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Hydroxychloroquine binding to cytoplasmic domain of Band 3 in human erythrocytes: Novel mechanistic insights into drug structure, efficacy and toxicity

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ABSTRACT

Hydroxychloroquine (HCQ) is a widely used drug in the treatment of autoimmune diseases, such as arthritis and systemic lupus erythematosus. It has also been prescribed for the treatment of malaria owing to its lower toxicity compared to its closely related compound chloroquine (CQ). However, the mechanisms of action of HCQ in erythrocytes (which bind preferentially this drug) have not been documented and the reasons underlying the lower side effects of HCQ compared to CQ remain unclear. Here we show that, although the activity of erythrocyte lactate dehydrogenase (LDH), but not GAPDH, was inhibited by both HCQ and CQ *in vitro*, LDH activity in erythrocytes incubated with 20 mM HCQ was not significantly reduced within 5 h in contrast to CQ did. Using HCQ coupled Sepharose chromatography (HCQ-Sepharose), we identified Band 3, spectrin, ankyrin, protein 4.1R and protein 4.2 as HCQ binding proteins in human erythrocyte plasma membrane. Recombinant cytoplasmic N-terminal 43 kDa domain of Band 3 bound to HCQ-Sepharose and was eluted with 40 mM (but not 20 mM) HCQ. Band 3 transport activity was reduced by only 23% in the presence of 20 mM HCQ. Taken together, these data demonstrate that HCQ binds to the cytoplasmic N-terminal domain of Band 3 in human erythrocytes but does not inhibit dramatically its transport activity. We hypothesize that the trapping of HCQ on Band 3 contributes to the lower side effects of the drug on energy production in erythrocytes.

1. Introduction

Hydroxychloroquine (HCQ), a 4-aminoquinoline, is widely used to treat autoimmune diseases, such as arthritis and systemic lupus erythematosus. It has also been prescribed for the treatment of malaria infection [1–3] owing to its lower toxicity compared to its closely related compound chloroquine (CQ) yet achieving an equal activity against *Plasmodium falciparum* [1,4]. The mechanism of action of CQ on *P. falciparum* is thought to involve the inhibition of erythrocyte lactate dehydrogenase (LDH, EC 1.1.2.27), this inhibition leading to erythrocyte lysis as a result of their inability to produce ATP by anaerobic glycolysis in absence of LDH activity [4]. Studies suggest that HCQ exerts the same inhibitory effect on LDH activity. However, surprisingly, unlike CQ, HCQ does not cause anemia [2,3], suggesting a much lower toxicity of HCQ compared to CQ. The mechanisms underlying this important therapeutic difference are still unclear.

Since mammalian erythrocytes do not have intracellular organelles, the maintenance of the cell shape is strongly dependent on the architecture of the plasma membrane and its associated cytoskeleton. The major proteins involved in maintenance of cell shape have been identified. They include transmembrane proteins, such as the anion exchanger Band 3, and membrane skeletal proteins, such as spectrin and actin [5].

Gaining a better understanding of how drugs interact with these protein complexes is of great interest to better evaluate drug

Abbreviations: CBB, Coomassie Brilliant Blue R-250; CQ, chloroquine; DAC, Drug affinity column chromatography; HCQ, hydroxychloroquine; IOVs, inside-out-vesicles; LDH, lactate dehydrogenase; B3cd, recombinant N-terminal 43 kDa Band 3 cytoplasmic domain; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.

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activity and toxicity. Drug affinity chromatography (DAC) is a powerful tool to explore candidate drug binding proteins in target tissues or cells. For example, a novel 25 kDa puromycin aminonucleoside-binding protein in porcine kidney, and its homolog quinone reductase 2 in human kidney, have been identified [6]. However, very few studies have attempted to identify HCQ binding proteins in the erythrocyte membrane using DAC despite the fact that HCQ is a widely prescribed drug in major indications. Our study provides novel insights into the pharmacological activity of HCQ in human erythrocytes and into the mechanisms contributing to its lower toxicity compared to CQ.

In the present study, we sought to characterize the effect of HCQ on the activity of LDH, a key cytoplasmic protein whose function is associated with energy metabolism and to identify the HCQ-binding sites in the erythrocyte plasma membrane. We found that, although HCQ inhibited the oxidation of NADH by LDH *in vitro*, LDH activity in human erythrocytes exposed to the drug was not significantly changed. HCQ coupled Sepharose column chromatography enabled us to identify the N-terminal cytoplasmic domain of Band 3 as the main binding site for HCQ in the human erythrocyte membrane. We also demonstrated that binding of HCQ to Band 3 did not reduce dramatically Band 3 exchange activity. Taken together, we conclude that HCQ is primarily retained by the N-terminal cytoplasmic domain of band 3, thus precluding the drug to affect significantly the activity of cytoplasmic enzymes (such as LDH).

2. Materials and methods

2.1. Materials

2.1.1. Reagents

HCQ and CQ (biochemical grade) were purchased from Tokyo Kasei Kōgyo Ltd. (Tokyo, Japan). Epoxy-activated Sepharose was purchased from GE-Healthcare (GE Healthcare UK Ltd., Amersham Place, England). Trypsin and all reagents were of chemical grade (including purified rabbit muscle LDH) and obtained from Wako Pure Chemical Industries (Ōsaka, Japan) or Dojindo Molecular Technologies, Inc. (Kyoto, Japan). Antibodies to CD47, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ankyrin and spectrin (recognizing both α and β polypeptides) were purchased from Santa Cruz Biotechnology Co. Ltd. (Santa Cruz, CA, USA). Preparation of antibodies to the N-terminal 30 kDa domain of protein 4.1R was described in one of our previous reports [7].

2.1.2. Human erythrocytes

Human erythrocytes were prepared from blood collected on heparin. Blood was obtained from healthy adult donors at Akita University after informed consent and under an institutionally approved protocol.

2.1.3. Erythrocyte membrane and inside-out-vesicles (IOVs)

Whole blood was passed through LeukoCatchII™ resin (WATOSON Co. Ltd., Tokyo, Japan) to remove leucocytes according to the manufacturer's instructions. Blood was then washed with PBS (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, containing 0.15 M NaCl) to remove plasma. Erythrocytes were resuspended in and washed with an excess of 5 mM Na_2HPO_4/NaH_2PO_4, pH 7.4 containing 1 mM EDTA. The erythrocyte plasma membrane fraction ("ghosts") was collected by centrifugation (21,000 *g*) for 20 min at 4 °C [8]. Ghosts were incubated with an excess of 5 mM EDTA (inside-out-vesicles: IOVs) and then adjusted at pH11 with 1 M NaOH for 30 min at 37 °C. The resulting IOVs (pH11-IOVs) were neutralized with 50 mM Tris-HCl, pH 7.4 containing 0.15 M NaCl (Buffer A). In some instances, trypsinized pH11 treated IOVs (T-pH11-IOVs) were prepared according to our previous reports [8]. Briefly, IOVs were incubated with 15 μg trypsin/ml packed IOVs for 30 min at 4 °C. At the end of the trypsinization step, erythrocytes were immediately washed with an excess of PBS to remove the enzyme.

2.1.4. Recombinant cytoplasmic domain of Band 3

We used recombinant cytoplasmic domain of Band 3 in the binding assays described in this study. Expression and purification of this recombinant protein has been described in one of our

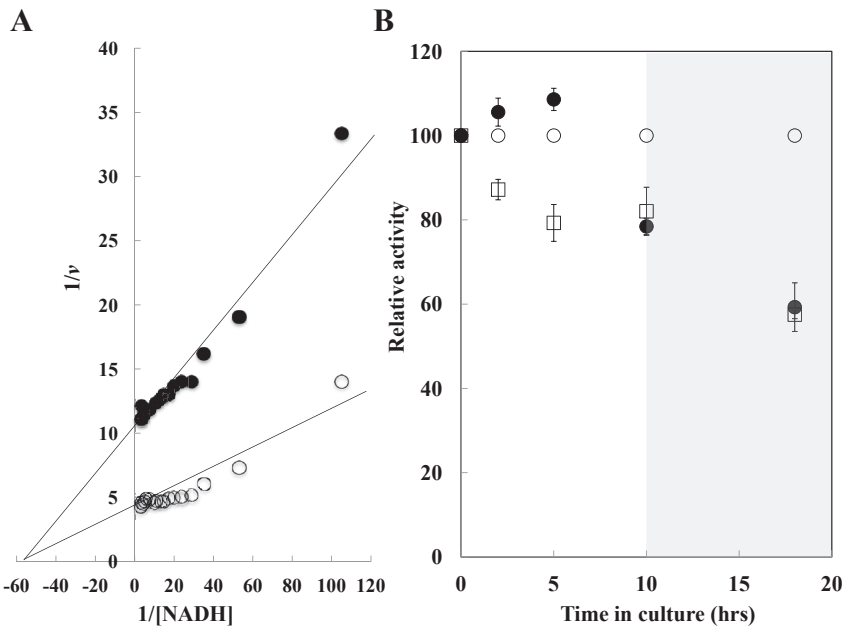

Fig. 1. Effect of HCQ and CQ on human erythrocyte LDH activity *in vitro*. (A) Lineweaver–Burk plot of human erythrocyte LDH activity in the absence (○) or presence (●) of 100 μM HCQ. LDH activity was assessed by monitoring of NADH oxidation at 370 nm as described in the "*Methods 2.2.1*" section. (B) LDH activity in human erythrocytes incubated in the absence (○) or presence (●) of 20 mM HCQ and in the presence of 20 mM CQ (□). Light grey shedding represents the region where hemolysis was observed.

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