Polyunsaturated Fatty Acids Are Cerebral Vasodilators via the TREK-1 Potassium Channel

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Abstract—Vessel occlusion is the most frequent cause for impairment of local blood flow within the brain resulting in neuronal damage and is a leading cause of disability and death worldwide. Polyunsaturated fatty acids and especially α-linolenic acid improve brain resistance against cerebral ischemia. The purpose of the present study was to evaluate the effects of polyunsaturated fatty acids and particularly α-linolenic acid on the cerebral blood flow and on the tone of vessels that regulate brain perfusion. α-Linolenic acid injections increased cerebral blood flow and induced vasodilation of the basilar artery but not of the carotid artery. The saturated fatty acid palmitic acid did not produce vasodilation. This suggested that the target of the polyunsaturated fatty acids effect was the TREK-1 potassium channel. We demonstrate the presence of this channel in basilar but not in carotid arteries. We show that vasodilations induced by the polyunsaturated fatty acid in the basilar artery as well as the laser-Doppler flow increase are abolished in TREK-1−/− mice. Altogether these data indicate that TREK-1 activation elicits a robust dilation that probably accounts for the increase of cerebral blood flow induced by polyunsaturated fatty acids such as α-linolenic acid or docosahexanoic acid. They suggest that the selective expression and activation of TREK-1 in brain collaterals could play a significant role in the protective mechanisms of polyunsaturated fatty acids against stroke by providing residual circulation during ischemia. (Circ Res. 2007;101:0-0.)

Key Words: cerebral ischemia □ α-linolenic acid □ CBF □ vasodilatation □ two pore-domains channel TREK-1

After stroke, the function of cerebral arteries is critical to maintain cerebral perfusion and preserve neuronal integrity. The duration and intensity of the blood flow deficit are associated with the severity of brain damage. The level of cerebral blood flow (CBF) in brain tissue is coupled to an evident extent with vascular function and beyond neuronal consequences. It is well known that cerebral ischemia is associated with functional impairment of vascular tone within the occluded artery.

Polyunsaturated fatty acids (PUFAs) and particularly α-linolenic acid (ALA) and docosahexanoic acid (DHA) are potent protectors against focal and global ischemia.2–4 The molecular mechanism of PUFA-induced neuroprotection has been recently clarified.5 The main PUFA target seems to be the potassium channel, TREK-1, which belongs to the new family of two-pore domain potassium channels (K2P) and is known to be potently activated by PUFA.6–8 The importance of the TREK-1 channel in cerebral protection against ischemia has been validated by the fact that the protective effects of PUFA are drastically decreased in TREK-1−/− mice.5

The vascular wall represents the primary compartment of ischemic stroke. PUFA application confers neuronal protection,2–4 but little is known about TREK-1 channel in cerebral vessels. A previous report shows the presence of the TREK-1 channel in mesenteric rat arteries but not in pulmonary arteries, suggesting that the expression pattern of this channel depends on the type of arteries.9 On the other hand, Bryan et al report the presence of K2P channels in vascular smooth muscle cells of rat middle cerebral arteries,10 and we have recently described the crucial role of the TREK-1 channel in both mesenteric arteries and skin microvessels.11 In these vessels the TREK-1 deletion leads to a dysfunction of endothelial factors production, particularly NO, that are responsible for smooth muscle relaxation.11 The aim of this work was to determine whether TREK-1 could also play a role in the cerebral circulation. We have addressed the following questions: (1) is there a crucial role for the TREK-1 channel in the cerebral vasculature, especially in the basilar artery? (2) could the PUFA-induced activation of vascular TREK-1 channels result in cerebral vasodilation, which would result into a protective mechanism?

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This study examines the effect of PUFAs on cerebral laser-Doppler blood flow and vessel tone. It also analyzes TREK-1 distribution in different arteries and establishes with TREK-1−/− mice that the increased cerebral blood flow and vasodilation of basilar arteries produced by PUFAs is due to an action on TREK-1 channels.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement available at http://circres.ahajournals.org.

Artery Vasoreactivity
Dissected rat carotid arteries were quickly placed in ice-cold physiological saline solution (PSS), cut into ring segments of 2 to 3 mm length and mounted for standard tension-recording in isolated organ baths (Radnoti Glass Technology) containing 40 mL PSS bubbled with 95% O2/5% CO2, then, carotid segments were equilibrated 30 minutes at 37°C and underwent a passive force normalization procedure to determine for each vessel a passive length-tension curve and the resting tension leading to optimal responses (8 mM/min). Isometric tension was recorded with a force-displacement transducer connected to a data acquisition system (Workbench PC software).

Segments of dissected arteries of rats (basilar) and mice (basilar and carotid) were mounted in a small vessel arteriograph (LSI) on two glass cannulas, perfused with PSS equilibrated with 20% O2/5% CO2/balance N2, and maintained at 37°C and pH 7.4. Briefly, the proximal cannula was connected to a pressure transducer, a miniature perfusion pump, and a servomotor controller that continually measured and adjusted transmural pressure (TMP), and then the lumen diameter was analyzed in the no-flow condition through a camera coupled to a video dimension analyzer. Basilar arteries were equilibrated at a TMP of 75 mm Hg for 1 hour before experimentation, at which time basilar arteries spontaneously developed pressure-induced myogenic tone.

Responses to fatty acids were determined by successive additions of linoleic acid (ALA; 10, 100 μmol/L), docosahexanoic acid (DHA; 10, 100 μmol/L), and palmitic acid (PAL; 10 μmol/L) on both basilar and carotid arteries. The smooth muscle relaxation was assessed with sodium nitroprusside (SNP; 10 μmol/L) and papaverine (10 μmol/L), respectively, for active and passive relaxation. The smooth muscle constriction was tested with serotonin (5-HT; 1 μmol/L) for rat arteries and endothelin-1 (ET-1; 10 μmol/L) for mouse arteries. The mice endothelium-induced relaxing response was evaluated by acetylcholine (ACh; 10 μmol/L) application after preconstriction by ET-1 (10 μmol/L).

The PSS ionic composition was as follows (mmol/L): 119 NaCl, 24 NaHCO3, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4·7H2O, 10 glucose, 1.6 CaCl2, pH 7.4. All fatty acid (Avanti Polar Lipids) and vasoactive drugs (ACh, ET-1, Papaverine, 5-HT, SNP, and KC1; Sigma-Aldrich). Ach, ET-1, 5-HT, and SNP were made fresh daily in stock solutions of 0.1 mol/L and stored at 4°C.

Percent of dilation was calculated according to the following equation: [ΔDia-Db]/Index VC*100, where D is the diameter on stabilization, after vehicle or fatty acid administrations (fa), baseline (b) and the index of vessel capability (VC index) is provided applying papaverine (10 μmol/L) on rat serotonin-precontracted vessels or mice endothelin-1-precontracted vessels. The VC index corresponds to the difference between the maximum diameter and the basal precontracted diameter.

Results
Are Fatty Acids Active on Rat Basilar Artery Tone?
Myogenic tone and vasoactive responses to serotonin (5-HT) or sodium nitroprusside (SNP) were indications of a viable basilar artery (data not shown). The fatty acid–induced relaxation was studied in arteries that possessed an intact endothelium equilibrated with 20% O2/5% CO2/balance N2 and maintained at 37°C and pH 7.4.

Alpha-linolenic acid (ALA) and docosahexanoic acid (DHA), which are known to potentiate the TREK-1 channel and which protect the brain against stroke, were tested (Figure 1). The 10-μmol/L and 100-μmol/L concentrations of ALA correspond to concentrations at which this PUFA is a potent activator of the TREK-1 channel. ALA induces an increase of the basilar artery diameter (Figure 1A). The maximal relaxation is of 54.8±8.9% at 100 μmol/L (n=10; Figure 1C). The relaxation induced by 100 μmol/L DHA was in the same range, at 42.9±9.7% (data not shown). At 10 μmol/L, ALA and DHA induced relaxations are 32.2±3.9% and 23.4±5.7%, respectively. Palmitic acid (PAL), a saturated fatty acid that does not activate the TREK-1 channel and does not protect neurons, had no effect on the basilar artery (Figure 1A and 1C). The basilar vasodilation induced by 10 or 100 μmol/L of ALA (30.9±3.1% and 38.2±6.8%, respectively) is not affected by the presence of Nω-nitro-L-arginine (L-NNA, 10 μmol/L), a blocker of nitric oxide synthase, reflecting that ALA-induced vasodilation is mostly independent of the nitric oxide (NO) pathway. Because cycloxygenase metabolites of PUFA, especially of arachidonic acid are vasoactive and generally potent dilators, we performed the same type of experiments on rat basilar artery in presence of L-NNA (10 μmol/L) and indomethacin (10 μmol/L) to inhibit both NO and prostanooids pathways. This treatment did not affect the relaxation induced by 10 μmol/L ALA (33.9±7.1%, n=7, data not shown).

Are Fatty Acids Active on Rat Carotid Artery Tone?
Using a carotid segment, the main artery supplying blood, oxygen, and glucose to the brain, we analyzed whether this blood vessel also responds to ALA. ALA (10 μmol/L) was unable to dilate the carotid segment (n=10), and PAL (10 μmol/L) failed to produce relaxation (Figure 1B and C).

ALA Injection Induces Laser-Doppler Flow (LDF) Changes in MCA Territory
Laser-Doppler flowmetry was used for continuous estimation of hemodynamic changes in the cerebral microcirculation. The variation of the regional LDF in the rat MCA territory was measured 30 minutes after either a vehicle (n=6) or a 500 nmoles/kg ALA injection (Figure 1D). The dose of 500 nmoles/kg was selected based on our previous studies, where this dose offers the best protection against global ischemia. Although not statistically significant, LDF associated to MCA has a tendency to decrease in the 30 minutes after initial vehicle injection. During the experiment, normocapnia was not maintained by mechanical ventilation of the rat lungs and it is known that isolurane tends to induce hypocapnia that could reduce global LDF up to 30%. The LDF of ALA-injected rats increased almost by 20% in the 30 minutes after injection. In all ALA-injected rats, a marked increase was noted as soon as 10 minutes after injection (data not shown). In our experiment, no significant difference in the continuous monitoring of mean arterial blood pressure was observed (data not shown).
TREK-1 Channel Distribution in Different Rat and Mouse Arteries Delivering Blood to Brain

We determine the presence of TREK-1 channels in the different rat and mouse arteries, which represent the 2 delivery pathways for cerebral blood flow. TREK-1 mRNA expression was assessed not only in rat basilar and carotid arteries (Figures 2 and 3), that are relevant for stroke, but also in femoral artery as an independent control not related to Figure 1.

Figure 1. In vitro and in vivo effects of PUFA administration. ALA and DHA induce vasodilation of the rat basilar artery but not of the carotid artery. A, Diameter recordings using Halpern arteriography technique show ALA and PAL effects on rat basilar artery. 10 μmol/L PAL had no effect whereas ALA at the same concentration induced a dilation that is fully reversible. B, Diameter converted from tension-recordings on rat carotid artery. Neither ALA nor PAL were able to dilate a carotid at the same concentration. C, Relaxation induced by SNP as control of relaxation and different fatty acids on rat basilar and carotid artery. ALA induced a marked relaxation on the basilar, but not on the carotid artery. L-NNA as NO inhibitor treatment did not modify the relaxation induced by ALA (10 and 100 μmol/L). PAL had no effect on these arteries. Data were expressed as mean±SEM of n animal (n=9). Statistically different from the vehicle treatment, ***P<0.001 and **P<0.01. Statistically different from the PAL treatment, $$$P<0.001. Statistically different from the same treatment applied in carotid arteries, ###P<0.001. D, ALA injection increases the cerebral blood flow in rat. Laser-Doppler flow (LDF) variations are measured 30 minutes after vehicle injection (n=6) and 500 nmoles/kg ALA injection (n=9). Data were expressed as mean±SEM of n animal. Statistically different from the basal level ##P<0.01. Statistically different from the vehicle-injected animal, ###P<0.001.
brain perfusion (data not shown). RT-PCR experiments demonstrate the presence of mRNA encoding for TREK-1 only in basilar arteries (Figure 2A and 2B).

Expression of the TREK-1 channel protein was also analyzed by Western blots using a well-characterized antibody. Under reducing conditions, in the presence of β-mercaptoethanol (β-Me), anti-TREK-1 antibodies labeled in the basilar artery a major band at approximately 45 kDa (Figure 2A and 2B) corresponding to the size predicted for the TREK-1 subunit. TREK-1 was hardly detectable in carotid (Figure 2A and 2B) and in femoral arteries (data not shown).

The subcellular location of TREK-1 mRNA was examined first by in situ hybridization in both basilar and carotid arteries. TREK-1 mRNA is expressed throughout the myocyte and the endothelial cell layer of the basilar artery (Figure 2C). TREK-1 mRNA is absent in the carotid and femoral arteries (not shown). Immunohistochemical labeling on basilar arteries sections (Figure 2C) indicates that TREK-1 expression is widespread throughout the basilar wall in both myocytes and endothelial cell layers. The TREK-1 channel is colocalized with antigen CD31 (also called PECAM), a specific marker of...
endothelial cells in blood vessels (Figure 2C, merged panel).

In mice arteries, TREK-1 mRNA was only present in basilar arteries (Figure 3A and 3B). The quantitation of the TREK-1 transcriptional expression showed that TREK-1 mRNA remained poorly detectable in carotid artery compared with the basilar artery \((P < 0.001)\). Protein expression of TREK-1 by Western Blotting indicated a band of approximately 45 kDa corresponding to the predicted size of the TREK-1 protein. This band was only detectable in the basilar artery (Figure 3A and 3B).

Effects of Different Fatty Acids on Mouse Basilar and Carotid Arteries

As for rat arteries, mice basilar and carotid arteries were studied with an intact endothelium in PSS equilibrated with 20% O\(_2\)/5% CO\(_2\)/balance N\(_2\) and maintained at 37°C and pH 7.4. Vasoactive responses such as spontaneous contraction (myogenic tone), endothelin-1-induced contraction, and sodium nitroprusside–induced relaxation were assessed as indicative controls of the basilar viability in conditions of intraluminal pressure of 75 mm Hg (supplemental Table 1). ALA or DHA at 10 \(\mu\)mol/L and 100 \(\mu\)mol/L induced a marked relaxation on basilar, but not on carotid artery. L-NNA as NO-inhibitor treatment did not modify the relaxation induced by ALA (10 and 100 \(\mu\)mol/L). PAL had no effect on these arteries. SNP-induced active relaxation was not affected throughout the experiment. Data were expressed as mean \pm SEM of \(n\) animal (\(n = 9\) for the basilar artery and \(n = 5\) for the carotid artery). Statistically different from the vehicle treatment, *** \(P < 0.001\) and ** \(P < 0.01\). Statistically different from the PAL treatment, $$$ \(P < 0.001\) and $$ \(P < 0.01\). Statistically different from the same treatment applied in carotid arteries, ## \(P < 0.01\) and # \(P < 0.05\).

Effects of Different Fatty Acids on Mouse Basilar Artery Tone in TREK-1\(^{+/+}\) and TREK-1\(^{-/-}\) Mice

PUFA effects on mice basilar artery tone were then studied in wild-type and TREK-1 knock-out mice (TREK-1\(^{-/-}\)). As for the rat basilar artery, L-NNA treatment (10 \(\mu\)mol/L) did not modify the relaxation induced by ALA (48.3 \(\pm\) 4.6%, \(n = 9\)) or DHA (62.4 \(\pm\) 10.8%, \(n = 9\)) (Figure 3C).
before, vasoactive responses to pressure (myogenic tone) and to endothelin-1 (contraction) and sodium nitroprusside (relaxation) indicated the viability of the mouse mounted segments. They were not different in TREK-1/H11001/H11001/H11001 and TREK-1/H11002/H11002/H11002 basilar arteries (supplemental Table I), indicating that the basic vascular properties were conserved in both genotypes. Supplemental Figure II shows that the endothelium was present and intact in basilar arteries of knock-out mice. Although treatment with 10 \(\mu\)mol/L and 100 \(\mu\)mol/L ALA increased the basilar diameter in TREK-1\(^+\)/+ mice (Figure 4A), they had essentially no effect on the basilar artery of TREK-1\(^-\)/- mice (Figure 4B). Palmitic acid was without effect on the diameter of both TREK\(^+\)/+ and TREK-1\(^-\)/- arteries (Figure 4B). The basilar artery relaxation induced by 10 \(\mu\)mol/L and 100 \(\mu\)mol/L DHA was also markedly decreased if not abolished in TREK-1\(^-\)/- vessels (Figure 4C). The lack of ALA or DHA-induced relaxation in vessels isolated from TREK-1\(^-\)/- mice strongly suggests a central

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**Figure 4.** ALA-induced vasodilation is abolished in the basilar artery of TREK-1\(^-\)/- mice. A, Diameter recordings on TREK-1\(^+\)/+ mice basilar artery. PAL (10 \(\mu\)mol/L) had no effect on the basilar artery (n=10), whereas ALA at a similar concentration induced dilation. B, Diameter recordings on TREK-1\(^+\)/+ mice basilar artery showing the lack of effect of 10 \(\mu\)mol/L ALA or PAL. C, Percent of relaxation induced by different fatty acids on basilar artery of TREK-1\(^+\)/+ and TREK-1\(^-\)/- mice. PUFA such as ALA (10 \(\mu\)mol/L, n=13; 100 \(\mu\)mol/L, n=13) and DHA (10 \(\mu\)mol/L, n=6; 100 \(\mu\)mol/L, n=5) induced a marked relaxation of the basilar artery of TREK-1\(^+\)/+ but not of TREK-1\(^-\)/- mice (ALA, 10 \(\mu\)mol/L, n=6; 100 \(\mu\)mol/L, n=5; DHA, 10 \(\mu\)mol/L, n=6; 100 \(\mu\)mol/L, n=5). Endothelium-mediated vasodilation using ACh was drastically attenuated in TREK-1\(^-\)/- mice basilar artery. Palmitic acid (PAL, n=12 per genotype) had no effect on the basilar artery of both genotypes. Data were expressed as mean±SEM of n animal. Statistically different from the vehicle treatment, ***P<0.001. Statistically different from the same treatment applied in the TREK-1\(^-\)/- mice, ###P<0.001 and ##P<0.01. D, Diameter recordings show ACh effects on mice basilar artery. ACh (10 \(\mu\)mol/L) induced an endothelium-mediated dilation of the ET-1 preconstricted TREK-1\(^+\)/+ basilar artery, whereas it had no effect on the TREK-1\(^-\)/- basilar artery.
role of the TREK-1 channel in PUFA effects on basilar artery tone. A classical way to evaluate endothelium-mediated vasodilation is to use acetylcholine (ACh). Application of ACh on TREK-1−/− basilar artery resulted in a relaxation of 65.3±6.4% (Figure 4D), which was classically decreased after L-NNA alone or L-NNA + indomethacin incubation (data not shown). In TREK-1−/− mice, the ACh-induced vasodilation was strongly attenuated (3.3±4.8%) (Figure 4D).

Laser-Doppler Flow (LDF) Changes After PUFA Injection Are Linked to TREK-1

Figure 5 shows the LDF variation in the MCA territory of TREK-1−/− and TREK-1−/− mice measured 30 minutes after either a vehicle or a 500 nmoles/kg ALA or DHA injection. TREK-1−/− LDF associated with the MCA area statistically increased in the 30 minutes after initial ALA or DHA injection. Cerebrovascular reactivity to ALA (or LDF responses to PUFA) looks clearly different from those induced by standard vasodilator stimuli (acetazolamide and hypercapnia). This LDF increase occurred while the continuous monitoring of systolic blood pressure displayed no change after ALA injection in the TREK−/− mice (supplemental Table II). Autoregulation depends on the ability of resistance vessels to dilate when mean arterial blood pressure (MABP) falls and to constrict when MABP rises. In our experiment the MABP did not change. In addition, blood flow in TREK−/− mice had a tendency to decrease in the 30 minutes after initial ALA or DHA injection, but this decrease was not statistically significant from the vehicle injection. In addition, CBF responses to ALA and DHA injections were drastically different in TREK-1−/− and TREK-1−/− animals (**P<0.001) suggesting again a central role of the TREK-1 channel in PUFA effects on CBF regulation. As control, we also compared the MABP between TREK-1 KO and WT mice. There was no significant difference (TREK+/* mice: 72.5±2.6 and TREK-1−/−: 73.1±2.9 mm Hg, n=6 per phenotype).

**Discussion**

The purpose of this study was to gain more insight into the mechanism of PUFA-induced brain resistance to ischemic stroke. In ischemic stroke, the neurovascular unit is damaged primarily by the reduction of blood flow and the cerebral infarct size is inversely related to CBF. This paper reports for the first time the action of PUFAs on CBF as well as on cerebral arteries dilatation, a property that may be related to their protective effects against brain ischemia. LDF-monitoring has shown that an acute injection of ALA significantly increased the local cerebral blood flow in the rat MCA area. This ∼20% increase of the flow is related to an increased vasodilation. Indeed, ALA acts as a vasoactive compound and leads to a ∼30% increase of the diameter of the basilar artery. This effect appears to be specific to cerebral resistance arteries, because ALA does not dilate carotid arteries with elastic properties. The results are consistent with the idea that ALA induces vasodilation only in resistance arteries such as those of the cerebral vascular bed, without decreasing systemic blood pressure. A vasoactive effect in the basilar artery is also observed with the other classical polyunsaturated fatty acid DHA, whereas it is not seen with a saturated fatty acid such as PAL. We propose that the increased capacity of brain arteries to dilate after PUFA treatment increases collateral flow, reduces CBF loss in the periphery of the ischemic zone, and contributes to the PUFA-induced protection against ischemic stroke. Improved collateral flow might lead to an increase of cerebral tissue perfusion especially in the penumbra and might limit the spread of the infarct.

The TREK-1 channel has been previously demonstrated to be involved in the ALA-induced resistance of the brain against ischemia. This channel is a member of the family of potassium channels with two pore-forming domains having the properties of background K+ channels. This channel is potently activated by PUFA such as ALA and DHA, but not by the saturated fatty acid PAL. Recent work has provided evidence for the presence of two-pore domain K+ channels in rat mesenteric, cerebral, and pulmonary arteries, suggesting that some of these channels could play a physiological role in regulating basal membrane potential and tone in these vessels. This article shows that TREK-1 channels are well expressed in the basilar vasculature of both rats and mice whereas they are essentially absent in the carotid artery. There is a strict parallelism between the localization of TREK-1 channels in these different arteries and the vasodilation effect of PUFAs, which is observed only with basilar but not carotid arteries. This work supports the idea that TREK-1 plays an important role in the vasodilator responses to PUFAs. Indeed, the lack of PUFA-induced vasodilation in TREK-1−/− mice indicates clearly that PUFA-induced relaxation on arterial basal tone is related to their action on the TREK-1 channel.

Our laboratory has recently shown an important effect of TREK-1 deletion on microvascular function in mesenteric...
acetylcholine, bradykinin, and cutaneous pressure-induced vasodilation were drastically altered in mice lacking the TREK-1 channel. Deletion of the TREK-1 channel was shown to be associated with an impairment in the cascade producing NO, leading to endothelial dysfunction.11 In cerebral basilar arteries, the deletion of TREK-1 did not alter smooth muscle vasodilation in response to the NO-donor SNP, but it abolished the response to ACh. These new observations are in line with our previous work.11 They indicate an impairment of endothelial function in TREK-1−/− mice basilar arteries. Thus, to gain insight into the mechanism by which TREK-1 deletion alters PUFA-induced relaxation, we first investigated the hypothesis of a NO pathway alteration in the TREK-1−/− mice. If PUFA-induced vasorelaxation only relies on NO production by the endothelial cell, then L-NNa, an inhibitor of NO synthase, should drastically decrease ALA- or DHA-induced vasodilation of basilar arteries. This is not what we have observed, suggesting the involvement of an NO-independent mechanism in the vasorelaxation process. To ensure that prostanoids, which are also important regulators of vascular tone, were not responsible for the observed vasodilation, we also combined L-NNa and indomethacin, a nonselective inhibitor for cyclooxygenases. This treatment again did not alter ALA- or DHA-induced vasodilation of basilar arteries; PUFA-induced vasodilation of cerebral vessel is a non–NO-dependent and non–prostanoid-dependent mechanism. This result leaves us with 2 possible mechanisms to explain PUFA-induced vasodilation in relation with TREK-1 channels: The first one would involve an endothelial component, distinct from NO and prostanoids, which could be EDHF.25 The second one would involve a direct effect of PUFAs on smooth muscle creating an hyperpolarizing-relaxing response. Bryan et al10 have recently demonstrated the presence of an “atypical” K+ channel, insensitive to classical potassium channels inhibitors, with biophysical properties similar to K+ channels of the TREK-1 family, that, in response to arachidonic acid, hyperpolarizes smooth muscle and dilates MCA artery.10

The NO-independent PUFA-induced vasodilation reported here is a particularly interesting observation considering the drastic alteration of endothelium-dependent NO-mediated vasodilation in cerebral arteries after stroke.26,27 The consequence of ALA- and DHA-mediated vasodilation is an increased LDF. We have previously demonstrated a neuronal protection induced by PUFA treatment in models of ischemia.5,5 In a model of focal ischemia, the ALA treatment was found to be efficient to protect from ischemic damage up to 6 hours after reperfusion, ie, within a clinically interesting time window. The beneficial effects of ALA have been reported to be attributable in part to its activating effect on neuronal TREK-1 channel, preventing an excessive release of excitotoxic glutamate and favoring a Mg2+ block at postsynaptic NMDA receptors.2 This article indicates that the protection is potentially also attributable to ALA-induced vasodilation, also via TREK-1 channels. In the pathobiology of ischemic stroke, anoxic depolarization and perinfarct spreading depression are 2 major events that have been linked to worsening focal ischemic damage.1 The reduction of oxygen delivery in both core and penumbra is attributable to the additional metabolic burden imposed on the ischemic brain but also to the ischemic depolarization consequences on the brain vascular network causing vasoconstriction. This secondary reduction in CBF probably expands the infarct during stroke. PUFA activation of TREK-1 channels would be expected to generate resistance to depolarization of the collateral vessels and lead to a better tissue oxygenation during acute focal cerebral ischemia and reperfusion. PUFAs would be expected to increase the resistance to vasoconstriction induced by ionic changes produced by the depolarization wave, leading to spreading ischemia.28 We did not examine the role of this channel in CBF autoregulation reflecting the fundamental property of the cerebral circulation that enables it to maintain stable brain perfusion in face of blood pressure changes.

To our knowledge, this work is the first demonstration that the TREK-1 channel is involved in the control of brain circulation and that PUFAs that are so popular (particularly omega-3s) in preventive automedication29,30 have a beneficial effect on brain blood flow via this particular type of channel.

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Disclosures
None.

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**Material and Methods**

**Animals**

All experiments were performed according to policies on the care and use of laboratory animals of European Community legislation. The local Ethics Committee approved the experiments (protocol numbers NCA/2006/10-1 and NCA/2006/10-2). We used adult male C57/Bl6 and deficient-TREK-1 mice as well as adult male Wistar rats. The animals housed under controlled laboratory conditions with a 12-hour dark-light cycle, a temperature of 21 ± 2°C, and a humidity of 60 to 70% for at least one week prior to drug treatment or surgery. Rodents had free access to standard diet and tap water.

**Laser-Doppler flow measurement in the middle cerebral artery territory**

Rats or mice were anesthetized with 2% isoflurane (Baxter SA) mixed to 30% oxygen and 70% nitrous oxide. Core temperature was continuously monitored and maintained at physiological temperatures using an heating blanket (Harvard Apparatus). The femoral artery was catheterized with polyethylene tubing for continuous monitoring of mean arterial blood pressure with a blood pressure transducer (Harvard Apparatus). The temporal muscle was retracted to attach to the intact skull overlying the region of the left middle cerebral artery (MCA) territory a flexible 0.5-mm fiber optic probe. Then the transcranial monitoring of the cerebral blood flow (CBF) was performed before and during drug administration by laser-Doppler flowmetry (PF5000, Perimed). The left MCA CBF during the experiment was presented as a percentage of the flow before drug administration.

Data were expressed as mean ± SEM of n animal. Comparison between different groups was evaluated by one-way analysis of variance (ANOVA) followed by a PLSD (protected least significant difference) Dunnet's test. Significance was assumed at a value of **P<0.05 or ***P<0.001.

**RT-PCR**

Total RNA was isolated from different arteries with RNeasy mini kit plus proteinase K (Qiagen) according to the manufacturer’s instructions. For cDNA synthesis, 16µl reaction mixture containing total RNA 100ng, oligo dT 500ng and RNA inhibitor X IU were heated to 60°C for 10 min. Then dNTP 10 mM of each, buffer 4µl, dTT 2µl, and SuperScript II RNase H RT 200 IU (Invitrogen) were added and reverse transcription mixture was incubated 1 h at
42°C. PCR amplification was carried out with 6 µl cDNA product in 100 µl reaction volume containing: dNTP 200mM, MgCl2 2.5 mM mmol Taq DNA polymerase 2.5 IU (Qiagen) and specific oligonucleotide primers 20pmol. The primer for TREK-1 (KCNK2) (accession number U73488) were 5’-GCTGGGAATTCCCTCTTTGG-3’ and 5’-CGCTCTGAACTCTCCCACCTC-3’ and those for GAPGH were 5’-CGGGAAGCTCAGTCAGGTGACCACCCCTG-3’. PCR was performed for 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. 20 µl PCR products were electrophoresed in 2% agarose gel, visualized by staining with ethidium bromide, and scanned for quantitation. Raw values of gene products were relative to the endogenous standard (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) and then expressed as a percentage of TREK-1/GAPDH in basilar artery. Control reactions using DNAse treated but not reverse-transcribed total RNA or performed in the absence of a cDNA template were all negative (data not shown).

**Western Blotting**

Protein extraction was performed from different arteries (n=6 per group). Fresh arteries were cut into pieces and homogenized in four volumes of cold lysis buffer (2% SDS, 5% glycerol and protease inhibitor cocktail) on ice. The homogenates were centrifuged at 12,000 g for 30 min at 4°C and supernatants were stored at -70°C until further use. Protein concentrations were measured using conventional Bradford’s method. 50 mg proteins from each experimental group were applied to 12% SDS Page gels and electrophoresed for 2 hours at 60 mA. Proteins were transferred onto Hybond-P PVDF membrane (Amersham Biosciences) in blotting buffer (156 mmol/L Tris, 1 mol/L glycine, PBS) for 90 min at 80 mA and blocked with 5% skim milk (Regilait) in PBS for 2 hours at room temperature. Membranes were incubated with the rabbit polyclonal TREK-1 antibody directed against residues 1 to 44 and 71 to 114, overnight at 4°C. After washing in 0.1% Tween/PBS (four times, 15 min each), peroxidase-conjugated donkey anti-rabbit. IgG antibody was used as the second antibody (Amersham Biosciences, diluted 1/10000) for 1 h at room temperature. Stripped membranes were rehybridized with a α-tubulin antibody (Sigma-Aldrich) to control the sample loading. Detection was performed using ECL plus Western blotting detection reagents (Amersham Biosciences, Orsay, France) with a Las-3000 imaging system (Fujifilm).
**Immunohistochemistry**

Frozen artery sections (12 µm thick) were post-fixed with 4% paraformaldehyde/PBS, permeabilized in 0.3% polyoxyethylensorbitan monolaurate (Tween 20, Sigma) for 10 min and blocked with 3% horse serum/PBS for 1 h at room temperature. Sections were incubated with an anti-TREK-1 antibody (1:5000)² overnight. After 3 washes in PBS, sections were incubated in anti-IgG Alexa 488-coupled antibodies (Molecular Probe) in 3% normal horse serum for 1 h, washed three times in PBS for 5 min each. Then, sections were incubated with a goat polyclonal CD-31 antibody (diluted 1/100, Chemicon) or a rabbit polyclonal von Willebrand factor antibody (diluted 1/3000, Chemicon) in 3% normal horse serum for 1 h at room temperature, rinsed three times in PBS, and then incubated in anti-IgG Alexa 594-coupled antibodies (Molecular Probes) for 1 h at room temperature. Confocal microscopy observations were performed with a Laser Scanning Confocal Microscope (TCS SP, Leica) equipped with a DMIRBE inverted microscope, using a Plan Apo 63x/1,4 NA oil immersion objective. Signal specificity was assessed in negative control sections by omitting primary antibody, directed against the TREK-1 protein. The control sections show an autofluorescence background of the tissue, particularly at the level of the internal elastic lamina (see Online Figure I). Confocal images of TREK-1-Alexa-488 antibody labeling were then obtained after spectral correction of the autofluorescence background. Acquisition of the confocal images was designed to minimize collagen and internal elastic lamina autofluorescence background of the tissue. The photomultiplier gain and offset values were adjusted to obtain black background images on the negative control (basilar artery tissue section without any labeling) and kept for TREK-1 imaging. Spectral acquisitions were done using the confocal laser scanning microscope Leica SP5 (Rueil Malmaison, France) on a basilar artery tissue section without any labeling, and on the TREK-1-Alexa-488 labeled specimen, in the same spectral conditions (laser excitation 488 nm, acquisition 500-600nm every 10nm). Spectral unmixing was applied on this second set of images by elimination of the autofluorescence component using the spectrum obtained on the non-labeled tissue section. Corrected “FITC” images of TREK-1 labeling were thus obtained. The double-labeling observations were performed with the same parameters (such as the laser power, scanning speed and the photomultiplier gain).

**In-Situ Hybridization and Electron microscopy**

Tissue-Tek embedded vessels were frozen in isopentane at −40°C. To obtain a fairly good immunocytochemical signal, tissues were only fixed with formaldehyde (made freshly
from paraformaldehyde). Cryostat sections (30µm) were treated with 4X SSC/1X Denhart (2 times 30 min), 1X SSC (10 min), and acetylated in 0.1M triethanolamine (3 min, RT) followed by 0.1M triethanolamine/acetic anhydride (100/0.25, 10 min, RT). Hybridization of DIG-dUTP labeled oligonucleotide probes (CAC AAT GGT CCT CTG GGA AAT CTC CTG AGG) was performed at 42°C overnight in the presence of 50% formamide. Hybridization specificity was assessed by treating sections with 40 mg/ml RNAse in TE buffer for 30 min at 37°C followed by 2 washes in 0.5x SSC. The sections were then washed in 1X SSC (2 times 30 min), 0.1X SSC (2 times 20 min), blocked in 1X PBS/BSA (100/5, 20 min, RT). They are then immunogold-labeled with an anti-DIG antibody (diluted 1/100 in 1X PBS/BSA (100/5, 60 min, RT) and silver enhanced (20 min) (BBI International). Sections are then embedded in araldite resin. The localization of region of interest was realized under the light microscope after a light staining with a mixture of methylene blue, Azur B Blue. Ultrathin sections were obtained with an ultramicrotome and a diamond knife, stained with aqueous uranyl acetate and Reynold's lead citrate and then transferred onto 300 mesh carboned Formvar coated grids. They were examined using a JEOL 6700F field emission scanning electron microscope.

Online Table I: Basilar arteries of TREK-1<sup>+/+</sup> and TREK-1<sup>-/-</sup> mice conserve the same basic vascular properties.

<table>
<thead>
<tr>
<th>Mice artery</th>
<th>Constriction (%)</th>
<th>Relaxation (%)</th>
<th>Basal tone (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myogenic tone at 75 mmHg</td>
<td>ET-1 (10 nM)</td>
<td>SNP (10 µM) 0 min 60 min After PUFA incubation</td>
</tr>
<tr>
<td>TREK-1&lt;sup&gt;+/+&lt;/sup&gt; basilar</td>
<td>14.0 ± 2.6</td>
<td>36.9 ± 4.1</td>
<td>53.2 ± 7.4</td>
</tr>
<tr>
<td>TREK-1&lt;sup&gt;-/-&lt;/sup&gt; basilar</td>
<td>13.4 ± 2.3</td>
<td>37.5 ± 4.8</td>
<td>61.1 ± 15.0</td>
</tr>
</tbody>
</table>

Vasoactive responses were assessed as an indicative control of the basilar viability at 75mmHg for the different mouse phenotypes. Spontaneous contraction (myogenic tone) was calculated according to the following equation: \( [(D_{Pav-Db}) / D_{Pav}]\times100, \) where \( D \) is the diameter upon stabilization, after papaverin administration (Pav, 10 µM), baseline (b).
Endothelin-1-induced contraction was calculated according to the following equation: 
\[ \frac{(D_{Pav}-D_{Et-1})}{D_{Pav}} \times 100, \text{ where } D \text{ is the diameter upon stabilization, after papaverin administration (Pav), endothelin-1 (ET-1, 10 nM).} \]

Sodium nitroprusside-induced relaxation was calculated according to the following equation: 
\[ \frac{(D_{SNP}-D_{b})}{Index\ VC} \times 100, \text{ where } D \text{ is the diameter upon stabilization, after sodium nitroprusside administration (SNP, 10 µM), baseline (b) and the index of vessel capability (index VC) is provided applying papaverin on endothelin-1-precontracted vessels. No statistical difference was noticeable between genotypes. Data were expressed as Mean ± SEM of 10 animals.} \]

Basal tone diameter values were given in micrometer at different time points of experimental procedure: at the beginning point after 1 hour stabilization (0 min); at the middle point during physiologic saline solution washout (60 min); at the last point after washout PUFA incubation (without L-NNA and indomethacin incubation).

**Online Table II : Linolenic acid injection does not alter the systolic blood pressure and then does not induce a systemic hypotension.**

<table>
<thead>
<tr>
<th>Systolic blood pressure</th>
<th>Baseline</th>
<th>Delay post-ALA-injection (500 nmoles/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREK-1+/+ mice</td>
<td>72.5 ± 2.6</td>
<td>71.9 ± 2.7</td>
</tr>
</tbody>
</table>

Systolic arterial blood pressure (SBP) in response to bolus injection of 500 nmoles/kg linolenic acid (ALA) in TREK-1+/+ mice (n=3). Each time point represents the systolic arterial blood pressure before (baseline) and 5, 15 and 30 minutes following ALA-injection. No statistical difference was noticeable following ALA-injection.

**References**


Online Figure I

Immunohistochemistry depicting negative control of TREK-1 and CD31 in cross sections of the rat basilar artery. Red fluorescence indicates the presence of CD31 protein, while green is the autofluorescence in the TREK-1 negative control, where the primary antibody directed against the TREK-1 channel was omitted. (*) indicates the dense autofluorescence of the internal elastic lamina. Scale bar represents 25 μm and pertains to all panels.
Online Figure II
Immunohistochemistry depicting the endothelium integrity of basilar arteries of TREK-1-/- mice. Green and red fluorescence indicate the presence of von Willebrand factor VIII and CD31 protein respectively, while the blue one is the Hoescht counter-staining. Scale bar represents 10 µm and pertains to all panels.