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MODULATION OF PROSTACYCLIN/THROMBOXANE FORMATION BY MOLSIDOMINE DURING PLATELET–ENDOTHELIAL CELL INTERACTIONS

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Abstract—Platelet and endothelial cell metabolism of both exogenous and endogenous arachidonic acid, via the cyclooxygenase pathway, was evaluated according to thromboxane and prostacyclin formations. This was investigated in platelets, endothelial cells alone or during their interactions, in the presence or absence of SIN-1, the active anti-anginal metabolite of molsidomine. This revealed that, in contrast to the generation of thromboxane, which was decreased in the presence of endothelial cells, especially from endogenous arachidonate, that of prostacyclin increased under basal conditions as well as from endogenous arachidonate to a lesser extent. SIN-1 reduced thromboxane formation solely from endogenous arachidonate, and this was more pronounced when both cell populations interacted. In contrast, SIN-1 failed to decrease prostacyclin formation, which would emphasize its anti-aggregating potential. We conclude that the liberation of arachidonic acid leading to prostanoid synthesis may be differently regulated in platelets and endothelial cells, and that molsidomine might be a potential anti-aggregating drug in altering specifically thromboxane formation.

When stimulated by thrombin or incubated with arachidonic acid (AA), platelets (PL) and endothelial cells (EC) produce various AA metabolites, in particular thromboxane A2 (TxA2) and prostacyclin (PGI2), respectively, which are the major derivatives. TxA2 is a potent pro-aggregating and vasoconstricting agent, whereas PGI2 has completely opposite activities. These mediators are short lived molecules and are readily degraded into stable metabolites in biological media, namely thromboxane B2 (TxB2) and 6-keto-prostaglandin F1α (6K-PGF1α) [1, 2].

It has been also suggested that platelets may provide a substantial amounts of prostaglandin H2 (PGH2) to endothelial cells during the interactions between both cell populations, then increasing PGI2 production [3, 4].

Molsidomine (*N*-ethoxy-carbonyl-3-morpholino-sydnonimine), a nitric oxide generator, is a vasodilator [5] as well as an inhibitor of platelet activation [6]. Its bioactive metabolite, SIN-1 or 3-morpholino-sydnonimine exhibits anti-aggregating activities *in vitro*. The mechanism of antiplatelet activity remains unclear, however, studies on AA oxygenation revealed that SIN-1 fails to inhibit the oxygenation of exogenous AA whereas it is able to depress that of endogenous AA when thrombin is the stimulator [7], suggesting that SIN-1 might exhibit an anti-phospholipase activity.

In this study, the effect of SIN-1 on AA metabolism in platelets and endothelial cells alone or in combination was examined.

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MATERIALS AND METHODS

Materials and reagents. Collagenase type II, human fibronectin and culture media were purchased from Intermed Biopro (Strasbourg, France). Culture glassware were obtained from Falcon Becton-Dickinson (Grenoble) and AB human serum from the local blood transfusion centre. Antibiotics provided from Gibco Bio-Cult Ltd (Paisley, U.K.). [^{3}H] labelled prostaglandins were yielded from Amersham International, (Amersham, U.K.). Human thrombin and arachidonic acid were furnished by Sigma (St Louis, MO). Other reagents and organic solvents were obtained from Prolabo, Paris and from SDS (Peypin, France).

Cell cultures. Endothelial cells from human umbilical veins were cultivated according to the method of Jaffe *et al.* [8], slightly modified. Briefly, cells obtained by collagenase treatment were seeded in Medium 199 containing 100 U/ml streptomycin, 100 μg/ml penecillin, 0.25 μg/ml amphotericin B and suplemented with 20% pooled AB human sera. Cells were cultivated in 24 wells plates coated with human fibronectin. Confluent cell monolayers were obtained after five or six days.

Blood platelet preparation. Human blood from healthy donors was taken onto acid citric-citrate-dextrose (ACD). Blood was centrifuged at 100 *g* for 15 min, and platelet-rich plasma was decanted and acidified to pH 6.4 with 0.15 M citric acid. After centrifugation of PRP at 900 *g* for 10 min, platelet poor plasma was removed, and platelets were resuspended into Tyrode HEPES Buffer (THB), pH 7.35 [9] and adjusted to 3×10^8/ml.

Experimental design and sample preparation. Confluent endothelial cell monolayers, washed once with

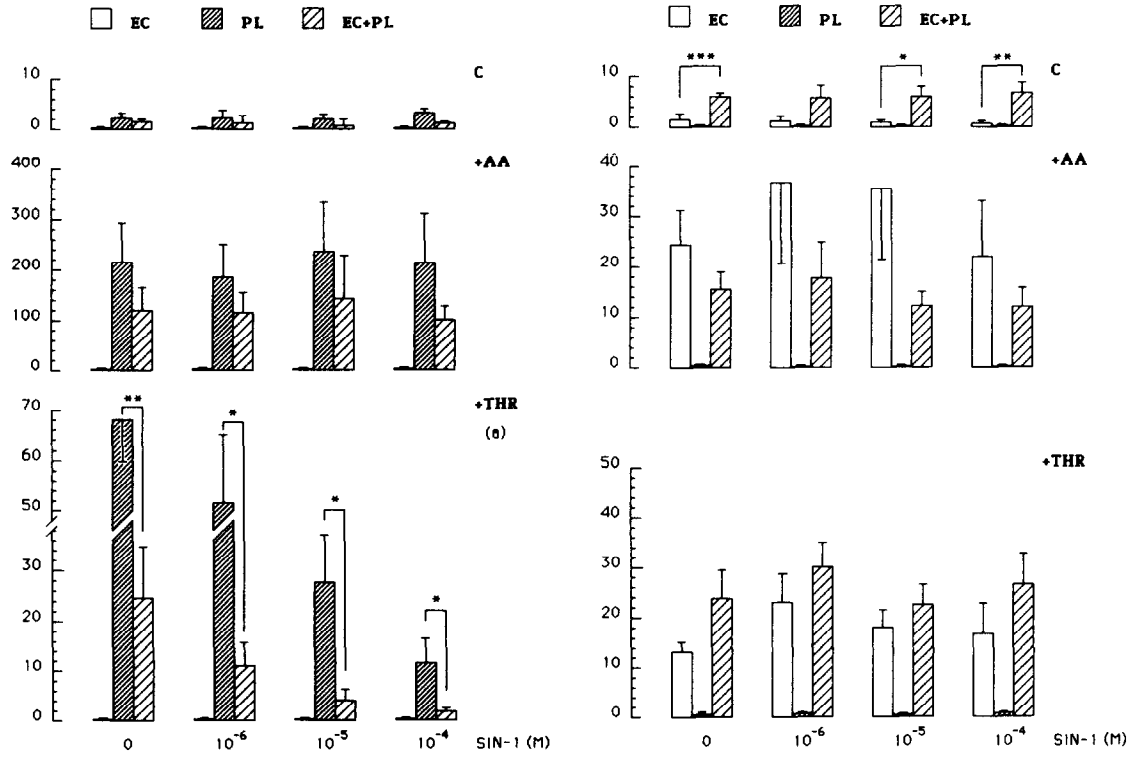
THB, were incubated with 400 μl of THB or 400 μl of platelets. SIN-1 or its vehicle was then pre-incubated for 2 min at 37° before adding 10 μM arachidonic acid or 0.1 U/ml human thrombin for 5 min under gentle stirring. Control cells received THB instead. Incubations were terminated by acidification to pH 3 with 1 N HCl and prostanoids were extracted with 2 × 5 ml of chloroform: acetonitrile (72/28, v/v) containing 50 μM butylated hydroxytoluene, 2 nCi [^{3}H]-TxB2 and 2 nCi [^{3}H]-6K-PGF1α for yield recovery of endogenous prostanoids. 6K-PGF1α and TxB_2 were then purified by thin-layer chromatography (TLC) using the organic phase of the mixture: ethylacetate/isooctane/acetic acid/water (110/50/20/100) as eluent. These prostanoids were localised according to the migration of 20 nCi [^{3}H]-PGF2α(R_f: 0.33) added before the TLC step; this localization was done with a radiochromatograph (Berthold, Siléna). Gel areas corresponding to TxB2 (R_f: 0.44) or 6K-PGF1α (R_f: 0.28) were scraped off and respectively extracted with diethylether: methanol (9:1) and acetonitrile:water (9:1). Routinely, the yield recovery of prostanoids was about of 86% for TxB2 and of 72% for 6K-PGF1α. These products were then quantitated by radioimmunoassays [10].

RESULTS AND DISCUSSION

Platelet–endothelial cell interactions

TxB2 and 6K-PGF1α, the main metabolites of platelet and endothelial cell cyclooxygenase, respectively, were measured after incubation with exogenous AA or thrombin. The presence of the second cell population affected markedly the formation of the main cyclooxygenase product in the first one. Endothelial cells inhibited TxB2 formation in unstimulated and AA- or thrombin-stimulated platelets. However, solely the decreased TxB2 production when stimulated by thrombin was significant. Since phospholipase activity is involved in TxB2 generation induced by thrombin, but not by exogenous AA, the significant decrease is likely to be due to platelet cAMP raised by endothelial cell-derived prostacyclin [11, 12].

On the contrary, endothelial cell production of prostacyclin was significantly enhanced by platelets in the absence of specific stimulation, in agreement with previous data [3, 4]. However, such enhancement was not significant when cells were stimulated by thrombin. Prostacyclin production was even reduced, although not significantly, when AA was the stimulating agent. The reason of such a reduction



Figs 1 and 2. TxB2 (1) and 6K-PGF1α (2) synthesized by isolated platelets (PL) or endothelial cells (EC) or both during their interaction (EC+PL), after 2 min incubation with the indicated concentrations of SIN-1, and stimulation by 10 μM arachidonic acid (AA) or 0.1 U/ml thrombin (THR) for further 5 min. C represents control unstimulated cells. TxB2 and 6K-PGF1α were measured by RIA after purification by TLC (see Materials and Methods). Results (means ± SEM) provide from five different batches of cells. Statistical analysis were evaluated by the Student's *t* test: * $P \leq 0.05$; ** $P \leq 0.025$; *** $P \leq 0.01$. (a) In the presence of SIN-1, the inhibition of TxB2 production by thrombin-stimulated platelets was significant (variance analysis: $P \leq 0.05$ for PL alone, $P = 0.05$ in EC + PL).

is not clear. It could, however, reflect the decreased PGH2 transfer from platelets to endothelial cells, as judged by the reduction of thromboxane formation (Fig. 1).

Effects of SIN-1 on thromboxane formation

SIN-1 did not affect the basal production of TxB2 in platelets alone or platelets in the presence of endothelial cells. Similarly, TxB2 remained constant in both situations from exogenous AA. In contrast, TxB2 produced under thrombin stimulation was significantly reduced by SIN-1 treatment in a dose-dependent manner (Fig. 1). Interestingly, the decrease was more pronounced when endothelial cells were present during platelet stimulation by thrombin. Thromboxane formation in the presence of SIN-1 10^{-4} M was even completely abolished. The IC_{50} evaluated with the three concentrations of SIN-1 used was 8.5×10^{-6} M and 4.8×10^{-7} M in the absence and presence of endothelial cells, respectively. This indicates that SIN-1 would be around 18-fold more potent for inhibiting thrombin-induced TxB2 formation in the presence of endothelial cells, which would be of therapeutic relevance. The inhibition of platelet TxB2 formation when induced by thrombin, but not exogenous AA, confirms what we found earlier, e.g. that both cyclooxygenase and lipoxygenase products were decreased under thrombin stimulation [7]. Such inhibition suggests that SIN-1 may act at the phospholipase rather than at the oxygenase level. The mechanism of action is not known but one may speculate that the increase of cyclic GMP induced by SIN-1 in platelets [6] would be responsible for the inhibition observed. As a matter of fact, cyclic GMP has been reported to decrease thrombin-induced phosphoinositide breakdown, protein phosphorylation [13] and calcium mobilization [14], then allowing to reduce phospholipase A2 activity [15].

Effects of SIN-1 on prostacyclin formation

In contrast to what was observed on thromboxane inhibition, SIN-1 did not reduce prostacyclin formation induced by thrombin. No reduction could be detected in thrombin or AA-induced prostacyclin generation nor in stimulated cells (Fig. 2). Even, prostacyclin tended to increase in the presence of the lowest concentrations of SIN-1. This was not significant but agrees with preliminary data from Astoin *et al.* [16]. No explanation could be given for this tendency, although SIN-1 being a nitric derivative, we may speculate that it would mimic the stimulation of prostacyclin synthesis, already described with nitroglycerin by Levin *et al.* [17], although this is controversial [18].

Nitric derivatives enhance cGMP content in endothelial cells but not that of cAMP [19]. Whereas such an increase does not affect PGI2 formation in endothelial cells, it induces an inhibition of TxA2 in platelets. In such a way, SIN-1 reproduces the effects of the endothelial-derived relaxing factor of EDRF [20, 21].

CONCLUSION

SIN-1, the active metabolite of molsidomine, an anti-anginal drug, may alter specifically the balance of platelets–endothelium in favoring the anti-aggregating side by decreasing thromboxane but not prostacyclin formation from endogenous stores of arachidonic acid. This also reveals that the mechanism leading to the liberation of endogenous arachidonic acid might be quite different in platelets and endothelial cells.

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