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PARP-1-Dependent 3-Nitrotyrosine Protein Modification After DNA Damage

E. Siles,[1] E. Martinez-Lara,[1] M.I. Núñez,[2] J.A. Muñoz-Gámez,[2,3] D. Martín-Oliva,[2,3] M.T. Valenzuela,[2] M.A. Peinado,[1] J.M. Ruiz de Almodóvar,[2] and F. Javier Oliver[3]*

[1]Dpto. Biología Experimental, Universidad de Jaén, Spain
[2]Instituto de Biopatología y Medicina Regenerativa, Facultad de Medicina, Universidad de Granada, Granada, Spain
[3]Instituto López Neyra de Parasitología y Biomedicina, CSIC, Granada, Spain

Abstract 3-nitrotyrosine (NO_2-Tyr) is thought to be a specific marker of cell injury during oxidative damage. We have evaluated the role of poly(ADP-ribose)polymerase-1 (PARP-1) in protein nitration after treatment of immortalized fibroblasts *parp-1*+/+ and *parp-1*−/− with the alkylating agent 2′-methyl-2′-nitroso-urea (MNU). Both cell lines showed increased iNOS expression following MNU treatment in parallel with a selective induction of tyrosine nitration of different proteins. PARP-1 deficient cells displayed a delayed iNOS accumulation, reduced number of nitrated proteins, and a lower global nitrotyrosine ''footprint.'' We have identified the mitochondrial compartment as the major site of oxidative stress during DNA damage, being MnSOD one of the NO_2-Tyr-modified proteins, but not in *parp-1*−/− cells. These results suggest that NO-derived injury can be modulated by proteins involved in the response to genotoxic damage, such as PARP-1, and may account for the limited oxidative injury in *parp*-1 knockout mice during carcinogenesis and inflammation. J. Cell. Biochem. 96: 709–715, 2005.

Key words: PARP-1; DNA damage; protein nitration; nitric oxide; MnSOD

The presence of damaged DNA in the cell activates repair mechanisms as well as signal transduction pathways leading to cell cycle arrest and programmed cell death. Poly(ADP-ribose)polymerase-1 (PARP-1) is a nuclear, zinc-finger, DNA-binding protein that detects specifically DNA-strand breaks generated by different genotoxic agents [D'Amours et al., 1999]. PARP-1 is associated in vivo with XRCC1, a DNA repair protein involved, together with DNA polymerase β and DNA ligase III, in the base excision repair of DNA [Masson et al., 1998]. Treatment of *parp-1*−/− mice with either alkylating agents or γ irradiation reveals an extreme sensitivity and a high genomic instability to both agents [de Murcia et al., 1997; Samper et al., 2001].

In previous studies it has been established that the absence of PARP-1 is able to slow down tumor formation and to increase the lifespan of p53 deficient mice by a mechanism dependent on the inhibition of oxidative stress and partial restoration of the G_1/S checkpoint after DNA damage [Conde et al., 2001; Martin-Oliva et al., 2004]. On the other hand, we and others have shown that PARP-1 is a transcriptional co-activator of NF-κB and PARP-1 knockout mice are much less sensitive to inflammatory stress [Oliver et al., 1999; Hassa et al., 2003] as a result of a diminished release of pro-inflammatory mediators, including nitric oxide (NO).

Reactive oxygen species (ROS), and specially O_2^-, are important modulators of NO activity under various pathophysiological conditions [Grisham et al., 1999] and are thought to be involved in cellular injury through the action of NO-derived radicals species such as the powerful oxidant peroxynitrite [Beckman et al., 1993]. 3-nitrotyrosine (NO_2-Tyr) is a modified amino

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*Correspondence to: F. Javier Oliver, PhD, Instituto López Neyra de Parasitología y Biomedicina, CSIC, Campus de Ciencias de la Salud. Avda. del Conocimiento s/n, 18100-Armilla, Granada, Spain. E-mail: joliver@ipb.csic.es

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acid formed by nitric oxide-derived species and has been implicated in the pathology of diverse human diseases including atherosclerosis, reperfusion injury, amyothrophic lateral sclerosis, septic lung, and rheumatoid arthritis [Macmillan-Crow and Cruthirds, 2001]. Free NO_2-Tyr is produced in abundant concentrations under pathological conditions and its capacity to alter protein structure and function at the translational or post-translational level is well known [Beal, 2002]. The aim of this study was to evaluate the impact of the absence of PARP-1 in the NO-mediated oxidative stress response during the DNA damage.

MATERIALS AND METHODS

Cell Culture and Treatments

Immortalized murine embryonic fibroblasts expressing or lacking *PARP-1* (*parp-1*+/+ and *parp-1*−/−) were obtained as described previously [Boczkowski et al., 2001]. They were grown in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's medium (FBS-DMEM, Sigma, St. Louis, MO) and incubated at 37°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Fibroblasts were treated for different periods of time with the monofunctional alkylating agent 2′-methyl-2′-nitroso-urea (MNU) at 0.5 mM or with lipopolysaccharide (LPS) at 15 μg/ml for 24 h.

Western Blotting Assay

At the end of each incubation period, cells were collected, washed with cold PBS, and lysed for 20 min at 4°C in 20 mM phosphate buffer pH 7.5, 0.1 mM EDTA, 0.5% Triton X-100, 12.5 mM β-mercaptoethanol, and protease inhibitors. After centrifugation at 14,000*g* for 15 min at 4°C, supernatant was collected and protein was quantified. Western blotting was carried out using standard procedures. Equal amounts of protein extracts were loaded into each well of a 7.5% or 10% SDS–polyacrylamide gel (NO synthases and NO_2-Tyr-modified proteins analysis, respectively). Proteins in the gel were transferred to a PVDF membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) and then blocked. Monoclonal antibodies to inducible and endothelial NO synthases (iNOS and eNOS respectively) (Transduction, BD), polyclonal antibodies to neuronal NOS and 3-nitrotyrosine (generous gifts from V. Riveros-Moreno of Welcome Research Laboratories, Berkenhem, UK and Dr. Rodrigo, Instituto Cajal, C.S.I.C, Madrid) [Alonso et al., 2002] and a monoclonal antibody to α-tubulin, as internal control (Sigma) were used for detection of the respective proteins. Antibody reaction was revealed with chemioluminescence detection procedures according to the manufacturer's recommendations (ECL kit, Amersham Corp., Buckinghamshire, UK).

Immunoprecipitation

For immunoprecipitation, cell lysates (1.2 mg) were precleared by constant mixing for 4 h with protein A-Sepharose (Pharmacia). The beads were removed by centrifugation, and the supernatant was mixed constantly overnight with 5 μg of monoclonal antibody against 3-nitrotyrosine (Upstate Biotechnology, Waltham, MA) or p53 (Novocastra, Newcastle, United Kingdom) as an immunoprecipitation control. Immune complex were adsorbed (2 h, 4°C) onto protein A-Sepharose, washed several times in a saline buffer (20 mM Tris-HCl, pH 8; 150 mM NaCl, 1 mM EDTA, 0.5% NP-40), boiled and electrophoresed on 15% polyacrylamide gels. For detection of manganese-containing superoxide dismutase (MnSOD), electrotransferred proteins were incubated with a polyclonal anti-MnSOD antibody (1/4,000 dilution, StressGen Biotechnologies Corp., San Diego, CA). Immunoreactive proteins were detected as described above.

3-Nitrotyrosine Indirect Immunofluorescence

Cells were grown on slides and immunofluorescence was performed 24 h following MNU exposure. Cells were washed three times in PBS, fixed in fresh cold methanol-acetone (1:1) and then washed again with PBS-Tween 0.1%. The primary antibody for 3-nitrotyrosine analysis was used diluted 1/50 in PBS-Tween 0.1% and bovine serum albumin (BSA) 1%. Cells were incubated for 90 min at room temperature and then washed three times in PBS-Tween 0.1%. For colocalisation studies an antibody against cytochrome *c* (Pharmingen, San Jose, CA) was used. The secondary antibodies used were linked to the FITC (to reveal nitrotyrosine) or Cy3 (to reveal cytochrome *c*) (Sigma) and were diluted 1/400 in PBS-Tween 0.1%, BSA 1%. The cells were incubated 1 h at room temperature in the dark. Finally cells were washed three times in PBS-Tween 0.1% and stained with 4′,6′-diamidino-2-phenylindole dihydrochloride

(DAPI) 1/1,000 10 min. Slides were prepared using the Dako mounting medium (Dako Corporation, Glostrup, Denmark), coverslipped and stored in the dark at 4°C.

RESULTS

Induction of iNOS and Tyrosine Nitrated Proteins After MNU Treatment

Firstly we established a sublethal dose of MNU (0.5 mM) which allows for the long incubation periods without inducing cell death (not shown). Inactivation of PARP-1 has been associated with a reduced induction of iNOS [Oliver et al., 1999]. To test the oxidative stress response as function of the presence or absence of PARP-1, we have determined iNOS induction and 3-nitrotyrosine-dependent protein modification following MNU treatment. After treatment iNOS accumulates gradually in both cell types (Fig. 1) reaching the maximum value after 24 h in both cases, however in *parp-1*−/− cells this accumulation is strongly delayed. The presence of eNOS and nNOS was also evaluated and no signal was found for these two isoforms (results not shown).

MNU treatment also results in the selective induction of tyrosine nitration of three different electrophoretic bands of approximately 44, 56, and 65 kDa (Fig. 2A). Constitutive nitration was found in a 31 kDa protein in parp-1+/+ cells but not in *parp-1* mutant cells (Fig. 2B). Bulk tyrosine nitration increases gradually mainly in wt fibroblasts. If we compare the different electrophoretic bands in the two genotypes we find striking differences: *parp-1*+/+ fibroblasts accumulate nitrated tyrosine residues in the 65, 56, and 44 kDa bands until the end of the treatment (Fig. 2A). Nevertheless, the 31 kDa band does not seem to change. However, the most striking differences were found in *parp-1*−/− cells in which only two bands (of 56 and 44 kDa) were present, representing a clear global decrease in protein nitration levels (Fig. 2B).

Increased Nitration at Mitochondrial and Microtubule Compartments During DNA-Damage Derived Oxidative Stress

To support the biochemical evidence of MNU-induced nitration of tyrosine residues of different proteins, confocal microscopy studies were conducted using an anti-3-nitrotyrosine antibody. A treatment of 24 h with 0.5 mM MNU was used because at this time the highest levels of protein nitration were found (Fig. 2). Untreated *parp-1*+/+ and *parp-1*−/− cells displayed minimal immunoreactivity (Fig. 3). After MNU treatment, 3-nitrotyrosine immunoreactivity increased in *parp-1*+/+ cells, but again, *parp-1*−/− cells were minimally affected.

Peroxynitrite generation may have critical implications on mitochondrial functions

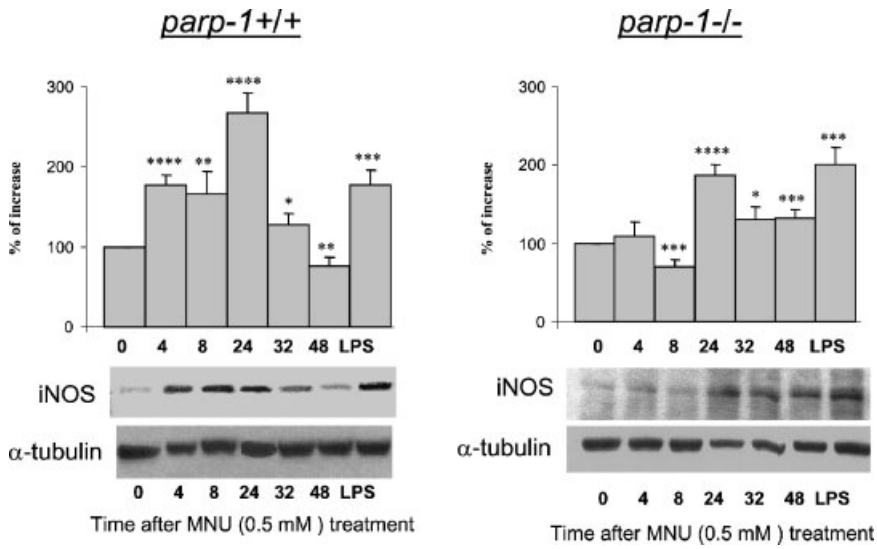

Fig. 1. Western blot analysis of iNOS expression in *parp-1*+/+ and *parp-1*−/−3T3 cell extracts. The experiments were carried out at different times (ranging from 0 to 48 h) after MNU treatment or 24 h after LPS treatment. Expression of α-tubulin is shown as a loading control. Results are from one representative experiment. Densitometries are shown in parallel and represents the average of three separate experiments ±SEM. (*) $P < 0.05$; (**) $P < 0.02$; (***) $P < 0.01$; (****) $P < 0.001$.

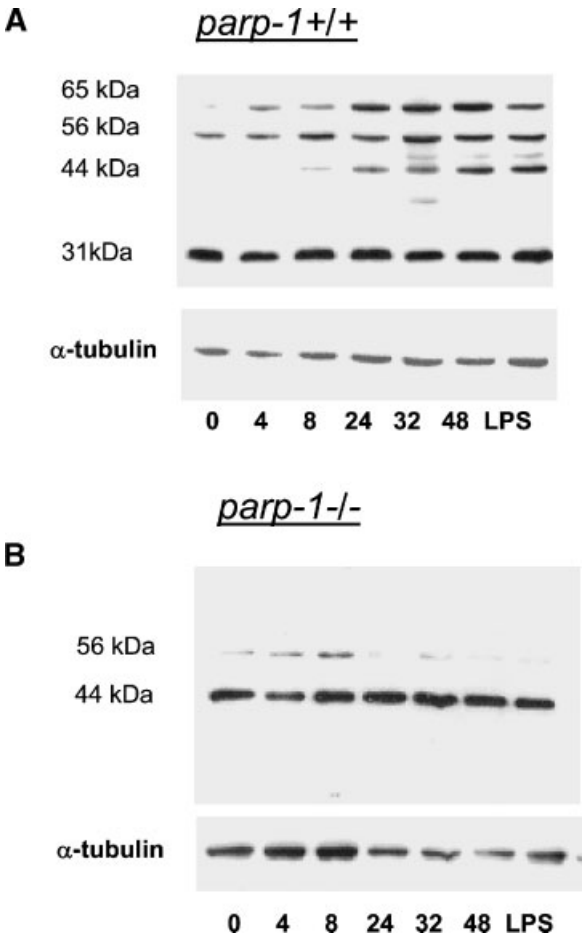

Fig. 2. **A**, **B**: NO_2-Tyr protein modification is dependent on the status of *parp*-1 after MNU treatment. Expression of α-tubulin is shown as a loading control. These results are representative of three different experiments.

[Boczkowski et al., 2001]. Figure 3 shows a double confocal immunofluorescence using anti-NO_2-Tyr and anti-cytochrome *c* as mitochondrial marker. These results suggest that the "footprint" of NO-derived oxidants is particularly high in the mitochondrial compartment and the intensity of the mitochondria-associated 3-nitrotyrosine signal is notably reduced in the absence of PARP-1 (Fig. 3).

MnSOD Is Modified by NO_2-Tyr in Parental Cells but not in parp-1 Single Mutant Cells

In order to identify a NO_2-Tyr mitochondrial target we have focused on MnSOD that has been described as a substrate of NO-derived free radicals with critical pathological implications [Macmillan-Crow and Cruthirds, 2001]. Figure 4A shows a similar presence of MnSOD in both *parp-1*+/+ and *parp-1*−/− (lanes 3 and 4) compared with the levels of NO_2-Tyr modification for both genotypes respectively (lanes 1 and 2 in Fig. 4A and Fig. 2). A clear modification of MnSOD by NO_2-Tyr in parental cells but not in *parp-1* single mutant cells (Fig. 4B) has been demonstrated by immunoprecipitation. Reverse immunoprecipitation studies (IP with anti-MnSOD and revealed with anti-nitrotyrosine) confirmed the modification of MnSOD by nitrotyrosine (results not shown). These results are in agreement with those in Figure 2 showing a complete absence of the 31 kDa band in the western blot of NO_2-Tyr modified proteins, thus identifying this band as MnSOD. A non related monoclonal antibody was used as control in lanes 1 and 3 of Figure 4B.

DISCUSSION

Increasing evidences suggest that oxidative damage to proteins and inflammation are prominent features in the development of cancer [Bentz et al., 2000; Ekmekcioglu et al., 2000].

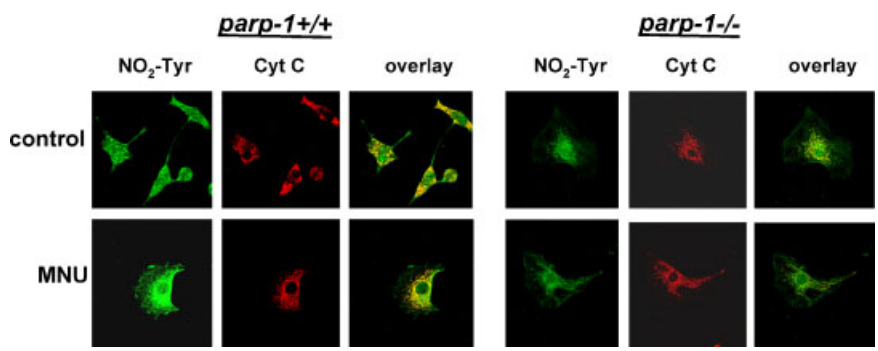

Fig. 3. Confocal immunofluorescence of NO_2-Tyr-modified proteins and cytochrome *c* as a mitochondrial marker in the different genotypes. The experiments were done 24 h after 0.5 mM MNU treatment and the extent of colocalisation is indicated by the merged images (yellow). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

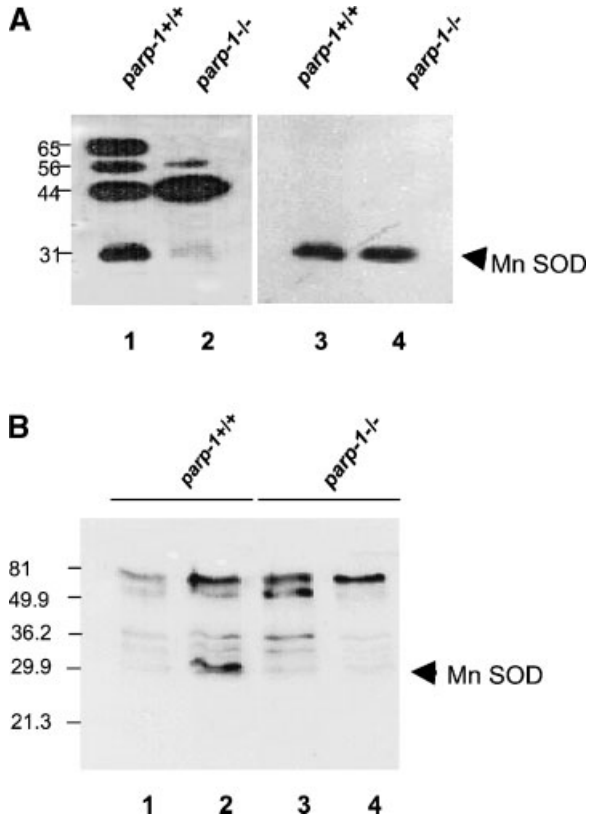

Fig. 4. MnSOD is the protein modified by NO_2-Tyr corresponding to the 31 kDa band with anti-nitrotyrosine. **A**: Expression levels of MnSOD in *parp-1*+/+ and *parp-1*−/− following 24 h of 0.5 mM MNU treatment. For better comparison the expression of NO_2-Tyr-modified proteins in the same cells has been included in parallel (**lanes 1** and **2**). **B**: Immnunoprecipitation of cell extracts (treated with 0.5 mM MNU) with an antibody against nitrotyrosine and revealed with anti-MnSOD. **Lanes 2** and **4** correspond to *parp-1*+/+ and *parp-1*−/− respectively immunoprecipitated with anti-nitrotyrosine and an irrelevant monoclonal antibody (against p53) was used as control in **lanes 1** and **3**.

Establishing the source of oxidants is key to understanding the pathogenesis of tumors. Our results in this study demonstrate that: (1) treatment with a DNA damaging agent up-regulates iNOS protein expression in 3T3 cells; (2) this increased iNOS expression parallels with the accumulation of 3-nitrotyrosine modified proteins; (3) both the accumulation and the number of nitrated proteins following MNU treatment depend largely on the status of PARP-1. These data as a whole suggest that the magnitude of cellular injury induced by DNA damage is connected with the NO-dependent pathway of oxidative stress and conversely, the functional status of a key components of the cell's genome surveillance machinery (such as PARP-1) influences the extent of NO-derived oxidative damage, in such a way that the absence of PARP-1 represents a benefit in order to avoid cellular damage. The nature of the mechanism bridging PARP-1 with NO-derived oxidative damage is not clear yet. There are a number of studies where pharmacological inhibition or genetic deletion of PARP-1 results in a diminished inflammatory response and reduced nitrogen-derived oxidative species (reviewed in [Shall and de Murcia, 2000]). Different mechanisms have been proposed to explain that PARP-1 inactivation improve the outcome of a variety of pathophysiological conditions associated with an exacerbated tissue damage or systemic inflammation in the animal [Szabo and Dawson, 1998]. The most extended model implicates PARP-1 in the following pathway: after an oxidative stress (as MNU treatment induces in our model), different cells activate a massive synthesis of NO, which is in turn converted into a cytotoxic derivative, peroxynitrite. Rapid DNA single-stranded breaks are induced by peroxynitrite, leading to over-activation of PARP-1 and depletion of cellular energy resulting in mitochondrial free radical generation and cell necrosis [Szabo et al., 1997]. On the other hand, PARP-1 may promote cell injury through its effects on NF-κB activation that results in massive iNOS induction during inflammation [Oliver et al., 1999; Conde et al., 2001]. One or both of these mechanisms might also explain the resistance of *parp-1*−/− mice to brain ischemia, where synthesis of NO by different isoforms of NOS, TNF-α upregulation and NF-κB activation play a crucial role [Iadecola et al., 1995; Barone et al., 1997; Schneider et al., 1999].

Peroxynitrite is known to initiate oxidative modification of proteins and the evidence for the in vivo formation of peroxynitrite has been derived from immunohistochemical detection of 3-nitrotyrosine in different human tissues. Protein modification by 3-nitrotyrosine is increased in different pathologies and is used as a marker of cellular injury. So far several proteins have been identified to become nitrated both in vivo and in vitro including MnSOD [van der Loo et al., 2000]. Our data show a pattern of protein nitration, corresponding approximately to molecular weights 65, 56, 44, and 31 kDa in *parp-1*+/+ cells; the only modified bands in *parp-1*−/− are the 44 kDa, constitutively modified, and the 56 kDa, with a very faint appearance. In our study, the identity of one of

the nitrated proteins (MnSOD) has been determined by co-immunoprecipitation.

Mammalian mitochondria are one of the most important targets of the cytotoxicity of peroxynitrite. A mechanism for peroxynitrite-mediated dysfunction of mitochondria has been proposed as follows. When mitochondria are exposed to NO, NO diffuses easily through the membranes and reversibly inhibits cytochrome oxidase. This inhibition causes inactivation of the mitochondrial respiratory chain and as a consequence increases mitochondrial O_2^- formation leading to peroxynitrite formation [Cassina and Radi, 1996; Hsu et al., 1996]. Peroxynitrite then irreversibly inhibits complexes I and II in the mitochondrial respiratory chain [Hsu et al., 1996]. Mitochondrial MnSOD has a function to eliminate O_2^- from the mitochondrial matrix space and to scavenge ROS. Different groups have shown that nitration and inhibition of this enzyme is linked to increased rates of production of peroxynitrite [Szabo and Dawson, 1998]. In our study we have identified the 31 kDa band of NO_2-Tyr modified proteins as MnSOD and, more importantly, we have shown that PARP-1 single deficient cells lack this modification.

Nonetheless, the physiological implications of the absence of MnSOD nitration in these cells remain to be elucidated. The mechanism by which PARP-1 single mutant cells are less sensi-tive to peroxynitrite-induced mitochondrial dysfunction (as shown in Fig. 3 by the decreased coincident (yellow) immunofluorescence signal) is probably related with the higher efficacy of these cells and knockout mice to cope with oxidative damage during inflammation and ischemia-reperfusion injury acting together with the inhibition of NAD^+ depletion [Szabo and Dawson, 1998] and by the inhibition NF-κB dependent acute inflammatory response [Oliver et al., 1999].

In conclusion, the data presented here, as a whole, demonstrate that PARP-1 is involved in the regulation of the NO-induced oxidative stress leading to protein modification by nitration, and indirectly in the cell injury derived from the activation of this pathway by a DNA damaging agent. This finding might help to improve current therapeutic strategies based in chemotherapy trying to minimize adverse inflammatory and cell injury responses of tumors using proper PARP-1 inhibitors [Szabo et al., 1998]. In any case, all the approaches which aim to eliminate *parp-1* or PARP-1 activity from the cell or the organism should take into account that this enzyme is involved in genomic surveillance, and its long term inhibition might lead to the accumulation of DNA damage, mutations and oncogenic transformation.

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