TNF alpha production in human monocytes is NF-κB and p38 dependent

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Abstract

In chronic diseases such as rheumatoid arthritis, intracellular signalling cascades involving p38 and IκB-kinases are considered to play important roles in regulating inflammatory cell activation and responses. We have investigated involvement of these pathways in LPS-induced production of TNFα in human THP-1 cells using specific p38 and IKK2 kinase inhibitors. Assessment of p38 activation was based on phosphorylation of hsp27, a downstream substrate of MAPKAP-K2, while activation of NF-κB (p65) was used to monitor IKK2 activation. Both p38α and IKK2 inhibitors blocked LPS-induced TNFα production by the monocytic cells. However, while LPS-induced hsp27 phosphorylation was reduced by p38 inhibition little effect was seen with the IKK2 inhibitor. Conversely, LPS-induced NF-κB activation was reduced by IKK2 inhibition but not by p38 inhibition. Sorbitol, an activator of p38, but not NF-κB failed to induce TNF production. These observations indicate that both p38 and NF-κB pathways are required to regulate TNF production in LPS-stimulated THP-1 cells.

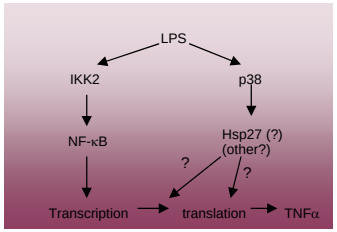

Figure 1. Schematic diagram of p38 and NF-κB activation

Methods

Cells and inhibitors

THP-1 cells were routinely grown in RPMI medium containing 10% FCS. Cells were differentiated with 4ng/ml vitamin D3 for 3 days at 37°C then washed before use. Human neonatal foreskin fibroblasts (Biowhittaker) were routinely grown in DMEM containing 10% FBS. The p38 inhibitor (SB239063) and IKK2 inhibitor (compound X), (GlaxoSmithKline), were diluted in dmso and added to cells for 1 hour before stimulation.

TNFα Assay

1x10^5 cells were stimulated with 100ng/ml LPS in a total volume of 250μl. After 4 hours incubation at 37°C, supernatants were harvested and tested for TNFα using commercially available ELISA kits (ImmunoKontact).

Phosphorylation of p38 and hsp27

Differentiated THP-1 cells were diluted in DMEM containing 10% FCS and 2.5x10^5 cells added/well of a 96-well plate. After addition of compound, cells were stimulated with 0.3M sorbitol (Sigma) for 30 minutes or 250ng/ml LPS (Sigma E.coli) for 1 hour at 37°C (total volume/well was 150μl). Cells were then lysed on ice with 50μl/well of 4x RIPA buffer (50mM Tris, 300mM sodium chloride, 1% v/v NP40, 0.5% deoxycholate, 50mM sodium fluoride, 1mM sodium vanadate, 1mM EDTA, protease inhibitors) and frozen at -80°C before assay. Lysates were tested for phospho hsp27 in a washed chemiluminescent assay based on MSD technology (Figure 2). Sample was captured on blocked avidin coated MSD plates using a biotinylated anti total hsp27 (MBL) and an anti phospho hsp27 antibody (Cell signalling). The phospho antibody was detected using a ruthenium labelled anti sheep IgG (IGEN).

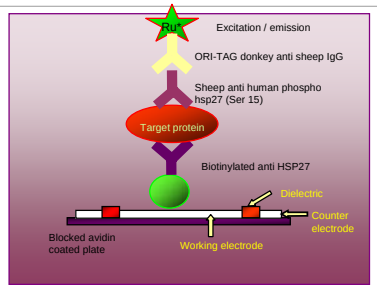

Figure 2. phsp27 assay using Meso-Scale Discovery technology.

NF-κB Assay

Cells were prepared as described above but lysed using nuclear extract solution (Active Motif) and NF-κB measured using commercially available kits (TransAM NF-κB p65, Active Motif).

Results

The p38 and IKK2 inhibitors used in these studies were found to be highly specific for their respective kinases in enzyme assays (Table 1). The p38 inhibitor reduced p38α with an IC50 of 60nM compared to an IC50 of >15.8μM for IKK2. The IKK2 inhibitor had little effect on p38α but was highly potent against IKK2 (IC50 36nM).

Inhibitor	IKK2 IC50 (μM)	P38 IC50 (μM)
SB239063	>15.8	0.060
Inhibitor X	0.036	>15.8

Table 1. Enzyme activity of p38 and IKK2 inhibitors

TNFα production in human blood monocytes is p38 and NF-κB dependent

LPS induced high levels of TNFα but sorbitol failed to induce TNF production (Figure 3A). LPS induced TNFα was inhibited by both p38α and IKK2 inhibitors (p38 IC50 18nM); (IKK2 IC50 215nM) (Figure 3B).

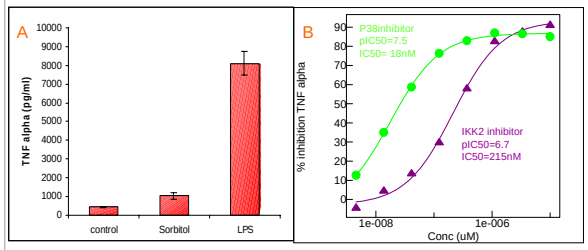

Figure 3. LPS versus sorbitol induced TNFα production (A), effect of p38 and IKK2 inhibitors on LPS stimulated TNFα (B)

HSP27 is a marker of the p38 pathway.

Both LPS and sorbitol induced phosphorylation of hsp27 (Figure 4A). The p38 inhibitor potently blocked LPS and sorbitol induced phosphorylation of hsp27 with IC50 values close to those characteristic for inhibition of TNFα production and enzyme inhibition (mean IC50's for 2 experiments 12±13nM for LPS and 60±33nM for sorbitol. Phsp27 was weakly inhibited by high concentrations of the IKK2 compound (mean IC50's of 1.4±0.3μM (LPS) and 1.1±0.4μM (sorbitol). This effect is unlikely to be due to inhibition of p38 or MK2 as *in-vitro* IC50's against these enzymes are >16uM. It is possible that the compound may weakly inhibit an enzyme involved in p38 activation or that inhibition of IKK may modulate hsp27 phosphorylation by p38.

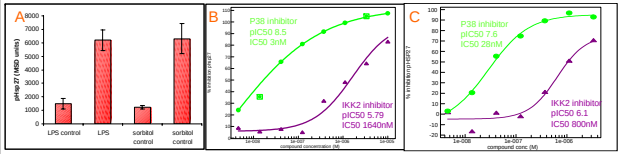

Figure 4. Stimulation of phsp27 by LPS and sorbitol (A), effect of inhibitors on LPS (B) and sorbitol induced phsp27 (C)

NF-κB activation is p38 independent

LPS but not sorbitol induced NF-κb (p65) activation in THP-1's. The IKK2 inhibitor (IC50~1μM) but not the p38 inhibitor (at concentrations up to 10μM) suppressed LPS-induced activation of p65. Results suggest that activation of NF-κB is required for LPS-induced TNFα production and that this event is regulated independently of the p38 pathway.

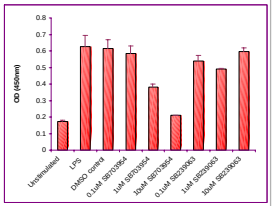

Figure 5. Effect of compounds on LPS induced NF-κB-p65

Conclusions

- LPS induced TNFα production in THP-1 cells was found to be dependent on both p38 and IKK2/NF-κB pathways
- Activation of the p38 pathway by sorbitol (without concurrent induction of NF-κB) was not sufficient to induce TNFα production
- Selective inhibitors of p38 or IKK2 activation were each capable of blocking TNFα production
- These results are consistent with evidence from the literature that IKK2/NF-κB and p38 regulate transcriptional and post-transcriptional mechanisms required for TNFα production by LPS stimulated monocyte/macrophages.

Refs

K-J Park, R Gaynor, YT Kwak, JB; J Biol Chem (June 25, 2003) in press.

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