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Melanin potentiates kanamycin-induced inhibition of collagen biosynthesis in human skin fibroblasts

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Ototoxicity is one of the well known side effects of kanamycin. The mechanism underlying the organ specificity of the side effect is not understood. Since many pharmacologic agents are known to form complexes with melanin and melanin is an abundant constituent of the inner ear, we investigated whether kanamycin interacts with melanin and how this process affects biosynthesis of collagen in cultured human skin fibroblasts. We found that kanamycin forms complexes with melanin. The amount of kanamycin bound to melanin increases with increase of initial drug concentration. The Scatchard plot analysis of the drug binding to melanin has shown that at least two classes of independent binding sites are implicated in the kanamycin-melanin complex formation: strong binding sites with the association constant $K_1 \sim 3 \times 10^5\ M^{-1}$, and the weak binding sites with $K_2 \sim 4 \times 10^3\ M^{-1}$. The number of total binding sites ($n_1 + n_2$) was calculated as about 0.64 μmol kanamycin per 1 mg melanin. We found that kanamycin induced inhibition of collagen and DNA biosynthesis ($IC_{50} \sim 5$ μM). Melanin at 100 μg/ml produced about 25% inhibition of DNA synthesis, but it had no effect on collagen biosynthesis in cultured fibroblasts. However, the addition of melanin (100 μg/ml) to kanamycin-treated cells (5 μM) augmented the inhibitory action of kanamycin on collagen and DNA biosynthesis. We have suggested that IGF-I receptor expression, involved in cell growth and collagen metabolism, may be one of the targets for kanamycin-induced inhibition of these processes. As shown by Western immunoblot analysis melanin augmented kanamycin-induced decrease in the expression of IGF-I receptor as well MAP kinases expression: ERK1 and ERK2. The obtained results demonstrate that melanin potentiates the inhibitory effect of kanamycin on IGF-I receptor-dependent signaling pathway in cultured fibroblasts. The data suggest a potential mechanism for the organ specificity of kanamycin-induced hearing loss in patients which may result from melanin-induced augmentation of the inhibitory effects of kanamycin on collagen and DNA biosynthesis.

1. Introduction

Kanamycin is aminoglycoside antibiotic, that still remains as a first-line antibiotic in gram-negative infections (Reiss and Reiss 2003). Several untoward side effects however, accompany the high effectiveness of kanamycin therapy (de Jager and van Altena 2002; Seligmann et al. 1996; Bamonte et al. 1980). One of them is ototoxicity (Govaerts et al. 1990; Zhuravskii et al. 2002; Bates 2003). The precise mechanism underlying the organ specificity of kanamycin-induced toxicity has not been fully established. Several toxins and pharmacologic agents (antibiotics, psychotropic, antirheumatic and anesthetic agents) may undergo a specific interaction with melanin, leading to the accumulation of these agents in melanin-rich tissues and to an increase in their toxicity (Sarna 1992). Neuropathy, retinopathy, skin hyperpigmentation and hearing loss (Larsson and Tjälve 1979; Larsson et al. 1988) are symptoms of their accumulation in melanin-rich tissues.

It seems reasonable to suspect that the specificity of kanamycin toxicity may result from its ability to form a complex with melanin that is an abundant constituent of the inner ear. The phenomenon may contribute to accumulation of the antibiotic in the inner ear and facilitation of toxic effects on surrounding cells. Fibroblasts, the main collagen synthesizing cells, may represent the target for the action of kanamycin (Chernikov et al. 2003). In view of the fact that collagen is the major constituent of the hearing organ it seems possible that kanamycin-induced hearing loss may result from its ability to inhibit collagen biosynthesis in the tissues of the inner ear.

Since insulin-like growth factor I (IGF-I) is one of the most potent stimulators of collagen biosynthesis acting through the IGF-I receptor (Goldstein et al. 1989, Peterkofsky et al. 1991) we decided to examine the kanamycin effects on IGF-I receptor expression in fibroblasts and the role of melanin in this process. Signal transmitted by activated IGF-I receptor induces phosphorylation of MAP ki-

nases. Disturbances in the expression of IGF-I receptor may therefore affect expression of phosphorylated MAP kinases: ERK1 and ERK2 (Misawa et al. 2000).
In this study we examined the ability of kanamycin to form a complex with melanin, the stability constants of this complex and the effect of melanin on kanamycin-induced inhibition of DNA and collagen biosynthesis, IGF-I receptor expression and phosphorylated MAP kinases expression in cultured human skin fibroblasts treated with melanin, kanamycin and both substances.

2. Investigations and results

Present studies show that melanin binds kanamycin (Fig.1). The amount of kanamycin bound to melanin increases with increasing initial drug concentration. It can be seen from the binding isotherm (Fig. 1A) that the amount of kanamycin bound to the melanin polymer reached a plateau at about 0.5 μmol kanamycin/mg melanin, which reflects an initial concentration of the drug equal to 8×10^{-4} M. The data were analyzed by the Scatchard method (Scatchard et al. 1957; Kalbitzer and Stehlik 1979). Analysis of kanamycin-melanin binding showed that the Scatchard plot was curvilinear with an upward concavity (Fig. 1B), indicating that at least two classes of independent binding sites are implicated in complex formation (Larsson 1993). The calculated binding parameters for the interaction of kanamycin with melanin were as follows: one class of binding sites with the association constant $K_1 = 3.1 \times 10^5\ M^{-1}$ and a second class of binding sites with $K_2 = 4.3 \times 10^3\ M^{-1}$. The number of binding sites was $n_1 = 0.30$ and $n_2 = 0.34$ μmol kanamycin per milligram melanin.
The effect of kanamycin on collagen biosynthesis was tested in confluent human skin fibroblasts. It is well known that increased cell density is accompanied by increased collagen production by these cells (Makela et al. 1990). Collagen biosynthesis was measured in fibroblasts treated for 24 h with different concentrations of kanamycin. The drug contributed to the decrease of collagen biosynthesis in confluent human skin fibroblasts in a dose-dependent manner (Fig. 2A). The concentration of the drug required for 50% inhibition (IC_{50}) of collagen biosynthesis was found at about 5 μM.
A similar effect of kanamycin on DNA synthesis was found. The drug induced decrease of fibroblast DNA synthesis (Fig. 2B) in a dose-dependent manner. IC_{50} for DNA synthesis was found at about 5 μM. At 10 μM of kanamycin, DNA synthesis was decreased to about 25% of control value. In the experiments IC_{50} value was calculated on the basis of kanamycin concentration in the medium of cultured cells.

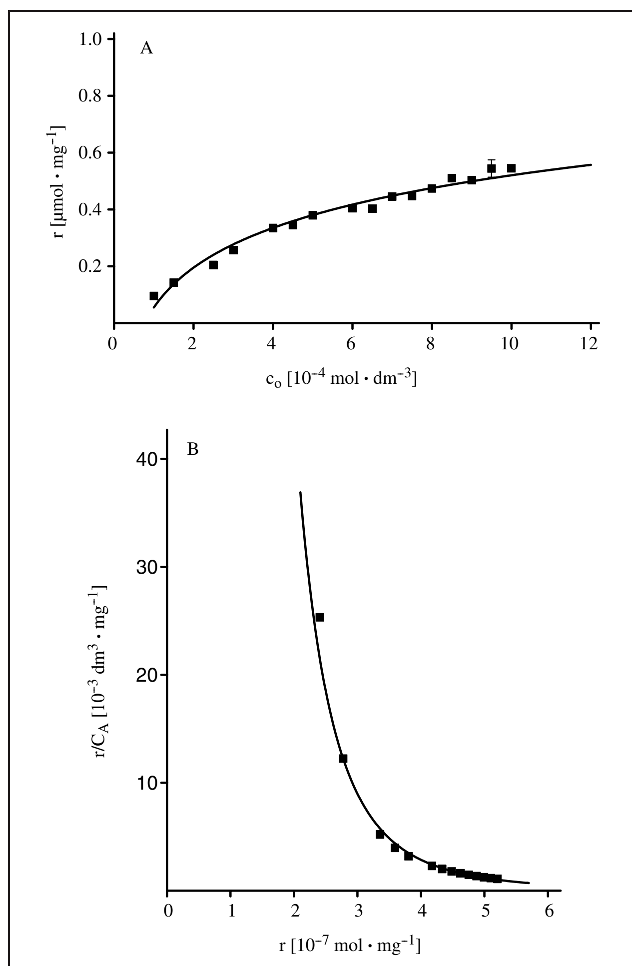

Fig. 1: Binding isotherm (A) and Scatchard plot (B) for the kanamycin-melanin complexes obtained after 24 h incubation; c_o-initial concentration of kanamycin, r-amount of kanamycin bound per 1 mg melanin, c_A-concentration of unbound kanamycin. Mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

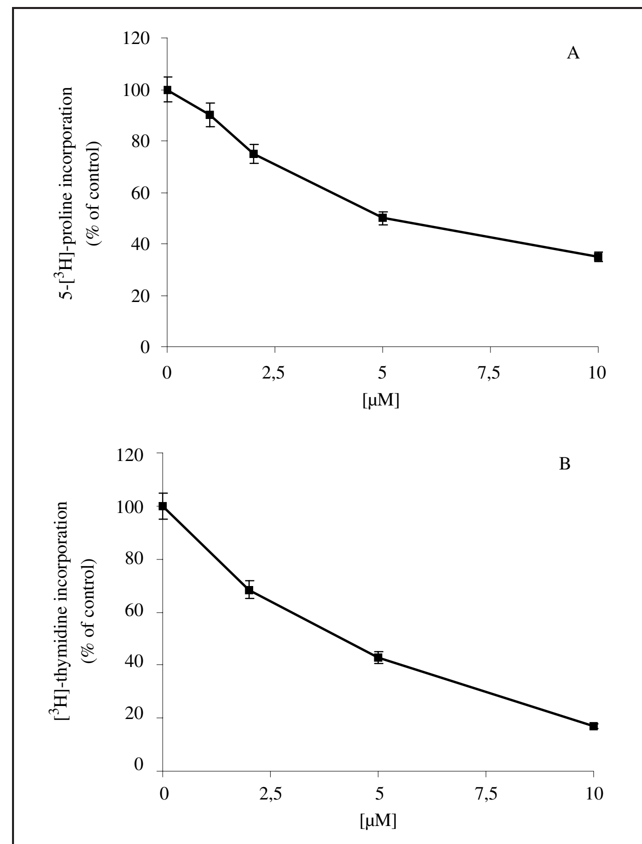

Fig. 2: A: Collagen biosynthesis (measured as 5[^{3}H]proline incorporation into proteins susceptible to the action of bacterial collagenase) in confluent human skin fibroblasts incubated for 24 h with different concentrations of kanamycin. Mean values ± SD from six independent experiments are presented
B: DNA biosynthesis (measured as [^{3}H]thymidine incorporation into DNA) in semiconfluent human skin fibroblasts incubated for 24 h with different concentrations of kanamycin. Mean values ± SD from six independent experiments are presented

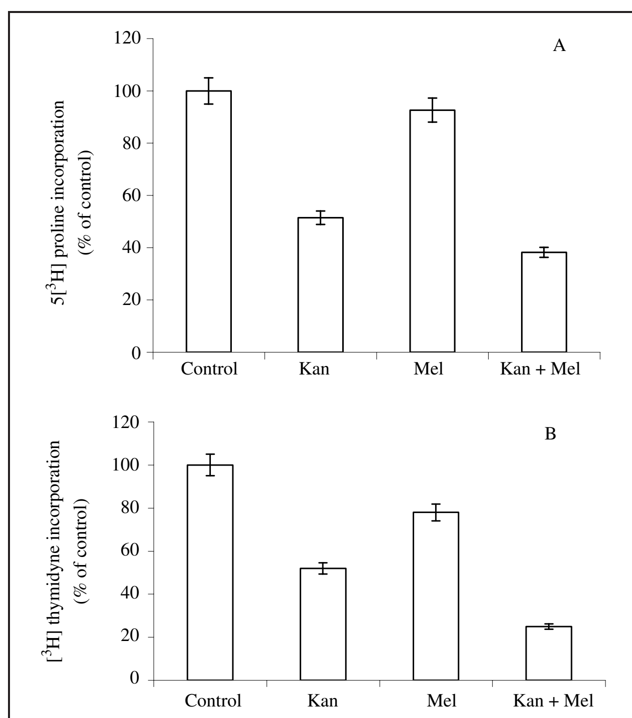

Fig. 3: A: Collagen biosynthesis in confluent human skin fibroblasts (control) cultured for 24 h with 5 μM kanamycin (Kan), 100 μg/ml melanin (Mel) or both (Kan + Mel). Mean values ± SD from three independent experiments done in duplicates are presented
B: DNA biosynthesis in semiconfluent human skin fibroblasts (control) cultured for 24 h with 5 μM kanamycin (Kan), 100 μg/ml melanin (Mel) or both (Kan + Mel). Mean values ± SD from three independent experiments done in duplicates are presented

Since kanamycin was found to form a complex with melanin, we determined the effects of both compounds (added simultaneously) on collagen and DNA biosynthesis in confluent fibroblasts. In these experiments, the kanamycin was used at IC_{50} (5 μM) for the respective processes together with 100 μg/ml of melanin. As can be seen from Fig. 3A, the addition of melanin to kanamycin-treated cells further decreased collagen biosynthesis in fibroblasts, to about 40% of control values. DNA biosynthesis (Fig. 3B) was decreased in these cells, in a similar manner, to about 25% of control values.

Insulin-like growth factor I (IGF-I) is one of the most potent stimulators of collagen biosynthesis and important metabolic and mitogenic factor involved in cell division (Oyamada et al. 1990; Phan et al. 2003). It has been found that IGF-I stimulates collagen biosynthesis through interaction with IGF-I receptor (Goldstein et al. 1989). Since disturbances in the expression of IGF-I receptor evoke disregulation of collagen biosynthesis and cell division, we determined IGF-I receptor expression in fibroblasts treated for 24 h with kanamycin (5 μM), melanin (100 μg/ml) and both substances. Western blot analysis, with specific antibody against human insulin-like growth factor-I receptor (IGF-I sR) showed high expression of IGF receptor in control cells. In these cells the expression of two bands (Fig. 4, lane 1) of about 130 kDa and 95 kDa were observed. In the presence of 5 μM kanamycin (Fig. 4, lane 2) the amount of receptor protein was decreased, compared to control cells and in the presence of melanin (100 μg/ml) (Fig. 4, lane 3) it was similar, compared to control cells. When both substances were added simultaneously, no band staining for IGF receptor was observed (Fig. 4, lane 4). Supporting data came from studying MAP kinases expres-

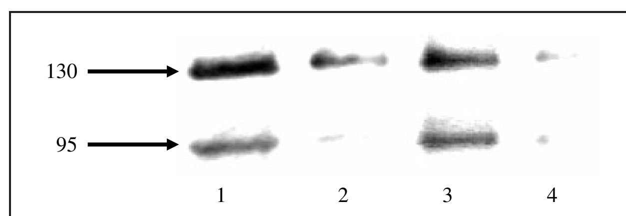

Fig. 4: Western immunoblot analysis of IGF receptor in control human skin fibroblasts (lane 1) and cultured for 24 h with 5 μM kanamycin (lane 2), 100 μg/ml melanin (lane 3), or both (lane 4). The mean values of 6 pooled cell homogenate extracts are presented. The same amount of supernatant protein (20 μg) was run in each lane

Fig. 5: Western immunoblot analysis for MAP kinases (ERK1 and ERK2) in control human skin fibroblasts (lane 1) and incubated for 24 h with 5 μM kanamycin (lane 2), 100 μg/ml melanin (lane 3), or both (lane 4). The mean values of 6 pooled cell homogenate extracts are presented. The same amount of supernatant protein (20 μg) was run in each lane

sion. Western blot analysis, with specific antibody against phosphorylated human ERK1 and ERK2 showed high expression of the MAP kinases in control cells. In these cells we observed expression of two bands (Fig. 5, lane 1), ERK1 of about 42 kDa and ERK2 of about 44 kDa. A slight decrease in the expression of the MAP kinases was observed in melanin-treated cells (Fig. 5, lane 3). In the presence 5 μM of kanamycin (Fig. 5, lane 2) or kanamycin with melanin (Fig. 5, lane 4) no band staining for MAP kinases was found in these cells.

3. Discussion

It is now well known that many drugs are markedly accumulated and retained for a considerable time by pigmented tissues and that the retention of these compounds is proportional to degree of melanin pigmentation (Larsson 1993). *In vivo* studies documented that ototoxicity of kanamycin was different in pigmented and albino guinea pigs. In pigmented animals a high kanamycin dose (200 mg per kilogram of body weight) resulted in hearing loss together with loss of both inner and outer hair cells. The albino animals in the same dose group showed significantly less hearing loss and hair cell degeneration (Wasterstrom et al. 1986). However, the mechanism for the process is not known. In view of the fact that melanin is an abundant constituent of the inner ear, we determined whether kanamycin interacts with melanin and how this process affects biosynthesis of collagen (an important structural and functional constituent of hearing organ) in cultured fibroblasts. The data presented here show that melanin forms complexes with kanamycin and that at least two classes of independent binding sites are implicated in complex formation. The two calculated binding sites in the kanamycin-melanin complex may be due to a different accessibility of the melanin binding sites. This may be explained by differences between the surface and the interior of the melanin polymer with regard to steric hindrance and physico-chemical conditions, as was earlier suggested (Larsson and Tjälve 1979; Larsson 1993). Melanins are

polyanions with a relatively high content of carboxy groups and o-semiquinones (Ito 1986; Prota 1992), which are negatively charged at physiological pH. Substances with cationic properties (e.g., metal ions, some drugs) are thus bound to melanin mainly by ionic interaction, which also may be strengthened by other forces such as van der Waals attraction, charge-transfer reactions and hydrophobic interactions (Larsson and Tjälve 1979; Larsson et al. 1988).

The ability of melanin to bind kanamycin was accompanied by the melanin-dependent potentiation of the inhibitory action of kanamycin on collagen and DNA biosynthesis. One possible explanation for this phenomenon may be that the kanamycin-melanin complex gradually dissociates during the 24-h incubation of cultured cells and that the released kanamycin constantly affects the metabolism of the cells.

A similar mechanism for melanin-dependent augmentation of gentamicin-induced inhibition of collagen biosynthesis was previously demonstrated (Wrzesniok et al. 2002).

Insulin-like growth factor I (IGF-I) is one of the most potent stimulators of collagen biosynthesis acting through IGF-I receptor (Goldstein et al. 1989). Interestingly, melanin potentiated the inhibitory effect of kanamycin on IGF-I receptor expression, suggesting the mechanism for augmentation of kanamycin-induced inhibition of collagen and DNA biosynthesis.

Signal transmitted by activated IGF-I receptor induces MAP kinases expression (Wang and Sun 2002). Disturbances in the expression of IGF-I receptor is known to decrease MAP kinases expression: ERK1 and ERK2 (Galvan et al. 2003). The data presented here show that the inhibitory effect of the kanamycin-melanin complex on IGF-I receptor expression is accompanied by simultaneous inhibition of MAP kinases expression.

Other studies also postulate that the uptake of kanamycin into the hair cells of the inner ear, initiates a cascade of irreversible changes in signaling processes, possibly through inhibition of the phosphoinositide second-messenger system (Schacht 1986; Williams et al. 1987).

Another interesting point arises from the data, namely, whether the melanin-kanamycin complex may serve as a pro-drug with a prolonged duration of action. If this holds true, one would expect that similar therapeutic effects of kanamycin would be achieved with much lower doses of the melanin-kanamycin complex.

The data suggest that the ability of kanamycin to form complexes with melanin may contribute to the accumulation of the drug in melanin-rich tissues (e.g. inner ear). Since the stability of the complexes is not high it seems that the drug undergoes subsequent slow dissociation from the complex, leading to augmentation of its inhibitory action on collagen and DNA biosynthesis in fibroblasts. The phenomenon may be responsible for the organ specificity of kanamycin-induced hearing lesions occurring sometimes in patients receiving this drug.

4. Experimental

4.1. Materials

Kanamycin (kanamycin sulphate), bacterial collagenase (type VII), trypsin, bovine serum albumin, L-3,4-dihydroxyphenylalanine (L-DOPA), anti-human insulin-like growth factor-I receptor (IGF-I sR) antibody, alkaline phosphatase conjugated anti-goat IgG, monoclonal anti-MAPK antibody, anti-mouse immunoglobulin G (whole molecule) alkaline phosphatase conjugated antibody, Fast BCIP/NBT reagent were purchased from Sigma Chemical Co. (USA), as were most other chemicals used. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum used in cell culture were obtained from Life Technologies (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). Nitrocellulose membrane (0.2 μm), sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), molecular weight standards and Coomassie Briliant Blue R-250 were received from Bio-Rad Laboratories, USA. L-5[^{3}H] proline (28Ci/mmol) was purchased from Amersham (UK). [^{3}H]thymidine (6.7 Ci/mmol) was obtained from NEN (USA).

4.2. Preparation of melanin

Synthetic melanin was obtained by oxidative polymerization of L-DOPA solution (1 mg/ml) in 0.067 mol/l phosphate buffer (pH 8.0) for 48 h, according to the Binns et al. (1970).

4.3. Preparation of kanamycin-melanin complex

Kanamycin-melanin complexes were obtained as follows: 5 mg of melanin were placed into plastic test-tubes, to which drug solutions in 0.067 mol/l phosphate buffer at pH 7.0 were added to a final volume of 5 ml. The initial concentration of kanamycin ranged from 1×10^{-4} to 1×10^{-3} mol/l. Control samples contained 5 mg of melanin and 5 ml of buffer without drug. All samples were incubated for 24 h at room temperature, and then filtered.

4.4. Analysis of kanamycin binding to melanin

The concentration of kanamycin remained in each filtrate after incubation with melanin was determined spectrophotometrically, using chloranil as a reagent (Rizk and Younis 1984). The amounts of kanamycin bound to melanin (calculated as difference between the initial amount of the drug added to melanin and the amount of unbound drug in the filtrate after incubation) are expressed in μmoles of bound drug per mg melanin. A qualitative analysis of kanamycin-melanin interaction was performed using the Scatchard plot of the experimental data according to Kalbitzer and Stehlik (1979). The number of binding sites (n) and the values of association constant (K) were calculated.

4.5. Fibroblast cultures

Normal human skin fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C under 5% CO_2 in an incubator. The cells were used between the 12th to 14th passages. The fibroblasts were subcultivated by trypsinization. Subconfluent cells from Costar flasks were detached with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in calcium-free phosphate-buffered saline (PBS). For the experiments, cells were counted in hemocytometers and cultured at 1×10^5 cells per well in 2 ml of growth medium in 6-well plates (Costar). Cells reached confluence at day 6th and in most cases such cells were used for the experiments. Confluent cells were treated for 24 h with the studied drug (kanamycin), melanin or both added to the growth medium.

4.6. Collagen production

Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells in growth medium with various concentrations of kanamycin, melanin or both for 24 h with 5[^{3}H]proline (5 μCi/ml, 28 Ci/mmol) as described by Oyamada et al. (1990). Incorporation of tracer into collagen was determined by digesting proteins with purified *C. histolyticum* collagenase according to the method of Peterkofsky et al. (1982). Results are shown as combined values for cell plus medium fractions.

4.7. DNA biosynthesis assay

To examine the effect of the substances on fibroblast proliferation, the cells were seeded in 24-well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth medium. After 48 h ($1.6 \pm 0.1 \times 10^5$ cells/well), the plates were incubated with various concentrations of melanin or kanamycin with or without melanin and 0.5 μCi of [^{3}H] thymidine for 24 h at 37 °C. Cells were rinsed 3 times with phosphate buffered saline (PBS), solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% sodium dodecyl sulfate (SDS), scintillation liquid (9 ml) was added and radioactivity incorporated into DNA was measured in a scintillation counter.

4.8. SDS-PAGE

Slab SDS/PAGE was used, according to the method of Laemmli (1970). Samples of cell supernatants (20 μg of protein) were electrophoresed. The following Bio-Rad's unstained high molecular weight standards were used: myosin (200 kDa), galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa).

4.9. Western blot analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transfered to 0.2 μm pore-sized nitrocellulose at 100 mA for 1 h using a LKB 2117 M

ultiphor II electrophoresis unit. The nitrocellulose was incubated with: anti-human insulin-like growth factor-I receptor at a concentration of 1 : 1,000 or monoclonal antibody against MAPK at a concentration of 1 : 5,000 in 5% dried milk in TBS-T (20 mmol/l Tris-HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20) for 1 h. In order to analyze insulin-like growth factor-I receptor alkaline phosphatase conjugated antibody against goat, IgG (whole molecule) was added at a concentration of 1 : 1,000, in order to analyze MAPK second antibody-alkaline phosphatase conjugated, against mouse IgG (whole molecule) was added at a concentration of 1 : 7,500 in TBS-T. Incubation was continued for 30 min shaking slowly. Then nitrocellulose was washed with TBS-T (5 × 5 min) and submitted to Sigma-Fast BCIP/NBT reagent.

4.10. Determination of protein

Protein concentration was measured by the method of Lowry et al. (1951).

4.11. Statistical analysis

In all experiments, the mean values for six experiments ± standard deviations (S.D.) were calculated, unless otherwise indicated.

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