厚生科学研究費補助金(生活安全総合研究事業)
分担研究報告書

表面プラズモン共鳴高速分析によるデータの高速取得技術及びHTPSに特化するための試験

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研究要旨
内分泌かく乱物質の生体への影響を評価するにあたり、各種化合物と核内受容体分子との結合を定量的に測定することが求められる。一方で、核内受容体分子と外来性化合物の多様性を考慮した場合に、定量測定系にはハイ・スルー・プットスクリーニングが可能であることが求められる。本研究は表面プラズモン共鳴高速分析法を用いることで、迅速かつ定量的に両者の結合を測定する新たな解析法を確立することを目的とする。初年度においてはハイ・スルー・プット性能を考慮した際にアッセイ系確立に必要な基礎データを得るために、エストロジェン受容体を用いた予備実験を行った。

A.　研究目的

内分泌系の確立が多細胞生物の生存にとって極めて重要なステップであったことは、個々の独立した組織が協調的に機能することで個体としての生存を可能にしていることを考えれば容易に理解されるところである。この調節系では、種々の組織から分泌されるホルモンがメッセンジャーとしての機能を通じまたホルモンに対する特異的なレセプターがメッセージを受け取ることで、それぞれ重要な働きを担っている。ホルモンを広義に捉えた場合には、すなわち一群の細胞増殖因子をホルモンの一種と見なすならば、このような調節系は単に組織の機能調節に関する寄与のみならず、組織形成過程における寄与も極めて重要であると言える。本来、内分泌かく乱物質はこのような広義のホルモン調節系に関わる問題であると解釈する必要があるかもしれないが、多くの場合ホルモンの中でも、とりわけステロイドホルモンに対するレセプター(核内受容体)に親和性を有する物質を議論する場合が多い。本研究で対象とするレセプターも核内受容体であるが、今後細胞増殖因子などのレセプターを対象とした研究が必要であろうと思われる。

種々の人工化合物がホルモン様作用を有することが明らかになっているなかで、人工の化合物は年々増加の一途をたどっており、内分泌かく乱物質としての潜在的能力を有する化合物も同時に増加している可能性がある。一方で、当初ステロイドホルモンレセプターとして発見されたエストロジェン受容体やアンドロジェン受容体などの核内受容体も、その後新たな受容体分子の発見に伴い、その数が増加している。現在ではステロイドホルモンだけでなく、これまでステロイドホルモン産生過程での中間代謝産物と思われていた物質や分解産物をリガンドとする核内受容体や、ステロイド以外の、プロスタノイドやレチノイドなどをリガンドとする核内受容体分子種の存在が明らかにされてきた。これらのリガンド分子が明らかな核内受容体に加え、核内受容体としての構造上の特長を有しながらそのリガンドが不明であるいわゆるオーファンレセプターと総称される一群の核内受容体も数多く存在する。これらのオーファンレセプターに結合する化合物も内分泌かく乱物質として機能する可能性があり、化合物とレセプターの両面から広範で大規模なアッセイが必要であることは明らかである。

本研究ではビアコア社が開発した表面プラズモン共鳴センサーを用いて、核内受容体をターゲットにした内分泌かく乱候補物質のアッセイ系の確立をめざす。また、このアッセイ系をもとに高速分析法とそれに基づくハイスループット化のための技術開発を行い、大規模なスクリーニングが可能なアッセイ系の確立をめざす。実験にはリガンドが既知の

エストロジェン受容体(α型とβ型)とアンドロゲン受容体の他にリガンドが未知の核内受容体であるAd4BP/SF-1とDax-1をも対象に加えるが、本研究で構築されることが期待される実験系は広く他の核内受容体へ応用が可能であると思われる。

B. 研究方法

1)ホルモンレセプターの発現と精製(α型とβ型エストロゲジェン受容体、アンドロジェン受容体とオーファンレセプターであるAd4BP/SF-1とDax-1)はビアコアとの委託研究により行う。

2)エストロジェン受容体とDNA及び、内分泌かく乱候補物質の結合活性に関する予備実験

アッセイでは受容体の標的配列をもつ DNA 短鎖を結合させたビアコアセンサーチップをもちいる。具体的には図1に示すように、ビオチン標識した標的配列を持つ DNA をあらかじめストレプトアビジンコートしたセンサーチップ上に結合させる。このチップ上で DNA と受容体の複合体が形成されることになるが、ビアコアを用いたアッセイでは複合体の形成をセンサーチップの重量の変化として検出することが可能である。本年度は、受容体と標的配列の結合に影響を与える種々の条件の解析、センサーチップを再生する際に用いる条件の検討のため、溶媒の組成、インキュベーション条件、チップ再生試薬の条件を検討した。

C. 研究結果

1)エストロジェン受容体とDNA及び、内分泌かく乱物質の結合活性に関する予備実験

本実験では以下に示すように、スウェーデンのビアコア本社と連絡を取り合い、種々の条件を検討した。

1, Initial experiments and optimization of regeneration

Initially we made scouting experiment where the buffersubstance, pH, KCl, MgCl, TWEENcontents in the buffers were varied. The reproducibility in the initial experiments was poor probably due to improper regeneration. The initial reagent used for regeneration -SDS 0.05%- gave a drifting baseline and the time between cycles was therefore kept long. The regeneration conditions were optimised. X agents (a, b, c, d) were tested and 0.1% sds and then washed by TE buffer was found to be best for regeneration.

2, Screening

The experiments shown in Table 1 were designed to fix the buffer condition which showed the best selectivity in the scouting experiments. Four parameters, pH, KCl, MgCl, and Tween, in the assay were varied. The experiments were run in both HBs and Tricine buffers. The binding of ER to oliga-x was studied in the presence of Estra-diol, BPA and without a lmw-binder. ER concentration was kept 10 nM). Concentrations of compounds were setted as ER is occupied with chemicals completely, i.e. 10^{-6}M Estradiol and 10^{-4}M BPA were used respectively. The binding level after 10 sec in the dissphase was used as response (D1). The binding level varied between 10-300 RU depending on the buffer composition.

The selectivity, calculated as D1Est-D1non /D1est*100, varied between -96 % in buffer-1 to 95 % in buffer 8 (Fig1. cube1-2). A pareto plot from the regression models forthe relative selectivity for estradiol (RSest) and BPA (RSbpa) is shown in Fig 2 (pareto a,b). The regression coefficient pattern between the buffer composition and the relative selectivity for estradiol and BPA induced binding are different. BPA selectivity seems to significantly increase with increased pH. The relative selectivity for estradiol (Rsest) is significantly influenced by pH, KCl, the interaction effect between KCl and pH and TWEEN.The coefficient pattern is similar for estradiol in

HBS and Tricine buffers (REH and RET), while the BPA shows some difference between the buffers (RBH, RBT). In Tricine the pH and KCl and their interaction was ranked as highest effects but in HBS-buffer only KCl gave an significant effect. A simple scatterplot between the relative selectivity for BPA in the two buffers show that HBS gives a higher selectivity (up to 60%)compared with Tricin (approx 20%). The high design settings in all buffer parameters gave the highest selectivity for EST (95%) and BPA(60%), however for thelatter it was also accompanied by a low binding signal of 30 RU. A general conclusion: an increase in KCl and pH increases selectivity but the strong negative interaction effect between these factors (KCl and pH) decrease the selectivity. BPA and Estradiol induced binding responds differently to the variation in assay buffers. KCl and pH was selected as parameters useful for the next optimisation run.

3, Optimization

The design used for the detailed study of the effects of KCl and pH, and the response parameters determined are shown in table 2. A general conclusion is that the selectivities in this experiment are generally higher in comparison with experiment

1. The experiments were performed at higher pH and KCl concentrations. The square plot in Fig. Square displays the relative selectivity for and the binding signal in presence of or estradiol in the 9 buffers using HBS-buffer as vehicle (one missing experiment - air-injection). The binding level/signal decreases as function of increased pH and KCl concentration. The effect on the relative selectivity was more complex due to interaction effects and a nonlinearity (quadratic effect) Fig a-b. The strong interaction effect manifest it self by the fact that at high KCl concentrations (300 mM) the increse in pH do not give any Effect on selectivity. Furthermore at high pH an increase in KCl gives decreased selectivity and diminished binding level. In HBS-buffer the highest selectivity's(estradiol, 60 %; BPA 51%) and acceptable binding signal (290-240 RU) was achieved in experiment-8 (pH 9.0; KCl 100mM). In tricin buffer the selectivity was betterat higher KCl concentrations but the binding signal was low. The best compro -mise for HBS-buffer, i.e. highest possible selectivity and a high estradiol and BPA induced signal is at pH 9.0 using 100mM KCl. The triplicate experiments using intermediate settings for all assay parameters showed for the Tricin-buffer a high variability. The relative selectivity inc reased and the binding signal decreased. This correlated with increased time after mixing of the ER with the compounds. Therefore a separate experiment was erform -ed where the incubation time of the ER/ compound mix was varied at different temperatures (Fig Time) In the final assay the samples were incubated 1h at room tempera -ture and stored at 4 degrees before analysis.

4, Binding kinetics in different buffers

The shapes of the binding curves vary consi derably in different buffers (Fig.sensorgrams).

In buffer-1 the association phase is linear mass-transport limited binding giving a very stable complex with low off-rate (Koff $10^{-3?}$). The selectivity is poor.In buffer-2 the selectivity is higher and estradiol/BPA induced bindings are linear with a slightly increased dissociation rate ($5*10^{-3?}$). The nonactivated ER binds slower indicating that increasing the interaction time (or dilution) can increase selectivity. In buffer-3 the on-rate is much slower and off rate higher in comparison with the above mentioned

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