

Secretogranin II: Novel Insights into Expression and Function of the Precursor of the Neuropeptide Secretoneurin

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Abstract SgII is an acidic secretory which belongs to the family of chromogranins. It is present in the large-dense cored vesicles of the regulated secretory pathway of many neurons and endocrine cells and it is well conserved during evolution. Like chromogranin A, SgII can induce granulogenesis in endocrine cells but also in cells typically lacking secretory vesicles like fibroblasts. In the secretory vesicles SgII is processed to smaller peptides, e.g. secretoneurin, EM66 and manserin. For secretoneurin several biological effects like induction of neurotransmitter release, chemotactic activity towards immune-, endothelial- and muscle cells, and potent angiogenic and vasculogenic properties have been established. Thus, SN displays potent hormonal and paracrine effects, which help to orchestrate development, maintenance, physiologic activity and repair of the surrounding tissue. In addition, SgII has been established as valuable biomarker for endocrine tumours and cardiovascular diseases.

1 Introduction

Secretogranin II (SgII) – also named chromogranin C - is an acidic secretory protein expressed in many neurons and endocrine tissues. It was initially isolated from rat PC12 and the soluble content of bovine chromaffin granules as third main secretory protein and belongs thus to the family of chromogranins. The chromogranins are typically widely expressed throughout neuronal and endocrine tissues. They are stored within the cell in large dense secretory vesicles and released via the regulated pathway in a calcium-dependent manner. The chromogranins represent

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proteins of 50–120 kD, which share several features, such as acidic nature, heat-stability and proteolytic processing to smaller peptides. The so-called granin motif (D/E-S/N-L-S/A/N-X-X-D/E-X-D/E-L) present in the C-terminus of SgII represents a moderately homologous stretch of 10 amino acids also found in chromogranins A and B. It was postulated when the primary amino acid sequence of these proteins became available (Huttner et al. 1991) and is most likely without physiological relevance. This is underlined by the absence of this motif in secretogranins III and V as well as its presence in unrelated proteins like BRCA1, BRCA2, Golgin-245 or *trans*-Golgi p230, an acidic protein localized to the cytosolic side of Golgi membranes (Erlich et al. 1996). The chromogranins share some physicochemical properties like acidic pI and binding of calcium with low affinity but high capacity, which is responsible for the electron-dense core of large dense secretory vesicles. They are cleaved at pairs of consecutive basic amino acids to multiple smaller peptides and thus give rise to the characteristic pattern of multiple immunoreactive bands of intermediate size seen in immunoblots with monospecific antisera. Chromogranins have recently been shown to be ultimately involved in vesicle formation or biogenesis, to contribute to packaging and sorting of hormones and enzymes into LDVs and they can function as precursors of small peptides generated by prohormone convertases from chromogranins A, B, and SgII. Furthermore, chromogranins have been established as valuable biomarkers for the characterisation of neuroendocrine tumours and cardiovascular diseases. Several excellent reviews have recently been published, which provide further details on the structure and physiologic aspects of this protein family (Bartolomucci et al. 2011; Conlon 2010; Fischer-Colbrie et al. 2005, 1995; Helle 2010; Huttner et al. 1991; Portela-Gomes et al. 2010; Stridsberg et al. 2008; Taupenot et al. 2003).

2 Structure & Posttranslational Modifications of Secretogranin II

Human and mouse SgII consist of 617 amino acids (aa) whereas the bovine homolog is 4 amino acids shorter. SgII contains 20% acidic amino acids in its primary amino acid sequence (Fischer-Colbrie et al. 1990), which causes its untypical physicochemical properties like a random-coil structure and heat-stability. Even though a molecular weight of 67 kDa can be deduced from the primary sequence, a band with an apparent M_r of 86,000 is seen in SDS gels. This abnormal migration in SDS electrophoresis is probably due to the high amount of acidic amino acids found in the primary sequence. After co-translational cleavage of a 27 amino acid signal peptide at a typical cleavage site (Ala-X-Ala/Ala), (see (Fischer-Colbrie et al. 1990) for discussion), SgII is further posttranslationally modified in the Golgi apparatus (Fig. 1): it is sulphated at Tyr-151 and phosphorylated at Ser-532 (Lee et al. 2010). SgII is not or only marginally glycosylated. The primary amino acids sequence of bovine SgII contains no consensus sequence for N-glycosylation and SgII does not bind to concanavalin A lectin, in addition no significant O-glycosylation was detected

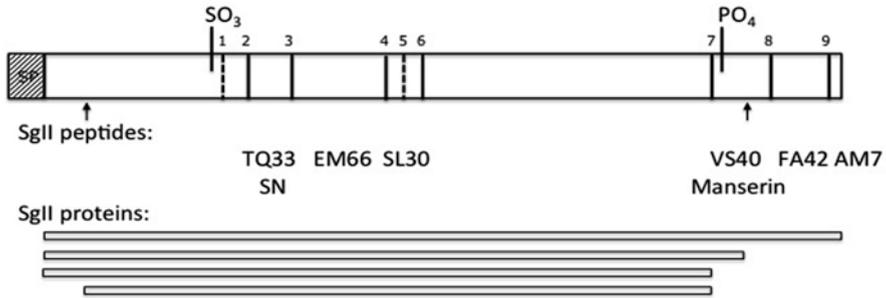


Fig. 1 Structure and processing of secretogranin II (*SgII*). Human *SgII* contains 617 amino acids including a 27 amino acid signal peptide (*SP*). One potential tyrosine sulfation and phosphorylation site is shown. Sites of two consecutive basic amino acids are labelled by numbers. These sites with the exception of site 1 and 5 are used by prohormone convertases (PCs) for endoproteolytic processing. Proteins and peptides generated by PCs from the *SgII* precursor are shown below. Two non-dibasic cleavage sites are indicated by arrows. The name of the peptides represent their first and last amino acid in the single letter code plus the total length. SN, secretoneurin

(Fischer-Colbrie et al. 1995). In the secretory granules *SgII* is processed to small peptides and proteins of intermediate size by prohormone convertases PC1 and PC2, two proteases belonging to the furin-like prohormone convertase group. *SgII* contains 9 pairs of basic amino acids (7 KR and 2 RK sites). Five peptides (secretoneurin (SN), EM66, SL30, manserin, FA42, AM7; see Fig. 1) flanked by KR sites have been isolated from different tissues and are thus generated *in vivo* (Anouar et al. 1996; Kirchmair et al. 1993; Lee et al. 2010; Tilemans et al. 1994; Vaudry and Conlon 1991; Yajima et al. 2004), whereas the RK sites are probably not used for processing. In addition to these small peptides four longer processing intermediates of 20–90 kDa size have been identified by mass spectroscopy (Fournier et al. 2011; Gupta et al. 2010). These proteins include full-length *SgII*, two proteins cleaved at the C-terminal end at position 547 and 524 and one cleaved N-terminally at position 59. Position 527 represents dibasic cleavage site #7 (see Fig. 1) whereas cleavage sites 59 and 547 are non-dibasic sites. Cleavage site 547 is located in the manserin peptide and represents an Ala-IsoLeu site, the second non-dibasic cleavage site is located between Ala-58/Leu-59 in the N-terminal fragment of *SgII* (see Fig. 1). The proteolytic enzymes cleaving at these sites have not been characterized yet.

Proteolytic processing of *SgII* is pronounced in most tissues, especially the central nervous system, resulting in more than 90% of *SgII* processed to small peptides including SN. In contrast, in the adrenal medulla, processing is more limited with the majority of *SgII* immunoreactivity existing as high molecular weight proteins of 20–86 kD (see Fig.1 in (Fischer-Colbrie et al. 1995)). This limited processing is due to the high amount of catecholamines stored in adrenomedullary vesicles, which are potent inhibitors of prohormone convertases PC1 and PC2 (Wolkersdorfer et al. 1996). Also, in astrocytes *SgII* is mainly unprocessed (Fischer-Colbrie et al. 1993) reflecting the relative absence of prohormone convertase activity in these cells.

3 Subcellular Localization

In neurons and endocrine cells SgII is localized to the so-called large dense-cored synaptic vesicles (LDV). It is not found in the cytoplasm or other subcellular organelles like lysosomes. The subcellular localisation to the LDV was established by several means including subcellular fractionation techniques, as well as by immunohistochemistry and immune electron-microscopy. In fact, due to this well-established subcellular localisation SgII is considered and has been used in numerous studies as marker molecule for LDVs. In two studies SgII-immunoreactivity was also found in the nucleus by immune-electron microscopy and immunohistochemical techniques (Yajima et al. 2008; Yoo et al. 2007). It is currently not known whether epitopes shared by a nuclear protein cross-react or whether small amounts of SgII are indeed translocated to the nucleus. This for a secreted protein untypical nuclear localisation can only unequivocally be established when tissue from SgII knock-out mice becomes available. In any case the presence of a well-defined signal peptide rather argues against a nuclear form of SgII.

The N-terminal 27 amino acids of SgII comprise a signal peptide, which cause its co-translational translocation to the rough endoplasmatic reticulum. From there SgII is sorted to the LDV of the regulated pathway. Two regions of SgII, ie a putative α -helix at the very N-terminus (SgII 25–41) and 16 amino acids in the middle region (SgII 334–348) have been identified as sorting motifs (Courel et al. 2008). Sorting of SgII depends on a saturable machinery which is greatly uncharacterized still. It has been suggested that sorting occurs via binding to secretogranin III (Hotta et al. 2009), another granin. However, in secretogranin III knock-down cells SgII was still secreted in a regulated manner (Sun et al. 2013) in line with a proposed sorting mechanism operating mainly by retention (Kuliawat and Arvan 1994; Tooze 1998).

4 Tissue Expression and Secretion from Cells

SgII is constitutively and abundantly expressed throughout the endocrine and nervous system. SgII has been identified in the adrenal medulla, all 3 lobes of the pituitary, the endocrine pancreas, in C-cells of the thyroid and endocrine cells of the whole gastrointestinal tract whereas the parathyroid gland is devoid of SgII (for details see (Fischer-Colbrie et al. 1995)). In the nervous system SgII is distributed in the phylogenetically older parts of the brain with high densities of immunoreactive terminals and fibers found in the hypothalamus, extended amygdala, hippocampus, lateral septum, medial thalamic nuclei, locus coeruleus, nucleus tractus solitarii and substantiae gelatinosae of the spinal cord (Fischer-Colbrie et al. 1995). In the peripheral nervous system SgII is expressed in sensory as well as sympathetic and parasympathetic neurons. In the eye SgII/SN immunoreactivity is expressed in amacrine cells of the retina (Overdick et al. 1996) and capsaicin-sensitive sensory neurons innervating the iris/ciliary complex (Troger et al. 2005).

Under pathological conditions SgII is induced in tissues, which *per se* do not express SgII. Ischaemia induces SgII expression in muscle cells of the hindlimb (Egger et al. 2007; Theurl et al. 2015) and the heart (Røsjø et al. 2012; Theurl et al. 2015). A similar phenomenon was seen in animal models of cerebral ischemia for neurons of the central nervous system (Kim et al. 2002; Marti et al. 2001). Furthermore, epithelial cells from adenocarcinomas of the prostate and the gastrointestinal tract can acquire a so-called endocrine phenotype by the induction of SgII synthesis (Pruneri et al. 1998).

Like other neuroendocrine secretory proteins SgII is released *en bloc* with co-stored classical transmitters, other granins and neuropeptides and biosynthetic enzymes from endocrine cells and neurons into circulation or the synaptic cleft, respectively. This secretory cocktail, however, contains only small amounts of intact, full-length SgII whereas the majority of SgII immunoreactivity comprises small SgII-derived peptides, i.e. SN or EM66, generated in the vesicles by proteolytic processing prior to release. Following depolarisation of cells by action potentials or hormonal signals SgII-derived peptides are exocytotically released in a calcium-dependent manner (Troger et al. 1994).

SN released from endocrine cells and neurons was detected in several body fluids. Serum steady-state SN levels of 22 fmol/ml originate from enterochromaffin cells of the gastrointestinal tract since other sources like the adrenal medulla, pituitary and the endocrine pancreas contribute only little (Ischia et al. 2000a). This is corroborated by the fact that high-emetogenic chemotherapy, which potently stimulates secretion from gastro-intestinal enterochromaffin cells, leads to a 50% increase of SN serum levels (Ischia et al. 2000a). Similarly, serum chromogranin A, which is co-stored together with SgII in these vesicles, is increased 2.5-fold (Cubeddu et al. 1995). SN levels are elevated 5-fold in childhood and are positively correlated with serum creatinine suggesting an influence of renal clearance on SN serum levels. This can be readily explained by the low molecular weight of SN. The half-life of SN was established experimentally as 2.5 h by analysing its decline in serum following removal of SN secreting tumors (Stridsberg et al. 2008). In the urine 80 fmol/ml have been found.

SN is furthermore released in significant amounts into the cerebro-spinal fluid (1500 fmol/ml (Eder et al. 1998; Miller et al. 1996), the aqueous humor (500 fmol/ml (Stemberger et al. 2004)), synovia (16 fmol/ml (Eder et al. 1997)) and faeces (Wagner et al. 2013).

5 Phylogenetic Conservation of Secretogranin II

SgII is well conserved during evolution. It is expressed in the entire vertebrate lineage including mammalia, birds, reptilia, amphibia and fish. SN is even found in agnatha like the lamprey (Trudeau et al. 2012) but has not been detected in any invertebrates so far. In concordance with the teleost-specific whole-genome duplication, teleost bony fish contain two moderately different SgII gene products. In some teleost fish like salmon even 4 SgII gene products exist (unpublished observation).

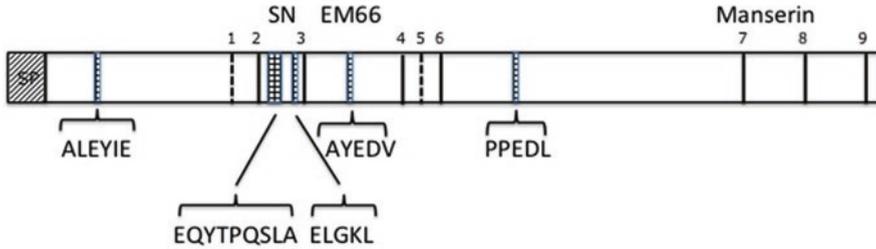


Fig. 2 Phylogenetic conservation of secretogranin II (*SgII*). The primary amino acid sequence of human and zebrafish *SgII* were compared. Homologous regions with a minimum of 5 identical amino acids are given in the single letter code. Only the region of *SgII* comprising the secretoneurin (SN) peptide is significantly conserved

Table 1 Conservation of SN

Human (1)	TNEIVEEQYTPQSLATLESVVFQELGKLTGPNNQ
Primates (21)	TNEIV EQYTPQSLATLESVVFQELGKL PN Q
Mammalia (84)	TNE EQYTPQ LATLESVVFQELGK
Birds (62)	TNEIVEEQYTPQSLATLES FQELGK
Reptilia (19)	TNEI EEQYTPQSLATLESVVFQELGK P
Amphibia (6)	T EIVE QYTPQ LATL SVF ELGK N
Cartilage fish (3)	TNEIVEEQYTPQSLATLES F ELG
Pre-Teleost bony fish (2)	TNEIVEEQYTPQSLATLESVF ELGKL P
Teleost fish - gene A (46)	E YTPQ LA L S F EL
Teleost fish - gene B (44)	E E YTPQ L S EL
Agnatha (1)	E E YTPQ LA L QELG

The primary amino acid sequence of human SN is given in the single letter code. For the various taxa shown only conserved amino acids are listed and variable ones are blanked out. The number of species analyzed per taxa is given in brackets

To date, *SgII* sequence information of up to 300 species has been deposited in the GenBank sequence database. The entire *SgII* precursor is highly conserved between mammalia, whereas between mammalia and fish only the region of *SgII* comprising the SN peptide is significantly homolog (see Kähler and Fischer-Colbrie 2000). Apart from SN only three additional regions with stretches of 5 or more amino acids conserved between human and zebrafish *SgII* can be identified (Fig. 2) suggesting that SN is indeed the physiological relevant peptide present within the *SgII* precursor.

Table 1 presents the high degree of phylogenetic conservation of SN peptide with 16 out of its 33 amino acids identical between human, mammalian, bird, reptile, amphibian, cartilage fish and pre-teleost bony fish. In teleost bony fish still 12 (gene A) and 10 (gene B) are identical with human homolog and SN is even found in agnatha like the lamprey. The middle part of SN comprises the best-conserved region, whereas the C-terminus varies greatly (see Table 1). It is interesting to note that the highly conserved part of SN is composed of 2 contiguous α -helices as determined by two-dimensional ^1NMR analysis (Oulyadi et al. 1997).

6 Gene Organisation and Gene Regulation

The secretogranin II gene is organized into 2 exons only, which are separated by a 3 kb intron (Schimmel et al. 1992). It is located as a single copy gene on human chromosome 2q35-q36 and mouse chromosome 1, respectively (Mahata et al. 1996). The entire open reading frame plus 15 nt of the 5' untranslated region are located on exon 2, exon 1 contains only 5' untranslated region.

In the SgII promoter region several regulatory elements have been identified. A functional cyclic AMP response element (CRE) is located 74 bp upstream of the transcription site (Scammell et al. 2000). This site is conserved during evolution and found in the human, rat and mouse SgII promoter and confers induced gene-expression in response to several stimuli including nicotine and PACAP (Mahata et al. 1999), histamine (Bauer et al. 1993), gonadotropin-releasing hormone (GnRH) (Song et al. 2003) and NO (Li et al. 2008). In addition, the SgII promoter contains 2 TRE-like elements (Li et al. 2008) and a serum-response element (SRE) (Mahata et al. 1999), which is present in the mouse and rat promoter but not conserved in human. Nevertheless, the inactivation of this SRE decreased SgII expression in response to nicotine and PACAP (Mahata et al. 1999). Recent studies demonstrated that SgII is a genuine target gene for the RE-1 silencing transcription factor (REST) (Hohl and Thiel 2005; Watanabe et al. 2004). Since REST is significantly repressed by hypoxia (Liang et al. 2014; Lin et al. 2016) this might explain the potent up-regulation of SgII under ischemic conditions (Egger et al. 2007) despite the absence of a hypoxia-response element (HRE) in the SgII promoter.

7 Biomarker and Disease

7.1 Tumors

Twenty years after the detection of chromogranin A as main secretory protein of adrenergic chromaffin cells its pan-endocrine expression was discovered (Cohn et al. 1982; O'Connor et al. 1983). This led to its rapid application as marker for an endocrine phenotype of normal and malign tissues. In the following years this concept was extended to other members of the chromogranin family, namely chromogranin B and SgII.

SgII has been identified in pituitary adenomas, gastro-enterohepatic carcinomas, pheochromocytomas, Merckel cell carcinoma, midgut carcinoids and oat cell lung and prostate carcinomas (Conlon 2010; Guillemot et al. 2006; Ischia et al. 2000a, b; Portela-Gomes et al. 2010; Wiedenmann et al. 1988). In general, the expression of chromogranin B and SgII in tumors comprising an endocrine phenotype is more restricted than that of chromogranin A. Only in the appendix

the majority of carcinoids (94%) stained positive for SgII-immunoreactivity, whereas chromogranin A was expressed less frequent there (83%, (Prommegger et al. 1998)).

In the serum, elevated levels of SgII can be expected if tumors secrete actively. In general however, serum levels vary greatly between patients and tumor types depending on their renal clearance and the degree of proteolytic processing and secretory capacity of the individual tumor cells. For two peptides generated from SgII, i.e. SN and EM66 data are available. In gut carcinoids and endocrine pancreatic tumors SN serum levels were elevated 20- and 15-fold, in pheochromocytomas, oat cell carcinomas of the lung and neuroblastomas up to 2.5–4.5-fold (Guillemot et al. 2006; Ischia et al. 2000a).

7.2 *CNS Diseases*

In the CSF SN levels are on average 70-fold higher compared to serum. However, there is a marked inter-individuality limiting its usefulness as potential biomarker for neurological and psychiatric diseases. On average, SgII levels are decreased by 15% in patients with multiple sclerosis (Mattsson et al. 2007) and not altered in schizophrenic patients (Miller et al. 1996) and patients with Parkinson's or Alzheimer's disease (Eder et al. 1998). Post-mortem studies demonstrated that SgII levels are decreased in brains of patients with tauopathies (Lechner et al. 2004) and amyotrophic laterals sclerosis due to a loss of presynaptic large dense core vesicles (Schrott-Fischer et al. 2009).

7.3 *Heart Failure*

Serum levels of SN were increased 2.4-fold in patients with cardiac arrest in the first 7 days and back to normal levels after another week (Hasslacher et al. 2014). These findings were corroborated in patients with acute heart failure although in this study the increase was less pronounced (1.2-fold, (Ottesen et al. 2015; Hasslacher et al. 2014)). In both studies SN serum levels were significantly correlated with a poor clinical outcome. The source of increased serum SN levels after heart failure has not unequivocally been established. It has been proposed that under severe ischemic conditions SN leaks from the cerebrospinal fluid into circulation due to an impaired tightness of the blood brain barrier (Hasslacher et al. 2014). In accordance, SN levels in umbilical cord blood was elevated in neonates with hypoxic-ischaemic encephalopathy (Wechselberger et al. 2016).

8 Physiologic Function of Secretogranin II

8.1 Biogenesis of Secretory Granules

Chromogranins induce biogenesis of secretory granule in neuronal but also non-neuronal cells. After the initial discovery that gene ablation of chromogranin A leads to a reduced granule biosynthesis (Kim et al. 2005) a similar function was established for SgII. Expression of full-length SgII stimulated biogenesis of secretory granules in fibroblast cells (Beuret et al. 2004) and a secretory-deficient PC12 cell-line (Courel et al. 2010). From these newly formed secretory granules cargo was released in a calcium dependent manner (Beuret et al. 2004). *Vice versa*, silencing of SgII expression led to a decrease in the number and size of large dense vesicles (Courel et al. 2010). Thus, induction of SgII in non-neuronal tissues typically lacking secretory granules sorting of SgII into the trans-Golgi network might first induce granule biogenesis whereas later on after storage and proteolytic processing in these vesicles SgII might function in addition as a neuropeptide precursor protein. It seems justified to speculate that this scenario is initiated by ischemic conditions in skeletal muscle cells (Egger et al. 2007) or under endocrine differentiation of various adenocarcinomas (Courel et al. 2014).

8.2 Sorting and Release of Secretory Proteins

SgII facilitates sorting of proopiomelanocortin (POMC) to secretory vesicles and its release from AtT-20 cells (Sun et al. 2013). It remains to be demonstrated whether SgII promotes sorting of LDV secretory proteins like POMC *per se* or if its established granulogenic activity alone is sufficient to mediate this effect. Also, SgII alters release of viral particles from infected cells. Depending on the type of virus, shRNA mediated down-regulation of SgII can either decrease or increase the amount of viral particles excreted into medium (Berard et al. 2015).

8.3 Precursor of the Neuropeptide/Cytokine Secretoneurin

In recent years, numerous important physiological functions of SN in the nervous, immune and endocrine system as well as on blood vessels were unravelled (for a review see (Fischer-Colbrie et al. 2005)). In the nervous system SN stimulates dopamine release from rat striatal slices and basal ganglia in vivo. It stimulates neurite outgrowth and survival of cerebellar granules cells (Fujita et al. 1999; Gasser et al. 2003). In concordance, SgII following up-regulation by REST promotes

differentiation and maturation of adult hippocampal progenitor cells (Kim et al. 2015). In the immune system SN displays potent chemotactic activity toward monocytes, eosinophils and dendritic cells, which has also been shown for smooth muscle cells and fibroblasts (Dunzendorfer et al. 1998, 2001; Kähler et al. 1997a, b; Reinisch et al. 1993). In the endocrine system SN inhibits melatonin release from melanocytes and stimulates gonadotropin II release from goldfish pituitary as well as food intake (Blázquez et al. 1998; Mikwar et al. 2016).

8.3.1 Angiogenesis and Vasculogenesis

In 2004 a potent angiogenic and vasculogenic property of SN was discovered. Due to biologic actions of SN on vascular cells such as induction of chemotaxis in endothelial or vascular smooth muscle cells (Kähler et al. 1997a, b) and the fact that SN-containing nerve fibers are closely associated with blood vessels in the uterus (Collins et al. 2000) we hypothesized that SN might induce the growth of new blood vessels out of the pre-existing vasculature, a process called angiogenesis. We observed that SN indeed induced angiogenesis in the mouse corneal neovascularization assay and that newly generated vessels are covered by smooth muscle cells what might indicate durable, stable vessels. In vitro SN induced angiogenesis in a matrigel assay, stimulated proliferation and inhibited apoptosis of endothelial cells (ECs) and activated prominent intracellular signal transduction pathways like Akt or MAPK (Kirchmair et al. 2004a). Beside angiogenesis new blood vessels might also be generated by circulating progenitor cells, a process called vasculogenesis. We could show that SN also stimulates incorporation of these cells into new blood vessels and activates these cells in vitro (Kirchmair et al. 2004b).

Regulation by Hypoxia. Angiogenic factors typically are up-regulated by hypoxia to counteract lack of oxygen by generation of new blood vessels. In the case of SN it was already shown that induction of hypoxia in the central nervous system by ligation of the carotid artery leads to up-regulation of SN in neurons of the hippocampus or the cerebral cortex (Marti et al. 2001). We could show that also muscle cells (a cell type that normally doesn't produce SN) in the ischemic hindlimb after ligation of the femoral artery express SN. In vitro L6 myocytes also increased SN after prolonged hypoxia but this effect was indirect as the promoter region of the gene encoding the SN precursor secretogranin-II does not contain a hypoxia-responsive element and hypoxia did not increase SN in the absence of serum. We could show that SN increase by hypoxia was dependent on hypoxia-inducible factor 1 and basic fibroblast growth factor (FGF) in contrast to the direct regulation of vascular endothelial growth factor (VEGF) (Egger et al. 2007).

SN Gene Therapy. To explore if SN might possess therapeutic potential in the treatment of ischemic limb and heart disease we generated a plasmid gene therapy vector and could demonstrate biologic activity of recombinant SN by EC chemotaxis. After injection of plasmid into ischemic hindlimbs in mice SN improved clinical outcome (less limb necrosis) and increased tissue perfusion and density of capillaries and arteries compared to control plasmid. SN gene therapy additionally

increased numbers endothelial progenitor cells in this ischemia model. These findings indicate induction of angiogenesis, arteriogenesis and vasculogenesis by SN gene therapy. We could also show that SN increased other potent angiogenic factors in ECs (basic fibroblast growth factor and platelet-derived growth factor B) and stimulated the nitric oxide pathway (Schgoer et al. 2009).

In the heart SN gene therapy improved systolic function and inhibited scar formation and remodelling of the left ventricle after an experimental myocardial infarction (permanent LAD ligation). Again, density of capillaries and arterioles/arteries in the infarct border zone was increased, consistent with induction of angiogenesis and arteriogenesis also in this ischemia model.

In arterial coronary ECs SN inhibited apoptosis, stimulated proliferation and in-vitro angiogenesis and activated Akt and MAPK. Interestingly, in vitro effects were blocked by a neutralizing antibody against VEGF, indicating that SN effects depend on VEGF. We indeed could demonstrate that SN stimulates activation of VEGF receptor-2 in a receptor tyrosine kinase (RTK) array. Further experiments elucidated that SN stimulates binding of VEGF to its co-receptors neuropilin-1 and heparan-sulfate proteoglycans. In RTK assays also activation of receptors for FGF and insulin-like growth factor-1 (IGF-1) was observed by SN. This activation of several potent angiogenic growth factor receptors by SN might be the reason for the robust effect of SN we observed on growth of new blood vessels as a complex biological effects like angiogenesis probably is mediated by different factors (Albrecht-Schgoer et al. 2012). A similar effect was shown recently in airway epithelial cells where it was demonstrated that SN stimulates mucus secretion by enhancing binding of epidermal growth factor (EGF) to neuropilin-1 (Xu et al. 2014).

These observations also might indicate that SN acts via binding to growth factors thereby stimulating binding of these factors to and activation of respective tyrosine kinase receptors instead of acting via an own specific cell surface receptor. Indeed, no specific SN receptor was detected so far. In this respect it is also of interest that SN was considered to act via a G-protein coupled receptor (GPCR) in cell migration experiments as effects were blocked by pertussis toxin. It will be interesting to elucidate if, in analogy to RTK, also GPCRs are activated by SN via classical chemotactic factors.

8.3.2 Wound Healing

To investigate potential effects of SN gene therapy we investigated wound healing in diabetic mice (db/db mice). Application of SN accelerated wound closure in this model and increased density of capillaries and arterioles in the wound. In microvascular dermal endothelial cells SN stimulated proliferation and in vitro angiogenesis in a basic-FGF dependent manner. We could show that FGF receptor-3 mediates SN-induced effects and that SN stimulates binding of basic FGF to heparan-sulfate proteoglycans on dermal endothelial cells (Albrecht-Schgoer et al. 2014). This finding corroborates our observation on coronary endothelial cells that SN stimulates receptors of potent angiogenic cytokines.

8.3.3 Effects of SN on Cerebral Ischemia

Systemic (intra-venous) application of SN in a rat model of cerebral ischemia reduced infarct area, enhanced motor performance and increased brain metabolic activity. In ischemic areas of the brain in this animal model as well as in human samples after stroke SN was increased in neuronal cells. In vitro SN inhibited apoptosis of neurons in cell culture after oxygen/glucose deprivation by stimulation of the Jak/Stat pathway. In this work it was also demonstrated that SN enhanced growth of blood vessels in the ischemic brain area and attracted neuronal stem cells (Shyu et al. 2008).

8.3.4 Effects of SN on Cardiomyocytes

Recently it was demonstrated that SN is taken up by cardiomyocytes and influences Calcium (Ca^{2+}) handling in these cells by reduction of Ca^{2+} /calmodulin (CaM)-dependent protein kinase II δ (CaMKII δ) activity. SN binds to CaM and CaMKII and attenuates CaMKII δ -dependent phosphorylation of the ryanodine receptor. SN also inhibits sarcoplasmic reticulum Ca^{2+} leak and augments sarcoplasmic reticulum Ca^{2+} content. SN also attenuates Ca^{2+} sparks and waves in cardiomyocytes (Ottesen et al. 2015). These findings indicate that SN might have a potential to inhibit arrhythmias.

8.3.5 Effects of SN Gene Therapy on Animal Models of High Vascular Risk

Over the last two decades a variety of endothelial growth factors have been investigated as promising novel therapeutic agents for the treatment of peripheral arterial disease or myocardial ischemia. Unfortunately, the promising results obtained in animal models could not be verified in human trials so far. One reason might be that, in pre-clinical models, often young and healthy animals were studied, whereas in clinical trials patients with severe, long-lasting atherosclerosis and impaired vascular response were treated. Nevertheless, available data from clinical trials suggest that intramuscular injection of DNA might be safe and not associated with an increased rate of cancer. Due to promising results of SN gene therapy in hindlimb and myocardial ischemia we investigated the efficacy of SN gene transfer in two animal models (type I diabetes mellitus and hypercholesterolemia) with impaired angiogenic response (Schgoer et al. 2013; Theurl et al. 2015). In both animal models therapy of hindlimb ischemia with a SN-encoding plasmid resulted in significant improvement of limb reperfusion. Moreover, animals treated with the SN-plasmid showed a significant reduction in tissue defects and amputation rate. In the Apo E $-/-$ mice, a model of severe hypercholesterolemia, we also evaluated the effect of SN-gene therapy in myocardial ischemia induced by permanent ligation of the left anterior descending artery. Similar to our data in rats SN treatment resulted in

significant improvement of cardiac function as shown by echocardiographic parameters. Immunofluorescence staining revealed a significant increase of capillary and arteriole density as possible underlying mechanism for the favourable outcome of SN-treated animals.

Despite the negative influence of hypercholesterolemia and hyperglycemia on vascular cells, SN showed beneficial effects on EC function like proliferation, *in vitro* angiogenesis, or activation of the MAPK-ERK1/2 signaling cascade.

The local injection of the SN-plasmid did not result in elevated SN-serum levels and did not influence plaque progression as described for systemic administration of VEGF. Therefore, to our current knowledge, SN-gene therapy seems to be an effective and safe treatment strategy for hind limb and myocardial ischemia. Large animal models should be the next step to bring SN-gene therapy from bench to bedside.

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