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Insulin-Like Growth Factor-1 Receptor Regulation in Activated Human T Lymphocytes

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Key Words
Insulin-like growth factor-1 receptor · T lymphocyte · Up-regulation · Down-regulation

Abstract
Objective: To investigate the kinetics of insulin-like growth factor-1 receptor (IGF-1R) expression in PHA-stimulated T lymphocytes. ***Methods:*** IGF-1R protein and mRNA were detected by flow cytometry and RT-PCR respectively, between 0 and 48 h after cell activation. ***Results:*** Few minutes after T lymphocytes were activated, internalization of the IGF-1R from the cell membrane was observed, achieving the lower level between 1 and 6 h and was accompanied by a reduction in its mRNA. This was followed by re-expression of IGF-1R on the cell surface and an increase in IGF-1R mRNA levels in the cytoplasm, reaching levels higher than those recorded initially after 48 h activation. ***Conclusion:*** This down- and up-regulation suggests that restoration of IGF-1R would be the result of receptor recycling and de novo synthesis and highlights its importance for T lymphocyte proliferation.

Introduction

The presence of IGF-1 mRNA in unstimulated peripheral blood mononuclear cells (PBMC) and T lymphocytes, and the detection of the hormone in the culture medium after 1 h of PHA stimulation, suggest that an autocrine/paracrine-stimulated release of IGF-1 is induced immediately after T-cell activation, causing the down-regulation of IGF-1R, which increased when IGF-1 is added to the medium [1]. This loss of IGF-1R corresponds to receptor activation, since IGF-1 in the culture medium increases the internalization and phosphorylation of IGF-1R, as well as the phosphorylation of mitogen-activated protein kinase (MAPK), leading to an increase in interleukin (IL)-2 and CD25 synthesis [1].

Receptor-mediated endocytosis of IGF-1 occurs after the binding of the ligand to its specific receptor, and while this process is linked to cellular responses and receptor degradation, some receptors are sorted for recycling to the cell surface [2]. Therefore, to investigate the origin of the increase in IGF-1R 36 h after T-cell activation that is described in many works [3–6], we examined the kinetics of IGF-1R expression on the T-cell membrane and the levels of IGF-1R mRNA, between 0 and 48 h after PHA

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activation, in the T-lymphocyte population within a culture of PBMC in medium supplemented with fetal calf serum (FCS). Results demonstrate that activation induces a decline in IGF-1R and its mRNA, followed by a recovery in the levels of IGF-1R in the cell membrane, and an increase in the levels of IGF-1R mRNA.

Material and Methods

Cells

Peripheral blood mononuclear cells were obtained from the blood of normal healthy donors, after they had given informed consent, using a Ficoll-hypaque gradient. Cells were cultured in RPMI 1640 medium with 5% FCS and stimulated with 5 μg/ml *Phaseolus vulgaris* agglutinin (PHA) (Sigma Chemical Co.).

RT-PCR Analysis

Total RNA was prepared from whole cells using Trizol reagent (GIBCO), and reverse transcription (RT) was performed with 1 μg RNA in a 25-μl reaction volume. The transcribed cDNA fragments were amplified in a 50-μl reaction volume with 100 μ*M* each dNTP, 0.4 μ*M* each primer, and 1 U *Taq* DNA polymerase (Promega Corp.). The primer sequences were:

Primer	Primer sequence	PCR product
IGF-1R (F) [7]	5′-ATTGAGGAGGTCACA-GAGAAC-3′	IGF-1R (889 or 892 bp)
IGF-1R (R)	5′-CACGACATACGGAGA-CACT-3′	
G3PDH (F) [8]	5′-TGAAGGTCGGAGT-CAACGGATTTG-3′	G3PDH (956 bp)
G3PDH (R) [8]	5′-CACCACCTGGAG-TACCGGGTGTAC-3′	

Amplification conditions for IGF-1R and glycerol-3-phosphate dehydrogenase (G3PDH) cDNAs are described in Quinn et al. [7]. PCR products were separated by electrophoresis on a 1.8% agarose gel, and visualized by ethidium bromide staining under ultraviolet transillumination.

Levels of mRNA were evaluated by densitometric analysis of the PCR products using Image Quant (Molecular Dynamics). Values were expressed as the ratio of arbitrary densitometric units of IGF-1R versus units of the G3PDH control band $\times 100$. A 100-bp ladder (GIBCO) was used to verify the sizes of the PCR products. The identity of the PCR fragment was confirmed by *Pst*1 cleavage.

Flow Cytometric Analysis

Determination of IGF-1R expression on the surfaces of T lymphocytes was evaluated by indirect immunofluorescence using α-IR3 antibody (Oncogene Science Inc.) and anti-mouse FITC-conjugated secondary antibody (Caltag Laboratory) and finally, T cells were identified in total cell samples by direct immunofluorescence using PE-conjugated anti-CD3 antibody (Becton Dickinson), as described previously [3]. Cells were examined using a FACScan cytometer (Becton Dickinson), and levels of background staining were determined in cells incubated with the FITC- or PE-conjugated isotype controls antibodies. A total of 10^4 cells/sample was analyzed. Data analysis was performed using Cellquest software (Becton Dickinson). The mean fluorescence intensity (MFI) was determined by subtracting the mean fluorescence of the isotype control cells, to the mean fluorescence of αIR3-treated cells. This value is the average of fluorescence intensity of each cell, which is proportional to IGF-1-R molecules/cell.

Sequence Analysis

Sequencing analysis of IGF-1R mRNAs was done with the AlFexpress II DNA Analysis System (Amersham Pharmacia Biotech), an automated DNA sequencer which uses dye-labelled ddNTP terminators.

Statistical Analysis

Paired comparisons of controls and treated samples were based on Student's t test, and results are presented as means ± SE.

Results and Discussion

The kinetics of IGF-1R expression in T lymphocytes from PBMC stimulated with PHA cultured in medium with 5% FCS is shown in figure 1. Although there was a rapid decrease in the number of IGF-1R after activation, they achieved the lower levels between 1 and 6 h, instead of the 15 min described previously for cells stimulated in FCS-free medium [1]. Afterwards, there was an increase in IGF-1R, which, 48 h after activation, reached levels higher than those recorded initially.

After endocytosis receptors can be degraded in lysosomes or recycled back to the cell surface [2]. Therefore, to investigate the nature of IGF-1R recovery during T-lymphocyte activation, we compared the levels of IGF-1R mRNA with simultaneous protein expression on the cell membrane. However, because Hartmann et al. [9] have described IGF-1R mRNA in unstimulated PBMC whereas Reiss et al. [10] found none, we initially looked for IGF-1R transcripts in PBMC, and in the T-lymphocyte population belong to those cells. T cells were isolated by the RosetteSep technique (StemCell, Vancouver, Canada) with >98% purity, as estimated by flow cytometric analysis, and using the same analysis, 70 ± 4% of the total PBMC were shown to be CD3+ cells. Results from five different experiments showed that the total IGF-1R mRNA values, expressed as the ratio of arbitrary densitometric units of mRNA vs. units of G3PDH per 100, was 100.0 ± 10.7 in T lymphocytes against 84.6 ± 11.8 in PBMC.

When the time course analyses were performed (fig. 2), we observed that the decrease in receptor expression on the T-cell membrane was accompanied by a decrease in IGF-1R mRNA. However, the recovery of IGF-1R on the cell surface was followed by the increase of its mRNA in

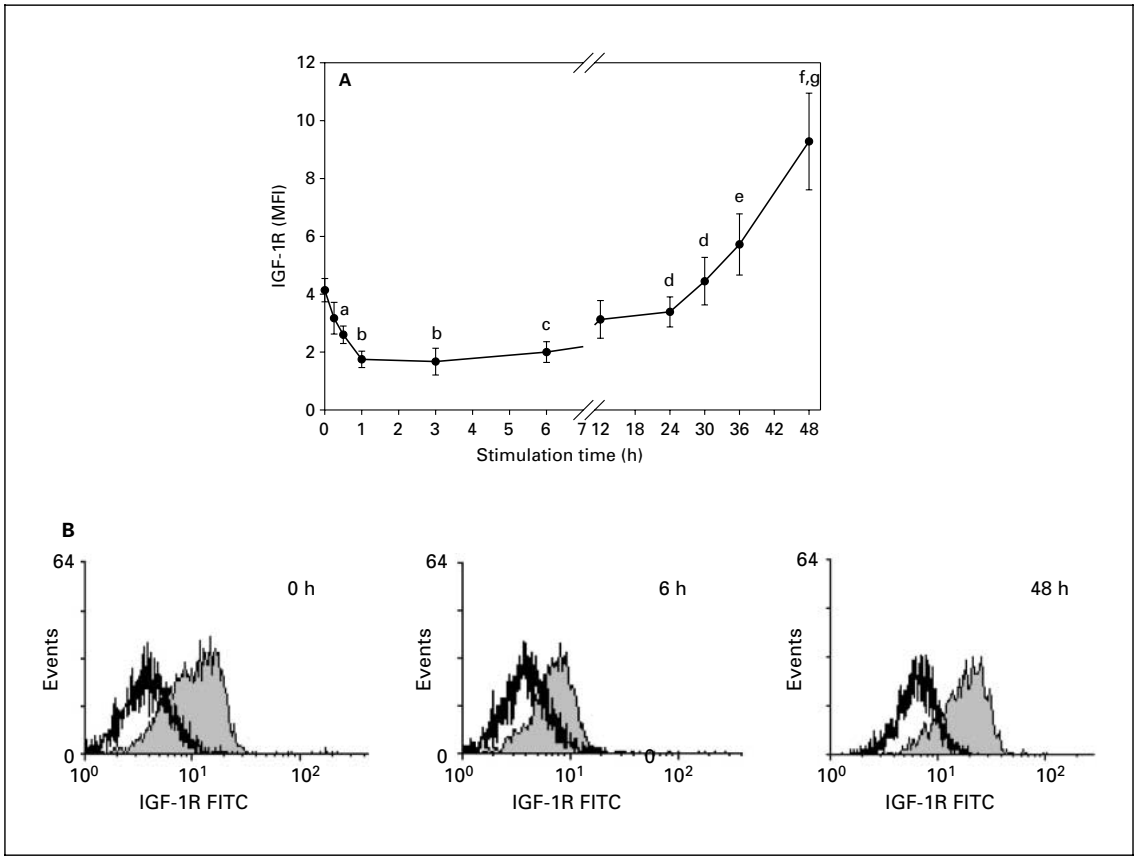

Fig. 1. Effects of PHA activation on the time-dependent expression of IGF-1R in T lymphocytes. **A** Samples were taken at 0, 15, 30 min, 1, 6, 12, 30, 36 and 48 h after stimulation of PBMC. IGF-1R was detected on CD3-positive cells, as described in 'Material and Methods'. Means ± SE corresponds to three or more determinations. [a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.01$ and [g] $p < 0.05$, against 0 min; and [d] $p < 0.05$, [e] $p < 0.02$ and [f] $p < 0.01$ against 6 h. IGF-1R are expressed as mean fluorescence intensity (MFI) determined by subtracting the mean fluorescence of the isotype control cells, to the mean fluorescence of αIR3-treated cells. **B** Representative histograms of IGF-1R expression on CD3-positive cells at 0, 6 and 48 h after stimulation of PBMC. Black lines define the isotype control area and gray areas define the IGF-1R expression.

the cytoplasm (fig. 2). These results show that the increase in IGF-1R after T-lymphocyte activation described in many works [3–6] is preceded by an internalization and a subsequent re-expression of the receptor on the cell membrane. This down- and up-regulation apparently occurs with similar changes in the levels of mRNA. However, the slower increase in the mRNA levels suggests that the earlier recovery of IGF-1R results from receptor recycling, followed by de novo synthesis.

On the other hand, two IGF-1R transcripts have been identified that differ by the deletion of three nucleotides of the coding region, which occurs in the extracellular portion of the receptor β-subunit. Both forms bind IGF-1 with similar affinity, but the shorter IGF-1R stimulates IRS-1 to a greater extent and is internalized about 50% less faster in comparison with the larger one [11]. Therefore, by sequencing analysis we looked for both mRNAs isoforms in the three assays of figure 2. Results demonstrated that in unstimulated and cells stimulated during 48 h both isoforms were present, however, we were not able to detect quantitative differences between both groups (data not shown). If it is considered that not all the mRNA detected is transcripted, it would be interesting to know the total amount of both proteins expressed on the cell membrane, but we could not do this because there are no antibodies available for each of the isoforms. Biological effects triggered by IGF-1R phosphorylation and internalization during T-lymphocyte activation induces the

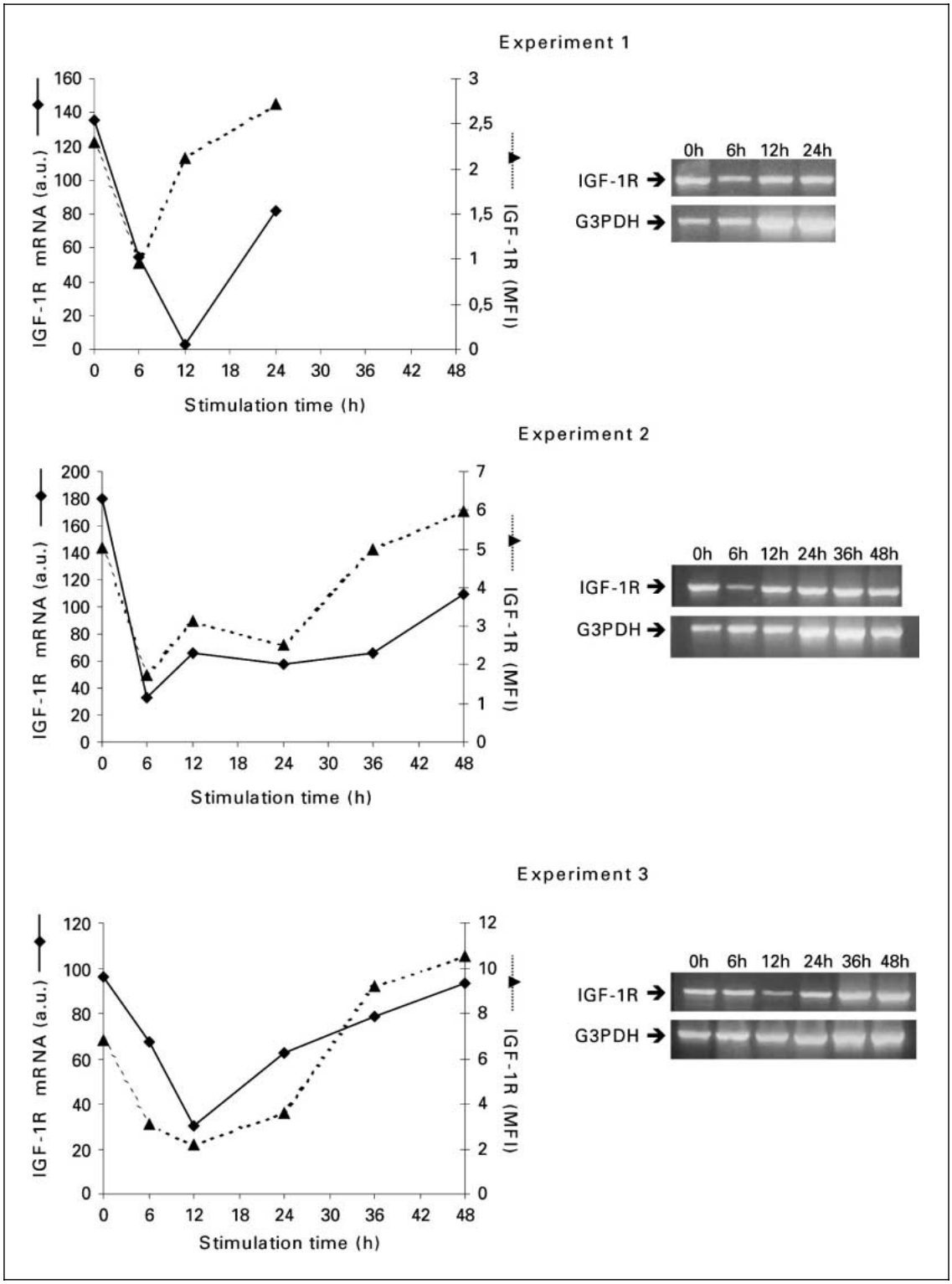

Fig. 2. Relationship between IGF-1R and IGF-1R mRNA during T-lymphocyte activation. Values of IGF-1R mRNA detected by RT-PCR are expressed as the ratio of arbitrary densitometric units (a.u.) of IGF-1R versus units of G3PDH per 100. IGF-1R are expressed as mean fluorescence intensity (MFI) determined by subtracting the mean fluorescence of the isotype control cells, to the mean fluorescence of αIR3-treated cells. Three representative experiments out of five are shown.

increases in MAPK phosphorylation and the synthesis of CD25 and IL-2 [1]. The re-expression of IGF-1R in the membrane of these cells, as reported here, suggests that the IGF-1R could play a role not only during T-cell activation but also in later events, as the antiapoptotic effect described by Walsh et al. [5], although this remains to be determined.

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