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EFFECT OF CHEMICAL MODIFICATION OF *N*-ACETYLNEURAMINIC ACID ON HORMONAL ACTIVITY OF ERYTHROPOIETIN *IN VIVO* AND *IN VITRO*

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Summary

Sheep, rabbit and human erythropoietins (EP) were oxidized by periodate and then reduced by potassium borohydride, yielding EP containing analogues of N-acetylneuraminic acid (NANA) shortened by 1 or 2 carbon atoms. The EP activity of the modified preparations was assayed ***in vivo*** *by* ^{59}Fe *incorporation in starved rats and* ***in vitro*** *incorporated in bone marrow cells. The modification enhanced the* ***in vivo*** *activity by 2–3 times, possibly due to an increased resistance of the hormone to endogenous neuraminidase. However, the* ***in vitro*** *activity of the modified EP was reduced by about 20 per cent from native preparations.*

Introduction

Erythropoietin (EP) is the hormone produced by kidney[1], which stimulates the production of red blood cells in bone marrow[2]. It has been isolated from blood and urine of a number of species and characterized as a glycoprotein[3-5]. *N*-acetylneuraminic acid (NANA) has been shown to be essential for the biological activity of the hormone[6]. Several chemical modifications have been reported to decrease or destroy EP activity[6]. Suttajit *et al.*[7] have shown that the 3-carbon polyhydroxy side chain of NANA in glycoproteins is susceptible to relatively mild periodate oxidation

which, after further reduction with borohydride, results in the generation of the 8- or 7-carbon analogues. It was shown that an intact 3-carbon polyhydroxy side chain in NANA is not required for full activity of follicle stimulating hormone[8]. However, the optimum activity of neuraminidase and influenza virus binding capacity still require the intact structure of polyhydroxy side chain of NANA[9]. It is of interest to study the effect of chemical modification of NANA on the biological activity of EP. This investigation has shown that EP activity is increased in the *in vivo* system after modification of NANA molecule by shortening of its side chain.

Materials and Methods

Five male sheep weighing 30–40 kg, 20 rabbits in both sexes weighing from 2 to 5 kg and several groups of albino rats, 120 to 250 g in weight were kindly given by the animal house of Faculty of Medicine, Chiang Mai University.

Radioisotope iron (^{59}Fe) as ferric citrate was directly purchased from the Radiochemical Center, Amersham. Cobalt chloride as $CoCl_2.6H_2O$ crystals was taken from May and Baker Ltd. Sodium metaperiodate was obtained from Fisher Scientific, and potassium borohydride from the British Drug Houses Ltd. Neuraminidase from *Clostridium perfringens* was purchased from Sigma Chemical Company.

Bone marrow cells were obtained from normal children recovered from protein-calorie malnutrition with the sincere co-operation of the Anemia and Malnutrition Research Center, Faculty of Medicine, Chiang Mai University.

Erythropoietin preparations

The phenylhydrazine-anemic plasma[10] of sheep and rabbits was employed. The purification process of erythropoietin was modified from the method of Goldwasser *et al.*[11].

The 24-hour urine samples were collected from anemic patients in Chiang Mai Hospital; and from the patients with anemia or protein calorie malnutrition in the Anemia and Malnutrition Research Center, Faculty of Medicine, Chiang Mai University. A few drops of toluene were added as a preservative. The purification procedure of urine was followed the steps of Graham *et al.*[12], Espada and Gutniskey[13], and of Fish *et al.*[14].

***In vivo** assay of erythropoietic activity*

The EP assay was slightly modified from the method of Graham[12], requiring at least 4 rats per group for testing materials and with two remaining groups for a control normal saline solution and standard cobalt chloride solution[15]. It took five days to be completed as below:

Day 1. All food was removed from the cages and the rats were weighed out and starved. Only deionized water was given throughout the assay.

Day 2. Two ml of testing materials were intraperitoneally injected to the starved rats.

Day 3. About 24 hours after the first injection of testing materials, the same rats were intraperitioneally injected with two more ml in addition.

Day 4. 0.5 ml of redioisotope ferric citrate containing one μCi of ^{59}Fe was intravenously introduced into the rats via the tail vein.

Day 5. About 18 hours after the ^{59}Fe injection, the rats were placed under light ether anaesthesia to facilitate decapitation, they were decapitated and gradually bled. One ml of blood was collected with few drops of ACD solution as anticoagulant. The blood was washed at least 3 times. The red blood cells were packed by centrifugation at the speed of 3,500 rpm. ^{59}Fe incorporation into the cells was counted by a Packard Liquid Scintillation Counter, model 3320.

In vitro assay of erythropoietic activity

Bone marrow cell cultures were performed by using the method of Krantz[16]. The Fe^{59} incorporation into the nucleated red blood cells (NRBC) was finally determined.

Chemical modification of bound NANA in EP

Purified erythropoietin was periodate-oxidized and borohydride-reduced by the method of Suttajit and his colleagues[8,9]. An amount of 0.005 M sodium periodate was added to the aqueous solution of EP yielding molar ratio of periodate to NANA varied from 0 to 4. The reaction mixtures were vigorously mixed and allowed to stand in the dark at 4°C for 2 hours. Excess periodate was destroyed by the addition of a few drops of 10% glycerol. The periodate treated samples were dialyzed, and to these were added 0.1 M bicarbonate buffer, pH 9.6. The mixtures were reduced by potassium borohydride, the excess of which was destroyed by glacial acetic until pH 4.5. The treated samples were dialyzed and lyophilized. The NANA content in modified EP was analyzed by direct Ehrlich method[17]. The increase in absorbancy after modification indicated that the intact NANA has been converted to its 8-or 7-carbon analogues[18].

Results

In vivo study

One mg of highly purified EP from anemic sheep plasma and human urine was incubated with 1 unit of *Clostridium perfringens* neuraminidase in 0.1 M acetate buffer, pH 5.0. The incubation mixture was dialyzed against 20 ml distilled water at 37°C for 10 hours. The result of EP activity affected by the enzymatic action is shown in Table I.

TABLE I: EFFECT OF NEURAMINIDASE ON EP ACTIVITY *IN VIVO*

Substrate	Before enzymatic treatment		After enzymatic treatment	
	EP activity (Co units/mg)[a]	NANA content in the substrate (μg/mg)	EP activity (Co units/mg)	NANA content in the dialysate (μg/mg)
Sheep EP	369.37	18.5	7.68	17.5
Human EP	686.80	29.5	9.06	28.5

[a]One Co unit is equal to the activity by which 5 μmoles of $CoCl_2.6H_2O$ as a total dose were injected into starved rats. From the experiments, the average ^{59}Fe in corporation caused by 5 μmoles of $CoCl_2.6H_2O$ was 7.76%

Table II, III and IV represent the *in vivo* effect of chemical modification on sheep, rabbit and human erythropoietin respectively. The NANA chromophore in the modified EP determined by the direct Ehrlich method was raised up to approximately 2–3 times of native and control preparations. This indicated that the intact NANA in EP had been converted to its 8- or/and 7-carbone analogues[18]. The EP activity of modified hormone was readily increased up to at least double of that native EP. Even though the amount of rabbit EP was not quite sufficient for this (Table III), the available data also indicated a trend of increasing the hormonal activity of the rabbit modified preparations.

TABLE II: EFFECT OF CHEMICAL MODIFICATION ON SHEEP EP *IN VIVO*

Samples	Percent of NANA (apparent)[a]	EP activity (Co units/mg)
Native	1.85	369.37
Treated, IO_4^-: NANA molar ratio		
0 : 1	1.73	326.00
1 : 1	2.02	309.44
2 : 1	1.93	370.48
3 : 1	3.38	492.31
4 : 1	5.07	676.97

[a]An increase indicates modification to analogues with higher extinction coefficient. See text for details

TABLE III: EFFECT OF CHEMICAL MODIFICATION ON RABBIT EP *IN VIVO*

Samples	Percent of NANA	EP activity (Co units/mg)
Native	1.60	441.88
Treated, IO_4^-: NANA molar ratio		
0 : 1	N.D.[a]	443.33
1 : 1	N.D.	476.00
2 : 1	N.D.	N.D.
3 : 1	N.D.	645.33
4 : 1	N.D.	1,186.00

[a]N.D. = Not determined

TABLE IV: EFFECT OF CHEMICAL MODIFICATION ON HUMAN EP *IN VIVO*

Samples	Per cent of NANA (apparent)[a]	EP activity (Co units/mg)
Native	2.95	686.80
Treated, IO_4^-: NANA molar ratio		
0 : 1	2.23	640.95
1 : 1	2.91	679.23
2 : 1	3.50	699.33
3 : 1	5.16	944.61
4 : 1	6.43	1,056.69

[a]An increase indicates modification to analogues with higher extinction coefficient. See text for details.

***In vitro* study**

The EP preparation (0.3 unit of NIH) was treated with 0.018 unity of *Clostridium perfringens* neuraminidase in 0.2 ml. of 0.1 M acetate buffer, pH 3.0 at 37°C for 10

TABLE V: EFFECT OF NEURAMINIDASE ON EP ACTIVITY *IN VITRO*

Samples	Average per cent of stimulation
Native	51.40
Neuraminidase-treated	40.67

Normal saline solution was used as control. EP activity was expressed in term of % ^{59}Fe incorporation per nucleated red blood cells $\times 10^7$

hours. The enzymatic hydrolysate was used as a testing material for EP activity assay. The result was shown in Table V. It was observed that the hormonal activity of enzyme treated EP was slightly decreased, about 21% from that of native preparation.

Table VI represents the effect of chemical modification on human EP activity *in vitro*. It indicated that the treated EP samples have slightly decreased hormonal activity. About 20% of the activity was lowered from that of the native EP.

TABLE VI: EFFECT OF CHEMICAL MODIFICATION ON EP ACTIVITY *IN VITRO*

Human bone marrow cells	Samples	% ^{59}Fe incorporation per NRBC $\times 10^7$ (over control)	% stimulation
	Native EP[a]	2.40	63.0
Pool I	Modified EP (1:1)[b]	2.06	52.2
	Modified EP (4:1)	2.17	55.0
	Native EP	2.82	77.5
Poor II	Modified EP (1:1)	1.97	55.0
	Modified EP (4:1)	2.23	62.0

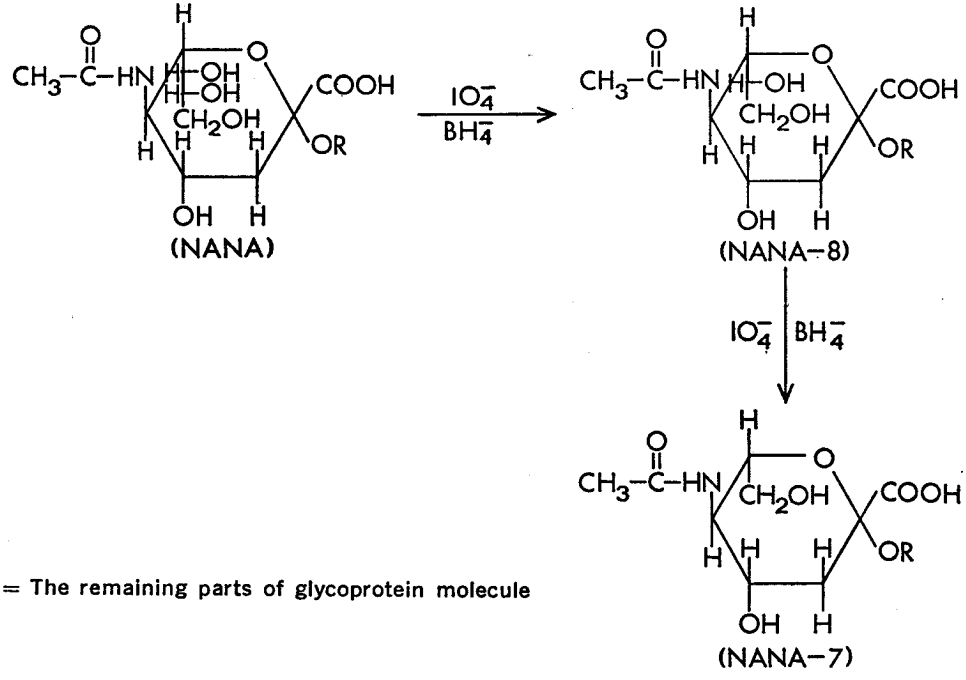
[a]Normal saline solution was used as control

[b]Figures in parentheses indicate the molar ratios of IO_4^- to NANA

Discussion

The erythropoietins were incubated with *Clostridium perfringens* neuraminidase both *in vivo* and *in vitro* for 10 hours. After the enzymatic treatment was completed, NANA moiety in EP had mostly been cleaved off. The results show that NANA residues are apparently essential for only *in vivo* EP activity, but the *in vitro* effect is rather insignificant. The results confirm the works of Lowy *et al.*[19], and Krantz[20]. Goldwasser and Kung[21] suggested that NANA might not function *in vitro* as the active site of the hormone. It is probably required for specific binding with a carrier in the circulation, or it may act as a protective structure in EP molecule.

A stepwise shortening of the polyhydroxy side chain of NANA in EP from C-9 to C-8 and from C-8 to C-7 analogues had been achieved by using sodium periodate and potassium borohyride under controlled conditions, and it was previously reported in other glycoproteins[7,9]. The structure of NANA and its modified intermediates and products are illustrated below:



R = The remaining parts of glycoprotein molecule

The first mole of periodate converted NANA to the aldehyde form of the C-8 analogues (NANA-8), which were then further degraded to the C-7 analogues (NANA-7) by the second mole of periodate. The oxidized intermediates in aldehyde form of NANA-8 and NANA-7 residues were further reduced to the more stable alcohol form of the corresponding analogues. According to Suttajit and his coworkers[8,9,18], results from modification of NANA in other glycoproteins including follicle stimulating hormone (FSH) and human chorionic gonadotropin (HCG) indicated that NANA-7 has an extinction coefficient 2–3 times that of native NANA in the Ehrlich assay. As previously shown by others[9,22,23], the chromogenic reactions were dependent on the chain length. Periodate is a specific chemical for oxidation of the vicinal hydroxy groups of compounds[24]. It was mentioned that the rate constant for hydroxy oxidation of other sugars, especially in the pyranose ring, is slower than the free terminal side chain of NANA under the controlled condition[25]. The *in vivo* activity of chemically modified EP preparations was significantly enhanced from that of the native and control ones. Suttajit *et al.*[8] described that glycoproteins containing C-7 analogue of NANA moiety were resistant to the neuraminidase naturally present in bacteria and various animal tissues[26,-28]. Our results may be due to the higher resistance of the treated EP to the endogenous neuraminidase. The hormone containing C-7 or/and C-8 analogues of NANA may have a prolonged life in the circulation, and in turn, can continuously stimulate the red blood cell production in term of ^{59}Fe incorporation in rats as well. Surprisingly, the *in vitro* EP activity of modified hormon is slightly decreased, and so does that of NANA-free EP. The mechanism of the *in vitro* hormonal action is still unclear. It can be finally concluded that the whole intact polyhydroxy side chain of NANA molecule is definitely not important

in the *in vitro* activity, but its role is ambiguous for the *in vitro* effect. Further investigation in the latter system is needed.

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บทคัดย่อ

การเปลี่ยนแปลงโครงสร้างทางเคมีของฮอร์โมนอีริโธรปอยอีตินที่แยก และทำให้บริสุทธิ์ได้จากแกะ กระต่าย และคน กระทำโดยปฏิกริยา oxidation-reduction ด้วย sodium periodate และ potassium borohydride ตามลำดับ ซึ่งได้ผลิตผลเป็นฮอร์โมนที่มีโมเลกุลของ *N*-acetylneuraminic acid (NANA) ที่มีจำนวนคาร์บอนน้อยลง 1 และ/หรือ 2 อะตอม เมื่อนำฮอร์โมนที่ถูกเปลี่ยนแปลงโครงสร้างแล้วนี้ไปวัด activity ใน *in vivo* system ซึ่งทำโดยวิธี ^{59}Fe incorporation เข้าไปในหนูที่อดอาหาร ปรากฏว่า activity จะสูงขึ้นประมาณ 2-3 เท่าตัว อาจจะอธิบายว่า ฮอร์โมนที่ถูกเปลี่ยนแปลงโครงสร้างนี้มีความทนต่อ endogenous neuraminidase ได้ดีกว่าฮอร์โมนปกติ ส่วนการศึกษาใน *in vitro* system ซึ่งทำใน bone marrow cells พบว่า activity ลดลงจากเดิมประมาณ 20 เปอร์เซนต์