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Protein kinase Cα and ζ regulate nitric oxide-induced NF-κB activation that mediates cyclooxygenase-2 expression and apoptosis but not dedifferentiation in articular chondrocytes☆

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Abstract

Nitric oxide (NO) regulates differentiation, survival, and cyclooxygenase (COX)-2 expression in articular chondrocytes. NO-induced apoptosis and dedifferentiation are mediated by p38 kinase activity and p38 kinase-independent and -dependent inhibition of protein kinase C (PKC)α and ζ. Because p38 kinase also activates NF-κB, we investigated the functional relationship between PKC and NF-κB signaling and the role of NF-κB in apoptosis, dedifferentiation, and COX-2 expression. We found that NO-stimulated NF-κB activation was inhibited by ectopic PKCα and ζ expression, whereas NO-stimulated inhibition of PKCα and ζ activity was not affected by NF-κB inhibition. Inhibition of NO-induced NF-κB activity did not affect inhibition of type II collagen expression but did abrogate COX-2 expression and apoptosis. Taken together, our results indicate that NO-induced inhibition of PKCα and ζ activity is required for the NF-κB activity that regulates apoptosis and COX-2 expression but not dedifferentiation in articular chondrocytes.
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Keywords: Nitric oxide; Protein kinase C; NF-κB; Apoptosis; Dedifferentiation; COX-2

Nitric oxide (NO) produced via inducible NO synthase in articular chondrocytes plays a central role in cartilage diseases such as osteoarthritis and rheumatoid arthritis. NO causes cartilage destruction by inducing apoptosis, loss of differentiated phenotype (i.e., dedifferentiation), and inflammatory responses such as cyclooxygenase (COX)-2 expression and prostaglandin E_2(PGE_2) production in articular chondrocytes [1–4].

Our previous studies in primary articular chondrocytes indicated that NO caused apoptosis and dedifferentiation, which are mediated by mitogen-activated protein (MAP) kinase subtypes extracellular signal-regulated protein kinase (ERK) and p38 kinase [5]. These MAP kinases play opposing roles, with activated ERK-1/-2 inducing dedifferentiation and inhibiting NO-induced apoptosis, while p38 kinase signaling maintains the differentiated status and induces apoptosis. We also demonstrated that p38 kinase activity induced apoptosis via p53 accumulation through NF-κB-dependent transcription and stabilization by serine-15 phosphorylation [6]. In addition to MAP kinase signaling, NO also causes inhibition of protein kinase C (PKC)α and ζ activity that is required for NO-induced apoptosis and dedifferentiation [7]. Inhibition of PKCα activity occurs through inhibition of its expression independent of MAP kinase signaling, while PKCζ activity is inhibited by p38 kinase activation that follows proteolytic cleavage by caspase-3 [7].

Although it is clear that p38 kinase activates NF-κB, which is required for NO-induced apoptosis, it is not known whether inhibition of PKCα and ζ activity is linked to NF-κB activation, nor is it known whether NF-κB regulates NO-induced dedifferentiation, COX-2

☆ *Abbreviations:* COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated protein kinase; IKK, IκB kinase; MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; PGE_2, prostaglandin E_2; PKC, protein kinase C; SNP, sodium nitroprusside.

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expression, and subsequent PGE_2 production. Therefore, in the present study we investigated the functional relationship between PKC and NF-κB signaling, and the role of NF-κB in apoptosis, dedifferentiation, and COX-2 expression. We report that inhibition of PKCα and ζ is necessary for NF-κB activation that regulates NO-induced apoptosis and COX-2 expression, but not dedifferentiation, in articular chondrocytes.

Materials and methods

Culture of articular chondrocytes. Articular chondrocytes were isolated from joint cartilage of 2-week-old New Zealand white rabbits, as described previously [8]. Briefly, chondrocytes were dissociated from cartilage slices using 0.2% collagenase type II and plated on culture dishes at 5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Medium was replaced every two days after seeding and cells were confluent after approximately 5 days in culture. Day 3 or 4 cultures were treated with the indicated pharmacological reagents, such as SN-50 peptide (Biomol, Plymouth Meeting, PA), for 1 h prior to treatment with the NO donor sodium nitroprusside (SNP). In some experiments, chondrocytes at day 3 were transfected with dominant negative forms of IKKα or IKKβ or infected with either control adenovirus or adenovirus coding for wild-type PKCα or ζ, as described previously [6,7]. Infected cells were cultured for 24 h and then treated with 1 mM SNP for an additional 24 h.

Assay of caspase-3 and apoptosis. We have previously shown that NO-induced death of articular chondrocytes is due to apoptosis [5]. In this study, apoptotic cells were quantified by analyzing 1×10^4 to 2×10^4 cells on a FACSort flow cytometer using the Cellquest analysis program (Becton) Dickinson, Mountain View, CA). Activation of caspase-3 was determined by measuring absorbance of the cleaved caspase-3 synthetic substrate Ac–Asp–Glu–Val–Asp–chromophore *p*-nitroaniline, using a Clontech A ApoAlert CPP32 colorimetric assay kit [5].

NF-κB reporter gene assay. Activation of NF-κB was determined indirectly by examining IκB degradation using Western blot analysis or directly using a reporter gene assay. For the reporter gene assay, chondrocytes were transfected with a plasmid containing the luciferase coding region and three tandem repeats of the serum response element. Following incubation in complete medium for 24 h, cells were left untreated or treated with the indicated pharmacological reagents and luciferase activity was determined using an assay kit (Promega, Madison, WI) [6].

Immunoprecipitation and kinase assays. Cell lysates were prepared in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, protease inhibitors [10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride], and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). Cell lysates were precipitated with antibodies against PKCα (BD Transduction Laboratories, Lexington, KY) or PKCζ (Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were collected using protein A–Sepharose beads and kinase reactions were performed in 20 μl reaction buffer containing 25 mM Tris–HCl (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, [γ-^{32}P]ATP, and 1 μg myelin basic protein (MBP). Phosphorylation of MBP was determined using autoradiography [7].

PGE_2 assay. PGE_2 production in articular chondrocytes was determined by using a PGE_2 assay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Briefly, chondrocytes were seeded in standard 96-well microtiter plates at 2×10^4 cells/well. Following addition of the indicated pharmacological reagents, total cell lysate was used to quantify the amount of PGE_2, according to the manufacturer's protocol. PGE_2 levels were calculated using a PGE_2 standard curve.

Western blot analysis. Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and protease and phosphatase inhibitors (see above). Proteins were size-fractionated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following antibodies were used: anti-type II collagen (Chemicon, Temecula, CA), anti-p53 (New England Biolabs, Beverly, MA), anti-PKCα and -PKCζ (BD Transduction Laboratories), anti-COX-2 (Cayman Chemical, Ann Arbor, MI), and anti-IKKα and -IKKβ (Cell Signaling, Beverly, MA). Blots were developed using a peroxidase-conjugated secondary antibody and a chemiluminescence system.

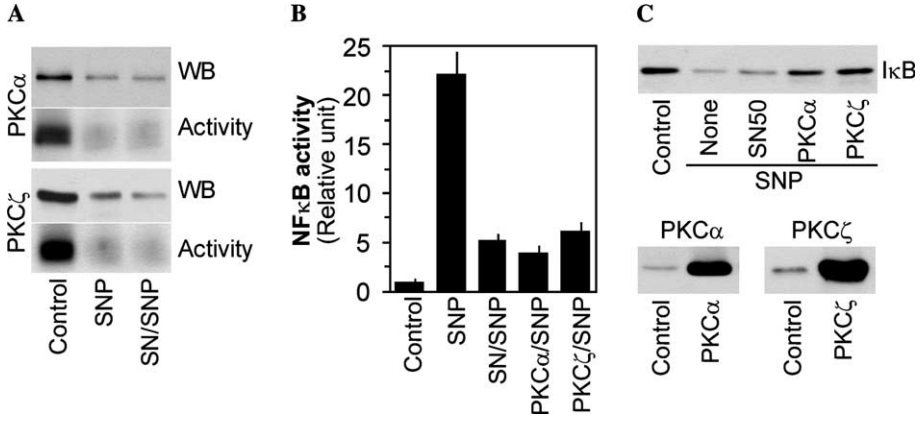

Fig. 1. Inhibition of PKCα and ζ is required for NO-induced NF-κB activation. (A) Articular chondrocytes were left untreated (Control) or were treated with 1 mM SNP alone (SNP) or SNP plus 50 μg/ml SN-50 peptide (SN/SNP) for 24 h. Expression and activity of PKCα and ζ were determined by Western blotting and in vitro kinase assay, respectively. (B,C) Articular chondrocytes were untreated (Control) or treated with 1 mM SNP alone (SNP) or SNP plus 50 μg/ml SN-50 peptide (SN50/SNP) for 24 h. Alternatively, chondrocytes were infected with adenovirus coding for wild-type PKCα (PKCα/SNP) or PKCζ (PKCζ/SNP), cultured in complete medium for 24 h, and treated with 1 mM SNP for 24 h. NF-κB activity in (B) was determined by reporter gene assay, while IκB, PKCα, and PKCζ protein levels in (C) were determined by Western blot analysis. The data represent either results of typical experiments (A,C) or mean and SD (B) ($n = 4$).

Results

NO-induced inhibition of PKCα and ζ is required for NF-κB activation

The addition of the NO donor SNP to primary articular chondrocyte cultures was shown to inhibit PKCα and ζ activity (Fig. 1A) and activate NF-κB (Fig. 1B), which is consistent with our previous reports [6,7]. We further investigated the relationship between PKC and NF-κB signaling. NO-induced inhibition of PKCα and ζ activity was not affected by the addition of 50 μg/ml SN-50 peptide (Fig. 1A), which inhibits NF-κB activation, as determined by reporter gene assay (Fig. 1B). However, ectopic expression of PKCα or ζ by adenovirus infection, a condition that prevents NO-induced inhibition of PKCα and ζ activity [7], inhibited NO-induced NF-κB activation, as determined by both reporter gene (Fig. 1B) and IκB degradation (Fig. 1C) assays. These data indicate that NO-induced inhibition of PKCα and ζ activity is required for NF-κB activation in articular chondrocytes.

NF-κB regulates NO-induced apoptosis and COX-2 expression but not dedifferentiation

Because both inhibition of PKCα and ζ and activation of NF-κB are necessary for NO-induced apoptosis [6,7], we investigated whether inhibition of PKCα and ζ regulates NO-induced apoptosis via NF-κB signaling. Ectopic expression of PKCα or ζ, which inhibited NO-induced NF-κB activation, blocked NO-induced apoptosis (Fig. 2A). NO-induced apoptosis was also blocked by direct inhibition of NF-κB activation by SN-50 peptide (Fig. 2A). Inhibition of apoptosis was accompanied by inhibition of upstream apoptotic events, such as accumulation of p53 (Fig. 3C) and activation of caspase-3 (Fig. 3B). Similar to the effects on apoptosis, NO-induced COX-2 expression (Fig. 3A) and subsequent PGE_2 production (Fig. 3B) were blocked by direct inhibition of NF-κB with SN-50 peptide, or by indirect inhibition using ectopic expression of PKCα or ζ. These data indicate that NO inhibition of PKCα and ζ regulates NO-induced apoptosis and inflammatory responses (i.e., COX-2 expression and PGE_2 production) through NF-κB activation.

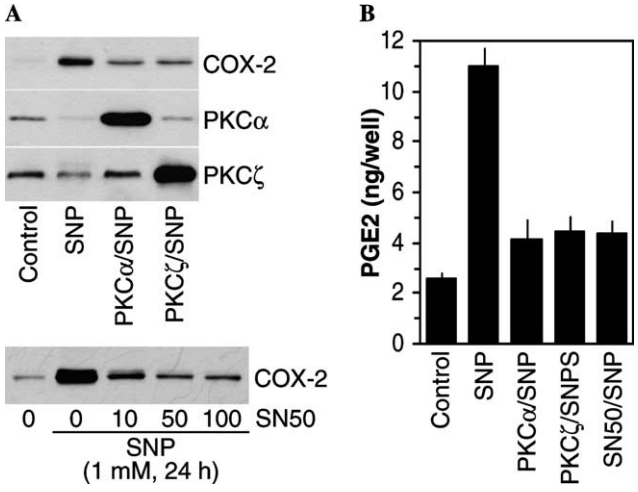

Fig. 3. PKCα and ζ regulate NO-induced COX-2 expression and PGE_2 production via NF-κB signaling. Articular chondrocytes were infected with control adenovirus or adenovirus coding for wild-type PKCα or ζ. Cells were cultured in complete medium for 24 h and then left untreated or treated with 1 mM SNP for an additional 24 h. Alternatively, chondrocytes were untreated (control) or treated with 1 mM SNP for 24 h with the indicated concentrations (μg/ml) (A) or 50 μg/ml (B) SN-50 peptide. Expression of COX-2, PKCα, and PKCζ (A), and PGE_2 production (B) were determined as described in Materials and methods. The data represent either typical results (A) or means and SD (B) ($n = 4$).

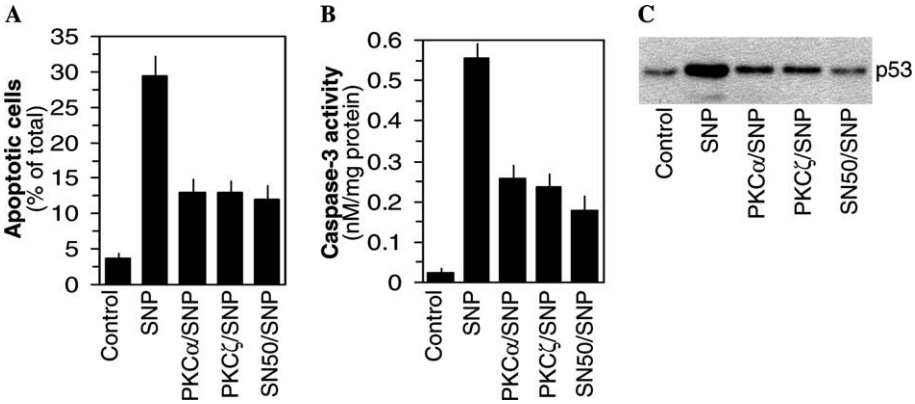

Fig. 2. PKCα and ζ regulate NO-induced apoptosis via NF-κB signaling. Articular chondrocytes were left untreated (Control) or were treated with 1 mM SNP alone (SNP) or with SNP plus 50 μg/ml SN-50 peptide (SN50/SNP) for 24 h. Alternatively, chondrocytes were infected with adenovirus coding for wild-type PKCα (PKCα/SNP) or PKCζ (PKCζ/SNP), cultured in complete medium for 24 h, and treated with 1 mM SNP for 24 h. Apoptotic cells (A), caspase-3 activity (B), and p53 protein levels (C) were determined as described in Materials and methods. The data represent either means and SD (A,B) ($n = 4$) or are results of typical experiments (C).

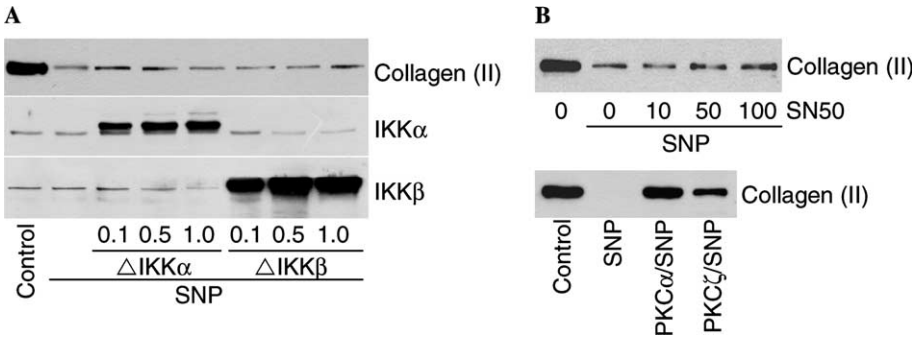

Fig. 4. PKCα and ζ regulate NO-induced dedifferentiation independent of NF-κB signaling. (A) Articular chondrocytes were transfected with control vector or vector coding for dominant negative IKKα or IKKβ. Following incubation in complete medium for 24 h, cells were treated with 1 mM SNP for a further 24 h. (B) Chondrocytes were untreated as a control or treated with 1 mM SNP for 24 h with the indicated concentrations (μg/ml) of SN-50 peptide (upper panel). Alternatively, chondrocytes were infected with control adenovirus or adenovirus coding for wild-type PKCα or ζ. Following incubation in complete medium for 24 h, cells were treated with 1 mM SNP for an additional 24 h (lower panel). Expression of type II collagen, IKKα and IKKβ was determined by Western blot analysis. The data represent results of typical experiments.

Type II collagen expression is a marker for dedifferentiation of articular chondrocytes [8]. In contrast to apoptosis and COX-2 expression, NO inhibition of type II collagen expression was not affected by inhibition of NF-κB caused by transient expression of dominant negative IκB kinases (IKKα and IKKβ) (Fig. 4A) or by SN-50 (Fig. 4B). However, ectopic expression of PKCα and ζ prevented NO inhibiting type II collagen expression (Fig. 4B), indicating that chondrocyte dedifferentiation caused by inhibition of PKCα and ζ is independent of NF-κB signaling.

The above results indicating that NO inhibition of PKCα and ζ is required for NF-κB activation, and that NF-κB mediates apoptosis, COX-2 expression, and PGE_2 production, but not dedifferentiation, in articular chondrocytes, are summarized schematically in Fig. 5.

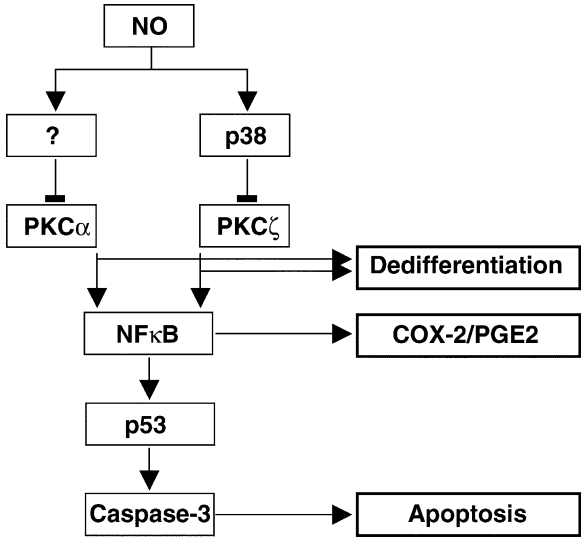

Fig. 5. Schematic summary of a signaling pathway describing NO-induced apoptosis, COX-2 expression, and dedifferentiation in articular chondrocytes. See text in Discussion for details.

Discussion

We characterized signaling pathways involved in NO-induced dedifferentiation, apoptosis, and COX-2 expression in articular chondrocytes, focusing on the functional relationship between PKC and NF-κB signaling. Based on results obtained in this study and our previous reports [5–7] we propose the following pathway: NO activation of p38 kinase inhibits PKCζ activity and NO also inhibits PKCα activity independent of MAP kinase signaling. This inhibition of PKCα and ζ is necessary for NO-induced dedifferentiation and is also required for NF-κB activation. Activated NF-κB has dual functions, one being induction of COX-2 expression and subsequent PGE_2 production, and the other being induction of apoptosis by stimulating p53 transcription (see diagram in Fig. 5).

Our previous study [6] indicated that NO activation of p38 kinase activates NF-κB through IKK, based on the observation that expression of dominant negative IKKα or β inhibited p38 kinase-induced NF-κB activation. We demonstrate in this study that inhibition of PKCζ and α is required for NF-κB activation. While the involvement of PKC isoforms in NF-κB activation is well known, previous studies indicated that activation of PKC isoforms is required [9–12]. For instance, PKCζ is known to stimulate IKKβ to activate NF-κB [12]. Therefore, our observation that PKCα and ζ activity prevents NO activation of NF-κB in articular chondrocytes is the first report indicating a negative role of PKC in NF-κB activation. Although the mechanisms by which PKCα and ζ inhibit NF-κB activation were not investigated in this study, it may be that PKC isoforms inhibit either IKK or its upstream signaling molecules to block NO-induced NF-κB activation. Regarding NF-κB activity, the results of the present study demonstrate that activated NF-κB mediates NO-induced COX-2

expression, subsequent PGE_2 production, and apoptosis, but not dedifferentiation, in chondrocytes.

Arthritic joints produce large amounts of prostaglandin via COX-2 [1–4]. Our data indicating that NF-κB regulates COX-2 expression are consistent with the structure of the *COX-2* gene promoter, which contains κB binding sites [13–15]. In addition to transcriptional regulation, COX-2 expression also appears to be regulated at post-transcriptional levels, since p38 kinase activity stabilizes COX-2 mRNA [16–18]. Therefore, it appears that NO-stimulated p38 kinase activity regulates COX-2 expression at both transcriptional (via NF-κB activation) and post-transcriptional (via stabilization of mRNA) levels. In addition to induction of COX-2 expression, NO-stimulated NF-κB activity is also required for induction of apoptosis caused by inhibition of PKCα and ζ. This is based on the observation that ectopic expression of PKCα and ζ blocked both NF-κB activation and apoptosis. The requirement for NF-κB activity in apoptosis is consistent with p53 transcription and subsequent activation of caspase-3 [6].

Unlike regulation of COX-2 expression and apoptosis, our current results indicated that dedifferentiation of chondrocytes caused by inhibition of PKCα and ζ is independent of NF-κB activation. This was concluded on the basis of the observation that inhibition of NF-κB did not affect dedifferentiation, whereas ectopic expression of PKCα and ζ blocked NO-induced dedifferentiation. We considered the possibility that NO-induced COX-2 expression regulates apoptosis and dedifferentiation, because COX-2-mediated PGE_2 production is known to regulate differentiation [19–21] and apoptosis [22,23] in chondrocytes, depending on the experimental system. However, we did not observe any modulation of apoptosis or dedifferentiation by inhibiting COX-2 activity (data not shown). Therefore, further study is necessary to elucidate the mechanisms involved in the NF-κB-independent regulation of NO-induced dedifferentiation in articular chondrocytes.

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