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Erection capability is potentiated by chronic sildenafil treatment: role of blood flow-induced endothelial nitric oxide synthase phosphorylation

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Running title: Chronic sildenafil potentiates erection

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Nonstandard abbreviations: cGMP, guanosine monophosphate; eNOS, endothelial nitric oxide synthase; ICP, intracavernosal pressure; MAP, mean arterial pressure; MYPT1, myosin phosphatase target subunit 1; PDE, cyclic nucleotide phosphodiesterase ; PI3-kinase, phosphatidylinositol 3-kinase

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ABSTRACT

Despite demonstrated clinical efficacy of sildenafil for the temporary treatment of erectile dysfunction, the possibility that sildenafil used long-term durably augments erectile ability remains unclear. We investigated whether continuous long-term administration of sildenafil at clinically relevant levels to aged rats "primes" the penis for improved erectile ability and involves nitric oxide (NO) or RhoA/Rho-kinase signaling pathways. In aged, but not young rats, sildenafil prolonged erection and increased the protein expressions of phosphorylated endothelial NO synthase (eNOS) at Serine-1177 and phosphorylated Akt at Serine-473 in penes. Only in the young rat penis, protein expressions of phosphodiesterase-5 and phospho-myosin phosphatase target subunit 1, a marker of Rho-kinase activity, were increased by sildenafil. Sildenafil inhibited phosphodiesterase-5 activity in penes of young and aged rats coincident with assayed free plasma levels of the drug equivalent to clinically therapeutic measurements. We conclude that erectile ability can be enhanced under preconditions of erectile impairment by chronic inhibition of phosphodiesterase-5 and the effect is mediated by Akt-dependent eNOS phosphorylation. The lack of erectile ability enhancement in young rats by chronic phosphodiesterase-5 inhibition may relate to restrained NO signaling by phosphodiesterase-5 upregulation, lack of incremental Akt and eNOS phosphorylation, and heightened Rho-kinase signaling in the penis.

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Penile erection involves relaxation of the blood vessel supply of the penis and the trabecular meshwork of smooth muscle that constitutes the corpora cavernosa. The main mediator of erection is nitric oxide (NO) (Ignarro et al., 1990; Burnett et al., 1992). NO derived from neuronal NO synthase in the nerves supplying the penis initiates this process, while sustained production of NO from endothelial NO synthase (eNOS) within the vascular and trabecular endothelium of the penis is responsible for full erection and maintenance of erection (Hurt et al., 2002; Burnett 2004). The classic concept of neuronal NO synthase and eNOS activation by calcium/calmodulin accounts for rapid and transient production of NO. However, long-term constitutive NO production occurs upon phosphorylation of eNOS at Serine (Ser)-1177 (human sequence) by a mechanism that does not require maintained intracellular calcium levels (Dimmeler et al., 1999; Fulton et al., 1999; Michell et al., 1999). Via shear stress stimuli associated with blood flow increases, NO is constitutively generated in the penis through phosphorylation and activation of phosphatidylinositol 3-kinase (PI3-kinase)/Akt and eNOS (Hurt et al., 2002). NO then activates soluble guanylyl cyclase in adjacent smooth muscle cells and increases the production of 3',5'-cyclic guanosine monophosphate (cGMP). Subsequent activation of protein kinase G reduces contractile activity and promotes relaxation of smooth muscle cells, resulting in increased arterial blood inflow, veno-occlusion, and erection (Ignarro et al., 1990; Burnett 1997).

In contrast to vasorelaxation mediated mostly by the NO pathway, vasoconstriction, which maintains the penis in the nonerect state, is mediated substantially by calcium sensitization mediated by the RhoA/Rho-kinase pathway (Chitaley et al., 2001; Wang et al., 2002; Chang et al., 2003). Rho-kinase is activated by RhoA, a small GTP-binding protein. Activated Rho-kinase (α and ß isoforms) phosphorylates the regulatory myosin phosphatase target subunit 1 (MYPT1)

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of myosin light chain phosphatase at Thr-696 and inhibits its activity, promoting smooth muscle contraction (Feng et al., 1999). During erection, this pathway is inhibited, most likely by NO (Mills et al., 2002). Substantial evidence demonstrates that NO/cGMP/protein kinase G inhibits RhoA activity by phosphorylation of RhoA at Ser-188 which prevents its translocation to the membrane and activation (Sauzeau et al., 2000). In addition, RhoA/Rho-kinase suppresses eNOS gene expression and enzyme activity in the penis (Bivalacqua et al., 2004a). In human endothelial cells, the RhoA/Rho-kinase pathway inhibits Akt-dependent eNOS activity/phosphorylation (Ser-1177) (Ming et al., 2002). The degree of contraction of the smooth muscle of the corpora cavernosa is, thus, controlled by a balance between these 2 major signaling pathways in the penis.

The levels of cGMP available for transduction of NO signaling are regulated by the availability of NO and activities of guanylyl cyclase, protein kinase G, and specific phosphodiesterases (PDEs). Degradation of cGMP in the penis to an inactive 5'-GMP, which terminates NO signaling pathway and returns the penis to the flaccid state, is catalyzed primarily by PDE5 (Boolell et al., 1996; Corbin and Francis, 1999; Moreland et al., 1998). Three alternatively spliced PDE5 isoforms are encoded by a single PDE5A gene (Lin et al., 2002). Inhibition of PDE5 by the commercially available PDE5 inhibitors, including the prototype sildenafil, which thus facilitates NO-mediated corpus cavernosum relaxation, has proven clinically efficacious in multiple clinical studies for patients with erectile dysfunction of various etiologies. Sildenafil is a competitive, highly selective and potent inhibitor of PDE5 (Boolell et al., 1996; Moreland et al., 1998; Turko et al., 1999; Ballard et al., 1998). In both in vitro (Ballard et al., 1998; Gemalmaz et al., 2001) and in vivo animal studies (Gemalmaz et al., 2001; Andersson et al., 1999; Ueno et al., 2002), sildenafil acutely increases the amplitude and the

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duration of erection induced by nerve stimulation or agents that release NO such as endothelium-dependent vasodilators (Behr-Roussel et al., 2005), nitric oxide donors (Thompson et al., 2001) or gene transfer of eNOS (Bivalacqua et al., 2004b).

Despite the demonstrated clinical efficacy of PDE5 inhibitors for the treatment of erectile dysfunction, it is understood that the medication temporarily augments penile erection and is standardly used on a periodic basis prior to planned sexual activity. The possibility that PDE5 inhibitors used as long-term therapy may potentiate spontaneous erectile ability has been postulated (Burnett 2005), but scientific support in this regard is lacking. The aim of the present study was to determine whether continuous long-term administration of sildenafil at therapeutically relevant levels to aged rats, serving as a major paradigm for erectile dysfunction, "primes" the penis for improved erectile ability and involves eNOS activation mechanisms and/or Rho-kinase signaling.

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MATERIALS AND METHODS

Animal Model. All animal procedures were conducted in accordance with the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Animals. Male Fischer 344 "young" (4-month-old, erection intact) and "aged" (19-month-old, erection impaired) rats (Garban et al., 1995; Musicki et al., 2005) were purchased from the National Institute of Aging, Bethesda, MD. Rats were injected with soluble sildenafil mesylate (20 mg/kg), provided by Pfizer, Global Research and Development, Sandwich, UK, or saline subcutaneously every 8 hours for 3 weeks. All the experiments were performed after a 1, 3, or 7 day wash-out period (intervals after the termination of treatment). At each time point, a group of vehicle- and sildenafil-treated rats underwent physiologic erection studies, while for another group of identically treated rats penes were collected for molecular and biochemical studies. Blood was taken by cardiac puncture at necropsy and plasma was subsequently sent to Pfizer (Sandwich, UK) for measurements of sildenafil.

Physiologic Erection Studies. Animals were anesthetized with 40 mg/kg pentobarbital (Abbott Laboratories, Abbott Park, Illinois). To monitor intracavernosal pressure (ICP), the shaft of the penis was denuded of skin and fascia, and the left corpus cavernosum was perforated with a 27-gauge needle connected to a pressure transducer (DI-190; Dataq Instruments, Akron, OH). The right carotid artery was cannulated with polyethylene tubing-50 for continuous monitoring of mean arterial pressure (MAP). For electrically stimulated penile erections, a bipolar electrode attached to a Grass Instruments S48 stimulator (Quincy, MA) was placed around the cavernous nerve as described previously (Burnett et al., 1992). Stimulation parameters were 4 V at a

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frequency of 16 Hz with square-wave duration of 5 msec for 1 min. The submaximal stimulation parameter (4 V) was chosen based on our previous studies showing that maximal voltage may obscure pharmacologic effects on physiologic and molecular parameters including measurements of phosphorylated eNOS (Ser-1177) in penes (Musicki et al., 2004). Response parameters were calculated using MATLAB software (Mathworks, Natick, MA), and were expressed per MAP. Statistical analysis was performed on: 1) ICP area above baseline pressure, defined as the area under the curve which corresponds to the duration of electrical stimulation; 2) detumescence time, defined as the period from the end of electrical stimulation to the point exhibiting 50% of maximal ICP, and 3) detumescence area, defined as the area under the ICP curve during this interval.

Western blot analysis. For immunoblot studies, penes were excised at baseline. Minced penile tissue was homogenized and partially purified for NOS as described previously (Hurt et al., 2002). Purified NOS or 15-100 µg of proteins in crude homogenate (for phospho-Akt, Rho-kinases α and ß, phospho-MYPT1, and PDE5 analyses) were resolved on 7.5%, 12%, or 4-15% Tris gels under reducing conditions and transferred to polyvinylidene fluoride membrane. The membranes were stained with Ponceau Red to ascertain equal loading of proteins (except for purified eNOS) and probed with polyclonal antibodies against phospho-eNOS (Ser-1177) at 1:450, phospho-Akt (Ser-473) at 1:1,000 (Cell Signaling Technology, Beverly, MA), phospho-MYPT1 (Thr-696) at 1:1,000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and Rho-kinase α at 1:1,000, Rho-kinase ß at 1:500 dilutions, or monoclonal antibody against PDE5 at 1:450 (BD Transduction Laboratories, San Diego, CA). Membranes used for phospho-eNOS and phospho-Akt were stripped and reprobed for eNOS (1:1,000, BD Transduction Laboratories) or

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Akt (1:1,000, Cell Signaling Technology). Bands were quantified by densitometry. Phospho-Akt and phospho-eNOS densities were normalized relative to their corresponding total protein forms. Band densities for PDE5, Rho-kinases α and ß, and phospho-MYPT1 were normalized relative to ß-actin (Sigma Chemical, St. Louis, MO). Results were expressed relative to the ratio for saline-treated young animal samples prepared and blotted at the same time.

Assay of PDE5 activity. Penile extracts were assayed for total cGMP-dependent PDE activity and sildenafil (0.1-1 μM)-, tadalafil (50 nM)-, and 3-isobutyl-1-methylxanthine (50 μM)-inhibited PDE activity at 1 μM of substrate using a fluorescence polarization assay (Molecular Devices, Sunnyvale, CA) and a Fluorescence polarization plate reader (Perkin-Elmer Victor 3, Wellesley, MA), or 2 step radiolabeled method (Champion et al., 2005). The difference between cGMP hydrolytic activity in the presence and absence of 100 nM sildenafil was used as a measure of PDE5 activity.

Assay of sildenafil in plasma. Sildenafil was measured by liquid chromatography/tandem mass spectrometry following separation with a Chromolith Speedrod column (50x4.6 mm, Merck KGaA, Darmstadt, Germany) (Walker et al., 1999). Data were expressed as ratios to the internal standard, and the unknown sample results were calibrated to a standard in control plasma.

Statistics. Statistical analysis was performed by using one-way ANOVA followed by Newman-Keuls multiple comparison test or by t-test when appropriate using SigmaStat version 2.03 (SPSS Inc., Chicago, IL). The data are expressed as the mean ± SEM. A value of $p < 0.05$ was considered to be significant.

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RESULTS

Chronic sildenafil treatment increases poststimulation erectile response (delays detumescence) in aged rats. Erectile response to electrical stimulation of the cavernous nerve has been shown by others (Garban et al., 1995) and by us (Musicki et al., 2005) to be decreased in aged compared to young rats. Sildenafil given continuously for 3 weeks did not affect the magnitude of the ICP/MAP response during electrical stimulation of the cavernous nerve in young or aged rats at any time point after the termination of the treatment (Figure 1A). However, in aged rats chronic sildenafil treatment increased the duration of erectile response after the termination of neurostimulation: detumescence time was significantly increased up to day 3 wash-out (Figure 1B), and the corresponding detumescence area was significantly increased up to day 7 wash-out (Figure 1C). In young rats the improvement of erectile response was minimal: detumescence time was significantly increased through day 1 wash-out only, while detumescence area did not show any significant changes.

Chronic sildenafil treatment resulted in a significant ($p<0.05$) decrease in MAP in young rats through day 1 (58.8 ± 7.5 mmHg) and day 3 (85.3 ± 4.4 mmHg) wash-outs compared to values after vehicle treatment (101.8 ± 3.8 mmHg). In aged rats after vehicle treatment, MAP (78.5 ± 5.1 mmHg) was significantly ($p<0.05$) lower than that of young rats (101.8 ± 3.8 mmHg), while it was not affected by sildenafil treatment.

Penes from aged rats have decreased eNOS (Ser-1177) and Akt (Ser-473) phosphorylation: reversal by chronic sildenafil treatment. The ratios of phospho-eNOS (Ser-1177) and phospho-Akt (Ser-473) to total eNOS and Akt, respectively, were significantly reduced in penes of aged

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rats relative to levels in penes of young rats after vehicle treatment only (Figures 2 and 3). Chronic sildenafil treatment did not change the phosphorylation of these phospho-enzymes in penes of young rats. In contrast, it significantly increased phospho-eNOS and phospho-Akt expression in penes of aged rats up to day 3 wash-out to levels comparable to basal levels of young rats; these elevated levels returned to control values by day 7 wash-out.

Penes from aged rats have increased Rho-kinase activity. Chronic sildenafil treatment increases Rho-kinase activity in penes of young, but not aged rats. To assess whether chronic sildenafil treatment affects the RhoA/Rho-kinase signaling pathway, responsible substantially for vasoconstriction in the penis, we measured expression levels of Rho-kinases α and ß, and phospho-MYPT1 (Thr-696), a marker of Rho-kinase activity (Feng et al. 1999), in rat penes. Protein expressions of Rho-kinases α and ß were not changed by the treatment (data not shown). Phospho-MYPT1 levels were significantly increased in the aged rat penis relative to levels in the young rat penis after vehicle treatment (Figure 4). Chronic sildenafil treatment significantly increased MYPT1 phosphorylation in penes of young rats through day 1 wash-out and had no effect on the levels of MYPT1 phosphorylation in penes of aged rats.

Penes from aged rats have increased PDE5 protein expression and activity. Chronic sildenafil treatment increases PDE5 expression in penes of young, but not aged rats, and inhibits PDE5 activity in penes of both young and aged rats. Protein expression of PDE5 was significantly increased in the aged compared to the young rat penis after vehicle treatment (Figure 5). Chronic sildenafil treatment significantly increased PDE5 protein expression in penes of young rats

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through day 7 wash-out (on day 3 the increase of approximately 2-fold did not reach statistical significance), but had no effect on the levels of PDE5 expression in penes of aged rats.

PDE5 activity was also significantly increased in the aged compared to the young rat penis after vehicle treatment (Figure 6). Chronic sildenafil treatment significantly inhibited PDE5 activity in penes of young rats (through day 3 wash-out) and aged rats (through day 1 wash-out). The activity then returned to control levels.

Plasma concentrations of sildenafil. Mean free plasma concentrations of sildenafil decreased with time after the termination of sildenafil treatment (Table 1), and were approximately 40-50% higher in aged compared to young rats at all examined time points, which is in agreement with reduced sildenafil clearance with increasing age (Salonia et al., 2003).

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DISCUSSION

This is the first study to demonstrate the effect of long-term treatment of sildenafil on erectile capability and the underlying mechanism of the effect. Erectile ability is durably enhanced in aged, but not young, rats by chronic inhibition of PDE5, and the effect involves Akt-dependent phosphorylation of eNOS (Ser-1177) in the penis. The lack of erectile ability enhancement by chronic PDE5 inhibition in the young "healthy" penis may relate to restrained NO signaling by PDE5 and Rho-pathway upregulations, while relatively high basal levels of phosphorylated Akt and eNOS cannot be further increased by the treatment. The findings led us to propose a model for chronic sildenafil effects in young and aged rats, summarized in Figure 7. These effects were observed at sildenafil free plasma concentrations which resembled the therapeutic range in men after standard sildenafil dosing (Boolell et al., 1996). A 3 week dosing interval in the rat approximates a 2 year duration in humans, such that we believe the results are transferable to long-term treatment clinically. Whether similar mechanisms operate in other vascular beds awaits further investigation.

Age-associated erectile dysfunction is characterized by a decrease in NO production, increased contractility of the smooth muscle of the penile corpora, and corporal veno-occlusive dysfunction. Endothelial dysfunction has been implicated in the pathogenesis of erectile dysfunction associated with aging and a variety of vascular disorders such as diabetes mellitus, hypertension, heart disease, hypercholesterolemia, and atherosclerosis (Bivalacqua et al., 2003). Herein, we found decreased basal phosphorylation of eNOS (Ser-1177) in the aged compared to the young rat penis, despite an increase in total eNOS expression in the aged penis (Musicki et

al., 2005). In addition, PDE5 expression and RhoA/Rho-kinase pathway activity are increased in the aged penis, also contributing to erectile dysfunction.

The increase in the post-stimulation erectile response by chronic sildenafil is presumably due to the delayed decline of cGMP to basal levels when nerve stimulation is terminated. Sildenafil increases cGMP accumulation by competing with cGMP for PDE5 catalysis, while simultaneously potentiating its own binding to PDE5 (Corbin et al, 2003). PDE5 activity was decreased in penes of young and aged rats, as expected, in the presence of unmetabolized drug, although in aged rats this effect was pronounced only at relatively higher sildenafil free plasma concentrations. In the continuous presence of sildenafil increased levels of cGMP then promote cavernous relaxation and increase penile blood flow resulting in shear stress on endothelial cells. Shear stress promotes constitutive activation of eNOS in the penis of aged rats by increasing phosphorylation of the enzyme on Ser-1177. Increased NO release, while opposing RhoA/Rho-kinase mediated contraction, further stimulates cavernous tissue relaxation, thus durably enhancing erectile capability in aged rats. This effect of sildenafil appears to be mediated by phosphorylation/activation of Akt, as the levels of phospho-Akt were increased in parallel with that of phospho-eNOS (Ser-1177). Phosphorylation of eNOS on Ser-1177 and Akt on Ser-473 is coincident with the activation of the enzymes and thus the ratio of phospho- to total enzyme expression represents activated forms of the enzymes. At a delayed interval after the discontinuation of chronic sildenafil treatment when free plasma concentrations of sildenafil had declined, PDE5 activity and both Akt and eNOS phosphorylation levels returned to baseline. This effect coincided with complete (as measured by detumescence time) or partial (as measured by detumescence area) regression of augmented poststimulation erectile responses in aged rats.

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In addition to its role in mediating eNOS (Ser-1177) phosphorylation, increased levels of the active form of Akt in penes of aged rats treated long-term with sildenafil may indicate increased antiapoptotic capability of the penis. A considerable level of apoptosis is observed in the aged rat penis with a loss of smooth muscle cells and collagen deposition in the corpora cavernosa (Ferrini et al., 2001). Decreased apoptosis in response to chronic sildenafil treatment may improve the imbalance between smooth muscle/connective tissue and contribute to increased corporal smooth muscle relaxation.

In contrast to findings in aged rats, chronic sildenafil treatment had only minimal long-term proerectogenic effects in young rats. The young rat penis distinctively exhibited increased PDE5 expression in response to chronic sildenafil treatment. cGMP responsive sequences in the PDE5 promoter have been identified and both cGMP and sildenafil may upregulate the PDE5 promoter (Lin et al., 2002; Lin et al., 2003). Increased PDE5 protein expression in the young rat penis may result from a negative feedback in response to chronic exposure to sildenafil and sustained elevation of cGMP, preventing excessive accumulation of cGMP and excessive erection. This conclusion is strengthened by our findings that the young rat penis exhibits relatively high basal levels of phospho-Akt and phospho-eNOS (Ser-1177), which are not further increased by sildenafil. Higher levels of phosphorylated eNOS (Ser-1177) in the young “healthy” penis may generate more NO, but its signaling appears to be restrained by PDE5 upregulation in response to chronic sildenafil. The resulting decreased availability of endothelial NO/cGMP coupled with increased Rho-kinase activity in the penis of young rats apparently prevents eNOS over-activation and excessive erection in young rats, especially when free plasma concentrations of sildenafil are relatively high. These findings also corroborate clinical data showing the

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proerectile effect of sildenafil in men with erectile dysfunction only, and not in young healthy men (Mondaini et al., 2003).

Sildenafil is known to cause a moderate transient decrease in blood pressure, which reflects the presence of PDE5 in vascular smooth muscle cells and the role of NO/cGMP pathway in the regulation of systemic blood pressure (Salonia et al., 2003). In comparison with young rats, aged rats exhibited somewhat lower blood pressure basally, and sildenafil did not reduce it further, presumably due to higher basal levels of PDE5 in the systemic vasculature.

We acknowledge that our rat model of aging does not represent the phenotype of truly senescent rats, i.e., 25 months or older rats, which have been applied in other studies of erectile dysfunction (Garban et al., 1997; Magee et al., 2002). We reasoned that senescent rats may exhibit severe erectile impairment possibly refractory to this form of rehabilitative treatment. Further studies using senescent rats or other models of erectile dysfunction may be done to perceive the potential recovery of erectile ability by chronic PDE5 inhibition therapy for late aging and various other conditions associated with erectile impairment.

In conclusion, this study suggests that erectile ability can be enhanced under preconditions of erectile impairment by chronic inhibition of PDE5 and the effect is mediated by Akt-dependent eNOS phosphorylation. Through its effect on penile vascular homeostasis, the chronic use of sildenafil may be beneficial to patients with erectile dysfunction. On the other hand, chronic PDE5 inhibitor therapy would appear unhelpful in potentiating erectile ability in the absence of erectile dysfunction. This may be explained by counteractive increases in both RhoA/Rho-kinase pathway activity and levels of NO/cGMP-inhibitable PDE5 after this treatment, as well as by basally maximal levels of phosphorylated Akt and eNOS, which cannot be further increased by this treatment.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Effect of chronic sildenafil treatment on erectile response. Young and aged rats were injected with sildenafil or saline subcutaneously every 8 hours for 3 weeks. After a 1, 3, or 7 day wash-out period, a group of vehicle- and sildenafil-treated rats underwent electrical stimulation of the cavernous nerve to induce erection. Response parameters were expressed per MAP. ICP area is the area under the curve which corresponds to the duration of electrical stimulation; detumescence time is the period from the end of electrical stimulation to the point exhibiting 50% of maximal ICP; detumescence area is the area under the ICP curve during this interval. Values are normalized to young vehicle-treated; n=5-6. *, $p<0.05$ compared with young + vehicle; #, $p<0.05$ compared with aged + vehicle. Veh, vehicle-treated; Sd1, Sd3, Sd7 are responses on day 1, 3, and 7 wash-out after chronic sildenafil treatment.

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Figure 2. Effect of chronic sildenafil treatment on eNOS (Ser-1177) phosphorylation [P-eNOS (Ser-1177)] in rat penes. Young and aged rats were injected with sildenafil or saline subcutaneously every 8 hours for 3 weeks. After a 1, 3, or 7 day wash-out period, penes were excised. P-eNOS was examined in partially purified homogenates by Western blotting. (A) and (B) are 2 representative Western immunoblots of P-eNOS (Ser-1177) and total eNOS on days 1 and 3 (A) and on day 7 (B) wash-out; (C) quantitative analysis of P-eNOS (Ser-1177) in penes of young and aged rats on days 1, 3, and 7 wash-outs. Results are expressed as the ratio of P-eNOS (Ser-1177) to total eNOS relative to the ratio for saline-treated young animal samples prepared on the same blot; n=6; *, $p<0.05$ compared with young + vehicle; #p, $p<0.05$ compared with aged + vehicle. Abbreviations are explained in the legend to Figure 1.

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Figure 3. Effect of chronic sildenafil treatment on Akt (Ser-473) phosphorylation [P-Akt (Ser-473)] in rat penes. Young and aged rats were injected with sildenafil or saline subcutaneously every 8 hours for 3 weeks. After a 1, 3, or 7 day wash-out period, penes were excised for Western blot analysis. (A) and (B) are 2 representative Western immunoblots of P-Akt (Ser-473) and total Akt on days 1 and 3 (A) and on day 7 (B) wash-out; (C) quantitative analysis of P-Akt (Ser-473) in penes of young and aged rats on days 1, 3, and 7 wash-outs. Results are expressed as the ratio of P-Akt (Ser-473) to total Akt relative to the ratio for saline-treated young animal samples prepared on the same blot; n=6; *, $p<0.05$ compared with young + vehicle; #, $p<0.05$ compared with aged + vehicle. Abbreviations are explained in the legend to Figure 1

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Figure 4. Effect of chronic sildenafil treatment on MYPT1 (Thr-696) phosphorylation [P-MYPT1 (Thr-696)], a marker of Rho-kinase activity. Western blot analysis was performed on penile homogenates. (A) and (B) are 2 representative Western immunoblots of P-MYPT1 (Thr-696) and β-actin on days 1 and 3 (A) and on day 7 (B) wash-out; (C) quantitative analysis of P-MYPT1 (Thr-696) in penes of young and aged rats on days 1, 3, and 7 wash-outs. Results are expressed per β-actin relative to the ratio for saline-treated young animal samples prepared on the same blot; n=6; *, $p<0.05$ compared with young + vehicle; #, $p<0.05$ compared with aged + vehicle. Abbreviations are explained in the legend to Figure 1.

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Figure 5. Effect of chronic sildenafil treatment on PDE5 protein expression in rat penes. Western blot analysis was performed on penile homogenates. (A) and (B) are 2 representative Western immunoblots of PDE5 and β-actin on days 1 and 3 (A) and on day 7 (B) wash-out; (C) quantitative analysis of PDE5 in penes of young and aged rats on days 1, 3, and 7 wash-outs. Results are expressed per β-actin relative to the ratio for saline-treated young animal samples prepared and blotted at the same time; n=6; *, $p<0.05$ compared with young + vehicle; #, $p<0.05$ compared with aged + vehicle. Abbreviations are explained in the legend to Figure 1.

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Figure 6. Effect of chronic sildenafil treatment on PDE5 activity in penes of young and aged rats on days 1, 3, and 7 wash-out. Penile extracts were assayed for cGMP-dependent PDE activity. Values are normalized to young vehicle-treated; n=4-5; *, $p<0.05$ compared with young + vehicle; #, $p<0.05$ compared with aged + vehicle. Abbreviations are explained in the legend to Figure 1.

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Figure 7. A model for improved erectile capability in aged but not young rats by chronic inhibition of PDE5 by sildenafil. Sexual stimuli elicit short-lived bursts of NO from neuronal NO synthase (nNOS) in nerve fibers of the penis, which initiates relaxation of cavernous smooth muscle. The resulting increase in blood flow/shear stress activates PI3-kinase/Akt, which phosphorylates eNOS (Ser-1177) in vascular and sinusoidal endothelial cells resulting in a sustained increase in NO, thereby causing continued relaxation and maximal erection. In young rats chronic treatment with sildenafil upregulates PDE5 expression in the penis, decreasing the availability of neuronal and endothelial NO/cGMP. The resulting reduced suppression of RhoA/Rho-kinase-mediated contraction, coupled with increased Rho-kinase activity and unchanged Akt and eNOS (Ser-1177) phosphorylation, negatively affects cavernous smooth muscle relaxation and prevents potentiation of erection. In contrast, chronic treatment of aged rats with sildenafil did not change PDE5 expression or RhoA/Rho-kinase pathway activity, while it spurs Akt and eNOS (Ser-1177) phosphorylation in the penis. The increased availability of NO/cGMP, while opposing RhoA/Rho-kinase-mediated contraction, promotes cavernous smooth muscle relaxation and potentiates erectile capability. The difference in the response to chronic sildenafil between young and aged rats may relate to differences in baseline expression of mediators of penile erection. Aging is characterized by elevated basal levels of PDE5 expression and RhoA/Rho-kinase-pathway activity and decreased basal levels of phosphorylated Akt and eNOS (Ser-1177).

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TABLE 1.

Mean free plasma concentrations of sildenafil in young and aged rats on day 1, 3, and 7 wash-outs.

Plasma sildenafil (free nM)

	Young	Aged
day 1	26.6 ± 1.1	35.4 ± 3.7
day 3	13.4 ± 0.9*	24.3 ± 4.5*
day 7	0.9 ± 0.1*	2.4 ± 0.2*

Mean free plasma concentrations of sildenafil decreased with time after the termination of 3-week continuous sildenafil treatment of young and aged rats; n=9-15. *, $p<0.05$ compared with day 1 wash-out.

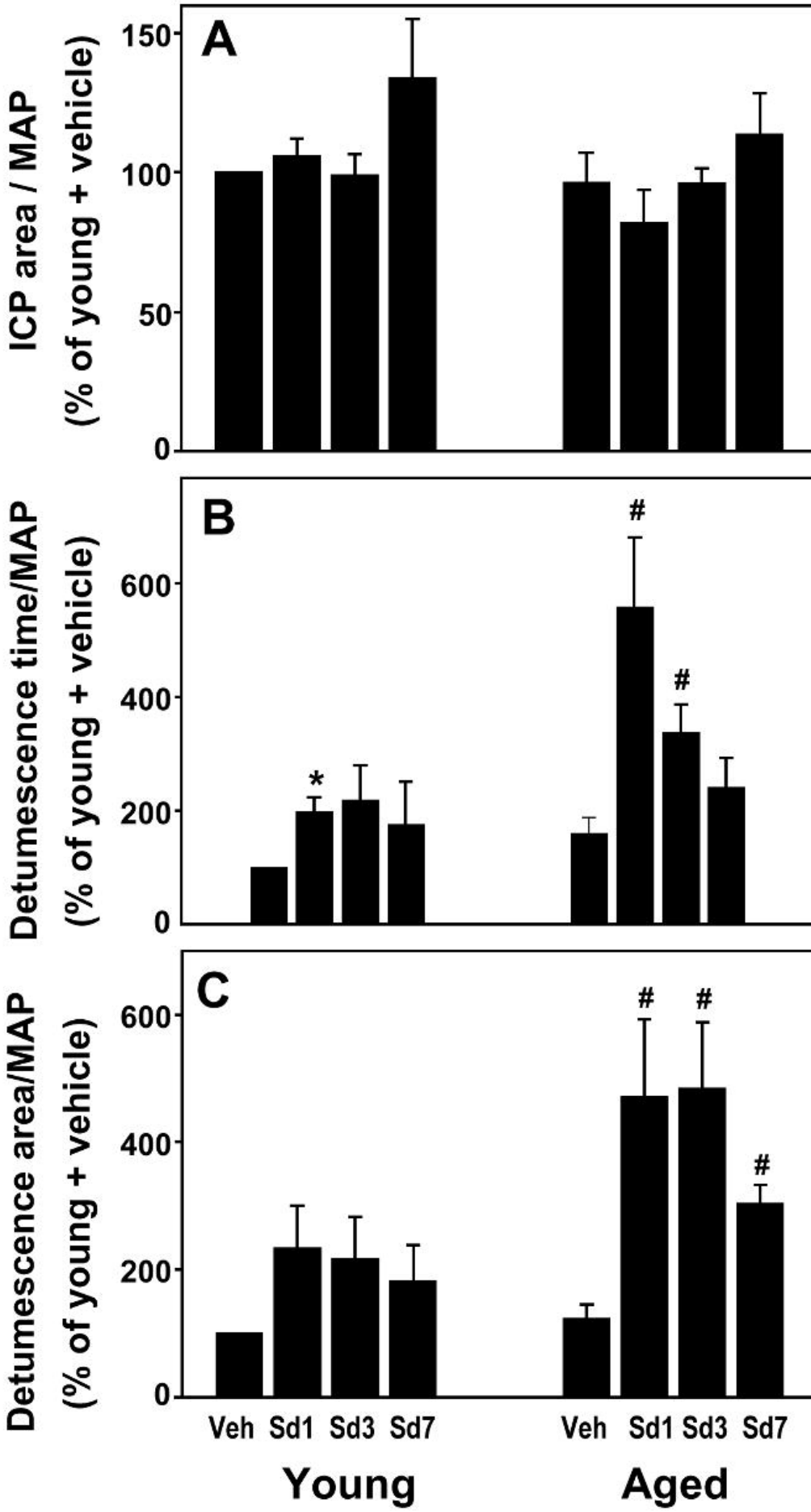

A
ICP area / MAP
(% of young + vehicle)
150
100
50
0
B
Detumescence time/MAP
(% of young + vehicle)
600
400
200
0
*
#
#
C
Detumescence area/MAP
(% of young + vehicle)
600
400
200
0
#
#
#
Veh Sd1 Sd3 Sd7
Young
Veh Sd1 Sd3 Sd7
Aged

Figure 1

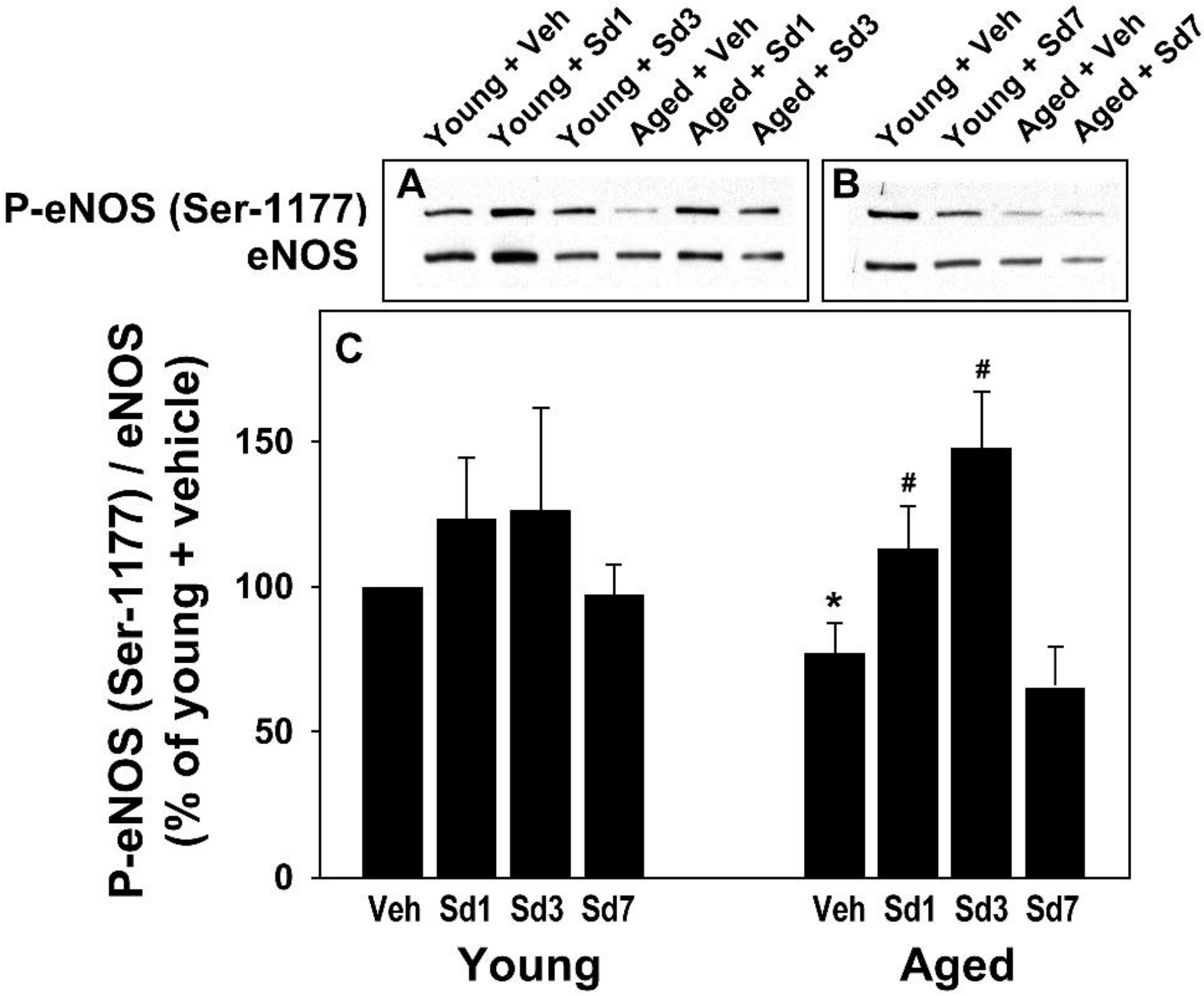

Young + Veh
Young + Sd1
Young + Sd3
Aged + Veh
Aged + Sd1
Aged + Sd3
Young + Veh
Young + Sd7
Aged + Veh
Aged + Sd7
P-eNOS (Ser-1177)
eNOS
A
B
C
P-eNOS (Ser-1177) / eNOS
(% of young + vehicle)
150
100
50
0
*
#
#
Veh Sd1 Sd3 Sd7
Young
Veh Sd1 Sd3 Sd7
Aged

Figure 2

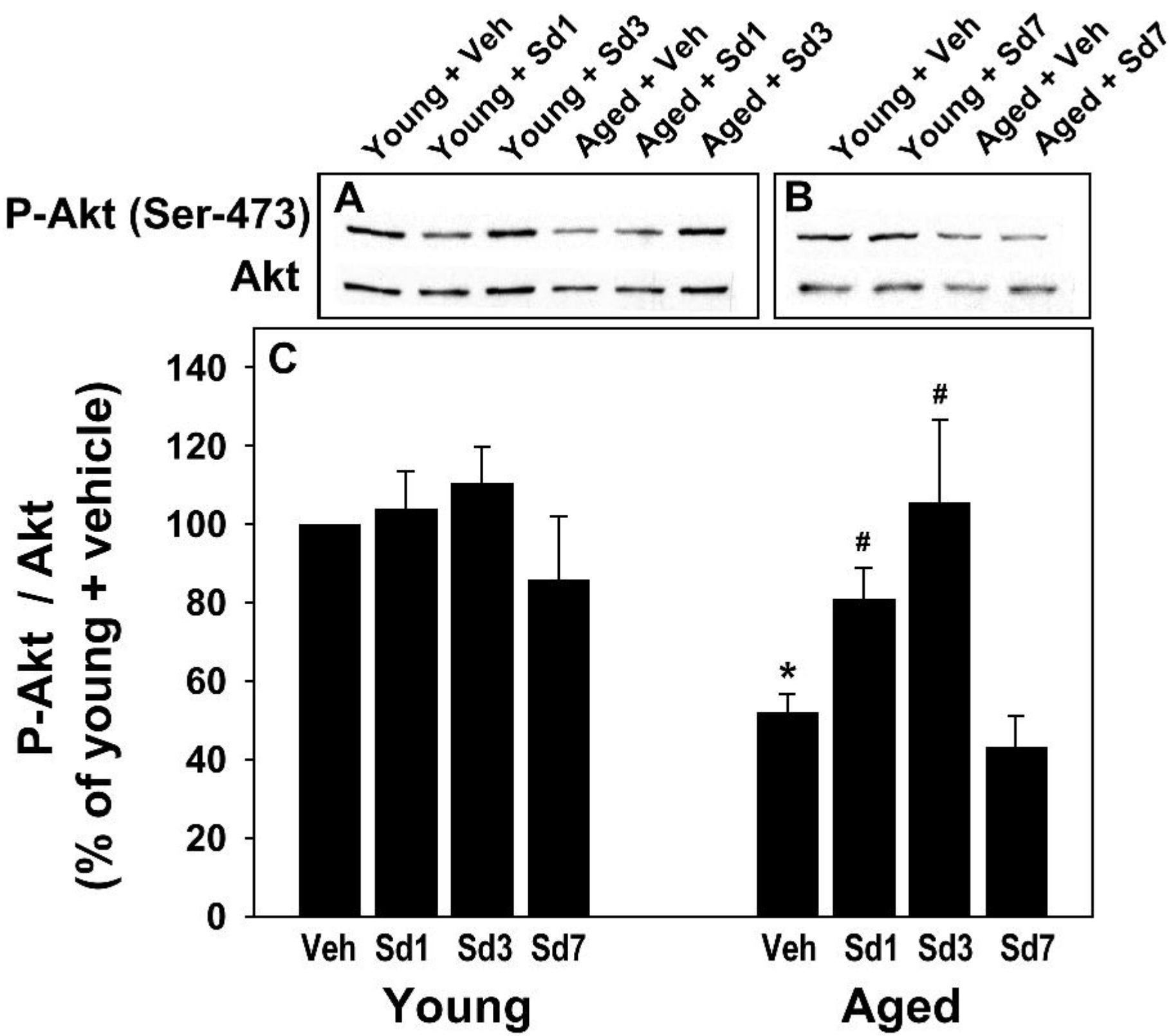

Young + Veh
Young + Sd1
Young + Sd3
Aged + Veh
Aged + Sd1
Aged + Sd3
Young + Veh
Young + Sd7
Aged + Veh
Aged + Sd7
P-Akt (Ser-473)
Akt
A
B
C
P-Akt / Akt
(% of young + vehicle)
140
120
100
80
60
40
20
0
*
#
#
Veh
Sd1
Sd3
Sd7
Young
Veh
Sd1
Sd3
Sd7
Aged

Figure 3

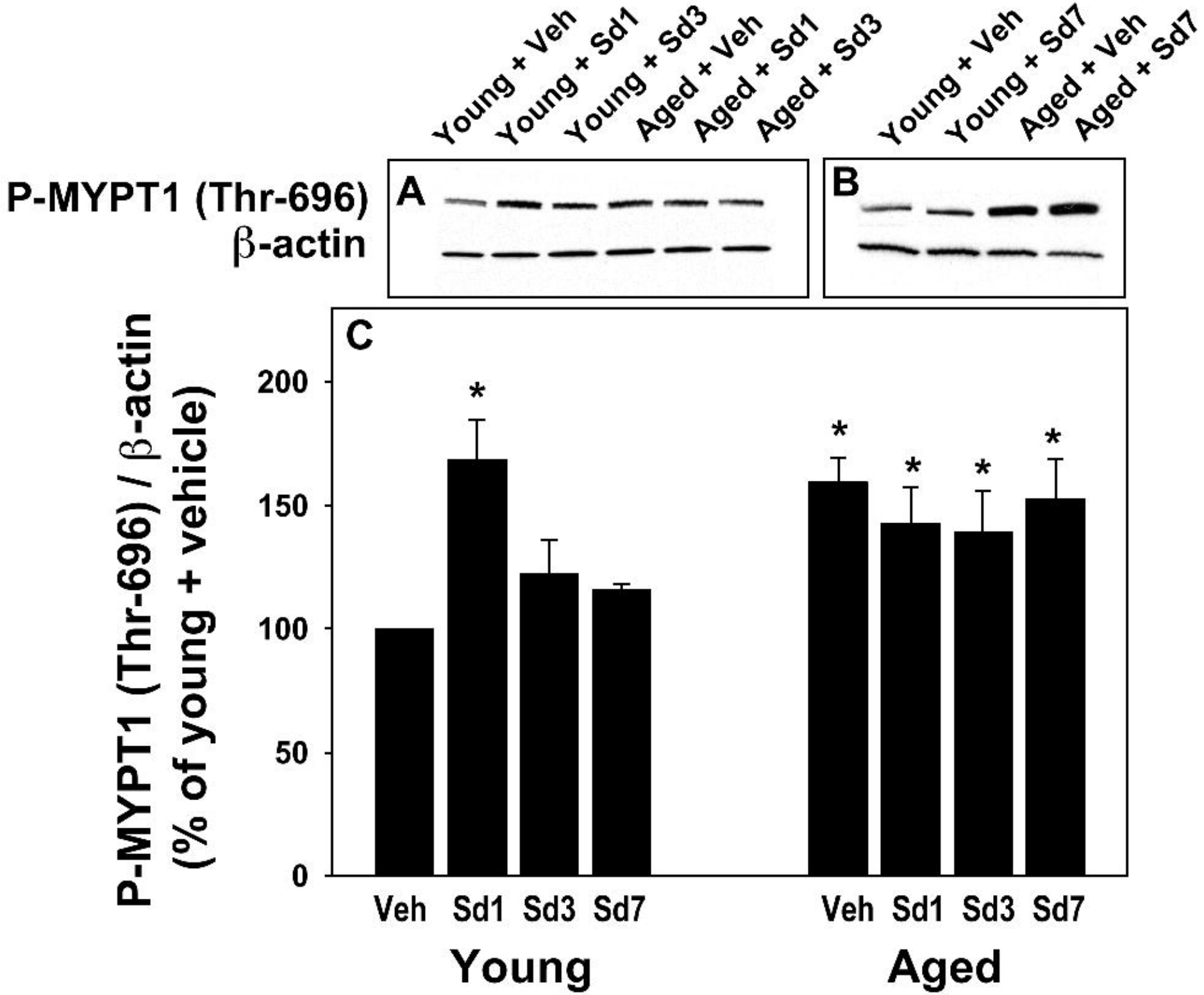

Young + Veh
Young + Sd1
Young + Sd3
Aged + Veh
Aged + Sd1
Aged + Sd3
Young + Veh
Young + Sd7
Aged + Veh
Aged + Sd7
P-MYPT1 (Thr-696)
β-actin
A
B
C
P-MYPT1 (Thr-696) / β-actin
(% of young + vehicle)
200
150
100
50
0
*
Veh
Sd1
Sd3
Sd7
Young
Aged

Figure 4

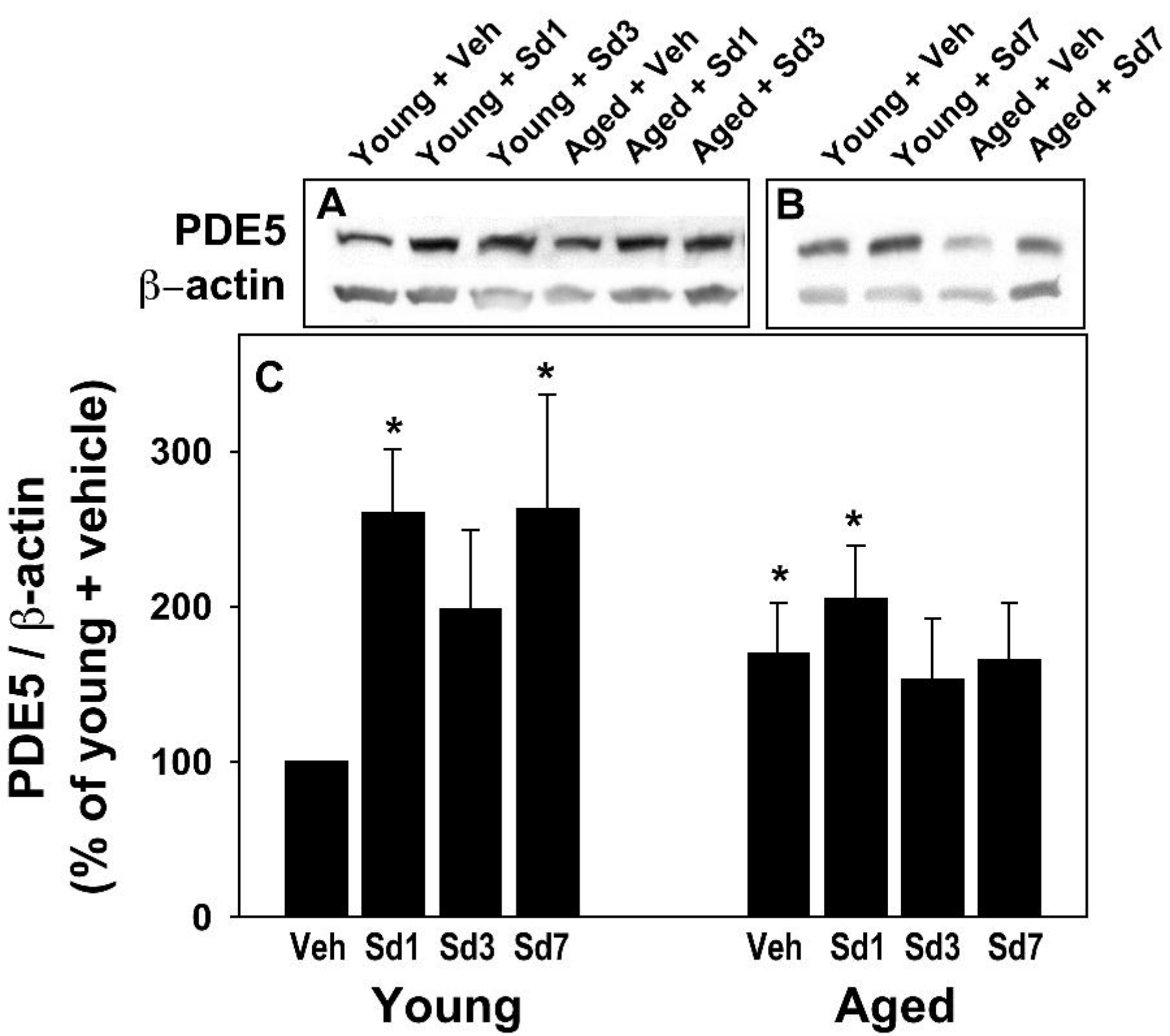

Young + Veh
Young + Sd1
Young + Sd3
Aged + Veh
Aged + Sd1
Aged + Sd3
Young + Veh
Young + Sd7
Aged + Veh
Aged + Sd7
A
B
PDE5
β–actin
C
PDE5 / β-actin
(% of young + vehicle)
300
200
100
0
*
*
*
*
Veh
Sd1
Sd3
Sd7
Young
Veh
Sd1
Sd3
Sd7
Aged

Figure 5

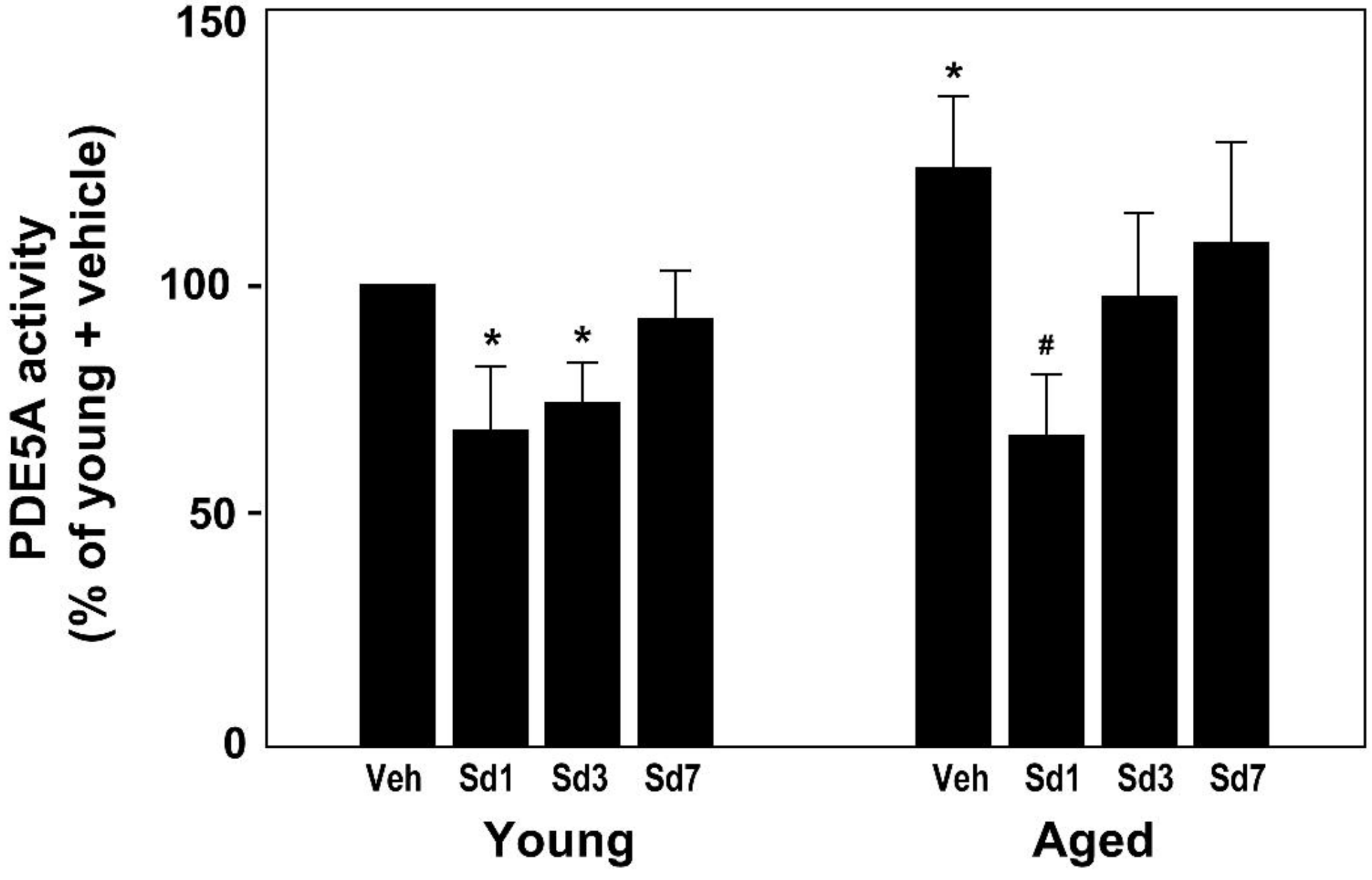
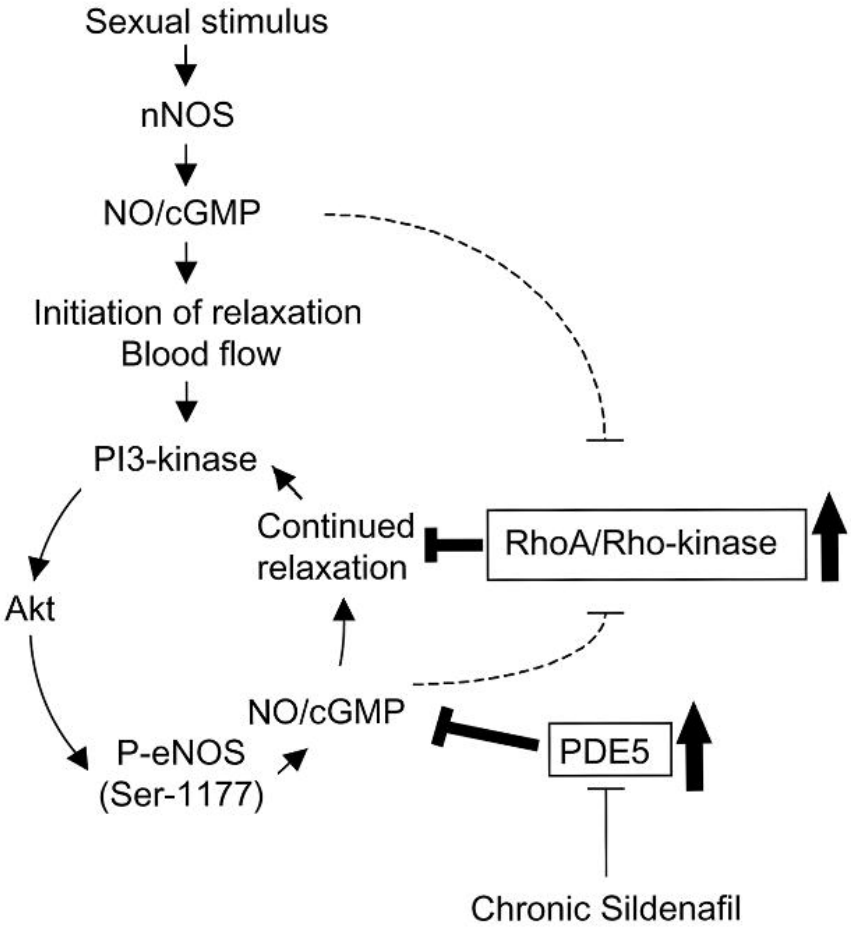
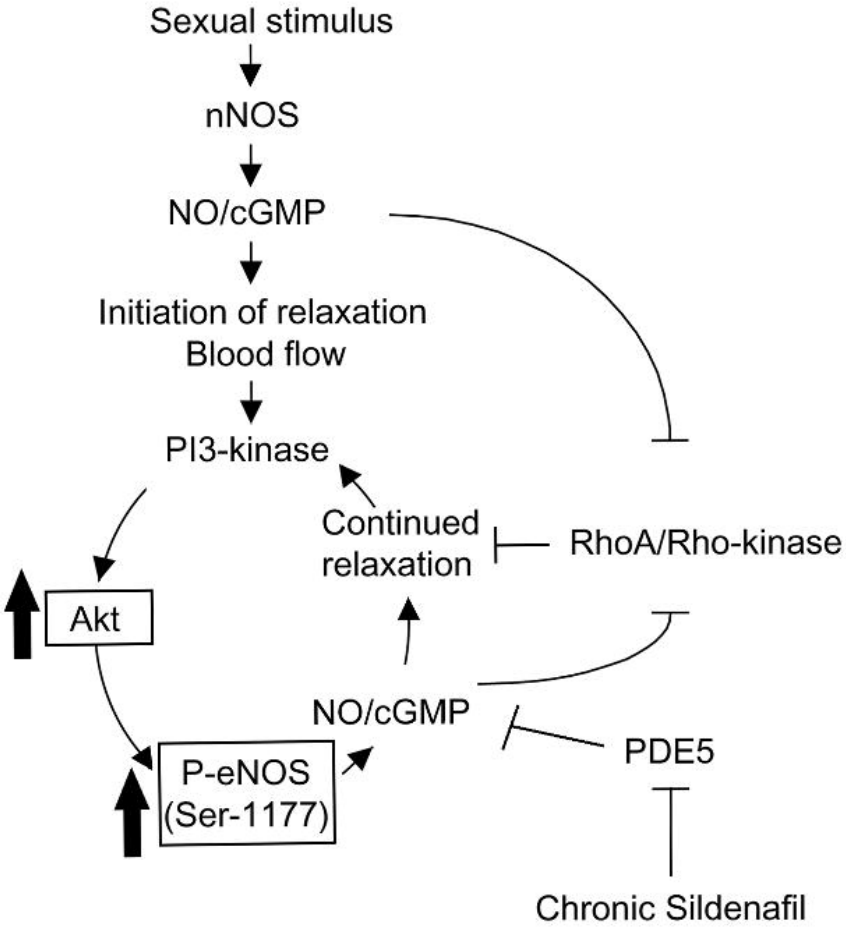

150
100
50
0
PDE5A activity
(% of young + vehicle)
*
*
*
#
Veh
Sd1
Sd3
Sd7
Young
Veh
Sd1
Sd3
Sd7
Aged

Figure 6



Young: no effect on erection



Aged: increased erectile capability

Figure 7