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Poly(ADP-Ribose) Polymerase-1 Regulates Activation of Activator Protein-1 in Murine Fibroblasts

Teresa L. Andreone, Michael O’Connor, Alvin Denenberg, Paul W. Hake, and Basilia Zingarelli

Poly(ADP-ribose) polymerase (PARP)-1 is activated in response to DNA injury in the nucleus of eukaryotic cells and has been implicated in cell dysfunction in inflammation. We investigated the role of PARP-1 on the AP-1 pathway, which is involved in the signal transduction of the inflammatory process. In murine wild-type fibroblasts, oxidative challenge by peroxynitrite and hydrogen peroxide or immunological challenge by IL-1 and 20% FCS induced phosphorylation of the mitogen-activated protein kinase kinase-4, activation of c-Jun N-terminal kinase (JNK), and DNA binding of AP-1. In comparative experiments, peroxynitrite induced DNA binding of heat shock factor-1. Pretreatment of wild-type cells with 5-iodo-6-amino-1,2-benzopyrone, a PARP-1 inhibitor, inhibited JNK activation and DNA binding of AP-1. In parallel experiments in PARP-1-deficient fibroblasts, DNA binding of AP-1 was completely abolished. Activation of JNK was significantly elevated at basal condition, but it exhibited a lesser increase after oxidative or immunological challenge than in wild-type fibroblasts. Nuclear content of phosphorylated mitogen-activated protein kinase kinase-4 was observed in PARP-1-deficient cells after peroxynitrite challenge only. Western blotting analysis for AP-1 subunits indicated that c-Fos was similarly expressed in wild-type and PARP-1-deficient cells. Phosphorylated c-Jun was expressed after oxidative or immunological challenge, but not in basal condition, in wild-type cells; however, it was significantly elevated at basal condition and further enhanced after oxidative or immunological challenge in PARP-1-deficient cells. No DNA binding of heat shock factor-1 was observed in PARP-1-deficient cells. These data demonstrate that PARP-1 plays a pivotal role in the modulation of transcription. The Journal of Immunology, 2003, 170: 2113–2120.

Poly(ADP-ribose) polymerase (PARP)-1 is a highly abundant nuclear protein with one molecule of enzyme per 1000 bp of DNA. It is activated by ss- or dsDNA nicks and breaks in damaged cells, and it modifies nuclear proteins through attachment of poly(ADP-ribose) units (1, 2). Transient and extensive poly(ADP-ribosylation) can be induced by a wide variety of environmental stimuli including reactive oxygen and nitrogen species such as hydrogen peroxide, hydroxyl radical, NO, and peroxynitrite, as well as ionizing radiation and genotoxic agents (2–6). Activated PARP ADP-ribosylates a wide variety of proteins including histones, topoisomerases I and II, molecule Ap4A that associates with DNA polymerase, DNA polymerases α and β, DNA ligase, and PARP itself (2, 7–10). However, the role of PARP and/or the effects of ADP-ribosylation on functions of nuclear proteins and transcription factors are not completely understood.

Recently, it has been reported that PARP is not an enzyme alone, but a family of proteins composed of six members: PARP-1 (11, 12), short PARP-1 (13), tankyrase (14), PARP-2 (15, 16), vault PARP (17), and PARP-3 (18). In vitro and in vivo experimental studies implicate PARP-1 as an active participant in regulation of genomic stability, gene expression and gene amplification, cellular differentiation and malignant transformation, and DNA replication (19–23). Furthermore, evidence has accumulated that activation of PARP-1 is a major cytotoxic pathway of tissue injury in different pathologies associated with inflammation. Genetic deletion of PARP-1 has been shown to attenuate tissue injury after ischemia and reperfusion, streptozocin-induced diabetes, arthritis, endotoxic and hemorrhagic shock, and localized colon inflammation. These beneficial effects have been confirmed also with the use of pharmacological inhibitors of poly(ADP-ribosylation) (6, 25–30).

It has been proposed that the mechanism by which oxidants and cytokines trigger an inflammatory process is related to their ability to activate mitogen-activated protein kinase kinase-4 (MEK-4) and, subsequently, c-Jun N-terminal kinase (JNK). In turn, the stress-regulated JNK phosphorylates c-Jun, thus allowing its homodimerization or heterodimerization with c-Fos to form the active transcription factor AP-1 (31–33). Activation of transcription by AP-1 enables the gene expression of several proinflammatory mediators and adaptive modifications of the damaged cells such as the early process of inflammation and cellular death by apoptosis (34). In eukaryotes, oxidative injury and ATP depletion also lead to activation of the heat shock factor-1 (HSF-1), a transcription factor which modulates the adaptation and cytoprotective response of the cell through gene expression of heat shock proteins (35, 36).

These observations have led us to propose that PARP-1 activation may modulate the inflammatory process through regulation of the signal transduction mediated by the AP-1 pathway. In the present study, we provide evidence that activation of AP-1 is dependent on PARP-1 activation in fibroblasts, as genetic or pharmacological inhibition of PARP-1 altered the phosphorylative activity of JNK, the nuclear binding of AP-1, and the content of phosphorylated c-Jun. Furthermore, our data demonstrate that the
nuclear presence of PARP-1, but not its catalytic activity, is required for the DNA binding of HSF-1.

Materials and Methods

Cell culture

Mouse fibroblasts from a strain genetically deficient in PARP-1 and fibroblasts from the corresponding wild-type controls were created by immortalization by a standard 3T3 protocol (37). Cells were grown in monolayer at 37°C and 5% CO2 using DMEM (Life Technologies, Grand Island, NY) containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). When cells reached 75–80% confluence, culture medium was replaced by fresh DMEM containing 0.25% FCS, and the cells were incubated for 16 h. These serum-starved monolayers were then stimulated with peroxynitrite (600 µM), hydrogen peroxide (100 µM), mouse IL-1 (1 ng/ml), and 20% FCS in the presence or absence of the PARP-1 inhibitor 5-ido-6-aminol-1,2-benzopyrone (INH2 BP; 100 µM). These concentrations were established following preliminary experiments to determine the maximal stimulation of total PARP activity by these reagents over a prolonged time course extending from 5 min to 2 h (data not shown). The pretreatment time with INH2BP was 30 min in all experiments.

Total PARP activity

Serum-starved fibroblasts (80% confluence in 6-well plates) were treated with peroxynitrite, hydrogen peroxide, IL-1, and 20% FCS. After 10 min, medium was aspirated and replaced with buffer containing 56 mM HEPES (pH 7.9), 28 mM KCl, 28 mM NaCl, 2 mM MgCl2, 0.01% digitonin, and 0.5 mM EDTA, and 1.5 mM boric acid, and 1 mM EDTA) for 1 h. The protein that was ribosylated with [3H]NAD was precipitated with 200 µl of cold 50% TCA. Samples were refrigerated for 24 h, and the pellet was recovered by centrifugation at 10,000 rpm for 10 min at 4°C. Pellets were washed twice with 5% TCA and solubilized overnight in 250 µl of 2% SDS, 0.1 N NaOH at 37°C. The radioactivity was determined by a Wallac 1450 Microbeta Plus scintillation counter (Wallac, Gaithersburg, MD). Results are expressed as cpm.

Preparation of nuclear extracts

Nuclear extracts were prepared as previously described (38). Briefly, after treatment, cells were washed twice with cold PBS and scraped into a minimal volume of the same buffer. The cell suspension was centrifuged at 6000 rpm at 4°C for 5 min. The pellet was resuspended in 50–200 µl of lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 0.2% Nonidet P-40, 1 mM DTT, and 0.5 mM PMFS, and then incubated on ice for 5 min. Cell lysates were centrifuged at 6000 rpm for 5 min at 4°C, and the pellet was resuspended in a buffer containing 10 mM HEPES (pH 7.9), 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl2, 25% glycerol, 1 mM DTT, and 0.5 mM PMFS. After incubation on ice for 15 min, the pellet was recovered by centrifugation at 14,000 rpm at 4°C for 15 min. The supernatant (nuclear extract) was collected and stored at −70°C. Amount of protein was quantitated by Bradford assay.

EMSA

EMSA was performed as previously described (38). An oligonucleotide probe corresponding to an AP-1 consensus sequence (5'–GCC TCG ATG ACT CAG CCG GAA-3') and an oligonucleotide probe corresponding to the known heat shock-responsive element (5'–GCC TCG ATT GTT CGC GAA GGT TCG-3') were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Life Technologies) and purified in Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Ten micrograms of nuclear protein were preincubated with EMSA buffer (12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 25 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 117 µg/ml poly(dI-dC) (1:1), 12% glycerol, 0.2% PMFS) on ice for 30 min before addition of the radiolabeled oligonucleotide for an additional 30–60 min. Excess of unlabeled oligonucleotide was added in some samples for competition to verify the specificity of AP-1 or HSF-1 binding. Protein-nucleic acid complexes were resolved using a nonde- naturating polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide:bisacrylamide) and run in 0.5× TBE (45 mM Tris-HCl, 45 mM boric acid, and 1 mM EDTA) for 1–3 h at constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under a vacuum at 80°C for 1 h, and exposed to photographic film at −70°C with an intensifying screen. Densitometric analysis was performed using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Western blotting analysis

Nuclear content of PARP-1, poly(ADP-ribose), c-Fos, phosphorylated c-Jun, and phosphorylated MEK-4 were determined by immunoblot analyses. Nuclear extracts were boiled in equal volumes of loading buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% ME) and 50 µg of protein/lane were loaded on an 8–16% Tris-glycine gradient gel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 5% nonfat dried milk in TBS for 1 h and then incubated with primary Abs against PARP-1, poly(ADP-ribose), c-Fos, phosphorylated c-Jun, or phosphorylated MEK-4 for 1 h. The membranes were washed in TBS with 0.1% Tween 20 and incubated with secondary peroxidase-conjugated Ab. Immunoreaction was visualized by chemiluminescence. Densitometric analysis of blots was performed using ImageQuant (Molecular Dynamics).

JNK activity assay

Fibroblasts, cultured and treated in T-175 flasks, were washed and scraped with PBS containing 1 mM PMFS, 100 µM Na2VO3, 2 mM ρ-nitrophenyl phosphate, and 210 µM/ml aprotinin. The cells were then centrifuged at 5000 rpm at 4°C for 5 min.

FIGURE 1. Effect of oxidative or immunological stress on PARP-1 expression (A), total PARP activity (B), and poly(ADP-riboseylation) of nuclear proteins (C). Wild-type (PARP-1+/+) and PARP-1-deficient (PARP-1−/−) cells were stimulated with peroxynitrite (ONOO−, 600 µM), hydrogen peroxide (H2O2, 100 µM), IL-1 (1 ng/ml), and 20% serum for 15 min. Autoradiographs of Western blot analysis for PARP-1 (A) and poly(ADP-ribose) formation (C) in nuclear proteins are representative of four similar separate experiments. B, Catalytic activity of total PARP was evaluated by [3H]NAD incorporation. The data represent the average ± SE of three separate experiments in triplicate, * p < 0.05 vs respective basal control activity in cells with the same genotype; #, p < 0.05 vs PARP-1+/+ cells after similar treatment.
3000 rpm for 5 min at 4°C. The pellet was suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 100 μM Na3VO4, 2 mM p-nitrophenyl phosphate, 1 mM PMSF, and 210 mM/ml aprotinin. The lysates were rocked for 30 min at 4°C and centrifuged at 10,000 X g for 10 min at 4°C. The supernatant was then collected, quantitated for protein by Bradford assay, and stored at −70°C.

Activity of JNK was determined by immune complex kinase assay and was estimated as the ability to phosphorylate GST-c-Jun (39). After immunoprecipitation of lysates with specific Ab directed to JNK1, the immunoprecipitate was incubated for 30 min at 30°C in 40 μl of reaction buffer containing 25 mM HEPES (pH 7.6), 20 mM MgCl2, 20 mM glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM DTT, 25 μM ATP, and 5 μCi of [γ-32P]ATP. GST-c-Jun (1–79) (1 μg) was used as substrate for JNK. Reaction products were separated by SDS-PAGE and visualized by autoradiography. Densitometric analysis was performed using ImageQuant (Molecular Dynamics).

Materials

The primary Abs directed at c-Fos, phosphorylated MEK-4, JNK1, and the oligonucleotide for AP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The primary Ab directed at c-Jun phosphorylated at serine 63 and 73 was obtained from New England Biolabs (Beverly, MA). The primary Abs directed at PARP-1 and poly(ADP-ribose)-ribose were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Mouse rIL-1α was obtained from Roche Molecular Biochemicals (Indianapolis, IN). The oligonucleotide probe for HSF-1 corresponded to a previously published heat shock element consensus sequence (36) and was synthesized by the University of Cincinnati DNA core facility. All other chemicals were from Sigma-Aldrich (St. Louis, MO). INH2BP was a kind gift from Prof. E. Kun of the Octamer Research Foundation (Berkeley, CA). Peroxynitrite was a kind gift of Dr. H. Ischiropoulus of the Children’s Hospital of Philadelphia (Philadelphia, PA).

Data analysis

All values in the figures and text are expressed as mean ± SE of n observations (n = 3–4). The results were examined by ANOVA followed by the Bonferroni’s correction post hoc test. A value of p < 0.05 was considered significant.

Results

Oxidative or immunological stress induce activation of PARP and poly(ADP-ribo-sylation) of nuclear proteins in wild-type fibroblasts

Because of recent reports of PARP-2 or short PARP genetic material in PARP-1-deficient animals and cell lines (13), we investigated the differences of PARP-1 expression and total PARP activity between wild-type and PARP-1-deficient fibroblasts. To this end, both wild-type and PARP-1-deficient cells were subjected to oxidative stress, as obtained by treatment with peroxynitrile or hydrogen peroxide, or to immunological stress, as obtained by treatment with IL-1 or 20% serum. Western blotting analysis revealed the presence of PARP-1 enzyme at a band at ~113 kDa only in wild-type cells. A second band was also visualized at 86 kDa, corresponding most probably to a cleavage product of PARP-1 after stimulation with peroxynitrile (Fig. 1A). As shown in Fig. 1B, a 15-min exposure of wild-type cells to peroxynitrile, hydrogen peroxide, IL-1, or 20% serum induced a significant increase of total PARP activity above baseline levels (345.2 ± 26.2 cpm) of control untreated cells. The increase of the catalytic activity was associated by a massive poly(ADP-ribo-sylation) of a nuclear protein with a molecular mass in the range of 110–131 kDa, corresponding most probably to the automodified PARP-1 (Fig. 1C). In contrast, PARP-1-deficient cells exhibited a significantly lower baseline catalytic activity (165.8 ± 8.5 cpm; p < 0.05), which was not affected by stimulation with hydrogen peroxide, IL-1, or 20% serum when compared with wild-type cells.

The absence of the increase in catalytic activity paralleled with absence of detectable nuclear poly(ADP-ribo-sylation) in the range of 110–131 kDa. However, when PARP-1-deficient cells were treated with peroxynitrile, a slight but significant increase in PARP activity (216. 9 ± 10.9 cpm; p < 0.05) was observed when compared with baseline levels.

Pretreatment of wild-type cells with INH2BP, a specific potent inhibitor of PARP, completely blunted the catalytic activity of total PARP after oxidant or immunological stimulation. Pretreatment of PARP-1-deficient cells with INH2BP did not alter the low residual PARP activity (Fig. 2). Thus, these experiments suggested that PARP-1 is the major effector of poly(ADP-ribo-sylation) after cellular stress.

DNA binding of AP-1 is depressed in the absence of PARP-1

As activation of AP-1 has been implicated in oxidative stress, we further determined the nuclear activation of this factor. In wild-type fibroblasts, DNA binding of AP-1 increased steadily in a time-dependent manner after peroxynitrile stimulation. However, in PARP-1-deficient cells peroxynitrile-induced DNA binding of AP-1 was virtually abolished (Fig. 3). To obtain further evidence of the involvement of PARP-1 in the regulation of AP-1 activation,
we investigated the effect of pharmacological inhibition of PARP by INH2BP in wild-type fibroblasts. In this experiment, preincubation for 30 min with INH2BP significantly reduced the DNA binding of AP-1 induced by peroxynitrite (Fig. 3).

In parallel experiments, both hydrogen peroxide and the inflammatory cytokine IL-1 enhanced DNA binding of AP-1 in a time-dependent manner in wild-type fibroblasts. In contrast, in PARP-1-deficient cells DNA binding of AP-1, either induced by oxidative stress or by immunological challenge, was significantly reduced when compared with that of wild-type cells (Fig. 4).

**Nuclear content of AP-1 components is altered in the absence of PARP-1**

Because we observed an absence of AP-1 DNA binding in PARP-1-deficient cells, we further investigated whether genetic deficiency of PARP-1 affected the content of AP-1 components and, therefore, composition of the dimer. To this purpose, Western blotting analyses were performed to detect c-Fos and phosphorylated c-Jun in nuclear extracts from oxidant-stimulated or immunostimulated wild-type and PARP-1-deficient cells. As shown in Fig. 5, wild-type cells exhibited high content of c-Fos in basal control condition as well as after oxidative or immunological challenge. Under basal control condition, wild-type cells exhibited no content of phosphorylated c-Jun, which was expressed with phosphorylated sites at both serine 73 and 63 after oxidative or immunological challenge. PARP-1-deficient cells exhibited similar content of c-Fos in basal control condition as well as after oxidative or immunological challenge when compared with wild-type cells. Interestingly, under basal control condition, PARP-1-deficient cells exhibited high content of c-Jun phosphorylated at serine 73 only, which was further increased by oxidative or immunological challenge (Fig. 5). Thus, these data suggest that, in the absence of PARP-1, the nuclear content and phosphorylation of c-Jun are abnormally incremented.

**FIGURE 3.** DNA binding of AP-1 in wild-type (PARP-1+/+) and PARP-1-deficient (PARP-1−/−) cells stimulated with peroxynitrite with or without a 30-min preincubation with the PARP inhibitor INH2BP (100 μM). A, Autoradiographs of EMSA for AP-1 are representative of four similar separate experiments. NS, nonspecific binding. B, Densitometric analysis of autoradiographs was performed using ImageQuant (Molecular Dynamics). Fold increase was calculated vs respective basal control binding set to 1.0. The data represent the average ± SE of four separate experiments in triplicate. *p < 0.05 vs respective basal control activity (time 0) in cells with the same genotype; #, p < 0.05 vs PARP-1+/+ cells. Cells were stimulated with peroxynitrite (600 μM) and harvested at the times indicated.

**FIGURE 4.** DNA binding of AP-1 in wild-type (PARP-1+/+) and PARP-1-deficient (PARP-1−/−) cells stimulated with hydrogen peroxide (H2O2; A) or IL-1 (B). Densitometric analysis of EMSAs was performed using ImageQuant (Molecular Dynamics). Fold increase was calculated vs respective basal control binding set to 1.0. The data represent the average ± SE of three separate experiments in triplicate. *p < 0.05 vs respective basal control activity (time 0) in cells with the same genotype; #, p < 0.05 vs PARP-1+/+ cells after similar treatment. Cells were stimulated with H2O2 (100 μM) or IL-1 (1 ng/ml) and harvested at the times indicated.
Activation of JNK is altered in the absence of PARP-1

Because phosphorylation of c-Jun by JNK represents an important event for the stability and activation of the transcription factor AP-1 (32–34), we further determined the nuclear activity of JNK. In this experiment, wild-type and PARP-1-deficient fibroblasts were exposed to oxidative and immunological stress and the activity of JNK in nuclear extracts was estimated, using exogenously added GST-c-Jun as substrate. As shown in Fig. 6, in wild-type cells, 15-min stimulation with peroxynitrite induced a 5-fold increase in JNK activity, which was reduced significantly by 30-min pretreatment with INH2BP. In contrast, PARP-1-deficient cells exhibited higher levels of JNK activity in basal control condition, i.e., in the absence of oxidative or immunological stress, when compared with basal levels of wild-type cells (see representative autoradiograph in Fig. 6A). The fold increase of basal activity of JNK in PARP-1-deficient cells was 3.56 ± 0.56 vs basal levels of wild-type cells (set to 1), thus confirming the excessive basal expression of phosphorylated c-Jun. Stimulation with peroxynitrite further increased JNK activity although to a lesser degree than in wild-type cells (Fig. 6).

In parallel experiments in wild-type fibroblasts, both hydrogen peroxide and IL-1 induced activation of JNK, which was significantly reduced by preincubation with the PARP inhibitor INH2BP. In contrast, in PARP-1-deficient cells, stimulation with hydrogen peroxide and IL-1 induced a significantly lesser increase in JNK, which was not affected by preincubation with the PARP inhibitor INH2BP (Fig. 7). Taken together, these data strongly suggest that, under conditions of cellular stress, PARP-1 is an important regulator of JNK phosphorylative activity.

Activation of MEK-4 is altered in the absence of PARP-1

Because the upstream kinase responsible for the activation of JNK has been identified as MEK-4 (31), we further examined the nuclear content of the active phosphorylated MEK-4. Western blotting analyses showed low content of phosphorylated MEK-4 under basal conditions in wild-type cells. Exposure to peroxynitrite, hydrogen peroxide, IL-1, or 20% serum induced a notable increase of phosphorylated MEK-4. PARP-1-deficient cells exhibited low

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**FIGURE 6.** Activity of JNK in wild-type (PARP-1+/+) and PARP-1-deficient (PARP-1−/−) cells stimulated with peroxynitrite. A, Autoradiograph of activity assay for JNK is representative of four similar separate experiments. Immunoreactive c-Jun was detected with c-Fos-specific Ab. Immunoreactive c-Jun was detected with a c-Fos-specific Ab. Immunoreactive c-Jun was detected with a c-Fos-specific Ab. B, Densitometric analysis of autoradiographs was performed using ImageQuant (Molecular Dynamics). Fold increase was calculated vs respective basal control binding set to 1.0. The data represent the average ± SE of four separate experiments in triplicate, *p < 0.05 vs respective basal control activity in cells with the same genotype; †p < 0.05 vs hydrogen peroxide-stimulated cells with the same genotype; †p < 0.05 vs PARP-1−/− cells after similar treatment. Cells were stimulated with peroxynitrite (ONOO−, 600 μM) for 15 min with or without a 30-min preincubation with the PARP inhibitor INH2BP (100 μM). JNK activity was estimated as the ability to phosphorylate GST-c-Jun after immunoprecipitation of proteins with specific anti-JNK1 Ab.
content of phosphorylated MEK-4 under basal conditions or after exposure to hydrogen peroxide, IL-1, and 20% serum. However, when PARP-1-deficient cells were treated with peroxynitrite, a marked increase was observed when compared with basal condition. Pretreatment with INHBP of both wild-type and PARP-1-deficient cells did not affect the content of phosphorylated MEK-4 under basal conditions or after oxidative or immunological stress (Fig. 8).

**PARP-1-deficient cells show lack of activation of HSF-1**

Stimuli leading to activation of HSF-1 have been suggested to regulate the cytoprotective response during inflammation (35, 36). Thus, in comparative experiments, we examined whether PARP-1 would modulate the activation of this anti-inflammatory pathway. In wild-type cells, exposure to peroxynitrite induced DNA binding of HSF-1 in a time-dependent manner. As previously reported (35), a constitutive binding of HSF-1 was also observed under basal conditions in wild-type cells. PARP-1-deficient cells exhibited high constitutive binding under basal conditions. However, no DNA binding activity could be observed after stimulation with peroxynitrite. Pretreatment with INHBP of both wild-type and PARP-1-deficient cells did not affect the HSF-1 binding under basal conditions or after oxidative stress (Fig. 9).

**Discussion**

In the present study, we have demonstrated that, under in vitro conditions of oxidative or immunological stress, PARP-1 is an important modulator of the AP-1 signaling pathway. We have shown that, in the absence of a functional gene for PARP-1, the AP-1 DNA binding is completely abolished and is associated with alterations in the activation of MEK-4 and JNK. Furthermore, we have demonstrated that peroxynitrite-induced activation of HSF-1 requires PARP-1 but not the polymerase activity.

PARP-1 is a highly conserved 113-kDa protein that consists of an N-terminal catalytic domain containing two zinc finger motifs, an internal automodification region, and a C-terminal catalytic domain. Under normal conditions, catalytically inactive PARP-1 resides in the nucleoplasm. Binding of PARP-1 to DNA strand breaks activates PARP in the presence of NAD+, promoting extensive poly(ADP-ribosylation) on nuclear proteins (9, 10). Several in vitro studies have demonstrated that this binding is a very early and transient event after genotoxic or inflammatory stress. For example, we have demonstrated that, in murine peritoneal macrophages, DNA breakage occurs and total PARP is maximally activated within 5–10 min after oxidative stress (40). In support of these previous findings, in the present study, we show that in wild-type fibroblasts total PARP activation is a very early event, because the enzyme was activated within 15 min after oxidative or immunological challenge. Although PARP-1-deficient cells possess other enzymes capable of some poly(ADP-ribosylation) (13–18), we found that the catalytic activity was maximally expressed in wild-type cells only. This suggests that, among the PARP homologous enzymes, PARP-1 is the major active enzyme to modulate poly(ADP-ribosylation) and the events leading to DNA repair. According to our data, this transient and acute nature of poly(ADP-ribosylation) could be useful in regulating the cellular process of signal transduction, rather than DNA repair only.

Our data indicate that blocking PARP-1 by genetic or pharmacological inhibition significantly inhibited DNA binding of AP-1 and reduced activation of JNK induced by immunological or oxidative stress, while levels of c-Fos were unaltered. AP-1 is a collective term referring to dimeric transcription factors composed of Jun, Fos, or activating transcription factors subunits that bind to a common DNA site. AP-1 activity is regulated at two major levels: the abundance and stability of the AP-1 proteins. The abundance of AP-1 proteins is regulated at the transcriptional level of the respective genes. In addition, the stability of c-Jun subunit can be modulated through phosphorylation on serine 63 and 73 by JNK, which is in turn activated by the upstream kinase MEK-4 (32, 33, 39, 41). Thus, our data suggest a scenario in which PARP-1 modulates AP-1 pathway by regulating the activation of nuclear kinases. Interestingly, we found that genetic ablation of PARP-1 increased basal content of phosphorylated c-Jun and basal activity of JNK. The abnormal basal activity of JNK and content of c-Jun may reflect an alteration of the genomic stability. It has been demonstrated that primary fibroblasts derived from PARP-1-deficient mice show an elevated frequency of sister chromatid exchanges and micronuclei formation in response to DNA damaging agents (42). For example, it has been shown that deletion of the PARP-1 gene in mice is associated with increased tissue expression of the oncogene Jun as evaluated by microarray genomic analysis (42).

**FIGURE 8.** Western blot analyses for phosphorylated MEK-4. Western blot analyses in nuclear proteins are representative of three similar separate experiments. Immunoreactive MEK-4 was detected with a specific Ab against MEK-4 phosphorylated at threonine 261. Cells were stimulated with peroxynitrite (ONOO⁻, 600 μM), H₂O₂ (100 μM), IL-1 (1 ng/ml), and 20% serum for 15 min with or without a 30-min preincubation with the PARP inhibitor INHBP (100 μM).

**FIGURE 9.** DNA binding of HSF-1 in wild-type (PARP-1+/+) and PARP-1-deficient (PARP-1−/−) cells stimulated with peroxynitrite. Autoradiograph of EMSA for AP-1 is representative of two similar separate experiments. CB, Constitutive binding; NS, Nonspecific binding. Cells were stimulated with peroxynitrite (600 μM) with or without a 30-min preincubation with the PARP inhibitor INHBP (100 μM) and harvested at the times indicated.
Thus, these data, together with our present findings, further implicate PARP-1 in the maintenance of genomic integrity. However, the abnormal JNK activation and the increased availability of phosphorylated c-Jun as subcomponent of the AP-1 dimer at basal condition was not associated with increased DNA binding of AP-1 in PARP-1-deficient cells either at basal condition or after cellular stress, thus suggesting that the presence of PARP-1 is required for the activation of AP-1. Although our study did not address the precise mechanism by which PARP-1 and/or poly(ADP-riboseylation) may modulate AP-1 activation, our data suggest a multiple role of PARP-1. Although upstream pathways may be activated during the inflammatory challenge, PARP-dependent modulation may be important at the nuclear level. It is possible that PARP-1 is required to maintain a critical balance between JNK activity in basal condition and its phosphorylative activity during stress conditions. In support of this hypothesis, in a two-hybrid bacterial system, we have shown that JNK interact with PARP-1 (B. Zingarelli and M. O’Connor, unpublished observations). PARP-1 may also be required for the direct modulation of the dimer composition. In this regard, it has been demonstrated in mouse epidermal cells that Fos protein is weakly poly(ADP-ribosylated) in response to active oxygen (43). The hypothesis of PARP-1-dependent direct modulation of the subunits may also explain the absence of AP-1 DNA binding in PARP-1-deficient cells even in the presence of highly activated JNK under basal conditions. Whether the reduction of c-Jun phosphorylation at serine 63 may affect coupling with c-Fos is also not known. Further studies are also needed to investigate whether other inducers may induce AP-1 binding in these cells. Similar to our findings, Ha et al. (44) have recently demonstrated that PARP-1-deficient glial cells lack the ability to induce AP-1 after endotoxin or TNF-α challenge. Nevertheless, taken together, our data suggest for the first time that the AP-1 pathway requires poly(ADP-ribosylation).

A plethora of physiological and pathological stimuli induce activation of AP-1. Downstream targets for the transcription initiated by AP-1 are genes controlling the expression of most immunomodulatory and inflammatory mediators, such as chemokines and cytokines. For example, it has been shown that oxidative stress up-regulates intercellular adhesion molecule-1 expression in endothelial cells via activation of AP-1 (45). Thus, a dysregulation of the AP-1 pathway in PARP-1-deficient cells may explain our previous data demonstrating that genetic ablation or pharmacological inhibition of PARP completely abolished intercellular adhesion molecule-1 expression in vitro immunostimulated fibroblasts and in vivo myocardial reperfusion injury (25).

Our findings are in line with other reports demonstrating a role of poly(ADP-ribosylation) in signal transduction. It has been demonstrated that PARP-1-deficient cells are also defective in NF-κB-dependent transcriptional activation and show a down-regulation of inducible NO synthase (iNOS) after genotoxic stress (46, 47). Similarly, expression of iNOS is abolished by inhibitors of PARP (48).

Interestingly, in comparative experiments, pharmacological inhibition of PARP-1 catalytic activity was unable to affect DNA binding of HSF-1. Similar to our findings, Ha et al. (44) have recently demonstrated that genetic deletion of PARP-1, but not pharmacological inhibition, reduced NF-κB binding in glial cells. However, pharmacological inhibition of PARP-1 was able to reduce gene expression of iNOS. These variable findings may reflect a dual role of PARP-1, poly(ADP-ribosylation)-dependent and poly(ADP-ribosylation)-independent, in the modulation of transcription. For example, PARP-1 has been shown to modulate transcription by direct interaction with transcription factors such as activating protein-2 (49), octamer transcription factor-1 (50), Yin-Yang-1 (51), transcription enhancer factor-1 (52), and NF-κB (53, 54) independently of its catalytic activity. In contrast, it has been demonstrated in rat testis that PARP activity and poly(ADP-ribosylation) are associated with regions of chromatin that are transcriptionally active (55).

In conclusion, we propose a critical role for PARP-1 in the modulation of the signal transduction mediated by AP-1. The identification of this regulation may explain the critical inflammatory role of PARP-1. Nevertheless, further studies are required to identify the precise interactions between PARP-1 and components of the inflammatory signaling pathways.

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References


