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Calpain activation by ROS mediates human ether-a-go-go-related gene protein degradation by intermittent hypoxia

Institute for Integrative Physiology, Biological Sciences Division, University of Chicago, Chicago, Illinois

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Wang N, Kang HS, Ahmmed G, Khan SA, Makarenko VV, Prabhakar NR, Nanduri J. Calpain activation by ROS mediates human ether-a-go-go-related gene protein degradation by intermittent hypoxia. Am J Physiol Cell Physiol 310: C329–C336, 2016. First published December 9, 2015; doi:10.1152/ajpcell.00231.2015.—Human ether-a-go-go-related gene (hERG) channels conduct delayed rectifier K\(^+\) current. However, little information is available on physiological situations affecting hERG channel protein and function. In the present study we examined the effects of intermittent hypoxia (IH), which is a hallmark manifestation of sleep apnea, on hERG channel protein and function. Experiments were performed on SH-SY5Y neuroblastoma cells, which express hERG protein. Cells were exposed to IH consisting of alternating cycles of 30 s of hypoxia (1.5% O\(_2\)) and 5 min of 20% O\(_2\). IH decreased hERG protein expression in a stimulus-dependent manner. A similar reduction in hERG protein was also seen in adrenal medullary chromaffin cells from IH-exposed neonatal rats. The decreased hERG protein was associated with attenuated hERG K\(^+\) current. IH-evoked hERG protein degradation was not due to reduced transcription or increased proteasomal/lysosomal degradation. Rather it was mediated by calcium-activated calpain proteases. Both COOH- and NH\(_2\)-terminal sequences of the hERG protein were the targets of calpain-dependent degradation. IH increased reactive oxygen species (ROS) levels, intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), calpain enzyme activity, and hERG protein degradation, and all these effects were prevented by manganese-(111)-tetrakis-(1-methyl-4-pyridyl)-porphyrin pentachloride, a membrane-permeable ROS scavenger. These results demonstrate that activation of calpains by ROS-dependent elevation of [Ca\(^{2+}\)]\(_i\) mediates hERG protein degradation by IH.

sleep apnea; oxidative stress; arrhythmia; apnea of prematurity; adrenal medullary chromaffin cells

HUMAN ETHER-A-GO-GO-RELATED gene (hERG) channels conduct delayed rectifier K\(^+\) current (23). The hERG K\(^+\) channel has been implicated in a variety of cellular functions including ion channel function (11, 15). However, people living at sea level most often experience intermittent hypoxia (IH) as a consequence of sleep apnea (i.e., brief, repetitive cessation of breathing) (22). IH, mimicking O\(_2\) profiles encountered during sleep apnea, affects various cellular functions including activation of early genes (31), hypoxia-inducible factor (HIF)-1\(\alpha\) (33), and voltage-gated Ca\(^{2+}\) channels (14). Sleep apnea patients and IH-exposed rodents exhibit cardiovascular abnormalities, including hypertension and cardiac arrhythmias (18, 30). Given that hERG K\(^+\) channels are implicated in cardiac arrhythmia (5, 23), we examined the effects of IH on hERG channel protein and function in neuroblastoma (SH-SY5Y) cells, which express high levels of endogenous hERG protein (2, 3). Our results demonstrate that IH decreases hERG protein and hERG-mediated K\(^+\) current and these effects are mediated by reactive oxygen species (ROS)-dependent activation of Ca\(^{2+}\)-activated calpain proteases.\(^1\)

MATERIALS AND METHODS

Exposure of cell cultures and rats to intermittent hypoxia. Neuroblastoma (SH-SY5Y) cells were obtained from ATCC (no. CRL-2266). Cells were grown in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin and maintained at 37°C in 10% CO\(_2\). Cell cultures were exposed to IH 60 cycles (IH\(_{60}\)) or 120 cycles (IH\(_{120}\)) with each cycle consisting 1.5% O\(_2\) for 30 s followed by 20% O\(_2\) for 5 min at 37°C as described previously (33). Ambient O\(_2\) levels in the IH chamber were monitored by an O\(_2\) analyzer (Alpha Omega Instruments). Cells were pretreated for 30 min with either drug or vehicle in all experiments involving drug treatment.

Studies were performed on Sprague-Dawley rats of both sexes. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago. Rat pups (ages P0–P10) along with their dams were exposed to IH between 9:00 AM and 5:00 PM for 5 days as described previously (19, 25, 26). Control experiments were performed on age-matched rat pups exposed to alternating cycles of room air instead of hypoxia.

hERG NH\(_2\)-terminal; and/or COOH-terminal deletion plasmid constructs and transfections. pcDNA3-full-length [wild-type (WT)] HA-tagged hERG plasmid was used as a template for PCR and cloned into pcRII-T/A vector (Invitrogen). The amino (NH\(_2\)) terminus deletion construct pcDNA3-HA-ΔN300 was generated by amplifying ~300-bp NH\(_2\)-terminal flanking regions using primers HindIII-hERG-ΔN-F (5’-GCG GCG GAG ACC ATG TAT CCA TAC GAT GTA CCT GAC TAC GCA CGC CAC GAG ACC GGG GCC ATG CAC-3’) and BstE2-hERG-ΔN-R (5’-CAG GAG CAT GTG GAC ATC TTC CTC AGT GAT GGT TCG C-3’).

Address for reprint requests and other correspondence: J. Nanduri, Institute for Integrative Physiology, Biological Sciences Division, The University of Chicago, Chicago, IL 60637 (e-mail: jnanduri@uchicago.edu).

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The NH2/carboxy (COOH)-terminal deletion construct pcDNA3-HA-ΔN300/Ct679 was generated by amplifying ~900-bp COOH-terminal flanking regions using the primers hERG-Cid-Xhol-Stop Codon-F (5′-CGG CTC GAG TAG ATG AAT AAT ACT TCC AGC) and hERG-Cid-XbaI-R (5′-GGG TCT AGA GTG GGA CAC TCC TGA GAA GGC GCC-3′). The PCR product was cloned into pCRII-T/A vector. This plasmid was cut by Xhol and XbaI and then ligated into pcDNA3-HA-hERG predigested by Xhol and XbaI. All plasmid DNA sequences were verified by DNA sequencing at the core facility of the University of Chicago. SH-SY5Y cells were transiently transfected with different plasmid constructs using TransT-2020 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer’s instructions.

**Immunoblot assay.** SH-SY5Y cells or adrenal medullary tissues were solubilized at 4°C in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations were determined, and equal amounts of proteins were separated on a 6% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (PVDF). Membranes were blocked in 5% milk overnight and immunoblotted with specific primary antibodies, followed by horse-radish peroxidase-conjugated secondary antibody. ECL (GE Healthcare)-care were used to develop the blots. Image densities were quantified using ImageJ (National Institutes of Health) by integrating pixel densities of individual protein bands.

**Immunocytochemistry.** Neuroblastoma cells coated on coverslips were fixed with 4% formalin, blocked, and permeabilized in 5% BSA and 0.1% Triton X-100 for 30 min at room temperature. Rat pups were anesthetized with urethane (1.2 g/kg ip), and perfused transcardially with ice-cold heparinized PBS followed by 4% paraformaldehyde. Adrenal glands were harvested and mounted in OCT compound (Tissue Tek; VWR Scientific), and 8-μm sections were cut and stored at −80°C till further analysis. Sections were blocked with 20% normal goat serum in PBS with 0.2% Triton X-100 for 30 min. Permeabilized cells or adrenal gland tissues were incubated with anti-hERG antibody (1:2,000; Alamone Labs) for 2 h at 37°C, washed with PBS, and incubated with Alexa Fluor conjugated secondary antibody (1:250; Molecular Probes) for 1 h at 37°C. Cells were mounted in DAPI containing media and analyzed using a Nikon fluorescence microscope.

**Real-time RT-PCR assay.** SH-SY5Y cells exposed to normoxia or IH were used to generate total RNA followed by first-strand cDNA. Aliquots of cDNA were used in quantitative real-time reverse transcription polymerase chain reaction (rt RT-PCR) with rat-specific primers detecting either hERG or 18S using SYBR green as a fluorophore. Aliquots of cDNA were used in quantitative real-time reverse transcription polymerase chain reaction (rt RT-PCR) with rat-specific primers detecting either hERG or 18S using SYBR green as a fluorophore. Relative mRNA levels were calculated based on the comparative threshold method known as the 2−ΔΔCt method, where [ΔΔCt] = ΔCt (IH) − ΔCt (normoxia). The ΔCt values of both normoxia and IH are normalized to an internal standard (18S RNA).

**Measurements of hERG K+ currents.** hERG currents were monitored in SH-SY5Y cells at room temperature by whole cell configuration of the patch-clamp technique as described previously (15). hERG currents were recorded using patch pipettes filled with solution containing (in mM): 100 K+ aspartate, 20 KCl, 2 MgCl2, 1 CaCl2, 10 EGTA, and 10 HEPEs (pH 7.2). The extracellular solution was the following (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPEs, and 10 glucose (pH 7.4). Currents were elicited from a holding potential of −90 mV with depolarizing pulses to +50 mV in 10-mV step range. The current density was analyzed after maximal activation of hERG currents in a 2-s pulse. Maximal tail current amplitudes measured at −90 mV were normalized to cell capacitance to compute hERG current densities.

**Measurements of calpain activity.** Calpain enzyme activity was measured using Immunoxy Calpain I/2 Kit (no. CBA054; Calbiochem) as described previously (17). Briefly, following exposure to IH, cells were lysed in 50 mM Tris-Cl (pH 7.4), 0.5 mM EDTA, 0.05% β-mercaptoethanol, 1 mm PMSF, and protease inhibitor cocktail (Sigma). Calpain activity was measured using purified calpain as standard. Enzyme activity was normalized to protein content and expressed as percentage of normoxic controls.

**Measurements of intracellular Ca2+ concentration.** Intracellular calcium levels were monitored in SH-SY5Y cells using fura-2 AM as described previously (13). Background fluorescence was subtracted from signals. Image intensity at 340 nm was divided by 380-nm image intensity to obtain the ratiometric image. Ratios were converted to free intracellular Ca2+ concentration ([Ca2+]i) using calibration curves constructed in vitro by adding fura-2 (50 μM, free acid) to solutions containing known concentrations of Ca2+ (0–2,000 nM).

**Measurement of malondialdehyde levels and aconitase enzyme activity.** Malondialdehyde (MDA) levels were determined as described previously (20) and expressed as nanomoles per milligrams of protein. Mitochondrial and cytosol fractions were isolated by differential centrifugation and aconitase activity was determined using aconitate assay kit (no. 705502; Cayman Chemical) as described previously (20). Protein concentration was estimated in the cell extracts using Bio-Rad protein assay kit. The enzyme activities were expressed as nanomoles per milligram of protein per minute.

**Data analysis.** Data are expressed as means ± SE from three to five independent experiments. Statistical analysis was performed by one-way ANOVA, and P values <0.05 were considered as significant.

**RESULTS**

**IH reduces hERG protein expression.** Previous studies showed that cell cultures exposed to 60 cycles of IH (IH60) activates early genes (31) and HIF-1-mediated transcriptional activity (33). Based on these studies, we chose to treat SH-SY5Y cells with IH60 and hERG protein expression was analyzed by immunoblot assay. Cells treated with IH60 showed ~40% reduction in hERG protein compared with normoxia-treated control cells (Fig. 1A). Increasing the IH exposure to 120 cycles of IH (IH120) further decreased hERG protein by 80% (Fig. 1A), suggesting that IH decreases hERG protein in a stimulus-dependent manner.

Catecholamine secretion from adrenal medullary chromaffin cells (AMC) is augmented in rat pups exposed to 5 days of IH (25, 27). Since neonatal rat AMC express hERG protein (10), we investigated whether 5 days of IH affects hERG protein. As shown in Fig. 1B, hERG protein decreased by 65 ± 12% in IH-treated AMC compared with control tissue derived from rats reared under normoxia.

Immunocytochemical analysis showed localization of hERG-like protein primarily in the plasma membrane of both SH-SY5Y and AMC. The membrane expression of hERG decreased following IH exposure in both cell types (Fig. 1C).

Based on these results, all further experiments were performed on SH-SY5Y cells to further assess the effects of IH on hERG K+ current and to delineate the mechanisms underlying hERG protein degradation.

**IH reduces hERG-mediated K+ current.** To assess the effect of IH on hERG K+ channel function, K+ currents were recorded in SH-SY5Y cells in the presence and absence of 2 μM E-4031, a potent and selective blocker of hERG K+ channels (29). E-4031 blocked 70 ± 4% of the K+ current (n = 15 cells; P < 0.01), suggesting hERG channel mediates the majority of the observed K+ current. IH progressively decreased the hERG K+ current in a stimulus-dependent manner (Fig. 2A). Normalizing the current values to cell capacitance showed that hERG K+ current density decreased signif-
Fig. 1. Intermittent hypoxia (IH) downregulates human ether-a-go-go-related gene (hERG) protein expression. A: representative immunoblot of hERG protein in SH-SY5Y cells exposed to either normoxia (N) or 60 and 120 cycles of IH (IH60 and IH120; top). Densitometric analysis (means ± SE; n = 5 independent experiments) of hERG protein normalized to tubulin and expressed as percentage of normoxia (bottom). B: representative immunoblot of hERG protein in adrenal medullary chromaffin cells (AMC) harvested from rat pups exposed to either normoxia or 5 days of IH (IH5d; top). Densitometric analysis (means ± SE; Normoxia and IH; n = 8 rat pups each) of hERG protein normalized to tubulin and expressed as percentage of normoxia (bottom). C: immunolocalization of hERG (red) in SH-SY5Y cells and AMC exposed to normoxia or IH. Blue: DAPI staining of nuclei. Arrows denote membrane localization of hERG. **P < 0.01, compared with normoxia.

Mechanisms underlying decreased hERG protein by IH. To assess the mechanisms underlying decreased hERG protein expression by IH, experiments were performed with SH-SY5Y cells exposed to IH120, which produced maximal changes in hERG protein expression (Fig. 1A) and hERG K+ current (Fig. 2, A and B). We first analyzed hERG mRNA expression by real-time quantitative PCR. hERG mRNA levels increased in IH120-exposed cells compared with control cells (Fig. 3A), suggesting that transcriptional changes are unlikely to account for down regulation of hERG protein by IH. Given that hERG protein can be degraded by either proteasome or lysosome (4, 9), we tested the effects of MG-132, a proteasomal...
 inhibitor or bafilomycin, a lysosomal inhibitor on hERG protein degradation by IH. Neither MG-132 nor bafilomycin were able to prevent degradation of hERG protein by IH (Fig. 3B).

**ROS-dependent Ca^{2+} signaling mediates hERG degradation by IH.** IH activates ROS signaling in cell cultures and in intact animals (21). We therefore examined whether ROS signaling contributes to IH-induced hERG protein degradation. To this end, we first determined ROS levels in IH_{120}-treated SH-SY5Y cells by two approaches: one by measuring MDA levels, which represents oxidized lipids (20), and the other by determining the aconitase enzyme activity, an established biochemical marker of ROS (20), in cytosolic and mitochondrial fractions. IH-exposed cells showed increased MDA levels and decreased aconitase enzyme activity in the cytosolic and mitochondrial fractions (Fig. 4, A and B), demonstrating increased ROS levels. MntMpyP, a membrane permeable ROS scavenger, prevented increased ROS levels and hERG protein degradation by IH (Fig. 4, A–C).

We then investigated how ROS contributes to IH-induced hERG degradation. We previously reported that ROS generated by IH increases [Ca^{2+}]i; levels in rat pheochromocytoma PC12 cells (17). To determine whether Ca^{2+} signaling contributes to hERG protein degradation, we first monitored [Ca^{2+}]i levels in IH_{120}-treated SH-SY5Y cells. Basal [Ca^{2+}]i levels were elevated in IH-treated cells, and this effect was absent following treatment with MnTmPyP, a ROS scavenger (Fig. 4D). BAPTA-AM, a chelator of Ca^{2+} prevented hERG degradation by IH, whereas thapsagargin and ionomycin, a Ca^{2+} ionophore, which elevate [Ca^{2+}]i, decreased hERG protein levels under normoxia, mimicking the effects of IH (Fig. 4F).

**Calpain proteases mediate IH-induced hERG degradation.** Elevation of [Ca^{2+}]i activates several downstream effector molecules including Ca^{2+}–activated proteases, calpains (8). To determine the role of calpains in IH-induced hERG protein degradation, calpain enzyme activity was determined in IH_{120}-treated SH-SY5Y cells. As shown in Fig. 5A, calpain activity was elevated in IH-treated cells. N-Acetyl-Leu-Leu-Methioninal (ALLM), a potent inhibitor of calpains, prevented IH-evoked calpain activation and hERG degradation and restored hERG-mediated K+ currents (Fig. 5, A–D).

To further establish that hERG is a substrate for calpains, cell lysates from normoxic cells were incubated with purified calpain-1 in presence of CaCl2 with and without EDTA, a chelator of Ca^{2+} ions. hERG protein was completely degraded when cell lysates were incubated as brief as 15 min with calpain-1 and CaCl2, and addition of EDTA prevented this effect (Fig. 5E).

**Identification of calpain binding sites in hERG protein.** Calpain cleavage prediction using multiple Kernel learning (6) from Calpain Modulatory Proteolysis Data Base (CaMPDB) identified potential calpain binding sites in the hERG protein both at the NH2 terminus containing a PER-ARNT-SIM (PAS) domain and the COOH terminus containing a cyclic nucleotide binding (cNBD) domain (28). The contributions of NH2 and COOH terminus to IH-induced hERG degradation were determined. To this end, SH-SY5Y cells were transfected with HA-tagged hERG plasmids with deletions of either NH2 or COOH terminus (Fig. 6A) and then were exposed to IH_{120}. Control experiments were performed on cells transfected with full length WT plasmid. hERG protein, synthesized in the endoplasmic reticulum, as an immature core glycosylated (cg) protein, is exported to the Golgi apparatus for glycosylation and subsequent insertion into the plasma membrane as a fully glycosylated (fg) mature protein (34). Immunoblot analysis of cells transfected with WT hERG (Fig. 6A, plasmid 1) revealed two bands corresponding to cg and fg forms under normoxia (Fig. 6B). IH-exposed cells showed 81% reduction in the fg form, and 48% reduction in the cg form (Fig. 6, B and C, lane 1). The effects of IH on cells expressing NH2-termina deletion of 300 amino acids including the PAS domain (Fig. 6A, plasmid 2) were similar to those seen in cells expressing WT hERG (Fig. 6, B and C, lane 2). In cells expressing COOH-terminal deletion with intact cNBD (Fig. 6A, plasmid 3), IH exposure resulted in near absence of the fg form (90% reduction) with modest reduction (39% reduction) in the cg form (Fig. 6, B and C, lane 3). Combined deletion of NH2 or COOH terminus either with cNBD (Fig. 6A, plasmid 4) or without cNBD (Fig. 6A, plasmid 5) showed no detectable expression of
experiments. 

Cytosolic and mitochondrial aconitase activity (E) and densitometric analysis (bottom; means ± SEM from 4 independent experiments). **P < 0.01; n.s = not significant, P > 0.05, compared with normoxia.

DISCUSSION

The results of the present study demonstrate that IH degrades hERG protein in SH-SY5Y neuroblastoma cell cultures. Similar degradation of hERG protein was also seen in AMC from IH-treated neonatal rats, suggesting that the effects of IH are not cell selective and can be seen in intact rodent models of IH. The finding that IH exposure reduces hERG-mediated K⁺ current further demonstrates the functional importance of hERG protein degradation by IH.

Analysis of the underlying mechanisms revealed that the reduced hERG protein was not due to transcriptional down-regulation, because IH increased hERG mRNA levels. The immature core glycosylated protein (cg) of hERG protein is degraded by proteasomes with a half-life of 4 h (9), and the fully glycosylated mature protein (fg) is degraded by the lysosomes with a half-life of 10–12 h (4, 7). Although cg and fg forms were not always distinguishable by Western blot assay in native SH-SY5 neuroblastoma cells, immunocytochemical analysis showed that IH selectively degrades the membrane fg form of hERG. However, both MG-132 and baflomycin were unable to prevent the reduced hERG protein expression caused by IH, demonstrating that neither proteasome nor lysosome contribute to hERG protein degradation by IH.

The following observations demonstrate that ROS signaling mediates hERG protein degradation by IH: 1) ROS levels were elevated in IH-exposed neuroblastoma cells as evidenced by increased MDA levels as well as decreased aconitase activity in the cytosolic and membrane fractions; and 2) MnTmPyP, a membrane permeable ROS scavenger, prevented IH-induced ROS generation as well as the degradation of hERG protein. Previous studies reported that IH increases ROS levels by activating several prooxidant enzymes including NADPH oxidases 2 and 4 (32), xanthine oxidase (16), and mitochondrial electron transport chain at the complex I (12) as well as by

Fig. 4. Reactive oxygen species (ROS)-dependent elevation of intracellular Ca²⁺ concentration ([Ca²⁺]) is necessary for IH-induced hERG degradation. Malondialdehyde (MDA) levels (A) and cytosolic and mitochondrial aconitase activity (B) were monitored as indexes of ROS generation in SH-SY5Y cells exposed to normoxia or IH120 with and without MnTmPyP (50 μM), a membrane permeable ROS scavenger. Data shown are means ± SEM from 4 independent experiments. C: effect of MnTmPyP on hERG protein in cells exposed to normoxia or IH120 (IH). Representative immunoblot (top) and densitometric analysis (bottom; means ± SEM from 3 independent experiments). D: basal [Ca²⁺]i levels in cells exposed to either normoxia or IH120 with and without MnTmPyP. Data shown are means ± SEM from 3 independent experiments. E: BAPTA-AM (10 μM), a chelator of Ca²⁺, prevents IH120-induced hERG protein degradation. Ionomycin (Ion; 1.4 μM), a Ca²⁺ ionophore, and thapsagargin (Thaps; 1.5 μM), which elevates [Ca²⁺], degrade hERG protein in cells exposed to normoxia. Representative immunoblot (top) and densitometric analysis (bottom; means ± SEM from 3 independent experiments). **P < 0.01; n.s = not significant, P > 0.05, compared with normoxia.

the fg form under normoxia, and the reduction of cg form by IH was comparable with WT hERG (Fig. 6, B and C, lanes 4 and 5).
Fig. 5. Calpains mediate IH-induced hERG degradation. A: calpain activity was determined in SH-SY5Y cells exposed to either normoxia (N) or to IH120. Calpain activity increased in cells exposed to IH120 which was inhibited by the calpain inhibitor N-Acetyl-Leu-Leu-Methional (ALLM; 10 μM; means ± SE from 8 independent experiments). B: representative immunoblot of hERG in cells exposed to normoxia or IH120 with and without pretreatment with ALLM (10 μM; top). Densitometric analysis (bottom; means ± SE from 3 experiments). C and D: representative hERG currents (C) and hERG K+ current density (D) in normoxia, IH120, and IH120 + ALLM-treated cells. Current density in D are represented by box charts from cells exposed to normoxia (n = 13 cells) and IH120 with (n = 11 cells) and without (n = 12 cells) pretreatment with ALLM (10 μM). Current density was derived by dividing the peak tail currents measured at −90 mV by the cell capacitance. E: representative hERG immunoblot (top) in SH-SY5Y cell lysates incubated for 15 min with purified calpain-1 (3 mg/ml) in presence of 1 mM CaCl2 with and without 2 mM EDTA. Densitometric analysis (bottom; means ± SE from 3 individual experiments). **P < 0.01; n.s = not significant, P > 0.05 compared with normoxia.

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...decreasing antioxidant enzymes such as superoxide dismutase-2 (17). Further studies are needed to determine which of these ROS-generating sources contribute to hERG degradation by IH.

How might ROS contribute to hERG degradation by IH? We found that 1) IH-exposed cells exhibit elevated [Ca2+]i, and ROS scavenger prevents this response; and 2) BAPTA-AM, a membrane permeant Ca2+ chelator, prevents hERG degradation by IH, whereas thapsagargin or ionomycin, which increase [Ca2+]i, decrease hERG protein levels in neuroblastoma cells exposed to normoxia, mimicking the effects of IH. These findings demonstrate that ROS-dependent [Ca2+]i elevation is essential for hERG degradation by IH.

The novel finding of the present study is the demonstration that Ca2+-activated proteases, calpains, mediate hERG protein degradation by IH. Calpain enzyme activity increased in IH-exposed cells, and ALLM, a potent inhibitor of calpains, not only blocked IH-induced calpain enzyme activity but also prevented the degradation of hERG protein. More importantly, the calpain inhibitor restored the hERG-mediated K+ currents in IH-exposed cells. The finding that hERG is degraded in presence of exogenous calpain and Ca2+, which could be prevented by EDTA, a Ca2+ chelator, further establishes that hERG protein is one of the substrates for calpains during IH. While both NH2- and COOH-terminal regions of the hERG protein contain putative calpain binding sites, COOH-terminal deletion was ineffective in preventing IH-induced degradation, whereas NH2-terminal deletion resulted in modest degradation of fg form of hERG in IH-exposed cells. Cells with forced expression of combined NH2- and COOH-terminal deletion constructs showed only the cg form of hERG, which was relatively resistant to IH-induced degradation. We did not measure K+ currents in cells treated with various NH2- and COOH-terminal deletion constructs for two reasons: 1) cells transfected with these constructs expressed predominantly the cg form and very little or none of the fg form, which is the
main hERG form expressed in the membrane and important for 
K⁺ currents; and 2) these experiments were performed with 
forced expression of HA-tagged constructs in neuroblastoma 
cells expressing native hERG protein, which complicates the 
interpretation of results. Despite these limitations, our obser-
vations suggest that calpain-mediated hERG protein degrada-
tion by IH is complex and likely involves multiple calpain 
binding sites. Nonetheless, collectively these findings demon-
strate a hitherto uncharacterized role for calpains in regulating 
hERG protein expression.

Although hERG is an important K⁺ channel in many cell 
types, arguably it is most important for regulating the heart-
beat. A recent study reported that obstructive sleep apnea 
(OSA) patients with congenital long-QT syndrome (LQTS) 
exhibit increased QT prolongation and sudden cardiac death 
(24). Given that hERG is implicated in long-QT syndrome 
(24), it would be of interest to assess whether downregulation 
of hERG protein by IH contributes to heart rate abnormalities 
associated with OSA. Recurrent apnea is also a major clinical 
problem in infants born preterm (1). Neonatal rats exposed to 
IH exhibit markedly augmented catecholamine secretion from 
AMC (25). hERG protein is expressed in neonatal AMC, and 
the hERG-mediated K⁺ current is important for catecholamine 
secretion (10). Given that IH increases calpain enzyme activity 
in the adrenal medulla (17), it would be interesting to deter-
mine whether calpain-mediated hERG degradation contributes 
to IH-induced augmented catecholamine secretion from neo-
natal AMC.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: N.W., H.S.K., S.A.K., G.A., and V.V.M. performed 
experiments; N.W., S.A.K., G.A., V.V.M., and J.N. analyzed data; N.R.P. and 
J.N. edited and revised manuscript; J.N. conception and design of research;
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