Activation of a Cytosolic ADP-ribosyltransferase by Nitric Oxide-generating Agents*

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Sodium nitroprusside is a vasodilator and an inhibitor of platelet activation. It is thought that these effects are mediated by the spontaneous release of nitric oxide and stimulation of cytosolic guanylate cyclase. We have found that sodium nitroprusside (5–200 μM) greatly increased a cytosolic ADP-ribosyltransferase that ADP-ribosylates a soluble 39-kDa protein. This activity causes the mono-ADP-ribosylation of the 39-kDa protein, since digestion with snake venom phosphodiesterase releases 5'-AMP. This enzyme is present in platelets, brain, heart, intestine, liver, and lung. The effect of sodium nitroprusside is not related to stimulation of soluble guanylate cyclase and the production of cyclic GMP because cyclic GMP, dibutyryl cyclic GMP, and 8-bromo-cyclic GMP are ineffective. 3-Morpholinosydnonimine (commonly known as SIN-1) (20–1000 μg/ml), another compound that acts through the spontaneous formation of nitric oxide as does sodium nitroprusside, also stimulates ADP-ribosylation of the 39-kDa protein. Hemoglobin, which binds nitric oxide, inhibits sodium nitroprusside's activation of the cytosolic ADP-ribosyltransferase. These studies demonstrate a novel action of nitric oxide related to the activation of an endogenous ADP-ribosyltransferase. The physiological role of this ADP-ribosylation needs further exploration.

Adenosine diphosphate-ribosyltransferase (EC 2.4.2.30) catalyzes the transfer of ADP-ribose from NAD to proteins. Poly(ADP)-ribosylation is involved in DNA repair (1–3), cell differentiation (4, 5), and malignant transformation (6, 7), while mono(ADP)-ribosylation is similar to the action of bacterial toxins (8, 9). Mono(ADP)-ribosyltransferase is present in a large number of animal tissues (10–13), and it is an important post-translational protein modification mechanism (14–16).

Recent evidence suggests the existence of cytosolic ADP-ribosyltransferase in human platelets (17, 18). We have now found that compounds that liberate nitric oxide as does sodium nitroprusside and 3-morpholinosydnonimine can greatly enhance this activity in the cytosolic fraction of different tissues. It is widely known that nitric oxide, which has been identified as endothelium-derived relaxing factor (19), can stimulate soluble guanylate cyclase (20). However, here we show that the effect of nitric oxide on the activation of ADP-ribosyltransferase is totally independent of the activation of guanylate cyclase.

**EXPERIMENTAL PROCEDURES**

Isolation of Human Platelets—Human blood (100–200 ml) was drawn from healthy volunteers, using trisodium citrate (0.38%, w/v) as an anticoagulant, and centrifuged at 180 × g for 25 min to yield platelet-rich plasma. Platelets were obtained by centrifuging the platelet-rich plasma at 1200 × g for 15 min in the presence of prostacyclin (0.1 μg/ml) and were washed once by centrifugation at 800 × g for 10 min in 50 ml of a Hepes-buffered Tyrode’s solution (138 mM NaCl, 0.36 mM NaH2PO4, 2.9 mM KCl, 1 mM MgCl2, 1 mM EGTA, 10 mM glucose, 20 mM Hepes, pH 7.4) containing prostacyclin (0.3 μg/ml). At this stage the platelets were either resuspended in cold hypotonic buffer for fractionation or in Tyrode’s solution for experiments with different agonists. In the latter case, incubations were performed at 37 °C for the times indicated, and platelets were centrifuged at 800 × g for 10 min and resuspended in hypotonic buffer for fractionation.

**Fractionation of Platelets**—A modification of a published method (21) was used. The platelet pellet was quickly resuspended at 4 °C in 1 ml of hypotonic buffer (5 mM Tris-HCl; 5 mM EDTA, pH 7.5) and frozen in liquid nitrogen. Four cycles of rapid freezing and thawing lysed the cells, and after centrifugation at 800 × g for 10 min to remove any unbroken cells, the homogenate was centrifuged at 160,000 × g for 15 min in an air-driven microcentrifuge. The resulting supernatant, representing the platelet cytosol fraction, was removed and stored in liquid nitrogen.

**Preparation of Cytosolic Fraction from Different Rat Tissues**—A female rat was decapitated, and the organs were immediately withdrawn, extensively washed in cold hypotonic buffer, homogenized, and centrifuged at 800 × g for 10 min to remove unbroken cells. The homogenate was centrifuged at 160,000 × g for 20 min in an air-driven microcentrifuge. The supernatant was used as the cytosolic fraction.

**[32P]ADP-ribosylation**—ADP-ribosylation was carried out by generally following the procedure of Ribeiro-Neto et al. (22). The reaction was performed in 1 ml containing 10 mM thymidine, 1 mM ATP, 2 mM dithiothreitol, 0.1 mM GTP, 138 mM NaCl, 0.36 mM NaH2PO4, 2.9 mM KCl, 12 mM NaHCO3, 5 mM HEPES, 1 μM NAD, 3 mM MgCl2, 200 mM potassium phosphate (pH 7), 1.5 μCi of [32P]NAD/assay, and 30–38 μg of platelet protein. After a 30-min incubation (or otherwise as indicated) at 37 °C, samples were precipitated with 1 ml of cold 10% (w/v) trichloroacetic acid and centrifuged for 10 min at 2000 × g. The pellets were finally washed twice with 2 ml of cold water-saturated ether, and proteins were resolved in 11% sodium dodecyl sulfate-polyacrylamide gels. Gels were subjected to autoradiography, and the bands were cut and counted using a liquid scintillation counter. Representative results of at least three similar experiments are shown.

**Snake Venom Phosphodiesterase Digestion**—The ADP-ribosylation of the 39-kDa protein was carried out as described above, but 200 μg of protein/assay was used. The radioactive protein in the sodium dodecyl sulfate gels was located by autoradiography, and bands corresponding to two assays were removed from the dried gels and subjected to electroelution according to the method of Hunkapiller et al. (23). The eluted protein was dialyzed against 160 mM Tris (pH 8) containing 10 mM MgCl2 and lyophilized. Snake venom phosphodiesterase cleavage of the [32P]ADP 39-kDa protein was done as previously reported (24–26).

**Materials**—Prostacyclin was from Wellcome (Beckenham, United Kingdom) and [32P]NAD (800 Ci/mmol) from Du Pont-New England Nuclear. *1-C-Methylated rainbow protein molecular weight markers

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RESULTS

We have studied the effects of different platelet agonists and known platelet inhibitors on cytosolic ADP-ribosyltransferase activity. We found that sodium nitroprusside stimulates the ADP-ribosylation of a cytosolic 39-kDa protein. Fig. 1, A and B, shows the time course of this reaction, which is linear up to 120 min. Activation by sodium nitroprusside is observable at 10 μM and increases up to 200 μM (Fig. 2), although it was not observed in the particulate fraction (not shown).

ADP-ribosylation of the 39 kDa is not affected when a 200-fold excess of nonradioactive NAD is added at the end of the incubation which suggests a covalent modification of the 39-kDa protein. Moreover, the increase of the concentration of nonradioactive NAD (1–100 μM) resulted in a gradual decrease of the labeling of the 39-kDa protein. A concentration of 100 μM NAD produced 80% inhibition of the labeling. The covalent modification of the 39-kDa protein was also confirmed by snake venom phosphodiesterase cleavage of the [32P]ADP 39-kDa electroeluted protein, which caused only the release of [32P]-labeled 5'-AMP as shown in Fig. 3. The release of [32P]-labeled 5'-AMP paralleled the loss of radioactivity associated with the [32P]-labeled ADP 39-kDa protein. Sodium nitroprusside did not produce degradation of [32P]NAD in our assay system.

It is known that sodium nitroprusside liberates nitric oxide,
which in turn activates soluble guanylate cyclase. Therefore, we studied whether cyclic GMP, 8-bromo-cyclic GMP, dibutyryl cyclic GMP, and other agents that increase cellular cyclic GMP levels such as hydroxylamine and aniline influence the ADP-ribosylation of the 39-kDa protein. Fig. 4 illustrates that the activation of ADP-ribosyltransferase is not related to cyclic GMP. This activity is lost when the cytosolic fraction is boiled (not shown). We have also treated intact platelets for 2 min with sodium nitroprusside and then prepared the cytosolic fraction to assess endogenous ADP-ribosyltransferase activity. Under this condition, sodium nitroprusside stimulated ADP-ribosylation of the 39-kDa protein, but hydroxylamine and aniline were much less active while 8-bromo-cyclic GMP was not active (not shown).

3-Morpholinosydnonimine (29) is another compound that releases nitric oxide. 3-Morpholinosydnonimine stimulates the ADP-ribosylation of the 39-kDa protein (Fig. 5A). Hemoglobin binds nitric oxide (30) and inhibits, in this way, its effects. Fig. 5B shows that hemoglobin inhibits the effect of sodium nitroprusside on the ADP-ribosylation of the 39-kDa protein in a concentration-dependent manner.

The effect of sodium nitroprusside on the activation of cytosolic ADP-ribosyltransferase was also shown in different rat tissues. Fig. 6A shows this activation in rat brain, liver, intestine, heart, and lung. The basal enzymatic activity varies in the different tissues but in all cases is activated by sodium nitroprusside. In brain and heart (Fig. 6B), the effect of sodium nitroprusside is not mimicked by agents that increase the levels of cyclic GMP.

It has previously been shown that sodium cyanide can stimulate a mitochondrial ADP-ribosyltransferase (31). Therefore, we have also determined the effect of thiocyanate (100–500 μM) and sodium cyanide (100–500 μM) on the ADP-ribosylation of the 39-kDa protein; however, activity was not affected (not shown).

**DISCUSSION**

These studies describe the stimulation of an endogenous ADP-ribosyltransferase in various tissues by nitric oxide-releasing compounds. The enzyme mono-ADP-ribosylates a specific 39-kDa protein in all tissues analyzed. This cytosolic protein has not been characterized, but we determined that it is not recognized by G\textsubscript{common, o} (A-569) antiserum (17) in platelets, brain, or heart. Therefore, this protein is not one of the known G\textsubscript{i} subunits of the GTP-binding proteins that have similar molecular masses.

**FIG. 4.** The effect of compounds that affect cyclic GMP levels on the ADP-ribosylation of a cytosolic platelet protein. 1, radioactive protein standards; 2, control; 3, 500 μM sodium nitroprusside; 4, 500 μM hydroxylamine; 5, 500 μM aniline; 6, 500 μM 8-bromo-cyclic GMP; 7, 500 μM dibutyryl cyclic GMP; 8, 500 μM cyclic GMP. This figure also shows that sodium nitroprusside increases the labeling of a high molecular weight protein. This only occurred when assays were carried out with a high concentration of sodium nitroprusside and a long incubation time (compare to Fig. 1).

**FIG. 5.** Effect of 3-morpholinosydnonimine and hemoglobin on the ADP-ribosylation of a cytosolic platelet protein. A, effect of different concentrations of 3-morpholinosydnonimine. Radioactivity (cpm) after subtraction of the control value is shown. B, inhibition of the stimulation of ADP-ribosylation by sodium nitroprusside (50 μM) by different concentrations of hemoglobin. Other details as in Figs. 1 and 2.

Sodium nitroprusside and 3-morpholinosydnonimine are known nitrovasodilators and inhibitors of platelet aggregation (32, 33). This action is mediated through the spontaneous release of nitric oxide that stimulates soluble guanylate cyclase (34). Nitric oxide is also produced by stimulation of cells, and it is recognized as an endothelium-derived relaxing factor (19, 35). Our results now indicate that the release of nitric oxide can affect another enzymatic activity, an ADP-ribosyltransferase. This effect of nitric oxide is independent of guanylate cyclase activation, because cyclic GMP or agents that increase cellular levels of cyclic GMP do not cause stimulation of this ADP-ribosyltransferase. Sodium nitroprusside and 3-morpholinosydnonimine produce a spontaneous nonenzymatic production of nitric oxide, while formation of nitric oxide by hydroxylamine and aniline is not spontaneous. This could explain why hydroxylamine and aniline are not stimulators of the cytosolic ADP-ribosyltransferase.

Sodium nitroprusside and 3-morpholinosydnonimine can produce vasodilation and the inhibition of the physiological responses of platelets. It has been assumed that this was the result of the activation of guanylate cyclase and the subsequent protein phosphorylation induced by cyclic GMP-dependent protein kinase. It should also be considered that
Cytosolic ADP-ribosyltransferase Stimulation

Pertussis toxin, cholera toxin, diphtheria toxin, and botulinum toxin are known to have intrinsic ADP-ribosyltransferase activity that can ADP-ribosylate specific cellular proteins. This protein modification has been associated with the changes of specific biochemical reactions and physiological responses (36, 37). Endogenous ADP-ribosyltransferase has not as yet been associated with specific cellular changes. It is now imperative to study the possible influences of physiological agonists on this activity and the functional consequences related to this ADP-ribosylation.

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FIG. 6. Endogenous ADP-ribosylation induced by the cytosolic fractions of different tissues. A. the cytosolic fraction of human platelets (Plat.) and various tissues from rat such as brain, liver (Liv.), intestine (Int.), heart, and lung were prepared. ADP-ribosylations were carried with 35 μg of protein in all cases in the absence and presence of 100 μM sodium nitroprusside. B, effect of 500 μM sodium nitroprusside (SNP), 500 μM hydroxylamine (NH₂OH), 500 μM 8-bromo-cyclic GMP (8-Br-cGMP), and 500 μM cyclic GMP (cGMP) on the ADP-ribosylation caused by the cytosolic fractions (35 μg of protein) of brain and heart.

nitric oxide release by sodium nitroprusside and 3-morpholinosydnonimine also affects the ADP-ribosylation of a cellular protein and that this covalent modification might explain, at least in part, the physiological action of nitric oxide. Prostacyclin, which increases cellular cyclic AMP levels and consequent protein phosphorylation, also causes vasodilation and inhibition of platelet responses. Contrary to sodium nitroprusside and 3-morpholinosydnonimine, prostacyclin does not activate the ADP-ribosylation of the 39-kDa protein (not shown). Therefore, the specific contributions of the ADP-ribosylation and phosphorylation of proteins to physiological responses induced by nitric oxide remain to be elucidated.