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Bradykinin Increases Ceramide and Sphingosine Content in Human Fibroblasts: Possible Involvement of Glycosphingolipids

Elisabetta Meacci,[1] Valeria Vasta, Marta Farnararo, and Paola Bruni

Department of Biochemical Sciences, University of Florence, Viale G.B. Morgagni 50, 50134 Florence, Italy

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Sphingolipid-derived products are recognized as second messengers able to mediate the action of extracellular signals such as cytokines and growth factors. In the present study it is shown that also bradykinin (BK), a pro-inflammatory peptide acting through G-protein-coupled receptors, is able to activate sphingolipid metabolism. Fibroblast treatment with the peptide provokes a rapid and significant increase in ceramide followed by a transient rise in sphingosine content. Sphingomyelin does not appear to be the source of ceramide since BK was unable to decrease the [^{3}H]sphingomyelin pool and no increase in neutral or acidic sphingomyelinase activities could be detected after treatment with the peptide. The observation that the labeled glycosphingolipid pool is decreased upon BK stimulation would rather suggest that the peptide increases ceramide cellular content by rapidly mobilizing neutral glycolipids. Even though the physiological relevance of ceramide and sphingosine increase induced by BK is not known, it is noteworthy that glycosphingolipids may participate in this lipid signalling pathway.

Sphingolipids represent a family of complex lipids ubiquitously found in eukaryotic cell membranes. In the last few years sphingolipid derivatives have been identified as endogenous signal-transducing molecules (1–3). In this regard it has been demonstrated that in a variety of cell systems the signalling pathway of the major sphingolipid, sphingomyelin, is regulated by extracellular signals and that sphingomyelin-released products act as second messengers. Indeed, ceramide, the direct product of sphingomyelinase (SMase) action on sphingomyelin and precursor of all sphingolipids, can exert a variety of biological effects participating to central biological processes such as cell cycle regulation (4), cell differentiation (5), apoptosis (6), secretion (7) and endocytosis (8), in particular, ceramide has been recently recognized as a key second messenger able to mediate the action of cytokines such as tumor necrosis factor-α and interleukin-1β in target cells (9). Although at present the molecular mechanisms responsible for ceramide segnalatory action are not completely elucidated, it has been recently shown that it activates a specific protein phosphatase involved in the control of c-myc expression (10) as well as a proline-directed serine/threonine kinase which can in turn activate the mitogen activated protein kinase cascade through Raf phosphorylation (11).

Additionally, the activation of sphingomyelin hydrolysis can also result in an increase in sphingosine cellular content, as consequence of ceramidase action on ceramide, sphingosine, together with sphingosine 1-phosphate formed by the action of sphingosine kinase, has been shown to be involved in the control of cell proliferation (1,2). In particular, it has been demonstrated that sphingosine acts as second messenger in the mitogenic signal triggered by platelet-derived growth factor and other growth factors (12,13), likely through activation of multiple signalling pathways such as mobilization of intracellular calcium (14), activation of phospholipase D (15) and enhancement of the DNA binding activity of transcriptional factor AP-1 (16). Other sphingolipids, besides sphingomyelin, are also regarded as signal molecules: glycosphingolipids are indeed able to modu-

[1] Fax: 39-55-4222725.

Abbreviations used: BK, bradykinin; SMase, sphingomyelinase; BODIPY FL-sphingomyelin, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosyl-phosphocholine; BODIPY FL-ceramide, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosine; PMSF, phenylmethylsulfonyl fluoride.

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late transmembrane signalling, mediate cellular interactions and regulate cell cycle (17). In this regard it has been recently shown that the specific inhibition of glucosylceramide synthase is responsible for cell cycle arrest by increasing ceramide cellular content (18).

Although a number of agonists acting through distinct signalling pathways, including cytokines (9), growth factors (13) and vitamin D_3 (19), are able to activate the sphingomyelin pathway, presently no information is available concerning the ability of agonists acting through G-protein-coupled receptors to affect sphingolipid turnover. In the present study this latter possibility has been investigated by examining the effect of the pro-inflammatory peptide bradykinin (BK) on fibroblast sphingolipid metabolism.

MATERIALS AND METHODS

BK, *S. aureus* SMase, DMEM, PBS, fetal bovine serum (FBS) were obtained from Sigma. *E. Coli* diacylglycerol kinase was from Calbiochem. [λ-^{32}P]ATP (111 TBq/mmol), [^{3}H]serine (803 MBq/mmol) were obtained from New England Nuclear (Dreiech, Germany). The Silica gel 60 plates for TLC were obtained from Merck (Darmstadt, Germany). N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosylphosphocholine (BODIPY FL-sphingomyelin) and N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine (BODIPY FL-ceramide) were obtained from Molecular Probes (Leiden, The Netherlands). Sphingosine 1-phosphate was kindly provided by Prof. L. Riboni (Department of Medical Chemistry and Biochemistry, University of Milan). Galactosylceramide was kindly given by Prof. G. Mugnai (Institute of General Pathology, University of Florence).

Cell culture. Human IMR-90 fibroblasts were obtained from Institute for Medical Research (Camden, NJ, USA). Cells were maintained in DMEM containing antibiotics and 10% FBS at 37°C in an atmosphere of 5% CO_2. Cell cultures were routinely tested for contaminating mycoplasma using a kit from Boehringer (Mannheim, Germany).

Measurement of ceramide and sphingosine content. Fibroblasts grown to confluence in 100 mm dishes were made quiescent by incubation in DMEM without serum for 48 h, washed with PBS and incubated for 90 min in fresh DMEM before the addition of the agonist. The incubations were terminated by aspirating the medium, washing twice with ice-cold PBS and immediately adding 1 ml of ice-cold methanol. Cells were collected by scraping and lipids were then extracted by adding 2 vol of chloroform and 1/4 vol of 0.1N HCl, vortexing and incubating at room temperature for 5 min. The phases were separated by centrifugation at 2,800 × g for 10 min. The extraction procedure was repeated, the organic phases were pooled and used for ceramide or sphingosine quantitation; an aliquot was always utilized to quantify total phospholipids by measuring the phosphate content after acid hydrolysis (20).

For ceramide determination aliquots containing approximately 150 nmol of phospholipids were dried under a stream of nitrogen and dissolved by sonication in 20 μl of 1 mM DETAPAC, pH 7.0 containing 7.5% octyl-β-D-glucoside and 5 mM cardiolipin; ceramide content was evaluated by diacylglycerol kinase-catalyzed conversion of ceramide to [^{32}P]ceramide-1-P in the presence of [γ-^{32}P] ATP and subsequent separation of [^{32}P]ceramide-1-P by TLC as reported in (21). Known amounts of ceramide were processed similarly to the samples to produce a standard curve.

Sphingosine content was determined essentially as described (22) using sphingosine kinase prepared from NIH-3T3 fibroblasts, to generate [^{32}P]sphingosine-1-P in the presence of [γ-^{32}P]ATP; [^{32}P]sphingosine-1-P was then separated by TLC with 1-butanol/methanol/acetic acid/water (80:20:10:20). Spots corresponding to sphingosine 1-phosphate standard were scraped into scintillation vials and radioactivity was measured after addition of 0.5 ml of methanol and liquid scintillation cocktail. Sphingosine was quantified using the standard curve generated by processing, in parallel with the samples, known amounts of sphingosine added with 200 nmol phosphatidylcholine.

Sphingolipid metabolism. Fibroblasts grown to confluence in 6-well dishes were made quiescent by incubation in DMEM without serum for 48 h and labeled for the last 24 h with [^{3}H]serine (2.5 μCi/ml). The monolayers were washed twice with PBS and incubated for 5 h with unlabeled serine (5 mM). The cells were washed as before and incubated with BK or *S. Aureus* SMase. The incubations were terminated by aspirating the medium, washing twice with PBS and cells were collected in 1 ml ice-cold methanol containing 20 μg/ml each of sphingomyelin, sphingosine, and ceramide. Lipids were extracted by incubation with chloroform/methanol (1:2, v/v) for 1 h and then with chloroform/methanol (2:1, v/v) for further 30 min. The phases were separated by addition of 1.5 ml of water and the organic phase was incubated with 0.1 M methanolic KOH (final concentration) for 1 h at 37°C, dried, solubilized in chloroform/methanol (2:1). Samples were separated by sequential TLC in chloroform/methanol/ammonia/water (80:20:1:1) to 9.5 cm, followed by solvent containing dietylether/methanol (99:1). Unlabeled standards were run together with samples and lipid spots corresponding to sphingomyelin, ceramide and sphingosine were localized by iodine staining and scraped into scintillation vials. The samples received 0.5 ml of methanol and liquid scintillation cocktail to measure radioactivity.

For [^{3}H]glycosphingolipid determination, fibroblasts were labeled with [^{3}H]serine (2.5 μCi/ml) for 48 h and lipids were extracted as described above. Samples together with standard sphingomyelin, ceramide, sphingosine and galactosylceramide utilized as standard were separated by TLC in chloroform/methanol/water (65:25:5) and the lipid spots were quantified as described above.

Measurement of SMase activity. SMase activity was assayed essentially according to Wiegmann et al. (23) in cell homogenates prepared from quiescent human fibroblasts, incubated or not in the presence of BK for different time intervals. Approximately 5×10^6 cells were washed twice in ice-cold PBS, scraped and collected by brief centrifugation at 4°C. To measure neutral SMase, pellets were resuspended in a buffer containing 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mM Na_3VO_4, 0.2% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were homogenized using Dounce homogenizer (30 strokes) and centrifuged at 800 × g for 10 min. The protein concentration in the supernatant was measured according to Bradford (24). An aliquot of supernatant containing 200 μg of protein was incubated for 2 h at 37°C in 100 μl of buffer containing 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$ and 60 nmol sphingomyelin prepared essentially as described in (25): BODIPY FL-sphingomyelin (3 nmol per assay) and sphingomyelin (57 nmol per assay) in chloroform/methanol (2:1) were mixed, dried under nitrogen and the residue dispersed in 20 mM Hepes pH 7.4. The reaction was linear within the examined time; the BODIPY FL-sphingomyelin spontaneous hydrolysis did not exceed 10% of the total amount of the fluorescent compound.

To measure the acidic SMase, cells were homogenized in a buffer containing 250 mM sodium acetate (pH 5), 1 mM EDTA, 0.2% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 1 mM PMSF and the supernatant obtained by centrifuging at 14,000 × g for 5 min was used for enzyme assay. An aliquot of supernatant corresponding to 50 μg of protein, was incubated for 2 h at 37°C in the same buffer (100 μl final volume) containing 60 nmol sphingomyelin prepared as above and resuspended in 250 mM sodium acetate, pH 5. All the reactions were terminated by adding 1.5 ml heptane and 0.45 ml isopropyl alcohol. The phases were separated adding 1.5 ml of water, vortexing and centrifuging at 2,800 × g for 10 min. The upper phase, heptane-rich, was transferred to a second tube and washed with 0.35 ml water. The fluorescence intensity of the upper phase was determined in a Perkin Elmer RF-5000 spectrofluorophotometer. The excitation and emission wavelengths corresponding to BODIPY FL-ceramide were 490 nm and 515 nm. The compound was quantified using a standard curve generated by measuring the fluorescence intensity of known amounts of BODIPY FL-ceramide processed as the samples.

RESULTS

To examine the potential action of BK on sphingolipid-derived products in human IMR-90 fibroblasts, ceramide and sphingosine content were determined in cells stimulated with the agonist. Basal ceramide content in confluent quiescent fibroblasts (1.9 ± 0.17 pmol/nmol phospholipid (n = 9)) was significantly increased by addition of 100 nM BK as early as at 30 sec of treatment; ceramide content attained a maximal value of 4.6 pmol/nmol phospholipid at 5 min of incubation (Fig. 1) and it decreased thereafter returning to control values at 30 min of stimulation. In the same

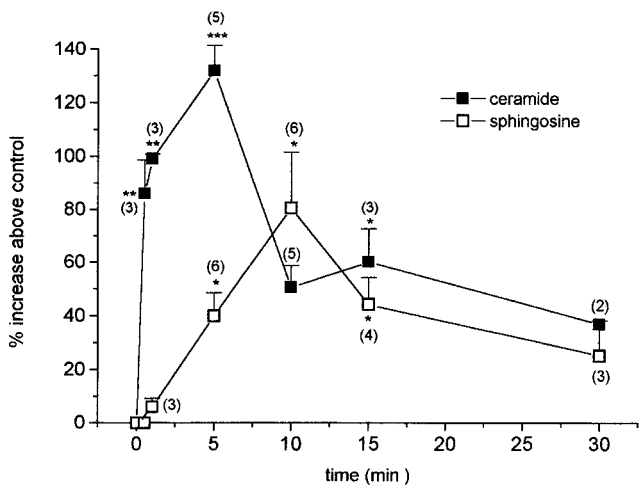

FIG. 1. Time-course of BK effect on ceramide and sphingosine content in fibroblasts. Quiescent fibroblasts were stimulated with 100 nM BK. Ceramide (■) and sphingosine (□) were measured as described in Materials and Methods section. Basal levels of ceramide and sphingosine were 1.9 ± 0.17 (n = 9) pmol/nmol of phospholipid and 0.73 ± 0.13 (n = 11) pmol/nmol of phospholipid, respectively. Data are means ± SEM of (n) experiments performed in duplicate. Statistical significance was determined by Student's *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

figure it is shown that agonist treatment resulted also in the appreciable rise of sphingosine content that was delayed compared to that of ceramide, reaching the maximal value at 10 min of incubation.

With the aim of better understanding the effect of BK on sphingolipid metabolism, the products of sphingolipid hydrolysis were analyzed in human fibroblasts in which the sphingolipid pool had been labeled by incubation for 24 h in the presence of 2.5 μCi/ml of [^{3}H]serine. As it is shown in Fig. 2A, BK treatment of prelabeled cells was responsible for a relevant increase in [^{3}H]ceramide

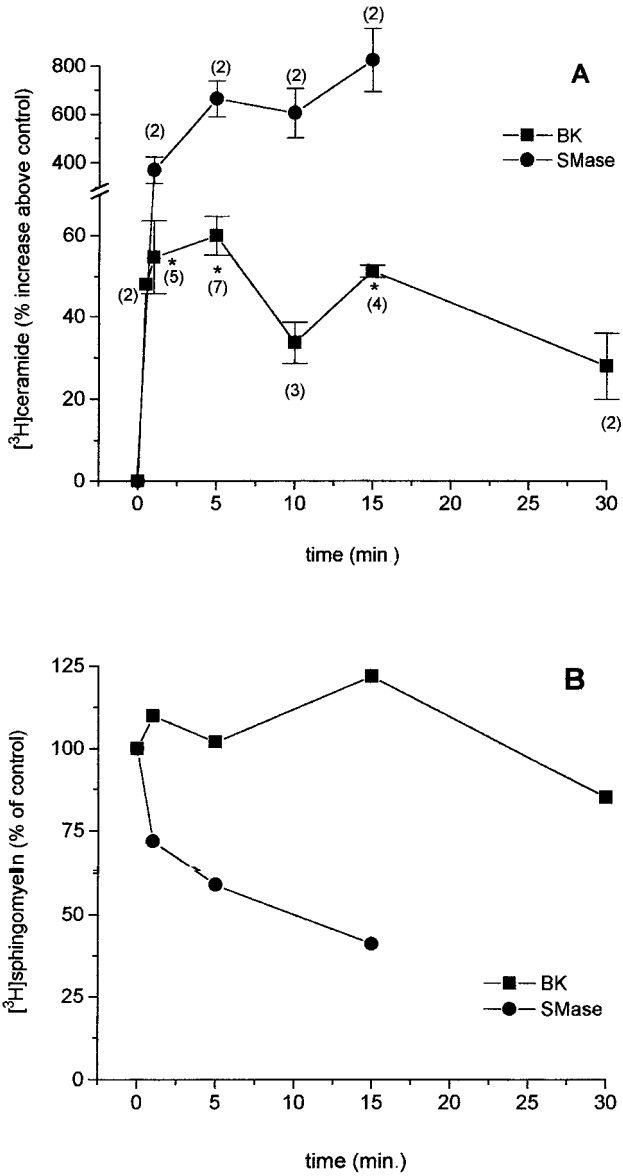

FIG. 2. Time-course of the effect of BK and SMase on [^{3}H]ceramide (A) and [^{3}H]sphingomyelin (B) in human fibroblasts. Quiescent fibroblasts, labeled with [^{3}H]serine, were stimulated with 100 nM BK (■) or 0.1 U/ml *S. Aureus* SMase (●). [^{3}H]ceramide and [^{3}H]sphingomyelin were measured as described in Materials and Methods section. The radioactivity incorporated in the controls was 2,600 ± 462 d.p.m. (n = 8) for [^{3}H]ceramide and 33,317 ± 3035 d.p.m. (n = 8) for [^{3}H]sphingomyelin. Data are means ± SEM of (n) experiments performed in duplicate (A) or means of a representative experiment (B). Statistical significance was determined by Student's *t* test. (*$p < 0.05$).

which was not accompanied by an appreciable reduction of radioactivity associated with sphingomyelin (Fig. 2B); from the same figure it can be observed that the parallel incubation of fibroblasts with *S. Aureus* SMase (0.1 U/ml) provoked a larger increase in [^{3}H]ceramide and a concomitant diminution of the [^{3}H]sphingomyelin pool. The absence of [^{3}H]sphingomyelin reduction by BK could indicate either that the increase of ceramide content induced by BK does not involve SMase activation or that the enzyme is activated by the peptide, but the decrease in the sphingomyelin pool is not detectable with this experimental procedure.

To further clarify this point, neutral and acidic SMase activities, both demonstrated to participate in the generation of sphingolipid-derived signals (23), were measured in cell extracts prepared from fibroblasts incubated with BK for different time-intervals. Values for neutral and acidic SMase activities, determined utilizing as substrate the fluorescent BODIPY-derivative of sphingomyelin, were in accordance with those measured by others in the same cell type (26); in particular, acidic SMase was approximately 200 times more active than the neutral form. As reported in Table I, both enzymatic activities were not significantly affected by fibroblast treatment with BK for 1–5 min. The present results strongly suggest that BK is unable to activate SMase activity although it can remarkably increase ceramide content. Glycosphingolipids were then examined as potential alternative source of ceramide released by BK. Results reported in Fig. 3 indicate that after 5 min of BK treatment a detectable decrease of the labeled glycosphingolipid pool was observed, suggesting that glycosphingolipid hydrolysis could be responsible for the observed increase in ceramide content induced by the peptide.

DISCUSSION

BK, a peptide released during the inflammatory response, is known to act through its specific G-protein-coupled receptors, by activating multiple phospholipid-derived signalling pathways through activation of phospholipase C, phospholipase A_2, phospholipase D and protein kinase C (27,28). In the present study we report that BK is also able to rapidly activate sphingolipid metabolism, by increasing ceramide and sphingosine cellular content. This finding, in view of the diverse biological roles exerted by ceramide and sphingosine, may imply that the variation in their cellular content participates to the signalling pathways triggered by the peptide and has a role in the overall biochemical changes induced by the agonist. This hypothesis is sustained by the recent observation that exogenous ceramide is able to interfere in the signalling pathway of BK by inhibiting protein kinase C-a translocation and PLD activation (28). In addition, it must be noted that the observed increase in ceramide content is rapid, similarly to that of diacylglycerol (29), a key transducer molecule of BK action in human fibroblasts.

The other major finding of the present study is that the rise in ceramide and sphingosine induced by BK is not due to the activation of SMase, as demonstrated by in vivo experiments in which the

TABLE I
Effect of BK on Neutral and Acidic SMase Activities in Human Fibroblasts

time	1 μM BK	neutral SMase activity (pmol/min/mg protein)	acidic SMase activity (pmol/min/mg protein)
—	−	2.62 ± 0.41	471.7 ± 28.3
30 sec	+	3.38 ± 0.8	n.d.
1 min	+	2.92 ± 0.6	n.d.
5 min	+	2.44 ± 0.5	578.3 ± 38.3

Human fibroblasts were stimulated with BK and neutral SMase and acidic SMase were assayed as described in Materials and Methods section. Data are means ± SEM of at least three independent experiments performed in duplicate. Statistical significance was determined by Student's *t* test. Abbreviation: n.d. not determined.

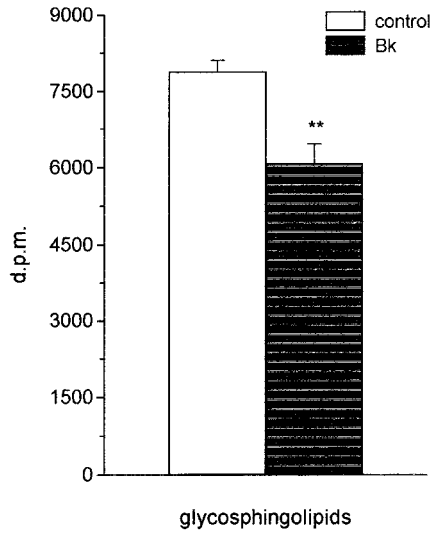

FIG. 3. Effect of BK on [^{3}H]glycosphingolipid in human fibroblasts. Quiescent fibroblasts, labeled with [^{3}H]serine, were stimulated with 100 nM BK for 5 min. [^{3}H]glycosphingolipids were measured as described in Materials and Methods section. Data are means of a representative experiment performed in triplicate. Statistical significance was determined by Student's *t* test (**$p < 0.02$).

radioactive sphingomyelin pool was unaffected by BK treatment, as well as by the in vitro assay of neutral or acidic SMase activities in extracts of cells incubated with the peptide. These results are in agreement with a recent study in which another agonist known to act through G-protein-coupled receptors, endothelin-1, failed to activate SMase in mesangial cells (13). The finding that neutral glycosphingolipid pool is reduced by BK treatment, provides an alternative explanation for the source of increased ceramide upon peptide stimulation. So far little information is available on the potential segnalatory role exerted by glycosphingolipids. In this regard it has been observed that in fibroblasts glycosphingolipids undergo a rapid recycling process, analogous to that demonstrated to occur to sphingomyelin (30) and that they contribute to the maintenance of ceramide levels since the inhibition of glycosylceramide synthase provokes the arrest of cell cycle by increasing ceramide content (18). Finally, in Madin-Darby canine kidney cells it has been shown that the inhibition of glycosylceramide synthase potentiates BK-stimulated inositol trisphoshate formation (32), whereas the inhibition of the glycosylceramide β-glucosidase activity, which generates ceramide, causes a reduction in inositol trisphoshate formation (33) suggesting a modulatory role of glycosphingolipids in cell signalling. In addition, the observation that interferon-γ is able to decrease cell surface expression of galactosylceramide in epithelial cells (31) suggests that glycosphingolipid metabolism is regulated, similarly to sphingomyelin metabolism, by extracellular agonists. In this respect we here provide evidence for a direct involvement of neutral glycosphingolipids in short-term formation of lipid-generated signals, this finding supporting the concept that all sphingolipids may participate to cell membrane signalling.

At present no information is available on the molecular mechanisms involved in the regulation of glycosphingolipid pathway, but in view of the recent observation that the glycosylceramide β-glucosidase activity is regulated in vitro by changes in Ca^{2+} concentration (35), it is tempting to speculate that the increase in cytosolic Ca^{2+} induced by BK (34) may be involved.

Besides the relevant increase in ceramide levels, BK treatment also provokes a remarkable rise in sphingosine content; the temporal shift of the two phenomena suggests that the formation of sphingosine is dependent on the previous rise in ceramide and its subsequent hydrolysis by ceramidase to form sphingosine. In view of the role of sphingosine in cell proliferation (12) and the

reported mitogenic action of BK in cultured fibroblasts (36), it is tempting to speculate that the rise in sphingosine may be a component of the cellular signalling pathway that mediates the mitogenic action of the peptide.

In conclusion, we have demonstrated that ceramide and sphingosine content of human fbroblasts are significantly increased by BK and that rapid glycosphingolipid hydrolysis induced by the peptide is involved in such event.

Further investigation on the individual segnalatory pathways triggered by BK in the presence of specific inhibitors of the sphingolipid metabolism will help to clarify the physiological relevance of the detected changes in sphingolipid derived products induced by the agonist.

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