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INTERFERENCE OF TRANSMETHYLATION INHIBITORS WITH THROMBOXANE SYNTHESIS IN RAT PLATELETS

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SUMMARY. In order to determine whether a methylation reaction is involved in the platelet metabolism of arachidonic acid (AA), we investigated the effect of the transmethylase inhibitors 3-deazaadenosine (DZA) and L-homocysteine-thiolactone (Hcy) on the production of immunoreactive thromboxane (TX) B2 by rat platelets. Incubation for at least one hour of the platelet-rich plasma with DZA and Hcy led to an inhibition of TX synthesis induced by collagen (5 $\mu g.ml^{-1}$). Platelets in plasma were then preincubated for 4 hours with DZA ($10^{-3}M$) in association with Hcy($5x10^{-4}M$), washed, resuspended in buffer, and stimulated with 3 different activators. The formation of TXB2 in response to collagen (25 $\mu g.ml^{-1}$) was markedly reduced, whereas no inhibition occurred when AA ($5x10^{-6}M$) or the calcium ionophore A 23,187 ($5x10^{-6}M$) were used. In addition labelled AA was incorporated into the platelet phospholipids (PL). Its release induced by collagen (25 $\mu g.ml^{-1}$) was inhibited when platelets were preincubated with DZA and Hcy under the same experimental conditions. By contrast, the release of AA induced by A 23187 ($10^{-6}M$) was unaffected. This results strongly suggest the association of a methylation reaction with platelet activation, at a calcium-independent step of endogenous AA metabolism, before the cyclo-oxygenase level. Its precise biochemical nature remains to be determined.

1 - INTRODUCTION. 3-deazaadenosine (DZA) and L-homocysteine-thiolactone (Hcy) are known to inhibit methyl-transferases (1,2). We recently demonstrated that their combination interfered with collagen-induced rat platelet aggregation and release of ATP (3). As might be expected, these reagents also inhibited the intra-platelet synthesis of phosphatidylcholine from phosphatidylethanolamine via the N-methylation pathway. However marked differences were noted between the suppression of platelet functions and that of phospholipid methylation. First of all, the former required at least a 2 hours incubation with both compounds to occur whereas the latter had a rapid (5 min.) onset. Secondly, each compound by itself blocked the phospholipid methylation but failed to inhibit platelet activation. We therefore concluded that a methylation reaction was probably involved in platelet activation, but that it did not concern phospholipids as the methyl acceptors.

There is compelling evidence that platelet activation is closely linked to the metabolism of endogenous arachidonic acid (AA) particularly

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into the potent aggregating substance, thromboxane A_2 (TXA_2) (4). The hydrolysis of AA from phospholipids is the prerequesite for the generation of AA metabolites and is thought to be the rate-limiting step, and the site of the main regulatory processes (5). HIRATA et al. suggested that phospholipid methylation could be a critical event in AA release (6), since they found that inhibitors of methyl-transferases blocked this release in rabbit leukocytes (7) and rat leukemic basophils (8). It has also been reported that the same kind of inhibitors interfered with the generation of prostacyclin and TXA_2 by rat macrophages (9) and the phospholipase activities in human monocytes (10).

In an attempt to determine whether the interference of DZA and Hcy with platelet aggregation was related to changes in AA metabolism, we investigated the effects of these drugs on the production of thromboxane, as measured by means of a radioimmunoassay (RIA) for thromboxane B_2 (TXB_2), and on the turnover of exogenous ^{14}C-labelled AA, incorporated into platelet phospholipids prior to stimulation.

2 - MATERIALS AND METHODS.

2.1. Preparation of rat platelets. Male Wistar rats (350-450 g) were anesthetized with 30 mg.kg-1 of sodium pentobarbital i.p. and exsanguinated via a cannula inserted into the carotid artery. Platelet-rich plasma (PRP) was prepared by differential centrifugation of blood anticoagulated with heparin (final concentration : 10 U.ml^{-1} blood) and adjusted at about 500,000 platelets.µl^{-1} with platelet-poor plasma. Washed platelets were prepared from blood collected on sodium citrate (0.38%, final concentration), according to ARDLIE et al. (11), as modified by BENVENISTE et al., using a Tyrode's modified solution (12).

2.2. Chemical. The following reagents were used : 3-deazaadenosine (Southern Research Institute, Birmingham, USA) L-homocysteine-thiolactone (Sigma), collagen (Horm, Hormon Chemie, München), arachidonic acid (Sigma) and the divalent cation ionophore A 23187 (Boehringer Mannheim).

2.3. Effects of the inhibitors of methyl-transferases on TXB2 production. Platelets in plasma were preincubated at room temperature with DZA, Hcy, or both, or with an equivalent volume of saline. 400 µl aliquots were withdrawn at each indicated time and placed at 37°C under constant stirring (1100 rpm) in an aggregometer. PRP was then challenged with collagen (5 and 25 µg.ml^{-1}) and aggregation was recorded for 3 min. The aliquots were allowed to stand at 37°C for 7 additional minutes. Finally 20 µl were collected and processed for TXB2 determination by RIA according to a schedule previously described (13,14). In another set of experiments, washed platelets were prepared from rat PRP preincubated with the drugs for four hours. Pelleted platelets were resuspended with Tyrode's modified solution, centrifuged and resuspended in the same solution once again, and stimulated, in the presence of Ca^{++}. The platelet activators were collagen (25 µg.ml^{-1}), AA ($5x10^{-6}$M) and A 23187 ($5x10^{-6}$M). Tyrode's solution did not contain the inhibitors, but the blockade of collagen-induced aggregation remained identical four hours after the removal of the drugs. Hence the insensitivity to methyl-transferases inhibitors of platelet production of TXB2 in response to AA and A 23187 cannot be related to reversibility of the blockade.

Table I. Formation of TXB2 by rat PRP stimulated with different agents.

AA		COLLAGEN		A 23,187
10^{-3}M	$5x10^{-4}$M	25 $\mu g.ml^{-1}$	5 $\mu g.ml^{-1}$	$2.5x10^{-5}$M
514±61	433±42	79±8	12±2	12±1
(5)	(4)	(15)	(14)	(6)

Rat PRP was incubated with the activator for 10 min. and TXB2 was measured by RIA as described in the text. Results are expressed in ng of TXB2 per ml PRP (mean±S.E.M.). The figures in brackets denote the number of separate experiments.

2.4. Release of AA from platelet phospholipids. Rat platelets in plasma, at the concentration of about $1.1x10^6$ μl^{-1}, obtained as described above, were incubated in the presence of 1-^{14}C arachidonic acid (Amersham, 60 mCi/mmol^{-1}) previously evaporated to dryness under a stream of nitrogen. The final plasma concentration of labelled AA was 6.7 x 10^{-6}M. After 15 min., PRP was divided into two parts. One of them was incubated with DZA (10^{-3}M) and with Hcy (5 x 10^{-4}M) and the other one with an equivalent volume of saline at room temperature for four hours. The inhibitors of methyl-transferases did not interfere with the incorporation of labelled AA into the platelets. Platelets were then washed twice and resuspended (500,000 μl^{-1}) with Tyrode's modified solution. 2.4 ml samples of the suspension were stimulated with either collagen (25 $\mu g.ml^{-1}$) or AA 23187 (10^{-6}M) under aggregating conditions for 20 min. The reaction was stopped with 5 x 10^{-3}M EGTA and the platelets pelleted by a centrifugation at 4000 xg for 20 min. The supernatant, corresponding to the released radiolabelled arachidonate and metabolites, as well as the pellet containing the residual radioactivity in the phospholipids, were counted after addition of a scintillation mixture.

3 - RESULTS. Since in preliminary experiments formation of TXB2 by rat platelets stimulated with AA, collagen or the calcium ionophore A 23187 was completed within 10 min., this interval was chosen for all experiments. Arachidonic acid was the most potent stimulator of thromboxane synthesis (Table I). By contrast, ADP did not always induce a detectable formation, though aggregation was present.

3.1. Impairment by DZA and Hcy of TXB2 formation by rat PRP in response to collagen. DZA (10^{-4}M) or Hcy ($5x10^{-4}$M) used alone slightly reduce the synthesis of TXB_2 induced by 5 $\mu g.ml^{-1}$ of collagen; this inhibition is however never above twenty per cent. By contrast, the combination of both agents diminishes markedly the formation of TXB_2 in a time-dependent manner. The plateau is reached after 2 hours (Fig. 1).

Similar results are obtained when platelets are stimulated with a five-fold higher concentrations of collagen, except that the onset of inhibition is more delayed (unshown data).

DZA alone, 10^{-3}M, preincubated with rat platelets for four hours, interferes with thromboxane synthesis. The combination of DZA (10^{-3}M) with Hcy ($5x10^{-4}$M) is even more effective, inducing a decrease in TXB_2 formation up to about eighty per cent (Table II).

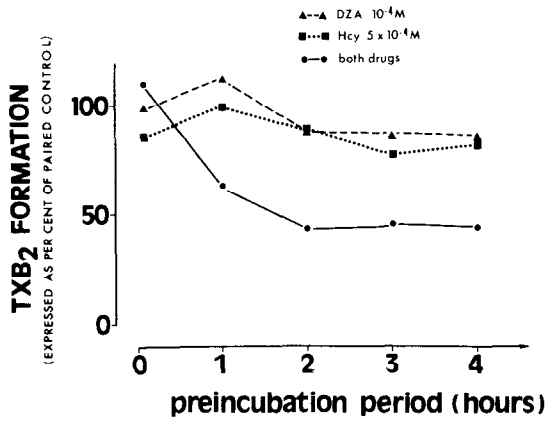

Fig.1. Collagen induced synthesis of TXB2 by rat PRP incubated with DZA and/or Hcy prior to stimulation.

Rat PRP was preincubated at room temperature, either with each drug alone, or with both together, or at last with an equivalent volume of saline. Aliquots were withdrawn after five minutes, 1,2,3,4 hours and challenged with collagen (5 $\mu g.ml^{-1}$) under aggregating conditions. Samples were then processed for RIA for TXB2 as described in the text.

Control TXB2 formation is arbitrarly expressed as 100 per cent to correct variations in the response of platelets from different preparations. Each point is the mean of three to seven separate experiments. The samples of PRP obtained from a given experiment were assayed in the same batch.

From the data gathered in the table II, it clearly appears that Hcy potentiates the inhibitory effect of DZA (10^{-4} or 10^{-3}M).

3.2. Effect of DZA in association with Hcy on the synthesis of TXB2 by rat washed platelets stimulated with collagen, AA or A 23187. When rat platelets are preincubated in plasma for four hours with the combination of DZA (10^{-3}M) and Hcy ($5x10^{-4}$M) and then washed and stimulated with collagen, the thromboxane production is largely diminished. By contrast, when AA or A 23187 are used as platelet activators, the impairment of TXB_2 formation is comparatively very low, or non existent (Table III).

Table II. Effect of two concentrations of DZA, with or without Hcy, on the production of TXB2 by rat PRP challenged with collagen.

	INHIBITORS ADDED				
Collagen	Hcy	DZA 10^{-4}M	DZA 10^{-4}M + Hcy	DZA 10^{-3}M	DZA 10^{-3}M + Hcy
5 $\mu g.ml^{-1}$	82±9 *(6)*	85±6 *(3)*	44±3 *(7)*	55± 4 *(5)*	26±7 *(4)*
25 $\mu g.ml^{-1}$	85±2 *(6)*	111±5 *(3)*	58±6 *(7)*	58±12 *(5)*	18±4 *(4)*

Rats PRP was preincubated for four hours with the inhibitors or with the same volume of saline and challenged with collagen. TXB2 was measured by means of a RIA as described in the text. Results are expressed as per cent of paired controls (mean ± S.E.M.). The figures in brackets denote the number of separate experiments. (Hcy = 5 x 10^{-4}M).

Table III. Effect of DZA + Hcy on TXB2 formation by washed platelets stimulated with collagen, arachidonic acid or A 23,187.

Experiment number	STIMULATING AGENT		
	Collagen 25 $\mu g.ml^{-1}$	AA $5x10^{-6}M$	A 23,187 $5x10^{-6}M$
1	15%	-	69%
2	3%	128%	144%
3	1%	88%	62%
4	1%	115%	162%

Platelets were preincubated for four hours with DZA ($10^{-3}M$) + Hcy ($5x10^{-4}M$) in plasma, washed and stimulated in drug free medium. Thromboxane synthesis was measured as described in the text and is indicated as per cent of paired controls. On the same line are the results of a given experiment. Under these experimental conditions, AA is as potent as collagen to trigger TXB2 formation.

3.3. Effect of DZA + Hcy on the release of labelled AA in response to collagen or to A 2387. The release of (1.^{14}C)AA is markedly inhibited when the platelets are preincubated in the presence of the drug combination and then challenged with collagen (25 $\mu g.ml^{-1}$). On the contrary, when the calcium ionophore A 23187 ($10^{-6}M$) is used the hydrolysis of phospholipids is not affected (table IV).

4 - DISCUSSION. The present investigation was carried out to determine whether the interference of inhibitors of methyl-transferases with platelet aggregation and secretion (3) is related to modifications in the metabolism of AA, and, if so, what step is affected. Two different parameters, thromboxane production from endogenous or exogenous AA, and release of labelled AA in response to two platelet activators, namely collagen and the ionophore A 23187, were studied.

Collagen, the calcium ionophore A 23187 and AA trigger TXB_2 formation by rat PRP as measured by RIA. It is time dependent and inhibited by aspirin

Table IV. Effect of DZA + Hcy on 1.^{14}C AA release from platelet phospholipids.

	Control platelets	Platelets preincubated with DZA + Hcy
Unstimulated platelets	1.4 ± 0.3	1.5 ± 0.3
Collagen 25 $\mu g.ml^{-1}$	11.2 ± 1.9	2.6 ± 0.3
A 23,187 $10^{-6}M$	20.2 ± 0.6	19.8 ± 0.6

Platelets were incubated in plasma with 1.^{14}C AA, in the presence or absence of DZA ($10^{-3}M$) and Hcy ($5x10^{-4}M$) for four hours at room temperature. They were then washed, resuspended in a drug-free medium, stimulated for 20 min., and finally pelleted by centrifugation. Results are expressed as the loss of radioactivity from the phospholipids, in percentage (mean ± SEM, 3 to 5 separate experiments).

(unshown data). We report here that inhibitors of methyl-transferases impair the formation of TXB_2 by rat platelets stimulated with 5 $\mu g.ml^{-1}$ of collagen. This inhibition induced by the combination of DZA ($10^{-4}M$) and Hcy ($5x10^{-4}M$), with respect to its delayed onset and to the requirement of both drugs for the effect to appear, parallels that of platelet aggregation and differs from the blockade of phospholipid methylation. By contrast, a higher amount of collagen (25 $\mu g.ml^{-1}$) triggers aggregation even though thromboxane synthesis is still reduced to approximately fifty per cent of the paired control value. However these high concentrations of collagen always trigger, despite the presence of the inhibitors (DZA $10^{-4}M$ + Hcy $5x10^{-4}M$), the production of more TXB_2 than that obtained in response to 5 $\mu g.ml^{-1}$ of collagen, in the absence of the drugs : respectively 52 $\pm$ 8 and 14 $\pm$ 3 $ng.ml^{-1}$ (mean $\pm$ S.E.M. of the results of seven separate experiments). These amounts of TXB_2 correspond to thromboxane synthesis which may be sufficient to trigger aggregation. Anyway, platelet aggregation in response to high concentrations of collagen under conditions where thromboxane synthesis is impaired has already been reported (15).

HOTCHKISS et al. failed to demonstrate inhibition by DZA plus Hcy of the production of malondialdehyde by washed human platelets stimulated with collagen (16). In their experiments, the incubation of platelets with the drugs lasted only 35 min. and might be insufficient for inhibition, and collagen was used at higher concentrations (360 $\mu g.ml^{-1}$) than in our experiments. On the other hand, species differencies can be ruled out since our findings are similar with either rat or human PRP (unshown data).

The availability of drugs that interfere with methyl-transfer reactions allows us to study their involvement in cell physiology. Even though it is possible that these drugs alter platelet activation through a mechanism different from the inhibition of transmethylation, the synergism between DZA and Hcy, as shown here, is highly suggestive of the contrary (17). It is also unlikely that an increase in cellular cyclic AMP content occurs as has been reported for lymphocytes (18), since such an increase would inhibit the response of platelets irrespective of the kind of activator.

In an attempt to localize the methylation-dependent step of the activation of thromboxane synthesis, the response to three different platelet stimulating agents was investigated. Collagen is thought to interact with the external leaflet of the membrane; then the signal is transduced to the phospholipase enzyme, leading to the release of AA. The ionophore A 23187 induces platelet aggregation, the release reaction (19) and thromboxane synthesis (20). It initiates phospholipase A_2 activity, and therefore leads to AA release, by promoting Ca^{++} entry into the cell (21,22). Finally, aggregation triggered by exogenous AA (23) is due to its conversion to endoperoxides and then to thromboxane A_2 (4). Hence, these three platelet activators act at different

levels of platelet metabolism of AA; A 23187 and AA bypassing the plasma membrane receptor step.

With respect to its sensitivity to DZA plus Hcy, the platelet thromboxane formation in response to collagen can be dissociated from that occurring in response to AA and to the calcium ionophore. Thus TXB_2 formation in response to exogenous AA is not affected, but is reduced in collagen stimulated platelets which metabolize their own AA. This result suggests that cyclo-oxygenase and thromboxane synthetase activities are only marginally impaired by the inhibitors, if at all. The site of inhibition of platelet activation therefore takes place before the cyclo-oxygenase level. Since DZA and Hcy fail to impair TXB_2 formation when platelets are challenged with the calcium ionophore A 23187, we conclude that a methyl-transfer reaction is involved in an early calcium-independent step preceding AA release in activated platelets, before the intervention of phospholipase A_2. Our findings can be compared to those of MORITA et al., who found that histamine released by human basophils challenged with the calcium ionophore is not sensitive to methyl-transferase inhibitors whereas the release mediated by IgE is blocked (17). Similar results were reported with rat peritoneal mast cells (24).

According to our results (3) and to those of KANNAGI et al. (25), HOTCHKISS et al. (16), SHATTIL et al. (26), it is unlikely that platelet activation is directly related to phospholipid methylation or that phosphatidylcholine synthesized from phosphatidylethanolamine through the N-methylation pathway is a preferential substrate for phospholipases. Consequently, if the observations reported here strongly suggest the association of a methylation reaction with platelet aggregation and phospholipase A_2 activation, its precise nature remains to be determined.

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