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Beneficial effect of capsaicin via TRPV4/EDH signals on mesenteric arterioles of normal and colitis mice

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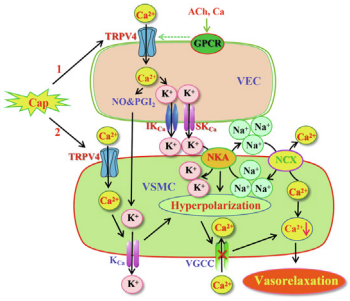
HIGHLIGHTS

- Capsaicin induced vasorelaxation of human colonic submucosal arterioles *in vitro* and *in vitro*.
- Capsaicin induced an endothelium-dependent vasorelaxation of human submucosal arterioles.
- Capsaicin induced an endothelium-dependent vasorelaxation of mouse mesenteric arterioles.
- Capsaicin induced vasorelaxation minily by TRPV1-mediated endothelial nitric oxide release.
- Capsaicin induced vasorelaxation mainly by TRPV4/endothelium-dependent hyperpolarization.
- Capsaicin exerted anti-colitis action in wide-type mice, but not in TRPV4 knock-out mice.
- Capsaicin rescued the impaired endothelium-dependent vasorelaxation via TRPV4/EDH pathway.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Although capsaicin has long been used as food additive and medication worldwide, its actions on gastrointestinal tract as its most delivery pathway have not been well addressed.
Objectives: In the present study, we aimed to study GI actions of capsaicin on mesenteric arterioles in normal and colitis mice and to elucidate the underlying mechanisms.
Methods: Vasorelaxation of human submucosal arterioles and the mesenteric arterioles from wide-type (WT) mice, TRPV1$^{-/-}$ and TRPV4$^{-/-}$ (KO) mice were measured. The expression and function of TRPV channels in endothelial cells were examined by q-PCR, immunostaining, Ca^{2+} imaging and membrane potential measurements.
Results: Capsaicin dose-dependently induced vasorelaxation of human submucosal arterioles and mouse mesenteric arterioles *in vitro* and *in vivo* through endothelium-dependent hyperpolarization (EDH), nitric

NE, norepinephrine; AUC, area under curve; Rmax, max of relaxation; EC_{50}, 50% maximal effect; CRC, cumulative concentration-response curve.

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oxide (NO), and prostacyclin (PGI_2). Using TRPV1 and TRPV4 KO mice, we found that capsaicin-induced vasorelaxation was predominately through TRPV4/EDH, but marginally through TRPV1/NO/PGI_2. Capsaicin induced hyperpolarization through activation of endothelial TRPV4 channels and intermediate-conductance of Ca^{2+}-activated K^+ channels to finally stimulate vasorelaxation. Importantly, capsaicin exerted anti-colitis action by rescuing the impaired ACh-induced vasorelaxation in WT colitis mice but not in TRPV4 KO colitis mice.

Conclusions: Capsaicin increases intestinal mucosal blood perfusion to potentially prevent/treat colitis through a novel TRPV4/EDH-dependent vasorelaxation of submucosal arterioles in health and colitis. This study further supports our previous notion that TRPV4/EDH in mesenteric circulation plays a critical role in the pathogenesis of colitis.

Introduction

Vascular endothelium is critical not only for maintaining the structural integrity of blood vessels but also for regulating their functional activities. It has long been known that endothelial cells generate three endothelium-derived relaxing factors: nitric oxide (NO), endothelium-dependent hyperpolarization (EDH), and prostacyclin (PGI_2) [1,2]. NO is dominant in regulating conduit arterial tone (like aorta), but EDH is dominant in regulating resistant arterial tone (like mesenteric arterioles), highlighting the essential role of EDH in resistant vessel physiology [3]. However, when NO generation is reduced in endothelial dysfunction in some chronic diseases, such as hypertension and diabetes, EDH signals may be stimulated to serve as a compensatory mechanism that maintains the endothelium-dependent vasodilator tone of resistance vessels, implying its therapeutic potential for some vascular disease [4]. However, it is totally unknown about potential contribution of EDH in mesenteric circulation to digestive disease.

Chili pepper has long been used as flavoring as well as medication worldwide since 7000BC [5]. The numerous beneficial roles of capsaicin have been established for prevent/treatment of several human disorders, including pain, inflammation, cancer [6], and haematopoietic disorders [7]. As a food additive, dietary capsaicin is quickly absorbed into the circulation system by the stomach and the small intestine with a great efficiency [8]. After capsaicin is absorbed into the mesenteric vascular system, it initiates several gastrointestinal (GI) actions, including stimulation of intestinal mucosal nerve and mesenteric blood flow rate [9]. However, in comparison with the effects of capsaicin on the outside of GI tract [10], its exact actions within GI tract and vasculature have not been fully understood.

Numerous studies revealed that capsaicin has pleiotropic actions in mammalian cells, including the transient receptor potential vanilloid 1 (TRPV1)-dependent and -independent actions. Capsaicin can activate TRPV1 channels highly expressed in peripheral nerve terminals and in non-neuronal cells [11,12], but it can also act in a TRPV1-independent manner, such as nuclear factor-κB (NF-κB) inactivation and suppression on the inflammatory responses [13]. Little is known about the TRPV1-independent actions of capsaicin in digestive system. Although capsaicin was reported to improve endothelial TRPV1/NO-dependent vasorelaxation to prevent hypertension [14], it has not been explored if capsaicin could induce vasorelaxation via EDH to prevent digestive disease. Furthermore, TRPV4 channel, another important member of TRPV subfamily, is predominantly expressed in endothelial cells [15]. TRPV4 channel plays a role in regulating vasorelaxation through endothelial cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$)-mediated EDH [16,17], but it is currently unknown if capsaicin could modulate resistance vascular function via the TRPV4/EDH signals.

Inflammatory bowel disease (IBD) includes ulcerative colitis and Crohn's disease, and its hallmarks are immune cell infiltration and activation in the mucosa leading to inflammation and ulceration of the intestinal wall [18]. There is no effective therapy for IBD so far due to the lack of in-depth understanding of its pathogenesis. Although numerous studies on IBD focused on immune cell infiltration and compromised intestinal epithelial barrier function [19,20], little is known about the involvement of mesenteric circulation in IBD. It was reported that intestinal submucosal arterioles from colitis patients did not dilate in response to acetylcholine, and that blood flow to chronically inflamed regions of gut in colitis patients was reduced [21,22]. In addition, a comparative study of human healthy subjects and the patients with IBD revealed that blood flow was decreased in the affected intestinal lesions in IBD [23]. The patients with chronic intestinal inflammation exhibit microvascular abnormalities, such as vasculitis characterized by decreased intestinal perfusion and limited oxygen supply [24]. Therefore, emerging evidence supports the critical role of mesenteric circulation in the pathogenesis and progression of IBD. We previously reported that endothelial TRPV/EDH signals in mesenteric circulation may be critical in colitis [25]; however, this notion and underlying mechanisms need further investigation.

Since capsaicin has long been used as daily flavoring worldwide and is orally delivered to GI tract, it is therefore important to investigate the novel action of capsaicin on the mesenteric circulation in GI health and disease. Particularly, the effects of capsaicin on colitis and the underlying mechanisms need to be verified. Although clinical epidemiological studies indicate that the incidence rate of IBD (including ulcerative colitis and Crohn's disease) is much lower in the high chili consuming regions in China (such as Chengdu and Xian) compared to the light/no chili consuming regions (such as Guangzhou and Hong Kong) [26,27], capsaicin was reported to alleviate [28,29], exacerbate [30], or not affect the severity of colitis in mice. Moreover, it has not been explored if capsaicin could affect mesenteric circulation during colitis; and if so, what the underlying mechanisms are. Surprisingly, we found in the present study that capsaicin could ameliorate colitis by acting on submucosal arterioles via novel TRPV4/EDH signals.

Materials and methods

Isometric tension recordings of human colonic submucosal arterioles and mouse mesenteric arterioles

The human submucosal arterioles and the second-order branch of mouse mesenteric arterioles with 2 mm length were mounted in a Mulvany-style wire myograph (Model 520A, DMT, Aarhus, Denmark) for functional assessment. Please see supplementary materials.

The perfused vessel density (PVD) measurements of mouse mesenteric arterioles in vivo

PVD was measured by a LH-SDF-1 side-stream dark field imager (Lihua Electronic Technology Co., Ltd., Xuzhou, China) controlled by AVA 3.0 software. Please see supplementary materials.

DSS-induced colitis of mouse model

The mice were administered drinking water with 2.5% DSS or a combination of 2.5% DSS plus capsaicin (10 mg/kg/day, po) for 7 days. Please see supplementary materials.

Measurements of $[Ca^{2+}]_{cyt}$ and membrane potential in HUVEC

$[Ca^{2+}]_{cyt}$ and membrane potential in HUVEC were measured with calcium-sensitive fluorescent dye Fura-2/AM and voltage-sensitive fluorescent dye DiBAC4(3). Please see supplementary materials.

Quantitative PCR analysis on TRPV iso format

The total RNA from the HUVEC in Trizol reagent was determined by StepOnePlus (ThermoFisher Scientific, USA). Please see supplementary materials.

Ethics statement

The human colon samples of patients were obtained according to the full consent informed in the study. The study of clinical samples was under the approvement of the Clinical Research Ethics Committee of the Army Medical University (AMU, Chongqing, China, AMUWEC2020368).

Results

Capsaicin induction of human colonic submucosal arterioles via EDH predominantly

Human submucosal arterioles play an important role in controlling blood flow perfusion in intestinal mucosal circulation to maintain barrier function in healthy subjects [31]. First, we examined the direct effect of capsaicin (1–10 μM) on the basal tone of human colonic submucosal arterioles, but it did not (n = 6, data not shown). Second, we examined if capsaicin induced vasorelaxation. Capsaicin induced a dose-dependent vasorelaxation of the arterioles pre-constricted by NE, but barely induced a vasorelaxation of the arterioles pre-constricted by high K^+. There were significant differences in capsaicin-induced CRC, R_{max}, AUC, and EC_{50} between the arterioles pre-constricted by NE or high K^+ (Fig. 1A), indicating capsaicin induced much greater vasorelaxation of the arterioles pre-constricted by NE than by high K^+. Third, to test if capsaicin depended on endothelium to induce vasorelaxation, we compared capsaicin-induced vasorelaxation of endothelium-intact with endothelium-denuded arterioles. Without endothelium arterioles that was verified by the loss of CCh-induced vasorelaxation, capsaicin induced marginal vasorelaxation (Fig. 1A), indicating capsaicin predominantly depended on endothelium to induce vasorelaxation.

Fourth, we elucidated the underlying mechanisms of capsaicin-induced vasorelaxation of human submucosal arterioles. When either N^{ω}-nitro-L-arginine (L-NNA, 100 μM) or indomethacin (INDO, 10 μM) was applied to inhibit NO and PGI_2, respectively, capsaicin-induced vasorelaxation was attenuated by each of them (Fig. 1B). The inhibitory effect of either L-NNA or INDO on capsaicin-induced CRC, R_{max}, AUC, and EC_{50} suggests that capsaicin induced vasorelaxation through NO and PGI_2. Furthermore, when L-NNA and INDO were combined to eliminate both NO and PGI_2, about 80% of capsaicin-induced vasorelaxation was remained (Fig. 1B). This remaining component in the presence of L-NNA and INDO was further attenuated by TRAM-34 and apamin which selectively blocker IK_{Ca} and SK_{Ca} (Fig. 1C); but potentiated by a selective IK_{Ca} and SK_{Ca} channel activator (Fig. 1C), SKA-31 which can promote EDH-mediated vasorelaxation [32]. Therefore, capsaicin induced endothelium-dependent vasorelaxation of human submucosal arterioles through EDH predominantly but NO and PGI_2 marginally.

The second-order branch of mouse mesenteric arterioles represents human colonic submucosal arterioles in capsaicin-induced endothelium-dependent vasorelaxation

Since we previously verified that the last branch of human colonic mesenteric arterioles and the second-order branch of mouse superior mesenteric arterioles shared similar characterization in glucose-induced endothelium-dependent vasorelaxation [25], we examined if human colonic submucosal arterioles and the second-order branch of mouse superior mesenteric arterioles also have similar characterization in capsaicin-induced vasorelaxation. Indeed, first, capsaicin did not affect basal tone of mouse mesenteric arterioles (n = 6, data not shown). Second, capsaicin induced a marked vasorelaxation in the arterioles pre-constricted by NE, but not in those pre-constricted by high K^+ (Fig. 2A&B), indicating that capsaicin induces much greater vasorelaxation of mouse arterioles pre-constricted by NE than by high K^+. Third, capsaicin-induced vasorelaxation was attenuated by either L-NNA or INDO (Fig. 2C). Finally, when L-NNA and INDO eliminated NO and PGI_2, the remaining component of capsaicin-induced vasorelaxation was attenuated either by TEA or by apamin plus TRAM-34; but potentiated by SKA-31 (Fig. 2D). Since the second-order branch of mouse mesenteric arterioles were verified to truly mirror human colonic submucosal arterioles in capsaicin-induced endothelium-dependent vasorelaxation, the former was used to replace the latter in all following experiments unless otherwise stated because human submucosal arterioles are valuable but they are very difficult to be obtained.

Capsaicin induction of vasorelaxation largely in a TRPV1-independent manner

Since capsaicin is a well-known activator of TRPV1 channel, TRPV1 knockout (KO) and wild-type (WT) mice were used to compare capsaicin-induced vasorelaxation of mesenteric arterioles. In the presence or the absence of L-NNA alone or L-NNA plus INDO, capsaicin-induced CRC, R_{max}, AUC, and EC_{50} of mesenteric arterioles were altered similarly in WT mice (Fig. 3A). However, capsaicin-induced vasorelaxation was remained largely in TRPV1 KO mice but differently altered by same treatments (compare Fig. 3A and B), suggesting that although capsaicin/TRPV1 induces vasorelaxation via NO and PGI_2, they may not play a major role.

We further compared the capsaicin-induced vasorelaxation between WT and TRPV1 KO mice. Capsaicin-induced CRC, R_{max}, AUC, and EC_{50} were marginally altered in TRPV1 KO compared to those in WT mice (Fig. 3C). The capsaicin-induced vasorelaxation was similar between TRPV1 KO and WT mice in the presence of L-NNA (Fig. 3D), but was significantly altered in the presence of L-NNA plus INDO (Fig. 3E), suggesting that capsaicin/TRPV1-induced vasorelaxation via NO and PGI_2 unlikely plays a major role, but other TRPV1-independent mechanisms via non-NO and non–PGI_2 may be involved.

Capsaicin was previously reported to stimulate CGRP release from perivascular sensory nerve terminals in the first-order branch of mouse mesenteric artery [33]. As shown in Suppl Fig. 1 A&B, capsaicin-induced vasorelaxation of the first-order branch was significantly attenuated by the CGRP receptor antagonist CGRP8-37 (3 μM) alone, and further attenuated by a combination of CGRP8-37 and L-NNA plus INDO. However, surprisingly, capsaicin-induced vasorelaxation of the second-order branch was not

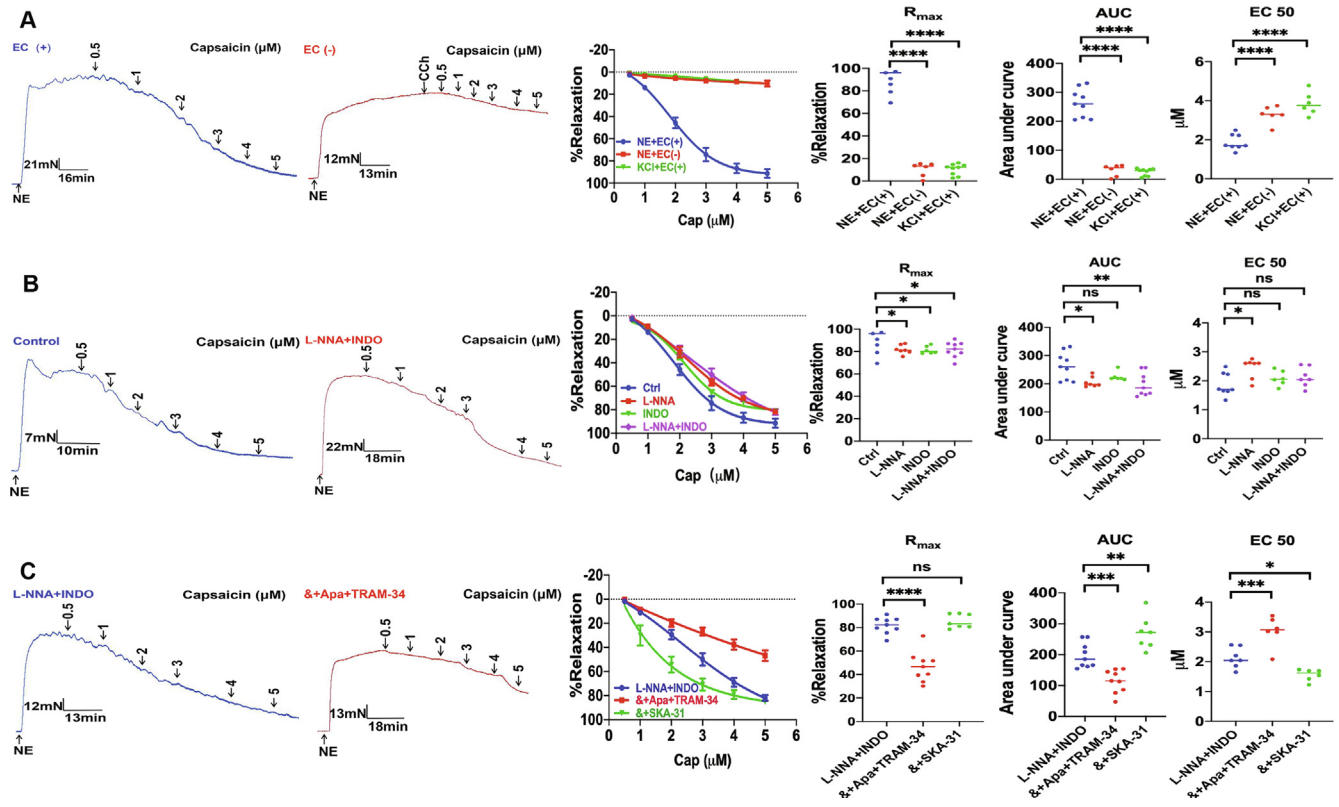

Fig. 1. Capsaicin-induced vasorelaxation of human colonic submucosal arterioles via EDH predominately. (A) Representative tracings and generalize data showing capsaicin (Cap)-mediated dose-dependent vasorelaxation of human submucosal arterioles with endothelium-intact (EC+, n = 9) or endothelium-denuded (EC-, n = 6) pretreated with noradrenalin (NE) or high K^+ (KCl, n = 9) in the CRC, R_{max}, AUC and EC_{50}. **(B)** Representative tracings and generalize data showing capsaicin-mediated vasorelaxation of the arterioles without (control, n = 9) or with 100 μM L-NNA (n = 7), 10 μM INDO (n = 6), or L-NNA plus INDO (n = 9) in the CRC, R_{max}, AUC and EC_{50}. **(C)** Representative tracings and generalize data showing capsaicin-mediated vasorelaxation of the arterioles with L-NNA + INDO (n = 9), L-NNA + INDO (&) + 0.3 μM SKA-31 (n = 7), or L-NNA + INDO (&) + 3 μM apamin + 30 μM TRAM-34 (n = 9) in the CRC, R_{max}, AUC and EC_{50}. Data are represented as percentage of NE (5 μM)- or KCl (80 mM)-mediated contractions and expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns: no significance.

affected by CGRP8-37 (3 μM) alone, but attenuated by a combination of CGRP8-37 and L-NNA plus INDO (Suppl Fig. 2A&B). Therefore, CGRP plays a role in capsaicin-induced vasorelaxation of the first-order branch of mesenteric artery but not the second-order branch.

Since capsaicin is widely-used reagent for denervation which could affect vascular activity, we applied capsaicin (10 μM) pretreatment for denervation to examine this possibility [34]. As shown in Suppl Fig. 3A&B, the denervation significantly attenuated the capsaicin-induced vasorelaxation of the first-order branch of mesenteric arteriole but not the second-order branch. This result is consistent with our data shown in Suppl Fig. 1 A&B and a previous report [33] that capsaicin induced CGRP release in the first-order branch of mesenteric arterioles to cause vasorelaxation rather than the second-order branch. Taken together, our data suggest a regional difference of mesenteric arterioles in response to capsaicin: that is, perivascular sensory nerve terminals and CGRP play a role in capsaicin-induced vasorelaxation of the first-order branch of mesenteric arterioles but not the second-order branch.

Capsaicin induction of vasorelaxation predominately in a TRPV4-dependent manner

Besides TRPV1, other TRPV family members TRPV3, TRPV4 and TRPV6 have been reported to be expressed in vascular endothelial cells [35]. We first compared the mRNA expression levels of TRPV family members in HUVEC by q-PCR. Suppl Fig. 4A shows the TRPV expression levels in HUVEC with the following orders: TRPV4≫TRPV1≫TRPV3 = TRPV6, in which TRPV4 was almost 10-fold higher than TRPV1 and 100-fold higher than TRPV3 and TRPV6. Second, carvacrol (10 μM), a selective TRPV3 activator [36], neither altered endothelium-dependent vasorelaxation nor affected capsaicin-induced vasorelaxation (Suppl Fig. 4B&C). Third, ruthenium red (10 μM) and 2-APB (70 μM), two commonly used inhibitors of TRPV6 channels [37,38], did not influence capsaicin-induced vasorelaxation (Suppl Fig. 4D). Fourth, since capsaicin at high concentration (EC_{50} of 48 μM) can activate cystic fibrosis transmembrane conductance regulator (CFTR) also expressed on endothelium [39], we examined its possible involvement. However, CFTRinh-172 (3 μM), a selective CFTR inhibitor, did not influence capsaicin-induced vasorelaxation (n = 3, data not shown). Taken together, these data exclude the involvements of TRPV3, TRPV6 and CFTR channels in the capsaicin-induced vasorelaxation.

Since TRPV4 channel is the highest TRPV family member expressed in endothelial cells and to play a critical role in endothelium-dependent vasorelaxation [35], we examined if it is involved in capsaicin-induced relaxation. In the first set experiments, we performed immunofluorescence to confirm the protein expression of TRPV1 and TRPV4 channels in HUVEC. Both TRPV1 and TRPV4 proteins were predominantly localized on the plasma membrane, but also in the cytosol of HUVEC (Suppl Fig. 5A, B&C); however, the immunofluorescence staining was not observed without the primary antibodies against TRPV1 and TRPV4 in control, indicating specific staining on these proteins in HUVEC. To further investigate if capsaicin acts on TRPV channels in endothelial cells, we determined the function of TRPV1 and TRPV4 channels in HUVEC. GSK1016790A, a potent activator of TRPV4 channel, significantly stimulated $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-

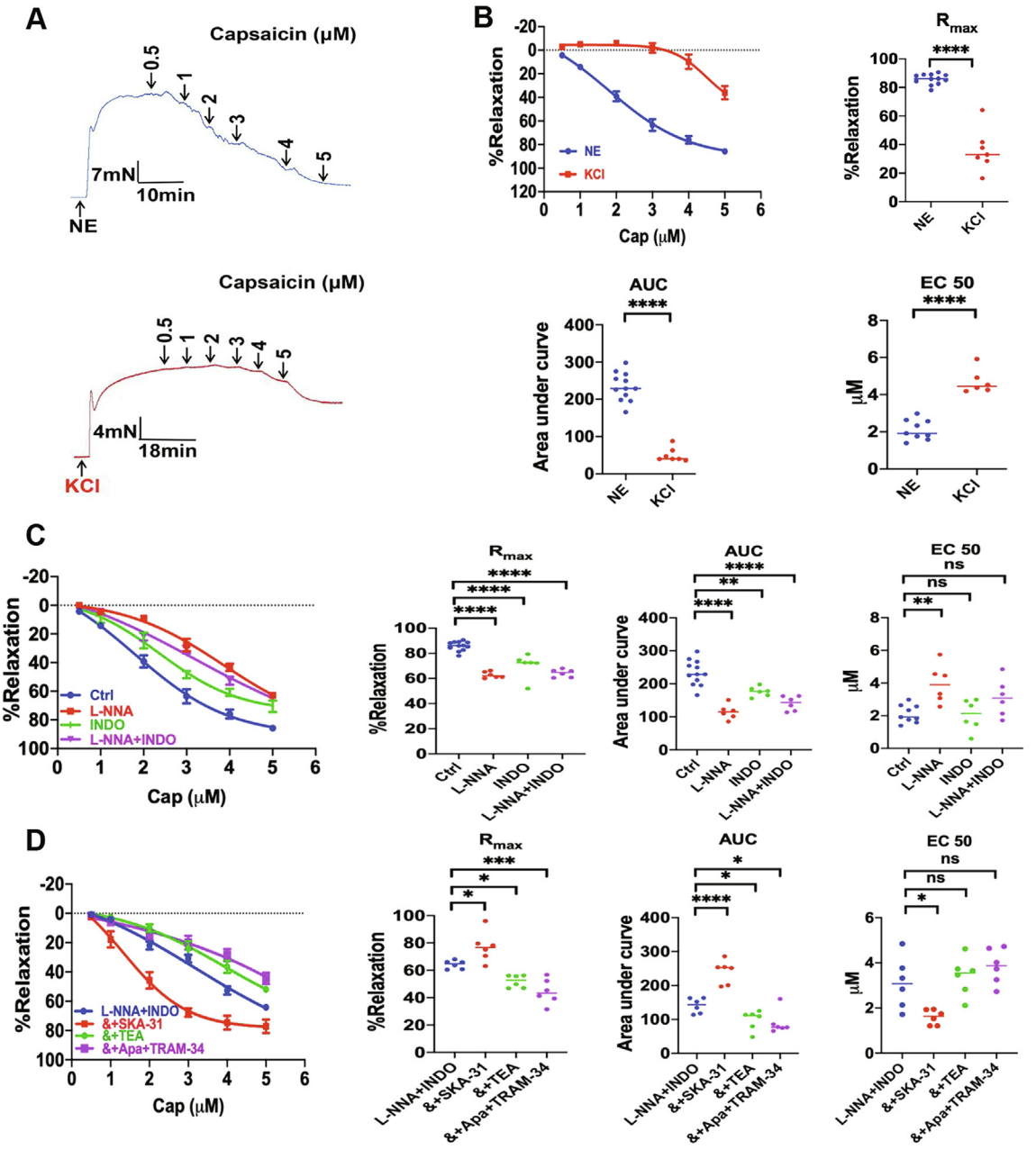

Fig. 2. The second-order branch of mouse mesenteric arterioles mirrors human submucosal arterioles in capsaicin-induced endothelium-dependent vasorelaxation. **(A)** Representative tracings of capsaicin (Cap)-mediated vasorelaxation of mouse mesenteric arterioles in a dose-dependent manner pretreated with noradrenalin (NE, the upper) or high potassium (KCl, the lower). **(B)** Generalize data showing capsaicin-mediated vasorelaxation of mesenteric arterioles pretreated with NE (n = 12) or KCl (n = 7) in the CRC, R_{max}, AUC and EC_{50}. **(C)** Generalize data showing capsaicin-mediated vasorelaxation of mesenteric arterioles pretreated with NE without (control, n = 12) or with either 100 μM L-NNA (n = 6), or 10 μM INDO (n = 6), or L-NNA plus INDO (n = 6) in the CRC, R_{max}, AUC and EC_{50}. **(D)** Generalize data showing capsaicin-mediated vasorelaxation with L-NNA + INDO (n = 6), L-NNA + INDO (&) + 0.3 μM SKA-31(n = 6), L-NNA + INDO (&) + 10 mM TEA (n = 6), or L-NNA + INDO (&) + 3 μM apamin + 30 μM TRAM-34 (n = 6) in the CRC, R_{max}, AUC and EC_{50}. Data are represented as percentage of NE (5 μM)- or KCl (80 mM)-mediated contractions and expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns: no significance.

containing solutions (Suppl Fig. 6A&D), but not in Ca^{2+}-free solutions, in which calcium still induced $[Ca^{2+}]_{cyt}$ signaling as a positive control (Suppl Fig. 6B&D). Moreover, GSK1016790A-induced $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-containing solutions could be abolished by HC-067047, a selective TRPV4 blocker that did not alter calcium-induced $[Ca^{2+}]_{cyt}$ signaling (Suppl Fig. 6C&D).

In the second set experiments, we examined the roles of TRPV channels in capsaicin-induced Ca^{2+} influx. Capsaicin stimulated marked $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-containing solutions (Suppl Fig. 6E), which could be significantly attenuated either by AMG-517 (Suppl Fig. 6F), a selective TRPV1 channel blocker, or by HC-067047 (Suppl Fig. 6G), a selective TRPV4 channel blocker. Moreover, capsaicin-induced $[Ca^{2+}]_{cyt}$ signaling was further inhibited by a combination of two TRPV1 and TRPV4 channel blockers (Suppl Fig. 6H), suggesting capsaicin activation of both channels in HUVEC (Suppl Fig. 6I). We revealed that capsaicin dose-dependently potentiated GSK1016790A-induced $[Ca^{2+}]_{cyt}$ signaling in HUVEC (Suppl Fig. 6J, K, L). Therefore, capsaicin indeed stimulates Ca^{2+} influx through TRPV4 channels in endothelial cells. Taken together, capsaicin can regulate TRPV4 channels to increase $[Ca^{2+}]_{cyt}$ signaling in HUVEC.

In the third set experiments, we further tested the function of capsaicin/TRPV4 on mesenteric arterioles. Indeed, capsaicin-induced CRC, R_{max}, AUC, and EC_{50} of mesenteric arterioles from

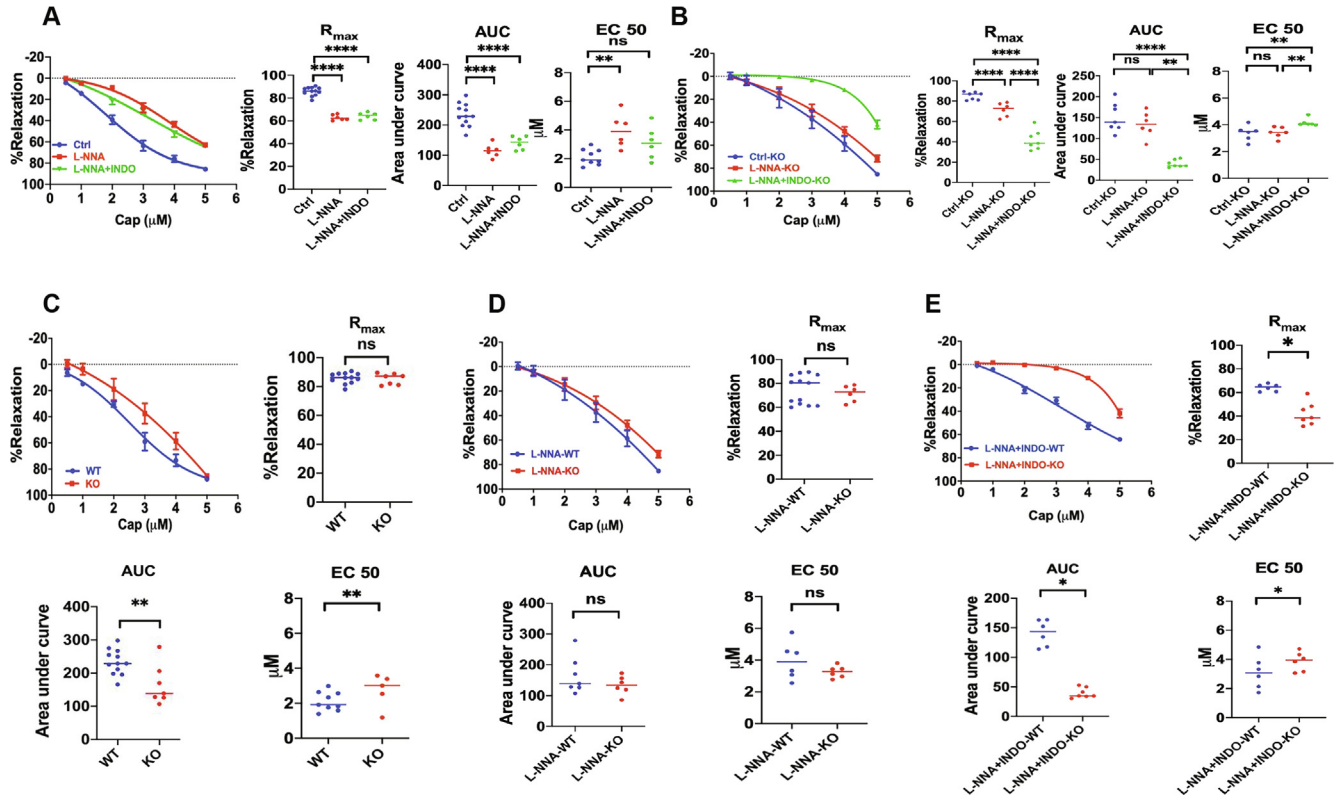

Fig. 3. Comparisons of capsaicin-mediated vasorelaxation in mouse mesenteric arterioles between WT mice and TRPV1 KO mice. **(A)** Generalize data showing capsaicin (Cap)-mediated vasorelaxation of mesenteric arterioles pretreated with NE without (control, n = 12) or with either 100 μM L-NNA alone (n = 6) or 100 μM L-NNA + 10 μM INDO (n = 6) in WT mice in the CRC, R_{max}, AUC and EC_{50}. **(B)** Generalize data showing capsaicin-mediated vasorelaxation of mesenteric arterioles without (control, n = 7) or with either 100 μM L-NNA alone (n = 6) or 100 μM L-NNA + 10 μM INDO (n = 6) in TRPV1 KO mice in the CRC, R_{max}, AUC and EC_{50}. **(C)** Comparisons the capsaicin-mediated vasorelaxation of WT mice (n = 12) with TRPV1 KO mice (n = 7) in CRC, R_{max}, AUC and EC_{50}. **(D)** Comparisons the capsaicin-mediated vasorelaxation of WT mice (n = 6) with TRPV1 KO mice (n = 6) with 100 μM L-NNA in CRC, R_{max}, AUC and EC_{50}. **(E)** Comparisons the capsaicin-mediated vasorelaxation of WT mice (n = 6) with TRPV1 KO mice (n = 6) with 100 μM L-NNA + 10 μM INDO in CRC, R_{max}, AUC and EC_{50}. Data are represented as percentage of noradrenalin (NE, 5 μM)-mediated contractions and expressed as means ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001 and ns: no significance.

TRPV4 KO mice were markedly attenuated compared to those from WT mice (Fig. 4A&B). Furthermore, in the presence of L-NNA and INDO, the capsaicin-induced vasorelaxation in WT mice was either potentiated by RN-1747, a selective of TRPV4 activator or inhibited by RN-1734, a selective TRPV4 blocker (Fig. 4C). Finally, the capsaicin-induced vasorelaxation was attenuated in TRPV4 KO mice (Fig. 4C), strongly suggesting that capsaicin induces endothelium-dependent vasorelaxation via TRPV4 channel activation.

Capsaicin induction of vasorelaxation via TRPV4/EDH signals

We focused on the capsaicin-induced vasorelaxation via EDH mechanism since it plays a predominate role in regulating endothelium-dependent vasorelaxation of mesenteric arterioles [40]. When L-NNA plus INDO inhibited the endothelium-dependent vasorelaxation via NO and PGI_2, the remaining portion of capsaicin-induced vasorelaxation is via EDH mechanism (Fig. 4C&D). First, TEA, a general K_{Ca} channel blocker, significantly attenuated capsaicin-induced vasorelaxation both in WT and TRPV4 KO mice in the presence of L-NNA plus INDO (Fig. 4D), but the vasorelaxation was further attenuated in TRPV4 KO mice in the presence of L-NNA, INDO and TEA (Fig. 4D). Second, ouabain, a selective Na^+-K^+ ATPase (NKA) inhibitor, significantly inhibited capsaicin-induced vasorelaxation in the presence of L-NNA plus INDO (Fig. 5A), and the inhibition was greater in TRPV4 KO mice than in WT mice (Fig. 5B). Finally, SN-6, a selective Na^+/Ca^{2+} exchanger (NCX) inhibitor, significantly attenuated capsaicin-induced vasorelaxation in the presence of L-NNA + INDO (Fig. 5C). Since K_{Ca} channels, NKA and NCX are well known to involve in EDH signals, these results confirm that capsaicin induces vasorelaxation via TRPV4/EDH signals. In addition, we investigated the action of capsaicin on membrane potential in HUVEC. Capsaicin induced marked hyperpolarization in HUVEC, which was significantly attenuated by selective TRPV4 channel blocker HC-067047 (Suppl Fig. 5D&E). The capsaicin–induced hyperpolarization was also significantly attenuated by selective IK_{Ca} channel blocker TRAM-34 (Suppl Fig. 5D&E). Therefore, capsaicin induces hyperpolarization through activation of TRPV4 and IK_{Ca} channels in HUVEC, supporting that capsaicin can regulate TRPV4/EDH to induce vasorelaxation.

Capsaicin-induced different endothelium-independent vasorelaxation via TRPV1 and TRPV4 channels

Since capsaicin-induced vasorelaxation was not completely abolished by L-NNA plus INDO and TEA or ouabain, we compared capsaicin-induced vasorelaxation between endothelium-intact and -denuded mesenteric arterioles to examine if the remaining portion is endothelium-independent. In endothelium-denuded arterioles (confirmed by losing response to CCh), capsaicin still induced vasorelaxation in WT mice (Fig. 6A). Interestingly, the capsaicin-induced endothelium-independent vasorelaxation was larger in TRPV1 KO mice (Fig. 6B), but disappeared in TRPV4 KO mice (Fig. 6C). Comparing to WT mice, capsaicin-induced R_{max} was significantly increased in TRPV1 KO mice; however, both R_{max}

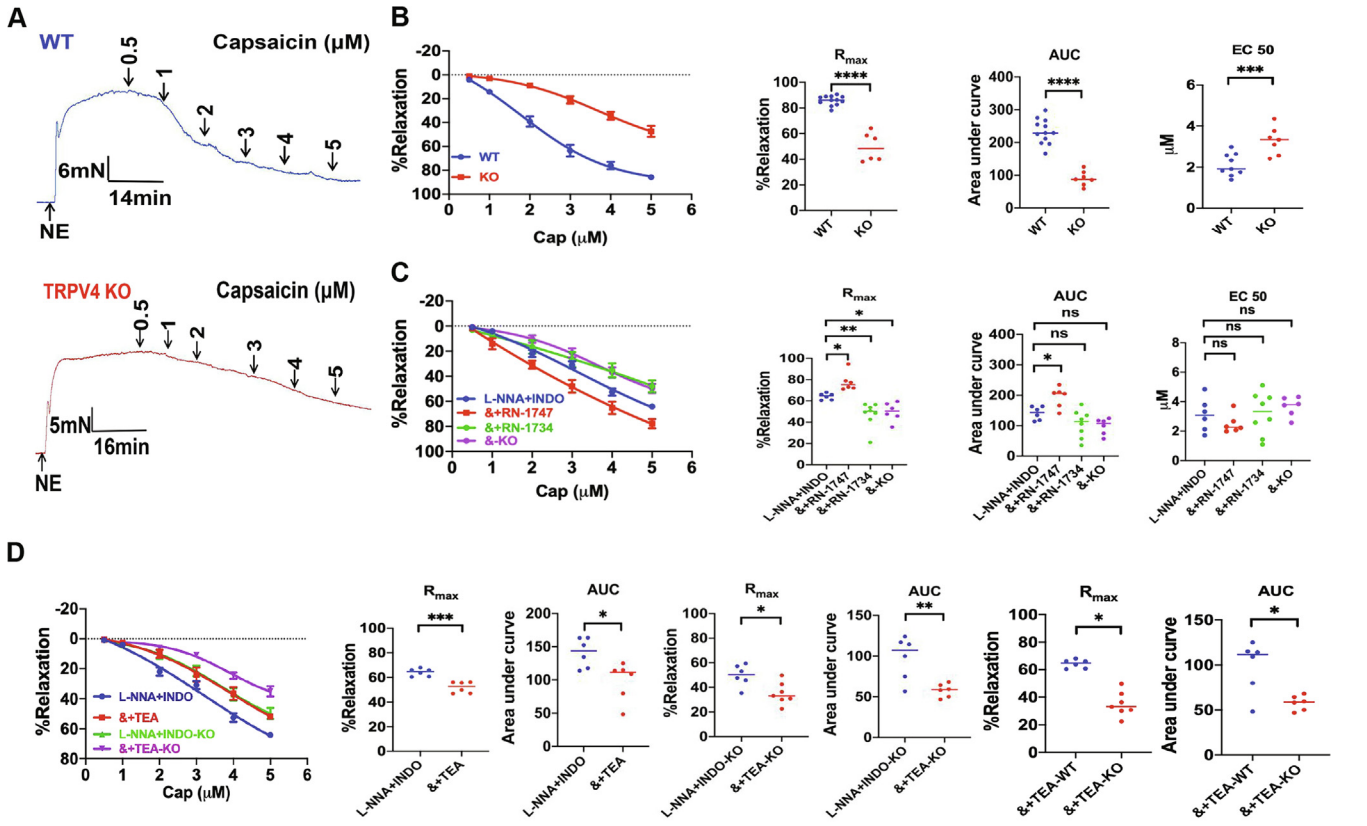

Fig. 4. Comparisons of capsaicin-mediated vasorelaxation in mouse mesenteric arterioles between WT mice and TRPV4 KO mice. (A) Representative tracings of capsaicin (Cap)-mediated vasorelaxation of mesenteric arterioles in a dose-dependent manner pretreated with noradrenalin (NE) between WT mice (the upper) and TRPV4 KO mice (the lower). **(B)** Comparisons the capsaicin-mediated vasorelaxation of WT mice (n = 12) with TRPV4 KO mice (n = 7) in CRC, R_{max}, AUC and EC_{50}. **(C)** Generalize data showing capsaicin-mediated vasorelaxation of WT mice with L-NNA + INDO (n = 6), L-NNA + INDO (&) + 5 μM RN-1747 (n = 6), L-NNA + INDO (&) + 40 μM RN-1734 (n = 8), or TRPV4 KO mice with L-NNA + INDO (n = 7) in the CRC, R_{max}, AUC and EC_{50}. **(D)** Generalize data showing capsaicin-mediated vasorelaxation of WT mice or TRPV4 KO mice with either L-NNA + INDO (n = 6) or L-NNA + INDO (&) + 10 mM TEA (n = 6–8) in the CRC, R_{max} and AUC. Data are represented as percentage of NE (5 μM)-mediated contractions and expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns: no significance.

and AUC were markedly decreased in TRPV4 KO mice (Fig. 6D). Therefore, capsaicin may exert different effects on VSMC via activation of TRPV1 and TRPV4 channels.

Beneficial effect of capsaicin via TRPV4 in colitis

We examined the effect of capsaicin in colitis. First, after treatment with DSS for 7 days, the body weight and colon length of WT mice were reduced (Fig. 7A&B&C), but the stool score and MPO were increased (Fig. 7D&E). Second, oral administration of capsaicin reversed not only the DSS-induced decrease in body weight and colon length (Fig. 7A&B&C), but also the DSS-induced increase in stool score and MPO in WT mice (Fig. 7D&E). However, capsaicin did not alter these experimental parameters in normal WT mice without DSS treatment (Fig. 7A-E). Therefore, capsaicin exerts a beneficial effect in WT colitis mice. Third, there was no difference in colon length between normal and TRPV4 KO mice (7F&G); however, oral administration of capsaicin could not anymore reverse the DSS-induced alteration in body weight, colon length (Fig. 7H&I&J), stool score and MPO (Fig. 7K&L) in TRPV4 KO mice, indicating capsaicin lost its actions in these mice. Therefore, capsaicin exerts a beneficial effect via TRPV4 channels in experimental colitis.

Capsaicin rescue of impaired endothelium-dependent vasorelaxation in colitis via TRPV4/EDH signals

Since mucosal microvascular dysfunction contributes to the pathogenesis of chronic colitis [21,22], we determined if capsaicin acts beneficially on mesenteric arterioles in WT colitis mice. The vagus neurotransmitter acetylcholine (ACh)-induced endothelium-dependent vasorelaxation via stimulation of endothelial muscarinic receptors was markedly impaired in WT colitis mice compared to those in control mice. However, oral administration of capsaicin significantly reversed the impairment of ACh-induced vasorelaxation in colitis (Fig. 7M). Similarly, the Ca^{2+}-induced vasorelaxation via stimulation of endothelial Ca-sensing receptors were markedly impaired in colitis, which were also reversed by capsaicin (Fig. 7N). Therefore, capsaicin exerts the beneficial action via rescue of the endothelium-dependent vasorelaxation impaired in WT colitis mice.

We determined if capsaicin acts beneficially on mesenteric arteriole in colitis via endothelial TRPV4 channels. Indeed, ACh-induced vasorelaxation was impaired in TRPV4 KO colitis mice compared to TRPV4 KO control mice (Fig. 8A); however, oral administration of capsaicin could not anymore reverse the impairment of ACh-induced vasorelaxation in TRPV4 KO colitis mice as it did in WT colitis mice (compare Fig. 7M with 8A). Furthermore, in the presence of L-NNA plus INDO, capsaicin could not reverse the impairment of ACh-induced vasorelaxation in TRPV4 KO colitis mice neither (Fig. 8B). Taken together, capsaicin prevents endothelium-dependent vasorelaxation from impairment in colitis via TRPV4/EDH signals.

Capsaicin in vivo rescue of the impaired vasodilation in colitis

We applied whole animal study to examine if capsaicin acts beneficially on mesenteric arteriole in colitis via TRPV4 channels.

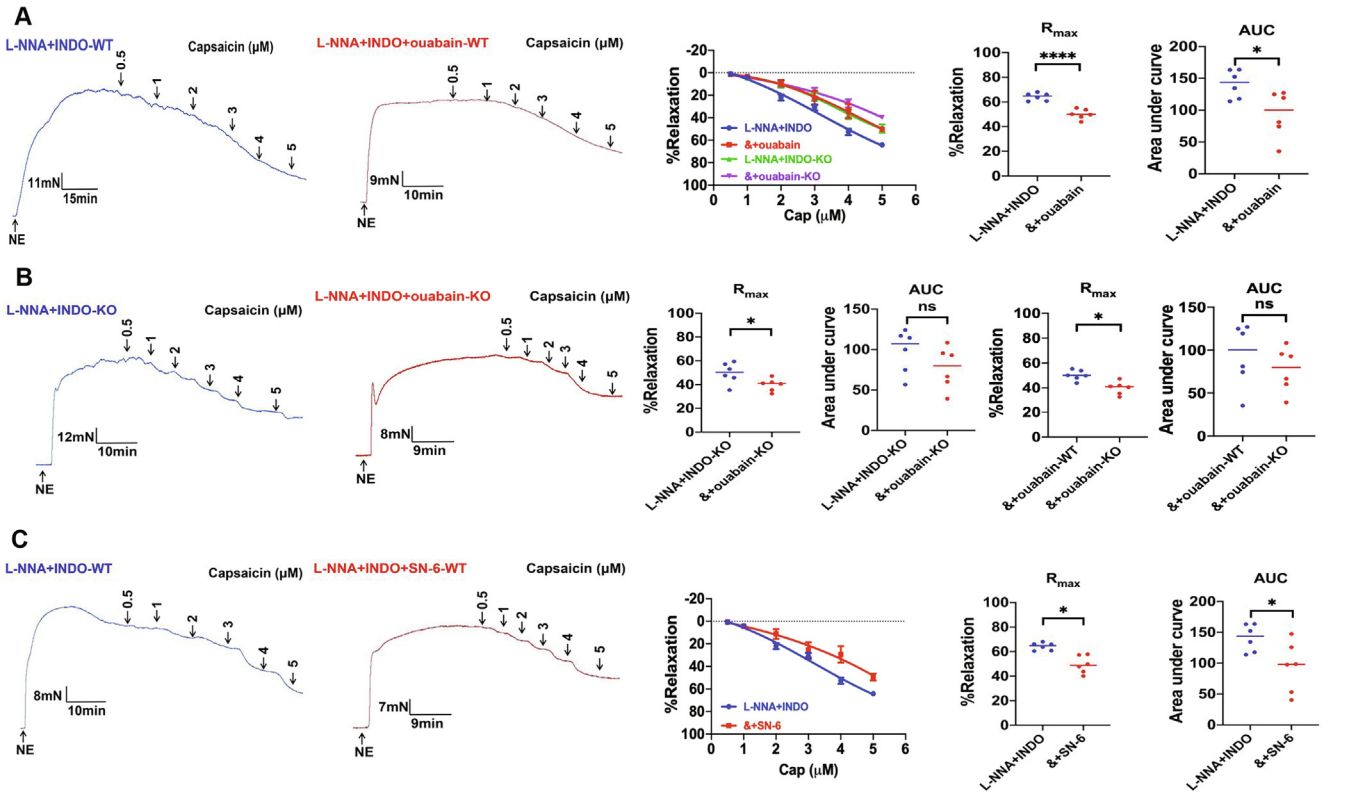

Fig. 5. Role of NKA and NCX in capsaicin-induced vasorelaxation of mouse mesenteric arterioles via EDH. (A-B) Generalize data showing capsaicin (Cap)-mediated vasorelaxation of WT mice or TRPV4 KO mice with either L-NNA + INDO (n = 6) or L-NNA + INDO (&) + 1 mM ouabain (n = 6–8) in the CRC, R_{max}, and AUC. **(C)** Generalize data showing capsaicin-mediated vasorelaxation of WT mice with either L-NNA + INDO (n = 6) or L-NNA + INDO (&) + 10 μM SN-6 (n = 6) in the CRC, R_{max}, and AUC. Data are represented as percentage of NE (5 μM)-mediated contractions and expressed as means ± SEM. *P < 0.05, ****P < 0.0001 and ns: no significance.

First, capsaicin (5 μM) induced PVD of mesenteric arteriole in WT mice but not in TRPV4 KO mice (compare Fig. 8C and D), and there was a significant difference in ΔPVD of WT and TRPV4 KO mice (Fig. 8E), indicating that capsaicin dilates mesenteric arterioles through activation of TRPV4 channels. Second, a stable analog of ACh, CCh-induced endothelium-dependent vasodilation was impaired in colitis mice (Fig. 8F), which was rescued by oral administration of capsaicin (Fig. 8G). There was a significant difference in ΔPVD of colitis mice with or without capsaicin treatment (Fig. 8H). Therefore, these *in vivo* findings confirm the evidence provided *in vitro* that capsaicin rescues the impairment of TRPV4-mediated microvascular vasodilation of in colitis.

Discussion

In the present study, we revealed that capsaicin has pleiotropic actions on the human submucosal arterioles and mouse mesenteric arterioles in health and colitis. The major novel findings of this study are: (1) capsaicin induces endothelium-dependent vasorelaxation of the arterioles predominately through TRPV4/EDH, but marginally through other TRPV family members, NO, PGI_2 and CGRP; (2) TRPV4/EDH signals play a critical role in the capsaicin-induced submucosal arterial relaxation to increase mucosal blood perfusion; and (3) capsaicin exerts anti-colitis action by rescuing the impaired endothelium-dependent vasorelaxation via TRPV4/EDH pathway.

Chili pepper and its major active compound capsaicin have long been used as a daily food additive and medication worldwide. Like in other human organs outside of GI tract, capsaicin has multiple actions in GI health and disease. Although capsaicin is a selective agonist for TRPV1 channel, it acts not only in the well-known TRPV1-dependent manner but also in the poorly understood TRPV1–independent manner in GI tract, such as apoptotic induction of gastric cancer cells via TRPV6 activation [41]. In the present study, we demonstrate for the first time that capsaicin induced endothelium-dependent relaxation of submucosal and mesenteric arterioles predominantly via endothelial TRPV4 rather than TRPV1 channels, which was further confirmed by using both TRPV1 and TRPV4 KO mice. Moreover, capsaicin-induced endothelium-independent vasorelaxation was increased in TRPV1 KO mice but decreased in TRPV4 KO mice, indicating that capsaicin activates TRPV1 and TRPV4 channels on VSMC to likely play different roles. Although further investigation is needed to elucidate the underlying mechanisms, it is reasonable to speculate that TRPV1 activation may induce global $[Ca^{2+}]_{cyt}$ increase in VSMC to trigger vasoconstriction, but TRPV4 activation may induce local $[Ca^{2+}]_{cyt}$ increase to activate K_{Ca} channels and to trigger vasorelaxation, which has been previously described as the TRPV4/Ca^{2+} sparklets [42].

The mesenteric resistance vascular plays an important role in regulation of blood pressure and mucosal perfusion. It has been widely accepted that endothelium regulates vascular tone via NO, PGI_2, and EDH [1,2]. NO activates soluble guanylate cyclase in VSMC to increase cyclic GMP production, resulting in vasorelaxation. PGI_2 activates inositol phosphate receptors on VSMC to relax most arterioles [43,44]. EDH has been considered as an endothelium-derived non-NO and non-PGI_2 factor [45]. Although the nature of EDH has not been identified, it is generally acknowledged that Ca^{2+} influx into vascular endothelial cells is critical for EDH signal that is mediated not only by IK_{Ca} and SK_{Ca} channels expressed predominantly in endothelial cells but also by NKA

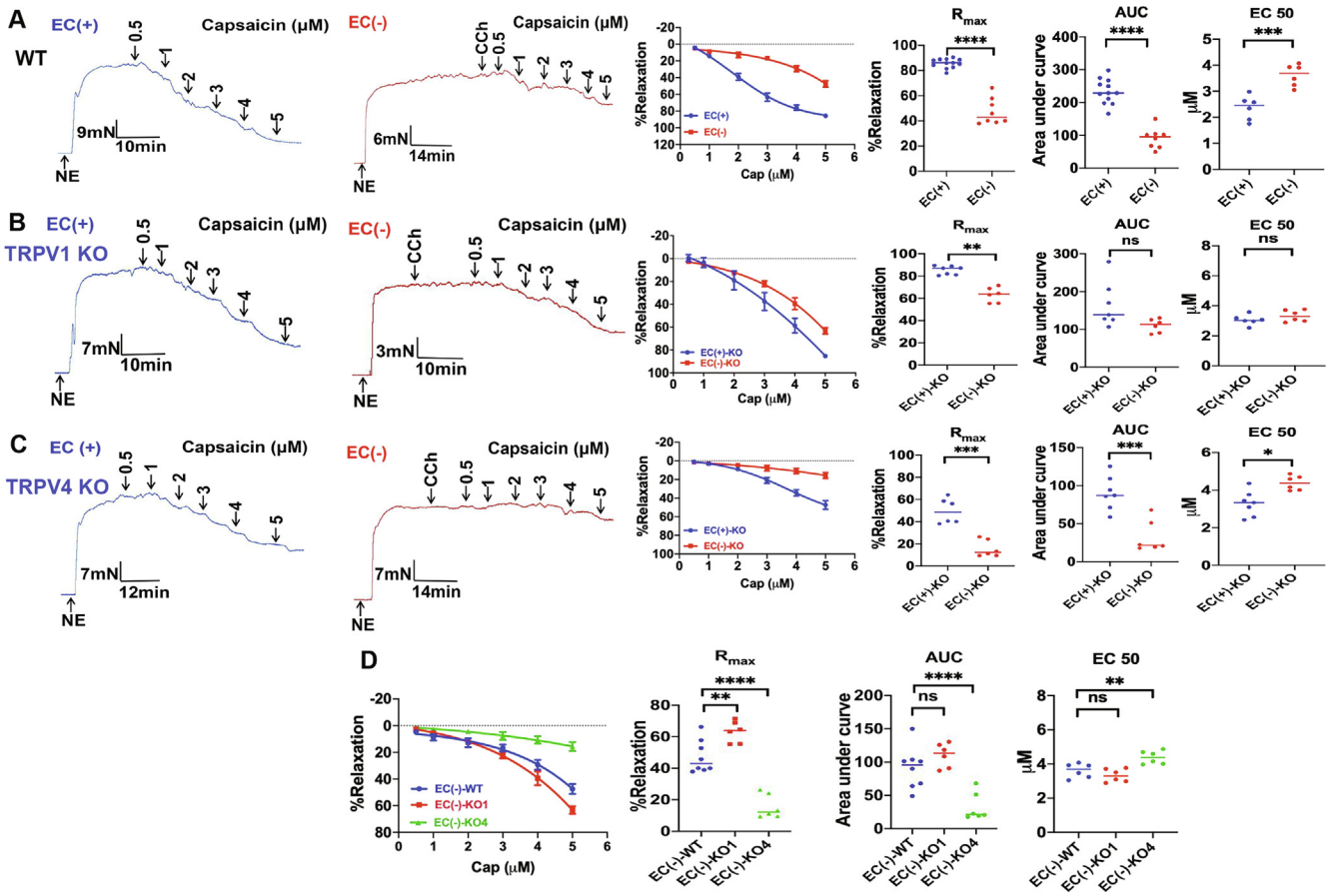

Fig. 6. Comparisons of capsaicin-induced endothelium-dependent and endothelium-independent vasorelaxation between WT mice and TRPV KO mice. (A) Representative tracings, CRC, R_{max}, AUC and EC_{50} of capsaicin (Cap)-mediated endothelium-dependent (EC+, n = 6) or endothelium-independent (EC-, n = 6) vasorelaxation in WT mice. **(B-C)** Representative tracings, CRC, R_{max}, AUC and EC_{50} of capsaicin-mediated endothelium-dependent (EC+, n = 6) or endothelium-independent (EC-, n = 6) vasorelaxation in TRPV1 or TRPV4 KO mice, respectively. **(D)** Summary data and comparison of CRC, R_{max}, AUC and EC_{50} of capsaicin- mediated endothelium-independent vasorelaxation between WT mice and TRPV KO mice. Data are shown as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns: no significance.

and NCX in VSMC [46]. In addition, the endothelial TRPV4 channels can participate in EDH-mediated responses by activating IK_{Ca} and SK_{Ca} in mesenteric arterioles of rats [47].

Capsaicin could increase NO production in HUVEC [48], and activate TRPV1 channels to induce PKA phosphorylation and eNOS in endothelial cells and plasma NO concentration, improve mesenteric arterial relaxation, and lower blood pressure in hypertensive rats. Similarly, using TRPV1 KO mice, we reveal that TRPV1 activation induces vasorelaxation through NO and PGI_2, but these mechanisms have few influences on capsaicin-induced vasorelaxation. It was previously reported that capsaicin induced vasorelaxation of the first-order branch of mesenteric arterioles via CGRP release [33]; however, we reveal that perivascular sensory nerve terminals and CGRP participate in capsaicin-induced vasorelaxation of the first-order branch of mesenteric arterioles but not the second-order branch. Therefore, consistently with the previous report that capsaicin-induced vasorelaxation of the first-order branch of mesenteric arterioles via NO and CGRP, we uncover a novel mechanism that capsaicin-induced vasorelaxation of the second-order branch of mesenteric arterioles predominately via EDH. Moreover, we found that the EC_{50} for capsaicin-induced vasorelaxation predominately via TRPV1 activation in the first-order branch of mesenteric arterioles was 55.0 ± 6.0 nM, but the EC_{50} for capsaicin-induced vasorelaxation predominately via TRPV4 activation in the second-order branch was 2.1 ± 0.2 μM. Therefore, for the first time we reveal a significantly regional difference of mesenteric arterioles in response to capsaicin through TRPV1/NO and CGRP pathway in the first-order branch of mesenteric arterioles but through TRPV4/EDH pathway in the second-order branch. We have also excluded the possible involvements of other endothelial ion channels in mesenteric arterioles, such as TRPV3, TRPV6 and CFTR although they may play roles in regulating vasorelaxation of cerebral arterioles [36].

Although EDH plays a major role in regulation of resistance arterial tone, it has not been explored if capsaicin induces EDH-mediated vasorelaxation. We have provided strong evidence for the important role of capsaicin-induced TRPV4/EDH signals in the endothelium-dependent vasorelaxation of small resistance arterioles: 1) capsaicin induced much greater endothelium-dependent relaxation of the arterioles pre-constricted by NE than by high K^+, and capsaicin-induced vasorelaxation remained mostly in the presence of L-NNA plus INDO, 2) the remaining portion of capsaicin-induced vasorelaxation was potentiated by opener of IK_{Ca} and SK_{Ca} channels, but attenuated by their blockers or by inhibitors of NKA and NCX; 3) the capsaicin-induced vasorelaxation mediated by EDH was inhibited by selective TRPV4 channel blocker or attenuated in TRPV4 KO mice in a similar pattern; 4) capsaicin promoted Ca^{2+} entry through endothelial TRPV4 channels, and induced hyperpolarization through activation of TRPV4 and IK_{Ca} channels.

Capsaicin has long been used to treat several human disorders outside of GI tract, such as neuropathic inflammation and pain; however, so far there are few studies on GI actions of capsaicin, and conflicting data exist in GI patients. An epidemiological study

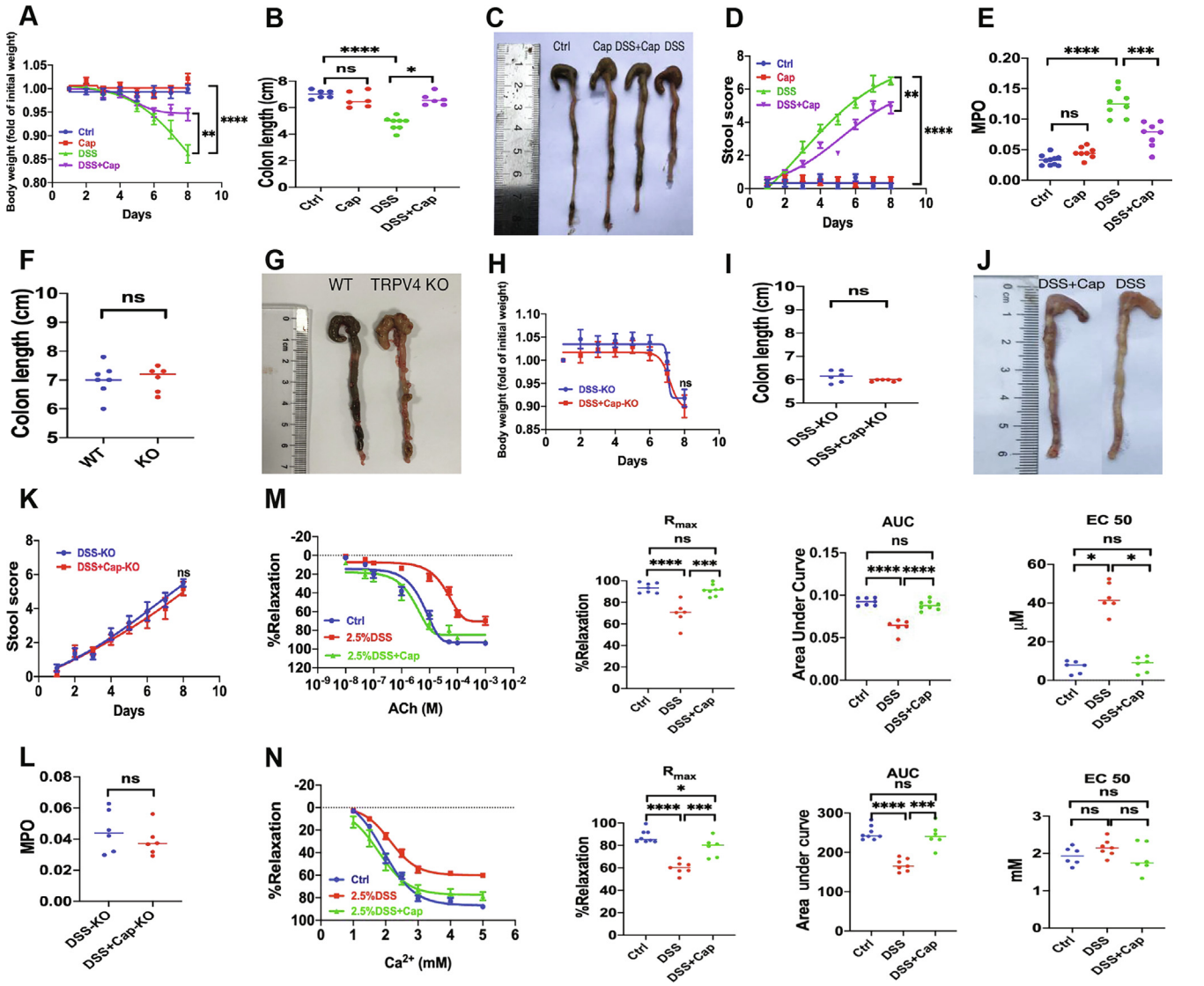

Fig. 7. Beneficial effect of capsaicin via TRPV4 channels in experimental colitis mouse. (A-C) Generalize data showing the time course of body weight and the colon length of WT mice after different treatments without (control) or with capsaicin (Cap), DSS, or DSS + capsaicin (n = 6). **(D-E)** Generalize data showing the time courses of stool score and MPO of WT mice after different treatments as described in A-C. **(F-G)** Generalize data showing the colon length between wild-type mice (WT) and TRPV4 KO mice (n = 6). **(H-J)** Generalize data showing the time course of body weight and the colon length of TRPV4 KO mice after different treatments with DSS alone or DSS + capsaicin (n = 6). **(K-L)** Generalize data showing the time courses of stool score and MPO of TRPV4 KO mice after different treatments as described in H-J. **(M)** Generalize data showing the CRC, R_{max}, AUC and EC_{50} of acetylcholine (ACh)-mediated vasorelaxation of WT mice after different treatments without (control) or with DSS or DSS + capsaicin (n = 6). **(N)** Generalize data showing the CRC, R_{max}, AUC and EC_{50} of calcium (Ca^{2+})-mediated vasorelaxation of WT mice after different treatments as described in M (n = 6). Data are shown as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns: no significance.

found three times higher peptic ulcer incidence among population who consumes less chili compared to those who consumes more [49], suggesting beneficial effect of chili and capsaicin in GI disease. Capsaicin also markedly reduced gastric mucosal injury induced by alcohol and aspirin [50]. Although capsaicin protects gastric mucosa likely through capsaicin-sensitive nerves, the underlying mechanisms are largely unclear. In contrast, some studies suggest that consuming more chili and capsaicin may be harmful to GI tract because they provoke a burning sensation. Since chili pepper and capsaicin have long been used as daily food additive and medicine worldwide, it is important to study in-depth their GI actions in health and disease.

As mentioned earlier, due to both TRPV1-dependent and TRPV1-independent actions of capsaicin, it is important to distinguish between the role of TRPV1 channels in colitis and the effect of dietary capsaicin on colitis. Some previous studies suggest that activation of TRPV1 channels in sensory neurons aggravates colitis [29,30]. Although the critical role of TRPV1 channels in the pathogenesis of IBD has been intensively studied in terms of immune cells and sensory neurons [51,52], surprisingly, so far little is known about the detailed action of capsaicin in colitis, except one study reported an increased visceral sensitivity to capsaicin in mouse colitis [53]. In the present study, we have provided strong evidence both *in vitro* and *in vivo* studies that dietary capsaicin exert an anti-colitis effect via novel TRPV4-mediated endothelium-dependent hyperpolarization of mesenteric arterioles. Our finding that capsaicin ameliorates experimental colitis is consistent with clinical epidemiological studies that the incidence rate of ulcerative colitis is much lower in high chili consuming regions in China [26,27].

It is well known that mesenteric microvascular function plays a critical role in maintaining intestinal mucosal barrier function and

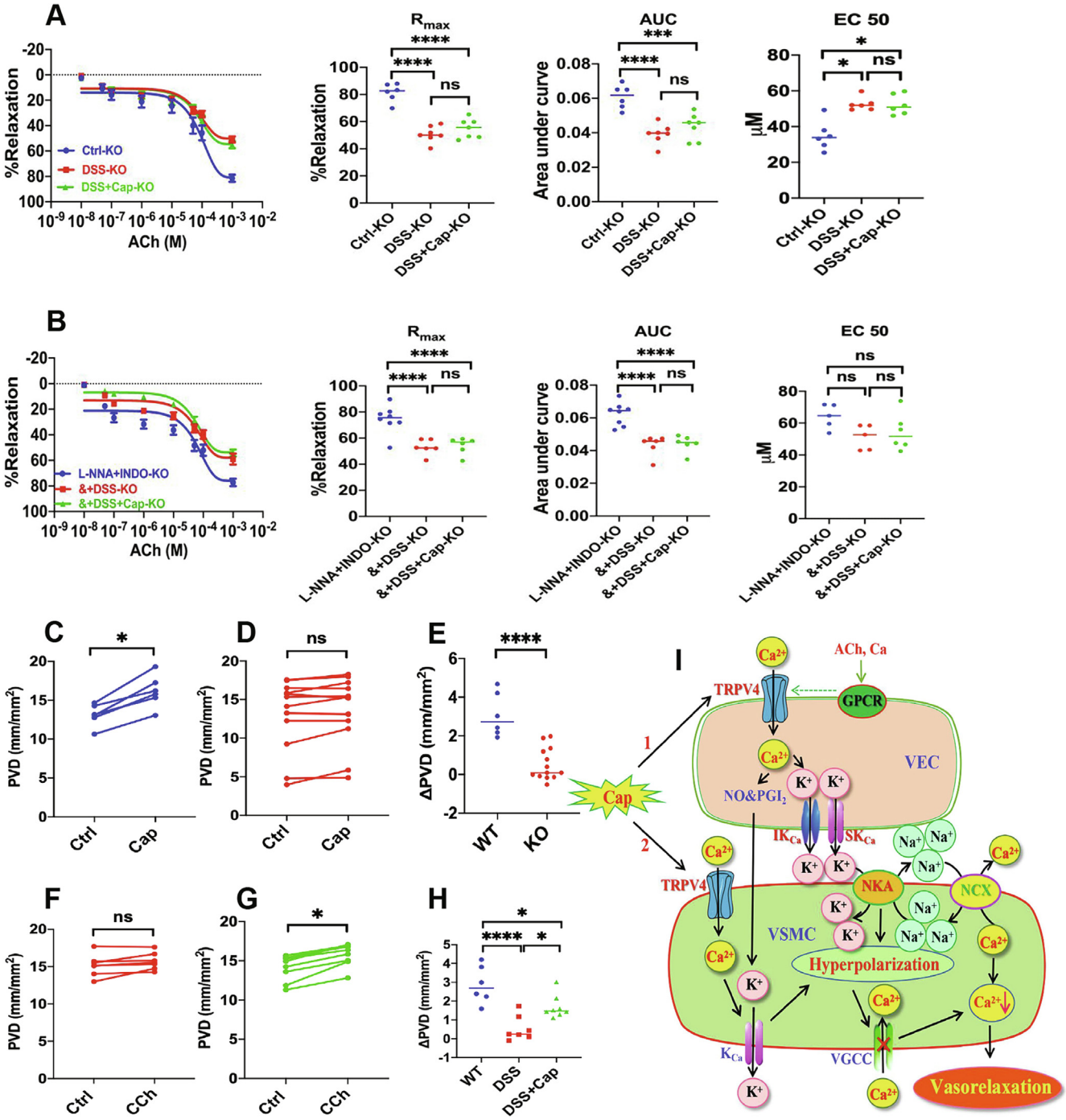

Fig. 8. Confirmation of capsaicin rescue of the impaired vasodilation *in vitro* and *in vivo* in colitis mice. **(A-B)** Generalize data showing ACh-mediated vasorelaxation of TRPV4 KO (n = 6) mice in the CRC, R_{max}, AUC and EC_{50} after different treatments as described in Fig. 7M without **(A)** or with L-NNA + INDO (&, **B**). **(C-E)** Generalize data showing the influence of capsaicin on PVD measured *in vivo* in WT mice (**C**, n = 6) and in TRPV4 KO mice (**D**, n = 13) and comparison of ΔPVD (the difference in PVD before and after drug treatment) between WT and TRPV4 KO mice **(E)**. **(F-H)** Generalize data showing the influence of CCh on PVD measured *in vivo* in WT DSS-colitis mice as control (**F**, n = 7) and in WT DSS-colitis mice with capsaicin treatment (**G**, n = 8) and comparison of ΔPVD among normal, DSS-colitis and colitis mice with capsaicin treatment **(H**, n = 6–8). (**I**) The schematic diagram depicting the endothelium-dependent and -independent mechanisms of capsaicin (Cap) and its prevention of mesenteric vasorelaxation impaired in colitis. Acetylcholine (ACh) and calcium (Ca) stimulate endothelial GPCR (M3 receptors and Ca-sensing receptors) to induce Ca^{2+} entry via endothelial TRPV4 channels. An increase in Ca^{2+} signaling not only produces NO and PGI_2, but also activates SK_{Ca} and IK_{Ca} channels in HUVEC, resulting in the activation of NKA and hyperpolarization that inhibited Ca^{2+} influx of VSMC by VGCC and finally causes vasorelaxation via EDH. Moreover, the activation of NKA reduces $[Na^+]_{cyt}$ in VSMC, which results in the reduction of $[Ca^{2+}]_{cyt}$ by NCX, leading to further vasorelaxation. Capsaicin induces: 1) the endothelium-dependent vasorelaxation predominately via the endothelial TRPV4/Ca^{2+}/EDH pathway described above and 2) the endothelium-independent vasorelaxation via the smooth muscle TRPV4/Ca^{2+}/K_{Ca} pathway. While the GPCR/TRPV4/EDH-mediated vasodilatation is impaired in colitis, capsaicin-induced endothelium-dependent and endothelium-independent vasorelaxation takes over.

histological structure, whereas an endothelial defect impaired vasodilatory response which leaded to microvascular dysfunction in colitis. The intestinal mucosal microvascular dysfunction may result in the refractory, mucosal ulceration. In colitis, endothelial GPCR muscarinic receptor (MR)- and Ca-sensing receptor (CaSR)-induced vasorelaxation were impaired, but for the first time we revealed that endothelial dysfunction was rescued by capsaicin. While GPCR-induced vasorelaxation is impaired in colitis, capsaicin-induced vasorelaxation takes over the responsibility in endothelium-dependent and -independent manners (Fig. 8I). Therefore, capsaicin induces microvascular relaxation to rescue the impairment of mucosal blood perfusion in colitis as examined in our *in vitro* and *in vivo* studies, and finally promote epithelial barrier function to cause post-injury recovery. Likewise, this study has provided further evidence to support our previous notion that EDH in mesenteric arterioles plays a critical role in the pathogenesis of colitis [25,54].

It was previously reported that endothelial TRPV4 channel increases colonic vascular permeability in colitis [55]; however, the experimental data were mostly related to TRPV4 expression rather than functional activities. Importantly, it has not been explored if the role of TRPV4 channels in regulating microvascular relaxation contributes to the pathogenesis of colitis. Not only has the present study verified a critical contribution of endothelial TRPV4 to the pathogenesis of colitis, but elucidated the underlying mechanism that capsaicin-mediated TRPV4/EDH beneficially rescues the impairment of microvascular vasorelaxation in colitis. Therefore, dietary capsaicin has potential to become a food additive and a safe drug for the prevention/treatment of colitis. Although further investigation is needed for the detailed mechanisms by which capsaicin ameliorates endothelial defect in colitis through TRPV4/EDH signals, here we offer new ideas of capsaicin in the microvascular actions in health, and develop a novel strategy to prevent/treat colitis as well.

Conclusion

In summary, capsaicin induced an obvious endothelium-dependent vasorelaxation of human colonic submucosal arterioles and mouse mesenteric arterioles *in vitro* and *in vivo*. Capsaicin induced vasorelaxation predominately through TRPV4-mediated endothelium-dependent hyperpolarization rather than TRPV1-mediated endothelial nitric oxide release. Capsaicin exerted anti-colitis action in wide-type colitis mice but not in TRPV4 knock-out colitis mice. Moreover, capsaicin could rescue the impaired endothelium-dependent vasorelaxation via TRPV4/EDH pathway. Capsaicin has potential to become a food additive and medication for the prevention/treatment of colitis by promoting mucosal blood perfusion via submucosal micro-arterial dilation.

Compliance with Ethics Requirements

All Institutional and National Guidelines for the care and use of animals were followed.

Animal studies were reported in compliance with the ARRIVE guidelines. The protocols were approved by the Army Military Medical University Committee on Investigations Involving Animal Subjects. All the animal care and experimental studies were conducted in accordance with the guidelines of the Animal Ethical Committee of the Army Military Medical University (AMU-WEC2020368) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.11.001.

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