

Expression of the PDE5 enzyme on human retinal tissue: new aspects of PDE5 inhibitors ocular side effects

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Abstract

Objective We tested the effect of two phosphodiesterase type-5 (PDE5) inhibitors, sildenafil and tadalafil, on ophthalmic artery (OA) blood flow velocity and investigated the presence of the PDE5 enzyme on human retinal tissue in comparison with the PDE6 enzyme localization.

Methods Using Colour Doppler ultrasonography (CDU) we investigated, in 30 healthy young subjects (27.8 years of age; range, 24.3–33.7 years), the effects of a single oral dose of sildenafil (100 mg), tadalafil (20 mg), and placebo on OA blood flow velocity. Western blot for PDE6 and PDE5 protein expression was performed on frozen samples of human retina, testis, sperm, skin, and corpus cavernosum.

Immunohistochemistry was performed on two ocular globes from dead donors.

Results CDU showed a relationship between the administration of PDE5 inhibitors and OA blood flow velocity modifications in a time-dependent manner. Western blot and immunohistochemical analysis showed PDE6 and PDE5 presence in human retinal tissue and gave a map of its distribution.

Conclusion We demonstrated that (a) tadalafil and sildenafil are able to modify the OA flux in a time-dependent manner; (b) the PDE5 enzyme is expressed on retinal and choroid vasculature (smooth muscle and endothelial cells), on ganglion and bipolar cells; (c) human retinal tissues express the PDE6 enzyme in the rod and cone photoreceptors; (d) visual side effects after PDE5 inhibitors administration may be linked to a specific effect on the PDE5 enzyme; and (e) the PDE5 enzyme may have a physiologic role on ganglion and bipolar cells that need to be further investigated.

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Introduction

Phosphodiesterase type-5 (PDE5) inhibitors are a widely used class of drugs initially studied for their potential effect on the cardiovascular system. It soon became evident that PDE5 inhibitors have considerable effects on the biochemical signalling pathway leading to erection. Since this discovery, the use of PDE5 inhibitors in the treatment of erectile dysfunction (ED) is now considered a cornerstone.^{1–4} Administration of PDE5 inhibitors has been associated with visual disturbances such as blue tinge, increased brightness of lights and blurred vision;⁵ however, the exact mechanism of these disturbances has not been definitively elucidated. We set out to study the effect of sildenafil and tadalafil on ophthalmic artery (OA) blood flow velocity. Furthermore, we investigated the expression of the PDE5 enzyme on human retinal tissue in comparison with the PDE6 enzyme localization.

Patients and methods

The research followed the tenets of the Declaration of Helsinki. After approval by Local Ethics Committee of the University of Padova, 30 healthy young subjects (27.8 years of age; range, 24.3–33.7 years) gave informed consent and were enrolled in this study (double-blind and placebo-controlled) to investigate the effects

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of a single oral dose of sildenafil (100 mg), tadalafil (20 mg), and placebo on OA blood flow velocity by using Colour Doppler ultrasonography (CDU). Subjects were randomly and blindly assigned to each group. Patients with coronary artery disease, diabetes mellitus, systemic hypertension, and ocular diseases were excluded from the study. All subjects underwent an ophthalmologist's clinical and instrumental examination to exclude degenerative or vascular ocular diseases. Intraocular pressure was below 21 mmHg in both eyes and none of the patients was under any continuous and ophthalmological medication or any treatment with nitrates. Systolic and diastolic blood pressure were measured before and 1 h after the dose of PDE5 inhibitor or placebo. The patients were also questioned about side effects and visual symptoms. For evaluation of ocular blood flow, CDU measurements were performed using a high-resolution Echo-Colour-Doppler (AU5, Esaote, Genoa, Italy) equipped with a 7.5 MHz linear probe with <0.2 mm axial resolution. After administration of a single tablet of sildenafil, tadalafil, or placebo, the OA was examined as follows. After 15 min rest in supine position in a quiet and semi-darkened room, ultrasound gel was applied to the closed eyelid of the orbits; Doppler probe was then placed and moved gently on the eyelid to avoid any pressure on the eye. The peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured on the OA⁶⁻⁸ in both orbits. Doppler examination was performed in each subject at baseline and after 1, 4, 12, 24, 36, and 48 h after the drug administration to evaluate the influence of PDE5 inhibitors at different circulating concentration. Immunoblotting assay and immunohistochemistry were performed on two ocular globes from different donors following cornea removal for transplantation. These two Caucasian donors, aged 45 and 51 respectively, were healthy and without ocular disease.

For immunoblotting assay, a total cellular proteins extract has been obtained by frozen samples of human retina, testis, sperm, skin, and corpus cavernosum, using the standard protocol of ReadyPrep Protein Extraction Kit (Biorad, Hercules, CA, USA). The final concentration of proteins has been determined by Bradford proteins assay. Primarily, the compatibility and functionality of primary (anti-PDE5 and anti-PDE6) and secondary (anti-rabbit) antibodies has been tested by dot-blot assay. Aliquots of the samples, containing equal amounts of proteins (20 µg), were suspended in reducing SDS-PAGE sample buffer and boiled for 5 min. Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes using the Mini-transblot system (both Biorad, Hercules, CA, USA). These membranes were incubated in blocking buffer (0.05% Tween-20 in phosphate-buffered saline (PBS) containing 0.5% casein) for 1 hour, followed by overnight

incubation with primary rabbit antibodies directed against PDE5 and PDE6 at 4°C (Abcam, Cambridge, UK). After incubation with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit polyclonal antibody (BD Transduction Laboratories, Franklin Lake, NJ, USA), immunoreactivity was detected by chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) (Figure 2a and b).

For immunohistochemistry, tissue was formalin-fixed and processed into paraffin wax using standard methods. Sections (3 µm) were cut, mounted onto polylysinated slides, dried at 40° ON, dewaxed, rehydrated in graded ethanols. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide containing sodium azide and levamisole for 6 min and then rinsed gently in PBS for 3 min. For the staining we used the Envision + Dual Link System-HRP (DAB +) (DakoCytomation, Fort Collins, CO, USA) that is based on an HRP-labeled polymer which is conjugated with secondary antibodies, this system does not require Normal Goat Serum incubation for nonspecific binding. The primary antibody was serially diluted (1:400, 1:800, and 1:1600) and incubated for 30 min at room temperature, then slides were washed in PBS for 3 min and stained with the peroxidase-labeled polymer for 30 min. After a wash in PBS the substrate-chromogen solution was applied and incubated for 5 min. At the end, slides were washed with distilled water and counterstained with hematoxylin. The primary antibody against PDE-6 enzyme was provided by Abcam (Cambridge, CA, UK). It was a polyclonal Ab raised in rabbit and immunogen affinity purified. The Abcam's synthetic peptide is 'MGEVTAEEVEKFLDSN', corresponding to amino acids 1-16 of human PDE6 α . This sequence is completely conserved in humans, mouse, and bovine and 94% conserved in canine. The primary antibody against PDE-5 was provided by Abcam (Cambridge, CA, UK). It was a polyclonal Ab raised in rabbit and immunogen affinity purified. Abcam's immunogen is a synthetic cyclic peptide (common to all PDE5A variants). Data are expressed as median (range). Comparison between baseline and at each end point was performed by the Wilcoxon sum ranks test for matched pairs. *P*-values <0.05 were regarded as statistically significant.

Results

No statistically significant differences in heart rate, systolic, and diastolic blood pressure were found between the values before and 1 h after drugs (sildenafil, tadalafil, and placebo) administration (*P* > 0.05). Functional studies on the PDE5 inhibitors and placebo influence on OA blood flow velocity are given in Table 1

and Figure 1. Measurements were done at each end point (baseline, 1, 4, 8, 12, 24, 36, and 48 h) for the three drugs (sildenafil, tadalafil, and placebo). Colour Doppler measurements of the right and left eyes were compared using the Mann–Whitney *U*-test before and after drug intake. No statistical differences were detected between the right and left eyes ($P > 0.05$). Administration of 100 mg sildenafil resulted in a significant increase ($P < 0.01$) in OA PSV and EDV at 60 min after drug uptake. At 4, 8, 12, 24, 36, and 48 h after sildenafil uptake OA blood flow velocity values no longer differed significantly from baseline (Table 1 and Figure 1). Tadalafil (20 mg) administration determined a significant increase ($P < 0.001$ and $P < 0.01$) in OA PSV and EDV values at 1, 4, 8, 12, 24, and 36 h after uptake (Table 1 and Figure 1). Placebo administration have no influence on OA blood flow velocity values (Table 1 and Figure 1). After PDE5 inhibitors administration, no cardiovascular events were found and side-effect rates were similar to those reported in literature: six patients (20%) reported side effects like headache (three patients), nasal congestion (one patient), flushing (one patient), and increased light sensitivity (one patient). Other visual side effects were not reported. To evaluate the possible direct influence of PDE5 inhibitors on retinal blood flow and function we performed an immunoblotting assay and an immunohistochemistry on retinal tissue for PDE5 and PDE6 expression.

Figure 2 provides Western blot for PDE6 and PDE5 protein expression (a and b) and immunohistochemistry for both enzymes (c and d). Western blot for both PDE5 and PDE6 demonstrate the presence of the two proteins in retinal tissues. The immunohistochemical analysis clearly revealed that the PDE6 enzyme is expressed on the photoreceptor layer both on rods and cones. Finally, we demonstrated the PDE5 localization on endothelial and smooth muscle cells of the vascular wall (retina and choroids vessels), on ganglion (III neuron), and bipolar cells (II neuron).

Discussion

Selective PDE5 inhibitors are widely used drugs for the treatment of ED, given that PDE5 is thought to be responsible of penis detumescence. Inhibition of PDE5 is known to increase the level of cyclic guanosine monophosphate (cGMP), an intracellular messenger effecting vasodilation by relaxation of smooth muscle in arterioles. Production of cGMP from guanosine triphosphate is mediated through the nitric oxide (NO) signalling pathway. By inhibiting PDE5, PDE5 inhibitors increase cGMP levels and thereby potentiate the NO-elicited effect on sinusoidal vessels of corpora cavernosa.^{9,10} The PDE5 enzyme is largely expressed in

Table 1 PSV and EDV values of the OA after placebo, sildenafil and tadalafil administration

	Baseline	1 h	4 h	8 h	12 h	24 h	36 h	48 h
PSV (cm/s) placebo no., 10 pts	69.0 (43.0–90.5)	60.5 (49.5–91.5)	65.5 (48.0–84.0)	69.5 (56.5–86.5)	73.5 (60.5–90.0)	65.5 (55.0–87.5)	60.0 (47.5–77.5)	65.0 (48.0–83.0)
EDV (cm/s) placebo no., 10 pts	22.0 (13.0–30.5)	19.5 (12.5–33.0)	22.5 (13.0–30.0)	25.0 (16.0–34.0)	23.0 (16–27.5)	23.5 (14.0–32.0)	25.5 (18.0–44.5)	23.0 (18.0–35.5)
PSV (cm/s) tadalafil (20 mg) no., 10 pts	63.0 (54.0–74.0)	106.5 (83.0–145.0)*	87.0 (80.0–105.0)*	93.5 (77.0–98.0)*	89.5 (81.0–100.0)*	84.0 (76.0–95.0)*	77.5 (63.0–100.0)**	63.0 (57.0–76.0)
EDV (cm/s) tadalafil (20 mg) no., 10 pts	27.5 (15.0–35.0)	43.5 (21.0–55.0)*	37.0 (19.0–46.0)*	40.0 (20.0–48.0)*	36.0 (17.0–47.0)**	36.0 (18.0–42.0)**	32.0 (16.0–38.0)	25.5 (14.0–32.0)
PSV (cm/s) sildenafil (100 mg) no., 10 pts	67.5 (44.5–88.5)	102.5 (74.5–120.0)**	69.5 (48.0–86.5)	65.0 (52.5–93.0)	66.0 (49.5–74)	70.0 (57.0–83.5)	70.5 (46.0–75–5)	71.0 (44–81.5)
EDV (cm/s) sildenafil (100 mg) no., 10 pts	19.5 (9.5–32.0)	31.5 (22.5–55.5)**	20.0 (11.0–24.5)	19.0 (16.5–25)	18.0 (11.5–22.0)	20.0 (14.5–24.5)	21.0 (15.0–29.0)	20.0 (16.0–27.5)

Abbreviations: EDV, end-diastolic velocity; OA, ophthalmic artery; pts, patients; PSV, peak systolic velocity. * $P < 0.001$ vs baseline; ** $P < 0.01$ vs baseline.

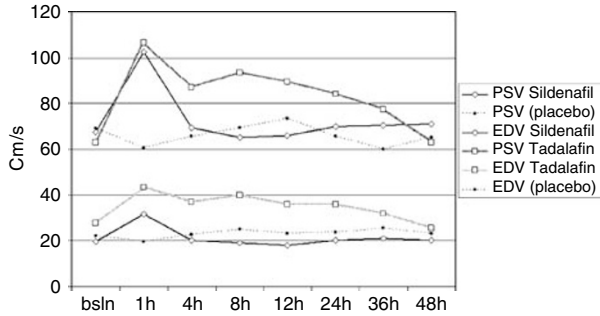


Figure 1 PSV and EDV values in ophthalmic artery after sildenafil (100 mg), tadalafil (20 mg) and placebo uptake. After sildenafil uptake, a significant increase ($P < 0.01$) in OA PSV and EDV was observed only at 60 min after drug uptake. After tadalafil uptake a significant increase in OA PSV was observed at 1, 4, 8, 12, 24 ($P < 0.001$), and 36 h ($P < 0.01$). A significant increase in OA EDV values was observed at 1, 4, 8 ($P < 0.001$), and 12 h, 24 h ($P < 0.01$) after drug uptake. EDV, end-diastolic velocity; OA, PSV; peak systolic velocity.

the corpora cavernosa of the penis, but many other organs express the PDE5 enzyme such as pulmonary and coronary vasculature, sympathetic nervous system, and Purkinje neurons,^{11–13} where its influence is less known. Minor adverse effects of PDE5 inhibitors administration such as headache, flushing, hypotension, dyspepsia, and visual disturbances may be due to this influence and inspired many studies.^{14–16} To the best of our knowledge, the presence of the PDE5 enzyme on the retinal tissue has not been documented and the reported visual side effects of the most studied molecule (sildenafil) has been related to its interaction with PDE6. PDE6 is intensely localized at the rod and cone cells and has an important role in the phototransduction cascade.^{17–20} PDE5 inhibitors show a PDE5 affinity only 10 times greater than PDE6, so some recent studies hypothesized that visual side effects of these drugs may be related to their cross-reactivity with PDE6.^{21–26}

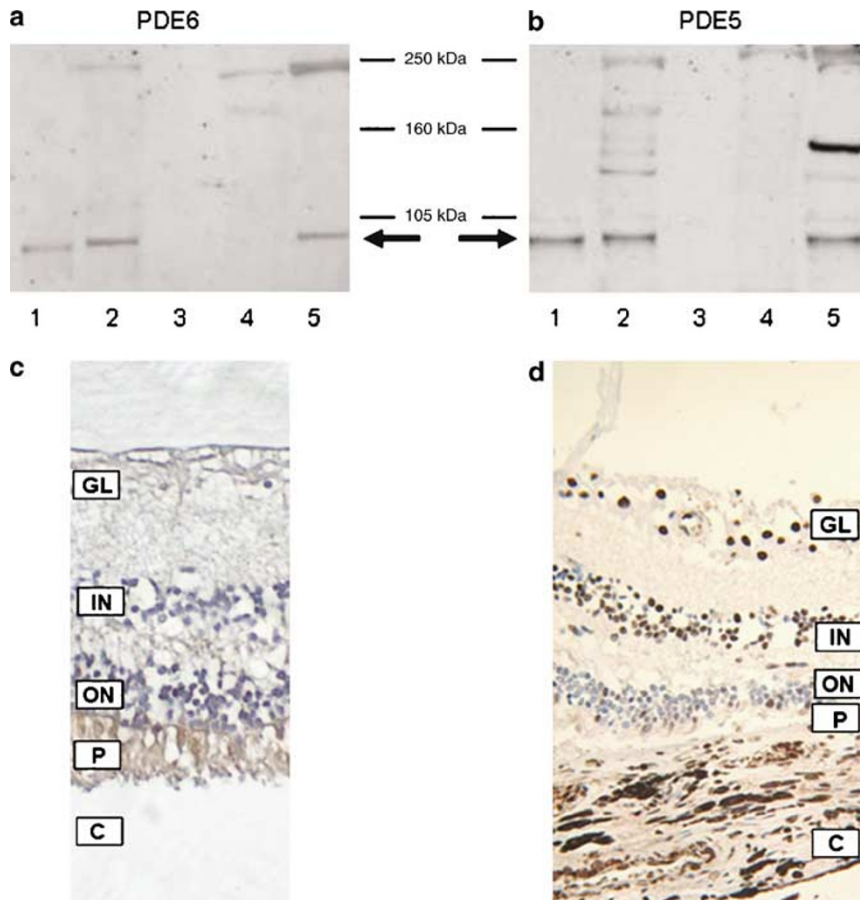


Figure 2 Western blot for both PDE6 and PDE5 (a and b) demonstrating the presence of the two proteins in human retinal tissues, testis, and corpus cavernosum (1: retina, 2: testis, 3: spermatozoa, 4: skin, 5: corpus cavernosum). The immunohistochemical analysis (c and d) clearly revealed that the PDE6 enzyme is expressed on the photoreceptor layer both on rods and cones. The PDE5 has been localized on endothelial and smooth muscle cells of the vascular wall (retina and choroids vessels), on ganglion cells (III neuron) and bipolar cells (II neuron). C, choroids; P, photoreceptors layer; ON, outer nuclear layer; IN, inner nuclear layer; GL, ganglion cell layer. PDE5, phosphodiesterase type-5; PDE6, phosphodiesterase type-6.

In addition, few data are available about alterations of ocular blood flow after PDE5 inhibitors administration, and all are about sildenafil.^{27–32} Sildenafil causes a significant increase in blood flow velocity in the ophthalmic artery, a significant dilation of retinal arteries and an increase in choroid and retinal vessels blood flow in healthy subjects.^{28,30–44} This influence may be due to the NO signalling pathway amplification. In fact, blood vessels are innervated by neurons producing NO that plays a very important role in the local regulation of ocular blood flow.^{45,46} The authors postulated that assuming the existence of PDE5 in the retinal vasculature, these effects may be justified. Recently, Koksals *et al*³⁰ found that sildenafil causes a significant increase in blood flow of OA, probably as a consequence of PDE5 inhibition on smooth muscle cells, in a time-dependent manner. The time course of the vasodilatory effect of sildenafil shows the same pattern as found in the corpus cavernosum and various other human tissue^{1,11} confirming that the mechanism of action is probably the same. Hypothesizing not only a drug-induced but also a class-induced effect on ocular arteries, we compared the blood flow modifications on the OA of a single dose of sildenafil with a single dose of tadalafil. The half-life of sildenafil is about 4 h (maximum observed plasma concentration within 30–60 min) and that of tadalafil is about 48 h (maximum observed plasma concentration within 30 min to 6 h). We chose to study the OA and not the central retinal artery and the ciliary artery because, for anatomical reasons, the OA is better investigatable (greater and easier to find) and because, from the literature, it seemed that the OA is the most responsive ocular artery after PDE5 inhibitors administration (sildenafil).^{28,30} After the assumption of the two PDE5 inhibitors (sildenafil and tadalafil), we showed an increase in OA blood flow velocity that keep pace with the time metabolization of the two drugs (Figure 1). These findings showed that observed ocular blood flow modifications are not drug-specific but class-specific. Then we investigated the presence of the PDE5 and PDE6 enzyme on human retinal tissues with Western blot and immunohistochemical analysis confirming their presence and giving a map of their distribution. Interestingly, we observed that the PDE5 enzyme has a different distribution with respect to PDE6. In accordance with animal findings, we demonstrate for the first time that the PDE6 enzyme is expressed on human retinal tissues (Western blot) and is located in rod and cone photoreceptors (immunohistochemistry) also in humans where it has a role on visual transduction (low-noise and high-gain signal amplification). The PDE5 enzyme showed a wide expression on retinal and choroidal vessels (Figure 2), confirming the direct influence of PDE5 inhibitors on their dilation. Finally, unexpectedly,

we found the presence of the PDE5 enzyme on ganglion (III neuron) and bipolar cell layers (II neuron) (Figure 2). Ganglion and bipolar cell layers act as a filter in the visual signal, giving a first codification of the neural signal.^{40–43} This is of interest because visual side effects after administration of PDE5 inhibitor might now be considered a consequence of a direct effect on the PDE5 enzyme. Therefore, our study has some limitations that deserve comment: first, the relatively small number of subjects; second, we measure CDU readings only in the OA even though measuring of the central retinal artery (CRA) and posterior ciliary arteries (PCA) would yield additional important information.

In conclusion, herein we have demonstrated that (a) tadalafil and sildenafil are able to modify the OA flux in a time-dependent manner; (b) the PDE5 enzyme is expressed on retinal and choroid vasculature (smooth muscle and endothelial cells), on ganglion and bipolar cells; (c) animals human retinal tissues express the PDE5 enzyme in the rod and cone photoreceptors; (d) visual side effects after PDE5 inhibitor administration may be linked not only to their affinity for the PDE6 enzyme, but also to a specific effect on the PDE5 enzyme; (e) the PDE5 enzyme may have a physiologic role on ganglion and bipolar cells that need to be further investigated.

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