Nicotinic Acid Activates the Capsaicin Receptor TRPV1
Potential Mechanism for Cutaneous Flushing

Linlin Ma, Bo Hyun Lee, Rongrong Mao, Anping Cai, Yunfang Jia, Heather Clifton, Saul Schaefer, Lin Xu, Jie Zheng

Objective—Nicotinic acid (also known as niacin or vitamin B3), widely used to treat dyslipidemias, represents an effective and safe means to reduce the risk of mortality from cardiovascular disease. Nonetheless, a substantial fraction of patients discontinue treatment because of a strong side effect of cutaneous vasodilation, commonly termed flushing. In the present study, we tested the hypothesis that nicotinic acid causes flushing partially by activating the capsaicin receptor TRPV1, a polymodal cellular sensor that mediates the flushing response on consumption of spicy food.

Approach and Results—We observed that the nicotinic acid–induced increase in blood flow was substantially reduced in Trpv1–/– knockout mice, indicating involvement of the channel in flushing response. Using exogenously expressed TRPV1, we confirmed that nicotinic acid at submillimolar to millimolar concentrations directly and potently activates TRPV1 from the intracellular side. Binding of nicotinic acid to TRPV1 lowers its activation threshold for heat, causing channel opening at physiological temperatures. The activation of TRPV1 by voltage or ligands (capsaicin and 2-aminoethoxydiphenyl borate) is also potentiated by nicotinic acid. We further demonstrated that nicotinic acid does not compete directly with capsaicin but may activate TRPV1 through the 2-aminoethoxydiphenyl borate activation pathway. Using live-cell fluorescence imaging, we observed that nicotinic acid can quickly enter the cell through a transporter-mediated pathway to activate TRPV1.

Conclusions—Direct activation of TRPV1 by nicotinic acid may lead to cutaneous vasodilation that contributes to flushing, suggesting a potential novel pathway to inhibit flushing and to improve compliance. (Arterioscler Thromb Vasc Biol. 2014;34:1272-1280.)

Key Words: cardiovascular diseases ■ ion channels ■ lipoproteins ■ vasodilation

Nicotinic acid (also commonly called niacin or vitamin B3) is a water-soluble small molecule that is converted in vivo to nicotinamide adenine dinucleotide, a coenzyme involved in the catabolism of fat. As one of the oldest lipid-lowering medications,1 nicotinic acid has been prescribed for >50 years. At a daily dosage of gram quantities, nicotinic acid (but not its derivative nicotinamide) lowers the serum concentrations of total cholesterol and low-density lipoprotein, whereas raises that of high-density lipoprotein, reducing the risk of mortality from cardiovascular disease.2 This beneficial effect is thought to be mediated, in part, by activation of hydroxy-carboxylic acid receptor 2 (HCA2) expressed in adipocytes, causing a drop in the intracellular cAMP level and inhibition of lipolysis.3,4

See accompanying editorial on page 1122

Despite its well-known antidyslipidemic effects, the clinical use of nicotinic acid has been significantly hindered by an unpleasant side effect called flushing, which is characterized by cutaneous vasodilation and symptoms of hot flashes and burning. A dose of 0.05 to 0.1 g of nicotinic acid is sufficient to elicit flushing of the face and upper body, whereas the rest of the body may be affected when higher doses (0.5–1.0 g) are used.6 Occurring in ≤90% of patients, flushing usually lasts for 30 to 90 minutes and is associated with intense erythema, tingling, itching, and elevation in skin temperature. Some patients have more severe skin reactions, such as urticaria, periorbital edema, conjunctivitis, or nasal congestion.6 Flushing was thought to be mediated by nicotinic acid–induced HCA2 activation in Langerhans cells and keratinocytes of the skin. The resulted activation of arrestin β1 and the downstream effector ERK1/2 MAP (extracellular signal-regulated kinase ERK1/2 mitogen-activated protein) kinase7 in turn leads to the activation of cyclooxygenase and release of vasodilatory prostaglandin D2 and E2. The flushing response (but not the antidyslipidemic effects) is subject to tolerance8–10; it markedly decreases after continuous treatment (a property called tachyphylaxis). Nonetheless, up to one third of patients refused to continue treatment mainly because of intolerable flushing.11,12

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To take advantage of the beneficial effects of nicotinic acid fully and to reduce the drop-off rate, a better understanding of the molecular events underlying flushing and potential treatments is of great practical importance. Interestingly, recent studies discovered that pharmacological blockade of cyclooxygenase (by aspirin) and prostaglandin D2 receptor 1 (by laropiprant) does not fully inhibit flushing.13,14 Meanwhile, research in both humans and animal models showed that nicotinic acid–induced flushing is a biphasic process.15,16 These findings, together with the selective tachyphylaxis behavior, indicate that flushing may be mediated by target(s) outside the beneficial HCA2 pathway, raising hope that flushing can be inhibited while preserving the clinical efficacy of nicotinic acid.

Intriguingly, capsaicin (the active compound of spicy chili peppers) also causes flushing symptoms closely resembling that caused by nicotinic acid.17 The capsaicin receptor TRPV1 is a heat-sensing ion channel that responds to many physical and chemical stimuli.18–20 The activation of TRPV1 causes hot and pain sensations and thermoregulatory responses, such as sweating and vasodilation.21 Noticeably, Langerhans cells and keratinocytes, the critical carriers of flushing reaction, respond to nicotinic acid with an increase in intracellular Ca2+22,23 whereas TRPV1 is a nonselective Ca2+-permeable cation channel richly expressed in keratinocytes and Langerhans cells.24,25 Furthermore, repetitive administration of capsaicin results in tachyphylaxis, similar to that seen with nicotinic acid.26 Taken together, these findings point to the possibility that TRPV1 may play a role in nicotinic acid–induced flushing. We were further drawn to this possibility by observations that a TRPV1-specific antagonist, AMG9810 ([E]-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylamide), can selectively inhibit the early phase of nicotinic acid–induced flushing response (Schaefer et al, unpublished data). Hence, in the present study, we tested TRPV1 for nicotinic acid response.

**Material and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Trpv1**−/− Knockout Mice Exhibited Much Reduced Response to Nicotinic Acid

To develop an animal model of nicotinic acid–induced vasodilation, we used laser Doppler perfusion imaging to examine the cutaneous perfusion increase (vasodilation) in mouse. Nicotinic acid (dissolved in physiological saline, pH 7.4) was administered subcutaneously to anesthetized mice at a dosage of 120 mg/kg. Change in the ear blood flow was measured with a laser Doppler flowmeter. As shown in Figure 1, wild-type mice responded to nicotinic acid treatment with a substantial increase in blood flow,27 which is similar to the nicotinic acid–induced flushing in humans.28,29 Noticeably, the nicotinic acid response was substantially reduced in Trpv1−/− mice. In addition, neither wild-type nor knockout mice exhibited detectable response to vehicle treatment. These observations are fully consistent with the hypothesis that TRPV1 serves as a target for nicotinic acid.

**Nicotinic Acid Directly and Potently Activates TRPV1**

The molecular structure of nicotinic acid loosely resembles the head-group of capsaicin (Figure 2A). To test whether nicotinic acid acts as a TRPV1 agonist, we first conducted patch-clamp recordings in either the cell-attached or the whole-cell configuration from mouse TRPV1 (mTRPV1)–expressing HEK293 cells. When applied from the extracellular side, no channel activation was observed acutely (Figure 2B, left). However, when applied to the intracellular side of inside-out patches, nicotinic acid strongly potentiated the channel, eliciting large currents at room temperature (Figure 2B, right). We measured the dose–response relationship for nicotinic acid, which yielded an estimated half maximal effective concentration (EC50) value of 62.34±0.75 mmol/L (n=4; Figure 2C). Because the EC50 value for capsaicin under the same conditions was 157.71±16.87 mmol/L (n=4), nicotinic acid is a much less potent agonist for TRPV1. The Hill coefficient of nicotinic acid response was estimated to be 2.87±0.09 (n=4), suggesting the binding of ≥3 nicotinic acid molecules that promote channel activation with positive cooperativity (Figure 2C).

Although the apparent binding affinity for nicotinic acid is low, we found surprisingly that its efficacy is even higher than capsaicin. At 130 mmol/L, nicotinic acid elicited a current >35% higher than 10 μmol/L capsaicin (a saturating
concentration; Figure 2D and 2E). Given that capsaicin at saturating concentrations activates TRPV1 to an open probability of ≈80%, the observation indicates that the larger current elicited by nicotinic acid could not simply be because of a higher maximum open probability; instead, there has to be an increase in single-channel conductance or both open probability and conductance. Single-channel recordings confirmed that indeed nicotinic acid activates TRPV1 dose dependently, reaching a high open probability at 130 mmol/L (Figure 2F). In addition, although the single-channel conductance at most nicotinic acid concentrations was similar to that of capsaicin-induced currents, at 130 mmol/L, the conductance increased significantly (Figure 2F and 2G). Correction for the change in single-channel conductance slightly shifted the dose–response curve (Figure 2C). Because the concentration required to exhibit a permeation effect was much higher than that for gating, nicotinic acid must bind to a distinct site (or sites, most likely close to the pore) to affect conductance.

In summary, our results confirmed that nicotinic acid dose dependently activates TRPV1. When compared with capsaicin, nicotinic acid exhibits a much lower apparent affinity but higher efficacy because of a combination of gating (higher open probability) and permeation (higher conductance) effects.

Human TRPV1 Exhibits High Nicotinic Acid Sensitivity

TRPV1 channels from different species exhibit distinct properties.20,30,31 To make sure that knockout mice and the mTRPV1 channel are suitable models for the study of nicotinic acid effects in humans, we repeated patch recordings from cells expressing human TRPV1 (hTRPV1) channels. We found that nicotinic acid could also potently activate hTRPV1 from the intracellular side (Figure 3A). Most properties tested, for example, the relative amplitudes between capsaicin and nicotinic acid–induced currents, were similar to those of mTRPV1. To test the sensitivity of hTRPV1 to nicotinic acid, the 0.13 mmol/L concentration (a clinically attainable concentration in the plasma during niacin treatment) was used, whereas the recording temperature was...
shifted the capsaicin dose–response curve, lowering the EC_{50} value for capsaicin by about one-half (from 157.71±16.87 to 81.04±16.4 nmol/L, \( P < 0.05 \); Figure 4B). As expected, this change makes capsaicin a more potent agonist in the presence of nicotinic acid. Interestingly, the Hill coefficient value. In summary, nicotinic acid and capsaicin mutually potentiate each other in activating TRPV1.

### Nicotinic Acid and Capsaicin Activate TRPV1 Synergistically

TRPV1 is a well-known molecular integrator of many chemical and physical stimuli that elicit pain. To understand how nicotinic acid, a new TRPV1 agonist, interacts with the channel, we first investigated the relationship between nicotinic acid and capsaicin. We observed that a combined application of low concentrations of nicotinic acid and capsaicin potentiates TRPV1 to a greater extent than either of them applied independently (Figure 3A). Because we did not observe detectable activity from the mTRPV1 at this concentration even at 37°C (data not shown), it seems that the hTRPV1 is more sensitive to nicotinic acid. However, because hTRPV1 current in inside-out patches inactivated rapidly (Figure 3A), biophysical analyses were performed using the mTRPV1.

![Representative current trace recorded from an inside-out patch exposed to capsaicin (Cap) and NA.](image)

**Figure 3.** Nicotinic acid (NA) activates human TRPV1 (hTRPV1) with higher sensitivity. **A**, Representative current trace recorded from an inside-out patch exposed to capsaicin (Cap) and NA. **B**, Current traces recorded at different temperatures in the presence of 0.13 mmol/L NA. **C**, Heat-dependent activation in the absence and in the presence of 0.13 mmol/L NA. Dotted lines indicate the baseline current and the channel current. Arrows indicate the activation threshold temperature. **D**, Comparison of activation threshold temperatures. n=4 for 0.13 mmol/L NA application and 12 for control condition, respectively. ***P<0.001***.

To test whether nicotinic acid binds to the same binding site for capsaicin, which is located at a pocket formed by S2-S3 linker, S3, S4, S4-S5 linker, S5 and S6,\(^{31,32}\) we used a TRPV1 antagonist, capsazepine (Figure 4D, top). As a capsaicin analog, capsazepine is known to compete for the same binding sites in TRPV1; however, being an antagonist, binding of capsazepine inhibits TRPV1 activity induced by capsaicin with a half maximal inhibitory concentration (IC_{50}) of 420 nmol/L,\(^{33}\) whereas inhibits nicotinic acid–induced TRPV1 currents with an IC_{50} of 2.42±0.66 μmol/L (n=3; Figure 4D, bottom). As expected, we observed that capsazepine at 30 μmol/L (a saturating concentration) almost completely inhibited capsaicin-evoked currents (Figure 4E and 4F, top). However, at this high concentration, capsazepine could only partially block channel activation triggered by 130 mmol/L nicotinic acid (Figure 4E and 4F, bottom). As a control, 20 mmol/L intracellular Ba+ (a pore blocker)\(^{34}\) fully inhibited TRPV1 currents induced by either capsaicin or nicotinic acid (Figure 4E and 4F). These results confirmed that nicotinic acid and capsaicin bind to distinct sites in TRPV1.

### Nicotinic Acid and 2-Aminoethoxydiphenyl Borate Also Activate TRPV1 Synergistically

2-Aminoethoxydiphenyl borate (2-APB; Figure 5A, top) is a common activator for TRPV1, TRPV2, and TRPV3 channels.\(^{35}\) For TRPV1, 2-APB exhibited an EC_{50} value of 157.2±17.9 μmol/L (n=4), making 1 mmol/L a saturating concentration (Figure 5C). We observed that nicotinic acid and 2-APB also exhibited synergistic effects in promoting TRPV1 activation. The current amplitude induced by 180 μmol/L 2-APB was significantly increased in the presence of
Nicotinic Acid Promotes Voltage- and Heat-Dependent TRPV1 Activation

Results described above demonstrated that nicotinic acid both directly activates TRPV1 and facilitates channel activation by other agonists. Because TRPV1 is a polymodal sensor for both chemical and physical stimuli, to understand how nicotinic acid promotes TRPV1 activity under physiological conditions fully, we investigated effects of nicotinic acid on voltage- and heat-dependent channel activation. As shown in Figure 6A, voltage-dependent activation was clearly boosted in the presence of 65 mM nicotinic acid. As a result, there is a significant left-shift of the $G-V$ curve, with half-activation voltage changing from 150±6.5 mV to 70±6.55 mV (Figure 6B). This shift would not only make depolarization more effective in activating TRPV1 but also confer a higher open probability at the resting membrane potential (−20 to −30 mV in keratinocytes; comparing with −60 to −70 mV in neurons). However, it seems that at clinically attainable concentrations, nicotinic acid cannot appreciably activate TRPV1 by shifting voltage-dependent activation toward the resting membrane potential range.

Importantly, we found that nicotinic acid can strongly affect the heat activation of TRPV1, as demonstrated by results shown in Figure 3. Gating of TRPV1 is strongly temperature dependent. The sharply defined temperature activation threshold is characteristic of the channel and can be modulated by factors such as chemical ligands and the phosphorylation state of the channel.20 This plasticity potentially confers a broader range of temperature sensitivity on TRPV1-expressing cells. We observed that, similar to other TRPV1 activators such as capsaicin, proton and Mg2+, nicotinic acid substantially left-shifted the temperature dependence of TRPV1, making it easier to open at lower temperatures.

Figure 4. Nicotinic acid (NA) and capsaicin (Cap) synergistically activate TRPV1 and have different binding sites. A, Representative currents activated by low concentrations of NA and Cap independently or jointly when compared with the fully activated current by a combination of 1 µmol/L Cap and 1 mmol/L 2-aminoethoxydiphenyl borate (2-APB). B, Cap dose–response curves with (green) or without (gray) 65 mM NA. C, NA dose–response curves with (green) or without (gray) 200 nM Cap. Parameters (EC50 and slope factor) for the Hill fits are Cap without NA, 157.71±16.87 nmol/L and 1.23±0.13; Cap with NA, 81.04±16.4 nmol/L and 2.4±0.37; NA without Cap, as in Figure 2D; NA with Cap, 31.04±2.59 nmol/L and 2.2±0.47. n=4 each. D, The molecular structure of capsazepine (CPZ) and dose–response curve of CPZ inhibition of 130 mM NA activated TRPV1 current. E, Representative currents sequentially stimulated by 130 mM NA and Cap, 31.04±2.59 mmol/L and 2.2±0.47. n=4 each. F, Nicotinic Acid Promotes Voltage- and Heat-Dependent TRPV1 Activation
temperatures (Figure 6C and 6D). In the absence of nicotinic acid, the heat activation threshold of TRPV1 was estimated to be 36.57±0.54°C (n=12). With a low concentration of 1.3 mmol/L, nicotinic acid lowered the threshold temperature significantly to 27.92±0.65°C (n=6; P<0.001). Further increasing the nicotinic acid concentration to 13 mmol/L shifted the threshold temperature to the room temperature range (22.47±1.5°C; n=10; P<0.005 compared with 1.3 mmol/L nicotinic acid). Therefore,
at clinically attainable concentrations, nicotinic acid will cause a substantial fraction of TRPV1 channel to be heat activated. Combining this strong sensitization effect on heat activation with potentiation effects on other activators, it is conceivable that nicotinic acid can substantially activate TRPV1 in vivo, leading to the flushing response.

Nicotinic Acid Activates TRPV1 From the Intracellular Side

Our data showed that nicotinic acid only activates TRPV1 from the intracellular side but not from the extracellular side (Figures 2B and 7A). This observation raised an important question, that is, could nicotinic acid from extracellular sources (blood supply) get in the cell to activate TRPV1? To address this question, we used live-cell fluorescence imaging to monitor intracellular pH level on extracellular application of nicotinic acid, which would drop if nicotinic acid enters the cell and acidifies the cytoplasm. We observed that, with increasing concentrations of extracellular nicotinic acid (all titrated to pH 7.4), intracellular pH dropped exponentially over time in a concentration-dependent manner and plateaued at 6.63±0.02 (n=3) with 130 mmol/L nicotinic acid (Figure 7B and 7C). The rate of intracellular pH reduction also increased exponentially when the nicotinic acid concentration was increased, reaching a saturated level of 64.4±6.3 s⁻¹ (n=3) at room temperature when nicotinic acid concentration was higher than 60 mmol/L (Figure 7D). The fact that nicotinic acid translocation saturates at high concentrations points to a transporter-mediated mechanism, which is consistent with previous reports in intestinal epithelia cells and liver cells.

Discussion

Nicotinic acid remains an underused therapy for dyslipidemias and cardiovascular disease because of the strong unwanted niacin flush side effect. Although nicotinic acid binds to HCA2, resulting in catabolism of arachidonic acid and release of prostaglandins, there are data suggesting that nicotinic acid may cause vasodilation by other mechanisms. Results from the present study on knockout animal physiology, electrophysiology, and pharmacology, as well as live-cell fluorescence imaging, collectively demonstrate that nicotinic acid directly and potently activates TRPV1. The concentrations needed to elicit an effect are within the range attainable in patient plasma. Our findings present a likely additional pathway for the niacin flushing response that substantially hinders the highly beneficial nicotinic acid treatment.

As a member of the B family vitamins, nicotinic acid at normal physiological concentrations regulates blood cholesterol and fat, in part, by interacting with HCA2 in adipocytes. Because under clinical settings the blood concentration of nicotinic acid is substantially raised, unintended targets, such as the polymodal cellular sensor TRPV1, are activated. The noticeable low apparent binding affinity of nicotinic acid to TRPV1 is consistent with this view. Indeed, although TRPV1 in nerve terminals under the skin is thought to serve as a primary temperature sensor, it is also abundantly expressed in internal organs where temperature variation is minimal. It is thought that TRPV1 serves its physiological role in these organs as a nociceptor through activation induced by endogenous ligands or extracellular H⁺ (eg, under inflammatory or ischemic conditions). Nicotinic acid seems to be yet another chemical compound that TRPV1 can sense. The flushing response to nicotinic acid is a multicomponent complex biological event that, in blood flow profiles, is presented as a biphasic increase in dermal blood flow. In a mouse model with repetitive capsaicin application-induced tachyphylaxis, acute exposure to nicotinic acid resulted in a much diminished initial flushing response when compared with control, thus significantly blunted the vasodilatory response of nicotinic acid (Schaefer et al, unpublished data, 2014). The observation further underpins the proposed process of TRPV1-mediated flushing response.

Our results further suggest that nicotinic acid activates TRPV1 under physiological conditions predominantly

![Figure 7](http://atvb.ahajournals.org/)

Figure 7. Nicotinic acid (NA) rapidly permeates to the intracellular side. A, No TRPV1 activation by extracellular NA in whole-cell recordings. n=4. B, Representative pH imaging recording with a NA (pH 7.4) concentration ladder applied in sequence, followed by a high potassium solution with nigericin (pH 7.0). Amplitude (C) or time constant (D) of intracellular pH change (pH) is plotted against the NA concentration and fitted to an exponential function. n=3 each.
through a shift of the heat activation threshold. Although the voltage-dependent activation and ligand-dependent activation are also affected, it seems that these processes are not capable of independently bringing the activity of TRPV1 to a level sufficient to produce a physiological response. Nonetheless, it is possible that these processes may contribute to the physiological effects of nicotinic acid through a multiallosteric mechanism.43 Further investigations under more physiological conditions are needed to elucidate the details on how nicotinic acid alters cellular physiology.

The emerging picture from the present study is as follows. When a patient undergoes treatment, the blood nicotinic acid concentration elevates significantly. Nicotinic acid is transported into Langerhans cells and keratinocytes by a transporter yet to be identified. Transporters for nicotinic acid have been functionally identified; however, their molecular identity remains unknown.38–40 As has been previously proposed, the ubiquitous Na+-coupled monocarboxylate transporters and unknown.38–40 As has been previously proposed, the ubiquitous Na+-coupled monocarboxylate transporters and Na+-coupled monocarboxylate transporters are interesting potential candidates missing the TRPV1 channel leads to vasoconstriction,48 supporting H+-coupled monocarboxylate transporters and Na+-coupled monocarboxylate transporters are interesting potential candidates underling nicotinic acid transportation.49,44–46 Monocarboxylate transporters are known to transport L-lactate, pyruvate, the ketone bodies, and many other monocarboxylic substances across the plasma membrane.37 Once entered into the cytosol, nicotinic acid binds to TRPV1 and causes the channel to be heat activated at physiological temperatures. Potentiation of ligand- and voltage-dependent activations may contribute to this process. The activation of TRPV1 leads to Ca2+ influx into Langerhans cells and keratinocytes, causing downstream vasodilation and nerve sensation. Studies with Trpv1+/– knockout mice suggested that missing the TRPV1 channel leads to vasoconstriction,48 supporting the notion that upregulation of TRPV1 activity by nicotinic acid or other means may lead to vasodilation.

Conclusions
Identification of TRPV1 activation as a likely candidate mediating nicotinic acid–induced flushing side effects opens up new ways to improve patient compliance of this beneficial treatment. Indeed, because TRPV1 is a polymodal receptor, its activity can be regulated by numerous physical and chemical methods. The channel thus presents ample opportunities to reduce the flushing response.

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Disclosures
None.

References
Nicotinic acid (also known as niacin or vitamin B3) is widely used for treating dyslipidemias to reduce the risk of mortality from cardiovascular disease. Nonetheless, a substantial fraction of patients discontinue the treatment because of a strong side effect of cutaneous vasodilation, commonly termed flushing. In the present study, we identified the polymodal capsaicin/heat receptor TRPV1 ion channel as a molecular target of nicotinic acid at the clinical dosage. We demonstrated that nicotinic acid directly and strongly activates TRPV1, by interacting with the intracellular side of the channel and lowering the channel’s heat activation threshold. Our observations suggest that TRPV1 is a potential target mediating vasodilation side effect of nicotinic acid, pointing to a novel pathway to inhibit flushing and improve compliance.
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**Materials and Methods**

**Laser Doppler Measurement of Blood Flow from Wildtype and Trpv1\(^{-/-}\) Mice**

Wild-type (WT) and TRPV1-deficient (Trpv1\(^{-/-}\)) C57B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were bred and genotyped at Model Animal Research Center of Nanjing University, China. Adult male mice (aged >15 weeks, weight ~25 g) were used. For blood flow measurements, mice were anesthetized using Nembutal (80 mg/kg) given by I.P. injection 30 min before experiments. Nicotinic acid (130 mM, dissolved in physiological saline, pH 7.4) or vehicle (physiological saline with osmolarity adjusted to the same level as nicotinic acid solution using glucose, pH 7.4) was administered subcutaneously to the abdomen at a dosage of 120 mg/kg. The change in the ear blood flow was measured using a laser Doppler flowmeter (LDF100C, BIOPAC Systems, Inc.) with fiber-optic based probe (TSD146, BIOPAC Systems, Inc.), an approach similar to that taken in previous studies of both human and murine niacin responses\(^1\)-\(^3\). The probe was placed against the dorsal side of the right ear of anesthetized mice to measure the blood flow at 5 min intervals before and after exposure to nicotinic acid or vehicle. Baseline blood flow was established by an average of measurements over 10 min prior to injection of drug or vehicle. Data were analyzed as the percentage change over the baseline blood flow in tissue perfusion units \([(TPU/TPU_{baseline}) \%]\).

**cDNA Constructs and Cell Transfection**

The constructs used in the present study were mouse TRPV1-4 and human TRPV1 cDNAs inserted into the mammalian expression vector pEYFP-N3 \(^4\), which provided an enhanced yellow fluorescence protein (eYFP) tag fused to the C-terminal end of each channel. tsA201 cells were cultured in a DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, at 37°C with 5% CO\(_2\). Cells were passaged 18-24 h before transfection by plating onto glass coverslips coated with 0.1 mg/ml poly-D-lysine to improve cell adhesion and subsequent patch or pH imaging recordings. Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions 24-to-48 h before recording.

**Electrophysiological Recordings**

Macroscopic and single-channel currents were recorded from channel-expressing cells using a HEKA EPC10 amplifier with PatchMaster software (HEKA). Unless stated otherwise, the recordings were done at room temperature using mouse TRPV1 (mTRPV1) with inside-out configuration. Patch pipettes were pulled from thin-wall borosilicate glass and fire-polished to a resistance of ~2 M\(\Omega\). For whole-cell recordings pipettes were pulled from thick-wall borosilicate glass to 3-5 M\(\Omega\). Membrane potential was held at 0 mV and, except for testing voltage-dependence of TRPV1 activation, currents were elicited by a 300-ms step to +80 mV followed by a 200-ms step to -80 mV at 1-s intervals. Data were filtered at 2.25 kHz and sampled at 12.5 kHz. For whole-cell recordings the capacity current was minimized by amplifier circuitry, and the series resistance was compensated by 65-80%. Standard symmetrical bath and pipette solutions
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contained 140 mM NaCl, 0.2 mM EGTA, 10 mM Glucose and 15 mM HEPES (pH 7.2). When desensitization was studied, 2 mM CaCl$_2$ was added whereas EGTA was removed from extracellular solution. Solution switching was achieved with a rapid solution changer RSC-200 (Biological Science Instruments).

**Temperature Control and Monitoring**

Temperature control and monitoring were achieved with the same approach as previously reported$^5$. Briefly, the perfusion solution was heated using an SHM-828 eight-line heater driven by a CL-100 temperature controller (Harvard Apparatus). To obtain a complete temperature-current relationship, in some experiments the solution was first cooled by embedding the perfusion solution reservoir in ice water. A TA-29 miniature bead thermistor (Harvard Apparatus) was placed about 1 mm from the pipette tip to monitor local temperature change. Temperature readout from the thermistor was fed into an analog input port of the HEKA patch-clamp amplifier and recorded simultaneously with channel current. When experimental temperature was not controlled, recordings were conducted at room temperature at ~24°C.

**Fluorescence Imaging of Intracellular pH**

TRPV1-expressing tsA201 cells grown on poly-D-lysine-coated 25 mm glass coverslips were washed twice with HEPES-buffered extracellular solution (ECS) that contained (in mM) 140 NaCl, 5 KCl, 1.0 MgCl$_2$, 1.8 CaCl$_2$, 10 D-glucose, and 15 HEPES (pH 7.4, ~300 mOsm). Cells were initially incubated in ECS containing 5 µM BCECF-AM (Molecular Probes) for 15 min at 37°C and then placed in a closed perfusion-imaging chamber (Warner) mounted on the stage of a Zeiss Axiovert inverted microscope. Before intracellular pH measurement, cells were perfused with ECS at a constant rate of 3 ml/min for >10 min to wash out extracellular BCECF-AM and to allow intracellular hydrolysis process of the AM ester to complete, which converts the non-fluorescent BCECF-AM into the fluorescent BCECF. After measurement of the baseline level of intracellular pH ($pH_i$), cells were subjected to ECS supplemented with different concentration of nicotinic acid (pH 7.4) until an equilibrium state was reached at each concentration. At the end of each experiment, the high-$K^+$-nigericin technique$^6$ was used to calibrate the ratio of fluorescence emissions to the pH level, with a high-$K^+$ medium (pH 7.0) containing (in mM) 135 KCl, 0.6 CaCl$_2$, 1.0 MgCl$_2$, 10.1 KOH, 5.1 D-glucose, 20 HEPES, and 3.2 NMDG-Cl, supplemented with 10 µM nigericin. $pH_i$ was calculated from the intensity ratio of fluorescence lights emitted at 535 nm upon excitation at 490 nm and 440 nm respectively ($F_{490}/F_{440}$). Images were collected every 10 s using an ICCD camera system (Stanford Photonics) and analyzed with the OpenLab image processing software. For each experiment, 20-30 cells were monitored per coverslip. All experiments were performed at room temperature.

**Data Analysis**

The dose-response relationship was quantified from macroscopic currents and fitted with the Hill equation:
\[
1 - \frac{I_{\text{min}}}{I_{\text{max}}} = \frac{[x]^n}{EC_{50}^n + [x]^n}
\] (1)

Where \(I\) and \(I_{\text{max}}\) are the steady-state currents in the presence of an agonist at concentration \([x]\) and saturating concentration, respectively, \(I_{\text{min}}\) is the leak current or, in the case of dual-agonist experiments, the total current elicited in the presence of the first agonist, \(EC_{50}\) is the agonist concentration at which activation is half-maximal, and \(n\) is the Hill coefficient.

\(G-V\) curves were fitted to a single-Boltzmann function:

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + e^{(V_{\text{half}} - V)/k}}
\] (2)

Where \(G/G_{\text{max}}\) is the normalized conductance, \(V_{\text{half}}\) is the half-activation voltage, \(k\) is the slope factor defined as \(RT/qF\), where \(q\) is the equivalent gating charge, \(T\) is temperature, \(R\) is the gas constant, and \(F\) is the Faraday’s constant.

Activation threshold temperature was determined from the raising phase of the current-temperature relationship recorded from cells expressing TRPV1 channels. The raising phase exhibited two temperature-dependent phases, a less temperature-dependent phase at lower temperatures (reflecting heat-dependent increase in background current) followed by a higher temperature-dependent phase at higher temperatures (reflecting heat-dependent activation of TRPV1). Each phase was fitted to a linear function. The temperature at the intersection of the two lines was defined as the activation threshold temperature (\(T_{\text{takeoff}}\)).

All values are given as mean ± SEM for the number of measurements (\(n\)). Statistical significance was determined using the Student’s \(t\) test or mixed-model ANOVA.

