## PERSPECTIVES

# Molecular Events of Acid-Induced Bone Pain

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**Abstract**

Pain is one of the most common and feared complications in patients with any disease. Pain causes discomfort, depression and anxiety and occasionally induces secondary diseases due to its immunosuppressive effects, making quality of life and prognosis worse. Pain is triggered following the recognition of local noxious stimuli by specialized afferent sensory neurons called nociceptors. The nociceptors can sense diverse noxious stimuli including thermal, mechanical and chemical agents that are released from inflammatory cells, immune cells, cancer cells and/or bone-destroying osteoclasts invading disease sites and injured tissues. Protons are one of these noxious stimuli and have long been known as a cause of pain. Sensory neurons innervating peripheral tissues can sense protons via acid-sensing nociceptors such as transient receptor potential channel vanilloid subfamily members. Noxious acid stimulus received by these nociceptors subsequently activates intracellular signaling pathways and transcription factors, leading to the release of neurotransmitters, including calcitonin gene-related peptide, in sensory neuronal cells. This *Perspective* describes intracellular molecular events propagated in sensory neurons as a consequence of acid activation of nociceptors, with a special emphasis on bone pain. Understanding the molecular events underlying acid-induced bone pain may lead to the design of novel mechanism-based approaches for the management of bone pain associated with inflammation and cancer metastasis. *IBMS BoneKEy*. 2011 April;8(4):195-204.
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## Introduction

By definition, pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (as defined by the International Association for the Study of Pain) and serves as a warning to detect and avoid harmful stimuli. Children with congenital insensitivity to pain due to loss-of-function mutations of pain-sensing receptors do not survive long (1). On the other hand, pain is one of the most common and feared complications in patients with any disease. It has been estimated that more than one-third of people in the world experience persistent or recurrent pain (2). Pain causes discomfort, depression and anxiety and has a significant influence on quality of life (QOL) and prognosis of patients. Of note, patients occasionally

become susceptible to secondary diseases due to the immunosuppressive effects of pain. It is estimated that 100 million people in the U.S. suffer from chronic pain, costing the U.S. approximately $79 billion each year due to caring for patients and loss in work productivity (3). Patients with advanced cardiopulmonary diseases, infection or renal failure suffer from deleterious acute pain, while individuals with back injuries, migraine headaches, arthritis, osteoporosis, herpes zoster, diabetic neuropathy, and temporomandibular joint syndrome suffer from chronic pain.

## Transduction of Pain

Noxious stimuli are recognized and converted into electrochemical signals by specialized afferent sensory neurons called "nociceptors." The nociceptors can sense diverse noxious stimuli including thermal, mechanical and chemical agents that are released by injured tissues or inflammatory cells and immune cells. In the case of bone pain associated with bone metastases, bone-destroying osteoclasts and metastatic cancer cells produce a variety of noxious stimuli as well. These peripheral algesic signals are finally transmitted to the central nervous system (CNS) and brain via the dorsal root ganglia (DRG, primary afferent neurons) and subsequently the spinal cord (secondary afferent neurons) (Fig. 1) (4;5).
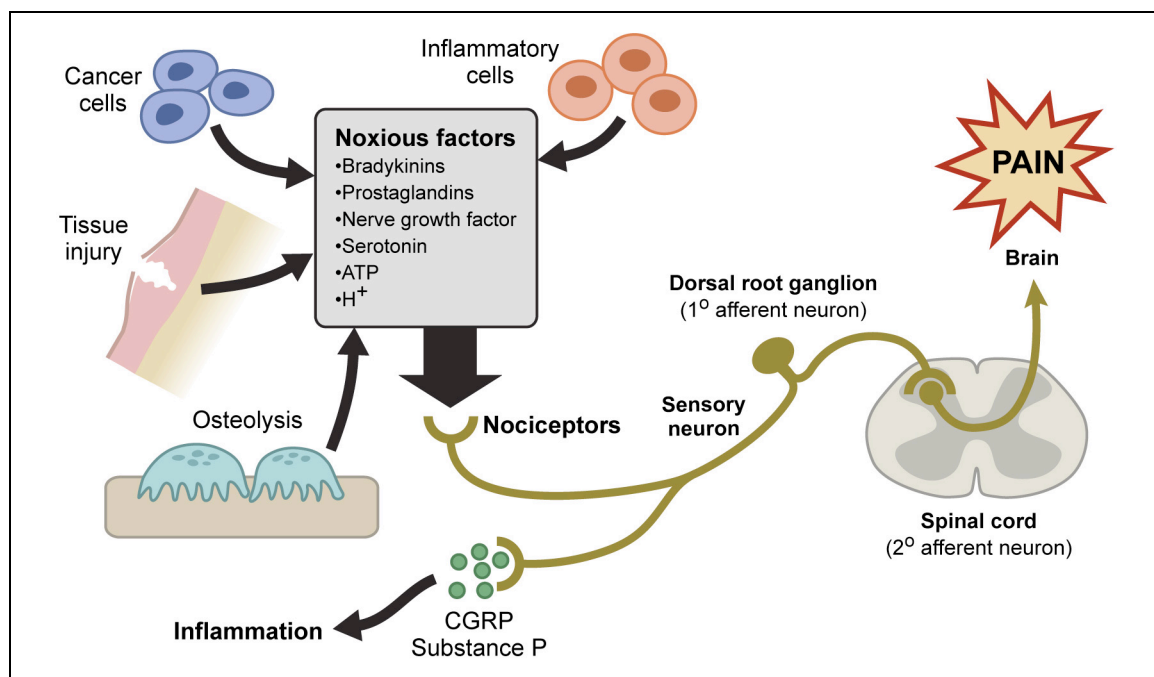


Fig. 1. Transduction of pain signals. Noxious factors released from cancer cells, inflammatory cells and bone-resorbing osteoclasts or injured soft tissues at peripheral local sites are sensed by nociceptors expressed on sensory neurons and are converted into electrochemical signals. Noxious signals are subsequently transmitted to the spinal cord (secondary afferent neurons) via the sensory neuron cell body dorsal root ganglia (DRG, primary afferent neurons) and finally reach the brain. Concomitantly, DRG produce neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P, which are released at peripheral sites via efferent sensory neurons and exacerbate inflammation. Adapted and modified from (4), with permission from Macmillan Publishers Ltd.

DRG are cell bodies that bi-directionally develop afferent and efferent sensory neuronal fibers and play a role as the gateway for peripheral noxious signals, relaying those signals to central neurons.

Accordingly, DRG can represent noxious events taking place at peripheral sites. In response to peripheral noxious stimuli, DRG produce neurotransmitters including calcitonin gene-related peptide (CGRP) and

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substance P, which in turn enhance transduction of pain signals to the CNS via afferent neurons and exacerbate inflammation at peripheral sites via efferent neurons.

*Calcitonin gene-related peptide*

CGRP is a neuropeptide that is widely distributed in the peripheral and central nervous systems. It has been proposed that the release of CGRP from perivascular nerves causes vasodilatation resulting in neurogenic inflammation. CGRP released into the dorsal spinal cord is associated with nociceptive transmission (6). CGRP levels in the circulation are elevated during migraines and cluster headache attacks (7;8), as well as in myocardial infarction (9). Interestingly, recent studies have demonstrated that CGRP knockout mice show an attenuated response to chemical pain and inflammation (10). Collectively, these results indicate the important role of CGRP in the regulation of pain. Consequently, CGRP has been used as a marker of pain. We have examined CGRP expression and production in sensory neurons following noxious agent stimulation of nociceptors as an indicator of pain.

**Bone Pain**

Bone pain is most frequently associated with bone metastases. Over 80% of cancer patients with bone metastases experience intolerable bone pain during the clinical course of the illness (5;11-13). Bone pain is most frequently seen in lumbar bones, the pelvis and femoral bones that bear mechanical or physical load. Of note, the anatomical location, size and number of tumors and the extent of bone destruction are not necessarily correlated with the severity of bone pain. Bone pain seriously and devastatingly affects QOL, causing diverse and complex problems in the management of cancer patients. Moreover, a recent study has reported that survival is worse in breast cancer patients with skeletal metastases and bone pain than in those without bone pain (14). However, our understanding of the molecular mechanisms underlying bone pain is still very poor. More importantly, therapies currently available for

bone pain are inadequate and ineffective or cause uncomfortable adverse effects. Elucidation of the mechanisms underlying bone pain is therefore of pressing importance.

There have been several classical mechanisms of cancer-associated bone pain. Microfractures resulting from decreased bone density and/or disrupted bone architecture due to increased osteoclastic bone resorption; stretching of the periosteum by tumor expansion in the bone marrow cavity that in turn irritates the nociceptive sensory neurons innervating periosteal surfaces; and direct nerve injury by cancer invasion have all been proposed (11). However, the observations that the severity of bone pain is not correlated with clinical and pathological features of cancer and the extent of osteolysis, and that not all bone cancers cause bone pain, indicate the heterogeneity and complexity of underlying mechanisms of bone pain. Furthermore, bone pain in patients with prostate cancers who develop osteosclerotic bone metastases (15) suggests a mechanism(s) other than cancer-associated osteolysis.

In addition to these classical mechanisms, excitement or sensitization of nociceptors by increased production of noxious substances by cancer cells and inflammatory cells has also been implicated in bone pain. We are particularly interested in protons as a noxious stimulus involved in bone pain associated with bone metastases.

**The Acidic Microenvironment in Bone Metastases**

One condition that progresses in bone metastases is local acidosis. Bone-resorbing osteoclasts (16), metastatic cancer cells (17-19) and inflammatory cells (4;20) all produce protons, making the bone microenvironment acidic. In fact, using acridine orange staining, we have found that the bone in which cancer cells progressively grew and osteoclastic bone destruction progressed was acidic (Fig. 2). Local acidosis is a well-known cause of pain (4;5).
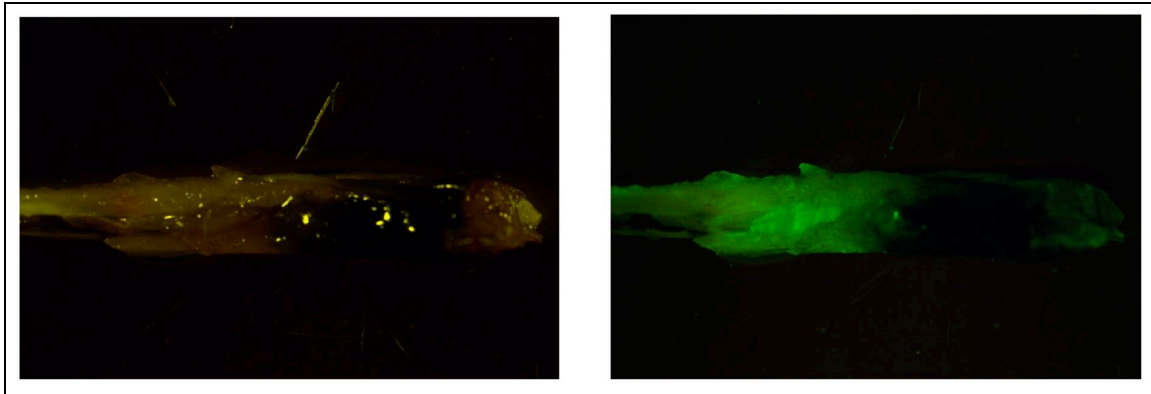
Fig. 2. The acidic microenvironment in bone colonized by cancer cells. B16-F10 melanoma cells were injected into the left cardiac ventricle in C57BL/6 mice. Two weeks after cell inoculation, acridine orange (1.0 mg/kg) was injected intravenously via the tail vein. Animals were sacrificed after 2 hours and excised bones were examined by fluorescence stereoscopic microscopy. Bone with cancer cell colonization (right side) and osteoclastic bone destruction shows green fluorescence indicating local acidosis, whereas control bone (left side) shows no fluorescence.

### Generation of an acidic microenvironment by osteoclasts

Osteoclasts are increased in number in bone metastases and are activated to destroy bone under the influence of metastatic cancer cells. Increased bone resorption, in turn, stimulates the growth, survival and metabolic activity of metastatic cancer cells, leading to the progression of bone metastases (an interactive process referred to as "the vicious cycle") (21-23). Honore *et al*. (24) have reported that bone cancer pain is different from inflammatory pain or neuropathic pain at the molecular level. The involvement of osteoclasts may be a reason why bone cancer pain is unique, difficult to treat and more deleterious than cancer pain in soft tissues (13).

Osteoclastic bone resorption consists of two steps including dissolution of bone mineral and degradation of bone matrix proteins (25;26). Dissolution of bone mineral is mediated by acidification of the resorption compartment (16). The decrease in pH is caused by active proton secretion into the extracellular space that is driven by the a3 isoform of vacuolar-$H^+$-ATPase (a3V-ATPase) localized in the ruffled border of osteoclasts (27). Suppression of a3V-ATPase by bafilomycin A1, a non-selective inhibitor of V-ATPase, impaired local acidosis and inhibited bone resorption *in vitro* (28). Furthermore, specific inhibitors of V-ATPase including SB242784 (29) and FR167356 (30) were also shown to inhibit local acidosis and prevent bone loss through inhibition of bone resorption *in vivo*. These results indicate that local acidosis due to proton secretion through V-ATPase is critical for osteoclasts to resorb bone.

Many clinical studies have demonstrated that a variety of structurally and mechanistically distinct inhibitors of osteoclasts such as bisphosphonates (BPs) (31-34) and calcitonin (35) significantly reduce bone pain in patients with cancer and osteoporosis. Moreover, experimental studies have reported that osteoprotegerin (OPG), a specific natural inhibitor of osteoclasts, also reduces bone cancer pain *in vivo* (36;37). Together these results suggest a potential role for osteoclasts in the pathophysiology of bone pain.

We have reported that zoledronic acid, alendronate and OPG decrease bone pain in chemical-induced inflammation (38). Zoledronic acid also inhibited bone pain caused by the presence of cancer cells in bone (39). Inflammation-associated pain was reduced in c-Src-deficient mice in which osteoclasts were dysfunctional (38). Furthermore, stimulation of osteoclastic bone resorption by parathyroid hormone-related peptide increased hyperalgesia and this increase was reduced by zoledronic acid (manuscript in

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preparation). More recently, we have found that bafilomycin A1 (38) and FR167356 (manuscript in preparation) significantly reduced inflammatory bone pain and bone cancer pain. Collectively, these results suggest that the a3V-ATPase in osteoclasts is a critical player in the pathophysiology of inflammatory bone pain and bone cancer pain.

*Generation of an acidic microenvironment by cancer cells*

Cancer cells express V-ATPase in the plasma membrane (40) and the extracellular pH of various human cancers is acidic (17-19). Highly metastatic human breast cancer cells displayed greater V-ATPase activity (41) and lower extracellular pH (42) than that of low-metastatic human breast cancer cells. Inhibition of V-ATPase activity by small interfering RNA suppressed growth and metastasis of human hepatocellular carcinoma (43).

We have recently demonstrated that metastatic B16-F10 melanoma cells show increased expression of a3V-ATPase compared with non-metastatic parental B16 cells (manuscript in preparation). The extracellular pH of metastatic B16 melanoma cells is acidic and knock-down of a3V-ATPase significantly inhibited distant metastases, suggesting that an acidic condition due to increased proton pump expression facilitates distant metastases. It needs to be shown whether the local acidosis created by bone-colonizing cancer cells plays a role in bone pain.

*Generation of an acidic microenvironment by inflammatory cells*

Accumulated lines of evidence indicate that there are close links between cancer and inflammation (44). Cancers frequently develop at the site of chronic inflammation and inflammatory cells are always present in cancers. Non-steroidal anti-inflammatory drugs decrease the risk of and mortality from some types of cancers.

Inflammatory cells, including neutrophils, express vacuolar $H^+$ATPase and pump protons, creating acidic conditions (20). Consistent with these earlier reports, using a mouse model of chemical-induced inflammatory bone pain, we recently observed that legs with inflammation were acidic by acridine orange staining. Furthermore, bafilomycin A1 inhibited bone pain in these legs (38). These results suggest that inflammatory cells are also involved in bone pain through creation of an acidic microenvironment by producing protons.

## Transient Receptor Potential Channel Vanilloid Subfamily Members

The nociceptors that sense local acidosis are the acid-sensing nociceptors (ASNs). An ASN that may be involved in sensing acid in bone metastases is the transient receptor potential channel vanilloid subfamily member 1 (TRPV1)/capsaicin receptor (45) that was originally identified by Caterina *et al*. (46). It is one of six subfamily members and a putative six-transmembrane-spanning protein with a pore loop and three ankyrin repeats in the N-terminal region. TRPV1 is a non-selective calcium-preferential cation channel that is activated by acid (pH < 6.0) and heat (> 42°C) and is the only channel that is excited by the vanilloid capsaicin (46).

*TRPV1 expression in bone*

CGRP-positive sensory neurons innervating bone co-expressed TRPV1 as assessed by immunohistochemistry (47). Similarly, co-expression of CGRP and TRPV1 was found in DRG in organ culture (47).

*TRPV1 activation by acid*

TRPV1 activation via phosphorylation by acid induces $Ca^{2+}$ influx into neurons, leading to activation of $Ca^{2+}$-mediated signaling pathways including $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) (48) and protein kinase C (PKC) isoenzymes (49). We have found recently that acidic stimulation elevated the activity of CaMK II and the transcription factor cAMP response element binding protein (CREB), followed by increased production of CGRP in DRG in organ culture (Fig. 3) (47). The

selective TRPV1 antagonist I-RTX and non-selective antagonist capsazepine significantly inhibited the increase in CGRP mRNA expression and protein production caused by acid stimulation (47). These results suggest that cascades of events including acid activation of TRPV1, $Ca^{2+}$ influx into the cytoplasm, CaMK II activation, stimulation of CREB transcription activity and increased CGRP production are critical in pain caused by acidic conditions such as bone metastases and inflammation.
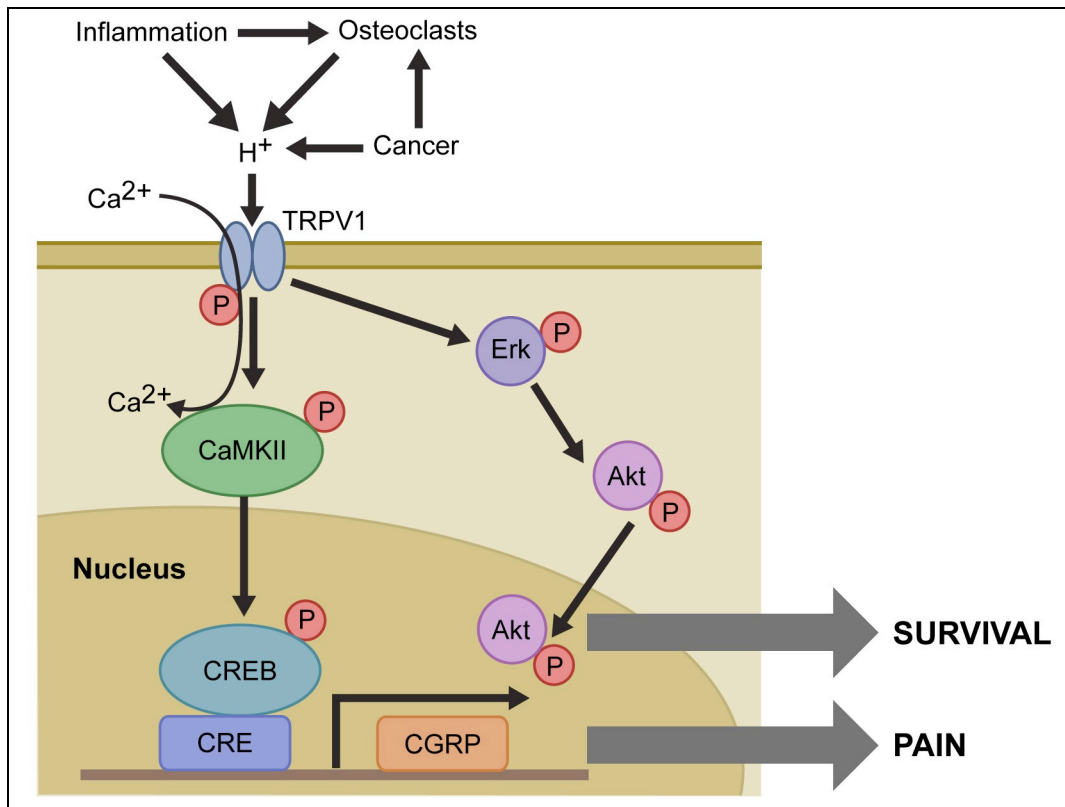


Fig. 3. Intracellular noxious events following acid stimulation. Activation of the TRPV1 acid-sensing nociceptor induces $Ca^{2+}$ influx causing activation of CaMK II by phosphorylation, followed by increased transcriptional activity of CREB, leading to up-regulation of CGRP production in DRG sensory neurons. CGRP is a neurotransmitter involved in nociceptive transmission via afferent sensory neurons and exacerbation of inflammation at peripheral sites via efferent sensory neurons.

### TRPV1 up-regulation by bone-derived growth factors

TRPV1 mRNA expression in DRG was up-regulated in the presence of cancer cells in bone (39). The mechanism of this up-regulation is unknown. Of interest, however, we have shown recently that conditioned medium harvested from resorbing calvarial bone increased TRPV1 mRNA expression in DRG in organ culture. This increase was diminished by treatment with AG1024, a specific inhibitor of IGF tyrosine kinase. Recombinant IGF-1, but not TGFβ, augmented TRPV1 mRNA expression in DRG (manuscript in preparation). These results raise the possibility that TRPV1 expression is increased by growth factors such as IGF-1 that are released from bone as a consequence of increased osteoclastic bone resorption. Up-regulation of TRPV1 expression by bone-derived growth factors might be a reason why bone pain is progressive, sustained and intolerable in some cancer patients.

## Conclusion

The breakthrough discovery of TRPV1 acid-sensing nociceptors in 1997 (46) enabled investigators for the first time to study the role of protons/acid in the pathophysiology of pain at cellular and molecular levels. Protons/acid are of particular importance in causing bone pain associated with bone metastases, since osteoclasts produce large amounts of protons during bone destruction and it has long been known that inhibitors of osteoclasts reduce bone pain. In addition, cancer cells metastasizing to bone and accompanying inflammatory cells also release protons. Thus, the study of bone pain associated with bone metastases will provide an opportunity to analyze the interactions between protons/acid and TRPV1 activation at the molecular level. The combination of suitable animal models of pain (50) with these molecular studies will enable investigators to establish a unique translational research field in cancer-related bone diseases. Moreover, it is also intriguing to study the role of protons/acid produced by bone-resorbing osteoclasts in the regulation of bone metabolism, since extracellular acidification has been shown to be essential for normal bone remodeling (51) and bone cell function (52).

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**Peer Review:** This article has been peer-reviewed.

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