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EXPLORING THE ROLE OF PHOSPHODIESTERASE PDE5 INHIBITION IN THE TREATMENT OF MUSCULAR DYSTROPHY

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SUMMARY

Duchenne muscular dystrophy (DMD) is caused by a mutation of a single gene, dystrophin, and is characterized by a slow progression and the increased turnover of myofiber degeneration and regeneration. Despite the long history of scientific research, the full mechanisms of muscle damage still need to be clarified and the therapeutic choices are limited. Recent studies on blood flow regulation have led to the discovery of a novel pharmacological treatment for muscular dystrophies. Phosphodiesterase PDE5 inhibitors were originally designed to improve blood circulation by inducing vasodilation in target organs and are widely used to treat male erectile dysfunction. Recently, the range of potential clinical applications for PDE5 inhibitors has widened, with increasing reports of their efficacy against various conditions, including pulmonary hypertension. The potential efficacy against DMD was reported in animal studies using the murine equivalent model. In this review, I discuss the recent understanding of the role of blood flow regulation in the pathogenesis of muscular dystrophy. Therapeutic approaches with PDE5 inhibitors will be discussed and include citations from the recent publications with animal studies. Future considerations of PDE5 inhibitor therapies will be examined, including the evaluation of drug efficacy and potential side effects.

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BRIEF HISTORY OF PHOSPHODIESTERASE PDE5 INHIBITORS

The discovery by Furchgott (1), Ignarro (2), Murad (3) and Moncada (4) that nitric oxide (NO) is a vasodilating agent released from endothelial cells led to the finding that various cell populations produce NO, which exerts a variety of cellular functions. NO increases the intracellular level of cyclic guanosine monophosphate (cGMP) (5) in target cells and leads to the activation of cGMP-dependent protein kinase 1 (cGK1; also known as protein kinase G, PKG) (6). This chain of signal transduction is responsible for the relaxation of the vascular smooth muscle cells and vasodilation (7, 8). Phosphodiesterases (PDEs) counteract this reaction by decreasing the amount of cGMP. Among the 11 PDE families so far identified (9), PDE5 resides in the lung, the skeletal/cardiac muscles and the penis (corpus cavernosum), and is responsible for modulating blood flow in these organs (Table I).

Sildenafil was first designed to target PDE5 in the cardiovascular system with the aim of alleviating the symptoms of ischemic heart disease, but its unexpected beneficial effects on male erectile dysfunction (ED) led to this becoming its leading clinical application (10). Following the commercial success of sildenafil, other PDE5 inhibitors have subsequently been designed (11-13), some of which have been approved for the treatment of ED, including tadalafil and vardenafil (14). It was only some time later when the drugs' beneficial effects on circulation were exploited in other tissues and tests were conducted for diseases other than ED, including pulmonary hypertension (15-17).

Recently, several reports have suggested the efficacy of PDE5 inhibitors in certain types of muscular dystrophy (18-20). Details of these studies will be reviewed in the sections below.

Table I. *Isoforms of phosphodiesterases, their substrate specificity and distribution patterns (modified from previous reports [115, 169, 170]).*

Isoform	Substrate	Tissue distribution	Selectivity of PDE5 inhibitors (IC_{50} nmol/L)		
			Sildenafil	Vardenafil	Tadalafil
PDE1	cAMP, cGMP	VSMC, CM, Br	280	70-180	> 30,000
PDE2	cAMP, cGMP	VSMC, CM, Br, CC	> 30,000	6,200	> 100,000
PDE3	cAMP, cGMP	VSMC, CM, Br, CC, Pl	16,200	> 1,000	> 100,000
PDE4	cAMP	VSMC, CM	7,680	6,100	> 100,000
PDE5	cGMP	CC, VSMC, SkM, CM, Pl, lung artery	3.5	0.14	6.7
PDE6	cGMP	Retina	34-38	0.6-3.5	1,260-1,300
PDE7	cAMP	Immune cells, EC, other	> 20,000	> 30,000	> 100,000
PDE8	cAMP	Immune cells, other	> 20,000	> 30,000	> 100,000
PDE9	cGMP	various	2,610	581	> 100,000
PDE10	cAMP, cGMP	Br, other	9,800	> 3,000	> 100,000
PDE11	cAMP, cGMP	SkM, VSMC, other	2,730	162	37

VSMC, vascular smooth muscle cells; CM, cardiomyocytes; Br, brain; CC, corpus cavernosum: Pl, platelet; SkM, skeletal muscle; EC, endothelial cell. Modified from Table-1. "Current Pharmaceutical Design (2006, 12, 3485-3494)", with permission from Bentham Science Publishers Ltd.

DUCHENNE MUSCULAR DYSTROPHY

The first academic descriptions of muscular dystrophy were published in the mid-19th century (21-23). Among the many types of muscular dystrophies, Duchenne muscular dystrophy (DMD) (21) is the most common form, affecting 1 in 3,500 male births (24), and has been most extensively investigated. In this review, discussion will focus mainly on DMD, although other types of muscular dystrophy will also be considered in the final section.

Patients with DMD lack dystrophin, which is mainly expressed in myofibers (25). Lack of dystrophin and its associated molecules in myofibers is thought to cause: 1) an abnormality in physical properties of the sarcolemma ("membrane vulnerability theory") (26-30); 2) a defect in blood flow response in the muscle tissues ("blood flow theory") (31, 32); and 3) abnormal calcium mobility ("calcium theory") (33, 34), among many other potential consequences (Fig. 1). The evidence to support each of these theories is increasing, and all three seem to be essential in the pathogenesis of DMD. How muscle cells die and due to which mechanism will depend on the types of stress encountered by a particular muscle. When muscles are subjected to a strenuous contraction, muscle membranes may not tolerate the mechanical stress, or oxygen supply to the tissue may be insufficient due to a poor blood flow response. When a patient physically hits a muscle against a hard obstacle, the vulnerability of the membrane will result in the membrane breach. On other occasions, it is possible that abnormal calcium leakage into the cytosol can directly lead to detrimental intracellular signals. The blood flow theory will be the main focus of this review, and is explained in detail in the following section.

The clinical onset of disease occurs around 2-5 years of age, when symptoms of muscle weakness become prominent (35). Myofiber damage is, however, continuously upregulated in DMD patients (or in the murine *mdx* model) beginning immediately after birth (36-39), or even before birth (40, 41). Even during the latency period in early life, when muscle weakness is not apparent, the disease progress is not quiescent; muscle damage continues to occur, as reflected by constantly elevated levels of serum creatine kinase (CK) (39), the enzymatic marker of muscle damage (42). The reason for the chronological discrepancy between enduring muscle damage and the late onset of clinical symptoms will be discussed in the sections below.

Clinical therapeutic choices are still limited for DMD and most of the established remedies can be considered supportive care.

Blood flow studies in DMD

There has been a long history of debate over whether a blood flow abnormality exists in subjects with muscular dystrophy. Duchenne (21) and Meryon (23) briefly discussed circulatory issues. Griesinger observed altered skin color and temperature of the affected site, especially during the patients' effort for muscle contraction (22). The atypical coloration and temperature of the patients' skin were later confirmed (43). More recent studies showed a pathological trait of muscular dystrophy involving perivascularly clustered distribution of muscle damage, suggestive of the involvement of vascular mechanisms in the pathogenesis of muscular dystrophy (44). Another line of evidence came from histological studies of the intramuscular vasculature. Excessive calcification (45), thickening of the endothelium of arterioles (46-50), platelet aggregation (48) and irregularity of the vascular wall (51, 52) were observed in the muscles of several patients with muscular dystrophy. Physiological experiments reported alterations in the contractility and reactivity of vascular smooth muscles in subjects with muscular dystrophy (53-59). Conflicting lines of evidence exist, however, supporting both the hypotheses of aberrant (60, 61) and unchanged (62, 63) blood flow regulation in muscular dystrophy. Such a dispute over the existence of a blood flow abnormality in DMD was finally put to an end by the discovery of "functional ischemia" in DMD patients and the murine equivalent, *mdx* mice (31, 32).

What is functional ischemia?

In response to a contractile workload, blood flow increases in normal muscles to meet increased muscular metabolic demand (64). However, when this blood flow response is attenuated, the muscles are at risk of ischemia, either due to a lack of a sufficient supply of oxygen and nutrients or to insufficient drainage of the accumulated

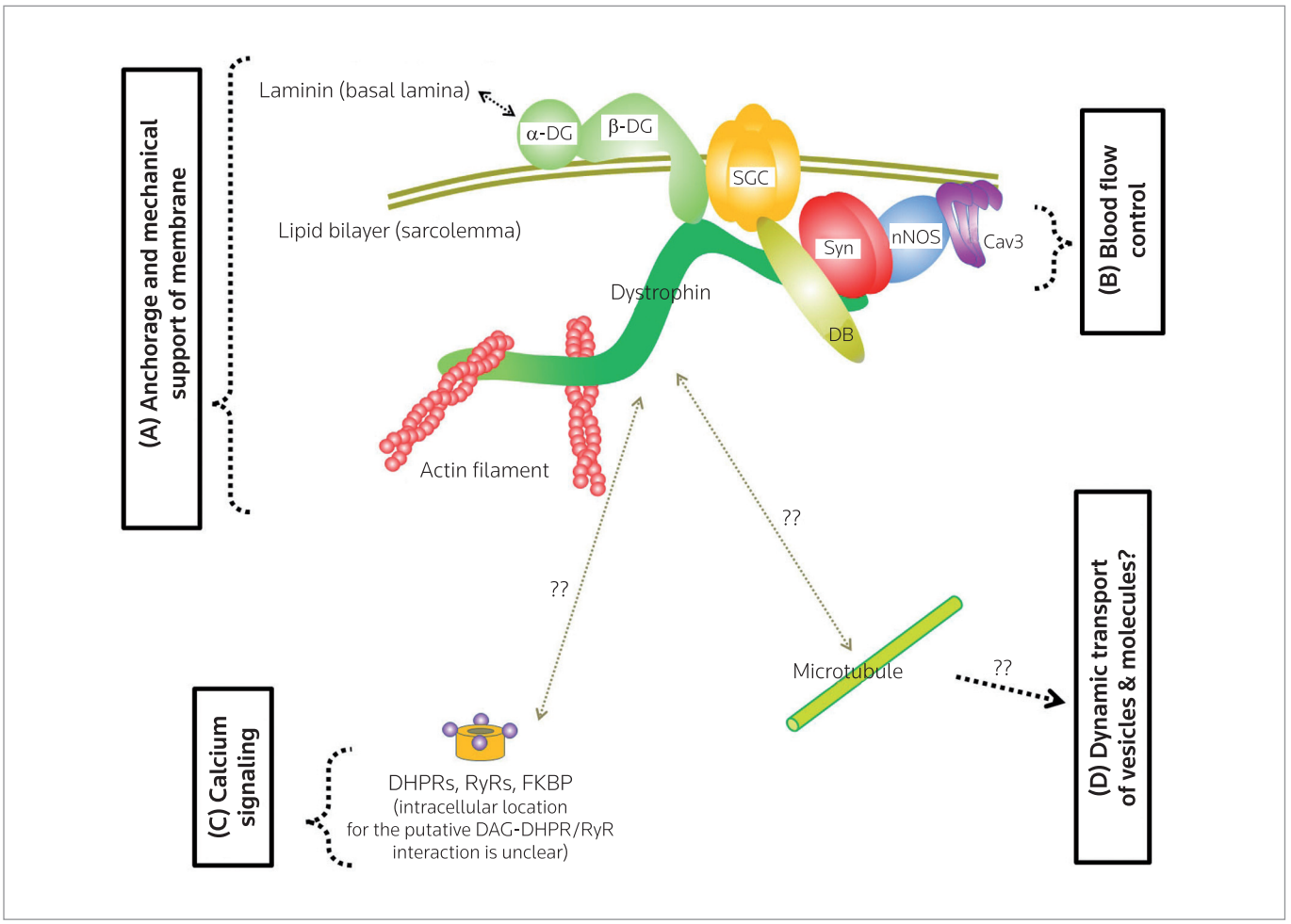


Figure 1. Schematic drawing of dystrophin-associated glycoprotein (DAG) complex and the presumed physiological roles it plays in normal muscles. This scheme is based on previously documented models of the DAG complex (71, 74). When dystrophin is absent in Duchenne muscular dystrophy (DMD), the physiological roles of the DAG complex shown in the figure are supposed to become defective. (**A**) The entire complex serves to link the actin cytoskeletal network to the sarcolemma and to the extracellular matrix, and is thought to maintain the mechanical strength of the myocyte membrane. Dystrophin is anchored to the membrane via β-dystroglycan (β-DG). β-DG is associated with the sarcoglycan complex (SGC). Extracellular matrix, or laminin, binds to α-dystroglycan (α-DG). (**B**) Neuronal nitric oxide synthase (nNOS) and its regulator caveolin-3 (Cav3) are bound to the complex at the *C*-terminus of dystrophin via syntrophins (Syn) and dystrobrevin (DB). nNOS is supposed to be responsible for many physiological regulations, including blood flow control. (**C**) Although the detailed interaction mechanism remains unclear, accumulating evidence suggests a functional, biochemical and immunohistological linkage between the DAG complex and dihydropyridine receptors (DHPR)/ryanodine receptors (RyR)/FK506-binding protein (FKBP), arguably the basis for the role of the complex in calcium signaling. (**D**) Recently, it has been shown that the microtubule network is associated with the complex (78). Microtubules are generally involved in the dynamic transport of vesicles and molecules..

metabolites. This pathological state is known as "functional ischemia" (31, 65). Ischemia is defined as the state of blood flow decrease due to structural vascular obstruction or vasoconstriction. Functional ischemia is a state in which blood flow cannot meet the metabolic demand of tissues despite the absence of vascular obstruction. In both cases, the balance between the demand and supply of blood flow is disturbed. What had long been unknown, however, was whether functional ischemia is the actual cause or a secondary result of muscular dystrophies.

Conventionally, macroscopic blood flow measurement documenting a "functional ischemia" demonstrated that under the stress of vasoconstrictors, the muscles are prone to a decrease in blood flow even during muscle contraction (31, 32) (lack of sympatholysis; Fig. 2A). The functional ischemia theory was further advanced by a recent in vivo microscopic study (18) that enabled a clear quantification analysis by a simple stimulation scheme (Fig. 2B), and demonstrated that functional ischemia is an essential cause of contraction-induced myofiber damage, and thus the cause of DMD. Briefly, normal contracting muscles require an increase in blood flow to meet metabolic demands. This response is lacking in *mdx* mice, leading to an increased risk for contraction-induced myofiber damage. When functional ischemia was abolished by pharmacological intervention,

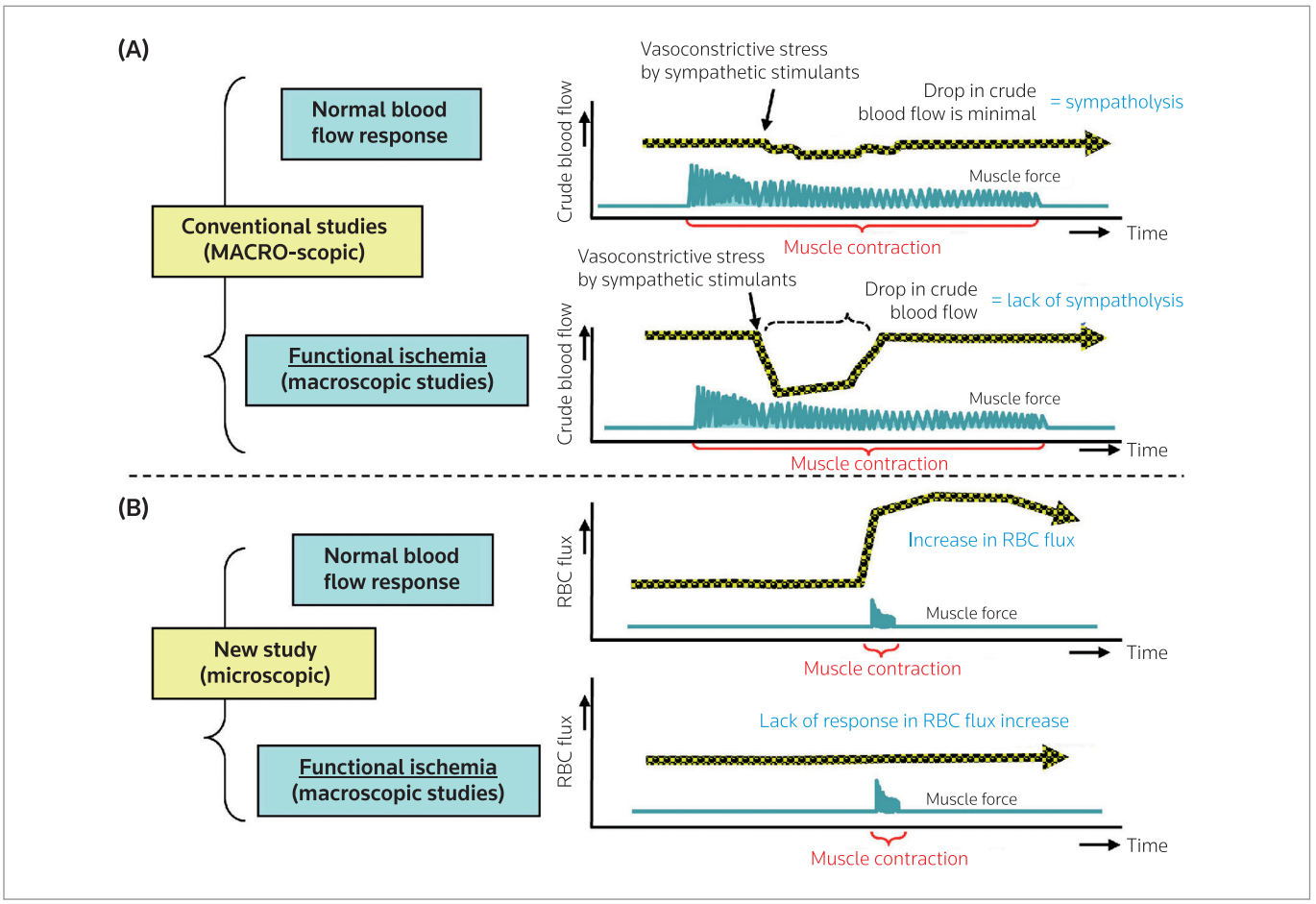


Figure 2. Illustration of functional ischemia by a conventional (MACRO-scopic) and by a new (microscopic) approach. (**A**) In order to observe functional ischemia, the original methods for measuring MACRO-scopic blood flow necessitated the muscles to be put under vasoconstrictive stress by sympathetic stimulants. Skeletal muscle contraction exempts the myofiber from the drop in the crude blood flow in normal muscles (a normal response of sympatholysis). Sympatholysis is absent in the affected muscles, where normal vascular regulation mechanisms are lacking. Thus, normal muscles are rescued from ischemia by sympathetic vasoconstriction when superimposed by contractile stress, but diseased muscles are put under the risk of ischemia. (**B**) In the new microscopic study, on the other hand, superimposition of muscle contraction with vasoconstrictive stress is unnecessary to observe functional ischemia. Red blood cell (RBC) flux (the number of RBCs passing by at the point of observation per unit time) increases after muscle contraction in normal subjects. This increase in RBC flux is perturbed in *mdx* mice. This observation is reasonable considering our understanding that the parameters from crude macroscopic blood flow measurement do not necessarily reflect peripheral (microscopic) RBC flux (165). Modified from PLoS One (18).

myofiber damage in *mdx* mice was almost completely prevented in an in vivo acute cell death experiment (18)[1]. Thus, functional ischemia is considered necessary as a cause of post-contraction myofiber damage. This finding is supported by a previous transgenic study that demonstrated that a vascular smooth muscle-specific overexpression of dystrophin reverted functional ischemia and ameliorated the muscular dystrophy phenotype of *mdx* mice (66). These lines of evidence support future therapeutic approaches using vasoactive molecules.

[1]Functional ischemia is supposed to derive from lack of neuronal nitric oxide synthase (nNOS) expression in the sarcolemma (see below), and thus it will be difficult to completely cure functional ischemia in a long-term experiment. In this study, three different vasoactive reagents (NO donor, β_2-adrenoceptor agonist and cGMP analogue) were applied separately, immediately after tetanic contraction was imposed, where otherwise functional ischemia would follow. Then, the extent of cell death attributable to the experimental stimulation was quantified and compared to the blood flow response. As a result of this short-term experiment (lasting up to 6 hours), cell death induced specifically by the contraction and functional ischemia was abolished (18).

Two-hit mechanism in DMD myofiber damage

The initial proposal hypothesizing a systematic theory of blood flow in DMD was met with controversy (44). Nor was the idea of vascular therapy in DMD readily accepted. Until recently, typical criticisms were as follows: 1) DMD myofibers are affected by an inherent fragility, and thus blood flow intervention cannot possibly cure such weakness of the myofiber; and 2) previous data suggest that blood flow abnormality to an extent similar to that seen in DMD patients does

not seem to cause prominent myofiber damage in control subjects. Therefore, blood flow abnormality cannot be the main cause.

The logical gap between the advocates and critics of the blood flow theory was filled by the "two-hit" concept (67)[2], which was quantitatively demonstrated by an in vivo comparative analysis between the blood flow response and the extent of myofiber damage (18). From these studies, it became clear that functional ischemia is one of the *necessary* causes of myofiber damage in *mdx* mice, but it alone is not a *sufficient* cause. Another *necessary* mechanism, which will be referred to operationally as "inherent weakness", confers myofiber susceptibility to damage. Only when both of these factors are involved do DMD subjects show myofiber damage (Fig. 3). This unappreciated complexity in the pathogenesis of DMD was one source of confusion in earlier days. The implication of this finding is significant in the development of therapeutics for DMD. Although interventions targeting the "inherent weakness" are challenging, there is a whole spectrum of vasoactive agents that improve the circulation disturbance. Appropriate drug administration can ease the

[2]As originally defined, the "two-hit" mechanism refers to the genetic process of oncogenesis or of developmental diseases where mutations of two (or multiple) genes are necessary to cause the disease (68, 69), but in this review, this term will be used in a broad meaning, also covering the pathogenesis of DMD. DMD is a disease with a single gene mutation of dystrophin, but the loss of functions of dystrophin disturbs at least two pathophysiological mechanisms. A recent study has concluded that one of them is functional ischemia (18). The other hit(s) remain to be identified.

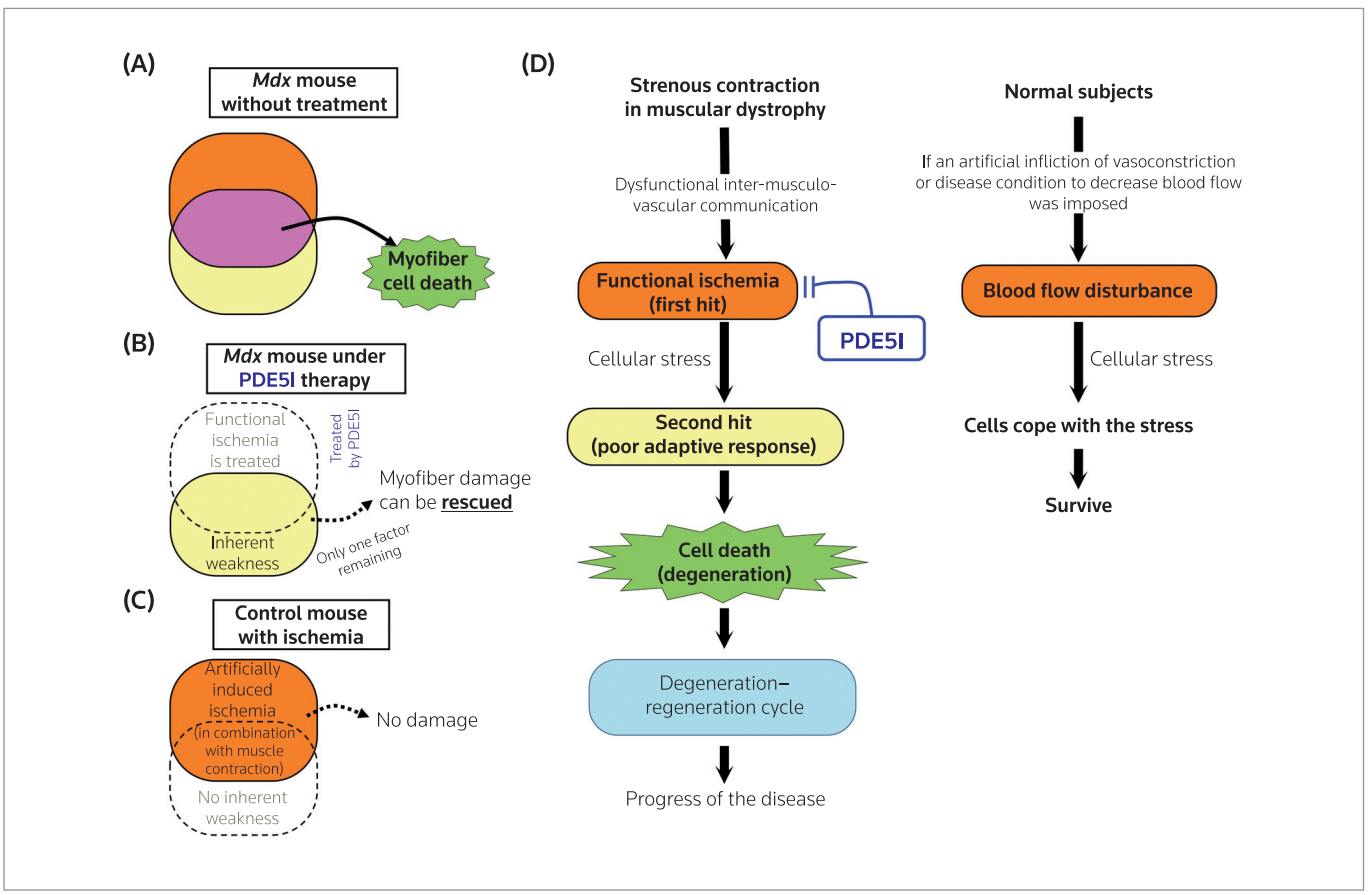


Figure 3. Schematic drawing of the relationship between functional ischemia and myofiber damage. Functional ischemia is thought to be a necessary but insufficient primary cause to induce myofiber damage. (**A**) In the *mdx* mouse without treatment, skeletal muscles are affected by both functional ischemia and inherent weakness, leading to contraction-induced myofiber damage. (**B**) Even if *mdx* myofibers are affected by inherent weakness, contractile stress does not kill the cell without functional ischemia. Thus, functional ischemia is an essential cause of cell death. In other words, if functional ischemia in the *mdx* mouse or Duchenne muscular dystrophy (DMD) patient is treated by a vascular therapy, myofiber damage can be prevented. (**C**) In the control mouse, even if the same extent of ischemia and contractile stress is inflicted, it does not lead to myofiber damage. This means that without the inherent weakness, a myofiber does not die from functional ischemia and under contractile stress. (**D**) The published experimental data currently available do not show which of the two hits are upstream and downstream. However, it is generally accepted that ischemic stress leads to a poor oxygen supply to the cell and causes mitochondrial disruption, oxidative stress, ATP depletion and disturbance of ionic homeostasis. Normal cells can tolerate these stresses. This adaptive response, however, is presumably missing in mdx mice. On the other hand, it is well accepted that the sarcoplasmic membrane of DMD/*mdx* muscle cells is inherently vulnerable. It is possible that this membrane vulnerability is the other hit. (*Note: Since functional ischemia seems to derive from the constitutive lack of nNOS from the sarcolemma, complete blockade of functional ischemia will be difficult. PDE5 inhibitors are likely to only improve the overall blood flow in the muscle tissue, and reduce the cellular stress imposed by functional ischemia)*. Modified from PLoS One (18).

cellular stress imposed by functional ischemia and delay progression of the disease[3].

Vasoregulatory role of NO in muscular dystrophy

When dystrophin is expressed in myofibers, it forms a scaffold underneath the sarcolemma, linking the membrane-bound dystrophin-associated glycoprotein (DAG) complex to other molecules (Fig. 1), many of which are related to cytoskeletal roles (70-74). Bolting of cytoskeletons to the sarcolemma is considered to form the basis for support of the muscle membrane, presumably by providing mechanical reinforcement. In addition to the scaffolding role of dystrophin in the cytoskeleton, recent evidence has suggested that regulatory proteins, including nNOS (75), caveolin-3 (76), or calcium/calmodulin-dependent protein kinase (CaMK) (77) interact with the dystrophin– DAG complex, and that dystrophin may mediate regulatory responses in the muscle. Recently, it has been demonstrated that cytoskeletal networks consisting of microtubules interact with dystrophin–DAG (78). This finding suggests that this molecular complex may be involved in the transport of various regulatory molecules and vesicles, in addition to providing mechanical fortification of the plasma membrane.

The sarcolemmal expression of nNOS is absent in DMD/*mdx* mice (75, 79). When the lack of nNOS is reverted by a transgenic overexpression, some of the pathological traits in *mdx* mice are ameliorated, indicating the key role of nNOS in pathogenesis (80, 81). In healthy subjects, muscle contraction increases local blood flow via the production of NO from myofibers, which is used for musculovascular communication. The post-contraction NO increase is, however, perturbed in *mdx* mice, leading to functional ischemia. Reversal of functional ischemia by applying exogenous NO or a cGMP analogue prevents the contraction-induced myofiber damage. It has also been demonstrated that the myofiber-protective effect of cGMP is indeed related to blood flow control (18).

MUSCULOVASCULAR COMMUNICATION

The role of NO in vasodilation was originally studied by evaluating the intercommunication between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), based on the ex vivo experimental model involving these two cell populations (1). In this context, ECs producing NO are considered upstream and VSMCs, the target cells of NO, downstream of this vasodilatory signal. Recently, a wide spectrum of different cell populations has been known to produce NO and induce vasodilation, including nonadrenergic, noncholinergic (NANC, or nitrergic) neurons in the brain and other tissues (82, 83), astrocytes (84) and skeletal myocytes (18), in addition to ECs. VSMCs also produce NO (85).

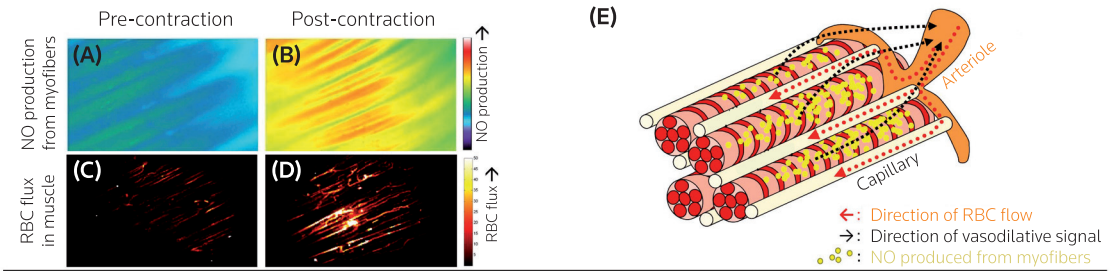
NO produced from myofibers appears to be another upstream signal in contraction-induced vasodilation, given that NO is produced from skeletal myofibers in response to muscle contraction (18) and that myofiber-specific knockout of nNOS perturbs the exercise-induced blood flow response (20). This finding led to the concept that musculovascular communication is essential in regulating the tissue microcirculation in skeletal muscles. The remaining question is how the signal initiated by the NO released from myofibers is conveyed to the artery or arteriole that supplies the blood flow to the muscle. It may be possible that released NO goes into the bloodstream, where it binds hemoglobin or carriers, circulates through the systemic blood flow and comes back to the skeletal muscle tissue. Although this model may have biological significance in a certain context, it will not impact the local microcirculation in a tissue-specific manner. Considering that local myofiber contraction increases the red blood cell flux only on the side of contraction, but not on the contralateral side (18), a more precise control model will be needed that accounts for the specialized musculovascular communication.

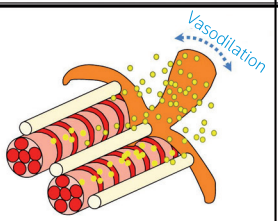
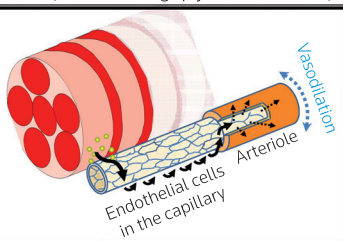
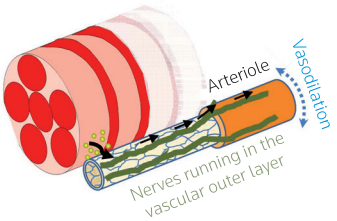
There are several feasible models (Fig. 4) of musculovascular communication. First, it is possible that NO produced from myofibers diffuses into the local vicinity, and directly reaches the arterioles and arteries (hypothesis 1 in Fig. 4F). Considering that NO is a gas molecule, this model is appealing, but by this mechanism, the released NO can control vasodilation only in a nonspecific manner, and the diffusion will take a considerable amount of time for the arterioles to respond to the signal. Secondly, it may involve the endothelial gap junction mechanism, as is arguably suggested for the regulation of vasomotion (86). In this model, signals initiated by myofibers will be transmitted to the nearest endothelial cell and the vasodilatory signal is conveyed through the EC-to-EC gap junction (hypothesis 2 in Fig. 4G). When the signal ascends to the arterioles or arteries, the EC underneath will release the signal, causing the adjacent VSMCs to dilate. Although accumulating evidence demonstrates that this model is essential in maintaining vascular tone, application of the same model to musculovascular communication raises a few questions: How do ECs control the direction of their signal? Why does the signal initiated from outside the blood vessels have to go to the innermost layer, and then come back to the middle layer? The third model predicts that nerves sitting on the outmost layer of blood vessels receive the NO signal released from myofibers and conduct the vasodilative signal to arterioles that feed the capillaries involved (hypothesis 3 in Fig. 4H). Although this model seems most specific and economical, there is no such nerve yet characterized in detail that controls contraction-induced vasodilation in skeletal muscles. In all of these models, the signal will ultimately reach the VSMCs in the arteries and arterioles. Thus, the target cell population of PDE5 inhibitors, when administered systemically, will include VSMCs. Depending on the models, however, PDE5 inhibitors may also exert their effect on ECs and the nerves. PDE5 inhibitors will counteract the PDE5 in these target cells, helping to increase the intracellular level of cGMP and causing vasodilation.

OTHER POSSIBLE MECHANISMS OF PDE5 INHIBITOR MYOFIBER PROTECTION

Most of the anti-cell death effect of cGMP seems to be mediated by its blood flow-modulating effect, especially in the acute in vivo experimental condition for tetanic contraction-induced skeletal myofiber cell death (18). However, it does not exclude the possibility that other mechanisms are involved in the PDE5 inhibitor-mediated muscle protection under different stress conditions, or in a more chronic fashion. PDE5 inhibitors have various in vivo effects other than the vasodilating

[3]Even with PDE5 inhibitors, it will be theoretically difficult to completely cure functional ischemia. PDE5 inhibitors are, however, expected to improve blood circulation and reduce the stress imposed by an insufficient blood supply.



(F) Hypothesis-1 (diffusion mode)	(G) Hypothesis-2 (endothelial gap junction model)	(H) Hypothesis-3 (neuronal transmission model)
		

Target of NO released from myocyte	Arterioles/arteries	Endothelial cells	Nerves
Target cell of PDE5Is	VSMC	EC (and VSMC)	Nerve (EC and VSMC)
Caveat	Non-specific regulation Not economical Too slow responses	Do the ECs "know" which direction to convey the message, especially at the bifurcation junctions? Why outside >> inside >> outside?	Such nerves not identified, not characterized

Figure 4. Possible models of musculovascular communication. (**A**) + (**B**) After tetanic contractile stimulation, skeletal myofibers produce nitric oxide (NO) (fluorescent image of a reporter dye, DAF-FM, with pseudocolor added; warm color pointing to increased production of NO). (**C**) + (**D**) After tetanic stimulation, microcirculation in the skeletal muscle tissue increases (pseudocolor is added with brighter color pointing to increased red blood cell [RBC] flux in the capillaries and arterioles). (**E**) In order for the NO released from myofibers to increase the blood flow, the signal must be transmitted in the opposite direction to that of the blood flow. (**F**) Hypothesis-1 suggests that the produced NO diffuses and reaches the arterioles that feed the blood flow to the muscle. (**G**) Hypothesis-2 assumes that the NO released from a myofiber reaches the adjacent endothelial cells (ECs). Then, ECs transmit the signal via the EC-to-EC gap junction. When it arrives at the level of the arteriole, the EC stimulates vascular smooth muscle cells (VSMCs). (**H**) Hypothesis-3 predicts that the NO from a skeletal myofiber stimulates the perivascular nerves. Then, the signal is transmitted via the nerve to the VSMCs at the level of the arterioles. Modified from PLoS One (18).

function discussed above. Given that administration of PDE5 inhibitors is known to have beneficial "preconditioning" (87) and "post-conditioning" (88) effects against ischemia–reperfusion injury in cardiac muscles, both acute and chronic effects may also work in concert in skeletal myofiber protection. To support this assumption, there have been accumulating studies linking the cell protective function of PDE5 inhibitors to their direct effect on the various cellular signals (see below; summarized in Fig. 5).

Although the compartmentalization of cGMP, or the detailed intracellular localization of where it is produced, seems to affect the downstream signals, it is generally accepted that an increased level of intracellular cGMP activates the pivotal kinase PKG (89). In cardiomyocytes, cGMP/PKG restricts calcium mobilization by inhibiting the L-type calcium channel ($Ca_v1.1$) (90). cGMP/PKG also phosphorylates phospholamban (91), the putative regulator of cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (92, 93), possibly leading to reuptake of calcium into the sarcoplasmic reticulum. Thus, the cGMP/PKG pathway may ameliorate calcium overload, preventing muscle contracture, myocyte overwork and calpain activation, among many other detrimental signals.

Increases in cGMP and PKG activation are known to protect cardiac myofibers from cell death via extracellular signal-regulated kinase ERK-1/2, protein kinase PKCε, protein kinase Akt and glycogen synthase kinase GSK-3β phosphorylation (94, 95). These signals are supposed to activate mitochondrial K_{ATP} channels, produce reactive oxygen species (ROS) and inhibit the permeability transition pore (96), leading to mitochondrial protection.

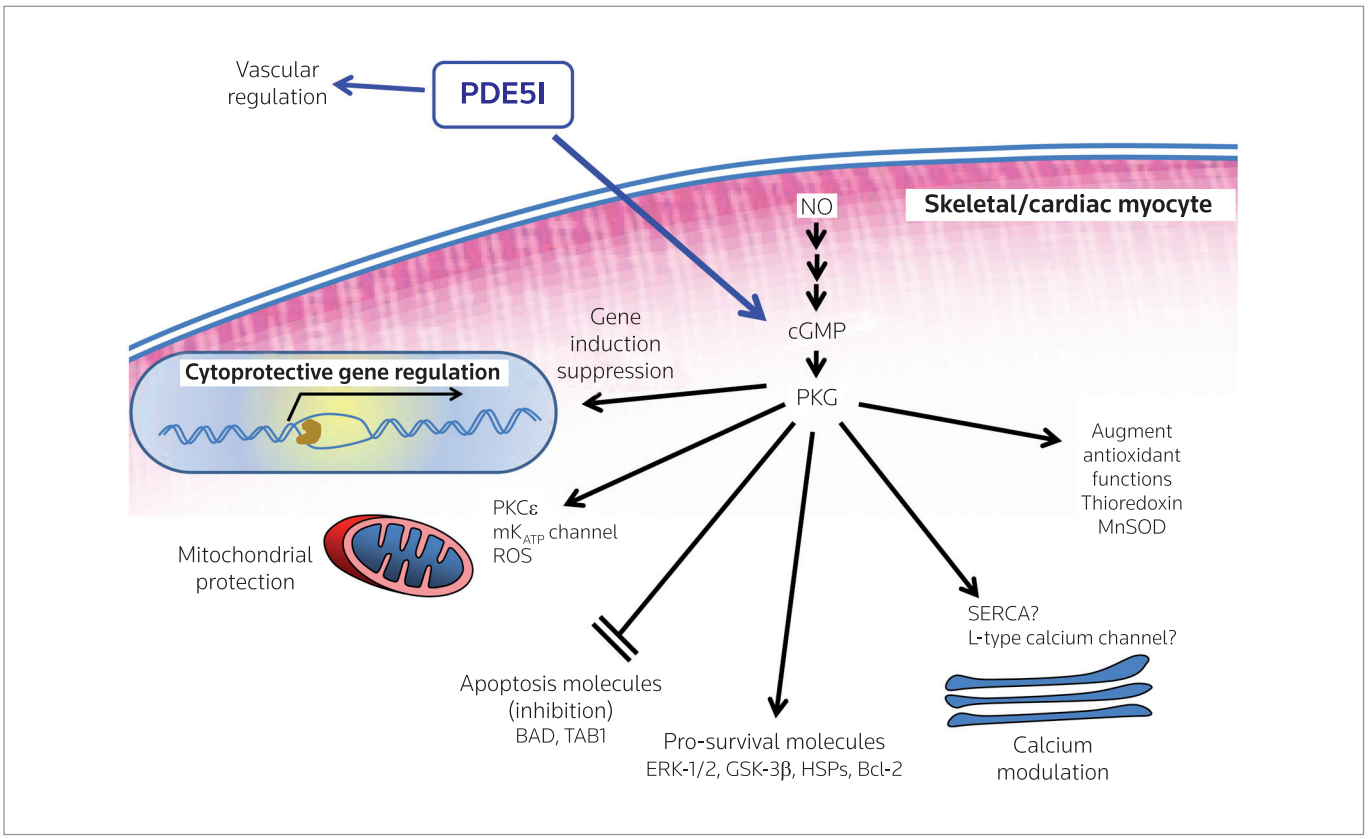


Figure 5. Non-vascular mechanisms of PDE5 inhibitor (PDE5I) protection of myofibers. In addition to vascular control, PDE5Is exert various cytoprotective functions. They can regulate proapoptotic or antiapoptotic gene transcription, contribute to mitochondrial protection, post-transcriptional regulation of pro- and antiapoptotic molecules, restrict calcium overload and augment antioxidative functions, among many other effects. NO, nitric oxide; cGMP, cyclic guanosine monophosphate; PKG, cGMP-dependent protein kinase 1; PKCε, protein kinase C epsilon; ROS, reactive oxygen species; MnSOD, Mn superoxide dismutase; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; HSPs, heat shock proteins.

PKG/cGMP is supposed to block apoptosis in some cell types by phosphorylating BAD (97), inhibiting TGF-beta-activated kinase 1-binding protein 1 (TAB1)-p38 signaling (98), and suppressing the expression of apoptosis-associated speck-like protein containing a CARD (PYCARD), Igtp and Tgtp (99). Conversely, it increases the expression of antiapoptotic molecules, including apoptosis regulator Bcl-2 (94), cAMP-dependent transcription factor ATF-3, growth arrest and DNA damage-inducible protein GADD45 gamma and F-box/WD repeat-containing protein 1A (99).

Given that cGMP/PKG signaling exerts a beneficial preconditioning effect by inducing an antioxidant function in endothelial cells (100) and by increasing the activity of thioredoxin and Mn superoxide dismutase (Mn-SOD) in neuronal cells (101), it is possible that a similar mechanism protects skeletal and cardiac myofibers.

It is also known that cGMP/PKG exerts its effects through gene regulation (99, 102).

THERAPEUTIC CONSIDERATIONS

NO or PDE5 inhibitor?

Although the precise mechanisms of the post-contraction blood flow control in muscles and the detailed roles that endogenous NO plays therein are not fully clarified, exogenously applied NO appeared to give beneficial effects in short-term experiments in *mdx* mice (18, 20). It is well known, however, that prolonged NO administration can result in adverse effects by perturbing various cellular functions (Fig. 6). The unregulated production/administration of NO can lead to deleterious effects, including hypotension (103, 104), insulin resistance (105-107) and cell death (108). Inducible NOS (iNOS) produces high concentrations of NO and is involved in the inflammatory response (109). Unregulated overproduction of NO by iNOS can also lead to hypotension in some sepsis models (110, 111). cGMP analogues and PDE5 inhibitors can bypass and/or reduce these unfavorable outcomes. Clinicians have gained accumulating knowledge regarding potential side effects and safety issues of administering PDE5 inhibitors during the past 15 years. Furthermore, recent and ongoing studies in pediatric patients (112, 113) will bring further assurance of safely administering these drugs to children.

Although further investigations are necessary to elucidate the precise mechanisms of PDE5 inhibitors on muscle blood flow modulation, evidence is accumulating that supports the beneficial effect of PDE5 inhibitors in patients with muscular dystrophy (18-20).

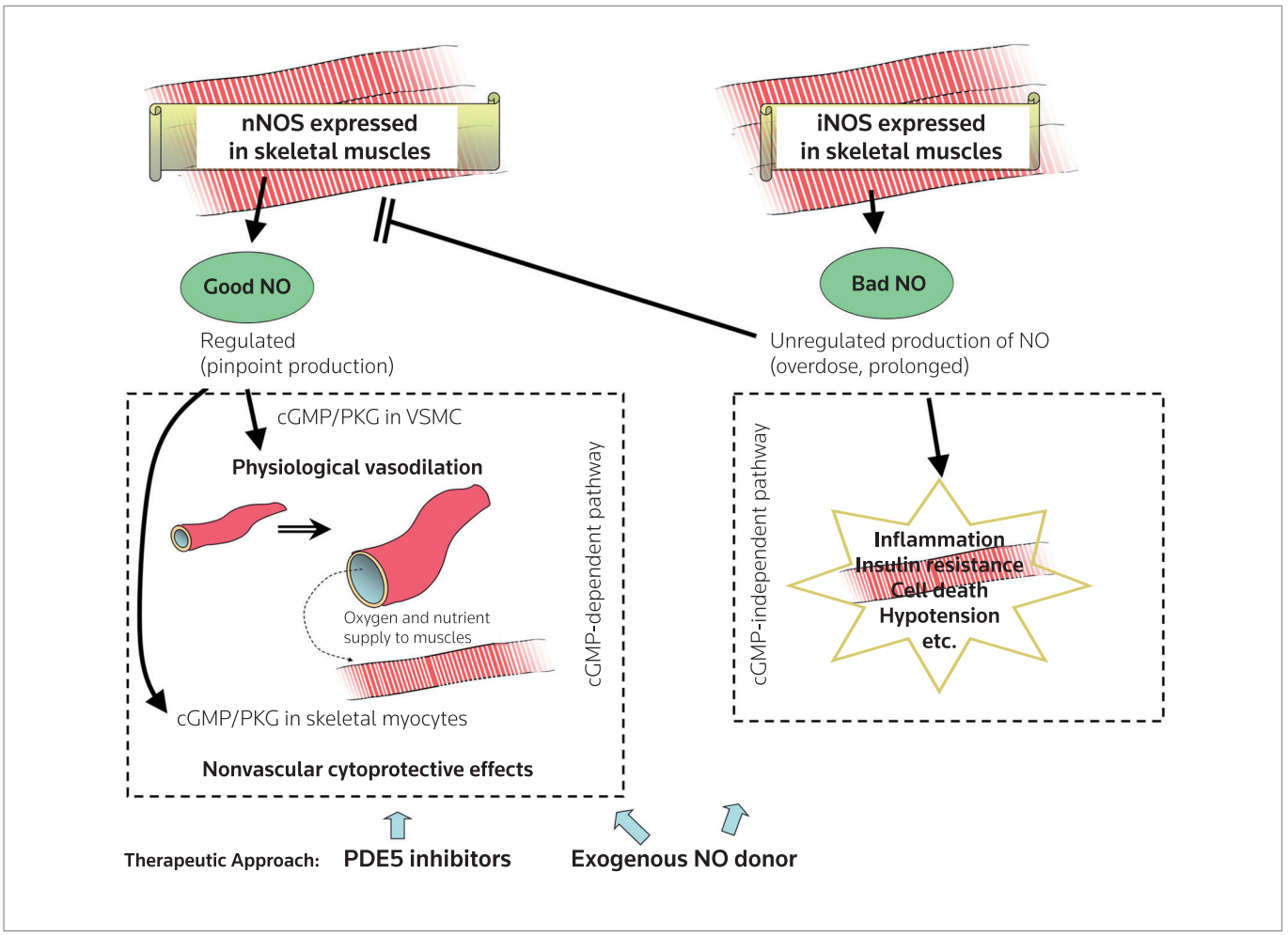


Figure 6. Comparison of the therapeutic advantage of PDE5 inhibitors over an exogenous nitric oxide (NO) donor to improve tissue circulation and reduce muscle damage. When endogenously produced NO reaches its target cells, or vascular smooth muscle cells (VSMCs), it increases the intracellular level of cyclic guanosine monophosphate (cGMP) and activates cGMP-dependent protein kinase 1 (PKG). Physiological vasodilation is thus caused by a cGMP/PKG-dependent pathway in VSMCs activated by regulated production of NO. On the other hand, when inducible nitric oxide synthase (iNOS) produces NO in an unregulated manner, it may lead to inflammation (109), insulin resistance (105-107) and cell death (108) by activating a cGMP-independent pathway. Unregulated production of NO by iNOS can also cause hypotension (110, 111) and downregulate the activity of endothelial nitric oxide synthase (eNOS)/neuronal nitric oxide synthase (nNOS). PDE5 inhibitors bypass the nonspecific effect of NO and stimulate a cGMP-dependent pathway by increasing the intracellular cGMP level.

Which PDE5 inhibitor to choose?

Since the development of sildenafil, many PDE5 inhibitors have been introduced. Most of these have a pyrazolopyrimidinone group in their chemical structure, which fits the Q-pocket of the PDE5 catalytic domain, as does the guanine group of cGMP (Fig. 7) (114). In addition, sildenafil has ethoxyphenyl and methylpiperazine groups, fitting H-pocket and L-regions of the enzyme, respectively. Tadalafil lacks the moiety for the L-region, but its NH-site and methylenedioxyphenyl group face the Q- and H-pockets, respectively. Most of the PDE5 inhibitors are specific for PDE5 with IC_{50} values on the order of nanomolar concentrations. Some traits of PDE5 inhibitors to consider for preparing a therapeutic scheme are presented below.

Long-acting vs. short-acting

Long-acting PDE5 inhibitors such as tadalafil alleviate the necessity of frequent oral intake and would be suitable for patients with low compliance, infants or animal models if frequent drug administration interferes with the study design. However, the disadvantage of long-acting PDE5 inhibitors is that they are slowly removed from the systemic circulation once any adverse side effect occurs.

Selectivity

Although all of the PDE5 inhibitors are designed to specifically target PDE5, some of the currently available PDE5 inhibitors have fairly high potency against other PDEs. For example, sildenafil, vardenafil and udenafil have relatively poor selectivity over PDE6, and

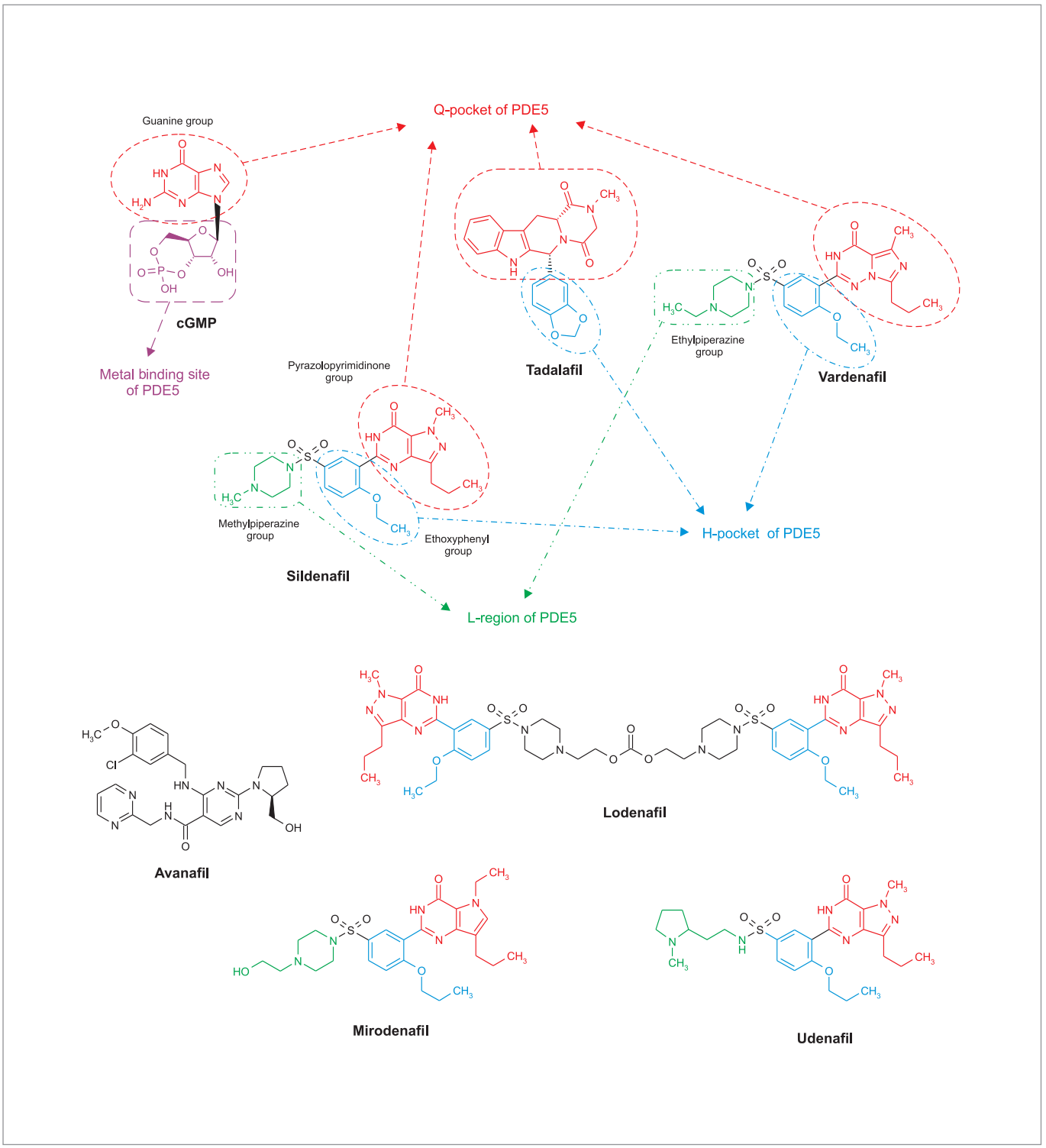


Figure 7. Chemical structures of representative PDE5 inhibitors are drawn together with the information obtained from the previous crystallography study (114). Groups fitting the Q pocket of PDE5 are encircled in red, including the guanine group of cyclic guanosine monophosphate (cGMP), the pyrazolopyrimidinone group of sildenafil and corresponding groups of tadalafil and vardenafil. Groups fitting the H pocket of PDE5 are encircled in blue, including the ethoxyphenyl and methylenedioxyphenyl groups of sildenafil, tadalafil and vardenafil. The methylpiperazine and ethylpiperazine groups of sildenafil and vardenafil fit the L region of PDE5 and are encircled in green, but tadalafil lacks the corresponding moiety. Only cGMP has a moiety that fits the metal binding site of PDE5. Color markings in avanafil, lodenafil, mirodenafil and udenafil are based on speculations from the chemical structure, but have not been confirmed by crystallography.

tadalafil over PDE11 (115). Further investigations are required, however, into whether such poor selectivity is related to some of the potential side effects of these PDE5 inhibitors. Given that the cGMP/PKG pathway can have beneficial effects directly on myocytes (see previous paragraph and Figure 5), this poor selectivity may have some merit, although more evidence is required for either scenario. The possibilities and specific issues of the side effects of PDE5 inhibitors will be discussed below.

Dosage

Despite the increasing reports of the efficacy of PDE5 inhibitors against muscular dystrophy in animal experiments, the optimal dosage in the experimental setting and in human clinical usage can be considerably different. It is not guaranteed that the doses used in animal studies are titrated appropriately. Many more animal experiments and preclinical studies are necessary to estimate the optimal dose of the PDE5 inhibitors of choice.

UPDATES OF RECENT PDE5 INHIBITOR STUDIES IN MUSCULAR DYSTROPHY PATIENTS

There are currently several animal studies with PDE5 inhibitors for which efficacy data are available, as summarized in Table II. All reports unanimously agree on the beneficial effect of the PDE5 inhibitors tested. Tadalafil or sildenafil administered p.o. reduced muscle contraction/exercise-induced damage, had beneficial effects in maintaining the overall histological structure, and improved muscle function (18, 20). Sildenafil administered i.p. improved cardiac function and metabolic parameters (19). All the animal studies listed here are based on short- to mid-term experiments with PDE5 inhibitor application up to 4-6 weeks. Regarding the timing of drug administration in most of the mid-term experiments, relatively young mice were used between 0 and 12 weeks of age. Notably, one study even initiated the intervention from the prenatal stage. The long-term study with oral administration of sildenafil demonstrated that the drug was effective in maintaining cardiac function in *mdx* mice and that it can even revert the diastolic dysfunction already present at 12 months of age (116). Further animal and preclinical studies are necessary to confirm the long-term effect on tissues other than the heart, search for the optimal dose and the potential administration scheme of the drugs. The value of early-stage intervention will be discussed below.

OTHER NONSELECTIVE PHOSPHODIESTERASE INHIBITORS AND VASOACTIVE AGENTS

The first phosphodiesterase inhibitor that came into human use was caffeine (117). Theophylline and pentoxifylline have also been used for a long time for treating asthma (118) and vascular diseases, respectively. Although these three xanthine derivatives have varying degrees of vasoregulatory effect, they have broad-spectrum inhibition against many types of PDEs. Caffeine causes calcium oversensitization and cardiac burden, and theophylline worsens oxygen saturation in DMD patients. Thus, they may not be suitable for vascular therapy for DMD/*mdx*. Pentoxifylline, however, is reported to have a beneficial effect in muscular dystrophy subjects when the dosage is carefully titrated (119), although the detailed mechanism of the therapeutic effects needs be further deciphered. ATP and other nucleotides (120-122), as well as other remedies, have also been tested and reviewed previously (123).

Table II. *Summary of the recent publications with PDE5 inhibitor treatment in muscular dystrophy patients.*

Choice of PDE5 inhibitor	Administration	Dose	Duration of treatment	Evaluation scheme	Efficacy	Ref.
Tadalafil	p.o.	5-50 mg/kg	4 weeks, and acute	Tissue fibrosis; extent of damage; post-mortem analysis of the entire tissue; in vivo workload damage analysis	Effective in reducing damage and fibrosis in the skeletal and diaphragm muscles	Asai (18)
Sildenafil	i.p., daily	0.7 mg/kg	6 weeks	Ex vivo functional study; ex vivo metabolic analysis; in vivo physiology and workload/damage test; gene analysis	Effective in improving the functions of the cardiac muscle. Workload-induced damage reduced	Khairallah (19)
Sildenafil and tadalafil	p.o.	100-500 mg/kg	Acute (1 day)	In vivo function test; blood flow analysis; serum CK; MRI	Function improved; edema and damage reduced; circulation improved	Kobayashi (20)
Sildenafil	p.o.	Dose attained the free plasma concentration of 70 nM	Long-term (up to 14-16 months), and various detailed time courses	In vivo cardiac function tests by echocardiography	Global cardiac function maintained in the long-term treatment group. Left ventricular dysfunction reversed in the group with late-stage treatment	Adamo (116)

All studies conducted in *mdx* mice and/or their genetic variants. CK, creatine kinase; MRI, magnetic resonance imaging.

POTENTIAL SIDE EFFECTS OF PDE5 INHIBITORS

Although the side effects of PDE5 inhibitors are considered to be relatively mild as compared to an NO donor or other vasodilators, there are potential adverse effects. Note that those listed below are not necessarily proven to have the causative mechanisms with PDE5 inhibitors or are accepted as statistically significant outcomes.

Retinopathy

Since the use of PDE5 inhibitors became prevalent, some case reports have documented the occurrence of retinopathy, including nonarteritic anterior ischemic optic neuropathy (NAION). Its etiology remains to be clarified, and various potential mechanisms of retinopathy are suggested, including the involvement of altered circulation, or the role of the relatively high activity of PDE5 inhibitors for the closely related isozyme PDE6 (124, 125). However, whether the poor selectivity over PDE6 causes retinopathy is arguable and requires further investigation.

Hypotension

PDE5 inhibitors are considered to have less risk for hypotension compared to conventional vasodilators. The combined use of PDE5 inhibitors and other vasodilators, however, increases the risk for hypotension (115).

Myocardial infarction

Although there have been some case reports of the occurrence of ischemic heart disease chronologically related to PDE5 inhibitor intake, sildenafil, tadalafil and vardenafil showed no increased risk for cardiac ischemia as compared to the placebo control (115, 126). Because the population treated for ED already has a higher incidence of vascular complications, the causative conclusion of this adverse effect is difficult to make. Given that muscular dystrophy patients often carry cardiac complications, however, careful monitoring of the patients would be necessary. Similarly, the preexisting vascular conditions of ED patients make it difficult to speculate the causative relationship of PDE5 inhibitors and retinopathy or neurological disorders.

Myalgia and myopathy

There are a few case reports of myalgia and myopathy when sildenafil and tadalafil are used in combination with statins (127, 128). Although the primary mechanism is still unclear, drug–drug interaction is likely an exacerbating factor. The relatively poor selectivity of tadalafil for PDE5 over PDE11 should also be noted, but wheter inhibition of PDE11 is involved in any adverse effect on the myofibers is currently unknown. Given that the cGMP/PKG signal has a cytoprotective effect directly on myofibers, whether the poor selectivity over PDE11 is associated with myalgia or myopathy requires investigation.

Neurological disorders

Both adverse and beneficial effects for PDE5 inhibitors have been reported from animal and clinical studies, including memory augmentation, seizures, ischemic episodes of the cerebrovascular system and brain hemorrhage, although none of the adverse effects on the neurological system are established in the causative relationship with PDE5 inhibitors (129).

In summary, most of the adverse effects listed in this section are not scientifically established issues of PDE5 inhibitor administration, but deliberate evaluations are required for various reasons. First, the treatment period for DMD could be considerably longer as compared to that for ED. Next, whether to start the drug intervention early in life is a key issue involving both scientific and social discussions. The theoretical value of starting early-stage treatment will be discussed in the next paragraph. Since infants cannot convey their subjective complaints and adverse effects can often be overlooked, careful monitoring is considered crucial, particularly if drug interventions are to start in early stages in DMD patients.

FUTURE THEMES IN PDE5 INHIBITOR THERAPIES

Evaluation scheme of efficacy 1 – understanding the progress of the disease

Over time, DMD patients lose their muscle power. The basis of functional tests widely used in the clinical setting (130-133) is the observation of the almost linear drop in the patients' contractile force between the ages of 5 and 13. In conventional studies, however, functional tests were not performed before age 4, because classically, the patients' parents would not recognize the disease before the onset, and patients could not follow the instructions of the studies before that age. After age 13, on the other hand, they would have already lost the great majority of the contractile force in many muscles. Therefore, most of the available data from human studies guarantee the linear drop in muscle force only between these ages.

How the disease progresses before this age remains to be investigated in detail, but from muscle function studies using laboratory animals, the gross muscle force generated by electrical stimulation does not show a dramatic decrease, at least for several months (Fig. 8A) (18, 134, 135), despite the fact that the tissue damage is constantly upregulated and the amount of tissue fibrosis steadily increases (trichrome staining in Fig. 8A). Also, in humans, the parents of a DMD patient do not always notice the disease during the early stages, suggesting that the decrease in muscle power at this stage is considerably slow (Fig. 8A; pre-onset period, or compensation period).

It can be safely assumed that the decline in contractile force, if any, is partly due to the decreased numbers of fully differentiated myofibers per tissue. The quantity of functional myofibers in skeletal muscle tissue depends on the balance between degeneration and regeneration of myofibers. Even if there is increased myofiber damage, the number of fully differentiated myofibers remains unchanged as long as increased regeneration compensates for the loss. In fact, the turnover rate of myofiber regeneration appears to be upregulated in *mdx* mice (136), canine muscular dystrophy (137) and DMD patients (138, 139), since significant amounts of regenerating fibers are consistently observed. This may be one of the reasons why DMD is not a rapidly progressive disease despite the constantly upregulated myofiber damage. In support of this notion, *mdx* mice showed exacerbated muscular damage, a reduction in the size of

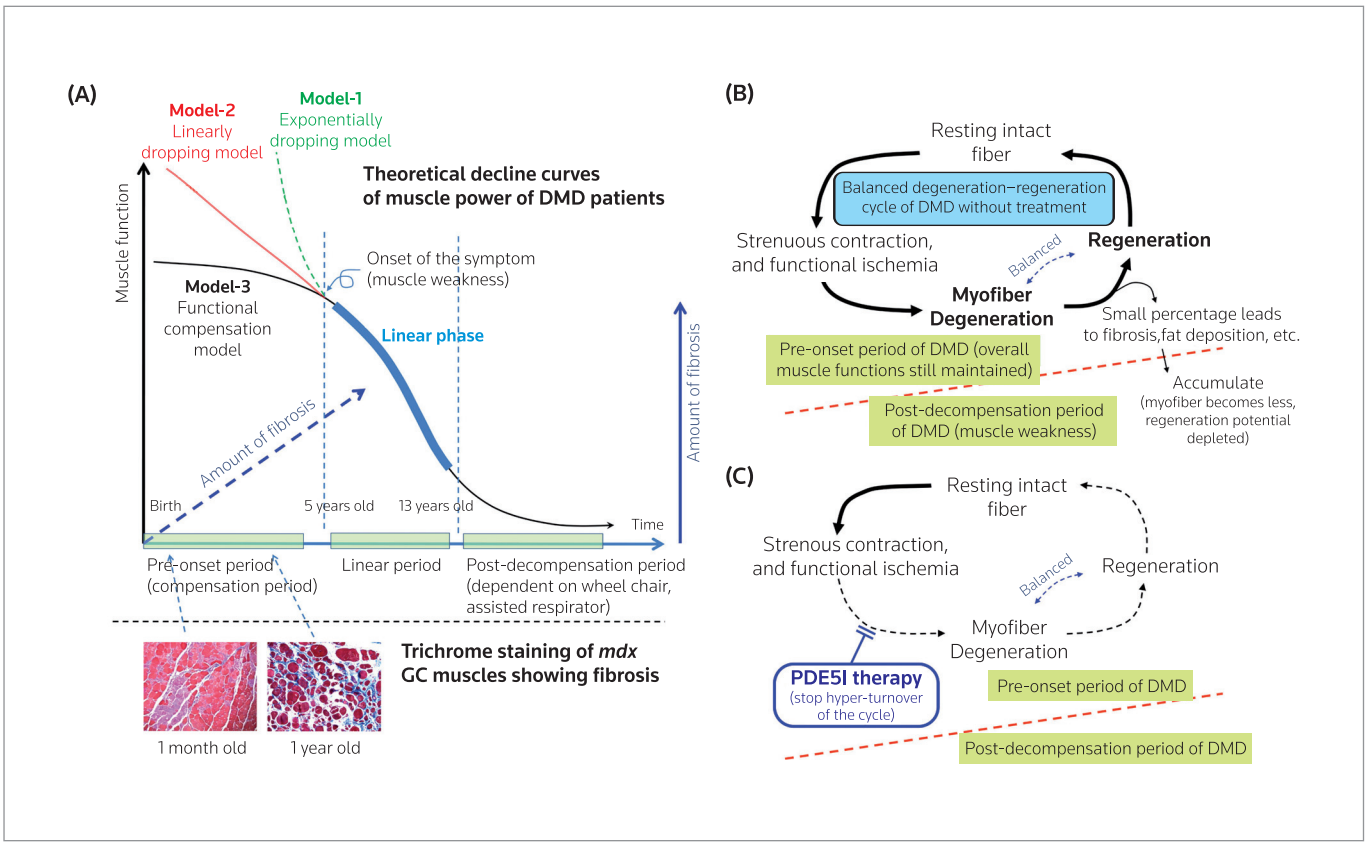


Figure 8. Decline curves of muscle force over the age (**A**) and the degeneration–regeneration cycle of myofiber damage in Duchenne muscular dystrophy (DMD) (**B** + **C**). (**A**) Previous functional studies in DMD patients show that contractile force drops almost linearly between the ages of 5 and 13 years ("linear period"). Little data are available, however, on how the muscle power is lost before the onset age. Drawn are three different possible models in the pre-onset period. First, if the human body were not designed with any regeneration capacity, a decreased rate of numbers of myofibers, and thus muscle contractile force, would mathematically follow the exponentially declining curve (Model-1 in green line). Next, it is possible that the linearity of the curve also holds true in this period (Model-2 in red). Observation of animal models, however, suggests the existence of a compensation period during this phase (Model-3 in black). In the lower panels, images of trichrome tissue staining from gastrocnemius (GC) muscles of *mdx* mice are shown (note the blue staining for fibrosis). Even during the early phase where contractile force is fairly maintained, fibrosis steadily accumulates. (**B**) Upregulated regeneration compensates the loss of myofibers from an increased rate of muscle damage in this disease. Especially during the pre-onset period, the amounts of regeneration and degeneration are well balanced. Degeneration and regeneration take place repeatedly, and form a cycle. A small portion of the damaged fiber, however, will lead to fibrotic change, fat deposition and vascular reorganization. When this misregeneration accumulates and the numbers of fully functional myofibers decrease and/or the regeneration capacity depletes from overusage (or satellite cells reach the senescence stage), it will proceed to the post-decompensation phase. (**C**) Myofiber protection therapy may stop the hyper-turnover of the regeneration–regeneration cycle and thus delay the transition into the post-decompensation phase. PDE5I, PDE5 inhibitor.

muscles and a rapid decrease in muscle power when regeneration was blocked by irradiation (140, 141). Conversely, beneficial treatment aimed at blocking the myofiber damage decreased the rate of temporary regeneration turnover in DMD cases (18, 142). It is therefore assumed that the quantity of myofibers depends on the dynamic balance between degeneration and regeneration, and that this turnover of degeneration and regeneration plays a role in preventing the decline in the muscle power.

This theory of the balanced degeneration and regeneration in DMD, however, raises fundamental questions: Does myofiber damage really affect the progress of the disease, if upregulated regeneration compensates the loss of myofibers from the increased degeneration of myofibers in *mdx* mice? Furthermore, wouldn't PDE5 inhibitor therapy for stopping the muscle damage be futile if the damaged tissue can heal by itself?

Observations of the liver and the pathogenesis of liver diseases provide an important insight. The liver is a highly regenerative organ. Although the actual regeneration is latent in healthy humans, once the liver is damaged, normal liver can perfectly regenerate to compensate for the lost tissue (143). In chronic hepatitis of various causes, where hepatocytes are constantly damaged, regeneration is always upregulated (144). Prolonged hyper-turnover of a degeneration–regeneration cycle, however, eventually results in adverse outcomes, including fibrosis, fatty replacement, and vascular reorganization and calcification, finally leading to cirrhosis. It is only towards the later phase that hepatic function is finally decompensated. The hepatocyte protective agents

glycyrrhizin and ursodeoxycholate are effective in preventing the progress of the disease when started at an early phase (145-147).

Similarly, muscular dystrophy is a slowly progressive disease despite the fact that there is always increased myofiber damage accompanied by upregulated regeneration. This suggests that in muscular dystrophies the increased rate of regeneration and degeneration turnover and gradual accumulation of mis-regeneration and/or fibrosis will lead to a decompensation period (Fig. 8A and B). Protecting myofibers from damage with PDE5 inhibitors will decrease the rate of degeneration–regeneration turnover (Fig. 8C), but might not immediately become reflected in the improvement of muscle contractile functions, especially during the compensation period. Stopping the hyperturnover of the cycle will, however, delay the progress of muscular dystrophy by slowing down the transition to the decompensation period.

In summary, relying solely on muscle contractile force may be useful when the patients are already in the linear period, but its value should be limited during the compensation period, or post-decompensation period, for judging the progress of the disease or the efficacy of the PDE5 inhibitor treatment.

Evaluation scheme of efficacy 2: towards establishing a systematic staging method

Multiple criteria for judging the efficacy of treatment will be necessary in addition to the muscle contractile properties. Although muscle biopsy in DMD patients is valuable for determining the significance of treatment in some special cases (148), evaluating the damage level of the observed pathology requires special deliberation. The distribution of damaged fibers in muscular dystrophy subjects is not uniform throughout the tissue (149), and biopsy is greatly affected by a sampling bias. Noninvasive imaging and electrophysiological techniques are established for the diagnosis of muscle diseases, using electromyography (EMG) (150), computed tomography (CT) (151), ultrasound (152) and magnetic resonance imaging (MRI) (153). MRI is con-

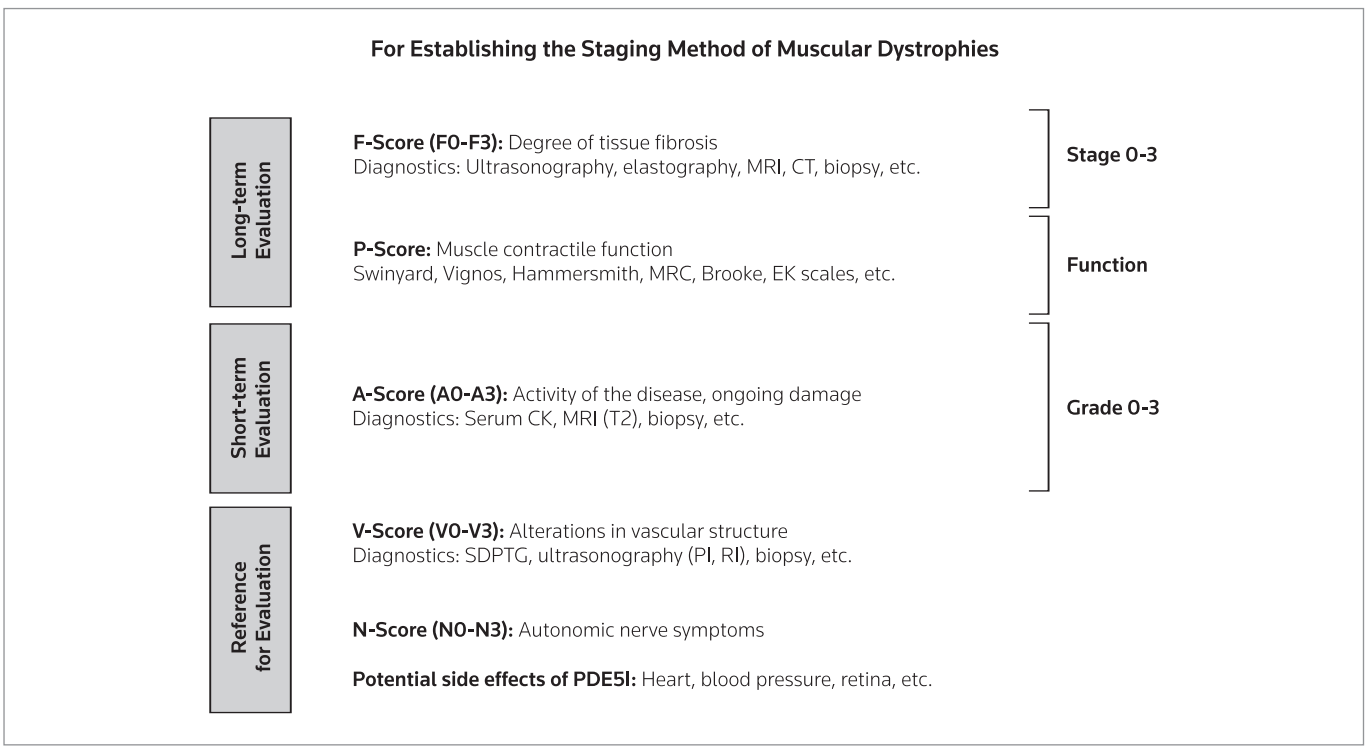


Figure 9. Example ideas for the systematic staging and grading scales for Duchenne muscular dystrophy (DMD) are presented based on those for other diseases (157-159). Fibrosis (F-score) can be diagnosed and quantified by ultrasonography, elastography, magnetic resonance imaging (MRI), computed tomography (CT), biopsy, etc. Especially during the early stage of the disease when the muscle function could be compensated (age < 5 years), scores related to tissue fibrosis would be valuable for long-term evaluation. Muscle power (P-score) can be evaluated according to the conventional or recently modified muscle function scaling tests, including the scoring systems of Swinyard (132), Vignos (lower extremity) (130), Hammersmith (131), Brooke (upper limb) (166), etc. Most of these tests are valuable for ambulatory patients, but it has been difficult to evaluate patients in the advanced stage where they have already lost most of their muscle power. Recently, scaling systems for nonambulatory patients (167) are introduced and expected to serve for the evaluation in the post-decompensation period. For the A-score, or the disease activity, serum creatine kinase (CK) levels are already known to reflect the extent of the ongoing damage in the patient's myofibers, and are useful for short-term evaluation. MRI with T2 enhancement detects tissue edema and serves the same purpose. Vascular changes (V-score) can be evaluated by the second derivative of the vascular plethysmogram for the arterial age assessment. Ultrasonography with the pulsatory index (PI) or resistance index (RI) can also be valuable. For the N-score, a number of studies have reported alterations in autonomic nerve function (168). It can be a useful parameter for the early to middle stage of the disease. The decline in power in DMD patients first becomes prominent in the proximal muscle and later on proceeds to other muscles. Ideally, if we can pick up a few muscles from each group (i.e., proximal, distal, etc.), this staging and grading system will cover a considerably broad range of patients at varying ages and stages. MRC, Medical Research Council; SDPTG, second derivative of plethysmography; PDE5I, PDE5 inhibitors.

sidered the forefront technology in medical diagnostic imaging, favored because it is radiation-free, and it is also vigorously used in the evaluation of muscular dystrophies (154). However, although widely used enhancement methods of MRI are suitable for detecting edema related to inflammation, it is not ideal for detecting the difference between fibrosis and the parenchymal muscle tissue. Given that fibrosis can reflect the long-term progress of DMD and edema can be related to the short-term activity of the disease, it is necessary to establish new enhancement methods for MRI that can be applied for a long-term evaluation of DMD. Diagnostic values of conventional imaging techniques and their variant modalities should never be underestimated, including CT and ultrasound (155, 156). Elastography combined with CT, MRI and ultrasound can also be useful. Most importantly, it is imperative to establish a systematic staging method of DMD for the evaluation of the long-term progress, as well as the short-term activity of the disease. Some examples are presented in Figure 9, based on the staging scheme of chronic hepatitis and cirrhosis (157-159).

POTENTIAL APPLICATION FOR OTHER MUSCULAR DYSTROPHIES AND MYOPATHIES

The theoretical background of PDE5 inhibitor therapy for DMD is the fact that muscular blood flow in DMD is disturbed due to the lack of nNOS expression and its proper localization on the sarcolemma. A similar, but sometimes milder pattern of nNOS abnormality is shared by other types of muscular dystrophies and myopathies, including Becker muscular dystrophy, limb-girdle muscular dystrophy (LGMD)-2B, LGMD-2D, LGMD-2E, LGMD-2I, Ullrich congenital muscular dystrophy, merosin-deficient congenital muscular dystrophy and nondystrophic myopathies such as polymyositis, and their animal models (20, 79, 160-162). LGMD-1C and rippling disease accompany abnormal behavior of caveolin-3 (163), a negative regulator of nNOS (164). An intervention with PDE5 inhibitors may prove effective in some of these diseases.

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DISCLOSURES

The author states no conflicts of interest.

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