INFLAMMATORY AND CATABOLIC SIGNALLING IN INTERVERTEBRAL DISCS: THE ROLES OF NF-κB AND MAP KINASES

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Abstract

Painful intervertebral disc disease is characterised not only by an imbalance between anabolic (i.e., matrix synthesis) and catabolic (i.e., matrix degradation) processes, but also by inflammatory mechanisms. The increased expression and synthesis of matrix metalloproteinases and inflammatory factors is mediated by specific signal transduction, in particular the nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinase (MAPK)-mediated pathways. NF-κB and MAPK have been identified as the master regulators of inflammation and catabolism in several musculoskeletal disorders (e.g., osteoarthritis), and recently growing evidence supports the importance of these signalling pathways in painful disc disease. With continuing research exploiting in vitro and in vivo model systems to elucidate the roles of these pathways in disc degeneration, it may be possible in the near future to specifically target these major inflammatory / catabolic signalling pathways to treat painful degenerative disc disease. In this perspective, we aim to summarise the current state of knowledge concerning the inflammatory and catabolic molecular pathways of intervertebral disc disease (IDD), with a detailed description of NF-κB and MAP kinase-mediated signal transduction in disc cells. Furthermore, we will discuss the emerging novel molecular treatment modalities for IDD using pharmacological inhibitors targeting these pathways.

Keywords: nuclear factor kappa B, NF-κB, MAP kinases, MAPK, intervertebral disc, IVD, signalling, target genes, activation, inhibition

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Abbreviations

ASIC: Acid-sensing ion channel
ASS: Acetylic salicylic acid
AF: Annulus fibrosus
COX: Cyclooxygenase
CPB: Creb-binding protein
EG-1: Early growth factor 1
EMSA: Electrophoretic mobility shift analysis
EP: Endplate
ERK: Extracellular signal-regulated kinase
bFGF: Basic fibroblast growth factor
Gluc AT-1: β-1,3-glucuronyl-transferase 1
IDD: Intervertebral disc disease
IGF-I: Insulin-like growth factor-I
IL: Interleukin
iκB: Inhibitor of κB
IKK: IκB kinase
iNOS: Inducible nitric oxide synthase
IVD: Intervertebral disc
JNK: Jun NH2-terminal kinase
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MCP-1: Monocyte chemotactic protein-1
MKK: MAPK kinase
MMP: Matrix metalloproteinase
NBD: NEMO binding domain
NEMO: NF-κB essential modulator
NF: Nuclear factor
NGF: Nerve growth factor
NO: Nitric oxide
NP: Nucleus pulposus
ODN: Naked decoy oligonucleotide
p75NTR: p75 neurotrophin receptor
PDGF: Platelet-derived growth factor
PGE: Prostaglandin E
PI3K: Phosphatydilinositol 3-kinase
PKC: Protein kinase C
RHD: Rel homology domain
TGF: Transforming growth factor
TIMP: Tissue inhibitor of metalloproteinases
TNF: Tumor necrosis factor
TonEBP: Tonicity-responsive enhancer binding protein
TSLP: Thymic stromal lymphopoietin
TWEAK: Tumor necrosis like weak inducer of apoptosis
VEGF: Vascular endothelial growth factor

Introduction

Degeneration of the intervertebral disc (VID) is a normal part of the ageing process, and is typically characterised
by a loss of disc extracellular matrix. This loss is due to perturbed matrix homeostasis, whereby matrix anabolism is decreased and matrix catabolism is increased. Aging is associated with increased cellular senescence and changes in disc cellular phenotype that result in cells with decreased matrix synthesis capacity and/or altered matrix production. Additionally, enzymes mediating matrix degradation, including matrix metalloproteinases (MMPs), are up-regulated during the process of IVD degeneration and aging, resulting in increased matrix degradation (Cui et al., 2010; Roberts et al., 2000; Weiler et al., 2002). Consequently, loss and remodelling of the extracellular matrix (ECM) can lead to the occurrence of clefts and tears and eventually complete disc structural failure.

Despite the large structural changes in their discs, patients with IVD degeneration often remain symptom-free. Nevertheless, a subgroup of individuals with IVD degeneration experience pain and thus can be categorised to have intervertebral disc disease (IDD). A recent systematic review indicated that the odds of chronic low back pain given the presence of disc degeneration (detected by magnetic resonance imaging changes) ranged from 1.8 to 2.8, meaning that the chances of suffering from back pain in people with degenerated discs was 2-3 times higher than in individuals without degenerated discs (Chou et al., 2011). Important in the context of disc-related back pain is the observed phenomenon of innervation of sensory nerve fibres in degenerated discs. These sensory nerves, containing nociception-related mediators such as substance P or calcitonin can penetrate not only into the peripheral annulus fibrosus (AF), but also into deeper zones of degenerated discs, especially if radial fissures and reduced pressure in the nucleus pulposus are present (Adams et al., 1996; Freemont et al., 1997; Hastreiter et al., 2001; Ozawa et al., 2006; Peng et al., 2006; Peng et al., 2005). Irritation of these sensory nerves has been described as a major underlying mechanism of discogenic back pain, which may occur via inflammatory processes (Goupille et al., 2007; Olmarker and Rydevik, 1998). Recently, surgically-removed human degenerative discs were shown to be actively inflammatory (Adams et al., 2010). Past research has also provided evidence that IDD is correlated to increased levels of pro-inflammatory cytokines in disc tissue, such as interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α): LeMaitre et al. (2007) demonstrated that herniated discs and degenerated discs from patients with chronic back pain showed higher expression of IL-1β and TNF-α than non-degenerated discs derived from post-mortem tissue from people without a history of back pain. In fact, not only IL-1β, but also IL-1α, type-I receptor of IL-1 and the IL-1β-converting enzyme were present in higher levels in degenerated samples compared to non-degenerated ones (Le Maitre et al., 2005). Furthermore, TNF-α expression increased continuously with age in the AF and up to the age of 60 years in the nucleus pulposus (NP) in a population study consisting of autopsy samples that did not have any medical notes concerning relevant back problems (Bachmeier et al., 2007). Importantly, surgical samples from patients with a low back pain history (protrusion, herniation, degenerative disc disease) showed higher level of TNF-α positively labelled cells than the autopsy group (Bachmeier et al., 2007). Similarly, Weiler et al. (2005) demonstrated that surgical disc tissue from symptomatic back pain patients contained more TNF-α positive cells than asymptomatic autopsy samples, with a positive correlation to the degree of degeneration for the AF. Burke et al. (2002) clearly demonstrated that disc tissue from patients with discogenic back pain revealed higher protein levels of IL-6 and IL-8 than patients with sciatica. A most recent immunohistochemical comparison of surgical disc tissue (degenerative disc disease, disc herniation) and non-degenerated autopsy discs showed higher expression of IL-4, IL-6 and IL-12 in surgical samples than in autopsy samples, but with highest levels in the cases of disc herniation (Shamji et al., 2010). In summary, these studies indicate that the inflammatory mediators play an important role in the processes of IDD and possibly IDD-related back pain.

During the past years, gene expression and function of these mediators in IDD have been a major topic of research interest. Furthermore, extensive therapeutic studies in the field of osteoarthritis and rheumatoid arthritis have highlighted the need to identify the underlying signalling pathways, prompting scores of IVD researchers to explore the molecular mechanisms leading to IVD inflammation and catabolism. This review describes two major intracellular pathways, nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), which potentially play vital roles in mediating the molecular events responsible for the initiation and progression of IDD. A graphical illustration of the NF-κB and MAP kinase pathways (as described in this review paper) is given in Fig. 1. Although this review focuses primarily on the NF-κB and MAPK pathways, the reader should keep in mind that the final effect of the activation of signalling pathways depends on their “crosstalk” with other activated pathways. For instance, a pathway that crosstalks with both the MAPK and the NF-κB pathways is the phosphatidylinositol 3-kinase (PI3K)/Akt axis (Conejo et al., 2002; Koh et al., 2006), which also seems to be involved in IVD homeostasis, e.g. by regulating aggrecan expression, glycosaminoglycan deposition and cell survival (Cheng et al., 2009; Rbisbud et al., 2005b).

**Transcription Factor NF-κB**

NF-κB is a central component in the cellular response to damage, stress and inflammation. NF-κB was first described in 1986 in B-lymphocytes and acquired its name as it was found in the nucleus where it bound to an enhancer element of the immunoglobulin kappa light chain gene (Sen and Baltimore, 1986). NF-κB proteins comprise a family of structurally related “rapid-acting” transcription factors, all sharing a common highly conserved 300-amino acid region, the Rel homology domain (RHD). In mammals, the ubiquitously expressed NF-κB family consists of five protein subunits, RelA or p65, c-Rel, RelB, p50 and p52. NF-κB exists either as a homodimer or a heterodimer, the most abundant being the p50-p65 heterodimer, which controls the expression of the majority of NF-κB-regulated genes (Baueuerle and Henkel, 1994).
Fig. 1. MAPK and NF-κB pathways. (A) Canonical cascades of the MAP kinases and NF-κB signalling pathway (Hacker and Karin, 2006; Roux and Blenis, 2004). Left panel: Representative modules of the pathway connections for the respective MAPK phosphorelay systems. The complexity of the system is evident in the multiple stimuli leading to various biological responses through the multi-component levels of MAKKks, MAKKs, and MAPKs. Right panel: Representative modules in the canonical NF-κB signalling. Specific cross talks between the components of MAPK and NF-κB pathways are included (red arrows). (B) Current reported literature of involvement of MAPK and NF-κB pathways in intervertebral discs. Left panel: Involvement of MAPK signalling in disc metabolism. Inflammatory stress perturbs disc matrix homeostasis through the ERK, p38, and JNK-mediated pathways, while both ERK and p38 are reported to mediate stimuli from mechanical stress and growth factors on disc cell apoptosis and senescence. Elevated in degenerated discs, cellular senescence might be mediated by p38 in disc cells (dashed line) as activation of p38a is required for stress-induced cellular senescence in other cell types (Freund et al., 2010). Right panel: Involvement of the NF-κB signalling in disc metabolism. Inflammatory and mechanical stress are known to activate NF-κB in disc cells, leading to expression of key catabolic factors such as MMPs. Elevation of NF-κB is also reported in an accelerated aging mouse model due to DNA repair deficiency as well as in the injury-induced IDD rabbit model, both of which correlate to disc matrix loss. In animal studies, blocking NF-κB activity using the NF-κB decoy ODN proved effective in partially restoring IVD height in a rabbit model of IDD induced by annular puncture (Akeda et al., 2005), or NBD (nemo binding domain) peptide which inhibits IKK formation ameliorates age-related disc matrix loss in the progeroid mouse model (Nasto et al., 2011). Consistent with these findings are reports of increased NF-κB activation and activity in aged and degenerated disc.
The transcription activity of NF-κB is tightly controlled by binding of inhibitor of NF-κB (IκB) proteins, resulting in sequestration of NF-κB in the cytoplasm. Depending on IκB modification, the NF-κB/IκB complex can either be retained in the cytoplasm or can be a constantly shuttled between cytoplasm and nucleus, but with a balance that is largely shifted towards nuclear export rather than import (Web ref. 1). Canonical activation of NF-κB is mediated by IκB kinase (IKK), a heterotrimer consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit termed IKKγ or NEMO (NF-κB essential modulator). In response to a variety of stimuli, including pro-inflammatory cytokines, pathogens, cellular stress, mechanical stress, radiation, and growth factors, IKK becomes activated and phosphorylates IκB at two specific serine residues (S32 and S36), leading to its ubiquitination and subsequent

<table>
<thead>
<tr>
<th>Gene</th>
<th>Signalling Pathway</th>
<th>Species</th>
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<th>Gene Function</th>
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<td>Bovine</td>
<td>(Kim et al., 2012)</td>
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<td>(Uchiyama et al., 2007)</td>
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<td>Mouse</td>
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<td>p38</td>
<td>Bovine</td>
<td>(Seguin et al., 2006)</td>
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proteosomal degradation (Bubici et al., 2006; Hacker and Karin, 2006; Hayden and Ghosh, 2008; Karin and Ben-Neriah, 2000; Ramana et al., 2004). IkB degradation allows NF-κB to translocate to the nucleus where it selectively binds to its cognate DNA consensus sequence, as well as co-activators such as Creb-binding protein (CBP)/p300, to induce gene expression (Furia et al., 2002). Stress-induced activation of NF-κB leads to secretion of inflammatory cytokines, increased expression of genes that regulate cell survival and growth, such as arrested proliferation or cell death, depending on the nature of the insult and extent of damage (Karin and Lin, 2002).

As NF-κB also activates expression of IκB itself, NF-κB activation is normally transient due to this negative feedback mechanism, lasting approximately 30-60 min in most cell types (Ghosh et al., 1998). However, this is only true for normal cells, whereas constitutive NF-κB activation could be observed in tumour cells (Sethi et al., 2008). Additionally, NF-κB regulation also takes place in the nucleus via acetylation or phosphorylation, which modulate its transcriptional activity (Ghosh and Karin, 2002). More than 150 genes are regulated by NF-κB, including several proinflammatory mediators such as TNF-α, IL-1β, IL-6, cyclooxygenase-2 (COX-2), MMPs and adhesion molecules (Barnes and Karin, 1997; May and Ghosh, 1998).

Role of Chronic Activation of NF-κB in Diseases

NF-κB is activated in response to numerous types of stress, including oxidative (Bubici et al., 2006), genotoxic (Wu et al., 2006), physical (Chen et al., 2001) and inflammatory stress (Web ref. 2), that have been implicated in the pathogenesis of many diseases. Indeed, chronic activation of NF-κB is associated with numerous diseases, including musculo-skeletal diseases such as osteoarthritis (Berenbaum, 2004; Marcu et al., 2010), osteoporosis (Kim et al., 2006), rheumatoid arthritis (Dai et al., 2004), and muscular dystrophy (Acharya et al., 2007). For instance, expression of MMP1, MMP3, and MMP13 in chondrocytes is mediated primarily by activated NF-κB (Elliott et al., 2002; Liacini et al., 2003; Mengshol et al., 2000). In addition, activation of the NF-κB pathway is required for IL-1 to inhibit the expression of SOX-9, a transcription factor involved in chondrocyte differentiation (Murakami et al., 2000). NF-κB signalling is found to be persistently elevated in immune cells and regenerative muscle fibres of patients with Duchenne muscular dystrophy, the disorder associated with dystrophin deficiency that results in chronic inflammation and severe skeletal muscle degeneration (Acharya et al., 2007). Oxidative stress-induced osteoelasticogenesis in osteoporosis is also mediated by NF-κB signalling (Altingad et al., 2008).

Overexpression of either the c-rel or p65/RelA subunit of NF-κB induces hallmark features of cellular senescence including decreased proliferation and morphologic changes, such as enlarged, multinucleated cells (Bernard et al., 2004; Bernard et al., 2001; Seitz et al., 2000). Moreover, NF-κB is up-regulated in tissues of aged rodents, specifically in the skin, liver, kidney, cerebellum, cardiac muscle and gastric mucosa (Bregegere et al., 2006; Giardina and Hubbard, 2002; Helenius et al., 1996a; Helenius et al., 1996b; Korhonen et al., 1997; Xiao and Majumdar, 2000). Cells derived from elderly persons and patients with Hutchinson-Gilford progeria, a disease of dramatically accelerated aging, also exhibited increased NF-κB signalling (Adler et al., 2007; Boland, 2001; Kriete et al., 2008). Growing evidence indicates that NF-κB becomes activated in aged tissues in response to accumulated damage and mediates the degenerative changes. Indeed, a recent modelling study identified NF-κB as the transcription factor most associated with mammalian aging, and demonstrated that expression of a subset of NF-κB effectors is increased with aging (Adler et al., 2007). The role of NF-κB in aging is supported by the fact that genetic inhibition of NF-κB in skin reversed histological features of aging and signs of cellular senescence (Adler et al., 2007).

Current Knowledge on the Role of NF-κB in the IVD

Role of NF-κB in IDD

While NF-κB is implicated in a large number of diseases, only limited information of its role in IDD has been generated so far. Compared to asymptomatic autopsy disc samples, symptomatic (surgical) discs are characterised by increased levels of pro-inflammatory cytokines that are considered typical NF-κB target genes, e.g. TNF-α, IL-1β, IL-6 and IL-8 (Adams et al., 2010; Bachmeier et al., 2007; Burke et al., 2002; Hoyland et al., 2008; Le Maître et al., 2005; Le Maître et al., 2007; Ulrich et al., 2007). It is therefore likely that NF-κB activation is involved in disc disease. In fact, evidence supporting the role of NF-κB in the IVD has been generated by immunohistochemical studies, demonstrating that activation of the NF-κB signalling system occurs in the human IVD in vivo, especially in the nucleus pulposus tissue (Nerlich et al., 2007). Furthermore, NF-κB activity in the IVD was shown to correlate with accumulated oxidative stress and increase with age and degeneration (Nerlich et al., 2007). Using a rabbit annular puncture model, intra-discal injection of ‘naked’ NF-κB decoy oligonucleotides (ODN) proved effective in partially restoring IVD height, indicating that activation of NF-κB is involved in matrix loss in this animal model of IDD (Akeda et al., 2005). Hence, NF-κB most likely plays an important mediatory role in the disc degenerative process. However, further investigation is needed to confirm if NF-κB is also involved in the development of a painful disc, as increased NF-κB activity is found in both symptomatic and non-symptomatic degenerative discs.

Target genes

Although more than 150 NF-κB-responsive genes have been identified in multiple cell types (Barnes and Karin, 1997; May and Ghosh, 1998), little is known about the gene targets of NF-κB in the IVD (Table 1). MMP1, MMP2, MMP3, MMP9 and MMP13 have been identified as NF-κB target genes in IVD cells, as their protein levels are reduced as NF-κB signalling is inhibited (Adler et al., 2007). MMP1, MMP3, MMP9 and MMP13 have been identified as NF-κB target genes in IVD cells, as their protein levels are reduced as NF-κB signalling is inhibited (Adler et al., 2007). Furthermore, ADAMTS4 and ADAMTS5, the two major aggregases in the IVD, were most recently shown to be NF-κB dependent.
Furthermore, it was shown that peroxynitrite, one of the most damaging reactive oxygen species, was able to induce mRNA levels of IL-1β, IL-6 and IL-8 in human IVD cells and this was correlated with sustained nuclear translocation of p65 in human IVD cells (Ohba et al., 2009). Similarly, tumour necrosis factor-like weak inducer of apoptosis (TWEAK) was also shown to induce NF-κB activation in mouse IVD tissue (Wako et al., 2008). In contrast, LPS treatment did not (or only very slightly) cause nuclear translocation of p65 in human IVD cells (Fig. 2), even though this has been described for p65 in the literature for other cell and tissue types (Crisostomo et al., 2008; Deshpande et al., 1997; Rodrigues et al., 2008).

Furthermore, it was shown that peroxynitrite, one of the most damaging reactive oxygen species, was able to induce nuclear translocation of NF-κB in human IVD cells (Oh et al., 2010; Wang et al., 2012), but our own data indicate that this effect is less persistent compared to IL-1β (Fig. 2). NF-κB-induced activation of NF-κB was also shown in murine disc tissue (Ohba et al., 2009). Similarly, tumour necrosis factor-like weak inducer of apoptosis (TWEAK) activation and the alterations of these mRNAs can only be assumed (Poveda et al., 2009). Depending on the type and magnitude of loading, mechanical signals have been shown to either activate or inhibit the NF-κB pathway in

**Table 2: Summary of NF-κB and MAPK activators in IVD cells**

<table>
<thead>
<tr>
<th>Signalling Pathway</th>
<th>Activator</th>
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<td>(Oh et al., 2010)</td>
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<td>(Wako et al., 2008)</td>
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<td>TNF-α</td>
<td>Bovine</td>
<td>(Seguin et al., 2006)</td>
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<td><strong>ERK</strong></td>
<td>bFGF</td>
<td>Bovine</td>
<td>(Pratsinis and Kletsas, 2007)</td>
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<td></td>
<td>Fibronectin frag.</td>
<td>Human</td>
<td>(Xia and Zhu, 2010)</td>
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<td>IGF-1</td>
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<td>TGF-β1</td>
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<td>TGF-β3</td>
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<td>TNF-α</td>
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<td>(Seguin et al., 2006; Seguin et al., 2008)</td>
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<td>TWEAK</td>
<td>Mouse</td>
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**Activation**

Different exogenous and endogenous stimuli can activate NF-κB in the IVD (Table 2). Stimulation of human disc cells with recombinant IL-1β caused nuclear translocation of p65 into the nucleus, as confirmed by immunoblotting and immunocytochemistry as well as by electrophoretic mobility shift analysis (EMSA) (Wuertz et al., 2011). Stimulation of human IVD cells with IL-1α also resulted in activation of NF-κB (detected by EMSA), even after 3 days (Yu et al., 2009). Similarly, TNF-α can also induce nuclear translocation of NF-κB in human IVD cells (Oh et al., 2010; Wang et al., 2012), but our own data indicate that this effect is less persistent compared to IL-1β (Fig. 2). NF-κB-induced activation of NF-κB was also shown in murine disc tissue (Ohba et al., 2009). Similarly, tumour necrosis factor-like weak inducer of apoptosis (TWEAK) activation and the alterations of these mRNAs can only be assumed (Poveda et al., 2009). Depending on the type and magnitude of loading, mechanical signals have been shown to either activate or inhibit the NF-κB pathway in
chondrocytes (Agarwal et al., 2003; Deschner et al., 2003). Similarly, hypoxia has been reported to enhance NF-κB activity (primarily through the canonical pathway) in low oxygen tissues, but no data is currently available with regard to the IVD (Oliver et al., 2009).

**Inhibition/Therapy**

Suppression of NF-κB has become a major research target to treat diseases in the past years. Different strategies to block NF-κB have been investigated, such as the use of proteasome inhibitors, which may inhibit degradation of IkB upon phosphorylation and ubiquitination (Ahn et al., 2007; Palombella et al., 1994). Other possibilities may be to block IKK, the upstream regulator that phosphorylates IkB (Ji et al., 2001), or to modify IkB at the phosphorylation or ubiquitination sites via gene transfer (Abu-Amer et al., 2001). In addition, a large group of anti-inflammatory drugs seem to have the potential to inhibit NF-κB. On the one hand, there are several traditional drugs that have been commonly used for decades, such as acetylsalicylic acid, ibuprofen or glucocorticoids, e.g. dexamethasone, which have NF-κB inhibitory activities (Auphan et al., 1995; Kopp and Ghosh, 1994). Furthermore, more “modern” pharmaceuticals, such as the COX-2 inhibitor rofecoxib, can influence NF-κB activity, e.g. in this case by inhibiting its DNA binding capacity (Niederberger et al., 2003).

On the other hand, there are multiple “natural” candidates that seem to inhibit NF-κB in certain cell types, such as resveratrol (a polyphenol found in wine) (Holmes-McNary and Baldwin, 2000), curcumin (the main component of curcuma) (Jobin et al., 1999; Surh et al., 2000) or capsaicin (found in red pepper) (Surh et al., 2000).

In human IVD cells, the well known anti-inflammatory steroid drug dexamethasone, which has been described to act as an inhibitor of NF-κB, was not able to inhibit the transcription of NF-κB stimulated by TNF-α in human IVD.
K Wuertz et al.  

NF-κB and MAPK in the intervertebral discs

...cells, as shown by the EMSA experiments (Oh et al., 2010). Similarly, the polyphenol resveratrol did not reverse IL-1β induced activation of NF-κB as demonstrated by EMSA, immunoblotting and immunocytochemistry (Wuertz et al., 2011), although this has been described for chondrocytes (Shakibaei et al., 2008). Furthermore, the effect of resveratrol on insulin resistance and inflammatory mediators in obese and type 2 diabetic subjects is being investigated in a clinical trial (see http://clinicaltrials.gov).

Curcumin, a potential biological NF-κB inhibitor, did not inhibit IL-1β-induced activity of nuclear translocation of p65 at an early time point as shown in Fig. 3 (results not yet published), while late IL-1β-induced activity of NF-κB (after 3 days) was reduced when treated with curcumin (Yu et al., 2009). As NF-κB is continuously shuttling between the nucleus and cytoplasm, assays measuring NF-κB activation by nuclear translocation (i.e. presence of p65 in the nucleus) can give variable results if the dose of inhibitor or time point of analysis are not optimally determined. It is also possible that the mechanisms of NF-κB activation and inhibition in disc cells are different from those found in other cell types. Hence, although time course experiments have been performed in most of the above-mentioned studies, a negative result cannot completely rule out inhibition of NF-κB by the tested substances. Nevertheless, the failure of NF-κB inhibition using well-established pharmaceuticals (e.g. dexamethasone) or natural substances at least challenges the efficacy of this therapeutic approach.

Aside from NF-κB inhibition with “traditional substances” (as described above), another strategy that targets NF-κB has been investigated recently: NF-κB Decoy transduction into dorsal root ganglion neurons in vivo has been tested in a rat lumbar disc herniation model with regard to its efficacy in altering nerve injury, mechanical allodynia and thermal hyperalgesia. This strategy reduced nerve injury, improved mechanical allodynia and thermal hyperalgesia in this animal model (Suzuki et al., 2009) and may thus be a promising new approach but one that will require more detailed investigations in the future. A recent study revealed that systemic inhibition of NF-κB activation by the inducible IKK via chronic administration of the Nemo Binding Domain peptide inhibitor, 8K-NBD, in a mouse model of progeria (Ercc1−/− mice) delayed the onset of age-related IDD (Fig. 4) (Nasto et al., 2011). For adults with mild to moderate atopic dermatitis, the use of NF-κB Decoy is being currently investigated in clinical trials (http://clinicaltrials.gov).

MAPK Signalling Pathways

MAPKs are a family of highly conserved signal transduction pathways, allowing the cells to respond to multiple extracellular inputs. MAPKs are activated by different stimuli, such as hormones and growth factors acting through tyrosine or serine/threonine kinases, inflammatory cytokines, peptides acting through G protein-coupled receptors, as well as environmental stresses such as ionising radiation or osmotic stress (Huang et al., 2010; Kyriakis and Avruch, 2001). In mammals, these diverse signals activate at least three major subfamilies of MAPKs, the extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinases (JNKs), and p38 isoforms (p38MAPKs) (Boutros et al., 2008; Wagner and Nebreda, 2009). All MAPKs are activated following a common cascading pat-
tern, i.e. each member of the family of MAPK is activated by specific upstream kinases (MAPKK) through phosphorylation on threonine and tyrosine residues, while each MAPKK is activated by a MAPKK kinase (MAPKKK) by phosphorylation on serine or threonine residues (Imajo et al., 2006).

ERK 1/2 was the first mammalian MAPK pathway to be identified. It is regulated by the activation of the GTPase Ras, which recruits MAPKKks of the Raf family and activate the MEK1 and MEK2 MAPKKs, which in turn activate the ERKs by phosphorylation (Chambard et al., 2007; Kyriakis and Avruch, 2001). Activation of the ERK pathway was found to control several cellular functions, including cell cycle progression. This is achieved by ERK-mediated regulation of components of the cell cycle machinery, such as c-Myc, cyclin D1 and cyclin-dependent kinases that phosphorylate the retinoblastoma protein, leading to the release of the E2F transcription factors and thus allowing the transition from the G1 to the S phase of the cell cycle (Chambard et al., 2007). JNKs are activated by various exogenous stresses, which lead to phosphorylation of tyrosine and threonine residues by the upstream kinases MMK4 and MKK7. JNKs are involved in the regulation of cell proliferation and survival (Wagner and Nebreda, 2009).

Finally, the p38 MAPK pathway is also activated by many diverse stresses, resulting in dual phosphorylation on its threonine and tyrosine residues by the upstream kinases MKK3/6. Activation of p38 MAPK is associated with cell growth and differentiation, cell death and inflammation (Kaminska, 2005; Kyriakis and Avruch, 2001; Pearson et al., 2001). There are four known isoforms expressed in mammalian cells, i.e. p38α, p38β, p38γ and p38δ, and the first two are expressed in most tissues, while p38γ and p38δ are exclusively found in muscle, skin and kidney cells (Han and Sun, 2007; Huang et al., 2010). Amongst the various stimuli activating p38 are inflammatory cytokines (e.g. interleukins and TNF-α), pathogenic stimuli (such as LPS, staphylococcal peptidoglycan, enterotoxin B and herpes simplex virus I) (Kaminska, 2005), and UV and gamma radiation (Dent et al., 2003). A summary of the known MAPK cascades, including ERK5, which has not been investigated in the disc so far, is given in Fig. 1.

Roles of MAPKs in Diseases

As MAPKs are activated by several exogenous stimuli commonly found in inflammatory diseases, e.g. inflammatory cytokines and growth factors, pathogenic components, reactive oxygen species, etc., these signalling pathways are closely involved in diseases such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, neurodegenerative diseases or cancer (Huang et al., 2010; Wagner and Nebreda, 2009; Zarubin and Han, 2005). Hence, MAPKs represent important targets for therapeutic interventions. In this context, several specific kinase inhibitors have been developed for the regulation of these pathways, with the aim to function as anti-inflammatory agents. A few compounds have been used in clinical trials. However, toxic side effects still represent a major obstacle (Huang et al., 2010; Kaminska, 2005), probably due to the pivotal roles of the MAPK pathways in normal cellular physiology.
Cellular senescence, which is increased in aged and degenerated discs, is regulated by the MAPK pathways (Gruber et al., 2010; Gruber et al., 2007; Kletsas, 2009; Roberts et al., 2006). In several cell types, activation of p38 MAPK, for example, is used as a marker of senescence (Chen and Ames, 1994; Iwasa et al., 2003; Papadopoulou and Kletsas, 2011), while constitutive activation of p38 via MKK3 or MKK6 is reported to induce premature senescence via the upregulation of cyclin-dependent kinase inhibitors (Wang et al., 2002; Wu, 2004). In addition, the MEK/ERK pathway is involved in the oncogenic Ras induced senescence (Lee et al., 1999; Maruyama et al., 2009), while JNK has been reported to inhibit or provoke cellular senescence under different settings (Maruyama et al., 2009).

**Current Knowledge on the Role of MAPKs in the IVD**

**Role of MAPKs in IDD**

MAP kinases have received greater attention in the IVD research community during the past few years. However, little information still exists on their expression and activity in relation to the progression of IDD. With laser capture microdissection (LCM) microarray, which can be used to identify cell-specific gene expression patterns, Gruber et al. (2010) provided evidence that p38 MAPK gene expression is upregulated in senescent human AF cells compared to non-senescent cells. Furthermore, in a rodent in vivo stab injury model, annular fibroblasts became immunopositive for the phosphorylated form of p38 and produced increased levels of proinflammatory factors, such as IL-1 and TNF-α (Ulrich et al., 2007).

The MAPK signalling pathways seem to play a crucial role in modulating both matrix synthesis and degradation in the IVD by altering expression of anabolic and catabolic genes, as well as by influencing proteoglycan degradation in the IVD. In particular, the p38 and ERK signalling pathways have been shown to play a role in proteoglycan metabolism, as treatment with chemical inhibitors of p38 or ERK significantly counteracted the cytokine-induced decrease in proteoglycan content, synthesis and release (Seguin et al., 2006; Studer et al., 2008). For the first time, ERK is recently reported to be involved in the activation of Wnt/b-catenin signals, which may contribute to the pathogenesis of IDD (Hiyama et al., 2011). Furthermore, activation of p38 by hyperosmotic conditions (as seen in the IVD during daily activity) has an inhibitory effect on cell proliferation via induction of a G2 arrest in bovine NP cells (Mavrogonatou and Kletsas, 2009). On the other hand, ERK activation plays an important role in cell adhesion by positively influencing α2-integrin expression, as well as cell adhesion to collagen II substrates in rat NP cells (Risbud et al., 2005b). In addition, activation of ERK and p38, which was shown to be higher in freshly isolated compared to expanded bovine IVD cells (Tsai et al., 2007b), can counteract apoptosis induced by mechanical stress (in the endplate (EP) and transitional zone of AF) (Ariga et al., 2003) or by serum starvation under hypoxic conditions (in the rat NP) (Risbud et al., 2005a). As mentioned above, several growth factors exert their mitogenic action by activating ERK by phosphorylation. On the other hand, several growth factors and growth factor receptors have been found to be over expressed in degenerated discs (Pratsinis and Kletsas, 2008). Likewise, classical growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) or basic fibroblast growth factor (bFGF), which are known to be overexpressed in degenerated disc tissue, can stimulate ERK and subsequent DNA synthesis in bovine AF and NP cells in vitro (Pratsinis and Kletsas, 2007), indicating that MAPKs are possibly involved in catabolic and anabolic processes in the IVD.

**Target genes**

MMP9, MMP13, MMP14, inducible nitric oxide synthase (iNOS), tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3, aggrecan, collagen II, SOX-9, acid-sensing ion channel (ASIC)3 and toxicity-responsive enhancer binding protein (TonEBP) all have been identified as target genes of ERK in IVD cells of various species (Kim et al., 2012; Seguin et al., 2006; Tsai et al., 2007a; Uchiyama et al., 2007; Xia and Zhu, 2010). Furthermore, the ERK pathway seems essential in regulating the enzymatic activation of MMP2 via early growth factor EG-1 (Seguin et al., 2008). The p38 pathway controls expression of MMP1, MMP3, MMP13, IL-6, IL-8, COX-2, iNOS, VEGF, IGF-1, transforming growth factor (TGF)-β and TonEBP (Kim et al., 2009; Kim et al., 2012; Seguin et al., 2006; Studer et al., 2008; Tsai et al., 2007a) and influences levels of prostaglandin (PGE)2, PGF2α and nitrite (Kim et al., 2009; Studer et al., 2008). Furthermore, expression of the anabolic and anti-catabolic genes aggrecan, collagen I, collagen II, versican, TIMP-1, TIMP-2 and TIMP-3 seems to be influenced by the p38 MAPK pathway as well (Kim et al., 2012; Studer et al., 2008). Finally, JNK was shown to regulate expression of MMP1, MMP3 and MMP13 (Seguin et al., 2006; Wako et al., 2008). All genes known to be regulated by MAPKs in disc cells are summarised in Table 1.

**Activation**

Several signalling molecules of MAPK pathways in IVD cells have been identified in the past years (Table 2). The proinflammatory cytokines TNF-α and IL-1β are able to induce activation of the ERK, p38 and JNK pathways in bovine, rabbit and human IVD cells (Klawitter et al., 2011; Seguin et al., 2006; Seguin et al., 2008; Studer et al., 2008; Wuertz et al., 2011). Treatment of rat IVD cells with nerve growth factor (NGF) resulted in a rapid increase in the levels of phosphorylated ERK, which was mediated through the low-affinity neurotrophin receptor (p75NTR) (Uchiyama et al., 2007). Furthermore, the fragmented form of the matrix protein fibronectin was shown to induce phosphorylation of ERK1/2 in a protein kinase C (PKC)-dependent manner (Xia and Zhu, 2010). Lactoferricin, a glycoprotein from the transferrin family, was also shown to activate the p38 and ERK signalling in bovine NP cells (Kim et al., 2011) and TGF-β-induced levels of phosphorylated ERK1/2 and p38 (Risbud et al., 2005a).
Other environmental factors also seem to play a crucial role in MAPK activation: hyperbaric oxygen for example reduced phosphorylation of p38 MAPK in human IVD cells (Niu et al., 2011). High osmolarity, to which NP cells are exposed during daily activities (Urban, 2002), resulted in p38 MAPK activation in bovine NP cells, while the activation of ERK and JNK was inhibited under these conditions (Mavrogonatou and Kletsas, 2009; Mavrogonatou and Kletsas, 2010; Mavrogonatou and Kletsas, 2011). Notably, this differential regulation of MAP kinases was observed after stimulation of cells with a salt solution (NaCl/KCl) or the osmolyte sorbitol but not by urea, indicating that these effects are due to changes in osmolarity and not to increased ionic strength (Mavrogonatou and Kletsas, 2011). It is well known that one of the characteristics of the disc NP cells' environment is high osmolarity due to the abundance of proteoglycans. This osmolarity can increase further during daily activities, but it is decreased in the degenerated discs. These changes affect also growth factor-mediated ERK activation. In particular, while ERK activation is decreased under hyperosmotic conditions in bovine NP cells, its activation is enhanced in hypoosmotic conditions, indicating that the conditions of the degenerated disc are more permissive for cell proliferation and repair (Mavrogonatou and Kletsas, 2010). In contrast, in rat NP cells, hyperosmotic culture conditions induced by NaCl supplementation of the medium were shown to increase phosphorylation and activation of ERK (Tsai et al., 2007a). Furthermore, hypoxic conditions induced phosphorylation of both, p38 and ERK MAPK in rat NP cells (Risbud et al., 2005a; Risbud et al., 2005b). Cellular responses to microenvironmental factors, i.e. alteration in oxygen levels and osmotic pressure, are inherent mediatory steps in the degenerative processes of the IVD. Activation of MAPK signalling pathways by exogenous stresses seems to affect several aspects of IVD homeostasis. Hypoxic environment activates ERK leading to the upregulation of α2 integrin and to an increased cell survival in discs (Risbud et al., 2005b). In addition, hypoxia increases the expression of β1,3-glucuronyltransferase 1 (GlucAT-1), a key enzyme in glucosaminoglycan synthesis, a process that is partly mediated by ERK activation (Gogate et al., 2011). Furthermore, hypertonicity activates ERK in rat NP cells, leading to the transactivation of TonEBP, a transcription factor that is involved in the adaptation to osmotic stress and in the regulation of aggregcan expression (Tsai et al., 2007a). This is in agreement with the effect of osmotic stress on aggregcan overexpression in chondrocytes (Peffers et al., 2010). Finally, exposure of disc cells to high osmolarity decreased the phosphorylation of ERK in response to serum or to isolated growth factors, such as PDGF or IGF-1, thus inhibiting bovine NP cell proliferation (Mavrogonatou and Kletsas, 2010; Pratsinis and Kletsas, 2007).

Inhibition/Therapy

Inhibition of MAPK activity under inflammatory stressful conditions has been demonstrated to be potentially beneficial in treating IDD by stimulating matrix protein expression (aggrecan, collagen-I, collagen-II, versican) and by inhibiting expression of inflammatory mediators (IL-1β, IL-6, COX-2, NO, PGE-2) and matrix degrading enzymes (MMP3) (Niu et al., 2011; Studer et al., 2008). So far, several chemical/biological therapeutics as well as external factors have been identified that can influence the activity of the specific MAPKs. While non clinical studies have been performed so far with regard to IDD, several MAPK inhibitors, especially p38 MAPK inhibitors, are currently being investigated for other inflammation-related diseases. For rheumatoid arthritis, the efficacy and safety of oral medication of the p38 MAP kinase inhibitors VX-702, RO4402257 or PH-797804 are being determined in ongoing clinical studies (http://clinicaltrials.gov).

Phosphorylation (and thus activation) of p38 can be reduced by link N peptide (the N-terminal peptide of link protein) (Petit et al., 2011) and triptolide (diterpenoid triepoxide form the Chinese herb, Tripterygium wilfordii Hook) (results not yet published) in human IVD cells, with an overall pro-anabolic, anti-catabolic and anti-inflammatory effect. Furthermore, triptolide also inhibited ERK activation, while curcumin (the principal curcuminoid in curcuma/tumeric) reduced activation of JNK in human disc cells (Fig. 3) (Klawitter et al., 2011). Application of the p38 chemical inhibitor SB202190 in human AF cells co-cultured with macrophages or stimulated with TNF-α caused a significant reduction in proinflammatory cytokines and prostaglandins, indicating that p38 blockade may be useful for the treatment of IDD (Kim et al., 2009). The anabolic, anti-catabolic and anti-inflammatory response of IVD cells treated with the p38 inhibitor SB202190 was confirmed in rabbit IVD cells exposed to inflammatory signals (Studer et al., 2008). On the other hand, the glycoprotein lactoferricin caused anabolic effects in bovine NP cells via activation of p38 and ERK, and this effect was reversed by treatment with the p38 inhibitor SB203580 or the ERK inhibitor PD98059 (Kim et al., 2011).

Importantly, multiple studies in the last decade suggest that MAPK inhibitors may also offer therapeutic potential for patients with sciatic nerve crush, spinal nerve ligation or disc herniation. In fact, MAP kinase activity has been shown to be elevated in conditions mentioned above (Doya et al., 2005; Ito et al., 2007; Jin et al., 2003; Kominato et al., 2003; Myers et al., 2003; Obata et al., 2004; Schafers et al., 2003; Zhuang et al., 2005; Zhuang et al., 2006). In light of this, asialo-erythropoietin, a nonerythropoietic cytokine, was recently shown to reduce levels of phosphorylated p38 and improve pain-related behaviour (as measured by von Frey filament testing) in a rat model of lumbar disc herniation (Sasaki et al., 2006). Furthermore, MAPK inhibitors seem to have the ability to reduce neuropathic pain. These include the use of JNK peptide inhibitor D-JNKI-1 in a model of spinal nerve ligation (Zhuang et al., 2006), p38 inhibitor SB203580 in a model of spinal nerve ligation (Schafers et al., 2003) and p38 inhibitor SD-169 in a model of sciatic nerve crush (Myers et al., 2003).

However, despite the promising potential of MAPK inhibitors, their risk-benefit ratio remains controversial. This is because inhibition of MAPKs, which are involved in a multitude of physiological processes, may lead to...
detrimental side effects. Inhibition of the ERK-mediated pathway (regulator of growth factor signalling) and JNK-mediated pathway (modulator of cell proliferation, differentiation and apoptosis) may cause general cytotoxicity. For instance, inhibition of the p38 MAPK pathway blocks chondrocyte differentiation (Jin et al., 2006; Li et al., 2010; Li et al., 2009). A careful screening will thus have to be done using MAPK inhibitors to treat IDD in order to rule out a potential anti-anabolic or toxic behaviour.

Summary

Based on existing information, NF-κB and MAP kinases appear to be potentially ideal therapeutic targets to treat IDD. P38 MAPK inhibition, either via natural compounds (e.g. curcumin, triptolide) or synthetic compounds (e.g. SB202190, PD38059, SB203580), was shown to reduce inflammatory and catabolic responses in intervertebral disc cells and ameliorate stress-induced loss of matrix anabolism. Reduction of NF-κB, either via genetic or pharmacologic intervention, also mitigates age-associated disc matrix proteoglycan loss. However, because these signalling pathways regulate a large number of genes in cellular response to a variety of stressors, general non-discriminating inhibition of these pathways may produce unanticipated deleterious side effects, such as general toxicity, cell proliferation, or inhibition of anabolism and differentiation. Therefore, much more basic research is needed to gain a better understanding of these complex pathways and how their interactions determine the outcome of IDD treatment through inhibiting these pathways. In fact, we are only beginning to explore the pathways that regulate intervertebral disc homeostasis, degeneration and inflammation. A number of important questions still remain unanswered. It is still unclear whether NF-κB or MAPK activation levels correspond to the different stages of IDD, which physiologic stressors trigger their activation, and how disc matrix homeostasis is affected as the result of such activation. Future research will also have to address the distinct roles of specific MAPK isoforms, as well as the complex interplay of the reviewed signalling pathways. These and other insights will be crucial for identifying specific therapeutic molecular targets within these pathways in order to minimise the toxic side effects in the treatment of IDD through inhibiting these pathways.

Acknowledgements

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Discussion with Reviewers
Reviewer I: Would there ever be any use of inhibitors of intracellular signalling pathways such as NFκB, in targeting disease, given their usually crucial role in other processes? Authors: The authors believe that NF-κB and/or MAP kinases are potentially promising drug targets, at least for diseases which are characterised by aberrant activation of these pathways, e.g. certain types of cancer or inflammatory diseases. Abnormal hyperactivation of these intracellular signalling pathways in diseased IVD tissue of patients with discogenic back pain needs to be confirmed prior to contemplating the use of inhibitors of these pathways as therapeutic treatment. However, in order to minimise potential side effects that would almost certainly occur with systemic delivery, methods for targeted delivery (i.e. limiting the action to the desired tissue, in this case the IVD) are needed. Furthermore, efficacy and safety need to be assessed and ascertained by tightly controlling the duration and magnitude of inhibition (e.g. by using an appropriate regulated slow release system).
Reviewer II: Many of the therapeutic agents targeted for inhibition of transcription factors have not been successful in clinical trials, primarily due to their diverse effects in various cells and situations. Do the authors believe that these agents will benefit patients with IVD disease? If so, what is the potential therapeutic targets and delivery methods?

Authors: As NF-κB or MAP kinases play essential roles in inflammatory responses, these signalling pathways may be relevant therapeutic targets. In fact, many drugs that have been used for decades have most recently been shown to interfere with these signalling pathways, e.g. glucocorticoids or acetylsalicylic acid. In addition, much research effort has been undertaken to develop novel NF-κB or MAPK inhibitors. However, a general inhibition of NF-κB or MAPK signalling when treating inflammation-related diseases such as IDD could cause undesirable, detrimental side effects in many other tissues or organs. Disc tissue-specific delivery system might be necessary to control the respective signalling pathway within the IVD, while not affecting other tissues. Thus, IVD-specific application of inhibitors, e.g. by intradiscal injection or via gene therapy approaches, may enable such a cell specific molecular intervention. Despite ongoing clinical trials that use either NF-κB (e.g. by PS-1145, SPC-839 or SC-514) or p38 MAPK (SB-681323, RO4402257, PH-797804) inhibitors for various diseases, such as atopic dermatitis and rheumatoid arthritis, many fundamental aspects will need to be evaluated both in *in vitro* studies and in appropriate animal models before these approaches become realistic therapeutic options for IDD patients. Despite these challenges, the NF-κB and MAPK signalling pathways remain the most relevant and promising therapeutic targets for inflammation-related diseases such as IDD.