

ARTICLE

Detection of Poly(ADP-ribose) Polymerase Activation in Oxidatively Stressed Cells and Tissues Using Biotinylated NAD Substrate

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SUMMARY Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme activated by DNA damage. Activated PARP cleaves NAD⁺ into nicotinamide and (ADP-ribose) and polymerizes the latter on nuclear acceptor proteins. Over-activation of PARP by reactive oxygen and nitrogen intermediates represents a pathogenetic factor in various forms of inflammation, shock, and reperfusion injury. Using a novel commercially available substrate, 6-biotin-17-nicotinamide-adenine-dinucleotide (bio-NAD⁺), we have developed three applications, enzyme cytochemistry, enzyme histochemistry, and cell ELISA, to detect the activation of PARP in oxidatively stressed cells and tissues. With the novel assay we were able to detect basal and hydrogen peroxide-induced PARP activity in J774 macrophages. We also observed that mitotic cells display remarkably elevated PARP activity. Hydrogen peroxide-induced PARP activation could also be detected in wild-type peritoneal macrophages but not in macrophages from PARP-deficient mice. Application of hydrogen peroxide to the skin of mice also induced bio-NAD⁺ incorporation in the keratinocyte nuclei. Hydrogen peroxide-induced PARP activation and its inhibition by pharmacological PARP inhibitors could be detected in J774 cells with the ELISA assay that showed good correlation with the traditional [³H]-NAD incorporation method. The bio-NAD⁺ assays represent sensitive, specific, and non-radioactive alternatives for detection of PARP activation.

(J Histochem Cytochem 50:91–98, 2002)

KEY WORDS

poly(ADP-ribose) polymerase
hydrogen peroxide
biotinylated NAD⁺
enzyme histochemistry
cell ELISA

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that becomes activated in response to DNA damage (de Murcia and Menissier de Murcia 1994). Activated PARP cleaves NAD⁺ to nicotinamide and ADP-ribose and polymerizes the latter on nuclear acceptor proteins such as histones, transcription factors, and PARP itself. Poly-ADP ribosylation contributes to DNA repair and to the maintenance of genomic stability (Wang et al. 1997; Simbulan-Rosenthal et al. 1999; Muiras and Bürkle 2000). During inflammation, ischemia–reperfusion, or shock, free radical/oxidant-induced DNA single-strand breakage triggers the

over-activation of PARP, leading to depletion of NAD⁺ (Szabó and Dawson 1998; Szabó 2000). In an effort to re-synthesize NAD⁺, ATP is also consumed, resulting in necrotic type cell death (Schraufstatter et al. 1986; Virág et al. 1998a,b). This PARP-mediated pathway of cell suicide has been implicated in the death of immune-stimulated macrophages as well as in peroxynitrite- or hydrogen peroxide-induced dysfunction or cell death of thymocytes, macrophages, endothelial cells, neuronal cells, and fibroblasts (Zingarelli et al. 1996; Szabó et al. 1998; Virág et al. 1998b; Soriano et al. 2001). Inhibition of PARP activity by pharmacological inhibitors or the absence of functional PARP enzyme in PARP knockout animals provided significant protection in animal models of a wide variety of diseases, including various forms of inflammation, shock, stroke, myocardial ischemia, dia-

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Received for publication April 23, 2001; accepted July 27, 2001 (1A5527).

betes, and diabetic endothelial dysfunction (Szabó and Dawson 1998; Szabó 2000).

For measurement of PARP activity, the incorporation of radioactivity from isotope-labeled NAD⁺ into TCA-precipitable proteins is considered the gold standard (Schraufstatter et al. 1986). Alternatively, poly(ADP-ribose), the product of the PARP-catalyzed reaction can be purified from cells and tissues by a tedious procedure and the polymer can be quantitated by HPLC (Kiehlbauch et al. 1993; Shah et al. 1995). A more convenient approach to assessment of PARP activation is the detection of poly(ADP-ribose) by a monoclonal anti-poly(ADP-ribose) antibody in Western blots, dot-blots, immunocytochemistry, and flow cytometry (Affar et al. 1998,1999). However, the use of the murine monoclonal anti-poly(ADP-ribose) antibody in mouse tissues, especially in inflamed tissues, often results in high background staining (unpublished observations). Moreover, the amount of the polymer synthesized does not necessarily reflect the degree of PARP activation, because in the cells and tissues poly(ADP-ribose) is rapidly metabolized by poly(ADP-ribose) glycohydrolase (PARG) (Ueda et al. 1972).

Recently, a novel non-radioactive assay has been marketed by Trevigen (Gaithersburg, MD) for screening of potential PARP inhibitors. The assay utilizes a novel PARP substrate, 6-biotin-17-nicotinamide-adenine-dinucleotide (bio-NAD⁺), which was originally developed to detect and isolate mono-ADP-ribosylated proteins (Zhang and Snyder 1993). In this assay, 96-well plates are coated with histones as acceptor proteins and biotinyl-ADP ribose is incorporated from bio-NAD⁺ into the histones by purified PARP. Biotin incorporation is then detected by streptavidin–peroxidase and a suitable peroxidase substrate. The potency of PARP inhibitors is assessed on the basis of their inhibition of biotinyl–ADP-ribose incorporation. We set out to investigate whether this commercially available novel PARP substrate, bio-NAD⁺, can be used to detect cellular PARP activation. We have developed a cellular ELISA (CELISA) assay to quantify PARP activation in cultured cells and an enzyme cytochemical/histochemical reaction to detect PARP activation in oxidatively stressed cells and tissues.

Materials and Methods

Materials

Biotinylated NAD⁺ and the TACS-Saphire substrate were purchased from Trevigen. Streptavidin–peroxidase was from Sigma (St Louis, MO). Peroxynitrite was from Cayman Chemical (Ann Arbor, MI). The PARP inhibitor PJ-34 was synthesized in our laboratory as described (Soriano et al. 2001). Cryoembedding medium was from Shandon (Pittsburgh, PA). Nuclear fast Red and Vectamount were obtained from Vector Laboratories (Burlingame, CA). Triti-

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ated NAD was from NEN Life Science Products (Boston, MA). All other chemicals were from Sigma.

Application of Hydrogen Peroxide to the Skin

Animal experiments conform with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health and the treatment protocol was approved by the Institutional Animal Care and Use Committee. C57/BL6 mice were used in the experiments and were allowed free access to food and water. Hair was removed from the back of mice ($n=4$) by Veet creme. Next day, hydrogen peroxide (250 nmol/50 μ l PBS, pH 7.4) was smeared onto the skin by a micropipette. Control animals ($n=4$) were treated the same way with PBS. After 30 min, mice were sacrificed and skin was excised. Samples were embedded in cryoembedding medium and immediately placed in a -70°C freezer.

Isolation of Peritoneal Macrophages

PARP-proficient (PARP^{+/+}) and PARP-deficient (PARP^{-/-}) mice generated by Wang et al. (1995) were sacrificed by an overdose of CO₂ and 10 ml of RPMI 1640 medium was injected IP. The medium was then withdrawn and spun down (250 \times g, 10 min). Cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and seeded on chamber slides. After 2-hr incubation at 37C, non-adherent cells were removed and cells were incubated in RPMI medium/10% FCS for 1 hr. Cells were pretreated with 5 μ M PJ34, 5 mM 3-aminobenzamide, or PBS for 30 min and then treated with 200 μ M hydrogen peroxide. Cytochemical PARP detection was carried out as described below.

Cytochemical PARP Detection

J774.1 cells were cultured on coverslips in RPMI medium supplemented with 10% FCS. At 20 min after treatment with H₂O₂ (500 μ M), the medium was removed and replaced with PARP reaction buffer (56 mM HEPES, 28 mM KCl, 28 mM NaCl, 2 mM MgCl₂, pH 8.0, complemented with 0.01% digitonin, 12.5 μ M biotinylated NAD⁺ immediately before use). Control reactions were carried out in the presence of the PARP inhibitors PJ34 (30 μ M) or 3-aminobenzamide (5 mM). After 60-min incubation at 37C, the cells were fixed in 95% ethanol (10 min at -20°C) followed by 10 min in 10% TCA (-20°C). Coverslips were rinsed in PBS, pH 7.4 (10 min), and endogenous peroxidase was blocked by 0.5% H₂O₂/methanol for 15 min. After two 5-min rinses with PBS, pH 7.4, coverslips were blocked in 1% BSA/PBS for 30 min followed by two rinses in PBS–Triton X-100 (0.1%). Incorporated biotin was detected by streptavidin–peroxidase (diluted 1:100 in PBS–Triton X-100 for 30 min at room temperature). Coverslips were washed four times for 5 min with PBS, pH 7.4–Triton X-100 and color was developed with cobalt-enhanced nickel–DAB substrate. Coverslips were mounted with glycerol on slides and viewed with a Zeiss Axiolab microscope. Pictures were taken with a Zeiss Axiocam digital camera.

PARP Enzyme Histochemistry

Cryosections (10 μ m) were fixed for 10 min in 95% ethanol at -20°C and then rinsed in PBS. Sections were permeabilized by 1% Triton X-100 in 100 mM Tris, pH 8.0, for 15

min. Reaction mixture (10 mM MgCl₂, 1 mM dithiothreitol, 30 μM biotinylated NAD⁺, in 100 mM Tris, pH 8.0) was then applied to the sections for 30 min at 37°C. Reaction mix containing PARP inhibitors (30 μM PJ34 or 5 mM 3-aminobenzamide) or biotinyl-NAD⁺-free reaction mix were used as controls. After three washes in PBS, incorporated biotin was detected by peroxidase-conjugated streptavidin (1:100, 30 min, RT). After three 10-min washes in PBS, color was developed with cobalt-enhanced nickel-DAB substrate: 4 min incubation in nickel-DAB solution (95 mg DAB, 1.6 g NaCl, 2 g nickel sulfate and 25 μl of 30% hydrogen peroxide in 0.1 M acetate buffer pH 6.0) followed by 5 min incubation in TRIS-cobalt solution (1.2 g TRIS base, 1 g cobalt chloride in 200 ml distilled water, pH 7.2). Sections were counterstained in Nuclear Fast Red, dehydrated, and mounted in Vectamount.

CELISA Method for Detection of PARP Activation

J774.1 cells were seeded in 96-well plates in RPMI/10% fetal bovine serum. Next day, cells were treated with the PARP inhibitor PJ34 (5 μM) for 30 min and then stimulated with hydrogen peroxide (100–400 μM). Medium was then replaced by PARP reaction buffer (56 mM HEPES, 28 mM KCl, 28 mM NaCl, 2 mM MgCl₂) containing 0.01% digitonin and 10 μM biotinylated NAD⁺. Plates were incubated for 30 min at 37°C. Buffer was then aspirated and cells were fixed by the addition of 200 μl/well pre-chilled 95% ethanol at –20°C for 10 min. Endogenous peroxidase activity was blocked by 15-min incubation in 0.5% hydrogen peroxide/methanol. Wells were washed once with 300 μl/well PBS and then blocked by 1% BSA in PBS (200 μl/well) for 30 min at 37°C. BSA solution was then aspirated and replaced by 50 μl/well peroxidase-labeled streptavidin (diluted 1:500 in 1% BSA-PBS). After incubation (30 min at 37°C), plates were washed three times with PBS and reaction was developed with TACS-Saphire (Trevigen) substrate (100 μl/well). The optical density was measured with a microplate spectrophotometer (Molecular Devices; Sunnyvale, CA). Data were expressed as mean ± SD of quadruplicate samples.

[³H]-NAD Incorporation Assay

The assay was carried out as described previously (Szabó et al. 2001). J774 cells were seeded in 12-well plates and were treated with the PARP inhibitor PJ34 (5 μM) for 30 min. Cells were then exposed to 50–400 μM hydrogen peroxide and incubated for 30 min at 37°C. Medium was then replaced by 0.5 ml PARP buffer [56 mM Hepes pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM MgCl₂, 0.01% digitonin, and 0.125 μM [³H]-NAD (0.5 μCi/ml)] and plates were incubated for 10 min at 37°C. Cells were then scraped and transferred into Eppendorf tubes. Two hundred μl ice-cold 50% trichloroacetic acid (TCA) was added to the samples and tubes were incubated for 4 hr at 4°C. Samples were then spun (10,000 × g, 10 min) and the pellets washed twice in ice-cold 5% TCA and solubilized overnight in 250 μl 2% SDS/0.1 N NaOH at 37°C. Contents of the tubes were added to 7 ml ScintiSafe Plus scintillation liquid (Fisher Scientific) and radioactivity was determined in a liquid scintillation counter (Wallac; Gaithersburg, MD). Data were expressed as mean ± SD of quadruplicate samples.

Statistical Analysis

PARP activity in the CELISA and [³H]-NAD assays was determined from quadruplicate samples. Data are given as mean ± SD of quadruplicate samples. Experiments were repeated three times. Statistical significance was calculated by unpaired Student's *t*-test.

Results

Enzyme Cytochemical Detection of PARP Activity in J774 Cells

J774 macrophages were stained for PARP activity with the bio-NAD⁺ substrate (Figure 1). All cells showed a predominantly nuclear staining (Figure 1A), indicating that the bio-NAD⁺-metabolizing enzyme localizes in the nucleus. The intensity of nuclear staining in the vast majority of cells was moderate, reflecting basal PARP activity. In sharp contrast to interphase cells, mitotic cells displayed an intense nuclear staining (Figures 1A and 1B). These cells appeared to be in the metaphase or ana-telophase of the mitotic cycle. Treatment of J774 cells with 500 μM H₂O₂ induced a marked enhancement of ADP ribosylating activity, as indicated by the strong nuclear staining of H₂O₂-treated cells (Figure 1C). Pretreatment of cells with the novel potent PARP inhibitor PJ34 (5 μM) (Abdelkarim et al. 2001; Soriano et al. 2001) 30 min before H₂O₂ exposure prevented enhancement of bio-NAD⁺ staining (Figure 1D). Similar results were also obtained with other types of cells, including fibroblasts and human keratinocytes (data not shown).

Lack of Hydrogen Peroxide-induced Bio-NAD⁺ Incorporation into PARP-1-deficient Macrophages

To prove the identity of the product synthesized by the cells from bio-NAD⁺, we used wild-type (PARP^{+/+}) and PARP-deficient (PARP^{-/-}) macrophages (Figure 2). Exposure of cells to 200 μM hydrogen peroxide induced bio-NAD⁺ incorporation into the nuclei of wild-type but not of PARP-deficient macrophages. Bio-NAD⁺ incorporation could be inhibited with PJ34 (Figure 2) or 3-aminobenzamide (not shown). These results indicate that PARP-1 is responsible for bio-NAD⁺ incorporation into hydrogen peroxide-treated macrophages.

Enzyme Histochemical Detection of PARP Activation in Hydrogen Peroxide-treated Mouse Skin

To demonstrate the ability of the bio-NAD⁺ method to detect PARP activation in tissues, we applied hydrogen peroxide (250 nmol/50 μl) to the skin of mice for 30 min. Skin was excised and immediately frozen in cryoembedding medium to preserve enzyme activity. Frozen sections (10 μm) permeabilized with Triton X-100 were incubated with the bio-NAD⁺ substrate followed by biotin detection with streptavidin-per-

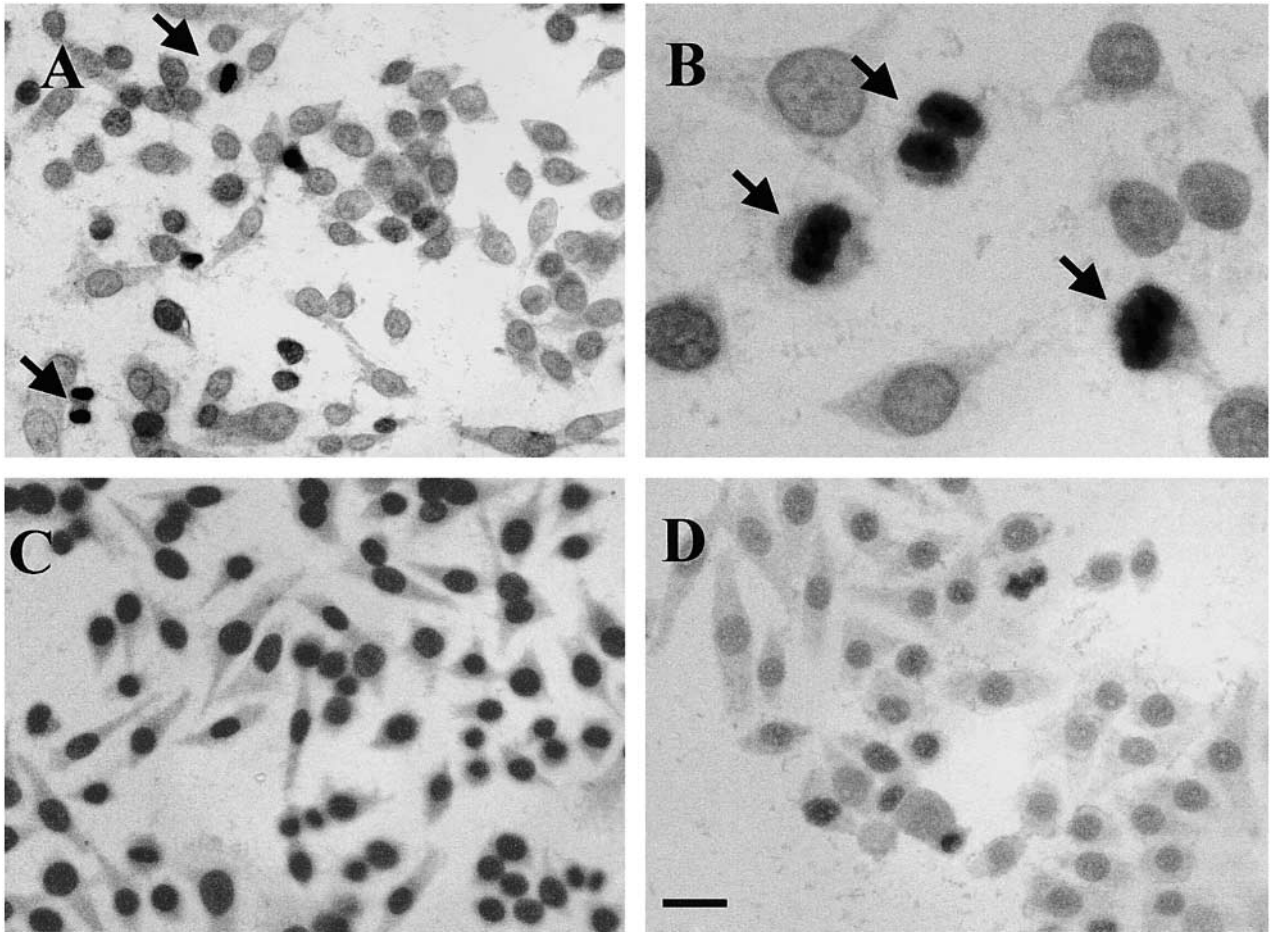


Figure 1 Basal and hydrogen peroxide-induced poly-ADP ribosylation in J774 macrophages. Untreated (A,B) and hydrogen peroxide-treated J774 cells were stained with the bio-NAD method as described in Materials and Methods. A low-intensity nuclear staining could be visualized with the assay in untreated cells, reflecting baseline PARP activity (A,B). Mitotic cells displayed increased poly-ADP ribosylating activity (arrows). In response to hydrogen peroxide (500 μ M), a markedly increased nuclear staining could be detected in J774 cells (C) but not in cells pretreated with the PARP inhibitor PJ34 (5 μ M) (D). Bars: A,C,D = 8 μ m; B = 3 μ m.

oxidase. In control (vehicle-treated) skin, no detectable ADP ribosylation was found (Figure 3A). Peroxynitrite treatment activated PARP in the skin, as indicated by the appearance of darkly stained cells (Figure 3B). Staining was nuclear and was most intense in keratinocytes. However, some scattered cells in the dermis also showed nuclear PARP activity (Figure 3B). The presence of the PARP inhibitor PJ34 (5 μ M) (Figure 3C) or 3-aminobenzamide (5 mM) (not shown) abolished peroxynitrite-induced bio-ADP-ribose incorporation, demonstrating that PARP activation was responsible for the staining.

Determination of Cellular PARP Activation with Bio-NAD⁺ Substrate in a CELISA Method

A cellular ELISA method allows the quantification of PARP activity. Furthermore, the potency of pharmacological PARP inhibitors in cells can also be deter-

mined in a CELISA. J774 macrophages seeded in 96-well plates were exposed to hydrogen peroxide (50–400 μ M) in the presence or absence of PJ34 (5 μ M). Hydrogen peroxide induced a dose-dependent PARP activation in J774 cells, and pretreatment with the PARP inhibitor PJ-34 suppressed hydrogen peroxide-induced PARP activation (Figure 4). As a reference method, [³H]-NAD incorporation was also used to measure PARP activity and the two assays showed good correlation ($r^2 = 0.92$) with the bio-NAD⁺ method, giving higher induction results.

Discussion

We have demonstrated that bio-NAD⁺ can be used as a substrate for PARP in cells and tissues. In untreated J774 macrophage, bio-NAD⁺ metabolizing activity could be detected in the nuclei. The nuclear staining

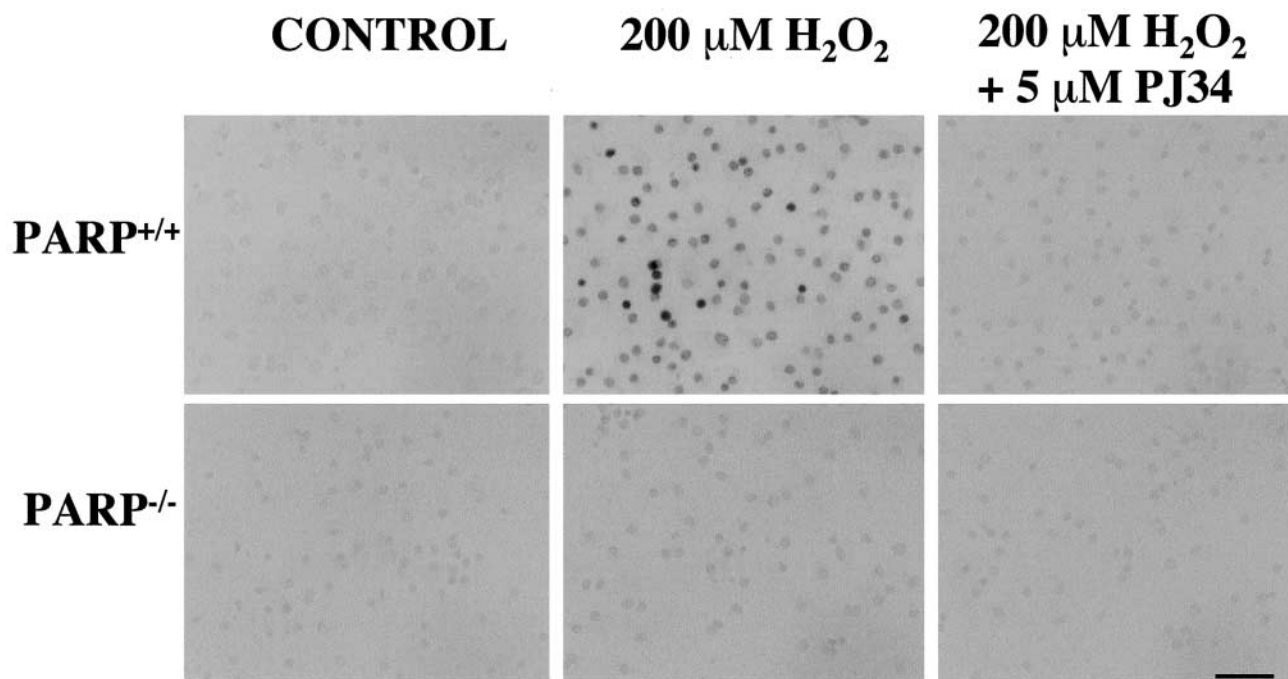


Figure 2 Hydrogen peroxide-induced poly-ADP ribosylation in mouse peritoneal macrophages. Macrophages were isolated from the peritoneal cavity of wild-type (PARP^{+/+}) and PARP deficient (PARP^{-/-}) mice. Cells were seeded onto chamber slides and treated with 200 μ M hydrogen peroxide in the presence or absence of PJ34 (5 μ M). Bio-NAD⁺ incorporation was detected as described in Materials and Methods. Bar = 15 μ m.

pattern indicates that bio-NAD⁺ is metabolized most likely by PARP, a nuclear enzyme polymerizing ADP-ribose units on nuclear acceptor proteins. It has been shown previously that bio-NAD⁺ can also serve as a substrate for mono-ADP ribosylation *in vitro* (Zhang and Snyder 1993). However, this G-protein-coupled process takes place in the plasma membrane. Because bio-NAD staining is localized to the nuclei, mono-ADP ribosylation is not likely to contribute to bio-NAD⁺ metabolism in our system. Furthermore, the polymeric nature of the PARP product, as opposed to mono-ADP-ribose, may also be responsible for its good detectability with streptavidin–peroxidase. We observed an interesting phenomenon in untreated J774 cells. Mitotic cells displayed strong nuclear positivity compared to interphase cells. Many cells representing various stages of mitosis could be found with strong reactivity localized to the condensed chromatin but not to the interchromatin areas. This observation, however, is not surprising in light of previous observations linking PARP to the process of replication. PARP has been shown to associate with and to regulate the activity of topoisomerase I, an enzyme-uncoiling DNA, by temporarily cutting into one of the DNA strands (Ferro et al. 1984; Bauer et al. 2000; Bauer and Kun 2000). Association of PARP with other proteins involved in replication, such as the DNA polymerase I–primase complex, has also been demonstrated (Dantzer et

al. 1998). Furthermore, it has also been reported that in PARP knockout cells, but not in cells treated with PARP inhibitors, a tetraploid population emerges, indicating that the presence of PARP protein but not PARP activity is required to maintain chromosomal stability (Simbulan–Rosenthal et al. 1999,2001). Although PARP protein has previously been shown to localize to centrosomes and chromosomes during cell division and in the interphase (Kanai et al. 2000), to our best knowledge this is the first morphological demonstration of increased poly(ADP-ribose) polymerase activity in mitotic cells.

In line with previous data reporting PARP activation in oxidatively stressed cells (Szabó et al. 1998; Virág et al. 1998b), treatment of J774 cells with hydrogen peroxide caused a marked enhancement of nuclear bio-NAD⁺ staining. Hydrogen peroxide-induced bio-NAD⁺ metabolism could be blocked by PJ34 or 3-aminobenzamide, inhibitors of PARP, providing further confirmation that PARP activity is responsible for bio-NAD⁺ staining in our system. The question arises as to what degree the different PARP isoenzymes contribute to bio-NAD⁺ incorporation under basal conditions and after oxidative stress. Until recently, PARP activity was believed to result from the function of a single enzyme. After the observation that PARP-deficient cells have some residual PARP activity (Shieh et al. 1998), intensive research began to identify enzymes

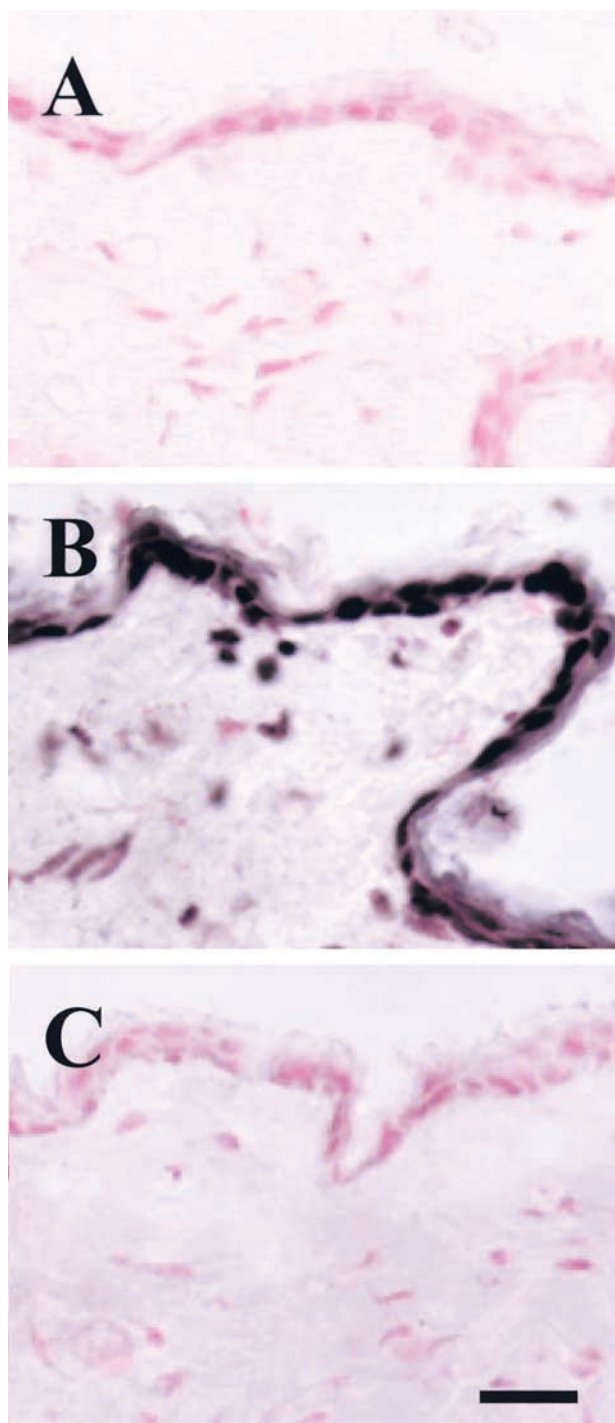


Figure 3 Enzyme histochemical detection of PARP activation in hydrogen peroxide-treated skin. Frozen sections were cut from untreated (A) and hydrogen peroxide-treated (B,C) mouse skin and were stained with the bio-NAD method. (C) A section stained in the presence of the PARP inhibitor 3-aminobenzamide (5 mM). Bar = 10 μ m.

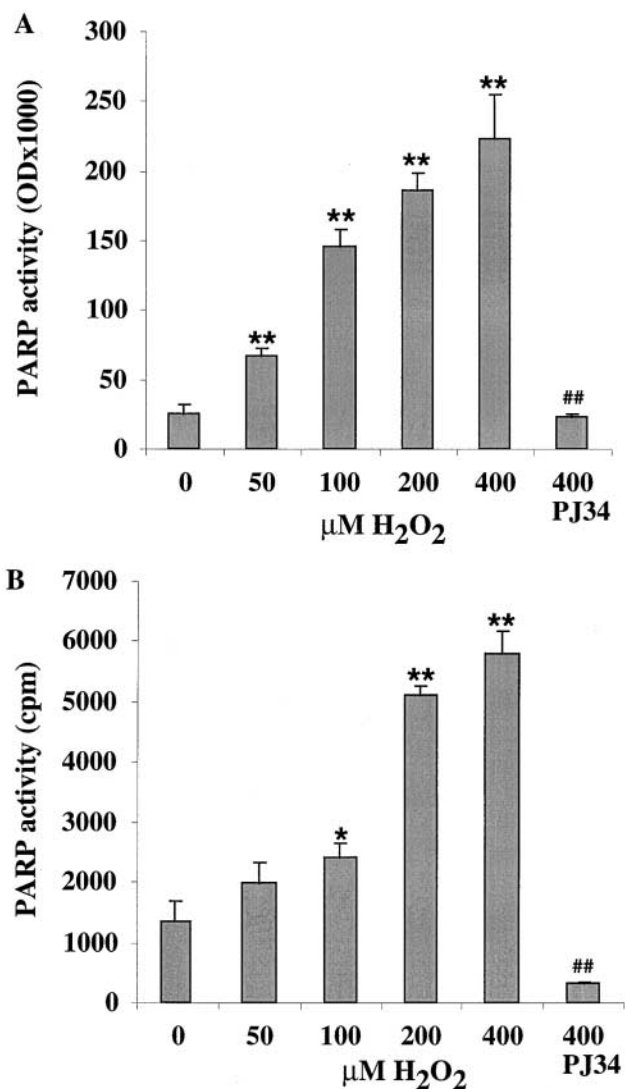


Figure 4 Detection of hydrogen peroxide-induced PARP activation in J774 cells with the PARP CELISA method. J774 cells were seeded in 96-well plates (for the CELISA method) or 12-well plates (for the $[^3H]$ -NAD⁺ assay) and were treated with the indicated concentration of hydrogen peroxide in the absence or presence of 5 μ M PJ34. PARP activity was detected with the CELISA method and with the $[^3H]$ -NAD⁺ assay, as described in Materials and Methods. Results are given as mean \pm SD of quadruplicate samples. Asterisks indicate significantly (* p <0.05; ** p <0.01) increased PARP activity compared to control; ## indicates significant (p <0.01) suppression of PARP activity by PJ34. p values were calculated by unpaired Student's t -test.

responsible for this activity. In the past 3 years, four other enzymes possessing poly(ADP-ribosylation) activity have been described (for review see Szabó 2000) and named PARP-2–5, with the founding member of the PARP enzyme family now designated as PARP-1. Although research on the biological role of these novel PARP enzymes is in the embryonal stage, interesting differences in domain structure, subcellular localiza-

tion, tissue distribution, and ability to bind to DNA have already been established. Our data showing increased bio-NAD⁺ incorporation in wild-type but not in PARP-1-deficient macrophages indicates that, at least under conditions of oxidative stress, PARP-1 is responsible for bio-NAD⁺-incorporating activity.

In situ immunohistochemical demonstration of PARP activation in tissues by detecting poly(ADP-ribose) was successfully demonstrated in some cases by our group (Liaudet et al. 2000) and by others (Eliasson et al. 1997). However, in mouse tissues, evaluation of stainings obtained by using the mouse monoclonal anti-poly(ADP-ribose) antibody is often difficult because of possible crossreaction of the secondary anti-mouse immunoglobulin-peroxidase conjugates with the endogenous immunoglobulins that are present in the mouse tissues. This problem may be very difficult to overcome in inflamed tissues, in which extravasation leads to increased immunoglobulin content. This technical problem can be circumvented by the use of bio-NAD⁺ enzyme histochemistry. Using this assay, PARP activation has been detected and localized in vascular tissues of diabetic mice in our recent studies (Soriano et al. 2001).

Another technical difficulty that must be considered is the removal of poly(ADP-ribose) by poly(ADP-ribose) glycohydrolase (PARG). This could theoretically also occur with biotinyl-poly(ADP-ribose). Bürkle and co-workers have previously reported that PARG can be inactivated by treating cells with trichloroacetic acid (Lankenau et al. 1999). Therefore, we included this step in our cytochemical procedure to prevent removal of the incorporated biotinylated-ADP-ribose. In tissues, however, we found that TCA treatment was unnecessary.

For a long time the only assay in which PARP inhibitors could be tested was a radioactive method using either ³²P- or ³H-labeled NAD⁺. This assay could be used both with purified PARP and in cellular systems. The appearance of the bio-NAD⁺ assay on the market provided a non-radioactive alternative to assess the efficacy of potential PARP inhibitors in a cell-free system. Our CELISA protocol has now extended the applicability of the bio-NAD⁺ substrate to the measurement of cellular PARP activity. The CELISA assay showed good correlation with the [³H]-NAD⁺ method. It is also important to emphasize here that, according to our experience, because of differences in cell permeability of different compounds the IC₅₀ values may dramatically differ in a cellular PARP assay compared to cell-free assays. Therefore, we believe that elaboration of a CELISA method for quantitation of cellular PARP activity represents an important advancement aiding more successful research in the PARP field.

In summary, we have developed three applications to detect or to measure PARP activation in cells and tissues. The assays are based on the use of biotinylated NAD⁺ as a commercially available PARP substrate.

The straightforward protocols described here allow a simple, cost-effective two-step detection of activated PARP in oxidatively stressed tissues and cells.

Acknowledgments

Supported by grants from the Hungarian Ministry of Health (ETT 104/99) and from the Hungarian National Science Research Fund (OTKA T 035182) to LV, by a grant from the National Institutes of Health to CS (R01GM60915), and by a grant from the Health Science Center of the University of Debrecen to ES (Mec 10/99). LV was supported by a Bolyai fellowship from the Hungarian Academy of Sciences.

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