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The inhibitor protein of the F_1F_0-ATP synthase is associated to the external surface of endothelial cells ☆

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Abstract

The ATPase inhibitor protein (IP) of mitochondria was detected in the plasma membrane of living endothelial cells by flow cytometry, competition assays, and confocal microscopy of cells exposed to IP antibodies. The plasma membranes of endothelial cells also possess β-subunits of the mitochondrial ATPase. Plasma membranes have the capacity to bind exogenous IP. TNF-α decreases the level of β-subunits and increases the amount of IP, indicating that the ratio of IP to β-subunit exhibits significant variations. Therefore, it is probable that the function of IP in the plasma membrane of endothelial cells is not limited to regulation of catalysis.

Keywords: ATP synthase; Inhibitor protein; Endothelial cells; TNF-α; Plasma membrane

The F_1F_0-ATP synthase of energy transducing membranes catalyzes the synthesis of ATP with the energy that derives from electron transfer in the respiratory or photosynthetic electron transport chains. F_1F_0 is a highly efficient rotary motor that couples proton conduction through F_0 to ATP synthesis in the F_1 moiety. The enzyme also has a central stalk that acts as a rotor and a peripheral stalk which is part of the stator (reviewed in [1,2]). Although F_1F_0 has been found in all energy transducing membranes, in the last years several reports have shown that some of its subunits localize in the plasma membrane of neoplastic [3], endothelial [4], and hepatic cells [5]. Moreover, it has been proposed that the enzyme may be a receptor for ligands such as angiostatin in endothelial cells [4,6]. Along the same line, it has been suggested that the activity of F_1F_0 in the plasma membrane contributes to regulation of signal transduction [7,8].

The basic rotor and stator of F_1F_0 consist of at least eight different subunits, five belong to F_1 (α, β, γ, δ, and ε), and three to F_0 (a, b, and c). In eukaryotic F_1F_0, there are at least eight additional subunits, most of them in F_0 [9,10]. It has not been established if all of the subunits of F_1F_0 are present in the plasma membrane. In mitochondria the function of F_1F_0 is regulated by a

☆ *Abbreviations:* BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; IP, the inhibitor protein of ATP synthase; PBS, phosphate-buffered saline; PE, phycoerythrin.

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protein of relatively low molecular weight which was originally described by Pullman and Monroy [11] as the natural ATPase inhibitor protein. The latter (here called "IP") inhibits synthesis and hydrolysis of ATP by interacting with α, β [12,13], γ, and ε subunits of F_1 [14,15], and thus, it probably blocks the rotatory mechanisms involved in catalysis [14,16].

Recently, it was shown that the plasma membrane of endothelial cells possesses IP [17]; on the basis of activity measurements, it was put forth that it regulates the catalytic activity of plasma membrane F_1F_0 [17]. In this work, we confirm the existence of IP in the plasma membrane of human umbilical vein endothelial cells (HUVECs). In addition, and because in mitochondria the regulation of the catalytic activity of F_1F_0 requires a stoichiometry of one IP per enzyme [13,18], we also explored if under different conditions, the ratio of IP to β-subunits is maintained constant, or whether it undergoes variations. A constant ratio would indicate that indeed IP regulates catalysis by F_1F_0 in the plasma membrane; otherwise, the data could suggest that IP exerts other functions different to regulation of catalysis.

Materials and methods

Cell culture, antibody production, and Western blotting. HUVECs were obtained from human umbilical cords by trypsin dissociation as previously described [19] and cultured as in [19] up to the third passage before use. Rabbit polyclonal antibodies raised against β-subunit and IP [20] were used at the indicated dilutions. Mouse monoclonal antibodies against bovine IP were used as described previously [21]. FITC- or PE-conjugated secondary antibodies against mouse or rabbit were obtained from Molecular Probes and used as indicated by the manufacturer. Western blot analyses were carried out described elsewhere [21].

Flow cytometry. HUVECs attached to culture plates were washed twice with PBS (containing $Ca^{2+} + Mg^{2+}$, 0.1 g/L each) to eliminate culture medium and incubated for 30 min with the indicated primary antibodies or pre-immune serum. Cells were washed twice with PBS Ca^{2+}/Mg^{2+} and incubated in the dark for 30 min with a FITC- or PE-conjugated secondary antibody, as indicated. All antibody incubations were carried out at 4 °C in PBS Ca^{2+}/Mg^{2+} with 2.5% bovine serum albumin (BSA, Sigma, fatty acid free). After washing twice with PBS, cells were detached from plates with ice-cold PBS containing 5 mM EDTA and pelleted. The cells were suspended in ice-cold PBS and the antibody-associated fluorescence was determined in a FACScalibur cytometer (using CellQuest software). Where indicated, prior to their incubation with primary antibodies, cells were fixed for 15 min in PBS that had 2% paraformaldehyde; thereafter, these cells were incubated with the indicated antibodies as described above.

Confocal imaging. HUVECs were plated in endothelial cell growth medium on glass coverslips. After 36 h of growth, cells were incubated with 250 nM Mitotracker Red (Molecular Probes) for 30 min at 37 °C, washed and incubated with antibodies as described for the flow cytometry experiments. They were analyzed by MRC-1024 confocal microscopy (Bio-Rad). Where indicated, prior to exposure to antibodies, the cells were fixed by incubation for 15 min in PBS with 2% paraformaldehyde or permeabilized in 100% ethanol for 5 min at room temperature before fixation.

Protein purification. The F_1-ATPase [22] and the F_1-IP complex (F_1 containing its endogenous inhibitor protein) were purified as described elsewhere [23]. Soluble IP was purified as described by [24], except that a Mono S column equilibrated with 50 mM sodium acetate, 0.001% PMSF and EDTA 1 mM (pH 5.0) was used instead of a Mono Q column. Elution was performed with a linear NaCl gradient.

Results and discussion

Intact HUVECs incubated with polyclonal and monoclonal antibodies against IP were analyzed by flow cytometry. The distribution of fluorescence intensities obtained after incubation with IP antibodies showed a significant increase relative to that observed when only the secondary antibody was used (Fig. 1). The fluorescence curves obtained with a monoclonal antibody specific for mitochondrial succinate dehydrogenase (SD70) were almost identical to those in which only secondary

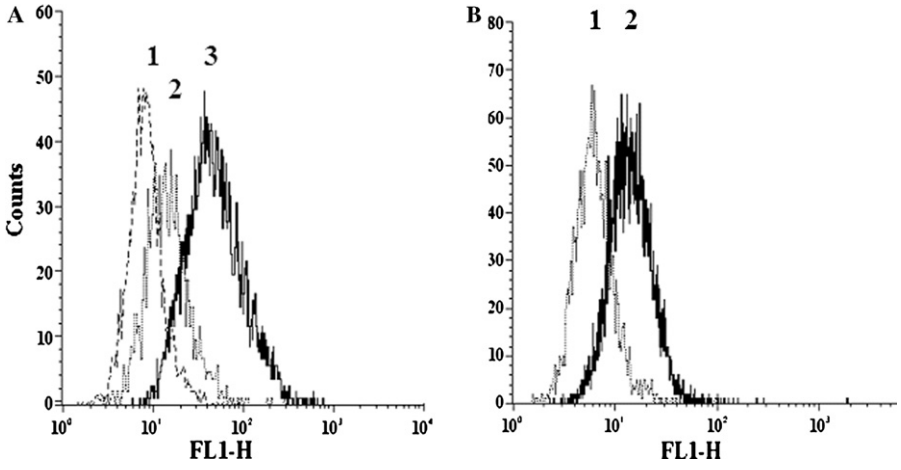

Fig. 1. Binding of antibodies directed against the inhibitor protein to the surface of intact HUVECs. Histograms of flow cytometry data. (A) Trace 1 shows cells incubated with pre-immune serum; trace 2 cells incubated with only the secondary antibody; and trace 3 cells incubated with polyclonal anti-IP antibodies (139 μg/ml). (B) Trace 1 depicts cells incubated only with secondary antibody; trace 2 was obtained after preincubating the cells with monoclonal anti-IP antibody (50 μg/ml). Figure made with FCSPress.