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International Journal of Pharmaceutical Research & Allied Sciences, 2017, 6(3):144-150

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Modulation of IGF-I Receptor Activation by the IGF-I peptides

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ABSTRACT

Background: Insulin-like growth factor (IGF-I) is a growth factor that modulates the growth and development of skeletal muscles. The E-peptide is the C-terminal part of the IGF-I growth factor, and its function remains unknown. Amino acid sequencing of E-peptides suggests that they are either EA or EB peptides. Aim: To establish the time course modulation effect of E-peptide on IGF-I receptor activation in skeletal muscle cells. Method: We tested IGF-IR activation by immunoblotting for Phospho-Protein Kinase B (P-AKT) and Phospho-Extracellular signal-Regulated KinasesP-ERKI/II, the signal proteins of the AKT/PK13 and MAPK/ERK pathways. The C2C12 muscle cell line was starved overnight in serum-freemedia, then treated with either IGF-I alone or IGF- I plus either EA or EB synthetic peptides. The final group received NoTx, (negative control). Cells were treated for different periods of time (15, 30, 60, and 120 min). For quantification of the protein in each sample, equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. Results: The potency of IGF-I receptor activation was increased in cells were treatedwith IGF-I plus EA peptide (for 30 min) compared to IGF-I alone. IGF-I plus EB managed to sustain the signal for a longer period. Conclusion: E-peptides have apotent modulating effect on IGF-I function and further studies are now needed to identify their exact function.

Key words: *Insulin, IGF-I Receptor, IGF-I peptides*

INTRODUCTION

Endodontics is the branch of dentistry that is responsible for the safety and the health of the dental pulp, and the therapeutic replacement for irreversible damage like necrosis or irreversible inflammation. To date there has been no synthetic material that could replace the dental pulp to the quality or efficacy desired. As bacteria and microorganisms present in caries progress towards the dental pulp, lytic activity of the acids secreted by these microorganism allow for several growth factors to be released from the dentin and the pre dentin layer including, TGF-β, insulin- like growth factor (IGF)-1 and IGF-2, BMPs, VEGF, and other factors. These growth factors attract and cause proliferation and differentiation of mesenchymal stem cells to form reparative dentin and new blood vessels [1].

Insulin-like growth factor-1 (IGF-I) is a highly mitogenic growth factor with paracrine and autocrine functions. IGF-I controls pre- and post-natal growth and development in most organs and tissues [2,3]. The main source of IGF-I is the liver 4, which secretes IGF-I into the circulation. However, many cell types, including skeletal muscle, produce

and respond to their own IGF-I. Skeletal muscle development depends on IGF-I as a main promoter and organizer for its growth. Mice with an inactive IGF-I gene are small because of reduced organ and muscle size and reduced bone mass. These mice have a 60% reduction in body weight, and more than 95% die prenatally. Thus, IGF-I is important for embryonic development [2,5].

A study done on dog pulp cells showed that Platelet derived growth factor-BB (PDGF-BB) and insulin-like growth factor-1 (IGF-1) when added to the human pulp cells both separately and in combination increased proliferative activity of cells [6].In rodents, there are two IGF-I isoforms, IGF-IA and IGF-IB. These isoforms have different E-peptide sequences. IGF-I without its E-peptide extension is known as mature IGF-I, whereas IGF-I with its E-peptide it is known as Pro-IGF-I [7]. Both IGF-I and Pro-IGF-I are present in the circulation but at different concentrations. Different degrees of hypertrophy in adult mice are seen with different degrees of IGF-IA and IGF-IB expression [8,9]. Multiple studies have investigated the exact function of the E-peptides and their dependence on IGF-I.

IGF-I works by activating the IGF-I receptor (IGF-IR) in various cell types and tissues. Quantification of the downstream signaling protein of various signal pathways, including the MAPK and the AKT/ P13K signal pathways, is often used as an indicator of IGF-IR activation. A recent study from the Barton lab aimed to determine whether the E-peptides act independently of IGF-I and IGF-I signaling, and to compare the biological actions of EA and EB. These authors found that the synthetic E-peptides works similarly or in concert with IGF-I. They also found that addition of synthetic E-peptide to C2C12 skeletal muscle cell line increased the activation of the MAPK signaling pathway. Immunoblotting analysis for phosphorylated and total ERK1/2 was performed following a 20 mintreatment with EA or EB. The maximum increase in ERK1/2 was seen15 min after the EA peptide was added and returned to control levels after 30 min. In contrast, when EB was added, the increased signaling was not seen until 15 min after E-peptide addition but remained elevated for at least 30 min9.

In a previous study, it was shown that E-peptide could modulate the effect of IGF-I on skeletal muscle cells. However, all data were collected from a single time point, and the prolonged effect of the E-peptide addition was never examined. Therefore, here we aimed to compare signal activations at multiple time points.

MATERIAL AND METHODS

Recombinant IGF-I and synthetic E-peptide signaling

On the first day, skeletal muscle C2C12 (ATCC, Manassas, VA) cells were maintained in growth media (Dulbecco`s Modified Eagle Media (DMEM) containing, 10% fetalbovineserum, streptomycin, and gentamicin).Six-well plates were seeded with 5 × 105cells in growth media, which were allowed to attach and grow overnight. Five plates were prepared for each time point.

On the second day, cells are starved overnight in growth media without serum. During the third day, cells are treated. The first two wells are treated with media alone and served as negative controls. The second pair of wells were treated with 2nM recombinant human IGF-I (Gemini Bio-Products West Sacramento, CA) alone. The third pair of wells were treated with 2nM IGF-I and synthetic E-peptide (either EA or EB). Murine EA and EB (based on Gen Bank AY878192 and AY878193, respectively) were synthesized by Bio-Synthesis Inc. Lewisville, TX, and purified to greater than 95% purity by High Performance Liquid Chromatography (HPLC). We confirmed the final products by Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometry (Wistar Proteomics Facility, University of Pennsylvania, Philadelphia, PA). Peptides were provided in 0.1mg lyophilized aliquots to avoid freeze-thaw cycles and stored at -80°C until the time of use. We added 155 µm and 124.5 µm of distilled water to the of EA and EB aliquots respectively, and 1:100 and 1:10000 dilutions of these were used to treating the cells, according to manufacturer's instructions and previous studies (Brisson et al. 2013). This produced concentration of 1 µM EA and 10 nM EB, which generated the maximum ERK1/2 phosphorylation.

We treated the cells for five different time points (5, 15, 30, 60 and 120 min) before they were processed by immunoblotting. Each experiment was done in triplicate (Fig. 1).

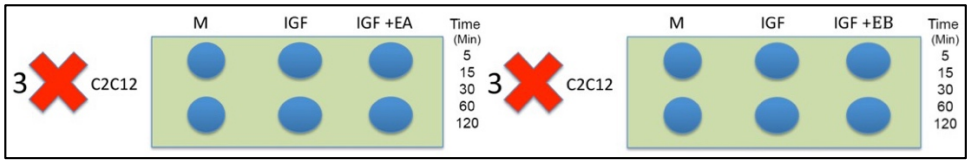

Figure 1. Study conditions for IGF and EA treatment similarly IGF and EB treatment.

Several studies have used the activation of the MAPK and AKT/PK13 pathways as a proxy measure of IGF-IR activation [10]. In C2C12 skeletal muscle cells, MAPK and AKT/P13K pathway activation were tested when cells were treated with 2nM rhIGF-I and compared to the pathway activation when norhIGF-I (NoTx) was added. To test the pathway activation, final signal protein quantification was done for ERKI/ERKII (for the MAPK pathway) and AKT (for the AKT/P13K pathway).

We predicted that the changes induced by E-peptide addition to IGF-I were likely to be minor. To properly evaluate the differences seen when E peptides are added to IGF-I, and to measure the exact effect it has on modulating the signal activation, we decided to compare everything to the 2nM rhIGF-I treatment, rather than the NoTx treatment. Comparing signals from cells treated with 2nM rhIGF-I plus E-peptides against those of activation induced by IGF-I alone makes evaluating the role played by the E-peptide more relevant.

Immunoblotting analysis

The activation of the MAPK and Akt/PI3K pathways was determined by immunoblotting. Cells were washed in cold PBS before incubation in 120 µL lysis buffer, referred to as RIPA buffer (50nMHEPES, 150nMNaCl, 5mM EDTA, 1nM EGTA, 15mM p- nitrophenyl phosphate disodium hexahydrate, 1% NP-40, 0.1% SDS, 1% deoxycholate, 0.025% sodium azide) with protease and phosphatase inhibitors (P8340, P5726, Sigma, St. Louis, MO). Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were blocked in 0.1% Tween 20 (TTBS) and 5% nonfat dry milk. Membranes were incubated in primary antibody diluted in 5% milk-TTBS overnight at 4°C. The following antibodies were used: phospho-Akt (no. 9271), total Akt (no.9270), phospho-ERK1/2 (no. 9101), and total ERK1/2 (no. 9102) (Cell Signaling, Beverly, MA). Membranes were washed in 5% milk-TTBS and incubated with horseradish peroxidase-conjugated secondary antibodies.Enhanced chemiluminescence and the ImageQuant (GE 64 Fairfield, CT) detection system were used for protein detection. Analysis of band intensity was performed using the associated image analysis software.

The 30-min time point experiment

The 30-min time point was redone using both the IGF-I+EA and IGF-I +EB treatments (Fig. 2). Each experiment was done once and gave rise to three different samples; performed as described above.

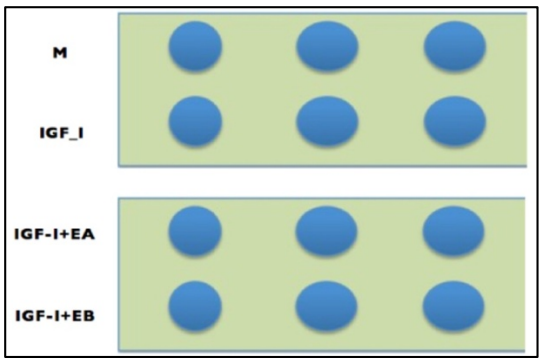

Figure 2. 30-min time point was redone using both the IGF-I+EA and IGF-I +EB treatment.

Immunocytochemistry

Coverslip slides were prepared for immunocytochemistry by incubating first in Triton 0.2% (GE HealthcarePlusOne Triton X-100) for 45 min, then blocking in 3%bovine serum albumin (BSA) (Gemini Bio-Products) for 2h. IGF-I was localized using antibodies against FLAG (polyclonal antibody [pAb] FLAG, catalog no. 2368; Cell Signaling Technology, Danvers, MA) at a dilution of 1:500 overnight at 4°C. On the followingday, slides were washed in saline and incubated in secondary anti-rabbit conjugated to Alexa 555 (Invitrogen) at a dilution of 1:500. After staining, the cells were covered in aqueous mounting media containing 4,6-diamidino-2-phenylindole (DAPI) (Vectashield Laboratories, Burlingame, CA) and sealed onto a coverslip for visualization on an epifluorescence microscope (DMR; Leica Microsystems, Deerfield, IL). Non overlapping microscopic fields were acquired at 200× using Open Lab software (Improvision, Coventry, United Kingdom).

Statistics

To compare results from cells treated with 2 nMrIGF-I and cells that received no treatment, we used unpaired t-tests with Welch's correction due to unequal variance. For the time course data, we used 2-way ANOVA followed by post-hoc multiple comparisons using Bonferroni adjustment to determine the differences in time and treatment at $P<0.05$.

RESULTS

IGF-I increases MAPK and P13K signaling

Compared to NoTx,MAPK and P13K signaling were significantly higherwhen recombinant IGF-I was added. At the 15-min time point, we detecteda250-fold increase in the AKT signal in IGF-I-treated cells compared to NoTx. Compared to NoTx,AKT and ERKI/IIwere upregulated at the 15-and 120-min time points (Fig.3).

At the 5-min time point, we detected nodifferences between the IGF-I and NoTxtreatments. The relatively high ERKI/ERKII signaling at the 5-min time point might be due tothe cells being excited by the addition of the media (DMEM, no serum, and streptomycin) (Fig.4). This effect disappears shortly after the 5-min time point. For this reason, the 5-min time point was removed from the analysis.

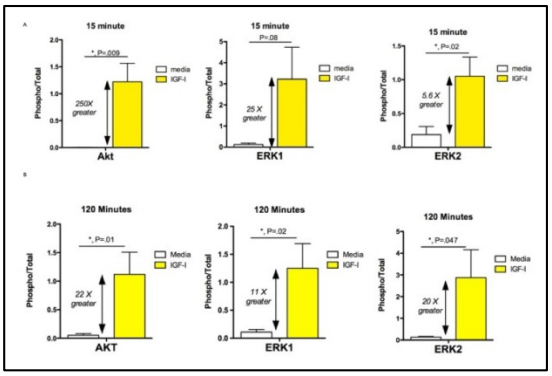

Figure 3. Comparison of cellular signaling activation in the IGF-I alone and NoTxtreatments, after 15 min (A) and 120 min (B). Asterisks indicate statistically significant differences.

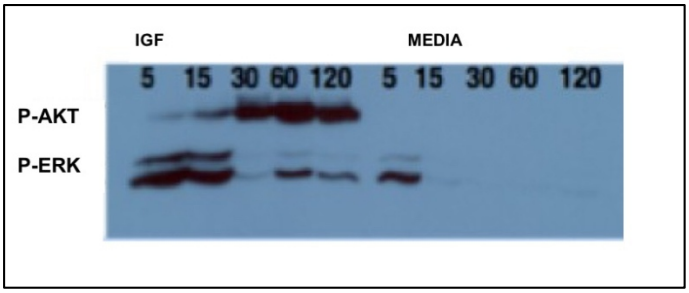

Figure 4. A Western blot showing an increase in ERKI/ ERKII signaling at the 5-min time point in theNoTxtreatment, which later disappears.

How does EA or EB alter IGF-I signaling?

We found that thesynthetic E-peptides modulate the action of IGF-I (Fig.5), indicated by increased phosphorylation of AKT and ERKI/II as a result of MAPK and AKT/PK13 pathway activation (Fig.6). Cells treated with IGF-I only were also collected at 15 min to obtain the time-course baseline of AKT and ERK1/2 phosphorylation.

Cells treated with IGF-I+EA hadan increased P-AKT signalat the 30-mintime point compared to the 15-min point or IGF-I alone at the 30-min time point. After 30 min, the P-AKT signal was reduced and was significantly lower than when the cells were treated with IGF-I+EB at the 120-min time point. Treatment with IGF-I+EA also caused an increase in the ERKI signal at the 30-mintime point, compared to cells treated with IGF-I alone or IGF-I+EB at the 15- and 30-min time points. The P-AKT signal was reduced after 30 min and reached the level of the IGF-I alone treatment by the 120-min time point. When cells were treated with IGF-I+EB, the greatest increase in P-ERKII was seen at 30 min; compared to the 15-min time point, the P-ERKII signal was increased throughout the time course. EA was more potent at stimulating the AKT/P13K and MAPK pathways when IGF-I was present; whereas EB

induced a more sustained signal activation. The IGF-I treatment at the 15-min time point was used to normalize the Western blots because the NoTx treatment often returned no signal and would be quantified to zero. Because we cannot divide by zero, we instead used the IGF-I 15-mintime point. The difference in signal seen at 30 min was statistically significant for all downstream signaling proteins. Therefore, we decided to investigate this time point further.

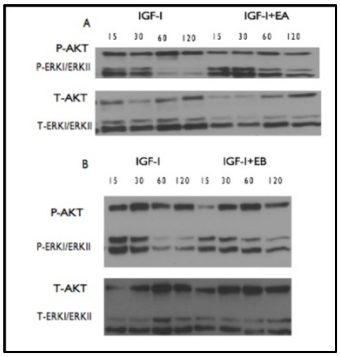

Figure 5. IGF-I + EA and EB increase MAPK and AKT/P13K signaling in C2C12

A. cell were starved in media without serum and treated with (2nM rhIGF-I) or (2nM rhIGF-I plus EA-peptides) for times indicated. Protein lysates were separted via SDS-PAGE and immunoblotted for P-AKT and P-ERK 1 and 2, stripped and blotted for TotalERK 1 and 2 (T-ERK1/2). B. The same as A except the cells were treated with either (2nM rhIGF-I) or (2nM rhIGF-I plus EB-peptides).

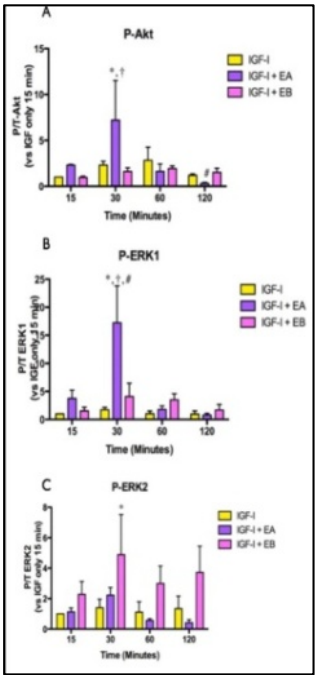

Figure 6. Quantification of all time course blots. IGF-I at 15 min was included in each experiment for normalization between blots. Bars represent means * is different than 15 min time point in same condition # is different at the same time point between EA and EB † is different at IGF-I time point.

The 30-min time point

In the 30-min time point follow-up experiments, when cells were treated with IGF-I+ EA, we detected an increase in the ATK and ERKI signal compared to IGF-I alone and IGF-I+EB. When cells were treated with IGF-I+EB, there was small increase in ERKI and ERKII, but not AKT, compared to IGF-I alone (Fig. 7). These findings are relatively consistent with the time course data presented above.

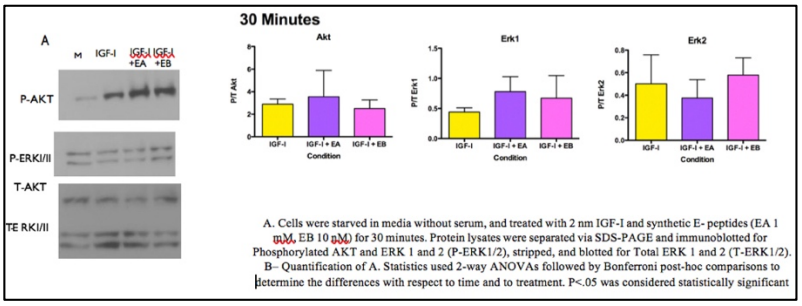

Figure 7. IGF-I + EA and EB increase MAPK and AKT/P13K signaling in C2C12 at 30 min time point.

DISCUSSION

The EA peptide was shown to augment IGF-I potency, which is consistent with our findings9. These authors also found that the E-peptide-induced increase in IGF-IR activation did not alter the AKT/P13kinase pathway. In contrast, we found that the addition of synthetic EA peptide to cells treated with IGF-I significantly increased the AKT signal after30 min, compared to cells that received IGF-I treatment alone. Also, we found that EB addition to IGF-I treatment seemed to sustain signal activation for a prolonged period.

We found that controlling several time points within a single experiment was technically difficult; it was easier to examine each time point on separate days, which reduces the opportunity for technical errors. The E-peptide affected the activity of IGF-I. However, this additional activity was minimal. Variability between the samples might have masked some of the differences or reduced the estimated activity. We attempted to plate equal numbers of cells into each plate, but this does not exclude the possibility that variable numbers of cells were actually plated, thereby reducing the accuracy of the experiment. Dental pulp regeneration is a new therapeutic goal in dentistry. It involves stem cells, scaffolds and growth factors. This v new direction to regenerate a vital dental pulp instead of replacing it with synthetic materials that will never match the nature of the dental pulp [11].

CONCLUSION

E-peptide has strong modulation effects on IGF-I and has been shown to augment tissue response to IGF-I in skeletal muscle cells. Other cells and tissue might respond in the same way and should be examined. How IGF-IR and other downstream signaling proteins are affected by the addition of E-peptide should be further investigated. These proteins might have therapeutic benefits in the treatment of muscle diseases and damage. It is still unclear how the IGF-I growth factors bind to the IGF-IR. Moreover, it is not clear how the E-peptide augments the receptor activations. Further E-peptide investigation is vital to the better understanding of functions of IGF- I.

ACKNOWLEDGMENTS

Special thanks for Dr. Barton for the lab facility, guidance, support, and help. All Dr. Barton fellow lab members for the continuous technical support.

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