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Mono ADP-ribosylation inhibitors prevent inflammatory cytokine release in alveolar epithelial cells

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Abstract A549, a type II alveolar epithelial cell line stimulated with LPS (10 μg/ml), released high levels of the inflammatory cytokines IL-6 and IL-8. Here, we have investigated whether ADP-ribosylation inhibitors block the LPS-triggered cytokine release in epithelial cells. When coincubating A549 with LPS and *meta*-iodobenzylguanidine or novobiocin, selective arginine-dependent ART-inhibitors, the release of IL-6 and IL-8 was inhibited in a concentration-dependent manner. This effect has been linked with the presence of a functionally active arginine ADP-ribosylating enzyme on the cell surface. To this aim, we amplified by RT-PCR the ART1 transcript and identified four ADP-ribosylated proteins likely substrate for ART1. The mechanism behind the cytokine inhibition in epithelial cells seems to be correlated with the presence of ART1, which behaves as an essential positive regulator of inflammatory cytokines. This novel observation indicates this enzyme as well as other novobiocin/MIBG sensitive ARTs as potential targets for the development of new therapeutic strategies.

Keywords LPS · TLR-4 · ADP-ribosylation inhibitors · Epithelial cells · Cytokines

Abbreviations

TLR-4	Toll like receptor 4
LPS	Lipopolysaccharide
Nam	Nicotinamide
ADP-ribose	Adenosine diphosphoribose
MIBG	*meta*-iodobenzylguanidine
novo	Novobiocin

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Introduction

Mono ADP-ribosylation is a covalent post-translational modification of proteins in which the ADP-ribose moiety of NAD is transferred to a protein substrate by ADP-ribosyltransferases (ARTs) [1]. The mammalian ARTs, which share structural homology with the family of bacterial toxins, consist of five members (ART1-5). ART1 and ART5 transfer ADP-ribose onto arginine-residues of proteins [2, 3], while ART3 and ART4 activity and acceptor have not been characterized yet. ART2, which is not expressed in human due to three premature stop codons [4], also recognizes arginine as an acceptor.

Many studies have used ADP-ribosylation inhibitors in order to determine the biological functions of ART-catalyzed reaction. Such studies have revealed a link between inhibition of ADP-ribosylation and reduction of chemotaxis and actin assembly [5], prevention of apoptotic body formation [6], suppression of proliferation, and differentiation of smooth [7] and skeletal muscle cells [8]. In airways, it has become increasingly apparent that the so-called non-immune epithelial cells contribute to immune response as regulators of inflammation since they are continuously exposed to invading particles and potential pathogens as a result of breathing [9]. These cells also respond to a range of bacterial products like lipopolysaccharide (LPS), which stimulates the release of biologically active mediators including cytokines [10, 11].

Inhibition of ADP-ribosylation correlated with suppression of inflammatory mediators in macrophages [12] and in monocytes [13]. However, a direct link between mono ADP-ribosylation and cytokine release in epithelial cells was not provided. Therefore, we decided to assess whether ART-inhibitors can prevent the LPS-induced production of cytokines in lung epithelial cells. The major outcome of this report is to show that *meta*-iodobenzylguanidine (MIBG) and novobiocin suppress the LPS-induced production of IL-6 and IL-8 in A549 cells, a lung epithelial cell line, with features of type II alveolar epithelial cells [14]. Moreover, since only ART inhibitors are effective in suppressing the cytokine release, we have correlated this inhibition with the presence of a functionally active ART1 on the cell surface of these cells.

Materials and methods

Cells and reagents

[*adenylate*-^{32}P]NAD (1,000 Ci/mmol) and [*adenine*-U-^{14}C]NAD (274 mCi/mmol) were purchased from Amersham; Dulbecco's modified Eagle's minimum essential medium (DMEM) was from Gibco BRL, Cholera toxin was from Biological laboratories INC, LPS from *E. coli* serotype R515 from Alexis, MHAB N45 filter plates were purchased from Millipore and A549 cells from ATCC. Novobiocin, MIBG, PD128763, and all other reagents used in this study were from Sigma Aldrich.

Cytokine measurements

Human IL-8 and IL-6 concentrations were measured in cell culture supernatants using the Cytometric Bead Array (BDA) human soluble Protein kit provided from Becton-Dickinson.

Cells and membrane purification

A549 were cultured following the vendor's instructions, trypsinized in the exponential growth phase and collected by centrifugation at 900*g* for 5 min, washed three times in PBS and lysed in hypotonic lysis buffer (10 mM Tris/HCl, pH 8, and 1 mM EDTA) containing a protease cocktail (Roche). Cell lysates were freeze-thawed once in dry ice for 10 min and then centrifuged at 100,000*g* at 4°C for 1 h. The membrane fraction obtained after centrifugation was suspended in the same lysis buffer and sonicated twice before assaying for transferase activity.

Arginine-dependent mono ADP-ribosyltransferase assay

Intact cells or purified membranes were assayed for ADP-ribosyltransferase using a filter plate-based assay with poly-arginine as substrate [15]. Assays were carried out in 0.3 ml containing 50 mM potassium phosphate, pH 7.5, 0.6 mg of poly-arginine and 0.1 mM [*adenine*-U-^{14}C]NAD (0.05 μCi). After incubation at 30°C, samples were precipitated with 0.3 ml of 50% TCA. After 30 min in ice, quadruplicate samples (100 μl) were applied under vacuum conditions in each well of a mixed cellulose esters filterplate (Millipore) and washed with 10 vol.5% TCA. The incorporated radioactivity was measured in a Packard Top counter.

RNA purification and semiquantitative PCR

Total RNA was purified from A549 (3×10^6 cells) using the RNAeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed in cDNA at 55°C for 2 h with Superscript III and 50 pmol each of ART1, ART5, and β-actin gene-specific primers (Table 1) using the One-step RT-PCR system from Invitrogen. The integrity of RNA and cDNA synthesis was verified by PCR measuring the amount of the housekeeping gene β-actin. The touch-down amplification was performed directly after retro transcription with an initial denaturation step at 95°C for 2 min, the initial annealing temperature was 70°C for 1 min followed by 15 cycles, in which temperature decreased 1°C per cycle. An annealing temperature of 55°C were repeated for 25 cycles for 90 s followed by an extension phase of 90 s at 72°C. A final extension phase at 72°C for15 min was also performed. PCR products were resolved on 1% Tris-acetate-EDTA (TAE)-agarose gels containing 0.5 μg/ml ethidium bromide. The forward and reverse primers of all intron spanning are shown in Table 1.

ADP-ribosylation of A549 surface proteins

A549 cells (0.5×10^6 cells from a confluent culture) were incubated in 50 mM potassium phosphate pH 7.4 containing 10 μM [*adenylate*-^{32}P] NAD (10 μCi/assay) and different effectors in 50 μl final volume. A control assay reaction containing all the above ingredients except [*adenylate*-^{32}P]NAD was performed. Reactions were started by the addition of NAD, incubated at 37°C for 1 h and stopped by adding 50 μl of 50% ice-cold TCA. The proteins were allowed to precipitate on ice overnight and were collected by centrifuging the samples at 13,000*g* at 4°C for

Table 1 ARTs and Actin gene-specific primers

Gene	Forward 5′-3′	Reverse 5′-3′	Product size (bp)
ART1	CAGATGCCTGCTATGATGTC	TCAAAGGAGGCCTGGACCAT	981
	AGCCAATGGCAGGAGCGTCA	TCAAAGGAGGCCTGGACCAT	735
ART5	ATCCTGCCCCTGAGCCTGGTT	GACACACAGCCACGTCTCTTC	741
	ATCCTGCCCCTGAGCCTGGTT	CGCAGTTAAAGTGGCTGCAGG	703
β-Actin	ATGGATGATGATATCGCCGCG	CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC	1003
	ATCTGGCACCACACCTTCTACAATGAGCTGCG	CGTCATACTCCTGCTTGCTGATCCACATC	822

30 min. Labeled pellets were solubilized in reducing sample buffer heated at 70°C for 10 min and separated by SDS-PAGE (10% NuPAGE gel system using MES as running buffer). Electrophoresed proteins were then electroblotted to nitrocellulose membrane. The radiolabelled proteins were detected by autoradiography exposing the membrane to X-ray film (Kodak) 24–48 h at −80°C. Molecular masses of the ADP-ribosylated proteins were calculated using the software Imaged Master 1D elite from Pharmacia.

Chemical stability of the protein-ADP-ribosyl linkages

After ADP-ribosylation, proteins from A549 cells were precipitated with 50 µl of 50% ice-cold TCA overnight at 4°C. The nature of the ADP-ribose-protein linkage was determined by incubating the precipitated proteins at 37°C in the presence of H_2O, 1 M NaCl, 0.1 M HCl, 10 mM $HgCl_2$ or 1 M NH_2OH (in 0.1 M Tris, adjusted to pH 7 with NH_4OH). After 4 h, the samples were precipitated with 50% TCA (25% final concentration), solubilized in reducing sample buffer and electrophoresed as described above.

Protein assay

Protein concentration was estimated using Bradford assay kit (Bio-Rad) with BSA (2 mg/ml Pierce) as standard.

Statistical analysis

Cytometric Bead array data and enzymatic activity assays were quantified and presented as means ± SD of single experiments performed in triplicate. One-way ANOVA was used to test for differences between means of independent groups. A typical threshold of P values < 0.05 was set for statistical significance. Means and P values are specified in figure legends.

Results and discussion

In human airways epithelial cells represent, with nose and mucus, the first line of defense against microbial attack. Similar to the immune cells, activation of epithelial cells releases pro-inflammatory mediators and cytokines suggesting that these cells, as well as immune cells, are active modulators of the immune response [10, 11]. To this aim, and based on the observation that production of inflammatory mediators and cytokines are inhibited by ADP-ribosylation inhibitors in macrophages [12] and monocytes [13], we were interested in verifying whether ART-inhibitors prevent cytokine release in alveolar epithelial cells. Experiments were carried out using A549, a cell culture system of type II alveolar epithelial cells known to produce IL-6 and IL-8 [16] stimulated with LPS (10 µg/ml) in the presence of increasing concentrations of novobiocin and MIBG, a functional analog of noradrenalin. Both compounds are ART-specific inhibitors that have no effects on poly (ADP-ribose) polymerase-1 (PARP-1) [17, 18]. As shown in Fig. 1 MIBG (black bars) led to a 90% reduction in cytokine response even at 0.05 mM. The inhibitory effect of novobiocin (white bars) was less prominent at 0.05 mM ∼50% inhibition but similar to MIBG at higher doses. As assessed by trypan blue staining, neither MIBG nor novobiocin affected the viability of the cells (data not shown). Nam (nicotinamide), a member of the benzamide family, has a dual inhibitory effect on PARP-1 with a K_i of 6 µM and on arginine-dependent ARTs with a K_i of 3.4 mM [17, 19]. At the concentration employed in this study (5 mM) Nam does not prevent IL-6 (Fig. 2A) and IL-8 (Fig. 2B) release, confirming as others reported for cells of the immune system, that the potent inhibitory effect on inflammatory cytokines is exerted by Nam at a higher concentration [12, 13, 20]. This supports the inference that poly ADP-ribosylation is not involved in this process. Further, PD128763, a more specific PARP-1 inhibitor [21] was found unable to inhibit cytokine release (Fig. 2) showing convincingly that ARTs and not PARP play a role in IL-6 and IL-8 release in epithelial cells.

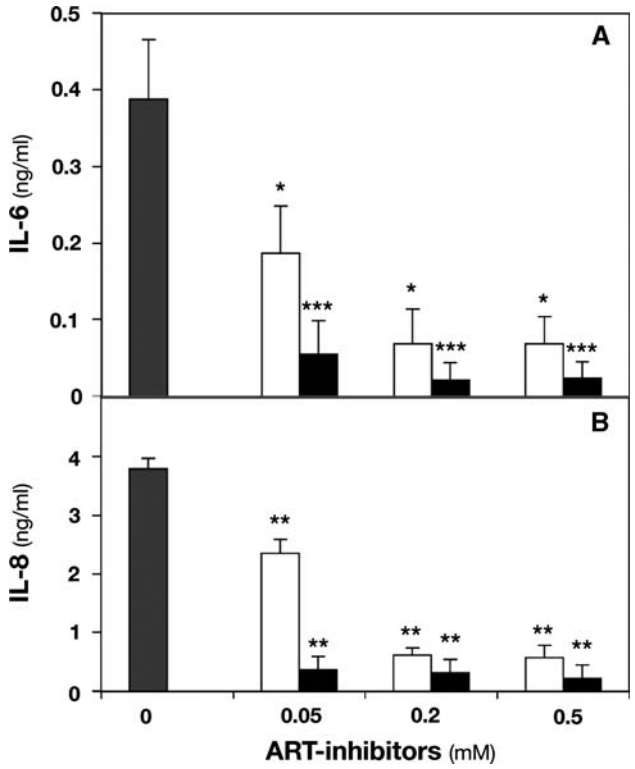


Fig. 1 Effect of novobiocin and MIBG on the release of IL-6 and IL-8. A549 cells were stimulated with LPS (10 μg/ml) without (grey bars) or with increasing concentrations (0.05, 0.2, 0.5 mM) of novobiocin (white bars) and MIBG (black bars). After 24 h of stimulation, supernatants were collected and IL-6 (**A**) and IL-8 (**B**) quantified as described in method section. Each values show means ± SD ($n = 3$) of separate experiments conducted with three different A549 cultures. *$P < 0.001$, **$P < 0.009$, ***$P < 0.0001$

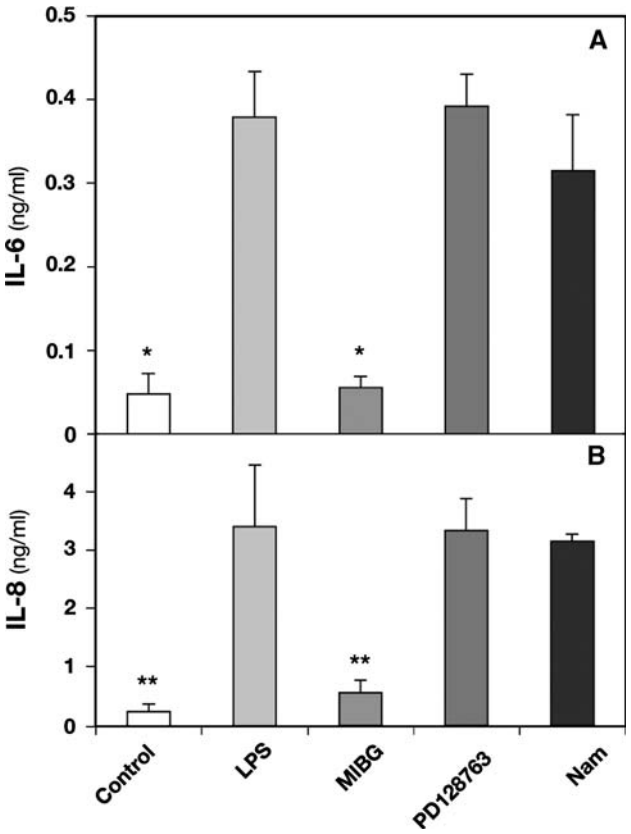
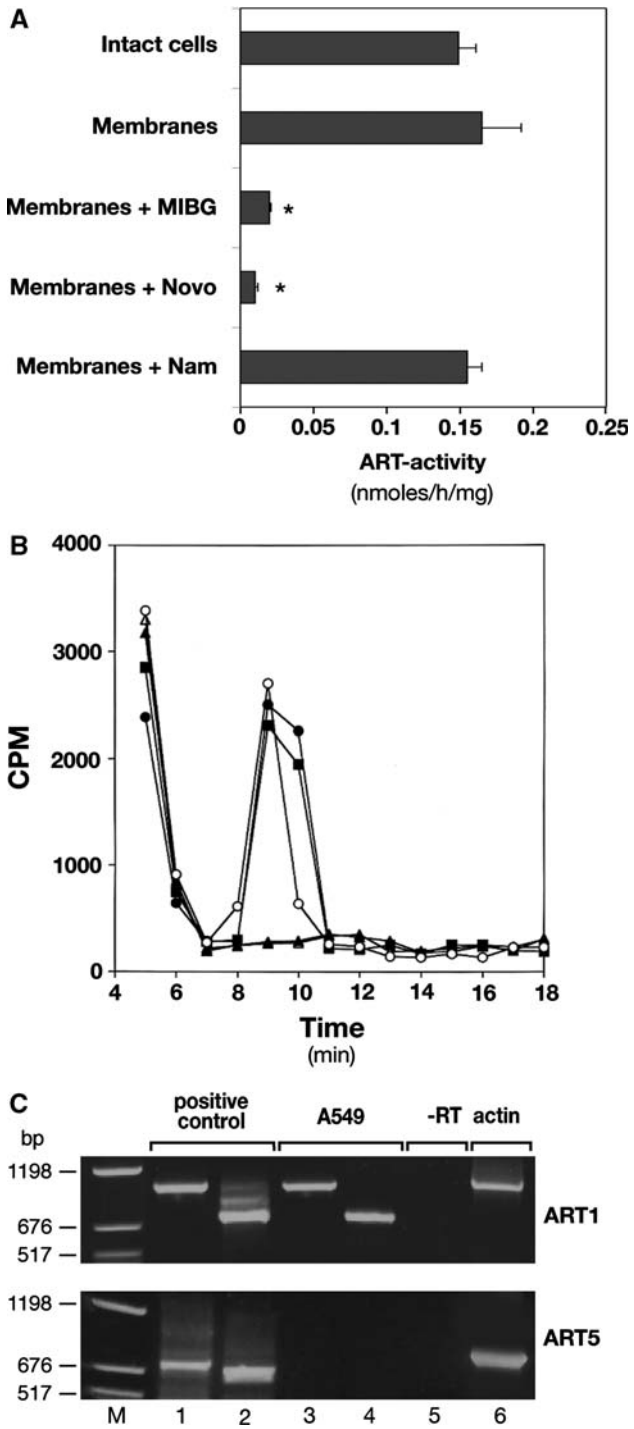


Fig. 2 Comparative effects of ADP-ribosylation inhibitors on cytokine release. A549 cells were cultured in 24-well dishes as described in methods and incubated with LPS (10 μg/ml) in complete medium for 24 h in the presence of Nam (5 mM), MIBG (50 μM) and PD128763 (10 μM). Inhibitors were added at the same time as LPS and supernatants were quantified for IL-6 (**A**) and IL-8 (**B**) as described in method section. Values show means ± SD ($n = 3$) using three separate cell cultures. *$P < 0.0004$, **$P < 0.002$

The capacity of ART inhibitors to inhibit the cytokine release suggests a link between cytokine production and ART1, an arginine-modifying ART, which might fulfill a role in epithelial cells. Evidence for an arginine-specific ART-activity expressed on cell surface of A549 was sought. A recently developed filter assay with poly-arginine as ADP-ribose acceptor [15] was used to localize an arginine MIBG and novobiocin-sensitive modifying ART on the A549 cell membranes (Fig. 3A). Alternative assays were run to confirm that A549 were indeed catalyzing the formation of a linkage between ADP-ribose and simple guanidine compounds. Membranes were incubated with [^{14}C]arginine and a saturating concentration of NAD and the product were separated and analyzed by HPLC. The assay was run in parallel with Cholera toxin, which is a known arginine-modifying bacterial enzyme [22]. [^{14}C]Arginine eluted at 4–5 min while a peak corresponding to ADP-ribosylarginine eluted at 9 min with Cholera toxin and 9–10 min with A549 membranes. We have previously demonstrated that a consistent NAD-glycohydrolase activity that cleaves NAD in free ADP-ribose and Nam and therefore produce a high concentration of free ADP-ribose, is associated with the A549 membranes [23]. Free ADP-ribose did not inhibit the ADP-ribosylation of arginine and there was no evidence of ADP-ribosylarginine formation when [^{14}C]arginine and 5 mM ADP-ribose were coincubated (Fig. 3B). Evidence in support of the expression of ART1 in these cells was obtained by the amplification of ART1 mRNA (Fig. 3C), using full-length (lane 3) and nested primers (lane 4). The amplified PCR products were shown to be ART1 by automated sequencing. Therefore, we checked if ART1 expression on A549 was accompanied by detectable ADP-ribosylation of cell-surface proteins. ADP-ribosylation was visualized by autoradiography after SDS-PAGE and Western blot of cell lysates obtained from intact cells incubated in the presence of [*adenylate*-^{32}P]NAD. As shown in lane 1 of Fig. 4, covalent and specific incorporation of the label occurred primarily in four proteins of approx molecular masses of 130, 88, 51, and 41 kDa. A large excess of unlabeled ADP-ribose over [*adenylate*-^{32}P]NAD, used to



◄ **Fig. 3** A549 cells express the cell-surface arginine-specific ART1. (**A**) A549 cells cultivated in three 75 cm^2 flasks containing approximately 2 × 10^6 cells each were trypsinized and resuspended in PBS. The arginine-specific ART-activity was assayed in intact cells, lysates, and membranes using a filter-based assay with poly-arginine as acceptor molecule, following the conditions described in Methods in the presence of 2 mM novobiocin or 5 mM Nam. The values presented are the means ± SD of three different preparations. *$P < 0.001$. (**B**) A549 cell membranes purified from three 75 cm^2 flasks were incubated in 50 mM Tris pH 7.5 with 2 mM NAD and 100 μM [^{14}C]arginine (~208,000 cpm) at 30°C for 3 h in a final volume of 150 μl in the absence (●) or in the presence (■) of 5 mM ADP-ribose. No ADP-ribosyl-[^{14}C]arginine was formed upon incubating [^{14}C]arginine with (△) or without (▲) 5 mM ADP-ribose in the absence of purified membranes. As positive control, 5 μg of cholera toxin were incubated under the same conditions as above plus 10 mM Mg^{++} and 20 mM DTT (◯). Samples from each reaction (100 μl) were separated on SAX-HPLC (SynChropak Q300, 250 × 4.6 mm) and eluted with 20 mM sodium phosphate, pH 4.5. Fractions (1 ml) were collected for radioassay in a liquid scintillation counter and their counts/min are shown. Similar results were obtained in three separate experiments. (**C**) Total RNA (1 μg) extracted from A549 was reverse transcribed following the procedure explained in methods. PCR was carried out with ART1 full-length (lane 3) and nested (lane 4) primers or with ART5 full length (lane 3) and nested (lane 4) primers. As positive control for ART1 and ART5 we used skeletal muscle and testis respectively, amplified with full-length (lane 1) and nested (lane 2) primers. In lane 5, PCR was performed with ART1 and ART5 full-length primers without previous RT. As positive control for A549, *β*-actin was amplified (lane 6). PCR products were 981 and 735 bp for ART1, 741, and 703 for ART5, 1003, and 838 for *β*-actin. Positions of molecular mass markers (M) are on the left

minimize the contribution of the non-enzymic addition of free [*adenylate*-^{32}P]ADP-ribose to protein acceptors, did not decrease the labeling (lane 2 vs. 1), while slightly increasing it at high molecular weight. This suggests that phosphodiesterases, which are inhibited by ADP-ribose [24], might be active on the cell membrane. Addition of Nam, a NADase inhibitor, did not decrease the intensity of the four bands, while the background observed at the high molecular weights was reduced (lane 3 vs. 1), indicating that this could be partly due to non-enzymic incorporation of ADP-ribose. The presence of ATP, which prevents the hydrolysis of NAD due to the action of phosphatases (lane 4), increases the intensity of radioactivity incorporated at 130, 88, and 41 kDa. On the other hand, the addition of radioactive ADP-ribose was blocked by novobiocin (lane 5) and by heat inactivation (lane 6) confirming the enzymatic transfer of the ADP-ribose unit onto the four target proteins.

Arginine and cysteine are recognized ADP-ribose acceptors, but histidine, lysine, and asparagine are also potential acceptors. To characterize the ADP-ribose-protein linkage, ^{32}P-labeled proteins from the ADP-ribosylation reactions were incubated with H_2O used as control, NaCl, HCl, $HgCl_2$, and NH_2OH. Radioactivity was completely released from the bands at 88 and 51 by hydroxylamine, consistent with an arginine linkage and likely substrate for ART1 (Fig. 5A), $HgCl_2$, which splits ADP-ribosyl-cysteine linkage, as well as NaCl and HCl had no effect on any of the four bands, showing that cysteine, glutamine or lysine are not involved. Interestingly, the bands at 130 and 41 kDa are NH_2OH-stable, suggesting that mono-ADP-ribosylation of these two proteins occurs at amino-acid acceptors different from arginine.

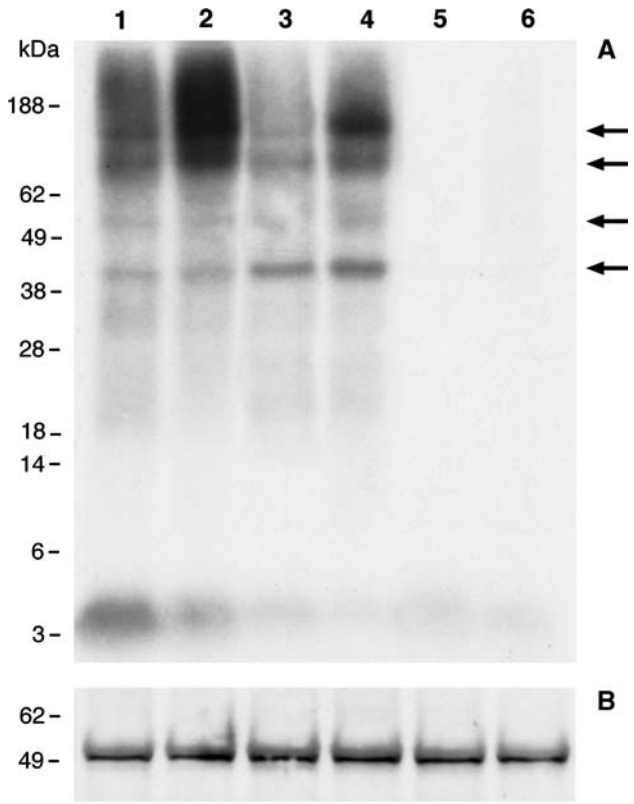


Fig. 4 ADP-ribosylation in intact A549 cells. 5×10^5 A549 cells were incubated in a final volume of 50 µl at 37°C for 1 h in the presence of the compounds indicated below or after heating the cells in 50 mM potassium phosphate pH 7.5 and 10 µM [*adenylate*-^{32}P]NAD (10 µCi/assay). After incubation, cells were pelleted and washed three times with buffer before precipitation with 50 µl of 50% TCA. An overnight after incubation at 4°C, the pellet was resuspended in lithium dodecyl sulfate (LDS) sample buffer as described in Methods and proteins separated with 10% NuPAGE gel using MES as running buffer and blotted on nitrocellulose. The autoradiograph of the blot after 48 h at −80°C is shown in panel A. Positions of molecular mass markers are on the left. Lane 1, no additions (control); lane 2, 10 mM ADP-ribose; lane 3, 10 mM Nam; lane 4, 10 mM ATP; lane 5, 5 mM novobiocin; lane 6, 5 min at 100°C. Panel B, the amount of β-actin present in each sample was evaluated by Western blotting of the same filter. Data shown are representative of two experiments performed with different cell preparations

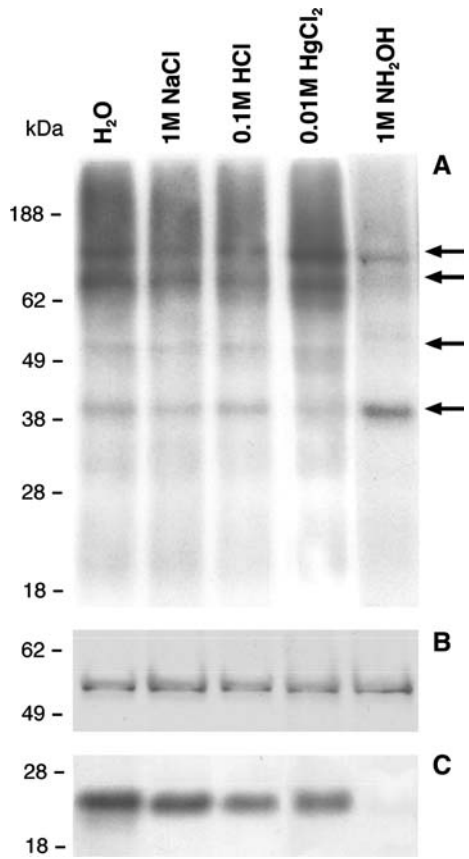


Fig. 5 Chemical stability of protein-ADP-ribosyl linkages. After incubation at 37°C for 1 h with 10 µM [*adenylate*-^{32}P]NAD (10 µCi/assay) 5×10^5 cells were washed three times with PBS and precipitated overnight at 4°C with 50 µl of 50% TCA. Samples were then incubated in 50 µl of H_2O, 1 M NaCl, 0.1 M HCl, 0.01 M $HgCl_2$, or 1 M NH_2OH for 4 h at 37°C (**A**). Proteins were precipitated again with TCA (25% final concentration) overnight at 4°C and then subjected to SDS-PAGE and Western blotting followed by autoradiography (Panel A). Data shown are representative of three separate experiments. (**B**) Control blotting with β-actin. (**C**) Control experiment with 3.7 µg of Cholera toxin

The precise mode of action and the different events leading to cytokine release have not been ruled out in this study. Further, despite the observation that ADP-ribosylation might be involved in LPS-triggered cytokine release, more detailed pharmacological studies are needed to evaluate the ability of MIBG and novobiocin to reduce inflammation in vivo. However, since inflammation is the net result of the interaction of many endogeneous mediators, the discovery of new pharmacological inhibitors may be of interest. Therefore, the present study may stimulate the search for novel compounds able to mimic the inhibitory effects of MIBG and novobiocin. Likewise, ART1 as well as other novobiocin/MIBG sensitive ARTs might be considered as potential novel targets for ART-specific inhibitors opening new avenues for the treatment of inflammatory diseases.

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