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European Journal of Pharmacology 486 (2004) 297–306
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Effects of a 20-HETE antagonist and agonists on cerebral vascular tone

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Received 15 September 2003; received in revised form 10 December 2003; accepted 13 January 2004

Abstract

This study examined the effects of a 20-hydroxyeicosatetraenoic acid (20-HETE) antagonist, 20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid (WIT002) and two agonists, 4-amino-*N*-(20-hydroxy-eicosa-5(*Z*),14(*Z*)-dienoyl) benzenesulfonamide (ABSA) and 20-hydroxyeicosa-5(*Z*),14(*Z*)-dienoic acid (WIT003), on the diameter of rat middle cerebral arteries in vitro and on cerebral blood flow in vivo. WIT003, ABSA and 20-HETE all had a similar effect to reduce the diameter of the middle cerebral artery by 26%. WIT003 and 20-HETE both increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle cells isolated from the middle cerebral artery. In contrast, WIT002 had no effect on the basal diameter of the middle cerebral artery but it attenuated the vasoconstrictor responses and the rise in $[Ca^{2+}]_i$ in vascular smooth muscle cells following administration of 20-HETE and 5-hydroxytryptamine (5-HT). WIT003 partially restored the vasoconstrictor response to 5-HT in the middle cerebral artery after administration of an inhibitor of the endogenous synthesis of 20-HETE. Infusion of the 20-HETE agonists, WIT003 and ABSA, into cisterna magna of rats reduced baseline cerebral blood flow by 20%, whereas administration of the 20-HETE antagonist, WIT002, had no effect. Intracisternal injection of WIT002 attenuated the fall in cerebral blood flow following injection of blood into the cisterna magna, whereas administration of the 20-HETE agonist, ABSA, potentiated this response. These findings indicate that the 20-HETE agonists, WIT003 and ABSA, increase cerebral vascular tone both in vivo and in vitro and suggest blocking the vasoconstrictor actions of 20-HETE may be useful to prevent the acute fall in cerebral blood flow following subarachnoid hemorrhage.

Keywords: 20-HETE; Agonist; Antagonist; Middle cerebral artery; Subarachnoid hemorrhage

1. Introduction

20-Hydroxyeicosatetraenoic acid (20-HETE) is a potent endogenous vasoconstrictor produced by cerebral (Harder et al., 1994; Gebremedhin et al., 2000), renal (Schwartzman et al., 1996; Carroll et al., 1999), mesenteric (Chu et al., 2000) and skeletal muscle arteries (Frisbee et al., 2001; Kunert et al., 2001). Previous studies have indicated that 20-HETE increases vascular tone by inhibiting the opening of Ca^{2+}-activated K^+ (K_{Ca}) channels (Harder et al., 1994; Zou et al., 1996; Obara et al., 2002) thereby depolarizing vascular smooth muscle cells (Ma et al., 1993; Cloutier et al., 2003) and by activating L-type Ca^{2+} channels (Gebremedhin et al., 1998). More recently, Randriamboavonjy et al. (2003) have reported that 20-HETE also may promote vasoconstriction by activating Rho-kinase to enhance the sensitivity of the contractile mechanism to Ca^{2+}. 20-HETE has been shown to play an important role in the myogenic response of cerebral, renal and mesenteric arteries to elevations in transmural pressure (Imig et al., 1999; Gebremedhin et al., 2000; Wang et al., 2001; Looft-Wilson et al., 2002). In addition, nitric oxide (NO) inhibits the formation of 20-HETE and a fall in 20-HETE levels appears to contribute to the vasodilator response to NO in both cerebral (Alonso-Galicia et al., 1999b; Sun et al., 2000; Yu et al., 2002) and renal arteries (Sun et al., 1998; Oyekan et al., 1999).

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doi:10.1016/j.ejphar.2004.01.009

Subarachnoid hemorrhage in man is often associated with an initial period of cerebral ischemia (Broderick et al., 1994; Bederson et al., 1998) and a high mortality rate of 32–67% (Hop et al., 1997). A large percentage of deaths occur within the first 2 days of the injury (Broderick et al., 1994). The factors that contribute to the initial fall in cerebral blood flow as well as delayed vasospasm that often develops 4–7 days following subarachnoid hemorrhage remain uncertain. Previous studies have indicated that the cerebral vasospasm in animal models is associated with activation of protein kinase C (Matsui et al., 1991; Nishizawa et al., 1996) and Rho-kinase (Wickman et al., 2003), diminished K^+ channel activity and depolarization of vascular smooth muscle cells (Harder et al., 1987; Sobey and Faraci, 1998). The acute fall in cerebral blood flow following subarachnoid hemorrhage has been linked with elevated levels of endothelin (Seifert et al., 1995; Juvela, 2000), thromboxane (Pickard et al., 1994), glutamate (Bederson et al., 1998) and 5-hydroxytryptamine (5-HT) in cerebrospinal fluid (Saida et al., 1997; Cambj-Sapunar et al., 2003) and a diminished response of cerebral vasculature to NO and other endothelial dependent vasodilators (Kim et al., 1992; Sobey et al., 1996; Yamamoto et al., 1997). There is also evidence for the enhanced release of fatty acids (Gewirtz et al., 1999; Pilitsis et al., 2002) and increased formation of metabolites of arachidonic acid following subarachnoid hemorrhage (D'Avella et al., 1990; Cook, 1995). In view of previous studies indicating that 20-HETE is one of the major metabolites of arachidonic acid produced by cerebral arteries (Harder et al., 1994; Gebremedhin et al., 2000) and that the vasoconstrictor response to 20-HETE shares many of the properties associated with cerebral vasospasm, we recently examined the contribution of 20-HETE to the acute fall in cerebral blood flow following subarachnoid hemorrhage in rats. We found that 20-HETE levels are elevated in cerebrospinal fluid following subarachnoid hemorrhage and that inhibitors of the formation of 20-HETE attenuate the fall in cerebral blood flow (Kehl et al., 2002). Subsequently, we demonstrated that 5-HT levels are also elevated in cerebrospinal fluid following subarachnoid hemorrhage and that 5-HT stimulates the synthesis and release of 20-HETE in cerebrospinal fluid by activating a 5-HT_{1B} receptor (Cambj-Sapunar et al., 2003). These studies suggest that elevations in the production of 20-HETE likely contribute to the fall in cerebral blood flow following subarachnoid hemorrhage.

More recently, we studied analogs of 20-HETE and found that 20-hydroxyeicosa-5(*Z*),14(*Z*)-dienoic acid (WIT003) and 4-amino-*N*-(20-hydroxyeicosa-5(*Z*),14(*Z*)-dienoyl) benzenesulfonamide (ABSA) had a similar effect as 20-HETE to constrict renal arteries of rats. We also found that 20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid (WIT002) had no effect on the diameter of the renal arteries, but it attenuates the vasoconstrictor effects of 20-HETE in these vessels (Alonso-Galicia et al., 1999a). However, little is know about the effects of these compounds on the cerebral circulation either in vivo or in vitro. Thus, the present study characterized the effects of WIT003, ABSA and WIT002 on the diameter of rat middle cerebral arteries in vitro and cerebral blood flow in vivo under control condition and following the induction of subarachnoid hemorrhage in rats.

2. Methods

Experiments were performed on 120 male Sprague–Dawley rats weighing between 270 and 350 g. The rats were housed in an Animal Care Facility at the Medical College of Wisconsin, which is approved by the American Association for the Accreditation of Laboratory Animal Care. All protocols comply with the European Community Guidelines for the use of experimental animals in research and were approved by the Animal Care Committee of the Medical College of Wisconsin.

2.1. Effects of WIT003, ABSA, 20-HETE and WIT002 on the diameter of rat middle cerebral arteries and basilar arteries in vitro

Middle cerebral arteries were dissected from the brains of rats, mounted on glass micropipettes with a 10–0 suture and pressurized to 80 mm Hg in a perfusion chamber as previously described (Yu et al., 2002). The vessels were bathed in a physiological saline solution (PSS) containing (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.17, Glucose 10, NaH_2PO_4 1.18, $NaHCO_3$ 12, EDTA 0.03, HEPES 10; that was saturated with 95% O_2/5% CO_2 mixture at 37 °C. Indomethacin (4 μM), a cyclooxygenase inhibitor, and 17-octadecynoic acid (17-ODYA, 10 μM), an inhibitor of the synthesis of 20-HETE, were added to the bath to block the endogenous synthesis of 20-HETE and the metabolism of 20-HETE by cyclooxygenase. The internal diameters of the vessels were measured with a video system composed of stereomicroscope (Carl Zeiss, Germany), a video camera (COHU-4815, COHU Electronics, Poway, CA, USA) and a video measuring system (VIA-100, Boeckeler Instrument, AZ, USA). After a 45-min equilibration period, the baseline diameter of the vessel was measured. Then, cumulative concentration–response curves to WIT002 (0.01–1 μM), WIT003 (0.01–1 μM), ABSA (0.01–1 μM) and 20-HETE (0.01–1 μM) were constructed by adding increasing amounts of each compound to the bath.

We also examined the ability of WIT002 to block the vasoconstrictor response to 20-HETE in both middle cerebral and basilar arteries of rats. In these experiments, a cumulative concentration–response curve to 20-HETE (0.01–1 μM) was constructed. Then, WIT002 (1 μM) or vehicle (equal volume of ethanol) was added to the bath. After 15-min equilibration, the response to 20-HETE was redetermined.

2.2. Effects of WIT003 and WIT002 on the vasoconstrictor response to 5-HT in rat middle cerebral arteries in vitro

Previous studies have indicated that inhibitors of the synthesis of 20-HETE attenuate the vasoconstrictor response to 5-HT in cerebral vasculature of rats both in vivo and in vitro (Cambj-Sapunar et al., 2003). In the present experiment, we compared the effect of the 20-HETE antagonist, WIT002, to that of a known inhibitor of the synthesis of 20-HETE, 17-ODYA, on the vasoconstrictor response of the middle cerebral artery of rats to 5-HT. Cumulative dose–response curves to 5-HT (0.01–100 μM) were constructed before and after addition of WIT002 (1 μM), WIT003 (1 μM) or 17-ODYA (10 μM) to the bath. We then studied whether addition of the 20-HETE agonist, WIT003 (1 μM), could restore the vascular responsiveness to 5-HT in vessels treated with 17-ODYA (10 μM) to block the endogenous synthesis of 20-HETE.

2.3. Effects of WIT002 and WIT003 on $[Ca^{2+}]_i$ responses in vascular smooth muscle cells isolated from rat middle cerebral arteries

The effects of WIT002 and WIT003 on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle cells isolated from the middle cerebral artery of rats were also studied. Vascular smooth muscle cells were freshly isolated from rat middle cerebral arteries and $[Ca^{2+}]_i$ was measured using an InCyt Im2 imaging system (Intracellular Imaging, Cincinnati, OH, USA) mounted on an inverted microscope (Nikon TS-100, Japan) as previously described (Yu et al., 2002). The cells were loaded with fura-2 AM (4 μM) for 45 min at room temperature and then transferred to a 1-ml perfusion chamber maintained at 37 °C. The cells were alternatively excited at wavelengths of 340 and 380 nm and images were recorded at emission wavelength of 510 nm using a 40× ultraviolet fluorescence objective. Indomethacin (4 μM) and 17-ODYA (10 μM) were included into the bath to block the endogenous synthesis of 20-HETE and its subsequent metabolism by cyclooxygenase.

Baseline $[Ca^{2+}]_i$ was measured and then 20-HETE (1 μM), WIT003, (1 μM) or vehicle (ethanol) was added to the bath. Changes in $[Ca^{2+}]_i$ were recorded during a 10-min experimental period. In other experiments, the effect of WIT002 on 20-HETE-induced changes in $[Ca^{2+}]_i$ was determined. WIT002 (1 μM) or vehicle (equal volume of ethanol) was added to the bath. After a 15-min equilibration period, the response to 20-HETE (1 μM) was determined.

We also examined the effect of the 20-HETE antagonist, WIT002, on the $[Ca^{2+}]_i$ response of middle cerebral vascular smooth muscle cells to 5-HT. In these experiments, the cells were pretreated with vehicle or WIT002 (1 μM) and after a 15-min equilibration period the response to 5-HT (10 μM) was determined.

2.4. Effects of WIT003, ABSA and WIT002 on cerebral blood flow in vivo

The effects of the 20-HETE analogs on cerebral blood flow was studied under control conditions and after induction of subarachnoid hemorrhage using laser Doppler flowmetry as previously described (Kehl et al., 2002; Cambj-Sapunar et al., 2003). Briefly, rats were anesthetized with Inactin (100 mg/kg), the trachea was cannulated and the rats were ventilated with 30% O_2/70% N_2 gas mixture. Arterial blood gases were monitored and maintained within physiological levels (pH = 7.4, $PO_2 = 156 \pm 6$ mm Hg; $PCO_2 = 37 \pm 2$ mm Hg) by adjusting ventilation. The left femoral artery and vein were cannulated for measurement of mean arterial pressure and for iv infusions. A 3 × 5 mm area of the parietal bone overlying the irrigation area of the middle cerebral artery was thinned with a hand-held drill until the pial arteries were visible through the intact skull. A laser Doppler probe (Perimed, Stockholm, Sweden) was placed above the cranial window for measurement of cerebral blood flow. The atlanto-occipital membrane was exposed and a 30-gauge needle attached to a PE-10 catheter was inserted into the cisterna magna. Cerebral blood flow, mean arterial pressure and pCO_2 were continuously monitored using analog-to-digital conversion hardware and software system purchased from Dataq Instruments (Akron, OH, USA).

2.4.1. Effects of WIT003, ABSA and WIT002 on baseline cerebral blood flow

After the surgery and a 30-min equilibration period, WIT002 (1.5 nmol), WIT003 (1.5 nmol), ABSA (1.5 nmol) or vehicle (1:1000 dilution of ethanol dissolved 50 μl of artificial cerebrospinal fluid containing: NaCl 132, KCl 3, $MgCl_2$ 1.38, $CaCl_2$ 1.5, $NaHCO_3$ 19, Urea 6.69 and Glucose 3.69 mM) was infused into cisterna magna at a rate of 5 μl/min over a 10-min period. Changes in cerebral blood flow and mean arterial pressure were then recorded at 5-min intervals for 30 min.

2.4.2. Effects of WIT003, ABSA and WIT002 on cerebral blood flow following subarachnoid hemorrhage

These experiments were performed on four groups of rats surgically prepared as described above. After a 30-min equilibration period, a 0.2 ml sample of cerebrospinal fluid was collected from the catheter in the cisterna magna for measurement of baseline 20-HETE levels. Then, the rats received an intracisternal infusion of WIT002 (1.5 nmol), ABSA (1.5 nmol) or vehicle in 50 μl of artificial cerebrospinal fluid at a rate of 5 μl/min over a 10-min period. Changes in baseline cerebral blood flow were recorded over a 20-min period. After this control level of cerebral blood flow was established, a subarachnoid hemorrhage was induced by injecting 0.3 ml of fresh autologous arterial blood in cisterna magna over a 10-min period at a rate of 30 μl/min. Changes in

cerebral blood flow were measured 10, 20, 30, 60, 90 and 120 min after the intracisternal injection of blood. In control rats, artificial cerebrospinal fluid instead of blood was injected into cisterna magna. Two hours following the injection of blood, a sample of cerebrospinal fluid (0.2 ml) was collected and the levels of 20-HETE were again measured using a fluorescent high-performance liquid chromatography (HPLC) assay as previously described (Maier et al., 2000).

2.5. Statistical methods

Mean values ± 1 S.E.M. are presented. Significance of differences in mean values between and within groups was evaluated using analysis of variance for repeated measurements followed by a Duncan multiple-range test. The significance of changes in $[Ca^{2+}]_i$ in response to 20-HETE and 5-HT in vascular smooth muscle cells pretreated with vehicle or WIT002 was determined using an unpaired *t*-test. A $P < 0.05$ was considered to be significant.

2.6. Drugs

WIT002 and WIT003 were synthesized by Taisho Pharmaceutical (Saitama, Japan). 20-HETE and ABSA were synthesized by Dr. John R. Falck (University of Texas, Southwestern Medical Center, Dallas, TX, USA). The methods used for the synthesis of 20-HETE and the 20-HETE agonists and antagonist have been described in detail previously (Yu et al., 2003). 5-HT and 17-ODYA were purchased from Sigma, USA. The solvent used to prepare the stock solutions of WIT002, WIT003, 20-HETE and 17-ODYA was ethanol. 5-HT was dissolved in physiological salt solution. The effect of each vehicle was examined in every experiment and is presented in the figures below.

3. Results

3.1. Effects of WIT002, WIT003 and ABSA on diameter of middle cerebral arteries in vitro

The effects of WIT002, WIT003 and ABSA on the diameter of rat middle cerebral arteries studied in vitro are presented in Fig. 1 (upper panel). The mean baseline diameters of the vessels subsequently treated with 20-HETE, WIT002, ABSA and WIT003 were not significantly different and averaged 93 ± 6 μm. Addition of 20-HETE (0.01–1 μM) to the bath dose-dependently reduced the diameter of the middle cerebral artery by 26%. Both ABSA (0.01–1 μM) and WIT003 (0.01–1 μM) mimicked the actions of 20-HETE and reduced the diameter of the middle cerebral artery by 25% and 26%, respectively. In contrast, WIT002 (0.01–1 μM) had no effect on the diameter of the middle cerebral artery of rats in vitro.

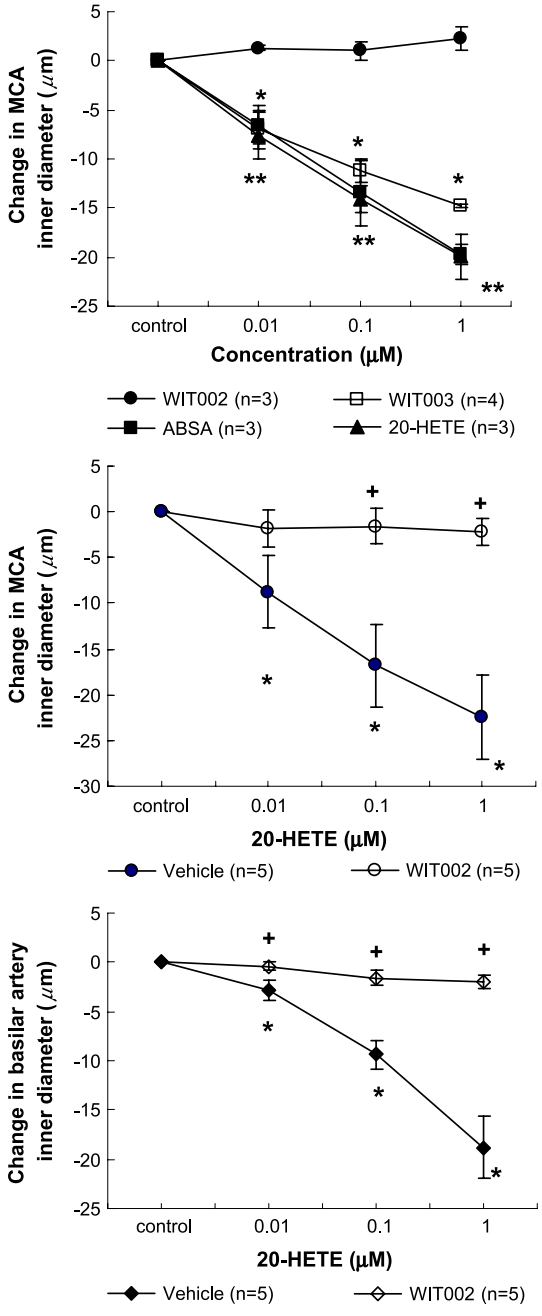

Fig. 1. The effects of 20-HETE analogs, WIT002, ABSA and WIT003, on the inner diameter of rat cerebral arteries. The upper panel presents cumulative dose–response curves comparing the effects of WIT002, WIT003, ABSA and 20-HETE on the inner diameter of the middle cerebral artery (MCA) of rats studied in vitro. The middle and lower panels compare cumulative concentration–response curves to 20-HETE on the inner diameter of the MCA (middle panel) and basilar artery (lower panel) before and after addition of WIT002 (1 μM) to the bath. *Indicates a significant difference from control values and + indicates a significant difference from the corresponding value in vessels pretreated with vehicle.

The effect of WIT002 on the vasoconstrictor response to 20-HETE in the middle cerebral and basilar arteries of rats is presented in Fig. 1 (middle and lower panels). Addition of vehicle or WIT002 to the bath had no effect

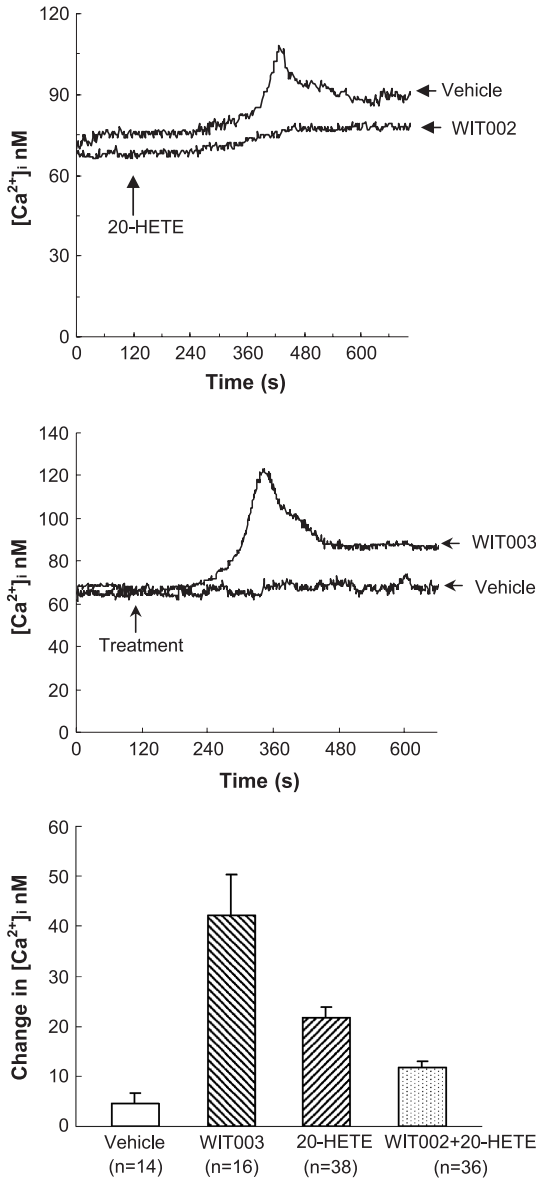

Fig. 2. The effects of 20-HETE analogs on changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in vascular smooth muscle cells isolated from rat middle cerebral arteries. The upper panel presents representative tracings comparing the effect of 20-HETE on $[Ca^{2+}]_i$ in the cells pretreated with vehicle or WIT002 (1 μM). The middle panel presents representative tracings illustrating the effect of WIT003 (1 μM) on $[Ca^{2+}]_i$. The lower panel presents a comparison of changes in $[Ca^{2+}]_i$ in cells treated with vehicle, WIT003, 20-HETE and 20-HETE plus WIT002. The control values of $[Ca^{2+}]_i$ in vascular smooth muscle cells were similar in all the groups and averaged 70 ± 2 nM (pooled data, $n = 104$ cells). + Indicates a significant difference from vehicle treatment, # indicates a significant difference from 20-HETE treatment alone.

on the inner diameter of either the middle cerebral or basilar artery, which averaged 128 ± 2 and 177 ± 5 μm, respectively. 20-HETE dose-dependently reduced the diameters of the middle cerebral artery and basilar artery by 22 ± 4 and 19 ± 3 μm, respectively. Pretreatment of these vessels with WIT002 (1 μM) abolished the constrictor response to 20-HETE (0.01–1 μM).

3.2. Effects of WIT002 and WIT003 on $[Ca^{2+}]_i$ in vascular smooth muscle cells isolated from middle cerebral arteries

The effect of WIT002 on the $[Ca^{2+}]_i$ response to 20-HETE in vascular smooth muscle cells isolated from the middle cerebral artery of rats is presented in Fig. 2. Baseline $[Ca^{2+}]_i$ was similar in cells pretreated with WIT002 and vehicle and averaged 70 ± 2 nM. 20-HETE (1 μM) produced a small but significant increase in $[Ca^{2+}]_i$ in the cells pretreated with vehicle. WIT002 (1 μM) prevented the rise in $[Ca^{2+}]_i$ following addition of 20-HETE to the bath.

The effect of WIT003 on $[Ca^{2+}]_i$ in vascular smooth muscle cells isolated from middle cerebral arteries of rats is also presented in Fig. 2. The control value of $[Ca^{2+}]_i$ in these cells averaged 70 ± 4 nM. WIT003 (1 μM) increased $[Ca^{2+}]_i$ and the response was greater than that seen in cells treated with 20-HETE (1 μM).

3.3. Effect of WIT002 and WIT003 on the vasoconstrictor and $[Ca^{2+}]_i$ responses to 5-HT

The effects of WIT002 and WIT003 on the vasoconstrictor response of the middle cerebral artery of rats to 5-HT are presented in Fig. 3. The baseline diameter of the middle cerebral arteries averaged 106 ± 5 μm. 5-HT at doses from 0.01 to 100 μM dose-dependently reduced the

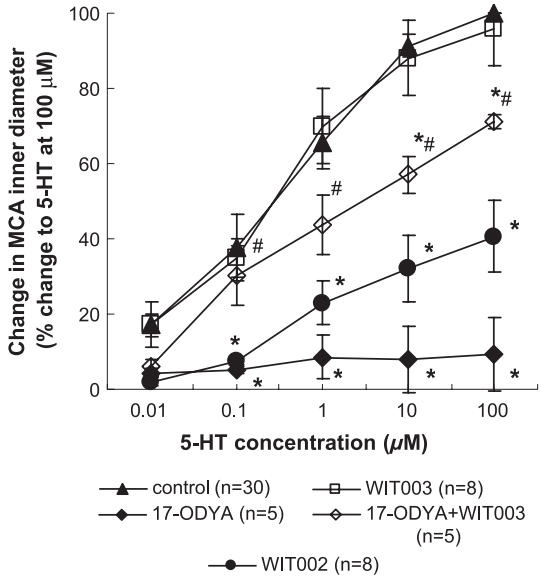

Fig. 3. The effects of WIT002, 17-octadecynoic acid (17-ODYA) and WIT003 on the vasoconstrictor response to 5-hydroxytryptamine (5-HT) in rat middle cerebral arteries (MCA). Baseline inner diameters of MCA were similar among all groups and averaged 106 ± 5 μm (pooled data, $n = 56$ vessels). The cumulative concentration–response curves were constructed to 5-HT before and after addition of WIT003 (1 μM), WIT002 (1 μM), 17-ODYA (10 μM) or 17-ODYA plus WIT003 to the bath. *Indicates a significant difference from the corresponding value in control vessels and # indicates a significant difference from the corresponding value in vessels pretreated with 17-ODYA alone.

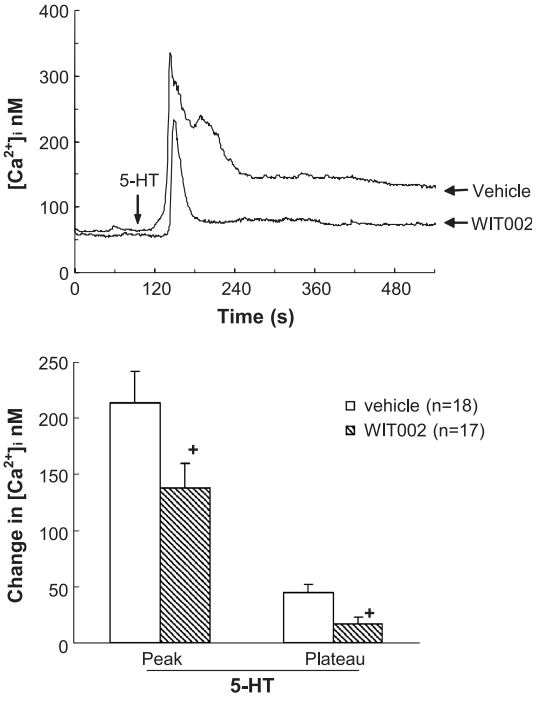

Fig. 4. The effect of WIT002 on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in vascular smooth muscle cells stimulated with 5-hydroxytryptamine (5-HT). The upper panel presents representative tracings of changes in $[Ca^{2+}]_i$ in the cells pretreated with vehicle or WIT002 (1 μM), an inhibitor of vasoconstrictor actions of 20-HETE, and stimulated with 5-HT (10 μM). The lower panel compares changes in $[Ca^{2+}]_i$ in cells pretreated with vehicle or WIT002 and stimulated with 5-HT. The baseline $[Ca^{2+}]_i$ in the cells was similar in vehicle and WIT002 pretreated cells and averaged 75 ± 5 nM. 5-HT produced a transit increase (Peak) and followed by a sustained steady-state elevation (Plateau) in $[Ca^{2+}]_i$. + Indicates a significant difference from the corresponding value in cells pretreated with vehicle.

diameter of the vessels by more than 70 μm. Pretreatment of the vessels with the 20-HETE antagonist, WIT003 (1 μM), had no effect on the vasoconstrictor response to 5-HT. However, blockade of the endogenous formation of 20-HETE with 17-ODYA (10 μM) or addition of the 20-HETE antagonist, WIT002 (1 μM), to the bath markedly attenuated the response to 5-HT by 90% and 65%, respectively. Addition of WIT003 (1 μM) to the bath partially restored the vasoconstrictor response to 5-HT in vessels treated with 17-ODYA to block the endogenous formation of 20-HETE.

The effect of WIT002 on $[Ca^{2+}]_i$ response to 5-HT in vascular smooth muscle cells isolated from middle cerebral arteries of rats is presented in Fig. 4. Pretreatment with WIT002 had no effect on baseline $[Ca^{2+}]_i$ in these cells, which averaged 75 ± 5 nM. 5-HT induced a transit increase (peak) and followed by a sustained steady-state elevation (plateau phase) in $[Ca^{2+}]_i$ in the cells treated with vehicle. Addition of WIT002 (1 μM) to the bath significantly attenuated both the peak and steady-state increases in $[Ca^{2+}]_i$ following administration of 5-HT.

3.4. Effects of WIT002, ABSA and WIT003 on cerebral blood flow in rats in vivo

The effects of intracisternal infusions of WIT002, WIT003 and ABSA on cerebral blood flow in rats are presented in Fig. 5. Intracisternal injection of vehicle (1:1000 dilution of ethanol in 50 μl artificial cerebrospinal fluid) had no significant effect on cerebral blood flow. Cerebral blood flow tended to increase following administration of the 20-HETE antagonist, WIT002; however, the change was not significant in comparison to that seen in rats treated with vehicle alone. In contrast, intracisternal injection of both the 20-HETE agonists, ABSA (1.5 nmol) or WIT003 (1.5 nmol), reduced cerebral blood flow by 20% and the cerebral blood flow remained significantly below the levels seen in rats treated with vehicle for the 20-min duration of the experiment.

The effects of WIT002 and ABSA on the changes in cerebral blood flow following subarachnoid hemorrhage are presented in Fig. 6 (upper panel). In control rats, injection of artificial cerebrospinal fluid into the cisterna magna of rats had no effect on cerebral blood flow during the 2-h course of the experiment. In vehicle treated rats, cerebral blood flow fell by 37%, 10 min after injection of blood into cisterna magna (i.e. induction of subarachnoid hemorrhage) and it remained at this level for the duration of the experiment. Pretreatment of the rats with the 20-HETE antagonist, WIT002 (1.5 nmol), markedly attenuated the initial fall in cerebral blood flow after induction of subarachnoid hemorrhage by 40%, and cerebral blood flow recovered fully within 90 min following induction of subarachnoid hemorrhage. In contrast, pretreatment of the rats with a 20-HETE agonist, ABSA (1.5 nmol), enhanced

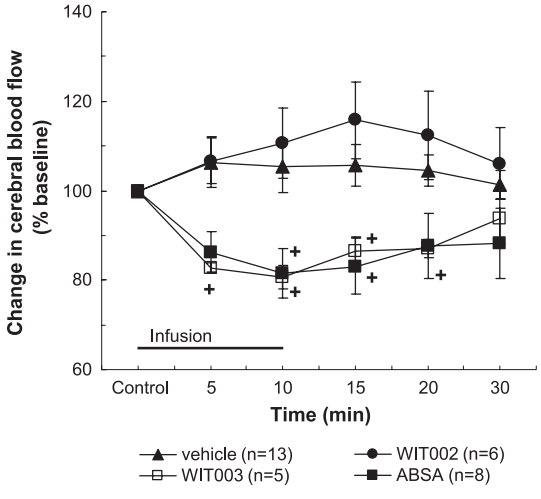

Fig. 5. The effects of WIT002, WIT003 and ABSA on cerebral blood flow in rats. Changes in cerebral blood flow were recorded using laser-Doppler flowmetry after intracisternal infusion of WIT002 (1.5 nmol), WIT003 (1.5 nmol), ABSA (1.5 nmol), or vehicle for 10 min. + Indicates a significant difference from the corresponding value measured in rats treated with vehicle.

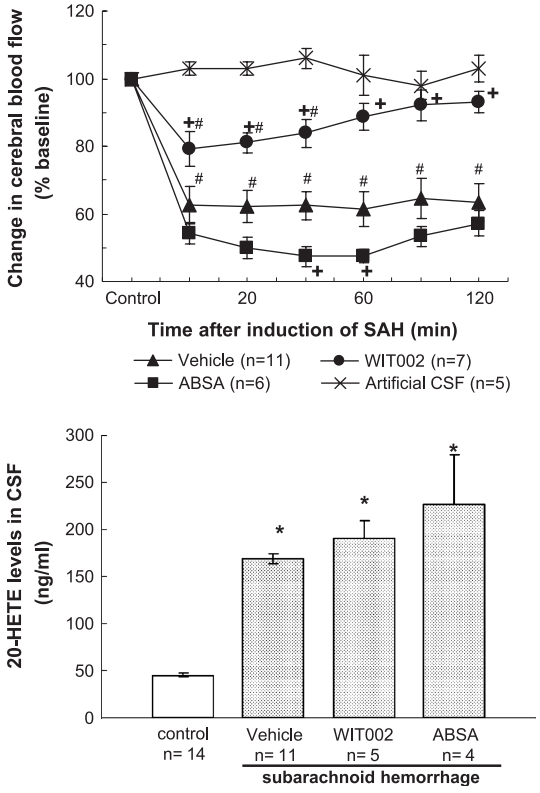

Fig. 6. The effects of 20-HETE analogs on cerebral blood flow and 20-HETE levels in cerebrospinal fluid (CSF) following the induction of subarachnoid hemorrhage (SAH) in rats. SAH was induced by injecting 0.3 ml fresh arterial blood into the cisterna magna of rats after the rats had received an intracisternal injection of WIT002 (1.5 nmol), a 20-HETE antagonist, ABSA (1.5 nmol), a 20-HETE agonist, or vehicle. To determine the role of blood in change in cerebral blood flow, artificial CSF was injected into the cisterna magna of one group of rats. Changes in cerebral blood flow were recorded using laser-Doppler flowmetry. CSF samples were collected before (control value) and 2 h after the induction of SAH. 20-HETE levels in CSF were measured using the fluorescent HPLC method. *Indicates a significant difference from control value, + indicates a significant difference from the corresponding value in rats treated with vehicle and # indicates a significant difference from the corresponding value in rats received artificial CSF.

the fall in cerebral blood flow following subarachnoid hemorrhage.

3.5. Effects of WIT002 and ABSA on 20-HETE levels in cerebrospinal fluid following subarachnoid hemorrhage

A comparison of the effects of the 20-HETE antagonist, WIT002 and the 20-HETE agonist, ABSA, on the concentration of 20-HETE in cerebrospinal fluid after induction of subarachnoid hemorrhage is presented in Fig. 6 (lower panel). The concentration of 20-HETE in cerebrospinal fluid before the induction of subarachnoid hemorrhage was similar in both groups and averaged 45 ± 3 ng/ml. Two hours after the induction of subarachnoid hemorrhage, 20-HETE levels rose to 169 ± 5 ng/ml in vehicle-treated rats. WIT002 and ABSA had no significant effect on 20-HETE levels in cerebrospinal fluid (190 ± 19 and 227 ± 68 ng/ml) after subarachnoid hemorrhage compared to the levels seen in rats treated with vehicle alone.

4. Discussion

The present study examined the effects of analogs of 20-HETE, WIT003 ABSA and WIT002 on the diameter of the middle cerebral artery of rats in vitro, $[Ca^{2+}]_i$ responses in vascular smooth muscle cells isolated from these vessels and on cerebral blood flow in rats in vivo before and after the induction of subarachnoid hemorrhage. The results indicate that both WIT003 and ABSA mimic the response to 20-HETE and constrict the middle cerebral artery of rats in vitro. WIT003 and ABSA both reduce cerebral blood flow when introduced into the cerebrospinal fluid of rats in vivo. We also found that WIT003 and 20-HETE have a similar effect to increase $[Ca^{2+}]_i$ in vascular smooth muscle cells isolated from the middle cerebral artery of rats. In contrast, WIT002 has no effect on the diameter of the middle cerebral or basilar artery of rats in vitro. However, it blocks the vasoconstrictor response to 20-HETE in both of these arteries and prevents the rise in $[Ca^{2+}]_i$ in cerebral vascular smooth muscle cells produced by 20-HETE.

Recent studies have indicated that the synthesis of 20-HETE in cerebral arteries is stimulated by 5-HT and that 20-HETE contributes to the vasoconstrictor actions of 5-HT in these vessels (Cambj-Sapunar et al., 2003). Therefore, we also studied the effects of the 20-HETE antagonist, WIT002, on the vasoconstrictor response of middle cerebral arteries to 5-HT and changes in $[Ca^{2+}]_i$ in vascular smooth muscle cells isolated from these arteries. WIT002 attenuated the vasoconstrictor response to 5-HT in the middle cerebral artery of rats by 65% and greatly reduced the increase in $[Ca^{2+}]_i$ in response to 5-HT in vascular smooth muscle cells isolated from these vessels. We also found that the 20-HETE agonist, WIT003, partially restored the vasoconstrictor response to 5-HT in vessels treated with 17-ODYA to block the endogenous synthesis of 20-HETE (Fig. 3).

Overall, the present results indicating that WIT003 and ABSA are equipotent with 20-HETE in promoting constriction of rat middle cerebral arteries are consistent with the previous findings that these compounds mimic the actions of 20-HETE in renal arteries (Alonso-Galicia et al., 1999a; Yu et al., 2003). We also confirmed that WIT002 is an inactive analog of 20-HETE that competitively blocks the vasoconstrictor effects of 20-HETE in cerebral arteries similar to its reported actions in renal arteries (Alonso-Galicia et al., 1999a; Yu et al., 2003). The new finding of the present study is that these analogs of 20-HETE are effective in vivo and can alter cerebral blood flow when introduced into cerebrospinal fluid.

The present results support the view that there is some sort of receptor that mediates the effects of 20-HETE on vascular tone (Gebremedhin et al., 2000), K^+ channel activity (Sun et al., 1999) and vascular growth (Muthalif et al., 2000). However, the nature of this receptor remains to be determined. The effects of 20-HETE on K^+ channel activity, vascular tone and growth, and sodium transport in the kidney have been linked to activation of protein kinase C, mitogen-activated protein kinase and tyrosine kinase signal transduction cascades (Frazier and Yorio, 1992; Ominato et al., 1996; Sun et al., 1999; Muthalif et al., 2000). Activation of these second messenger pathways are consistent with an action of 20-HETE on a traditional membrane bound receptor. However, there is also evidence that 20-HETE can inhibit K^+ channel activity in inside-out detached membrane patches (Sun et al., 1997), indicating that it may act intracellularly to activate various kinases involved in the phosphorylation of K^+ channels, rather than by stimulating an extracellular receptor.

Recently, the vasoconstrictor response to 20-HETE in small resistance arteries has been linked to sensitization of the contractile response to intracellular Ca^{2+} through a Rho-kinase dependent pathway (Randriamboavonjy et al., 2003). This mechanism is also consistent with an intracellular site of action of 20-HETE and may help explain why 20-HETE produced a slowly evolving but sustained increase in vascular tone in the present study with only a small increase in $[Ca^{2+}]_i$. Previous studies have also reported that the vasoconstrictor action of 20-HETE in some vascular beds is partially dependent on the formation of vasoconstrictor metabolites by cyclooxygenase in endothelium (Escalante et al., 1993; Randriamboavonjy et al., 2003). This is why in the present study the vessels were pretreated with indomethacin to block the activity of cyclooxygenase and to eliminate the possibility that the vasoconstrictor response in the cerebral arteries of rats was secondary to the formation of a cyclooxygenase metabolite of 20-HETE.

Recent studies have shown that 20-HETE levels increase in cerebrospinal fluid following subarachnoid hemorrhage and that inhibitors of the synthesis of 20-HETE (17-ODYA or HET0016) reduce the fall in cerebral blood flow (Kehl et al., 2002; Cambj-Sapunar et al., 2003). These studies suggested that inhibitors of 20-HETE may be useful in limiting cerebral ischemia and brain injury following subarachnoid hemorrhage and other conditions associated with intracranial bleeding. However, 20-HETE plays an important role in the regulation of vascular tone and autoregulation of blood flow throughout the body and in promoting sodium and water excretion in the kidney (Roman, 2002). Thus, it is likely that systemic administration of inhibitors of the synthesis of 20-HETE may have adverse effects on the control of blood pressure and renal function that would limit their usefulness in the treatment of subarachnoid hemorrhage. A potential solution to this problem would be to administer an antagonist of the vasoconstrictor actions of 20-HETE directly to cerebrospinal fluid. Since WIT002 is a lipid that is avidly bound to protein, its diffusion out of the cerebrospinal fluid would be limited thereby minimizing the potential adverse effects of this compound in systemic circulation. Thus, we examined the effects of intrathecal administration of two 20-HETE agonists and one antagonist on the fall in cerebral blood flow of rats following the induction of subarachnoid hemorrhage in rats. We found that administration of WIT002 into cerebrospinal fluid was just as effective as inhibitors of the synthesis of 20-HETE (Kehl et al., 2002; Cambj-Sapunar et al., 2003) to attenuate the fall in cerebral blood flow following subarachnoid hemorrhage. Unlike the 20-HETE synthesis inhibitor (Kehl et al., 2002), WIT002 did not prevent the elevation in 20-HETE levels in cerebrospinal fluid. These results are consistent with the view that WIT002 competitively blocks the vasoconstrictor actions of 20-HETE released into cerebrospinal fluid following subarachnoid hemorrhage in rats. We also found that ABSA, a 20-HETE agonist, potentiated the fall in cerebral blood flow following subarachnoid hemorrhage in rats, again without altering 20-HETE levels in cerebrospinal fluid. These results are also consistent with the view that 20-HETE contributes to the fall in cerebral blood flow following subarachnoid hemorrhage (Kehl et al., 2002) by blocking K^+ channels (Harder et al., 1994), depolarizing cerebral vascular smooth muscle and potentiating the vasoconstrictor response to 5-HT and other constrictors known to be released following subarachnoid hemorrhage (Cambj-Sapunar et al., 2003).

In summary, the results of the present study demonstrate that two 20-HETE analogs, ABSA and WIT003, mimic the actions of 20-HETE and constrict cerebral arteries both in vivo and in vitro. In contrast, WIT002 appears to act as an antagonist that opposes the vasoconstrictor actions of 20-HETE in vitro and attenuates the acute fall in cerebral blood flow in rats following subarachnoid hemorrhage. These results suggest that a stable analog of 20-HETE that antagonizes its vasoconstrictor actions, like WIT002, may have potential to minimize the acute fall in cerebral blood flow following subarachnoid hemorrhage. However, the contribution of 20-HETE to the development of delayed vasospasm following subarachnoid hemorrhage has yet to be established. Thus, further work will be needed to see if blockade of the actions of 20-HETE also has some potential for the treatment of this devastating condition as well.

Acknowledgements

This study was supported in part by Grants HL-59996, HL-29587 HL-36279 and GM-31278 from the National Institutes of Health and from the Robert A. Welch Foundation.

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