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Inhibitory Effects of Cordycepin (3'-Deoxyadenosine), a Component of *Cordyceps militaris*, on Human Platelet Aggregation Induced by Thapsigargin

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Abstract Cordycepin (3'-deoxyadenosine) is an adenosine analog, isolated from *Cordyceps militaris*, and it has been used as an anticancer and anti-inflammation ingredient in traditional Chinese medicine. We investigated the effects of cordycepin (3'-deoxyadenosine) on human platelet aggregation, which was induced by thapsigargin, a tumor promoter, and determined the cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) (an aggregation-stimulating molecule) and cyclic-guanosine monophosphate (cGMP) (an aggregation-inhibiting molecule). Cordycepin inhibited thapsigargin-induced platelet aggregation in a dose-dependent manner, and it clearly reduced the levels of $[Ca^{2+}]_i$, which was increased by thapsigargin (1 μM) or U46619 (3 μM). Cordycepin also increased the thapsigargin-reduced cGMP levels. Accordingly, our data demonstrated that cordycepin may have a beneficial effect on platelet aggregation-mediated thrombotic diseases through the $[Ca^{2+}]_i$-regulating system such as cGMP.

Keywords: Cordycepin, intracellular Ca^{2+}, cGMP, antiplatelet activity

Platelet aggregation is an essential part of the haemostatic process when blood vessels are injured. Platelet aggregation is brought about by many platelet-activating factors such as thrombin, collagen, ADP, serotonin, and the von Willebrand factor, which may be the cause of thrombosis in atherosclerosis and myocardial infarction [14, 15]. In particular, cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) plays a central role in the activation of platelet aggregation [11]. The $[Ca^{2+}]_i$ levels for platelet activation increase from the inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from internal stores *via* the IP_3 receptor [2]. The increased $[Ca^{2+}]_i$ activates both the Ca^{2+}/calmodulin-dependent phosphorylation of a myosin light chain (20 kDa) and the 1,2-diacylglycerol (DG)-dependent phosphorylation of cytosolic protein (40 or 47 kDa), in order to induce platelet aggregation [2, 8]. Thapsigargin is known to increase intracellular Ca^{2+} levels without having any direct interaction with the IP_3 receptor [3, 12]. Antiplatelet aggregating drugs, including verapamil, theophylline, molsidomine, and nitroprussside, elevate either cyclic-adenosine monophosphate (cAMP) or cyclic-guanosine monophosphate (cGMP) levels, and thus this decreases the $[Ca^{2+}]_i$ elevation, which is induced by collagen, thrombin, and ADP [7, 10].

Cordycepin (3'-deoxyadenosine) is an adenosine analog and was isolated from *Cordyceps militaris* [1, 20], which has been used as an ingredient in traditional Chinese medicine [21]. Cordycepin is known to have antitumor effects on cancers of the bladder, colon, and lungs, as well as fibrosarcoma [5], and it possesses anti-inflammatory effects regarding the production of inflammatory mediators [17, 19, 21]. It has been reported that cordycepin possesses an inhibitory effect on the adenylate cyclase activity in either a particulated platelet fraction or a platelet membrane [4, 9]. An adenosine analog, 2',5'-dideoxyadenosine, has been reported as effecting no change in either the levels of cAMP or cGMP in collagen-induced platelet aggregation [7]. Other reports have suggested that cordycepin, or 2',5'-dideoxyadenosine, did not have any effects on the inhibition of platelet aggregation. Recently, we have observed that cordycepin decreased collagen-elevated $[Ca^{2+}]_i$ without the inhibitory effect on IP_3 production (data not shown). It could be anticipated that cordycepin could inhibit $[Ca^{2+}]_i$ elevation, which was induced by thapsigargin [3, 6, 12]. In this study,

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we have investigated the effects of cordycepin on $[Ca^{2+}]_i$, which were increased by thapsigargin, and U46619, a TXA_2 analog, and on cGMP production, an aggregation-inhibiting intracellular molecule.

MATERIALS AND METHODS

Materials

Cordycepin from *Cordyceps militaris* was purchased from the Sigma Chemical Co. (St. Louis, U.S.A.). Fura 2-AM, thapsigargin, and other reagents were obtained from the Sigma Chemical Co. (St. Louis, U.S.A.). [^{3}H-cyclic GMP] and [^{3}H-cyclic AMP] radioimmunoassay kits were obtained from Amersham Bioscience (Buckinghamshire, U.K.). U46619 was purchased from Calbiochem (CN Biosciences, Inc., Germany).

Preparation of the Washed Human Platelets

Platelet-rich plasma (PRP) was obtained from the antecubital veins of normal healthy human volunteers. The collected blood was anticoagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) during preparation. PRP was centrifuged for 10 min at 125 $\times g$ to remove red blood cells, and it was centrifuged for 10 min at 1,300 $\times g$ to obtain the platelet pellets. The platelets were washed twice with a washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, and 1 mM EDTA, pH 6.5). The washed platelets were then suspended in a suspending buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, 5.5 mM glucose, 0.25% gelatin, pH 6.9). The platelet number was adjusted to 5×10^8/ml in the suspending buffer. All of the above-mentioned procedures were carried out at 25°C in order to avoid platelet aggregation from any effect of low temperatures.

Measuring Platelet Aggregation

Washed platelets (10^8/ml) were preincubated for 3 min at 37°C in the presence of exogenous 2 mM $CaCl_2$, either with or without cordycepin and then stimulated with thapsigargin for 5 min. Aggregation was monitored by using an aggregometer (Chrono-Log, Corp., Havertown, PA, U.S.A.) with gentle stirring. Each aggregation rate was evaluated as a percent of light transmission. The suspending buffer was used as a reference (transmission 0%). Cordycepin was dissolved in a platelet suspending buffer (pH 6.9). The light transmission (%) was not affected by 0.004% of ethanol, a vehicle of thapsigargin.

Determination of the $[Ca^{2+}]_i$

PRP was incubated with 5 μM of fura 2-AM for 60 min at 37°C. As fura 2-AM is light-sensitive, PRP was covered with aluminum foil during the fura 2 loading. The fura 2-loaded washed platelets were also prepared using the procedure described above. The fura 2-loaded washed platelets (10^8/ml) were preincubated for 3 min at 37°C with cordycepin, in the presence of 2 mM of $CaCl_2$. The platelets were then stimulated with thapsigargin or U46619 for 5 min. Fura 2 fluorescence was measured in a spectrofluorimeter (SFM 25, Bio-Tek Instrument, Italy) with an excitation wavelength that ranged between 340 and 380 nm, altering every 0.5 sec; the wavelength of emission was at 510 nm. The $[Ca^{2+}]_i$ was calculated by the method of Schaeffer and Blaustein [13]. $[Ca^{2+}]_i$ in cytosol=224 nM$\times$($F-F_{min}$)/($F_{max}-F$), where 224 nM is the dissociation constant of the fura 2-Ca^{2+} complex; F_{min} and F_{max} are the fluorescence intensity levels at very low and very high Ca^{2+} concentration amounts, respectively. In our experiment, F_{max} is the fluorescence intensity of the fura 2-Ca^{2+} complex at 510 nm, after the platelet suspension containing 2 mM of $CaCl_2$ had been solubilized by Triton X-100 (0.1%), and F_{min} is the fluorescence intensity of the fura 2-Ca^{2+} complex at 510 nm, after the platelet suspension containing 20 mM Tris/3 mM EGTA had been solubilized by Triton X-100 (0.1%); F is the fluorescence intensity of the fura 2-complex at 510 nm after platelet suspension was stimulated by U46619, either with or without cordycepin, in the presence of 2 mM of $CaCl_2$. U46619 was dissolved in platelet suspending buffer (pH 6.9). Ethanol (0.004%), a vehicle of thapsigargin, did not have an effect on the level of intracellular Ca^{2+}. U46619 was dissolved in a platelet suspending buffer (pH 6.9).

Measuring cGMP and cAMP

Washed platelets (10^9/ml) were preincubated for 3 min at 37°C, either with or without cordycepin in the presence of 2 mM of $CaCl_2$, and then the platelets were stimulated with 1 μM of thapsigargin for 5 min for aggregation. The aggregation was stopped by adding 80% ice-cold ethanol for cGMP and cAMP assays. The levels of cGMP and cAMP were measured by using [^{3}H-cGMP] radioimmunoassay and [^{3}H-cAMP] radioimmunoassay, respectively. Ethanol (0.004 %), a vehicle of thapsigargin, did not have an effect on the levels of cAMP and cGMP.

Statistical Analysis

All data are shown as means±SD. A Student's *t*-test was used for data analysis, and paired or unpaired comparisons were used when necessary.

RESULTS AND DISCUSSION

As shown in Fig. 1, thapsigargin (1 μM) strongly induced the aggregation of human platelets up to approximately 69±4%. This, however, was drastically inhibited by the presence of cordycepin in a dose-dependent manner. Since

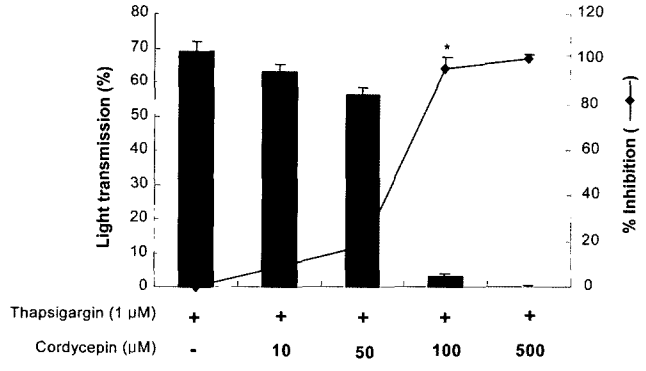

Fig. 1. The effects of cordycepin on thapsigargin-induced platelet aggregation.
Washed human platelets (10^8/ml) were preincubated either with or without cordycepin in the presence of 2 mM of $CaCl_2$ for 3 min at 37°C. The platelets were stimulated with thapsigargin for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by cordycepin was recorded as a percentage of the thapsigargin-induced aggregation rate. Data are given as means±SD (n=4). *P<0.05 compared with the thapsigargin-induced aggregation.

$[Ca^{2+}]_i$ is a critical regulator of platelet aggregation, it was used to examine the effects of cordycepin on $[Ca^{2+}]_i$ with regard to thapsigargin-induced platelet aggregation. When human platelets (10^8/ml) were stimulated with thapsigargin, $[Ca^{2+}]_i$ increased from 134±8 nM in intact platelets, to 1,002±38 nM (Fig. 2A). Although 500 μM of cordycepin inhibited almost all thapsigargin-induced platelet aggregation (Fig. 1), and reduced the 1,002±38 nM of thapsigargin-elevated $[Ca^{2+}]_i$ levels to 349±10 nM, the decreased levels of $[Ca^{2+}]_i$ were still higher than those of the control (134± 8 nM) (Fig. 2A). This means that cordycepin inhibited thapsigargin-elevated $[Ca^{2+}]_i$ levels by 76%. It is known that thapsigargin stimulated the formation of TXA_2 *via* the increase of $[Ca^{2+}]_i$, and the resulting TXA_2 increased $[Ca^{2+}]_i$ by itself [3]. At present, we do not know whether cordycepin inhibits TXA_2 production in thapsigargin-induced platelet aggregation. TXA_2, however, is a potent autacoidal agonist for platelet activation, and it is a potent vasoconstrictor. If cordycepin reduces the levels of $[Ca^{2+}]_i$ which were increased by TXA_2, cordycepin may be considered as a good candidate for inhibiting thrombosis. Therefore, we also investigated the effect of cordycepin on $[Ca^{2+}]_i$ increased by U46619, a TXA_2 analog. As shown in Fig. 2B, U46619 (3 μM) elevated the $[Ca^{2+}]_i$ from 134±8 nM in intact cells to 468± 25 nM. When platelets were incubated in the presence of both cordycepin (500 μM) and U46619 (3 μM), however, $[Ca^{2+}]_i$ potently decreased to 233±12 nM (Fig. 2B), and the degree of inhibition was calculated as 70%. It is not clear whether the inhibitory effects of cordycepin on $[Ca^{2+}]_i$ were due to the inhibition of Ca^{2+} mobilization from the endoplasmic reticulum, or by the inhibition of Ca^{2+} influx through the plasma membrane, so further studies are necessary. Considering that inducers of platelet aggregation, thapsigargin and collagen, normally increase the intracellular level of IP_3, an inducer of Ca^{2+} release from the endoplasmic reticulum, it is supposed that cordycepin may inhibit endoplasmic reticulum-derived Ca^{2+} mobilization.

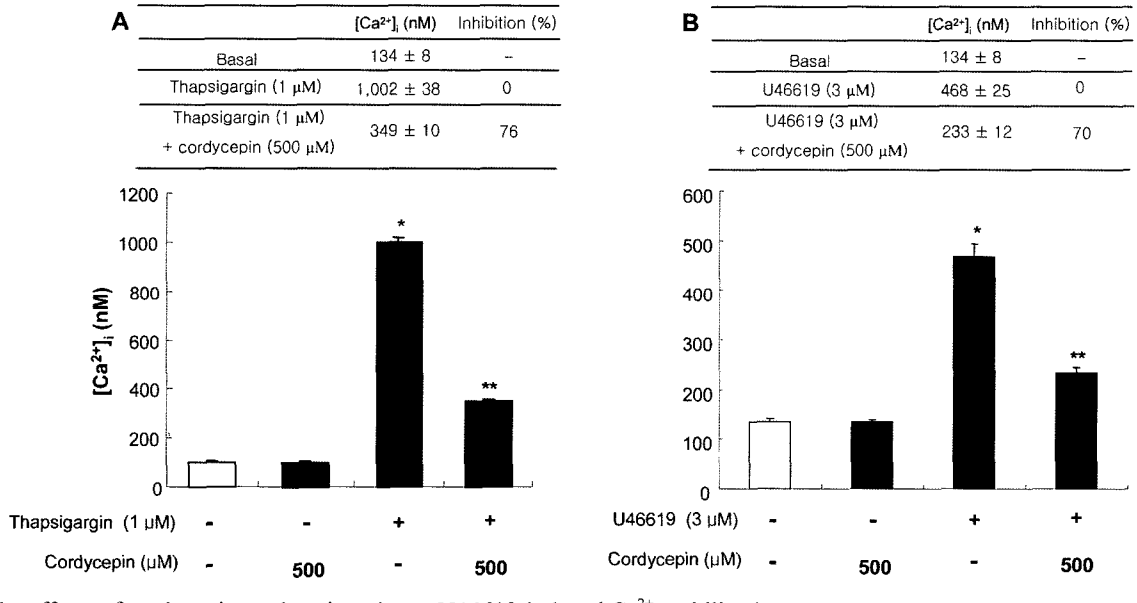

Fig. 2. The effects of cordycepin on thapsigargin- or U46619-induced Ca^{2+} mobilization.
Fura 2-loaded platelets (10^8/ml) were preincubated either with or without cordycepin in the presence of 2 mM of $CaCl_2$ for 3 min at 37°C. Then, either thapsigargin or U46619 was added. **A.** The effect of cordycepin on thapsigargin-induced $[Ca^{2+}]_i$. **B.** The effect of cordycepin on U46619-induced $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ levels were determined as described in Materials and Methods. Data are given as means±SD (n=4). *P<0.05 as compared with the resting platelets. **P<0.05 as compared with the thapsigargin- or U46619-induced platelets.

Table 1. The effects of cordycepin on cAMP in intact platelets.

	cAMP (pmol/10^9 platelets)
Intact cell	3.4±0.3
Cordycepin (500 μM) only	3.5±0.2

The levels of cAMP were determined as described in Materials and Methods. Data are given as means±SD (n=4). *P<0.05 as compared with intact cells.

The elevation of the platelet-activating reagent-induced $[Ca^{2+}]_i$ levels is lowered by either cGMP or cAMP via activating protein kinase G or protein kinase A, since these enzymes induce the phosphorylation of IP_3 receptor, leading to the blockade of Ca^{2+} transport [7]. Therefore, we examined whether cordycepin affected the production of cAMP in intact platelets and the production of cGMP in thapsigargin-activated platelets (Table 1, Fig. 3). The treatment of cordycepin did not affect the normal levels of cAMP in platelets, as compared with the control (Table 1), indicating that this compound may not modulate the intracellular cAMP-regulating enzymes such as adenylate cyclase or cAMP phosphodiesterase, under normal conditions. It is interesting to note that cordycepin modulated the production of cGMP in both intact and thapsigargin-stimulated platelets. Thus, cordycepin significantly upregulated cGMP production in intact platelets (2.2±0.3 pmol/10^9 platelets to 3.8± 0.1 pmol/10^9 platelets). More interestingly, co-treatment of cordycepin with thapsigargin dramatically enhanced the intracellular level of cGMP up to 6.0±0.5 pmol/10^9 platelets, even though the treatment of thapsigargin remarkably reduced its level (1.9±0.2 pmol/10^9 platelets). These results suggest that the cordycepin-mediated inhibition of $[Ca^{2+}]_i$ (Fig. 2A, 2B) might be associated with the enhanced production of cGMP. Although we do not have direct evidence at present, it is supposed that cordycepin-induced upregulation of cGMP may be linked to activating protein kinase G, which is able to negatively modulate the functional activation of the IP_3 receptor. In fact, a similar pattern was also shown in the case of TXA_2-induced Ca^{2+} elevation and platelet aggregation. Thus, upregulated cGMP in these events was known to be a crucial key molecule in their downregulation [16]. Up to date, we do not know how this compound is able to upregulate the cGMP level. Simply, the fact that a combination treatment of cordycepin with either a soluble guanylate cyclase inhibitor, ODQ, or cGMP phosphodiesterase inhibitor, zaprinast, enhanced cGMP levels seems to indicate that this compound may not directly affect the cGMP-producing enzyme activities (data not shown). Rather, it is thought that cordycepin may participate in upstream signaling events involved in upregulating the cGMP level. In fact, adenosine analogs with a structural similarity have been developed as an adenosine receptor agonist and, interestingly, these compounds also display the upregulating features regarding the intracellular cGMP level [18]. Whether cordycepin has adenosine receptor agonistic function should be followed in the future.

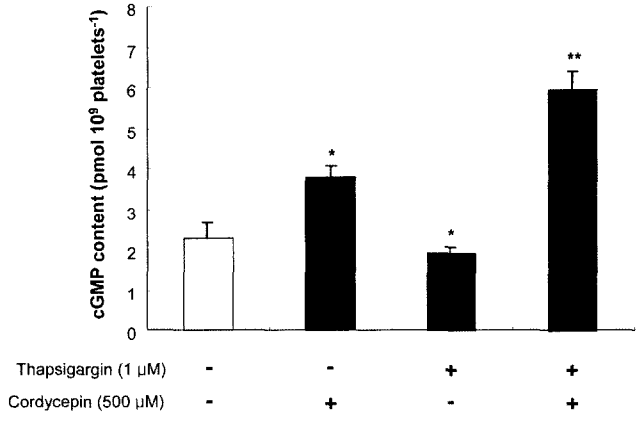

Fig. 3. The effects of cordycepin on cGMP production in thapsigargin-induced platelet aggregation.
Washed platelets (10^9/ml) were preincubated with cordycepin for 3 min and were stimulated with thapsigargin for 5 min at 37°C. The reactions were terminated by adding 80% ice-cold ethanol. The cGMP contents were measured by using a [^{3}H-cGMP] radioimmunoassay kit. Data are given as means±SD (n=4). *P<0.05 as compared with intact cell. **P<0.001 as compared with the thapsigargin-induced platelets.

In summary, cordycepin clearly inhibited platelet aggregation that was induced by thapsigargin. It was observed that cordycepin had a potent inhibitory effect on thapsigargin- and U46619-elevated $[Ca^{2+}]_i$. Intracellular calcium is a potent integrator of the downstream molecules of platelet aggregation. The elevation of $[Ca^{2+}]_i$ plays an important role in platelet aggregation, which is induced by a wide variety of ligands. In addition, the increase in cGMP production leads to a reduction in the levels of $[Ca^{2+}]_i$ that were stimulated by various agonists [7, 10]. Cordycepin also increased cGMP production in thapsigargin-induced platelet aggregation. In addition to its anticarcinogenic effect [5], therefore, our results suggest that cordycepin itself may be developed as a novel therapeutic agent, modulating Ca^{2+}- and platelet-mediated thrombotic diseases. Because the inhibitory potency was not drastic, a special formulation (*e.g.*, an injectable formulation), to reach the drug's pharmacologically effective concentrations, should be further studied.

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