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·Original Article·

Formaldehyde up-regulates TRPV1 through MAPK and PI3K signaling pathways in a rat model of bone cancer pain

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Abstract: Objective Our previous study showed that tumor tissue-derived formaldehyde at low concentrations plays an important role in bone cancer pain through activating transient receptor potential vanilloid subfamily member 1 (TRPV1). The present study further explored whether this tumor tissue-derived endogenous formaldehyde regulates TRPV1 expression in a rat model of bone cancer pain, and if so, what the possible signal pathways are during the development of this type of pain. **Methods** A rat model of bone cancer pain was established by injecting living MRMT-1 tumor cells into the tibia. The formaldehyde levels were determined by high performance liquid chromatography, and the expression of TRPV1 was examined with Western blot and RT-PCR. In primary cultured dorsal root ganglion (DRG) neurons, the expression of TRPV1 was assessed after treatment with 100 μmol/L formaldehyde with or without pre-addition of PD98059 [an inhibitor for extracellular signal-regulated kinase], SB203580 (a p38 inhibitor), SP600125 [an inhibitor for c-Jun N-terminal kinase], BIM [a protein kinase C (PKC) inhibitor] or LY294002 [a phosphatidylinositol 3-kinase (PI3K) inhibitor]. **Results** In the rat model of bone cancer pain, formaldehyde concentration increased in blood plasma, bone marrow and the spinal cord. TRPV1 protein expression was also increased in the DRG. In primary cultured DRG neurons, 100 μmol/L formaldehyde significantly increased the TRPV1 expression level. Pre-incubation with PD98059, SB203580, SP600125 or LY294002, but not BIM, inhibited the formaldehyde-induced increase of TRPV1 expression. **Conclusion** Formaldehyde at a very low concentration up-regulates TRPV1 expression through mitogen-activated protein kinase and PI3K, but not PKC, signaling pathways. These results further support our previous finding that TRPV1 in peripheral afferents plays a role in bone cancer pain.

Keywords: formaldehyde; TRPV1; cancer pain; mitogen-activated protein kinase; phosphatidylinositol 3-kinase

1 Introduction

Pain is one of the most common symptoms in cancer patients, 75%–90% of advanced or terminal cancer patients experiencing chronic pain[1]. Malignant bone tumors occur in patients with primary bone cancer, but are more common in distant metastases from other primary cancers, notably those of the breast, lung and prostate[2].

Formaldehyde is a consistent metabolic product of demethylation (N-, O-, and S-demethylation)[3]. Its concentration is increased in the urine of patients with prostate cancer and bladder cancer and in the expired air from tumor-bearing mice and breast cancer patients[4,5] who

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more frequently suffer from bone cancer pain[6]. Formaldehyde is considered a risk factor in the development of chronic bone cancer pain[7], and our recent research demonstrated for the first time that cancer tissue-derived low concentrations of formaldehyde induce cancer pain[8].

Transient receptor potential vanilloid subfamily member 1 (TRPV1), a nociceptive receptor in peripheral nerve fibers[9], plays a pivotal role in the development of cancer pain[10-16]. Our previous study found that formaldehyde at concentrations as low as 1 mmol/L induces TRPV1 currents in dorsal root ganglion (DRG) neurons[8,17]. It is well-known that TRPV1 expression increases in peripheral nerve fibers and DRG neurons in cancer pain. It is therefore of interest to determine whether formaldehyde modulates TRPV1 expression in peripheral afferents in bone cancer pain, and if so, reveal the intracellular signaling pathway underlying this modulation. Mitogen-activated protein kinases (MAPKs), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) play critical roles in the cell signaling of TRPV1 regulation. MAPKs have three major family members: extracellular signal-regulated kinase (ERK) (ERK1/2 or p44/42 MAPK), p38 and c-Jun N-terminal kinase (JNK), representing three different signaling cascades[18]. It was reported that inhibitors of ERK, p38 and JNK alleviate neuropathic pain[19,20], while PKC and PI3K are also involved in the regulation of pain[20,21]. These protein kinases, once activated, phosphorylate TRPV1, leading to rapid and dynamic changes in pain sensitivity[22-26]. Exposure of intestinal epithelial cells to low-dose formaldehyde results in MAPK activation[27], and formaldehyde induces apoptosis through decreased peroxiredoxin 2 via p38 MAPK in lung epithelial cells[28]. So, we set out to determine whether the protein kinase signaling pathway participates in the modulation of TRPV1 expression by formaldehyde in the development of bone cancer pain.

In the present study, a rat model of bone cancer pain and primary cultures of DRG neurons were used to determine whether tumor tissue-derived endogenous formaldehyde regulates TRPV1 expression and if so, the possible underlying signal pathways.

2 Materials and methods

2.1 Preparation of MRMT-1 rat mammary gland carcinoma cells MRMT-1 rat mammary gland carcinoma cells were a kind gift from Novartis Oncology Research Institute and were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, heat-inactivated) according to our previous description[8]. Cells in the culture dish were harvested using 0.25% (*w*/*v*) trypsin (Hyclone), centrifuged and then washed with phosphate-buffered saline (PBS). The final pellet was re-suspended in PBS, and cells were kept on ice until injection into the tibia. In the heat-killed control group, MRMT-1 cells were diluted to the same final concentrations but boiled in water for 20 min before injection. In the PBS control group, only PBS was injected.

2.2 Establishment of the rat model of bone cancer pain Female Sprague-Dawley rats weighing 250–300 g were used. Establishment of the bone cancer pain model has been described previously in detail[8,29]. Briefly, after anesthesia, the rat was placed supine. The left hindlimb was shaved and the skin disinfected with 75% (*v*/*v*) ethanol. A 1-cm rostro-caudal incision was made in the skin over the top half of the tibia. The tibia was carefully exposed with minimal damage to muscles or blood vessels. Using a 23-gauge needle, the bone was pierced 5 mm below the knee joint distal to the epiphyseal growth plate. The needle was inserted at an angle to enable it to be pushed down the intramedullary canal. Then it was removed and replaced by a long thin blunt needle. A volume of 3 μL (3×10^4 cells) MRMT-1 live cells, heat-killed MRMT-1 cells or PBS vehicle was injected into the bone cavity. As the plunger in the syringe was slowly depressed, the needle was simultaneously lifted out of the bone to enable the cells to fill the space left in the bone cavity, with no leakage of cells from the bone. Following injection, the site was closed using bone wax. The wound was then closed with suture silk and dusted with penicillin antibiotic powder. The experiments were approved by the Animal Research Committee of Peking University Health Science Center.

2.3 Primary culture of DRG neurons Two-week-old rats were decapitated after anesthesia. Lumbar 4 (L4)–L5

DRGs were dissected free. All DRGs on both sides from cervical, thoracic and lumbar levels were collected, placed in a 35-mm culture dish containing 1 mL collagenase (type I, 3 mg/mL; Sigma-Aldrich, St. Louis, MO, USA), and shaken for 45 min in a heated (37°C) chamber. After removing the collagenase and washing with PBS, 1 mL trypsin (0.25%) was added and the dish was shaken for 10 min in the same chamber. Digestion was inhibited by FBS, followed by spinning at 500 rpm for 5 min. Then trypsin was replaced by fresh DMEM containing FBS. The cells were dissociated with a polished Pasteur pipette and plated on poly-*L*-lysine-coated 35-mm plastic dishes or glass sheets for culture.

2.4 Drug administration Formaldehyde was dissolved and diluted in sterile PBS to the desired concentrations (30, 100 and 300 μmol/L). To assess the possible underlying signaling pathways for formaldehyde modulation of TRPV1, 10 μmol/L PD98059 (an ERK inhibitor), 10 μmol/L SB203580 (a p38 inhibitor), 10 μmol/L SP600125 (a JNK inhibitor), 10 μmol/L LY294002 (a PI3K inhibitor) or 1 μmol/L bis-indolylmaleimide I hydrochloride (BIM, a PKC inhibitor) was added to the culture dishes 60 min prior to formaldehyde incubation. All inhibitors were from Sigma-Aldrich Chemicals. Cells without formaldehyde and any inhibitor treatment were used as negative control, and those with formaldehyde but no inhibitor treatment were used as positive control.

2.5 Measurement of formaldehyde concentration with high performance liquid chromatography (HPLC) Formaldehyde concentrations in blood plasma, bone marrow, and tissue homogenates were assessed on day 14 following tumor cell inoculation and in the cultural media of cancer cell lines using HPLC (ESA Biosciences, Chelmsford, MA, USA) based on the previous descriptions with modifications[8,30].

2.6 Western blot detection of TRPV1 L4 and L5 DRGs or primary cultured DRG neurons were dissociated in lysis buffer containing (in mmol/L) 50 Tris-HCl, 40 NaF, 2 EDTA, 1 dithiothreitol and protease inhibitor, pH 7.6. Lysates were separated in SDS-PAGE gel and transferred on to polyvinylidene difluoride membranes. After blocking with 5% non-fat, dried milk in TBST (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20), membranes were incubated with rabbit anti-TRPV1 antibody (1:1 000, Abcam, Cambridge, UK) in 5% non-fat, dried milk in TBST overnight at 4°C. After washes with TBST, membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit antibody (Zhongshan Goldenbridge Biotechnology Co., Beijing, China) diluted with 5% non-fat dried milk in TBST and detected with enhanced chemiluminescence reagents (Amersham Biosciences, Arlington Heights, IL, USA). Blots were scanned with Spot Advanced, and the band densities were compared using TotalLab software (version 2.01 Bio-Rad, Hercules, CA, USA). Membrane protein was extracted following the method described previously[31].

2.7 RT-PCR detection of TRPV1 Total RNA was extracted from primary cultured neurons using total RNA kits (Fermentas, Thermo Fisher Scientific, Pittsburgh, PA, USA). First-strand cDNA was synthesized using oligo$(dT)_{15}$ primer and M-MuLV reverse transcriptase. PCR products were amplified again using the PCR primers (TRPV1 forward: 5'-GTGCCGGTTTATGTTCGTCT, reverse: 5'-GTTTACCTCGTCCACCCTGA; β-actin forward: 5'-ATCTGGCACCACACCTCC, reverse: 5'-AGCCAGGTCCAGACGCA). PCR products were separated in 2% agarose gel, stained by ethidium bromide, and visualized under UV light. The ratios of optical densities of DNA bands were measured by TotalLab software (version 2.01). All chemicals were from Invitrogen (Grand Island, NY, USA). Cells without formaldehyde and any inhibitor treatment were used as negative control, and those with formaldehyde but no inhibitor treatment as positive control.

2.8 Data analysis Data are expressed as mean ± SEM. For comparison among groups, ANOVA followed by *post hoc* analysis (Bonferroni test) was used. $P<0.05$ was considered as statistically significant.

3 Results

3.1 Formaldehyde concentration increased in tibia marrow and tissues from rats with bone cancer pain Formaldehyde concentrations by HPLC measurement are

Table 1. Formaldehyde concentrations in blood plasma and tissues from cancer pain rats compared with control group

Tissues	Control group (μmol/L)	Cancer pain group (μmol/L)
Cerebral cortex (L)	96 ± 17	93 ± 14
Cerebral cortex (R)	102 ± 21	115 ± 36
Hippocampus (L)	94 ± 16	92 ± 6
Hippocampus (R)	107 ± 33	98 ± 6
Thalamus (L)	86 ± 13	91 ± 11
Thalamus (R)	96 ± 21	96 ± 11
Spinal cord (L)	78 ± 14	257 ± 59 **
Spinal cord (R)	89 ± 14	97 ± 12
Bone marrow (L)	51 ± 9	657 ± 104 **
Bone marrow (R)	54 ± 15	96 ± 12 *
Lung	67 ± 15	85 ± 13
Cardiac muscle	109 ± 31	115 ± 41
Pancreas	75 ± 14	198 ± 46 *
Liver	104 ± 26	174 ± 102 *
Spleen	59 ± 12	100 ± 23 *
Kidney	74 ± 15	101 ± 16 *
Blood	76 ± 24	212 ± 84 *

Data are expressed as mean ± SEM. L, left (ipsilateral) side; R, right side. *P <0.05, **P <0.01 compared with control. n = 6.

shown in Table 1. In the control group, formaldehyde was detected in all of the collected tissues and blood plasma. Compared with the control group, formaldehyde concentrations significantly increased in the bone cancer pain group on day 14 in plasma, ipsilateral spinal cord, bone marrow, pancreas, liver, spleen and kidney, and especially in bone marrow and ipsilateral spinal cord.

3.2 Increase of TRPV1 protein expression in DRG in cancer pain rats Levels of TRPV1 in the DRGs at L4–L5 on days 1, 3, 7, 14, 21 and 28 after inoculation of MRMT-1 tumor cells into the tibia were measured by Western blot. Compared with the PBS and the heat-killed groups, TRPV1 protein expression increased significantly in the bone cancer pain group on day 21, and continued increasing to day 28. In addition, there was no difference in TRPV1 expression between the PBS and heat-killed groups at any of these time points (Fig. 1).

3.3 Formaldehyde treatment increased TRPV1 expression in primary cultured DRG neurons The expression of TRPV1 protein in primary cultured DRG neurons treated with formaldehyde (30, 100, and 300 μmol/L) was measured with Western blot at 4, 12, 24, 48 and 72 h of formaldehyde incubation. Compared with the control group, TRPV1 protein increased significantly at 48 h, and

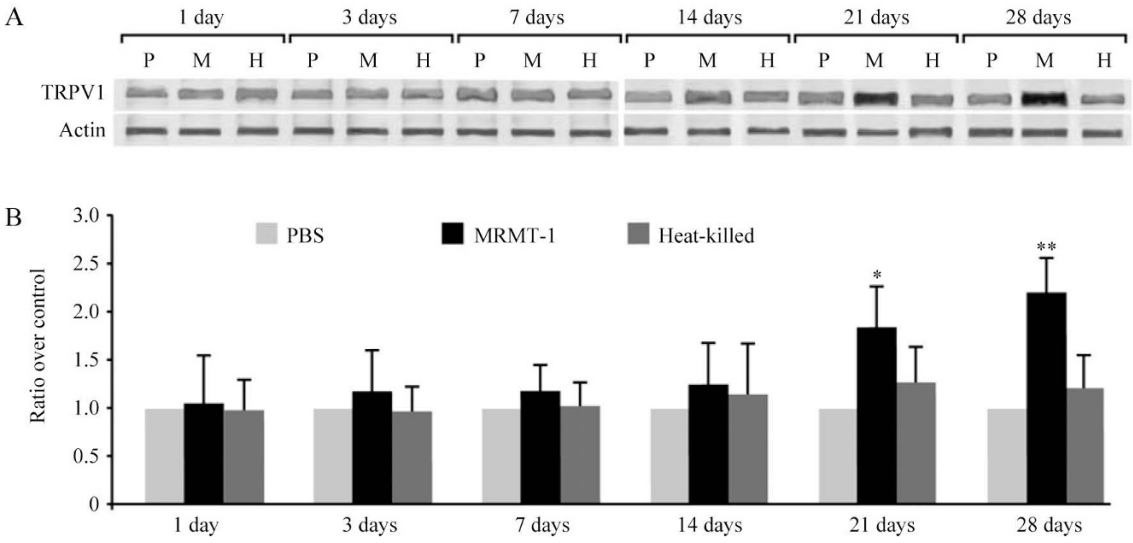

Fig. 1. TRPV1 protein expression increased in lumber (L) 4–L5 dorsal root ganglia in cancer pain rats, as detected by Western blot. A: Western blots of TRPV1 protein on days 1, 3, 7, 14, 21 and 28 following inoculation of MRMT-1 tumor cells into the tibia. P, PBS group; M, MRMT-1 live cells group; H, heat-killed MRMT-1 cells group. B: Statistical analysis of the relative densities of TRPV1 bands. Compared with the heat-killed or PBS group, TRPV1 protein increased significantly on day 21, and continued increasing to day 28 after inoculation of MRMT-1 cancer cells. *P <0.05, **P < 0.01 compared with control. n = 6 rats for each time point in each group.

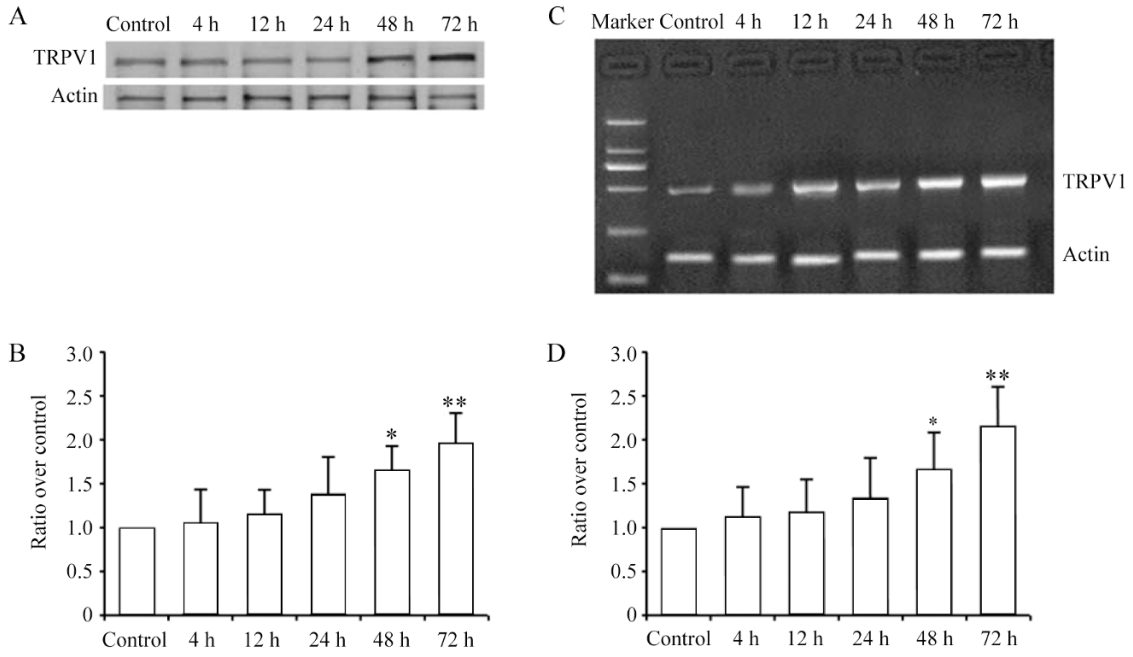

Fig. 2. Increase of transient receptor potential vanilloid subfamily member 1 (TRPV1) expression in primary cultured dorsal root ganglion neurons after formaldehyde incubation. A: Example of TRPV1 protein bands detected by Western blot. B: Statistical analysis of the relative amount of TRPV1 protein. C: Example of TRPV1 mRNA bands detected by RT-PCR. D: Statistical analysis of the relative amount of TRPV1 mRNA. Compared with the PBS control group, TRPV1 expression increased at 48 h of 100 μmol/L formaldehyde treatment, and further increased at 72 h. *P <0.05, **P <0.01 compared with control (n = 6).

further increased at 72 h of incubation in the 100 μmol/L formaldehyde group (Fig. 2A, B). Besides, there was no difference in TRPV1 protein expression between the groups treated with 30 and 300 μmol/L formaldehyde and the control group (data not shown).

In addition, TRPV1 mRNA levels detected by RT-PCR showed changes consistent with those of protein expression. In the 100 μmol/L formaldehyde group, TRPV1 mRNA increased at 48 h, and further increased at 72 h (Fig. 2C, D). Similarly, the expression of TRPV1 mRNA in the groups treated with 30 μmol/L and 300 μmol/L formaldehyde did not differ from that in the control group (data not shown).

3.4 Inhibitory effects of MAPK and PI3K inhibitors on formaldehyde-induced TRPV1 up-regulation in primary cultured DRG neurons After 72-h incubation of cultured DRG neurons with 100 μmol/L formaldehyde alone, TRPV1 protein expression increased significantly (Fig. 3). Compared with the formaldehyde-only group, the TRPV1 expression decreased after pre-incubation with the signal transduction pathway inhibitors PD98059, SB203580, SP600125 or LY294002. However, TRPV1 protein expression did not change after addition of BIM (Fig. 3A, B).

Furthermore, changes of TRPV1 mRNA expression in DRG neurons were consistent with those of TRPV1 protein expression. TRPV1 mRNA increased significantly 72 h after 100 μmol/L formaldehyde treatment. Compared with the formaldehyde-only group, TRPV1 mRNA expression decreased in the formaldehyde groups with pre-addition of the inhibitors PD98059, SB203580, SP600125 or LY294002, but did not change after addition of BIM (Fig. 3C, D).

4 Discussion

Formaldehyde is a consistent metabolic product of demethylation. Previous studies demonstrated that the formaldehyde level increases in some tissues and the blood of patients with bone cancer pain, but formaldehyde is

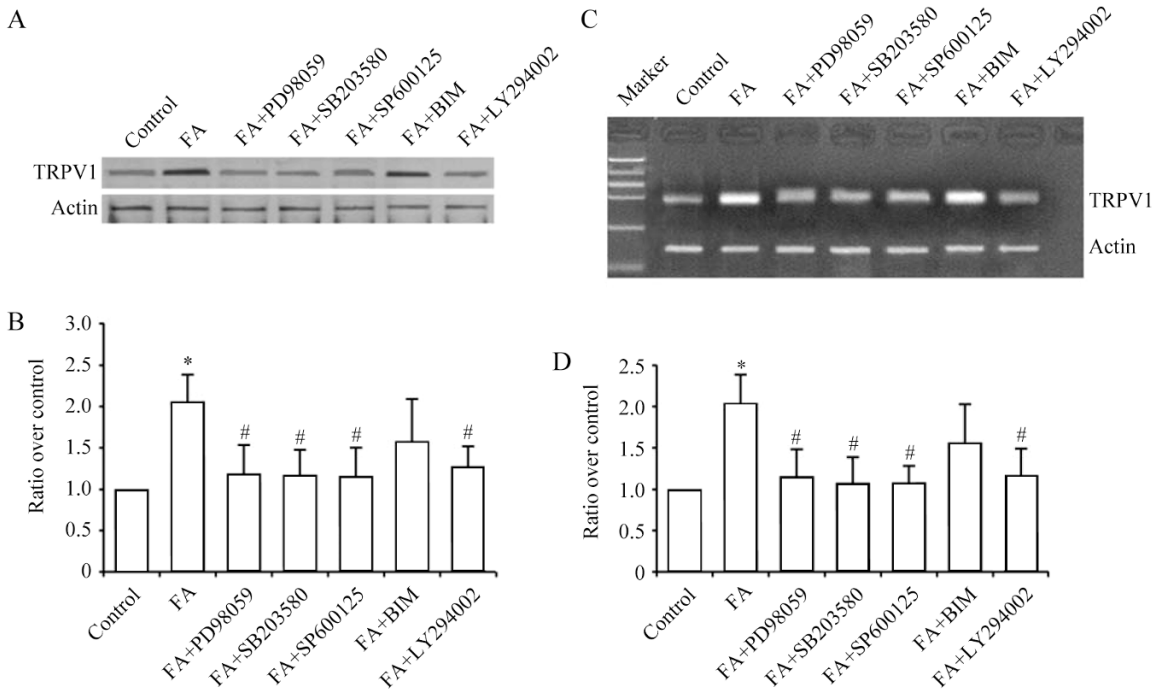

Fig. 3. Effects of signal transduction pathway inhibitors on formaldehyde (FA)-induced up-regulation of transient receptor potential vanilloid subfamily member 1 (TRPV1) expression in primary cultured dorsal root ganglion (DRG) neurons. A: Example of TRPV1 protein bands detected by Western blot. B: Statistical analysis of the relative band densities of TRPV1 protein. C: Example of TRPV1 mRNA bands detected by RT-PCR. D: Statistical analysis of the relative band densities of TRPV1 mRNA. TRPV1 expression increased significantly in DRG neurons 72 h after incubation with 100 μmol/L formaldehyde. Compared with formaldehyde alone, PD98059 (inhibitor for extracellular signal-regulated kinase), SB203580 (p38 inhibitor), SP600125 (inhibitor for c-Jun *N*-terminal kinase), and LY294002 (inhibitor for phosphatidylinositol 3-kinase) inhibited the formaldehyde-induced up-regulation of TRPV1 expression. BIM (inhibitor for protein kinase C) had no significant effect. *P <0.01 compared with control, $^{\#}P$ <0.05 compared with formaldehyde group (n = 6).

usually considered as a risk factor (a cause) in the development of cancer[4-7]. In the present study, formaldehyde concentrations in blood and tissues increased significantly in rats with bone cancer pain. This result is consistent with our previous finding that formaldehyde is increased in the cancer tissues of patients[8]. More importantly, this result in a rat model of cancer pain provides further support for the concept that the elevated formaldehyde is not only a "cause", but also, more interestingly, a "consequence" of the pathogenesis of cancer or bone cancer pain.

TRPV1 is expressed predominantly in peripheral nociceptors and participates in pain sensitization[32]. Our previous study found that cancer tissue-derived endogenous formaldehyde at low pathological concentrations in an acidic environment induces pain responses through TRPV1 activation in rats *in vivo*[8]. In the present study, our results showed that TRPV1 protein expression increased in rats with bone cancer pain, and formaldehyde incubation increased TRPV1 expression in cultured DRG neurons. Here, formaldehyde incubation with cultured DRG neurons mimics the contact of cancer tissue-derived formaldehyde with peripheral afferent nerve fibers innervating the tibia in the rat model of bone cancer pain. These results indicate that formaldehyde induces bone cancer pain not only by activating TRPV1 receptors (as in our previous report[8]), but also by up-regulating TRPV1 protein expression in peripheral nociceptors.

It is an interesting phenomenon that endogenous formaldehyde at very low concentrations up-regulates TRPV1 expression. To further investigate the mechanisms underlying this, we examined possible signaling transduction pathways involved in the process. The results showed that

application of MAPK and PI3K inhibitors suppressed the formaldehyde-induced increase of TRPV1 expression in cultured DRG neurons, while a PKC inhibitor did not. It is well known that MAPKs, PI3K and PKC play critical roles in cell signaling. MAPKs have three major family members, ERK, p38, and JNK, that represent three different signaling cascades[33]. TRPV1 expression can result from ERK and p38 signaling[34,35]. PI3K is another key mediator of central pain sensitization and of inflammatory heat hyperalgesia through TRPV1 sensitization[24,36]. So, the present study gives preliminary evidence that formaldehyde up-regulates TRPV1 expression through MAPK and PI3K signal pathways, but not the PKC signal pathway. However, more evidence is needed and related interesting questions, for example, interaction or cross-talk among these signaling pathways, are worthy of further investigation.

Previous studies showed that MAPKs, PI3K and their downstream kinases in primary sensory neurons are involved in pain[20,21]. MAPK and PI3K pathways are usually targets of activating factors in the tumor microenvironment, and increased formaldehyde activates MAPK and PI3K/AKT signal pathways[37]. PI3K in primary DRG sensory neurons is activated by inflammatory agents, and PI3K inhibitors inhibit chemical- and nerve injury-induced pain hypersensitivity, suggesting that PI3K activation contributes to pain[24,38].

We note possible differences between the responses of DRG neurons to formaldehyde in two-week-old rat culture and those in adult animals. We chose two-week-old culture because long-term culture of the adult DRG is technically very difficult.

In summary, on the basis of our previous report that tumor tissue-derived endogenous formaldehyde at very low concentrations induces cancer pain through activation of TRPV1, the present study provides further evidence that formaldehyde at a low pathological concentration up-regulates TRPV1 expression through MAPK and PI3K signaling pathways, but not the PKC pathway, in the pathogenesis of bone cancer pain.

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