J Anesth (2015) 29:186–190
DOI 10.1007/s00540-014-1903-x

ORIGINAL ARTICLE

Direct effects of Rho-kinase inhibitor on pial microvessels in rabbits

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Received: 19 March 2014 / Accepted: 31 July 2014 / Published online: 24 August 2014

Abstract
Purpose Rho-kinase inhibitor is widely used for prevention of cerebral vascular spasm. However, the cerebral pial vascular action of Rho-kinase inhibitor has not been investigated. We therefore evaluated the direct effects of Y-27632, a Rho-kinase inhibitor, on pial microvessels.
Method Experiments were performed on anesthetized rabbits. A closed cranial window was used to visualize the pial microcirculation. After baseline hemodynamic and pial vascular measurements, the cranial window was superfused with four increasing concentrations of Y-27632 (10^{-9}, 10^{-7}, 10^{-6}, 10^{-5} mol l^{-1}; $n = 7$) dissolved in artificial cerebrospinal fluid for 7 min each. We measured the diameters of pial vessels, mean arterial pressure (MAP), heart rate (HR), and rectal temperature at 7 min after application of each Y-27632 concentration.
Results MAP, HR, rectal temperature, arterial pH, $PaCO_2$, PaO_2, and plasma Na^+, K^+ and glucose concentrations did not change significantly during the experimental period. Y-27632 at 10^{-9} to 10^{-7} mol l^{-1} did not produce any significant change in pial arterioles. Topical application of Y-27632 at 10^{-6} and 10^{-5} mol l^{-1} produced pial large (8.4 ± 5.7 and 19.8 ± 12.7 %) and small (10.1 ± 8.5 and 18.1 ± 12.3 %) arterioles dilation. However, Y-27632 did not produce any change in pial large and small venules.
Conclusion We evaluated the direct effects of Y-27632 on pial microvessels. Y-27632 dilates only pial arterioles in a concentration-dependent manner, and most at a concentration of 10^{-5} mol l^{-1}. Y-27632 is a potent cerebral pial arteriolar dilator but is not a venular dilator.

Keywords Rho-kinase · Y-27632 · Pial microvessels

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Introduction

Particularly in brain surgeries, control cerebral microcirculation is a major concern for anesthesiologists. Because the Rho/Rho-kinase pathway plays a pivotal role in vascular constriction and dilation, it is important to understand the mechanisms.

Contraction of smooth muscle generally is mediated by intracellular Ca^{2+} concentration. Smooth muscle cells generate inositol triphosphate, IP_3, in response to stimulation by a vasoconstrictor such as angiotensin II. IP_3 opens an intracellular Ca^{2+} channel on the surface of the sarcoplasmic reticulum and facilitates Ca^{2+} release, thereby elevating intracellular Ca^{2+} concentration. IP_3 also opens a Ca^{2+} channel on the cell membrane and allows extracellular Ca^{2+} to flow into the cell. Elevated intracellular Ca^{2+} complexes with calmodulin and activates myosin light chain (MLC) kinase. Activated MLC kinase phosphorylates MLC; thereby, actin activates Mg^{2+}-ATPase on the myosin neck. Then, smooth muscle constriction occurs. In contrast, relaxation and contraction of smooth muscles mediated by Rho-kinase are Ca^{2+} independent [1–3]. Rho-kinase is a serine-threonine protein kinase identified as a target protein of the small GTP-binding protein Rho [4–6].

Y-27632, a Rho-kinase inhibitor, is a potent vasodilator and reported to dilate various arteries such as the pulmonary [7], radial [8], and ophthalmic [9] arteries. Nevertheless, the cerebral pial vascular action of Y-27632 has not been investigated. We therefore evaluated the direct effects of Y-27632 on pial microvessels in rabbits using a closed cranial window technique.

Materials and methods

The protocol was approved by the University of Yamanashi Animal Care Committee. Experiments were performed on Japanese white rabbits weighing 3.0–3.8 kg. After obtaining intravenous access in an ear vein, the animals were anesthetized with pentobarbital sodium (20 mg•kg^{-1} IV). Anesthesia was maintained with an infusion of pentobarbital sodium (5 mg/kg/h). The animals' core body temperature was maintained at 39 ± 0.5 °C using a heating blanket. The animals were tracheostomized and were connected to a ventilator. End-tidal CO_2 ($ETCO_2$) was continuously monitored (Vamos; Dräger Medical, Tokyo, Japan). Based on $ETCO_2$ measurements, the tidal volume and respiratory rate were adjusted to maintain arterial carbon dioxide tension ($PaCO_2$) between 35 and 45 mmHg. A femoral artery was catheterized for continuous monitoring of mean arterial blood pressure (MAP) and blood sampling.

A closed cranial window was used to visualize the pial microcirculation. Each rabbit was placed in the sphinx posture. The scalp was retracted and a 0.8-cm-diameter hole was made in the parietal bone. After bipolar coagulation of dural vessels, the dura and arachnoid membranes were cut, and a thin ring of glass was positioned over the hole and secured with bone wax and dental acrylic. The space under the window was filled with artificial cerebrospinal fluid (aCSF), and three polyethylene catheters were inserted into the ring. One catheter was attached to a reservoir bottle containing aCSF, which was continuously bubbled with 5 % CO_2 in air (pH 7.398 ± 0.0839, $PaCO_2$ 37.94 ± 0.73 mmHg, PaO_2 124.2 ± 5.81 mmHg). The composition of aCSF was Na^+ 151 mEq/l, K^+ 3.5 mEq/l, Ca^{2+} 2.5 mEq/l, Mg^{2+} 1.3 mEq/l, HCO_3^- 25 mEq/l, urea 40 mg/dl, and glucose 65 mg/dl. The aCSF was suffused at 0.1 ml/min. Two other catheters served as an inlet and an outlet for aCSF and study drug solutions. The level of the outlet was maintained approximately 5–6 cm above the window to maintain normal intracranial pressure. The volume of fluid below the window was between 0.5 and 0.7 ml. The diameters of pial arterioles and venules were measured using a digital video analyzer (VH Analyzer VH-H1A5; Keyence) on a personal computer that was attached to a microscope (VH-5000; Keyence) via video capture unit (VH-E500; Keyence) (Fig. 1). Data from the pial views were stored on the computer hard disk for subsequent analysis after the experiments.

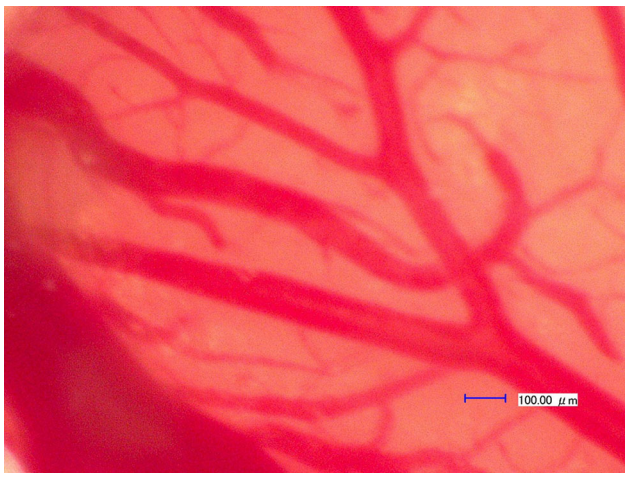

Fig. 1 A closed cranial window was used to visualize the pial microcirculation. The diameters of pial arterioles and venules were measured using a digital video analyzer (VH Analyzer VH-H1A5; Keyence, Osaka, Japan) on a personal computer attached to a microscope (VH-5000; Keyence, Osaka, Japan) via a video capture unit (VH-E500; Keyence, Osaka, Japan)

After baseline hemodynamic and pial vascular measurements, the cranial window was superfused with four increasing concentrations of Y-27632 (10^{-9}, 10^{-7}, 10^{-6}, 10^{-5} mol/l; $n = 7$) dissolved in artificial cerebrospinal fluid (aCSF) for 7 min each. We measured the diameters of pial vessels, MAP, heart rate, and rectal temperature 7 min after application of each Y-27632 concentration. The window was then flushed with aCSF for 40 min before the next concentration was administered. The sizes of blood vessels were divided on the basis of initial diameters into two subgroups: larger than 70 μm (large) and 70 μm and smaller (small). Values were represented as mean ± SD. Statistical analysis was performed via analysis of variance and Newman–Keuls post hoc comparisons. Unpaired *t* test was used to compare the differences of percent change in diameter between large and small arterioles. A *P* value less than 0.05 was considered statistically significant.

Results

MAP, HR, rectal temperature, arterial pH, $PaCO_2$, PaO_2, and plasma Na^+, K^+, and glucose concentrations did not change significantly during the experimental period. Further, MAP and HR were not affected by topical application of Y-27632 (Table 1).

Y-27632 at 10^{-9}–10^{-7} mol l^{-1} did not produce any significant change in pial arterioles. Topical application of

Table 1 Hemodynamic and laboratory data

	MAP (mm Hg)	HR (bpm)	pH	BE	$PaCO_2$ (mm Hg)	PaO_2 (mm Hg)	Na (mEq/l)	K (mEq/l)	Glucose (mg/dl)	Lactate (mmol/l)
10^{-9}	94 ± 14	261 ± 35	7.39 ± 0.03	1.4 ± 2.8	43 ± 4	260 ± 30	140 ± 3	3.5 ± 0.6	139 ± 19	2.2 ± 0.8
10^{-7}	95 ± 12	253 ± 46	7.38 ± 0.04	0.6 ± 2.4	43 ± 4	246 ± 36	139 ± 5	3.3 ± 0.4	139 ± 21	2.3 ± 0.9
10^{-6}	93 ± 12	285 ± 38	7.42 ± 0.03	1.0 ± 2.2	40 ± 4	250 ± 33	140 ± 4	3.2 ± 0.3	135 ± 20	2.4 ± 1.3
10^{-5}	93 ± 9	288 ± 37	7.42 ± 0.04	1.1 ± 2.3	39 ± 5	253 ± 29	141 ± 2	3.2 ± 0.3	134 ± 15	2.6 ± 1.0

Values are mean ± SD

There were no statistical differences among the concentrations

MAP mean arterial blood pressure, *HR* heart rate, *BE* base excess

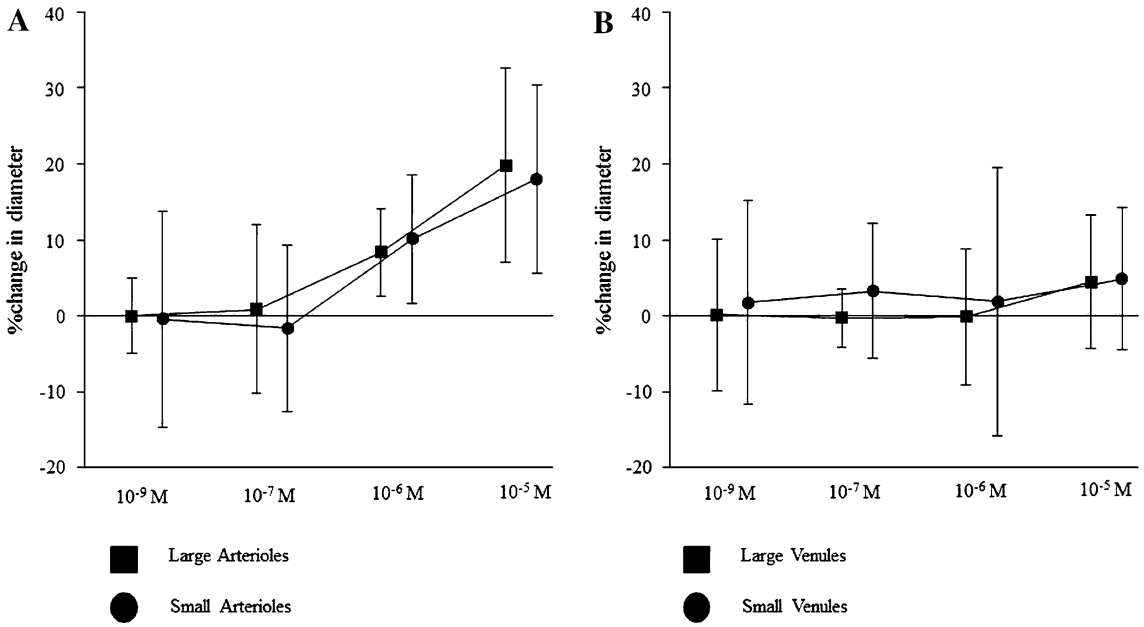

Fig. 2 Y-27632 at 10^{-9}–10^{-7} mol l^{-1} did not produce any significant change in pial arterioles. Topical application of Y-27632 at 10^{-6} and 10^{-5} mol l^{-1} produced pial large (8.4 ± 5.7 and 19.8 ± 12.7 %) and small arterioles (10.1 ± 8.5 and 18.1 ± 12.3 %) dilation (**a**). However, Y-27632 did not produce any change in pial large and small venules (**b**)

Y-27632 at 10^{-6} and 10^{-5} mol l^{-1} produced concentration-dependent dilation in both large (8.4 ± 5.7 % and 19.8 ± 12.7 %) and small (10.1 ± 8.5 % and 18.1 ± 12.3 %) pial arterioles. There was no statistical difference between percent changes of two subgroups (large and small arterioles). On the other hand, Y-27632 did not produce any change in large or small pial venules (Fig. 2).

Discussion

Rho-kinase inhibitor dilates constricted arteries [10]. Expression of endothelial nitric oxide synthase (eNOS) is suppressed in an ischemic situation, and Rho-kinase is considered to be related to this phenomenon [11]. Rho-kinase inhibitor increases expression of eNOS by stabilizing its mRNA [11]. Cerebral arterioles are constricted after the ischemia–reperfusion period [12], and Rho-kinase inhibitor exerts its action on the constricted arteries [10]. Because the vasodilative effect of Rho-kinase inhibitor on constricted cerebral arterioles was already known, we therefore investigated the effect of Rho-kinase inhibitor on normal vessels. In this study, Y-27632 exerted a vasodilatory action on normal pial arteries. Generally, myosin light chain (MLC) kinase is inactivated as a result of separation of Ca^{2+} and calmodulin when the intracellular Ca^{2+} concentration is reduced. Then, MLC is dephosphorylated by activated MLC phosphatase, and smooth muscle relaxes [13]. Rho-kinase mediates relaxation and contraction of smooth muscle independently of intracellular Ca^{2+} concentration [13]. Rho is activated by vasoconstrictors through G protein-coupled receptors,

whereby the Rho target protein Rho-kinase is activated. Activated Rho-kinase inactivates MLC phosphatase by phosphorylating the myosin-binding subunit. As a result, MLC is phosphorylated and smooth muscle contracts [1.3]. Y-27632, a Rho-kinase inhibitor, blocks this pathway and exerts a vasodilatory effect not only on constricted arteries but also on normal arteries.

The sizes of blood vessels were divided on the basis of initial diameters into two subgroups: larger than 70 μm (large), and 70 μm and smaller (small). The sympathetic contractile response to norepinephrine is substantial in first-order vessel branches but relatively less in second-order vessel branches in the rabbit middle cerebral tree [14]. The mean diameter of the second branch is 67 μm [15]. There was no statistical difference between percent changes of the two subgroups (large and small arterioles). It seems that the sympathetic nervous system is little, if ever, affected by the Y2 vasodilative effect.

Although Y-27632 produced pial arterial vasodilation, it did not affect the pial veins. The relationship between Rho-kinase and venous dilation has not been clarified. Both arteries and veins consist of three layers: vascular intima, media, and adventitia. The intima consists of simple squamous epithelium. The media includes a mature circular smooth muscle layer and is crucial in mediating the diameters of vessel and blood flow. The adventitia is the outermost connective tissue layer of vessels. The "small artery" has an internal elastic membrane in its intima but no external elastic membrane in the media. An artery that is smaller than a small artery is called an arteriole and does not have an internal elastic membrane. The arteriole consists of a single endothelial layer, several smooth muscle layers, and epithelial membrane. On the other hand, a vein consists of restricted intima, a few smooth muscle cells, and connective tissue. The quantity of smooth muscle cells is larger in arteries than in veins. Because Rho-kinase acts on smooth muscle and mediates its contraction, Y-27632 dilated only pial arterioles in our study. The Rho-kinase-involved pathway may participate in the mechanism of venous dilation. However, its participation should be minor.

Y-27632 has been widely used as a Rho-kinase inhibitor in various fields of research to reveal the mechanism of the Rho/Rho-kinase pathway [16–18]. Rho-kinase plays an important role not only in smooth muscle contraction but also in many cellular functions such as adhesion, morphology, proliferation, and motility [19]. Because Rho-kinase mediates various cellular functions and gene expression, a Rho-kinase inhibitor has various clinical effects on many disease states such as vascular spasm and stenosis, arteriosclerosis [20–24], and systemic and pulmonary hypertension [25–29]. Clinically, Rho-kinase inhibitor is used for prevention of cerebral vascular spasm. Many studies support the efficacy of Rho-kinase inhibitor for other diseases such as cerebral infarction [30, 31], asthma [32], and glaucoma [33]. In addition, there is some possibility that Rho-kinase inhibitor suppresses cancer invasion [34] and improves insulin resistance [35] and osteoporosis [36]. It is important to elucidate the mechanism of the Rho/Rho-kinase pathway and the effects of Rho-kinase inhibitor. Nevertheless, the effects of Rho-kinase inhibitor are not fully understood, and further study is necessary.

Conclusion

We evaluated the direct effects of Y-27632 on pial microvessels. Y-27632 dilates only pial arterioles, in a concentration-dependent manner, most at a concentration of 10^{-5} mol l^{-1}. Y-27632 is a potent cerebral pial arteriolar dilator but is not a venular dilator.

Acknowledgments This work was supported by JSPS KAKENHI Grant Numbers 19591784 and 23592246.

References

1. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Fenf J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science. 1996;3:245–8.
2. Kureishi Y, Kobayashi S, Amano M, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem. 1997;272:12257–60.
3. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem. 1996;271:20246–9.
4. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J. 1996;15:2208–16.
5. Leung T, Manser E, Tan L, Lim L. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem. 1995;270:29051–4.
6. Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 1996;15:1885–93.
7. Gupta V, Gupta N, Shaik IH, Mehvar R, McMurtry IF, Oka M, Nozik-Grayck E, Komatsu M, Ahsan F. Liposomal fasudil, a rho-kinase inhibitor, for prolonged pulmonary preferential vasodilation in pulmonary arterial hypertension. J Control Release. 2013;167:189–99.
8. Kun X, Lefeng W, Rongjing D, Xincun Y. RhoA/ROK pathway related to the mechanism of higher susceptibility to spasm in RA than IMA. J Card Surg. 2009;24:766–71.
9. Ito I, Jarajapu YP, Grant MB, Knot HJ. Characteristics of myogenic tone in the rat ophthalmic artery. Am J Physiol Heart Circ Physiol. 2007;292:H360–8.

10. Bagi Z, Feher A, Cassuto J, Akula K, Labinskyy N, Kaley G, Koller A. Increased availability of angiotensin AT1 receptors leads to sustained arterial constriction to angiotensin II in diabetes—role for Rho-kinase activation. Br J Pharmacol. 2011;163: 1059–68.
11. Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK. Rho-kinase mediated hypoxia-induced downregulation of endothelial nitric oxide synthase. Circulation. 2002;106:57–62.
12. Ishiyama T, Shibuya K, Ichikawa M, Masamune T, Kiuchi R, Sessler DI, Matsukawa T. Cerebral pial vascular changes under propofol or sevoflurane anesthesia during global cerebral ischemia and reperfusion in rabbits. J Neurosurg Anesthesiol. 2010;22:207–13.
13. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. Nature (Lond). 1994;372:231–6.
14. Bevan JA, Duckworth J, Laher I, Oriowo MA, McPherson GA, Bevan RD. Sympathetic control of cerebral arteries: specialization in receptor type, reserve, affinity, and distribution. FASEB J. 1987;1:193–8.
15. Hartl R, Joshi S, Levine S, Wang M, Sciacca RR. Pial arterial response to topical verapamil in acute closed cranial windows in rabbits. Anesth Analg. 2005;100:1140–6.
16. Shimokawa H, Takeshita A. Rho-kinase is an important therapeutic target in cardiovascular medicine. Arterioscler Thromb Vasc Biol. 2005;25:1767–75.
17. Masumoto A, Mohri M, Shimokawa H, Urakami L, Usui M, Takeshita A. Suppression of coronary artery spasm by the Rho-kinase inhibitor fasudil in patients with vasospastic angina. Circulation. 2002;105:1545–7.
18. Inokuchi K, Ito A, Fukumoto Y, Matoba T, Shiose A, Nishida T, Masuda M, Morita S, Shimokawa H. Usefulness of fasudil, a Rho-kinase inhibitor, to treat intractable severe coronary spasm after coronary artery bypass surgery. J Cardiovasc Pharmacol. 2004;44:275–7.
19. Eto Y, Shimokawa H, Hiroki J, Morishige K, Kandabashi T, Matsumoto Y, Amano M, Hoshijima M, Kaibuchi K, Takeshita A. Gene transfer of dominant negative Rho kinase suppresses neointimal formation after balloon injury in pigs. Am J Physiol Heart Circ Physiol. 2000;278:H1744–50.
20. Morishige K, Shimokawa H, Eto Y, Kandabashi T, Miyata K, Matsumoto Y, Kaibuchi K, Takeshita A. Adenovirus-mediated transfer of dominant-negative rho-kinase induces a regression of coronary arteriosclerosis in pigs in vivo. Arterioscler Thromb Vasc Biol. 2001;21:548–54.
21. Shimokawa H, Morishige K, Miyata K, Kandabashi T, Eto Y, Ikegaki I, Asao T, Kaibuchi K, Takeshita A. Long-term inhibition of Rho-kinase induces a regression of arteriosclerotic coronary lesions in a porcine model in vivo. Cardiovasc Res. 2001;51: 169–77.
22. Uehata M, Ishizaki T, Satoh H, Ono T, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature (Lond). 1997;389:990–4.
23. Masumoto A, Hirooka Y, Shimokawa H, Hironaga K, Setoguchi S, Takeshita A. Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. Hypertension. 2001;38: 1307–10.
24. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, Hattori T, Nakashima Y, Kaibuchi K, Sueishi K, Takeshita A. Long-term treatment with a rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. Circ Res. 2004;94:385–93.
25. Nagaoka T, Fagan KA, Gebb SA, Morris KG, Suzuki T, Shimokawa H, McMurtry IF, Oka M. Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. Am J Respir Crit Care Med. 2005;171:494–9.
26. Fukumoto Y, Matoba T, Ito A, Tanaka H, Kishi T, Hayashidani S, Abe K, Takeshita A, Shimokawa H. Acute vasodilator effects of a Rho-kinase inhibitor, fasudil, in patients with severe pulmonary hypertension. Heart. 2005;91:391–2.
27. James SE, Dunham M, Carrion-Jones M, Murashov A, Lu Q. Rho kinase inhibitor Y-27632 facilitates recovery from experimental peripheral neuropathy induced by anti-cancer drug cisplatin. Neurotoxicology. 2010;31:188–94.
28. Gauthaman K, Fong CY, Bongso A. Effect of ROCK inhibitor Y-27632 on normal and variant human embryonic stem cells (hESCs) in vitro: its benefits in hESC expansion. Stem Cell Rev. 2010;6:86–95.
29. Minase T, Ishima T, Itoh K, Hashimoto K. Potentiation of nerve growth factor-induced neurite outgrowth by the ROCK inhibitor Y-27632: a possible role of IP receptors. Eur J Pharmacol. 2010;648:67–73.
30. Satoh S, Utsunomiya T, Tsurui K, Kobayashi T, Ikegaki I, Sasaki Y, Asano T. Pharmacological profile of hydroxy fasudil as a selective rho kinase inhibitor on ischemic brain damage. Life Sci. 2001;69:1441–53.
31. Toshima Y, Satoh S, Ikegaki I, Asano T. A new model of cerebral microthrombosis in rats and the neuroprotective effect of a Rho-kinase inhibitor. Stroke. 2000;31:2245–50.
32. Iizuka K, Shimizu Y, Tsukagoshi H, Yoshii A, Harada T, Dobashi K, Murozono T, Nakazawa T, Mori M. Evaluation of Y-27632, a rho-kinase inhibitor, as a bronchodilator in guinea pigs. Eur J Pharmacol. 2000;406:273–9.
33. Honjo M, Inatani M, Kido N, Sawamura T, Yue BY, Honda Y, Tanihara H. Effects of protein kinase inhibitor, HA1077, on intraocular pressure and outflow facility in rabbit eyes. Arch Ophthalmol. 2001;119:1171–8.
34. Ito K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med. 1999;5:221–5.
35. Kanda T, Wakino S, Homma K, Yoshioka K, Tatematsu S, Hasegawa K, Takamatsu I, Suano N, Hayashi K, Saruta T. Rho-kinase as a molecular target for insulin resistance and hypertension. FASEB J. 2006;20:169–71.
36. Ohnaka K, Shimoda S, Nawata H, Shimokawa H, Kaibuchi K, Iwamoto Y, Takayanagi R. Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. Biophys Biochem Res Commun. 2001;287: 337–42.