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Effect of a Mitochondrial Peptide on Rat Liver Cell Cultures

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Abstract

We isolated the mitochondrial peptide fraction and hypothetizing that it could coordinate mitochondrial metabolism with the functions of other cellular compartments, we investigated the effect of this peptide fraction on the transmembrane potential generation in rat liver cells in cultures. We showed an increase of membrane potential with the mitochondrial peptide concentration of 10 ng/ml and 48 hours of incubation. These findings suggest effects of the peptide also on the membrane potential.

Keywords: mitochondrial peptide, rat hepatocytes, fluorescence, rhodamine 123
Abbreviations: Rho 123, rhodamine 123; DMEM, Eagle modified Dulbecco's medium.

1. Introduction

It is well known that the DNA of eucaryotic nuclei contains low molecular weight peptides[1]. Cohen et al.[2] hypothesized that these peptides act both as activators of protein transport through the mitochondrial membrane and as regulators of the nuclear transcription and cytoplasmic translation mechanisms in many tissues and in differentiated isolated cells[3]. As previously demonstrated the low molecular weight peptide fraction from calf liver mitochondrial DNA[4] exerts a regulatory role on the transcription and duplication activity of DNA. The same fraction also binds with mitochondrial DNA with a high affinity constant and stabilizes DNA against thermal denaturation[5]. Preliminary researches on rat hepatocytes cultures pointed out a positive effect of the peptide on cell viability both at long and short term[6-7]. Peptide fractions, which play a similar role have been isolated, purified and characterized from cromatin of calf thymus cells by other authors[8]. The aim of this research is to study the effect of the mitochondrial peptide fraction on the membrane potential and the mitochondrial role of ATP generation in liver cells.

2. Materials and methods

2.1 Isolation of hepatocytes

Primary liver cell cultures were obtained from 60 days old male rats, Wistar strain, according to the method of Seglen [9], by perfusion with collagenase type IVS in Hanks buffer solution (hepes 20 mM pH 7.4, added with EDTA 8mM). All previous procedures on the animals were performed according to the CEE directive n. 86/609 on animal experimentation. The viability of the obtained cells was at least 95% according to the trypan blue exclusion test[10]. The cells were plated in chamber slides (Nunc, Neperville, IL, USA) and incubated in Eagle modified Dulbecco's medium (DMEM) supplemented with 10% fetal calf serum, penicilline and streptomicine mixture (100 μg/ml), fungizone (2,5 μg/ml), PMSF (50 mg/L) and mitochondrial peptide at various concentrations at 37°C.
The peptide fraction from calf liver mitochondrial DNA was isolated using Drouin's method[11].

2.2 Study of membrane potential

According to Shapiro[12], membrane potential can be estimated from the distribution of lipophilic cationic indicators between cells and culture medium. The lipophilicity of the indicator enables dye molecules to pass freely through the membrane lipids; the concentration gradient of a lipophilic cationic dye across the membrane is determined by the transmembrane potential difference according to the Nernst equation. Once cells have been equilibrated with a cationic dye, a subsequent electrical depolarization of the cells will cause the release of the dye from cells into the medium, while a hyperpolarization will make the cells take up additional dye from the medium. The dye distribution will not adequately represent the new value of potential until equilibrium has again been reached. Among all lipophilic, cationic, fluorescent dyes tested thus far, the least toxic has been rhodamine 123 (Rho 123). It has a relatively stable yellowish-green fluorescence under blue excitation. The excitation spectrum of Rho 123 has a maximum at 500 nm; the maximum of the emission spectrum varies, depending on concentration. At low concentrations (10^{-8} M), the maximum is at 525 nm; at a higher concentrations (10^{-3} M), 545 nm. However, the adsorption spectrum doesn't change with dye concentration[13].

In the our study the Rho 123 is added at the cultures at the 50 ng/ml concentration before 4 hours of the analysis. The effects of mitochondrial peptide fraction at the 1, 5, 10, 20, 30, 50 ng/ml were studied. All chemicals were analytical grade from Sigma (St. Louis, MO, USA).

2.3 Fluorescence microscopy and image analysis

The membrane potential was studied according to the fluorescence microscopy method of Chen[14]. 4 hours before the observation, the Rho 123 at a concentration of 50 ng/ml was added to liver cell cultures. At the specified incubation times, the observations by fluorescence microscopy were performed. The chamber slide was removed from the incubator and the medium was removed to discard the Rho 123 which was not absorbed. To examine Rho 123, the filter system for fluoresceine was used. The objective was a Zeiss Plan-Neofluar 40X mounted on a Zeiss Axioskop microscope. To photograph the cells, the Kodak T-Max 400 ISO film was used with a 3200 ISO exposition index and Kodak T-Max RS developer at 2°C for 9.5 minutes. The negatives were acquired by the means of a Microtek 35t scanner and they were analysed with the Image 1.58 program (NIH Bethesda, USA) with the MacOS system. A gray scale of the photographs, observed in fluorescence, in standard conditions, was compared with the control pictures in the same conditions for the same series of experiments. The gray scale was converted into a linear 32 colors scale. For each color, an intensity value linearly variable between 1 and 255 was ascribed. The intracellular correspondent areas were obtained and their integrated density was divided by the correspondent area value. These values were referred to as relative percentages with respect to the reference tests, as a function of the mitochondrial peptide fraction concentrations.

3. Results

3.1 Effect of rhodamine 123 on membrane potential

The authors preliminarly determined the optimal concentration of Rho 123 fluorescent probe to study the membrane potential variations in rat liver cells in culture. In figure 1, relative percentage fluorescence values trends in isolated hepatocytes incubated for 2, 4 and 6 hours at 37°C with Rho 123 at 25, 50, 75 ng/ml are described. The final fluorescence of the probe, proportional to the transmembrane potential, is referred to data of the tests performed without dye at the same observation time points. The fluorescence data were almost constant beetwen 4 and 6 hours at the final concentration of 50 ng/ml (fig.1B). Therefore, for the following studies of characterization of hepatocytes capacity to generate transmembrane potential, this concentration was chosen for the 4 hours incubation period. Such time and concentration limits are obviously appropriate for our specific cellular type in the same conditions of culture medium. We also verified the diffusion of Rho 123 at -20°C in the same kind of preparations and with the same incubation buffer. Higher concentrations of the dye were tested but the viability trypan blue test demonstrated that the liver cells were almost completely poisoned at all the studied time points (data not shown).

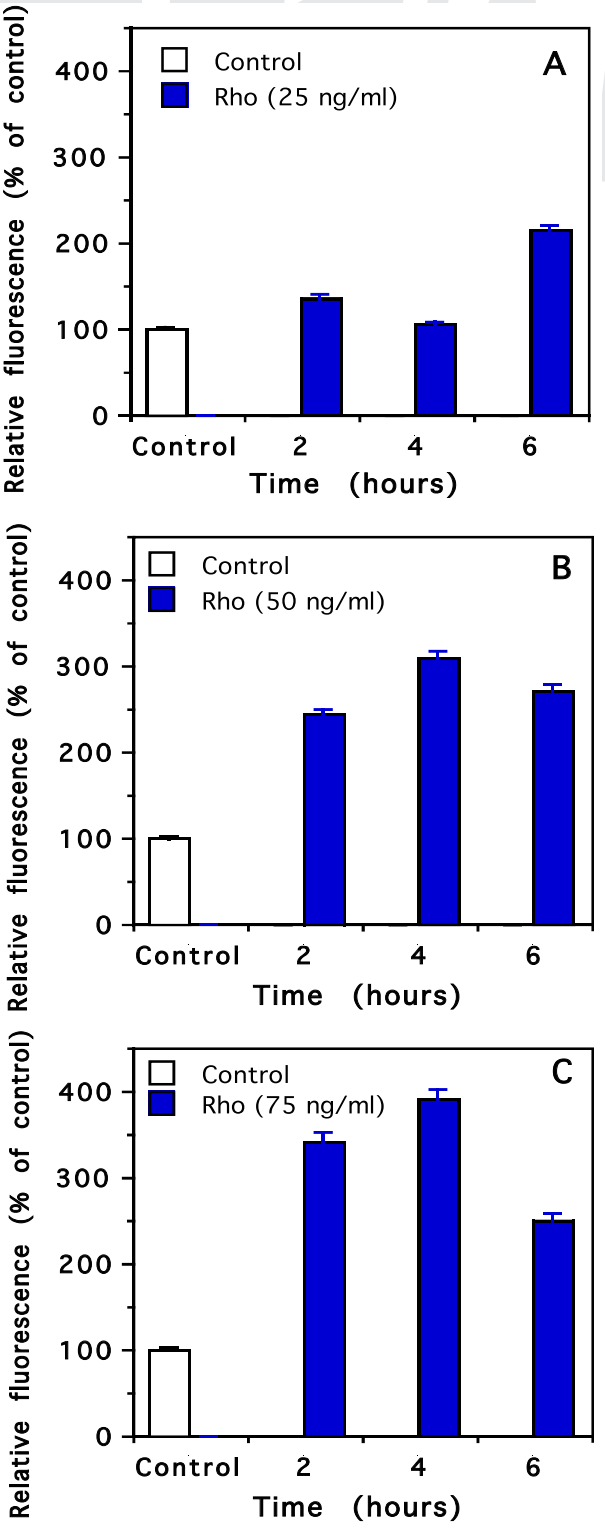

Fig. 1. Rhodamine 123 fluorescence expressed as relative % ratio of the integrated density of test cells and the same index of control ones, as a function of incubation time. A: cells treated with Rho 123, 25 ng/ml, final concentration. B: cells treated with Rho 123, 50 ng/ml, final concentration. C: cells treated with Rho 123, 75 ng/ml, final concentration. The cells were incubated with Rho 123 for 2, 4 and 6 hours at 37°C. All data are mean + S.E. (n=6)

3.2 Effect of mitochondrial peptide at short and medium term

In another series of experiments, to verify the action of the mitochondrial peptide fraction, the authors determined the

best culture time and peptide concentration to study the effects on membrane potential (fig. 2). The cultures incubated for 4 hours with the peptide fraction show that membrane potential increased at all the concentrations in comparison to controls and this was still more evident between 30 and 50 ng/ml.

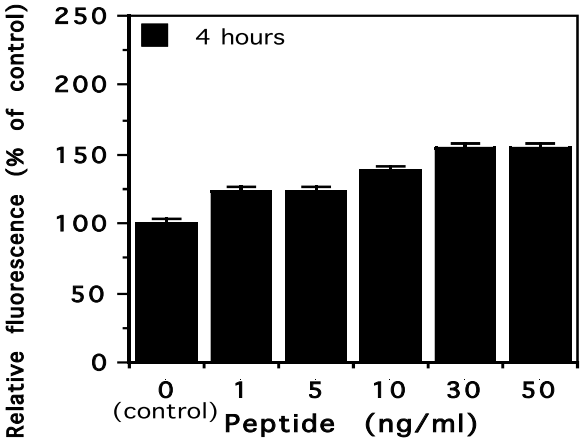

Fig. 2. Rhodamine 123 fluorescence expressed as relative % ratio of integrated density of test cells and the same index of control ones, as a function of peptide concentration expressed in ng/ml. The cells were incubated with peptide fraction and Rho 123 (50 ng/ml) for 4 hours at 37°C. All data are mean + S.E. (n=6)

After 24 hours of incubation (fig. 3), the peptide fraction had a significant effect at the concentration of 10 ng/ml. The comparison between the two experiments (fig. 2 and 3) demonstrated that the effect of the peptide fraction on membrane potential does not disappear at 24 hours, but it was more evident at the final concentration of 10 ng/ml. At short term, the potential variations were less evident but significant at all the studied concentrations.

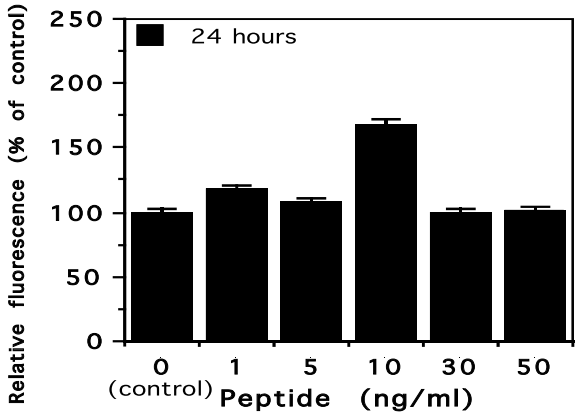

Fig. 3. Rhodamine 123 fluorescence expressed as relative % ratio of integrated density of test cells and the same index of control ones, as a function of peptide concentration expressed in ng/ml. The cells were incubated with the peptide fraction for 24 hours at 37°C. Fluorescence intensity was measured after 4 hours of incubation with 50 ng/ml Rho 123. All data are mean + S.E. (n=6)

3.3 Effect of mitochondrial peptide in hepatocytes incubated for 72 hours

The effect of the peptide fraction at the concentrations of 5, 10, 20 ng/ml for 72 hours of incubation was studied. In figure 4, the peptide fraction at all the studied concentrations caused, at short time, an increase of

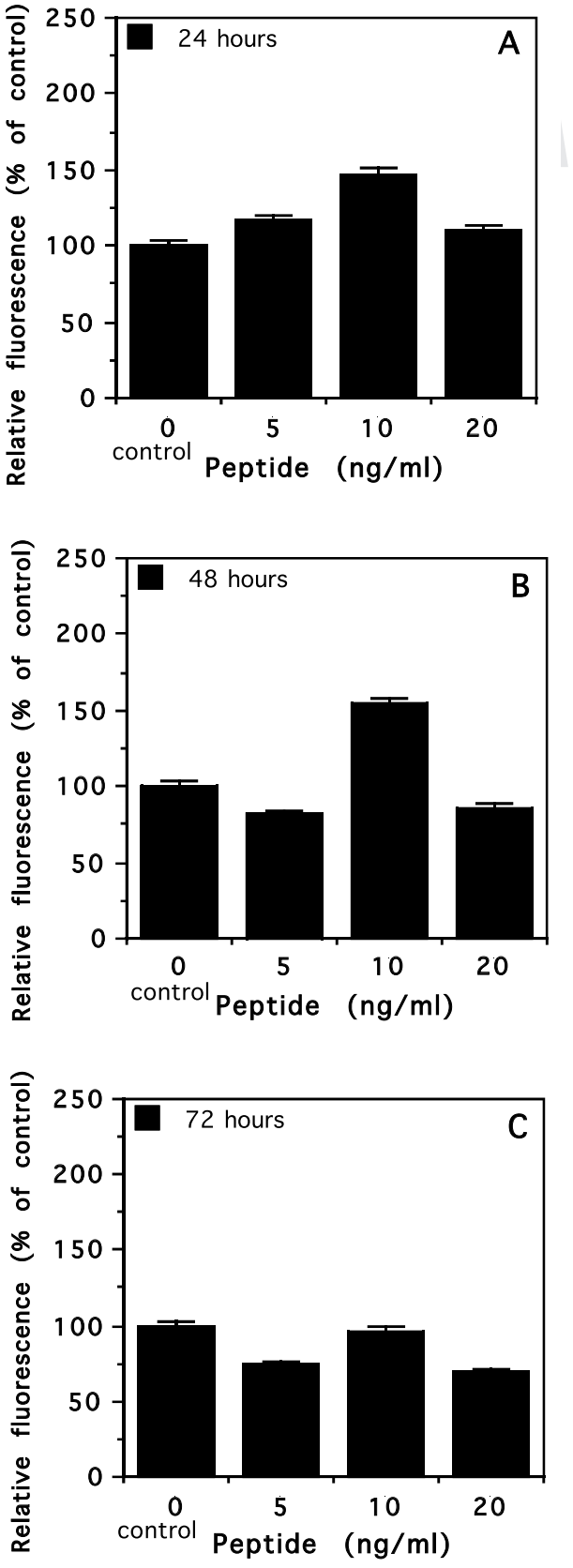

Fig. 4. Rhodamine 123 fluorescence expressed as relative % ratio of the integrated density of test cells and the same index of control ones, as a function of peptide concentration expressed in ng/ml. The cells were incubated with peptide fraction (5, 10, 20 ng/ml) for A: 24 hours; B: 48 hours; C: 72 hours at 37°C. Fluorescence intensity was measured after 4 hours incubation with 50 ng/ml Rho 123. All data are mean ± S.E. (n=6)

polarity with a maximum at 10 ng/ml and a decrease at 20 ng/ml. The relative values of membrane potential intensity at 10 ng/ml, were stronger at 24 and 48 than at 72 hours, but at 5 ng/ml at 48 and 72 hours, a decrease of polarity with respect to controls was observed. In figure 5, the Rho

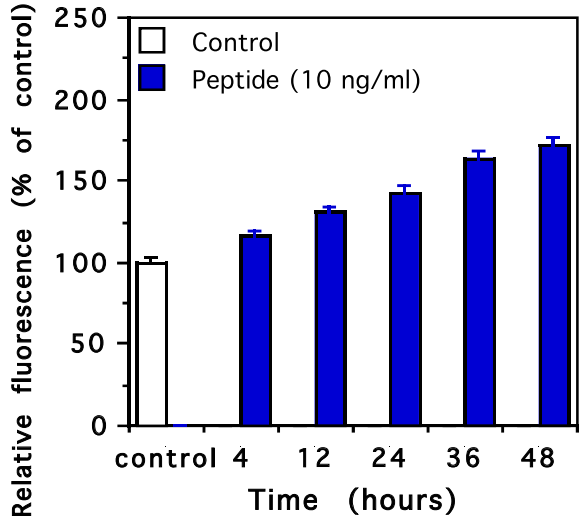

Fig. 5. Rhodamine 123 fluorescence expressed as relative % ratio of integrated density of test cells and the same index of control ones, as a function of incubation time. The cells were incubated with peptide fraction (10 ng/ml) for 4, 12, 24, 36 and 48 hours at 37°C. Fluorescence intensity was measured after 4 hours of incubation with 50 ng/ml Rho 123. All data are mean ± S.E. (n=6)

123 fluorescence relative intensity at 4, 12, 24, 36, 48 hours of incubation in rat liver cells treated with the mitochondrial peptide fraction at the concentration of 10 ng/ml are shown. The peptide always increases the hepatocytes potential with respect to controls. The same figure shows that this increase reaches a plateau between 36 and 48 hours, validating the previous data.

4. Discussion

As described in figure 1 the relative fluorescence % increased with increasing Rho 123 label concentrations, but only at 50 ng/ml was a plateau value obtained between 2 and 6 hours. According to data in figure 2, the optimal peptide concentration was between 30 and 50 ng/ml at 4 hours and 10 ng/ml at 24 hours, as described in figure 3. According to the described data, the authors studied only 5, 10, 20 ng/ml of the mitochondrial peptide, the effect at longer incubation times (fig. 4). At all the times studied, the optimal peptide concentration was 10 ng/ml, but the increase of membrane potential attained the maximum at 48 hours. At 72 hours all the concentrations of the peptide studied decreased membrane potential generation. In figure 5, the data show that at 10 ng/ml the plateau was reached between 36 and 48 hours. Thus, the hypothesis is that the peptide fraction effectively coordinates the plasma membrane function with mitochondrial energy supply. Therefore, the authors hypothesize that the mitochondrial peptide really has a physiological role in modulating the plasma active transport.

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