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Original article

Degradation of a connexin40 mutant linked to atrial fibrillation is accelerated

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ABSTRACT

Several Cx40 mutants have been identified in patients with atrial fibrillation (AF). We have been working to identify physiological or cell biological abnormalities of several of these human mutants that might explain how they contribute to disease pathogenesis. Wild type (wt) Cx40 or four different mutants (P88S, G38D, V85I, and L229M) were expressed by the transfection of communication-deficient HeLa cells or HL-1 cardiomyocytes. Biophysical channel properties and the sub-cellular localization and protein levels of Cx40 were characterized. Wild type Cx40 and all mutants except P88S formed gap junction plaques and induced significant gap junctional conductances. The functional mutants showed only modest alterations of single channel conductances or gating by trans-junctional voltage as compared to wtCx40. However, immunoblotting indicated that the steady state levels of G38D, V85I, and L229M were reduced relative to wtCx40; most strikingly, G38D was only 20–31% of wild type levels. After the inhibition of protein synthesis with cycloheximide, G38D (and to a lesser extent the other mutants) disappeared much faster than wtCx40. Treatment with the proteasomal inhibitor, epoxomicin, greatly increased levels of G38D and restored the abundance of gap junctions and the extent of intercellular dye transfer. Thus, G38D, V85I, and L229M are functional mutants of Cx40 with small alterations of physiological properties, but accelerated degradation by the proteasome. These findings suggest a novel mechanism (protein instability) for the pathogenesis of AF due to a connexin mutation and a novel approach to therapy (protease inhibition).
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1. Introduction

Gap junctions contain intercellular channels (formed of connexins) that allow the exchange of ions (and small molecules) between adjacent cells. In the heart, these channels are critical for normal electrical conduction. Abnormalities of connexins have been implicated in both atrial and ventricular arrhythmias.

Atrial fibrillation (AF) is the most common cardiac arrhythmia. It is characterized by a rapid and irregular electrical activation and the loss of atrial muscle contractility. The pathogenesis of AF involves initiating triggers (often rapidly firing ectopic foci located inside the pulmonary veins) and an abnormal atrial tissue substrate that maintains the arrhythmia [1,2]. It is likely that the abundance and distribution of atrial connexins contribute to that tissue substrate [3].

Two different connexins, Cx40 and Cx43, are abundantly expressed by atrial myocytes and determine the properties of conduction within this tissue [4,5]. Various alterations of both Cx40 and Cx43 have been observed in patients with AF (reviewed in [6]). The confounding results of these studies may have resulted from the different etiologies and durations of AF in these patients and the extent of failure and structural heart disease. About 15% of AF patients have “lone AF” which develops in apparently normal hearts in the absence of structural abnormalities.

Connexin abnormalities identified in patients with lone AF may help to elucidate the contribution of connexins (and gap junctions) to this disease. Several Cx40 mutants have been identified in patients with lone AF [7,8]. We have been working to identify any physiological or cell biological abnormalities of these mutants that might help to explain how they contribute to disease pathogenesis. Our general strategy is to express the wild type or mutant Cx40 by the transfection of communication-deficient cells and characterize their protein levels, sub-cellular localization, and biophysical channel properties.

Abbreviations: AF, atrial fibrillation; Cx, connexin; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; g_j, junctional conductance; G_j, normalized junctional conductance; $G_{j,max}$, maximum junctional conductance; $G_{j,min}$, minimum junctional conductance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I_j, junctional current; 3-MA, 3-methyl adenine; PDI, protein disulfide isomerase; PI, propidium iodide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; q, electron equivalents; wt, wild type; $V_{½}$, half-inactivation voltage; V_j, transjunctional voltage; V_m, membrane potential; z, gating charge valence; γ_j, gap junction channel conductance.

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In recent studies [9], we concentrated on studying two Cx40 mutants (G38D and M163V). Our electrophysiological experiments showed that these two mutants produced channels with only mildly altered conductance and gating properties when studied individually in transfected N2a cells. However, we found more dramatic alterations when the two mutants were co-expressed, suggesting that they interact with each other. Our new data contrasted with the initial report of G38D [7] which suggested that when expressed, this mutant did not produce a significant level of gap junctional conductance and that cells expressing this mutant showed only very low levels of immunoreactive Cx40 as detected by immunolabeling and microscopy.

In the current study, we have continued cellular/biochemical and physiological characterization of G38D and of several other reported Cx40 mutant proteins associated with lone AF, including P88S, V85I, and L229M. Our new data confirm that many AF-associated Cx40 mutants make gap junction plaques and functional gap junction channels. The channels may or may not have significant abnormalities. But, most strikingly, our new studies presented here suggest that G38D (and likely some of the other mutants) is unstable as compared to the wild type, and it supports reduced intercellular coupling due to accelerated degradation.

2. Material and methods

2.1. Connexin-expressing cells

HeLa and N2a cells were cultured as previously described [9–11]. The mouse HL-1 atrial cardiomyocyte cell line was generated and kindly provided by Dr. W.C. Claycomb (Louisiana State University Medical Center, New Orleans, LA). HL-1 cells were grown in Claycomb medium, supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM norepinephrine and penicillin/streptomycin 100 U/mL, 100 μg/mL (Sigma, St. Louis, MO) [12]. Human Cx40 DNA was subcloned into the pSFFV-neo or pTracer-CMV2 as described earlier [9]. Mutants of Cx40 containing substitutions: G38D [9], G38E, G38V, G38N, V85I, P88S, and L229M, were generated with the Quick Change Site-Directed Mutagenesis Kit (Agilent Technologies UK, Ltd., Cheshire, UK). The plasmid constructs were purified using the Plasmid Maxiprep Kit (OriGene, Rockville, MD) and the DNA sequences were confirmed to contain only the wanted mutations.

Cells were transiently or stably transfected with connexin DNA using Lipofectamine 2000 for HeLa cells or Lipofectamine 3000 for HL-1 cells (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. For immunofluorescence and immunoblot analysis of connexin expression, HeLa cells were transiently transfected with constructs in pSFFV-neo. For the initial assessment of the stability of mutant proteins in comparison to a wild type Cx40, HeLa cells were transiently transfected with connexin DNAs in pTracer-CMV2. For the electrophysiological experiments, N2a cells were transiently transfected with connexin DNAs in pTracer-CMV2.

For the cycloheximide experiments and experiments with inhibitors of protein degradation HeLa cells stably transfected with pSFFV-neo constructs were used. Stable clones were selected by culturing in medium containing G418 (1000 μg/mL) (Life Technologies). Clones were screened for Cx40 expression by immunofluorescence with anti-connexin antibodies.

2.2. Cell treatments

Hela cells expressing wild type or mutant Cx40 were treated with 40 μg/mL cycloheximide (EMD Millipore, Billerica, MA) for 0, 1, 3, 6 or 24 h or with 0.5 μM epoxomicin (Calbiochem-Novabiochem Corp.) [13], 0.1 mM chloroquine (Sigma, St. Louis, MO) [14], 5 mM 3-methyl adenine (3-MA, EMD Millipore, Billerica, MA) or DMSO (used as a solvent for stock solutions of epoxomicin or cycloheximide) for 18 h.

At the end of treatments cells were harvested for immunoblot analysis which was performed as described below.

2.3. Analysis of protein ubiquitination

Hela cells expressing wild type Cx40 or G38D were treated with 0.5 μM epoxomicin or DMSO alone for 18 h, harvested in PBS containing protease inhibitors [5], and centrifuged at 150 g for 7 min. Pelleted cells were resuspended in the same buffer, then disrupted by repeated passage through 20 and 27 gauge needles. Ubiquitinated proteins were isolated from the lysate using the UbiQaptureTM-Q Kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions with binding of proteins to the UbiQaptureTM-Q matrix beads over 4 h. Following washing, bound material was analyzed by immunoblotting.

2.4. Antibodies

Cx40 was detected using rabbit polyclonal antibodies directed against the carboxy-terminal domain of Cx40 (cat. no 36-4900 Life Technologies) at 1:250 dilution for all immunofluorescence and at 1:1000 dilution for most immunoblotting experiments. For blots of HL-1 cells and of ubiquitinated proteins, Cx40 was detected using goat polyclonal antibodies directed against the carboxy-terminal domain of Cx40 (cat. no sc-20499, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Mouse monoclonal anti-GFP antibodies were obtained from Life Technologies (cat. no 33-2600) and used at a 1:250 dilution for immunoblotting. Mouse monoclonal anti-β-tubulin antibodies were obtained from Sigma (cat. no T5283) and were used at 1:2000 dilution for immunoblotting. Mouse monoclonal anti-β-actin antibodies were obtained from Sigma (cat. no A2228) and were used at 1:2000 dilution for immunoblotting. Cy3-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-rabbit or anti-mouse IgG antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). HRP-conjugated donkey anti-goat IgG antibodies were obtained Santa Cruz Biotechnology, Inc. The ubiquitin-conjugate specific HRP-linked antibody from the UbiQaptureTM-Q Kit was used at a 1:1000 dilution.

2.5. Immunoblot analysis

Cell homogenates were prepared 48 h after transfection with connexin DNA as described by Gong et al. [15]. Immunoblots were performed as described earlier [5]. The protein concentrations of homogenates were determined using the method of Bradford (Bio-Rad, Richmond, CA) [16]. Aliquots containing 20 μg of protein were separated by SDS-PAGE on 10% polyacrylamide gels and blotted onto Immobilon-P membranes (Millipore, Bedford, MA). ProSieve QuadColor Protein Markers (Lonza Walkersville, Inc., Walkersville, MD) were used to calibrate the gels. Depending on the desired sensitivity, immunoblots were developed with ECL, ECL Prime (GE Healthcare Biosciences) or SuperSignal West Femto Chemiluminescence reagents (Thermo Fisher Scientific Inc., Rockford, IL) and exposure to X-ray film. Final figures for publication were assembled by cutting and cropping to include representative examples and to juxtapose panels to facilitate comparisons.

2.6. Immunofluorescence analysis of connexin expression

For microscopy, cells were cultured on multi-well slides. Cells were fixed in methanol/acetone (1:1) for 2 min. 27 h after they were transfected with connexin DNAs; then, they were stained as previously described [11]. Cells were studied using the 40× Plan Apochromat objective in an Axioplan 2 microscope (Carl Zeiss Meditec, Munich, Germany). Images were captured with a Zeiss Axiocam digital camera using Zeiss AxioVision software.

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