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Rapid paper

Rat Humanin is encoded and translated in mitochondria and is localized to the mitochondrial compartment where it regulates ROS production

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ABSTRACT

Evidence for the putative mitochondrial origin of the Humanin (HN) peptide has been lacking, although its cytoprotective activity has been demonstrated in a variety of organismal and cellular systems. We sought to establish proof-of-principle for a mitochondria-derived peptide (MDP) in a rat-derived cellular system as the rat HN sequence is predicted to lack nuclear insertions of mitochondrial origin (NUMT). We found that the rat HN (Rattin; rHN) homologue is derived from the mitochondrial genome as evidenced by decreased production in Rho-0 cells, and that peptide translation occurs in the mitochondria as it is unaffected by cycloheximide. Rat HN localizes to the mitochondria in cellular subfractionation and immunohistochemical studies. Addition of a HN analogue to isolated mitochondria from rat INS-1 beta cells reduced hydrogen peroxide production by 55%. In summary, a locally bioactive peptide is derived and translated from an open reading frame (ORF) within rat mitochondrial DNA encoding 16S rRNA.

1. Introduction

Humanin (HN) is a 24 amino acid putative mitochondrial-derived peptide (MDP) encoded within an open reading frame (ORF) found in the mitochondrial 16S ribosomal RNA (Capt et al., 2015; Hashimoto et al., 2001; Maximov et al., 2002). Antibodies were generated against the predicted peptide (Ikonen et al., 2003; Tajima et al., 2002), an ELISA developed (Chin et al., 2013), and HN levels assayed in a variety of biological fluids (Muzumdar et al., 2009). Since its discovery, studies have revealed diverse cytoprotective effects of HN against various cellular stressors in multiple organ systems, including recently described protective properties against chemotherapy-related side effects (Eriksson et al., 2014; Jia et al., 2015). In addition, HN improves survival of pancreatic beta cells and delays the onset of diabetes (Hoang et al., 2010); it also improves peripheral insulin sensitivity via a central mechanism (Muzumdar et al., 2009); and it increases glucose-stimulated insulin secretion (Kuliawat et al., 2013).

Perhaps the greatest amount of work that has been published on HN is its characterization as a neurosurvival factor capable of antagonizing Alzheimer's disease-related cellular insults (Hashimoto et al., 2001). Proposed mechanisms of action for HN include antagonistic effects on two mitochondrially-localized pro-apoptotic factors, insulin-like growth factor binding protein-3 and Bax (Guo et al., 2003; Ikonen et al., 2003).

Humanin-induced signaling has been demonstrated to occur via a trimeric cell surface receptor comprised of CNTFR, WSX-1, and gp130 (Hashimoto et al., 2009) that activates multiple signaling cascades including STAT3 (Hashimoto et al., 2005). In addition, the FPR-like-1 (FPRL1) receptor, which is a member of the G protein-coupled formylpeptide receptor (FPR) family that activates the ERK pathway has been proposed to be a functional receptor for HN action (Harada et al., 2004). These data suggest that HN may have a multiplicity of mechanisms of action.

The origin of the HN gene remains elusive as it is still unclear whether HN is translated in the mitochondria or the cytoplasm. Given that the mitochondrial genetic code for HN differs from the cytoplasmic genetic code, translation in the mitochondria would

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result in a shorter peptide (Bodzioch et al., 2009). Conversely, HN could originate from the nuclear genome (Guo et al., 2003). There are 13 nuclear loci that maintain the open reading frames of 15 distinct full-length HN-like peptides, and at least ten of them may be functional genes (Bodzioch et al., 2009). These sequences could be classified as nuclear insertions of mitochondrial origin (NUMTs) (Ramos et al., 2011). NUMTs are fragments of mitochondrial DNA that have been integrated into the nuclear genome throughout the evolutionary process by a potential mechanism involving transposable elements (Mishmar et al., 2004). There are hundreds of NUMTs in the human genome, and they come in various sizes with varying degrees of homology with the original sequences (Mishmar et al., 2004; Mourier et al., 2001). Nuclear HN NUMTs have also been found in other species; however, rats do not possess any coding nuclear isoforms for HN (Bodzioch et al., 2009), making rat-derived cells an attractive model to study the origin of the rat HN (Rattin; rHN) homologue. In our work, we identify the origin and translation site of rHN.

2. Results/discussion

Analysis of HN-like gene sequences in rodents revealed that rats apparently contain no coding nuclear HN genes but only a mitochondrially-encoded HN (Bodzioch et al., 2009). As an initial step in the identification of the origin and translation site of rHN, INS-1 cells were treated for 4 weeks with ethidium bromide (EtBr) to deplete them of mitochondrial DNA (mtDNA) as previously described (Miller et al., 1996). INS-1 cells treated with EtBr showed an expected reduction in COX I (a protein of mitochondrial genomic origin) by Western blot. rHN peptide was not detected in these mtDNA-depleted (Rho-0) cells (Fig. 1).

Translation localization of rHN was achieved through treatment of INS-1 rat insulinoma cells with the translation inhibitors chloramphenicol (mitochondria-specific), and cycloheximide (cytoplasmic-specific). Translation inhibition was confirmed by measuring levels of COX I, a respiratory chain protein translated in the mitochondria, and UQCRC2 (ubiquinol-cytochrome c reductase core protein II), a nuclearly-encoded respiratory chain protein that is transported into the mitochondria after cytoplasmic translation.

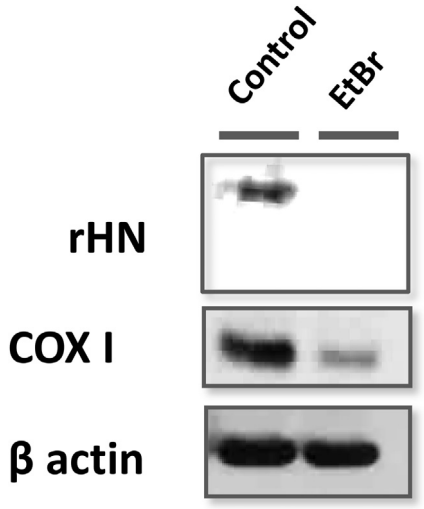

Fig. 1. rHN is derived from the mitochondrial genome. INS-1 cells were treated with ethidium bromide (EtBr) for 60 days. EtBr-induced loss of mitochondrial DNA was confirmed by decreased cytochrome C oxidase subunit I (COX I) signal. rHN was present in untreated INS-1 cells. However, after EtBr treatment, rHN was not expressed in cells that were depleted of mitochondrial DNA.

In INS-1 cells treated with chloramphenicol the rHN peptide signal decreased over time as detected by Western blot. However, rHN signal was preserved in cells treated with cycloheximide, suggesting mitochondrial translation (Fig. 2). Rat L6 myoblasts demonstrated a similar pattern (data not shown).

To identify the subcellular location of rHN peptide in insulin-producing beta cells, we analyzed subcellular fractions of INS-1 rat insulinoma cells by Western blot utilizing a polyclonal antibody against rHN. rHN was not detected in the microsomal fraction. rHN was found in fractions containing mitochondria as evidenced by co-localization with the mitochondrial peptides Hsp60 and cytochrome C oxidase subunit I (COX I); additionally, rHN co-localizes with the pro-apoptotic Bax (Fig. 3), a previously described HN binding-partner (Jia et al., 2013). Therefore, rHN peptide localizes to mitochondria in INS-1 cells. The apparent molecular weight difference in the synthesized rHN peptide and endogenous rHN may be due to post-translational modifications of the endogenous peptide. Clearly, sequencing of the endogenous peptide from various species will definitively answer many questions re: origin, translation location, and post-translation modifications.

Supporting the mitochondrial localization of HN, previous work has shown that HN localizes to the midpiece below the head of individual human sperm (Moretti et al., 2010), an area composed of a central filamentous core with many mitochondria spiraled around it, used for ATP production (Fig. 4A). In rats, rHN has been localized to various individual testicular cells, including Leydig cells, spermatocytes, and spermatids (Colon et al., 2006). Although mouse HN (mHN) has previously been detected in mouse testicular tissue (Tajima et al., 2002) we show via immunohistochemistry under 1000× magnification that mHN is detected at the midpiece of sperm, suggesting mitochondrial localization (Fig. 4B). mHN was also detected in the nucleolar regions of Sertoli cells and/or spermatogonia, visible at the basal membrane of the seminiferous tubules and nucleolar regions of cells outside the seminiferous tubules, comprised mainly of Leydig cells.

Recent studies have shown that HNG, a potent HN analog, induces a rapid and sustained activation of antioxidant defense systems and attenuates oxidative stress in rat cardiac myoblasts exposed to hydrogen peroxide (H_2O_2) (Klein et al., 2013). Though pancreatic beta cells have low antioxidant activity at baseline (Drews et al., 2010), an HNG-induced increase in glucose oxidation rate that results in increased ATP and increased production of reactive oxygen species (ROS), did not disrupt mitochondrial membrane integrity (Kuliawat et al., 2013). This finding suggests a role of HN in the regulation of oxidative stress processes in the beta cell. Therefore, we hypothesized that the cytoprotective properties of HN may be due to its ability to directly attenuate the production of mitochondrial ROS and thereby decrease oxidative stress of the cell. In order to exclude the effects of other antioxidant mechanisms present in the cell, we measured the direct effect of HNG addition on H_2O_2 production from isolated INS-1 mitochondria and show a 55% reduction in H_2O_2 production (Fig. 4C).

Recent insights on the role of mitochondria in disease suggest that the mitochondria may serve as a potential target for novel therapeutic approaches. In Type 1 diabetes, it has been proposed that production of ROS by mitochondria during immune destruction acts as a feed-forward activation loop for apoptosis (Szabadkai and Duchen, 2009). In Type 2 diabetes the acquired metabolic alterations, including hyperglycemia, hyperlipidemia and hyperinsulinemia, can promote the production of ROS and damage mitochondria in insulin responsive tissues (Szendroedi et al., 2012). Recent reports demonstrate that HN induces physiologic changes including increasing insulin sensitivity and enhanced insulin secretion in beta cells (Kuliawat et al., 2013). To date, several studies