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SHORT COMMUNICATION

Brain-Derived Neurotrophic Factor in Human Platelets

F. Bernardo Pliego-Rivero,† Nadhim Bayatti,† Xenophon Giannakoulopoulos,‡ Vivette Glover,‡ Henry F. Bradford,† Gerald Stern§ and Merton Sandler¶*

†Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK; ‡Department of Paediatrics, Queen Charlotte's & Chelsea Hospital, London W6 OXG, UK; §The Woolavington Private Patients Wing, The Middlesex Hospital, London W1N 8AA, UK; and ¶Department of Chemical Pathology, Queen Charlotte's & Chelsea Hospital, London W6 OXG, UK

ABSTRACT. A sensitive, capture enzyme-linked immunosorbent assay (CELISA) has been applied to the accurate and reproducible measurement of brain-derived neurotrophic factor (BDNF) protein in normal human blood platelets, a mean concentration of 1.03 ± 0.04 ng (SEM)/mg of platelet protein being observed. The method, which requires only 10 ml blood, is now suitable for the investigation of a variety of clinical disorders. BIOCHEM PHARMACOL **54**;1:207–209, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. brain-derived neurotrophic factor (BDNF); neurotrophins; human platelets; CELISA assay

When *in vivo* information about particular biochemical systems is required, direct examination of the tissue under investigation is usually the first option in experimental animals. In man, however, this approach is rarely possible, for ethical reasons, and the dilemma is strongest when the organ under scrutiny is as inaccessible as the brain. Indirect approaches are therefore indicated and, in practice, this frequently involves making inferences from measurements on the blood and its formed elements. The human blood platelet has been of particular interest, in this context, because of its accessibility. It has been discussed by some as a possible neuronal model [1, 2] and its 5-hydroxytryptamine concentration and uptake ability have been particularly well studied [see, for example, ref. 3], as has its content of monoamine oxidase B [4] and phenolsulphotransferase [5]. Significant amounts of brain-derived neurotrophic factor [BDNF]† have similarly been detected in these cells by Yamamoto and Gurney [6], although the large volumes of blood needed for their assay might preclude its routine clinical application.

Since nerve growth factor [NGF], the first of the neurotrophins, was identified [7] a family of these powerful growth promoting factors has emerged [for reviews, see ref. 8], BDNF being one of the most important [9]. These compounds are present and active in the nervous system and elsewhere in very low concentration. This is one reason they have been difficult to quantify and most claims to their presence in the literature have relied on measurements of mRNA. Recently, however, novel enzyme immunoassay techniques [see, for example, ref. 10] have made it possible to measure the actual neurotrophin protein, at least as far as BDNF in brain is concerned. In this paper, we describe how this methodological approach can be applied to the accurate and reproducible measurement of BDNF protein in platelets from small volumes of human blood.

MATERIALS AND METHODS

All general reagents used were either from Merck (Poole, Dorset) or Sigma Chemical Company (Poole, Dorset), and all procedures were carried out at 4°C unless otherwise stated. Human platelet samples from five normal subjects, prepared by the method of Summers et al. [11], were suspended in 1 ml of 3.0 M sucrose and stored at −70°C until assay. This method of platelet preparation requires only 10 ml blood and platelets can be harvested within 30 min of withdrawal. After thawing, a "cocktail" of protease inhibitors was added to the suspension, to a final concentration of 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM phenylmethylsulphonyl fluoride (PMSF), 100 pg/ml bestatin and 500 pg/ml cystatin, Platelets were centrifuged at 11000 × g for 15 min in a Sigma bench top centrifuge (Howe, Banbury, Oxfordshire) and the sucrose supernatant collected. The pellet of particulate material was resuspended in 0.5 ml 5% deoxycholate (DOC) in ice-cold (0–4°C) 100 mM Tris-HCl, pH 10.1, to which protease inhibitors had been added. The membranes were

* Corresponding author: Prof. M. Sandler, Department of Chemical Pathology, Queen Charlotte's & Chelsea Hospital, Goldhawk Road, London W6 OXG. UK. Tel. (0181) 740 3099; FAX (0181) 741 1948.

† *Abbreviations:* BDNF, brain-derived neurotrophic factor; hrBDNF, human recombinant BDNF; BSA, bovine serum albumin; CELISA, capture enzyme-linked immunosorbent assay; CNTF, ciliary neurotrophic factor; DC, detergent compatible; DOC, deoxycholate; EDTA, ethylenediamine tetraacetic acid; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; PBS, phosphate buffered saline; PMSF, phenylmethylsulphonyl fluoride; SDS/PAGE, sodium dodecylsulphate/polyacrylamide gel electrophoresis; SS, sucrose supernatant; TBS, tris buffered saline.

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homogenized thoroughly for 3 min with a hand-held Teflon homogeniser pestle designed to fit an 1.5 ml Eppendorf tube immersed in an ice-cold water bath (4°C). The DOC-extracted membranes were centrifuged at 150000 × g for 60 min, and the residual pellet re-extracted twice with 200 µl 5% DOC in 100 mM Tris-HCl, pH 10.1, containing 1 mM PMSF and 1 mM EDTA. Deoxycholate was used because it is a cationic detergent with a very strong surfactant and dispersing ability, capable of efficiently disrupting protein–protein and protein–lipid interactions and rendering them soluble in a monomeric form [12].

Capture Enzyme-Linked Immunosorbent Assay (CELISA)

All fractions were assayed in duplicate for their content of BDNF, using a CELISA procedure. Plastic multiwell plates (maxisorp, Nunc Inc., Naperville, IL) were coated overnight with rabbit anti-BDNF antibody [10] (50 µl/well, 1 µg/ml) diluted with phosphate buffered saline (PBS), 138 mM NaCl, 2.13 mM KH_2PO_4, 9.66 mM Na_2HPO_4, pH 7.5, plus 0.05% NaN_3. Blocking of non-coated plastic was then achieved with 1% bovine serum albumin (BSA) in PBS (PBS/BSA), applied for not more than 30 min. For the detection of BDNF in each subcellular fraction a sample of 50 µl was serially diluted 1:2 in triplicate and incubated with the capture antibody over a period of 48 hr at 4°C. After that, the plates were incubated for 24 hr with a primary turkey-anti BDNF antibody (Amgen, Thousand Oaks, CA) (1:1000) [13]. The secondary antibody, biotinylated rabbit anti-turkey (1 µg/ml), was then applied overnight. The secondary rabbit anti-turkey antibody was pre-adsorbed overnight with rat blood serum (1:10000) and protein (20 µg) taken from the soluble fraction of rat brain prior to its use for analysis. The last stage was a 1 hr incubation with streptavidin-alkaline phosphatase (Vector, Burlingame, CA) in 20 mM Tris-100 mM NaCl, pH 7.5 (Tris buffered-saline, TBS). Colour development due to the alkaline phosphatase reaction was carried out using p-nitrophenyl phosphate disodium tablets (Pierce Chemical Co., Rockford, IL) as substrate in 100 mM ethanolamine-10 mM $MgCl_2$, pH 9.6. All incubations and washings were done at 4°C on a rotary shaker. Unrelated protein, and unbound antigen and antibodies were removed by rinsing the wells with TBS and 0.1% BSA. Colour was detected with a plate-reader spectophotometer (Anthos Labtec Instruments, Salzburg) set at an absorption wavelength of 415 nm. The protein content of each fraction was determined using the DC protein assay (BioRad, Hemel-Hempstead, Herts) with BSA as standard, following the manufacturers instructions.

Fig. 1 demonstrates a standard curve produced with human recombinant BDNF (hrBDNF), showing linearity from approximately 0.1 ng/ml to 10 µg/ml. In every CELISA performed for the determination of BDNF, a standard curve was in fact constructed using hrBDNF. The hrBDNF protein was serially diluted 1:2, starting at a concentration of 125 ng/ml (saturating conditions) and provided a minimum level of detection of 1–4 ng/ml. Further dilution points served as baseline which was always very similar to levels of control samples to which no primary antibody had been added. The BDNF content of each sample was calculated from the linear portion of the standard curve generated by computer (GraphPad Prism version 2.01, GraphPad Software Inc., San Diego, CA).

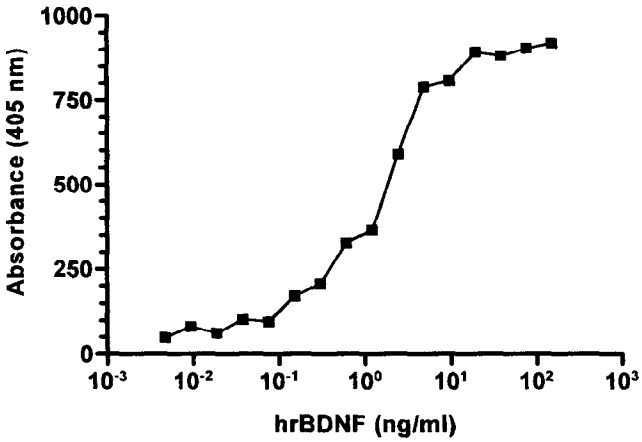

FIG. 1. Standard curve generated with human recombinant brain-derived neurotrophic factor (hrBDNF) showing linearity from approximately 0.1 ng/ml to 10 ng/ml. Every assay for BDNF content of platelet samples was run with its own hrBDNF standard curve.

Platelet protein was measured by the Biorad DC protein assay (see above). Cross-reactivity was not observed with NGF, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT4/5) or ciliary neurotrophic factor (CNTF), all at ten times the highest concentration of BDNF employed [14].

RESULTS AND DISCUSSION

A massive literature attests to the importance of the neurotrophins in early mammalian development [see ref. 8]. However, their role in the adult and, more particularly, in man [15], is relatively unknown. Even so, a continuous expression of BDNF has been identified and followed into adult life, in the monkey brain at least [16], and it seems likely that trophic actions, influencing brain development, are critical throughout life, mediating learning, memory and regrowth after injury [17].

Such measurements as those of Huntley et al. [16], mentioned above, had, of necessity to rely on quantification of BDNF mRNA, in the absence of suitable techniques for low concentrations of BDNF protein itself. However, the advent of CELISA techniques [10] has made such measurements possible and we now show, in Table 1, that when applied to normal human platelet samples, they are accurate and reproducible. An early study by Yamamoto & Gurney [6] focused on the development of a sensitive but highly complex assay for the measurement of BDNF in human platelets, which renders it inaccessible to the majority of those wishing to assay BDNF protein. These

TABLE 1. Results for BDNF-like protein concentration in human platelet preparations

	Sucrose supernatant (SS)		Deoxycholate extract (DOC)		Total BDNF-like content	
Subject	SS (±S.E.M.) ng/ml	SS/protein* ng/mg	DOC (±S.E.M.) ng/ml	DOC/protein* ng/mg	ng/ml	ng/mg*
1	0.75 (0.00)	1.03	2.09 (0.02)	1.28	1.79	1.12
2	0.75 (0.08)	0.60	1.23 (0.01)	2.20	1.36	1.00
3	0.79 (0.01)	0.93	1.60 (1.01)	1.01	1.59	0.96
4	0.66 (0.01)	0.65	1.61 (2.12)	2.12	1.46	0.95
5	0.63 (0.01)	0.95	1.84 (1.58)	1.58	1.55	1.14
Mean (±S.E.M.)	0.72 (0.03)	0.83 (0.09)	1.67 (0.23)	1.64 (0.23)	1.55 (0.07)	1.03 (0.04)

Values presented are the means for duplicates from each preparation.
* Values are corrected for protein content in each preparation.

investigators used ion-exchange columns partially to purify BDNF in human platelets obtained from 100 ml of blood, and a dorsal root ganglion assay and SDS-PAGE for qualitative detection of BDNF, but did not report quantitative levels of BDNF. To our knowledge, the only other purification from a similar source in the literature [18] does quote quantitative levels of BDNF in whole blood *and* serum (but not in purified platelets), obtained by an ELISA of similar sensitivity to our own, but the levels reported are 10-fold higher than those reported by us in purified platelets. Unfortunately, a very sensitive assay (lower limit of assay: 1 pg/ml) developed by Nawa and colleagues (6) requires that at least 10 ml of purified anti-BDNF serum are chromatographed through BDNF-linked affinity columns in order to isolate the antibody sub-fraction necessary for achieving this high sensitivity. This makes the procedure very costly, and increases extensively the overall run times.

Our report provides some reference data, obtained using a very specific and sensitive ELISA, requiring platelets from only 10 ml blood. Thus, the way is now open for platelet BDNF estimations to be made in such neurological conditions, for example, as depressive illness or migraine [15], where a possible disturbance in production has been adumbrated, and other functions, as in studying the previously reported pronounced increase in BDNF in blood during clotting [18].

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