

## Role of ADP-ribosylation in endothelial signal transduction and prostacyclin production

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ADP-ribosylation of proteins by the enzymatic transfer of ADP-ribose from NAD has been implicated in a number of biological processes. We report that inhibitors of ADP-ribosylation, most notably the novel inhibitor of arginine specific cellular mono(ADP-ribosyl) transferase, *meta*-iodo-benzylguanidine (MIBG) as well as nicotinamide, L-arginine methyl ester (LAME) and guanyltiramine, inhibit histamine-induced endothelial production of inositol phosphates, release of arachidonic acid and production of prostacyclin (PGI<sub>2</sub>). Those same responses were unaffected by MIBG when triggered by thrombin or leukotriene C<sub>4</sub>. These findings suggest that ADP-ribosylation serves a role in histamine-induced production of prostacyclin and imply differences in transduction pathways employed by the different agonists.

Endothelium; Mono ADP-ribosylation; Signal transduction; Prostacyclin

### 1. INTRODUCTION

ADP-ribosylation, i.e. posttranslational modification of proteins by the enzymatic transfer of ADP-ribose from NAD resulting in alteration of the functional properties of the respective proteins has been implicated in a number of biological processes [1,2]. ADP-ribosyltransferase activity has been demonstrated in various tissues and cells from many different species [2,3]. Furthermore, glycohydrolases capable of removing ADP-ribose from acceptor proteins have also been described in eukaryotic cells [2,4]. However, in most instances little is known about the physiological role of endogenous ADP-ribosylation and investigations have been hampered by the inability to introduce labeled NAD into cells. Recently Brüne and co-workers [5] were able to demonstrate agonist-induced ADP-ribosylation of a specific cytosolic 42-kDa protein in platelets and suggested that inhibition and stimulation of platelet responses are closely related to the ADP-ribosylation of different proteins. Smets and co-workers have demonstrated by the use of inhibitors that the cytolytic action of glucocorticoid hormones in leukemic cells is negatively controlled by mono-ADP-ribosylation while poly-(ADP-ribose)polymerase is not involved [6]. In cultured pulmonary endothelial cells ADP-ribosylation

of actin induced by botulinum C<sub>2</sub> toxin modifies the monomeric G-form of actin in such a way that its ability to polymerase is inhibited resulting in increased permeability of the pulmonary artery endothelial monolayer [7]. While it has been demonstrated that cholera toxin increases prostacyclin (PGI<sub>2</sub>) production in endothelial cells [8] no published information is available about the possible role of endogenous ADP-ribosylation in endothelial signal transduction mechanisms like those involved in production of PGI<sub>2</sub>. In chicken spleen membranes [9] and in platelets [10] it has recently been demonstrated that basal adenylyl cyclase activity is enhanced by endogenous ADP-ribosylation of the  $\alpha$ -subunit of the stimulatory GTP-binding protein G<sub>s</sub>. The implication is that basal adenylyl cyclase activity in eukaryotic cells may be controlled via endogenous ADP-ribosylation. Recently Nowicki and co-workers observed that nicotinamide and 3-aminobenzamide at high concentrations inhibited inositol phosphate generation and Ca<sup>2+</sup> mobilization in cytotoxic T-cells [11]. The high doses and relative potency of the inhibitors suggested involvement of a mono(ADP-ribosyl) transferase rather than a poly-(ADP-ribose) polymerase [12]. Using inhibitors of ADP-ribosylation, we have started to investigate the role of this form of modification of regulatory proteins on endothelial production of inositol phosphates, release of arachidonic acid and the production of PGI<sub>2</sub> in response to various stimuli including thrombin, histamine and leukotriene C<sub>4</sub>.

Of particular value is the availability of the novel inhibitor of cellular mono ADP-ribosylation, *meta*-iodo-benzylguanidine (MIBG). It can be used at lower concentrations than nicotinamide and L-arginine

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Abbreviations: LAME, L-arginine methyl ester; MIBG, *meta*-iodo-benzylguanidine; MIBA, *meta*-iodobenzylamine; PGI<sub>2</sub>, prostacyclin.

methyl ester (LAME) and has no effects on poly-(ADP-ribose) polymerase activity [6].

## 2. MATERIALS AND METHODS

### 2.1. Materials

[<sup>3</sup>H]arachidonic acid, *myo*-[<sup>3</sup>H]inositol and [<sup>3</sup>H]6-oxo-PGF<sub>1α</sub> were obtained from Amersham international; Morgan's medium 199, foetal calf serum and antibiotics were from Gibco; collagenase, bovine serum albumin, HEPES, LiCl, histamine, leukotriene C<sub>4</sub>, thrombin, nicotinamide and L-arginine methyl ester (LAME) were from Sigma; anion-exchange resin was from Bio-Rad; tissue culture plates were from Nunc; *meta*-iodo-benzylamine (MIBA) was from Aldrich Chemical, Milwaukee, WI; Anti-(6-oxo-PGF<sub>1α</sub>) antiserum was donated by Dr. Michael Dunn, Department of Medicine, Case Western Reserve University, Cleveland, OH, USA and *meta*-iodo-benzylguanidine (MIBG) was donated by Dr. L.A. Smets, Department of Experimental Therapy, The Netherlands Cancer Institute/Antoni v. Leeuwenhoekhuis, Amsterdam, The Netherlands.

### 2.2. Endothelial cell culture

Endothelial cells were cultured from human umbilical veins by the method of Jaffe et al. as previously described [13]. After harvesting the cells with 0.1% collagenase digestion, they were seeded on 35 mm culture dishes in Morgan's medium 199 containing 20% foetal calf serum and antibiotics (penicillin, 100 units/ml and streptomycin, 100 µg/ml). The medium was changed 24 h after seeding the cells and every 3–4 days thereafter until the cell culture reached confluence.

### 2.3. Formation of inositol phosphates

Cell cultures that had reached confluence were incubated for 30–36 h in Morgan's medium 199 containing 20% dialyzed foetal calf serum and 3 µCi of *myo*-[<sup>3</sup>H]inositol/ml. Before experiments took place, cells were washed twice with medium containing 20 mM LiCl. The cells were then placed in 0.9 ml of medium, containing 20 mM LiCl with inhibitors at the indicated concentrations. Twenty minutes later, 0.1 ml of concentrated agonist was added to reach the intended concentration and the cells incubated for additional 20 min. The medium was then removed for measurement of PGI<sub>2</sub> and 1 ml of ice-cold trichloroacetic acid (TCA) was added to terminate reactions. Inositol phosphates were separated as previously described [13] on columns of anion-exchange resin and then quantitated by liquid scintillation counting.

### 2.4. Prostacyclin production

To measure the PGI<sub>2</sub> production of the cells, the medium was subjected to a radioimmunoassay (RIA) for 6-oxo-prostaglandin F<sub>1α</sub>, a stable catabolite of PGI<sub>2</sub>, as previously described [13].

### 2.5. Arachidonic acid release

Confluent cell cultures were incubated for 24 h in Morgan's medium 199 containing 20% foetal calf serum, antibiotics and 1 µCi of [<sup>3</sup>H]arachidonic acid/ml. Before experiments were carried out, cells were washed twice with medium containing 0.1% bovine serum albumin and kept in this solution. Twenty minutes later a portion of the medium was removed and an equal volume of medium containing concentrated agonist added. After an additional 20 min another portion of the medium was removed. Both portions were then counted in a scintillation counter for quantitation of released activity.

## 3. RESULTS

The effects of the mono-ADP-ribosylation inhibitor MIBG on endothelial inositol phosphate production in response to histamine and thrombin are shown in Fig. 1. The response to histamine is almost totally inhibited

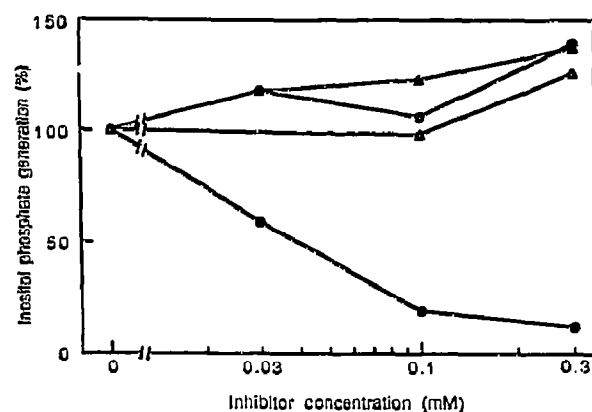


Fig. 1. Effects of MIBG (filled symbols) and MIBA (open symbols) on inositol phosphate generation in response to thrombin (triangles) or histamine (circles). The response is calculated as the increase in inositol phosphates as a fraction of the total acid-soluble counts. The response without an inhibitor is expressed as 100% for each agonist and was 3,264 cpm after thrombin stimulation and 3,442 cpm after histamine stimulation.

by MIBG at 0.1 mM whereas the response to thrombin is not affected. Figs. 2 and 3 show similar effects of MIBG on thrombin and histamine-stimulated arachidonic acid release and prostacyclin production, respectively. While the responses to histamine are almost totally inhibited, the thrombin-mediated responses are unaffected except for arachidonic acid release which at higher concentrations of MIBG is slightly inhibited.

Figs. 1–3 also show that MIBA, an analog of MIBG that does not inhibit cellular ADP-ribosylation [14], has no effects on inositol phosphate generation, or PGI<sub>2</sub> production in response to either histamine or thrombin. Arachidonic acid release is also unaffected except at the

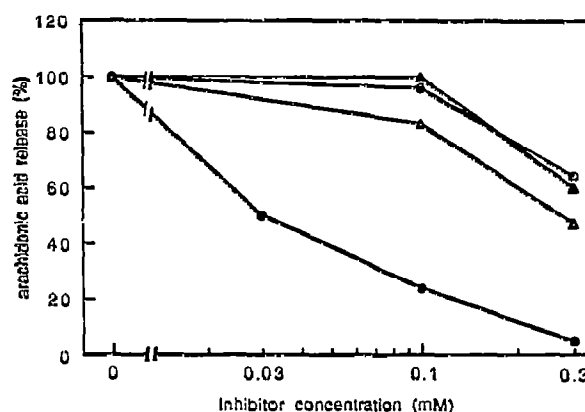


Fig. 2. Effects of MIBG (filled symbols) and MIBA (open symbols) on arachidonic acid release in response to thrombin (triangles) or histamine (circles). The response is calculated as the fraction of incorporated counts that are released in response to agonists. The response without inhibitor is expressed as 100% for each agonist and was 10,565 cpm after thrombin stimulation and 19,805 cpm after histamine stimulation.

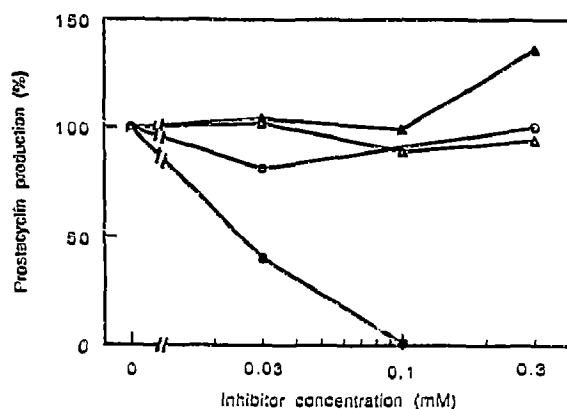


Fig. 3. The effects of MIBG (filled symbols) and MIBA (open symbols) on prostacyclin ( $\text{PGI}_2$ ) production in response to thrombin (triangles) or histamine (circles). The response without inhibitor is expressed as 100% for each agonist and was 71.4 ng/ml after thrombin stimulation and 41.2 ng/ml after histamine stimulation.

highest dose of MIBA which inhibits the thrombin and histamine responses to a similar extent.

To further examine the possibility that the effects of MIBG are unrelated to inhibition of cellular ADP-ribosylation, the effects of several other known inhibitors of ADP-ribosylation on inositol phosphate generation were tested. The results are summarized in Table I. Guanyltiramine is commonly used as a substrate for the arginine-specific ADP-ribosylation of cholera toxin [15]. At 3 mM it causes 65% inhibition of histamine-mediated inositol phosphate generation, but has negligible effects on the response to thrombin and leukotriene  $\text{C}_4$ . Nicotinamide, which at the high concentration used (100 mM) is an inhibitor of all ADP-ribosylation reactions [12], has similar effects. The arginine analog LAME at 50 mM inhibits the response to both histamine and thrombin, but has no effect on inositol phosphate generation in response to leukotriene  $\text{C}_4$ .

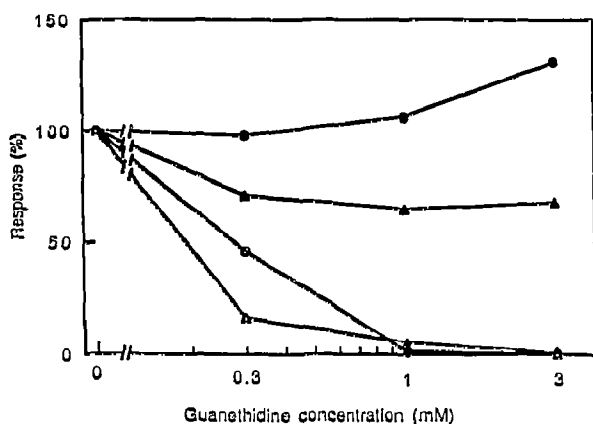


Fig. 4. The effects of guanethidine on inositol phosphate generation (circles) and prostacyclin production (triangles) in response to histamine (open symbols) or thrombin (closed symbols). The results are calculated and expressed as in Figs. 1 and 3.

Table I

Effects of several inhibitors of ADP-ribosylation on inositol phosphate generation in response to histamine, thrombin or leukotriene  $\text{C}_4$

| Stimulus                                | Inhibitor                 |                          |                          |                                  |
|-----------------------------------------|---------------------------|--------------------------|--------------------------|----------------------------------|
|                                         | Control<br>(no inhibitor) | Nicotinamide<br>(100 mM) | Guanyltiramine<br>(3 mM) | Arginine methyl ester<br>(50 mM) |
| Histamine (11 $\mu\text{M}$ )           | 100                       | 24                       | 35                       | 17                               |
| Thrombin (1 U/ml)                       | 100                       | 87                       | 86                       | 8                                |
| Leukotriene $\text{C}_4$<br>(150 ng/ml) | 100                       | 85                       | 94                       | 99                               |

The results are expressed as % of control for each agonist and are calculated as described in the legend to Fig. 1. Each number is the mean of duplicate cultures from a representative of several experiments that gave essentially the same results.

Apart from being an inhibitor of cellular ADP-ribosylation, MIBG is a functional analog of noradrenalin, derived from the neuron blocking agents guanethidine and bretyllium. We therefore studied the effects of isoprenaline and guanethidine on the response to thrombin and histamine. Isoprenaline at doses up to 1 mM had no effect on the response to either agonist (data not shown) whereas guanethidine at 1 mM almost totally inhibited both the inositol phosphate generation and prostacyclin production in response to histamine, but had little effect on the responses to thrombin (Fig. 4).

#### 4. DISCUSSION

This study demonstrates that inhibitors of ADP-ribosylation, most notably MIBG the novel inhibitor of arginine specific cellular mono(ADP-ribosyl) transferase, but also guanyltiramine, nicotinamide and LAME, inhibit histamine-induced endothelial production of inositol phosphates, release of arachidonic acid and production of  $\text{PGI}_2$ . In contrast, those same responses were unaffected by the inhibitors when triggered by thrombin or leukotriene  $\text{C}_4$ , except for LAME inhibiting the response to thrombin. Since LAME is an inhibitor of the protease activity of thrombin [16] which is necessary for the activation of the thrombin receptor, the effects of LAME on cellular responses to thrombin probably are unrelated to its activity as an ADP-ribosyl transferase inhibitor and are in no contradiction to the lack of effects of MIBG and nicotinamide on thrombin-induced endothelial responses. MIBG is a functional analog of norepinephrine and since it is selectively accumulated in storage granules of chromaffin tissues and chromaffin tissue-related tumors its radio-iodinated derivative has been used as a tumor-seeking radio-pharmaceutical for diagnosis and treatment of neuro endo-

crine tumors [17]. Recently Loesberg and co-workers [14] demonstrated that MIBG is an high-affinity substrate for the cholera toxin-catalyzed transfer of ADP-ribose from NAD to arginine-like residues. Furthermore, they showed that MIBG is a substrate for arginine specific mono(ADP-ribosyl) transferase which accumulates in intact mammalian cells and effectively competes with intracellular acceptors for endogenous enzymes [6]. It is metabolically stable and does not affect endogenous poly(ADP-ribose) polymerase. As suggested by Loesberg and co-workers, this agent appears to be a powerful tool to investigate the physiological function of mono(ADP-ribosyl)transferases in various cells. However, other effects of this agent on cellular functions, although possibly related to its ability to inhibit endogenous mono-ADP-ribosylation may introduce complexities into interpretation of experiments such as those presented in this paper. MIBG has been shown to inhibit mitochondrial respiration in several cell lines and consequently stimulate glucose consumption and lactic acid production [18]. It is possible that such changes affect inositol phosphate metabolism. However, the specificity of the inhibition, limited to the histamine responses, speaks against the possibility that such general phenomena are responsible for the inhibitory effects of MIBG reported in this paper.

Since histamine  $H_1$  receptor antagonists are known to inhibit endothelial inositol production and  $PGI_2$  production [13] the question arises if the inhibitory effects of MIBG found in the present study are mediated through histamine  $H_1$  receptor antagonism. Such a mechanism is highly improbable. The MIBG analog, MIBA, in which an amine group has been substituted for the ADP-ribosyl-accepting guanidine group had no effects on the histamine-induced endothelial responses thus supporting the notion that ADP-ribosyl transferase activity serves a role in those responses. Further support is derived from the effects of the other inhibitors of ADP-ribosylation, nicotinamide, LAME and guanlytyramine none of which is known to be a histamine  $H_1$  receptor antagonist.

G-proteins involved in signal transduction are the major natural substrates of pertussis- and cholera toxin-induced ADP-ribosylation. The latter toxin ADP-ribosylates arginine in Gs. GTP binding proteins generally have been thought to be the most important substrates of endogenous ADP-ribosyl transferases. However, in view of the multitude of unique mono-ADP-ribosyl transferases that have been purified it is possible that mono-ADP ribosylation plays a role in many different cellular functions. Actin is a substrate for arginine specific ADP-ribosylation induced by clostridial toxins [7]. A member of the 70 kDa heat shock family of stress proteins has been shown to be reversibly ADP-ribosylated during glucose starvation of mouse hepatoma cells [19]. Several other proteins have been found to be ADP-ribosylated in various in vitro prepa-

rations [1]. Studies are presently being carried out in our laboratory attempting to identify specific substrates of ADP-ribosyl transferases that may be activated during exposure of endothelial cells to histamine.

The selective effects of inhibitors of ADP-ribosylation on the endothelial responses to histamine are of particular interest. This suggests interference with specific steps in the signal transduction pathway. Jacobson and co-workers [20] have recently found differences in the protein phosphorylation in human umbilical vein endothelial cells stimulated by histamine compared to those stimulated with thrombin. In platelets, differences in signal transduction between thrombin and histamine were also suggested by the finding that antiserum against the  $\beta\gamma$  subunits of G-proteins inhibit the histamine response but not the thrombin response. Several reports have recently been published of different agonists mediating phospholipid breakdown through different G-proteins in the same cell type [22-24]. We have shown that histamine-induced production of inositol trisphosphate in human umbilical vein endothelial cells is more rapid than that induced by thrombin [13]. We have also found that pertussis toxin affects the responses to different agonists in different ways. There was amplification of both leukotriene  $C_4$  induced inositol phosphate production and  $PGI_2$  production, whereas the responses to thrombin and aluminium fluoride were not affected [25]. Finally we have observed differences in the calcium dependency of arachidonic acid release induced by different agonists. While the response to  $AIF_4$  was strictly calcium dependent, the leukotriene  $C_4$  induced arachidonic acid release was calcium independent [26].

The different effects of inhibitors of ADP-ribosylation on the responses to histamine compared to thrombin or leukotriene  $C_4$  fit into this maze of observations that suggest that different agonists employ different signal transduction pathways, involving different G-proteins, a variable role of calcium and possibly a variable role of endogenous mono-ADP-ribosylation.

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