Antiapoptotic Effect of Aurintricarboxylic Acid; Extracellular Action *versus* Inhibition of Cytosolic Protein Tyrosine Phosphatases

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Aurintricarboxylic acid (ATA) prevents apoptosis in a wide range of cell types, including PC12 cells. ATA is known to increase the phosphorylation level of IGF-1 receptor (IGF-1R) and downstream signaling proteins. ATA can translocate across the plasma membrane of PC12 cells and inhibit protein tyrosine phosphatases (PTPs) and, therefore, it is not clear whether ATA exerted its antiapoptotic effect through activation of IGF-1R or by inhibition of cytosolic PTPs. When PC12 cells, deprived of serum, were treated with Fab fragment of anti-IGF-1R antibody to prevent the binding of ATA to the extracellular domain of IGF-1R, ATA was found to penetrate into the cytosolic space of the cells. Under these conditions, the survival-promoting effects of ATA were abolished, and the increase of phosphorylation and characteristic cleavage of IGF-1R were not observed. These results indicate that the antiapoptotic effect of ATA in PC12 cells is due to the binding of ATA to the extracellular domain of IGF-1R and subsequent activation of the IGF-1R, not inhibition of cytosolic PTP(s).

Key Words : Apoptosis, Aurintricarboxylic acid, PC12 cells, Protein tyrosine phosphatase, Inhibitor

Introduction

Aurintricarboxylic acid (ATA) is a red dye composed of multiple salicylic acid moieties (Fig. 1). ATA is synthesized by the condensation of salicylic acid and formaldehyde and uncontrolled branching reactions result in a complex mixture of polymers in a molecular weight range of 138-6,500.[1,2] Because of its polyanionic character, ATA binds to and inhibits nucleic acid-binding enzymes, such as DNA and RNA polymerases, reverse transcriptase, nucleases, primases and topoisomerases.[3-10] ATA is also known to inhibit enzymes that do not bind to nucleic acids, such as Phe:tRNA ligase, aminopropyltransferase, phosphofructokinase and several enzymes required by NAD(H)/NADP(H).[11-14] More recently, a study conducted by our group as well as a study conducted by Liang *et al.* reported that ATA inhibits protein tyrosine phosphatases (PTPs).[15,16]

ATA has also been shown to prevent apoptotic cell death in a variety of cell types including breast cancer MDA-231 and MCF-7 cells, macrophage RAW 264.7 cells and rat pheochromocytoma PC12 cells.[17-20] Although the mechanism by which the antiapoptotic action of ATA occurs has been studied for two decades, it is not fully understood and only fragmentary information is available. Cell type-specific activation of numerous signaling proteins by ATA has been reported. ATA has been shown to increase the tyrosine phosphorylation of IGF-1R and downstream signaling proteins, such as IRS-1/2, PI3K, Akt, Shc and MAP kinases in MDA-231 and MCF-7 cells;[20-22] however, the activation pattern of the proteins in ATA-treated cells was not the same as those observed in IGF-1 treated cells. Additionally, ATA has been shown to induce proteolytic cleavage of the 95 kDa β-subunit of IGF-1R into a 75 kDa fragment,[20] however, this cleavage was not observed in IGF-1 treated cells.

The antiapoptotic effect and accompanying activation of signaling proteins by ATA have been explained by the binding of ATA to the extracellular domain of IGF-1R and resulting activation of the receptor.[20,21] However, we recently observed that ATA translocates across the plasma membrane of PC12 cells.[15] This observation, combined with the recognition that ATA is a potent inhibitor of protein tyrosine phosphatases (PTPs), raised the possibility that the antiapoptotic effect of ATA might be a result of inhibition of cytosolic PTPs. Because both IGF-1R activation and PTP inhibition are able to increase the tyrosine phosphorylation of IGF-1R and other proteins, it has been difficult to determine which of these events occurs for the anti-apoptotic effect in ATA treated cells. Furthermore, IGF-1R activation and PTP inhibition could be acting cooperatively. Therefore, this study was conducted to resolve the ambiguity regarding the mechanism by which the antiapoptotic action of ATA occurs.

Materials and Methods

Materials. ATA was purchased from Sigma (St. Louis, MO, U.S.A.) as triammonium salt and used without further purification. Hoechst was purchased from Sigma. RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from GIBCO-BRL (Gaithersbug, MD, U.S.A.). Chemical luminoscan for Western blot was obtained from Amersham Pharmacia (Uppsala, Sweden). Detection films were purchased from Eastman Kodak (Rochester, NY, U.S.A.).

Antibodies. Anti-phosphotyrosine antibody (4G10), anti-IGF-1R β-subunit antibody (C-20) and anti-IGF-1R α-subunit antibody (H-60) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated rabit anti-mouse IgG was obtained from Jackson ImmunoResearch Lab. (West Grove, PA, U.S.A.). Protein A/G agarose beads were obtained from Oncogene Research Products (San Diego, CA, U.S.A.).

Cell culture. The PC12, MDA231, NIH3T3 and Raw264.7 cells were obtained from the Korean Cell Line Bank. Cells were grown on collagen-coated dishes in RPMI 1640 medium (for PC12 and Raw264.7 cells) or DMEM (for MDA231 and NIH3T3 cells) supplemented with 10% fetal bovine serum. The cells were maintained at 37 °C in a humidified atmosphere under 5% CO_2 before further experiments under various conditions.

Assessment of the permeability of ATA by fluorescence microscopy. Experiments were performed as described previously.[15] Briefly, cells were grown for 24 h on the glass plate of a Chamber slide (Nunc, Naperville, IL, U.S.A.) in RPMI 1640 medium without Phenol Red supplemented with 10% fetal bovine serum. The cells were then washed in the same medium that has been amended with ATA (final concentration 500 μM). After incubation for 3 h in the presence of ATA, the cells on the glass plate were fixed with a 1:1 mixture of acetone-methanol, stained with Hoechst (0.05 μg/mL), and observed using a fluorescence microscope.

Immunoprecipitation and western blot analysis. Cells were collected and dissolved in RIPA lysis buffer (50 mM TrisHCl, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.25% Na-DOC, 1 mM NaF, 1 mM PMSF). Lysis was carried out at 4 °C for 30 minutes, and the lysates were then centrifuged at 15,000 rpm for 30 minutes. The protein concentration of the supernatant was then determined using the Bradford assay method.[23] For immunoprecipitation, cell lysates were incubated with anti-IGF-1R antibody C-20 in a 4 °C orbital shaker overnight, followed by incubation with immobilized protein A/G (Oncogene). Total cell lysates or immunoprecipitants were then electrophoresed under reducing conditions on 8% polyacrylamide gels. Next, proteins were electrophoretically transferred onto nitrocellulose membranes (Millipore), which were then blocked with 5% nonfat dry milk. The proteins were then incubated with primary antibodies against the IGF-1R β-subunit or phosphotyrosine, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody. The immunoblots were then visualized using an enhanced chemiluminescence (ECL) substrate (Amersham Pharmacia).

Fab fragment of anti-IGF-1R antibody (H-60). To obtain monovalent Fab of anti-IGF-1R antibody (H-60), the antibody solution (2 mg/mL antibody, 2 mM EDTA in PBS) was mixed with papain (1 mg papain/100 mg H-60), and then incubated at 37 °C for 30 min. The papain was then inactivated by addition of iodoacetamide to a final concentration of 30 μM, followed by incubation on ice for 60 min. Next, the mixture was incubated with 15 μL of protein A/G agarose beads at room temperature for 60 min, at which time the supernatant containing the Fab fragment was collected.

ATA treatment of PC12 cells pretreated with Fab of anti-IGF-1R antibody (H-60). Fab of the antibody against the α-subunit of IGF-1R was added to PC12 cells that had been maintained in the absence of serum for 24 h, and then incubated at 37 °C for 3 h. Immunoprecipitation and immunoblot analysis were then performed as described above.

Analysis of DNA fragmentation. For experiments on cells maintained in serum-free medium, cells that had been maintained in RPMI 1640 medium containing serum were washed twice with RPMI 1640 medium that did not contain serum, and the cells (5×10^5 cells) were then incubated at 37 °C for 3 h in the absence or presence of ATA or Na_3VO_4. The cells were then lysed and their DNA was isolated by centrifugation followed by ethanol precipitation. The DNA samples were then treated with RNase A, subjected to 1.2% agarose gel electrophoresis, stained with ethidium bromide and photographed by UV-transillumination.

Results

Permeability of ATA in NIH3T3, MDA231 and RAW264.7 cells. We previously reported that ATA translocates across the plasma membrane of PC12 cells after visualizing the native form of ATA using a fluorescence microscope.[15] Therefore, in this study, the same method was used to evaluate the cell permeability of ATA in NIH3T3, MDA231 and RAW264.7 cells. Cells grown on a glass plate and then treated with ATA were examined by fluorescence microscopy to detect the fluorescence of the dye. ATA was shown to permeate the RAW264.7 cells, but not the NIH3T3 and MDA231 cells (Fig. 2).

Effect of ATA in PC12, MDA231, NIH3T3 and RAW264.7

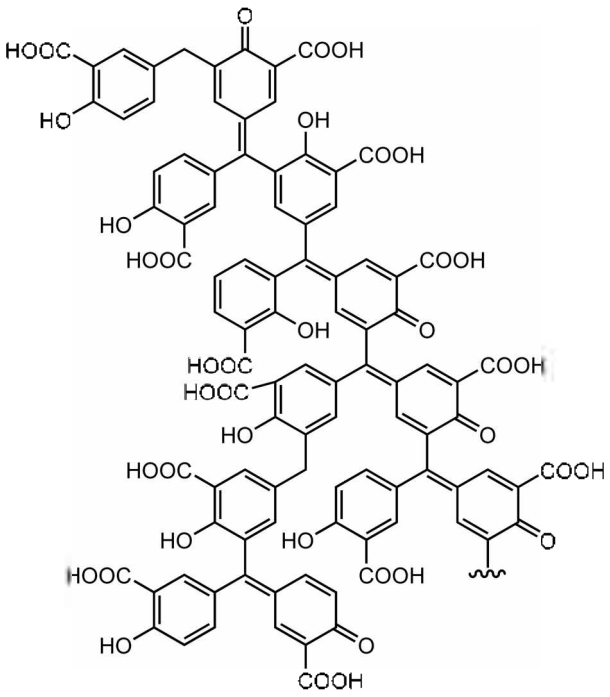

Figure 1. Structure of aurintricarboxylic acid (ATA).

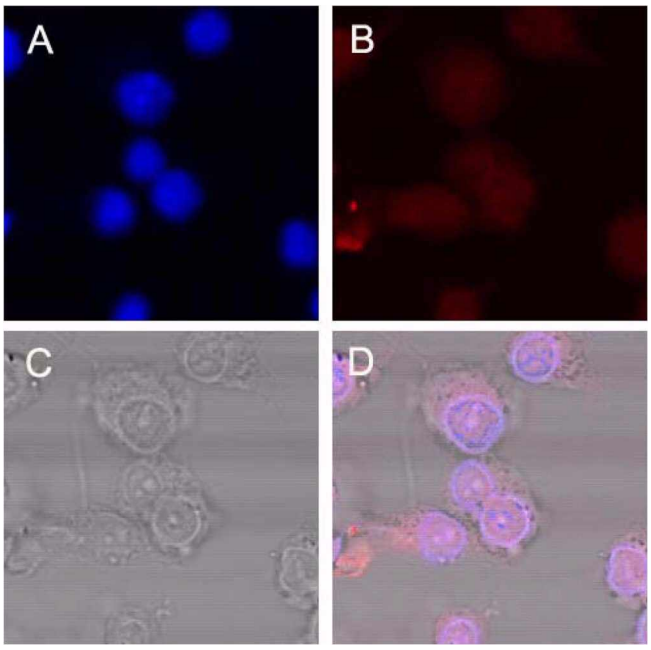

Figure 2. ATA penetrates into the cytoplasm of RAW264.7 cells. RAW264.7 cells grown on a glass plate were incubated in the presence of 500 μM ATA for 3 h, and then washed extensively to remove extracellular ATA. The fluorescence of the ATA was observed using a fluorescence microscope after simultaneous nuclear staining with Hoechst. Fluorescence signals of Hoechst (A) and ATA (B). (C) Phase contrast image of ATA-treated cells. (D) Fluorescence signals (A and B) were merged with a phase contrast image of ATA-treated cells.

cells; apoptosis and IGF-1R expression. ATA has been shown to suppress apoptosis in PC12 and MDA231 cells and, therefore, we evaluated ATA to determine if it also has an antiapoptotic effect in RAW264.7 and NIH3T3 cells. The results of a DNA ladder test indicated that ATA suppressed apoptosis in RAW264.7 cells, but not in NIH3T3 cells (data not shown). ATA is also known to activate IGF-1R and extend the phosphorylation of the β-subunit of IGF-1R in MDA231 cells.[20] therefore, we analyzed PC12, MDA231, RAW264.7 and NIH3T3 cells to determine if they could express IGF-1R on their plasma membrane. Western blot analysis of the cell lysates revealed that IGF-1R was present in PC12 and MDA231 cells, but IGF-1R was not detected in NIH3T3 or RAW264.7 cells (Fig. 3). If ATA prevents apoptosis via the IGF-1R signaling pathway, the absence of IGF-1R in NIH3T3 cells might explain the observation that ATA did not prevent apoptosis in NIH3T3 cells.

Cleavage of the IGF-1R β-chain by ATA treatment in PC12 cells. A recent study showed that ATA treatment of MDA231 cells resulted in increased tyrosine phosphorylation of the IGF-1R β-chain and cleavage of the IGF-1R β-chain, which resulted in the appearance of a 95 kDa form and a 75 kDa form of the chain.[20] Therefore, in this study, the effect of ATA on PC12 cells was examined. As shown in Figure 4, treatment of PC12 cells with ATA induced increased phosphorylation of the tyrosine residue, as well as the appearance of the 75 kDa form of the IGF-1R β-chain.

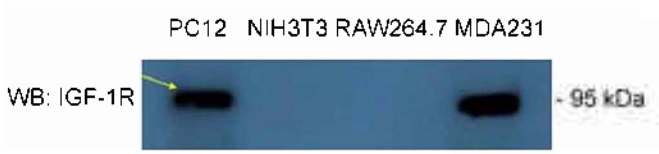

Figure 3. The abundance of IGF-1R was estimated in four cell lines. The same number (8×10^6) of cells was collected, analyzed by PAGE and the IGF-1R was then visualized by Western Blotting. IGF-1R was present in PC12 and MDA231 cells, but not in NIH3T3 and RAW264.7 cells.

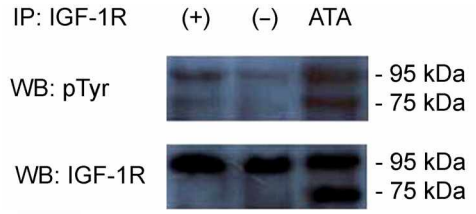

Figure 4. Effect of ATA on IRS-1R tyrosine phosphorylation and cleavage in PC12 cells. Cells were incubated in the presence (+) or absence (−) of serum for 27 h. Alternately, cells deprived of serum for 24 h were treated with ATA (300 μM) for 3 h. Cells were then lysed and immunoprecipitated with anti-IGF-1R antibody. The immunoprecipitates were then separated by SDS/PAGE and it immunoblotted with antibodies against pTyr (upper panel). The blot was then stripped and probed with anti-IGF-1R antibodies. IP, immunoprecipitation; WB, Western blotting; pTyr, phosphotyrosine.

When PC12 cells were treated with IGF-1, an increase in the phosphorylation level of IGF-1R was observed, however, cleavage of the IGF-1R β-chain was not observed (Fig. 4). These observations are consistent with those of MDA-231 cells.

Effect of ATA on PC12 cells pretreated with Fab fragments of the antibodies against the α-subunit of IGF-1R. The increased IGF-1R phosphorylation as a result of ATA treatment may be caused by either activation of protein tyrosine kinase (PTK) action or inhibition of PTP action. However, it is not clear if these processes are cooperative or exclusive. To determine if increased IGF-1R phosphorylation as a result of ATA treatment was caused by one of these processes exclusively, the binding of ATA to IGF-1R outside the cell was blocked by treatment of PC12 cells with Fab of the anti-IGF-1R antibody (H-60) prior to treatment with ATA. The Fab, which was prepared by the proteolytic cleavage of the antibody with papain, binds to the α-subunit of IGF-1R, preventing the ATA from binding to the extracellular domain of the IGF-1R. This binding of the receptors by monovalent antibodies does not result in activation of the IGF-1R signaling pathway as binding by divalent antibodies does. When PC12 cells treated with Fab of anti-IGF-1R antibody followed by ATA were observed under a fluorescence microscope, fluorescence was detected in cells, which indicates that ATA translocates across the plasma membrane even though the extracellular domain of IGF-1R was bound to the antibody (Fig. 5). Conversely, treatment of PC12 cells with Fab of anti-IGF-1R antibody (H-60), followed by treatment with ATA, did not result in augmen-

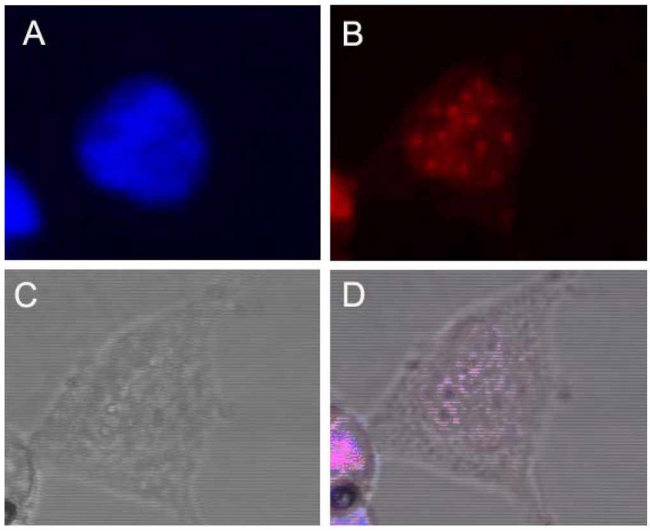

Figure 5. ATA penetrates into the cytoplasmic spaces of PC12 cells treated with Fab of anti-IGF-1R antibody (H-60) prior to the ATA treatment. PC12 cells grown on a glass plate were i) incubated in the presence of Fab of anti-IGF-1R antibody for 1 h and then washed to remove the unbound antibody and ii) incubated in the presence of 500 μM ATA for 3 h and then washed extensively to remove extracellular ATA. Fluorescence of ATA was observed using a fluorescence microscope with simultaneous nuclear staining with Hoechst. Fluorescence signals of Hoechst (A) and ATA (B). (C) Phase contrast image of ATA-treated cells. (D) Fluorescence signal of B was merged with the phase contrast image of C.

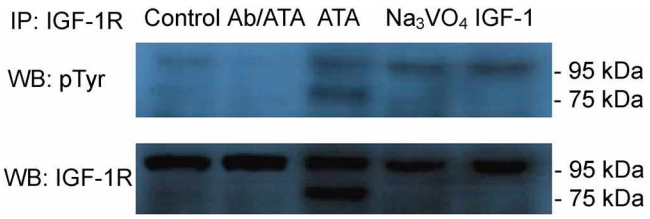

Figure 6. Effect of ATA, Na_3VO_4 and IGF-1 on IGF-1R tyrosine phosphorylation and cleavage. PC12 cells that has been serum starved for 24 h were treated H_2O (Control), Fab of anti-IGF-1R antibody (500 μg/mL) followed by ATA (300 μM) (Ab/ATA), ATA (300 μM), Na_3VO_4 (10 μM) or IGF-1 (5 ng/mL). Cells were then lysed and immunoprecipitated with anti-IGF-1R antibody. The immunoprecipitated proteins were then assayed as described in Fig. 4. IP, immunoprecipitation; WB, Western blotting; pTyr, phosphotyrosine.

tation of the phosphorylation level of the IGF-1R β-subunit or the characteristic cleavage of IGF-1R (Fig. 6). These results indicate that both the increased IGF-1R phosphorylation and the cleavage of IGF-1R by ATA treatment are the results of ATA binding to the extracellular domain of IGF-1R and not a result of ATA action in the cytosol. Treatment of PC12 cells with the antibody prior to ATA treatment also abolished the anti-apoptotic effect of ATA (Fig. 7). Additionally, treatment with ATA or a known PTP inhibitor, Na_3VO_4, prevented DNA fragmentation in PC12 cells cultured in the absence of serum, indicating that both ATA and Na_3VO_4 provide a protective effect against apoptosis

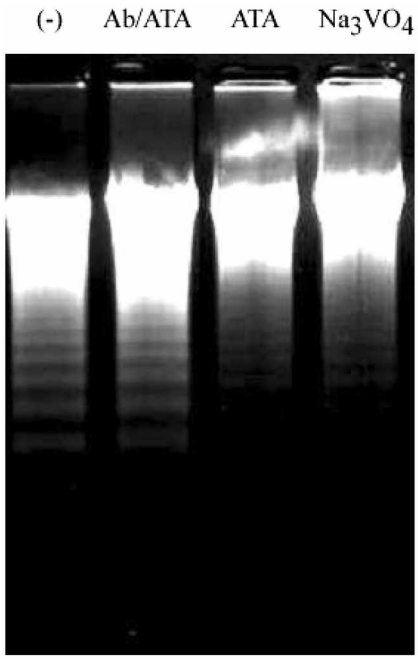

Figure 7. Agarose gel electrophoresis analysis of DNA extracted from PC12 cells cultured for 48 h in RPMI 1640 medium without serum in the presence of H_2O (lane 1), 100 μM ATA following treatment of the cell with Fab of anti-IGF-1R antibody for 1 h (lane 2), 100 μM ATA (lane 3) or 20 μM sodium orthovanadate (lane 4).

induced by serum deprivation.

However, when PC12 cells that had been deprived of serum were treated with Fab of anti-IGF-1R antibody prior to ATA treatment. DNA fragmentation was observed. Taken together, these results indicate that the antiapoptotic effect of ATA on PC12 cells in the absence of serum is the result of ATA binding to the extracellular domain of IGF-1R, and subsequent activation of the IGF-1R, not the inhibition of cytosolic PTP.

Discussion

The antiapototic effect of ATA has been widely utilized in biochemical experiments. It was initially assumed that survival-promotion by ATA was due to the inhibition of nucleases. However, subsequent studies revealed that ATA was not membrane-permeable in some cell types, therefore, the nuclease inhibition hypothesis was discarded. Although previous studies have shown that ATA is not membrane-permeable in some cell types, we recently found that ATA translocates across the plasma membrane in PC12 cells.[15] Additionally, ATA has been shown to increase tyrosine phosphorylation of IGF-1R and downstream signaling proteins in breast cancer cells and PC12 cells. Together, these findings show that ATA binds to the extracellular domain of IGF-1R while concurrently penetrating into the cytosolic space in PC12 cells. Because either of these events could cause the increased phosphorylation of IGF-1R and downstream signaling proteins, it was necessary to determine which of these mechanisms was responsible for ATA's

actions.

Therefore, in this study, we examined the membrane permeability, presence of IGF-1R and the antiapoptotic effect of ATA in four cell types. In PC12 cells, ATA was membrane-permeable and prevented apoptosis. However, in NIH3T3 cells, IGF-1R was not expressed, and ATA did not penetrate the membrane or exert any antiapoptotic effects. Based on these results, mechanism responsible for ATA's effects could not be distinguished. However, it is important to note that ATA did not permeate into the cytoplasm of MDA231 cells, which is where IGF-1R is expressed. This observation in MDA231 cells indicates that ATA's action may occur outside of the cell, not in the cytoplasm. Another interesting observation was that, although IGF-1R is not expressed in RAW264.7 cells, these cells are protected from apoptosis by ATA, which suggests that the mechanism of the antiapoptotic action of ATA might differ between cell types (Fig. 3 and data not shown).

To elucidate the site at which ATA action occurs, the extracellular binding of ATA was blocked without preventing the translocation of ATA across the plasma membrane of the cell. ATA treatment of PC12 cells under this condition, abolished all effects observed when cells were treated with ATA alone, which indicates that the antiapoptotic effect of ATA did not occur in the cytosol. The effect of ATA on cytoplasmic PTPs is yet to be explained and needs further study.

In conclusion, the results of this study indicate that the antiapoptotic effect of ATA on PC12 cells in the absence of serum is the result of ATA binding to the extracellular domain of IGF-1R, and subsequent activation of the IGF-1R, not the inhibition of cytosolic PTPs.

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