Direct interaction between the reductase domain of endothelial nitric oxide synthase and the ryanodine receptor

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Abstract **We have performed the recombinant expression and purification of the reductase domain of endothelial nitric oxide synthase (eNOS) and used it as a bait in search for interacting proteins present in endothelial cells. Using mass spectrometry of the bound proteins run in a PAGE–SDS gel, we were able to identify the ryanodine receptor (RyR) as a novel eNOS-binding partner. This interaction was confirmed through immunoprecipitation of both RyR and eNOS from endothelial cells and cardiac myocytes. Immunofluorescence data indicated that a subpopulation of eNOS associates with RyR in perinuclear regions of the cell, where eNOS might be responsible for the known nitrosylation of RyR.**

Keywords: Nitric oxide synthase; Calcium channels; Ryanodine receptors

1. Introduction

Due to its extreme reactivity and the modulation exerted on numerous biological processes, the synthesis of nitric oxide (NO) within the cell is a tightly controlled process. The regulation of the enzymatic activity of all three nitric oxide synthases is exerted through transcriptional and, post-translational modifications (phosphorylation, myristoylation, palmitoylation) together with an exquisite subcellular localization. In this context, the interactions of the three NOSs with cellular proteins always determine where and when NO is to be released. In the case of endothelial nitric oxide synthase (eNOS), at least 12 cellular proteins are known to bind and modulate its activity (see [1,2], and references therein). In addition to the well-characterized interactions with caveolin-1 and calmodulin, eNOS is also known to bind to the bradykinin B2 and angiotensin AT1 receptors, caveolin-3, dynamin-2, CAT1, porin, protein kinase B/Akt, heat shock protein of 90 kDa, NOS interacting protein and NOS traffic inducer [1,2]. However, in all the cases where the interacting region has been mapped, these interactions involve the N-terminal heme oxygenase domain of eNOS and never the C-terminal reductase domain.

Consequently, we have performed the recombinant expression and purification of a hexa-His tagged reductase domain of eNOS and used it as bait in order to identify novel interacting proteins present in endothelial cells. We clearly identified the ryanodine receptor (RyR) as a protein that was retained in a complex with eNOS. This direct association might be responsible for the known modulation of RyR exerted by nitric oxide.

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Abbreviations: BAEC, bovine aortic endothelial cells; eNOS, endothelial nitric oxide synthase; KLH, keyhole limpet hemocyanin; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NO, nitric oxide; PAGE–SDS, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PCR, polymerase chain reaction; PMSF, phenylmethyl sulfonyl fluoride; RyR, ryanodine receptor

2. Materials and methods

2.1. Molecular cloning, recombinant expression and purification of the reductase domain of eNOS

We have followed a similar procedure to the previously described cloning and recombinant expression of the full-length wild-type eNOS in *E. coli* [3]. Using the bovine eNOS as template, a *Nde*I site was introduced at amino acid position 489 and a *Xba*I at the 3′ end of the gene using polymerase chain reaction (PCR). The PCR product was ligated into the subcloning vector pGEMT (Invitrogen) and sequenced. We then double digested the reductase domain cDNA from pGEMT with *Nde*I plus *Xba*I and it was ligated into the corresponding site of the pCWori vector that possessed a hexa-His tag in the polylinker. This plasmid was used to routinely transform BL21 competent cells (Novagen). The recombinant expression of the reductase domain of eNOS was performed using 6 L of 2× YT medium at 30 °C. The bacteria were lyzed and the protein was purified using two affinity columns (the Ni-NTA resin and a 2′,5′-ADP-Sepharose) as previously described [3,4].

2.2. Interaction between the reductase domain of eNOS and endothelial cells

A minimum of fifteen 10 cm circular plates of fresh bovine aortic endothelial cells (BAEC) grown in DMEM supplemented with 10% fetal calf serum were scraped and resuspended in PBS. The cells were centrifuged at $10000 \times g$ at 4 °C and the pellet was homogenized in 100 mM Tris buffer, pH 7.5, in the presence of 10 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 2 μM phenylmethyl sulfonyl fluoride (PMSF) as protease inhibitors (buffer A) in a total volume of 10 mL. The cellular suspension was then passed several times through a syringe (0.5×16 mm) on ice. Unbroken cells and cellular debris were eliminated by a 10-min centrifugation at 5000

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rpm in a table-top microcentrifuge at 4 °C. This sample was loaded onto a Ni-NTA column that contained approximately 5 mL of affinity resin and the flow-through was collected. In this step, cellular proteins that interacted non-specifically with the Ni-NTA column were removed. This procedure was repeated twice in order to eliminate any trace of proteins that might bind to the Ni-NTA resin non-specifically following our published protocol [5]. After clarification of the total cell extract, 1 mg of hexa-His tagged pure recombinant reductase domain was added to the sample and allowed to interact with the cellular proteins for 15 min on ice. Afterwards, the mixture was loaded onto a new Ni-NTA resin pre-equilibrated in buffer A. The column was subsequently washed with 200 mL of 20 mM imidazole in buffer A and the complexes of reductase bound specifically to endothelial cells proteins were eluted with 200 mM imidazole. The absorbance of each collected fraction of eluate was measured at 280 nm. The fractions that contained protein were collected and the pool was extensively dialyzed (3 kDa cut-off) against 50 mM $(NH_4)HCO_3$, pH 8.0, in the presence of 2 μM PMSF. After the dialysis, the sample was lyophilized and stored at −80 °C.

2.3. SDS–PAGE and mass spectrometry (MS) analysis

The lyophilized sample was dissolved in SDS–PAGE loading buffer in the presence of 1% v/v β-mercaptoethanol, boiled for 5 min and resolved on a 15% acrylamide gel. Staining of the gel was performed with coomassie brilliant blue for 30 min. The coomassie-stained bands were excised with a sharp cutter and transferred to a clean Eppendorff tube. The proteomics analysis was performed using a Bruker Reflex™ IV matrix-assisted laser desorption/ionization (MALDI)-TOF mass spectrometer (Bruker-Franzen Analytic, Bremen, Germany) equipped with the SCOUT™ source in positive ion reflector mode using delayed extraction as previously described [5].

2.4. Development of a rabbit polyclonal serum against the RyR

We synthesized two peptides corresponding to the RyR and coupled them to keyhole limpet hemocyanin (KLH). The sequences that we chose were: EDEIQFLRTEDEWLQ (N-terminal end of all RyR1, RyR2 and RyR3) and the internal peptide KKAVWHKLLSQRKR.

2.5. Immunoprecipitation, immunodetection and immunofluorescence of fixed cells

Cell culture and all the cellular biology experiments were performed following our published protocols [6,7]. In each experiment we used two 10 cm circular plates of BAEC grown on DMEM in the presence of 10% serum. A minimum of two independent experiments were performed every time. The anti-eNOS rabbit polyclonal antibody was purchased from Sigma (cat. N3893) and the anti-caveolin-1 rabbit polyclonal antibody was purchased from BD Biosciences (cat. 610060). The anti-RyR rabbit polyclonal serum used in all the experiments was elicited in our laboratory and recognized the N-terminal end of RyR.

2.6. Molecular cloning and expression of the full-length eNOS-GFP construct

The full-length eNOS gene was amplified by PCR inserting an *Eco*RI site and a *Bss*HII at the 5′ and 3′ end, respectively, subcloned into pGEMT (Invitrogen), sequenced, double digested with *Eco*RI plus *Bss*HII and ligated into the corresponding sites of pCDNA3-GFP [6,7].

3. Results

3.1. Recombinant expression of the reductase domain of eNOS

The purification of the reductase domain of eNOS expressed in *E. coli* rendered an homogeneous protein band of 80.6 kDa according to PAGE–SDS, and displayed an absorption spectrum in the visible region characteristic of a flavoprotein (Fig. 1A). As expected, the absorbance spectrum showed two maxima, centered at 385 and 455 nm, characteristic of proteins with FAD and FMN. We were able to obtain approximately 2 mg of pure protein per liter of cell culture.

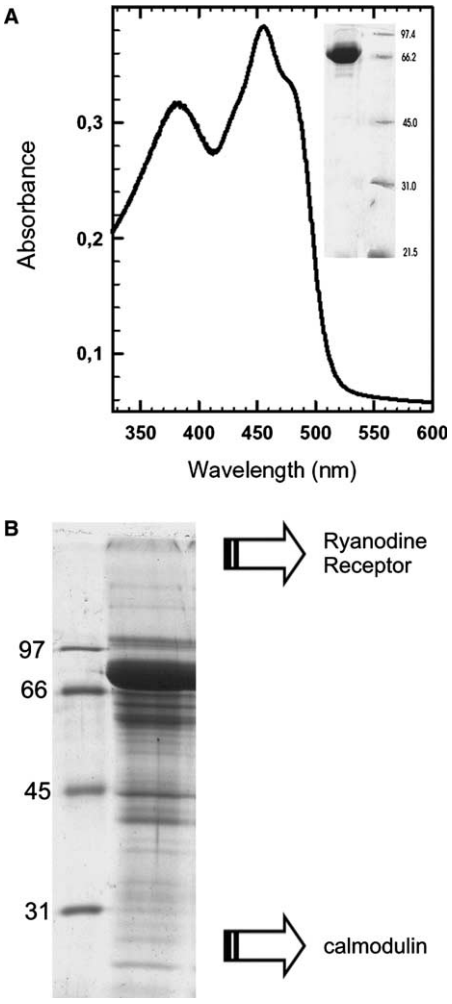

Fig. 1. Recombinant expression and purification of the reductase domain of eNOS and interaction with an endothelial cells extract. The C-terminal reductase domain of eNOS was recombinantly expressed, and purified using two affinity resins. The absorbance spectrum of the pure protein in the visible region together with a coomassie blue-stained PAGE–SDS gel (inset) are shown in (A). The purified protein was incubated with the clarified cell extract, bound to the Ni-NTA resin, eluted and analyzed by PAGE–SDS and coomassie blue staining (B). Twelve gel stripes were excised with a clean scalpel and subjected to MS analysis. The positions where calmodulin and RyR appear are shown by arrows.

3.2. Identification of reductase-interacting proteins by MS

In order to rule out non-specific interactions of the reductase domain of eNOS with proteins obtained from endothelial tissues, we first clarified the cell lysate loading it twice in the Ni-NTA agarose resin. The flow-through was then allowed to interact with the hex-His tagged reductase domain of eNOS and it was loaded again in the affinity column. After elution with 200 mM imidazole, dialysis and liophilization, the sample was run in a one-dimensional SDS gel and analyzed by MALDI-TOF (Fig. 1B). When we loaded over 30 μg of total protein in one lane, many bands could be identified after coomassie blue staining. We cut 10 bands ranging from 10 to over 500 kDa and we tried to identify them by MS. However, in most of the cases, we retrieved fragments of the eNOS reductase (probably resulting from proteases present in the cell lysate). Nevertheless, two proteins could be identified unambiguously,