Biochemistry (Moscow), Vol. 68, No. 11, 2003, pp. 1210-1216. Translated from Biokhimiya, Vol. 68, No. 11, 2003, pp. 1506-1513.

H_2O_2-Induced Platelet Aggregation and Increase in Intracellular Ca^{2+} Concentration Are Blocked by Inhibitors of Intracellular Signaling

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Received June 14, 2002
Revision received November 12, 2002

Abstract—Inhibitors of signaling enzymes such as guanosine-5′-O-2-thiodiphosphate, aristolochic acid, aspirin, indomethacin, and trifluoperazine block H_2O_2-induced platelet aggregation and H_2O_2-induced increase in the intracellular concentration of Ca^{2+}. These findings suggest that the effect of H_2O_2 on platelets is associated with activation of signal pathways responsible for increase in the concentration of intracellular Ca^{2+}. On H_2O_2-induced platelet aggregation, the concentration of cAMP in the cytoplasm decreases and that of cGMP increases.

Key words: hydrogen peroxide, platelets, platelet aggregation, intracellular Ca^{2+}, cAMP, cGMP, inhibitors, ADP, arachidonic acid

Platelets are blood cells that play a key role in the initial stage of thrombogenesis. Intravascular thrombus formation, which disturbs the blood microcirculation is, in particular, caused by an increased aggregational activity of platelets. The aggregational activity of platelets can be changed due to various factors, including reactive oxygen species (ROS). ROS (superoxide anion-radicals $O_2^{\cdot-}$, hydrogen peroxide H_2O_2, hydroxyl radicals $^{\cdot}OH$, and singlet oxygen $^1O_2$) are generated in the body under the influence of physicochemical factors, such as ionizing radiation, laser radiation, toxic compounds, drugs, and also during inflammatory processes and activation of neutrophils [1-4].

It has been established that the most stable ROS, H_2O_2, has a dual effect on the functional activity of platelets: on one hand, H_2O_2 induces platelet aggregation [5-7] and is synergic with other aggregating agents [8]; on the other hand, it inhibits platelet aggregation and release of Ca^{2+} [9-11]. The effect of H_2O_2 on platelet aggregation seems to be due to its effect on activities of various enzymes, especially those containing SH-groups. Thus, H_2O_2 activates phospholipase A_2 [12, 13], prostaglandin synthase [14], phospholipase C [15], protein kinase C [16, 17], G_i-proteins [18], Ca^{2+}-ATPase [19], guanylate cyclase [10, 20], etc. Many of these enzymes are involved in signal transduction in platelets. However, the involvement of enzymes in H_2O_2-induced platelet aggregation is insufficiently studied. It is known that aspirin inhibits H_2O_2-induced platelet aggregation, and cyclooxygenase is involved in H_2O_2-induced aggregation [7, 21]. In various cells H_2O_2 increases the intracellular concentration of Ca^{2+} by changing activities of calcium-transporting proteins [22-24]. An inhibitor of phospholipase C prevented increase in intracellular concentration of Ca^{2+} in H_2O_2-treated platelets [25]. The intracellular concentration of Ca^{2+} increased under the influence of ROS in aspirin-treated rabbit platelets [26]. However, the interrelation between the H_2O_2-induced signal pathway in platelets and changes in the intracellular concentration of Ca^{2+} remains unclear. To elucidate this question, in the present work the H_2O_2-induced platelet aggregation and changes in the intracellular concentration of calcium ions were studied in the presence of inhibitors of key enzymes of the known pathways of platelet activation.

Abbreviations: AA) arachidonic acid; ArA) aristolochic acid; ROS) reactive oxygen species; NEM) N-ethylmaleimide; PRP) platelet-rich plasma; TXA_2) thromboxane A_2; PLA_2) phospholipase A_2; PLC) phospholipase C.

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0006-2979/03/6811-1210$25.00 ©2003 MAIK "Nauka/Interperiodica"

MATERIALS AND METHODS

Studies on platelet aggregation. Fresh donors' blood stabilized with Glugitsir solution (Nesvizh Plant of Medical Preparations, Belarus) was from the Republican Blood Transfusion Station (Minsk, Belarus). Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 200*g* for 10 min with an OPN-03 centrifuge (Laboratory Equipment and Devices, Russia). Platelet-free plasma was prepared by centrifugation of the blood at 2000*g* for 15 min. The cell concentration in PRP was adjusted to $5 \cdot 10^8$ cells/ml by dilution with platelet-free plasma.

Platelet aggregation was determined by light transmission using an AP 2110 computerized analyzer of platelet aggregation (SOLAR, Belarus). To study platelet aggregation, the aggregometer cuvette was supplemented with 250 μl of phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, 1 mM $CaCl_2$) (pH 7.35), with Ca^{2+} and 250 μl of PRP (the final concentration of the cells was $2.5 \cdot 10^8$ cells/ml), incubated at 37°C with stirring for 3 min (in the absence and presence of inhibitors); then an aggregation inducer was added. Platelet aggregation was induced with 5 mM H_2O_2, 20 μM ADP, and 30 μM arachidonic acid. The platelet aggregation was recorded by changes in the light transmission of the cell suspension, the rate of aggregation (v, % light transmission per 1 min) was determined automatically from the aggregation curve. Inhibitory effects of the compounds were assessed by changes in the rate of platelet aggregation relatively to the control (v/v_0), where v was the rate in the presence of inhibitors under study, v_0 was the rate in the control.

Determination of intracellular Ca^{2+} concentration. Washed platelets were prepared by centrifugation of PRP at 2000*g* for 3 min. The cell precipitate was suspended in 10 mM MOPS buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM D-glucose, 0.1% BSA) (pH 7.4), without $CaCl_2$ and $MgCl_2$, with the final cell concentration of $2.5 \cdot 10^9$ cells/ml MOPS buffer.

Intracellular Ca^{2+} concentration in the platelets was determined using the Quin 2 calcium probe with an LSF 1211A spectrofluorimeter (SOLAR) as described in [27]. To the original platelet suspension (2 ml, $2.5 \cdot 10^9$ cells/ml) 16 μM Quin 2 was added, and it was incubated at 37°C for 20 min. To determine the intracellular Ca^{2+} concentration, 0.1 ml of suspension of the stained platelets with 1.9 ml of MOPS buffer containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2 \cdot 6\ H_2O$ were introduced into a fluorimeter cuvette, and kinetics of changes in the intracellular fluorescence of Quin 2 were recorded at 495 nm (λ_{ex} = 339 nm) at 37°C. The concentration of cytoplasmic Ca^{2+} was calculated according to [28].

Measurement of intracellular concentrations of cAMP and cGMP. Concentrations of cAMP and cGMP were determined radioimmunologically with a γ-counter (LKB-Wallac, Finland). Intracellular concentrations of cAMP and cGMP were determined in the presence of 0.5 mM papaverine in platelets not treated with H_2O_2 (control) and in platelets treated with 5 mM H_2O_2 (at the stage of the maximal aggregation). The platelets were precipitated by centrifugation at 2000*g* for 2 min and resuspended in 13.3 mM Tris-HCl buffer (120 mM NaCl, 15.4 mM KCl, 1.5 mM EDTA, 6 mM D-glucose) (pH 6.5) to the final platelet concentration of 10^9 cells/ml. Then the cells were broken with 1% Triton X-100. Then the specimens were analyzed using a commercial test-system elaborated at the Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus.

The following reagents were used: 3-[N-morpholino]propanesulfonic acid (MOPS), ionomycin, N-ethylmaleimide, nordihydroguaiaretic acid, trifluoperazine, indomethacin, arachidonic acid, guanosine-5′-O-2-thiodiphosphate, aristolochic acid, neomycin, and dipyridamole from Sigma (Germany); tris(hydroxymethyl)aminomethane (Tris) and ADP from Reanal (Hungary); Quin 2 acetoxymethyl ester from Calbiochem (Germany); aspirin from Bayer (Germany). Other reagents were from Reakhim (Russia) and Belmedpreparaty (Belarus).

The data are presented as mean value ± standard deviation of the mean value for five independent experiments.

RESULTS

H_2O_2-Induced platelet aggregation. Figure 1a presents changes in the light transmission of PRP (aggregogram) on addition of 5 mM H_2O_2. The curve shows that in response to H_2O_2 the platelets change their shape, aggregate, and then disaggregate. The aggregogram pre-

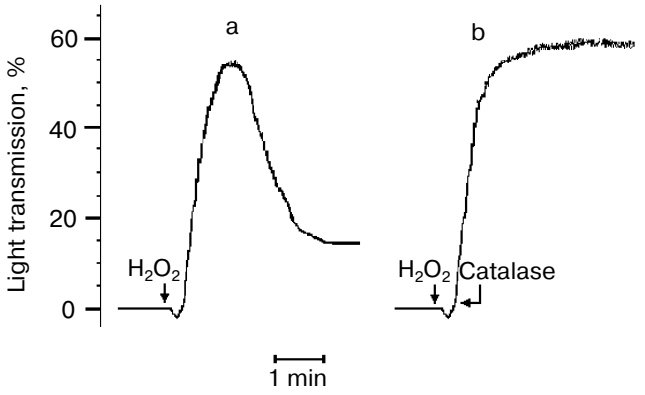

Fig. 1. Changes in light transmission of PRP under the influence of 5 mM H_2O_2 in the absence (a) and presence (b) of catalase (1000 units/ml).

sented in Fig. 1a is typical for PRP from various donors. Aggregograms of the H_2O_2-induced platelet aggregation are different from aggregograms of ADP-induced aggregation mainly by the delayed response of the platelets (lag-phase). In the curve presented the time interval between the H_2O_2 addition to PRP and beginning of changes in the light transmission is 15 sec. The aggregograms obtained under the influence of H_2O_2 on platelets were specified by reversibility of the aggregation. The platelet aggregates produced under the influence of hydrogen peroxide are unstable, destroyed spontaneously, and the aggregation is replaced by disaggregation.

Catalase (1000 units/ml) added before the introduction of H_2O_2 completely prevented the aggregating effect of hydrogen peroxide. Addition of catalase at the initial stage of the H_2O_2-induced aggregation prevented the disaggregation (Fig. 1b). These findings suggested that exogenous hydrogen peroxide should initiate the platelet aggregation, and its further presence in the extracellular medium is not required. To initiate disaggregation, the presence of H_2O_2 in the extracellular medium is needed. The addition of catalase at the initial stage of the platelet aggregation allowed us to prevent the disaggregation and study the irreversible H_2O_2-induced platelet aggregation.

Data presented in Fig. 2 show that the rate of the H_2O_2-induced platelet aggregation depends on the concentration of H_2O_2. Hydrogen peroxide induced the aggregation in a rather narrow range of concentrations. At H_2O_2 concentration lower than 2.5 mM the platelet aggregation was poor, and the aggregation rate was maximal at the concentration of 5 mM.

H_2O_2-Induced aggregation in the presence of specific inhibitors of aggregation. Data presented in the table show that an inhibitor of G-proteins, guanosine-5′-O-2-thiodiphosphate, which at the concentration of 100 μM affects the platelet aggregation induced with ADP and arachidonic acid also inhibited the H_2O_2-induced aggregation. The platelet aggregation was inhibited not only by aspirin and indomethacin, which are known inhibitors of H_2O_2-induced platelet aggregation, but also by aristolochic acid, which is an inhibitor of phospholipase A_2 (PLA_2), and by trifluoperazine, which is an inhibitor of protein kinase C. An inhibitor of phospholipase C (PLC), neomycin, which at concentrations of 1 and 2 mM suppresses the ADP-induced platelet aggregation, did not affect the aggregation induced by H_2O_2 and arachidonic acid. In the presence of 40 μM nordihydroguaiaretic acid the rate of platelet aggregation was virtually the same as in the control. H_2O_2-Induced platelet aggregation was not observed on addition into the incubation medium of 2 mM EDTA, which is a chelator of Ca^{2+}, and 200 μM verapamil, which is an inhibitor of calcium channels. An inhibitor of cGMP-phosphodiesterases, dipyridamole, at 200 μM concentration decreased the rate of platelet aggregation, suggesting the involvement of cGMP in regulation of the H_2O_2-induced platelet aggregation. The effect of the penetrating thiol reagent ethylmaleimide (NEM) on the H_2O_2-induced platelet aggregation depended on the reagent concentration. At concentrations of 10 and 15 μM, NEM accelerated the H_2O_2-induced platelet aggregation, and at concentrations of 50 and 100 μM, NEM inhibited it.

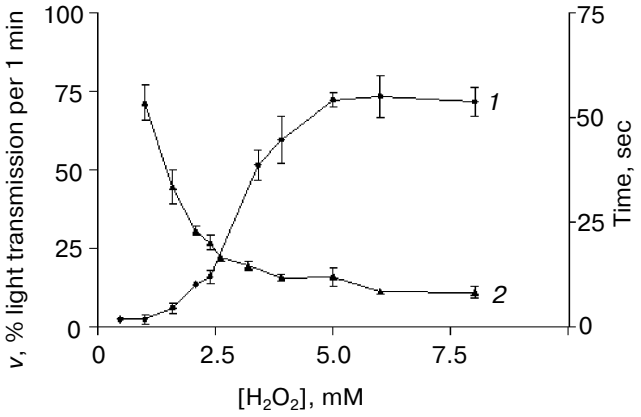

Fig. 2. Dependence of platelet aggregation rate (*1*) and lag-period (*2*) on the concentration of H_2O_2.

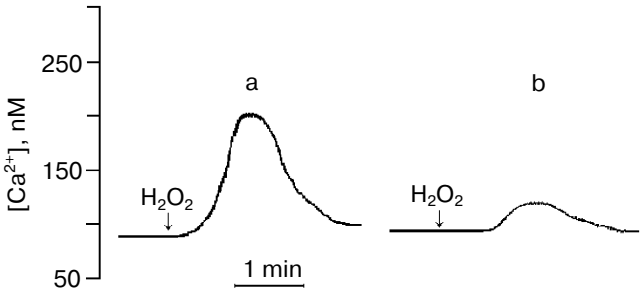

Fig. 3. Kinetics of changes in the intracellular concentration of Ca^{2+} in washed platelets suspended in Ca^{2+}-containing buffer under the influence of 5 mM (a) and 2.5 mM H_2O_2 (b).

H_2O_2-Induced increase in intracellular Ca^{2+} concentration. Figure 3 shows kinetics of changes in the Ca^{2+} concentration in the cytoplasm of platelets suspended in the Ca^{2+}-containing medium on addition into the suspension of H_2O_2 at concentrations of 5 and 2.5 mM. Immediately after the addition to the platelets of 5 mM H_2O_2, the intracellular concentration of Ca^{2+} did not change (the lag-period), but subsequently its concentration increased and then decreased to the basal level. In the calcium-containing buffer the intracellular concentration of Ca^{2+} increased 2.5-fold from the baseline (93 ± 11 nM) to 237 ± 58 nM (Fig. 4). In the absence of Ca^{2+} in the external medium the intracellular concentration of

Effects of inhibitors of signal pathways on platelet aggregation induced with H_2O_2, arachidonic acid (AA), and ADP

Substance	Target of the effect	Concentration	v/v_0		
			H_2O_2	AA	ADP
Guanosine-5′-O-2-thiodiphosphate	G-proteins	40 μM	0.91 ± 0.08	1	1
		100 μM	0.54 ± 0.15	0.61 ± 0.03	0.71 ± 0.16
Aristolochic acid	phospholipase A_2	40 μM	0.58 ± 0.11	0.61 ± 0.07	0.95 ± 0.07
		60 μM	0.41 ± 0.10	0.51 ± 0.13	0.80 ± 0.08
Aspirin	cyclooxygenase	2 mM	0.80 ± 0.05	0.86 ± 0.12	0.97 ± 0.04
		5 mM	0.32 ± 0.13	0.31 ± 0.20	0.85 ± 0.05
		7 mM	0	0	0.50 ± 0.10
Indomethacin	cyclooxygenase	40 μM	0.81 ± 0.11	0.84 ± 0.07	0.92 ± 0.08
		60 μM	0.26 ± 0.13	0.15 ± 0.07	0.77 ± 0.04
Nordihydroguaiaretic acid	lipoxygenase	40 μM	1	0.93 ± 0.05	0.90 ± 0.06
		60 μM	0.92 ± 0.05	0.77 ± 0.10	0.82 ± 0.09
		100 μM	0.74 ± 0.10	0.60 ± 0.07	0.66 ± 0,08
Neomycin	phospholipase C	1 mM	1	1	0.42 ± 0.12
		2 mM	1	0.91 ± 0.09	0.29 ± 0.08
Trifluoperazine	calmodulin	40 μM	0.39 ± 0.20	0.22 ± 0.14	0.85 ± 0.09
	protein kinase C	80 μM	0.17 ± 0.04	0	0.73 ± 0.07
EDTA	chelator of Ca^{2+}	100 μM	0.83 ± 0.10	0.91 ± 0.08	0.79 ± 0.12
		200 μM	0.67 ± 0.07	0.75 ± 0.11	0.53 ± 0.08
		1 mM	0.22 ± 0.05	0.10 ± 0.07	0
		2 mM	0	0	0
Verapamil	inhibitor of Ca^{2+}-channels	40 μM	0.63 ± 0.08	0.56 ± 0.08	0.64 ± 0.12
		100 μM	0.51 ± 0.17	0.30 ± 0.06	0.53 ± 0.14
		200 μM	0	0	0.51 ± 0.09
Dipyridamole	cGMP-phosphodiesterase	40 μM	1	0.82 ± 0.07	0.90 ± 0.10
		100 μM	0.67 ± 0.07	0.53 ± 0.10	0.73 ± 0.08
		200 μM	0.24 ± 0.09	0.25 ± 0.05	0.30 ± 0.06
Papaverine	cAMP-phosphodiesterase	5 μM	0.85 ± 0.06	0.51 ± 0.07	0.81 ± 0.10
		20 μM	0.76 ± 0.11	0.31 ± 0.13	0.58 ± 0.16
		100 μM	0.08 ± 0.02	0	0
N-Ethylmaleimide	SH-groups	2 μM	1	1	1
		10 μM	1.44 ± 0.08*	1.35 ± 0.07	1
		15 μM	1.18 ± 0.02*	1.23 ± 0.09	0.82 ± 0.06
		20 μM	1.21 ± 0.12	1.10 ± 0.06	0.54 ± 0.07
		50 μM	0.79 ± 0.10	0.73 ± 0.10	0.34 ± 0.09
		100 μM	0.42 ± 0.05	0.34 ± 0.09	0.14 ± 0.02

* $p < 0.05$.

Ca^{2+} was 62 ± 7 nM, and after the addition of 5 mM H_2O_2 it increased twofold, to 124 ± 17 nM. These data suggest the H_2O_2-induced release of Ca^{2+} from intracellular stores. Thus, under the influence of H_2O_2 the cytoplasmic concentration of Ca^{2+} increased in the platelets in both calcium-containing and calcium-free medium.

The H_2O_2-induced increase in the Ca^{2+} concentration was completely inhibited by aristolochic acid (60 μM), aspirin (2 mM), indomethacin (60 μM), and NEM (100 μM) (Fig. 5). Neomycin (1 mM) failed to affect the H_2O_2-induced increase in the Ca^{2+} concentration. However, this concentration of neomycin inhibited the ADP-induced increase in the intracellular concentration of Ca^{2+} (data not presented). Thus, compounds which inhibited the H_2O_2-induced platelet aggregation also inhibited the H_2O_2-induced increase in the intracellular Ca^{2+} concentration.

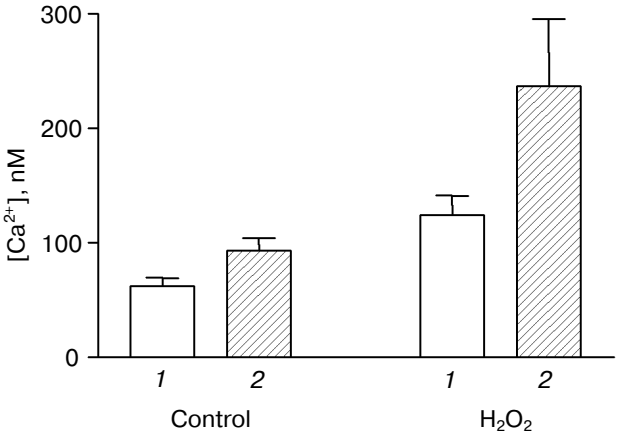

Fig. 4. H_2O_2-Induced increase in the intracellular Ca^{2+} concentration in washed platelets suspended in calcium-free (*1*) and Ca^{2+}-containing (*2*) buffers. The concentration of H_2O_2 was 5 mM.

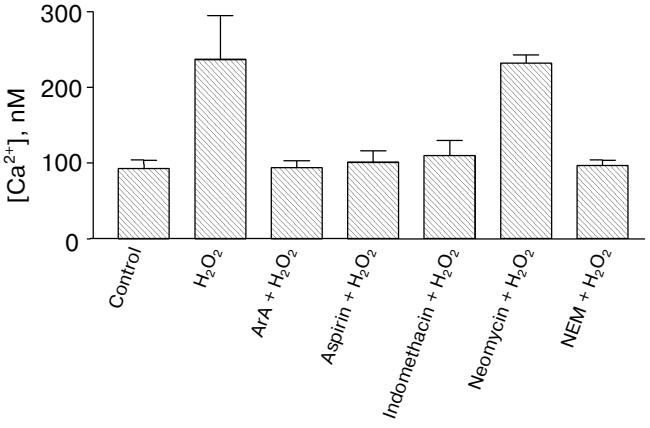

Fig. 5. Effects of inhibitors on H_2O_2-induced increase in intracellular Ca^{2+} concentration in washed platelets: 5 mM H_2O_2, 60 μM aristolochic acid (ArA), 2 mM aspirin, 60 μM indomethacin, 1 mM neomycin, 100 μM NEM.

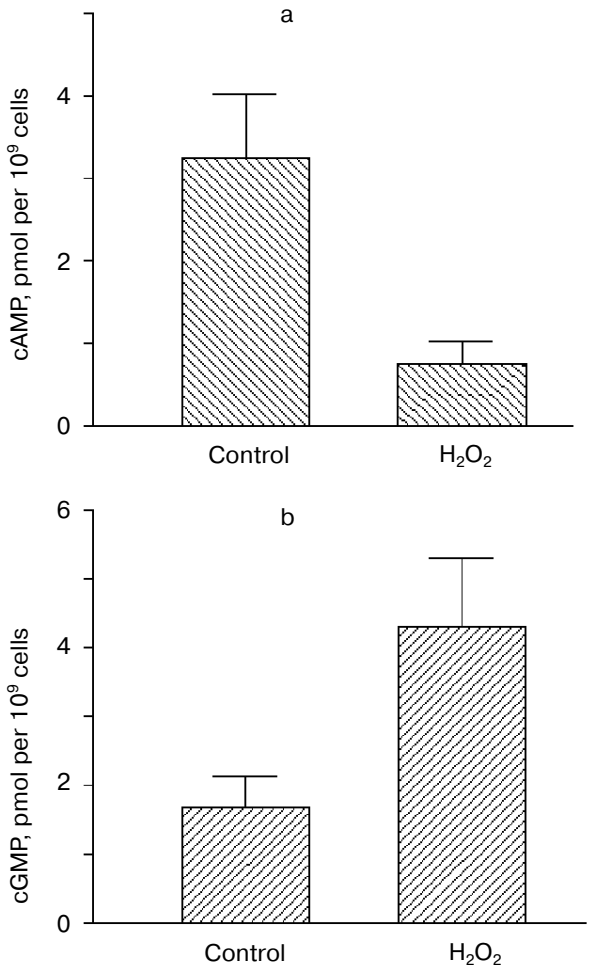

Fig. 6. H_2O_2-Induced changes in the intracellular concentration of cAMP (a) and cGMP (b). The concentration of H_2O_2 was 5 mM.

H_2O_2-Induced changes in the intracellular concentration of cyclic nucleotides. The intracellular concentration of cAMP in intact platelets was 3.25 ± 0.78 pmol per 10^9 cells, and the concentration of cGMP was 1.69 ± 0.45 pmol per 10^9 cells (Fig. 6). In the platelets activated with hydrogen peroxide (the maximum aggregation stage) the concentration of cAMP was 0.76 ± 0.28 pmol per 10^9 cells, and that of cGMP was 4.31 ± 1.01 pmol per 10^9 cells. Thus, during the platelet aggregation under the influence of hydrogen peroxide the intracellular concentration of cGMP increased and that of cAMP decreased.

DISCUSSION

Aggregation-inducing agents are known to activate in platelets two signal systems responsible for increase in the intracellular concentration of Ca^{2+}: the metabolic

pathways of arachidonic acid and phosphoinositide. Calcium and calcium-activated enzymes determine the response of platelets: changes in the shape, generation of aggregation receptors, and platelet aggregation. The findings of the present work suggest that H_2O_2 induces the release of Ca^{2+} from intracellular stores and entrance of Ca^{2+} from the external medium. It has been established by inhibitory analysis that hydrogen peroxide activates the signal pathways responsible for increase in the intracellular concentration of calcium in platelets. Because indomethacin and aspirin inhibited the H_2O_2-induced increase in the intracellular concentration of Ca^{2+} and the H_2O_2-induced platelet aggregation, it was suggested that the effect of H_2O_2 should be mediated by metabolites of the cyclooxygenase oxidation pathway of arachidonic acid. The Ca^{2+} response of platelets to H_2O_2 is similar to the effect of arachidonic acid produced on activation with PLA_2. Arachidonic acid regulates the Ca^{2+} response of platelets indirectly, via thromboxane A_2 (TXA_2). TXA_2 increases concentration of Ca^{2+} in the cytoplasm of platelets through activation of G_q-proteins, phospholipase C_β, and production of inositol triphosphate [29]. However, the intracellular concentration of calcium under the influence of TXA_2 increased in the presence of an antagonist of the receptor coupled with the G_q-protein [30]. In this work inhibition of PLC in the presence of neomycin did not affect the Ca^{2+}-response of platelets to H_2O_2. But neomycin concurrently inhibited the ADP-induced increase in the intracellular concentration of Ca^{2+}, and these finding suggested different mechanisms of the intracellular calcium mobilization in platelets under the influence of H_2O_2 and ADP; therefore, it was suggested that the phosphoinositide pathway should not be involved in the effect of H_2O_2.

Based on results of the inhibitory analysis, we consider H_2O_2 an inducer of platelet aggregation. However, the nature of H_2O_2 receptors remains unknown. Possibly, specific receptors for H_2O_2 are present on the plasma membrane of platelets. The H_2O_2-induced aggregation can also be associated with the mechanism of activation of G-proteins and phosphorylation of proteins. Phosphorylation/dephosphorylation of tyrosine residues is a common element in pathways of the intracellular signaling activated by different agents in different cells, including platelets [31]. H_2O_2 has been shown to inhibit the activity of tyrosine phosphatase [32, 33]. The role of tyrosine kinases and tyrosine phosphatases in the H_2O_2-induced aggregation in the presence of orthovanadate was shown in [34].

The data presented show that the H_2O_2-induced platelet aggregation occurs concurrently with decrease in the level of cAMP and increase in the level of cGMP. The increase in the cGMP content in the platelets under the influence of H_2O_2 is in agreement with data in the literature [10, 20]. The increase in [cGMP] can be explained by penetration of hydrogen peroxide into the platelets and direct activation of cytosolic guanylate cyclase. In contrast, the intracellular concentration of cAMP decreased and became below its basal level. H_2O_2 was shown to inhibit the increase in the cAMP concentration in platelets induced by prostaglandin precursors [35]. In [36] H_2O_2 was shown to inhibit adenylate cyclase of heart cell membranes. According to [37], the activity of phosphodiesterase was stimulated on increase in the concentration of cGMP. Taking into account the literature data, it is suggested that the decrease in the cAMP concentration during the H_2O_2-induced platelet aggregation should be associated with both inhibition of adenylate cyclase and increase in the activity of phosphodiesterase. The effect of classic inducers of aggregation, such as ADP and thrombin, is known to be also associated with inhibition of adenylate cyclase activity.

Thus, hydrogen peroxide is an inducer of platelet aggregation and switches on signal pathways resulting in an increase in the intracellular concentration of Ca^{2+}.

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