

# Effects of Adipokines and Insulin on Intracellular pH, Calcium Concentration, and Responses to Hypo-Osmolarity in Human Articular Chondrocytes from Healthy and Osteoarthritic Cartilage

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## Abstract

**Objective:** To evaluate the effects of adipokines and insulin on intracellular calcium concentration ( $[Ca^{2+}]_i$ ) and pH ( $pH_i$ ) in human articular chondrocytes from healthy (CHC) and osteoarthritic cartilage (COC). **Design:**  $pH_i$  and  $[Ca^{2+}]_i$  were measured using BCECF and Fura-2 fluorometric probes in CHC and COC under control conditions and following a hypotonic shock. The effects of interleukin-1 $\beta$  (IL1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), insulin, leptin, resistin, and adiponectin were assessed. **Results:**  $pH_i$  was lower in COC than in CHC. Only IL1 $\beta$  decreased  $pH_i$  in both cell types; all the agents enhanced  $pH_i$  recovery following an ammonium prepulse in CHC, effect that was attenuated by Na<sup>+</sup>-H<sup>+</sup> exchanger inhibitors, but they had no effect in COC. Hypotonic shock (HTS) caused a  $pH_i$  increase, which was significantly smaller in COC. All the hormones attenuated this response and the effect of IL1 $\beta$  was greater. The basal  $[Ca^{2+}]_i$  was similar in COC and CHC; IL1 $\beta$ , TNF $\alpha$ , and insulin increased the  $[Ca^{2+}]_i$ , but leptin, resistin, and adiponectin did not. These effects were greater in COC. This  $[Ca^{2+}]_i$  increase was dependent on extracellular Ca<sup>2+</sup> and attenuated by Na<sup>+</sup>-Ca<sup>2+</sup> exchanger inhibitors. HTS caused a  $[Ca^{2+}]_i$  increase, which was inhibited by transient receptor potential vanilloid blockers and attenuated by all the hormones tested with the exception of adiponectin. **Conclusions:** These findings may help explain the association between obesity and osteoarthritis, in which these hormones are altered. The responses of CHC and COC are different, which suggests that a modification of pH and Ca<sup>2+</sup> homeostasis is part of the osteoarthritis pathophysiology.

## Keywords

osteoarthritis, adipokines, insulin, calcium, obesity, pH

## Introduction

Osteoarthritis (OA) is a disabling disease that develops because of a misbalance in the synthesis and breakdown of the cartilage matrix, which is performed by articular chondrocytes<sup>1,2</sup> and is a key factor in maintaining the characteristics of cartilage, in response to a mechanical load, thus leading to a dysfunctional tissue, inflammation, pain, and the functional impairment of joints.<sup>3,4</sup> OA has a growing prevalence all over the world and represents a burden on health services.<sup>5,6</sup>

Osteoarthritis is associated with obesity,<sup>7,8</sup> and this condition worsens the clinical course of OA.<sup>5,9</sup> Furthermore, several adipokines have been related to OA. Obesity is associated with an increase in leptin and resistin levels, a decrease in adiponectin levels and an increase in pro-inflammatory cytokines, such as interleukin-1 $\beta$  (IL1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ).<sup>10,11</sup> The interplay of these hormones is an important issue in understanding the pathophysiological

process of obesity and metabolic syndrome<sup>12,13</sup> and all the other diseases associated with these conditions, such as OA.<sup>14</sup> For instance, pro-inflammatory cytokines have been clearly associated with OA pathophysiology because these cytokines activate matrix metalloproteases (MMPs), induce the production of free oxygen species and prostaglandins and reduce the expression of collagen type II and aggrecan genes.<sup>15</sup> Specifically, IL1 $\beta$  has been found in osteoarthritic synovial fluid and IL1 $\alpha$  may produce impairments in the homeostasis of intracellular calcium and pH in chondrocytes.<sup>16,17</sup>

Moreover, insulin increases collagen type II synthesis in human healthy and osteoarthritic chondrocytes,<sup>18</sup> but does

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not change aggrecan expression; insulin also enhances GLUT1 expression, particularly in normal chondrocytes.<sup>19</sup> In cartilage explants, insulin stimulates proteoglycan synthesis; inhibits aggrecanase, prostaglandin and nitric oxide production; and overcomes the inflammatory effects of IL1 $\beta$ .

Adipokines also play a role in the pathophysiology of OA.<sup>20,21</sup> A number of studies have found a relationship between increased plasma and articular levels of some adipokines and OA.<sup>22</sup> The cause of the increase of these adipokines in synovial fluid is not clear, although the increase could be attributable to various factors, such as greater synovial membrane permeability, the production of inflammatory agents by synoviocytes and the synthesis of adipokines by the infrapatellar fat pad, in the case of knee OA.

Leptin can be produced by chondrocytes and synoviocytes and can also act in these same cells.<sup>23,24</sup> At low doses, leptin induces the growth and proliferation of chondrocytes and extracellular matrix synthesis. At high doses, such as those found in obesity, leptin is proinflammatory, activates MMPs, induces the production of free oxygen species and alters chondrocyte metabolism, thereby inducing extracellular matrix catabolism.<sup>25</sup> Adiponectin also promotes matrix catabolism and has been correlated with the severity of knee OA and aggrecan degradation.<sup>26</sup> Intriguingly, in almost all tissues, adiponectin is anti-inflammatory and protective, even in cartilage<sup>27</sup>; there are some studies describing an inhibitory effect of adiponectin on MMPs and pro-inflammatory cytokines. Resistin has inflammatory effects in cartilage; induces the expression of other pro-inflammatory cytokines, MMPs, and free oxygen radicals; and promotes matrix catabolism.<sup>28</sup> This hormone has also been associated with a more severe OA. IL1 $\beta$ , a pro-inflammatory cytokine that is also released from adipocytes in obesity and is increased in the synovial fluid of osteoarthritic joints, produces an increase in intracellular calcium in chondrocytes, alters intracellular pH in these cells and may modify chondrocyte homeostasis in osteoarthritic cartilage.<sup>29</sup>

However, the effects of other adipokines on the ionic homeostasis of chondrocytes have not been explored although intracellular composition may regulate the extracellular matrix metabolism.<sup>30</sup> This study aims to evaluate the effects of a number of adipokines on  $[Ca^{2+}]_i$  and  $pH_i$ , and the pathways involved in those effects, in articular chondrocytes because these specific factors may regulate matrix turnover and are determining in the chondrocyte homeostasis.<sup>30-32</sup> The effects of these agents on the response to a hypo-osmotic challenge were also evaluated, given that chondrocytes are subjected to fluctuations in extracellular osmolarity during joint movement, which result from the fluid flux following changes in mechanical load.<sup>33</sup> Furthermore, changes in  $[Ca^{2+}]_i$  and  $pH_i$  can be produced in response to these osmotic challenges, as shown in bovine<sup>34-36</sup> and equine<sup>37</sup> chondrocytes.

## Method

### Cartilage and Isolation of Chondrocytes

This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and was approved by the Bioethics Committee of the Universidad Tecnológica de Pereira. Cells were isolated from knee or hip load-bearing cartilage obtained from patients who were undergoing joint replacement surgery because of OA and who signed an informed consent form, regardless of gender or age. The diagnosis of severe OA was made by the orthopedic surgeon via arthroscopy and was confirmed by the joint's macroscopic appearance during surgery; only the severely affected areas were sampled. The healthy cartilage was obtained from patients without macroscopic signs of degeneration who were subjected to surgery because of trauma, and the cartilage was extracted without any damage to the donor.

Cells were isolated using a method modified from the optimized low-temperature protocol described by Hidvegi *et al.*<sup>38</sup> Briefly, the cells were isolated by trypsin predigestion (0.5% in HEPES-buffered saline [HBS] for 15 minutes at 37°C) followed by digestion with a mixture of type I and type II collagenase (2000 U/mL each in HBS for 20 hours at 27°C); after the filtration of the isolation solution, the cells were resuspended in fresh 300 mOsm/L Dulbecco's modified Eagle's medium (DMEM) prior to resuspension in the experimental media. Previous studies have demonstrated that there are no functional alterations in human and bovine chondrocytes following the digestion protocol.<sup>17,35,38-41</sup> All the experiments were performed on freshly isolated cells.

### Media and Chemicals

All the chemicals and solutions were obtained from Sigma-Aldrich (St Louis, MO), unless otherwise stated. The cartilage slices and isolated chondrocytes were incubated in DMEM and supplemented with glutamine (2 mmol/L) and antibiotics (100  $\mu$ mol/L penicillin and 100  $\mu$ mol/L streptomycin; Life Technologies, Grand Island, NY).

The HBS solution contained (in mmol/L) 145 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 15 HEPES, and 10 glucose with the pH adjusted to 7.4 at 25°C using 1 M NaOH. The Ca<sup>2+</sup>-free solution lacked CaCl<sub>2</sub>, and EGTA (1 mmol/L) was added. For the Na<sup>+</sup>-free solution, NaCl was replaced with NMDG-Cl at the same concentration. Hypotonic shock (HTS), 290 to 145 mOsm/L, was induced by a 50% dilution of the solution with distilled water, which was abruptly added to the cell suspension.

Stock solutions of insulin (172 mmol/L), TNF $\alpha$  (2  $\mu$ g/mL), IL1 $\beta$  (0.1 mg/mL), and adiponectin (0.5 mg/mL) were dissolved in phosphate-buffered saline solution (PBS).

Resistin (0.5 mg/mL) was dissolved in a 25 mM Tris and 25 mM NaCl solution with a pH of 7.5, and leptin (1 mg/mL) was dissolved in a 15 mmol/L HCl and 7.5 mmol/L NaOH solution with a pH of 8.0. All the solutions were aliquoted and stored at  $-20^{\circ}\text{C}$ .

### Measurement of Intracellular pH

The pH was measured according to a method previously described using the pH-sensitive fluorescent dye BCECF.<sup>42</sup> Briefly, the isolated chondrocytes were preacidified with an ammonium prepulse (20 mmol/L  $\text{NH}_4\text{Cl}$  in HBS, 15 minutes), to evaluate the  $\text{pH}_i$  recovery, as described by Tattersall *et al.*,<sup>17</sup> the cells were then suspended in HBS and loaded by incubation with BCECF-AM (10  $\mu\text{mol/L}$  for 20 minutes at  $37^{\circ}\text{C}$ ) before the fluorescence emission was recorded for 300 seconds at  $37^{\circ}\text{C}$  with magnetic stirring (FP-6500 spectrophotometer, Jasco, Tokyo, Japan). The dye was alternately excited at 490 and 439 nm, and the fluorescence emission was measured at 535 nm. The 490/439 nm signal ratio was calibrated previously using the nigericin/high  $\text{K}^+$  method described by Thomas *et al.*,<sup>43</sup> before each experiment with a new set of samples. The  $\text{pH}_i$  recovery was measured in cells that had been pre-incubated for 1 hour with all the hormones tested.

The acid equivalent fluxes ( $J_{\text{H}^+}$ , mM/min) were calculated for the first 300 seconds of recovery using the following equation:

$$J_{\text{H}^+} = \Delta\text{pH}_i / \Delta t(\beta_i),$$

where  $\beta_i$  corresponds to the buffering power, which is  $-43.3\text{pH}_i + 377$  for  $\text{pH}_i$  values between 6.4 and 7.2 for this type of cell as described by Browning *et al.*<sup>44</sup>

To test the effects of the main inhibitors of the pathways involved in  $\text{H}^+$  transport, the cells were resuspended in a  $\text{Na}^+$ -free solution or in HBS supplemented with  $\text{Na}^+$ - $\text{H}^+$  exchanger (NHE) inhibitors, HOE694 (10  $\mu\text{mol/L}$ , specific for NHE1) or amiloride (1 mmol/L, a nonspecific dose) or with the  $\text{H}^+$ -ATPase inhibitors NBD-Cl (chloro-7-nitrobenz-2-oxa-1,3-diazole; 100  $\mu\text{mol/L}$ ) or bafilomycin A1 (200 nmol/L). In all cases, chondrocytes were loaded with BCECF-F before being exposed to drugs.

### Measurement of Intracellular Calcium Concentration ( $[\text{Ca}^{2+}]_i$ )

The previously isolated cells were loaded with Fura-2 (5  $\mu\text{mol/L}$ ) by incubation in HBS for 30 minutes at  $20^{\circ}\text{C}$  followed by 15 minutes at  $37^{\circ}\text{C}$ .<sup>45</sup> The cell suspension was then centrifuged, and the cells were resuspended in the appropriate experimental medium before being transferred to a cuvette. Fluorometer measurements were recorded for 300 seconds at  $37^{\circ}\text{C}$  with magnetic stirring (FP-6500

spectrophotometer, Jasco, Tokyo, Japan). The dye was alternately excited at 380 and 340 nm, and the fluorescence emission was measured at 510 nm. The 380/340 nm signal ratio was calibrated before every experiment using the Grynkiewicz *et al.* method.<sup>46</sup> Briefly, the fluorescence ratio was measured in HBS lacking  $\text{CaCl}_2$  and supplemented with EGTA (1 mmol/L) and also in a 2 mmol/L  $\text{Ca}^{2+}$  solution of HBS supplemented with ionomycin (300 nmol/L), a concentration of  $\text{Ca}^{2+}$  at which Fura-2 is saturated. Maximal and minimal ratios ( $R_{\text{max}}$  and  $R_{\text{min}}$ ) were obtained under these 2 conditions, and the  $[\text{Ca}^{2+}]_i$  values were derived using the following equation:

$$[\text{Ca}^{2+}]_i = K_d ([R - R_{\text{min}}] / [R_{\text{max}} - R]) (\text{Sf}_2 / \text{Sb}_2),$$

where  $K_d$  is the dissociation constant for Fura-2 (224 nmol/L),  $R$  is the experimentally measured ratio,  $\text{Sf}_2$  is the fluorescence measured at 380 nm under the  $\text{Ca}^{2+}$ -free condition, and  $\text{Sb}_2$  is the fluorescence measured at 380 nm with saturating  $\text{Ca}^{2+}$  (2 mmol/L).

The  $[\text{Ca}^{2+}]_i$  was measured in cells preincubated for 1 hour with all the hormones tested. In all cases chondrocytes were loaded with Fura-2 before being exposed to these agents.

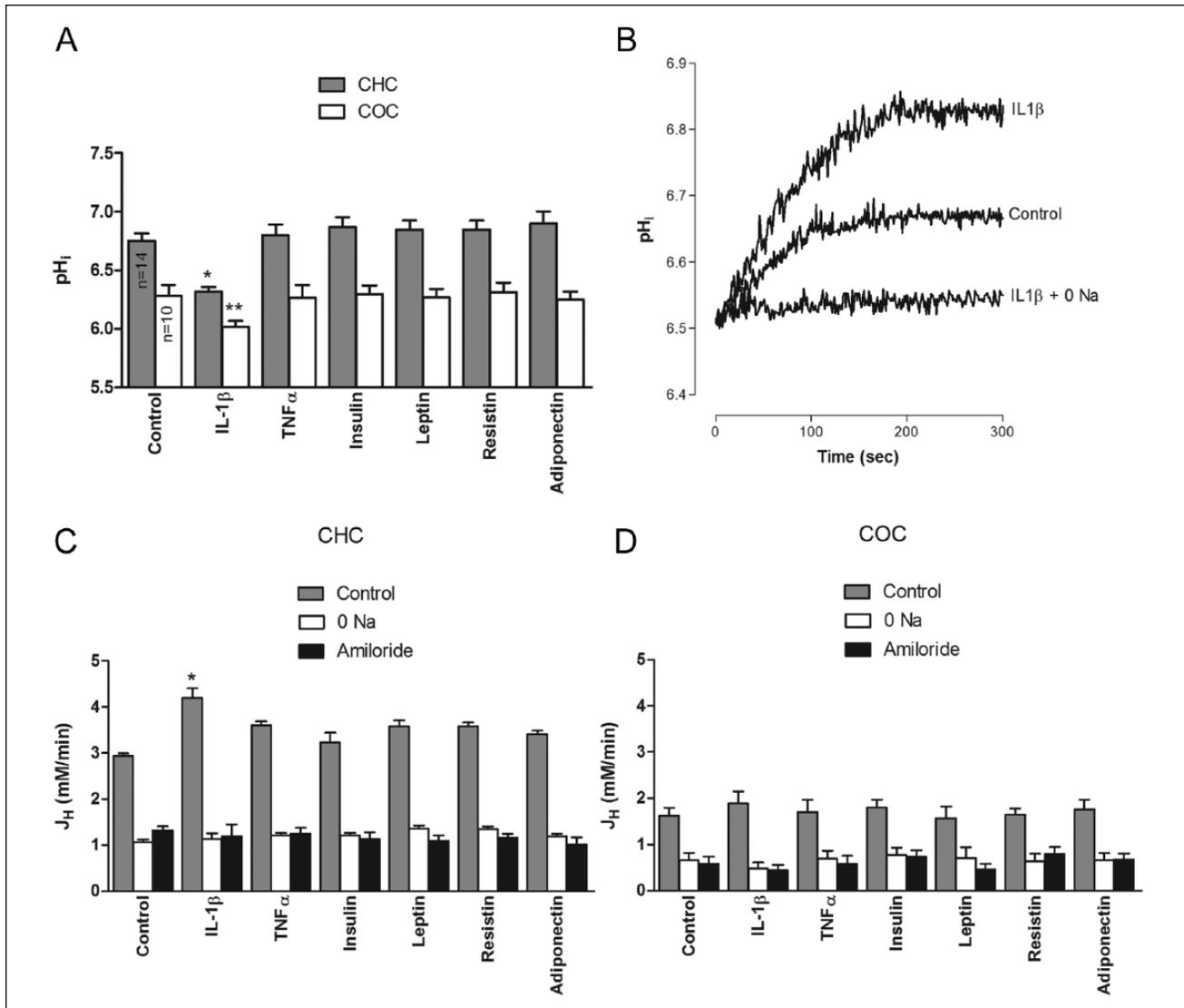
### Statistics

The results are presented as the mean  $\pm$  standard error of the mean, where  $n$  is the number of isolation batches. Each isolation batch represents the cells from the cartilage obtained from one patient's joint. Statistically significant differences were determined using a 2-way analysis of variance test. The Bonferroni method was employed as a post hoc test.

## Results

### Effects on $\text{pH}_i$ in CHC

The effects of a number of adipokines and insulin on  $\text{pH}_i$  were recorded over a 300-second period in basal conditions and after an acidifying ammonium prepulse in BCECF-loaded human chondrocytes from healthy cartilage (CHC) treated with  $\text{IL1}\beta$  (10 ng/mL),  $\text{TNF}\alpha$  (10 ng/mL), insulin (100  $\mu\text{mol/L}$ ), leptin (100 ng/mL), resistin (10 ng/mL), and adiponectin (100 ng/mL). The doses were those in the range used in several previous studies that explored the cellular effects of these agents.<sup>47-51</sup> In all cases, BCECF loading was performed prior to the incubation with these agents. The basal  $\text{pH}_i$  was  $6.74 \pm 0.21$  ( $n = 18$ ).  $\text{IL1}\beta$ , but not  $\text{TNF}\alpha$ , insulin, leptin, resistin or adiponectin, caused a significant decrease in  $\text{pH}_i$  before the ammonium prepulse (**Fig. 1A**), but all of the factors caused a significant increase in the  $\text{pH}_i$  recovery after the ammonium prepulse compared to the control (**Fig. 1B**); this effect was inhibited by the NHE inhibitor amiloride (1



**Figure 1.** (A) Comparison between the  $pH_i$  of human articular chondrocytes from healthy (CHC) and osteoarthritic cartilage (COC) on treatment with a number of hormones, as indicated. \* and \*\* denote significant differences from the respective control for both types of cells. In all cases, there were significant differences between CHC and COC  $pH_i$ . (B) Representative recordings of the  $pH_i$  recovery following an ammonium prepulse under control conditions and in a CHC under IL1 $\beta$  treatment in 140 mM extracellular  $Na^+$  and in  $Na^+$ -free extracellular solution (0 Na). (C) Comparison between the mean maximal acid equivalent fluxes ( $J_H$ ) following an ammonium prepulse with the different agents tested in CHC, in mM/L extracellular  $Na^+$  (control), in  $Na^+$ -free extracellular solution (0 Na) and under treatment with amiloride. \* denotes significant differences from the effect of all the other agents. In all cases, there were significant differences between  $J_H$  under the effect of amiloride and  $Na^+$ -free extracellular solution from the respective control. (D) Comparison between the mean maximal acid equivalent fluxes following an ammonium prepulse with the different agents tested in COC in the same conditions as C. In all cases, there were significant differences between  $J_H$  under the effect of amiloride and  $Na^+$ -free extracellular solution from the control.  $n = 8$  unless indicated otherwise.

mmol/L) and was dependent on the presence of extracellular  $Na^+$  (Fig. 1C). However, the effect on  $pH_i$  recovery was not affected by NBD-Cl<sup>-</sup> (100  $\mu$ mol/L), a H<sup>+</sup>-ATPase inhibitor or Zn<sup>2+</sup> (100  $\mu$ mol/L), a voltage-activated H<sup>+</sup> channel inhibitor (data not shown). When the  $J_H$  was compared, the effect of IL1 $\beta$  was significantly greater than those of all the other hormones (Fig. 1C).

### Effects on $pH_i$ in COC

When the experiments were repeated with the chondrocytes from osteoarthritic cartilage (COC), the effects were significantly different; the basal  $pH_i$  was lower,  $6.37 \pm 0.24$  ( $n = 10$ ), and the effects of the hormones had the same trend described for CHC (Fig. 1A). The  $pH_i$  recovery after an

ammonium prepulse in these cells was attenuated ( $2.936 \pm 0.059$  mmol/L/min in CHC,  $n = 14$  and  $1.618 \pm 0.173$  mmol/L/min in COC,  $n = 8$ ,  $P < 0.05$ ) and the tested agents failed to affect it; this effect was also amiloride-sensitive and dependent on extracellular  $\text{Na}^+$  (**Fig. 1D**) and was not affected by NBD- $\text{Cl}^-$  (data not shown).

### Effects on $\text{pH}_i$ Response to HTS in CHC

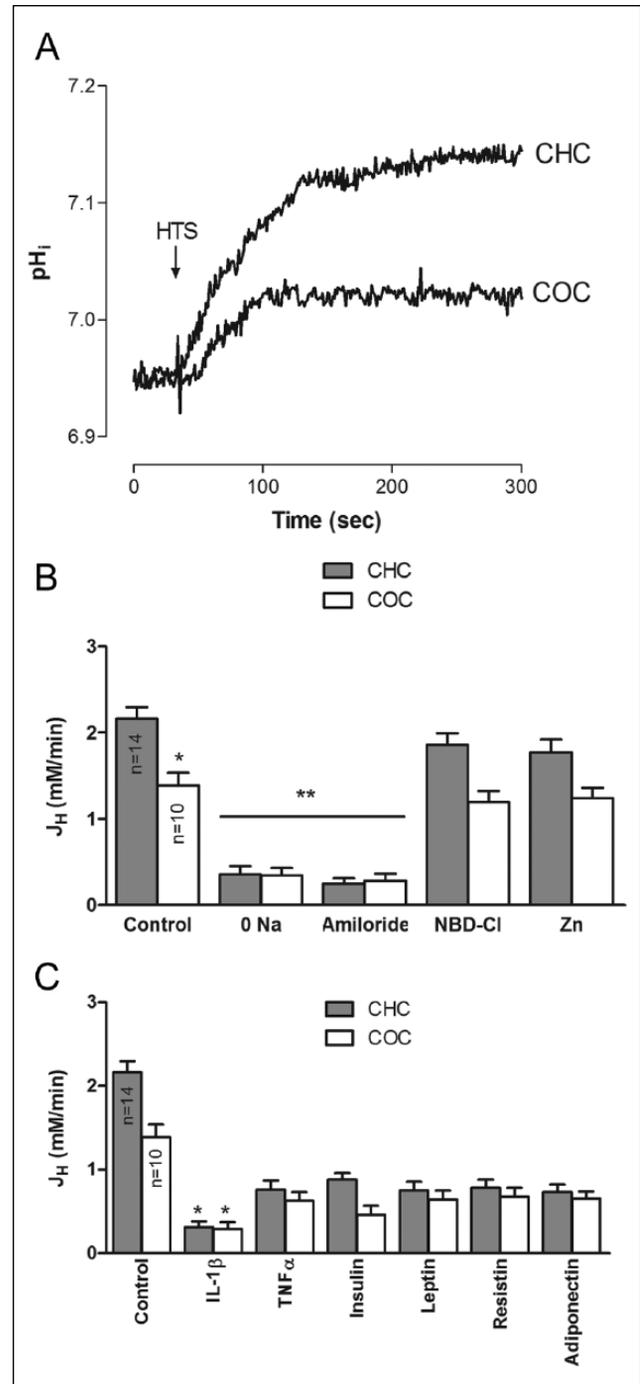
As demonstrated before in bovine chondrocytes,<sup>35</sup> an HTS caused a  $\text{pH}_i$  increase in both CHC and COC, but the effect on the later was significantly smaller (**Fig. 2A** and **B**). This increase was sensitive to amiloride and dependent on extracellular  $\text{Na}^+$ , but the  $\text{pH}_i$  increase did not respond to treatment with NBD- $\text{Cl}^-$  or  $\text{Zn}^{2+}$  (Figure 2B). Furthermore, all the hormones tested caused an attenuation of this response; however, the effect of IL1 $\beta$  was significantly greater than that of all the other factors (**Fig. 2C**).

### Effects on $[\text{Ca}^{2+}]_i$ in CHC

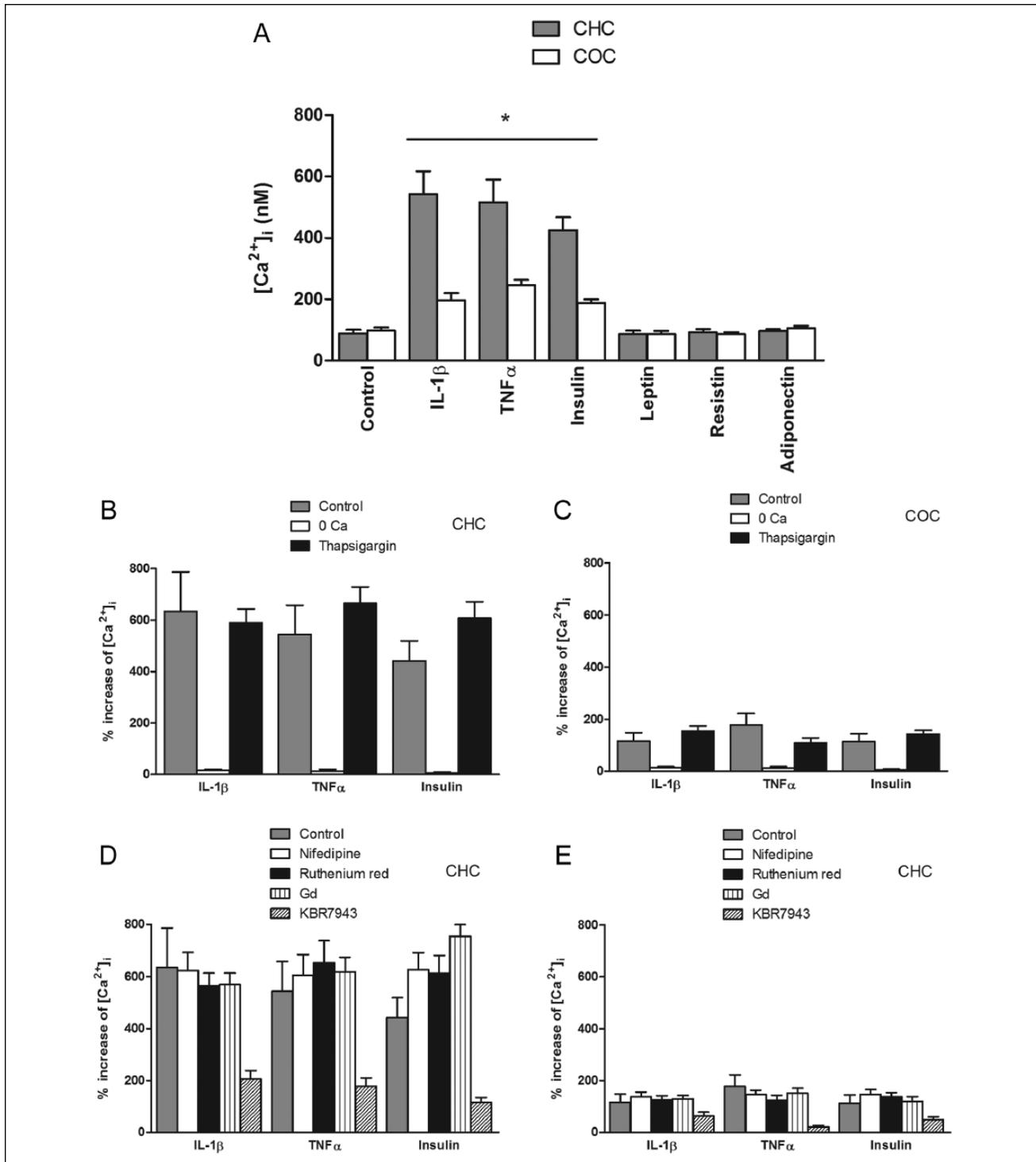
The effects of the same hormones at the same concentrations on  $[\text{Ca}^{2+}]_i$  were also evaluated over periods of 300 seconds in Fura-2-loaded CHC. In all cases, Fura-2 loading was performed prior the incubation with these agents. The control  $[\text{Ca}^{2+}]_i$  was  $96.5 \pm 17.2$  ( $n = 20$ ). Leptin, resistin, and adiponectin failed to affect basal  $[\text{Ca}^{2+}]_i$ . However, IL1 $\beta$ , TNF $\alpha$ , and insulin significantly increased  $[\text{Ca}^{2+}]_i$  after a 1-hour preincubation (**Fig. 3A**). To establish the origin of this rise in  $[\text{Ca}^{2+}]_i$ , the chondrocytes were treated with thapsigargin (1  $\mu\text{mol/L}$ , 30-minute preincubation in  $\text{Ca}^{2+}$ -free HBS) prior to the hormone treatment to deplete intracellular stores or were resuspended in  $\text{Ca}^{2+}$ -free extracellular solution. There was a significant attenuation of the increase following treatment with each hormone in  $\text{Ca}^{2+}$ -free extracellular solution, but thapsigargin treatment had no effect (**Fig. 3B**). Furthermore, this rise was not affected by nifedipin (1 mmol/L), a L-type voltage-activated  $\text{Ca}^{2+}$  channels inhibitor (LVACC); ruthenium red (10  $\mu\text{mol/L}$ ), a non-specific TRPV channels inhibitor;  $\text{Gd}^{3+}$  (10  $\mu\text{mol/L}$ ), a stretch-activated channels (SAC) inhibitor, or HC-067047 (100 nmol/L), a specific TRPV4 channel inhibitor, but it was significantly attenuated by KBR7943 (50  $\mu\text{mol/L}$ ), a specific  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (NCX) inhibitor, in all cases (**Fig. 3D**).

### Effects on $[\text{Ca}^{2+}]_i$ in COC

In COC, the basal  $[\text{Ca}^{2+}]_i$  was not significantly different from CHC, but the effects of the hormones on this parameter were different. The IL1 $\beta$ -, TNF $\alpha$ -, and insulin-induced  $[\text{Ca}^{2+}]_i$  increases were greater than those in CHC (**Fig. 3A**); these effects were dependent on extracellular  $\text{Ca}^{2+}$ , were not affected by thapsigargin treatment (**Fig. 3C**) and were inhibited by KBR7943 but not by the other inhibitors



**Figure 2.** (A) Representative recordings of the  $\text{pH}_i$  in human articular chondrocytes from healthy (CHC) and osteoarthritic cartilage (COC) following a hypotonic shock (HTS), indicated by the arrow. (B) Comparison between the mean maximal acid equivalent fluxes following HTS under treatment with a number of pharmacological agents, as indicated, in both types of cells. \* denotes significant differences from CHC. \*\* denotes significant differences from the respective control. (C) Comparison between the mean maximal acid equivalent fluxes following HTS in cells treated with different hormones as indicated. \* denotes significant differences from the effect of all the other hormones.  $n = 8$  unless indicated otherwise.



**Figure 3.** (A) Comparison between  $[Ca^{2+}]_i$  in human articular chondrocyte from healthy (CHC) and osteoarthritic cartilage (COC) under control conditions and with treatment with a number of hormones. (B) Comparison between the mean percentage increase in  $[Ca^{2+}]_i$  in CHC treated with thapsigargin or in  $Ca^{2+}$ -free extracellular solution (0 Ca) and under treatment with the agents that had an effect on  $[Ca^{2+}]_i$ . (C) Comparison between the mean percentage increase in  $[Ca^{2+}]_i$  in COC treated with thapsigargin or in  $Ca^{2+}$ -free extracellular solution (0 Ca) and under treatment with the agents that had an effect on  $[Ca^{2+}]_i$ . Note that the ranges and scales are maintained to allow a comparison to B. (D) Comparison between the mean percentage increase in  $[Ca^{2+}]_i$  in CHC under treatment with a number of inhibitors, as indicated, and under treatment with the same agents as in B and C. (E) Comparison between the mean percentage increase in  $[Ca^{2+}]_i$  in COC under treatment with a number of inhibitors, as indicated, and under treatment with the same agents as in B and C. Note that the ranges and scales are maintained to allow a comparison with D.  $n = 8$  in all cases.

(Fig. 3E). As in CHC, leptin, resistin, and adiponectin had no effect on the basal  $[Ca^{2+}]_i$  of these cells.

### Effects on $[Ca^{2+}]_i$ Response to HTS in CHC

Similar to bovine chondrocytes,<sup>34</sup> an HTS caused a  $[Ca^{2+}]_i$  increase in CHC (Fig. 4A); this effect was inhibited by ruthenium red and HC-067047, but was not affected by  $Gd^{3+}$ , nifedipin, or KBR7943 (Fig. 4B), which indicates a role for TRPV4 channels in this response but not for SAC, VACC, or NCX. Furthermore, all the hormones tested, with the exception of adiponectin, attenuated the HTS-induced rise in  $[Ca^{2+}]_i$ , although the effects of leptin and resistin were to a lesser extent (Fig. 4C). In COC, the response was smaller compared with CHC (Fig. 4A) and all the hormones tested with the exception of adiponectin, inhibited the response, which was also inhibited by ruthenium red,  $Gd^{3+}$  and HC-067047, but were not affected by nifedipin or KBR7943 as in COC (Fig. 4B and C).

## Discussion

The present study explored the effects of a number of adipokines and insulin on  $pH_i$  and  $[Ca^{2+}]_i$  in human articular chondrocytes from both healthy and osteoarthritic cartilage. The present study demonstrated that IL1 $\beta$  affected the basal  $pH_i$ , in contrast to TNF $\alpha$ , insulin, leptin, resistin, and adiponectin. However, all these agents did affect the  $pH_i$  recovery after an ammonium prepulse, which indicates that these hormones play a role in the regulation of this parameter. Because the effect in all cases was attenuated by amiloride and was dependent on extracellular  $Na^+$ , these effects can be attributed to an action on NHE, which has been shown to be one of the most significant regulators of  $pH_i$  in chondrocytes and other cells.<sup>52</sup> Furthermore, Tattersall *et al.*<sup>17</sup> demonstrated that IL1 $\beta$  modulates NHE, thereby affecting the  $pH_i$  recovery in bovine chondrocytes, and that this effect occurs after an acute exposure, findings that are in agreement with this study. However, there are no studies regarding the effects of other adipokines or insulin on  $pH_i$  in chondrocytes or other cells. Whether the effects of these agents on NHE are direct or through other intracellular mechanisms must be addressed by further research. All these hormones have receptors in chondrocytes<sup>19,28,53,54</sup> and have been related to the inflammatory process in which OA cartilage is immersed.<sup>15</sup> Chondrocyte  $pH_i$  is a key factor in the regulation of cartilage matrix synthesis as demonstrated by a number of studies.<sup>31</sup> It is worthwhile noting that the impairment of matrix metabolism is one of the most important determinants of the development and severity of OA.<sup>3</sup>

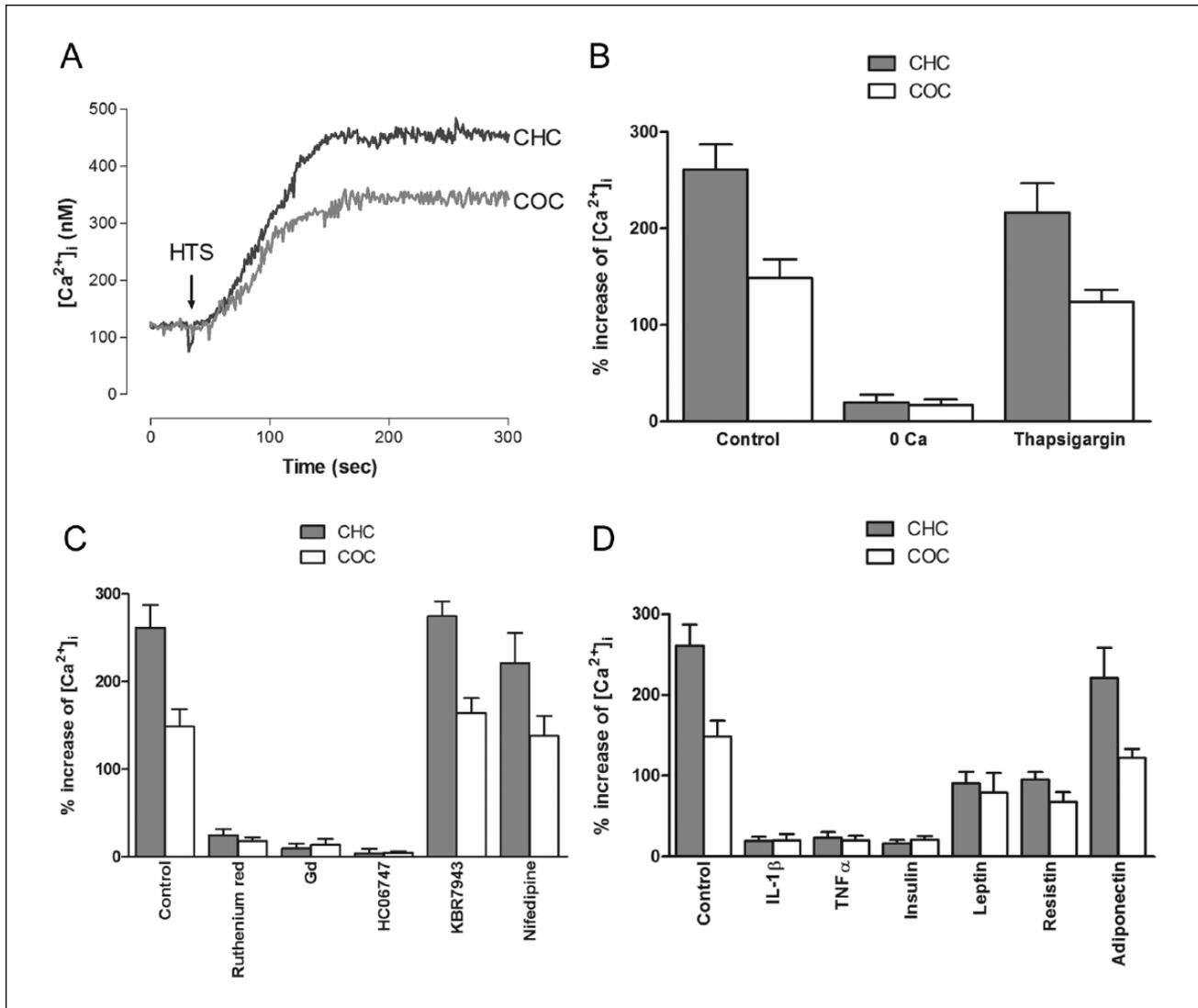
Hypotonic shock produced an increase in the  $pH_i$  of human articular chondrocytes, which is in agreement with the findings in bovine articular chondrocytes.<sup>35</sup> This effect is mediated by the activation of NHE, as suggested by the

inhibition by amiloride and the dependence on extracellular  $Na^+$ . In contrast to bovine chondrocytes, in which voltage-activated  $H^+$  channels seems to be responsible,<sup>35</sup> in human articular chondrocytes,  $Zn^{2+}$ , a  $H^+$ -channel inhibitor, failed to affect the  $pH_i$  increase.

Furthermore, when the effects of the tested agents on the HTS-induced  $pH_i$  increase were explored, all of the agents attenuated the HTS-induced effect, with the action of IL1 $\beta$  being significantly greater than the others.

The  $pH_i$  of chondrocytes from osteoarthritic cartilage was significantly lower than that of chondrocytes from healthy cartilage. This intracellular acidification may help to explain the impairment of matrix metabolism that is observed in osteoarthritic cartilage<sup>55</sup> and could reflect the alteration of the mechanisms that regulate the  $pH_i$  as a consequence of the pathophysiological process of OA. Furthermore, the response of chondrocytes isolated from osteoarthritic cartilage to insulin and cytokines was significantly different. These differential responses in CHC and COC could reflect alterations in chondrocyte homeostasis as a result of OA pathophysiological process; in other words, chondrocytes living in osteoarthritic cartilage behave in a different way when compared with chondrocytes living in healthy cartilage.<sup>3</sup> Articular chondrocytes alkalize in response to HTS<sup>35</sup> and that response may be a part of the adaptation to the osmotic challenges that these cells have to face physiologically; according to this study this response is impaired in COC. Given that  $pH_i$  regulates matrix metabolism,<sup>56</sup> alterations in chondrocyte  $pH_i$  adaptive response can alter matrix composition, leading to a dysfunctional cartilage and thus OA. According with the present study, the effects on  $pH_i$  are mediated by NHE; this transporter may be regulated by a number of agents and its function can affect significantly  $pH_i$ , particularly in articular chondrocytes.<sup>39,57</sup> The way in which NHE is affected in OA needs to be determined at a molecular or functional level by further studies.

IL1 $\beta$ , TNF $\alpha$ , and insulin increased the basal  $[Ca^{2+}]_i$ , but leptin, resistin, and adiponectin had no effect on this parameter; this effect, dependent only on extracellular calcium, is mediated by the activation of NCX as suggested by the pharmacological profile of the  $[Ca^{2+}]_i$  effect, which is in agreement with our findings in bovine articular chondrocytes.<sup>58</sup> However, all of these agents, except for adiponectin, inhibited the HTS-induced  $[Ca^{2+}]_i$  increase, which is mediated by TRPV4 channels as indicated by the effects of specific inhibitors of this osmosensitive cation channel; the effects of these inhibitors on the HTS-induced  $[Ca^{2+}]_i$  increase occurred similarly to what was observed in bovine articular chondrocytes.<sup>34</sup> The fact that adiponectin had no effects whatsoever on the basal  $[Ca^{2+}]_i$  or the HTS-induced  $[Ca^{2+}]_i$  increase cannot be easily explained because this adipokine also has pro-inflammatory effects on osteoarthritic joints. Again, whether these actions were a consequence of



**Figure 4.** (A) Representative recordings of  $[Ca^{2+}]_i$  in human articular chondrocytes from healthy (CHC) and osteoarthritic cartilage (COC) under control conditions and following a hypotonic shock (HTS), indicated by the arrow. (B) Comparison between the mean percentage increase in  $[Ca^{2+}]_i$  in steady-state conditions and following HTS indicated by the arrow, and under treatment with a number of inhibitors as indicated, in both type of cells. (C) Comparison between mean percentage increase in  $[Ca^{2+}]_i$  in both type of cells following HTS and under treatment with a number of hormones, as indicated.  $n = 10$  in all cases.

a direct effect of these hormones on the transporter or whether they occurred secondary to the activation of other mediators requires investigation.

The  $[Ca^{2+}]_i$  response of the chondrocytes from osteoarthritic cartilage was different from the chondrocytes from healthy cartilage. Although the basal  $[Ca^{2+}]_i$  was similar in both types of cells and the implied transporters are similar, IL1 $\beta$ , TNF $\alpha$ , and insulin induced a smaller  $[Ca^{2+}]_i$  increase and a HTS produced a larger  $[Ca^{2+}]_i$  increase in CHC than in COC. Furthermore, all the hormones tested, with the exception of adiponectin, attenuated this hypotonicity-induced  $[Ca^{2+}]_i$  increase in both types of cells, but the effect was more pronounced in COC. This is another example of a

dissimilar response in COC in comparison with CHC, which may help understand the role of  $Ca^{2+}$  homeostasis alterations in OA pathophysiology. NCX, the transporter involved in the  $[Ca^{2+}]_i$  increase induced by adipokines, and TRPV4, the pathway that was responsible for  $[Ca^{2+}]_i$  increase in response to a hypotonic challenge, may be regulated by a number of factors,<sup>59-62</sup> which may be altered in the development of OA and could lead to an impaired function of this channel in COC; since these 2 transporters can affect  $[Ca^{2+}]_i$ , which in turn regulates matrix synthesis, a deleterious effect on cartilage function will result. However, further studies need to be conducted in order to clarify the role of NCX and TRPV4 in the OA pathophysiological process.

In sum, all of these findings indicate that the responses of cells isolated from the cartilage of a patient with OA are different from those of cells from a healthy tissue, which suggests that an alteration of pH and  $[Ca^{2+}]$  homeostasis is part of the pathophysiological process of OA. These factors can affect matrix synthesis because intracellular ionic composition is a matrix modulator. Furthermore, the effects of adipokines and insulin revealed in the present study demonstrate that these hormones affect pH and  $[Ca^{2+}]$  homeostasis, which may explain the association between obesity and OA at a cellular level; however, the mechanisms by which these agents modulate  $pH_i$  and  $[Ca^{2+}]_i$  requires further research.

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