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IGF-I-induced VEGF expression in HUVEC involves phosphorylation and inhibition of poly(ADP-ribose)polymerase ☆

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Abstract

Insulin-like growth factor-I (IGF-I) has been shown to promote angiogenesis by enhancing vascular endothelial growth factor (VEGF) expression. However, how IGF-I-induces VEGF expression is not yet fully understood. With this investigation, we propose a new possible mechanism involving downregulation of poly(ADP-ribosyl)ation (pADPR). We first demonstrated that IGF-I increased VEGF protein expression in endothelial cells. Inhibitors of mitogen activated kinase (PD 98059), phosphatidyl-3-inositol-kinase (LY 294002), and protein kinase C (staurosporine) diminished the IGF-I effect suggesting the involvement of signal transduction. Since there is an established link between pADPR and transcriptional activity, we focused on a possible role of poly(ADP-ribose)polymerase (PARP). The inhibition of PARP by 3-aminobenzamide or nicotinamide enhanced VEGF expression. Additionally, IGF-I markedly decreased PARP activity. Furthermore, the IGF-I-mediated inhibition of PARP could be demonstrated as a result of protein phosphorylation since phosphorylation of PARP decreased its activity in vitro and IGF-I treatment of endothelial cells induced PARP phosphorylation. The IGF-I-mediated phosphorylation and inhibition of PARP represent a novel mechanism of VEGF protein expression.

Keywords: Insulin-like growth factor-I; Vascular endothelial growth factor; Poly(ADP-ribose)polymerase; Phosphorylation

Evidence that insulin-like growth factor-I (IGF-I) stimulates cellular proliferation was reported for the first time almost 25 years ago [1]. Later, beneficial effects of IGF-I on various regenerative processes such as neuroregeneration or gastric and cutaneous ulcer healing were described [2–4]. Angiogenesis is a major component of IGF-I action since IGF-I enhances Vascular endothelial growth factor (VEGF) expression in fibroblasts [5], osteoblasts [6], and several tumor cell lines [7–9].

It is accepted that the synthesis of VEGF is primarily controlled at the level of its gene transcription. However, the molecular mechanism for how IGF-I induces gene transcription is not yet elucidated. A large body of evidence demonstrates that the activation of the signal transduction pathway via phosphatidyl-3-inositol-kinase, MAP-kinase or protein kinase C leads to target protein phosphorylation, plays a key role in IGF-I signaling, and mediates its effect on gene transcription [10–12].

Poly(ADP-ribose)polymerase (PARP) is a nuclear enzyme that was initially discovered as a repair system for DNA strand breaks, in which it catalyzes the transfer of poly(ADP-ribose) units synthesized from β-nicotinamide adenine dinucleotide (NAD^+) to DNA nicks [13]. More recently, a link between poly(ADP-ribose) (pADPR) and gene transcription was indicated by the presence of

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poly(ADP-ribosyl)ated nucleoproteins in transcriptionally active and DNase 1-hypersensitive domains of chromatin [14]. Subsequently, several proteins involved in transcription and transcription-related processes were found to serve as $(ADP\text{-}ribose)_n$ acceptors [15]. In addition, inhibitors of PARP enhance the transcription of some genes, such as the proto oncogenes c-myc and c-fos [16]. Similarly, the PARP inhibitor 3-aminobenzamide (AB) decreases poly(ADP-ribosyl)ation on the high-mobility-group proteins 14 and 17, and enhances the level of mouse mammary tumor virus mRNA [17]. Poly(ADP-ribosyl)ation of a number of transcription factors, including SP-1, prevents their binding to DNA and decreases their transcriptional activity [18–22].

We hypothesize that IGF-I inhibits PARP activity through phosphorylation as upstream effect of signal transduction kinases, lowers poly(ADP-ribosyl)ation, and therefore increases VEGF protein synthesis. We plan to examine this hypothesis by studying the effect of IGF-I on the phosphorylation of PARP in human umbilical vein endothelial cells (HUVEC).

Experimental procedures

Materials. Cell culture materials (Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, and culture dishes) were obtained from Mediatech, Herndon, VA. All chemicals, unless otherwise stated, were purchased from Sigma–Aldrich.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were grown in six-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in a 37 °C, 90% air, 10% CO_2 atmosphere to subconfluent monolayers with a cell density of approximately 10^6 cells/well. After overnight serum starvation, cells were treated with IGF-I using LongR3-IGF-I (Diagnostic Systems Laboratories, Webster, TX) which has relatively low affinity for IGF binding proteins. For the phosphorylation experiments each well was incubated with 50 μCi of [^{32}P]phosphoric acid (Amersham Bioscience, Piscataway, NJ).

VEGF ELISA. Confluent HUVECs that had been cultured in six-well plates were treated in triplicate as indicated. Cell culture supernatant was harvested and cell count was performed after trypsinization. The concentration of $VEGF_{165}$ in the supernatant was determined using a commercially available ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

PARP immunoprecipitation. Aliquots of 1×10^7 HUVECs were washed with ice-cold PBS and collected by scraping into 1 ml PBS. After centrifugation at 1000*g* for 5 min, pellets were homogenized with equal volume of lysis buffer (20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl) (Cell Signaling, Beverly, MA) containing various protease inhibitors and incubated for 20 min on ice. After sonication for 15 s, samples were subjected to centrifugation at 15,000*g* for 20 min at 4 °C to separate membrane and cytosolic fractions. Equal amounts of the nuclear fraction of cell lysate were incubated with 10 μg PARP-1 antibody (Roche Diagnostics, Indianapolis, IN) at 4 °C overnight on a tube rotator. The immunocomplex was captured by adding 25 μl of washed protein G–Sepharose beads (Amersham Bioscience, Piscataway, NJ) and incubating the samples for 6 h at room temperature. Beads were then collected by pulsing (5 s in the microcentrifuge at 14,000*g*), while the supernatants were discharged. After washing three times with ice-cold lysis buffer, beads were incubated either with 10 μl phosphatase buffer (150 μM NaCl, 20 mM Mops, pH 7.5, 60 mM 2-mercaptoethanol, 0.1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, and 0.1 mg/ml serum albumin) containing 1 U of purified phosphatase PP1 enzyme (Upstate, Charlottesville, VA) or with phosphatase buffer alone for 60 min at 37 °C. Then the samples were resuspended in 50 μl of 2× SDS sample buffer (Cell Signaling, Beverly, CA), boiled for 5 min, and separated by SDS–page electrophoresis on a 7.5% Tris–HCl gel. The gel was stained with Coomassie blue, dried for 3 h, and exposed to X-ray film (Kodak X-Omat AR Film, Eastman Kodak, Rochester, NY) for 48 h at −70 °C. The film was developed using a standard automatic film developer.

Poly(ADP-ribose)polymerase assay. Poly(ADP-ribose)polymerase activity was determined by measuring the incorporation of [^{14}C]NAD for 15 min at 37 °C into an acid-insoluble product[23]. The standard reaction mixture contained in 0.2 ml, 50 mM Tris–HCl, pH 7.5, 15 mM $MgCl_2$, 10 mM NaF, 0.1 mM DTT, 3 mM 3′,5′-cyclic AMP, 10 μg DNA, and 96 μM [^{14}C]NAD^+, and incubation was carried out at 37 °C for 15 min. The reaction was stopped by adding 5 ml of cold 10% (v/v) trichloroacetic acid. After 10 min on ice, the acid-insoluble material was collected on a Millipore filter (HAWP, 0.45 μm) and washed four times with 5 ml of cold 5% trichloroacetic acid to remove all acid-soluble radioactivity. The filter was dissolved in 15 ml of Bray's scintillation fluid and assayed for radioactivity. The value was subtracted from the radioactivity obtained when incubation was carried out with the boiled supernatant. The specific activity of the enzyme was expressed as picomoles of [^{14}C]NAD incorporated per milligram of DNA for 15 min.

Phosphorylation of PARP in vitro. Cell lysates were prepared as described above. One hundred microgram of nuclear proteins was incubated with the test substance for 1 h at 37 °C. Then PARP activity was assessed, as described above.

Statistical analysis. Results are given as means ± SD. Differences between the groups were calculated by ANOVA followed by a post hoc test when appropriate. A value of $p < 0.05$ was considered significant.

Results

IGF-I stimulates expression of VEGF protein

IGF-I, dose-dependently, increased the concentration of VEGF protein in cell culture supernatants over a 24-h period with a maximum stimulation occurring at 100 ng/ml. A time course experiment showed that a single treatment with 100 ng/ml IGF-I produced a sustained increase in VEGF expression over 48 h (Fig. 1). As a positive control we used phorbol myristate acetate (PMA) that increased VEGF protein approximately five times over control (data not shown).

Stimulation of VEGF expression by IGF-I is mediated via PI3-K, Mek 1/2, and PKC signaling

To determine whether PI3-K, Mek 1/2 or PKC pathway signaling mediated the stimulatory effect of IGF-I on VEGF expression, the pharmacological inhibitors LY 294002 (PI3-K), PD 98059 (selective Mek 1), and staurosporine (PKC) were used. All three components inhibited VEGF expression, indicating their involvement in its regulation. Moreover, the expression of VEGF was inhibited even in the absence of IGF-I stimulation, suggesting that constitutive activity in these pathways contributes to basal VEGF production (Fig. 2). These findings provide further evidence that phosphorylation is an important part of IGF-I signaling.

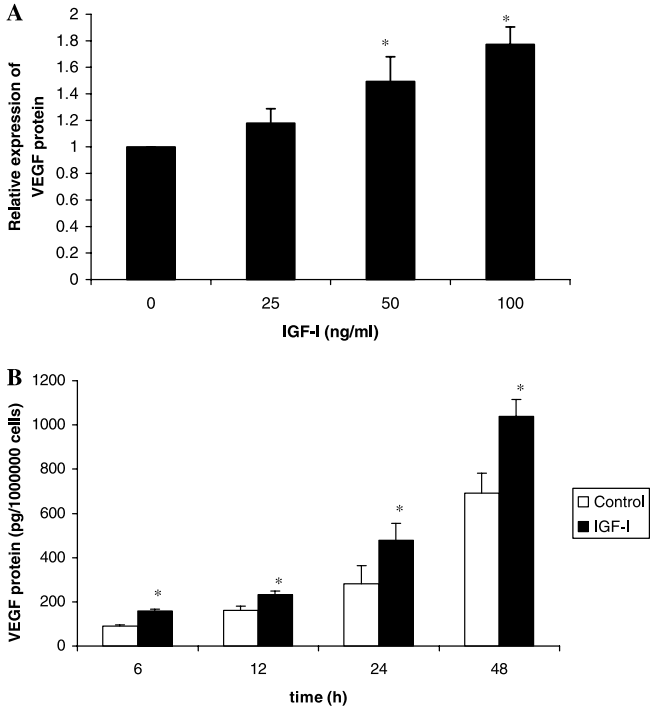

Fig. 1. IGF-I stimulates VEGF expression in HUVEC. (A) Confluent HUVEC cultures were treated with the indicated concentrations of LongR3-IGF-I or vehicle for 24 h. The concentration of $VEGF_{165}$ in the supernatant was measured by ELISA and normalized to control. (B) The concentration of VEGF in the media was measured after treatment with 100 ng/ml LongR3-IGF-I for the indicated times. The concentration of $VEGF_{165}$ in the supernatant was measured by ELISA and normalized 10^6 cells. $*p < 0.01$.

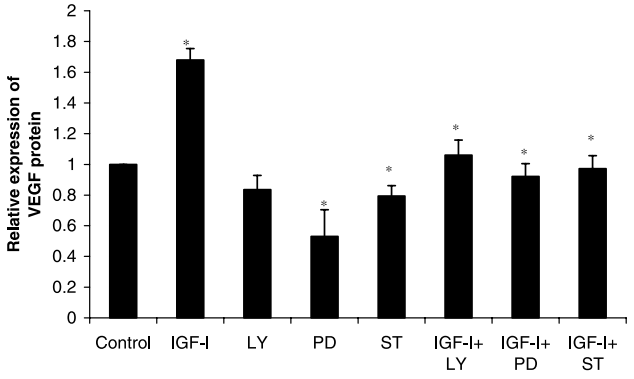

Fig. 2. PI3-K and MEK-1 mediate the effect of IGF-I on VEGF expression in HUVEC. HUVEC cultures were pretreated with the PI3-K inhibitor LY 294002 (LY, 50 μM), or the MEK-1 inhibitor PD 98059 (PD, 20 μM) or the PKC inhibitor staurosporine (ST, 3 nM) for 1 h before treatment with 100 ng/ml LongR3-IGF-I for 24 h in the continued presence of the inhibitor. $*p < 0.01$.

VEGF expression is inversely correlated to poly(ADP-ribose)polymerase (PARP) activity

PARP was originally found to be part of the mechanism by which DNA double strand breaks are repaired; it acts by transferring ADP-ribose units from β-nicotinamide ade-

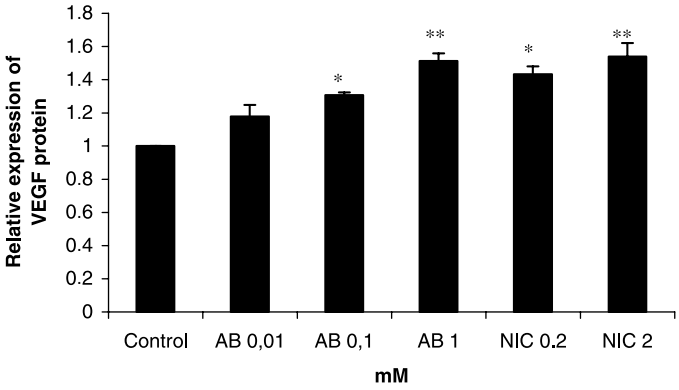

Fig. 3. Inhibition of PARP increases VEGF protein expression. Confluent HUVEC cultures were treated either with 3-aminobenzamide (AB) or nicotinamide (NIC) at the indicated concentrations for 24 h. VEGF protein in the supernatant was measured by ELISA and results normalized to controls. $*p < 0.05$ and $**p < 0.01$.

nine dinucleotide (NAD^+) onto nuclear protein acceptors. Recently, a link was established between poly(ADP-ribose) and gene transcription through the presence of poly(ADP-ribosyl)ated nucleoproteins in transcriptionally active and DNase 1-hypersensitive domains of chromatin.

Accordingly, we investigated whether the competitive inhibition of PARP is linked to an increased VEGF protein expression. PARP was inhibited by 3-aminobenzamide (AB) or nicotinamide (NIC), which act as analogs of NAD^+. They, dose-dependently, stimulated VEGF expression after a time period of 20 h (Fig. 3). AB and NIC also inhibited purified PARP in vitro (data not shown).

Phosphorylation decreases PARP activity in vitro

It has been previously reported that the activity of PARP can be modified by phosphorylation in vitro [25,26]. Here, we conducted in vitro studies to assess whether the activity

Table 1
Effect of phosphorylation on HUVEC PARP in vitro

Additions	PARP activity [^{14}C]NAD^+ incorporated (dpm/mg)
None	5530 ± 322
+ATP (10 mM)	3447 ± 264*
+ATP (20 mM)	3447 ± 213*
+ATP (20 mM) + phosphatase PP1	5385 ± 387
+IGF-1 (100 ng/ml)	4706 ± 296*
+IGF-1 (100 ng/ml) + staurosporine (4 nM)	5390 ± 346
+IGF-1 (100 ng/ml) + phosphatase PP1	5411 ± 382
+Staurosporine (4 nM)	5131 ± 306
+HCNS (10 μM)	2235 ± 210*
+TPA (20 ng/ml)	2148 ± 304*

HUVEC lysates (100 μg protein) were treated with indicated concentrations of ATP or IGF-I for 1 h at 37 °C. Additionally, cell lysates were incubated with phosphatase PP1 (PP1) (1 U/ml), staurosporine (ST) (4 nM), *N*-heptyl-5-chloro-1-naphthylenesulfonamide (HCNS, 10 μM), and 12-*o*-tetradecanoyl phorbol-13-acetate (TPA, 20 ng/ml). PARP activity was assessed by the incorporation of radiolabeled NAD^+ as described in Materials and methods.

* $p < 0.01$.

of cellular PARP can be affected by treatments known to depress or stimulate PKC. Table 1 shows the effect of in vitro treatments known to affect phosphorylation reactions on PARP in HUVEC. HUVEC homogenates contain a considerable amount of PARP demonstrated by the incorporation of ADP-ribose units into the substrate from NAD^+. As shown in the table, the addition of exogenous ATP (phosphate donor) facilitated the phosphorylation of PARP by the putative PKC and considerably lowered PARP activity. The decline of PARP activity was sensitive to the presence of phosphatase PP1 indicating that the kinase reaction was responsible for the PARP decline. On the other hand, 100 ng/ml IGF-I alone depressed PARP activity by 15%. This inhibition was obliterated by staurosporine, a PKC inhibitor, while HCNS (*N*-heptyl-5-chloro-1-naphthylenesulfonamide) and TPA (12-*o*-tetradecanoyl phorbol-13-acetate), activators of PKC, when added alone, lowered PARP activity by 62%. The addition of phosphatase PP1 to the homogenate also removed the inhibitory effect of IGF-1 and almost completely reversed PARP activity. These experiments show indirectly that phosphorylation of PARP may be responsible for the IGF-I-mediated depression of its activity.

IGF-I inhibits PARP in vivo by phosphorylation

We were then able to demonstrate that IGF-I depresses PARP activity in vivo by 40%. In order to eliminate the influence of newly synthesized PARP due to IGF-I-induced protein synthesis, the experiment was performed in the presence of cycloheximide. IGF-I again showed a significant decrease in PARP activity (Fig. 4).

To test the hypothesis that IGF-I inhibits PARP via phosphorylation, we incubated HUVEC with ^{32}P-labeled phosphoric acid in the presence of 100 ng/ml IGF-I for 20 h. After extraction of nuclear proteins, an aliquot was incubated with 1 unit of phosphatase PP1 for 60 min at 37 °C. Proteins were then separated by SDS–PAGE and the gel was exposed to autoradiograph film. After 48 h, the autoradiography film showed bands at 120 and 85 kDa indicating the presence of phosphorylated proteins. These bands corresponded to full-length PARP (120 kDa) and its major breakdown product (85 kDa). This assay demonstrated that even in controls a major part of full-length and 85 kDa PARP is phosphorylated. However, treatment with IGF-I increased phosphorylation in both full-length and 85 kDa PARP. The intensity of the bands in the PP1-treated sample was markedly reduced (Fig. 5).

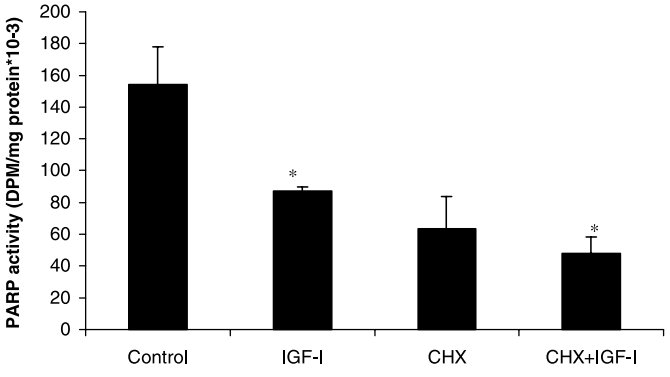

Fig. 4. IGF-I inhibits PARP. Confluent HUVEC cultures were treated with 100 ng/ml IGF-I for 24 h. PARP activity was measured by the incorporation of radiolabeled NAD^+. In some experiments, cycloheximide (CHX, 28 μg/ml) was added as indicated to block protein synthesis. *$p < 0.05$.

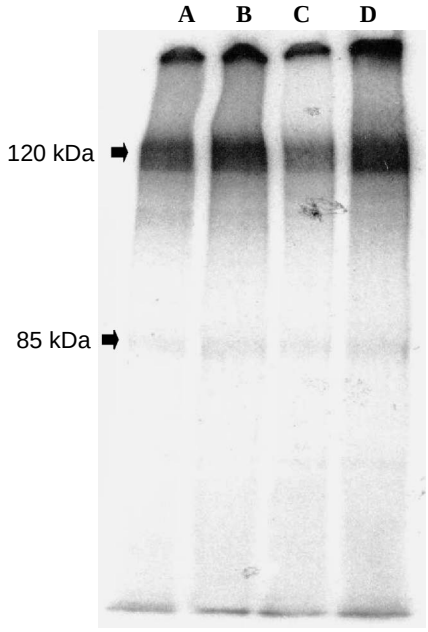

Fig. 5. ^{32}P-labeled immunoprecipitation of PARP. HUVEC cultures were incubated with 50 μCi [^{32}P]orthophosphate/well for 24 h in the presence or absence of 100 ng/ml IGF-I. Equal quantities of protein of lysed cells (20 μg) were incubated either with buffer containing 1 U phosphatase PP1 or buffer alone for 60 min at 37 °C, separated by electrophoresis and visualized by autoradiography, as described in Materials and methods. (A) Control, cells not treated with IGF-I, protein extract not incubated with phosphatase PP1. (B) Cells treated with IGF-I, protein extract not incubated with phosphatase PP1. (C) Cells treated with IGF-I and protein extract incubated with phosphatase PP1. (D) Equivalent to (B).

Discussion

The main signaling pathways activated by binding of IGF-I to its receptor (IGF-IR) are the ERK arm of MAPK- and the phosphoinositol-3-kinase (PI3-K) cascade [27]. The activated IGF-IR is linked to the ERK pathway through adapter proteins. Subsequent phosphorylation and activation of transcription factors trigger changes in gene expression patterns [28]. However, precisely how IGF-I instigates transcription is not yet completely understood.

In this manuscript, we describe a new possible mechanism for IGF-I affecting gene transcription. We show that IGF-I increases VEGF expression through inhibition of poly(ADP-ribose)polymerase (PARP) activity. In previous in vitro investigations phosphorylation was shown to be a possible regulatory step of controlling PARP function

[25,26,29]. In this study, we demonstrate that IGF-I phosphorylates PARP in vivo as a part of its signal transduction pathway. As a result, VEGF protein expression is increased.

Compared with other investigators, in our culture conditions, even unstimulated human endothelial cells produce a significant amount of VEGF protein [30,31]. As a positive control for VEGF protein production we used phorbol-myristate-acetate (PMA) since it has been shown to instigate VEGF mRNA synthesis in HUVEC [32].

First, we demonstrate that the basal and IGF-I-induced VEGF synthesis is dependent on signal transduction since inhibitors of PKC, PI3-K or ERK almost completely blocked the effect of IGF-I, suggesting that IGF-I mainly induces VEGF gene transcription rather than post-translational modification of VEGF. A relationship between PKC and VEGF expression in human endothelial cells has been described already in previous studies [32].

PARP has been thought to be a DNA repair and "housekeeping" enzyme since it was first found to be predominantly activated by DNA nicks. However, more recently a "background" physiological role for PARP was also proposed after it was demonstrated that certain undamaged linear and stem-loop DNA structures are more potent stimulators of PARP than DNA breaks [33]. We show here that in human endothelial cells, VEGF production is inversely correlated with PARP activity and the IGF-I-mediated phosphorylation of PARP depresses its activity. Even when PARP is blocked by inhibitors like 3-aminobenzamide (AB) or nicotinamide (NIC), VEGF expression increases. It has been reported that the binding of PARP with certain promotors enhances transcription [34,35]. On the other hand, poly(ADP-ribosyl)ation of transcription factors can also inhibit their binding to DNA [13,36] showing that conditions that inhibit PARP and the resultant down-regulation of poly(ADP-ribosyl)ation enhance transcription. It has already been shown that phosphorylation is a regulatory step for PARP activity [25,26]. With this investigation, we are able to demonstrate that IGF-I is capable of phosphorylating PARP through PKC in vitro and in vivo, and decreases its activity. We suggest that as a result, the transcription factors involved in VEGF transcription are less poly(ADP-ribosyl)ated and therefore would bind more efficiently with VEGF promotor. Consequently, VEGF transcription is activated. It is well accepted that PKC plays a key role in the regulation of cellular signaling [37]. Our results also indicate a possible link between PKC and IGF-I action.

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