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Short communication

Inhibitors of phosphatidylinositide 3-kinase: effects on reactive oxygen species and platelet aggregation

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Abstract:
Phosphoinositide 3-kinase (PI 3-kinase) exists in cells as a family of isoforms. The enzymes are important regulators of fundamental metabolic processes, such as energy utilization, growth, cell proliferation and survival. They are activated by cell surface receptors for hormones, and by G-protein coupled receptors. Enzyme p110 gamma, in particular, catalyzes production of second messengers from inositol phospholipids, including phosphoinositide (3,4,5) triphosphate or PtdIns (3,4,5) P3, PtdIns (3,4) P2 and Ptdins (3) P. The objective of this study was to corroborate the role of PI 3 kinase in ROS generation and in platelet aggregation through the use of four chemically unrelated inhibitors of PI 3 kinase: wortmannin, LY-294002, resveratrol and quercetin. In this study, we describe the effects of four PI 3-kinase inhibitors on the production of reactive oxygen species (ROS) and platelet aggregation induced by a diversity of agonists. Neutrophils and platelets were obtained from human blood and macrophages from mouse peritoneal cavity. ROS production was measured by a luminol-enhanced chemiluminescence assay; aggregation was measured in platelet-rich plasma (PRP) with a Chronolog Dual Channel Lumi-Aggregometer. Effects of graded concentrations of four enzyme inhibitors (wortmannin, LY-294002, resveratrol and quercetin) were evaluated. All inhibitors caused concentration-dependent depression of ROS generation and human platelet aggregation. They differed only in their potencies as revealed by concentration-response data. Moreover, inhibitors blocked activity of three chemically unrelated stimulants of aggregation: ADP, collagen and epinephrine. We conclude that inhibition of PI 3-kinase would appear to be a useful therapeutic goal in those conditions where the activities of platelets and/or phagocytes become aberrant.

Key words:
phosphatidylinositide 3-kinase, platelet aggregation, wortmannin, LY 294002, resveratrol, quercetin, luminol-enhanced chemiluminescence assay

Introduction

Reactive oxygen species (ROS) are a diverse group of chemically unstable molecules or ions, such as hydrogen peroxide and superoxide anion radical. These normal products of metabolism, if allowed to accumulate, can peroxidize membrane fatty acids, oxidize sulfhydryl groups in proteins, and alter genetic information by disrupting DNA. Numerous metabolic path-

ways are normally available to maintain the concentrations of ROS below cytotoxic levels. ROS have been detected in a variety of cell types, including platelets [1], which has led to the suggestion that they meet physiologic needs in certain cells by providing redox signaling [5, 6]. "Respiratory bursts", in which ROS are produced in a surge of oxygen consumption, were described initially in phagocytes where the bursts are thought to represent coordinated chemical attacks on microorganisms trapped inside the cells.

A number of enzymatically driven reactions produce ROS as byproducts of metabolism, but their physiologic functions in non-phagocytic cells are often uncertain. ROS in blood platelets are of interest from a functional viewpoint because they have been reported to participate in platelet aggregation [1, 2, 4, 8]. Moreover, bursts of ROS are generated in platelets exposed to thrombin [18]. An ROS generating reaction of interest in the present study is that catalyzed by PI 3-kinase. In platelets, this enzyme is found in the plasmalemma, a strategic location for sensing and reacting to events in the environment such as contact with collagen or thrombin, transmembrane release of multiple mediators from vesicles, adhesion to other platelets. We reported earlier that wortmannin, an inhibitor of PI 3-kinase, prevented human platelet aggregation [12]. Moreover, Wachowicz et al. [18] found that wortmannin depressed resting levels and bursts of ROS in platelets obtained from swine.

Wortmannin is an antifungal compound originally isolated from soil bacteria. Irreversible inhibition of PI 3-kinase is produced by binding of wortmannin to an ATP binding site [17]. However, wortmannin also affects other enzymatic pathways which renders definitive conclusions about its mode of action in platelets difficult.

The objective of this study was to corroborate the role of PI 3 kinase in ROS generation and in platelet aggregation through the use of four chemically unrelated inhibitors (wortmannin, LY-294002, resveratrol and quercetin). These compounds have multiple pharmacological properties besides inhibiting PI 3 kinase; however, we used only those concentrations of inhibitors at which they are reported to inhibit PI 3 kinase.

In this report, we describe experiments on human platelet aggregation, with ROS chemiluminescence in neutrophils, macrophages and whole blood as endpoints. Four different PI 3-kinase inhibitors were employed, each of which has a distinctive pharmacologic profile, thus reducing the likelihood of misinterpretation of mechanisms.

Materials and Methods

Wortmannin, LY-294002, resveratrol, quercetin, ADP, epinephrine bitartrate, collagen, zymosan and luminol were obtained from Sigma Chemical Co. (St. Louis, MO USA). Ficoll-Paque^{TM} PLUS was purchased from Amersham Biosciences AB, Sweden and lymphocyte separation medium 1077 (LSM) came from PAA Laboratories GmbH, Austria. All other chemicals used were of the highest purity grade available.

Whole blood assay

Using the whole blood assay, the effects of PI 3-kinase inhibitors on ROS production were measured by Luminol enhanced-chemiluminescence assay as follows. The PI 3-kinase inhibitors at the following concentrations: **a)** wortmannin (0.06–0.48 μM) **b)** resveratrol (7–56 μM) **c)** quercetin (3–24 μM) and **d)** LY-294002 (8–64 μM) were incubated with whole blood (diluted 1:50 with $HBSS^{++}$) for 30 min. Addition of 50 μl (2 mg/ml) of zymosan was followed by 50 μl (7×10^5 M) of luminol. The chemiluminescence peaks were recorded with the Luminometer (Luminoskan RS Labsystem, Finland) for 50 min.

Neutrophil assay

Human blood samples (20 ml) were withdrawn from healthy volunteers into heparinized tubes and mixed with $HBSS^{++}$ buffer. This was followed by addition of Ficoll-Paque at a ratio of 1:1 and allowed to sediment. The buffy layer was taken out and centrifuged at 400 × g at 22°C for 20 min. The resulting neutrophil disc was mixed with distilled water to lyse RBCs and centrifuged at 300 × g at 4°C for 10 min to obtain neutrophils. The neutrophil pellet was suspended in $HBSS^{++}$ buffer to give a final neutrophil concentration of 1×10^6 cells/ml.

Peritoneal macrophage preparation

Macrophages were obtained from mice (25–30 g) injected intraperitoneally with 1 ml of fetal bovine serum (FBS). Seventy two hours later, the mice were killed by cervical dislocation and the peritoneum was injected with 10 ml of Roswell Park Memorial Institute (RPMI) 1640 media. This was diluted with FBS to a concentration of 10% (v/v). After 2–3 min mas-

sage, the abdominal cavity was opened, and the abdominal exudate was withdrawn and centrifuged at 1100 × g rpm at 4°C for 25 min. Distilled water was added to lyse the RBCs followed by the addition of RPMI 1640 media and centrifugation at 1000 × g rpm at 4°C for 10 min to obtain macrophages. The macrophage pellet was diluted in $HBSS^{++}$ buffer to concentration of 1×10^6 cells/ml.

Platelet aggregation

Platelet effects were studied in human platelets by taking blood *via* venipuncture from normal human volunteers reported to be free of medication for 7 days. Blood sample were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 × g for 15 min at 20°C to obtain platelet-rich plasma (PRP). The remaining blood samples were centrifuged at 1200 × g for 10 min to obtain platelet-poor plasma (PPP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having platelet counts between 2.5 and 3.0×10^8 ml^{-1} of plasma. Aggregation was monitored with a Dual Channel Lumi-Aggregometer (Model 400, Chronolog Corporations Chicago, USA) using 450-µl aliquots of PRP [13]. The light transmission was adjusted to 0 and 100% with PRP and PPP, respectively. The PRP was pre-incubated with an appropriate amount of the PI 3-kinase inhibitors (wortmannin, LY-294002, resveratrol, quercetin) for 1 min before challenge with the aggregation agent. Aggregation was induced by ADP (5.0 µM), collagen (5.0 µg/ml) and epinephrine (20 µM). The resulting aggregation was recorded and expressed as percentage inhibition compared with control at 4 min after the challenge. Test compounds were tested at four concentrations in duplicate. Statistical analysis was done using Student's *t*-test and $p < 0.05$ was considered significant.

Results

The effects of PI 3-kinase inhibitors on ROS production was measured by luminol enhanced-chemiluminescence assay in (A) whole blood (B) neutrophils and (C) macrophages. The results are shown in Figure 1A, B and C. All four inhibitors of PI 3-kinase suppressed ROS production in a concentration-related manner. The data given in Table 1 (mean ± SEM n = 6) are presented as half maximal effect (IC_{50}) of the inhibitors. The IC_{50} (drug concentration producing 50% inhibition of control value) are given in Table 1. The results show that wortmannin, resveratrol, quercetin and LY-294002, in decreasing order of potency inhibited ROS production with wortmannin being the most potent compound.

The IC_{50} values for anti-platelet effects against three inducers of platelet aggregation are given in Table 2. The data show a slightly different order of potency against ADP-, collagen- or epinephrine-induced human platelet aggregation (Fig. 2). This may be due to structural differences between different aggregating agents. It is known that most aggregating agents (ADP, collagen or epinephrine) stimulate aggregation *via* the formation of thromboxane A_2 (TXA_2) from arachidonic acid (AA) and this effect is inhibited by aspirin [14].

Tab. 1. Comparative effect of PI 3-kinase inhibitors on ROS production as measured by luminol based chemiluminescence assay in whole blood, neutrophils and macrophages

	IC_{50} µM ± SEM			
	Wortmannin	Resveratrol	Quercetin	LY-294002
Whole Blood	0.250 ± 0.04	8 ± 0.6	9 ± 0.7	41 ± 1.8
Neutrophils	0.053 ± 0.001	17 ± 0.9	14 ± 2	57 ± 1.3
Macrophages	0.338 ± 0.002	13 ± 0.2	15 ± 1	31 ± 2.4

Data are the means ± SEM (n = 6) and are presented as half maximal effect (IC_{50}) of the inhibitors

Tab. 2. Inhibitory effects of PI3-kinase inhibitors on human platelet aggregation induced by various platelet agonists, presented as half maximal effect (IC_{50}) of the inhibitors

Agonist	(IC_{50}) µM ± SEM			
	Wortmannin	Resveratrol	LY-294002	Quercetin
ADP	*110 ± 0.01	85 ± 1.1	107 ± 3.4	N.A
Collagen	*280 ± 0.3	27 ± 0.6	25 ± 0.9	185 ± 9
Epinephrine	2.5 ± 0.5	44 ± 0.8	47 ± 1.2	238 ± 4

Data are the means ± SEM (n = 4–5) and are presented as half maximal effect (IC_{50}) of the inhibitors.* Concentrations are expressed in nM; N.A = not active

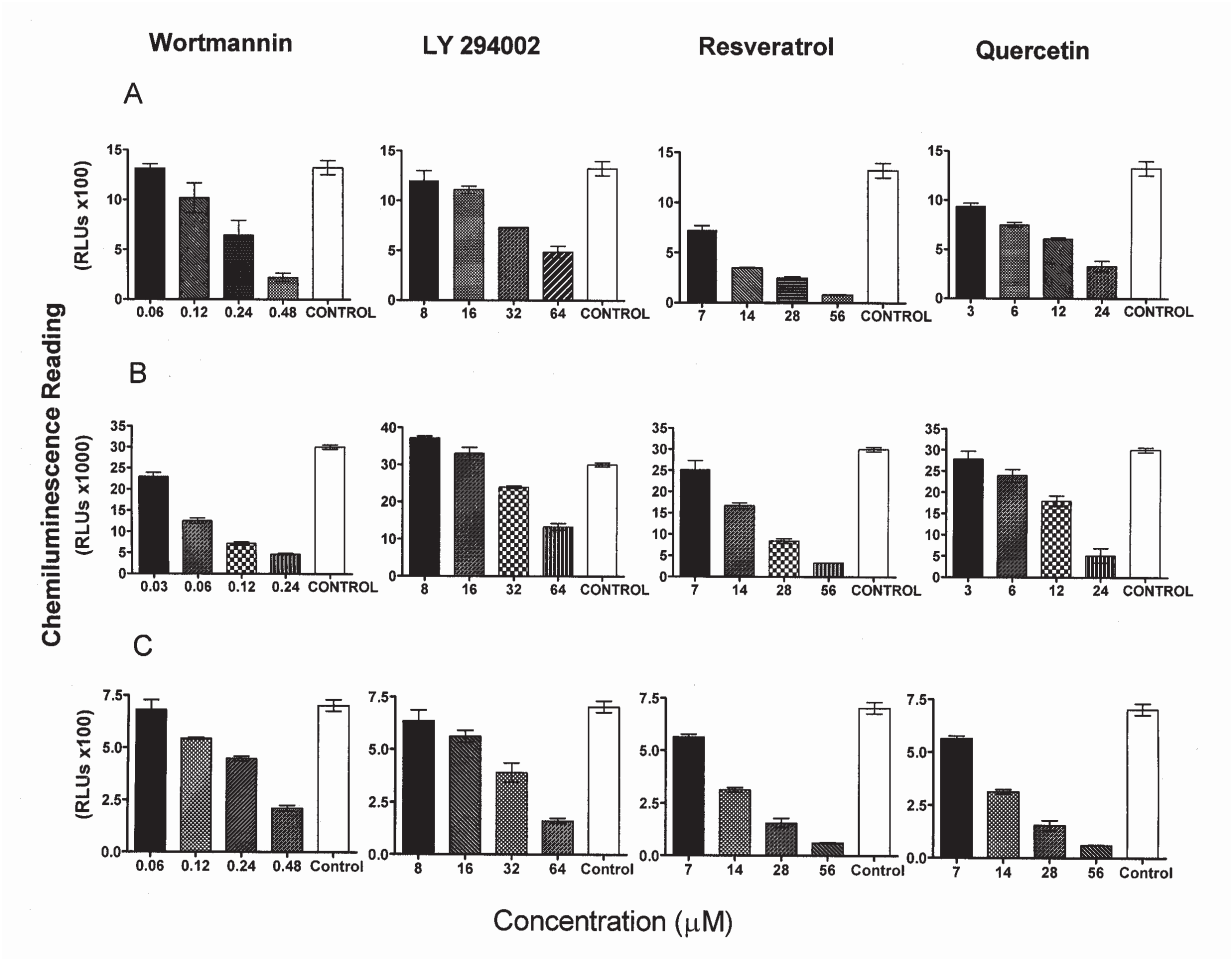

Fig. 1. Effects of four PI 3-kinase inhibitors on ROS production by human neutrophils *in vitro* as measured by luminol-enhanced chemiluminescence assay Relative Light Units (RLUs) in (**A**) whole blood (**B**) neutrophils and (**C**) macrophages. Bars are the means ± SEM of n = 6 independent experiments

Discussion

PI 3-kinase exists in cells as a family of isoforms that regulate fundamental processes, such as energy utilization, growth and proliferation [3, 17]. The enzymes are activated by cell surface receptors for hormones [11], and by G-protein coupled receptors [7] PI 3-kinase gamma (P110γ), which has a single P101 adapter, in particular, catalyzes production of second messengers from inositol phospholipids, including PtdIns (3,4,5) P3 [9]. The latter mediates specialized responses associated with cell function. In neutrophils, products of PI 3-kinase are thought to be important in chematoxis, motility, and phagocytosis. In platelets, PtdIns are utilized in release of mediators from vesicles, adhesion and modification in the cytoskeleton which contribute to aggregation [15].

Four chemically unrelated PI 3-kinase inhibitors were chosen for this study because these compounds have additional effects which might complicate interpretations. For example, resveratrol has antioxidant properties and is also an ROS scavenger [9]. Quercetin interferes with calcium metabolism in some cells [16]. Exposures of platelets to LY-294002 or wortmannin have been reported to block synthesis of several phosphorylated inositides [15]. LY-294002 inhibits casein kinase at concentrations similar to those that block PI 3-kinase. Casein kinase participates in a number of transcription/proliferation reactions. However, as shown in the present study, two responses common to all four PI 3-kinase inhibitors were interference with aggregation and suppression of ROS.

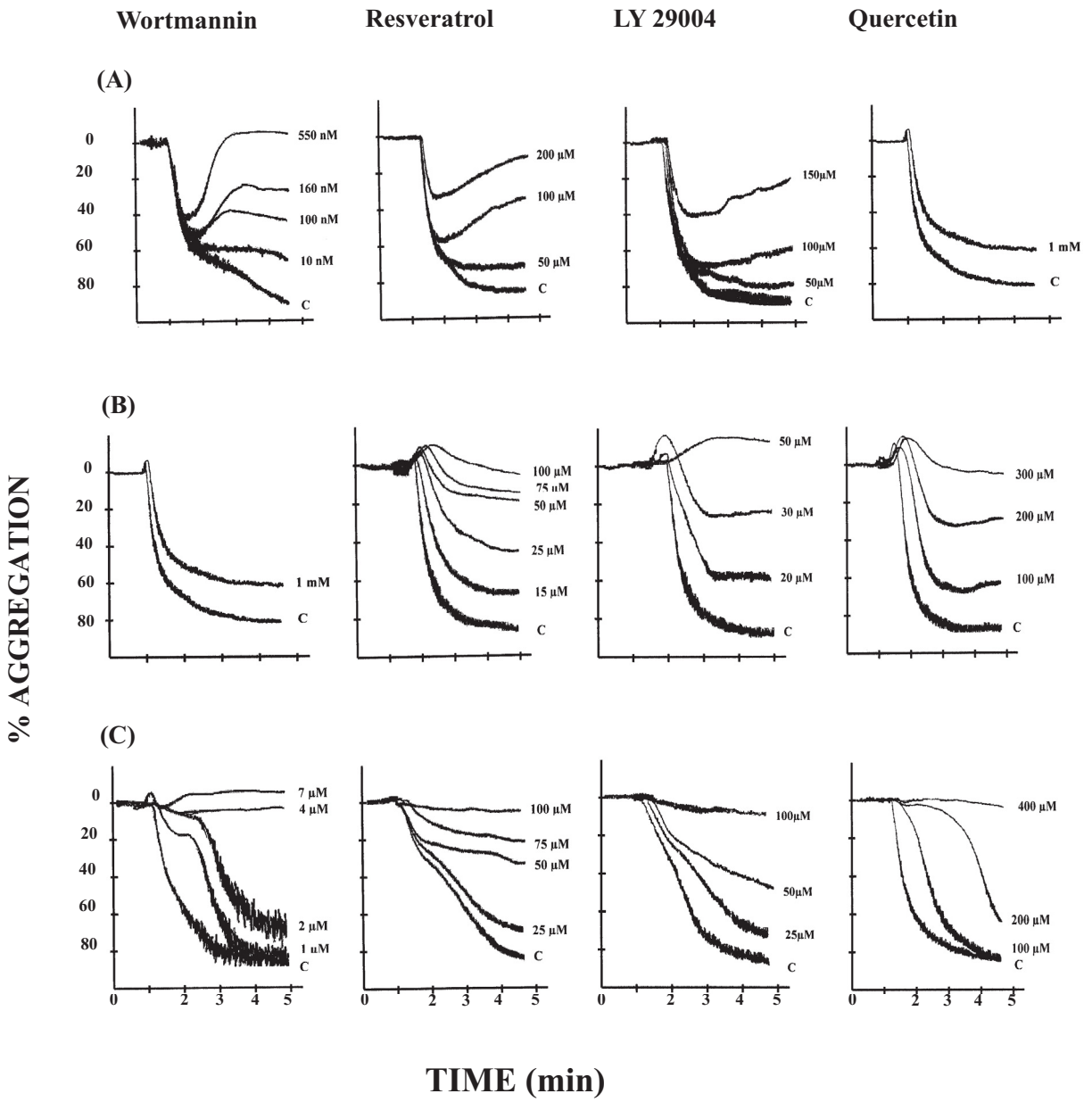

Fig. 2. Tracings showing the effect of PI3-kinase inhibitors on human platelet aggregation induced by (**A**) ADP, (**B**) collagen, (**C**) epinephrine

The identity of the reactive radical(s) involved in these reactions remains unclear. With respect to platelets, Ambrosio et al. showed that aggregation impaired by H_2O_2 was accompanied by a 10-fold increase in cGMP [1]. Since G-proteins are influenced by PtdIns which, in turn, arise from the action of PI 3-kinase, it may be that PI 3-kinase inhibition is related to H_2O_2. Alternatively, Wachowicz et al. measured superoxide anion production in resting and thrombin-stimulated platelets. Wortmannin decreased superoxide anion concentrations in both circumstances of metabolic activity. One of several ROS generators, including arachidonate release by phospholipase A_2 and membrane oxidase, both of which are responsive to PtdIns derived from PI kinases, was thought to be the source of superoxide anion.

With respect to neutrophils and macrophages, ROS and respiratory bursts have been studied extensively.

Several lines of investigations point to PI 3-kinase as a key component of inflammatory and immune responses that require phagocyte participation. For example, respiratory bursts in neutrophils induced by a commonly used stimulant peptide, f-met-leu-phe, require the presence of active PI 3-kinase [19]. Moreover, several proinflammatory cytokines, including tumor necrosis factor, activate PI 3-kinase [10]. Interpretation of data on ROS generation by whole blood is more problematic, since the cellular sources are not immediately apparent; however, the results indicate the ROS are a common feature in blood. A possible link that could explain the antioxidant and antiplatelet actions PI 3-kinase inhibitors is that a decrease in oxidative stress improves the availability of nitric oxide (NO) which in turn inhibits platelet aggregation. In summary, interactions between a variety of receptors and signaling pathways downstream of PI 3-kinase are clearly important for the functions of platelets and phagocytes.

Our results support a conclusion that generation of ROS under the partial control of PI 3-kinase is important in the maintenance of the activities of these cells, even in a basal, unstimulated state. Further studies of ROS, antioxidants and PI 3-kinase inhibitors will add to our current understanding of platelet disorders, such as the sequence of events in atherosclerosis. Similarly, the relationship between ROS and PI 3-kinase inhibition in neutrophils and macrophages suggests that there is a potential for development of novel approaches to the therapy of inflammation and problems of immunity.

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