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The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts

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P2Y12, a G protein-coupled receptor that plays a central role in platelet activation has been recently identified as the receptor targeted by the antithrombotic drug, clopidogrel. In this study, we further deciphered the mechanism of action of clopidogrel and of its active metabolite (Act-Met) on P2Y12 receptors. Using biochemical approaches, we demonstrated the existence of homooligomeric complexes of P2Y12 receptors at the surface of mammalian cells and in freshly isolated platelets. *In vitro* treatment with Act-Met or *in vivo* oral administration to rats with clopidogrel induced the breakdown of these oligomers into dimeric and monomeric entities in P2Y12 expressing HEK293 and platelets respectively. In addition, we showed the predominant association of P2Y12 oligomers to cell membrane lipid rafts and the partitioning of P2Y12 out of rafts in response to clopidogrel and Act-Met. The raft-associated P2Y12 oligomers represented the functional form of the receptor, as demonstrated by binding and signal transduction studies. Finally, using a series of receptors individually mutated at each cysteine residue and a chimeric P2Y12/P2Y13 receptor, we pointed out the involvement of cysteine 97 within the first extracellular loop of P2Y12 in the mechanism of action of Act-Met.

mechanism of action | platelet | antiaggregant

Many G protein-coupled receptors (GPCRs) have been shown to assemble as homodimers, heterodimers, as well as larger oligomers (1, 2). The existence of such oligomeric entities raises questions as to their functional consequences as well as their physiological relevance. Heterologous expression systems have provided a variety of answers concerning ligand-dependent regulation of GPCR oligomeric states. Ligand binding, depending on the GPCR studied, can promote (3–10) or inhibit (11–13) dimer formation, as well as having no effect on preexisting constitutive homo- or heterodimers (14–25). The fact that heterodimerization may alter the pharmacological properties of a GPCR along with its internalization and signal transduction behavior is of critical importance (26, 27).

Clustering, even for nonheptahelical receptors, now appears as a common feature of cell signaling. Specialized structures such as clathrin-coated pits, caveolae, and lipid rafts contain high concentrations of signaling molecules. Rafts represent dynamic assemblies of proteins and lipids, mostly sphingolipids and cholesterol (28, 29). Proteins such as glycophosphatidylinositol-anchored proteins, nonreceptor tyrosine kinases, Gα subunits of heterotrimeric G proteins, and palmitoylated proteins appear to localize to these microdomains (30). In addition, recent studies have shown that partitioning of proteins in and out of rafts can depend on their state of activation or dimerization (31–33). A variety of GPCR have also been identified in caveolae or rafts. These include α and β-adrenergic receptors (34, 35), adenosine A1 receptor (36), angiotensin II type I receptor (37), muscarinic receptor (38), EDG1 receptor (39), bradykinin B1 and B2 receptors (33, 40, 41), endothelin receptor (43), rhodopsin (44), and *N*-formyl peptide receptor (45). In the majority of cases, this location was found to be sensitive to ligand stimulation, clustering in raft being either increased or decreased.

Platelets are key elements in hemostasis and thrombosis. Diverse agonists are known to activate platelet aggregation and fibrinogen binding to the subsequently activated integrin GPIIb-IIIa complex. In this process, ADP is of particular importance, because it is released by damaged cells and activated platelets, thus enhancing the action of many platelet activators. ADP mediates platelet aggregation through its binding to two GPCRs P2Y1 and P2Y12, acting together to achieve complete aggregation (46). P2Y12 is expressed in platelets, megakaryocytes, and neuronal cells (47). Upon activation, P2Y12 triggers a cascade of signaling events including adenylyl cyclase inhibition and PI3K activation (48). P2Y12 knockout mice are particularly protected against thrombosis (49). In humans, two genetic P2Y12 deficiencies have been described, associated with a hemorrhagic phenotype and a pronounced impairment of ADP-induced platelet aggregation (50, 51).

P2Y12 is the target of clopidogrel, a well known antithrombotic compound that has demonstrated its efficacy and favorable safety profile in an extensive clinical program, by preventing ischemic events such as cardiovascular death, myocardial infarction, or stroke in atherothrombotic patients, on top of standard treatment (52). Clopidogrel does not, by itself, exhibit direct antiaggregant activity *in vitro*. Indeed, *in vivo* studies have demonstrated that clopidogrel has to undergo hepatic metabolization to obtain an active metabolite (53). This active metabolite (Act-Met) has been isolated, and its structure has been elucidated (54). It contains a free thiol function, and its activity is lost when the thiol is derivatized (55), suggesting its possible interaction with cysteine-containing sequences. *In vitro*, Act-Met inhibits the binding of 2MeS-ADP to platelets and ADP-induced aggregation of platelets. In a recent study, Act-Met was found to inhibit the binding of 2MeS-ADP to P2Y12 (56). This inhibition was shown to be irreversible and selective for P2Y12 (57, 58).

Here, we have further determined the mechanism of action of clopidogrel and of its active metabolite on the P2Y12 receptor. We have found that these compounds act on this receptor by an original mechanism, by interfering with P2Y12 assembly and its localization in lipid rafts. This allowed us to demonstrate the importance of oligomerization and membrane localization on the function of this

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Abbreviations: GPCR, G protein-coupled receptor; HA, hemagglutinin.

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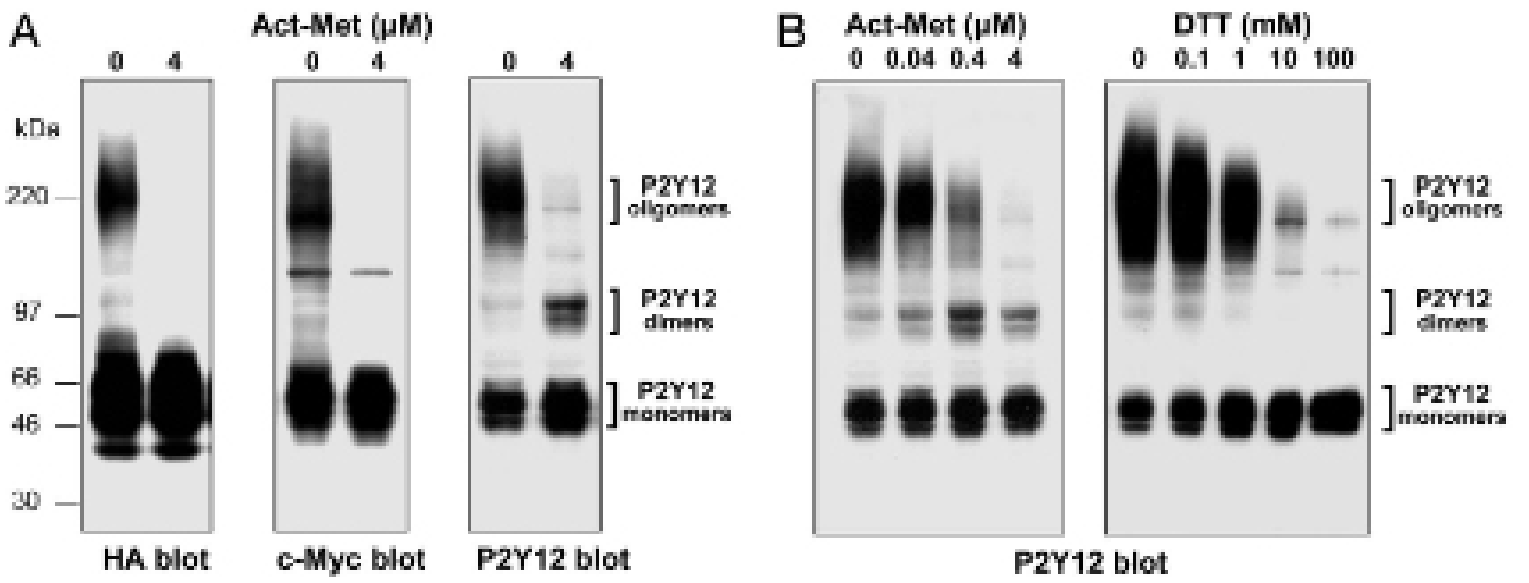

Fig. 1. Disruption by Act-Met of oligomeric forms of P2Y12 expressed in HEK293 cells. HEK293 cells coexpressing HA- and cMyc-tagged P2Y12 receptors were incubated for 1 h at room temperature with the indicated concentration of Act-Met, and whole-cell extracts were prepared in octyl-glucoside buffer, as described in *Materials and Methods*. (*A*) Cell extracts were subjected to SDS-PAGE under nonreducing conditions followed by anti-HA, anti-cMyc or anti-Cterm P2Y12 immunoblotting. (*B*) Extracts from Act-Met-treated cells (*Left*) were subjected SDS/PAGE under nonreducing conditions, whereas extracts from untreated cells were subjected to SDS/PAGE under reducing conditions (*Right*, increasing concentrations of DTT). Anti-P2Y12 immunoblotting was then performed.

receptor. Finally, we provide evidence for the molecular interaction between Act-Met and P2Y12.

Results

Effect of the Active Metabolite of Clopidogrel on the Oligomeric Forms of P2Y12. P2Y12 receptors tagged at their NH_2 termini with either hemagglutinin (HA) or the c-myc epitope were transiently expressed in HEK293T cells to investigate their state of multimerization by using immunoblotting analyses. As illustrated in Fig. 1*A* (left lanes), anti-HA, anti-cMyc and anti-P2Y12 antibodies all identified monomeric as well as oligomeric receptor species in whole cell lysates. It should be noted that dimers were only detected with the anti-P2Y12 antibody, and not with the anti-tag antibodies. This may be due to a lesser accessibility of NH_2-located tags by anti-tag antibodies in dimeric structures, as opposed to the accessibility by the anti P2Y12 antiserum to the COOH-located peptidic sequence used to raise the antibody. Upon treatment of the cells with the active metabolite of clopidogrel (Act-Met), the bands corresponding to the oligomeric state of P2Y12 receptors were no longer detected by all three antibodies (Fig. 1*A*, right lanes). The use of an anti-P2Y12 antiserum recognizing the COOH terminus of this receptor allowed for the immunodetection of dimers in whole cell lysates of Act-Met-treated cells. Using this antiserum, an increase in the intensity of the bands corresponding to dimeric and monomeric forms of P2Y12 was also observed, potentially reflecting a compensation for the loss of the oligomeric forms of P2Y12. As for the inability of the other two anti-tag antibodies to detect the P2Y12 dimers after Act-Met treatment, conformational changes at the NH_2 terminus of the receptors may eliminate recognition by the anti-HA and anti-c-Myc antibodies.

Because Act-Met exhibits a free thiol-reactive function (55), we compared the Act-Met-induced changes in oligomerization of P2Y12 with those induced by DTT, a commonly used thiol-reducing reagent (Fig. 1*B*). Both compounds strongly affected the high-molecular-mass species of P2Y12 in a concentration-dependent manner, Act-Met being at least three orders of magnitude more potent than DTT. However, the two compounds induced somewhat different effects on P2Y12 oligomeric organization. Act-Met appeared to preferentially disrupt oligomers into dimers, whereas treatment with DTT only generated monomers. The activity of Act-Met on P2Y12, when compared to DTT activity, seems to be restricted to a limited class of thiol-sensitive chemical functions.

Effect of Act-Met on the Localization of P2Y12 in Lipid Rafts. In the course of the biochemical characterization of P2Y12 receptors, we found that greater amounts of P2Y12 oligomeric forms were obtained when octyl-glucoside was used for cell lysis instead of Triton X-100 (data not shown). This observation led us to suggest that P2Y12 oligomers could be located in particular microdomains within the plasma membrane. These microdomains, e.g., lipid rafts (28), are known to be insoluble in Triton X-100 due to their lipid composition, rich in cholesterol and sphingolipids. To ascertain the presence of P2Y12 oligomers in lipid rafts, Triton X-100 cell lysates were fractionated by sucrose gradient centrifugation and gradient fractions were analyzed for the presence of P2Y12 by immunoblotting. Lipid raft-containing fractions were monitored by caveolin immunoblotting, these proteins serving as a convenient marker for caveolae, a subpopulation of lipid rafts (59). P2Y12 detection using the anti-P2Y12 antibody (Fig. 2*A Upper Left*) showed that oligomers were mainly located in the microdomain-rich fractions (fractions 4 and 5), whereas dimers and monomers were predominantly found in the microdomain-free fraction 6. This fraction contained the monomeric, dimeric, as well as the oligomeric form of P2Y12.

After treatment with Act-Met, oligomers were no longer detected in any of the gradient fractions (Fig. 2*A Upper Right*). This loss/disruption of the P2Y12 oligomeric entities was accompanied by an increase in monomers and dimers in fractions 5 and 6 of the sucrose gradient. The vast majority of monomers and dimers were redistributed outside microdomains as attested by their strong enrichment in the gradient fraction 6 that has a very poor content of caveolin.

We next wanted to verify the selectivity of the observed disruption and redistribution of P2Y12 complexes induced by Act-Met, and rule out any nonspecific effect on membrane microdomains that might result in artefactual partitioning of proteins out of lipid rafts. Microdomains prepared from HEK293T cells coexpressing c-Myc-P2Y12 and HA-P2Y13 were analyzed by anti-HA immunoblotting. In this heterologous expression system, P2Y13 existed as monomeric and oligomeric species, the latter in far lower amounts than P2Y12 expressed in those same cells (Fig. 2*A Lower Left*). Both monomeric and oligomeric P2Y13 species were mostly associated with lipid rafts (fractions 4 and 5). In nontreated HEK293T cells, P2Y13 monomers were targeted to cellular lipid microdomains, as opposed to P2Y12 monomers that were mainly localized outside such microdomains. When cells were treated with Act-Met, P2Y13 localization remained unchanged (Fig. 2*A Lower Right*) in contrast to P2Y12, thus demonstrating the selective action of the active metabolite of clopidogrel on P2Y12 oligomers.

To test the functionality of the various species, we performed binding of [^{33}P]2MeS-ADP to the proteins present in the sucrose gradient fractions 3–7. The P2Y12 protein content was evaluated by measuring the intensity on Western blots of bands migrated under reducing conditions (Fig. 2*B Upper*). In this experiment, significant specific binding was detected in fractions 3–5, identified as caveolin-containing fractions (Fig. 2*B Lower Left*), measured in the same amount of proteins deposited. In fraction 6, no binding was detected despite its high content in P2Y12. This was particularly obvious