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Cytoprotection by pyruvate through an anti-oxidative mechanism in cultured rat calvarial osteoblasts

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Summary. Although we have previously shown drastic cell death by pyruvate deficiency in osteoblasts at the proliferative stage, the exact mechanism remains unclear so far. Cell survivability was significantly decreased in rat calvarial osteoblasts cultured for 0 to 3 days *in vitro* (DIV) following replacement of the eutrophic α-modified minimum essential medium (α-MEM) with Dulbecco's modified eagle medium (DMEM) for cultivation. The addition of pyruvate enriched in α-MEM, but not in MEM, entirely prevented cell death induced by the medium replacement throughout a culture period from 0 to 3 DIV. Both cysteine and reduced glutathione protected cell death in cells cultured for 3 DIV without significantly affecting that in cells cultured for 1 DIV, however, while none of lactate, acetate and insulin significantly prevented the cell death irrespective of the culture period up to 3 DIV. A marked increase was detected in intracellular reactive oxygen species (ROS) levels 4 h after the medium replacement. In osteoblasts cultured in α-MEM for 3 DIV, but not in those for 7 DIV, hydrogen peroxide (H_2O_2) markedly decreased cell survivability when exposed for 2 to 24 h. Furthermore, H_2O_2 was effective in significantly decreasing cell survivability in osteoblasts cultured in DMEM for 7 DIV. Pyruvate at 1 mM not only prevented cell death by H_2O_2, but also suppressed the generation of intracellular ROS in osteoblasts exposed to H_2O_2. These results suggest that pyruvate could be cytoprotective through a mechanism associated with the anti-oxidative property rather than an energy fuel in cultured rat calvarial osteoblasts.

Key words: Pyruvate, Osteoblasts, Reactive oxygen species, Hydrogen peroxide, Viability

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Introduction

It is well known that the aliphatic alpha-ketoacid pyruvate ($CH_3COCOOH$) is synthesized from phosphoenolpyruvate by the catalytic action of pyruvate kinase during glycolysis in the cytoplasm, followed by incorporation into mitochondria for production of ATP in the TCA cycle with the respiratory chain through transformation to acetyl CoA under aerobic conditions in mammalian cells. Pyruvate is also converted to lactate ($CH_3CHOHCOOH$) by lactate dehydrogenase in the cytoplasm under anaerobic conditions (Holleman, 1904; Bunton, 1949). In our previous study, pyruvate deficiency leads to severe cell death in both primary cultured rat calvarial osteoblasts and the clonal murine osteoblastic cell line MC3T3-E1 at the proliferative stage but not in those at the differentiation and maturation stages (Hinoi et al., 2002). In cultured rat costal chondrocytes and stromal cell line ST2 cells, however, similar pyruvate deficiency is not cytotoxic at all (Hinoi et al., 2002).

On the other hand, hydrogen peroxide is a stable, unchanged and freely diffusible reactive oxygen species (ROS) with a putative second messenger role in intracellular and extracellular signaling mechanisms (Halliwell and Whiteman, 2004). Moreover, H_2O_2 can exert its toxic effects mainly through the ferrous iron-dependent formation of the highly reactive ROS hydroxyl radical ($OH^{\bullet}$), leading to destructive alterations of lipids, proteins and DNA (Fenton, 1894). In pathological situations, there is increasing evidence suggesting the role of free radicals in the pathogenesis of postmenopausal osteoporosis. There is a close negative

Abbreviations: ALP, alkaline phosphatase; α-MEM, α-modified minimum essential medium; DCFDA, dihidrodichlorofluorescein diacetate; DIG, digoxigenin; DIV, days *in vitro*; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

correlation between oxidative stress (antioxidant levels) and bone mineral density in women (Basu et al., 2001; Maggio et al., 2002), indeed, while H_2O_2 is suggested to be a ROS responsible for the bone loss seen in estrogen deficiency (Lean et al., 2005). In addition to postmenopausal osteoporosis, increased oxidative stress is supposed to play a role in mechanisms underlying osteopenia associated with other risk factors including diabetes mellitus (Christensen and Svendsen, 1999), atherosclerosis (Hamerman, 2005), smoking (Law and Hackshaw, 1997) and hypertension (Cappuccio et al., 1999).

In order to clarify the possibility that pyruvate may be an essential survival factor for proliferating osteoblasts, in the present study we have attempted to demonstrate the exact underlying mechanism as well as functional significance for the cytoprotection selective for osteoblasts.

Materials and methods

Materials

Both α-modified minimum essential medium (α-MEM) and Dulbecco's modified Eagle's medium (DMEM) were supplied by Gibco BRL (Gaithersburg, MD, USA). Cell counting kit was from Dojindo (Osaka, Japan). Dihydrodichlorofluorescein diacetate (DCFDA) was provided by Molecular probes (Eugene, OR, USA). Other chemicals used were all of the highest purity commercially available.

Culture of primary osteoblasts

Osteoblasts were prepared from calvaria of 1- to 2-day old Wistar rats by a sequential enzymatic digestion method as described previously (Hinoi et al., 2003). In brief, calvaria were gently incubated at 37°C for 10 min with 0.1% collagenase and 0.25% trypsin in calcium- and magnesium-free phosphate-buffered saline (PBS). After 10 min the supernatant was discarded, enzyme solution was further added, and incubation continued at 37°C. After 10 min, supernatant was collected, enzyme solution was further added, and incubation continued again. This process was repeated three more times. Cells obtained during the last 4 digestions were pooled in α-MEM containing 10% fetal bovine serum (FBS) and antibiotics, followed by centrifugation at 250 g for 5 min. The pellets were suspended in α-MEM containing 10% FBS. Cells were plated at a density of $2{,}5x10^3/cm^2$ to appropriate dishes, and then cultured at 37°C for different periods up to 28 days in vitro (DIV) under 5% CO_2. One day after plating, the culture medium was changed from α-MEM containing 10% FBS to α-MEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mM sodium ß-glycerophosphate for promotion of subsequent differentiation into osteoblasts. This day was defined as Day 0. The medium was changed every 2-3 days throughout cultivation.

Determination of cellular maturation

Both alkaline phosphatase (ALP) activity and Ca^{2+} accumulation were determined as described previously (Hinoi et al., 2003). In brief, osteoblasts were solubilized with 0.1% Triton X-100, followed by determination of ALP activity in lysates using p-nitrophenol phosphate as a substrate. The lysates were also treated with 2 M hydrochloric acid for 16 to 24 h, followed by centrifugation at 20,000g and subsequent determination of Ca^{2+} content in the supernatant using the C-TEST kit. Osteoblasts were also fixed with 4% paraformaldehyde, followed by incubation with 5% silver nitrate under exposure to ultraviolet and subsequent reaction with 5% sodium thiosulfate for detection of mineralization by von Kossa staining.

Northern blotting

Total RNA was extracted from cultured osteoblasts as described previously (Hinoi et al., 2003). The extracted RNA was resolved on 1% formaldehyde/agarose gel, and transferred onto positively charged nylon transfer membranes. After fixing RNA to the blot by UV crosslinking, the blotted membranes were prehybridized at 68°C for 1 h, and subsequently hybridized with a denatured digoxigenin (DIG)-labeled RNA probe of type I collagen, osteopontin and osteocalcin at 68°C for 16 h. The membranes were then washed and incubated with anti-DIG-AP-Fab, followed by incubation with CDP-star. The membranes were then exposed on X-ray film for appropriate periods to detect chemiluminescence.

Immunocytochemistry

Osteoblasts were fixed with 4% paraformaldehyde, followed by blocking with 10% bovine serum albumin containing 1% TritonX-100 and subsequent reaction with an antibody against osteocalcin adequately diluted. Cells were then reacted with fluorescein isothiocyanate conjugated with the anti-rabbit immunoglobulin G for subsequent observation under a confocal laser-scanning microscope.

Replacement of growth medium and determination of cell survivability

In calvarial osteoblasts cultured for 0 to 21 DIV, the standard eutrophic α-MEM culture medium was replaced with normally trophic DMEM in either the presence or absence of anti-oxidative agents or energy metabolism intermediates at appropriate concentrations. The earliest medium replacement was done at Day 0. Cell survivability was determined using cell counting kits according to the manufacturer's instructions 12 h after the medium replacement unless otherwise indicated (Hinoi et al., 2002). In addition, cultured osteoblasts were exposed to H_2O_2 at a concentration range of 10 to

1,000 μM in a-MEM or DMEM for different durations of 1.5, 2 and 24 h in either the presence or absence of pyruvate at 1 mM, followed by determination of cell survivability using cell counting kits. The cell counting kit used is composed of a highly water soluble tetrazolium salt that is reduced to a formazan dye by mitochondrial dehydrogenases. The kit might thus result in false positive survivability due to a direct reaction between anti-oxidative agents, including pyruvate, and tetrazolium salt to be reduced. To avoid such an experimental artifact, all media were invariably replaced with DMEM not containing any other reagents before the addition of kit reagents. The background value obtained under cell-free conditions was also subtracted from the total value to calculate the net value of tetrazolium reduction.

Determination of intracellular reactive oxygen species (ROS)

Osteoblasts were cultured for 3 or 7 DIV in α-MEM, followed by medium replacement with α-MEM or DMEM and additional cultivation for 3 to 4 h in either the presence or absence of H_2O_2 and pyruvate. Cells were then incubated with DCFDA at 10 mM in DMEM without FBS at 37°C for 30 min in 5% CO_2 incubator. In principle, DCFDA diffuses readily into cells, followed by hydrolysis of ester groups by intracellular esterases and subsequent release of the dichloro derivative. This derivative is then oxidized to the fluorescent parent dye by intracellular ROS. Cells were then washed with PBS twice, followed by determination of intracellular ROS under a confocal laser-scanning microscope (LSM 510; CarlZeiss, Jena, Germany) with excitation at 485 nm and emission at 525 nm, respectively.

Data analysis

Results are all expressed as the mean ± S.E. and the statistical significance was determined by the two-tailed and unpaired Students' t-test or the one-way analysis of variance ANOVA with Bonferroni/Dunnett post hoc test.

Results

Osteoblastic maturation

Under standard culture conditions using α-MEM, osteoblasts were in a proliferative phase between 1 to 6 DIV, with formation of several confluent cell layers. By 10 to 11 DIV, cells were more refractive and round than the background cell layers, and began to form nodules and clusters for creating a matrix. The matrix started to mineralize and calcify within 15 DIV (data not shown). As shown in Fig. 1a, ALP activity was markedly increased in proportion to the culture duration from 3 to 14 DIV with a plateau up to 28 DIV in cultured rat calvarial osteoblasts. Calcium was not detected in immature cells cultured for a period up to 7 DIV, but markedly increased with the culture period from 14 to 28

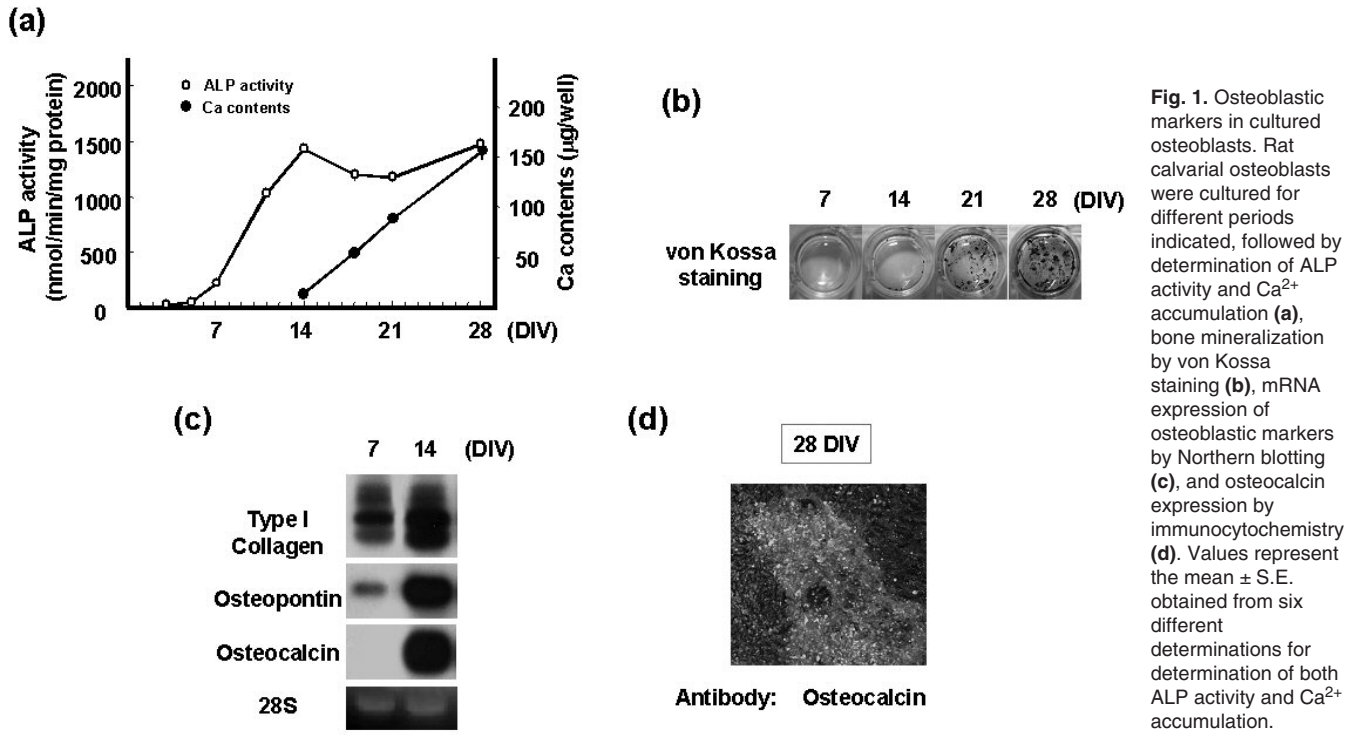

Fig. 1. Osteoblastic markers in cultured osteoblasts. Rat calvarial osteoblasts were cultured for different periods indicated, followed by determination of ALP activity and Ca^{2+} accumulation **(a)**, bone mineralization by von Kossa staining **(b)**, mRNA expression of osteoblastic markers by Northern blotting **(c)**, and osteocalcin expression by immunocytochemistry **(d)**. Values represent the mean ± S.E. obtained from six different determinations for determination of both ALP activity and Ca^{2+} accumulation.

DIV. The intensity of von Kossa staining for bone mineralization was also increased in proportion to the culture period up to 28 DIV (Fig. 1b). Moreover, mRNA expression of osteoblastic markers, including type I collagen, osteopontin and osteocalcin, was drastically increased from 7 to 14 DIV on Northern blotting analysis (Fig. 1c). The bone maturation marker protein osteocalcin was highly expressed in cells cultured for 28 DIV (Fig. 1d). No immunoreactivity was detected in cells not treated with the primary antibody against osteocalcin (data not shown).

Cell death by medium replacement

After medium replacement with DMEM, cellular viability was significantly decreased in cultured rat calvarial osteoblasts by approximately 90% on 0 and 1 DIV, and 75% on 3 DIV, respectively, compared to that in α-MEM (Fig. 2). However, no marked alternation was found when medium replacement was done on 5 DIV. Similarly, no significant change was seen in cellular survivability after the medium replacement on the day from 5 to 21 DIV (data not shown). Although the further addition of pyruvate did not significantly affect cell survivability in osteoblasts cultured for a period from 0 to 21 DIV in α-MEM (data not shown), the addition of pyruvate at the concentration present in α-MEM (1 mM) almost completely prevented cell death induced by medium replacement with DMEM in osteoblasts

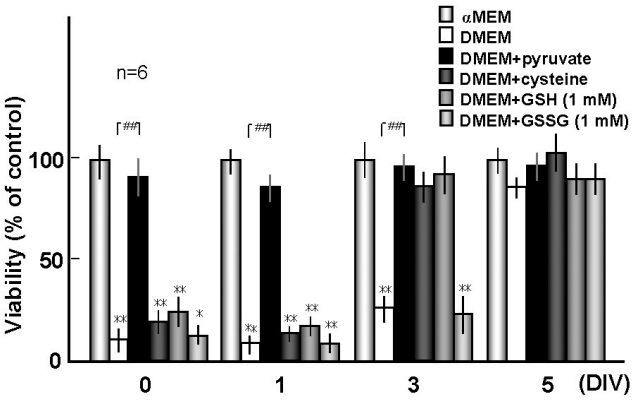

Fig. 2. Effects of pyruvate and related substances on cell survivability in cultured osteoblasts. Rat calvarial osteoblasts were cultured in eutrophic α-MEM for appropriate periods up to 5 DIV. Culture medium was replaced with α-MEM or DMEM on the day indicated, followed by additional cultivation in either the presence or absence of 1 mM pyruvate, 570 μM cysteine, 1 mM reduced glutathione (GSH) or oxidized glutathione (GSSG) for 12 h and subsequent determination of cell viability 12 h after replacement. Values are the mean ± S.E. from 6 different experiments. **P<0.01, significantly different from each control value obtained in osteoblasts cultured for an additional 12 h in α-MEM after medium exchange on the individual days indicated. ##P<0.01, significantly different from the value obtained in cells cultured for an additional 12 h in DMEM in the absence of added pyruvate after medium exchange on the respective days indicated.

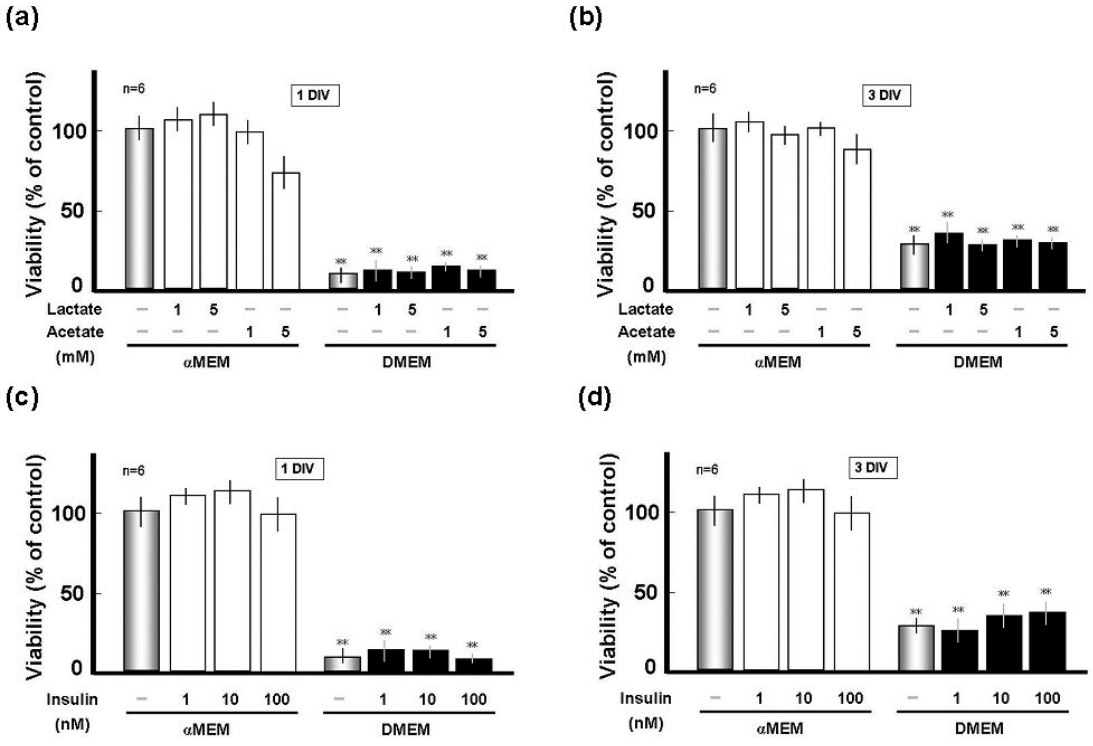

Fig. 3. Effects of several energy suppliers on cell survivability in cultured osteoblasts. Osteoblasts were cultured in α-MEM for 1 and 3 DIV, followed by medium exchange to α-MEM or DMEM and subsequent cultivation for an additional 12 h in either the presence or absence of lactate and acetate at 1 mM (**a, b**) and insulin at 1 to 100 nM (**c, d**). Cell viability was determined 12 h after medium exchange. Values are the mean±S.E. from 6 different experiments. **P<0.01, significantly different from each control value obtained in cells cultured for an additional 12 h in α-MEM after medium exchange on 1 or 3 DIV.

cultured for a period from 0 to 3 DIV. Although the addition of cysteine did not markedly affect the cell survivability in osteoblasts cultured for a period from 0 to 21 DIV in α-MEM (data not shown), the addition of cysteine at the concentration found in α-MEM (570 μM) significantly prevented cell death induced by the medium replacement done on 3 DIV but not on 0 and 1 DIV. Similarly, reduced glutathione (GSH) at 1 mM also almost completely prevented cell death induced by medium replacement done on 3 DIV but not on 0 and 1 DIV. The addition of oxidized glutathione at 1 mM, however, did not prevent the cell death induced by medium replacement done on the day from 0 to 3 DIV. Therefore, pyruvate was invariably protective against cell death by the medium replacement done on 0 to 3 DIV, in contrast to both cysteine and GSH which were effective on 3 DIV only.

Effects of pyruvate on cell viability according to cell maturity

In order to further confirm the protective property of exogenous pyruvate, the culture medium was replaced from α-MEM with DMEM in osteoblasts cultured for 0, 1 or 3 DIV in either the presence or absence of pyruvate added at a concentration range of 10 to 1,000 μM, followed by determination of cell survivability 12 h after the replacement. The addition of pyruvate significantly prevented the loss of cell survivability induced by medium replacement in a concentration-dependent manner at concentrations over 200 μM in cultured osteoblasts irrespective of the cellular maturity for a period from 0 to 3 DIV (Table 1).

Effects of lactate, acetate and insulin on cell viability

In order to evaluate the possibility that the protective effect of pyruvate may be attributed to a role as an

Table 1. Effect of pyruvate at different concentrations on cell survivability in cultured osteoblasts.

Culture medium	Pyruvate (μM)	Viability (% of control)		
		0 DIV	1 DIV	3 DIV
α-MEM	0	100.0±9.2	100.0±8.1	100.0±7.8
DMEM	0	10.2±3.5**	9.5±3.8**	32.2±3.2**
	10	7.4±3.7**	12.2±6.6**	27.7±5.1**
	100	11.3±3.2**	10.1±4.7**	50.3±6.3**
	200	24.8±6.8**	51.3±7.5**	71.8±4.1**
	400	46.4±12.6*	73.9±6.1*	95.3±6.7
	800	81.1±10.2	94.3±7.4	101.2±5.9
	1000	97.3±5.7	91.1±4.7	98.8±4.7

Osteoblasts were cultured in α-MEM for 0, 1 or 3 DIV, followed by medium replacement with α-MEM or DMEM and subsequent cultivation for an additional 12 h in either the presence or absence of pyruvate at different concentrations of below 1 mM. Values are the mean ± S.E. from 6 different experiments. *P<0.05, **P<0.01, significantly different from each control value obtained in osteoblasts cultured for an additional 12 h in α-MEM after medium exchange at the indicated time windows.

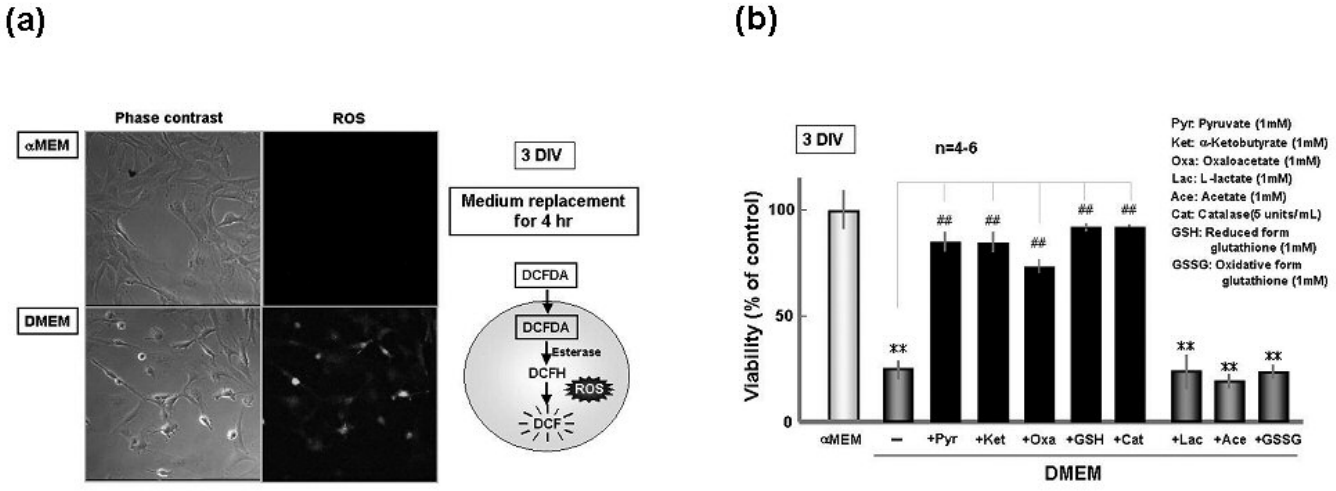

Fig. 4. Possible participation of ROS in cell death after medium replacement. **a.** Cells were cultured in α-MEM for 3 DIV, followed by medium replacement with α-MEM or DMEM and subsequent cultivation for an additional 4 h. Cells were then incubated with DCFDA in α-MEM or DMEM for 30 min, followed by determination of intracellular ROS under a confocal laser-scanning microscope. Typical pictures are shown here, with similar results in three independent determinations. **b.** Osteoblasts were also cultured in α-MEM for 3 DIV, followed by medium replacement with α-MEM or DMEM and subsequent cultivation for an additional 12 h in either the presence or absence of different antioxidants. Cellular viability was determined 12 h after medium exchange. Values are the mean ± S.E. from 4 to 6 different experiments. **P<0.01, significantly different from the control value obtained in cells cultured for an additional 12 h in α-MEM after medium replacement. $^{\#\#}$P<0.01, significantly different from the value obtained in cell cultured for an additional 12 h in DMEM after medium replacement.

energy fuel in osteoblasts, we next examined the effect of energy metabolism intermediates including lactate and acetate on cell death after medium replacement. The addition of lactate and acetate at 1 to 5 mM did not significantly affect cellular viability in osteoblasts cultured in eutrophic α-MEM for 1 (Fig. 3a, left columns) to 3 (Fig. 3b, left columns) DIV, and failed to significantly prevent cell death by replacement of α-MEM with DMEM in osteoblasts cultured for 1 (Fig. 3a, right columns) and 3 (Fig. 3b, right columns) DIV. Moreover, the addition of insulin at 1 to 100 nM neither affected cell viability in osteoblasts cultured in eutrophic α-MEM for a period from 1 (Fig. 3c, let columns) to 3 (Fig. 3d, left columns) DIV, nor prevented cell death seen after medium replacement with DMEM in osteoblasts cultured for a period from 1 (Fig. 3c, right columns) to 3 (Fig. 3d, right columns) DIV.

Protection of cell viability by α-ketoacids and antioxidative enzymes from oxidative stress

As the protection was invariably seen for compounds with anti-oxidative properties, an attempt was next made to determine whether intracellular ROS is indeed generated on medium replacement of eutrophic α-MEM with DMEM by using the DCFDA probe. Osteoblasts were cultured for 3 DIV in α-MEM, followed by replacement of culture medium with α-MEM or DMEM and subsequent additional cultivation for 4 h. Cells were then incubated with DCFDA at 10 mM in DMEM without FBS for 30 min for determination of intracellular ROS levels on fluorescence image. Under these experimental conditions, highly fluorescent cells were observed after medium replacement with DMEM, but not with α-MEM (Fig. 4a). In contrast, the addition of aliphatic α-ketoacids other than pyruvate, such as α-ketobutyrate and oxaloacetate, at 1 mM significantly prevented the decreased cell viability induced by medium replacement in osteoblasts cultured for 3 DIV, in addition to catalase at 5 units/mL (Fig. 4b). GSH was also effective in preventing the cell death after medium replacement, with lactate, acetate and oxidized glutathione being ineffective, at 1 mM.

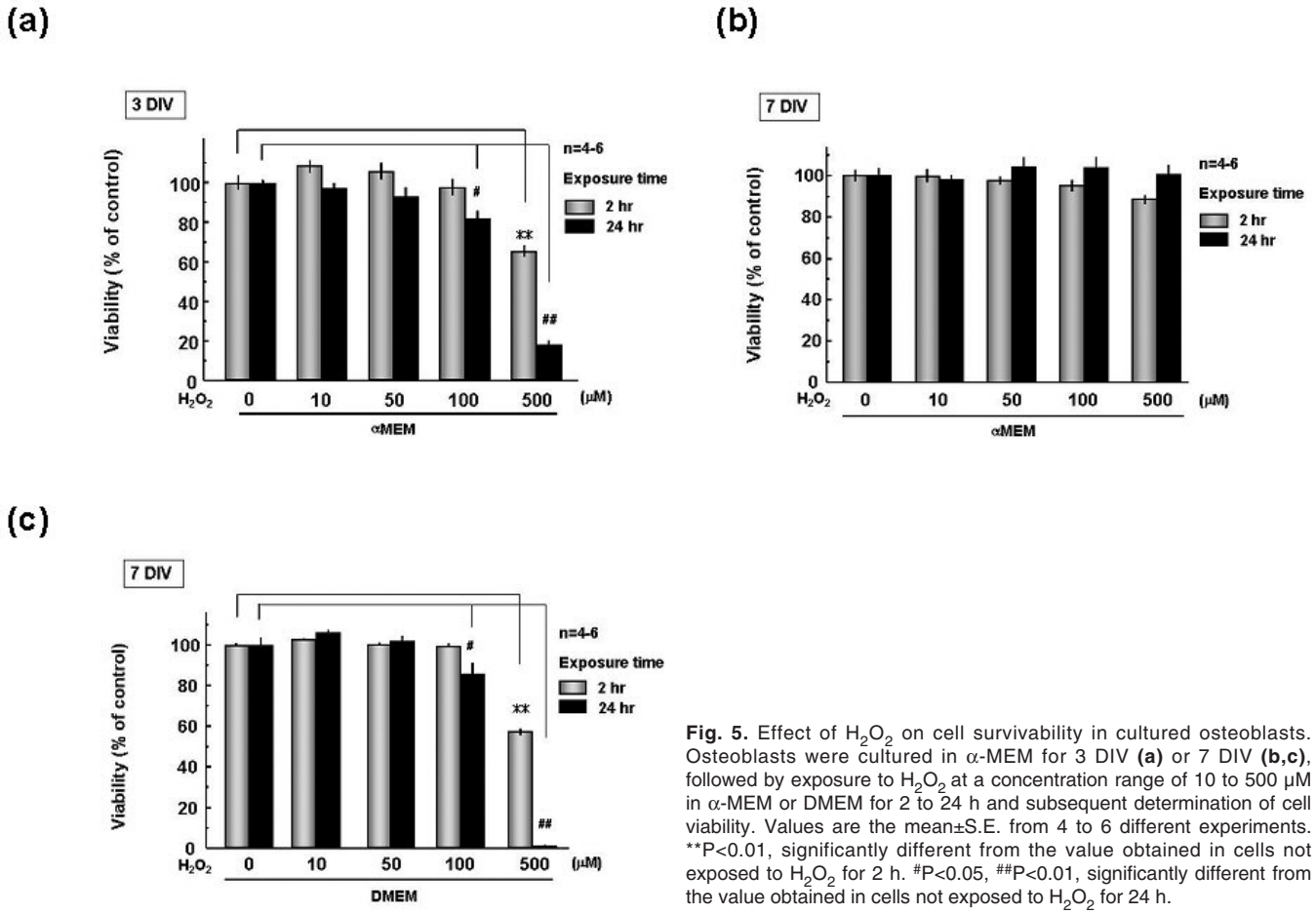

Fig. 5. Effect of H_2O_2 on cell survivability in cultured osteoblasts. Osteoblasts were cultured in α-MEM for 3 DIV **(a)** or 7 DIV **(b,c)**, followed by exposure to H_2O_2 at a concentration range of 10 to 500 μM in α-MEM or DMEM for 2 to 24 h and subsequent determination of cell viability. Values are the mean±S.E. from 4 to 6 different experiments. **P<0.01, significantly different from the value obtained in cells not exposed to H_2O_2 for 2 h. #P<0.05, ##P<0.01, significantly different from the value obtained in cells not exposed to H_2O_2 for 24 h.

H_2O_2-induced oxidative stress

In order to evaluate the vulnerability to oxidative stress, cultured osteoblasts were exposed to H_2O_2 at a concentration range of 10 to 500 μM in α-MEM or DMEM for different durations of 2 to 24 h, followed by determination of cell survivability. The exposure to H_2O_2 in α-MEM for 24 h led to a more potent decrease in cell survivability in a concentration-dependent manner than that for 2 h on 3 DIV (Fig. 5a), while no marked reduction of cell survivability was observed even after exposure for 24 h to 500 μM H_2O_2 in α-MEM on 7 DIV (Fig. 5b). However, the exposure to H_2O_2 in DMEM resulted in a significant reduction of cell survivability in a concentration dependent manner even on 7 DIV (Fig. 5c).

Protective effects of pyruvate on cell viability against oxidative stress

In order to further confirm the protective property of exogenous pyruvate, osteoblasts cultured for 3 or 7 DIV were exposed for 1.5 h to H_2O_2 at different concentrations of up to 1 mM in either the presence or absence of pyruvate at 1 mM. The addition of pyruvate significantly prevented the loss of cell viability induced by H_2O_2 at different concentrations in osteoblasts cultured for 3 (Fig. 6a) and 7 DIV (Fig. 6b). Moreover, the addition of pyruvate at 1 mM almost entirely abolished the generation of intracellular ROS in osteoblasts exposed to 500 μM H_2O_2 for 3 h when determined by DCFDA in DMEM on 7 DIV (Fig. 6c).

Discussion

The major findings in this study are: (1) that pyruvate could have a protective action against cell death after medium replacement of eutrophic α-MEM with normally nutritional DMEM in cultured osteoblasts through a mechanism associated with the anti-oxidative property rather than an energy fuel; (2) that medium replacement with DMEM could lead to generation of different species of cytotoxic ROS in a manner dependent on the culture stage; and (3) that the vulnerability to oxidative stress could be decreased in proportion to the cellular development in cultured

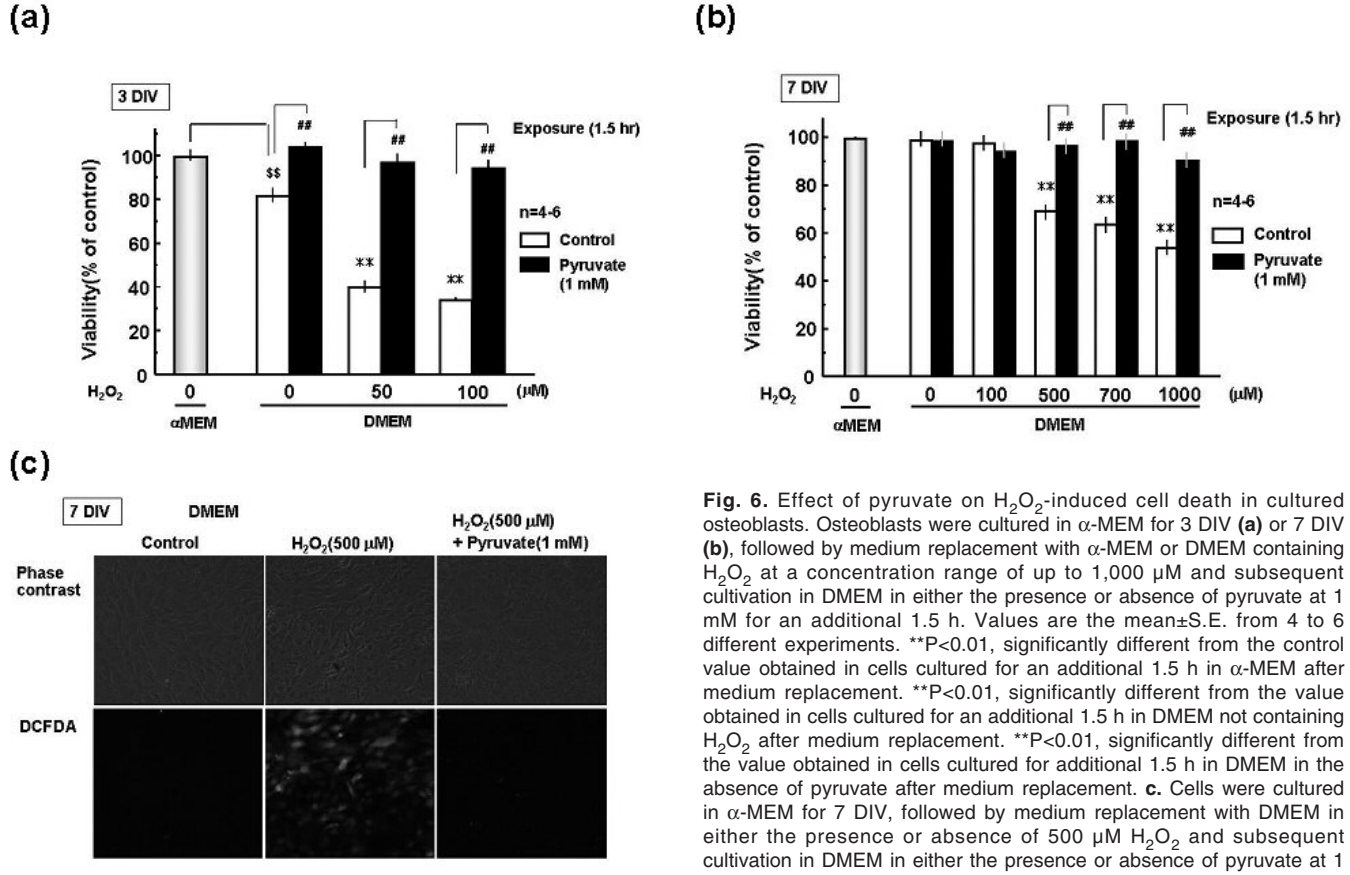

Fig. 6. Effect of pyruvate on H_2O_2-induced cell death in cultured osteoblasts. Osteoblasts were cultured in α-MEM for 3 DIV **(a)** or 7 DIV **(b)**, followed by medium replacement with α-MEM or DMEM containing H_2O_2 at a concentration range of up to 1,000 μM and subsequent cultivation in DMEM in either the presence or absence of pyruvate at 1 mM for an additional 1.5 h. Values are the mean±S.E. from 4 to 6 different experiments. **P<0.01, significantly different from the control value obtained in cells cultured for an additional 1.5 h in α-MEM after medium replacement. **P<0.01, significantly different from the value obtained in cells cultured for an additional 1.5 h in DMEM not containing H_2O_2 after medium replacement. **P<0.01, significantly different from the value obtained in cells cultured for additional 1.5 h in DMEM in the absence of pyruvate after medium replacement. **c.** Cells were cultured in α-MEM for 7 DIV, followed by medium replacement with DMEM in either the presence or absence of 500 μM H_2O_2 and subsequent cultivation in DMEM in either the presence or absence of pyruvate at 1 mM for an additional 4 h. Cells were then incubated with DCFDA in DMEM for 30 min, followed by determination of intracellular ROS under a confocal laser-scanning microscope. Typical pictures are shown here, with similar results in three independent determinations.

osteoblasts. Indeed, significant cytoprotection was seen with pyruvate, but not with cysteine or GSH, against cell death after the medium replacement in osteoblasts cultured for a period from 0 to 1 DIV. Although we have previously demonstrated that pyruvate deficiency induces cell death in cultured calvarial osteoblasts at the proliferative stage, the exact mechanisms for this effect have still remained unknown until this study. To our knowledge, therefore, this paper deals with the first direct demonstration of the essentiality of pyruvate for cell survival through the cytoprotection against oxidative stress in immature cultured rat calvarial osteoblasts.

Several independent lines of evidence indicate the potential cytoprotection by different endogenous constituents against cellular damage by H_2O_2 through two major mechanisms: enzymatic and non-enzymatic processes. The enzymatic defense against H_2O_2 includes catalase and glutathione peroxidase (Simonian and Coyle, 1996), whereas non-enzymatic mechanisms can also contribute to the cellular defense against H_2O_2-induced cytotoxicity. Pyruvate is, for instance, abundant in mammalian cells with a property to non-enzymatically react with H_2O_2. Pyruvate is converted to lactate by lactate dehydrogenase in the cytoplasm under anaerobic conditions, while pyruvate but not lactate is shown to play a role other than a metabolic energy fuel in mechanisms associated with protection of different cells against oxidative damage through non-enzymatic scavenging of ROS including H_2O_2. Bioactivities of polyphenols, non-enzymatic agents, are known to be closely related to their molecular structure containing hydroxyl, keto and gallate groups or double bonds. The ROS-scavenging activity of pyruvate might be involved partly in the structural property. Indeed, pyruvate is shown to chemically react with H_2O_2 to yield carbon dioxide, water and carboxylic acid as follows: $CH_3COCOO^- + H_2O_2 \rightarrow CH_3COO^- + CO_2 + H_2O$, preventing the formation of hydroxyl radical ($OH^\bullet$) from H_2O_2 (Holleman, 1904; Bunton, 1949). In cultured mouse striatal neurons, for example, pyruvate is effective in strongly preventing neurotoxicity of H_2O_2 extracellularly added and intracellularly formed from the quinone derivative menadione (Desagher et al., 1997). Similar cytoprotection by pyruvate is seen against apoptosis induced by H_2O_2 in bovine pulmonary endothelium (Kang et al., 2001) and human endothelial cells (Lee et al., 2004), cell death after ischemic injury in cultured rat astrocytes (Sharma et al., 2003), neurotoxicity by 6-hydroxydopamine, 1-methyl-4-phenylpyridinium and H_2O_2 in murine brain neuroblastoma cells (Mazzio and Soliman, 2003), and zinc toxicity in cultured mouse cortical neurons *in vitro* and in rat hippocampus and cortex in vivo (Sheline et al., 2000; Lee et al., 2001).

In this study, both cysteine (570 μM) and GSH (1 mM) had a protective effect on cell death after medium replacement in osteoblasts cultured for 3 DIV, without significantly affecting that in cells cultured for a period from 0 to 1 DIV. On Day 0 and Day 1, furthermore, decreased cell survivability was still observed even when 1 mM cysteine and 5 mM GSH were added in normally trophic DMEM in cultured osteoblasts (unpublished data). These findings give rise to an idea that medium replacement would lead to severe cell death in osteoblasts cultured for 3 DIV through molecular mechanisms different from those underlying the cell death in cells cultured for a period from 0 to 1 DIV. The protection by pyruvate could occur via intracellular and/or extracellular anti-oxidative defense mechanisms in cells cultured for 3 DIV, whereas the protective effect of cysteine or GSH may be attributed to an alternative action other than an anti-oxidative property including a role in energy metabolism in mitochondria. In both mouse neurons and astrocytes, in fact, tricarboxlic acid cycle substrates such as pyruvate and α-ketoglutarate, but not glucose, prevent cell death due to NAD depletion caused by a DNA alkylating agent (Ying et al., 2002). However, the addition of energy fuels, including lactate and acetate, did not significantly prevent osteoblastic cell death after medium replacement irrespective of the cellular maturity in the present investigation.

In addition, the inhibitor of mitochondrial pyruvate transporter α-cyano-3-hydroxycinnamate not only failed to affect cellular viability in osteoblasts alone, but also was ineffective in altering the protection by pyruvate (our unpublished data). As mentioned above, pyruvate could selectively react with H_2O_2 to prevent the generation of the hydroxyl radical ($OH^\bullet$), while both cysteine and GSH are known as a general scavenger of ROS including H_2O_2 (Holleman, 1904; Bunton, 1949). The aforementioned paradox between pyruvate and cysteine (or GSH) could be accounted for by taking into consideration a possible difference in species of ROS generated after medium replacement in osteoblasts cultured for different periods. Whether extracellular pyruvate is cytoprotective after the incorporation into intracellular locations, however, remains to be elucidated in future studies. From this point of view, it should be noted that a monocarboxylate transporter inhibitor attenuates the protective property of exogenous pyruvate against cell death by H_2O_2 in cultured mature rat neocortical neurons without markedly affecting that in immature neurons (Nakamichi et al., 2005).

It thus appears that pyruvate could invariably protect against the cell death induced by ROS at least in part through a direct scavenging action in cultured osteoblasts. If osteoblasts indeed express a particular isoform of pyruvate transporters, the transport activity may be a determinant of cellular survivability through modulation of the endogenous level of intracellular pyruvate that plays a dual role as an antioxidant and an energy fuel. Cell surface and/or mitochondrial pyruvate transport systems would therefore be a novel target for the discovery and development of drugs useful for the therapy and treatment of a variety of bone diseases associated with the cytotoxicity of ROS including

osteoporosis.

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