



Poly(ADP-ribose) polymerase-1-induced NAD⁺ depletion promotes nuclear factor- κ B transcriptional activity by preventing p65 de-acetylation

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ABSTRACT

NF- κ B is a transcription factor that integrates pro-inflammatory and pro-survival responses in diverse cell types. The activity of NF- κ B is regulated in part by acetylation of its p65 subunit at lysine 310, which is required for transcription complex formation. De-acetylation at this site is performed by sirtuin 1 (SIRT1) and possibly other sirtuins in an NAD⁺ dependent manner, such that SIRT1 inhibition promotes NF- κ B transcriptional activity. It is unknown, however, whether changes in NAD⁺ levels can influence p65 acetylation and cellular inflammatory responses. Poly(ADP-ribose)-1 (PARP-1) is an abundant nuclear enzyme that consumes NAD⁺ in the process of forming (ADP-ribose)polymers on target proteins, and extensive PARP-1 activation can reduce intracellular NAD⁺ concentrations. Here we tested the idea that PARP-1 activation can regulate NF- κ B transcriptional activity by reducing NAD⁺ concentrations and thereby inhibiting de-acetylation of p65. Primary astrocyte cultures were treated with the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to induce PARP-1 activation. This resulted in sustained acetylation of p65 and increased NF- κ B transcriptional activity as monitored by a κ B-driven eGFP reporter gene. These effects of MNNG were negated by a PARP-1 inhibitor, in PARP-1^{-/-} cells, and in PARP-1^{-/-} cells transfected with a catalytically inactive PARP-1 construct, thus confirming that these effects are mediated by PARP-1 catalytic activity. The effects of PARP-1 activation were replicated by a SIRT1 inhibitor, EX-527, and were reversed by exogenous NAD⁺. These findings demonstrate that PARP-1-induced changes in NAD⁺ levels can modulate NF- κ B transcriptional activity through effects on p65 acetylation.

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1. Introduction

NF- κ B is a transcription factor that integrates pro-inflammatory and pro-survival responses in diverse cell types. NF- κ B is activated by numerous stimuli including DNA damage, which can occur as a direct secondary effect of infection, ischemia, trauma, and other stressors [1]. The NF- κ B family includes five members, p65 c-Rel, RelB, p105/p50 and p100/p52 that form homo- or heterodimers [2]. In most cell types, the most abundant heterodimer consists of p65 and p50. The regulation of NF- κ B activity is complex, and occurs at multiple levels [2]. One level of regulation in canonical NF- κ B activation involves the release of p65/p50 dimers from the I κ B subunit in the cytosol, and subsequent translocation to the nucleus. A second

layer of regulation occurs within the nucleus, with the formation of an active transcription complex. p300/CBP mediated acetylation of various lysine sites (at least 5 sites reported) of the p65 subunit has been established as a key regulatory event in this process. In particular, acetylation at lysine 310 of p65 is required for full transcriptional activity of p65, while acetylation at lysine 218/221 enhances DNA binding and impairs assembly with I κ B α [3,4]. Acetylation of these lysine sites can be reversed by histone deacetylase (HDAC) corepressor proteins [5,6]. Deacetylation of p65 lysine 218/221 does not require NAD, but Lysine 310 of p65 is deacetylated in the nucleus by the atypical class III HDACs, SIRT1 and possibly other sirtuins in a reaction that requires NAD⁺ [3,4,7,8].

NAD⁺ is also required by the abundant nuclear enzyme, poly(ADP-ribose) polymerase-1 (abbreviated as PARP-1). Upon activation by DNA damage, cytokines, or other signals, PARP-1 consumes NAD⁺ to generate branched ADP-ribose polymers on chromatin and other acceptor proteins [9,10]. These polymers have diverse functions in DNA repair and other nuclear processes [11,12]. Extensive PARP-1 activation can deplete the cytosolic (non-mitochondrial) NAD⁺ pool ([13–16]. PARP-1 also regulates NF- κ B activity [17,18], but there remains uncertainty as to the role of PARP-1 activity in this process. Several studies

Abbreviations: BSS, balanced salt solution; HDAC, histone deacetylases; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; PAR, poly(ADP-ribose); PARP-1, poly(ADP-ribose) polymerase-1; SIRT1, sirtuin 1

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indicate that inhibition of PARP-1 enzymatic activity impairs NF- κ B-mediated responses [19–24], whereas others have found that PARP-1 influences NF- κ B-mediated responses independent of its enzymatic activity [17,25–27].

Given that both SIRT1 and PARP-1 require NAD⁺, one mechanism by which PARP-1 activation could influence NF- κ B activity is by reducing the amount of NAD⁺ available for SIRT1 de-acetylation of p65 [28]. Here we tested this hypothesis using primary astrocyte cultures in which PARP-1 activation was induced by the DNA alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Astrocytes are experimentally convenient because they are non-neoplastic cells that can be prepared in homogeneous cultures, and because prior work has established key aspects of PARP-1 activation and regulation in this cell type. Results of the present studies confirm that extensive PARP-1 activation promotes NF- κ B transcriptional activation by a mechanism involving p65 acetylation.

2. Materials and methods

2.1. Materials

Cell culture reagents were obtained from Cellgro/Mediatech (Herdon, VI). 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2h)-isoquinolinone (DPQ) was obtained from Calbiochem (San Diego, CA). All other reagents were from Sigma/Aldrich (St. Louis, MO) except where otherwise stated.

2.2. Cell cultures

All animal studies were approved by the San Francisco Veterans Affairs Medical Center animal studies committee and follow the NIH guidelines for humane care of animals. PARP-1^{-/-} mice were obtained from the Jackson Labs and out bred for more than 10 generations to the CD1 background. Wild type mice were from the closely related Swiss Webster strain and were obtained from Simonsen Labs (Gilroy, CA). Astrocyte cultures were prepared from mouse pups of both sexes in 24-well plates as described previously [29]. At confluency (12–14 days in vitro), the cultures were treated with 22 μ M cytosine β -D-arabinofuranoside for 2 days to inhibit the proliferation of microglia. The cultures were subsequently maintained in Eagle's minimal essential medium (MEM) supplemented with 3% fetal bovine serum (FBS).

2.3. Cell culture experiments

Studies were initiated by placing the astrocyte cultures in a physiological balanced salt solution (BSS) containing 3.1 mM KCl, 134 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 0.5 mM KH₂PO₄, 15.7 mM NaHCO₃ and 2 mM glucose, pre-equilibrated to pH 7.2 in a 5% CO₂ atmosphere. Experiments were performed at 37 °C in 5% CO₂ with test compounds prepared as concentrated stock solutions in BSS. Stimulations with 100 μ M MNNG for 10 min were terminated by complete medium exchange and replacement with BSS. Control wells received medium exchanges only.

2.4. Immunostaining

Fixation and immunostaining of cell cultures were performed as previously described [23]. Incubations were performed with a 1:1000 dilution of rabbit anti-poly(ADP-ribose) (Trevigen, Gaithersburg, MD), or with 1:30 dilution of rabbit polyclonal anti-NF- κ B p65 (Cell Signaling Technology, Inc., Danvers, MA) at 4 °C for 24 h, followed by incubation with Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR), 1:500 dilution, for 2 h. For evaluation of NF- κ B nuclear translocation, nuclei were stained with propidium iodide. Confocal photomicrographs were obtained from single layer scan with pinhole

of 1 Airy (~100 μ m). Controls prepared in the absence of primary antibody showed no staining under the conditions described (not shown).

2.5. Western blotting

Western blots from whole cell samples were prepared and quantified as described [23]. Membranes were incubated with 1:200 dilutions of rabbit anti-NF- κ B Ac-p65 (acetyl K310; Abcam, Inc. Cambridge, MA), 1:2500 dilution of rabbit polyclonal anti-NF- κ B (p65; Calbiochem/EMD Bioscience, Inc., La Jolla, CA) or with a 1:500 dilution of mouse monoclonal anti-poly(ADP-ribose) (clone 10HA; Trevigen, Gaithersburg, MD). After washing, the membranes were incubated for 2 h with peroxidase-conjugated anti-rabbit or anti mouse IgG (Vector Laboratories, Burlingame, CA) diluted 1:7500. The protein bands were visualized using ECLTM Plus WB Detection kit (Amersham-Pharmacia Biotech) and X-OMAT AR film (Kodak). To quantify protein loading, the membranes were re-probed with mouse monoclonal anti β -actin at a 1:10,000 dilution, followed by peroxidase-conjugated anti-mouse IgG (1:10,000 dilution). Controls performed in the absence of primary or secondary Abs showed no signal (data not shown). Band densities were quantified with the NIH Image J program.

2.6. Cell transfections

Wild-type and mutant human PARP-1 constructs were prepared as previously described [30]. PARP-1^{-/-} mouse cells were transfected at 8–9 days in vitro, at about 90% confluency, using Lipofectamine 2000 (Invitrogen) at a 2:1 ratio with DNA. Cells were used for experiments 40–48 h after transfection. The lentivirus κ B-dEGFP construct was prepared as described previously [31] and astrocytes were treated with the lentivirus 2–3 days prior to experiments. eGFP expression was evaluated by fluorescence microscopy at the designated time points after MNNG exposure by counting the number of eGFP-expressing cells in 5 randomly selected fields within each culture well.

2.7. Statistics

Each "n" denotes an independent experiment comprised of 3–4 parallel treatments per condition. Results are presented as a mean \pm standard error. Statistical significance was evaluated by one-way ANOVA followed by the Dunnett's test for multiple comparisons against a common control group.

3. Results

3.1. PARP-1 inhibition prevents NF- κ B p65 acetylation

Astrocyte cultures treated for 10 min with the alkylating agent MNNG (100 μ M) developed a robust activation of PARP-1 (Fig. 1). PAR immunoreactivity appears as a smear in these Western blots, indicating poly(ADP-ribosylation) of many different protein targets [32,33]. This signal was completely blocked by the addition of the PARP inhibitor DPQ, but was unaffected by the addition of NAD⁺ (Fig. 1). MNNG treatment also induced acetylation of p65 NF- κ B subunit (Fig. 2A). The increase in p65 acetylation was blocked by both pharmacological PARP inhibition (DPQ) and by genetic PARP-1 deletion (Fig. 2B, C, D).

3.2. NAD⁺ influences NF- κ B acetylation

Our prior studies have established that MNNG-induced PARP-1 activation under the conditions of these experiments causes a rapid and sustained depletion of cytosolic NAD⁺ (from 9.51 \pm 0.72 to 2.85 \pm 0.93 nmol/mg protein) [14]. We also previously demonstrated that this depletion can be reversed by medium supplementation with 2.5 mM NAD⁺, which gains entry into astrocytes through

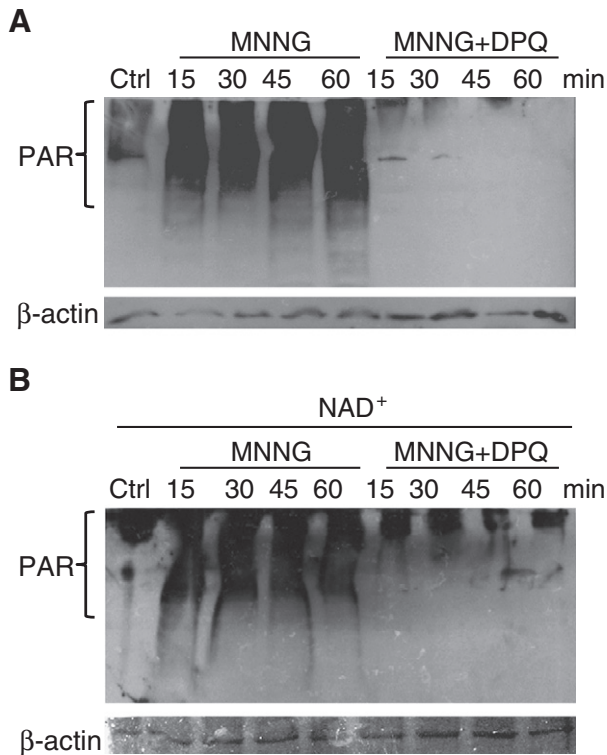


Fig. 1. MNNG-induced PARP-1 activation is blocked by DPQ, but not NAD⁺. Immunoblots show formation of poly(ADP-ribose)-conjugated proteins (PAR). A, Ten-minute incubations with 100 μ M MNNG induce robust PAR formation that is prevented by the PARP-1 inhibitor, DPQ (25 μ M). Time points denote intervals following washout of MNNG. B, NAD⁺ (2.5 mM) does not prevent PAR formation. Representative of $n = 3$.

P2X7 receptor-gated channels [15,32,34]. Since NAD⁺ is a requisite substrate for SIRT1 de-acetylation of p65, we evaluated the possibility that PARP-1 activation increases p65 acetylation and resultant NF- κ B transcriptional activation by impairing the ability of SIRT1 or other NAD⁺-dependent sirtuins to de-acetylate p65 (Fig. 3A). Consistent with this idea, 2.5 mM NAD⁺ was found to prevent the MNNG-induced increase in p65 acetylation (Fig. 2C, D). This effect cannot be attributed to PARP-1 inhibition by NAD⁺ or NAD⁺ metabolites because NAD⁺ supplementation did not block PARP-1 activity (Fig. 1B). Moreover, NAD⁺ was found to have no effect on p65 acetylation when SIRT1 activity was blocked by 10 μ M EX-527 [35,36] (Fig. 3B).

3.3. PARP-1 enzymatic activity is required for NF- κ B transcriptional activation

Activation of NF- κ B signaling was further evaluated in astrocytes transfected with a κ B reporter gene that responds to NF- κ B (p65) transcriptional activation by expressing enhanced green fluorescent protein (eGFP) [31]. Inhibition of SIRT-1 activity with EX-527 increased eGFP expression in concert with increased p65 acetylation (Fig. 3C, D). MNNG exposure similarly increased NF- κ B transcriptional activity in wild-type astrocytes, but not in PARP-1^{-/-} astrocytes or wild-type astrocytes treated with the PARP inhibitor DPQ. MNNG also failed to induce NF- κ B activation in cultures supplemented with 2.5 mM NAD⁺ (Fig. 4A, C).

The role of PARP-1 enzymatic activity in NF- κ B activation was further evaluated by using previously developed PARP-1 mutants containing modifications at phosphorylation sites that influence enzymatic activity [30]. PARP-1^{-/-} cells transfected with wild-type PARP-1 and treated with MNNG showed eGFP expression in about 30% of the cells. Prior studies have shown comparable transfection efficiency attained with all 3 constructs used [30]. PARP-1^{-/-} cells

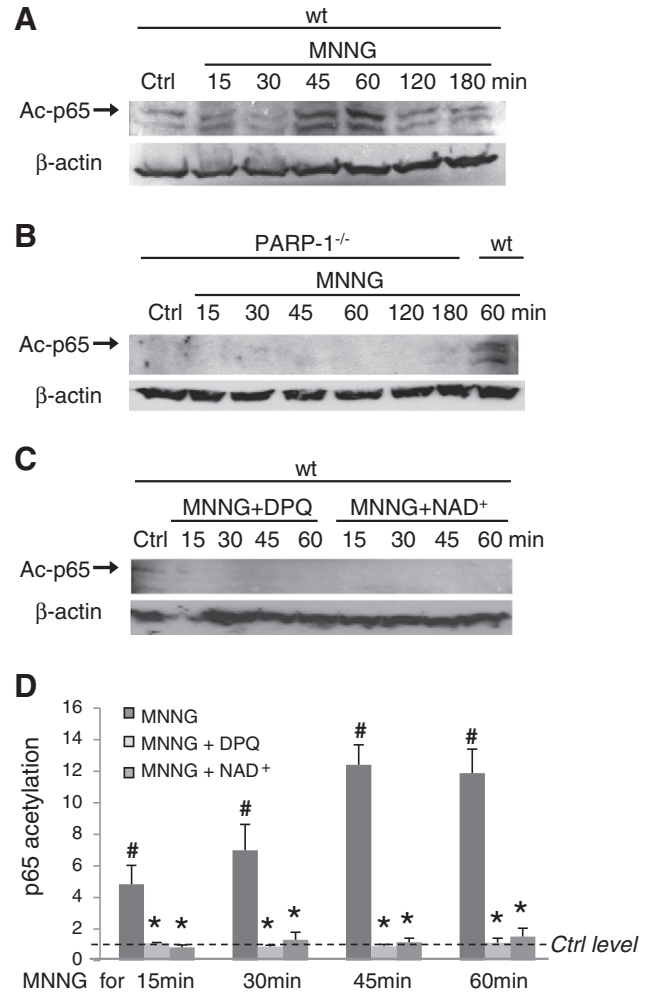


Fig. 2. NF- κ B subunit p65 acetylation is affected by PARP-1 activation and NAD⁺ levels. A, MNNG (100 μ M for 10 min) induces acetylation of NF- κ B p65 in wild-type (wt) astrocytes. B, MNNG does not induce p65 acetylation in PARP-1 deficient astrocytes. C, MNNG induced p65 acetylation in wt astrocytes is blocked by both the PARP inhibitor DPQ (25 μ M) and by medium supplementation with NAD⁺ (2.5 mM). Time points denote intervals following washout of MNNG. D, Graph shows quantified data of p65 acetylation in wt astrocytes (A and C) (# $p < 0.05$ compared to control, * $p < 0.05$ compared to MNNG at each time point, $n = 3-4$).

transfected with S372A & T373A PARP-1, which lacks phosphorylation sites required for enzymatic activation, failed to show NF- κ B activation after MNNG stimulus. Conversely, PARP-1^{-/-} cells transfected with the S372E PARP-1 mutant, which mimics constitutive phosphorylation at this site, showed increased NF- κ B activation even in the absence of MNNG stimulation (Fig. 4B, D). Additional studies confirmed that the impaired NF- κ B transcription activity in PARP-1^{-/-} cells is not due to deficient p65 protein expression (Fig. 5A), and that PARP-1 depletion does not either interfere with NF- κ B p65 nuclear translocation (Fig. 5B).

4. Discussion

These results show that extensive activation of PARP-1 can promote the transcriptional activity of NF- κ B by a mechanism involving p65 acetylation. Reversal of this effect by exogenous NAD⁺, in conjunction with previously demonstrated effects of PARP-1 and exogenous NAD⁺ on intracellular NAD⁺ levels, identify this as an NAD⁺ dependent mechanism. At present, the only enzymes known to perform NAD⁺-dependent de-acetylation of lysine residues are the class III histone de-acetylases, also known as sirtuins [37]. SIRT1 in particular can catalyze de-acetylation of the lysine 310 of p65 [31]. SIRT2 has also been reported to do so [8], but SIRT2 is not usually

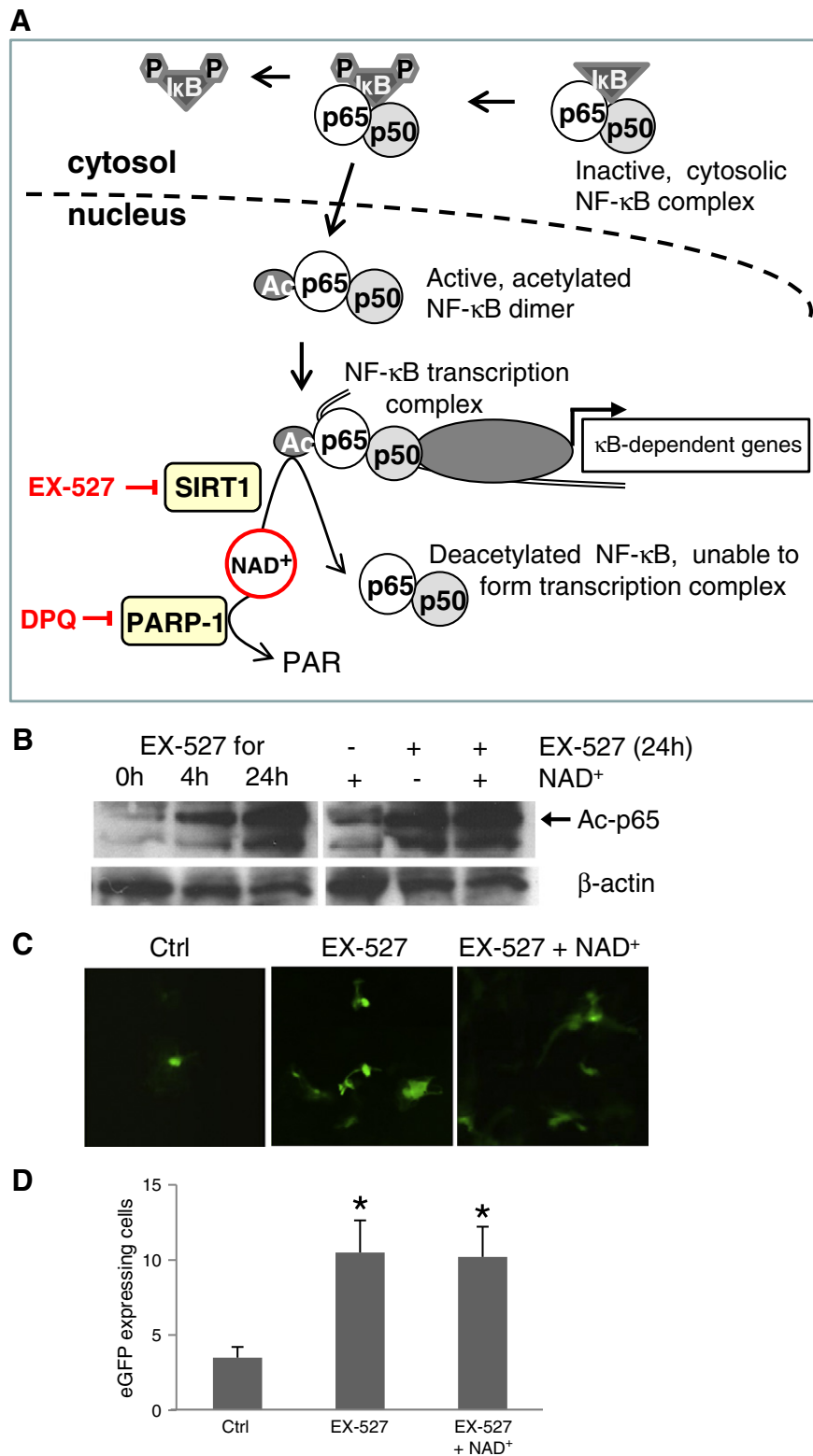


Fig. 3. NF- κ B subunit p65 acetylation is increased by SIRT1 inhibition. **A**, Diagram showing the proposed relationships between PARP-1, SIRT1, NAD⁺, and NF- κ B. NF- κ B is normally sequestered in the cytosol. In canonical NF- κ B activation [2], phosphorylation of the I κ B subunit permits the p65/p50 dimer to translocate to the nucleus, where it is acetylated and binds with other proteins to form an activated transcription complex on gene promoter regions. The activated transcription complex is normally deactivated by NAD⁺-dependent deacetylation of the p65 subunit, catalyzed by SIRT-1. NAD⁺ levels are reduced by PARP-1 activation, thereby preventing this de-acetylation step and promoting gene transcription. This effect can be negated by either PARP-1 inhibition (DPQ) or by NAD⁺ repletion. The SIRT-1 inhibitor EX-527 also blocks de-acetylation of NF- κ B, but this effect cannot be negated by NAD⁺. **B**, Astrocytes treated with the SIRT1 inhibitor EX-527 (10 μ M) show accumulation of acetylated p65, and this effect is not blocked by NAD⁺. Representative of $n = 3$. **C**, Photomicrographs show NF- κ B transcriptional activity in astrocytes transfected with a κ B reporter gene driving eGFP expression. eGFP expression is increased by EX-527, and this effect is not blocked by NAD⁺. **D**, Graph shows quantification of eGFP expression. (* $p < 0.05$, $n = 3$).

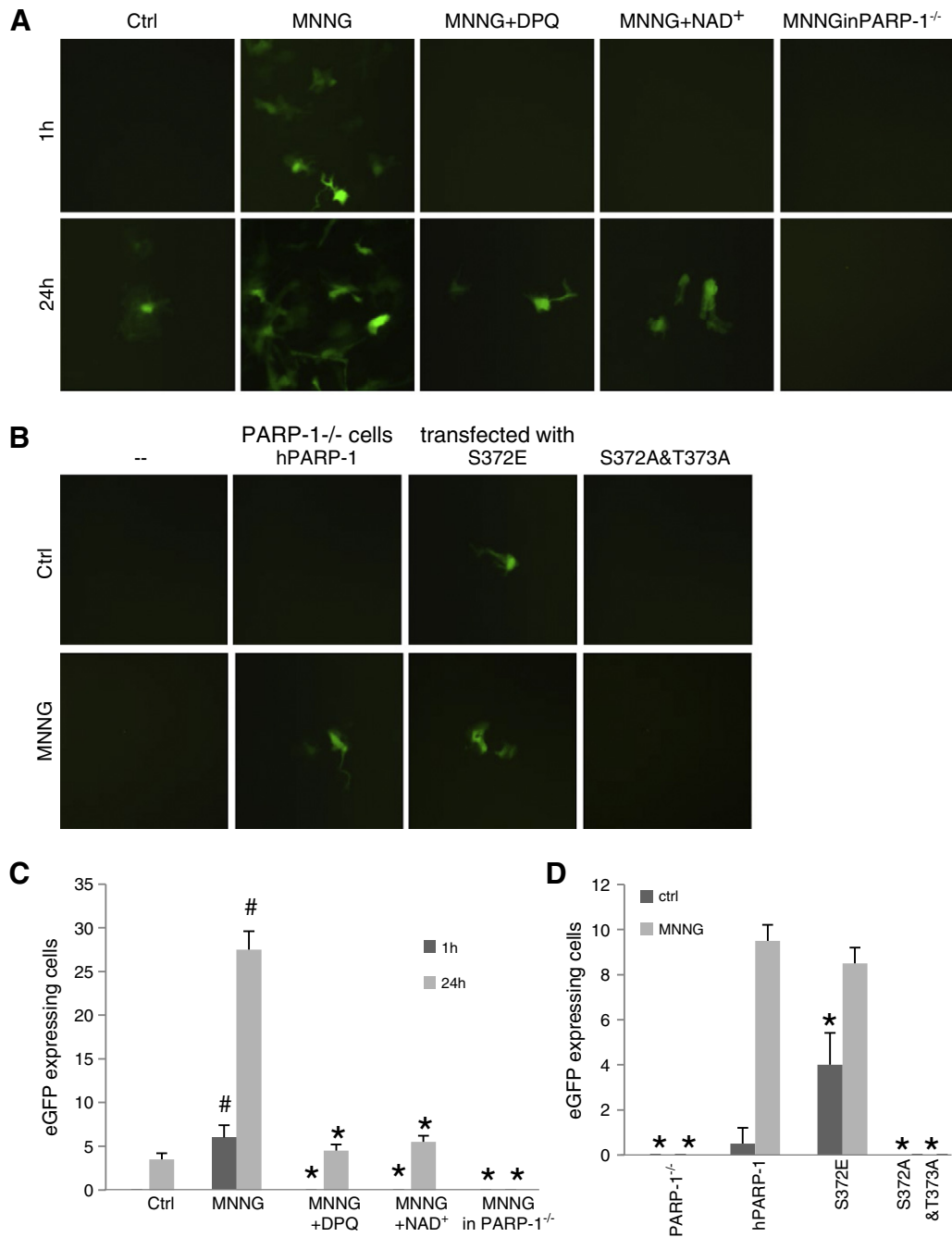


Fig. 4. PARP-1 enzymatic activity is required for NF- κ B transcriptional activation. **A**, NF- κ B transcription activity detected in astrocytes transfected with an eGFP-expressing κ B reporter gene. Photomicrographs were prepared at 1 and 24 h after MNNG exposures (100 μ M for 10 min). MNNG triggers NF- κ B activation in wild-type cells, but not in PARP-1^{-/-} cells or in wild-type cells treated with 25 μ M DPQ or 2.5 mM NAD⁺. **B**, PARP-1^{-/-} cells exhibit NF- κ B transcriptional activation when transfected normal human PARP-1 (hPARP-1) or with hPARP-1 containing the S372E phosphomimetic mutation, but not when transfected with hPARP-1 containing the S372A and T373A mutations that prevent enzymatic activation. **C** and **D**, eGFP expression quantification. For **C**, #p < 0.05 compared to control, *p < 0.05 compared to MNNG, n = 3. For **D**, *p < 0.05 compared to PARP-1^{-/-} cultures transfected with hPARP-1, n = 3. Where no bar is visible, there was no detectable eGFP expression.

localized to the nucleus. However, there is presently no way to directly evaluate in situ SIRT1 activity (other than by p65 acetylation), so we cannot exclude the possibility that NAD⁺ levels influence p65 acetylation by some other, as yet unrecognized mechanism.

PARP-1 activation not only consumes NAD⁺, but also produces nicotinamide as a product of NAD⁺ cleavage. The production of nicotinamide has been suggested as another possible way that PARP-1 and sirtuins may interact, because many sirtuins (including SIRT1) are inhibited by nicotinamide [37,38]. The expected effect of nicotinamide in this instance would be prolonged p65 acetylation and

enhanced NF- κ B transcriptional activation, as was observed here. However, the additional observation that exogenous NAD⁺ supplementation did not inhibit PARP-1 activity while was still able to reverse the effects of PARP-1 on both p65 acetylation and NF- κ B transcriptional activity argues against the idea that cleavage of NAD⁺ to nicotinamide or other metabolites is a significant regulatory factor under these conditions. This notion is further supported by our previously published data, in which NAD⁺ supplementation (5 mM) failed to increase intracellular levels required for PARP-1 inhibition [15].

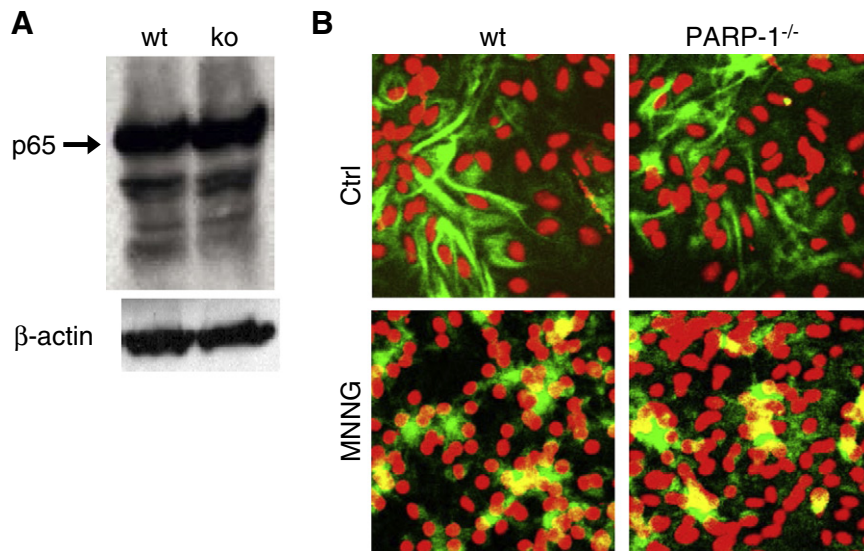


Fig. 5. PARP-1 depletion does not affect NF- κ B p65 expression or nuclear translocation. A, Immunoblots showing p65 expression in wild-type and PARP^{-/-} cells. B, Confocal images demonstrate NF- κ B p65 subunit nuclear translocation 30 min after MNNG stimulation (100 μ M for 10 min) in both wild-type and PARP-1^{-/-} cells. Representative of $n = 3$.

Canonical NF- κ B transcriptional activation requires both translocation of NF- κ B dimers to the nucleus and the subsequent formation and maintenance of a DNA-bound transcriptional complex. Under the conditions of MNNG-induced DNA damage employed here, NF- κ B translocation is likely induced by the ATM/NEMO pathway leading to I κ B phosphorylation [39–41]. The role of PARP-1 activation in NF- κ B signaling has been demonstrated in many levels, PARP-1 promotes complex formation [19,22,42], participates in DNA binding of p65 subunit [26], is involved in IKK complex activation [43], and regulates NF- κ B translocation between cytosol and nucleus [44–46]. In our model PARP-1 depletion did not prevent cytosol-to-nucleus translocation of NF- κ B (p65) but NF- κ B transcriptional activity was prevented. This finding underscores the fact that p65 nuclear translocation is not functionally equivalent to transcriptional activation. Our results point to the ability of PARP-1 to affect activity of other enzymes regulating NF- κ B signaling. This finding is consistent with the model proposed (Fig. 3A), and underscores the importance of NAD⁺ in PARP-1 mediated NF- κ B regulation.

The role of PARP-1 enzymatic activity in the regulation of NF- κ B is also addressed by these studies. Astrocytes transfected with the catalytically inactive PARP-1 construct failed to show increased NF- κ B transcriptional activity in the presence of MNNG, and wild type astrocytes treated with the PARP inhibitor, DPQ, similarly failed to show increased NF- κ B transcriptional activity. These results agree with those of several prior studies that demonstrate that catalytic PARP-1 inhibition suppresses variety of NF- κ B-mediated responses [17,20,22,23,45,47–51]. However, these results do not exclude the possibility that PARP-1 may also interact with NF- κ B by non-enzymatic mechanisms. Strong evidence for this possibility exists [46,52], including evidence for a physical association between PARP-1 and the p65 subunit [26]. Such a mechanism has already been demonstrated with p53 [53], which shares intriguing similarities with PARP-1 mediated NF- κ B regulation [45].

The present studies were performed under conditions of extensive PARP-1 activation, such as those that occur during oxidative stress or excitotoxicity. As such, they provide proof of principle that PARP-1 mediated depletion of NAD⁺, as occurs during genotoxic stress, is capable of influencing NF- κ B transcriptional activity. However, it remains to be established whether this is an active mechanism during more physiological PARP-1 activation. The SIRT1 Km for NAD⁺ is near the estimated cytosolic NAD⁺ concentration [54], suggesting that even modest levels of PARP-1 activation could influence NF- κ B transcriptional activity by

this mechanism. Recent work evaluating the effects of PARP-1 and SIRT1 on fat and muscle metabolism supports this contention [55].

5. Conclusions

PARP-1 activation can regulate NF- κ B transcriptional activity by reducing NAD⁺ concentrations and thereby inhibiting deacetylation of p65. This observation identifies a novel mechanism by which DNA damage can promote NF- κ B transcriptional activity.

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