Effects of copper-aspirin complex on platelet-neutrophil interactions[1]

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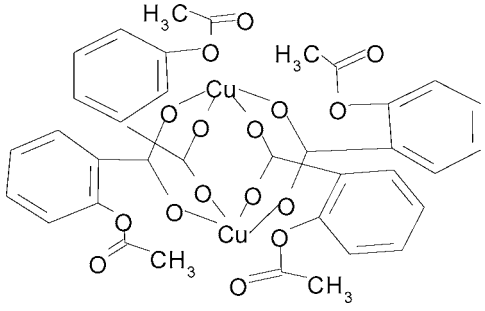
KEY WORDS copper; aspirin; thrombosis; platelets; neutrophils; adhesion; platelet aggregation

ABSTRACT

AIM: To investigate the effects of copper-aspirin complex on rat thrombosis and the interaction between platelets and neutrophils. **METHODS:** The model of electrically stimulated carotid artery thrombosis in Sprague Dawley rats was used; the effects of copper-aspirin complex on rat platelet-neutrophil adhesion and platelet aggregation stimulated by activated neutrophils were observed by rosette assay and Born's method, respectively. **RESULTS:** Intragastric copper-aspirin complex (5, 7, and 10 mg/kg) dose-dependently prolonged the occlusion time; it significantly decreased the rosette number formed between thrombin-activated platelets and neutrophils; the 50 % of inhibitory concentration (IC_{50}) was (54.6±4.3) μmol/L. Copper-aspirin complex markedly inhibited rat platelet aggregation induced by either cell free supernatant of activated neutrophils or by activated neutrophil suspension. The values of IC_{50} were (224.5±16.2) μmol/L and (820.5±21.4) μmol/L, whereas aspirin had no influence. **CONCLUSION:** Copper-aspirin complex inhibited platelet-neutrophil interactions through a different property from aspirin and resulted in a more potent antithrombotic activity.

INTRODUCTION

Platelets were considered to be the main cells involved in the pathogenesis of thrombosis, whereas only a minor role was attributed to neutrophils. However, many recent investigations realized that neutrophils not only participated in thrombus formation but also exerted a direct action on the extension of myocardial infarction by releasing several cytotoxic factors[1,2]. The interaction of platelet and neutrophil may be one of the key factors in the development of thromboembolic diseases[3]. It is more valuable, therefore, to develop an antithrombotic drug especially at an angle of influencing multiple cellular interactions.



The structure of copper-aspirin complex

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Copper-aspirin complex[4], a copper salt of aspirin, was investigated to show more potent antiplatelet activity through elevating 6-keto-prostaglandin $F_{1\alpha}$ level while decreasing thromboxane B_2 generation in plasma[5]. Based on our previous experiments, we further investigate the effects of copper-aspirin complex, as compared with aspirin, on platelet-neutrophil interactions and electrically stimulated carotid artery thrombosis.

MATERIALS AND METHODS

Animals Male Sprague Dawley rats weighing 250-300 g were used in this study in accordance with the Ethics Committee of our Laboratories (Grade II, Certificate No 9804).

Chemicals and drugs Copper-aspirin complex (Cu 14.99 %, C 51.21 %, H 3.32 %, and O 30.48 %; purity >98 %) was synthesized by Kunming Institute of Precious Metals. It was dissolved in 5 % propylene glycol and 1.4 % polyvinyl alcohol in water (pH 6.5). Crystalline aspirin was dissolved in 1 % Na_2CO_3 before use. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and human thrombin were from Sigma Chemical Co.

Electrically stimulated rat carotid artery thrombosis The animals were randomly divided into 5 groups of 8 rats each. Group A: the solution of 5 % propylene glycol and 1.4 % polyvinyl alcohol (control); group B: 10 mg/kg aspirin, and groups C–E: copper-aspirin complex 5, 7, and 10 mg/kg . The rats were administered intragastrically the above substances. One hour after administration, the rats were anesthesized by 30 mg/kg sodium pentobarbital injected intraperitoneally. Charlton's method[6] was modified. Briefly, the left carotid artery was exposed and placed over a bipolar silver electrode and ultrasonic flow probe was positioned distally. The artery was electrically stimulated (1.5 mA for 7 min) using an Electronic Stimulator (model SEN-7203, Nihon Kohden Corporation, Japan). The time to the formation of an occlusive thrombus (zero arterial flow) was recorded with a Direction Volume Meter (model DVM-4200, Hayashi Denki Corporation, Japan). Where appropriate, recording was continued for up to 60 min after stimulation. If the vessels were still patent at the end of this observation period, the duration of 60 min was ascribed to allow statistical analysis of the data.

Preparation of platelets Blood sample from rat carotid artery was collected into plastic tubes, anticoagulated with 2.7 % edetic acid (for the binding of platelets to neutrophils) or 3.8 % sodium citrate acid (for platelet aggregation). This sample was spun for 10 min at 180×*g* to obtain platelet-rich plasma. The later was further spun to pellet platelets at 1000×*g* for 10 min. Platelet pellets were washed three times and resuspended in phosphate buffer solution (PBS, containing 1.0 % bovine serum albumin, 1.4 mmol/L edetic acid, and 1 mmol/L $CaCl_2$). Cell viability by Trypan blue exclusion was above 95 % and cell counter was adjusted to 1×10^{11} cell/L.

Preparation of neutrophils Neutrophils were isolated from the blood (anticoagulated by 2.7 % edetic acid, from which platelet-rich plasma was removed) by dextran sedimentation and followed by Ficoll-Hypaque (specific density 1077 g/L) and hypotonic lysis of erythrocytes. The cell pellet was resuspended in an erythrocyte lysis buffer composed of NH_4Cl 155 mmol/L, $KHCO_3$ 2.96 mmol/L, and edetic acid 3.72 mmol/L. The tube was gently inverted and after 5 min the suspension was centrifuged at 350×*g* for 10 min, and the cell pellet was washed in PBS lacking calcium; then resuspended in Hanks' solution (containing $CaCl_2$ 1 mmol/L or egtazic acid 5 mmol/L in vehicle, reflecting the situation with or without external calcium). Cells were adjusted to a count of 2×10^9 cell/L (for adhesion) or 0.5×10^{10} cell/L (for platelet aggregation). Cells prepared in this manner containing 98 % neutrophils and 96 % cells were viable.

Rosette assay The method of rosette assay[7] was modified. Briefly, 50 μL aliquots of platelet suspension were placed in microtiter wells and exposed to human thrombin (0.2 kU/L) for 15 min at room temperature without stirring. Control solution 50 μL (the solution of 5 % propylene glycol and 1.4 % polyvinyl alcohol for copper-aspirin complex; 1 % Na_2CO_3 foe aspirin) or drug solution (copper-aspirin complex or aspirin) was added and incubated for 15 min at 37 ºC. Then 100 μL of neutrophils was added to the platelet suspension and incubated for 30 min at 4 ºC under rocking condition. One hundred neutrophils were scored for the presence (two or more platelets per neutrophil) or absence (zero or one platelet per neutrophil) of platelets. Neutrophils bearing two or more platelets were thus defined as rosettes. For each assay, done in triplicate, the rosetting score was assessed by two different observers. Percentage inhibition by drugs was calculated by use of the following equation and 50 % of inhibitory concentration (IC_{50}) was obtained though nonlinear regression.

Inhibition of adhesion=(A-B)/A ×100 %

Where A is the maximum change of adhesion when the control is added and B is the maximum change of adhesion when the drug (copper-aspirin complex or aspirin) is added.

Platelet aggregation

Induced by the supernatant of activated neutrophils Neutrophils were activated by fMLP (2 μmol /L) for 10 min at 37 ºC, then centrifuged at 3000×*g* for 1 min to obtain cell-free supernatant of neutrophils. Washed platelets were incubated with vehicle or different concentrations of drug (copper-aspirin complex or aspirin) for 10 min at 37 ºC, then 3 μL of the above cell-free supernatant of neutrophils was added. The maximum increase in light transmission was recorded within 5 min according to Born's method[8].

Induced by the suspension of activated neutrophils Platelets 100 μL (1×10^{11} cell/L) were mixed with 100 μL of neutrophils (5×10^{11} cell/L), stirring at 37 ºC for 2 min. The vehicle or drug solution was added and incubated for 5 min, then 2 μmol/L of fMLP was added. Platelet aggregation was observed as the above described.

RESULTS

Effect of copper-aspirin complex on electrically-stimulated arterial thrombosis in rats Copper-aspirin complex at 5, 7, and 10 mg/kg significantly prolonged the occlusion time in a dose-dependent manner. The reference compound aspirin also delayed the occlusion time (Tab 1).

Effect of copper-aspirin complex on the binding of thrombin-activated platelets to neutrophils The percentage of rosettes in vehicle was 64.5 % or 11.8 % in a condition of external $CaCl_2$ 1 mmol/L or egtazic acid 5 mmol/L. Copper-aspirin complex and aspirin significantly decreased the binding of platelets to neutrophils with 1 mmol/L external Ca^{2+}, obtaining the IC_{50} of (54.6±4.3) μmol/L and (62.9±3.7) μmol/L, respectively (Tab 2).

Tab 1. Effect of intragastric copper-aspirin complex on electrically stimulated carotid artery thrombosis in male Sprague-Dawley rats. *n*=8 in each group. Mean±SD. $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* aspirin.

Drug	Dose/ mg·kg^{-1}	Time to blood vessel occlusion/min
Control	-	17.7±0.8
Copper-aspirin complex	5	27.0±1.2^c
	7	30.8±2.1^c
	10	42.2±1.2^{cf}
Aspirin	10	34.2±2.3^c

Control: the solution of 5 % propylene glycol and 1.4 % polyvinyl alcohol.

Tab 2. Effect of copper-aspirin complex on the binding of thrombin-stimulated platelets to neutrophils *in vitro*. *n*=8 rats in each group. Mean±SD. $^cP<0.01$ *vs* control.

Drug/ μmol·L^{-1}	Copper-aspirin complex Adhesion/%	IC_{50}/ μmol·L^{-1}	Aspirin Adhesion/%	IC_{50}/ μmol·L^{-1}
Control	64.5±6.4		58.5±4.2	
37.5	40.2±3.6^c		36.4±3.4^c	
75	25.4±4.1^c	54.6±4.3	31.5±3.1^c	62.9±3.7
150	14.9±2.8^c		20.3±1.6^c	
300	5.9±4.7^c		12.8±2.5^c	

Control: the solution of 5 % propylene glycol and 1.4 % polyvinyl alcohol for copper-aspirin complex; 1 % Na_2CO_3 for aspirin; IC_{50}: 50 % of inhibitory concentration

Effect of copper-aspirin complex on platelet aggregation induced by the supernatant of activated neutrophils Copper-aspirin complex markedly inhibited platelet aggregation induced by the supernatant of fMLP-activated neutrophils. The IC_{50} was (224.5±16.2) μmol/L. Aspirin at 3000 μmol/L, however, had no inhibitory activity, obtaining (57.4±2.7) % of aggregation rate (Tab 3).

Tab 3. Effect of copper-aspirin complex on platelet aggregation stimulated by the supernatant of fMLP (2 μmol/L)-activated neutrophils *in vitro*. *n*=8 rats in each group. Mean± SD. $^cP<0.01$ *vs* control.

Drug/μmol·L^{-1}	Platelet aggregation/%
Control	58.1±2.5
45	53.3±3.3
90	42.2±7.0^c
180	25.4±3.8^c
360	18.9±3.5^c

Control: the solution of 5 % propylene glycol and 1.4 % polyvinyl alcohol.

Effect of copper-aspirin complex on platelet aggregation induced by the suspension of activated neutrophils Copper-aspirin complex suppressed platelet aggregation induced by activated neutrophils themselves, and its IC_{50} was (820.5±21.4) μmol/L (Tab 4). Aspirin at 3000 μmol/L, however, had no influence, obtaining an aggregation of (73.5±3.8) %.

Tab 4. Effect of copper-aspirin complex on platelet aggregation stimulated by the suspension of fMLP-activated neutrophils *in vitro*. *n*=8 rats in each group. Mean±SD. $^cP<0.01$ *vs* control.

Drug/μmol·L^{-1}	Platelet aggregation/%
Control	77.4±2.7
187.5	56.9±2.7^c
375	41.3±3.3^c
750	33.2±3.2^c
1500	22.6±3.7^c

Control: the solution of 5 % propylene glycol and 1.4 % polyvinyl alcohol

DISCUSSION

Neutrophils and platelets, but not platelets alone, are closely associated with thromboembolic disorders. Interactions of these two kinds of blood cells are involved in the process of thrombomodulation[9]. Activation of platelets increases neutrophil adhesion to foreign surfaces, neutrophile aggregation, and lysosomal enzyme release, *etc*. Platelet-derived products are able to promote neutrophil chemotaxis, enzyme release, and phagocytosis and to inhibit oxidative burst[10]. On the other hand, neutrophil-derived products can enhance platelet aggregation, serotonin release, and cytoplasmic Ca^{2+} movement[11]. Thus it is more advantageous to develop an antithrombotic drug especially at an angle of influencing multiple cellular interactions.

Thrombus formation is mediated by the platelet-neutrophil interactions, including cell binding and platelet aggregation. In the present study, copper-aspirin complex was more active than its parent compound — aspirin against carotid artery thrombosis. In vehicle containing external $CaCl_2$ 1 mmol/L or egtazic acid 5 mmol/L, the percentage of rosettes was 64.5 % or 11.8 %, suggesting a calcium-dependent relationship between platelet-neutrophil adhesion. Both copper-aspirin complex and aspirin showed a significant inhibition on rosetting between thrombin-activated platelets and neutrophils, and was more slightly active than aspirin based on the IC_{50} values. It was suggested that copper-aspirin complex showed more potent antithrombotic activity than aspirin due to its activity on platelet-neutrophil adhesion.

In vehicle group, platelet aggregating rate induced by the supernatant from fMLP-activated neutrophils was 75 % of the aggregation induced by fMLP-activated neutrophil suspension. It is suggested that platelet activation by neutrophils not only is induced by one or more soluble transferable factors, but also that cell-cell contact may play an important role. It can not be excluded, however, that some activity is lost during cell-free supernatant preparation. Nevertheless, copper-aspirin complex exerted a concentration-dependent inhibitory effect on platelet aggregation induced by either the cell-free supernatant from activated neutrophils or by activated neutrophils themselves. Aspirin even at 3000 μmol/L, however, had no influence on platelet aggregation in both the situations suggesting that cyclooxygenase might not be directly involved in platelet aggregation induced by activated neutrophils. This is in accordance with the report that the products of platelet arachidonate cyclooxygenase pathway are not required to mediate platelet activation by neutrophils, although TXB_2 is formed in significant amounts[10]. It is speculated that the increased prostacyclin by copper-aspirin complex[5] may be contributed to the inhibition of platelet aggregation induced by activated neutrophils.

Toxic oxygen radicals and serotonin had been suggested to act as mediators of neutrophil-dependent platelet activation, and platelet aggregation induced by activated neutrophils was accompanied by intracellular calcium increase[11]. Copper-aspirin complex inhibited serotonin release, suppressed cytosolic calcium[12,5], and reported to elevate the activity of copper- and zinc-dependent superoxide dismutase (Cu_2Zn_2SOD)[13]. Taken together these characteristics, copper-aspirin complex could suppress platelet aggregation challenged by activated neutrophils while aspirin could not.

These results indicated that copper-aspirin complex as compared with aspirin, showed different mechanism in inhibiting platelet aggregation induced by activated-neutrophils, and resulted in more potent antithrombotic effects.

In conclusion, copper-aspirin complex may have more potential prospects in treating thromboembolic dis-

eases due to its particular inhibition of platelet-neutrophil interactions.

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