

Inhibition of poly(ADP-ribose) glycohydrolase by gallotannin selectively up-regulates expression of pro-inflammatory genes

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List of Abbreviations: activator protein-1, AP1; cyclooxygenase-2, COX-2; gallotannin, GLTN; interleukin-1 β , IL-1 β ; inducible nitric oxide synthase, iNOS; interferon responsive factor-1, IRF-1; interferon- γ , IFN- γ ; poly(ADP-ribose) polymerase-1, PARP-1; nuclear factor-kB, NF-kB; poly(ADP-ribose) glycohydrolase, PARG; tumor necrosis factor- α , TNF- α .

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 expression of several genes, including those of the inflammatory response. In this study, we asked whether poly(ADP-ribose) glycohydrolase (PARG), the sole de-poly(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 iNOS and COX-2, but not of IL-1 β and TNF- α , in cultured RAW 264.7 macrophages. Remarkably, silencing of PARG by means of siRNA 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 due to activation of transcription factors such as NF- κ B, AP-1, STAT-1 or IRF-1, nor to mRNA stabilization. Overall, data provide the first evidence that pharmacological inhibition of PARG leads to PAR-dependent alteration of gene expression profiles in macrophages.

Introduction

Poly(ADP-ribose)ation is a post-translational modification of proteins operated by poly(ADP-ribose) polymerases (PARPs). These are nuclear and cytoplasmic enzymes metabolizing β -nicotinamide adenin dinucleotide (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 due to 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- κ B, AP-1, AP-2, YY-1, Oct-1, p53, and HSF-1 (Chiarugi, 2002b; Ha *et al.*, 2002; Hassa and Hottiger, 2002; Kraus and Lis, 2003; Zingarelli *et al.*, 2003b; Ziegler and Oei, 2001). In addition, PARP-1 is the general transcription factor TFIIC (Slattery *et al.*, 1983) and binds a 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 expression of several genes including those of the inflammatory response (Szabo, 1998; Ha *et al.*, 2002; Chiarugi, 2002a; 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 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, albeit experimental evidence indicates the enzyme 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, the present study investigated the effect of PARG inhibition on expression of pro-inflammatory 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 (DMEM) supplemented with 2mM glutamine, 10% bovine serum and antibiotics. Cultures were incubated at 37°C in a water-saturated 5% CO₂ / 95% air atmosphere and usually brought to 50-70% confluence. Gallotannin (GLTN) (Fluka Chemie, CH) and the other drugs were directly dissolved in the incubating medium.

Western Blotting

For Western blotting, cells were scraped, collected in eppendorf tubes, centrifuged (1500 *g*/5 min/4°C) and resuspended in lysis buffer [50 mM Tris pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 µg/ml aprotinin and leupeptin, 1% SDS]. 20-40 µg of protein/lane were loaded. After 4-20% SDS-PAGE and blotting, membranes (Hybond-ECL, Amersham, 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 (Vinci, Italy), the anti-inducible NO synthase (iNOS), anti-

interferon regulatory factor-1 (IRF-1), anti-interleukin (IL)-1 β , anti tumor necrosis factor (TNF)- α were polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the anti-cyclooxygenase-2 (COX-2) polyclonal antibody was from Cayman Chemicals (Ann Arbor, MI, USA), the anti p38 and phospho-p38 were polyclonal antibody from BD Transduction Laboratories (Lexington, KY, USA), whereas the polyclonal anti phospho STAT-1 antibody was from Cell Signaling (Beverly, MA, USA). Membranes were then washed with TPBS and incubated 1 h in TPBS/5% milk containing the corresponding peroxidase-conjugated secondary antibody (1:2000). After washing in TPBS, ECL (Amersham, UK) was used to visualize the peroxidase-coated bands.

Electrophoretic mobility shift assay

The DNA binding activity of NF- κ B and AP1 was investigated in cells scraped, pelleted and then resuspended in buffer "A" containing 10 mM Hepes pH 7.8, 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1mM PMSF and 4 μ g/ml aprotinin and leupeptin. Cells were kept on ice for 15 min, vortexed every 3 min and then centrifuged (5000 *g*/5 min/4°C). The nuclear pellet was resuspended in 50 μ l of buffer "B", analogous to "A" plus 400 mM NaCl and incubated for 10 min on ice. The mixture was centrifuged (14,000 *g*/10 min/4°C) and the supernatant aliquoted and stored at -80°C. The DNA binding activity was tested by incubating 10 μ g of proteins of the nuclear extract in 20 μ l of a buffer containing 10 mM Tris pH 7.4, 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 0.05 mg/ml poly(dI-dC), and 10,000 cpm of specific ³²P-labeled oligonucleotide for 20 min at room temperature. The mixture was electrophoresed in 6% non-denaturing polyacrylamide gels that, after drying, were exposed to x-ray films (Amersham, Little Chalfont Buckinghamshire, UK). The double-stranded oligonucleotides 5'-AGTTGAGGGGACTTTCCAGGC-3' (NF- κ B) and 5'-CGCTTGATGAGTCAGCCGGAA-3' (AP1) were used. For supershift experiments, 2 μ l of the antibodies raised against p65/RelA or c-Jun (Santa Cruz, CA, USA) were added to the binding mixture during incubation.

Semiquantitative RT-PCR

One μg of total RNA extracted with Trizol (GIBCO-BRL, Rockville, MD, USA) 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'-ACACACTCTACTACTGGCACC-3' (sense) and 5'-TTCAGGGAGAAGCGTTTGC-3' (antisense); $\text{TNF}\alpha$, 5'-TTCTGTCTACTGAACTTCGGGGTGATCGGT-3' (sense) and 5'-GTATGAGATAGCAAATCGGCTGACGGTGTG-3' (antisense); IL-1 β , 5'-CAGGACAGGTATAGATTCTTTCCTT-3' (sense) and 5'-ATGGCAACTGTTCTGAACTCAACT-3' (antisense); PARP-1, 5'-CTGATGTTGAGGTTGATG-3' (sense) and 5'-CACTTGCTGCTGGTTGAA-3' (antisense); PARG, 5'-CCACCTCGTTTGTGTTTCA-3' (sense) and 5'-CCAACATCTGGCAAAGGA-3' (antisense); β -actin, 5'-GACCTGACAGACTACCTC-3' (sense) and 5'-AGACAGCACTGTGTGTTGGC-3' (antisense). The number of PCR cycles (94°C 30 s, 58°C 30 s, 72°C 1 min, 5 min for the last extension) for the amplification of RT products, selected after determining the linear working range for the reaction, was 24 (β -actin), 31 (iNOS), 28 (COX-2), 31 (IL1 β), 28 ($\text{TNF}\alpha$), 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.* (Shah *et al.*, 1995). Briefly, cells grown in a 48 well plate were killed with 50 μl HClO₄ 1N and then neutralized with an equal volume of KOH 1N. 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 $\mu\text{l}/\text{ml}$ ethanol, 0.17 mg/ml MTT, 0.57 mg/ml fenazine ethosulfate and 10 μg alcohol dehydrogenase. Mixture

was kept at room temperature for 20' and then absorbance a 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 MgCl₂, 0.01% digitonin and 20 μM biotinylated NAD (bio-NAD, Trevigen, Gaithersburg, MD, USA) (Bakondi *et al.*, 2002). GLTN (100 μM), benzamide (BZD, 1 mM) or hydrogen peroxide (1mM) were added to the incubation buffer. Reaction was stopped after 30'-60' by fixing cells in cold ethanol for 5'. Later on, cells were dried and exposed to trichloroacetic acid (10% w/v in water) for 10' and washed twice with PBS. Biotinylated PAR was revealed by means of the ABC kit (Vector Laboratories, Burlingame, CA, USA).

RNA decay assay

For mRNA decay assay, cells were stimulated with 0.3 μg/ml LPS for 2h, 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' 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'-AACGCCACCTCGTTTGTTC-3' (QIAGEN, Milan, Italy). The sequence of negative control siRNA (non silencing) was 5'-UUCUCCGAACGUGUCACGU-3' (QIAGEN, Milan, Italy). RNA was dissolved in the accompanying buffer and then in the oligofectamine (Invitrogen, San Giuliano Milanese, Italy) containing medium w/o 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 DMEM+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, non toxic 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 (1h) to concentrations of GLTN consistent with the drug's IC₅₀ on PARG ($\approx 30\mu\text{M}$). At these concentrations, GLTN did not induce cell toxicity even after 12 h treatment (as revealed by MTT reduction and LDH release assays, not shown). Interestingly, PARP-1, histone H1 and high mobility group proteins (HMGs) are among the most poly(ADP-ribosyl)ated proteins in the cell (D'Amours *et al.*, 1999) and have molecular weight of 113, 37 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 as well as HMGs, and 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 benzamide (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 reduction. 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 auto-poly(ADP-ribosyl)ation of PARP-1 and ensuing inactivation (Ying *et al.*, 2001). The reduced ability of GLTN to inhibit NAD depletion compared to that of PHE and BZD (Fig. 1C) was conceivably due to 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 (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, assessment of PAR content by Western-blotting revealed increases of polymer after GLTN exposure higher than that detected in non-permeabilized cells (compare Fig. 2B with 1A). This is probably due to the facilitated entrance of GLTN by digitonin. Importantly, BZD prevented GLTN-induced PAR accumulation, indicating that it was dependent on PAR neo-synthesis. Similar increases in PAR contents were detected in cells exposed to hydrogen peroxide, a classical trigger of PARP-1 activation and automodification (Fig. 2A).

Effect of GLTN on expression of pro-inflammatory mediators

Because PAR promotes the inflammatory response (Szabo, 1998; Ha *et al.*, 2002; Chiarugi, 2002a; 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 pro-inflammatory mediators in resting macrophages. Interestingly, 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-1 β and

TNF- α (not shown). Of note, 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 pro-inflammatory mediators by GLTN was dependent on PAR neo-formation and subsequent accumulation due to 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 pro-inflammatory mediators by GLTN, we silenced PARG by means of siRNAs. Pilot experiments aimed at determining oligofectamine transfection efficiency by using fluorescein-labeled oligoRNAs (QIAGEN, Milan, Italy) showed that roughly 50% of cells were transfected after 4 h exposure (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). Notably, a negative control siRNA (QIAGEN, Milan, Italy) 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 to oligofectamine-treated controls (Fig. 4B). Of note, 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 that PARG downregulation did not impair protein expression in a non specific manner. Silencing of PARG *per se* did not induce expression of pro-inflammatory mediators (not shown).

Effect of GLTN on inflammatory signal transduction pathways

Polymixin B, a 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 expression of pro-inflammatory mediators. We also studied the effects of GLTN on activation of transcription factors classically involved in expression of iNOS and COX-2. In extracts of RAW macrophages exposed to LPS (1 μ g/ml/1h), the DNA binding activities of NF- κ B or AP1 appeared as retarded bands that were reduced by the addition to the binding mixture of an antibody raised against p65/RelA (NF- κ B) or c-Jun (AP1). The bands were also reduced by 50-fold molar excess of cold probe, thereby indicating specificity of binding (Fig. 5Ba and b). Surprisingly, GLTN inhibited the constitutive binding of NF- κ B to the oligoprobe in gel shift assays (Fig. 5Ba). This is consistent with the ability of polyphenols to suppress NF- κ B activation (Pan *et al.*, 2000). As for AP1, we found constitutive DNA binding activity in RAW macrophages which 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 pro-inflammatory mediators

We next analyzed mRNA levels of iNOS, COX-2, IL-1 β and TNF- α in RAW cells exposed to GLTN. Consistent with data obtained with Western blotting, the drug increased the transcript levels of iNOS and COX-2 after 4 h exposure, but not those of TNF- α and IL-1 β (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 GLTN-dependent increase of iNOS and COX-2 mRNA transcripts (Fig. 6Ab), suggesting that expression of pro-inflammatory mediators prompted by GLTN, although apparently independent 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, a mRNA decay assay was carried out. As shown in Fig. 6B, LPS-induced iNOS transcripts decreased after 5 h 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 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-ribose)ylation regulates assembly of transcription-regulating multiprotein complexes [for reviews see: (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-ribose)ylation at specific promoter elements coordinates transcriptional activation (Butler and Ordahl, 1999; Zhang *et al.*, 2002; Nirodi *et al.*, 2001; Akiyama *et al.*, 2001; Soldatenkov *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 (Zingarelli *et al.*, 2003a) proteins. In keeping with this scenario, our findings demonstrate that nuclear accumulation of PAR due to PARG inhibition triggers selective transcription of pro-inflammatory genes in macrophages. The apparent spontaneous (i.e. activator independent) nature of GLTN-induced transcription is in line with a prior report showing that PAR promotes 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*. Remarkably, inhibition of PAR formation rescues the plant wild-type phenotype (Panda *et al.*, 2002).

After GLTN exposure, we have been 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 AP1, 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 classical membrane receptor-dependent macrophage activators is also indicated by GLTN ability to induce, at variance with LPS, selective and delayed transcription of pro-inflammatory 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 pro-inflammatory 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 (see Fig. 4Aa), thereby allowing to speculate 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 downregulation 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 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 silencing, while 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 auto-poly(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 due to PARG inhibition is associated with PARP-1 auto-modification (Fig.1A, C and 2B) and apparent, *spontaneous* transcriptional activation (Fig. 3A and 6Aa). Taken together, 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 induced iNOS and COX-2 also in other mononuclear phagocyte cell lines such as NR 8383 (rat alveolar macrophages) and N11 (mouse microglia) (not shown). In human monocyte-derived macrophages, GLTN only induced COX-2 (not shown), in line with the well-known difficult induction of human iNOS *in vitro*.

In conclusion, the present study points to 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 pro-inflammatory transcription factors and cytokines, it is worth of mention that the PAR-dependent induction of iNOS and COX-2 without concomitant activation of NF- κ B, AP1, STAT-1 or IRF-1 as well as IL-1 β 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 to therapeutic intervention.

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Footnotes

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Legends for figures

Figure 1. GLTN and PARP-1 inhibitors modulate poly(ADP-ribosylation) and NAD content in RAW macrophages. (A) GLTN exposure (1h) increases poly(ADP-ribosylation) of proteins with approximate molecular weights of 15, 36, 110 and >150 kDa in a concentration-dependent manner. β -actin is shown as loading control. (B) Exposure (1h) of cells to the PARP-1 inhibitors PHE (100 μ M) or BZD (1mM) increases their NAD contents (basal content 18 \pm 3 nmol/mg prot). (C) GLTN (100 μ M/1h) has no effects on the basal NAD content whereas, similarly to 100 μ M PHE or 1mM BZD, counteracts nucleotide depletion triggered by the PARP-1 activator MNNG. * p <0.05, ** p <0.01, *** p <0.001 vs control (B) or MNNG (C) (ANOVA + Tukey's *w*-test). An experiment representative of three is shown in (A), whereas the mean of two or three experiments performed in duplicate is shown in (B) and (C), respectively.

Figure 2. Modulation of bio-PAR formation in RAW macrophages. (A) Immunocytochemistry of bio-PAR. Under control conditions bio-NAD (20 μ M/1h) 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 μ M/1h). 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-ribosylated) proteins reminiscent of that shown in Fig.1A. The PAR content is highly increased in GLTN-exposed cells. 1mM BZD inhibits GLTN-dependent PAR accumulation. 1mM hydrogen peroxide, a classical trigger of PARP-1 activation and auto poly(ADP-ribosylation), increases polymer formation. β -actin is shown as loading control. One experiment representative of six or two is shown in (A) and (B), respectively. Bar=10 μ m.

Figure 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 μ M) and BZD (1mM) 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 exposure. β -actin is shown as loading control. One experiment representative of at ten (A) or three (B, C) is shown.

Figure 4. PARG silencing impairs GLTN- but not LPS-dependent induction of iNOS and COX-2 in RAW macrophages.

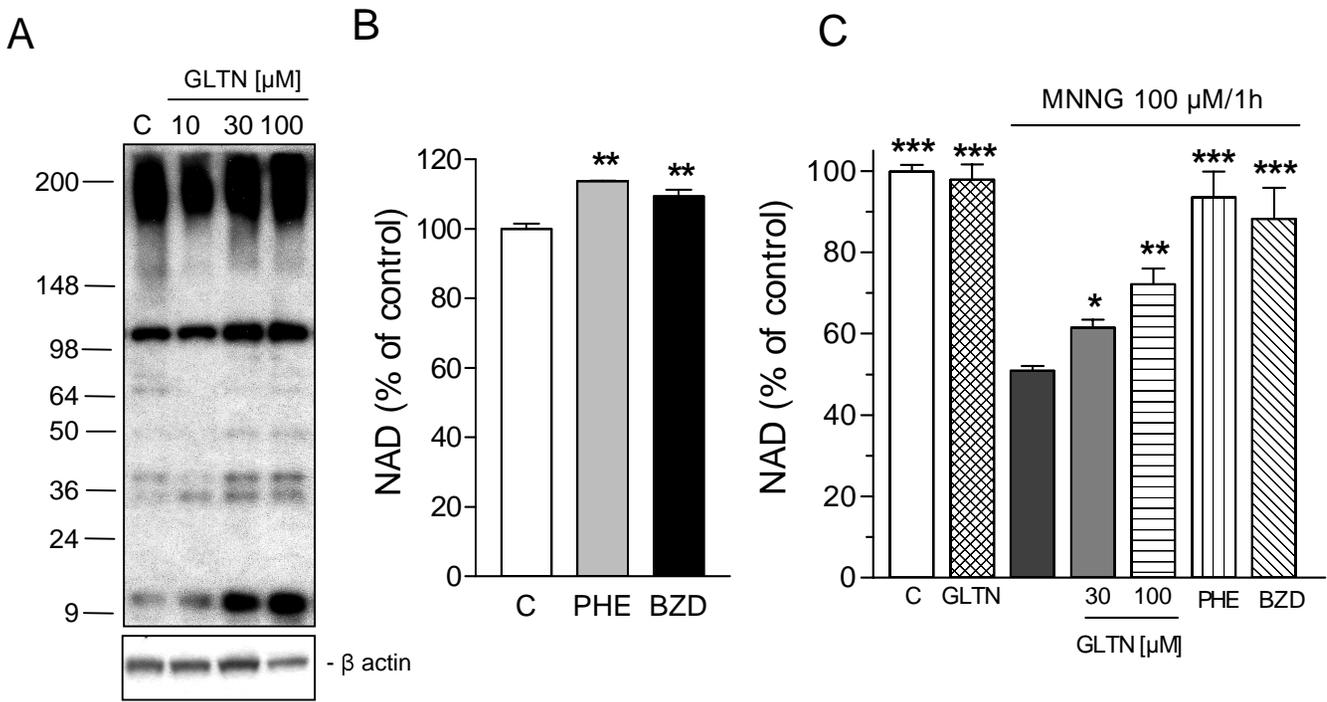
(Aa) Transfection of cells with siRNA for PARG reduces PARG transcript levels without affecting those of PARP-1 and β -actin after 24 h. (Ab) Negative control siRNA does not affect PARG or β -actin transcript levels. (B) 24 h after exposure to siRNAs, expression of iNOS and COX-2 by GLTN (100 μ M/6h) is impaired in cells with silenced PARG compared to oligofectamine-treated controls (43 \pm 16% iNOS and 30 \pm 12% COX-2 reduction vs control). Histogram represents the mean \pm SEM of three experiments. (C) PARG silencing does not affect induction of iNOS or COX-2 by LPS. One experiment representative of three (A-B) or two (C) is shown.

Figure 5. Effects of GLTN on inflammatory signaling pathways in RAW macrophages.

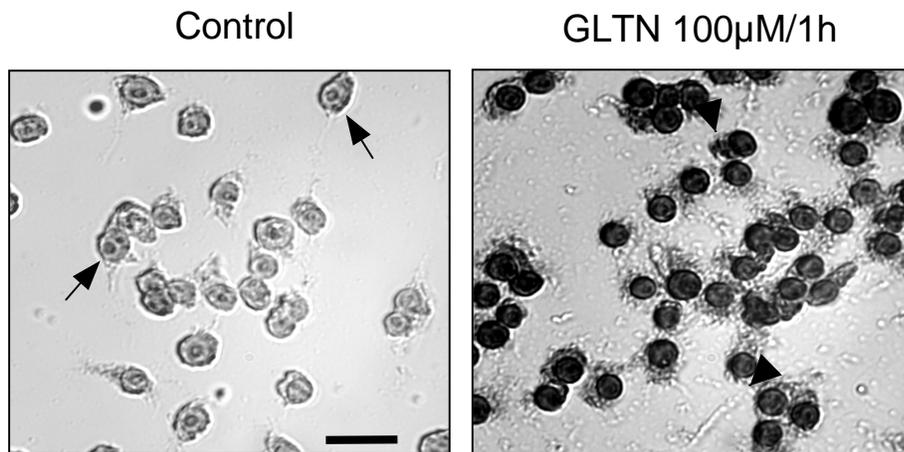
(Aa) Polymixin (plmx, 10 μ g/ml) prevents LPS (0.3 μ g/ml) but not GLTN induction of iNOS after 6 h exposure. (Ab) LPS (0.3 μ g/ml) but not GLTN (100 μ M) induce p38 phosphorylation. p38 levels are shown as loading control. (B) The LPS-induced DNA binding activities of NF- κ B (a) and AP1 (b) appear as retarded bands reduced by antibodies raised against p65 (NF- κ B) or c-Jun (AP1). The addition of 50-fold molar excess of cold probe also reduced band intensity. (Ba) GLTN (2h) inhibits basal binding activity of NF- κ B in gel shift assays. (Bb) Constitutive DNA binding activity of AP-1 is not affected by GLTN (2h). In (Ba) and (Bb) the effect of LPS is shown as positive control. (C) IFN γ (100 U/ml) but not GLTN (100 μ M) triggers STAT-1 phosphorylation and IRF-1 expression. β -actin is shown as loading control. One experiment representative of two (A) or three (B-C) is shown.

Figure 6. Effects of GLTN on transcripts of pro-inflammatory mediators in RAW macrophages.

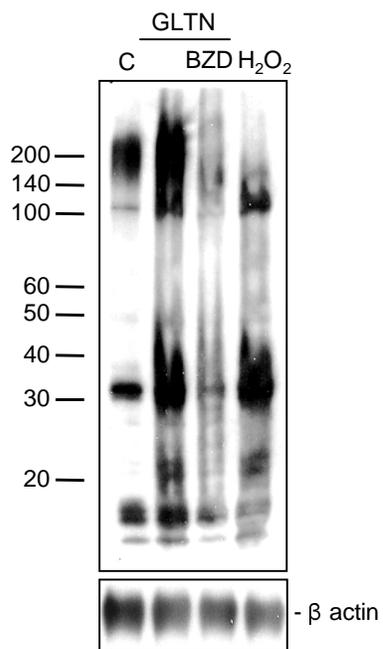
(Aa) GLTN induces synthesis of iNOS and COX-2 but not of IL-1 β transcripts. The drug has no effects on constitutive TNF- α transcript levels. LPS induces prompt increases of mRNAs of all the four proteins. β -actin is shown as loading control of RT products. (Ab) 10 μ g/ml Act-D abrogates GLTN-dependent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

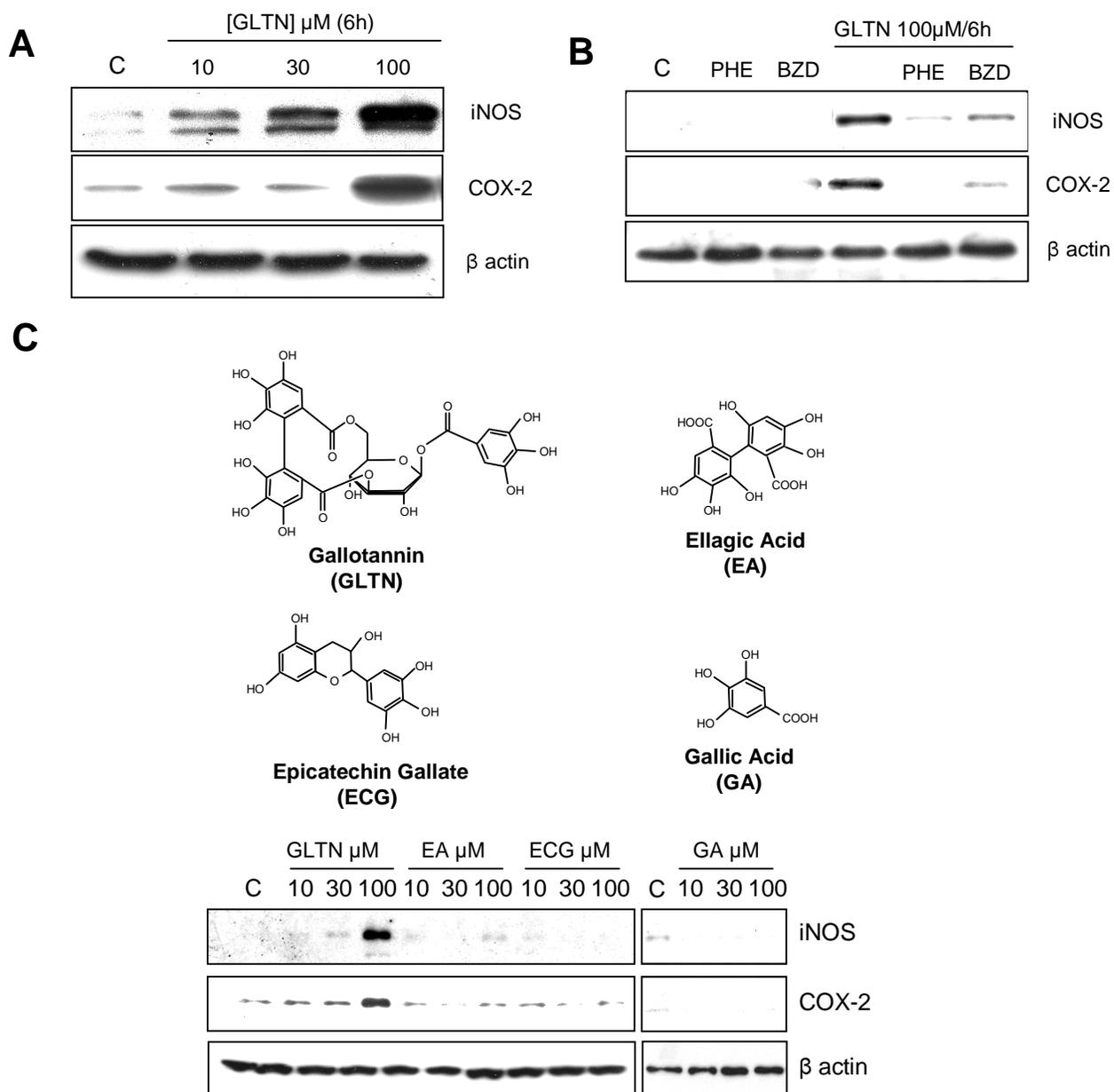


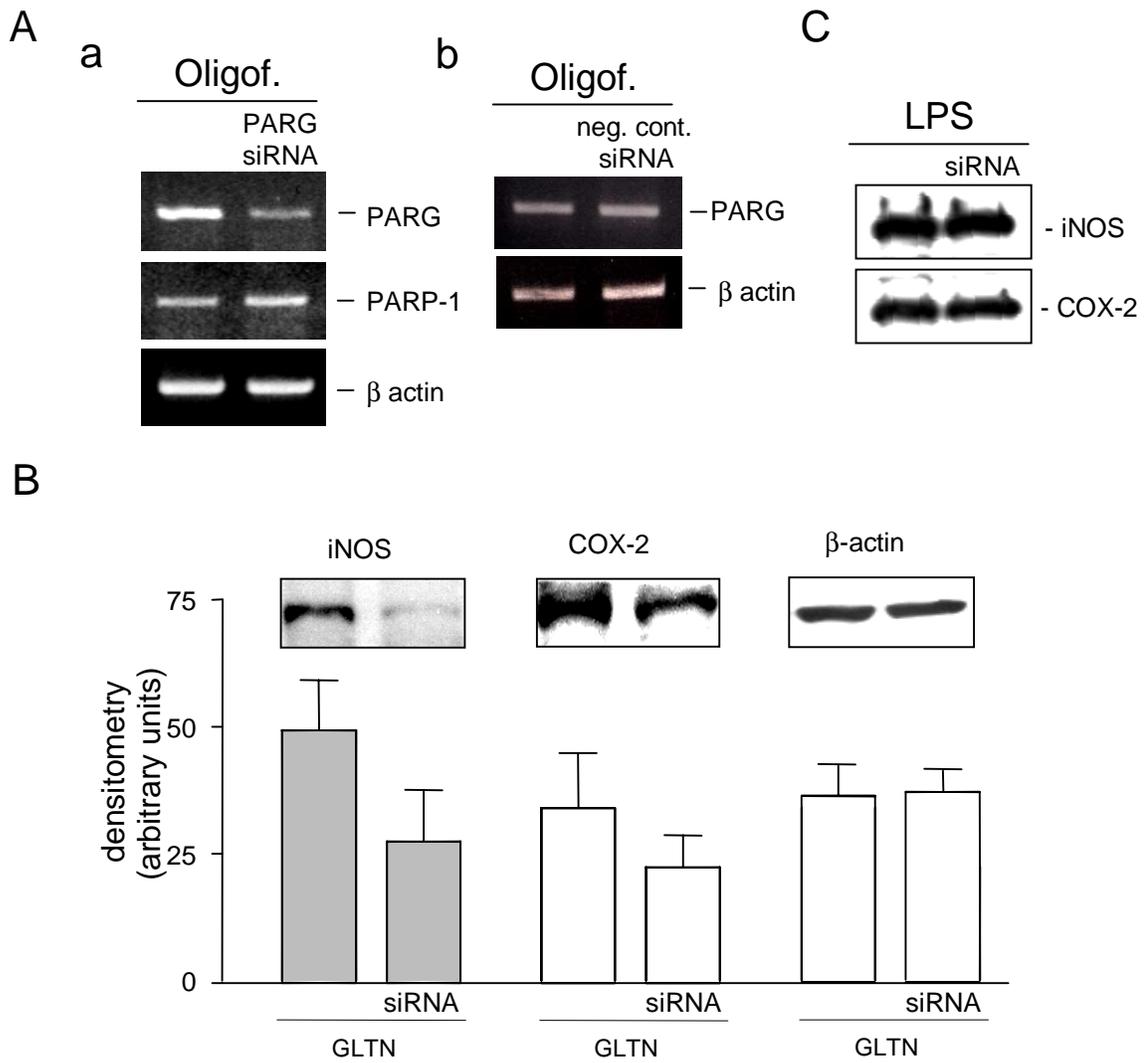
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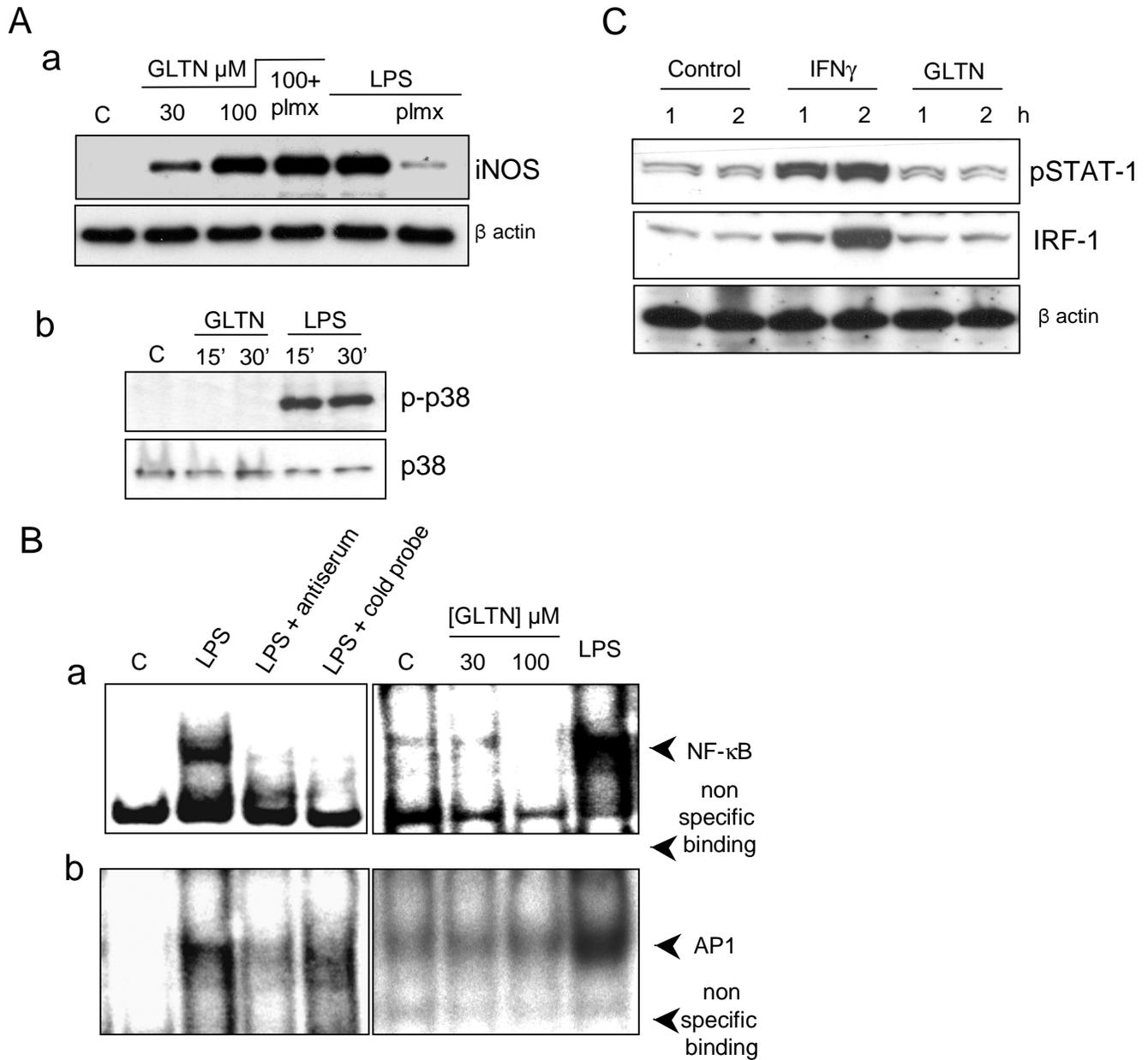


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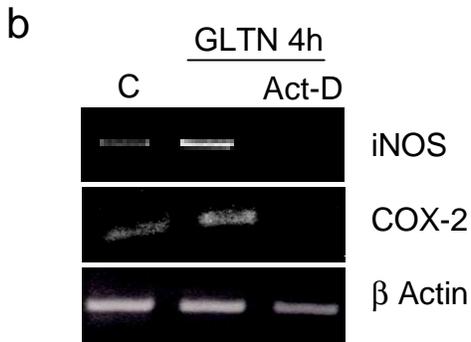
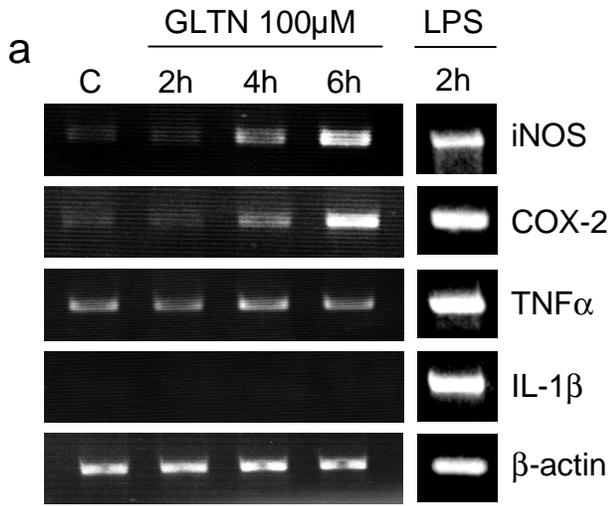




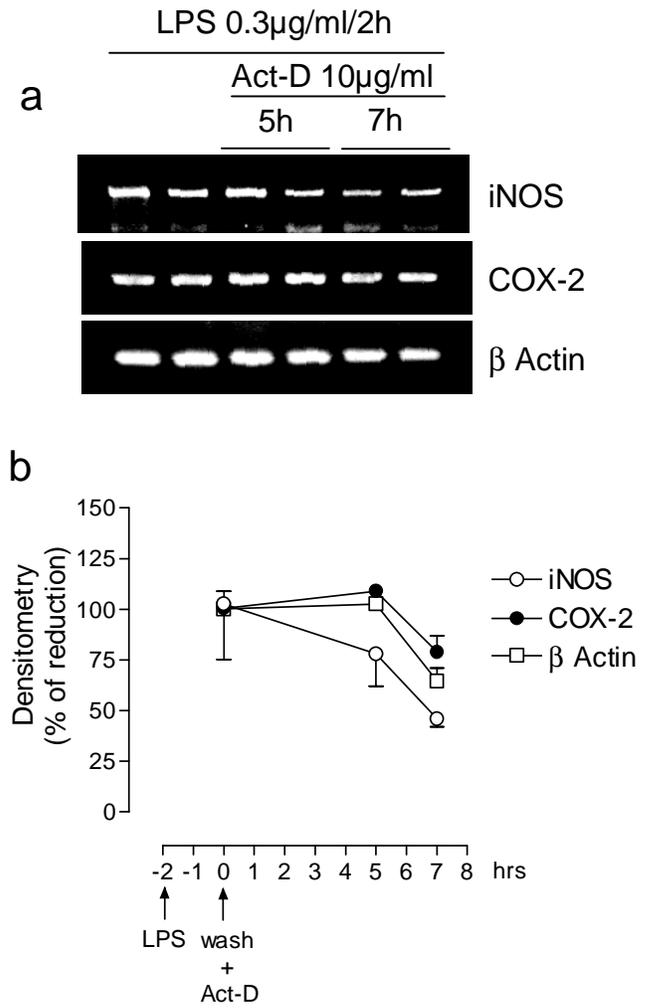




A



B



C

