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Inhibition of Platelet Aggregation by Carbon Monoxide is Mediated by Activation of Guanylate Cyclase

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SUMMARY

Carbon monoxide (CO) inhibits human platelet aggregation triggered with threshold levels of agonists like arachidonate, ADP, collagen, thrombin, or the prostaglandin endoperoxide analogue U46619. This inhibition is counteracted by illumination with light above 400 nm indicating the involvement of a ferrous hemoprotein. An earlier suggestion that the mechanism of CO inhibition involves the cytochrome P450 protein thromboxane A_2 synthase was ruled out as well as the involvement of the iron containing enzymes like cyclooxygenase or 12-lipoxygenase. In the presence of CO, no arachidonate was released from phospholipids, no increase of intracellular calcium levels was observed, and phospholipase C was not activated suggesting that the transducing mechanisms from the receptors to phospholipase C was effected in the presence of CO. cAMP levels were also unchanged but cGMP levels showed an increase of about 30%. By comparison with the guanylate cyclase stimulator nitroprusside, it was shown that such levels could block aggregation. In a 10,000 × *g* supernatant, CO enhanced guanylate cyclase activity 4-fold, supporting the view that CO acts by increasing platelet cGMP levels. With respect to the mechanism of guanylate cyclase action, the binding of CO to the regulatory subunit of guanylate cyclase must be responsible for the observed activation. It is concluded that cGMP is an important feedback regulator of the PI response and that already a 25% increase in its steady state levels can cause inhibition of platelet aggregation.

Platelet aggregation is triggered by a variety of extracellular signals such as ADP, collagen, thrombin, or the synthetic prostaglandin endoperoxide analogue U46619. These agonists interact with a specific receptor which is coupled to a signal amplifying cascade known as the "PI response" (1–4). Interactions with platelet receptors result in activation of phospholipase C, which by hydrolysis of phosphatidylinositol 4,5-bisphosphate, leads to the formation of 1,2 diacylglycerol and IP_3 in a bifurcating pathway. IP_3 releases $[Ca^{2+}]$ from intracellular stores (5–7) and thus causes the physiological response of platelet aggregation. This so-called first wave of aggregation is followed by a second wave initiated by TxA_2. This eicosanoid derivative is formed in a chain of events derived from the increase of intracellular $[Ca^{2+}]$ which activates phospholipase A_2 to liberate arachidonic acid. Free arachidonate is converted to the prostaglandin endoperoxides PGG_2 and PGH_2 and further transformed by TxA_2 synthase (EC 5.3.99.5) to TxA_2 (8, 9). It had been considered that TxA_2 is one of the most active agonists of platelet aggregation also responsible for the second wave of aggregation. Parallel to the onset of aggregation release of secretory granule occurs, a process which completes the aggregatory process (10, 11).

According to this complex series of events, an inhibition of aggregation can be mediated by a large and diverse group of compounds interfering at different steps of the activation cascade.

Among platelet aggregation inhibitors, one also finds CO (12, 13), which in a study by Cinti and Feinstein (14) was suggested to act on a cytochrome P450 protein present in platelet microsomes. We have recently isolated this cytochrome P450 and identified it as thromboxane synthase (15). Studying its mechanism of action, it became apparent that the isomerization of PGH_2 to TxA_2 by this enzyme is catalyzed in the ferric state of the hemoprotein and that, unlike cytochrome P450-dependent monooxygenases, thromboxane synthase never requires reduction to the ferrous state under physiological conditions. Since CO inhibition can only involve a ferrous hemoprotein, we have reinvestigated the phenomenon of inhibition of platelet aggregation by CO.

In this study we have measured the various biochemical parameters accessible during the aggregation process and conclude on a different and novel mechanism of action of CO.

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ABBREVIATIONS: IP_3, inositol-trisphosphate; PrP, platelet-rich plasma; CO, carbon monoxide; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2; HHT,12(L)-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12(*S*)-hydroxy-5,8,10,14-eicosatrienoic acid; $[Ca^{2+}]_i$, intracellular calcium; EDRF, endothelium-derived relaxing factor; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; IBMX, 3-isobutyl-1-methylxanthine. Hepes, *N*-2-hydroxethylpiperazine-*N*′-2-ethansulfonic acid; DTT; dithiothreitol; TCA, trichloroacetic acid.

Materials and Methods

Materials. Apyrase (grade I, from potato), heparin, Hepes, imipramine, IBMX, phosphatidic acid, TxB_2, aspirin, epinephrine, EGTA, DTT, and glutathione were all obtained from Sigma (Deisenhofen, W. Germany) ADP was purchased from Boehringer Mannheim (Mannheim, W. Germany), thrombin from Hoffman-La Roche (Basel, Switzerland), UK-37248 from Pfizer (Sandwich, U.K.), and the prostaglandin endoperoxide analogue U46619 (15S-hydroxy-11,9-[epoxymethano]prosta-5Z, 13E-dienoic acid) from Paesel (Frankfurt a.M., W. Germany). Radiochemicals were bought from Du Pont (Dreieich, W. Germany). All other chemicals were delivered by E. Merck (Darmstadt, W. Germany), Aldrich (Steinheim, W. Germany), or Riedl-de Haén (Hannover, W. Germany) in p.a. quality or in the highest quality available. Prostacylin was a gift from Schering (Berlin, W. Germany), the cGMP antibody was kindly provided by Prof. Dr. Hertting (Freiburg, W. Germany), the protein kinase as well as the thromboxane antibody was a gift from Prof. Dr. Schrör (Düsseldorf, W. Germany), and 8-octylamino-cGMP was provided by Prof. Dr. Wurster (Konstanz, W. Germany).

Preparation of PrP and washed platelets. Blood (200 ml) was obtained from healthy volunteers not having taken any drug known to interfere with platelet responses in the previous 2 weeks. Blood coagulation was prevented with 10% (v/v) 3.8% trisodiumcitrate, and PrP was obtained by centrifugation at 200 × *g* for 20 min using a Heraeus-Christ Digifuge GL (Heraeus-Christ, Osterode, W. Germany). PrP (50–90 ml) was either used directly for aggregation studies or for the preparation of washed human platelets. In order to prepare washed platelets the pH of the PrP was adjusted to pH 6.5 by using acidic citrate-dextrose (Biotest, Frankfurt, W. Germany). Isolation of platelets was performed at room temperature, using only polypropylene material throughout the preparation. Prostacyclin (300 ng/ml) was added and platelets were centrifuged for 10 min at 800 × *g*, and resuspended in an equal amount of washing buffer (113 mM NaCl, 4 mM $Na_2HPO \times H_2O$), 4 mM KCl, pH 6.5) containing 1 mg/ml glucose, potato apyrase (200 μg/ml), heparin (25 units/ml), and prostacylin (300 ng/ml). After centrifugation for a second time (10 min; 800 × *g*), platelets were resuspended in the desired volume of Tyrode-Hepes buffer (134 mM NaCl, 12 mM $NaHCO_3$, 29 mM KCl, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM Hepes, 5 mM glucose, pH 7.4) containing potato apyrase and sometimes 5% platelet-poor plasma (as indicated). Before starting the aggregation assays, platelets were kept for 15 min in a water bath adjusted to 37°. Experiments were carried out between 20 and 80 min after final resuspension.

Labeling of platelets with [1−^{14}C]arachidonic acid. PrP was labeled with [1−^{14}C]arachidonic acid (52 mCi/mmol; 0.3 μCi/ml) for 70 min at 37°. The platelets were separated from plasma after addition of prostacyclin (300 ng/ml).

Platelet aggregation. Aggregation studies were carried out in 0.25 ml or 0.5 ml using a two-channel Elvi 840 aggregometer from Logos (Logos, Milano, Italy). Zero transmission was set for PrP or washed platelets and 100% transmission was adjusted with platelet-poor plasma or Tyrode-Hepes buffer containing 5% platelet-poor plasma or omitted, respectively. Samples were placed in aggregometer tubes and incubated under stirring (1000 rpm) for 2 min at 37° with or without inhibitors, and then exposed to the different agonists for the times given. Shape change and aggregation were recorded (16). In the case of CO, PrP, or suspensions of washed platelets were bubbled for 15 to 30 s with the pure gas or with gas mixtures.

Platelet lipid metabolism. Incubations (0.5 ml) were stopped by addition of 0.5 ml of methanol and transferred into 1.4 ml of chloroform/methanol 1:2 (v/v). Samples were partitioned after the addition of 0.6 ml of chloroform and 0.6 ml of formic acid in order to reach pH 3. The organic phases were evaporated under a stream of nitrogen. Residues, dissolved in 50 μl of chloroform, were spotted on silica gel 60 plates (E. Merck, Darmstadt, W. Germany) in order to separate phosphatidic acid, TxB_2, HHT, and 12-HETE.

The plates were developed as described (2), using the upper phase of ethylacetate/2,2,4-trimethylpentane/acetic acid/H_2O (90:50:20:100, v/v). Substances were either localized by cochromatography with unlabeled standards (visualized by iodine vapor) or by autoradiography. Radioactive spots, corresponding to the compounds mentioned, were scraped off, transferred into scintillation vials, and ^{14}C-radioactivity was measured by scintillation counting using a Philips Liquid Scintillation Counter PW 4700 from Isomess (Straubenhardt, W. Germany). The same set-up was used in order to measure the metabolism of exogenous arachidonic acid (Larodan, Malmö, Sweden) diluted with [1−^{14}C]arachidonic acid ($1.7 \cdot 10^6$–$4.5 \cdot 10^6$ dpm/assay) using washed platelets ($3–5 \cdot 10^5$ platelets/μl).

Measurement of [^{3}H]serotonin release. Platelets labeled with [^{3}H]serotonin (1 μCi/ml PrP, 37°, 45 min, 26.2 Ci/mmol) received imipramine (0.5 μmol/l) at the end of incubation. Measurement and calculation were mainly performed according to Holmsen and Setkowsky-Dangelmaier (17).

Measurement of TxB_2 formation in PrP and washed platelets using a TxB_2 radioimmunoassay. At the end of each test period, 60 μmol/l of indomethacin was added to the sample and platelets were sedimented by centrifugation at 10,000 × *g* for 3 min. Measurement of TxB_2 was done according to the experimental protocol of Smith *et al.* (18).

Determination of cyclic AMP. The incubation (0.5 ml) was terminated by addition of 15% TCA (3:1, v/v). Cell debris were removed by centrifugation at 10,000 × *g* for 5 min and the TCA in the supernatant by repeated washing with water-saturated diethyl ether. The supernatant was then lyophilized, redissolved in sodium acetate buffer, and the cyclic AMP content was determined according to Gilman (19).

Determination of cyclic GMP. PrP (0.5 ml) was incubated with 0.1 mmol/l of IBMX for 2 min before adding any other reagent. Incubations were stopped after another 2 min by adding 1 ml of 6% TCA. After centrifugation at 10,000 × *g* for 3 min, 100 μl of ether-extracted TCA extract was assayed as described by Ortmann (20) without further purification.

Measurement of cytosolic free calcium. The development of the fluorescent Ca^{2+} indicator dye Fura-2 by Tsien and colleagues (21) allowed $[Ca^{2+}]_i$ measurements in intact platelets. PrP was centrifuged as described. The resulting pellet was resuspended in 0.5 volume of platelet-poor plasma. Fura-2-acetoxymethyl (Calbiochem, Frankfurt a.M., W. Germany) dissolved in dimethylsulfoxide was incubated with the concentrated PrP at a final concentration of 3 μmol/liter at 37° for 30 min. Afterwards, platelets were collected by centrifugation at 800 × *g* for 10 min after the addition of prostacyclin (300 ng/ml) and gently resuspended in Tyrode buffer. Samples (1.2 ml) were placed into a Sigma ZWS-II spectrophotometer (Biochem, Puchheim, W. Germany).

External Ca^{2+} was adjusted to 1 mmol/liter with $CaCl_2$. The fluorescence above 450 nm was monitored under constant stirring. $[Ca^{2+}]_i$ was calculated as described for Fura-2 (21) using the Ca^{2+}-dye dissociation constant of 224 nM. Excitation was at 335 and 362 nm, respectively.

Photochemical experiments. In order to test the photoreversibility of CO inhibition during exposure to light, 5 ml of PrP were gassed under continuous shaking in a 100-ml siliconized round bottom flask with 800 ml of a gas mixture (30% CO/60% O_2/10% N_2) in 7 to 10 min. Platelets were placed in the cuvets and reversibility of the CO effect was ascertained by exposing the platelets to light from a slide projector (250 W quartz lamp) positioned as close as possible to the platelet surface. In most cases the whole region of the visible light was used or sometimes an interference filter of 450 nm was employed.

Determination of guanylate cyclase activity. Guanylate cyclase (EC 4.6.1.2.) activity was determined in a total volume of 0.2 ml with 30 mM triethanolamine/HCl buffer, pH 7.4, containing 3 mM $MnCl_2$, 0.1 mM EGTA, 1 mM IBMX, 3 mM glutathione, 5 mM creatine phosphate, 5 units creatine kinase (EC 2.7.3.2.), and various amounts of platelet homogenate (10,000 × *g* supernatant containing 1 mM DTT, and 1 mM EGTA). The reaction was started by adding 0.1 mM GTP, 500,000 cpm [α-^{32}P]GTP, 0.1 mM cGMP, and sometimes nitroprusside (dissolved immediately before use in 1 mM sodium acetate buffer, pH

5.5). After incubation at 37° the reaction was stopped by adding 0.45 ml of 120 mM zinc acetate followed by 0.6 ml of 120 mM sodium carbonate. cGMP was purified (with slight modifications) by zinc carbonate coprecipitation of 5′-nucleotides and subsequent chromatography on neutral aluminum oxide (Woelm, Eschwege, W. Germany) as described (22). Recovery of cGMP throughout this purification was about 60% as determined by [^{3}H]cGMP.

Data presentation. Results are expressed as mean values of number of determinations ± standard deviation of individual experiments from different blood donors. Statistical comparison of the data was calculated by the paired Student's t test with p values of $p < 0.02$. In the case of dose response curves showing per cent aggregation versus concentration of the agonist the maximum response was adjusted as 100% effect. In some cases, only one typical experiment out of several others (minimum of five different experiments) is shown.

Results

Effect of CO on platelet aggregation induced by several agonists. Since arachidonic acid is converted to PGH_2 and TxA_2 by platelets it can be used as an aggregatory substance. When PrP is treated by gassing with CO and then incubated with arachidonate, a complete inhibition of aggregation is observed (Fig. 1A). Under these conditions, the secretion of [^{3}H]serotonin is prevented (Fig. 1A). Using U46619 which acts on the prostaglandin endoperoxide/thromboxane receptor gives similar results (Fig. 1B).

The effect of CO gassing is not due to a lack of oxygen required for the cyclooxygenase reaction since a mixture of 90% CO and 10% O_2 has the same effect. Gassing with nitrogen or argon was ineffective in blocking aggregation, stating further, that the effect is not due to a lack of oxygen. Replacing the amount of CO in the different gas mixtures by nitrogen and leaving the amount of oxygen constant shows also no inhibition of aggregation.

Complete inhibition by CO was also observed when PrP was stimulated with thrombin or collagen (data not shown). The agonist epinephrine is known to cause a slow aggregation response without a preceding platelet shape change. With epinephrine, a complete inhibition was never observed and higher concentrations caused a full aggregatory response (Fig. 2A).

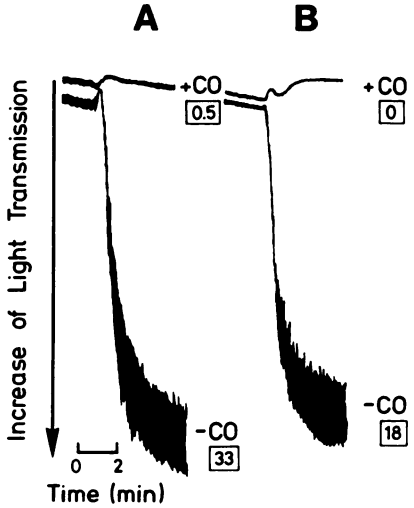

Fig. 1. Effect of CO on the arachidonic acid or U46619 induced platelet aggregation and serotonin release. PrP was incubated for 2 min at 37° without (−CO) or with (+CO) CO as described, before stimulation with 0.48 mmol/liter of arachidonic acid (A) or 0.48 μmol/liter U46619 (B). Release of [^{3}H]serotonin (% of total) is indicated in rectangles.

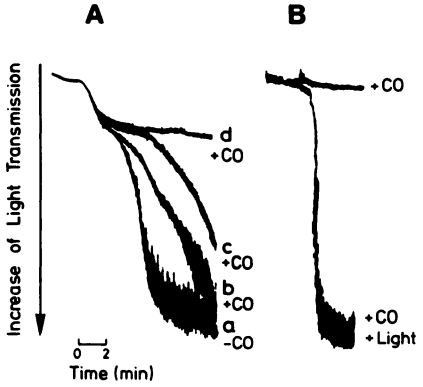

Fig. 2. Recovery of the normal platelet response in CO-treated samples by raising the agonist concentration or by illumination. PrP treated with (+CO) or without (−CO) CO was stimulated with epinephrine (A; a, d, $2 \cdot 10^{-6}$ mol/liter; b, $8 \cdot 10^{-6}$ mol/liter; c, $4 \cdot 10^{-6}$ mol/liter), or with arachidonic acid (B; 0.64 mmol/liter). One cuvet in B was illuminated with light (450 nm) shortly after adding the agonist.

Employing different agonists together results in an additive or even synergistic action. Likewise, arachidonate and thrombin, concentrations which were separately inhibited by CO were resistant to CO when added together (data not shown).

Fig. 2B contains the important proof that the CO effect is light reversible, since illumination of a CO-treated assay with light above 400 nm leads to immediate onset of aggregation.

No platelet response was noticed with light alone. Unfortunately, the aggregation reaction is difficult to quantitate and therefore it is impossible to obtain a photochemical action spectrum. By the addition of oxy-hemoglobin to CO-treated PrP it is possible to show the reversibility of the CO effect. In this case oxy-hemoglobin is added to a CO-treated assay. After a few minutes the hemoglobin is removed by centrifugation and the resulting PrP is used for aggregation studies. By using this specially treated PrP it is possible to show that low concentrations of various agonists are now able to induce aggregation again, showing the reversibility of the CO effect. Under exactly defined conditions an onset of CO inhibition can be measured, although the minimal concentrations of CO used, greatly depend on the remaining oxy-hemoglobin in the assay. Keeping these limitations in mind an onset of CO inhibition can be seen using 20–80 μM CO.

As with epinephrine, the CO effect was only seen below a certain level of the agonists. Above those concentrations CO proved to be ineffective. Several dose response curves, showing the inhibition of platelet aggregation by CO versus the concentration of different agonists, are depicted in Fig. 3 (A–C).

In all three cases, CO inhibits platelet aggregation although the degree of inhibition depends on the agonist used. Using arachidonic acid, ADP, or the endoperoxide analogue U46619 as the stimulating agent, the graphics show that aggregation is inhibited to a certain extent by CO, while allowing the full aggregatory response in control samples. Each agonist caused a dose related decrease in CO induced inhibition of platelet aggregation.

Working with 8-octylamino-cGMP it was confirmed that this lipophilic cGMP analogue is also able to inhibit platelet aggregation. The same results have been shown by Matsuoka *et al.* (23) working with 8-bromo cyclic GMP.

Effect of CO on the metabolism of arachidonic acid. Although from our previous investigations on thromboxane

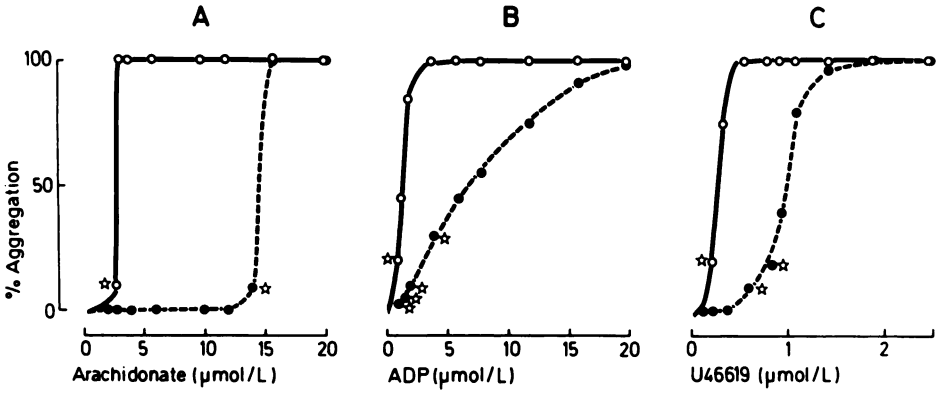

Fig. 3. Dose response curves for arachidonic acid, ADP, and U46619 induced aggregation in the presence and absence of CO. PrP (B, C) or washed platelets suspended in Tyrode-Hepes buffer containing 5% platelet-poor plasma (A) were incubated without (○) or with (●) CO before stimulation with various concentrations of each agonist (A, arachidonic acid; B, ADP; C, U46619). ☆, reversible aggregation.

synthase we could exclude an interaction of CO at this enzyme, it was less clear whether cyclooxygenase or 12-lipoxygenase was also unaffected. This can be studied best by incubation of platelets with ^{14}C-labeled arachidonate and by measuring the pattern of products with and without CO. Taking the fatty acid binding capacity of albumin into consideration, these experiments were performed with washed platelets. For comparison, two other inhibitors of platelet aggregation, sodium nitroprusside and prostacyclin, were investigated in parallel (Table 1).

It turned out that neither CO nor nitroprusside or prostacyclin inhibited the formation of TxB_2 or other prostaglandins derived from PGH_2. Only a slight inhibition of 12-HETE formation could be seen, although platelet aggregation was fully prevented with all three substances. Using a TxB_2 radioimmunoassay and unlabeled arachidonate, there was also no decreased formation of TxB_2 in the presence of CO. CO-treated assays showed 97 ± 6% (means ± standard deviation, n = 3) TxB_2 formation compared to controls.

In order to definitely exclude an inhibition of thromboxane synthase by CO, a different assay system was employed, which uses the concomitant formation of 12-hydroxyheptadecatrienoic acid in the isomerization of PGH_2 to TxA_2 (24). Solubilized platelet microsomes were used and HHT was measured photometrically. No effect was seen with CO although the specific thromboxane synthase inhibitor UK-37248 completely blocked the activity. Control experiments (without inhibitors) showed an activity of 0.192 ± 0.002 A/min (means ± standard deviation; n = 3). Gassing the buffer plus microsomes for 10 min with pure nitrogen or CO did not reduce the activity of the thromboxane synthase. The activity for the nitrogen assay was 0.192 ± 0.003 A/min (means ± standard deviation; n = 3) and 0.189 ± 0.003 A/min (means ± standard deviation; n = 3) for the CO assay, respectively. Using UK-37248 (6.7 μg/assay) we were unable to detect any activity.

A different picture emerged when TxB_2 formation from arachidonate was measured in PrP. At threshold levels of arachidonate (0.4 mmol/l) CO reduced TxB_2 levels to 43 ± 7% (values as per cent of controls, means ± standard deviation of 10 separate experiments). If the arachidonate concentration was increased above threshold levels to induce aggregation even in the presence of CO, then again 80% or more of the TxB_2 levels were reached.

The reason for the decrease in PrP is at present not evident, but by establishing dose-response curves of platelet aggregation and the associated TxB_2 formation from arachidonate, we could conclude that even 43% of the TxB_2 formed would be sufficient for platelet aggregation (Fig. 4).

Therefore, reduced TxB_2 formation is unlikely to be the reason for CO inhibition of aggregation in PrP and can certainly be excluded for washed platelets.

As could be expected, the formation of TxB_2 from endogenous arachidonate, liberated as a consequence of stimulation of PrP with thrombin, was completely inhibited in the presence of CO (2 ± 1% TxB_2, per cent of control, means ± standard deviation, from three separate experiments).

These results indicate that CO was able to block arachidonate release in platelets probably by not allowing the activation of phospholipase A_2, which itself must have been a consequence of a lack of activation of phospholipase C.

TABLE 1

Effects of different platelet inhibitors on the conversion of [1-^{14}C] arachidonic acid by intact platelets

Suspensions of washed platelets in Tyrode-Hepes buffer were incubated with or without different inhibitors of aggregation for 2 min before stimulation with [1-^{14}C] arachidonic acid as indicated under Materials and Methods. Results are expressed as per cent of controls of number of determinations (n). Assays without inhibitors are used as references.

Additions	n	TxB_2	HHT	12-HETE
CO	5	102 ± 7	100 ± 8	95 ± 7
Prostacyclin (2 ng/ml)	3	101 ± 2	104 ± 5	85 ± 5
Nitroprusside (10 μM)	3	102 ± 3	103 ± 4	94 ± 5

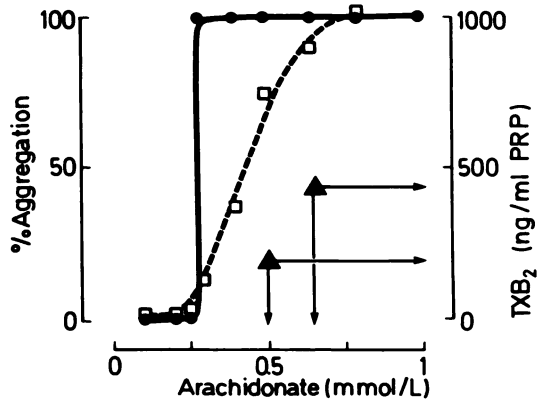

Fig. 4. TxB_2 formation and platelet aggregation following stimulation of PrP with increasing doses of arachidonic acid. PrP was stimulated with arachidonic acid (mmol/l); aggregation (●) and TxB_2 formation (□) was determined. ▲, situation for two CO-treated samples; no aggregation and reduced TxB_2 formation compared to the controls; after stimulation with 0.5 and 0.64 mmol/liter of arachidonic acid, respectively.

Platelet cytosolic free calcium. Since phospholipase A_2 is known to depend on $[Ca^{2+}]_i$ for activation, the effects of CO on intracellular $[Ca^{2+}]_i$ levels were investigated. When Fura-2 loaded platelets were stimulated by threshold concentrations of arachidonate, a rapid increase of Ca^{2+} from a resting level of 90 nM to about 400 nM within a few seconds was registered. This rise was short-termed and the $[Ca^{2+}]_i$ levels declined from this maximum without fully reaching the resting level again. According to the optical density (without recording) the platelets were aggregated.

Treating the platelets with CO before adding arachidonate greatly reduced the fluorescence and sometimes no increase at all was observed (Fig. 5).

This was paralleled by inhibition of platelet aggregation proving that CO prevented the rise of intracellular $[Ca^{2+}]_i$ which is considered to be a prerequisite of platelet aggregation.

Platelet cyclic nucleotide levels. The antiaggregatory effect of prostacyclin is linked to the increase in cAMP levels (25). It was therefore tested whether CO could lead to a similar rise either due to stimulation of adenylate cyclase activity or a decrease in phosphodiesterase activity.

Using a protein binding assay for cAMP, a pretreatment with CO under threshold concentrations of arachidonate resulted in average values of 101 ± 9% (per cent of control, means ± standard deviation from five separate experiments). As expected, prostacyclin significantly elevates cAMP levels dose dependently. cGMP also has been shown to be connected with inhibition of platelet aggregation since sodium nitroprusside causes a severalfold increase in cGMP and inhibits platelet aggregation (23, 26, 27). The phosphodiesterase inhibitor IBMX had to be added in order to determine cGMP. IBMX itself inhibits aggregation and causes an elevation of cGMP levels to 111 ± 5% (per cent of control, means ± standard deviation of seven separate experiments). As a positive control, addition of nitroprusside at 10 μM in the presence of IBMX caused an increase to 163 ± 17% compared to the controls (means ± standard deviation; n = 7) (Fig. 6).

This increase was dependent on the incubation time. Surprisingly, CO gassing (also in the presence of IBMX) resulted

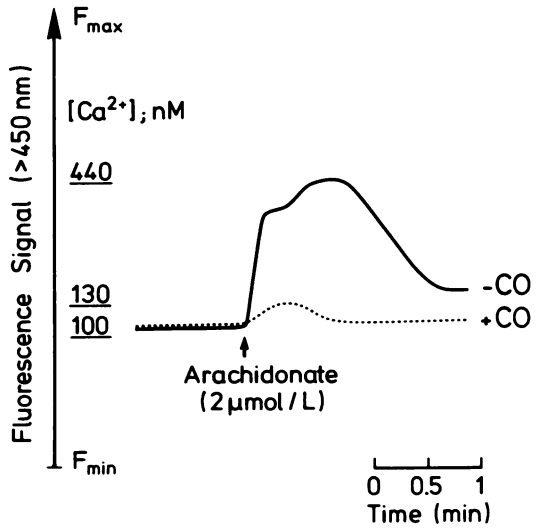

Fig. 5. Measurement of cytosolic free calcium. $[Ca^{2+}]_i$ was measured and calculated as described under Materials and Methods. Samples were treated without (−CO) or with (+CO) CO and stimulated with arachidonic acid (2 μmol/liter).

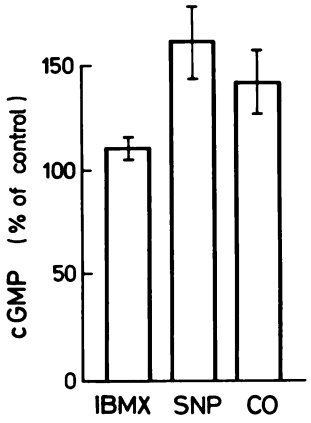

Fig. 6. Platelet cyclic GMP levels. Platelet cGMP was determined as indicated. Values are mean ± standard deviation for seven different experiments (per cent of control). Nitroprusside (SNP) (10 μmol/liter), IBMX (100 μmol/liter), CO was bubbled for 20 s.

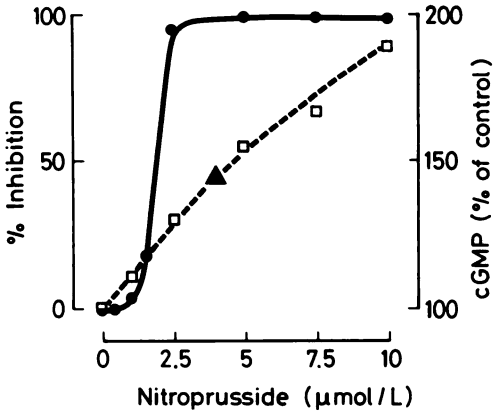

Fig. 7. Effects of nitroprusside and CO on inhibition of platelet aggregation and cGMP levels. Inhibition of platelet aggregation by nitroprusside (μmol/liter) stimulated by threshold concentrations of arachidonic acid (●) and the corresponding cGMP levels (□) are shown representatively out of five similar experiments. ▲, situation for a CO-treated sample (no aggregation and the corresponding cGMP level).

in a 143 ± 10% increase (means ± standard deviation of seven separate experiments) compared to the control levels. This increase, induced by nitroprusside or CO, was significant ($p < 0.02$) compared to controls. In order to clarify whether this effect would be sufficient to explain the inhibition of aggregation under these conditions, we established a dose-response curve for nitroprusside (Fig. 7).

The result shows an extreme sigmoid curve according to which platelet aggregation is already blocked at levels above 125 ± 7% cGMP compared to controls, having received IBMX (controls with IBMX set as 100%). The IC_{50} for nitroprusside was determined to be 2.1 ± 0.6 μM (means ± standard deviation of 10 additional experiments). Experiments were carried out in parallel determinations for cGMP (in the presence of IBMX) and for aggregation with identical threshold concentrations of arachidonic acid. It seemed reasonable to conclude from these results that CO inhibits aggregation by increasing cGMP levels.

Effect of CO on soluble platelet guanylate cyclase. It is generally accepted that nitroprusside exerts its effects by releasing NO in a reductive process and that NO by an unknown mechanism activates guanylate cyclase. Guanylate cyclase contains a heme regulatory subunit which could combine with NO. Nevertheless, it appeared attractive that CO could

activate guanylate cyclase by combining with the ferrous form of the heme regulatory subunit. Without giving experimental details, such a stimulation of guanylate cyclase activity by CO has been mentioned twice in literature (28, 29).

For the purpose of our investigation it was sufficient to prepare a 10,000 × *g* supernatant from human platelet homogenates as an enzyme source to establish the proposed stimulatory role of CO. Using ^{32}P-labeled GTP, we found a basal activity of guanylate cyclase of 11 ± 2.3 pmol/min × mg of protein. Nitroprusside activated in a concentration-dependent fashion (50 μM nitroprusside raised the basal activity about 2-fold, depending on the natural occurring inhibitors of guanylate cyclase in homogenates). When the reaction was performed under an N_2 atmosphere the enzyme was stimulated slightly (132 ± 8%, mean ± standard deviation, n = 3) compared to controls. Gassing the incubation mixture with CO instead of N_2 raised the activity to 402 ± 58% (means ± standard deviation, n = 3, N_2 value set as 100%). This confirms that guanylate cyclase is subject to stimulation by CO, probably by binding to the heme subunit. Unlike with NO which alternatively has been suggested to directly interact with an essential SH group, no other mechanism seems feasible.

Platelet phospholipid metabolism. Even if an increase in cGMP levels could be a reason for the antiaggregatory action of CO, its influence on the platelet regulatory mechanisms remained unclear. After having shown that intracellular $[Ca^{2+}]_i$ did not increase under CO, it remained to show whether this was due to blocking the release from intracellular stores and/or to an enhanced sequestration process into the stores or into the extracellular medium.

In order to measure the activation of phospholipase C as one of the first events of the PI-response, we used washed human platelets labeled with [^{14}C]arachidonic acid and determined the formation of phosphatidic acid after stimulation with cold arachidonate (10^{-6} M; Fig. 8A) or U46619 (8×10^{-8} M; Fig. 8B).

The concentration of these agonists were chosen in order to reach a maximum aggregation response in control assays in contrast to full CO inhibition under the same conditions. With 10^{-6} M arachidonate, the formation of ^{14}C-labeled TxB_2 could be measured in control assays using ^{14}C-labeled platelets, while CO was again active in preventing the formation of labeled TxB_2 (Fig. 9).

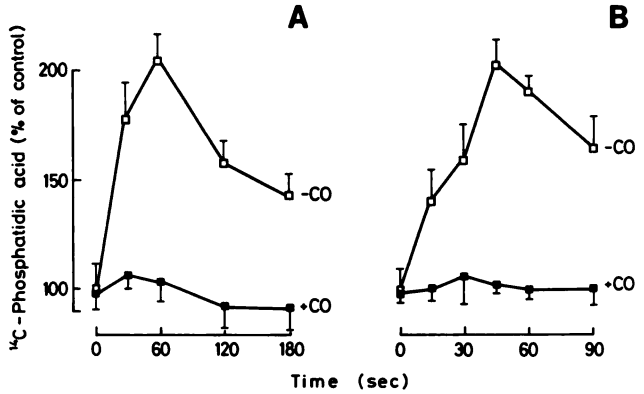

Fig. 8. Effects of arachidonic acid or U46619 on platelet phosphatidic acid production in the presence and absence of CO. Suspensions of washed human platelets ($2 \cdot 10^8$ platelets/ml) labeled with [1−^{14}C]arachidonic acid were exposed to cold arachidonic acid (1 μM; A) or to U46619 (0.08 μM; B). Phosphatidic acid was determined as given under Material and Methods in the absence (−CO) or presence (+CO) of CO.

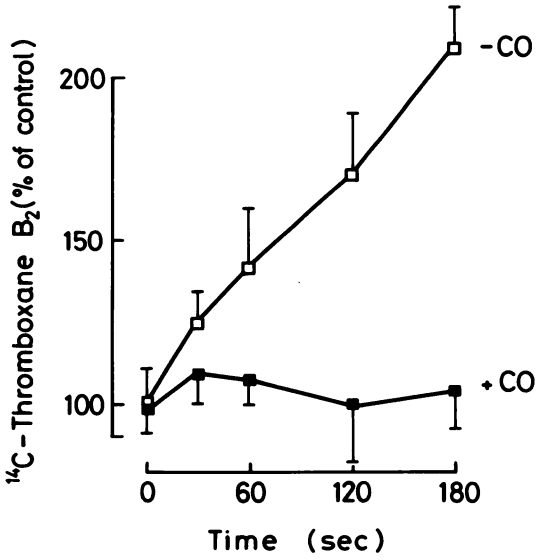

Fig. 9. Arachidonic acid induced the formation of [^{14}C]TxB_2 in the presence and absence of carbonmonoxide. Experimental details as in Fig. 8. Platelets were treated with cold arachidonic acid (1 μM) in the absence (−CO) or presence (+CO) of CO and the formation of [^{14}C]TxB_2 was determined.

It can be concluded from these results that in the presence of CO the complete sequence of the PI response is suppressed.

Discussion

Our studies confirmed earlier observations that CO can inhibit platelet aggregation. Although this finding has neither physiological nor major toxicological implications, it may shed light on the mechanism of aggregation since effects of CO are usually linked to catalytic processes involving ferrous hemoproteins. However, we could clearly exclude an inhibition of the cytochrome P450 enzyme thromboxane synthase since its mechanism does not require the ferrous state as in monooxygenase reactions. The finding by Cinti and Feinstein (14) that TxB_2 formation in platelet microsomes can be inhibited by CO is at variance with our results.

On the other hand, the action of CO at a reduced hemoprotein is likely since light above 400 nm could reverse the inhibition of platelet aggregation in the presence of CO. Due to the difficult quantitation of aggregation as an "all or none" process, a photochemical action spectrum could not be obtained.

In order to pinpoint the hemoprotein in question, we have measured most of the essential parameters involved in platelet aggregation. Not only thromboxane synthase was uneffected by CO, also the hemoprotein cyclooxygenase remained active as shown by the conversion of arachidonate to TxB_2. Aspirin-treated platelets stimulated with the prostaglandin endoperoxide analogue U46619 were also accessible to CO inhibition which presents further proof of a noninvolvement of the cyclooxygenase pathway. Similarly, 12-HETE formation was not affected by CO being in accordance with the mechanism of 12-lipoxygenase.

From the finding that CO prevented TxB_2 and 12-HETE formation in platelets stimulated by agonists like thrombin, collagen, ADP, or U46619, it follows that the liberation of arachidonate by phospholipase A_2 is prevented in the presence of CO. This again becomes understandable due to the lack of a $[Ca^{2+}]_i$ increase and a lack of phosphatidic acid formation.

Thus, phospholipase C, as the key enzyme for the PI response, remained inactive in the presence of CO. Above a certain threshold level, all agonists overcame the inhibition by CO. This, together with the additive or even synergistic action of agonists, suggested that the sensitivity of the transducing mechanisms from the receptor binding to phospholipase C was effected by some unknown mechanism, and that this inhibitory action is overruled by stimulating with different agonists simultaneously.

Our results give good reasons to assume that this transducing process is influenced by cGMP. The levels of this cyclic nucleotide were significantly increased, whereas the cAMP-levels remained unchanged. Comparative studies with sodium nitroprusside, which is known to activate guanylate cyclase, confirmed that platelet aggregation is sensitive to even minor increases in cGMP levels. This result is surprising since nitroprusside was reported to stimulate the activity of guanylate cyclase severalfold up to a factor of 50 or 100. It seems, however, that the physiological range of regulation is much lower. Guanylate cyclase activity was found to be increased in the platelet supernatant by 400% in the presence of CO, which led to a 40% increase of the cGMP steady state levels.

The additional question arises as to which mechanism CO increases the activity of guanylate cyclase when added. It is likely to think that CO is bound to the ferrous heme in the regulatory subunit of guanylate cyclase. This would imply that the enzyme is either present in the ferrous state or that CO shifts a preexisting ferric state to the ferrous. In conjunction with a postulated regulatory SH group (30), such internal redox shifts could provide a basis for enzyme regulation.

This question is not the only one remaining open for further investigations. Another one is related to the missing link between cGMP levels and phospholipase C activity. Such a regulatory mechanism was already proposed by Takai *et al.* (31) with a phosphorylation step as the most likely mechanism. Possible targets of a phosphorylation by cGMP dependent kinases are the 22, 24, or 50 kDa proteins which are known to be increased by cGMP. Corresponding studies with CO-treated platelets have revealed only small differences (Brüne, B., and U. Walter, unpublished) probably due to unfavorable labeling conditions with [^{32}P]phosphate. It can be speculated that the physiological significance of guanylate cyclase activation would represent a suitable mechanism of feedback inhibition of the PI response. Whether arachidonate itself, derived hydroperoxides like PGG_2 or 12-HPETE, are responsible for guanylate cyclase activation or whether EDRF or additional EDRF-like autacoids are required, is still under debate. The function of CO can possibly add to the understanding of the role of heme in this activation process.

A puzzling and not yet explainable result concerns the different degree of CO inhibition of washed human platelets compared to PrP. Already in the presence of 5% platelet-poor plasma washed platelets show a decrease in TxB_2 formation, whereas working with washed human platelets (without the addition of plasma) no inhibition is observed. Certainly, the high binding capacity of serum proteins could play a role which is supported by the improvement at higher arachidonate concentrations. Additional factors, however, must be involved since it was also mentioned by Fitzpatrick and Gorman (32) that agents which increase platelet cAMP levels reduce TxB_2 formation. A plausible explanation could be altered pathways of arachidonate utilization at increased cAMP or cGMP levels. At limiting levels of arachidonate, all competing pathways, like reacylation, would diminish the availability of arachidonate for cyclooxygenase. Irrespective of the validity of the underlying mechanism, it could be shown that even a diminished formation of TxB_2 could not have been the reason for the inhibitory action of CO.

Summarizing the main conclusion from our results, it seems evident that platelet aggregation involves a carefully balanced equilibrium between PI response-dependent agonists and their feedback regulatory mechanisms among which cGMP seems to play a major role. CO can be used as a rather selective probe to desensitize the response of the platelet to various agonists and CO may be a suitable tool to study the mechanism of activation of guanylate cyclase.

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