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Biochemical Pharmacology 64 (2002) 813–817

Biochemical Pharmacology

A homogenous assay to monitor the activity of the insulin receptor using Bioluminescence Resonance Energy Transfer

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Received 22 February 2002; accepted 30 April 2002

Abstract

Insulin exerts its biological effects through a plasma membrane receptor that possesses a tyrosine kinase activity. Binding of insulin to its receptor induces a conformational change that stimulates the autophosphorylation of the receptor on tyrosine residues. This autophosphorylation stimulates the tyrosine kinase activity of the receptor toward intracellular substrates involved in the transmission of the signal. The discovery of pharmacological agents that specifically activate the tyrosine kinase activity of the insulin receptor will be of great importance for the treatment of insulin-resistant or insulin-deficient patients. We have developed a procedure based on Bioluminescence Resonance Energy Transfer (BRET) to monitor the activation state of the insulin receptor. Human insulin receptor cDNA, was fused to either Renilla luciferase or yellow fluorescent protein coding sequences. Fusion insulin receptors were partially purified by wheat-germ lectin chromatography from HEK-293 cells co-transfected with these constructs. The conformational change induced by insulin on its receptor could be detected as an energy transfer (BRET signal) between Renilla luciferase and yellow fluorescent protein. BRET signal paralleled insulin-induced autophosphorylation of the fusion receptor. Dose-dependent effects of insulin, insulin-like growth factor 1 and epidermal growth factor on BRET signal were in agreement with known pharmacological properties of these ligands. Moreover, an antibody, which activated the autophosphorylation of the receptor, had similar effects on BRET signal. This methodology allows for rapid analysis of the effects of agonists on insulin receptor activity and could, therefore, be used in high-throughput screening for the discovery of molecules with insulin-like properties.

Keywords: Insulin receptor; Diabetes; Obesity; Bioluminescence Resonance Energy Transfer; High-throughput screening

1. Introduction

Insulin, a polypeptide hormone produced by the β cell of the pancreas, plays a crucial role in the regulation of energy metabolism. It stimulates the uptake of nutrients (glucose, amino acids and fatty acids) by cells, accelerates their conversion into macromolecules (glycogen, proteins and triglycerides) and inhibits the degradation of these macromolecules. At the cellular level, insulin acts on its target tissues (liver, muscle and adipose tissue) by binding to a plasma membrane receptor. The insulin receptor is a glycoprotein composed of two α-subunits and two β-subunits linked by disulfide bonds (Fig. 1). Binding of insulin to the α-subunits of the receptor induces the autophosphorylation of the β-subunits on tyrosine residues. This autophosphorylation stimulates the tyrosine kinase activity of the receptor toward intracellular substrates, thereby allowing the transmission of the signal [1]. In pathological states, such as diabetes and obesity, insulin effect on its target tissues is markedly reduced. This insulin resistance is often associated with a decrease in the tyrosine kinase activity of the receptor, resulting in alterations in insulin signalling. The discovery of new molecules, capable of stimulating the tyrosine kinase activity of the receptor, may be of considerable importance for the treatment of insulin-resistant or insulin-deficient patients. In order to discover such molecules, it is important to have a fast and convenient method to monitor the activity of the insulin receptor. Classically, the tyrosine kinase activity of the insulin

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Abbreviations: BRET, Bioluminescence Resonance Energy Transfer; YFP, yellow fluorescent protein.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01143-7

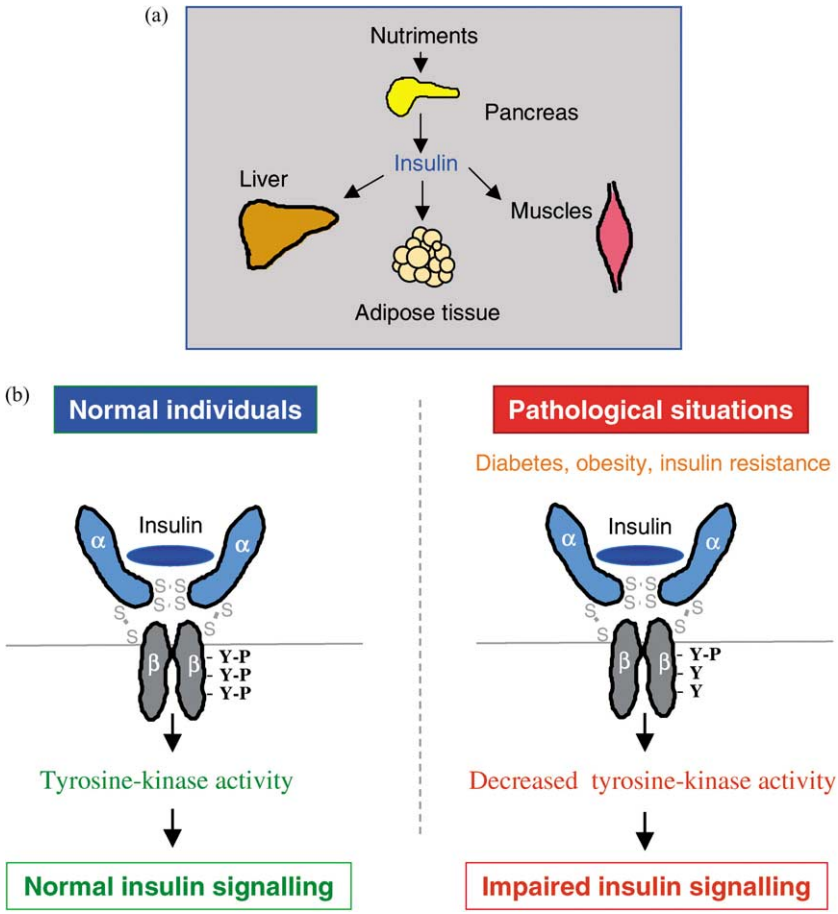

Fig. 1. Insulin regulates energy metabolism through a tyrosine kinase receptor. Insulin is a pancreatic hormone that controls energy metabolism in liver, muscle and adipose tissue (a). Binding of insulin to the α-subunit induces the phosphorylation of three tyrosines located in the kinase domain of the β-subunit of the receptor. In insulin-resistant patients, the autophosphorylation and the kinase activity of the receptor are decreased, resulting in alterations in insulin signalling (b).

receptor is assessed by determining its ability to phosphorylate substrate peptides on tyrosines. This can be achieved either by measuring the incorporation of radioactive phosphate into the substrate or by immunological techniques, such as ELISA, using anti-phosphotyrosine antibodies. However, the former method presents all the drawbacks associated with the handling of radioactive isotopes, and the latter implies many time-consuming incubation and washing steps. Therefore, the existing techniques for measuring the activity of the insulin receptor are not particularly convenient for the search of new molecules in high-throughput screening assays. For these reasons, we decided to determine whether we could develop a new assay, based on the BRET methodology [2,3], to monitor the activity of the insulin receptor.

2. The Bioluminescence Resonance Energy Transfer methodology

In order to study the interaction between two partners, the first partner is fused to Renilla luciferase. The second partner is fused to the yellow variant of the green fluorescent protein. The luciferase is excited using a specific substrate, cœlenterazine. If the two partners do not interact, only one signal, emitted by the luciferase at 485 nm, can be detected. If the two partners interact (if the distance between the two partners is comprised between 10 and 100 Å), an energy transfer occurs between the luciferase and the yellow fluorescent protein (YFP), resulting in the emission of a fluorescent signal by the YFP at 530 nm (Fig. 2).

Binding of insulin to the α-subunits of the receptor is believed to induce a conformational change that brings the two β-subunits in close proximity, allowing trans-phosphorylation of one β-subunit by the other β-subunit [4]. We reasoned that if we could make a chimeric insulin receptor with one of the β-subunit fused to the luciferase and the other β-subunit fused to the YFP, we should be able to detect the conformational changes of the receptor using the BRET methodology (Fig. 3a). For this purpose, we have fused the cDNA coding for the human insulin receptor to either Renilla luciferase or YFP. These constructs are co-transfected in HEK cells. The cells are extracted and the

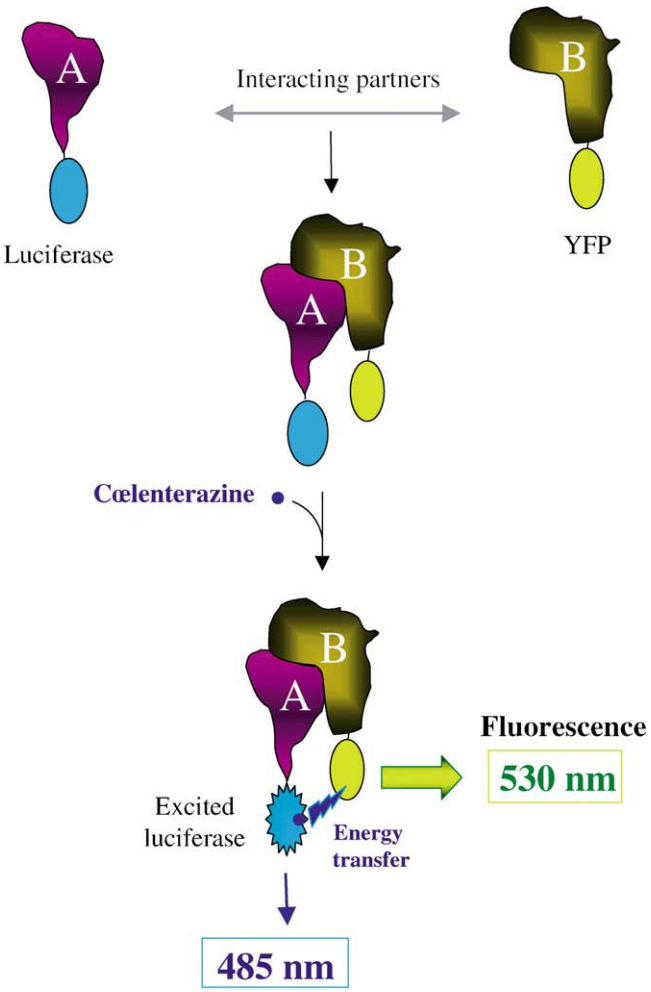

Fig. 2. Principle of the BRET methodology. The partners of interest (proteins A and B) are fused to either Renilla luciferase or to the yellow fluorescent protein. The luciferase is excited using a specific substrate, cœlenterazine. If the two partners are in close proximity (10–100 Å), an energy transfer occurs between the luciferase and the YFP, and a fluorescent signal, emitted by the YFP, can be detected.

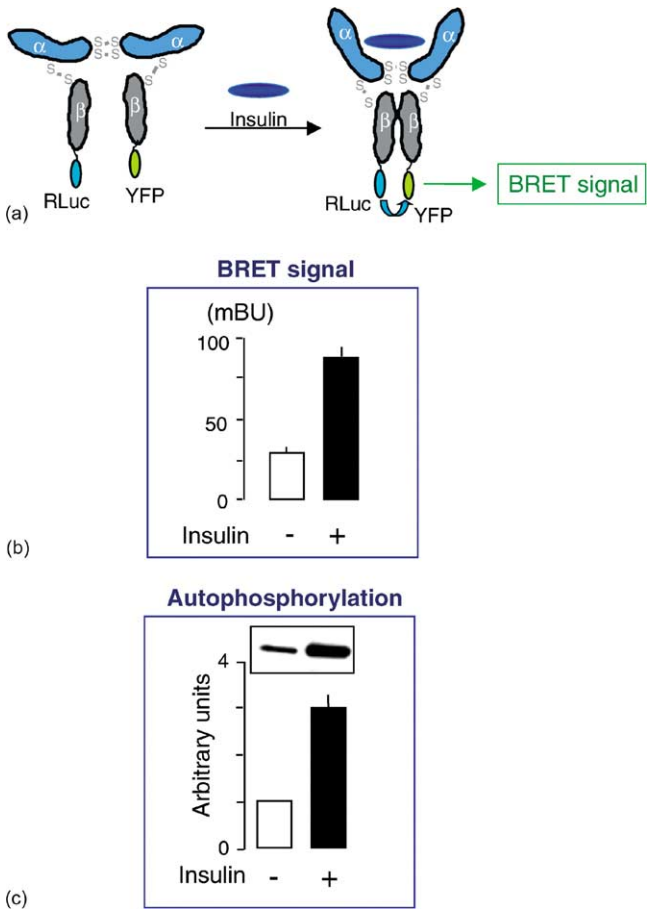

Fig. 3. The BRET methodology can be used to monitor the activity of the insulin receptor. The conformational change induced by insulin (a) can be detected as a BRET signal (b). This BRET signal reflects the activation state of the insulin receptor, as measured by its autophosphorylation on tyrosine residues using an anti-phosphotyrosine antibody (c). Adapted from [5].

chimeric receptors are partially purified by wheat-germ lectin chromatography [5].

3. Monitoring the activation of the insulin receptor with BRET

Partially purified chimeric receptors are incubated in 96 well-microplates in absence or presence of insulin. Cœlenterazine is added to stimulate the luciferase, and light emission acquisition at 485 and 530 nm is started immediately. We observed that a BRET signal could be readily detected in the basal state (Fig. 3b). This signal probably reflects random interactions between Renilla luciferase and YFP. Binding of insulin to its receptor resulted in a conformational change that brings closer together the two β-subunits, thus allowing trans-phosphorylation to occur. In this conformation, the probability of having Renilla luciferase and YFP in close proximity will increase, resulting in a stronger BRET signal (a 2–3-fold increase). This effect was obtained using receptors incubated in the absence of ATP. No further increase could be detected if the chimeric receptors were submitted to an autophosphorylation reaction [5]. Therefore, the BRET signal corresponds to the conformational change induced by insulin prior to any phosphorylation event.

We have also measured, in the same experiments, the effect of insulin on the autophosphorylation of the chimeric receptor, by immunoblotting using an anti-phosphotyrosine antibody (Fig. 3c). Densitometric analysis of the blots showed that the effect of insulin on BRET signal was very similar to the effect of insulin on the autophosphorylation of the chimeric receptor. Therefore, the BRET signal indeed reflects the activation state of the insulin receptor (Fig. 3c).

We also performed dose–response experiments [5] and we observed that insulin dose-dependently stimulated BRET signal, with a half-maximal effect at about 5 nM of insulin. Insulin-like growth factor 1 also dose-dependently stimulates BRET signal, but with a half-maximal effect at about 200 nM. Epidermal growth factor had no effect on the BRET signal. These results are in agreement

with known pharmacological properties of these ligands toward the insulin receptor.

In order to determine whether our assay could detect the effect of molecules with insulin-like activities, we have used a monoclonal antibody, directed against an epitope located on the α-subunit of the receptor [6]. It has been shown that 83-14 antibody has insulin-like effects on adipocyte metaboblism [7], and that it stimulates the autophosphorylation of the receptor [8]. It is known that 83-14 antibody acts on the insulin receptor through an epitope that is distinct from the insulin binding site. Moreover, it has been shown that this antibody can stimulate the tyrosine kinase of receptors from a diabetic patient with a rare mutation that impairs insulin binding [9]. We observed that this antibody also strongly stimulates BRET signal, indicating that our procedure should allow to detect molecules that activate the insulin receptor independently of the insulin binding site [5].

4. BRET vs. conventional techniques

Fig. 4 compares BRET to classical techniques employed to monitor the activity of the insulin receptor. The BRET assay is much faster, as it does not require any phosphorylation reaction, washing step or separation procedure. Such a homogenous assay is clearly advantageous to evaluate the activity of molecules or ligands towards the insulin receptor. However, like any methodology, it has its

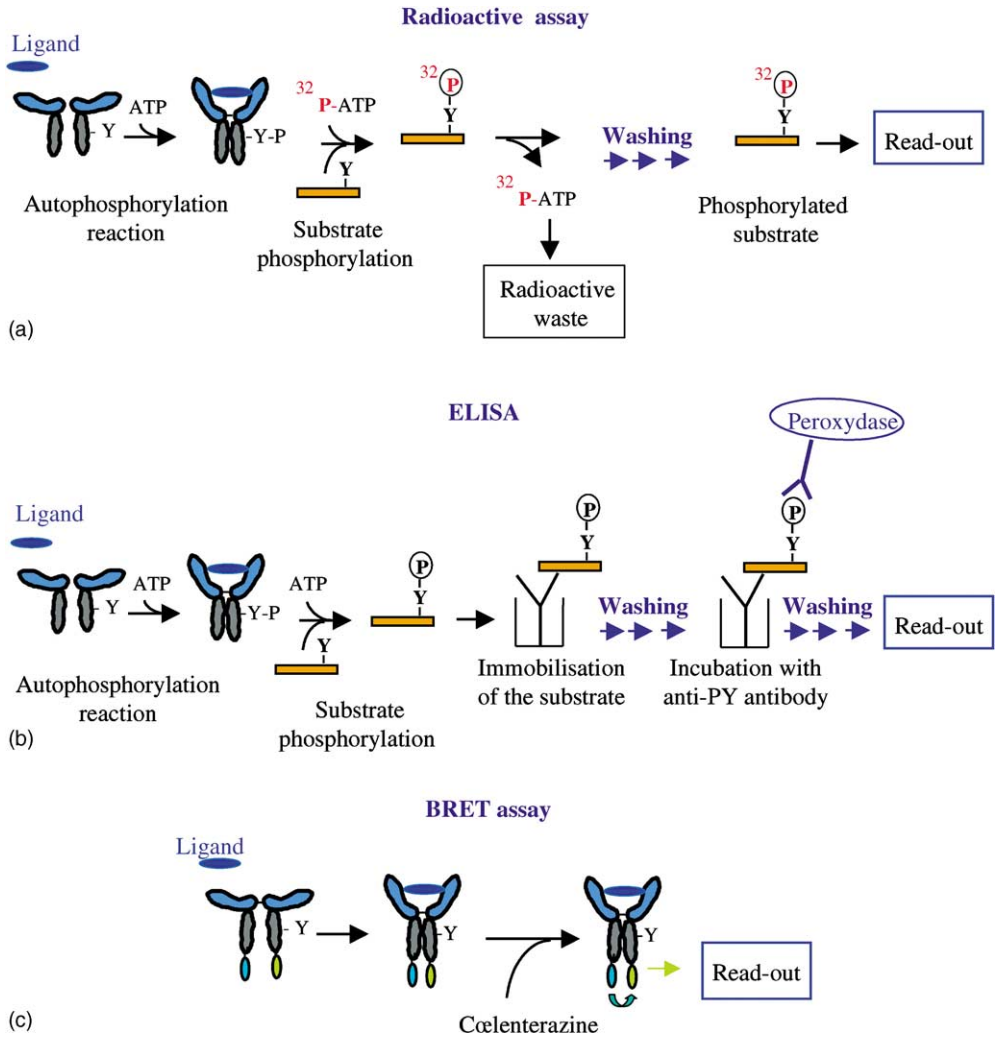

Fig. 4. Comparison of different methods used to assay the activity of the insulin receptor. The activity of the insulin receptor can be assessed by measuring the incorporation of radioactive phosphate into a substrate peptide. To this end, it is first necessary to perform an autophosphorylation reaction, in order to activate the kinase of the receptor. Then, exogenous substrate and radioactive ATP are added, and the phosphorylation reaction is performed. The reaction mixture is spotted onto phosphocellulose paper squares, which retain the peptide. After several washing steps, the paper squares are dried and transferred into a radioactive counter to quantify the incorporation of ^{32}P into the peptide (a). The activity of the insulin receptor can also be assessed using immunological techniques, such as ELISA. An autophosphorylation reaction is still necessary. The exogenous substrate is then added, and the phosphorylation of the substrate is performed. The phosphorylated substrate is immobilised by incubation with an anti-substrate antibody precoated on a microtitre plate. After several washing steps, the immobilised peptide is incubated with an anti-phosphotyrosine antibody coupled to peroxydase. After additional washing steps to remove unbound peroxydase labelled antibody, the signal can be measured (b). The BRET assay is entirely homogenous. It does not involve any phosphorylation reaction, separation or washing steps. The only reagent needed is cœlenterazine (c).

own limitations. Indeed, this assay is entirely based on artificial receptors with one β-subunit fused to luciferase and the other β-subunit fused to YFP. Therefore, it obviously cannot be employed for the study of the functional activity of the insulin receptor in different pathophysiological situations, for instance in animal models of insulin resistance or in human obese or diabetic patients. For such studies, the use of more conventional techniques remains necessary.

5. Conclusion

We have developed a fast and convenient assay, based on BRET methodology, which can be used for the detection of molecules with insulin-like activity. This is a homogenous assay, which requires neither phosphorylation reaction nor washing steps. Moreover, the fact that this assay is performed in very small volumes (we routinely use 5 μL of partially purified receptors) and that the only reagent needed is cœlenterazine, makes it a particularly economical procedure.

This method is obviously amenable to high-throughput screening assay. Indeed, partially purified chimeric receptors can be prepared on a large scale by wheat-germ lectin chromatography, aliquoted and stored at -80° for several months. At any time, these aliquots can be used to study the effects of molecules on the insulin receptor activity. The receptor solution can be distributed in an automated way in 96 well-microplates, and libraries of molecules can be rapidly screened for the search of new drugs with insulin-like activity that may be used for the treatment of insulin deficiency or insulin resistance.

Acknowledgments

This work was supported by the Association pour la Recherche sur le Cancer, the Ligue contre le Cancer and a Roche-Pharma-ALFEDIAM research grant.

References

[1] Combettes-Souverain M, Issad T. Molecular basis of insulin action. Diabetes Metab 1998;24:477–89.
[2] Xu Y, Piston DW, Johnson CH. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc Natl Acad Sci USA 1999;96:151–6.
[3] Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci USA 2000;97:3684–9.
[4] Hubbard SR. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO J 1997;16:5572–81.
[5] Boute N, Pernet K, Issad T. Monitoring the activation state of the insulin receptor using bioluminescence resonance energy transfer. Mol Pharmacol 2001;60:640–5.
[6] Soos MA, O'Brien RM, Brindle NP, Stigter JM, Okamoto AK, Whittaker J, Siddle K. Monoclonal antibodies to the insulin receptor mimic metabolic effects of insulin but do not stimulate receptor autophosphorylation in transfected NIH 3T3 fibroblasts. Proc Natl Acad Sci U S A 1989;86:5217–21.
[7] Taylor R, Soos MA, Wells A, Argyraki M, Siddle K. Insulin-like and insulin-inhibitory effects of monoclonal antibodies for different epitopes on the human insulin receptor. Biochem J 1987;242:123–9.
[8] O'Brien RM, Soos MA, Siddle K. Monoclonal antibodies to the insulin receptor stimulate the intrinsic tyrosine kinase activity by cross-linking receptor molecules. EMBO J 1987;6:4003–10.
[9] Krook A, Soos MA, Kumar S, Siddle K, O'Rahilly S. Functional activation of mutant human insulin receptor by monoclonal antibody. Lancet 1996;347:1586–90.