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# Altered acetylcholine, bradykinin and cutaneous pressure-induced vasodilation in mice lacking the TREK1 potassium channel: the endothelial link

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**The TWIK related K<sup>+</sup> channel TREK1 is an important member of the class of two-pore-domain K<sup>+</sup> channels. It is a background K<sup>+</sup> channel and is regulated by hormones, neurotransmitters, intracellular pH and mechanical stretch. This work shows that TREK1 is present both in mesenteric resistance arteries and in skin microvessels. It is particularly well expressed in endothelial cells. Deletion of TREK1 in mice leads to an important alteration in vasodilation of mesenteric arteries induced by acetylcholine and bradykinin. Iontophoretic delivery of acetylcholine and bradykinin in the skin of TREK1<sup>+/+</sup> and TREK1<sup>-/-</sup> mice also shows the important role of TREK1 in cutaneous endothelium-dependent vasodilation. The vasodilator response to local pressure application is also markedly decreased in TREK1<sup>-/-</sup> mice, mimicking the decreased response to pressure observed in diabetes. Deletion of TREK1 is associated with a marked alteration in the efficacy of the G-protein-coupled receptor-associated cascade producing NO that leads to major endothelial dysfunction.**

Keywords: skin; microcirculation; two-pore-domain K<sup>+</sup> channel  
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## INTRODUCTION

The TWIK related K<sup>+</sup> channel TREK1 belongs to a new class of mammalian K<sup>+</sup> channels with two pore regions (Lesage & Lazdunski, 2000). The TREK1 channel behaves as a background

channel; it is highly regulated by a variety of mediators, including neurotransmitters acting through receptors that are coupled to G<sub>s</sub> and G<sub>q</sub> proteins (Fink *et al*, 1998; Patel *et al*, 1998; Chemin *et al*, 2003, 2005), and is activated by intracellular acidification (Maingret *et al*, 1999). One of the important properties of this channel is that it is mechanosensitive. This channel is present in the vascular system (Gardener *et al*, 2004; Bryan *et al*, 2006) in which its role is not well understood. We thus decided to analyse the role of TREK1 in microvascular function.

The first system that we studied concerns mesenteric arteries. Pressure-induced tone (myogenic tone) is a characteristic of small resistance arteries (Bevan & Laher, 1991; Johnson, 1991; Osol, 1991; D'angelo & Meininger, 1994). It is opposed by flow-induced dilation, which can be observed *in vitro* and *in vivo* (Smiesko & Johnson, 1993; Bevan & Henrion, 1994; Segal, 1994; Davies, 1995). These two mechanical stimuli participate in maintaining a constant basal tone in resistance arteries and allow rapid adaptation to changes in flow and pressure.

The second system that we analysed concerns the control of the cutaneous circulation, particularly the vasodilator response to local pressure application (Fromy *et al*, 1998, 2000; Demiot *et al*, 2006b). The pressure-induced increase in cutaneous blood flow delays the occurrence of tissue ischaemia, thereby protecting the skin against pressure. Subjects deprived of pressure-induced vasodilation (PIV) are exposed to severe ischaemia, which can lead to pressure ulcers. Diabetic patients lack this rapid increase of cutaneous blood flow in response to locally applied pressure (Fromy *et al*, 2002; Koitka *et al*, 2004).

The endothelium has a key role in the control of vascular tone. We have studied *in vitro* and *in vivo* endothelium-mediated vasodilation using acetylcholine and bradykinin, which are the most commonly used agents. These two important effectors of vasodilation produce effects on the vascular endothelium through their specific G-protein-coupled receptors, leading to a sequence of Ca<sup>2+</sup>-dependent events that induces the production of endothelial factors, mainly nitric oxide (NO). NO then diffuses

to underlying vascular smooth muscle cells, where it activates guanylate cyclase to produce cyclic GMP, thus inducing vascular smooth muscle cell relaxation.

The results presented in this paper, from studies using mice with a deletion of the *TREK1* gene, show that *TREK1* is not essential in mesenteric resistance arteries for pressure-induced tone and flow-induced dilation. By contrast, *TREK1* deletion decreases vasodilation in response to acetylcholine and bradykinin. In the cutaneous microcirculation, *TREK1* deletion also markedly decreases PIV as well as vasodilation induced by acetylcholine and bradykinin. All these effects of *TREK1* deletion are interpreted to result from a marked alteration of the NO pathway in endothelial cells.

## RESULTS

### Localization and function of *TREK1* in mesenteric arteries

*TREK1* was localized using a polyclonal antibody that has been used previously for mapping the channel in the central nervous system and in sensory cells of dorsal root ganglions (Maingret *et al*, 2000). Immunohistochemical labelling on mesenteric artery sections (Fig 1A) indicates that *TREK1* expression is distributed throughout the mesenteric wall in both myocytes and endothelial cell layers. The extensive colocalization with antigen CD31 (also called platelet endothelial cell adhesion molecule), a specific marker of endothelial cells in blood vessels (Ilan & Madri, 2003), showed that the *TREK1* channel is located in endothelial cells in blood vessels.

In isolated mesenteric resistance arteries, stepwise increases in intraluminal pressure induced the development of basal (myogenic) tone (Fig 1B). Myogenic tone was not affected significantly by the absence of *TREK1*. Pressure-induced tone was antagonized by flow (shear stress)-induced dilation. Stepwise increases in intraluminal flow induced a progressive dilation (Fig 1C). *TREK1* deletion had no effect on flow-induced dilation of mesenteric arteries (Fig 1C). Contractions associated with anti-adrenergic receptor activation by phenylephrine were identical in mesenteric arteries from *TREK1*<sup>+/+</sup> and *TREK1*<sup>-/-</sup> mice (Fig 1D), indicating an intact contractile function of the smooth muscle. Vasodilation induced by increasing concentrations of the NO donor sodium nitroprusside (SNP) was also essentially identical in *TREK1*<sup>+/+</sup> and *TREK1*<sup>-/-</sup> mice (Fig 1E), indicating that endothelium-independent vasodilation is not affected and also reflecting an intact vasodilator function of the smooth muscle.

We then analysed acetylcholine-induced dilation of mesenteric arteries (Fig 1F). This dilation was strongly attenuated in *TREK1*<sup>-/-</sup> mice. As expected, inhibition of NO synthesis by L-NAME (*N*<sup>G</sup>-nitro-L-arginine methyl ester) decreased acetylcholine-induced vasodilation in arteries from *TREK1*<sup>+/+</sup> mice (Fig 1F), whereas it did not modify acetylcholine-induced vasodilation in arteries from *TREK1*<sup>-/-</sup> mice (Fig 1G), indicating an alteration in the NO pathway. Bradykinin-induced dilation of mesenteric arteries was also strongly attenuated in *TREK1*<sup>-/-</sup> mice (Fig 1H), indicating that an alteration in the NO pathway was not restricted to muscarinic receptor activation and could be ascribed to changes in the transduction pathways coupling the G-protein receptors. By contrast, vasodilation of mesenteric arteries induced by the Ca<sup>2+</sup> ionophore A23187 was not attenuated in *TREK1*<sup>-/-</sup> mice (Fig 1I), indicating that the events subsequent to Ca<sup>2+</sup> increase in endothelial cells are unaltered in *TREK1*<sup>-/-</sup> mice.

### Localization and function of *TREK1* in cutaneous arteries

Immunochemical experiments showed that the *TREK1* channel is expressed in these particular microvessels. Figure 2A indicates that *TREK1* is colocalized with the antigen CD31, a specific marker of endothelial cells (Ilan & Madri, 2003). A particularly strong staining was observed in the blood vessel network surrounding and interconnecting hair follicles. Electron microscopy analysis of *TREK1* distribution by using *in situ* hybridization confirmed the presence of *TREK1* in endothelial cells (Fig 2A).

We then analysed the functional effects of *TREK1* deletion. Basal laser Doppler flow (LDF) was identical in *TREK1*<sup>+/+</sup> mice (152.6 ± 23.4 a.u.) and in *TREK1*<sup>-/-</sup> mice (147.7 ± 15.2 a.u.). By contrast, local pressure-induced vasodilation (PIV) in *TREK1*<sup>+/+</sup> mice was decreased in *TREK1*<sup>-/-</sup> mice (Fig 2B). A statistically significant decrease was observed for locally applied pressures ranging from 0.13 to 0.33 kPa. At 0.13 kPa, the maximal increase for *TREK1*<sup>+/+</sup> mice, PIV was reduced from 40.8 ± 6.3% (*P* < 0.05) for *TREK1*<sup>+/+</sup> mice to 12.6 ± 6.0% for *TREK1*<sup>-/-</sup> mice. How is *TREK1* involved in PIV? Its putative role in cutaneous endothelium-independent vasodilation was assessed using SNP. Basal LDF did not differ between *TREK1*<sup>-/-</sup> mice (53.2 ± 14.6 a.u.) and *TREK1*<sup>+/+</sup> mice (57.2 ± 14.4 a.u.) before SNP delivery, and SNP-induced vasodilation did not differ significantly between the two strains of mice (Fig 2D). This reflects an intact vasodilator function of the smooth muscle in cutaneous microvessels.

The role of *TREK1* in cutaneous endothelium-dependent vasodilation was assessed using iontophoretic deliveries of acetylcholine and bradykinin in the skin. Baseline LDF values did not differ between *TREK1*<sup>+/+</sup> mice (47.8 ± 6.0 a.u.) and *TREK1*<sup>-/-</sup> mice (40.4 ± 3.7 a.u.) before acetylcholine (or bradykinin) delivery. However, although acetylcholine application produced vasodilation in *TREK1*<sup>-/-</sup> mice (34.7 ± 7.8%), this response was strongly attenuated compared with *TREK1*<sup>+/+</sup> mice (72.2% ± 15.5%, *P* < 0.05; Fig 2E), indicating an endothelial dysfunction associated with *TREK1* deletion. Similarly, although bradykinin application produced vasodilation in *TREK1*<sup>-/-</sup> mice (15.8% ± 5.2%), this response was strongly attenuated compared with *TREK1*<sup>+/+</sup> mice (73.9% ± 7.6% *P* < 0.001; Fig 2F).

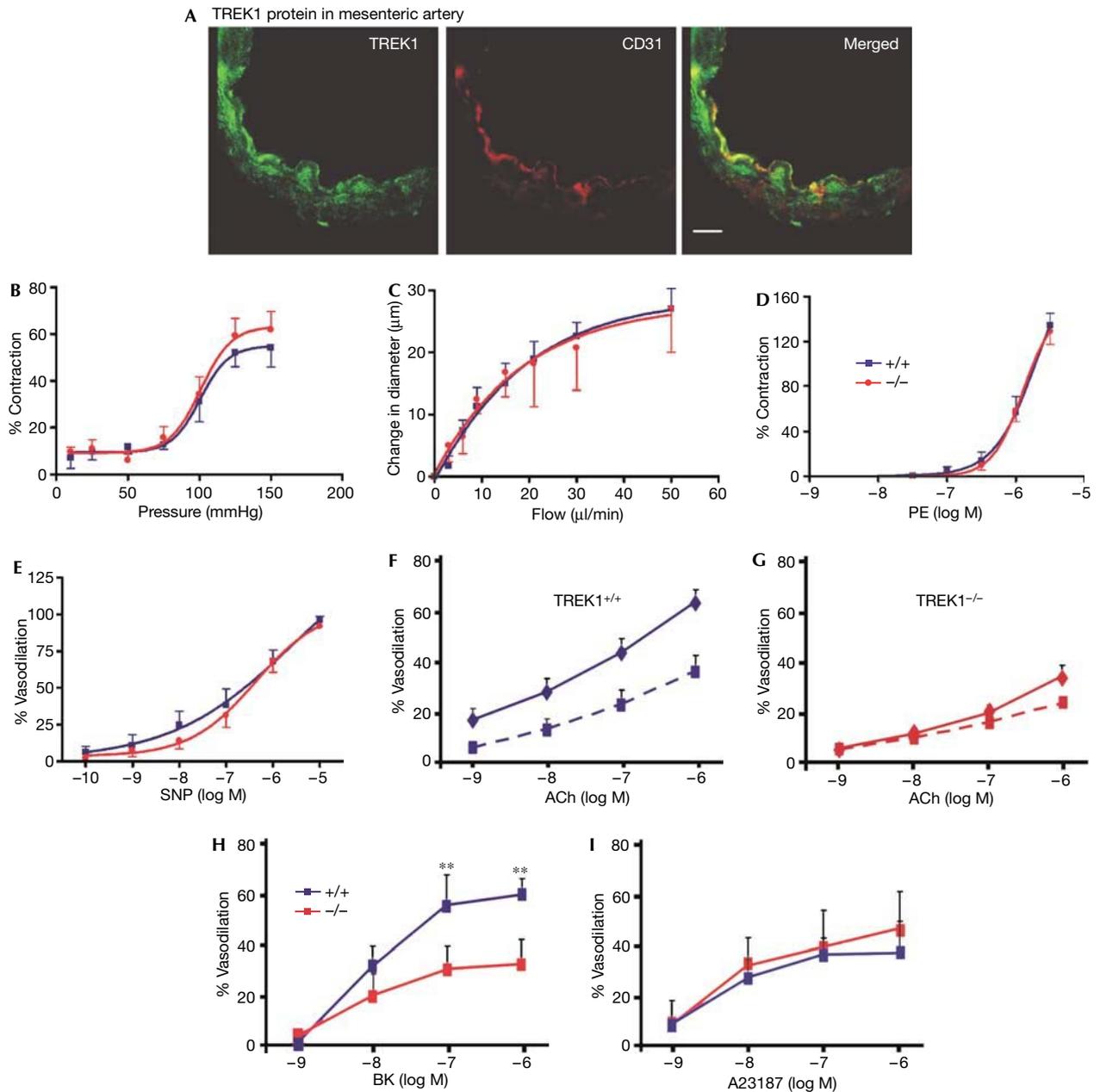
Cutaneous temperature and mean arterial blood pressure remained stable throughout all *in vivo* experiments (PIV and experiments with SNP, acetylcholine and bradykinin iontophoresis).

### Is there a role for *TREK1* in blood pressure response?

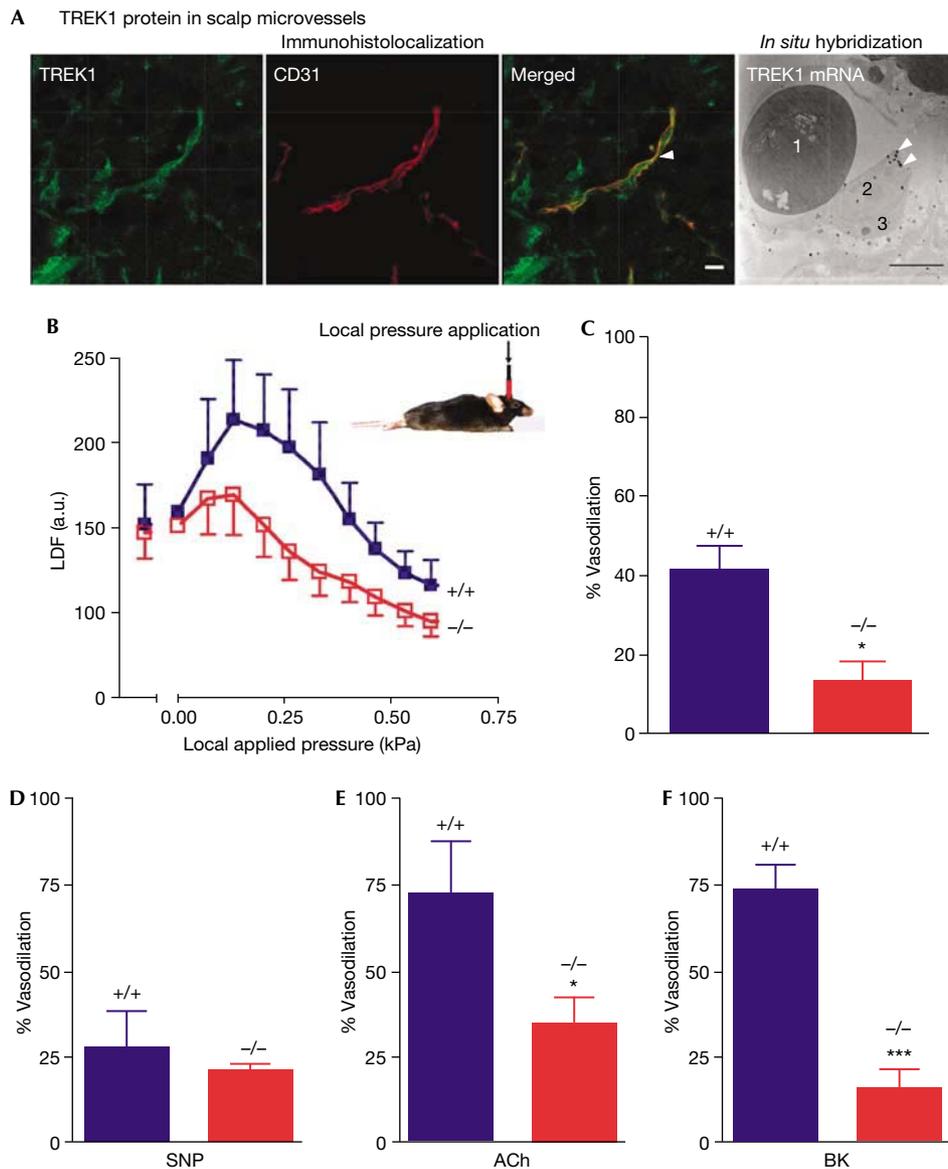
Blood pressure was modified by stress induced by pinching the tail (supplementary Fig 1A online) and by angiotensin II injection (supplementary Fig 1B online). Both treatments increased systolic arterial blood pressure to a similar degree in *TREK1*<sup>-/-</sup> and *TREK1*<sup>+/+</sup> mice. SNP injection decreased systolic blood pressure to a similar degree in *TREK1*<sup>-/-</sup> and *TREK1*<sup>+/+</sup> mice (supplementary Fig 1C online). These results show that the *TREK1* channel is not involved in blood pressure response to different stimuli.

## DISCUSSION

Immunocytochemistry and *in situ* hybridization clearly identify the *TREK1* channel in both mesenteric arteries and in skin microvessels. This particular K<sup>+</sup> channel, which behaves as a background channel and which is activated by mechanical and osmotic stimuli (Patel *et al*, 1998; Maingret *et al*, 1999), as well as by polyunsaturated fatty acids (Fink *et al*, 1998), is particularly well expressed in endothelial cells.



**Fig 1** | Vasodilation and contraction in mesenteric resistance arteries of TREK1<sup>+/+</sup> mice and TREK1<sup>-/-</sup> mice. (A) TREK1 protein localization in mesenteric artery. TREK1 immunoreactivity is shown in green, the endothelial cell marker CD31 in red or both in yellow merged. The double staining indicates that TREK1 channels are localized to endothelial cells in the mesenteric artery. (B) Myogenic tone determined in mesenteric resistance arteries isolated from TREK1<sup>-/-</sup> mice ( $n=10$ ) and TREK1<sup>+/+</sup> mice ( $n=10$ ). (C) Flow-induced dilation in arteries isolated from TREK1<sup>-/-</sup> mice ( $n=7$ ) and TREK1<sup>+/+</sup> mice ( $n=7$ ). (D) Percentage of contraction in response to increasing concentrations of phenylephrine in mesenteric resistance arteries isolated from TREK1<sup>-/-</sup> mice ( $n=10$ ) and TREK1<sup>+/+</sup> mice ( $n=8$ ). (E) Percentage of vasodilation in response to increasing concentrations of SNP in mesenteric resistance arteries isolated from TREK1<sup>-/-</sup> mice ( $n=7$ ) and TREK1<sup>+/+</sup> mice ( $n=6$ ). (F,G) Percentage of vasodilation in response to increasing concentrations of acetylcholine in mesenteric resistance arteries isolated from TREK1<sup>+/+</sup> mice (F,  $n=10$ ) and TREK1<sup>-/-</sup> mice (G,  $n=10$ ), with (dashed line) and without (100  $\mu$ M) L-NAME. (H) Percentage of vasodilation in response to increasing concentrations of bradykinin in mesenteric resistance arteries isolated from TREK1<sup>-/-</sup> mice ( $n=4$ ) and TREK1<sup>+/+</sup> mice ( $n=4$ ). (I) Percentage of vasodilation in response to increasing concentrations of the Ca<sup>2+</sup> ionophore A23187 in mesenteric resistance arteries isolated from TREK1<sup>-/-</sup> mice ( $n=4$ ) and TREK1<sup>+/+</sup> mice ( $n=4$ ). ACh, acetylcholine; BK, bradykinin; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; SNP, sodium nitroprusside.



**Fig 2** | TREK1 protein in scalp microvessels and its role in *in vivo* vasodilation in response to pressure, SNP, acetylcholine and bradykinin. (A) TREK1 localization by immunohistochemistry and *in situ* methods in blood vessels from skull skin. Sections were stained with TREK1 antibody (green), CD31 (red), an endothelial cell marker, or both (yellow; Merged). Scale bar, 10  $\mu$ m. The arrow-head shows that TREK1 channels are localized to endothelial cells in the microcirculation blood vessels. The high magnification of an *in situ*-labelled endothelial cell of a microvessel shows that TREK1 messenger RNA (arrowheads) is localized in the endothelial cell cytoplasm (label 3). The numbers identify an erythrocyte (1), the endothelial cell nucleus (2) and the endothelial cell cytoplasm (3). Scale bar, 5  $\mu$ m. (B) *In vivo* changes in cutaneous blood flow in response to a progressive local pressure applied at 2.2 Pa/s (PIV) in TREK1<sup>-/-</sup> mice ( $n=8$ ) and TREK1<sup>+/+</sup> mice ( $n=8$ ) (a.u., arbitrary units). Inset: representation of the mechanical device used to apply progressive pressure on the skin and simultaneously measure cutaneous blood flow with a laser Doppler probe at the same site. \* $P<0.05$  and \*\* $P<0.01$  versus TREK1<sup>+/+</sup> mice. (C) Maximal percentage of vasodilation in response to a local pressure of 0.13 kPa in TREK1<sup>-/-</sup> mice ( $n=8$ ) and TREK1<sup>+/+</sup> mice ( $n=8$ ). \* $P<0.05$  versus TREK1<sup>+/+</sup> mice. (D) Percentage of vasodilation following iontophoretic delivery of SNP in TREK1<sup>-/-</sup> mice ( $n=8$ ) and TREK1<sup>+/+</sup> mice ( $n=7$ ). (E) Percentage of vasodilation after iontophoretic delivery of acetylcholine in TREK1<sup>-/-</sup> mice ( $n=13$ ) and TREK1<sup>+/+</sup> ( $n=11$ ). \* $P<0.05$  versus TREK1<sup>+/+</sup> mice. (F) Percentage of vasodilation after iontophoretic delivery of bradykinin in TREK1<sup>-/-</sup> mice ( $n=7$ ) and TREK1<sup>+/+</sup> ( $n=7$ ). \*\*\* $P<0.001$  versus TREK1<sup>+/+</sup> mice. ACh, acetylcholine; BK, bradykinin; LDF, laser Doppler flow; PIV, pressure-induced vasodilation; SNP, sodium nitroprusside.

The important effect of TREK1 deletion on microvascular function described in this paper appears to be linked directly to its presence in endothelial cells. Indeed, the absence of TREK1 did

not alter the smooth muscle contractile response to  $\alpha$ -adrenergic stimulation by phenylephrine or smooth muscle vasodilation in response to NO (SNP). The function of the smooth muscle to

contract and relax in TREK1<sup>-/-</sup> mice was confirmed as by a intact response to stimulations that elevate or decrease systolic blood pressure. Conversely, vasodilator responses of mesenteric arteries induced by acetylcholine and bradykinin were strongly attenuated in TREK1<sup>-/-</sup> mice. This suggests that production of endothelial factors, particularly NO, that are responsible for smooth muscle relaxation requires TREK1. This view was confirmed by the fact that inhibition of NO synthesis by L-NAME abolished differences in acetylcholine-induced vasodilation between TREK1<sup>+/+</sup> and TREK1<sup>-/-</sup> mice. The mechanism by which *TREK1* deletion alters endothelial function in response to acetylcholine and bradykinin in both mesenteric arteries and skin microvessels remains to be explained. It might be related to the fact that this channel has a role in the Ca<sup>2+</sup>-dependent process (internal Ca<sup>2+</sup> mobilization and/or Ca<sup>2+</sup> influx in the endothelial cells after agonist binding of G-protein receptors) that is crucial in the activation of endothelial NO synthesis (eNOS) (Feron *et al*, 1998; Ungvari *et al*, 2001; Schneider *et al*, 2003). We observed that the vasodilation response to the Ca<sup>2+</sup> ionophore A23187, which elicits NO release by promoting Ca<sup>2+</sup> influx into the endothelium independent of receptor activation, was not altered in TREK1<sup>-/-</sup> mice. This indicates that *TREK1* deletion does not affect on the cascade of events after Ca<sup>2+</sup> increase in the endothelial cell. The results point to alterations in the NO pathway owing to the absence of TREK1, associated with a step between endothelial G-protein receptor activation by the vasodilator (acetylcholine or bradykinin) and Ca<sup>2+</sup> increase in the endothelial cell. This indicates TREK1, a K<sup>+</sup> channel regulated by effectors acting through G-protein-coupled receptors (Lesage & Lazdunski, 2000; Chemin *et al*, 2003; Murbartian *et al*, 2005), as one of the main targets in signal transduction associated with acetylcholine and bradykinin action in endothelial cells.

Flow-induced dilation is highly dependent on the production of NO by the endothelium (Kuo *et al*, 1992; Hecker *et al*, 1993; Bevan & Henrion, 1994; Segal, 1994; Davies, 1995). However, in this case, the cascade of events that ultimately leads to vasodilation does not involve receptor activation and does not involve a Ca<sup>2+</sup>-dependent pathway for activation of eNOS (Fleming *et al*, 1998; Fisslthaler *et al*, 2000; Fleming & Busse, 2003). Therefore, it is not surprising that *TREK1* deletion does not modify flow-induced dilation. In addition, it makes sense that myogenic tone, which is essentially independent of endothelial factors (Bevan & Laher, 1991; Osol, 1991; D'angelo & Meininger, 1994), is not altered in mesenteric resistance arteries of TREK1<sup>-/-</sup> mice.

Local PIV is a vasodilator response to non-nociceptive externally applied pressure in the skin. This cutaneous vasodilation is caused by a succession of steps: first, the release of mediators, particularly calcitonin-gene-related peptide (CGRP), from afferent sensory nerve endings in response to pressure stimulation; then NO liberation from the endothelium in response to CGRP; and, last, vascular smooth muscle relaxation in response to endothelial NO (Fromy *et al*, 2000). This mechanism was confirmed in TREK1<sup>+/+</sup> mice by the total elimination of PIV after NOS inhibition (N(ω)-nitro-L-arginine, 20 mg/kg, i.p.; data not shown). TREK1 is present in skin microvessels, particularly in endothelial cells. The physiological exploration of mice with a deletion of the *TREK1* gene indicates that they have a markedly reduced vasodilator response to pressure and that TREK1 is important in PIV mainly the endothelial level. This is strongly

suggested by experiments with transcutaneous iontophoretic delivery of acetylcholine and bradykinin to produce vasodilation of cutaneous microvessels. Although TREK1<sup>+/+</sup> and TREK1<sup>-/-</sup> mice respond identically to SNP application, indicating that there is no difference in their response to exogenous NO, they differ in their response to acetylcholine and bradykinin, with a much attenuated response in TREK1<sup>-/-</sup> mice. The simplest interpretation of the PIV data is that the response of vasodilating factors induced by pressure and acting at G-protein-coupled receptors was altered in the absence of TREK1, leading to endothelial dysfunction that markedly reduces NO production.

PIV impairment in TREK1<sup>-/-</sup> mice, as well as in streptozotocin-induced diabetic mice (Sigaudo-Roussel *et al*, 2004) and type I diabetic patients, is associated with endothelial dysfunction. Similarly to the present results in TREK1<sup>-/-</sup> mice, the non-endothelial-mediated response to iontophoretic delivery of SNP is preserved, whereas the endothelial-mediated response to acetylcholine is impaired in diabetic conditions (Koitka *et al*, 2004; Sigaudo-Roussel *et al*, 2004; Demiot *et al*, 2006a,b). In humans, PIV exists at the foot level in healthy subjects but is absent in type I diabetic patients (Koitka *et al*, 2004), suggesting a relationship between PIV absence and the high risk of foot ulcers in these patients. It is interesting to establish, in type I diabetic patients whether there are alterations in a signalling pathway that involves the TREK1 channel. Because elimination of TREK1 leads to a disease state, it might be that the activators of TREK1, such as polyunsaturated fatty acids (Fink *et al*, 1998; Patel *et al*, 1998; Heurteaux *et al*, 2004), facilitate PIV.

## METHODS

**Animals.** TREK1<sup>+/+</sup> and TREK1<sup>-/-</sup> mice were generated as described previously (Heurteaux *et al*, 2004).

**Study of pressure- and flow-dependent tone.** Myogenic tone was determined in increasing pressure by steps from 10 to 150 mmHg (Henrion *et al*, 1997; Matrougui *et al*, 1999). Pressure was then set at 100 mmHg until diameter stabilization. Flow was subsequently increased stepwise and the arterial dilation was measured.

**Pharmacology in isolated arteries.** Mesenteric arterial segments were placed in the chamber of a wire myograph (Danish Myo Technology A/S, Aarhus, Denmark). Passive tension was set to 1 mN and tension was recorded (Biopac, Santa Barbara, CA, USA). Viability of the vessel was assessed by contracting the vessel with 1 μM phenylephrine, followed by relaxation induced by 10 μM acetylcholine. Cumulative concentration–response curves to PE, SNP, acetylcholine (with or without L-NAME, 100 μM), bradykinin and A23187 were constructed.

**Assessment of PIV.** A weighbridge was adapted to hold a laser Doppler probe (PF408, Perimed, Järfälla, Sweden) that had a 7 mm<sup>2</sup> circular contact surface area. This allowed simultaneous local pressure application and cutaneous blood flow measurements at the local pressure application site. Data collection began with a 1-min basal period before the onset of increasing local pressure. Externally applied pressure was then progressively increased at 2.2 Pa/s for 15 min in eight TREK1<sup>-/-</sup> mice and eight TREK1<sup>+/+</sup> mice.

**Assessment of iontophoresis.** Cutaneous blood flow was recorded using a laser Doppler probe (481-1, Perimed) during transcutaneous iontophoresis carried out in the centre of the hairless area of the back. The endothelium-independent response was assessed by using iontophoretic delivery of SNP (2%) with a cathodal current

application of 100  $\mu$ A for 20 s in eight TREK1<sup>-/-</sup> mice and seven TREK1<sup>+/+</sup> mice. The endothelium-dependent responses were assessed by using iontophoretic delivery of acetylcholine (2%) with an anodal current application of 100  $\mu$ A for 10 s in 13 TREK1<sup>-/-</sup> mice and 11 TREK1<sup>+/+</sup> mice and by using iontophoretic delivery of bradykinin (0.5%) with a cathodal current application of 100  $\mu$ A for 90 s in seven TREK1<sup>-/-</sup> mice and seven TREK1<sup>+/+</sup> mice. The iontophoresis technique was chosen to assess *in vivo* skin microvascular function to avoid any systemic effects.

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