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Biochemistry

Glyceraldehyde-3-phosphate dehydrogenase is required for the transport of nitric oxide in platelets

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ABSTRACT **Nitric oxide (NO) or NO-generating compounds like sodium nitroprusside (SNP) increase cellular levels of cGMP and produce S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase [GAPDH; D-glyceraldehyde-3-phosphate:NAD$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12]. In search of a reagent that could discriminate between these two effects, we used the sesquiterpene antibiotic koningic acid, which binds to GAPDH at the Cys-149 of the active site. Koningic acid inhibited basal and sodium nitroprusside-stimulated NAD-dependent covalent modification of purified rabbit muscle GAPDH in a dose-dependent manner. Furthermore, we tested the effect of koningic acid on human platelets. Approximately 90% of GAPDH is present in the cytosol of human platelets, and the exposure of platelet cytosol to koningic acid inhibited GAPDH activity, while the soluble guanylyl cyclase (basal and sodium nitroprusside-stimulated) activity remained unaltered. Pretreatment of intact platelets with koningic acid slowed the rate of aggregation induced by a submaximal concentration of thrombin. In addition, the antibiotic also inhibited the cGMP increases triggered by SNP, *S*-nitroso-*N*-acetylpenicillamine (SNAP), and 3-morpholinosydnomidine (SIN-1) but failed to prevent an increase in cGMP caused by nitrosylated albumin. Under the same conditions, koningic acid also inhibited basal and SNP- SNAP-, and SIN-1-stimulated NAD-dependent modification of GAPDH and its enzymatic activity. These results suggest that the mechanism of delivery of NO from SNP, SNAP, and SIN-1 to platelets may require the active form of GAPDH. When NO is delivered by nitrosylated albumin, active GAPDH was not necessary.**

Glyceraldehyde-3-phosphate dehydrogenase [GAPDH; D-glyceraldehyde-3-phosphate:NAD$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12] reversibly catalyzes the oxidation and phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate. Thus, it is a key enzyme in the glycolytic conversion of glucose to pyruvic acid and represents an important pathway for carbohydrate metabolism in most organisms. The binding of NAD$^+$ to GAPDH has been extensively studied (1), and ADP-ribose bound to the enzyme has been found as a result of GAPDH degradation of NAD$^+$ (2). Sodium nitroprusside (SNP), which generates nitric oxide (NO), has been shown to form nitrosylated heme (3, 4), which stimulates guanylyl cyclase (5, 6). It also increased the cytosolic ADP-ribosyltransferase activity of different tissues (7, 8).

Brüne and Lapetina have previously shown that NO produced a covalent modification of a 39-kDa protein (7) that was subsequently identified as GAPDH (9–11). The covalent modification was defined as a mono-ADP-ribosylation because treatment of the modified GAPDH with snake venom phosphodiesterase produced formation of radiolabeled 5′ AMP (7, 9) and because a preparation of human erytrocyte GAPDH failed to incorporate labeling from [*nicotinamide*-^{14}C]NAD (9). Furthermore, NO-induced S-nitrosylation of GAPDH inhibited its enzymatic activity. S-nitrosylation also seemed to cause the increased labeling of radioactive preparations of GAPDH loaded with [^{32}P]NAD but with a rather low stoichiometry (0.1%) (12). However, it has been shown recently that the NO-stimulated modification of rabbit muscle GAPDH with NAD might not be ADP-ribosylation but rather a covalent binding of NAD through a NO-dependent thiol intermediate (13). This observation was supported by equal incorporation of radiolabel from the adenine, phosphate, and nicotinamide moieties to the extent of ≈0.02 mol of NAD per mol of GAPDH (13).

It has been assumed that the target for covalent modification is a cysteine residue of GAPDH, since the ADP-ribose–cysteine bond is sensitive to mercuric ions (9, 13, 14). In addition, it was suggested that the sulfhydryl group at Cys-149 functioned as the reactive nucleophile involved in GAPDH catalysis (15). We (12) have proposed that Cys-149 in the active site of the enzyme is the putative site of S-nitrosylation. Therefore, while the nature of the moiety covalently attached to GAPDH remains controversial, it probably is safe to say that S-nitrosylation is the cause of the inactivation of GAPDH (12, 13). This reaction is followed by oxidation, ADP-ribosylation, or NAD binding (12, 13).

To learn more about the role of Cys-149 present in GAPDH, we used koningic acid, a sesquiterpene antibiotic that has been shown to react specifically with residue Cys-149 of rabbit muscle GAPDH (16). In the presence of NAD$^+$, koningic acid irreversibly inactivated GAPDH in a time-dependent manner when a maximum of two sulfhydryl groups were modified (17). In this study, we present evidence that koningic acid inhibits the NO-induced covalent modification of GAPDH. In addition, we show that in human platelets pretreated with koningic acid, cytosolic GAPDH activity was inhibited. We also show that the ability of SNP, 3-morpholinosydnomidine (SIN-1), and *S*-nitroso-*N*-acetylpenicillamine (SNAP) to induce cGMP increases and stimulation of covalent modification of GAPDH was inhibited. That was not the case when nitrosylated albumin was used as the NO donor.

MATERIALS AND METHODS

SDS/PAGE Analysis of Modified GAPDH. Reactions were performed in a final volume of 60 μl containing 10 mM thymidine, 1 mM ATP, 2 mM dithiothreitol, 0.1 mM GTP, 138 mM NaCl, 0.36 mM NaH_2PO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 5 mM Hepes, 1 μM NAD$^+$, 3 mM $MgCl_2$, 150 mM potassium phosphate (pH 7.2), 1.5 μCi of nicotinamide [*adenylate*-^{32}P]adenine dinucleotide di(triethylammonium) salt ([^{32}P]NAD; DuPont/New England Nuclear; 1 μCi = 37 kBq) per assay, and 50 μg of platelet cytosol or 2 μg of

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SIN-1, 3-morpholinosydnomidine.

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purified GAPDH from rabbit muscle. After a 2-hr incubation at 37°C, samples were precipitated with 1 ml of cold 10% (wt/vol) trichloroacetic acid and centrifuged for 10 minutes at 2200 × *g*. The pellets were washed twice with 2 ml of cold water saturated with diethyl ether and resuspended in SDS sample buffer and resolved on SDS/12% polyacrylamide gels (NOVEX, San Diego). Gels were subjected to autoradiography, and the corresponding 37-kDa bands were excised from the gel and assayed for Cerenkof radiation in a liquid scintillation counter.

Isolation of Human Platelets. Human blood was obtained from healthy volunteers, mixed with 10% (vol/vol) ACD anticoagulant (85 mM trisodium citrate/111 mM dextrose/71 mM citric acid), and centrifuged at 200 × *g* for 25 min to yield platelet-rich plasma (PRP). The PRP was incubated for 15 min with 1 mM aspirin (this drug was not present in platelet preparations used for platelet aggregation assays) and 5 mM EDTA, and platelets were obtained by centrifuging the PRP at 900 × *g* for 15 min. The cells were resuspended and washed once by centrifugation at 900 × *g* for 15 min in 40 ml of Hepes-buffered Tyrode's solution (138 mM NaCl/0.36 mM NaH_2PO_4/2.9 mM KCl/12 mM $NaHCO_3$) containing 5 mM EDTA.

Distribution of GAPDH in Human Platelets. Isolated platelets were disrupted by resuspending the washed platelets in 5 mM Hepes (pH 7.4) containing 5 mM EDTA, leupeptin at 10 μg/ml, and phenylmethylsulfonyl fluoride (PMSF) at 100 μg/ml. The suspension was subjected to two cycles of freezing in liquid nitrogen and thawing at room temperature followed by centrifugation at 170,000 × *g* for 10 min to render particulate and cytosolic fractions. The particulate fraction was washed once with hypotonic buffer and centrifuged at 170,000 × *g* for 10 min. The supernatant was added to the cytosolic fraction. Fractions of platelet homogenate and cytosol were resolved in 10–20% SDS/PAGE gradient minigels (ISS Emprotech, Natick, MA) and electrophoretically transferred to nitrocellulose sheets. The amount of GAPDH present in the fractions was determined as described (12).

Effects of Koningic Acid on Guanylyl Cyclase and GAPDH Activity From Platelet Cytosol. Platelet cytosol was prepared as indicated above and used within 30 min. Cytosol was divided into two 1-ml fractions and incubated with and without 10 μM koningic acid for 20 min. Koningic acid was a gift from Akira Endo of the Department of Applied Biological Sciences, Tokyo Noko University (Tokyo). Each cytosolic sample was loaded on a Sephadex PD10 G-25 (Pharmacia LKB) sizing column preequilibrated with 20 mM Tris·HCl, pH 7.5/100 mM NaCl/100 μM phenylmethylsulfonyl fluoride to separate proteins from free koningic acid. The void volume of each column was collected, and aliquots were used for the determination of guanylyl cyclase and GAPDH activities.

Incubations for cGMP formation were initiated by addition of 10 or 20 μg of platelet cytosol to prewarmed (37°C) buffer to give final concentrations of 25 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM GTP, and 1 mM 3-isobutyl-1-methylxanthine (pH 7.2). After 20 min of incubation, the reaction was terminated by addition of 50 μl of 2.5 M $HClO_4$; the samples were then neutralized with 90 μl of 1.08 M K_3PO_4, and after centrifugation at 2200 × *g* for 10 min, the cGMP in the mixture was determined by radioimmunoassay (18) with a kit from Amersham.

GAPDH activity was measured as described (12).

Synthesis of *S*-Nitroso-Albumin. It was carried out following a previously published procedure (19) slightly modified (12).

Effects of Koningic Acid on Platelets. For platelet aggregation studies, washed platelets from 100 ml of blood were resuspended in 10 ml of Tyrode's buffer containing 10 mM glucose. Aliquots of 500 μl were incubated for different times with koningic acid, and platelet aggregation was triggered with a submaximal concentration of thrombin (2 nM). Increases in light transmittance were measured with an aggregometer (Chrono-Log, Havertown, PA).

To study the effects of koningic acid on cGMP levels, washed platelets from 150 ml of blood were resuspended in 2 ml of Tyrode's solution containing 5 mM EDTA (buffer B), divided in 150-μl aliquots, and incubated with different concentrations of koningic acid for 15 min at 37°C. The platelets were washed twice by centrifugation in buffer B and resuspended in 200 μl of buffer B containing 1 mM 3-isobutyl-1-methylxanthine (buffer C). Each aliquot was further incubated for 20 min in the presence or absence of 200 μM SNP with or without dithiothreitol, SIN-1, SNAP, albumin, or nitrosylated albumin; in the last two cases, the excess of albumin or nitroso-albumin at the end of the incubation was removed by centrifugation and two washes with buffer C. All samples were extracted twice with cold ethanol at a final concentration of 65%. The ethanol-precipitated samples were centrifuged at 2000 × *g* for 10 min, and the resulting supernatants were dried under a stream of nitrogen and resuspended in assay buffer (50 mM sodium acetate, pH 5.8) prior to radioimmunoassay for the determination of cGMP as detailed above.

A similar protocol was followed to study the effect of koningic acid on GAPDH activity and on the NO-induced covalent modification of the enzyme. After preincubation with koningic acid and washing, each aliquot of platelets was resuspended in hypotonic buffer containing 20 mM Tris·HCl and 0.25 mM NaCl and was subjected to the procedure described above for the preparation of platelet cytosol. Aliquots were used to study the NAD linkage and GAPDH activity as described above.

RESULTS

Inhibition of [^{32}P]NAD^+ Labeling of GAPDH. To analyze whether Cys-149 was one of the sites of the covalent modification on GAPDH, we used koningic acid, which is known to interact with this residue (20). The effect of koningic acid was measured on the incorporation of radioactivity from [^{32}P]NAD^+ into purified rabbit muscle GAPDH in the presence or absence of the NO generator SNP. Fig. 1 shows that increasing concentrations of koningic acid inhibited both the basal and the NO-induced covalent modification of GAPDH at concentrations of 10 μM and 100 μM, respectively. These results suggested that Cys-149 on GAPDH is one of the sites of covalent modification.

Effects of Koningic Acid on Guanylyl Cyclase. To learn if GAPDH and guamylyl cyclase were present in the same cellular compartment, we studied first the distribution of GAPDH in platelets and second the effects of koningic acid on the activity of the platelet cytosolic guanylyl cyclase and GAPDH.

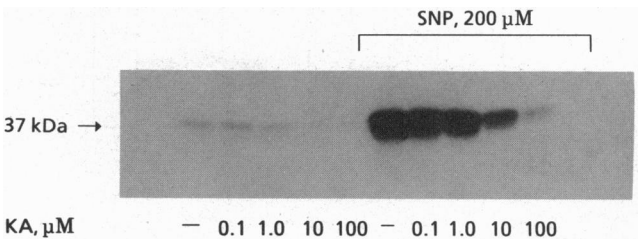

FIG. 1. Koningic acid inhibits [^{32}P]NAD labeling of GAPDH. Rabbit muscle GAPDH was preincubated for 15 min with or without different concentrations of koningic acid (KA), and the samples were labeled with radioactive [^{32}P]NAD in the presence or absence of SNP. The arrow denotes the 37-kDa subunit of GAPDH.

Table 1. Distribution of GAPDH in human platelets

Fraction	GAPDH	
	% total protein	% distribution
Homogenate	7	100
Cytosol	11	92
Particulate	4	8

The results show (Table 1) that GAPDH is predominantly present in the cytosol, where 92% of the enzyme is found. It is also quite abundant in platelets where GAPDH represents 11% of the cytosolic protein. For these reasons, and also because the form of guanylyl cyclase that can be stimulated by SNP is cytosolic, the differential effects of koningic acid on GAPDH and guanylyl cyclase were studied in the cytosolic fraction.

Platelet cytosol was preincubated with 10 μM koningic acid as described in *Materials and Methods*. The results of the experiment (Fig. 2) show that 10 μM koningic acid did not change the basal or SNP-stimulated activity of guanylyl cyclase. The sample pretreated with the antibiotic lacked detectable GAPDH activity, while the control sample showed an activity of 2.35 units/mg. To rule out any direct effect of free koningic acid on the decomposition of SNP, we determined that the cytosolic samples used for measurements of guanylyl cyclase and GAPDH activities were free of koningic acid by performing the following experiment. A known amount of rabbit muscle GAPDH was added to platelet cytosol previously treated with koningic acid and further purified by molecular-sieving. The activity of the spiked GAPDH was fully recovered, which strongly suggested that the cytosol was free of koningic acid (not shown).

Actions of Koningic Acid in Platelets. To study the effects of koningic acid on platelet function, we first investigated its action in the absence of NO. Fig. 3*A* shows a typical profile of platelet aggregation when thrombin is used at submaximal concentrations. Preincubation of the cells with koningic acid produced a change in the velocity of platelet aggregation (not in the aggregation itself) triggered with thrombin (Fig. 3*B*) and with collagen and ADP (not shown). This effect was not different when the time of preincubation with koningic acid was 15 min (not shown). No effect on the velocity of platelet aggregation was observed when thrombin, collagen, and ADP were used above threshold concentrations (not shown). Pretreatment of platelets with SNP caused a similar effect on the rate of platelet aggregation triggered with the same concentration of thrombin (Fig. 3*C*). When koningic acid was added to the platelet preparation prior to SNP, thrombin was unable to trigger platelet aggregation (Fig. 3*D*). It was not possible then to describe an inhibitory action of koningic acid on the effects of NO at the level of platelet aggregation.

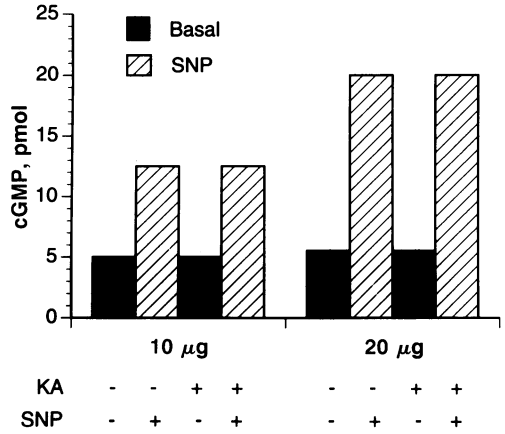

FIG. 2. Effect of koningic acid (KA) on the soluble guanylyl cyclase in platelet cytosol. Platelet cytosolic fractions were preincubated with or without 10 μM koningic acid and loaded on sizing columns. Guanylyl cyclase activity present in the void volume was measured in the presence or absence of SNP.

To characterize the biochemical effects of koningic acid in platelets, intact washed human platelets were incubated with different concentrations of koningic acid for 15 min followed by stimulation of guanylyl cyclase with the NO-generating compound SNP. The cytosolic fractions of these platelets were obtained, and the levels of cGMP were determined. The results in Fig. 4*A* show that the stimulatory effects of SNP on platelet guanylyl cyclase were inhibited in a dose-dependent manner by the antibiotic. We could not attribute this effect of koningic acid to direct inhibition of guanylyl cyclase, since we have previously demonstrated in the experiment described in Fig. 2 that koningic acid does not inhibit guanylyl cyclase. To study whether the effects of koningic acid were related to inhibition of the reductive activation of SNP by thiols (21), we performed the same experiment in the presence of physiological concentrations of reducing equivalents (2 mM dithiothreitol).

The results in Fig. 4*B* show that the presence of thiol did not change the inhibitory effects of koningic acid on SNP-stimulated production of cGMP. In addition, we assayed in parallel the effects of SNP, SNAP (which generates NO catalyzed by membrane components of the cell) (22), and SIN-1 (which rapidly releases NO without the need of any cofactor such as thiol) (23). The ability of these NO generators to induce increases in cGMP levels was again inhibited

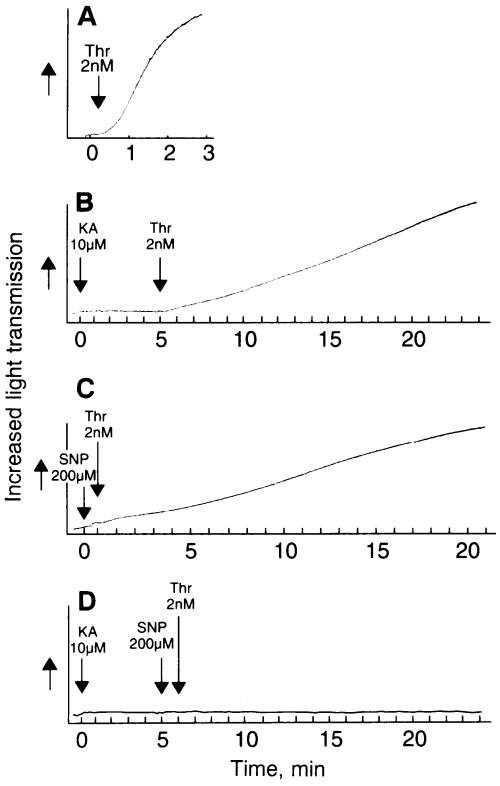

FIG. 3. Action of koningic acid (KA) on platelet function. (*A*) Platelet response to a submaximal concentration of thrombin (Thr). Washed human platelets were stimulated with thrombin, and the aggregation was measured as increased light transmission. (*B*) Effect of koningic acid on platelet aggregation. Platelets were preincubated for the time indicated in the figure with koningic acid and were stimulated with thrombin. (*C*) Effect of SNP on platelet aggregation. SNP was added to the cells followed by a submaximal concentration of thrombin. (*D*) Action of koningic acid and SNP on platelet aggregation. Platelets were preincubated with koningic acid and SNP followed by thrombin.

in a dose-dependent manner in platelets pretreated with koningic acid (Fig. 4*C*).

Surprisingly, under similar conditions, when nitrosylated albumin was the donor of NO, the elevation in cGMP levels was not affected by koningic acid (Fig. 4*D*).

At this point, it was important to assess the effect of koningic acid on the basal and NO-induced incorporation of radioactivity from [^{32}P]NAD to platelet GAPDH using the same experimental design. As Fig. 5*A* shows, pretreatment with 10 μM koningic acid totally abolished the incorporation of radioactivity to GAPDH from platelets treated and not treated with SNP and also with SNAP and SIN-1. We also tried to stimulate the NAD-dependent modification of GAPDH with nitrosylated albumin, but our efforts were unsuccessful (not shown), which indicated that only the heme present in guanylyl cyclase is the target of the NO originated in nitrosylated albumin under these experimental conditions.

In a parallel experiment, platelets were incubated with koningic acid, and the enzymatic activity of cytosolic GAPDH was determined (Fig. 5*B*). This activity was also inhibited by 10 μM koningic acid. Therefore, koningic acid penetrates intact platelets and has an inhibitory effect on GAPDH activity and on the incorporation of radioactivity from [^{32}P]NAD to GAPDH.

DISCUSSION

Our results have shown that the sesquiterpene antibiotic koningic acid inhibited the NO-induced covalent modification of purified rabbit muscle GAPDH and also of cytosolic GAPDH from platelets. It has been demonstrated (20) that koningic acid is a potent and specific inhibitor of GAPDH activity and consequently of ATP generation in the glycolytic pathway. Its ability to interact with Cys-149 in the active site of GAPDH (16, 17) was used to assess the site of NO-induced covalent modification of GAPDH. The inhibition of the covalent modification of GAPDH may be explained in the following way: the ADP-ribose or nicotinamide moieties present in NAD could react with the same free sulfhydryl group of Cys-149 that reacts with koningic acid (16). Therefore, it could be assumed that koningic acid inhibited the covalent attachment of NAD or ADP-ribose to GAPDH by formation of a thioether linkage with Cys-149.

Since treatment of platelet cytosol with the antibiotic inhibited GAPDH activity while the catalytic activity of guanylyl cyclase (basal and SNP stimulated) was not affected, we thought that inhibitors like koningic acid could discriminate between the effects of NO generation on guanylyl cyclase and on S-nitrosylation and further covalent modification of GAPDH. The importance of those reactions is not yet known, but they seem to occur at the cellular level where NO production inhibits GAPDH activity and stimulates S-nitrosylation (12) and endogenous ADP-ribosylation of the enzyme (24). To study if koningic acid would block the NO-induced inhibition of platelet aggregation, we first tried to determine the effect of the antibiotic on platelet function. Koningic acid induced a decrease in the velocity of platelet aggregation triggered with thrombin. The same kind of response was obtained with SNP, and when added in the same preparation, both reagents completely inhibited platelet aggregation. We could not then study the actions of koningic acid against SNP effects mediated by NO in platelet aggregation assays.

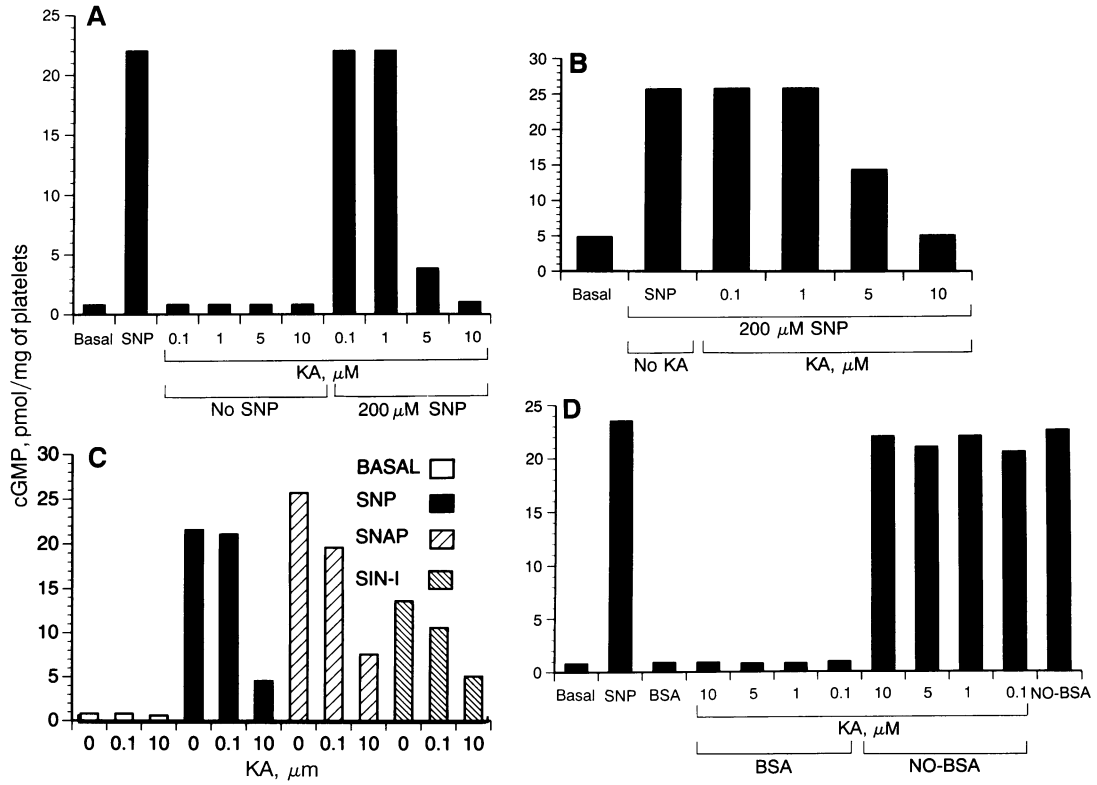

FIG. 4. Effects of koningic acid in platelet cGMP levels. (*A*) Effect on cGMP levels in platelets stimulated with 200 μM SNP. Platelets treated with various concentrations of koningic acid (KA) for 15 min (lanes 3–10) were further stimulated with 200 μM SNP (lanes 7–10) and cGMP levels present in cytosolic fractions were determined as described in text. (*B*) The same experimental design was followed, but this time dithiothreitol was present at 2 mM during the incubations. (*C*) Effect of koningic acid on the action of 200 μM SNP, SNAP, and SIN-1. Platelets were preincubated in the absence or presence of different concentrations of koningic acid for 30 min. The cells were washed and further incubated with the NO donors for 15 min, and levels of cGMP were determined. (*D*) Effect on cGMP levels in platelets exposed to nitrosylated albumin. Koningic acid-treated platelets were incubated with 30 μM albumin or nitrosylated albumin, and cGMP levels were measured in the cytosolic fractions.

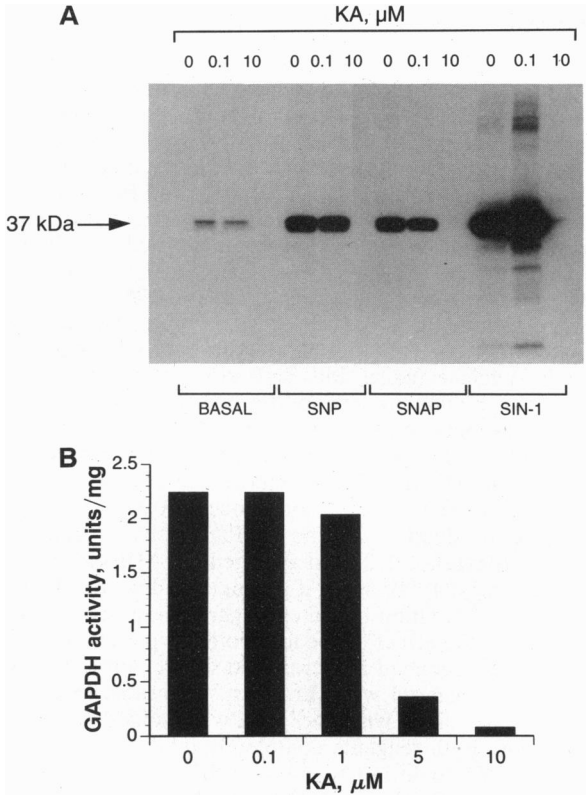

FIG. 5. Inhibition of [^{32}P]NAD labeling of platelet cytosolic GAPDH and GAPDH activity after preincubation of platelets with koningic acid (KA). (*A*) Cytosol was prepared from platelets preincubated with different concentrations of koningic acid, and [P^{32}]NAD labeling were performed in the absence (BASAL) or presence of 200 μM SNP, SNAP, or SIN-1. (*B*) Inhibition of platelet cytosolic GAPDH activity. Platelets were washed and lysed to obtain a cytosolic fraction, and cytosolic GAPDH activity was measured.

However, it was most intriguing that pretreatment of platelets with koningic acid only inhibited the cGMP increases induced with SNP, SNAP, and SIN-1 and not with S-nitroso-albumin. It has been assumed that NO freely diffuses in aqueous media and across cell membranes (25), but its phospholipid membrane permeability has not been reported. Our results are probably related to the existence of alternative mechanisms of transport and delivery of NO that go beyond spontaneous release and diffusion. Along these lines, the effects of koningic acid in platelet cytosol and in whole platelets are very interesting. Thiol compounds reduce nitroprusside to the corresponding metal-nitroxyl radical apparently via a thiol adduct that yields S-nitrosothiol derivatives (21), and we previously have observed S-nitrosylation of GAPDH *in vitro* (12). In addition, NO was produced from SNP in the presence of vascular tissue, plasma, and proteins but was not released spontaneously from the nitroprusside anion (26). In platelet cytosol, many proteins and thiol-containing compounds could provide the necessary environment for SNP decomposition independently of the thiol groups present in GAPDH. In fact, the results in Fig. 2 showed that after pretreatment of cytosol with koningic acid, the NO generated from SNP stimulated guanylyl cyclase.

In whole platelets we thought that koningic acid, by blocking the reactive thiol group of Cys-149 of GAPDH (in particular the ones present in the plasma membrane), impaired the decomposition of SNP and SNAP, but this hypothesis is not consistent with the fact that similar results are obtained in the presence of dithiothreitol (Fig. 4*B*). The effect of koningic acid on platelets treated with SIN-1, which seem to release NO spontaneously (23), is an additional argument against this hypothesis (Fig. 4*C*).

Interestingly, nitrosylated albumin delivered NO to intact platelets and increased cGMP levels by a mechanism that was not affected by koningic acid.

In conclusion, different mechanisms of NO transport are probably involved in the actions of SNP, SNAP, SIN-1, and nitrosylated albumin. The effects of koningic acid suggest that the free thiol of Cys-149 may be an essential component or intermediary of pathways of transport of NO from SNP, SNAP, and SIN-1 and not from nitrosylated ablumin. Alternatively high levels of ATP may be required for the transport of NO from certain NO generators, and koningic acid by impairing energy production could affect the mechanism of transport of NO. Finally, Cys-149 is most probably the site of S-nitrosylation and further covalent binding of NAD or ADP-ribose to GAPDH.

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