^2$ x 1000.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total cytoplasmic RNA was prepared from OA chondrocytes using a commercially available kit (Qiagen, Valencia, CA). Real-time RT-PCR with fluorescent labeled internal probes was performed using an ABI Prism 7700 detection system (ABI/Perkin Elmer Biosystems, Foster City, CA) as previously

described (Singh et al., 2002). Following primers and probes specific for MMP-13 (Sun and Yokota, 2002) were used: PCR primers. 5'-AAGGAGCATGGCGACTTCT-3', 5'-TGGCCCAG GAGGAAAAGC-3'; Probe. 5'-FAM-CCCTCTGGCCTGCTGGCTCA-TAMRA-3'. The PCR primers were designed to amplify a 71 bp fragment of the MMP-13 mRNA (GenBank Accession Number NM_002427.2) and the probe was specific for the internal region flanked by the PCR primers. Primers and fluorescent probes for quantifying the human β-Actin mRNA were obtained commercially (ABI/Perkin Elmer).

***In vitro* kinase assay.** Kinase assays were performed using a non-radioactive kinase assay kit according to the instruction of the manufacturer (Cell Signaling Technologies, Beverly, MA). Briefly, IL-1β treated cells were washed twice with ice-cold PBS and scrapped directly into 0.5 ml of lysis buffer (20mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and freshly added 1mM PMSF) and homogenized by passing ten times through a 21-gauge needle. The homogenate was microcentrifuged for 10 min at 10,000 rpm at 4 °C, and the supernatant was used for immunoprecipitation of the kinases. For immunoprecipitating activated JNK, 200μg of total proteins were incubated overnight with 2μg of c-Jun fusion protein beads. Activated ERKp44/p42 and p38-MAPK were immunoprecipitated by incubating 200 μg of total protein with 2 μg of immobilized anti-phospho ERK p44/p42 or anti-phospho-p38 MAPK antibody respectively, with gentle rocking at 4°C. In all cases, after centrifugation for 30 sec (10,000 g at 4 °C), the pellets were washed twice with cold lysis buffer and then twice with kinase buffer (25 mM Tris, pH 7.5, 1 mM β-glycerophosphate, 1 mM sodium orthovenadate, 2 mM DTT and 10 mM $MgCl_2$) and then dissolved in the kinase buffer. Finally, the kinase activity was determined in the presence or absence of PBN (10mM) according to the instructions

provided by the kit manufacturer. Samples were boiled for 5 min, vortexed and 30µl of sample was resolved on a 4-20% gradient SDS/PAGE gel. Western blots were probed with anti-phospho-c-Jun, c-Jun, anti-phospho Elk-1 and anti-phospho-ATF-2 antibodies (Cell Signaling Technologies).

ELISA assay for AP-1 DNA binding activity. The DNA binding activity of c-Jun was determined using a highly specific and sensitive AP-1 ELISA kit essentially according to the instructions of the manufacturer (Active Motif, Carlsbad, CA). Briefly, chondrocytes were treated with PBN and IL-1β alone or IL-1β + PBN for various time points, washed with cold PBS and lysed in kit supplied lysis buffer. Lysates were centrifuged at 14,000 rpm for 10 min and the nuclear fraction was transferred to a new tube and nuclei were lysed in the kit supplied nuclear lysis buffer. Five µg of total protein from each nuclear lysate was added to the wells of an ELISA plate precoated with an oligonucleotide containing the sequence 5'-TGAGTCAG-3' (TRE site) and incubated for 1 hr at room temperature with moderate shaking. Wells were washed three times with washing buffer (PBS and 0.1%Tween-20). Anti-phospho-c-Jun antibody was diluted in antibody binding buffer (100mM phosphate buffer, pH 7.5, 0.5M NaCl, 27mM KCl and 10% BSA) and added to the wells and the plate was incubated at room temperature for 1 hr. After washing as above, HRP-conjugated anti-rabbit IgG was diluted 1:1000 in antibody binding buffer and added to the wells and incubated for an additional 1 hr at room temperature without agitation. Finally, wells were washed four times with wash buffer and 200µl of developing solution was added to each well and incubated for 10 min at room temperature. Reactions were stopped by adding 200µl of kit supplied stop solution and the intensity of the yellow color developed was read on an ELISA reader (Bio-Tek) at 450 nm with a reference

wavelength of 655 nm. Wild type and mutated c-Jun oligonucleotides supplied in the kit were used as specificity controls.

Statistical analysis. All measurements were made in triplicate and experiments were repeated twice to ensure for reproducibility. The results are expressed as the mean ± S.E. of the values calculated from specified number of determinations. The level of significance between different groups is based on Dunnett's *t* test followed by analysis of variance.

RESULTS

Effect of PBN on the bioactivity of IL-1β *in vitro*. Using a bioassay we determined the effect of PBN on the ability of IL-1β to stimulate thymocytes proliferation *in vitro*. In this assay cellular proliferation is dependent on IL-2 released by IL-1 stimulated T cells and is measured by the cellular uptake of MTT. When thymocytes were stimulated with IL-1β in the presence of different concentrations of PBN, no significant difference ($p > 0.001$) was found in their proliferation compared to thymocytes stimulated with IL-1β alone (Figure-1). PBN on its own did not induce the proliferation of thymocytes and the values were very similar to the values obtained with medium alone. These results indicate that the bioactivity of IL-1β was not inhibited in the presence of PBN, at least *in vitro*.

PBN inhibited the IL-1β-induced expression of MMP-13 in OA chondrocytes. Because of the ability of MMPs to degrade most components of the extracellular matrix, inhibition of cytokine-induced expression and production of MMPs has been a prime target for the development of new therapies for the treatment of arthritis. In the present studies, Western blotting showed that IL-1β stimulated the production and release of MMP-13 by OA chondrocytes in culture supernatants (Figure-2). Importantly, as shown in Figure-2, IL-1β-induced release of MMP-13 was dose-dependently inhibited by PBN and the difference was statistically significant ($p<0.001$). In contrast, treatment with freshly prepared N-Acetylcysteine (NAC), a known antioxidant, showed no inhibition of MMP-13 expression in OA chondrocytes culture supernatants. This suggests that the PBN-mediated inhibition of MMP-13 expression was merely not an antioxidant effect and likely involves interference with biochemical pathways associated with MMP-13 expression.

PBN inhibited the IL-1β-induced activity of MMP-13 in OA chondrocytes. Using a highly specific ELISA assay, we studied the effect of PBN on the activity of MMP-13 present in culture supernatant of OA chondrocytes stimulated with IL-1β. Results showed that in OA chondrocytes cultures stimulated with IL-1β, the activity of MMP-13 was enhanced approximately 6.4-folds ($p<0.001$) over the basal level detected in untreated chondrocytes cultures (Figure-3). However, co-treatment of OA chondrocytes with PBN (5 and 10 mM) inhibited the IL-1β-induced up-regulation of MMP-13 activity by 43 and 70%, respectively, when compared to the activity levels detected in IL-1β treated samples and this difference was statistically significant ($p<0.005$). Interestingly, NAC (10mM) did not inhibit the IL-1β-induced MMP-13 activity in OA chondrocytes; rather enhanced MMP-13 activity levels were seen in samples cotreated with NAC (Figure-3).

PBN inhibited the IL-1β-induced increase in the expression of MMP-13 mRNA in OA chondrocytes. To determine the effect of PBN on IL-1β-induced MMP-13 mRNA levels, RNA from OA chondrocytes treated with IL-1β alone or with PBN (5 and 10 mM) for 24 hours was used and the MMP-13 mRNA expression level was determined by real-time quantitative PCR. We found that IL-1β stimulated the expression of MMP-13 by several folds in OA chondrocytes (Figure-4). This increase in IL-1β-induced MMP-13 mRNA levels was dose-dependently inhibited by PBN in OA chondrocytes (Figure-4). These results indicate that treatment with PBN inhibit the IL-1β–stimulated increase in the expression level of MMP-13 mRNA in OA chondrocytes.

PBN inhibited the IL-1β-induced activation of MAPKs in OA chondrocytes. Studies have shown an essential role of MAPK cascade in transducing signals from cell surface to nucleus in response to a variety of stimuli (for recent reviews see Chang and Karin, 2001; Choy and Panayi,

2001). In this study, we analyzed the effect of PBN on IL-1β-induced phosphorylation of ERK p44/p42 (Figure-5A), p38-MAPK (Figure-5C) and JNKp54/p46 (Figure-6B) in OA chondrocytes. We found that OA chondrocytes stimulated with IL-1β showed rapid phosphorylation of ERK p44/p42 reaching the peak at 15 mins and started to decline by 30 mins post treatment (Figure-5A). The maximum inhibitory effect of co-treatment with PBN was seen at 15 min post treatment when phosphorylation of ERK was 64% less compared to the chondrocytes treated with IL-1β alone. However, the intensity of phosphorylation was higher at 30 min post treatment (Figure-5A) indicating that the inhibition did not last very long. PBN or IL-1β did not modulate the non-phosphorylated levels of ERK p44/p42 in any of the groups (Figure-5B).

OA chondrocytes treated with IL-1β showed a rapid activation of p38-MAPK reaching the peak within 15 min of stimulation (Figure-5C). These results also showed that the IL-1β-induced phosphorylation of p38-MAPK was approximately 44% less at 15 min post treatment in cultures co-treated with PBN (Figure-5C). No inhibitory effect of PBN on IL-1β-induced phosphorylation of the upstream kinases MKK3/6 was seen (Figure-5E) indicating that PBN acted downstream of MKK3/6 to inhibit the phosphorylation of p38-MAPK. No difference in the non-phosphorylated levels of p38-MAPK in any of the groups was seen (Figure-5D).

Stimulation of OA chondrocytes with IL-1β induced the phosphorylation of JNKp54/p46 with maximum phosphorylation at 30 min post treatment (Figure-6B) confirming that, as previously reported, IL-1β activates JNKp54/p46 later than p38-MAPK in OA chondrocytes (Geng et al., 1996). However, co-treatment of OA chondrocytes with PBN and IL-1β showed almost 50% and 37% inhibition of phosphorylation of the JNK isoforms-JNK p-46 and JNK p-54-at 15 min post treatment (judged by the intensity of the signal on the blot) compared to the

phosphorylation levels of JNK p-46 and JNK p-54 in OA chondrocytes treated with IL-1β alone (Figure-6B). At 30 min post treatment both the JNK isoforms appeared to be recovering from the inhibition as the phosphorylation intensity of the bands was higher compared to the intensity at 15 min post treatment (Figure-6B). No inhibitory effect of PBN on the IL-1β-induced phosphorylation of MKK4/7 (Figure-6A) was seen at any of the time intervals tested suggesting that the IL-1 receptor to MKK-4/7 pathway was not inhibited by PBN. This further suggests that PBN interfered with some step(s) in the MKK-4/7 to JNK activation pathway, exact nature of which remains to be investigated. No change in the non-phosphorylated levels of JNKp54/p46 was seen in any of the groups. These blots were also probed with anti-β-Actin antibody (Santa Cruz) to ensure equal loading of proteins in each lane (Figure-6D).

PBN inhibited the IL-1β**-induced accumulation of phospho-c-Jun in OA chondrocytes.** Since JNK preferentially phosphorylate c-Jun, we also determined whether inhibition of JNK activation had any effect on the IL-1β-induced accumulation of phosphorylated c-Jun in OA chondrocytes. Results shown in Figure-7 demonstrated that in IL-1β-stimulated OA chondrocytes, maximum accumulation of phosphorylated c-Jun was observed at 30 min post-treatment (Figure-7A). However, in OA chondrocytes stimulated with IL-1β in the presence of PBN, levels of phosphorylated c-Jun were 43% and 29% lower at 15 min and 30 min post treatment respectively compared to the levels detected in OA chondrocytes stimulated with IL-1β alone (Figure-7A). This suggested that the phosphorylation of c-Jun was inhibited but not abolished and an alternate pathway or the residual activity of JNK may be phosphorylating c-Jun in OA chondrocytes at the time points analyzed. No modulation of the non-phosphorylated levels of total c-Jun was seen at any of the time points analyzed (Figure-7B).

PBN blocked the c-Jun phosphorylating activity of JNK *in vitro*. We used the *in vitro* kinase assay to determine whether the observed low levels of phosphorylated c-Jun (Figure-7A) were due to the inhibition of c-Jun phosphorylating activity of JNK by PBN. OA chondrocytes were stimulated with IL-1β, lysed and the phosphorylated JNK was immunoprecipitated. c-Jun phosphorylating activity of the immunoprecipitated JNK was measured with a GST-c-Jun substrate in the presence and absence of PBN. Results shown (Figure-8A) demonstrate that the c-Jun phosphorylating activity of JNK was completely blocked by the exogenously added PBN, at least *in vitro*.

Phosphorylation of c-Jun by p38-MAPK. Both p38-MAPK and JNK are activated by the same stimuli and it is reported that p38-MAPK can phosphorylate c-Jun (reviewed in Bode and Dong, 2000). Therefore, using the *in vitro* kinase assay we tested (1) whether activated p38-MAPK in OA chondrocytes can phosphorylate c-Jun; and (2) whether PBN had any effect on the c-Jun phosphorylating activity of p38-MAPK. In these experiments, OA chondrocytes were stimulated with IL-1β, lysed and the p38-MAPK was immunoprecipitated with antibodies specific for phospho-p38-MAPK. Our results showed that indeed c-Jun was phosphorylated by activated p38-MAPK and that PBN, at the concentration tested, inhibited but did not abolished the c-Jun phosphorylating activity of p38-MAPK *in vitro* (Figure-8B). However, the low level of GST-c-Jun phosphorylation observed (compare lanes 1 in Figure-8A and Figure-8B) may be due to inherent inefficiency of p38-MAPK in phosphorylating c-Jun. Importantly, PBN showed no inhibitory effect on the ATF-2 phosphorylating activity of the immunoprecipitated p38-MAPK or Elk-1 phosphorylating activity of activated ERK p44/p42, at least *in vitro* (Figure-8C & 8D). Taken together, these results indicate that PBN preferentially interferes with the phosphorylation of c-Jun.

PBN inhibited the IL-1β-induced DNA binding activity of AP-1 in human chondrocytes. The JNKs are the dominant Ser/Thr kinases responsible for the activation of AP-1 in response to inflammatory stimuli. As shown in Figure-7, treatment with PBN significantly inhibited the IL-1β-induced accumulation of phospho-c-Jun in OA chondrocytes. As phosphorylation of c-Jun play a regulatory role in the transcriptional activation of AP-1, we determined whether inhibition of IL-1β–induced phosphorylation of c-Jun by PBN had any impact on the AP-1/c-Jun DNA binding activity in the nuclei of OA chondrocytes. OA chondrocytes were stimulated with or without IL-1β and PBN (10mM) for different time periods and analyzed for the AP-1/c-Jun DNA binding activity using a highly sensitive and specific ELISA method as described above. The results (Figure-9) demonstrated that IL-1β-induced increase in the DNA binding activity of AP-1 reached its maximum at 30 min post treatment after which it declined. However, in OA chondrocytes co-treated with PBN, the IL-1β-induced DNA binding activity of AP-1 was significantly ($p<0.001$) decreased at both the time points analyzed when compared to the DNA binding activity levels detected in OA chondrocytes treated with IL-1β alone.

DISCUSSION

Spin trap agent PBN has been shown to possess a variety of anti-inflammatory and antioxidant properties (reviewed in Kotake, 1999; Floyd, 1999). Earlier studies demonstrated that PBN inhibits ROS-mediated oxidation of critical brain proteins and lipid to improve cognitive performance (Floyd and Hensley, 2000). There is excessive production of ROS and NOS in arthritic joints and this may lead to protein oxidation and lipid peroxidation resulting in the accumulation of modified carbonyl derivative that triggers cell death and sustain the inflammatory response (Tiku et al., 2000; Grazioli et al., 1998). In addition, due to the loss of tissue cellularity with aging, chondrocytes may lose the capability to repair cartilage damage consequential to oxidativestress and other degradative stimuli (Corvol, 2000). A number of *in vitro* and *in vivo* studies have shown that PBN inhibits the cytokine-induced production of nitric oxide (reviewed in Kotake, 1999). Thus, it is tempting to suggest that PBN may be useful in inhibiting the pro-inflammatory cytokine-induced oxidativestress in an arthritic joint thereby protecting cartilage against peroxidation-mediated damage. This is supported by studies showing that vitamin E supplementation reverses the toxic manifestation of oxidativestress and protects cartilage against cellular peroxidation (Watkins et al., 1996).

Matrix metalloproteinases (MMPs) is a family of enzymes that are normally required for the degradation of ECM components during tissue remodeling (reviewed in Nagase and Woessner, 1999). An increase in the expression of MMPs in arthritic joints represents the end result of activation of the MAPK-mediated signal transduction pathways by inflammatory cytokines such as IL-1 and TNF-α (Liacini, et al., 2002; Kyriakis and Avruch, 1996). It is well known that MAPKs are activated through a "phosphorelay" arrangement in which the binding of the inflammatory cytokine to its receptor activate a MAPK kinase kinase (MKKK), which in turn

phosphorylates a MAPK kinase (MKK). Activated MKKs recognize and phosphorylate specific MAPKs, which then phosphorylate the transcription factors and other protein kinases (Johnson, 2002; Chang and Karin, 2001; Kyriakis and Avruch, 1996). Recent studies have demonstrated that transcriptional and post-translational expression of MMP-13, which is found elevated in arthritic joints (Mitchell et al., 1996) and also has the ability to hydrolyze type II collagen more efficiently than the other collagenases (Nagase and Woessner, 1999), is regulated by the MAPK sub-group JNK and requires the activation of the transcription factor AP-1 for its expression (Vincenti and Brinckerhoff, 2002). Furthermore, it has been shown that the inhibition of JNK by the novel inhibitor SP600125 also inhibited the bone resorption in adjuvant-induced arthritis model (Han et al., 2001) further supporting the role of JNK cascade in cartilage/bone damage in an arthritic joint. Thus, agents that block/modulate signaling pathways that regulate MMP-13 expression may have therapeutic potential in joint disorders. Our results showed that PBN was an effective inhibitor of MMP-13 induction and expression in OA chondrocytes stimulated with IL-1β. Suppression of MMP-13 expression correlated with the inhibition of MAPKs subgroups JNKp54/p46 and p38-MAPK that are implicated in the induction and expression of MMP-13 in chondrocytes (Liacini et al, 2000; Mengshol et al 2002).

Results of *in vitro* kinase assays showed that PBN was not effective in inhibiting the phosphorylation of ATF-2 by activated p38-MAPK (Figure-8) although it inhibited the phosphorylation of p38-MAPK (Figure-5). These results are interesting as they point out that PBN inhibit the activation of p38-MAPK but does not interfere with the downstream phosphorylation of target molecules, except the c-Jun, by the activated p38-MAPK. Similarly, PBN inhibited the IL-1β-induced phosphorylation of ERKp44/p42 in OA chondrocytes (Figure-5A) but did not inhibit the Elk-1 phosphorylating activity of the activated ERKp44/p42 *in vitro*

(Figure-8) indicates that PBN is not an inhibitor of the activity of ERKp44/p42. In other studies, PBN has been shown to stimulate the phosphorylation of ERKp44/p42 in PC 12 cells (Tsuji et al 2001), and IL-1 is known to stimulate the phosphorylation of ERKp44/p42 in OA chondrocytes (Geng et al. 1996), we did not find a synergistic stimulatory effect of IL-1 and PBN on ERKp44/p42 in OA chondrocytes (Figure-5A). The stimulation of phosphorylation of ERKp44 /p42 by PBN alone may be an inherent general property of the antioxidants related to their ability to enhance the survival of stressed cells while the inhibition of IL-1β-induced phosphorylation may be a specific effect on chondrocytes related to the anti-inflammatory property of PBN. However, this need to be investigated further.

The JNKs are the dominant Ser/Thr kinases responsible for the activation of AP-1 in response to inflammatory stimuli (Karin, 1995). AP-1 components are organized into Jun-Jun, Jun-Fos or Jun-ATF dimers and the presence of Jun family members enables AP-1 to bind *cis* acting elements (Angel and Karin, 1991). Only Jun protein can form transcriptionally active homodimers with other AP-1 members and bind to DNA on a TPA-response element (TRE) with the 5'-TGA(C/G)TCA-3' sequence (Trenies et al., 1999). Phosphorylation of c-Jun in its activation domain at serine 63 and 73 prolongs its half-life and potentiates the ability of c-Jun to activate transcription as either a homodimer or as a heterodimer with c-Fos (Karin, 1995). It is known that the transcriptional activity of AP-1 depends not only on the abundance of AP-1 component and their ability to bind DNA but also on the degree of phosphorylation of these components (Chang and Karin, 2001). The inhibition of accumulation of phospho-c-Jun in chondrocytes cotreated with PBN and the results of *in* vitro kinase assays indicates that PBN inhibits the c-Jun phosphorylating activity of JNK. This would result in the decreased availability of activated c-Jun and concomitant inhibition of the DNA binding activity of AP-1/c-Jun

complexes in OA chondrocytes. Inhibition of AP-1 activity may seriously hamper the transcription of genes, such as the MMP-13 gene, that require binding of AP-1 in their promoters to initiate transcription.

The accumulation of phosphorylated c-Jun and the DNA binding activity of AP-1 in OA chondrocytes stimulated with IL-1β and PBN was inhibited, but not abolished, indicating that an alternate pathway was phosphorylating c-Jun and activating AP-1. Using *in vitro* kinase assays we found that PBN completely blocked the c-Jun phosphorylating activity of JNK, but not of p38-MAPK, although it was reduced. These results suggest that p38-MAPK could be the source of low levels of phosphorylated c-Jun found in OA chondrocytes stimulated with PBN and IL-1β (Figure–7). However, phosphorylation of c-Jun by activated p38-MAPK may not be efficient and thus would fail to provide the threshold level of phosphorylated c-Jun required for optimal activity of AP-1. Our results are supported by a previous study that showed decreased AP-1 DNA binding activity correlated with the inhibition of IL-1β-induced phospho-c-Jun accumulation and c-Jun phosphorylating activity in synoviocytes (Han et al., 2001). Non-phosphorylated levels of c-Jun were not inhibited either by IL-1β or by PBN indicating that these agents do not affect the expression of c-Jun in OA chondrocytes (Figure-7B). To our knowledge, phosphorylation of c-Jun by activated p38-MAPK from OA chondrocyte has not previously been shown. This finding could be important in interpreting the results of JNK inhibitors.

Thus, based on our results it is tempting to suggest that PBN inhibited the IL-1β-induced expression of MMP-13 by inhibiting the DNA binding activity of AP-1/c-Jun complex primarily by decreasing the availability of activated c-Jun to form transcriptionally active complexes in OA chondrocytes. These results provide a basis for future studies on the potential use of PBN or compounds derived from it in inhibiting the cartilage degradation in arthritis.

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Footnotes.

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Legends For Figures.

Figure-1. PBN did not inhibit the bioactivity of IL-1β *in vitro*. IL-1β-stimulated proliferation of mouse thymocytes in the presence or absence of PBN (1, 5, 10 mM) was measured by the MTT assay. Results shown are representative of two experiments and the values are Mean ±S.D.

Figure-2. PBN inhibited the IL-1β-induced expression of MMP-13 in OA chondrocytes. OA chondrocytes were stimulated with IL-1β (5ng/ml) alone or in combination with PBN (5 and 10 mM) and NAC (10mM) for 24 hours. Following stimulation, 1 mL of culture medium was precipitated with 10% TCA at 4°C for 1 hr. Protein pellets were resuspended in 5X sample buffer and Western immunoblotting was performed with an MMP-13 specific antibody. In the remaining culture medium, MMP-13 activity was measured.

Figure-3. PBN inhibited the IL-1β-induced activity of MMP-13 in OA chondrocytes. MMP-13 activity was measured using the Biotrak MMP-13 Activity ELISA kit. OA chondrocytes stimulated in the presence of PBN had decreased levels MMP-13 activity (**$p<0.001$; *$p<0.05$) when compared to values obtained in IL-1β treated cultures. Activity inhibition by this method is probably reflective of the inhibition of MMP-13 production in these cultures. Results shown are representative of three independent experiments performed with age and sex matched samples.

Figure-4. PBN i**nhibited the IL-1β-induced expression of MMP-13 mRNA in OA chondrocytes.** OA chondrocytes were stimulated with IL-1β (5ng/ml) alone or in combination with PBN (5 and 10 mM) for 24 hours. Total RNA was prepared and the expression of MMP-13 mRNA was determined by real-time quantitative RT-PCR. Values were expressed relative to the level of β−Actin mRNA. Results shown are representative of 3 independent experiments.

Figure-5. PBN inhibited the IL-1β-induced phosphorylation of ERK p44/p42 and p38-MAPK in OA chondrocytes. OA chondrocytes were stimulated with IL-1β (5ng/ml) and PBN

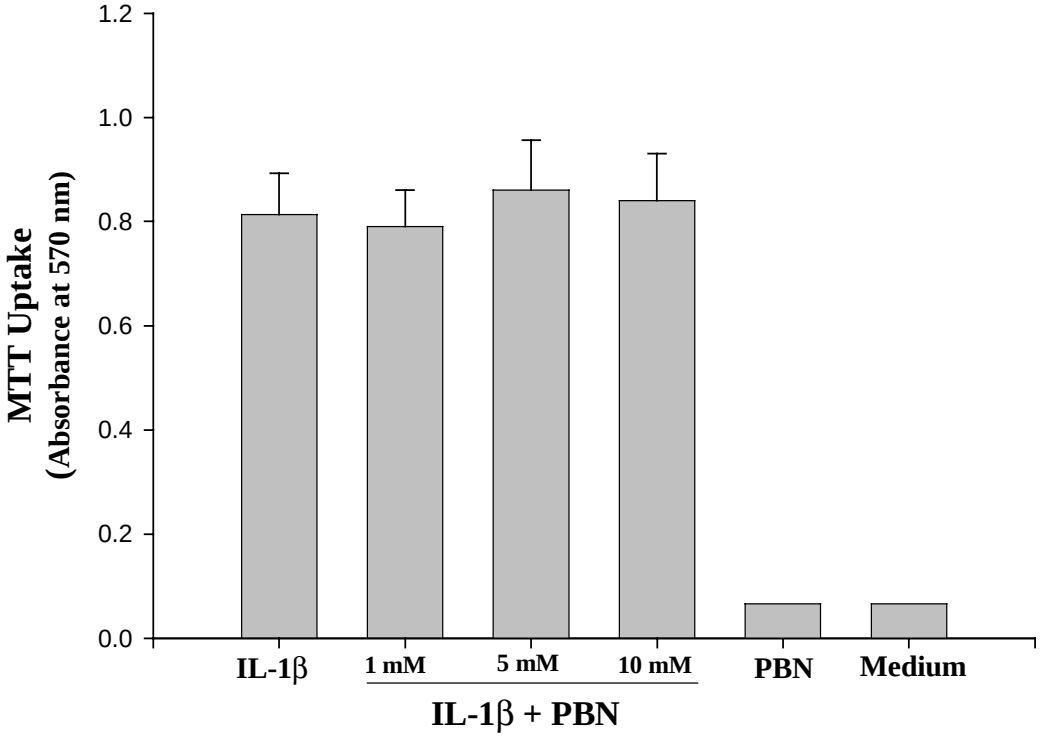
(10mM) for various time points and phosphorylation was determined by Western immunoblotting. Western blots were probed with **(A)** antibodies specific for phosphorylated-ERKp44/p42; **(C)** antibodies specific for phosphorylated p38-MAPK, or **(E)** antibodies specific for phosphorylated MKK-3/6. Blots were scanned and the intensity of the bands was analyzed using the Imagequant Software and expressed in arbitrary O. D. Units. **(B and D)** Blots shown in A & C were stripped and probed with antibodies reactive with non-phosphorylated ERKp44/ p422, or p38-MAPK. Results shown are representative of three independent experiments.

Figure-6. PBN suppressed the IL-1β-induced phosphorylation of JNKp54/p46 in OA chondrocytes. OA chondrocytes were stimulated with IL-1β (5ng/ml) alone or in combination with PBN (10mM) for various time points and phosphorylation of MKK4/7 and JNKp54/p46 was determined by Western immunoblotting using antibodies specific for (A) phosphorylated MKK4/7 and (B) phosphorylated JNKp54/p46. No modulation of non-phosphorylated levels of JNKp54/p46 or β-Actin was detected (C and D respectively). Blots were scanned and the intensity of the bands was analyzed using the Imagequant Software and expressed in arbitrary O. D. Units. Blots shown are representative of three independent experiments.

Figure-7. PBN inhibited the IL-1β-induced accumulation of phosphorylated c-Jun in OA chondrocytes. OA chondrocytes were stimulated with IL-1β (5ng/ml) alone or with IL-1β + PBN for 5, 15, and 30 min *in vitro* and the levels of phosphorylated c-Jun were determined by Western immunoblotting. **(A)**. In OA chondrocytes co-treated with IL-1β and PBN, levels of phosphorylated c-Jun were markedly reduced as compared to the levels detected in OA chondrocytes stimulated with IL-1β alone. **(B)**. Same blot probed with antibodies reactive with non-phosphorylated c-Jun. Results indicated that levels of c-Jun were not affected by treatment with IL-1β and PBN. Results shown are representative of two independent experiments.

Figure-8. Effect of PBN on the substrate phosphorylating activity of activated JNK, p38-MAPK, and ERK p44/p42 *in vitro*. OA chondrocytes were stimulated with IL-1β (5ng/ml) for 30min and activated JNK, ERKp44/p42 and p38-MAPK were immunoprecipitated. The ability of activated JNK, ERKp44/p42 and p38-MAPK to phosphorylate GST-c-jun, Elk-1 and ATF-2 *in vitro*, respectively, in the presence of PBN was determined by the *in vitro* kinase assay. **(A)** PBN completely blocked the c-Jun phosphorylating activity of JNK; **(B)** PBN only partially inhibited the c-Jun phosphorylating activity of p38-MAPK; **(C)** PBN had no effect on the ATF-2 phosphorylating activity of p38-MAPK; and **(D)** PBN had no effect on the Elk-1 phosphorylating activity of ERK p44/p42. Results shown are representative of two independent experiments. (Lane 1. No PBN in the assay. Lane 2. PBN (10 mM) in the assay).

Figure-9. PBN inhibited the IL-1β–induced DNA binding activity of AP-1/c-Jun complex in OA chondrocytes. OA chondrocytes were stimulated with IL-1β (5 ng/ml) and PBN (10 mM) for varying time points and AP-1/c-Jun DNA binding activity was determined by a highly specific ELISA method. Results shown are cumulative of two independent experiments and the values shown are Mean ± S.D. (*$p<0.005$ compared to treatment with IL-1β alone). Positive control used was kit supplied nuclear lysate of activated WI 38 cells.



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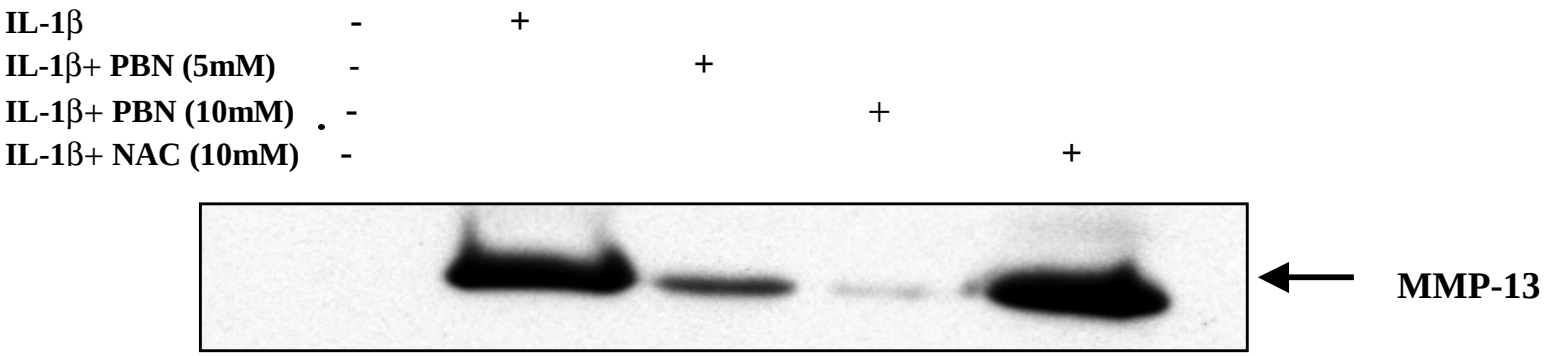

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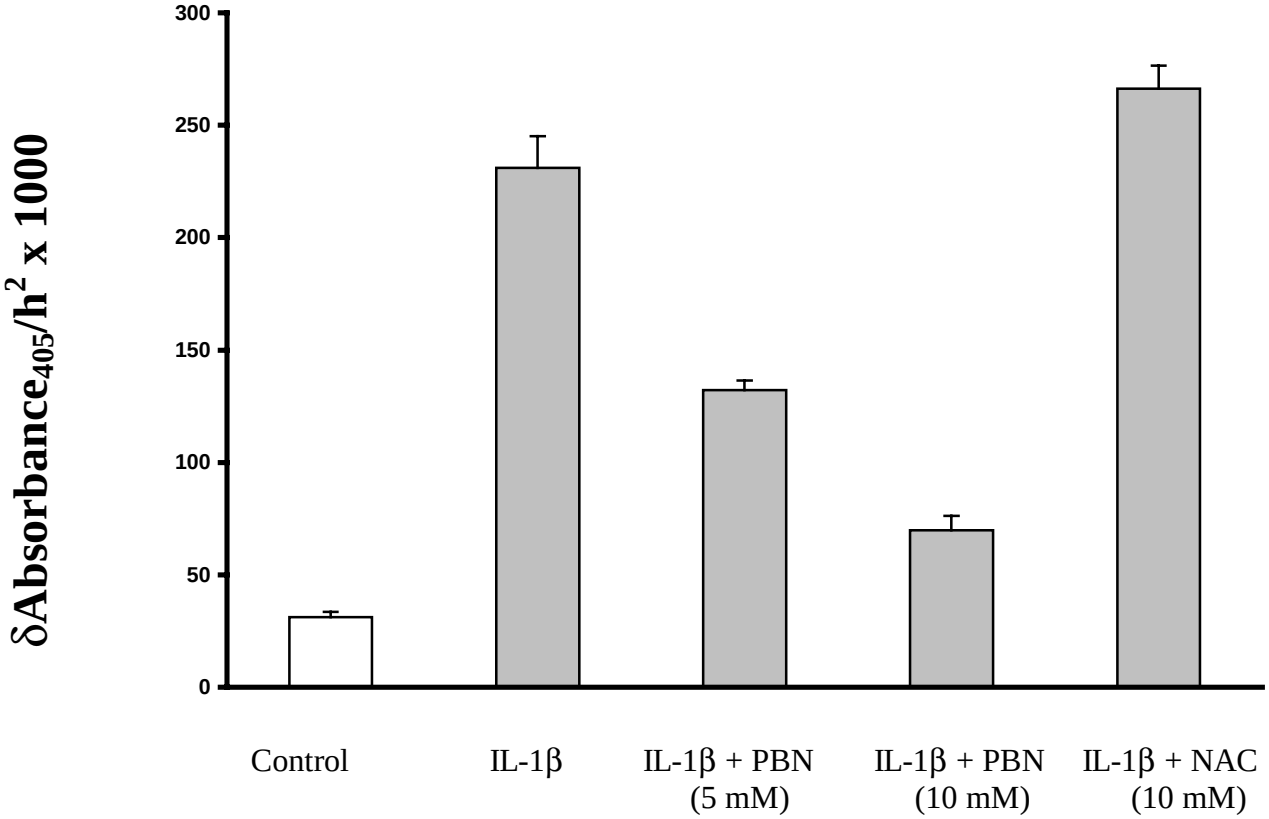

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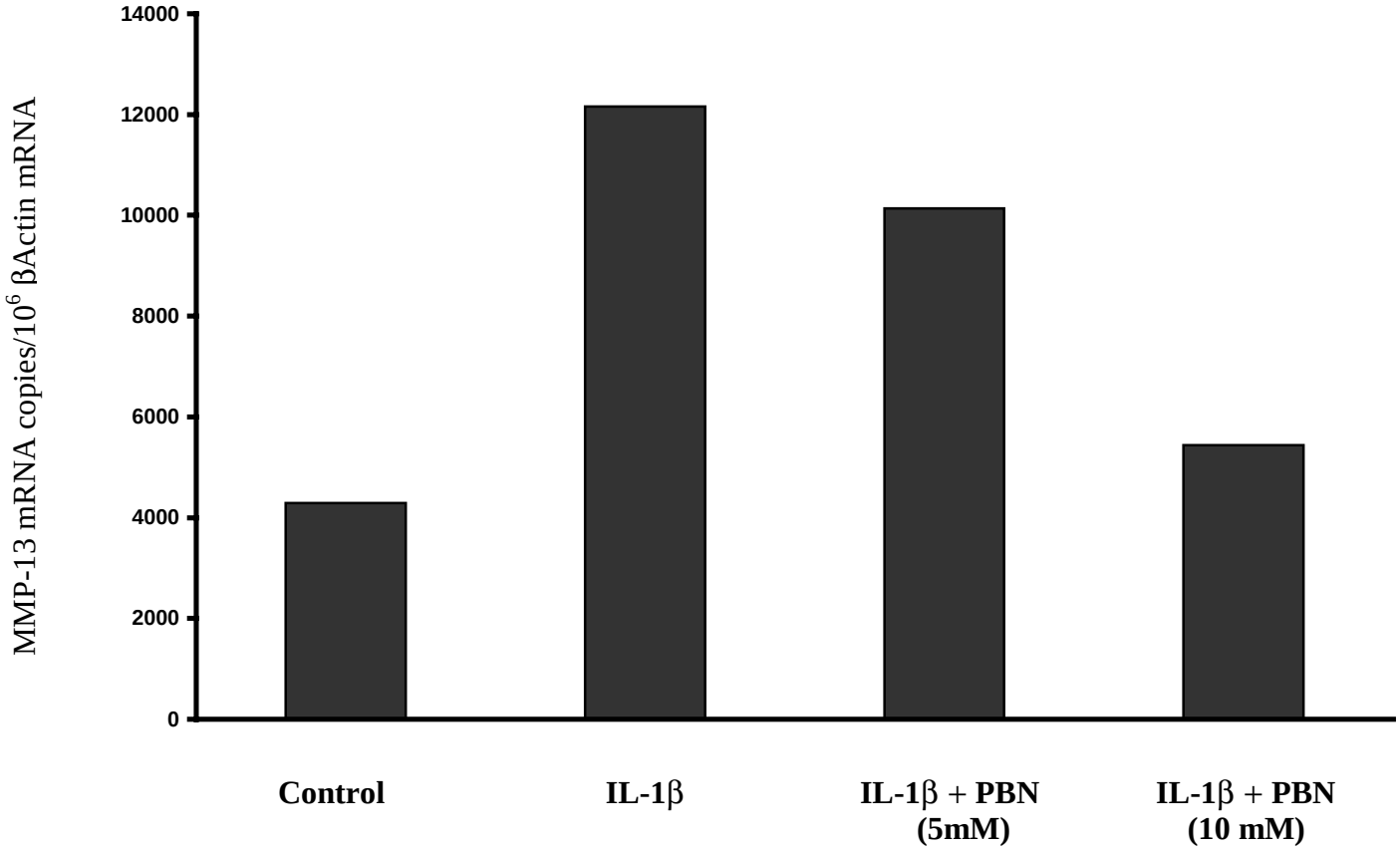

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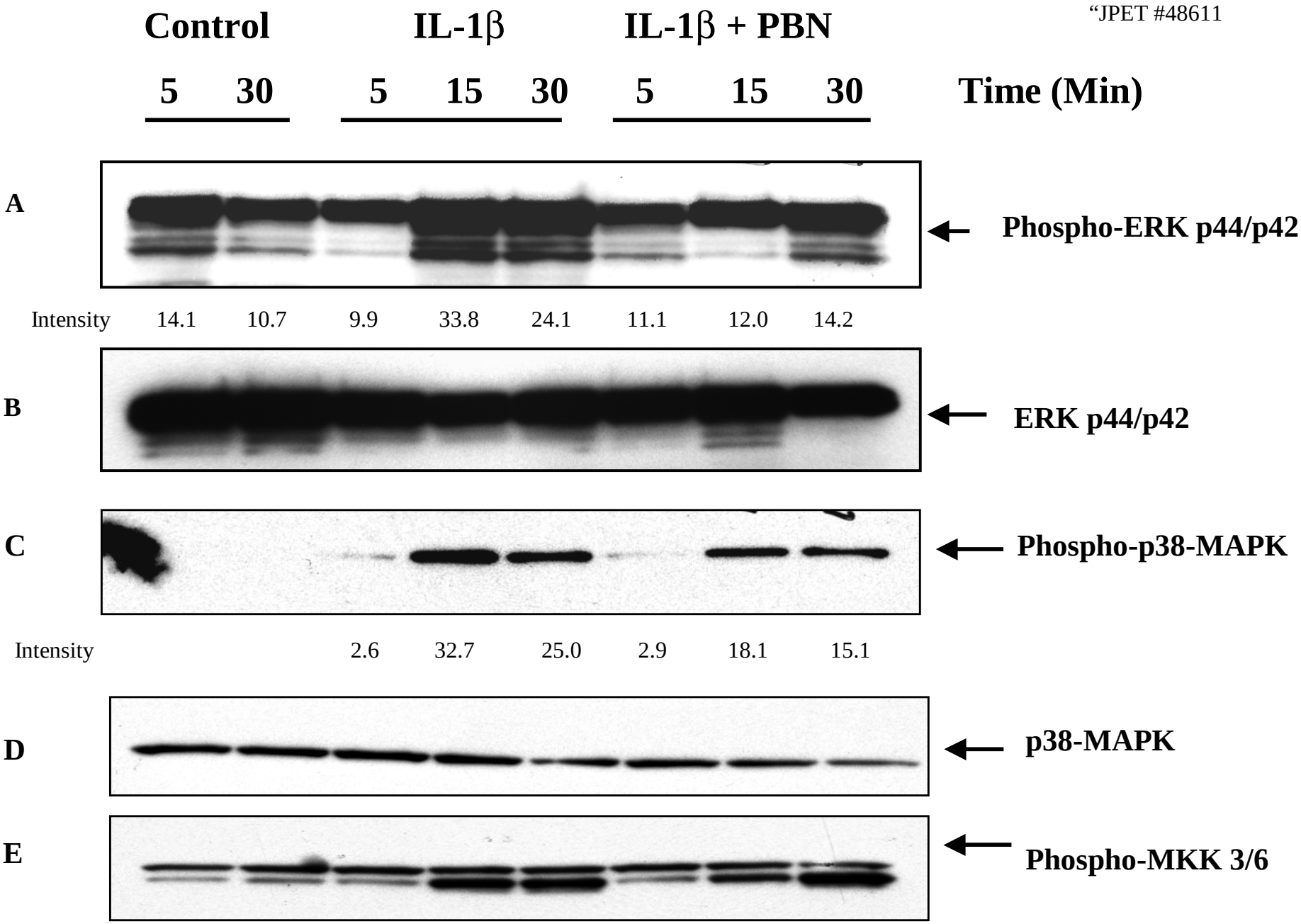

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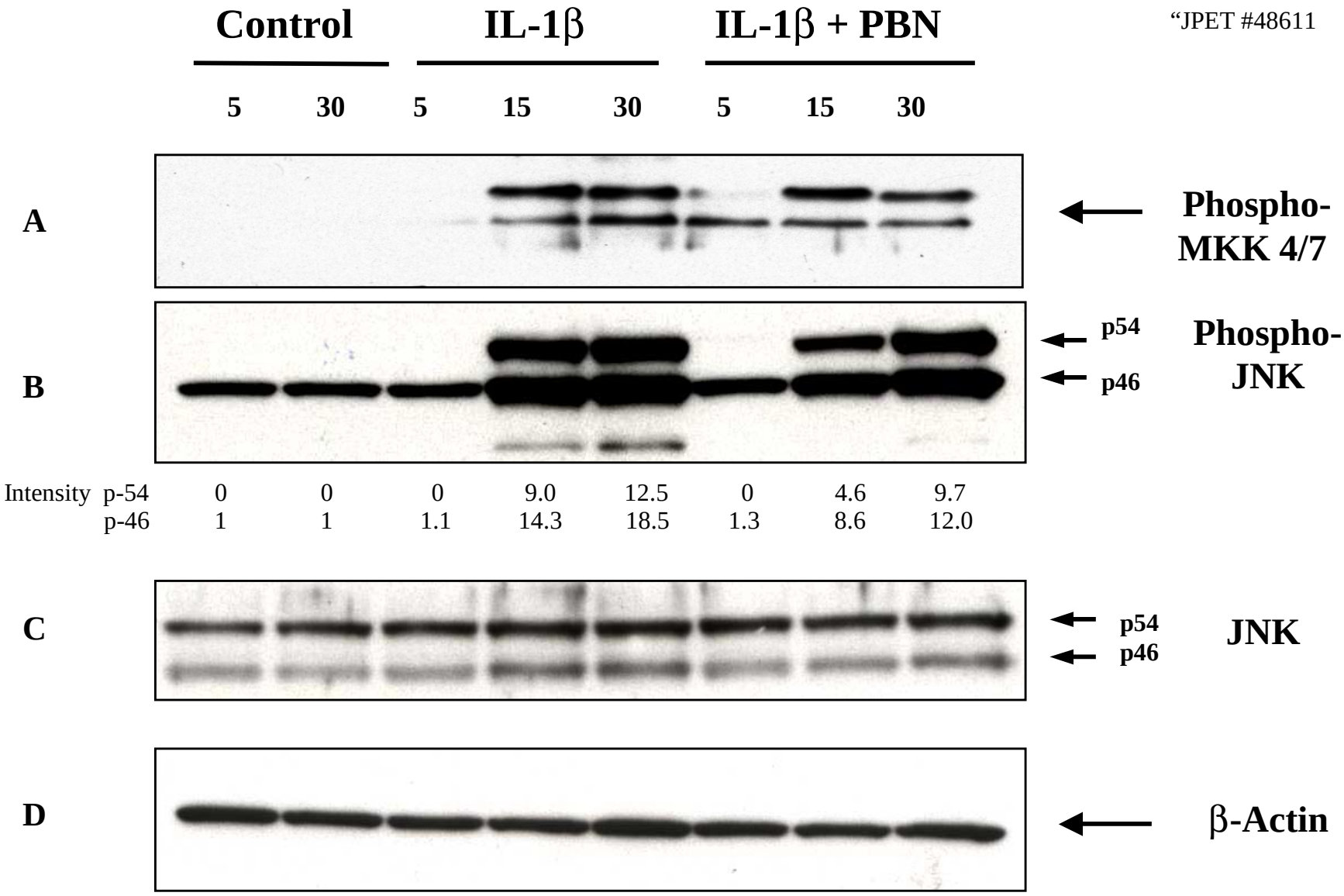

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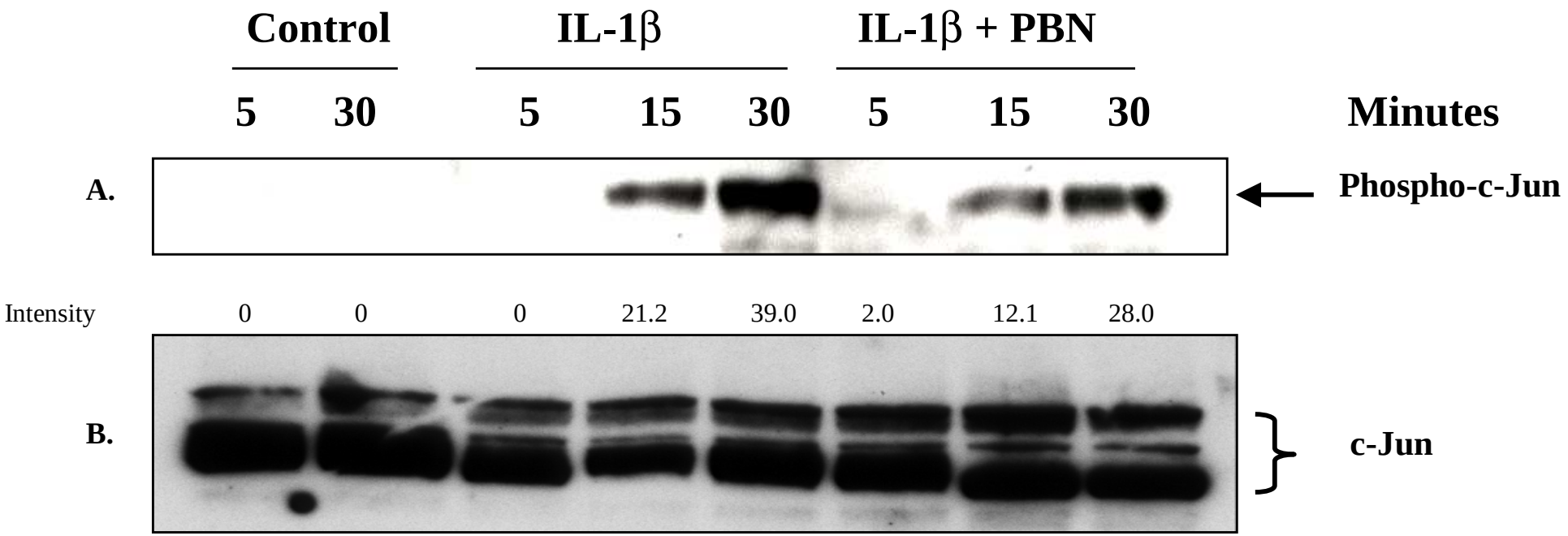

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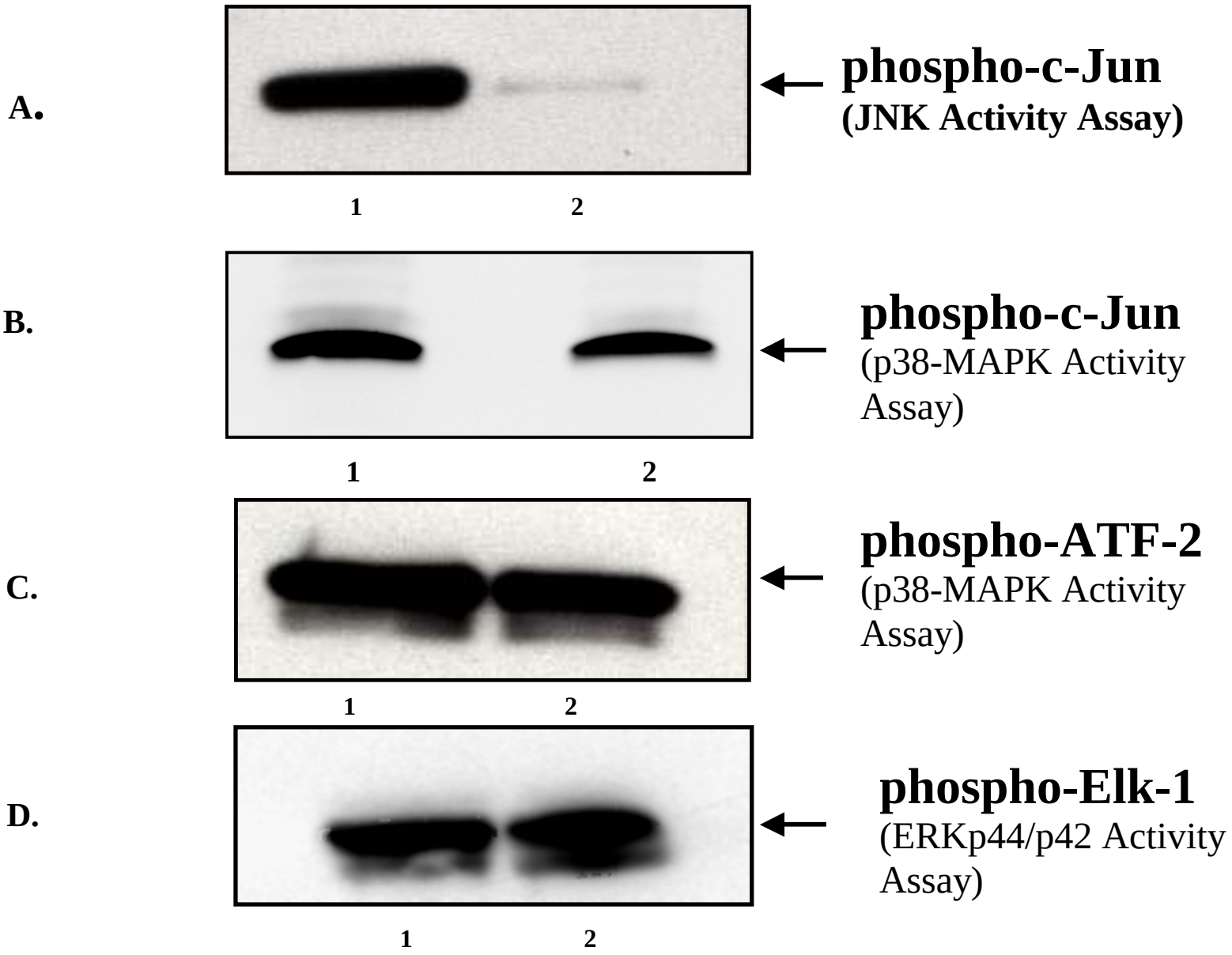

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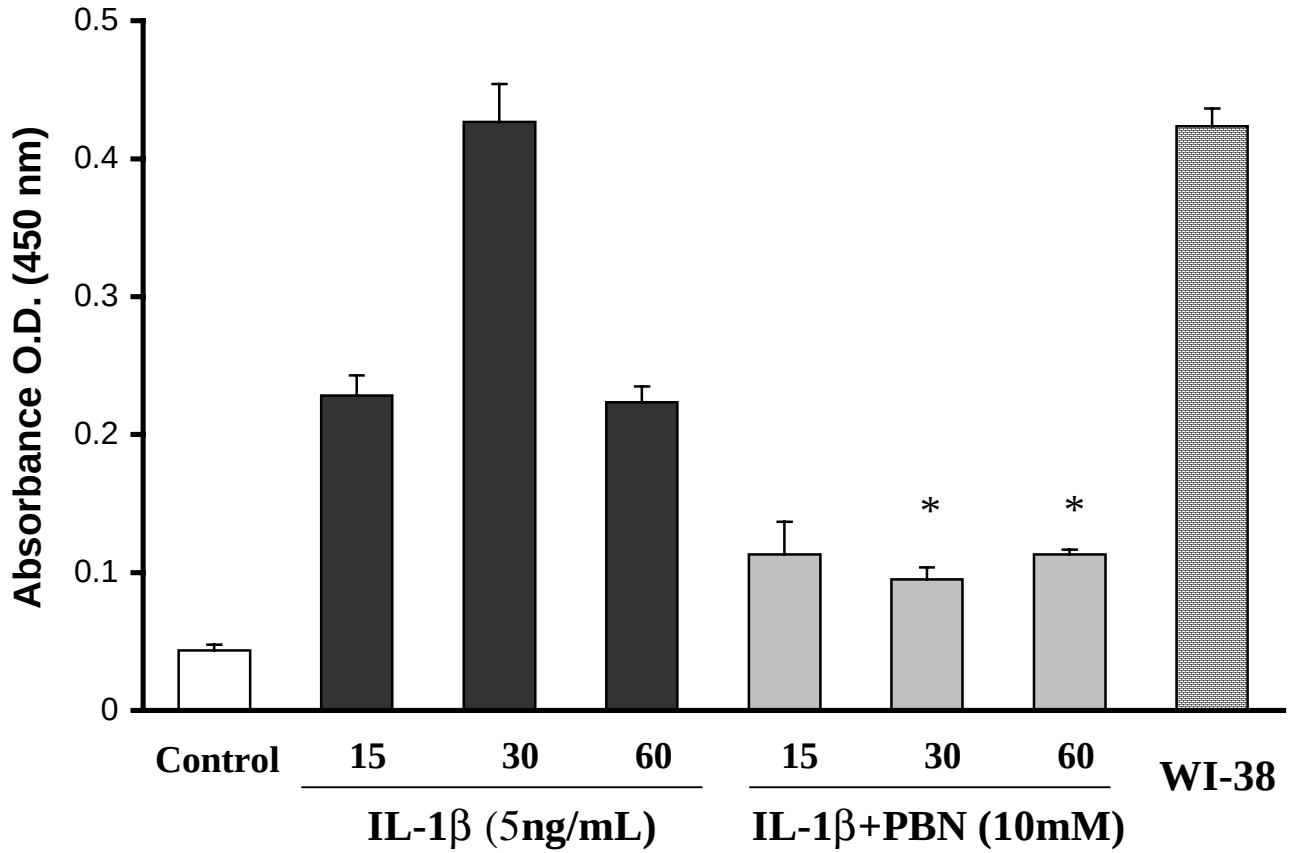

Figure-9.