Effect of metabolic and respiratory acidosis on intracellular calcium in osteoblasts

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Frick KK, Bushinsky DA. Effect of metabolic and respiratory acidosis on intracellular calcium in osteoblasts. Am J Physiol Renal Physiol 299: F418–F425, 2010. First published May 26, 2010; doi:10.1152/ajprenal.00136.2010.—In vivo, metabolic acidosis (decreased pH from decreased bicarbonate concentration ([HCO3\(^-\)]) increases urine calcium (Ca) without increased intestinal Ca absorption, resulting in a loss of bone Ca. Conversely, respiratory acidosis (decreased pH from increased partial pressure of carbon dioxide (PCO2)) does not appreciably alter Ca homeostasis. In cultured bone, chronic metabolic acidosis (Met) significantly increases cell-mediated net Ca efflux while isohydric respiratory acidosis (Resp) does not. The proton receptor, OGR1, appears critical for cell-mediated, metabolic acid-induced bone resorption. Perfusion of primary bone cells or OGR1-transfected Chinese hamster ovary (CHO) cells with Met induces transient peaks of intracellular Ca (Ca\(_i\)). To determine whether Resp increases Ca\(_i\), as does Met, we imaged Ca\(_i\) in primary cultures of bone cells. pH for Met = 7.07 ([HCO3\(^-\)] = 11.8 mM) and for Resp = 7.13 (PCO2 = 88.4 mmHg) were similar and lower than neutral (7.41). Both Met and Resp induced a marked, transient increase in Ca\(_i\) in individual bone cells; however, Met stimulated Ca\(_i\) to a greater extent than Resp. We used OGR1-transfected CHO cells to determine whether OGR1 was responsible for the greater increase in Ca\(_i\) in Met than Resp. Both Met and Resp induced a marked, transient increase in Ca\(_i\) in OGR1-transfected CHO cells; however, in these cells Met was not different than Resp. Thus, the greater induction of Ca\(_i\) by Met in primary bone cells is not a function of OGR1 alone, but must involve H\(^+\) receptors other than OGR1, or pathways sensitive to PCO2, HCO3\(^-\), or total CO2 that modify the effect of H\(^+\) in primary bone cells.

Osteoblasts and osteoclasts appear to respond to changes in extracellular pH through a specialized family of G protein-coupled receptors (GPR). This small family (40–50% shared homology) includes the ovarian cancer G protein-coupled receptor 1, OGR1 (51), GPR4 (51), TDAG8 (41, 62), and G2A (41, 53). These receptors are coupled either to phosphoinositide metabolism and increase intracellular Ca (Ca\(_i\); OGR1 and G2A) (51, 53) or alteration in adenylyl cyclase activity (GPR4 and TDAG8) (41, 51, 62). Ludwig et al. (51) reported that OGR1 is expressed in mouse osteoblasts and that a reduction in pH (increase in [H\(^+\)]) leads to an accumulation of phosphoinositide metabolites, making OGR1 a prime candidate for an osteoblastic H\(^+\) sensor. We established that perfusion of primary murine bone cells with medium modeling Met acidosis rapidly induces transient peaks of Ca\(_i\) (36), which could serve as the second messenger conveying the change in pH. To determine whether OGR1 is involved in this signaling, we utilized Chinese hamster ovary (CHO) cells, which normally do not increase Ca\(_i\) in response to Met acidosis. We found that after transfection with mouse OGR1, CHO cells had a robust increase in Ca\(_i\) in response to Met acidosis. We also determined that culture of bone with the OGR1 inhibitor CuCl2 suppresses acid-induced bone Ca efflux. These data implicate OGR1 as a critical mediator in the osteoblastic response to changes in [H\(^+\)].

In contrast to the profound biological effects of Met acidosis on urine Ca excretion and bone Ca efflux, a similar decrease in pH mediated by a primary increase in PCO2 [respiratory acidosis (Resp)] has a minimal effect on body Ca homeostasis. In some (27) but not all (49, 57) studies, there is a detectable increase in urinary Ca excretion, although any increase appears far less than that observed during Met acidosis (27). There are clear distinctions between the effects of Met and Resp acidosis on cultured bone (3, 5, 7, 15–18, 20, 29, 32, 55, 58). In acute studies, there was a greater net bone Ca efflux during culture in medium modeling Met acidosis than during culture in an isohydric model of Resp acidosis (22). During more chronic incubations, there is cell-mediated net Ca efflux from bone during models of Met, but not Resp, acidosis (5, 22). A
number of studies showed that Met acidosis stimulates osteoclastic resorption (7, 19, 22, 48). Resp acidosis does not alter osteoclastic β-glucuronidase release, osteoclastic collagen synthesis, or alkaline phosphatase activity as does Met acidosis (7). Medium PGEx levels and net calcium efflux from bone were increased in parallel with Met, but not Resp, acidosis (20).

Given the marked difference in the response of bone to isohydric Met and Resp acidosis, we tested the hypothesis that Resp acidosis would not increase Cai to the same extent as observed in isohydric Met acidosis. We also tested the hypothesis that any differential response to Met and Resp acidosis was mediated by OGR1.

MATERIALS AND METHODS

Primary Bone Cell Culture

Primary bone cells, which are almost exclusively osteoblasts (46), were isolated from neonatal CD-1 mouse calvariae immediately after dissection as described previously (44, 46). Briefly, bones were washed in PBS containing 4 mM EDTA for 10 min at 37°C and then incubated in HEPES (25 mM), pH 7.4, containing 2 mg/ml collagenase (Wako Pure Chemicals, Dallas, TX) and 90 μM Nα-tosyl-L-lysyl chloromethyl ketone for three sequential 20-min digestion periods at 37°C in a shaking water bath. At the end of each digestion, released cells were collected and resuspended in HEPES buffer containing 1 mM MgSO₄, and all three digests were pooled in DMEM (Lonzar, Walkersville, MD) containing 15% heat-inactivated horse serum (Invitrogen, Carlsbad, CA), heparin (10 USP U/ml; Baxter Healthcare, Deerfield, IL), and penicillin (100 U/ml) for plating on 8-mm glass coverslips (Warner Instruments, Hamden, CT). Coverslips were used for imaging 2 to 4 days after initial plating.

Cell Imaging

To reduce background, DMEM without phenol red (Invitrogen) was used for imaging. To closely replicate physiological conditions, only the HCO₃⁻/CO₂ buffer system was used; no other buffers were added to the medium. For neutral conditions (Ntl), the partial pressure of medium carbon dioxide (PCO₂) was maintained by gentle bubbling through a continuous flow of equilibrated medium was established through a manually operated perfusion valve system (Aquastream, Romulus, MI). Each cell was visualized every 2 s for a total of 600 s. Cai levels were measured using a dual wavelength system (TILL Photonics, Munich, Germany) by excitation at 340 and 380 nm and quantitating fluorescent emissions at 510 nm. Fluorescence data were interpreted and analyzed with TILLVision software (TILL Photonics). As calculations of absolute Cai require many assumptions and remain estimates, ratios of 340 nm/380 nm were used in this study (30, 43).

pH and PCO₂

Medium was sampled from the imaging chamber with a syringe and pH and PCO₂ were determined with a blood-gas analyzer (ABL5, Radiometer, Copenhagen, Denmark) and the [HCO₃⁻] was calculated from pH and PCO₂ as described previously (3, 15).

Animals and Cells

CD-1 mice were obtained from Charles River (Wilmington, MA). CHO fibroblasts were a gift from P. Hinkle (Univ. of Rochester, Rochester, NY) and grown in MEM-α (Invitrogen) containing 5% heat-inactivated FBS (Invitrogen) and penicillin (100 U/ml). CHO cells were transfected with full-length mouse OGR1 cDNA and stable transfectants were selected as previously described (36).

Reagents

All reagents, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Statistical Analysis

All values were expressed as means ± SE. Tests of significance were calculated using Student’s t-test (means) or Kolmogorov-Smirnov test (distributions) using conventional programs (Statistica) on a personal computer. P < 0.05 was considered significant.

RESULTS

Measurement of Cai in Primary Bone Cells

Met acidosis. Changes in Cai were measured by loading primary bone cells with Fura-PE3, excitation at 340 and 380 nm and quantitating fluorescent emissions at 510 nm. Bone cells were continuously perfused with Ntl medium (pH = 7.41 ± 0.008, PCO₂ = 44.6 ± 0.9 mmHg, [HCO₃⁻] = 27.6 ± 0.3 mM). We previously showed that perfusion with Ntl medium does not increase Cai.

The perfusion was then switched to medium acidified by a decrease in [HCO₃⁻] to model Met acidosis (pH = 7.07 ± 0.003, PCO₂ = 42.7 ± 0.6 mmHg, [HCO₃⁻] = 11.8 ± 0.1 mM). Met induced a substantial, rapid increase in Cai in multiple cells. The increase in Cai in response to Met was asynchronous, and varied in magnitude and duration.

Changes in Cai were quantified by the peak-to-nadir ratio. The ratio of the maximum-to-minimum Cai for each cell during the initial Ntl and subsequent Met phases of perfusion was determined for all 300 time points for each cell and the distribution of values was plotted. The peak-to-nadir ratio for cells during Met (Fig. 1A; n = 3,432 individual cells analyzed) was significantly greater than that for Ntl for all ratios greater than 1.1.

Resp acidosis. In other cells, in random order, the perfusion with Ntl was then switched to medium acidified by an increase in the PCO₂ to model Resp acidosis (pH = 7.13 ± 0.005, PCO₂ = 88.4 ± 1.7 mmHg, [HCO₃⁻] = 28.2 ± 0.5 mM). As with Met,
Resp induced a substantial, rapid increase in Cai in multiple cells. The increase in Cai in response to Resp was again asynchronous, occurred in individual cells at various times after the initial exposure to Resp, and varied in magnitude and duration.

The ratio of the maximum-to-minimum Cai for each cell during the neutral (Ntl) and Met phases of perfusion was determined and the distribution of values was plotted. The distributions were significantly different, \( P < 0.01 \) (n = 3,412 cells individually analyzed). The ratio of the maximum-to-minimum Cai for each cell during the Met and Resp phases of perfusion was determined and the distribution of values was plotted. The distributions were significantly different, \( P < 0.01 \) (n = 3,779 cells individually analyzed).

**Comparison of Met with Resp acidosis.** The percentage of cells with an increase in Cai above a specific threshold (1.1) was then determined. A peak in cell Cai was defined as a greater than 1.1-fold increase in area under the curve of Cai for each 2-s time interval, compared with the mean area under the curve for the previous five time intervals for that cell. This calculation was repeated for threshold values of 1.2, 1.3, 1.4, and 1.5. Compared with the initial Ntl phase of imaging, there was a significant increase in the percentage of cells with Cai peaks >1.1, >1.2, >1.3, >1.4, and >1.5 during Met and during Resp (Fig. 2; as above n = 3,432 cells for Met and n = 3,779 cells for Resp, all individually analyzed). For each threshold level, there were significantly more cells that demonstrated an increase in Cai with Met than with Resp.

**Measurement of Cai in OGR1-Transfected CHO Cells**

To determine whether expression of the H\(^{+}\) receptor OGR1 in a cell line not derived from bone would also induce a greater increase in Cai with Met compared with Resp, we examined the Cai response of OGR1-transfected CHO cells to perfusion with Met or with Resp. We previously showed that perfusion with Ntl or Met does not increase Cai in non-OGR1-transfected CHO cells (36).

**Met acidosis.** Changes in Cai were measured by loading OGR1-transfected CHO cells with Fura-PE3. Cells were continuously perfused with Ntl (pH = 7.39 ± 0.003, PCO\(_2\) = 43.3 ± 1.1 mmHg, [HCO\(_3^{-}\)] = 25.5 ± 0.7 mM) medium. Perfusion with Ntl did not increase Cai in OGR1-transfected CHO cells. The perfusion medium was then switched to Met (pH = 7.07 ± 0.007, PCO\(_2\) = 40.0 ± 0.6 mmHg, [HCO\(_3^{-}\)] = 11.1 ± 0.3 mM). Again, Met induced a substantial, rapid increase in Cai in multiple cells and the increase in Cai was asynchronous, occurred in individual cells at various times after the initial exposure, and varied in magnitude and duration.

Changes in Cai were quantified by the peak-to-nadir ratio. The ratio of the maximum-to-minimum Cai for each cell during
the initial Ntl and subsequent Met phases of perfusion was determined for all 300 time points for each cell and the distribution of values was plotted. The peak-to-nadir ratio for cells during Met (Fig. 3A; n = 1,953 individual cells analyzed) was significantly greater than that for Ntl for all ratios greater than 1.6.

Resp acidosis. In other OGR1-transfected CHO cells, in random order, the perfusion with Ntl was then switched to

Fig. 2. Comparison of percentage of primary bone cells responding above selected threshold levels to Met and Resp acidosis. Changes in Ca were quantified by the percentage of cells with an increase in Ca above specific threshold values for Ntl, Met, and Resp. The peak in cell Ca2+ was defined as an increase in Ca2+ of greater than the specified threshold (1.1–1.5) over the mean of the previous 5 time points (time point taken every 2 s) for that cell (n = 3,412 cells for Ntl and respective Met, n = 3,772 for Ntl and respective Resp; * different from respective Ntl, \( P < 0.05 \); +, Resp different from Met, \( P < 0.05 \)).

Fig. 3. Quantitation of Ca2+ in Chinese hamster ovary (CHO) cells transfected with mouse OGR1 in response to Met and Resp acidosis. Changes in Ca2+ were quantified by determining the distribution of the number of cells as a function of the peak-to-nadir ratio. A: ratio of the maximum-to-minimum Ca2+ for each cell during the Ntl and Met phases of perfusion was determined and the distribution of values was plotted. The distributions were significantly different, \( P < 0.01 \) (n = 1,953 cells individually analyzed). B: ratio of the maximum-to-minimum Ca2+ for each cell during the Ntl and Resp phases of perfusion was determined and the distribution of values was plotted. The distributions were significantly different, \( P < 0.01 \) (n = 1,982 cells individually analyzed).
Resp medium (pH = 7.13 ± 0.008, PCO₂ = 81.5 ± 2.2 mmHg, \([\text{HCO}_3^-]\) = 25.6 ± 0.2 mM). As with Met, Resp induced a substantial, rapid increase in Caᵢ in multiple cells. The increase in Caᵢ in response to Resp was again asynchronous, occurred in individual cells at various times after the initial exposure to Resp, and varied in magnitude and duration.

The ratio of the maximum-to-minimum Caᵢ for each cell during the initial Ntl and subsequent Resp phases of perfusion was determined for all 300 time points for each cell and the distribution of values was plotted. The peak-to-nadir ratio for cells during Resp (Fig. 3B; n = 1,982 individual cells analyzed) was significantly different than that for Ntl for all ratios greater than 1.6.

Comparison of Met with Resp acidosis. The percentage of cells with an increase in Caᵢ above a specific threshold (1.1) was then determined. Compared with the initial Ntl phase of imaging, there was no consistent direction in the differences between Met and Resp (Fig. 4; as above n = 1,953 cells for Met and n = 1,982 cells for Resp, all individually analyzed). For values >1.1, Met was greater than Resp, for >1.2 and >1.3 Resp was greater than Met, for values >1.4 Met was greater than Resp, and for >1.5 there was no difference between Met and Resp.

DISCUSSION

Met acidosis induces a marked increase in urine Ca excretion with little to no concomitant increase in intestinal Ca absorption resulting in a loss of total body Ca presumably from the largest repository of Ca in the body, bone (8, 42, 50). Resp acidosis has minimal, if any, effects on urine Ca excretion (27, 49, 57). In vitro models of Met acidosis increase net Ca efflux from bone through a marked increase in levels of PGE₂ leading to increased osteoclastic bone resorption (47). Again, in vitro isohydric Resp acidosis has minimal effects on bone resorption and levels of PGE₂ (20). We found that Met acidosis induces a marked increase in Caᵢ apparently mediated through the proton receptor OGR1 (36). The data presented in this study indicate that in primary cultures of bone cells, which are principally osteoblasts (46), this early intracellular signal, increased Caᵢ, is stimulated to a greater extent by a model of Met compared with isohydric Resp acidosis. However, when CHO cells were transfected with mouse OGR1, the response to both Met and Resp acidosis was of similar magnitude. The differences in response between primary bone cells and OGR1-transfected CHO cells may reflect the expression of other G protein-coupled proton receptors in bone cells (36) or an additional effect of other factors such as \([\text{HCO}_3^-]\), PCO₂, or total CO₂ in these cells.

In primary cells, the increases in Caᵢ were significantly and consistently larger with Met than with Resp; however, the increases with Resp were still significantly greater than during Ntl. This observation suggests that the magnitude of the Caᵢ increase may be of importance in eliciting biological responses. It is possible that the increase in Caᵢ (per cell or in the total mass of cells) must exceed some threshold value to induce downstream biological effects. It is also possible that increases in Caᵢ may be necessary, but not sufficient for biological effects, and that other early events, which are differentially regulated by Met and Resp acidosis, remain to be identified. In particular, bone cells in culture also express the H⁺-sensing receptors TDAG8 and GPR4 (36) and the action of these receptors on cAMP may further modulate cellular activity. However, in unpublished preliminary studies, we have not detected changes in cellular cAMP induced by Met acidosis, but perhaps any changes are below the limit of our detection, transitory or localized specifically within subcellular compartments.

Nontransfected CHO cells do not alter Caᵢ in response to either Met or Resp acidosis. Transfection with OGR1 results in CHO cells that respond equally to these two types of acidosis while primary bone cells have a differential Caᵢ response. This suggests that additional proton-sensing receptors, such as TDAG8 and GPR4 (36), which we showed are expressed in primary bone cells, may not be in the OGR1-transfected CHO cells. Further studies, perhaps utilizing CHO cells transfected with other proton receptors such as TDAG8 and/or GPR4, will be necessary to determine why primary bone cells and OGR1-transfected CHO cells respond differently to Met and Resp acidosis.

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**Fig. 4.** Comparison of percentage of CHO cells transfected with mouse OGR1 responding above selected threshold levels to Met and Resp acidosis. Changes in Caᵢ were quantified by the percentage of cells with an increase in Caᵢ above specific threshold values for Ntl, Met, and Resp. The peak in cell Caᵢ was defined as an increase in Caᵢ of greater than the specified threshold (1.1–1.5) over the mean of the previous 5 time points (time point taken every 2 s) for that cell (n = 1,953 cells for Ntl and respective Met, n = 1,982 for Ntl and respective Resp; * different from respective Ntl, P < 0.05; +, Resp different from Met, P < 0.05).
As we reported previously (36), the increase in Ca after exposure to Met medium with either primary bone cells or OGR1-transfected CHO cells was heterogeneous with regard to the time from when the cells were exposed to acid to their maximal increase in Ca, and in the magnitude of the increase. Similar heterogeneity was seen with exposure to Resp medium. In primary cells, it is unclear whether specific cells respond only to Met and not to Resp, or if all responding cells are equally capable of responding to either type of acidosis and it is just the magnitude of the response that differs. By visual examination, the responding cells were morphologically indistinguishable from cells that did not respond. The primary bone cells used in this study, while predominantly osteoblasts, represent a heterogeneous population at various levels of maturation (28, 39, 59). The responses of these osteoblasts to acidosis may be related to their level of maturation. In the case of the OGR1-transfected CHO cells, cell-to-cell variations in the Ca response to H + may be the result of differences in expression of OGR1.

Previously, we studied the effects of Met and Resp on Ca using an osteosarcoma cell line and quantitated the results with a fluorimeter that collected data en masse from cell monolayers (55). In contrast to the results presented here where Met induces a greater increase in Ca than does Resp in primary bone cells, in the previous study, using the rat osteosarcoma line UMR-106, Resp was found to transiently increase Ca to a greater extent than Met in the initial 20 to 100 s after medium change; after 100 s, Ca for Ntl, Met, and Resp was indistinguishable. The differences in results likely reflect the different cells used. While the UMR-106 cells possess many properties of osteoblasts [e.g., increased cAMP in response to parathyroid hormone, high alkaline phosphatase activity, mineralization in culture (63)], as a cell line they likely represent a narrow range of osteoblast maturation while the calvarial osteoblasts represent a more heterogeneous population of cells (28, 39, 59). There are technical differences between the two reports as well: in this study, we imaged individual cells, as opposed to measuring the mean response of a large number of cells on a slide in a fluorimeter cuvette. In the current study, we also changed pH by perfusion as opposed to moving the cells to medium with a different pH.

We previously reported the effects of variations in [HCO3−] and PCO2, to achieve isohydric pH conditions, on net Ca efflux from bone during an acute incubation. Over this 3-h time period, there is no cell-mediated bone resorption, only physicochemical mineral dissolution (21). We found that during acidosis, physicochemical mineral dissolution is critically dependent on [HCO3−]; the lower the [HCO3−], the greater the Ca efflux. These findings parallel the osteoblastic cell signaling described in the current paper in which Met acidosis, with a low medium [HCO3−], induced greater Ca signaling than did isohydric Resp acidosis, which has a higher [HCO3−].

To replicate physiologic conditions, to the extent possible with an in vitro system, we utilized only the HCO3− /CO2 buffer system; no other H + buffers were added to the medium. In the model of Met acidosis, HCl was utilized to lower [HCO3−] and thus pH at a constant, physiologic PCO2 and in the model of Resp acidosis, PCO2 was increased to lower pH at a constant, physiologic [HCO3−]. We made every effort to limit preperfusion CO2 loss though the actual perfusion chamber was open and undoubtedly PCO2 escaped. We did, however, measure and report the actual pH and PCO2 measurements from chamber samples, and the resultant calculated [HCO3−], which were consistent with values observed for clinical Met and Resp acidosis (4, 6, 14).

The medium modeling Met acidosis and that modeling Resp acidosis differ only in [HCO3−] and PCO2 and the sum of all CO2 present that is termed total CO2. While H2CO3 may be an intermediate in the equilibrium between CO2 and HCO3−, its half-life is extremely short and its concentration exceedingly low in biological systems (1). Thus, any difference in the cellular response to Met compared with Resp acidosis must be due to an interaction with either [HCO3−], PCO2, or total CO2 alone or in combination. Whereas CO2 appears to freely diffuse into cells (52), HCO3− must enter through transporters or channels or through intracellular formation catalyzed by carbonic anhydrase. The evolutionarily conserved bicarbonate-sensing soluble adenylyl cyclase (sAC; adenylyl cyclase 10) appears central to pH control in animals (61). Activation of sAC by HCO3− increases cAMP levels in cells expressing this enzyme. Increases in [HCO3−] enhance the transcription of the cystic fibrosis transmembrane conductance regulator (CFTR) in human pulmonary cells through activation of sAC (2). sAC is also present and upregulated by [HCO3−] in carotid bodies, which possess peripheral arterial chemoreceptors thought to be important in regulating CO2 homeostasis (54). In the renal collecting duct, an important site for electrolyte reabsorption, sAC activation elevates transepithelial Na + currents through Na + -K + -ATPase (38) and stimulates H + extrusion via increased V-ATPase accumulation (56). In bone, increased [HCO3−] inhibits osteoclast proliferation and differentiation through sAC, as demonstrated by siRNA knockdown of sAC (37). Expression of sAC may have consequences on bone health: a polymorphism in the adenylyl cyclase 10 gene has been reported to “modestly” associate with bone mineral density (40).

Perhaps basal activity of osteoblastic sAC in Ntl medium is necessary for normal cell function, while the decreased [HCO3−] in Met acidosis reduces the activity of sAC altering osteoblast physiology resulting in increased bone resorption. Conversely, Resp acidosis, with a similar level of [HCO3−] as Ntl medium, but a higher concentration of PCO2 and total CO2, might stimulate sAC resulting in inhibition of bone resorption. Other G protein-coupled proton-sensing receptors, TDAG8 and GPR4, are also linked to nonbicarbonate-sensitive adenylyl cyclase, indicating that their action could modulate intracellular signaling. In addition to sAC, Townsend et al. (60) presented evidence that a mammalian cell line, HEK 293T, expresses a CO2-sensitive adenylyl cyclase that is distinct from sAC.

Thus, in primary cultures of osteoblasts, Ca is increased to a greater extent by a model of Met compared with isohydric Resp acidosis. However, when CHO cells are transfected with mouse OGR1, the response to both Met and Resp acidosis is similar. Further studies are necessary to determine what factors, such as [HCO3−], PCO2, or total CO2, or the presence of other H + receptors in primary bone cells, are responsible for this differential response to Met and Resp acidosis in primary osteoblasts that is not present when the H + receptor OGR1 is expressed in CHO cells.
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REFERENCES


