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Review Article

Beneficial effects of spermidine on cardiovascular health and longevity suggest a cell type-specific import of polyamines by cardiomyocytes

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Recent and exciting *in vivo* studies show that supplementation with the polyamine spermidine (Spd) is cardioprotective and prolongs lifespan in both mice and humans. The mechanisms behind Spd-induced cardioprotection are supposed to involve Spd-evoked stimulation of autophagy, mitophagy and mitochondrial respiration and improved the mechano-elastical function of cardiomyocytes. Although cellular uptake of Spd was not characterized, these results suggest that Spd is imported by the cardiomyocytes and acts intracellularly. In the light of these new and thrilling data, we discuss in the present review cellular polyamine import with a special focus on mechanisms that may be relevant for Spd uptake by electrically excitable cells such as cardiomyocytes.

Introduction

In an impressive study, Eisenberg et al. showed that supplementation with the naturally occurring polyamine spermidine (Spd) is cardioprotective and prolongs lifespan in mice [1,2]. These authors also demonstrated that high intake of dietary Spd correlates with attenuated blood pressure and reduced incidence of cardiovascular disease in humans [1]. Recently, this group provided data in a human cohort of 829 individuals showing that high intake of Spd is associated with reduced mortality [3]. The possible mechanisms behind Spd-evoked cardioprotection are thought to involve direct cardiac effects such as increased autophagy and mitophagy which, in turn, leads to increased mitochondrial activity, reduced hypertrophy and enhanced titin phosphorylation and thereby improved mechano-elastical properties of the cardiomyocytes (Figure 1). Eisenberg et al. [1] propose that stimulation of autophagy by Spd may activate cardiac stem cells to produce new and functional cardiomyocytes. Spd supplementation also seems to have systemic effects involving lowered blood pressure and attenuated systemic inflammation demonstrated by reduced salt-induced hypertension and lowered levels of circulating TNF-α [1]. Treatment with Spd attenuates salt-induced hypertension in the Dahl salt-sensitive rat through a mechanism which is thought to involve elevated NO production accomplished by increased arginine bioavailability [1]. NO activates soluble guanylyl cyclase and PKG, the latter causing phosphorylation and activation of titin and thereby improved cardiomyocyte mechano-elastical properties. Reduced systemic levels of TNF-α is also regarded to increase titin phosphorylation via improved NO bioavailability. The exact molecular mechanism responsible for Spd-induced activation of autophagy in the cardiomyocytes is not known. Eisenberg et al. [1] showed that the cardiac tissue of mice supplemented with Spd is enriched in Spd, but they do not study and characterize uptake of Spd by cardiomyocytes, although it is assumed that cardiomyocytes from Spd-supplemented mice, rats and humans are enriched in Spd and that this polyamine acts intracellularly. Hence, it is of great importance to demonstrate that exogenous Spd is internalized/imported by cardiomyocytes and clarify the underlying molecular mechanism behind Spd import.

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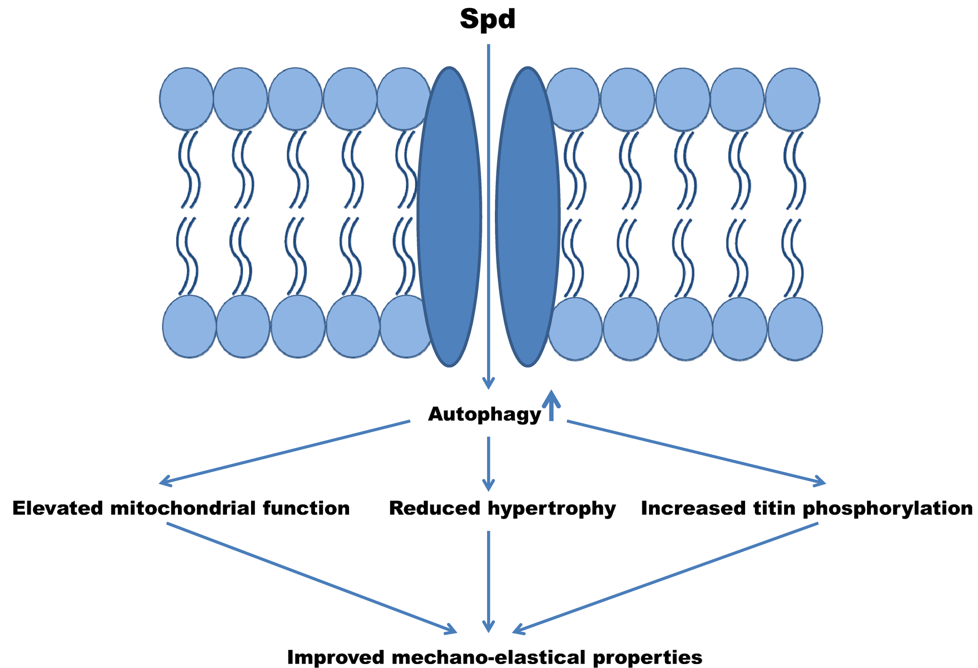

Figure 1. Schematic figure showing the proposed effects of exogenous Spd leading to improved mechano-elastical properties in cardiomyocytes as suggested by Eisenberg et al. [1].

Spd is internalized by the cardiomyocytes through a transporter, endocytosis or via diffusion through an unknown polyamine channel and acts intracellularly by stimulation of autophagy. Enhanced autophagy leads to elevated mitochondrial function, reduced hypertrophy and increased titin phosphorylation which in turn causes improved mechano-elastical properties.

Polyamine metabolism

Mammalian polyamines usually comprise putrescine, Spd and spermine (Figure 2). The first step in the biosynthesis of the mammalian polyamines is the conversion of ornithine into putrescine, which is catalyzed by the enzyme ornithine decarboxylase (ODC) [4]. Spd and spermine are produced by the sequential addition of an aminopropyl group from decarboxylated *S*-adenosylmethionine (dcAdoMet) to putrescine and Spd, catalyzed by the enzymes spermidine synthase (SPDSY) and (SPMSY), respectively. The enzyme *S*-adenosylmethionine decarboxylase (AdoMetDC) catalyzes the synthesis of dcAdoMet from *S*-adenosylmethionine. The substrate for polyamine production, ornithine, is derived from arginine in a step catalyzed by arginase (ARG1). Both ODC and AdoMetDC are strongly regulated and considered to catalyze rate-limiting steps in the biosynthesis of mammalian polyamines [4]. Difluoromethylornithine (DFMO) is a highly specific irreversible inhibitor of ODC. Addition of exogenous polyamines to mammalian cells rapidly down-regulates both ODC and AdoMetDC as part of a feedback control of cellular polyamine homeostasis, which may shift arginine metabolism from polyamine production to NO formation [5].

Spd may also be derived from the interconversion of spermine, which can occur via two different pathways (Figure 2) [4]. In one of the pathways, spermine is first converted into N^1-acetylspermine, a reaction catalyzed by spermidine/spermine N^1-acetyltransferase (SSAT), followed by an oxidative deamination catalyzed by a polyamine oxidase (PAOX) (Figure 2). Spd can similarly be converted into putrescine by the action of SSAT and PAOX. Like ODC and AdoMetDC, SSAT is a strongly regulated enzyme [4]. Spermine can also be converted into Spd in a single step catalyzed by spermine oxidase (SMOX). Unlike PAOX, which is considered to be a constitutive enzyme, SMOX is highly induced by a variety of stimuli [6].

Uptake of polyamines

Besides biosynthesis, polyamines may be provided through cellular uptake. The major extracellular sources for polyamines are polyamines synthesized by the intestinal microbiota and food-derived polyamines [7,8]. Polyamines are imported from the lumen of the gut to the portal circulation via the intestinal epithelial cells

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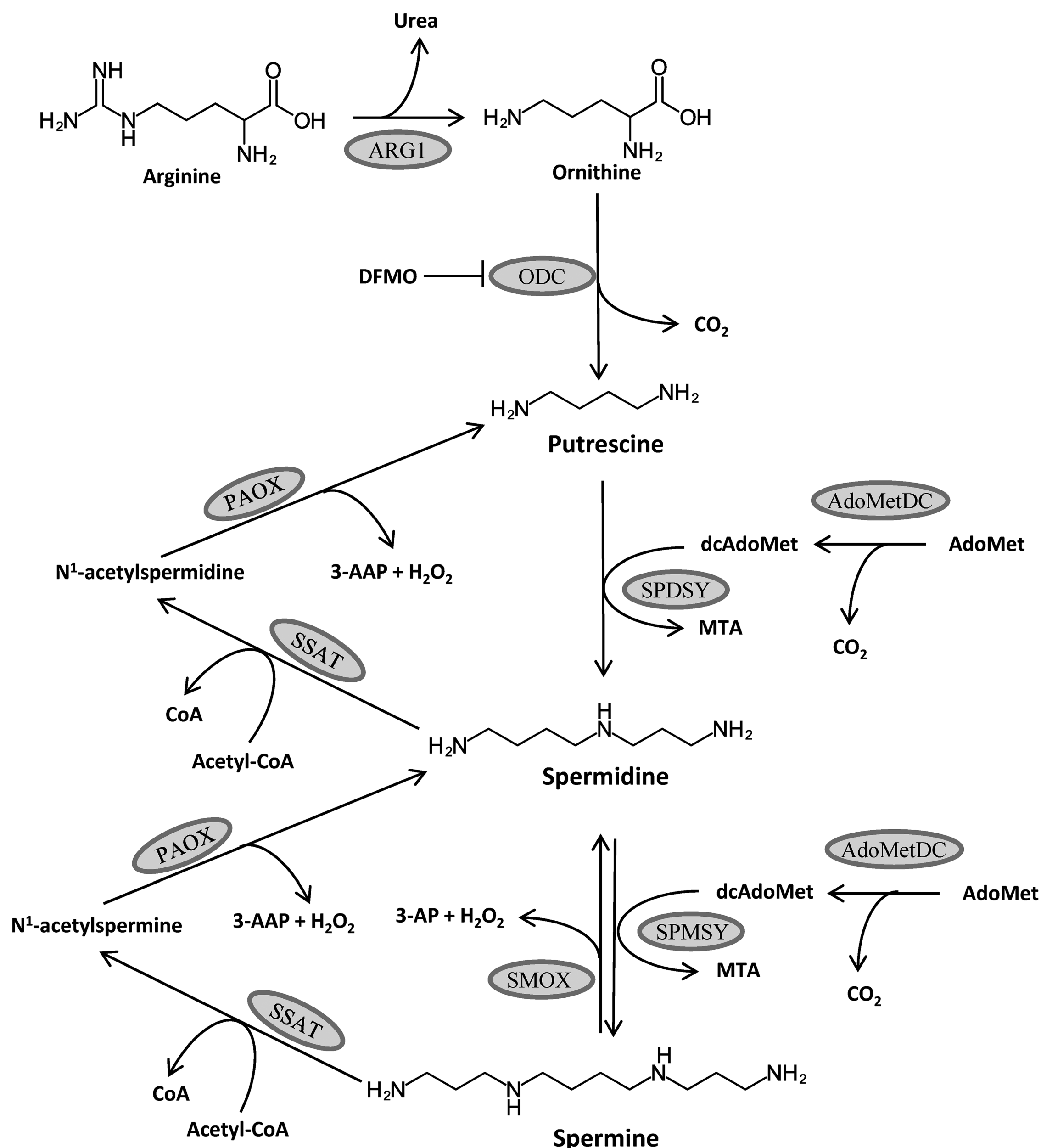

Figure 2. Metabolic pathway of polyamines.

ARG1, arginase; ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; SPDSY, spermidine synthase; SPMSY, spermine synthase; DFMO, difluoromethylornithine; AdoMet, S-adenosylmethionine; dcAdoMet, decarboxylated S-adenosylmethionine; MTA, methylthioadenosine; SSAT, spermidine/spermine N^1-acetyltransferase; CoA, coenzyme A; PAOX, polyamine oxidase; 3-AAP, 3-acetamidopropanal; SMOX, spermine oxidase; 3-AP, 3-aminopropanal.

through a process which is thought to be temperature-dependent but Na^+-independent [9,10]. For putrescine, intestinal diamine oxidase represents a barrier between the gut-lumen and the portal blood, but no such barrier seems to exist for Spd [11]. The concentration of Spd in whole blood is estimated to be ∼6 μM, and most of Spd occurs in blood cells while the plasma concentration of Spd is very low [12,13].

Polyamines are protonated at physiological pH which hampers passive transport over the plasma membrane. Instead, active transport of polyamines through polyamine transporters, characterized by energy and temperature dependence as well as saturation at low μM concentrations of polyamines, is suggested [14,15]. Putrescine

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and Spd import show different pH optimum with putrescine uptake being more sensitive than Spd uptake to acidification (pH 6.0–7.0) in human breast cancer cells, indicating that putrescine and Spd are imported through separate transporters in these cells [16]. In a study where the authors characterize a cation-Cl-cotransporter (*SLC12A8A*) in HEK-293 cells, they also showed that polyamine transport is independent of extracellular Na^+, K^+ and Cl^- [17]. In fact, polyamine transport was long believed to be a co-transport with Na^+ using the inward Na^+ gradient as the driving force, but later studies strongly argue that polyamine transport is Na^+-independent [15]. Different transporters for polyamines and molecular mechanisms for polyamine import have been proposed but the exact pathway is not understood. In Table 1, we summarize plausible polyamine transporters and mechanisms for polyamine uptake in mammalian cells as suggested in the literature. Polyamines have been suggested to be imported via caveolae which are Ω-shaped cholesterol-rich invaginations of the plasma membrane. Caveolin-1 has a critical role for both the structure and function of caveolae, and moreover, caveolin-1 seems to be involved in cell proliferation [18]. In intestinal epithelial cells, polyamine uptake has been shown to be regulated via a caveolin-1-dependent import via the solute carrier transporter *SLC3A2* (DAX) and in colon cancer cells uptake of polyamines is mediated via caveolin-1-regulated endocytosis, suggesting that caveolin-1 regulates polyamine uptake via different mechanisms [19,20]. Vascular smooth muscle cells lacking the caveolin-1 gene show higher uptake of polyamines than wild-type cells, suggesting that caveolin-1 negatively regulates polyamine uptake in these cells [21]. The enhanced uptake of polyamines in caveolin-1-deficient vascular smooth muscle cells is associated with increased expression of *SLC7A1* and *SLC43A1*. Polyamine uptake has also been suggested to be dependent on extracellular binding to the heparan sulfate proteoglycan glypican-1 and uptake via endocytosis [22–24]. However, uptake of polyamines is also observed in receptor-mediated endocytosis deficient CHO cell mutants, as demonstrated using fluorescence-labeled Spd, arguing that import of Spd is at least partly dependent on non-endocytosis-dependent mechanisms as well [25]. The identity of mammalian polyamine transporters is not known although members of the solute carrier transporter family (*SLC* gene family) have been suggested to facilitate polyamine uptake and represent polyamine transporters [15,26].

The solute carrier transporter (SLC) family and polyamine import

As mentioned above, the SLC family is suggested to be associated with the cellular import of polyamines acting as polyamine transporters [26]. The *SLC7A1* (CAT-1) transporter is proposed to mediate polyamine uptake in vascular smooth muscle cells [21]. *SLC7A1* belongs to the system y^+ that also includes *SLC7A2* (CAT-2A and CAT-2B) and *SLC7A3* (CAT-3) genes and this system is supposed to transport basic amino acids. The system y^+ is reported to import also polyamines but *SLC7A1* alone cannot be responsible for uptake of polyamines, but rather these different *SLCs* acting together and forming the y^+ system according to Sharpe and Seidel [27]. The rat *SLC22A1* (OCT1) transporter, expressed in Xenopus oocytes, transports polyamines via an electrogenic and pH-independent mechanism [28]. This transporter also uses cations, other than polyamines, and choline as substrates. In HEK-293 cells, *SLC12A8A* (CCC9A) has been shown to promote polyamine transport through a Na^+, K^+ and Cl^- independent mechanism which is inhibited by the antimicrobial agent pentamidine and the loop diuretic furosemide [17]. The carnitine transporter *SLC22A16* (CT2, OCT6) has been shown to mediate import of Spd via a high affinity ($K_m = 2.7 \pm 0.6\ \mu M$) and saturable ($V_{max} = 15.9 \pm 1.4$ pmol/10^7 cells/h) transport in HCT116 human colon cancer cells [29]. Thus, studies in various experimental systems suggest that not only one but many different SLCs may play a role in polyamine transport in mammalian cells.

Besides the different SLCs, ion channels have also been suggested to be responsible for polyamine transport [15], mainly because polyamine import seems strongly dependent on the resting membrane potential [16,28,30–32]. Another argument speaking for polyamine transport through channels is that polyamines are protonated at physiological pH and thus they are attracted by negatively charged molecules in the intracellular compartment. Intracellular polyamines have been shown to block inwardly rectifying K^+ channels which are crucial for setting normal electrical activity, implying that polyamines may be involved in modulation of cardiac excitability through this mechanism [33–35]. Furthermore, exogenous polyamines reduce inward Ca^{2+} currents through voltage-sensitive Ca^{2+} channels, attenuate intracellular Ca^{2+} concentration and cause relaxation of intestinal smooth muscle, suggesting that polyamines may act as plasma membrane Ca^{2+} channel blockers [36,37]. Down-regulation of intestinal smooth muscle cell Ca^{2+} channel activity is observed both when polyamines are applied from the outside and inside of the plasma membrane [37]. Polyamine-induced inward

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Table 1 Putative transporters and mechanisms responsible for polyamine uptake by mammalian cells

Transporter/mechanism	Cells/tissue/experimental model	References
Caveolin-1-dependent *SLC3A2*	Intestinal epithelial cells	[19]
Caveolin-1-regulated endocytosis	Colon cancer cells	[20]
Caveolin-1-dependent *SLC7A1* and *SLC43A1*	Vascular smooth muscle cells	[21]
Heparan sulfate proteoglycan glypican-1 and endocytosis	Fibroblasts, CHO cells, T24 bladder carcinoma cells	[22–24]
System y^+ amino acid carrier	Intestinal epithelial cells	[27]
Electrogenic and pH insensitive *SLC22A1*	Rat *SLC22A1* expressed in Xenopus oocytes	[28]
Na^+, K^+, Cl^- independent *SLC12A8A*	HEK-293 cells	[17]
Carnitine transporter *SLC22A16*	HCT116 colon cancer cells	[29]

rectification and inhibition of Ca^{2+} channel activity will presumably influence both cardiac excitability and systemic blood pressure. Thus, supplementation with dietary Spd, causing elevated levels of this polyamine in cardiac tissue as reported by Eisenberg et al. [1], may modulate the electrical activity and excitability of cardiovascular cells through these mechanisms.

Polyamine transport inhibitors (PTIs) have recently been synthesized showing low toxicity and high potency in inhibiting Spd uptake by DFMO-treated L3.6pl human pancreatic cancer cells [38]. In mouse aortic and human coronary vascular smooth muscle cells, these PTIs antagonize both basal and DFMO-stimulated polyamine uptake [39]. The PTI-induced down-regulation of polyamine import is associated with reduced proliferation and this effect seems potentiated when PTI is administered together with DFMO, suggesting that PTI and DFMO act in synergy [39,40]. It is an open question if these newly developed PTIs may hamper the beneficial effects of Spd supplementation observed in cardiomyocytes.

Driving force responsible for cellular polyamine import

What is the driving force for polyamine fluxes through polyamine transporters/channels over the plasma membrane from the extracellular to the intracellular space? This is a natural follow-up question after discussing possible polyamine transporters/channels and it is an important issue for the mechanistical understanding of polyamine transport over the plasma membrane. One possibility is that polyamines, through their cationic properties, act similar to monovalent and divalent cations and follow their electrochemical gradient. Depolarization of bovine lymphocytes using high extracellular concentration of K^+ (20 mM) is necessary to observe inhibition of polyamine import in the presence of the antibiotic compound valinomycin, indicating that the negativity of membrane potential is important for polyamine uptake [30]. Interestingly, these authors also demonstrated that the Ca^{2+} ionophore A23187, which allows Ca^{2+} to flow along its concentration gradient from the outside to the inside of the plasma membrane and thereby causes a strong depolarization, completely prevented polyamine uptake. The resting membrane potential in lymphocytes is −60 mV, whereas it is considerably more negative (−85 to −90 mV) in electrically excitable cells such as cardiomyocytes and skeletal muscle cells [41–43]. Hence, it may be argued that import of polyamines via a mechanism driven by the level of negativity of the membrane potential is more important in cardiac cells and skeletal muscle cells when compared with lymphocytes. Inhibition of polyamine uptake (both putrescine and Spd) was observed in human breast cancer cells subjected to high extracellular concentration of K^+ providing further evidence with the membrane potential drives polyamine import [31]. Strong evidence that the membrane potential and electrogenic mechanisms are involved in polyamine uptake was provided by Busch et al. [28] in Xenopus oocytes expressing the putative rat polyamine transporter *SLC22A1* (OCT1). They demonstrated that this transporter uses both Spd and spermine as substrate and that the uptake of polyamines is electrogenic and shows reduced activity upon depolarization. Polyamine uptake was characterized in cerebellar astrocytes by Dot et al. [32]. These authors showed that polyamines are imported via a single polyamine transport system which is saturable for putrescine ($K_M = 3.2\ \mu M$), Spd ($K_M = 1.8\ \mu M$) and spermine ($K_m = 1.2\ \mu M$) and not mediated via a co-transport with Na^+. Importantly, polyamine uptake by the astrocytes is antagonized by both the Na^+ ionophore gramicidin and the Ca^{2+} ionophore ionomycin implying that membrane potential is critical for polyamine import in these cells as well [32].

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Many reports conclude that polyamine transport systems are saturated with K_m for putrescine, Spd and spermine in the μM concentration range speaking for polyamine transport through transporters in the plasma membrane and against diffusion via a channel permeable for polyamines. Polyamines are relatively large when compared with ions representing another argument against polyamine diffusion through plasma membrane channels permeable for polyamines. However, it has recently been shown that the large and negatively charged ATP molecule is released through plasma membrane channels permeable for ATP [44]. There are indeed five groups of channels proposed as ATP-release channels. Moreover, ATP has a molecular mass of ~507 g/mol, i.e. higher than Spd (~145 g/mol). Hence, the relatively large size of the polyamine molecules may not be a very strong argument against the existence of cardiomyocyte channels permeable for polyamines allowing for inward polyamine transport.

Are polyamines imported by cardiomyocytes through channels using the electrochemical gradient as driving force?

Eisenberg et al. [1] showed *in vivo* that cardiomyocyte functional properties are improved by supplementation with Spd and that cardiac tissue levels of Spd are higher in mice given Spd for 4 weeks in the drinking water compared with control animals. These data suggest that exogenous Spd accumulates in the cardiac tissue and in the cardiomyocytes, indicating a preferential Spd uptake by cardiomyocytes. However, Spd is probably imported globally by all cells of the mouse, but perhaps uptake is more pronounced in cardiomyocytes than in other cells. Spd is most likely internalized/diffusing to the cytosol of cardiomyocytes of mice supplemented with Spd, but Eisenberg et al. [1] do not characterize the import of Spd. High-resolution microscopy of fluorescence-labeled Spd may reveal the intracellular distribution and trafficking of Spd imported by the cardiomyocytes.

Interestingly, it has been reported that heart tissue of both humans and rats contains low levels of Spd and spermine compared with, for example, pancreas, liver and spleen [45,46]. Skeletal muscle tissue, a tissue harboring electrically excitable cells similar to the heart, also shows low levels of polyamines compared with other tissues [45,46]. It is well recognized that polyamine contents are higher in rapidly growing tissues with high cell turnover than in tissues with cells showing no or low activity of cell proliferation [47]. Interestingly, it has been reported that polyamine formation, assessed by measuring ODC activity, is higher in the immature heart and decrease with age in rats [48]. Although these studies provide no information about the proportion of intracellular bound and free concentrations of polyamines, respectively, it is reasonable to suggest that mature cardiac and skeletal muscle tissues have relatively low intracellular concentrations of polyamines, and thus there may be a reasonable chance to build a concentration gradient for polyamines from outside to inside establishing a driving force for Spd from the extracellular to the intracellular compartment acting together with the inward electrical gradient for Spd.

Summary

Recent studies reporting beneficial effects of exogenous administration of the polyamine Spd on cardiovascular health and reduced mortality as a result of high intake of Spd-rich diet urge for more studies and information about the mechanism behind the uptake of Spd in electrically excitable cells such as cardiomyocytes. However, *in vitro* studies in isolated cells and cell lines, models where the cardiomyocytes lack their natural tissue-context and supply with neuronal and hormonal input, may not be sufficient to provide a complete answer to the complex matter on how polyamines are imported by these cells. Therefore, *in vivo* studies are probably necessary in order to establish the identity, underlying mechanisms and importance of polyamine uptake by cardiomyocytes. Here, we highlight that Spd may be imported by cardiomyocytes via a channel permeable for Spd and that the uptake of this polyamine is driven by the electrochemical gradient in these cells. Deepened and broadened knowledge on this important issue will probably provide targets which may be very interesting for future pharmacological intervention.

Abbreviations

AdoMetDC, *S*-adenosylmethionine decarboxylase; ARG1, arginase; dcAdoMet, decarboxylated *S*-adenosylmethionine; DFMO, difluoromethylornithine; MTA, methylthioadenosine; ODC, ornithine

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decarboxylase; PAOX, polyamine oxidase; PTIs, polyamine transport inhibitors; SMOX, spermine oxidase; SPDSY, spermidine synthase; SPMSY, spermine synthase; SSAT, spermidine/spermine N^1-acetyltransferase.

Author Contribution

B.-O.N. and L. P. planned and designed this manuscript and wrote the paper together.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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