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Detectability of secretagogin in human erythrocytes

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HIGHLIGHTS

- Eight male participants were recruited for this study.
- Secretagogin was probed for within erythrocytes and peripheral mononuclear cells.
- Secretagogin mRNA was detected in both erythrocytes and peripheral mononuclear cells.
- Secretagogin protein was only detected in erythrocytes.
- Secretagogin mRNA was observed to be significantly negatively correlated with age.

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ABSTRACT

Secretagogin is a six EF-hand calcium-binding protein that can identify granule cells in the dentate gyrus of hippocampus. The aim of this study was to determine if secretagogin can be detected in human blood cells. Eight adult males were recruited for blood analysis. Whole blood was separated into plasma, peripheral mononuclear cells and erythrocytes with Ficoll–Paque and probed for secretagogin using reverse-transcription polymerase chain reaction and Western blot. While secretagogin mRNA was detected in both peripheral mononuclear cells and erythrocytes using reverse-transcription polymerase chain reaction, SCGN protein was only detected in erythrocytes. Interestingly, peripheral mononuclear cells secretagogin mRNA expression levels showed significant negative correlation with age. This begets the question on the function of secretagogin in blood cells and if it is correlated to neurodegeneration associated with ageing. This remains our impetus for further research.

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1. Introduction

Secretagogin (SCGN), a recently discovered 32 kDa protein, was found to be ubiquitously expressed in the human pancreatic β cells, mammalian cone bipolar cells, sectors CA2-4 of the human hippocampus and recently discovered, in the human olfactory bulb and tract [2,4,15,19]. The presence of six EF-hand domains allow SCGN to bind to calcium (Ca^{2+}) [9]. It is suggested to be part of the 'sensor' family of calcium-binding proteins, where it may play a part in modulating exocytosis signalling via vesicle secretion by binding with SNAP-25 [1,5,16]. Although SCGN has a lower affinity for Ca^{2+}($K_D > 1\ \mu M$) as compared to other calcium binding proteins like calbindin, its Ca^{2+} binding sites may still confer on it limited buffering capacity [5]. At high Ca^{2+} concentrations, Ca^{2+} sensors can also act as Ca^{2+} buffer [17]. Additionally, Ca^{2+} induces conformational changes in SCGN, which may lead to possible intracellular signalling [1,16]. SCGN and hyperphosphorylated Tau protein (Tau) was found to rarely co-localise in the pyramidal neurones of the hippocampus of patients suffering from Alzheimer's disease [4]. This may hint that the presence of SCGN may either confer a certain amount of protection against Tau, or its expressions were inhibited by Tau [3,4]. Being found in the dentate gyrus of the hippocampus also means that SCGN may play a role in encoding memory or protecting memory encoding neurons from Ca^{2+} insults [1].

In this study, we screened the blood of eight healthy male participants and tested its individual components for SCGN using RT-PCR and Western blot. It was shown that SCGN was only present in the serum of patients with hypoxic neuronal damage, but not in healthy volunteers [9]. We would like to propose that SCGN might also be expressed in the blood cells, where it may contribute to the elevated levels in serum when released during trauma.

2. Materials and methods

2.1. Blood sampling

Participation in the study was opened to men between the ages of 17 and 45 years old, but specifically targeted at undergraduates at the National University of Singapore (NUS). Ethical approval was

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obtained from the NUS Institutional Review Board (reference no. 11-157) before recruitment commenced. Eight physically fit male participants with an average age of 23.75 ± 1.49 years old, height 171.88 ± 4.85 cm, weight 64.75 ± 15.16 kg were recruited for this study. All participants were given details of the study and had to sign a university approved informed consent form. To rule out possible dietary confounders, the participants had to fast overnight and were adequately hydrated the night before. The latter is essential to facilitate the blood drawing process. After 30 min of rest from arrival, about 8 ml of blood was drawn from the median cubital vein by a phlebotomist (J.J.L.) into BD vacutainers® catalog no. 364606.

2.2. Blood separation

The blood was diluted 1:1 with PBS and separated into plasma (supernatant), peripheral mononuclear cells (buffy layer) and erythrocytes (pellet) using Ficoll Paque™ with centrifugation at 1500 rpm for 30 min at 19 °C. The components were kept in the −80 °C freezer until ready for further experiments.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

RNA from the various blood components was isolated using Qiagen RNeasy® Mini Kit Catalog no. 74104. Invitrogen™ Superscript® first strand synthesis system was used for reverse transcription of RNA to cDNA. RNA and cDNA samples were quantified using Thermo Scientific Nanodrop™ spectrophotometer ND-1000 with a 260/280 absorbance ratio of ∼2.0 for "pure" RNA and ∼1.8 for "pure" DNA. The sequence for SCGN (NM_006998.3) forward primer is 5″-CTGTTAGATGGCTCTGCCTGTC-3″ and the reverse primer is 5″-GTTACAGGATTGCCATGAATGC-3″ was taken from a previous study [6]. Similarly, the sequence for GAPDH (NM_002046) forward primer is 5″-CTCTCTGCTCCTCCTGTTCGAC-3″ and the reverse primer is 5″-TGAGCGATGTGGCTCGGCT-3″ was adopted from another study [7]. Quantitect® SYBR® green PCR mix was used and this reaction mixture underwent a denaturation cycle of 95 °C for 15 min, followed by 45 cycles of amplification at 94 °C for 15 s, 60 °C for 25 s and 72 °C for 12 s. A melting curve was generated from 55 °C to 95 °C. Relative fluorescence SCGN at 530 nm, normalised to GAPDH was used for data analysis.

2.4. Western blot

2.4.1. Lysate preparation

Cell counting was done by loading 10 μl of sample into a haemocytometer and counted under a light microscope at 100× magnification. The amount of RIPA lysis buffer used was estimated based on number of cells present in sample (1 ml complete RIPA per 2.0×10^7 cells). Lysates from erythrocytes and PMCs were prepared using Santa Cruz RIPA lysis complete buffer. Amersham protease inhibitor mix was added to retard protein degradation further. Protein quantification was done using Thermo Scientific Pierce Coomassie plus protein assay in a 96-well plate. XFlour4 was used to read the plate at a wavelength of 595 nm.

2.4.2. Electrophoresis

Exactly 30 μg of protein samples (1:1 sample to sample buffer 2×) were loaded into the wells. Samples were boiled with sample buffer 2× for 5 min at 95 °C on a heating block. Gel electrophoresis was ran at 80 V for 2 h. Transfer onto PVDF membrane (0.2 μm pores) was done at 15 V for 45 min using Bio-rad semi-dry transfer cell. Membrane was blocked with 5% milk blocking buffer. The membrane was then incubated with primary rabbit anti-SCGN Ab (a gift from Dr. Ludwig Wagner, University of Vienna, Austria) (1:10,000) or GAPDH ab followed by secondary anti-rabbit HRP ab. GAPDH has been shown to be a reliable single-protein loading control, present in erythrocytes [8,20]. After which, the membrane was incubated with Thermo Scientific SuperSignal® west pico chemiluminescent substrate and exposed to an X-ray film.

2.4.3. Data analysis

All data analysis was done with Microsoft Excel 2007. Pearson correlation was used to measure statistical dependence of different variables. Significance was set at $p < 0.05$, but $p < 0.1$ was also highlighted in the data.

3. Results

In order for proper analysis, peripheral blood from the human subjects was successfully separated into plasma, PMCs and erythrocytes.

3.1. RT-PCR

Total RNA was isolated from PMCs and erythrocytes, and the mRNA converted into cDNA using oligo(dT)$_{20}$ for PCR. Specifically, the cDNA was subjected to real-time PCR and the expression of SCGN mRNA was normalised to GAPDH mRNA expression for comparison. We were able to detect SCGN mRNA expression in PMCs (Fig. 1A) and in erythrocytes (Fig. 1B). However, for the erythrocytes, not all samples yielded SCGN or GAPDH mRNA expression. A single peak obtained from the melting curve (Fig. 1C) showed that a single product was formed from the PCR [18].

As mRNA expression does not necessarily imply protein expression, SCGN protein was probed for in the plasma, PMC lysate and erythrocyte lysate using Western blot.

Western blot bands at 32 kDa were only observed for SCGN in the erythrocyte lysates (Fig. 2), but not in the plasma or PMC lysates. This indicated that SCGN protein could only be found in human erythrocytes. Despite our effort to ensure that the same amount of protein was loaded into each well, there was still a fluctuation in the GAPDH levels between individuals. As our study is to show the presence of SCGN in erythrocytes, no further control on the protein load was done.

Using Pearson correlation (Table 1), it was observed that there was a significant negative correlation between age and PMC SCGN mRNA expression levels.

4. Discussion

Calcium is a ubiquitous bivalent ion that functions as a secondary molecule for signalling, co-factor for enzymes and maintenance of membrane potentials. Dysregulation in calcium homeostasis has been suggested to manifest itself in neurodegenerative diseases and other pathophysiology [12]. As SCGN is a fairly novel calcium binding protein, not much is known about it, and its expression in blood cells had never been established. Proving its presence in the different components of blood would be the first step. From this, we could then determine where it would best serve as a suitable biomarker for various purposes, ranging from clinical diagnosis to monitoring fitness levels.

4.1. Isolation of mRNA

Although erythrocyte is known to enucleate during its final maturation stage, studies have shown that RNA can still be found in it, supporting the idea of nucleus independent protein synthesis [11]. However, isolation of total RNA from erythrocytes was harder than from PMC, where the quantity and quality extracted were lower. SCGN mRNA was not detected in all of the samples and

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