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Mitochondrial Inhibitory Factor Protein 1 Functions as an Endogenous Inhibitor for Coupling Factor 6

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ABSTRACT

Coupling factor 6 (CF6) forces a counter-clockwise rotation of plasma membrane F_1F_o complex unlike a proton-mediated clockwise rotation in the mitochondria, resulting in ATP hydrolysis, proton import, and apoptosis. Inhibitory peptide 1 (IF1) inhibits a unidirectional counter-clockwise rotation of F_1F_o complex without affecting ATP synthesis by a clockwise rotation. We tested the hypothesis that IF1 may antagonize the biological action of CF6 in human embryonic kidney 293 cells. We generated mature and immature IF1 expression vectors and those labeled with GFP at the C-terminus. In the immature IF1-GFP overexpressing cells, the mitochondrial network of IF1-GFP was newly found at the plasma membrane after peripheral translocation, whereas in mature IF1-GFP transfected cells, a less punctuate rather homogenous pattern was found in the cytoplasm. IF1 protein was detected in the exosome fraction of culture media, and it was enhanced by mature or immature IF1 transfection. Extracellular ATP hydrolysis was enhanced by CF6, whereas immature or mature IF1 transfection suppressed ATP hydrolysis in response to CF6. Intracellular pH was decreased by CF6 but was unchanged after immature IF1 transfection. CF6-induced increase in apoptotic cells was blocked by immature or mature IF1, being accompanied by protein kinase B (PKB) phosphorylation. IF1 antagonizes the pro-apoptotic action of CF6 by relief of intracellular acidification and resultant phosphorylation of PKB. Given the widespread biological actions of CF6, the physiological and pathological functions of IF1 may be expected to be complex. J. Cell. Biochem. 117: 1680–1687, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: COUPLING FACTOR 6; INHIBITORY PEPTIDE 1; ECTO-F_1F_o COMPLEX; ATP; APOPTOSIS; INTRACELLULAR ACIDOSIS

In ATP synthase (F_1F_o complex), the molecular rotary motor F_1-ATPase rotates in a counterclockwise direction to hydrolyze ATP [Yoshida et al., 2001], whereas its partner motor F_o, which is embedded in an inner membrane, rotates in a clockwise direction by a proton flux. In the mitochondria, F_o forces the backward (clockwise) rotation of F_1, resulting in ATP synthesis [Itoh et al., 2004]. In the plasma membrane, F_1 can hydrolyze ATP and inversely rotate F_o to pump protons in the opposite direction when the free energy of ATP hydrolysis is large in the extracellular space, leading to intracellular acidosis [Osanai et al., 2005]. The whole F_1F_o-ATP synthase complex localizes on the outer surface of the plasma membrane in hepatocytes, and is shown to catalyze both ATP synthesis and reverse ATP hydrolysis [Mangiullo et al., 2008]. Under conditions of acidosis induced by glycolysis, hepatocytes synthesize extracellular ATP in the presence of ADP and inorganic phosphate. The plasma membrane ATP synthase (ecto-F_1F_o complex) stimulation by apoprotein A-I triggers the endocytosis of high density lipoprotein particle by a mechanism that depends on the generation of ADP [Martinez et al., 2003]. In endothelial cells, ATP synthesis activity is detected on the cell surface after addition of ADP and inorganic phosphate [Arakaki et al., 2003], and the interaction of β-subunit of F_1 motor with angiostatin attenuates ATP generation and angiogenesis [Moser et al., 1999, 2001]. In C6 glioma cells, the whole respiratory chain localizes on the cell surface, and is involved in ATP synthesis coupled to oxygen consumption in isolated plasma membrane [Ravera et al., 2011].

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We recently identified a circulating peptide coupling factor 6 (CF6) as a novel ligand for ecto-F_1F_o complex, and after binding to protrusive F_1, CF6 forces the backward rotation of F_o, thereby stimulating proton import at the plasma membrane [Osanai et al., 2005]. Protons regulate cellular function by modulating the charge and structure of macromolecules, and CF6 has been disclosed to exert a widespread action of vascular biology. CF6 suppresses the synthesis of prostacyclin in vascular endothelial cells via inhibition of cytosolic phospholipase A_2, and injected CF6 induces an increase in arterial blood pressure in rats [Osanai et al., 1998, 2001]. In CF6-overexpressing transgenic (TG) mice, we showed that a high salt intake induces cardiac systolic dysfunction and nicotinamide adenine dinucleotide phosphate oxidase upregulation [Ashitate et al., 2010]. We further showed that CF6 attenuates prostacyclin generation via inhibition of cytosolic phospholipase A_2 [Osanai et al., 1998] and nitric oxide (NO) generation via upregulation of asymmetric dimethylarginine or inhibition of endothelial NO synthase phosphorylation [Tanaka et al., 2006; Kumagai et al., 2008]. CF6 also elevates arterial blood pressure and enhances angiotensin II-induced vasoconstriction in resistance arterioles [Osanai et al., 2001, 2009]. In the clinical settings, we and others showed that circulating CF6 is elevated in patients with hypertension, acute myocardial infarction, end-stage renal disease, stroke, and diabetes [Osanai et al., 2003a,b, 2010; Ding et al., 2004; Chai et al., 2007; Li et al., 2007], all of which predisposed to the progression of atherosclerosis.

Since the plasma level of CF6 is elevated in various states, it should be emphasized that the antagonizing action against CF6 may be important and useful to prevent atherosclerosis and its related diseases in humans. ATP synthase has a number of discrete inhibitor binding sites including peptides and other inhibitors, and recently its enzyme is thought to be a molecular therapeutic target for antimicrobial and antitumor peptides, and dietary polyphenols [Ahmad and Laughlin, 2010; Laughlin and Ahmad, 2010; Ahmad et al., 2013]. Asp residues of βDELSEED-motif are required for peptide binding and inhibition in ATP synthase [Ahmad et al., 2015]. Inhibition of counterclockwise rotation is available to antagonize the CF6 action, whereas suppression of clockwise rotation disturbs ATP generation. Unidirectional inhibitory peptide for ATP synthesis was reported previously and it was called as mitochondrial inhibitory factor peptide 1 (IF1) [Pullman and Monroy, 1963]. IF1 is a small basic protein found in the matrix of mitochondria, and inhibits hydrolysis of ATP in mitochondrial F_1-ATPase. It is intriguing that this protein has no ability to inhibit ATP generation in the presence of a proton motive force [Runswick et al., 2013]. Hence, IF1 is a unidirectional inhibitor of ATP hydrolysis only. Given the antagonizing action against CF6, IF1 may inhibit the onset and progression of cardiovascular disease and atherosclerosis. In the present study, we investigated whether IF1 functions as an endogenous inhibitor for CF6.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

Human embryonic kidney (HEK)-293 cells (HPA Culture Collections, catalogue No. 85120602) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. The cells from the 4th to 7th passages were used for the study. The transfection of DNA was carried out using Lipofectamine 3000 Transfection Kit (Invitrogen™ L3000-015), according to the manufactures' protocol. To over-express IF1 in HEK-293 cells, the cells were transfected with mature and immature IF1 vectors, and those labeled with green fluorescent protein (GFP). After treatment with various molecules, the viability of the cells, which was determined by trypan blue excretion, was generally >95%.

MATURE IF1-pcDNA3 AND IF1-pAcGFP1-C3 PLASMID CONSTRUCTIONS

The full sequence of mature IF1 gene carrying *Hind III* and *EcoRI* was synthesized by polymerase chain reaction (PCR) using pCMV-SPORT6 (immature IF1 vector, Open Biosystems, Invitrogen™ MHS1010-73732) as the template; forward oligomer: 5′-CCCAAGC TTATGGGCTCGGATCAGTCCGAGAAT-3′, reverse oligomer: 5′-GC GAATTCTTAATCATCATGTTTTAGCAT-3′. Mature IF1 gene and pcDNA3 vector were digested by *Hind III* and *EcoRI* restriction enzymes. After isolation and purification, they were ligated with T4 DNA ligase (Promega).

The full sequence of mature or immature IF1 genes carrying *Bam*H I and *Xho* I sites, which were synthetized by PCR, were inserted into pAcGFP1-C3 vector (Clontech Laboratories, Inc, Mountain View, CA) to construct IF1-pAcGFP1-C3. IF1 gene and pAcGFP1-C3 vector were digested by *Bam*H I and *Xho* I restriction enzyme, and then ligated with T4 DNA ligase. Ligation products were transformed to *Escherichia coli*, and then spread on 2×YT medium culture plate with kanamycin, which were cultured at 37°C overnight. Positive clones were picked up and screened by PCR, identified via digesting by *Bam*H I and *Xho* I restriction enzyme and sequencing the inserted fragments.

FLUORESCENT MICROSCOPY

Cell morphology was imaged using GFP targeted to mature and immature IF1. We generated mature and immature IF1 expression vectors, which are labeled with GFP at the C-terminus. The transfection of DNA was carried out using Lipofectamine 3000 Transfection Kit (Invitrogen™ L3000-015), according to the manufactures' protocol. Images were acquired using a BZ-X710 Fluorescence microscope with a oil immersion objective lens (Keyence, Osaka, Japan). IF1-GFP was excited with a 488 nm wavelength laser. Laser power was kept as low as possible (0.1–2%), to avoid bleaching of the signal. In order to control for observer bias, the identity of the transfected cells was blinded and analysis was carried out twice, with an average for each cell taken.

ATP CONTENT ANALYSIS

The ATP concentration in the culture medium was determined by the bioluminescent procedure according to the protocol supplied with the ATP bioluminescent somatic cell assay kit (Sigma Chemical, St. Louis, MO). Briefly, the culture medium was swirled with ATP releasing reagent, mixed working solution, and then the amount of light emitted with a luminometer was measured and expressed as RLU (relative light unit).

WESTERN BLOT ANALYSIS

The cells were harvested, pelleted, and treated for 30 min at 50°C in a sample-treating solution containing 3% sodium dodecyl sulfate (SDS) and 7.5% β-mercaptoethanol. Protein was applied to SDS-polyacrylamide gel electrophoresis (4–20% gradient gel) and transferred to a polyvinylidene difluoride membrane. After blocking for 1 h, the membranes were incubated with each primary antibody at 4°C overnight and followed by 1 h incubation with the secondary antibody of goat anti-mouse IgG for α- and β-subunits of ATP synthase, PKB, and phosphorylated PKB, and that of goat anti-rabbit IgG for glyceraldehyde 3-phosphate dehydrogenase. Immunoreactive bands were detected by ECL plus detection system. Densitometric analysis was performed with NIH image software.

MEASUREMENT OF INTRACELLULAR pH

HEK-293 cells were harvested and resuspended in phosphate buffered saline (PBS) containing BCECF (2′-7′-bis(carboxyethyl)-5(6)-carboxyfluorescein)-AM (1 mg/ml, Molecular Probes, Eugene, OR), and then incubated for 30 min at 37°C. Excess dye was removed by centrifugation and resuspension of the cells in nominally HCO_3^--free Krebs-Henseleit buffered with 20 mM Hepes containing 0.2% fatty acid-free BSA. The suspension was transferred to a cuvette that was placed in the temperature-controlled chamber of a Shimazu RF-5000 luminescence spectrophotometer, maintained at 37°C. Using a dual excitation fast filter accessory, the sample was excited at 495 nm and 440 nm successively, and the fluorescence emission was measured at 535 nm. The ratio of the fluorescence intensity measured using the 2 excitation wavelengths (495/440 nm) provides a quantitative measure of pH.

ANNEXIN V AND PROPIDIUM IODIDE (PI) ASSAY FOR APOPTOSIS

Flow cytometric analysis with a detection apoptosis kit was used for assessment of apoptotic cell. Briefly, the cells were trypsinized, washed with cold PBS twice, and incubated in the binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) with annexin-V-FITC and PI for 10 min on ice in the dark. The sample was analyzed using a dual-laser fluorescence activated cell sorting VantageSE flow cytometer (Becton Dickinson, Mountain View, CA) within a 1 h period. The percentage of apoptotic cells for each sample was estimated.

STATISTICAL ANALYSIS AND ETHICAL CONSIDERATIONS

Results were expressed as mean ± SEM. Differences between groups were examined for statistical significance using analysis of variance with Bonferroni's test.

RESULTS

SUBCELLULAR LOCALIZATION AND RELEASE OF MATURE AND IMMATURE IF1

In order to explore whether mature or immature IF1 may antagonize the action of CF6, we first examined the subcellular localization of the fusion protein IF1-GFP after transient transfection. In the case of immature IF1, the cleavage of the N-terminus 25 amino acids forms mature IF1 that is selectively localized to the mitochondria. Hence, we used mature and immature IF1-GFP distribution within the cell to estimate the relative amount of mitochondrial, cytoplasmic, and plasma membrane-bound IF1.

As shown in Figure 1A, in the immature IF1-GFP overexpressing cells, a dense widely distributed mitochondrial network was observed around the nucleus 24 h after transfection. Conversely, when the mitochondrial network was unstained in mature IF1-GFP transfected cells because of no mitochondrial import signal, a less punctuate rather homogenous pattern implying a lower level of mitochondrial IF1-GFP was found in the cytoplasm. In the immature IF1-GFP overexpressing cells, the

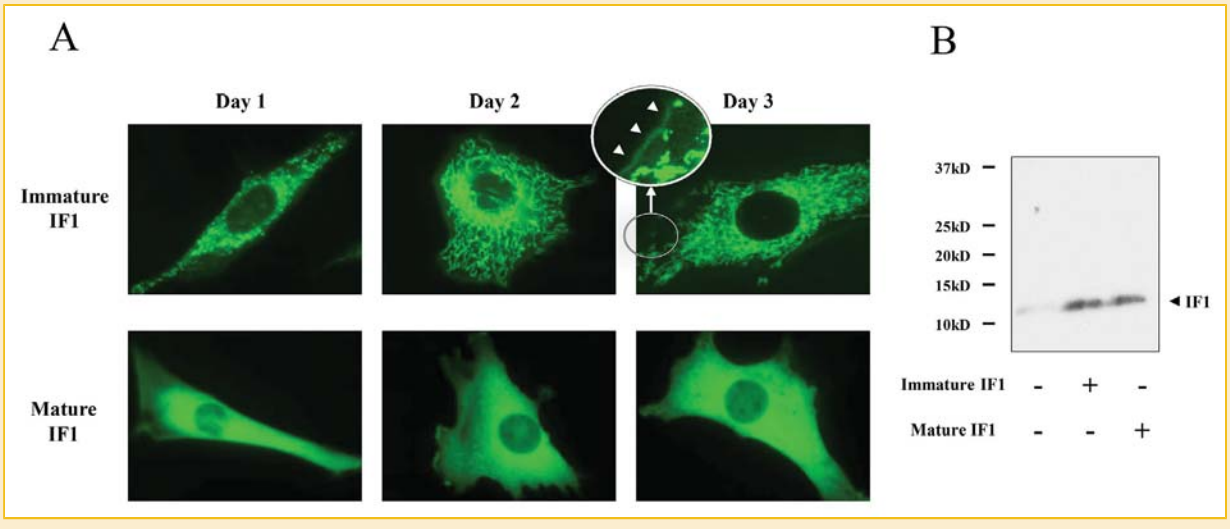

Fig. 1. Representative pictures of subcellular localization of mature or immature IF1 and representative bands of released IF1. A: Chronological changes in IF1-GFP distribution in HEK-293 cells expressing mature or immature IF1 labeled with GFP at C-terminus. Arrows show that the mitochondrial network of IF1-GFP was newly found at the plasma membrane in the immature IF1-GFP overexpressing cells. B: Released IF1 protein in the exosome fraction of culture media.

newly formed plasma membrane-bound IF1-GFP was created by the peripheral translocation at 48–72 h, showing a punctate pattern (arrows).

Figure 1B illustrates the representative bands for IF1 protein in the culture media 24–48 h after transfection of mature or immature IF1. The immunoreactive band for IF1 protein was detected in the exosome fraction of the cells after transfection of both mature and immature IF1, suggesting that IF1 is released through the mitochondria where immature IF1 is carried by N-terminal sorting signal or through the cytoplasm where mature IF1 is directly expressed.

EFFECT OF IF1 ON CF6-INDUCED DECREASE IN ATP AND INTRACELLULAR pH

CF6 converts the rotation of plasma membrane bound ATP synthase, ecto-F_1F_o complex, from a clockwise mode of proton export to a counter-clockwise mode of proton import [Itoh et al., 2004], resulting in the increase in intracellular protons only through ecto-F_1F_o complex but not through pumping from the intermembrane space with proton gradient, and the decrease in extracellular ATP. As shown in Figure 2A, ATP concentration in the culture media was unchanged until 60 min, and then declined at 300 min irrespectively of the presence or absence of CF6 and/or immature IF1. When the levels of extracellular ATP at 300 min were analyzed in more details, the declination of extracellular ATP was enhanced by CF6, whereas it was abolished by the overexpression of immature IF1 but rather enhanced compared with baseline (Fig. 2B), or by mature IF1 without enhancement compared with baseline (Fig. 2C). Consistent with the data of extracellular ATP, intracellular pH showed a slight but sustained decrease after addition of CF6 at 10^{-7} M in HEK-293 cells, whereas the transfection of immature IF1 blocked it (Fig. 2D).

EFFECT OF IF1 ON CF6-INDUCED APOPTOSIS IN HEK293

As shown in Figure 3A and B, the percentage of apoptotic cells was slightly but significantly increased from $4.5 \pm 1.2\%$ to $6.4 \pm 1.3\%$ by CF6 at 10^{-7} M for 24 h ($P < 0.05$). CF6-induced increase in apoptotic cells was blocked by transfection of immature or mature IF1.

MOLECULAR MECHANISM FOR THE ANTI-APOPTOTIC ACTION OF IF1

We examined the CF6 receptor expression level in HEK-293 cells after chronic exposure to CF6 and IF1 transfection. As shown in Figure 4A, the protein expression of CF6 receptor α and β subunits of ATP synthase was unaffected by CF6 at 10^{-7} M and IF1 transfection.

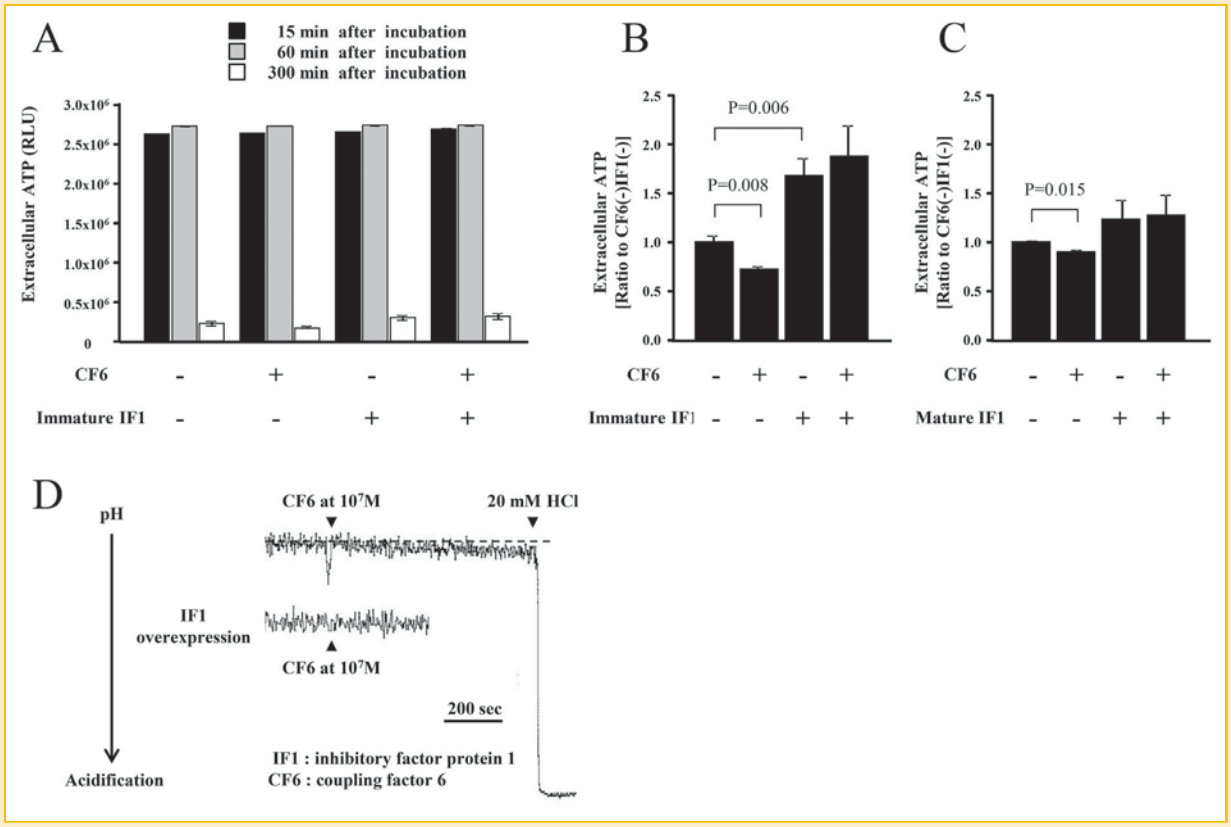

Fig. 2. Antagonizing effect of IF1 on CF6-induced decrease in extracellular ATP and intracellular pH. A: Chronological changes in extracellular ATP concentration with or without CF6 at 10^{-7} M in HEK-293 cells overexpressing immature IF1 ($n = 3$). RLU: relative light unit. B: Extracellular ATP at 300 min after the addition of CF6 at 10^{-7} M in HEK-293 cells overexpressing immature IF1. Ratio to extracellular ATP in the absence of CF6 and IF1 transfection ($n = 6$). C: Extracellular ATP at 300 min after the addition of CF6 at 10^{-7} M in HEK-293 cells overexpressing mature IF1. Ratio to extracellular ATP in the absence of CF6 and IF1 transfetion ($n = 6$). D: Intracellular pH after the addition of CF6 at 10^{-7} M in HEK-293 cells with or without transfection of immature IF1.

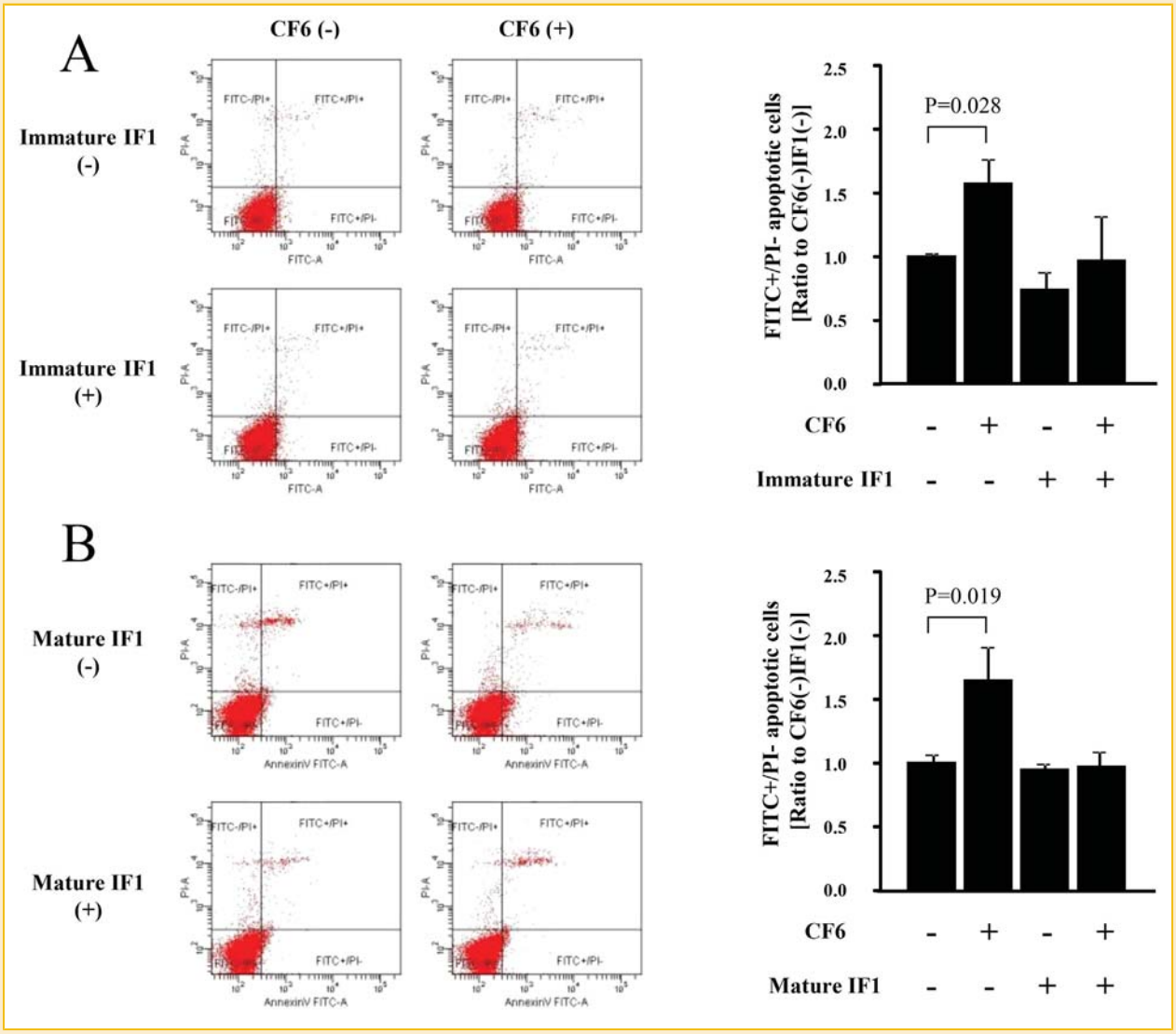

Fig. 3. Flow cytometric analysis of apoptosis with annexin-V-FITC and propidium iodide (PI) label. A: Left panel: Representative charts with or without CF6 at 10^{-7} M and transfection of immature IF1 in HEK-293 cells, Right panel: Ratio to the percentage of apoptotic cells in the absence of CF6 and IF1 transfection. (n = 6). B: Left panel: Representative charts with or without CF6 at 10^{-7} M and transfection of mature IF1 in HEK-293 cells, Right panel: Ratio to the percentage of apoptotic cells in the absence of CF6 and IF1 transfection. (n = 6).

CXC chemokine receptor 4 (CXCR4) activation by the chemokine CXCL12 (stromal cell-derived factor-1, SDF-1) could lead to the activation of anti-apoptotic signaling pathways; $G_{i\alpha}$ protein-dependent activation of PKB and extracellular signal-regulated protein kinase (ERK) [Vlahakis et al., 2002]. Since we showed that CF6 induces apoptosis by suppression of CXCR4 signaling in endothelial cells [Suzuki et al., 2014], we focused on one of the anti-apoptotic signaling PKB in the presence or absence of IF1. Figure 4B illustrates the representative bands for total and phosphorylated PKB in HEK-293 cells transfected with or without IF1. Phosphorylated PKB was decreased by CF6 at 10^{-7} M for 24 h as previously reported in endothelial cells. Transfection of mature or immature IF1 canceled CF6-induced attenuation of phosphorylated PKB without affecting the amount of total PKB. The ratio of phosphorylated PKB to total PKB was decreased by 52 ± 13% by CF6 at 10^{-7} M ($P < 0.05$), but was unchanged by CF6 under mature and immature IF1 transfection.

DISCUSSION

The major findings of this study were as follows. IF1 is released and detected in the exosome fraction of the cells after transfection of both mature and immature IF1. Transfection of mature and immature IF1 canceled CF6-induced decrease in extracellular ATP and increase in intracellular protons. The percentage of apoptotic cells was increased by CF6, and it was blocked by the transfection of mature and immature IF1, being accompanied by PKB phosphorylation.

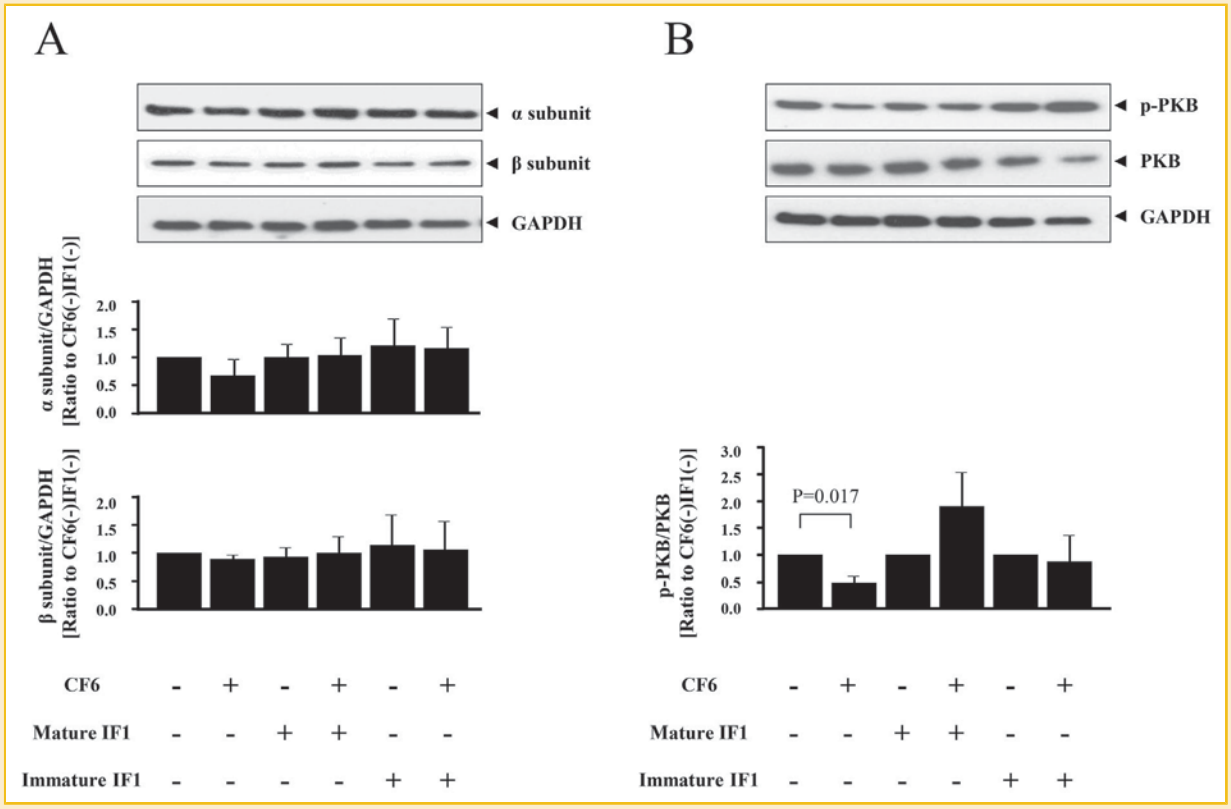

Fig. 4. Protein expression of CF6 receptor α and β subunits of ATP synthase and anti-apoptotic phosphorylated protein kinase B (PKB). A: Representative bands for CF6 receptor α and β subunits of ATP synthase in HEK-293 cells and the ratio to each protein in the absence of CF6 at 10^{-7} M and IF1 transfection (n = 3). B: Representative bands for total and phosphorylated PKB in HEK-293 cells and the ratio of phosphorylated to total PKB in the presence or absence of CF6 at 10^{-7} M and IF1 transfection (n = 3).

SUBCELLULAR LOCALIZATION OF IF1 AND ANTAGONIZING EFFECT ON INTRACELLULAR pH

The function of IF1 is well characterized in the mitochondria. When mitochondria lose proton motive force, the interior of the mitochondria becomes acidic and IF1 blocks wasteful ATP consumption by binding to ATP synthase [Hashimoto et al., 1990]. IF1 is abundantly expressed in human cancer cells, and the overexpression of IF1 inhibits the ATP hydrolysis activity of the mitochondrial F_1F_o complex. Thus, it is likely that IF1 plays a critical role in the metabolic shift from oxidative phosphorylation to enhanced aerobic glycolysis [Sanchez-Cenizo et al., 2010; Domenis et al., 2011]. Recently, IF1 was found in human serum and was proposed to contribute to cholesterol metabolism [Giorgio et al., 2010; Genoux et al., 2011], especially HDL-cholesterol. IF1 is present in the systemic circulation and functions as a unidirectional inhibitor of ATP hydrolysis, whereas CF6 is present in the systemic circulation and forces the unidirectional backward rotation of F_o, ATP hydrolysis [Osanai et al., 2005]. Thus, it is likely that IF1 antagonizes the action of circulating CF6 without affecting the mitochondrial ATP production. In the present study, we tested the hypothesis that IF1 may be an endogenous inhibitor for circulating CF6.

To investigate whether CF6-induced stimulation of ecto-F_1F_o complex is attenuated by IF1, we generated the expression vectors of mature and immature IF1 and those labeled with GFP at the C-terminus. We explored the subcellular localization of mature and immature IF1-GFP, and found that the newly formed plasma membrane bound IF1-GFP was present at 48–72 h with the peripheral translocation in the immature IF1 overexpressing cells. Furthermore, IF1 protein was detected in the exosome fraction of the cells after transfection of mature and immature IF1. These findings suggest that IF1 is released outside of the cells and may antagonize the action of circulating CF6.

To indicate the direct evidence that IF1 antagonizes the action of CF6, we analyzed the relationship between ATP consumption and proton import in the cells overexpressing mature or immature IF1. The result showed that ATP concentration in the culture media was decreased by CF6, but it was abolished after transfection of mature or immature IF1. Corresponding to the ATP data, which suggest the reduced backward rotation of F_1, the decrease in intracellular pH in response to CF6 was blocked in the cells after transfection of immature IF1. Thus, IF1 could antagonize the action of circulating CF6 at the site of plasma membrane bound F_1F_o complex.

We previously showed that CF6 attenuates prostacyclin generation via inhibition of cytosolic phospholipase A_2 [Osanai et al., 1998] and NO generation via upregulation of asymmetric dimethylarginine or inhibition of endothelial NO synthase phosphorylation [Tanaka et al., 2006; Kumagai et al., 2008]. CF6 elevates arterial

blood pressure and enhances angiotensin II-induced vasoconstriction in resistance arterioles [Osanai et al., 2001, 2009], and induces apoptosis of vascular endothelial cells through the decrease in CXCR4-SDF-1 axis [Suzuki et al., 2014]. We further demonstrated that in TG mice, intracellular pH measured by ^{31}P-MRS was reduced in the skeletal muscle and the liver, and the phenotype of TG mice showed salt-sensitive hypertension via activation of Rac1, and type 2 diabetes via suppression of insulin signaling [Izumiyama et al., 2012; Osanai et al., 2012]. Additionally, the apoptotic cells in the vessel wall were greater in TG mice compared with wild type mice [Suzuki et al., 2014], suggesting that IF1 may antagonize a number of biological actions of CF6.

ANTI-APOPTOTIC EFFECT OF IF1

Out of these widespread actions, we investigated whether IF1 may antagonize the pro-apoptotic action of CF6 using HEK-293 cells. The result clearly showed that exposure of the cells to CF6 increased the number of apoptotic cells, and it was abolished by transfection of mature or immature IF1. CXCR4 is a key receptor regulating a number of downstream effectors. CXCL12 binding to CXCR4 promotes activation of multiple G protein-dependent signaling pathways, resulting in diverse biological responses such as cell survival. CXCR4 activation by its chemokine SDF-1 could lead to the activation of anti-apoptotic signaling pathways; $G_{i\alpha}$ protein-dependent activation of PKB and ERK [Vlahakis et al., 2002]. We recently showed that apoptosis was exacerbated by CF6, and it was abolished by pretreatment with either siRNA for HIF-1α or CXCR4 ligand in endothelial cells [Suzuki et al., 2014]. Thus, we explored one of the anti-apoptotic signaling PKB in the presence or absence of IF1. In the present study, transfection of mature or immature IF1 canceled CF6-induced attenuation of phosphorylated PKB without affecting the total amount of PKB, suggesting that the recovery from apoptosis may be attributable to the increase in phosphorylated PKB, an anti-apoptotic molecule.

CLINICAL IMPLICATIONS

In the clinical settings, we and others showed that circulating CF6 is elevated in patients with hypertension, acute myocardial infarction, end-stage renal disease, stroke, and diabetes [Osanai et al., 2003a,b, 2010; Ding et al., 2004; Chai et al., 2007; Li et al., 2007], all of which predisposed to the development of atherosclerosis. Given the present finding and the widespread biological actions such as inhibition of prostacyclin and NO [Osanai et al., 1998; Tanaka et al., 2006; Kumagai et al., 2008], reduction in CF6 level may be important and useful to prevent cardiovascular events. To date, we have reported that salt restriction, vitamin C, and vitamin B_{12} plus folic acid lowered the plasma level of CF6 in patients with hypertension and stroke [Osanai et al., 2003a, 2010], and that peroxisomal proliferator-activated receptor γ ligand attenuated shear-induced CF6 release, that is dependent on reactive oxygen-related activation of nuclear factor-kappa B, from cultured vascular endothelial cells [Tomita et al., 2005]. The present finding might provide new insights into our understanding of the pivotal role of IF1 in inhibition against the action of CF6, and shed light on a novel therapeutic target in vascular medicine.

In conclusion, the present study showed that IF1 antagonizes the pro-apoptotic action of CF6 through activation of the anti-apoptotic signaling PKB by inhibiting a counter-clockwise mode of proton import in the plasma membrane F_1F_o complex. Given the widespread biological actions of CF6, the physiological and pathological functions of IF1 may be expected to be complex.

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