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SPR detection of human hepcidin-25: A critical approach by immuno- and biomimetic-based biosensing

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ABSTRACT

The human hepcidin-25 hormone has a key role in iron regulation in blood. The clinical relevance of this hepatic ~2.8 kDa cysteine-rich peptide is rapidly increasing, since altered levels can be associated with inflammatory events and iron dysfunctions, such as hereditary hemochromatosis and iron overload. Moreover, hepcidin has also attracted the anti-doping field for its possible role as indirect marker of erythropoietin blood doping. Methods currently reported are based on immunoassays (ELISA and RIA), or various types of mass spectroscopy (MS)-based protocols, semi-quantitative or quantitative. Despite the great effort in optimizing robust and simple assays measuring hepcidin in real matrices, at present this challenge remains still an open issue. To explore the possibility to face hepcidin detection through the development of affinity-based biosensors, we set up a comparative study by surface plasmon resonance (SPR) technology. An immuno-based, on anti-hepcidin-25 IgG, and a biomimetic-based, on a synthetic peptide corresponding to the hepcidin-binding site on ferroportin (HBD), biosensors were developed. Here we report behaviors and analytical performances of the two systems, discussing limits and potentialities.

1. Introduction

Mature hepcidin is a cysteine-rich peptide hormone of 25 amino acids secreted by the liver in response to iron loading and inflammation. Decreased hepcidin leads to tissue iron overload, whereas hepcidin overproduction leads to hypoferremia and anemia of inflammation diseases (Bartnikas, 2012; Ganz, 2011). Hepcidin performs the regulatory activity by binding the iron transporter ferroportin and causing its degradation (De Domenico et al., 2007; Nemeth et al., 2004; Kemna et al., 2008). Moreover, hepcidin has also attracted the anti-doping field for its possible role as an indirect marker of erythropoietin blood doping (Kroot et al., 2009; Lainé et al., 2012; Piperno et al., 2009; Robach et al., 2009). Despite the undisputed interest in detecting human hepcidin, its determination in real matrices, mainly urine and plasma, has been hampered by the lack of validated, fast, and low cost assays. The production of specific antibodies for human hepcidin was hampered by several features of hepcidin-25, i.e. its small size (2789 Da), its cysteine-rich and complex structure (Hunter et al., 2002; Jordan et al., 2009), and its highly conserved sequence among species (De Domenico et al., 2008). Nevertheless, advances have been recently made in this direction. As reported by Schwarz et al. (2011), a novel monoclonal antibody for ELISA-based measurements allowed reliable quantification of serum hepcidin in the range 0.9–140 ng ml^{-1}. Hepcidin mRNA expression was also investigated as a quantitative method (Sasu et al., 2010) but, due to the requirement of invasive sampling, it is foreseeable that this approach will be applicable only on animal and cell cultures. Other methods are mainly based on various types of mass spectroscopy (MS)-based protocols (including surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS; Altamura et al., 2009; Bozzini et al., 2008; Castagna et al., 2010; Kemna et al., 2005), matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Anderson et al., 2010, 2011) and liquid chromatography tandem–MS techniques (LC-MS/MS; Bansal et al., 2009; Li et al., 2009; Mansion et al., 2011), and allow semi-quantitative or quantitative hepcidin determination. Despite the number of results currently obtained by these last methods, their routine applicability for clinical purposes will be limited, since they are expensive and require highly trained personnel.

Results obtained by available techniques were compared in an extensive round-robin study, demonstrating how difficult the goal of having reliable and reproducible hepcidin quantification in real samples remains (Kroot et al., 2009).

In this framework, rapid methods allowing label-free and real-time detection of the active form of human hepcidin-25 could represent an interesting possibility for further application in

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clinical and anti-doping fields (Banfi et al., 2010). Among systems, affinity-based biosensors (ABBs) are interesting for their possibility of exploiting a variety of affinity interactions (nucleic acid/nucleic acid, antigen/antibody, receptor/binding protein, etc.) by using different recognition probes, natural and synthetic, able to selectively bind the analyte of interest (Rogers and Mulchandani, 1998).

In this work we present the possibility to face human hepcidin detection through the development of affinity-based biosensors, by surface plasmon resonance (SPR) technology. In particular, two different affinity receptors were chosen for biosensor development, and the relative performances in terms of analytical parameters were evaluated in standard conditions. A commercially available polyclonal anti-hepcidin antibody, on one side, and a biomimetic receptor consisting of a 19 aa-synthetic peptide mimicking the hepcidin-binding site on ferroportin (HBD; De Domenico et al., 2008) were selected to this aim. Particular attention was paid to determination of a proper management of hepcidin, because we observed that this is a fundamental aspect in minimizing problems of reproducibility, which are consistent even when working with standard samples.

Our preliminary results showed the possibility to go further in the direction of biosensing-based methods for hepcidin-25 controls, for their possible application in clinical and/or anti-doping fields.

2. Materials and methods

2.1. Reagents

Human hepcidin-25 trifluoroacetate salt and rabbit anti-human hepcidin-25 (T-4819) were from Bachem (Weil am Rhein, Germany). Hepcidin Binding Protein (biotin-PEG-RR-FDCITT-GYAYTQGLSGSILS-RR, namely biotin-HBD) was purchased from EspiKem (Florence, Italy). N-hydroxysuccinimide (NHS) was from Fluka (Milan, Italy); 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) was from Merck-Calbiochem (Darmstadt, Germany); sodium hydroxide, hydrochloric acid, Tween 20, bovine serum albumin (BSA, 98% purity), ethanolamine hydrochloride (EA), and streptavidin from *Streptomyces avidinii* were all from Sigma Aldrich (Milan, Italy). Water was obtained from a Millipore unit. Other chemicals were purchased from standard commercial sources at analytical grade.

2.2. Buffer solutions

Binding buffer was phosphate buffer saline solution (PBS), pH 7.4 containing 0.2 M NaCl and 10 mM phosphate. Streptavidin was immobilized on an activated carboxylated dextran layer by using 10 mM acetate buffer pH 4.5. Running buffers used in Biacore X® experiments were HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20) and PBS buffer added with 0.2 g l^{-1} BSA. All salts used for buffers were analytical grade and were purchased from Merck (Darmstadt, Germany). Deionized water (Millipore) was used throughout the preparations and buffer solutions were finally filtered (22 μm) before use.

2.3. Surface plasmon resonance measurements

SPR measurements were all carried out on Biacore X™, by using carboxylated dextran CM5 biochips (General Electric Healthcare Bio-Sciences AB; Uppsala, Sweden). For the immobilization of the biotin-HBD, the procedure was based on streptavidin/biotin reaction as follows: at a constant flow rate of 5 μl min^{-1}, the dextran layer was modified with streptavidin (35 μl, 200 μg ml^{-1} in 10 mM acetate buffer, pH 5.0) after an activation step (35 μl) with 50 mM NHS and 200 mM EDAC. For non-biotinylated anti-hepcidin IgG, the immobilization protocol was based on direct amino coupling on the active dextran layer. A blocking step by EA for 20 min to saturate unreacted sites was eventually performed for both receptors. The biochip surface was then let under flow of the proper running buffer until equilibration of the baseline. All experiments were performed atleast three times at a temperature of 25.0 °C, to determine reproducibility and standard deviations. The flow rate was set at 5 μL min^{-1} for all measurements, and different injected volumes (2–100 μL per injection) allowed varying the time of contact of different analytes on the biochip. Each biochip can work on two flow cells (0.06 μL per cell), separately or in series. In this work, measurements were conducted on both cells working in serial mode, one of them used as the reference cell. After each measurement, regeneration solutions were flowed for 30 s–2 min on the biochip to release hepcidin bound to the receptor. Sensorgrams were elaborated by the BIAevalutation 3.1 software.

3. Results and discussion

3.1. Optimization of hepcidin handling

Proper hepcidin handling for analytical purposes is still quite unclear, and this is one of the reasons why its accurate detection for clinical purposes is still a challenge (Castagna et al., 2010). The main issues to take into consideration for analytical purposes are the hepcidin tendency to break one or more disulfide bonds, and to oxidize the methionine residue to the sulfoxide form; to form non-well-defined intermolecular aggregates; to change its secondary structure with temperature; and to adsorb to tube walls at low concentrations. If not properly controlled, these features lead to macroscopic errors in quantitative analyte estimation, especially in biorecognition assays such as ABBs, where the stability of the active form of the analyte in solution is a mandatory requirement.

3.1.1. Effect of temperature and BSA

It was previously showed how strongly the secondary structure of hepcidin-25 is influenced by changes in temperature, forming dimers at low temperatures. In particular, this change seems to be promoted by the amino terminus of hepcidin-25 (Jordan et al., 2009), since data report that hepcidin-20 does not change its structure or form dimers at low temperature (De Domenico et al., 2008). In our experiments, hepcidin was stocked in water aliquots (2000 g L^{-1}) at −20 °C until analysis in PBS buffer. To minimize absorption of hepcidin on tube walls, low-binding tubes were used. During measurements samples were kept at room temperature, if near 25 °C, or thermostated in a thermoblock at the same temperature. As reported in Fig. 1, experiments confirmed that samples giving high SPR responses if kept at room temperature (TRoom series), rapidly lose their binding ability when moved for a few minutes in ice-bath (ice-bath series). Similar behavior was observed if thawed samples were directly kept in ice-bath. In this context, as reported in Fig. 1, we obtained good results in stabilizing hepcidin-25 samples with addition of 0.2% (w/v) BSA in working buffers, mainly in the case of HBD receptor (see Section 3.3.1). A possible explanation of this behavior could rely on two different behaviors at the tested temperatures: on one hand, the BSA addition could prevent the formation of intermolecular aggregates of hepcidin (mainly dimers, favored at $T < 15$ °C), leading to more reproducible SPR signals than in the absence of BSA as a stabilizing agent. Moreover, BSA could exert an additive action in preventing