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Inhibition of Poly(ADP-Ribose) Glycohydrolase by Gallotannin Selectively Up-Regulates Expression of Proinflammatory Genes

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ABSTRACT

Poly(ADP-ribose)-polymerase-1 (PARP-1) and poly(ADP-ribose) (PAR) are emerging key regulators of chromatin superstructure and transcriptional activation. Accordingly, both genetic inactivation of PARP-1 and pharmacological inhibition of PAR formation impair the expression of several genes, including those of the inflammatory response. In this study, we asked whether poly(ADP-ribose) glycohydrolase (PARG), the sole depoly(ADP-ribosyl)ating enzyme identified so far, also regulates gene expression. We report the novel finding that inhibition of PARG by gallotannin triggered nuclear accumulation of PAR and concomitant PAR-dependent expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), but not of interleukin-1β and tumor necrosis factor-α, in cultured RAW 264.7 macrophages. Remarkably, silencing of PARG by means of small interfering RNA selectively impaired gallotannin-induced expression of iNOS and COX-2. Consistent with a PAR-dependent transcriptional activation, increases of iNOS and COX-2 transcripts were not caused by activation of transcription factors such as nuclear factor-κB, activator protein-1, signal transducer and activator of transcription-1 or interferon regulatory factor-1, nor by mRNA stabilization. Overall, our data provide the first evidence that pharmacological inhibition of PARG leads to PAR-dependent alteration of gene expression profiles in macrophages.

Poly(ADP-ribosyl)ation is a post-translational modification of proteins operated by poly(ADP-ribose) polymerases (PARPs). These are nuclear and cytoplasmic enzymes metabolizing β-NAD^+ into polymers of ADP-ribose (Smith, 2001), which, once targeted to proteins, serve as key regulators of their functions (D'Amours et al., 1999). The majority of poly(ADP-ribose) (PAR) formation is caused by the activity of nuclear PARP-1, a DNA damage-dependent enzyme with active roles in cell death (Pieper et al., 1999; Herceg and Wang, 2001; Yu et al., 2003). Mounting evidence, however, indicates that PAR is synthesized also in the absence of DNA damage (Kun et al., 2002), playing key roles in the homeostatic regulation of chromatin functions (de Murcia et al., 1988; D'Amours et al., 1999; Zlatanova et al., 2000). For example, PARP-1 and PAR assist chromatin decondensation (de Murcia et al., 1988; Tulin and Spradling, 2003) and regulate activity of transcription factors such as NF-κB, AP-1, AP-2, YY-1, Oct-1, p53, and heat shock factor-1 (Ziegler and Oei, 2001; Chiarugi, 2002b; Ha et al., 2002; Hassa and Hottiger, 2002; Kraus and Lis, 2003; Zingarelli et al., 2004). In addition, PARP-1 is the general transcription factor TFIIC (Slattery et al., 1983) and binds an RNA polymerase II domain of relevance to epigenetic regulation (Carty and Greenleaf, 2002), suggesting an important role of the enzyme in the regulation of the transcriptional machinery. In keeping with this, PARP-1 deletion alters basal gene expression profiles (Simbulan-Rosenthal et al., 2000), and inhibition of PAR formation impairs the expression of several genes, including those of the inflammatory response (Szabo, 1998; Chiarugi, 2002a; Ha et al., 2002; Chiarugi and Moskowitz, 2003). This is clearly exemplified by the resistance of PARP-1−/− mice to endotoxic shock (Oliver et al., 1999).

It is well-established that PAR has a very short half-life (~1 min), being promptly degraded by the constitutively active poly(ADP-ribose) glycohydrolase (PARG). PARG is a 110-kDa protein ubiquitously expressed in mammalian cells

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ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose); AP, activator protein; COX, cyclooxygenase; GLTN, gallotannin; IL, interleukin; iNOS, inducible nitric-oxide synthase; IRF-1, interferon responsive factor-1; PARP-1, poly(ADP-ribose) polymerase-1; NF-κB, nuclear factor κB; PARG, poly(ADP-ribose) glycohydrolase; TNF-α, tumor necrosis factor-α; siRNA, small inducible RNA; STAT, signal transducer and activator of transcription; LPS, lipopolysaccharide; MNNG, methyl-nitrosoguanidine; BZD, benzamide; PHE, phenathridinone; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; TPBS, phosphate-buffered saline containing 0.1% Tween 20 and 5% skimmed milk; Act-D, actinomycin D.

with endo- and exoglycosidic activity, cleaving PAR into free ADP-ribose units (Davidovich et al., 2001). Although PARG resides in the cytoplasm, nuclear localization and exportation signals in its amino acid sequence allow nuclear shuttling and regulation of PAR content (Bonicalzi et al., 2003). Little is known, however, about the role of PARG in cell homeostasis, although experimental evidence indicates that the enzyme is involved in development (Hanai et al., 2004), differentiation (Di Meglio et al., 2003), and cell death (Affar et al., 2001; Ying et al., 2001). These findings, along with the apparent cell-cycle–dependent nuclear localization of PARG (Ohashi et al., 2003), suggest an important role for this latter in the control of cell functioning.

Given the importance of PARG in PAR metabolism and considering the relevance of PAR to transcription and immune activation, this study investigated the effect of PARG inhibition on the expression of proinflammatory mediators in macrophages.

Materials and Methods

Cells and Culture Conditions. Macrophages of the murine RAW 264.7 cell line were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% bovine serum, and antibiotics. Cultures were incubated at 37°C in a water-saturated 5% CO_2/95% air atmosphere and usually brought to 50 to 70% confluence. Gallotannin (GLTN) (Fluka Chemie, Buchs, Switzerland) and the other drugs were directly dissolved in the incubating medium.

Western Blotting. For Western blotting, cells were scraped, collected in Eppendorf tubes, centrifuged (at 1500*g* for 5 min at 4°C), and resuspended in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin and leupeptin, and 1% SDS). The, 20 to 40 μg of protein per lane was loaded. After 4 to 20% SDS-polyacrylamide gel electrophoresis and blotting, membranes (Hybond ECL; Amersham Biosciences, UK, Ltd., Little Chalfont, Buckinghamshire, UK) were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% skimmed milk (TPBS/5% milk) and then probed overnight with primary antibodies (1:1000 in TPBS/5% milk). The anti-PAR monoclonal antibody (10H) was from Alexis Corporation (Vinci, Italy); the anti-inducible NO synthase (iNOS), anti-interferon regulatory factor-1 (IRF-1), anti-interleukin (IL)-1β, and anti-tumor necrosis factor (TNF)-α were polyclonal antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); the anti–cyclooxygenase-2 (COX-2) polyclonal antibody was from Cayman Chemical (Ann Arbor, MI); the anti-p38 and phospho-p38 were polyclonal antibodies from Transduction Laboratories (Lexington, KY); and the polyclonal anti–phospho-STAT-1 antibody was from Cell Signaling Technology Inc. (Beverly, MA). Membranes were then washed with TPBS and incubated for 1 h in TPBS/5% milk containing the corresponding peroxidase-conjugated secondary antibody (1:2000). After washing in TPBS, ECL (Amersham Biosciences) was used to visualize the peroxidase-coated bands.

Electrophoretic Mobility Shift Assay. The DNA binding activity of NF-κB and AP-1 was investigated in cells, scraped, pelleted, and then resuspended in buffer A (10 mM Hepes, pH 7.8, 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 4 μg/ml each aprotinin and leupeptin). Cells were kept on ice for 15 min, vortexed every 3 min, and then centrifuged (at 5000*g* for 5 min at 4°C). The nuclear pellet was resuspended in 50 μl of buffer B, which was analogous to buffer A plus 400 mM NaCl, and incubated for 10 min on ice. The mixture was centrifuged (at 14,000*g* for 10 min at 4°C), and the surnatant was aliquoted and stored at −80°C. The DNA binding activity was tested by incubating 10 μg of protein of the nuclear extract in 20 μl of a buffer containing 10 mM Tris, pH 7.4, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 0.05 mg/ml poly(dI-dC), and 10,000 cpm of specific ^{32}P-labeled oligonucleotide for 20 min at room temperature. The mixture was electrophoresed in 6% nondenaturing polyacrylamide gels that, after drying, were exposed to X-ray films (Amersham Biosciences). The double-stranded oligonucleotides 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (NF-κB) and 5′-CGCTTGATGAGTCAGCCGGAA-3′ (AP-1) were used. For supershift experiments, 2 μl of the antibodies raised against p65/RelA or c-Jun (Santa Cruz Biotechnology) was added to the binding mixture during incubation.

Semiquantitative RT-PCR. Total RNA (1 μg) extracted with TRIzol (Invitrogen, Carlsbad, CA) was reverse-transcribed into DNA and subjected to PCR using the following software-designed oligonucleotide primers: iNOS, 5′-GGCTGTICAGAGCCTCGTGGCTTTGG-3′ (sense) and 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (antisense); COX-2, 5′-ACACACTCTATCACTGGCACC-3′ (sense) and 5′-TTCAGGGAGAAGCGTTTGC-3′ (antisense); TNF-α, 5′-TTCTGTCTACTGAACTTCGGGGTGATCGGT-3′ (sense) and 5′-GTATGAGATAGCAAATCGGCTGACGGTGTG-3′ (antisense); IL-1β, 5′-CAGGACAGGTATAGATTCTTTCCTT-3′ (sense) and 5′-ATGGCAACTGTTCCTGAACTCAACT-3′ (antisense); PARP-1, 5′-CTGATGTTGAGGTTGATG-3′ (sense) and 5′-CACTTGCTGCTGGTTGAA-3′ (antisense); PARG, 5′-CCACCTCGTTTGTTTTCA-3′ (sense) and 5′-CCAACATCTGGCAAAGGA-3′ (antisense); and β-actin, 5′-GACCTGACAGACTACCTC-3′ (sense) and 5′-AGACAGCACTGTGTGTTGGC-3′ (antisense). The number of PCR cycles (94°C for 30 s, 58°C for 30 s, 72°C for 1 min and then 5

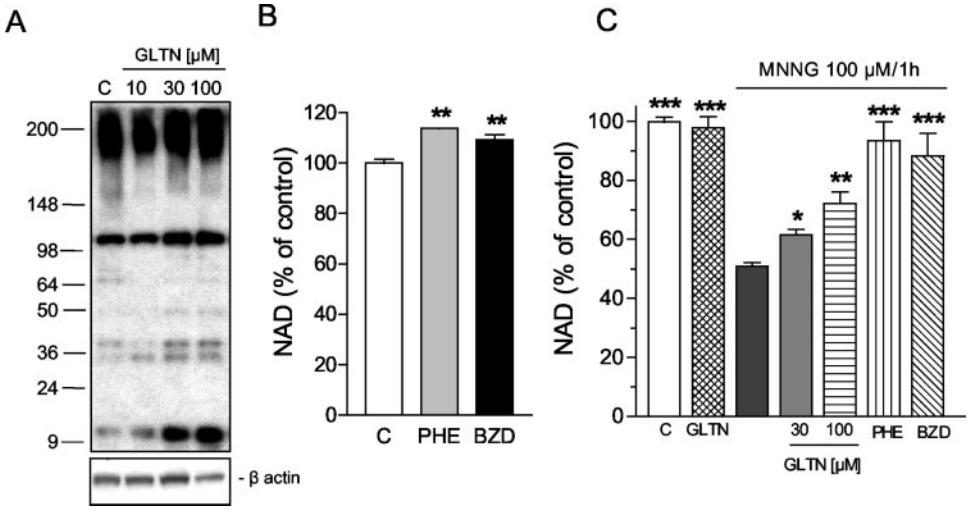

Fig. 1. GLTN and PARP-1 inhibitors modulate poly(ADP-ribosyl)ation and NAD content in RAW macrophages. A, GLTN exposure (1 h) increases poly(ADP-ribosyl)ation of proteins with approximate molecular masses of 15, 36, 110, and >150 kDa in a concentration-dependent manner. β-Actin is shown as loading control (C, control). B, exposure (1 h) of cells to the PARP-1 inhibitors PHE (100 μM) or BZD (1 mM) increases their NAD contents (basal content, 18 ± 3 nmol/mg protein). C, GLTN (100 μM/1 h) has no effects on the basal NAD content, whereas similarly to 100 μM PHE or 1 mM BZD, it counteracts nucleotide depletion triggered by the PARP-1 activator MNNG. ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ versus control (B) or MNNG (C) (analysis of variance plus Tukey's w test). An experiment representative of three is shown in A, whereas the mean of two or three experiments performed in duplicate in shown in B and C, respectively.

min for the last extension) for the amplification of reverse transcriptase products, selected after determining the linear working range for the reaction, was 24 (β-actin), 31 (iNOS), 28 (COX-2), 31 (IL-1β), 28 (TNF-α), and 28 (PARP-1 and PARG). PCR amplification products were separated on a 1.8% agarose gel.

NAD Measurement. NAD^+ contents were quantified by means of an enzymatic cycling procedure according to Shah et al. (1995). Briefly, cells grown in a 48-well plate were killed with 50 μl of 1 N $HClO_4$ and then neutralized with an equal volume of 1 N KOH. After the addition of 100 μl of bicine 100 mM, pH 8, 50 μl of the cell extract was mixed with an equal volume of the bicine buffer containing 23 μl/ml ethanol, 0.17 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, 0.57 mg/ml fenazine ethosulfate, and 10 μg alcohol dehydrogenase. The mixture was kept at room temperature for 20 min, and then absorbance at 550 nm was measured. A standard curve allowed quantification of NAD.

Biotinylated NAD Immunocytochemistry. Cells were grown on coverslips up to 50% confluence, washed with PBS, and then incubated with buffer containing 56 mM HEPES, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 20 μM biotinylated NAD (bio-NAD; Trevigen, Gaithersburg, MD) (Bakondi et al., 2002). GLTN (100 μM), benzamide (BZD, 1 mM), or hydrogen peroxide (1 mM) were added to the incubation buffer. Reaction was stopped after 30 to 60 min by fixing cells in ice-cold ethanol for 5 min. Later on, cells were dried and exposed to trichloroacetic acid (10% w/v in water) for 10 min and washed twice with PBS. Biotinylated PAR was revealed by means of the ABC kit (Vector Laboratories, Burlingame, CA).

RNA Decay Assay. For mRNA decay assay, cells were stimulated with 0.3 μg/ml LPS for 2 h, washed twice with PBS, and exposed to 10 μg/ml actinomycin-D (Act-D) dissolved in culture medium (Korhonen et al., 2002). GLTN was added to cultures 30 min after Act-D exposure to avoid direct interaction between the two drugs. At different incubation times, mRNA was extracted from cells for RT-PCR.

RNA Interference. The sequence of the double-stranded small interfering RNA (siRNA) fragments used for PARG silencing was 5′-AACGCCACCTCGTTTGTTTTC-3′ (QIAGEN, Milan, Italy). The sequence of negative control siRNA (nonsilencing) was 5′-UUCUCCGAACGUGUCACGU-3′ (QIAGEN). RNA was dissolved in the accompanying buffer and then in the oligofectamine (Invitrogen, San Giuliano Milanese, Italy) containing medium without serum according to the manufacturer's instruction. Cells (50–70% confluence) were exposed to siRNA for 4 h at 37°C, and then an amount of Dulbecco's modified Eagle's medium plus serum was added to bring the serum concentration to 10%. After 24 h, total RNA was extracted for RT-PCR, or GLTN was added to the incubation medium for 6 h for Western blotting.

Results

Effect of GLTN on PAR Metabolism. We first investigated the effects of PARG inhibition on PAR contents in RAW 246.7 macrophages. Among PARG inhibitors (Zhang and Li, 2002), hydrolyzable tannins are potent compounds, with GLTN being the only cell-permeable, nontoxic, and commercially available one (Tsai et al., 1992; Ying et al., 2001).

Under control conditions, electrophoresed poly(ADP-ribosyl)ated proteins of macrophages distributed in bands of approximately 12, 36, 110, and >150 kDa (Fig. 1A). Hardly detectable, faint bands were also present. Levels of poly(ADP-ribosyl)ation increased when cells were exposed (1 h) to concentrations of GLTN consistent with the drug's IC_{50} with PARG (≈30 μM). At these concentrations, GLTN did not induce cell toxicity even after 12 h of treatment [as revealed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium reduction and lactate dehydrogenase release assays] (data not shown). Interestingly, PARP-1, histone H1, and high-mobility group proteins are among the most poly(ADP-ribosyl)ated proteins in the cell (D'Amours et al., 1999) and have molecular masses of 113 kDa, 37 kDa, and in the range of 12 kDa, respectively. Taken together, these findings suggest that, in addition to highly modified proteins, PAR is constitutively targeted to PARP-1, histone H1, and high-mobility group proteins and are promptly hydrolyzed by PARG in macrophages. In line with the constitutive poly(ADP-ribosyl)ation in RAW cells, their NAD^+ content increased after exposure to PARP inhibitors such as phenathridinone (PHE) and BZD (Fig. 1B). We also considered the possibility that GLTN increased the content of PAR by activating PARP(s) instead of inhibiting PARG. However, as shown in Fig. 1C, GLTN did not decrease NAD^+ content at variance with the PARP-1–activating compound methyl-nitrosoguanidine (MNNG). On the contrary, GLTN counteracted MNNG-induced NAD re-

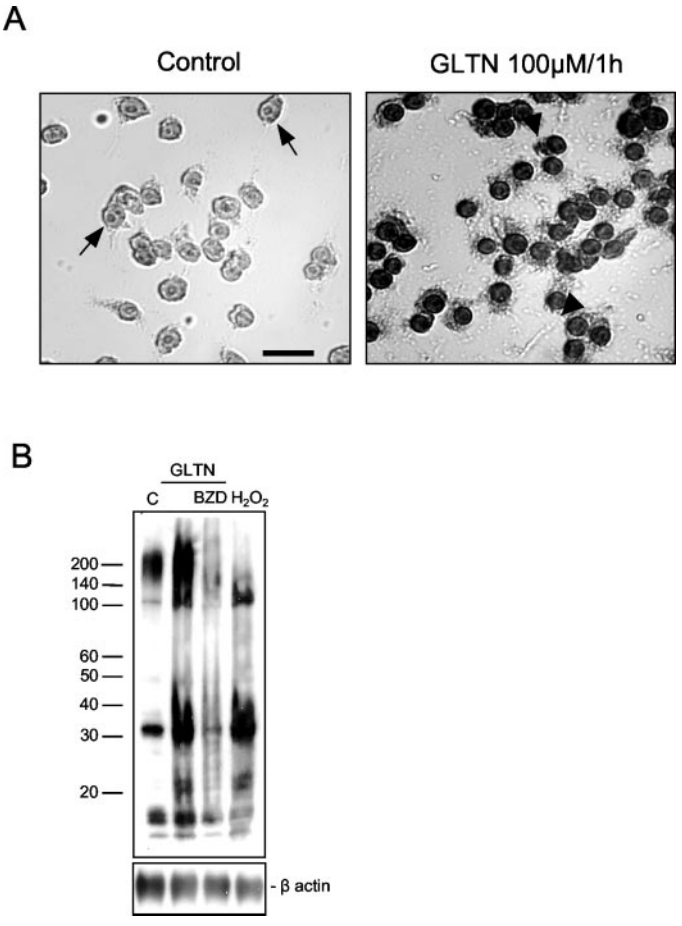

Fig. 2. Modulation of bio-PAR formation in RAW macrophages. A, immunocytochemistry of bio-PAR. Under control conditions, bio-NAD (20 μM/1 h) is incorporated into bio-PAR in the periphery of both nucleus and nucleolus (arrows) of digitonin-permeabilized RAW cells. Massive nuclear bio-PAR accumulation is present in cells exposed to GLTN (100 μM/1 h). Significant incorporation is also present in the cytoplasm (arrowheads). B, in digitonin-permeabilized cells under control conditions, Western blotting reveals a pattern of poly(ADP-ribosyl)ated proteins reminiscent of that shown in Fig. 1A. The PAR content is highly increased in GLTN-exposed cells. BZD (1 mM) inhibits GLTN-dependent PAR accumulation. Hydrogen peroxide (1 mM), a classic trigger of PARP-1 activation and autopoly(ADP-ribosyl)ation, increases polymer formation. β-Actin is shown as loading control. One experiment representative of six or two is shown in (A) and (B), respectively. C, control; Bar, 10 μm.

duction. This finding further supports the hypothesis that GLTN inhibited PARG in our experimental setting. Indeed, PARG activity is necessary to preserve PARP-dependent NAD^+ consumption by preventing excessive -poly(ADP-ribosyl)ation of PARP-1 and ensuing inactivation (Ying et al., 2001). The reduced ability of GLTN to inhibit NAD depletion compared with that of PHE and BZD (Fig. 1C) was conceivably caused by the fact that direct inhibition of PARP-1 by PHE or BZD is more efficient than its indirect inhibition by GLTN.

Further evidence that inhibition of PARG by GLTN increased PAR in RAW macrophages came from data obtained with immunocytochemistry of bio-PAR, formed by PARPs using bio-NAD as substrate (Bakondi et al., 2002). Bio-NAD incorporation under control conditions was weak and mainly occurred in the periphery of both nucleus and nucleolus. After exposure to GLTN, massive bio-NAD incorporation occurred in the nucleus (Fig. 2A) in a time-dependent manner (data not shown). Significant incorporation also occurred in the cytoplasm. These findings suggest that PARG hydrolyzes PAR synthesized by both nuclear and cytoplasmic PARPs. In digitonin-permeabilized macrophages, an assessment of PAR content by Western blotting revealed increases of polymer after GLTN exposure higher than that detected in nonpermeabilized cells (compare Figs. 2B and 1A). This is probably caused by the facilitated entrance of GLTN by digitonin. BZD prevented GLTN-induced PAR accumulation, indicating that it was dependent on PAR neosynthesis. Similar increases in PAR contents were detected in cells exposed to hydrogen peroxide, a classic trigger of PARP-1 activation and automodification (Fig. 2A).

Effect of GLTN on Expression of Proinflammatory Mediators. Because PAR promotes the inflammatory response (Szabo, 1998; Chiarugi, 2002a; Ha et al., 2002; Chiarugi and Moskowitz, 2003) and no reports are available on the effects of GLTN on immune cells under resting conditions, in this study we sought to investigate whether GLTN-dependent PAR accumulation altered the expression of

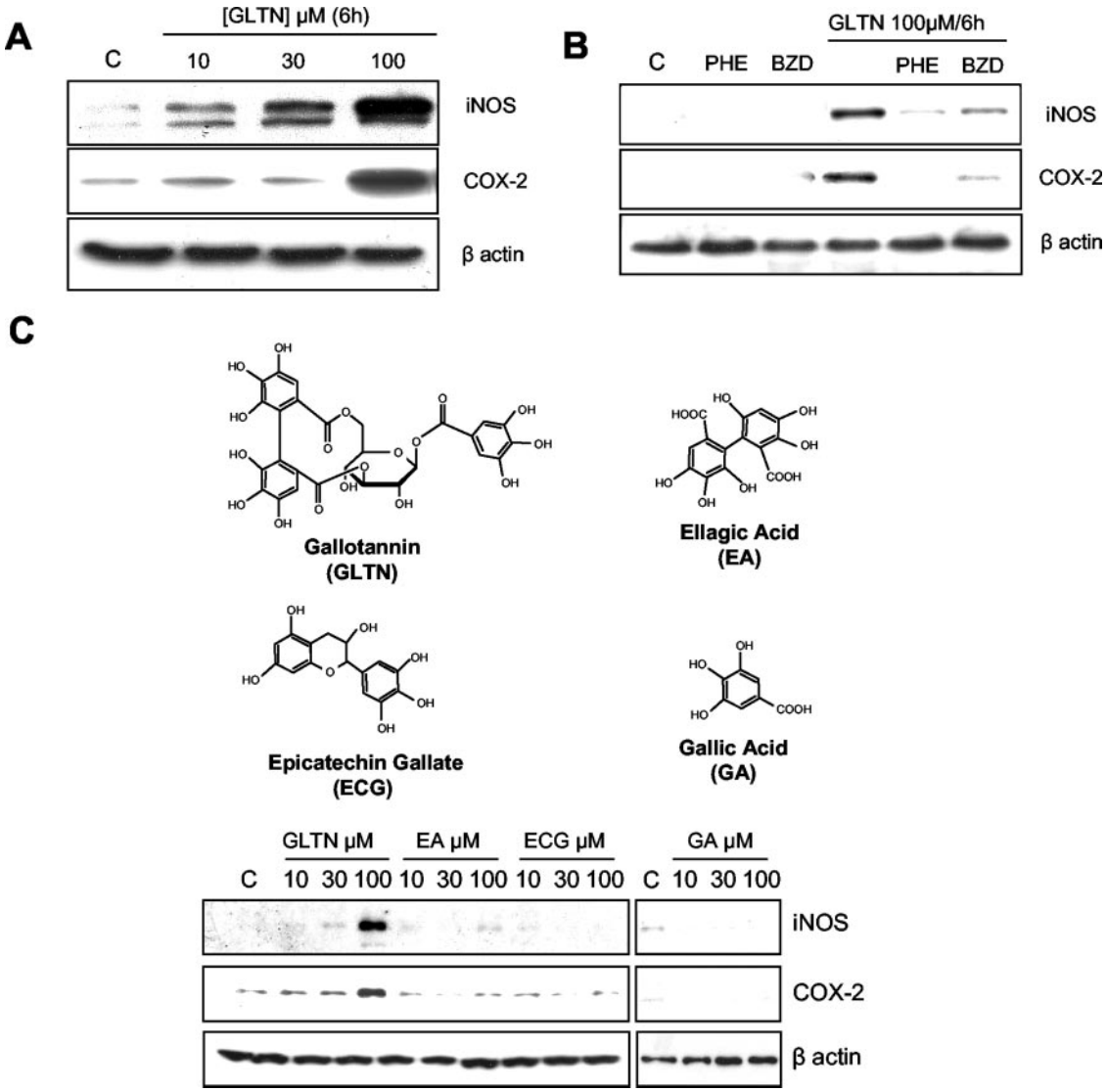

Fig. 3. Effect of GLTN and structurally related tannins on expression of iNOS and COX-2 in RAW macrophages. A, GLTN induces iNOS and COX-2 expression in a concentration-dependent fashion. B, PARP-1 inhibitors PHE (100 μM) and BZD (1 mM) prevent iNOS and COX-2 expression when present during GLTN exposure. C, tannins structurally related to GLTN but unable to affect PARG activity do not induce iNOS and COX-2 expression after 6 h of exposure. β-Actin is shown as loading control. One experiment representative of 10 (A) or 3 (B and C) is shown.

proinflammatory mediators in resting macrophages. When used at concentrations inhibiting PARG, GLTN induced expression of iNOS and COX-2 in RAW macrophages (Fig. 3A). The drug, however, did not induce expression of IL-1β and TNF-α (data not shown). It is noteworthy that iNOS and COX-2 expression by GLTN was almost completely reduced by inhibitors of poly(ADP-ribosyl)ation such as PHE and BZD (Fig. 3B). This indicates that expression of proinflammatory mediators by GLTN was dependent on PAR neoformation and subsequent accumulation caused by PARG inhibition. This assumption is further corroborated by the finding that tannins structurally related to GLTN but unable to affect PARG activity, such as gallic acid, ellagic acid, and epicatechin gallate (Tsai et al., 1992), did not induce iNOS and COX-2 expression (Fig. 3C).

To further strengthen the causal link between PARG activity and expression of proinflammatory mediators by GLTN, we silenced PARG by means of siRNAs. Pilot experiments aimed at determining oligofectamine transfection efficiency by using fluorescein-labeled oligonucleotide RNAs (QIAGEN) showed that roughly 50% of cells were transfected after 4 h of exposure (data not shown). siRNA for PARG reduced the enzyme's transcripts after 24 h, whereas mRNAs of PARP-1 or β-actin were not affected (Fig. 4Aa). It is noteworthy that a negative control siRNA (QIAGEN) did not change PARG transcript levels (Fig. 4Ab). Together, these findings indicate specificity of silencing by PARG siRNA. Because of the lack of commercially available anti-PARG antibodies, we could not evaluate the effects of siRNA on PARG protein expression levels. Regardless, GLTN-induced expression of iNOS and COX-2 was decreased (43 $\pm$ 16% iNOS and 30 $\pm$ 12% COX-2) in cells in which PARG was silenced compared with oligofectamine-treated control cells (Fig. 4B). PARG silencing affected neither β-actin expression nor induction of iNOS and COX-2 by LPS (Fig. 4C). This, on the one hand, indicates that GLTN induced expression of the two inflammatory mediators by interacting with PARG and, on the other hand, that PARG down-regulation did not impair protein expression in a nonspecific manner. Silencing of PARG per se did not induce the expression of proinflammatory mediators (data not shown).

Effect of GLTN on Inflammatory Signal Transduction Pathways. Polymixin B, an LPS-neutralizing antibiotic, suppressed LPS- but not GLTN-induced iNOS expression (Fig. 5Aa), ruling out the possibility that GLTN was contaminated by endotoxin. Similarly, LPS but not GLTN activated p38 (Fig. 5Ab), a kinase with key roles in the expression of proinflammatory mediators. We also studied the effects of GLTN on the activation of transcription factors traditionally involved in the expression of iNOS and COX-2. In extracts of RAW macrophages exposed to LPS (1 μg/ml/1 h), the DNA binding activities of NF-κB or AP-1 appeared as retarded bands that were reduced by the addition to the binding mixture of an antibody raised against p65/RelA (NF-κB) or c-Jun (AP-1). The bands were also reduced by 50-fold molar excess of nonradioactive probe, thereby indicating specificity of binding (Fig. 5B, a and b). Surprisingly, GLTN inhibited the constitutive binding of NF-κB to the oligoprobe in gel-shift assays (Fig. 5Ba). This is consistent with the ability of polyphenols to suppress NF-κB activation (Pan et

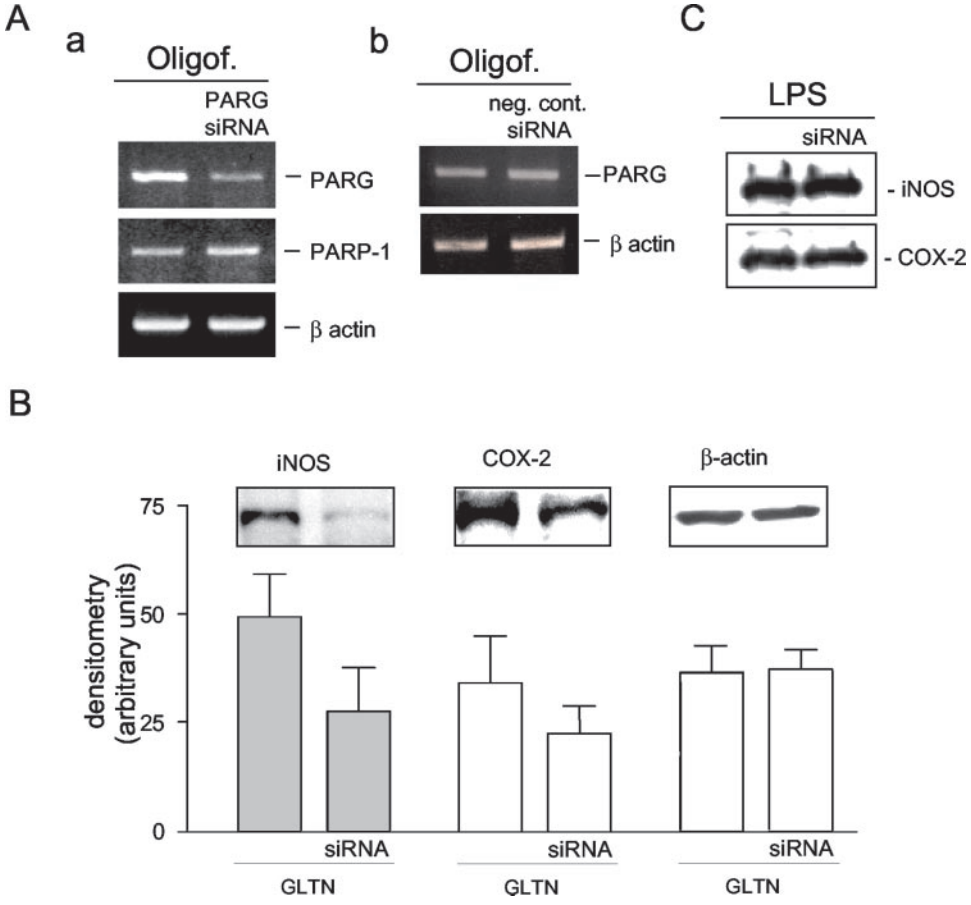

Fig. 4. PARG silencing impairs GLTN- but not LPS-dependent induction of iNOS and COX-2 in RAW macrophages. Aa, transfection of cells with siRNA for PARG reduces PARG transcript levels without affecting those of PARP-1 and β-actin after 24 h. Ab, negative control siRNA does not affect PARG or β-actin transcript levels. B, 24 h after exposure to siRNAs, expression of iNOS and COX-2 by GLTN (100 μM/6 h) is impaired in cells with silenced PARG compared with oligofectamine (oligof.)-treated controls (43 $\pm$ 16% iNOS and 30 $\pm$ 12% COX-2 reduction versus control). Histogram represents the mean $\pm$ S.E.M. of three experiments. C, PARG silencing does not affect the induction of iNOS or COX-2 by LPS. One experiment representative of three (A and B) or two (C) is shown.

al., 2000). As for AP-1, we found constitutive DNA binding activity in RAW macrophages that was not affected by GLTN exposure (Fig. 5Bb). Finally, GLTN induced neither STAT-1 phosphorylation nor IRF-1 expression, two events typically triggered by interferon-γ (Fig. 5C).

Effect of GLTN on Transcripts of Proinflammatory Mediators. We next analyzed mRNA levels of iNOS, COX-2, IL-1β, and TNF-α in RAW cells exposed to GLTN. Consistent with data obtained with Western blotting, GLTN increased the transcript levels of iNOS and COX-2 after 4 h of exposure but not those of TNF-α and IL-1β (Fig. 6Aa). mRNA induction of iNOS and COX-2 by GLTN was slower than that triggered by LPS (2 h). The discrepancy between basal levels of TNF-α mRNA (Fig. 6A) and lack of its constitutive expression (see above) is in keeping with the well-known instability of TNF-α transcripts under resting conditions. Of note, Act-D abrogated a GLTN-dependent increase of iNOS and COX-2 mRNA transcripts (Fig. 6Ab), suggesting that expression of proinflammatory mediators prompted by GLTN, although apparently not dependent on activation of specific transcription factors, was still dependent on functioning of RNA polymerase II. To further rule out the possibility that GLTN increased iNOS and COX-2 transcript levels by mRNA stabilization, an mRNA decay assay was carried out. As shown in Fig. 6B, LPS-induced iNOS transcripts decreased after 5 h of exposure to Act-D, whereas those of COX-2 and β-actin diminished only after 7 h. The long half-life of COX-2 transcripts argues against the mRNA-stabilizing properties of GLTN. Indeed, the drug did not affect iNOS or COX-2 transcript levels in cells challenged with LPS and exposed to Act-D (Fig. 6C).

Discussion

This study shows that pharmacological inhibition of PARG leads to nuclear accumulation of PAR, which in turn triggers the expression of iNOS and COX-2 in cultured macrophages. To our knowledge, this is the first evidence that reduced catabolism of PAR alters inflammatory gene-expression profile.

Increasing evidence supports a PAR-dependent model of transcriptional regulation. Indeed, poly(ADP-ribosyl)ation regulates assembly of transcription-regulating multiprotein complexes (D'Amours et al., 1999; Ziegler and Oei, 2001; Chiarugi, 2002b; Kraus and Lis, 2003) and interaction between transcription factors and the transcriptional coactivators HMG-I(Y) (Ullrich et al., 2001; Chiarugi and Moskowitz, 2003). In addition, poly(ADP-ribosyl)ation at specific promoter elements coordinates transcriptional activation (Butler and Ordahl, 1999; Akiyama et al., 2001; Nirodi et al., 2001; Soldatenkov et al., 2002; Zhang et al., 2002) and promotes expression of iNOS (Le Page et al., 1998), chemokines (Nirodi et al., 2001; Hasko et al., 2002), integrins (Ullrich et al., 2001), and muscle (Butler and Ordahl, 1999), as well as heat shock proteins (Zingarelli et al., 2004). In keeping with this scenario, our findings demonstrate that nuclear accumulation of PAR caused by PARG inhibition triggers selective transcription of proinflammatory genes in macrophages. The

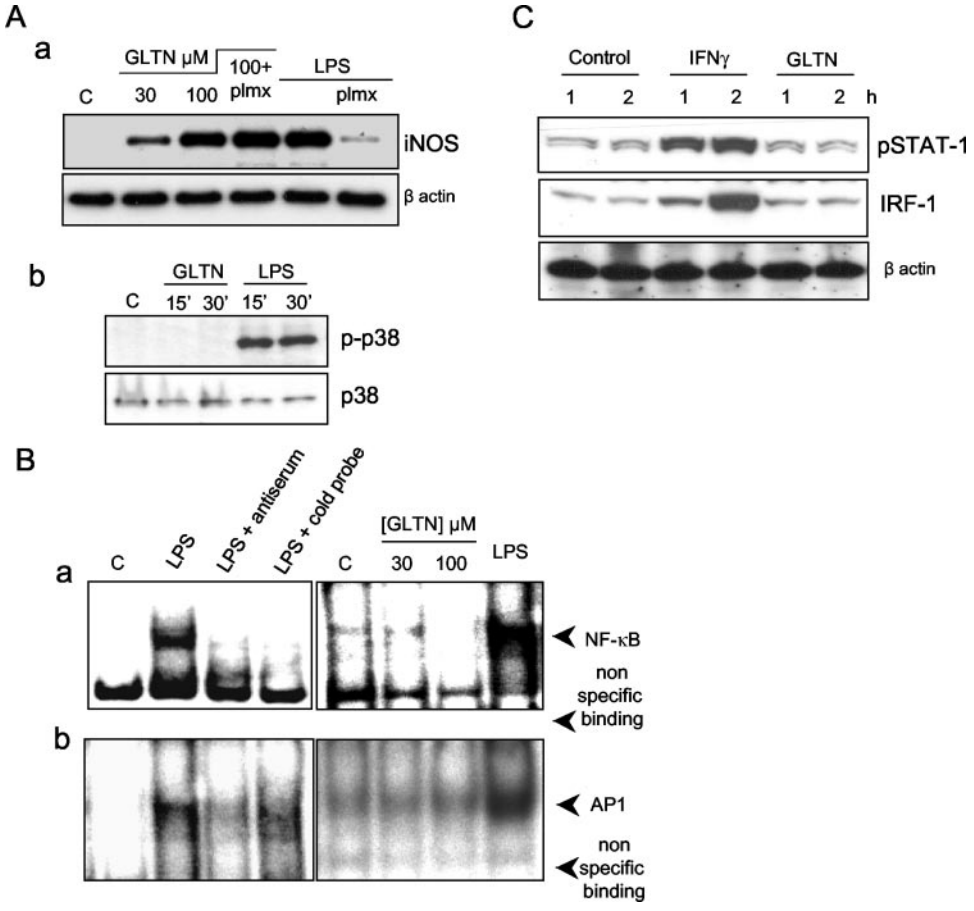

Fig. 5. Effects of GLTN on inflammatory signaling pathways in RAW macrophages. Aa, polymixin (plmx, 10 μg/ml) prevents LPS (0.3 μg/ml) but not GLTN induction of iNOS after 6 h of exposure. (Ab) LPS (0.3 μg/ml) but not GLTN (100 μM) induced p38 phosphorylation. p38 levels are shown as loading control. B, The LPS-induced DNA binding activities of NF-κB (a) and AP-1 (b) appear as retarded bands reduced by antibodies raised against p65 (NF-κB) or c-Jun (AP-1). The addition of 50-fold molar excess of cold probe also reduced band intensity. Ba, GLTN (2 h) inhibits basal binding activity of NF-κB in gel-shift assays. Bb, constitutive DNA binding activity of AP-1 is not affected by GLTN (2 h). In B, a and b, the effect of LPS is shown as positive control. C, interferon-γ (100 U/ml) but not GLTN (100 μM) triggers STAT-1 phosphorylation and IRF-1 expression. β-Actin is shown as loading control. One experiment representative of two (A) or three (B and C) is shown.

apparent spontaneous (i.e., activator-independent) nature of GLTN-induced transcription is in line with a prior report showing that PAR promotes the synthesis of mRNA (Vispè et al., 2000). Data are also consistent with a recent study showing that PARG mutation selectively alters transcription of genes involved in circadian rhythm regulation in *Arabidopsis thaliana*. Remarkably, the inhibition of PAR formation rescues the plant wild-type phenotype (Panda et al., 2002).

After GLTN exposure, we were unable to detect activation of signaling pathways typically triggered during iNOS and COX-2 induction in macrophages. However, it is worth noting that GLTN did not affect the low levels of basal DNA binding activity of AP-1, as well as constitutive STAT-1 phosphorylation and IRF-1 expression (Fig. 5). Furthermore, under control conditions, slight expression of iNOS and COX-2 was detected in some but not all experiments (compare blots in Fig. 3). We therefore hypothesize that PAR prompted a transcriptional machinery partially activated but unable, per se, to operate. That the drug acted through mechanisms different from those triggered by classic membrane receptor-dependent macrophage activators is also indicated by GLTN′s ability to induce, at variance with LPS, selective and delayed transcription of proinflammatory mediators (Fig. 6Aa). Although we cannot rule out the possibility that GLTN triggered cytoplasmic pathway(s) different from those investigated, our results, together with evidence that iNOS and COX-2 expression by GLTN was dependent on PAR formation (Fig. 3B), point to PAR accumulation as the cause of transcription of the two proinflammatory mediators. Furthermore, the important finding that PARG silencing impaired GLTN′s ability to induce expression of iNOS and COX-2 (Fig. 4B) corroborates the hypothesis that the drug acted via PARG inhibition and emphasizes the enzyme's role in transcriptional regulation. In principle, this assumption is at odds with the finding that iNOS and COX-2 expression was not induced by the sole PARG silencing. However, PARG silencing by siRNA was partial (Fig. 4Aa), thereby allowing for speculation that remaining PARG activity warranted homeostatic levels of de-poly(ADP-ribosyl)ation (and PARG-dependent transcriptional repression). In addition, one should consider that acute inhibition of PARG by GLTN might have different impacts on both chromatin superstructure and transcription than a slow down-regulation by siRNA. Finally, whereas siRNA only suppressed the known PARG isoform, it is conceivable that GLTN, as substrate analog, might have led to a more efficient suppression of de-poly(ADP-ribosyl)ation by inhibiting possible additional PARGs (Davidovich et al., 2001).

As for the molecular mechanisms through which the inhibition of PARG promotes gene expression, it is worth noting that the enzyme has been shown to trigger chromatin condensation (de Murcia et al., 1986), a hallmark of gene silenc-

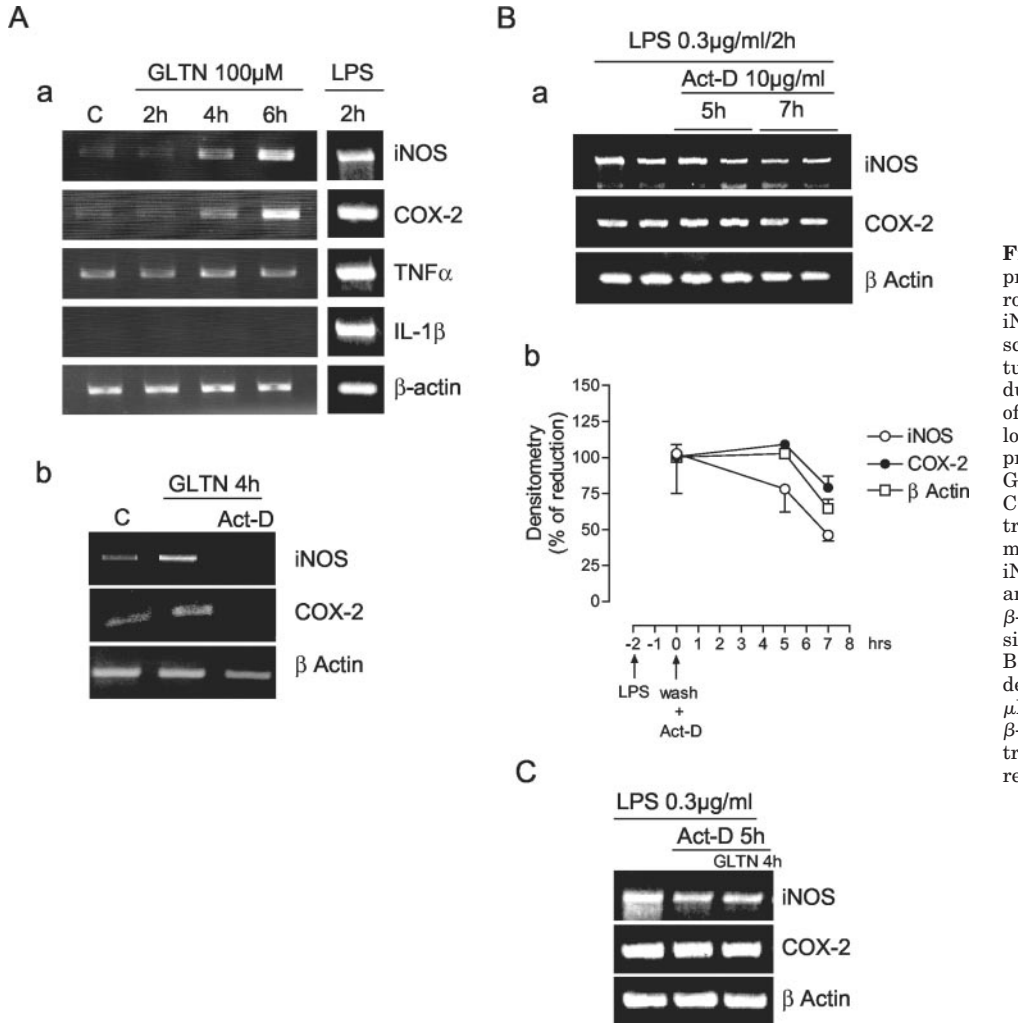

Fig. 6. Effects of GLTN on transcripts of proinflammatory mediators in RAW macrophages. Aa, GLTN induces synthesis of iNOS and COX-2 but not of IL-1β transcripts. The drug has no effects on constitutive TNF-α transcript levels. LPS induces prompt increases of mRNAs of all of the four proteins. β-Actin is shown as loading control of reverse transcriptase products. Ab, 10 μg/ml Act-D abrogates GLTN-dependent increase of iNOS and COX-2 mRNAs and also reduces β-actin transcript levels. Ba, in LPS-challenged macrophages, 10 μg/ml Act-D reduces iNOS and COX-2 mRNA levels after 5 and 7 h, respectively. Transcript levels of β-actin also decrease after 7 h. Bb, densitometric evaluation of data shown in Ba. C, when added during Act-D–dependent transcriptional block, GLTN (100 μM) does not alter iNOS, COX-2, and β-actin mRNA levels compared with cells treated with Act-D only. One experiment representative of three is shown in (A–C).

ing, whereas accumulation of its substrate (i.e., PAR) leads to chromatin loosening (de Murcia et al., 1988; Tulin and Spradling, 2003), a prerequisite of RNA polymerase II-driven transcription. However, an alternative or complementary mechanism may also be advanced. According to prior work from Satoh's group (Vispè et al., 2000), DNA damage-dependent autopoly(ADP-ribosyl)ation of PARP-1 detaches the enzyme from nascent mRNA and relieves constitutive transcriptional blockade. Likewise, we report here that hindrance of PAR catabolism caused by PARG inhibition is associated with PARP-1 automodification (Figs. 1, A and C, and 2B) and apparent spontaneous transcriptional activation (Figs. 3A and 6Aa). Taken together, these data suggest that, when exceeding a certain threshold, PAR selectively unleashes mRNA elongation. It is therefore important to establish whether PARG similarly regulates transcription in other cell types. In this regard, preliminary results demonstrate that GLTN also induced iNOS and COX-2 in other mononuclear phagocyte cell lines such as NR 8383 (rat alveolar macrophages) and N11 (mouse microglia) (data not shown). In human monocyte-derived macrophages, GLTN only induced COX-2 (data not shown), which in line with the well-known difficult induction of human iNOS in vitro.

In conclusion, this study indicates PARG as a novel player in epigenetics. Because PARG has a lower K_m with respect to PARP-1 (Davidovich et al., 2001), we reason that pharmacological modulation of the former rather than the latter should have a stronger impact on PAR-dependent transcription. In light of the role of PAR in the inflammatory response, PARG inhibition might boost immune cell activation and be exploited as an innovative immunomodulatory strategy. However, given the pleiotropic activities of proinflammatory transcription factors and cytokines, it is worth mentioning that the PAR-dependent induction of iNOS and COX-2 without concomitant activation of NF-κB, AP-1, STAT-1, or IRF-1 as well as IL-1β and TNF-α expression might have remarkable consequences on immunocompetence, survival, and cytotoxicity of macrophages. As a whole, these findings might be of relevance to inflammation and have important pathophysiological implications that could be harnessed for therapeutic intervention.

References

Affar EB, Germain M, Winstall E, Vodenicharov M, Shah RG, Salvesen GS, and Poirier GG (2001) Caspase-3-mediated processing of poly(ADP-ribose) glycohydrolase during apoptosis. *J Biol Chem* **276:**2935–2942.

Akiyama T, Takasawa S, Nata K, Kobayashi S, Abe M, Shervani NJ, Ikeda T, Nakagawa K, Unno M, Matsuno S, et al. (2001) Activation of Reg gene, a gene for insulin-producing B-cell regeneration: poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly(ADP-ribosyl)ation. *Proc Natl Acad Sci USA* **98:**48–53.

Bakondi E, Bai P, Szabo E, Hnuyadi J, Gergely P, Szabo C, and Virag L (2002) Detection of poly(ADP-ribose)polymerase activation in oxidatively stressed cells and tissues using biotinylated NAD substrate. *J Histochem Cytochem* **50:**91–98.

Bonicalzi ME, Vodenicharov M, Coulombe M, Gagne JP, and Poirier GG (2003) Alteration of poly(ADP-ribose) glycohydrolase nucleocytoplasmic shuttling characteristics upon cleavage by apoptotic proteases. *Biol Cell* **95:**635–644.

Butler AJ and Ordahl CP (1999) Poly(ADP-ribose)polymerase binds with transcription enhancer factor 1 to MCAT1 elements to regulate muscle-specific transcription. *Mol Cell Biol* **19:**296–306.

Carty SM and Greenleaf AL (2002) Hyperphosphorylated C-terminal repeat domain-associating proteins in the nuclear proteome link transcription to DNA/chromatin modification and RNA processing. *Mol Cell Proteomics* **1:**598–610.

Chiarugi A (2002a) Inhibitors of poly(ADP-ribose) polymerase-1 suppress transcriptional activation in lymphocytes and ameliorate autoimmune encephalomyelitis in rats. *Br J Pharmacol* **137:**761–770.

Chiarugi A (2002b) PARP-1: killer or conspirator? The suicide hypothesis revisited. *Trends Pharmacol Sci* **23:**122–129.

Chiarugi A and Moskowitz MA (2003) Poly(ADP-ribose) polymerase-1 activity promotes NF-KappaB-driven transcription and microglial activation: implication for neurodegenerative disorders. *J Neurochem* **85:**306–317.

D'Amours D, Desnoyers S, and Poirier GG (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J* **342:**249–268.

Davidovich L, Vodenicharov M, Affar EB, and Poirier GG (2001) Importance of Poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism. *Exp Cell Res* **268:**7–13.

de Murcia G, Huletsky A, Lamarre D, Gaudreau A, Poujet J, Daune M, and Poirier GG (1986) Modulation of chromatin superstructure induced by poly(ADP-ribose) synthesis and degradation. *Proc Natl Acad Sci USA* **261:**7011–7017.

de Murcia G, Huletsky A, and Poirier GG (1988) Modulation of chromatin structure by poly(ADP-ribosyl)ation. *Biochem Cell Biol* **66:**626–635.

Di Meglio S, Denegri M, Vallefuoco S, Tramontano F, Scovassi AI, and Quesada P (2003) Poly(ADPR) polymerase-1 and poly(ADPR) glycohydrolase level and distribution in differentiating rat germinal cells. *Mol Cell Biochem* **248:**85–91.

Ha HC, Hester LD, and Snyder SH (2002) Poly(ADP-ribose) polymerase-1 dependence of stress-induced transcription factors and associated gene expression in glia. *Proc Natl Acad Sci USA* **99:**3270–3275.

Hanai S, Kanai M, Ohashi S, Okamoto K, Yamada M, Takahashi H, and Miwa M (2004) Loss of poly(ADP-ribose) glycohydrolase causes progressive neurodegeneration in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **101:**82–86.

Hasko G, Mabley JG, Nemeth ZH, Pacher P, Deitch EA, and Szabo C (2002) Poly(ADP-ribose) polymerase is a regulator of chemokine production: relevance for the pathogenesis of shock and inflammation. *Mol Med* **8:**283–289.

Hassa PO and Hottiger MO (2002) The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-KappaB in inflammatory disorders. *Cell Mol Life Sci* **59:**1534–1553.

Herceg Z and Wang ZQ (2001) Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Biochem J* **477:**97–110.

Korhonen R, Lahti A, Hamalainen M, Kankaanranta H, and Moilanen E (2002) Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing MRNA in lipopolysaccharide-treated macrophages. *Mol Pharmacol* **62:**698–704.

Kraus WL and Lis JT (2003) PARP goes transcription. *Cell* **113:**677–683.

Kun E, Kirsten E, and Ordahl CP (2002) Coenzymatic activity of randomly broken or intact double-stranded DNAs in auto and histone H1 trans-poly(ADP-ribosylation), catalyzed by poly(ADP-ribose) polymerase (PARP I). *J Biol Chem* **277:**39066–39069.

Le Page C, Sanceau J, Drapier JC, and Wietzerbin J (1998) Inhibitors of ADP-ribosylation impair inducible nitric oxide synthase gene transcription through inhibition of NF-KB activation. *Biochem Biophys Res Commun* **243:**451–457.

Nirodi C, NagDas S, Gygi SP, Olson G, Ruedi A, and Richmond A (2001) A role for poly(ADP-ribose) polymerase (PARP) in the transcriptional regulation of the melanoma growth stimulatory activity (CXCL1) gene expression. *J Biol Chem* **276:**9366–9374.

Ohashi S, Kanai M, Hanai S, Uchiumi F, Maruta H, Tanuma S, and Miwa M (2003) Subcellular localization of poly(ADP-ribose) glycohydrolase in mammalian cells. *Biochem Biophys Res Commun* **307:**915–921.

Oliver FJ, Menissier-de Murcia J, Nacci C, Decker P, Andriantsitohania R, Muller S, de la Rubia G, Stoclet JC, and de Murcia G (1999) Resistance to endotoxic shock as a consequence of defective NF-KB activation in poly(ADP-ribose) polymerase-1 deficient mice. *EMBO (Eur Mol Biol Organ) J* **18:**4446–4454.

Pan MH, Lin-Shiau S-Y, Ho C-H, Lin J-H, and Lin J-K (2000) Suppression of lipopolysaccharide-induced nuclear factor K-B activity by theaflavin-3,3′-digallate from black tea and other polyphenols through down-regulation of IkB kinase activity in macrophages. *Biochem Pharmacol* **59:**357–367.

Panda S, Poirier GG, and Kay SA (2002) Tej defines a role for poly(ADP-ribosyl)ation in establishing period length of the *Arabidopsis* circadian oscillator. *Dev Cell* **3:**51–61.

Pieper AA, Verma A, Zhang J, and Snyder SH (1999) Poly(ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol Sci* **20:**171–181.

Shah GM, Poirier D, Duchaine G, Desnoyers S, Brochu G, Lageaux J, Verreault A, Hoflack J-C, Kirkland JB, and Poirier GG (1995) Methods for biochemical study of poly(ADP-ribose) metabolism in vitro and in vivo. *Anal Biochem* **227:**1–13.

Simbulan-Rosenthal CM, Ly DH, Rosenthal DS, Konopka G, Luo R, Wang ZQ, Schultz PG, and Smulson ME (2000) Misregulation of gene expression in primary fibroblasts lacking poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA* **97:**11274–11279.

Slattery E, Dignam JD, Matsui T, and Roeder RG (1983) Purification and analysis of a factor which suppresses nick-induced transcription by RNA polymerase II and its identity with poly(ADP-ribose) polymerase. *J Biol Chem* **285:**5955–5959.

Smith S (2001) The world according to PARP. *Trends Biochem Sci* **26:**174–179.

Soldatenkov VA, Chasovskikh S, Potaman VN, Trfimova I, Smulson ME, and Drithschilo A (2002) Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. *J Biol Chem* **277:**665–670.

Szabo C (1998) Role of poly(ADP-ribose)synthetase in inflammation. *Eur J Pharmacol* **350:**1–19.

Tsai YJ, Aoki T, Maruta H, Abe H, Sakagami H, Hatano T, Okuda T, and Tanuma S (1992) Mouse mammary tumor virus gene expression is suppressed by oligomeric ellagitannins, novel inhibitors of poly(ADP-ribose) glycohydrolase. *J Biol Chem* **267:**14436–14442.

Tulin A and Spradling A (2003) Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at *Drosophila* puff loci. *Science (Wash DC)* **299:**560–562.

Ullrich O, Diestel A, Eyupoglu IY, and Nitsch R (2001) Regulation of microglial expression of integrins by poly(ADP-ribose) polymerase-1. *Nat Cell Biol* **3:**1035–1042.

Vispè S, Yung TMC, Ritchot J, Serizawa H, and Satoh MS (2000) A cellular defense pathway to regulate transcription through poly(ADP-ribosyl)ation in response to DNA damage. *Proc Natl Acad Sci USA* **97:**9886–9891.

Ying W, Sevigny MB, Chen Y, and Swanson RA (2001) Poly(ADP-ribose) glycohydrolase mediates oxidative and excitotoxic neuronal death. *Proc Natl Acad Sci USA* **98:**12227–12232.

Yu SW, Wang H, Dawson TM, and Dawson VL (2003) Poly(ADP-ribose) polymerase-1 and apoptosis inducing factor in neurotoxicity. *Neurobiol Dis* **14:**303–317.
Zhang J and Li J-H (2002) PARP and PARG as novel therapeutic agents. *Drugs Future* **27:**371–383.
Zhang Z, Hildebrandt EF, Simbulan-Rosenthal CM, and Anderson MG (2002) Sequence-specific binding of poly(ADP-ribose) polymerase-1 to the human T cell leukemia virus type-1 tax responsive element. *Virology* **296:**107–116.
Ziegler M and Oei SL (2001) A cellular survival switch: poly(ADP-ribosyl)ation stimulates DNA repair and silences transcription. *Bioessay* **23:**543–548.
Zingarelli B, Hake PW, O'Connor M, Denenberg A, Wong HR, Kong S, and Aronow BJ (2004) Differential regulation of activator protein-1 and heat shock factor-1 in myocardial ischemia and reperfusion injury: role of poly(ADP-ribose) polymerase-1. *Am J Physiol* **286:**H1408–H1415.
Zlatanova J, Caiafa P, and Van Holde K (2000) Linker histone binding and displacement: versatile mechanism for transcriptional regulation. *FASEB J* **14:**1697–1704.

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