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Influence of Platelet Aggregation Modulators on Cyclic AMP Production in Human Thrombocytes

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Background: Cyclic AMP is a powerful inhibitor of platelet aggregation. In the present study we examined the effect of platelet aggregation modulators on cyclic AMP content in human thrombocytes. Of the agents we tested, lactoferrin, wortmannin, quercetin and amiloride are platelet aggregation inhibitors, whereas ouabain is a platelet activator.

Aim: To investigate the effect of lactoferrin, wortmannin, quercetin, ouabain and amiloride applied alone and in combination with lactoferrin on cyclic AMP production in human platelets.

Materials and methods: 'Direct cAMP ELISA kit' was used for cyclic AMP determination.

Results: The studied modulators, individually or in combination, stimulate cyclic AMP production in platelets.

Conclusions: Wortmannin, quercetin, ouabain and amiloride increase cyclic AMP level in human platelets. Lactoferrin also increases cyclic AMP level, but the effect is statistically insignificant, which shows that lactoferrin does not participate directly in the cyclic AMP signaling. Lactoferrin additionally augments the stimulating action of wortmannin, quercetin, ouabain and amiloride on the cyclic AMP production. This probably shows a synergetic interference of lactoferrin in signal pathways along with phosphatidylinositol 3-kinase (wortmannin), quercetin (control over protein kinases, the redox state of the cell and ion transport), ouabain and amiloride (mechanisms of ion transport and phosphorylation).

BACKGROUND

Cyclic AMP (cAMP) is a powerful inhibitor of platelet aggregation.[1] At least two signal-generating systems are involved in the actions of various hormonal factors in human platelets – the adenylate cyclase system and the phosphoinositide-metabolizing pathway. Apparently, there are several mutual interactions between these two signal-generating systems.[2] Endothelial prostacyclin and nitric oxide potently inhibit platelet functions. Prostacyclin and nitric oxide actions are mediated by platelet adenylate- and guanylate cyclases, which synthesize cAMP and cyclic GMP (cGMP), respectively. Cyclic nucleotides stimulate cAMP-dependent protein kinase (protein kinase A [PKA]) and cGMP-dependent protein kinase (protein kinase G [PKG]I) to phosphorylate a broad panel of substrate proteins. Substrate phosphorylation results in the inactivation of small G-proteins of the Ras and Rho families, inhibition of the release of Ca^{2+} from intracellular stores, and modulation of actin cytoskeleton dynamics.[1] There is evidence for a cross-talk between cAMP signaling and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) pathways, and cell-specific opposite effects of signal proteins from the cAMP-dependent signaling complex have been found[3], but no research has been done on platelets.

In a previous study, we have investigated the

Abbreviations used in this article

cAMP: cyclic adenosine monophospate; cGMP: cyclic guanosine monophospate; PKA: protein kinase A; PKG: protein kinase G; Ras, Rho: small G proteins; PI3K: phosphatidylinositol 3-kinase; PKB: protein kinase B; Lf: lactoferrin; Wort: wortmannin; Querc: quercetin; Ouab: ouabain; Amil: amiloride; EDTA: ethylenediaminetetraacetic acid; PBS: phosphate-buffered saline; Na(+)/K(+)-ATPase: sodium-potassium adenosine triphosphatase; NHE: Na+/H+-exchanger (sodium–hydrogen exchanger); KRDS: a tetrapeptide from human lactotransferrin; TX: thromboxane; GP IIb/IIIa: glycoprotein IIb/IIIa; RGDS: a tetrapeptide from human fibrinogen; PMA: 4 beta-phorbol-12-myristate-13-acetate; PKC: protein kinase C; MAPK: mitogen-activated protein kinase; Src: non-receptor protein tyrosine kinase

influence of signal transduction modulators, which have a proven effect on the platelet aggregation and thrombocyte glycolysis as well.[4,5] In the current study we use the same modulators – lactoferrin (Lf), wortmannin (Wort), quercetin (Querc), ouabain (Ouab) and amiloride (Amil). Lf, Wort, Querc and Amil are inhibitors of platelet aggregation[6-9], whereas Ouab activates platelets[10].

There is evidence that Querc exerts its biological effects on platelets through a change in cAMP content[8], but still, there is no thrombocyte related research for Lf, Wort, Amil and Ouab.

Querc significantly elevates cAMP level in collagen-stimulated platelets.[8] Moreover, Querc promotes neurite growth through enhancing intracellular cAMP level in N1E-115 cells.[11]

Lf elevates cAMP level in M21 human melanoma cell line.[12]

Wort treatment of *Aspergillus parasiticus* D8D3 increased intracellular cAMP levels up to 25-fold.[13]

Ouab has been shown to increase cAMP levels in rat optic nerve astrocytes[14], rat brain[15], and cultured renal papillary-collecting tubule cells[16].

AIM

The aim of the study was to explore the effect of Lf, Wort, Querc, Ouab and Amil, applied individually and in combination with Lf, on cAMP production in human platelets.

MATERIALS AND METHODS

Isolation of platelets

Platelets were obtained from human blood of up to 10 healthy donors using sodium citrate. Platelet-rich plasma was separated by centrifugation at 400 g for 10 min, followed by centrifugation for 15 min at 1100 g to obtain platelets.[17] After washing three times with 1 mM EDTA/phosphate-buffered saline (PBS), pH 7.4, and once with PBS, pH 7.4, platelets were suspended in 50 mM PBS[17] to a final concentration of 2.5×10^6 cells/ml. All the procedures described were carried out at room temperature.

cAMP determination

2.5×10^6 cells/ml were treated with modulators for 30 min at 37°C.[18] The untreated platelets (2.5×10^6) but incubated under the same conditions were regarded as control. The modulators were applied separately and in combination with Lf in the following concentrations: 100 nM Lf; 50 nM and 1 μM Wort; 1.5 μM and 100 μM Querc; 3 μM and 0.5 mM Ouab; 100 μM and 1 mM Amil; 100 nM Lf + 1 μM Wort; 100 nM Lf + 100 μM Querc; 100 nM Lf + 3 μM Ouab and 100 nM Lf + 100 μM Amil. After incubation, platelets were separated by centrifugation at 2500 rpm for 15 min and treated with 0.1 M HCl for 20 min at room temperature to ensure uniform lysis. After centrifuge at 4300 rpm for 10 min and sedimentation of the cell residues, the acquired supernatant is separated for quantitative determination of cAMP, which is performed according to the application protocol of 'Direct cAMP ELISA kit' by Enzo Life Sciences.

Statistical analysis

Comparison of quantitative variables was performed by independent T-test. The adopted significance level was $p < 0.05$.

RESULTS

The results are presented in **Table 1**, where it is obvious that all the modulators, applied separately in the listed concentrations, as well as in combination with 100 nM Lf, stimulate significantly cAMP production (from 2.16 up to 8.24 times). The lowest and most statistically insignificant stimulus of

Table 1. cAMP (pmol/mL) in the presence of modulators

Modulators (n=8)	cAMP, pmol/mL ($\overline{X}\pm SD$)	p
Without agent	0.025 ± 0.022	0
50 nM Wort	0.054 ± 0.016	< 0.01
1 μM Wort	0.057 ± 0.010	< 0.05
1.5 μM Querc	0.206 ± 0.008	< 0.001
100 μM Querc	0.072 ± 0.016	< 0.001
3 μM Ouab	0.078 ± 0.023	< 0.001
0.5 mM Ouab	0.122 ± 0.003	< 0.001
100 μM Amil	0.072 ± 0.008	< 0.001
1 mM Amil	0.178 ± 0.010	< 0.001
100 nM Lf	0.037 ± 0.021	> 0.05
100 nM Lf + 1 μM Wort	0.075 ± 0.024	< 0.001
100 nM Lf + 100 μM Querc	0.143 ± 0.007	< 0.001
100 nM Lf + 3 μM Ouab	0.150 ± 0.003	< 0.001
100 nM Lf + 100 μM Amil	0.136 ± 0.010	< 0.001

n – number of repetitions; $\overline{X}$ – mean value, SD – standard deviation; p – level of significance, Wort – wortmannin, Querc – quercetin, Ouab – ouabain, Amil – amiloride, Lf – lactoferrin.

cAMP production (1.48 times), as compared to the control, is observed solely with the separate addition of 100 mM Lf.

It is worth noting, that 50 nM and 1 μM Wort stimulate equally cAMP production **(Table 1, Fig. 1A)**; the low Querc concentration (1.5 μM) causes an almost 3-fold increase of cAMP production as opposed to the high one (100 μM), while the low concentrations of Ouab (3 μM) and Amil (100 μM) have less effect of stimulation than high ones (0.5 mM and 1 mM) **(Table 1, Fig. 1A)**.

Moreover, co-administration of 100 nM Lf to selected concentrations of the other modulators shows an additional increase of cAMP production **(Table 1, Fig. 1B)**, which suggests a synergism between Lf and each of the other agents.

DISCUSSION

Our results show that Wort, Ouab and Amil increase cAMP level in human platelets **(Table 1, Fig. 1A)**, for which there is no data in the literature. Moreover, the increase of cAMP level in platelets under the influence of Querc **(Table 1, Fig. 1A)** that we found corresponds to the effect of this particular modulator on the same type of cells, as observed by Oh et al. (2012).[8]

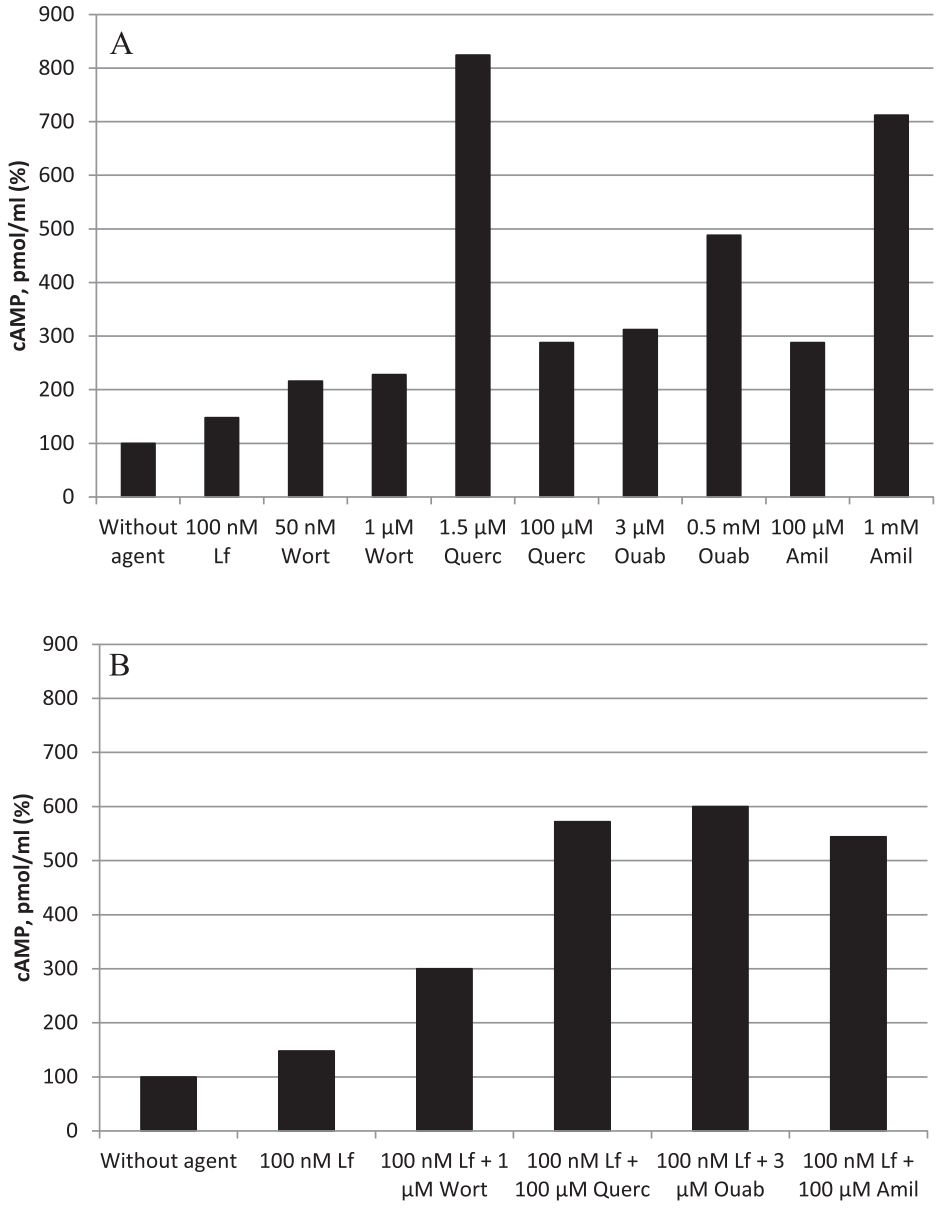

Figure 1. cAMP (%), under the influence of modulators, applied alone (A) and in combination with Lf (B). cAMP concentration in the absence of an agent is accepted as 100 %. Lf: lactoferrin, Wort: wortmannin, Querc: quercetin, Ouab: ouabain, Amil: amiloride.

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Wortmannin

Wort is a specific inhibitor of PI3K.[19] The amount of the produced cAMP doubles in the presence of Wort **(Table 1, Fig. 1A)**. This shows that PI3K is a negative regulator of adenylate cyclase in platelets, which coincides with the literary data for a cross-talk between cAMP signaling and PI3K/PKB pathways[3], as well as with the proven opposite effects of cAMP (platelet aggregation inhibitor) and PI3K (coagulation stimulator).[20]

Ouabain

Ouab is responsible for the increase of free cytosolic Ca^{2+} in platelets[10] – an event, leading to their activation, as well as for the increase of cAMP in other cell types.[14-16] Ouab may cause cell membrane depolarization, mediated by an increase of cAMP content, and this effect of Ouab is augmented in the presence of theophylline.[21] In contrast, cAMP regulates Ouab sensitive Na(+)/K(+)-ATPase activity in SH-SY5Y human neuroblastoma cells.[22] Obviously there is a correlation between Ouab and cAMP signals, but cAMP increase in the presence of Ouab **(Table 1, Fig. 1A)** is probably related to regulatory effects, which do not affect directly the platelet aggregation, because Ouab is a procoagulant, while cAMP is an anticoagulant.

Amiloride

There is no literary data for the cell signals, used by Amil, during platelet aggregation inhibition. According to our results, Amil increases cAMP content in platelets **(Table 1, Fig. 1A)**, which coincides with the observed anticoagulation effect of Amil.[9] It could be assumed that Amil activates a cAMP-PKA-dependent phosphorylation of serine residues, which leads to Na^+/H^+-exchanger (NHE) inhibition[23], and, consequently – to suppression of the platelet functions.[9]

Lactoferrin

Lf has platelet surface receptors[17] and is a natural anticoagulant[6]. Therefore, the interest of the researchers is aimed at clarifying the signal pathways with its participation. KRDS, a tetrapeptide from human lactotransferrin, inhibits thrombin-induced platelet aggregation, secretion and thromboxane (TX) synthesis without interfering with phospholipase C beta activation. KRDS strongly inhibits the tyrosine-phosphorylated substrates, in particular two 100-105 kDa substrates, which are related to glycoprotein IIb/IIIa (GP IIb/IIIa) activation and platelet aggregation. The absence of TX synthesis, observed in the presence of KRDS, could be due to the inactivation of cytosolic phospholipase A_2, since the latter needs tyrosine phosphorylation to be activated, thus explaining the inhibitory action of KRDS on platelet functions.[24] Other authors find, that KRDS and RGDS inhibit the 4 beta-phorbol-12-myristate-13-acetate (PMA)-induced aggregation and fibrinogen binding, while proteins were normally phosphorylated, which suggests an interference of protein kinase C (PKC) signals, leading to platelet aggregation.[25] There is no data for the role of Lf in cAMP-dependent signals in platelets. According to our data, there is a tendency of cAMP increase in the presence of Lf **(Table 1, Fig. 1)**. Casein kinase 2 is known to phosphorylate Lf via a PKA-dependent pathway, which leads to a decrease of its biological activity.[26]

Effect of the simultaneous action of agents and Lf

The separate application of Lf increases cAMP level **(Fig. 1)**, but the effect is statistically insignificant **(Table 1)**, which shows that Lf does not participate directly in the cAMP signaling. On the other hand, Lf additionally augments the stimulating action of Wort, Querc, Ouab and Amil on the cAMP production **(Table 1, Fig. 1B)**. This probably shows a synergetic interference of Lf in signal pathways along with PI3K (Wort), Querc (control over protein kinases, the redox state of the cell and ion transport), Ouab and Amil (mechanisms of ion transport and phosphorylation).

Flavonoids, such as Querc, are competitive inhibitors of many kinases – PI3K, Akt/PKB, tyrosine kinase(s), PKC, and mitogen-activated protein kinase (MAPK).[27] Up to now, there is no data reported whether or not Lf and flavonoids are using common signal pathways. Querc, as a redox system, is part of the electron transport chain, reducing extracellular ferric cyanide and at physiological conditions is an intercellular substrate of a transplasma oxidoreductase.[28] Some data exist about the participation of Lf in such a chain.[29] Donating electrons are necessary for the reduction of Fe^{3+} to Fe^{2+} during Lf binding with membrane receptors.[30] Flavonoids could facilitate the Lf binding because of their iron ion-reducing ability.[31]

The cell signals, used by Lf and PI3K, have common units – for instance, participants in MAPK-pathways.[32,33] It could be assumed that Lf increases the effect of Wort as PI3K competitor for substrates of protein kinases **(Table 1, Fig. 1B)**.

Ouabain-induced endocytosis of the Na(+)/K(+)-

ATPase depends on the activation of the Src kinase, clathrin-coated pits formation, and caveolin-1 (the major component of caveolae).[34] Lf carries signals along with Scr[35] and could enhance an Ouab-dependent pathway, leading to an increase of cAMP content **(Table 1, Fig. 1B)**.

CONCLUSIONS

In the present study we show that Wort, Querc, Ouab and Amil increase significantly the cAMP level in human platelets. The separate application of Lf also increases cAMP level, but the effect is statistically insignificant. This shows that Lf does not participate directly in the cAMP signaling. On the other hand, Lf additionally augments the stimulating action of Wort, Querc, Ouab and Amil on the cAMP production. This probably shows a synergetic interference of Lf in signal pathways used by Wort, Querc, Ouab and Amil.

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CONFLICT OF INTEREST

There is no conflict of interests.

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Влияние модуляторов агрегации тромбоцитов на циклический АМФ

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Введение: Циклический АМФ является мощным ингибитором агрегации тромбоцитов. В настоящем исследовании мы изучили влияние модуляторов агрегации тромбоцитов на содержание циклического АМФ в тромбоцитах человека. Из тестируемых агентов лактоферрин, вортманнин, кверцетин и амилорид являются ингибиторами агрегации тромбоцитов, тогда как уабаин является активатором тромбоцитов.

Цель: Исследовать влияние лактоферрина, вортманнина, кверцетина, уабаина и амилорида, применяемых самостоятельно и в сочетании с лактоферрином на циклическое производство АМФ в тромбоцитах человека.

Материалы и методы: «Набор ELISA для прямого анализа цАМФ» использовался для определения циклического АМФ.

Результаты: Изученные модуляторы, самостоятельно или в комбинации, стимулируют циклическое производство АМФ в тромбоцитах.

Заключение: Вортманнин, кверцетин, уабаин и амилорид увеличивают циклический уровень АМФ в тромбоцитах человека. Лактоферрин также увеличивает циклический уровень АМФ, но эффект статистически незначителен,

Ключевые слова: циклический АМФ; тромбоциты человека; лактоферрин

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что показывает, что лактоферрин не участвует непосредственно в циклической передаче сигналов АМФ. Лактоферрин дополнительно усиливает стимулирующее действие вортманнина, кверцетина, уабаина и амилорида на циклическое производство АМФ. Это, вероятно, показывает синергетическую интерференцию лактоферрина в сигнальных путях наряду с фосфатидилинозитол-3-киназой (вортманнином), кверцетином (контроль над протеинкиназами, окислительно-восстановительным состоянием клетки и переносом ионов), уабаин и амилорид (механизмы переноса ионов и фосфорилирования).