Apelin-13 induces ERK1/2 but not p38 MAPK activation through coupling of the human apelin receptor to the G_{i2} pathway

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Apelin-13 induces ERK1/2 but not p38 MAPK, which involves the coupling of APJ to the G_{i2} cascade. In conclusion, the ERK1/2, but not p38 MAPK pathway is activated by apelin-13 through coupling of human APJ to G_{i2}-protein, which contributes to cellular responses.

Keywords apelin receptor; apelin-13; ERK1/2; p38 MAPK; G-protein

Apelin, a novel endogenous peptide from bovine stomach tissue extracts discovered in 1998, orchestrates its functions by binding and activating the 7-transmembrane G-protein-coupled receptor, apelin receptor (APJ) [1,2]. Apelin and its receptor have been shown to be expressed not only in the central nervous system, but also in peripheral tissues and cells, such as stomach, gastrointestinal tract, heart, kidney, adipose, lung, and endothelial cells [3–6]. Apelin is originally synthesized as a 77 amino acid prepropeptide that is cleaved into biologically active C-terminal fragments of various sizes [5]. Apelin peptides containing 13 (65–77), 17 (61–77), and 36 (42–77) amino acids (carboxyl-terminal peptides) have been isolated in vivo, and all of them bind to APJ and activate the second messenger signaling cascades [1]. Apelin-36 and shorter C-terminal sequences have different potencies and efficacies in regulating these functions. Shorter sequences, especially apelin-13, are potent regulators of cardiovascular function, whereas longer peptides such as apelin-36 are more effective in inhibiting HIV infection by blocking the HIV co-receptor APJ. In addition to the control of water and food intake, apelin plays an important role in the
central and peripheral regulation of the cardiovascular system, the release of hormones and neuropeptides, as well as in the modulation of immune function [3,8–10]. Recent studies have shown that the endogenous peptide apelin is crucial to maintaining cardiac contractility in pressure overload and aging [11].

Recently, studies have indicated that apelin is able to trigger intracellular signaling cascades through APJ activation in transfected Chinese hamster ovary (CHO) cells expressing APJ, and in neural cells [2,12,13]. One of the central signaling molecules activated by APJ is the family of mitogen-activated protein kinases (MAPKs). Activation of MAPKs, in particular extracellular-regulated kinases (ERK1/2), has been implicated in mediating important pathophysiological processes, such as control of endothelial cell proliferation [13]. However, little is known about the activation of p38 MAPK by apelin, which also participates in the regulation of cell proliferation and differentiation as well as in apoptosis.

Further studies have shown that apelin-36 and apelin-13 activate ERK1/2 and inhibit forskolin-stimulated cAMP production through a pertussis toxin (PTX)-sensitive G-protein in CHO cells expressing APJ [12], suggesting that APJ might preferentially couple to G-protein. Furthermore, apelin-induced ERK1/2 activation is not mediated by the Gβγ subunits and is protein kinase C (PKC)-dependent and Ras-independent. However, the intracellular transduction pathways that provide the molecular link between activation of APJ by apelin fragments and their biological responses at the cellular level remain to be clarified.

In this study, we focused on the coupling of human APJ to G-protein leading to ERK1/2 and p38 MAPK activation in human embryonic kidney 293 (HEK293) cells overexpressing human APJ (HEK293-apelinR). Our results revealed that apelin-13 can activate the ERK1/2 pathway and inhibit forskolin-induced intracellular cAMP production through a PTX-sensitive G-protein, but does not activate the p38 MAPK pathway. The properties of apelin-13 in the activation of MAPKs might be involved in the modulation of different biological responses.

Materials and Methods

Materials

Human apelin-13 was obtained from Phoenix Pharmaceuticals (Belmont, USA). The amino acid sequence of apelin-13 is pGlu-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe. Lipofectamine 2000 was obtained from Invitrogen (Grand Island, USA). Human G-protein αi2 [dominant-negative (Q205L/D273N) Gαi2] was obtained from the UMR cDNA Resource Centre, University of Missouri-Rolla (Rolla, USA). Forskolin, 3-isobutyl-1-methylxanthine, and PTX were obtained from Sigma (St. Louis, USA). Polyvinylidene difluoride membranes and enhanced chemiluminescence (ECL) plus Western blotting detection reagents were purchased from Amersham Biosciences (Little Chalfont, UK). APJ antibody was purchased from Abcam (Cambridge, UK). Anti-phospho-ERK1/2 (Thr202/Tyr204) antibody, anti-ERK1/2 antibody, anti-phospho-p38 MAPK (Thr180/Tyr182) antibody, and anti-p38 MAPK antibody were purchased from Cell Signaling Technology (Danvers, USA).

Total RNA isolation and RT-PCR

To amplify the full-length human APJ cDNA, total RNA was extracted from human brain using the SV total RNA isolation kit (Promega, Madison, USA) and reverse-transcribed into cDNA. First-strand cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase and random hexamers as primers. The set of primers for the amplification of APJ was: sense, 5′-CCGG-AATT-CTGGAGGAAGTGTTGATT-3′; and antisense, 5′-CCGCTCGAGCTAGTCAACCAAGGTTCTC-3′ (GenBank accession No. NM005161). The 5′-ends of the APJ sense and antisense primers were designed with the cleavage sites by the restriction enzymes of EcoRI and Xhol (Invitrogen), respectively. PCR was carried out using a Taq DNA polymerase kit (MBI Fermentas, Hanover, USA). The reaction mixture (10× PCR buffer, 10 mM dNTP, 25 mM MgCl2, 200 ng/μl specific primer, cDNA, and Taq DNA polymerase) was placed in a thermal cycler to start PCR at 95°C (45 s), 56°C (45 s), and 72°C (1.5 min) for a total of 40 cycles, with a final extension step at 72°C for 10 min. The PCR product was purified using QIAquick gel extraction kit (Qiagen, Crawley, UK) for cloning.

Construction of human APJ with pcDNA3.1(-)

To create the recombinant pcDNA3.1(-)apelinR plasmid, the PCR product and pcDNA3.1(+)-apelinIR were digested with EcoRI and Xhol, followed by purification using agarose gel and the QIAquick gel extraction kit. The APJ cDNA was inserted between the EcoRI and Xhol sites of pcDNA3.1(+), and subsequently cloned as described previously [14]. In order to identify the construct, the recombinant plasmid was digested with two restriction enzymes and sequenced. The sequence data were analyzed using Blast Nucleic Acid Database searches from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Stable transfection of HEK293 cells

HEK293 cells were grown in culture medium containing high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Paisley, UK), 10% heat inactivated fetal calf serum, 200 U/ml penicillin, and 200 µg/ml streptomycin incubated at 37 °C in 5% CO₂. The cells were seeded in 25 cm² flasks until 50%–70% confluence and washed with fresh medium to remove any antibiotics. Ten micrograms of pcDNA3.1(+)-apelinR plasmid with 10 µL Lipofectamine 2000 reagent in Opti-MEM medium containing GlutaMax (Invitrogen) was added to HEK293 cells and incubated overnight at 37 °C. After incubation for 18 h, the transfection mixture was replaced by DMEM. For generation of cell lines stably expressing APJ (HEK293-apelinR), the transfected cells were cultured in DMEM in the presence of G418 (0.5 mg/ml) (Gibco BRL). The surviving cells were subcultured in the selective process for 8 weeks and used for all subsequent experiments.

Confocal microscopy for APJ expression in HEK293-apelinR cells

HEK293-apelinR cells, grown on poly-L-lysine pretreated coverslips until they reached 70%–80% confluence, were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. Non-specific binding was blocked by incubating cells with 3% bovine serum albumin in PBS-Triton X-100 (0.01%) for 1 h at room temperature. Cells were washed three times with PBS-Trition X-100 (0.01%) then incubated overnight with anti-APJ monoclonal antibody (1:100) in PBS-Trition X-100 (0.01%) at 4 °C. Cells were washed as before with PBS-Trition X-100 (0.01%), subsequently incubated in the dark for 1 h at room temperature with a 1:400 dilution of Alexa Fluor 633-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, USA). After three washes for 5 min in PBS-Trition X-100 (0.01%), cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, USA) with 4′,6′-diamidino-2-phenylindole (Vector Laboratories, Peterborough, UK) on microscope slides and observed under an oil immersion objective (×63) using a Leica model DMRE laser-scanning confocal microscope (Leica, Heidelberg, Germany).

Measurement of intracellular CAMP production

HEK293-apelinR cells cultured in 12-well plates coated with 10 µg/ml poly-L-lysine were pre-incubated with stimulation buffer including DMEM, 500 µM 3-isobutyl-1-methylxanthine, and 10 mM MgCl₂ at 37 °C for 20 min. Cells were then stimulated with stimulation buffer containing forskolin (10 µM) in the absence or presence of various concentrations of apelin-13 (0.01–1000 nM) at 37 °C for 15 min. The cAMP level was determined using a cyclic AMP assay kit (R&D Systems, Minneapolis, USA) according to the manufacturer’s instructions. Determination of optical density at 405 nm for each treatment was carried out immediately on a microplate reader with wavelength correction set to 570 nm.

SDS-PAGE and immunoblotting

Ten micrograms of cell lysate was separated by 10% SDS-PAGE, and then transferred onto polyvinylidene difluoride membranes at 100 V for 1 h. Non-specific binding was blocked by incubating membranes for 1 h in blocking buffer [5% (W/V) non-fat milk in 200 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 0.1% (V/V) Tris-buffered saline Tween-20 (TBST)] at room temperature. The membranes were incubated overnight with a 1:1000 dilution of phospho-ERK1/2 and p38 MAPK antibodies in 5% (W/V) bovine serum albumin and TBST (0.1%) at 4 °C. Phospho-ERK1/2 and phospho-p38 MAPK were detected using rabbit polyclonal antibodies. Membranes were subsequently washed four times in 1×TBST for 10 min, then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins in 5% (W/V) non-fat milk in TBST for 1 h at room temperature, followed by washing four times with 1×TBST as previously described. To detect activation of ERK1/2 and p38 MAPK, ECL reagents (Amersham Biosciences) were applied to generate light when the ECL system reacted with the horseradish peroxidase. After immersion into ECL solution for 5 min, the membranes were drained of excess solution and exposed to X-ray film in the dark room. The film was scanned and the bands were analyzed by densitometry using Scion Image (http://www.scioncorp.com). For standardization, the same membranes were submered in stripping buffer [final concentration: 2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 100 mM β-mercaptoethanol] and incubated at 50 °C for 30 min, followed by two washes with TBST at room temperature. The membranes were blocked with 5% non-fat dried milk in TBST for 1 h at room temperature then reprobed with total ERK1/2 and p38 MAPK antibodies as described above.

Statistical analysis

All quantitative data are presented as the mean±SEM. Statistical significance was evaluated using Student’s t-test. In the case of multiple group comparisons, ANOVA was adopted. P<0.05 was used for consideration of significance of the findings.
Results

Amplification and cloning of human APJ
To obtain the human full-length APJ cDNA, total RNA from human brain was reverse-transcribed and amplified with specific primers for human APJ using RT-PCR. As shown in Fig. 1(A), the band between 1 kb and 1.5 kb (marker) was shown to correspond to the size of 1143 bp APJ. The cloned recombinant cDNA was digested with two restriction enzymes, EcoRI and XhoI. As shown in Fig. 1(B), two bands were observed from the digested product in 1% agarose gel electrophoresis, one of them between 5.0 kb and 6.0 kb representing pcDNA3.1(+) plasmid, the other corresponding to the size of full-length APJ (1143 bp). The sequence data for APJ appeared to be identical to that of NM005161 (GenBank) in the Blast Nucleic Acid Database.

Characteristics of apelin-13-induced ERK1/2 activation in HEK293-apelinR cells
In order to characterize ERK1/2 activation involved in human APJ, HEK293-apelinR cells were treated with 100 nM apelin-13 for 5–60 min and stimulated with different concentrations of apelin-13 (0.01–1000 nM) for 15 min. The results showed that a significant activation of ERK1/2 by apelin-13 was seen after 5 min, with maximal activation at 15 min, and a return to the basal level after 60 min [Fig. 3(A)]. We also observed that treatment of HEK293-apelinR cells with different concentrations of apelin-13 for 15 min led to a dose-dependent increase of ERK1/2 activation, with a significant response to apelin-13 from a concentration of 10 nM, reaching maximal activation at 100 nM, with an EC50 value of 2.8 nM [Fig. 3(B)]. It is therefore suggested that the ERK1/2 pathway can be rapidly activated by apelin-13, and that ERK1/2 activation tends to decline at concentrations higher than 1000 nM.

Apelin-13 does not induce p38 MAPK activation in HEK293-apelinR cells
Given that apelin-13 can activate ERK1/2, it is interesting to determine whether apelin-13 could activate the p38 MAPK pathway. The results indicated that p38 MAPK was not activated by treatment with 100 nM apelin-13 within 60 min in HEK293-apelinR cells, although treatment with thrombin (0.1 U/ml) for 30 min resulted in remarkable p38 MAPK activation in the same cell line [Fig. 4(A)]. After stimulation with different concentrations of apelin-
13, as indicated in Fig. 4(B), for 10 min, the activation of p38 MAPK was not observed in HEK293-apelinR cells, suggesting that apelin-13 does not induce activation of the p38 MAPK pathway through APJ in the HEK293 cell line.

Apelin-13 inhibits forskolin-stimulated cAMP production through G<sub>i</sub>-protein

To determine the extent of adenylyl cyclase inhibition elicited by the different concentrations of apelin-13, we observed the effect of apelin-13 on forskolin-induced cAMP production in HEK293-apelinR cells. As shown in Fig. 5, the forskolin-induced cAMP production was significantly reduced when apelin-13 was added at 1 nM concentration. In addition, it was found that the inhibition of forskolin-stimulated cAMP production appeared to be dose-dependent, with saturation and maximum at 100 nM apelin-13 (EC<sub>50</sub>=3.43 nM), indicating that the inhibition of adenylyl cyclase might require the coupling of APJ to G<sub>i</sub>.

Apelin-13 induces ERK1/2 activation through coupling of APJ to G<sub>i</sub>

In view of these results, we next asked whether apelin-13

![Fig. 3 Activation of extracellular-regulated kinases 1/2 (ERK1/2) by apelin-13 in human embryonic kidney 293 cells overexpressing human apelin receptor (HEK293-apelinR)](image)

![Fig. 4 Activation of p38 mitogen-activated protein kinase (MAPK) by apelin-13 in human embryonic kidney 293 cells overexpressing human apelin receptor (HEK293-apelinR)](image)
activates ERK1/2 activation through coupling of APJ to G_i2 identical to that of inhibiting adenylyl cyclase. First, using dominant-negative techniques, we observed that the apelin-13-induced activation of ERK1/2 was completely inhibited by dominant-negative G_i2 in HEK293-apelinR cells [Fig. 6(A)], consistent with the inhibition of forskolin-stimulated cAMP production by apelin-13. Second, pre-treatment of HEK293-apelinR cells with PTX for 6 h or 12 h, which selectively deactivated G_i/o-protein through ADP ribosylation to prevent its interaction with the receptor, mostly abrogated the apelin-13-induced ERK1/2 activation [Fig. 6(B)], indicating that APJ preferentially couples to a PTX-sensitive G-protein. Taken together, it was suggested that the G_i2 pathway plays a predominant role in apelin-13-induced ERK1/2 activation.

Discussion

In the present study, we cloned the human APJ gene and established a stable HEK293 cell line (HEK293-apelinR) expressing human APJ. It was found that human APJ is intensely expressed at the plasma membrane of HEK293-apelinR cells. Thus, this cell line can be used to investigate
the profiles of intracellular signal transduction of APJ-mediated MAPK activation. We showed that apelin-13 is able to induce, in a dose-dependent manner, the activation of ERK1/2, but not p38 MAPK, through human APJ coupling to the Gαi pathway in HEK293-apelinR cells. This is the first study to provide evidence that the p38 MAPK pathway might not be activated by apelin-13 while the peak of ERK1/2 activation by apelin-13 occurs. As activation of ERK1/2 is related to cell survival and p38 MAPK is associated with induction of apoptosis [15,16], we speculate that the ERK1/2 and p38 MAPK pathways might be regulated differently by APJ, which contributes to different physiological responses to apelin during the cycle of cellular processes.

Previous studies have shown that in CHO cells expressing the murine APJ, ERK1/2 can be activated by apelin-13 and apelin-36 through Gαi and Gαq proteins [13]. In order to further shed light on the intracellular signal cascade from apelin-13 to MAPKs, the human G-protein dominant-negative technique was used in this study. Dominant-negative Gαi, which derives from the Q205L and D273N double mutations of human G-protein alpha i2 subunit, is conferred a preference for xanthine but not guanine nucleotide binding. It was reported that Gαo/Q205L/D273N was regulated by xanthine, not by guanine nucleotides, and bound xanthine diphosphate and xanthine triphosphate instead of guanine diphosphate or guanine triphosphate [17]. It has been shown that Gαo/Q205L/D273N protein retained the receptor binding specificity of the wild-type Gαo and was able to interact with Gα coupled receptors in transfected COS-7 cells. However, in the absence of xanthine triphosphate, Gαo/Q205L/D273N did not dissociate from the receptors and thus inhibited their activities. Because cells lack xanthine nucleotides, Gαo/Q205L/D273N might act as a dominant-negative mutant to disrupt Gαi-coupled receptor signal transduction pathways. Our results showed that the apelin-13-induced ERK1/2 activation is completely blocked by dominant-negative Gαi in HEK293 cells expressing human APJ. It is further supported by our study that the activation of ERK1/2 is abrogated by PTX, and that apelin-13 attenuates forskolin-stimulated cAMP production through inhibition of adenyl cyclase by the coupling of APJ to Gαi. These findings suggest that the Gαi pathway plays a crucial role in signal transduction from the stimulation of APJ to the intracellular kinase cascades. Similar to the δ-opioid receptor [18], APJ-mediated ERK1/2 activation requires Gαi-protein and does not involve Gβγ and Ras in CHO cells [12], although other G-protein-coupled receptors, like α2A-adrenergic receptor coupling to Gαi, mediate ERK1/2 activation through the Gβγ/Src/Ras cascade in HEK293 cells [19]. However, apelin-13-induced ERK1/2 activation is reduced by the PKC inhibitor [12], suggesting the involvement of PKC. In light of these observations, it is tempting to speculate that the apelin-13 signal to ERK1/2 through APJ might be tissue-specific and involves multiple signaling cascades through coupling to Gα subunits.

In addition to the APJ-mediated ERK1/2 activation, we also observed that apelin-13 slightly, but not significantly, decreased basal cAMP levels without forskolin at a concentration of 0.01–1000 nM in HEK293-apelinR cells. This prompted us to guess whether the activation of ERK1/2 by apelin-13 is affected by the change of cAMP. cAMP has already been reported to have either a stimulatory effect through B-Raf or an inhibitory effect through c-Raf-1 on ERK1/2 activation, depending on the cell types [20]. It is therefore possible that in HEK293 cells expressing both B-Raf and c-Raf-1, activation of ERK1/2 is modulated by the mechanisms of stimulation and inhibition of cAMP [20,21]. Additionally, in CHO cells expressing the human dopaminergic D2 receptor, the Gαo-induced ERK1/2 activation through Ras-independent and PKC- and phosphatidylinositol-3 kinase-dependent activation of B-Raf pathways is greatly enhanced by the Gαiβγ/Ras/Raf-1 cascade [22]. These speculations that the apelin signal from Gαi-protein subunits to the ERK1/2 cascade is mediated through activation of B-Raf or/and c-Raf-1 remains to be clarified in our future study.

Interestingly, apelin-13 at 100 nM can induce maximal activation of ERK1/2, whereas the activation of p38 MAPK was not seen at the same time points in response to 100 nM apelin-13 in HEK293-apelinR cells. It is in agreement with the study in human osteoblasts that apelin-13 does not result in activation of p38 MAPK within 60 min [23]. This raises the possibility that the coupling of APJ to Gαi does not activate the upstream effectors of p38 MAPK pathway, although APJ can mediate ERK1/2 activation via Gαi cascade. Previous studies have shown that p38 MAPK can be activated by muscarinic acetylcholine receptors and β-adrenergic receptor through coupling to Gαi, Gαq, and Gβγ subunits in HEK293 cells [24,25], and by adenosine A2h receptor through coupling to the Gα/AC/cAMP/PKA pathway in CHO cells [26], which is dependent on the expression of receptor and cells. Therefore, we speculate that the coupling of APJ to Gαi does not target the signaling molecules to p38 MAPK activation. It is suggested that the activation of different signaling pathways is tissue-specific or/and receptor-specific, and that the regulation of APJ for MAPKs might contribute to different physiological responses.
In conclusion, we showed that apelin-13 induces ERK1/2 activation through human APJ in a time-dependent and dose-dependent manner, and involves the $G_{13}$-dependent pathway. However, p38 MAPK is not activated upon stimulation by apelin-13 when a maximal activation of ERK1/2 occurs. These properties of apelin-13 for ERK1/2 and p38 MAPK activation could be useful for designing specific pharmacological tools to treat the pathological dysfunctions caused by lack of apelin signaling.

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