Enhancement of Erythropoietin Production in Recombinant Chinese Hamster Ovary Cells by Sodium Lactate Addition

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Abstract The stabilization of optimum pH for cells can cause a higher erythropoietin (EPO) production rate and a good growth rate with the prolonged culture span in recombinant Chinese hamster ovary (r-CHO) cells. Our strategy for stabilizing the optimum pH in this study is to reduce the lactate production by adding sodium lactate to a culture medium. When 40 mM sodium lactate was added, a specific growth rate was decreased by approximately 22% as compared with the control culture. However, the culture longevity was extended to 187 h, and more than a 2.7-fold increase in a final accumulated EPO concentration was obtained at 40 mM of sodium lactate. On the condition that caused the high production of EPO, a specific glucose consumption rate and lactate production rate decreased by 23.3 and 52%, respectively. Activity of lactate dehydrogenase (LDH) in r-CHO cells increased and catalyzed the oxidation of lactate to pyruvate, together with the reverse reaction, at the addition of 40 mM sodium lactate. The addition of 40 mM sodium lactate caused the positive effects on a cell growth and an EPO production in the absence of carbon dioxide gas as well as in the presence of carbon dioxide gas by reducing the accumulation of lactate.

Keywords: erythropoietin, high productivity, lactate, CHO, lactate dehydrogenase, pH

INTRODUCTION

Mammalian cell cultures including the culture of Chinese hamster ovary cells are used extensively to produce proteins for a therapeutic use [1-6]. The production process of recombinant proteins depends on a high cell yield, a prolonged culture span, steady productivity, and consistent glycosylation. The depletion of nutrients and the accumulation of cellular waste products are two major factors limiting cell growth in mammalian cell cultures. As a result, the cell density in a conventional batch culture is usually low and the culture span is short. Consequently, the amount of a product is small, which is undesirable for the production process of recombinant proteins. Both glucose and glutamine are the major carbon and energy sources used by mammalian cells, and these nutrients are often depleted during the cultivation. Periodic replenishment of glucose and glutamine through a fed-batch operation is commonly practiced to overcome their exhaustion. Waste products excreted by a mammalian cell itself have the significant effects on a cell growth and the production of metabolites. The major waste product is lactate, which is produced mainly from glycolysis for energy acquisition and in a small amount from glutamine metabolism, and ammonium, which is excreted when amino acids are metabolized in cells [7,8]. The concentration of lactate in mammalian cell cultures depends on the concentration of glucose, a type of cell, and a mode of bioreactor operation. Lactate inhibitions to mammalian cells are mainly attributed to the acidification of a medium. Namely, lactate accumulation often exceeds the buffering capacity of a medium, thereby lowering a culture pH value and inhibiting a cell growth. Because mammalian cells grow rapid *in vitro* only over a very narrow pH range within 0.2 to 0.4 pH units of the optimum, cell growth rates decrease at high lactate concentrations [9,10]. However, as compared to ammonium, the inhibition of cell growth by lactate occurs at relatively higher

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concentrations [11]. The toxic effects of lactate on mammalian cell cultures are well known and widely reported on a number of different cell lines [9,11-13]. For CRL-1606 hybridoma cells, lactate was found to be the only environmental parameter that significantly inhibited antifibronectin monoclonal antibody production, but the cell growth was slightly inhibited even at 40 mM lactate [14]. Stimulation of the cell growth by lactate concentrations up to 22 mM and inhibition above 28 mM lactate were reported in hybridoma cells (VII H-8) [15]. Miller *et al.* did not see any inhibition of lactate on the cell growth over the range of lactate concentration considered [16]. In addition, they observed almost no change in metabolic rates when lactate concentration was changed from 25 to 44 mM. Ozturk *et al.* reported that a specific growth rate of mouse hybridoma 167.4G5.3 cells was reduced by one-half at an initial lactate concentration of 55 mM [11]. A tolerance toward lactate is specific in cell lines [16]. A difference in the effects of lactate on different cell types may lie on the sensitivity of key enzymes to the glucose metabolic pathway and the metabolic shift in response to the adverse environment.

As previously mentioned, some cell lines such as hybridoma are characterized with respect to the effects of lactate. However, less information is available on the influence of lactate on the growth and productivity of Chinese hamster ovary (CHO) cells. CHO cells are the most frequently used mammalian host for biomedical products and they can express a wide variety of recombinant proteins. CHO cells have two significant advantages: (1) high productivity and (2) ability to synthesize oligosaccharide chain structures resembling those of the natural product. In addition, CHO cells are not apoptotic and eventually die by necrotic death [17]. The choice of nonapoptotic cells may, therefore, be beneficial to the production of proteins. EPO is a growth factor that stimulates the proliferation and differentiation of erythroid precursor cells to more mature erythrocytes [18]. This is one of the first recombinant proteins to make a successful trip from the laboratory to the clinical use. Recombinant human EPO has been approved for the clinical treatment of anemia associated with chronic renal failure, as well as nonrenal anemia [18,19]. Change in a pH value by the accumulation of lacate alters the protein processing, includeing glycosylation, and the secretion [20,21]. Many studies have described strategies to reduce the accumulation of lactate in mammalian cell cultures. The usual strategy is to replace the "spent" medium with a fresh medium. However, this strategy increases the use of serum, reduces the yield of a product on serum, and ultimately raises the cost of production. Glacken *et al.* introduced a strategy, which was to feed glucose into the cultures at a controlled low rate [22]. Because an increase in the concentration of glucose from 0.2 to 10 mM reduces the flux of glucose through the pentose-phosphate pathway by 50%, it preferably increases the flux of glucose through glycolysis to lactic acid [23]. In addition, Eagle *et al.* demonstrated that the substitution of more slowly utilized sugars, such as fructose, galactose, and maltose, results in a small amount of the accumulation of lactate [24]. Xie and Wang also reported that a production rate of lactate was significantly decreased through a rational medium design and a stoichiometric feeding of nutrients [25].

In this study, our objective is to decrease the formation of lactate by the design of an initial medium that would provide a starting environment to achieve an optimal cell growth and product production. We expected that the lactate added to a medium could alter cell metabolism. Because it was reported that cell metabolism was affected by the environmental conditions such as pH and the concentration of metabolites [26,27]. We investigated whether the addition of sodium lactate could cause higher EPO productivity in r-CHO cell cultures, and also examined effects of the lactate addition on lactate production and glucose consumption. Finally, the relationship between LDH activity and lactate addition was checked.

MATERIALS AND METHODS

Cells and Their Maintenance

r-CHO cell line producing EPO used in this study was provided by Dr. H. J. Hong from the Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. Minimum Essential Medium-α (MEM-α) (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 20 nM methotrexate (Sigma, USA) was used for the cultivation of r-CHO cells. No antibiotic was added to the medium. Cells were routinely cultured on 25-cm^2 T-flasks (Nunc, Denmark) and grown in a model MCO-175 incubator (Sanyo, Japan) at 37°C under atmosphere containing 5% CO_2 and 95% humid air. Cells in an exponential phase were used to seed at 10^5 cells/mL in 6-well plates (2.5 mL per well) in duplicate in media supplemented with various concentrations of indicated chemicals.

Experiments

For experiments in the addition of lactate, sodium lactate (Sigma) was dissolved in MEM-α at 400 mM, and then the pre-determined volume of 400 mM sodium lactate was added to a culture medium to obtain the required final concentration at the start of cell cultures. In this study, culture media with 0, 5, 10, 20, 40, and 80 mM sodium lactate were used. Using the same batch of cells, the culture with the same concentration of sodium chloride, which would give roughly the same osmolarity as that with sodium lactate, was also set up for the purpose of distinguishing between lactate effect and osmolarity effect. To test the effect of osmolarity, sodium chloride (Sigma) was added to a medium at the concentrations of 27.5, 47.5, 62.5, 72.5, and 87.5 mM. These concentrations of sodium chloride would give those osmolarities as 380, 420, 450, 470, and 500 mOsm/kg because the measured osmolarity of culture medium was found to be approximately 325 mOsm/kg. Cells cultured in the medium with normal osmolarity (325 mOsm/kg) were inoculated into media with varying osmolarities and cultured in 6-well plates (Nunc). Several aliquots of fresh medium were transferred to a series of conical tubes and their pH

were adjusted using sterile NaOH and HCl. An aliquot of a prepared suspension of cells was added to these tubes to achieve a final cell density of 10^5 cells/mL. Each cell suspension was pipetted in 2.5 mL into each well of 6-well plate. During the cultivation, the supernatant of cell cultures was taken at regular intervals and frozen for later analysis. Cells were counted for obtaining a growth profile. All experiments were duplicated.

Analyses

Concentrations of lactate and glucose in culture media were measured with a YSI model 2700 Biochemistry Analyzer (Yellow Springs Instruments, USA). Osmolarity was measured by a freezing point depression on an Osmomat 030 (Gonotec, Germany). To monitor an external pH, a sample of 1.0 mL was taken and immediately measured by a pH meter 440 (Corning, USA). Cell viability was determined by counting in a hemacytometer under a phase contrast microscope using the trypan blue exclusion.

Bioassay for EPO-Quantitative Analysis

Biological activity of EPO was examined by using an EPO-dependent cell line, human leukemia F-36E (Riken Cell Bank RCB0776, Japan), that could be maintained in the presence of EPO [28]. F-36E cell density in each well of 96-well plate was initially fixed to 1×10^5 cells/mL. The standard of EPO (Boehringer Mannheim, Germany) and the supernatant of each sample were diluted serially with F-36E cell broth and 100 μL of the dilution was assayed for the biological activity of EPO by measuring the viable cell density of EPO-dependent cells. After incubation for 4 days, we performed MTT assay to measure viability, proliferation, and activation of cells [29]. One hundred microliters of the cultured sample was incubated once more with 10 μL of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma) in 96-well plates at 37°C for 3 h. Then 100 μL of 0.04 M HCl in iso-propyl alcohol (Duksan, Korea) was added to each well, and the mixture was pipetted to dissolve the insoluble blue formazan crystals. The plate containing the dissolved blue formazan was read at an optical density of 570 nm. Sample was run in duplicate.

Electrophoretic Fraction and Colorimetric Detection of Lactate Dehydrogenase Isoenzymes

Cells for this assay were harvested, centrifuged (1,000 rpm, 2 min), and washed twice in ice-cold phosphate buffered saline (PBS). After the second washing, the viable cell density was determined. Intracellular components were extracted by ultra-sonicating the cell pellet in 0.5 mL of PBS. The obtained cell lysates were centrifuged at 13,000 × g for 5 min, and LDH isoenzyme present in the supernatants was separated by adding to 1% agarose (Bethesda Research Laboratories, USA) gel and electrophoresing in 50 V for 2 h using a mini gel migration system (Mupid-21, Cosmo Bio, Japan). An electrophoretic marker (LDH Isotrol, Sigma) containing LDH isoenzymes 1 to 5 was used as an aid in the identification of isoenzymes. After the electrophoresis, separated LDH isoenzymes were visualized by using a colorimetric detection (Procedure No. 705-EP, Sigma). The stained gel was fixed in a methanol-acetic acid solution (75 methanol : 5 glacial acetic acid : 20 water).

Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity was determined by a diagnostic kit (Sigma). There were two procedures for determination of LDH activity. The first procedure was based on the oxidation of lactate to pyruvate [lactate + NAD → pyruvate + NADH]. LDH catalyzes the oxidation of lactate to pyruvate with the simultaneous reduction of NAD. Formation of NADH results in an increase in absorbance at 340 nm. The increasing rate of absorbance at 340 nm is directly proportional to LDH activity in a sample. To determine LDH activity, the supernatant of cell lysates was prepared by following the procedure used in the detection of LDH isoenzyme. Assay reagent contained 50 mmol/L lactate, 7 mmol/L NAD, 0.05% sodium azide, and nonreactive stabilizer and fillers (pH 8.9). The prepared cell extract (50 μL) was added to a assay solution (1 mL, 30°C) and mixed by gentle inversion. After the incubation for 30 sec at 30°C, the absorbance was read at 340 nm by a spectrophotometer (Cary 300 Bio, Varian, Australia). This was initial absor-bance. Continuous incubation was performed and the absorbances of 30 and 60 sec following initial absorbance reading were recorded. The absorbance reading after 60 sec was final absorbance. Subtraction of initial absorbance from final absorbance was calculated to obtain 'absorbance change per minute'. LDH activity was determined as follows: LDH activity [U/L] = ('absorbance change per minute' × total reaction mixture volume × 1,000) / (millimolar absorptivity of NADH at 340 nm × sample volume × lightpath). One unit of LDH activity was defined as the amount of enzyme that would catalyze the formation of one micromole of NADH per minute. Specific activity of LDH was expressed as units per 10^6 cells. LDH activity was measured by monitoring the rate at which the substrate, pyruvate, is reduced to lactate [pyruvate + NADH → lactate + NAD]. Since the reduction is coupled with the oxidation of reduced form of NADH, this reaction is measured in terms of the decreasing rate of absorbance at 340 nm. Potassium phosphate buffer (2.85 mL, 0.1 mol/L, pH 7.5) and 50 μL cell extract were pipetted directly into a glass vial containing 0.2 mg reduced form of NAD. The mixture was mixed well, then left at 25°C for 20 min. This was transferred from a glass vial to a square cuvet (1-cm lightpath) capable of holding 3 mL, and 0.1 mL sodium pyruvate solution (22.7 mmol/L in 0.1 mol/L potassium phosphate buffer) was also added to that cuvet. The absorbance was read and recorded at 340 nm at 30 sec intervals for 3 min using water as reference. LDH activity in this second procedure was determined as follows: LDH activity [U/mL] = ('absorbance change per minute') / (0.001 × sample volume × lightpath), where: 0.001 = 'absorbance change per minute' equivalent to 1 unit of LDH activity in 3 mL volume with 1-cm lightpath at 25°C.

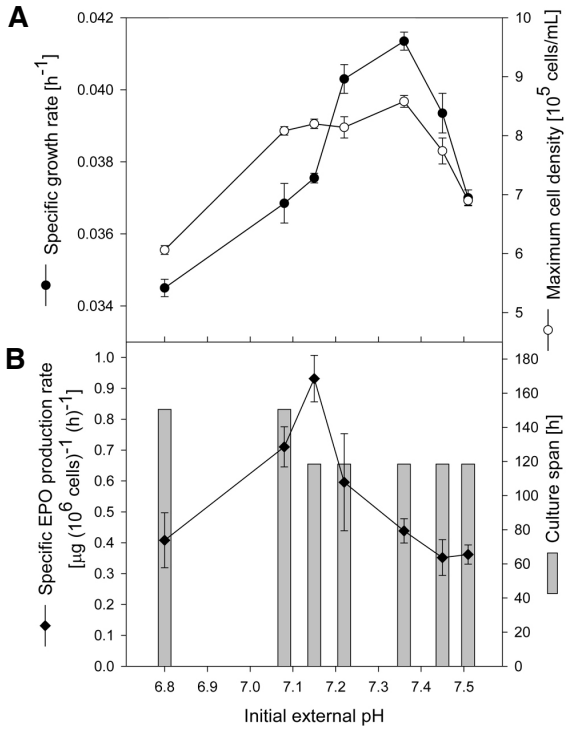

Fig. 1. Effect of initial external pH on cell growth (A) and EPO production (B) of r-CHO cells in MEM-α. Cells were inoculated at 1×10^5 cells/mL and grown in 6-well plates for 6 days.

RESULTS AND DISCUSSION

Inhibitory Effect of External pH on Cell Growth and Protein Productivity

The major by-product of energy metabolism is lactate, which arises from glycolysis. The accumulation of lactate often exceeds the buffering capacity of a culture medium, thereby lowering the culture pH. Subsequently, this may cause undesirable effects on cell growth, since mammalian cells grow rapidly *in vitro* only over a very narrow pH range within 0.2 to 0.4 pH units of the optimum. Unlike insect and primary mammalian cells, transformed mammalian cells convert a large amount of glucose to lactate [30]. A previously investigated CHO cell line was converted more than 97% of the consumed glucose into lactate. Effects of initial external pH on cell growth and protein productivity of r-CHO cells transfected for the human EPO synthesis were examined in batch cultures. Fig. 1A shows the growth results of r-CHO cells seeded at 10^5 cells per mL and grown over a 6 day-period at various pH values. Poor cell growth occurred at the extremes of tested pH. At pH 6.80, the specific growth rate decreased dramatically by the acidification of a culture medium, but the culture span was prolonged because of a retarded nutrient depletion. When initial culture pH was 7.51, cells had a long lag time initially, but they were able to adapt to pH 7.51 within a few days and subsequently their behavior was similar to that at pH 7.08. In addition, a variation of final cell density appeared. In result, an optimal plateau for good cell yield was obtained between pH 7.08 and 7.36, and a maximum specific growth rate was observed at pH 7.36, whereas the specific EPO production rate increased sharply above pH 6.80, reaching a maximum at pH 7.15 (Fig. 1B). Both higher EPO production rate and good cell growth were the cause of maximum EPO production. When the initial external pH decreased to 6.80, the specific EPO production rate was 43.8% of that at pH 7.15. The results from Fig. 1 verify the reports that pH has a direct effect on the cells producing EPO [20,31]. In general, external pH influences intracellular pH, and the change in intracellular pH can affect the activity of cytosolic enzymes or alter the metabolism of cells [32]. Moreover, the pH change in an endoplasmic reticulum and/or a Golgi apparatus could alter protein glycosylation and secretion [21]. Gramer and Goochee also reported that a sialidase activity in CHO cell lysates was active and stable at pH 7 [33]. Our results, together with these reports mentioned above, could account for the effect of initial external pH on the specific EPO production rate. In result, we concluded that the pH stabilization was necessary for good EPO production. The usual strategies for stabilizing the optimal pH in mammalian cell cultures are to replace the "spent" medium with fresh medium and to titrate the pH of culture media with alkaline. However, the one increases serum usage and reduces the yield of product on serum, thus increasing production costs, while the other increases media osmolarity, which ultimately causes cell lysis. Like this, the existing strategies have many problems. Therefore, in this study, we introduced a new strategy, which was to add sodium lactate as a culture supplement.

Increased EPO Production with Addition of Sodium Lactate

We examined effects of the added lactate on growth, metabolism, and protein productivity of r-CHO cells producing recombinant human EPO in batch cultures with sodium lactate of 0, 5, 10, 20, 40, or 80 mM because the addition of sodium lactate was considered as a new strategy for the stabilization of culture pH in the present work. In our study of r-CHO cells, it seems that the specific growth rate gradually decreases with an increase of lactate addition concentration. From Fig. 2A, however, no significant reduction in the specific growth rate was found up to 20 mM sodium lactate addition. The specific growth rate was not decreased by approximately 22% until the concentration of sodium lactate increased to 40 mM. We judged that the decreased specific growth rate in 40 mM would be profitable for EPO production because of the following reason. If a desirable product results from the expression of an amplified recombinant gene, the cellular energy required to synthesize this protein could be a substantial fraction of the total energy capacity. When cells grow rapidly, however, cellular energy sources may be diverted to the synthesis of materials necessary for cell growth rather than EPO production. Therefore, fast growth of r-CHO cells may lower EPO production. When

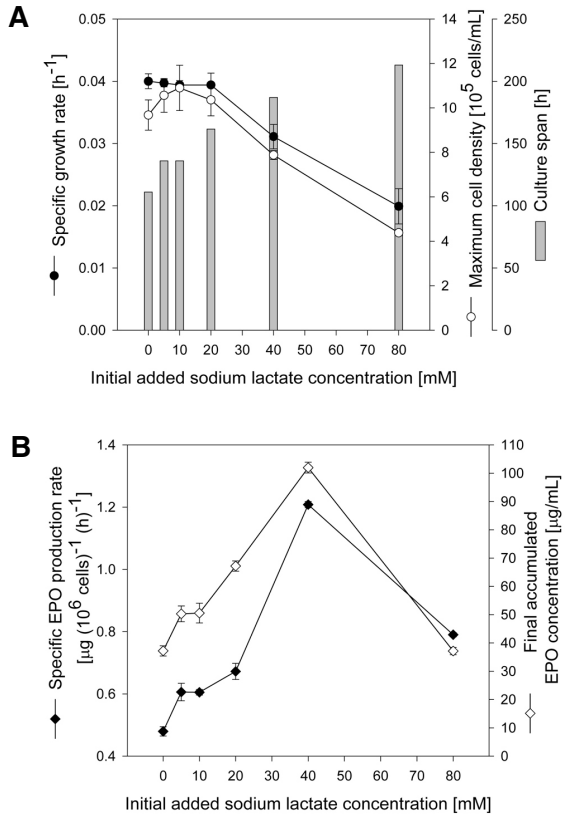

Fig. 2. Effect of sodium lactate concentration on cell growth (A) and EPO production (B) of r-CHO cells in MEM-α. All cultures were initiated at an inoculum of 1×10^5 cells/mL and performed in 6-well plates for 8 days. Sodium lactate was supplemented into cultures at the concentrations indicated.

the addition concentration increased further to 80 mM, much depressed growth rate was observed. In this result, the inhibitory concentration for a 50% decrease (IC-50) of r-CHO cells is estimated as 80 mM sodium lactate, which indicates that r-CHO cells are far less sensitive to lactate than other cells [11]. On the contrary, a culture span was extended in lactate-addition cultures compared with a control culture. According to Fig. 2A, the culture longevity was extended for between 50 and 100 h at high concentrations of lactate. Industrial trends toward high density cultures have required elongating a culture span. Therefore, it is valuable to find a way of elongating a culture span. From our study, we discovered that further prolonged culture span could be induced by the addition of sodium lactate.

All tested lactate concentrations except 80 mM produced EPO far more than that in the absence of sodium lactate, as illustrated in Fig. 2B. For 80 mM lactate addition, the specific EPO production rate was 1.65 times higher than that in a control. However, the apparent volumetric production of EPO was similar to that in a control because the specific growth rate was below 0.02 h^{-1}. The specific EPO production rate of 0.48 μg/10^6 cells per hour in a control increased significantly to 1.21 μg/10^6 cells per hour in the presence of 40 mM. Consequently, more than a 2.7-fold increase in final accumulated EPO concentration was obtained by adding 40 mM sodium lactate. The improvement of EPO production could be acquired by prolonged cell viability and increased specific EPO production rate. Therefore, 174% increase of final EPO concentration observed in 40 mM lactate addition was due to the elongated longevity of about 80 h and 152% increase of specific EPO production rate. In addition, the stimulation of EPO production observed here may be partially the result of reduced proliferation resulting in reduction of the cellular demand for ATP, because more than one post-transcriptional step in EPO synthesis may be affected by enhanced ATP availability. In conclusion, healthy cells, longer production period, and higher specific EPO production rate can be major factors to produce much EPO, and these factors can be accomplished by adding sodium lactate below 80 mM.

The entire cell metabolism could be altered in the presence of lactate, due to followed causes. The first cause is the intracellular acidification induced by lactate. Therefore, it has been believed that lowering lactate accumulation would enhance cell growth and protein production, and the reduction of lactate production has been considered as one of the important goals in mammalian cell culture technology. The second cause is the effect of lactate in itself, especially important in the disturbance of the metabolic pathways in which it is produced, or involved [34]. We observed only a marginal increase of external pH at 5 and 10 mM of the added lactate concentrations (Fig. 3A). However, the decrease rate of pH was lowered on the condition that more than 20 mM sodium lactate was added. This result implies that the addition of sodium lactate supports the stabilization of culture pH. The small change in external pH is profitable for long-term cultivation as well as EPO production. Fig. 3 also shows effects of the added lactate concentrations on specific rates in glucose consumption and lactate production. There was a significant decrease in specific glucose consumption rate at lactate concentrations varying from 0 to 80 mM, as shown in Fig. 3B. The specific consumption rate of glucose decreased by 23.2% as 40 mM sodium lactate was added, while the specific production rate of lactate decreased by 52% (Fig. 3C). These results from Figs. 3B and 3C may be mainly due to lactate effect in itself. In general, continuous mammalian cell lines have a metabolic disadvantage of being unable to completely oxidize glucose to CO_2 and H_2O, although they are well accepted as reliable and safe production hosts for manufacturing pharmaglycoproteins. In other words, most of the glucose (95 to 99.8%) is oxidized to pyruvate and finally to lactate [35-37]. The reason why continuous cells oxidize glucose incompletely that the enzymatic connection between glycolysis and tricarboxylic acid (TCA) cycle has yet to be known [38]. However, we expected that the added lactate could lead glucose to TCA cycle, because lactate could be converted into pyruvate which could enter the TCA cycle. The yield of lactate produced from glucose consumed, $Y_{L/G}$, is an important index of glycolysis. The effect of the added lactate concentrations on

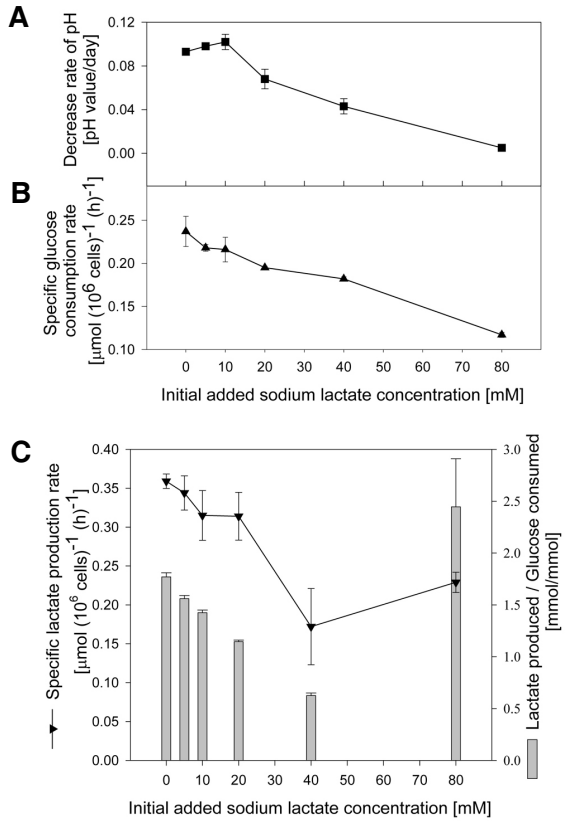

Fig. 3. Effect of sodium lactate concentration on decrease rate of pH (A), specific glucose consumption rate (B), and specific lactate production rate and ratio lactate produced to glucose consumed (C). r-CHO cells were inoculated at 1×10^5 cells/mL and cultured in 6-well plates for 8 days. Sodium lactate was supplemented into cultures at the concentrations indicated.

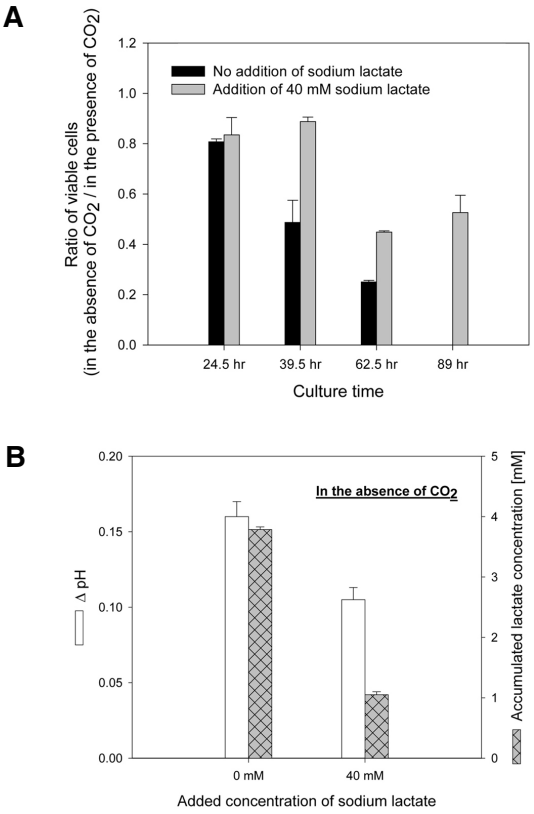

Fig. 4. Ratio of viable cells in the absence of atmospheric CO_2 to in the presence of atmospheric CO_2 (A), and change of pH (ΔpH) and accumulated lactate concentration of r-CHO cells in the absence of atmospheric CO_2 at the culture time of 39.5 h (B). r-CHO cells were inoculated at 2×10^5 cells/mL in 6-well plates and cultured in MEM-α supplemented with 0 or 40 mM sodium lactate for 4 days.

$Y_{L/G}$ is shown in Fig. 3C. $Y_{L/G}$ decreased progressively as the added lactate concentration increased up to 40 mM. The decreased $Y_{L/G}$ indicates that a larger proportion of consumed glucose may enter the TCA cycle [39]. Judging from results above, the action of lactate is likely to have a profound effect on glycolysis, so a good amount of glucose may enter the TCA cycle at high lactate concentrations. In result, an increase in energy efficiency may occur. However, the elevated energy is not likely to be diverted for cell growth. It is likely to be used for the synthesis of EPO instead. Flickinger *et al.* [40] and Leno *et al.* [41] also suggested that additional ATP could accelerate energy-consuming steps in biosynthetic pathway of protein.

Effect of 40 mM Sodium Lactate Addition on r-CHO Cells in the Absence of Carbon Dioxide

The pH of cell culture medium is controlled by CO_2/ bicarbonate buffer system ($H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$). The net result of increasing atmospheric CO_2 is to depress the pH, so the effect of elevated CO_2 tension is neutralized by increasing the bicarbonate concentration ($NaHCO_3 \leftrightarrow Na^+ + HCO_3^-$). The increased HCO_3 concentration forces the equilibrium of CO_2/bicarbonate buffer system toward the left until equilibrium is reached at pH 7.4. Due to the equilibrium attained between dissolved CO_2 and bicarbonate in this way, the pH of culture medium is maintained to the proper physiological level in buffer capacity. The absence of atmospheric CO_2 allows the equilibrium of CO_2/ bicarbonate buffer system to the left, eventually eliminating HCO_3^- from the medium. However, HCO_3^- is necessary for the good growth in CHO cells, because H^+ transport system of CHO cells requires Na^+, Cl^-, and HCO_3^- [42]. Therefore, the absence of atmospheric CO_2 inhibits cell growth. Nevertheless, it must be worth considering a free-CO_2 condition within the limits of the possibility, since the use of atmospheric CO_2 increases costs. In this study, we examined whether the addition of 40 mM sodium lactate could improve the growth of r-CHO cells in the absence of atmospheric CO_2. The ratio of viable cells in the absence of atmospheric CO_2 to in the presence of atmospheric CO_2 is shown in Fig. 4A. In the control experiment, where no so-

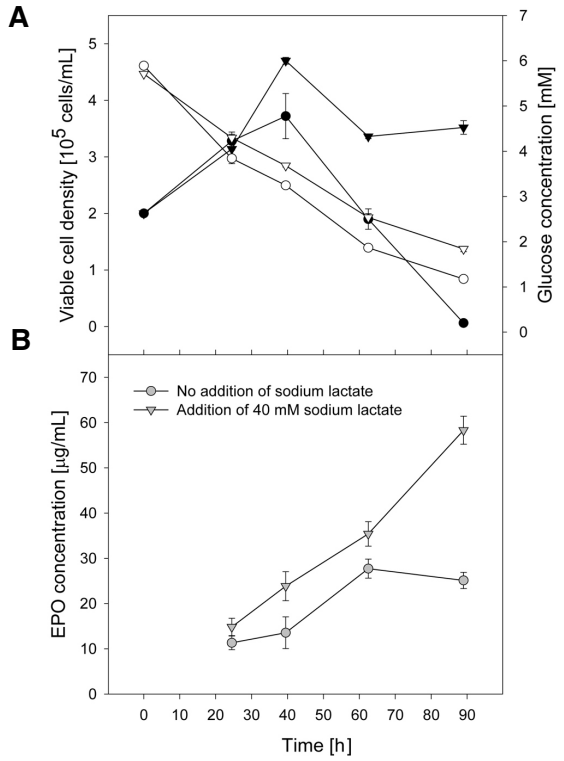

Fig. 5. Viable cell density (A, closed symbols), glucose concentration (A, open symbols), and EPO production (B) of r-CHO cells in MEM-α supplemented with 0 (circle symbol) or 40 (reversed triangle symbol) mM sodium lactate for 4 days. Cells were inoculated at 2×10^5 cells/mL in 6-well plates and cultured in the absence of atmospheric CO_2.

dium lactate was added, the viable cell density in the absence of atmospheric CO_2 decreased by 51.9% compared with that in the presence of atmospheric CO_2 at the culture time of 39.5 h and decreased significantly by 74.9% at 62.5 h. However, the viable cell density in the absence of atmospheric CO_2 was 88.7% of that in the presence of atmospheric CO_2 at 39.5 h on the condition that 40 mM of sodium lactate was present. The addition of 40 mM sodium lactate also caused the small change of external pH by reducing lactate production, as shown in Fig. 4B. In other words, the added sodium lactate is likely to replace the role of atmospheric CO_2 by decreasing lactate accumulation. When atmospheric CO_2 was absence, the poor viability in the medium without sodium lactate decreased dramatically to zero, as shown in Fig. 5A. This result indicates that the absence of CO_2 inhibits the growth of r-CHO cells. However, r-CHO cells in the culture medium containing 40 mM sodium lactate remained at the concentration of 3.52×10^5 cells/mL even at the culture time of 89 h. In addition, the glucose consumption in the presence of 40 mM sodium lactate was slower than that in the absence of sodium lactate. This result indirectly shows that the efficiency of energy production is increased by the addition of sodium lactate even on the condition without atmospheric CO_2. Moreover, because of the elongated culture span, EPO concentration in the presence of 40 mM sodium lactate was 232% of that in the absence of sodium lactate (Fig. 5B). These results imply the positive effect of added 40 mM sodium lactate on the growth and EPO production of r-CHO cells on the condition without atmospheric CO_2.

Comparison between Sodium Chloride and Sodium Lactate Addition in r-CHO Cell Cultures

The addition of sodium lactate increases the osmolarity, for example, at the addition of 40 mM sodium lactate, the osmolarity is approximately 400 mOsm/kg. However, the osmolarity in the range of 270~330 mOsm/kg is known to be quite acceptable for most cells [43]. In addition, growth depression in hypertonic medium has been reported for MCL1 cells and other hybridoma cells [44,45]. Therefore, we had to investigate whether the results in Figs. 2 and 3 were due to the sodium lactate addition itself or the osmolarity induced by the addition of sodium lactate. In advance of this investigation, we examined the effect of medium osmolarity on cell growth and EPO production of r-CHO cells over the range of 325 to 500 mOsm/kg. With an increase of the medium osmolarity, the specific growth rate and maximum cell density decreased (Fig. 6A). The specific growth rates at 380, 420, and 450 mOsm/kg decreased by 16, 31, and 46% as compared with the control (325 mOsm/kg), respectively. The inhibition of cell growth was extreme at 500 mOsm/kg. The result at 500 mOsm/kg could partially explain the exceptional phenomena observed in Figs. 2B and 3B when 80 mM sodium lactate (approximately 480 mOsm/kg) was added. The result of cell growth in Fig. 6A was somewhat different from the observation by Kurano *et al.* [46] that CHO cell growth was not affected by osmolarity below 390 mOsm/kg. In general, hybridomas are known to exhibit an increased specific antibody production rate when subjected to environmental stress, as hyperosmotic pressure [26,44, 45,47-56]. However, the increase of osmolarity decreases the specific growth rate [44,57,58]. Therefore, some "trade-off" relationship between cell growth and protein production is likely to exist [59]. We observed a similar response for the specific EPO production of r-CHO cells (Fig. 6B). However, Glacken *et al.* observed a decrease of specific antibody production rate in CRL-1606 cells under osmolarity conditions increased by the addition of sodium lactate [35]. The influence of osmolarity on protein productivity seems to be cell line dependent [60]. Increasing osmolarities from 325 to 420 mOsm/kg increased the specific EPO production rate, as shown in Fig. 6B. The specific EPO production rate in cells exposed to 420 mOsm/kg hyperosmotic stress was increased by 74% when compared with that of cells in the culture with 325 mOsm/kg, and the specific EPO production rates between 420 and 470 mOsm/kg formed a plateau. However, at 500 mOsm/kg, the specific EPO production rate dropped to 85.8% of 420 mOsm/kg. Oh *et al.* demonstrated that the osmolarity effect acted quite specifically on Na^+-dependent transport systems, while there was no significant increase in the uptake by Na^+-independent transport systems [50]. Accordingly, the increase in osmolarity might enhance the

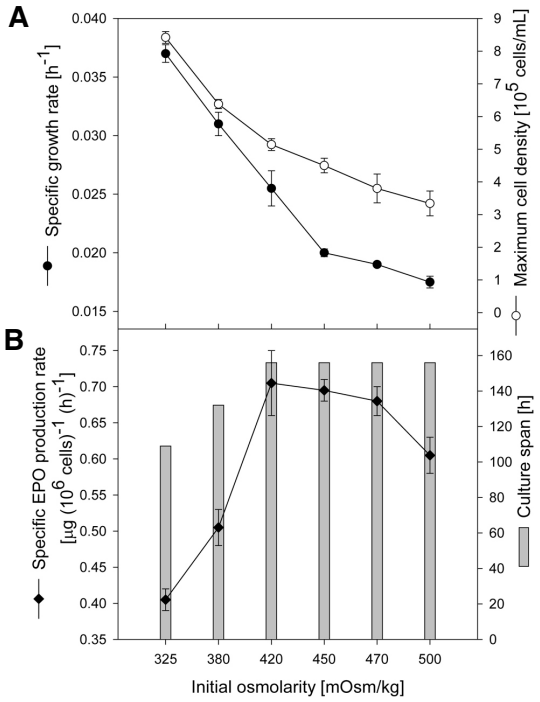

Fig. 6. Effect of medium osmolarity on cell growth (A) and EPO production (B) of r-CHO cells in MEM-α. Cells were inoculated at 1×10^5 cells/mL and grown in 6-well plates for 7 days. The growth medium became hyperosmotic through the addition of sodium chloride.

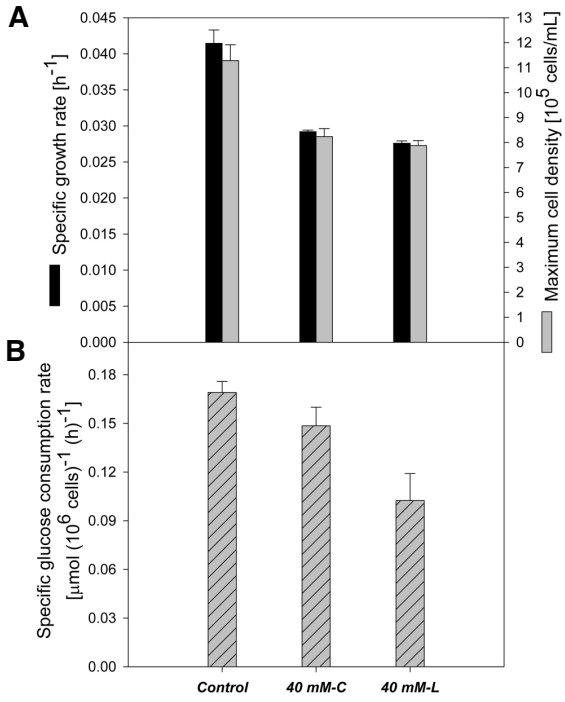

Fig. 7. Comparison of cell growth (A) and specific glucose consumption rate (B) of r-CHO cells in MEM-α supplemented with 40 mM sodium chloride (40 mM-C) or 40 mM sodium lactate (40 mM-L) for 7 days. No supplement including sodium chloride and sodium lactate was added in the control experiment. Cells were inoculated at 1×10^5 cells/mL in 6-well plates.

transport of nutrients, in particular, amino acids through Na^+-dependent transport systems [49]. In addition, stressed cultures contain the enhanced levels of total RNA, of which ~80% is ribosomal RNA (rRNA). Higher rRNA content could increase the translation rate of protein. Oh *et al.* also reported that RNA/DNA ratio was always higher for cultures with higher osmolarity. High RNA/DNA ratio gives us a crude indication that transcription levels might be elevated in osmotically stressed cells [50]. Furthermore, under the higher osmolarity, histones are more readily dissociated from DNA, thus allowing easier accessibility of the chromatin structure to RNA polymerase for transcription [61]. It was also reported that the rate of transcription increased through the nucleosome at high osmolarity [62]. These reports can partially explain the reason why the increase in osmolarity enhances the specific EPO production rate in our experiment.

To verify that the increase of specific EPO productivity observed in Fig. 2B was the effect of sodium lactate rather than the osmolarity effect, 40 mM sodium lactate and sodium chloride were used for adjusting medium osmolarity to approximately 400 mOsm/kg. We estimated that the effects of lactate observed in this study were mainly related to the alteration in cell metabolism by sodium lactate itself rather than the change in medium osmolarity induced by the added sodium lactate. Therefore, we attempted to separate the chemical effect of sodium lactate and the effect of osmolarity by comparing the results under the same osmolarity. In result, we obtained no significant difference of cell growth between lactate-containing culture and lactate-excluding culture of the same osmolarity, as illustrated in Fig. 7A. Thus, it is obvious that lactate itself has very little influence on growth and the inhibition to cell growth is mainly due to osmolarity. However, Fig. 7B showed that the specific glucose consumption rate decreased by 39.4% in addition of 40 mM sodium lactate and 12.1% in the same concentration of sodium chloride. In addition, specific lactate production rate was also found to be inhibited by 57.7% in 40 mM sodium lactate and 24.7% in 40 mM sodium chloride (Fig. 8B). Namely, when 40 mM sodium lactate was added, the specific glucose consumption rate and lactate production rate were considerably lower than that observed in the equivalent concentration of sodium chloride. This indicates that the effects of lactate on specific glucose consumption rate and lactate production rate are not due to the osmolarity induced by lactate addition alone. On any osmolarity adjusted by sodium chloride, $Y_{L/G}$ remained constant in the range of 1.8~2.0 (data not shown). This result agrees with that from the report of Ryu and Lee [63]. However, on the condition that sodium lactate was added, $Y_{L/G}$ decreased as concentration of sodium lactated increased, as shown in Fig. 3C. It is assumed that osmolarity drives the effect of lactate on cell

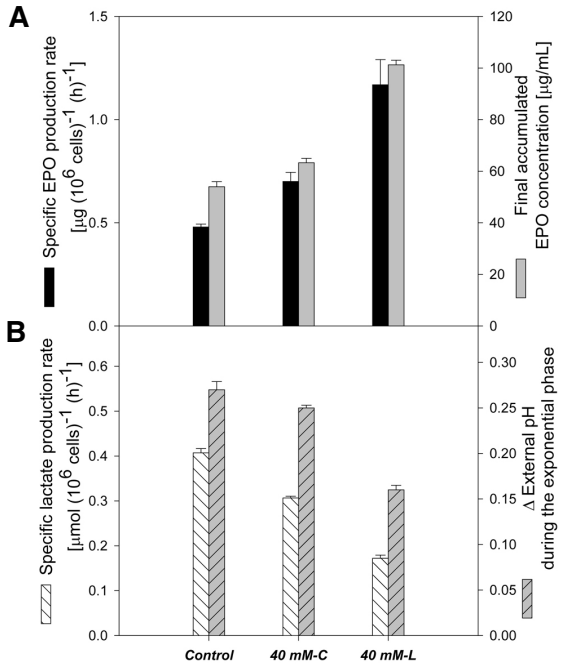

Fig. 8. Comparison of EPO (A) and lactate production (B) of r-CHO cells in MEM-α supplemented with 40 mM sodium chloride (40 mM-C) or 40 mM sodium lactate (40 mM-L) for 7 days. No supplement including sodium chloride and sodium lactate was added in the control experiment. Cells were inoculated at 1×10^5 cells/mL in 6-well plates.

growth, but lactate itself is the main reason for the specific glucose consumption rate and lactate production rate. In addition, the change of pH in culture medium during the exponential phase was 0.16 from initial pH in 40 mM sodium lactate and 0.25 in the same concentration of sodium chloride (Fig. 8B). This indicates that sodium lactate addition can prevent the big drop of medium pH. The pH stabilization induced by sodium lactate may be profitable for the long-term cultures of r-CHO cells. We also observed that the addition of sodium lactate had the highest level of specific EPO production rate among all the experiments tested, as shown in Fig. 8A. This increase in EPO productivity, on the condition that 40 mM sodium lactate was added, was not identical to the corresponding increase caused by the osmolarity. Specific EPO production rate increased by 144% in 40 mM sodium lactate and 46% in the same concentration of sodium chloride as compared with the control. In addition, the final accumulated EPO concentration from 40 mM sodium chloride supplemented culture showed the slightly higher level than that of the control. However, the accumulated EPO in 40 mM sodium lactate was 1.9 times higher than that in the control. This proves indirectly that the increased osmolarity of culture medium is not the only effector on EPO production. Although the exact mechanisms of actions by the two-stress factors, sodium lactate itself and osmolarity, are unknown, it is evident that the influences of lactate addition on EPO production and metabolisms are the combined effects of sodium lactate itself and osmolarity induced by it. Therefore, the effects of sodium lactate may be partly explained by the increased osmolarity in the case of r-CHO cells. After "correcting" for osmolarity effects, sodium lactate itself was found to increase the specific EPO productivity by 98%, and to decrease the specific glucose consumption rate and specific lactate production rate by 27 and 33%, respectively. If osmolarity was the only effect, the results of sodium lactate and sodium chloride observed in Figs. 7 and 8 would always be equal, but those of sodium lactate and sodium chloride did not coincide.

Relationship between Lactate Dehydrogenase Activity and Lactate Addition in r-CHO Cell Culture

Lactate dehydrogenase catalyzes the interconversion of lactate and pyruvate. The enzyme is a tetrameric protein [64] and gives rise to five isoenzymes. In massive documentation that has followed the reports of Dewey and Conklin [65] and Van der Helm [66], it is generally agreed that human tissue can be grouped into three categories based on LDH isoenzymes contents. LDH-1, -2, and -3 are prominent in tissues such as heart, kidney, brain, pancreas, erythrocytes, lymphocytes, platelets, and diaphragm. LDH-4 and LDH-5 are the major fractions of liver, skeletal muscle, and granulocytes. Prostate, thyroid, lung, adrenal, gastric mucosa, and ovary are characterized by preponderance of fractions intermediate to the other groups [67]. H represents the peptide associated with the main cardiac enzyme (LDH-1) and M the peptide from skeletal muscle (LDH-5). The intermediate isoenzymes LDH-2, -3, and -4 are hybrids formed by random association of H and M subunits into tetramers. Thus, LDH-2, -3, and -4 would contain H_3M, H_2M_2, and HM_3 subunits [68]. LDH-1 moves farthest toward the anode during electrophoresis at pH 8.6 and LDH-5 is the extreme cathodic enzymes. LDH-1 and LDH-2 are sometimes termed the fast isoenzymes and LDH-4 and LDH-5 the slow enzymes. LDH-3 is an enzyme with intermediate electrophoretic mobility. LDH-X migrates between LDH-3 and LDH-4 on agarose gel [69]. Agarose gel electrophoresis of LDH isoenzyme pattern in r-CHO cells is shown in Fig. 9. We observed no change of LDH isoenzyme pattern between lactate-containing culture and lactate-excluding culture of r-CHO cells. Thus, it is obvious that lactate itself has no influence on the isoenzyme pattern of LDH. Electrophoretic band for LDH isoenzyme type of r-CHO cells was present in the LDH-3 and LDH-4 regions, whereas Blanco and Zinkham [69] observed that human mature ovary tissue had five isoenzymes with the exception of extra isoenzyme, LDH-X. Taken together, our result obtained in r-CHO cells originated from Chinese hamster ovary tissue is quite different from that of human mature ovary tissue. We conclude from Fig. 9 that LDH isoenzyme type of r-CHO cells is LDH-X. It is generally known that H-type LDH is better adapted to function in the oxidation of lactate to pyruvate, whereas M-type LDH is more suited to catalyze the reverse reaction [70]. However, Brooks *et al.* [71] have reported that lactate oxidation exceeded pyruvate reduction by 10~40% in mitochondria isolated from liver, skeletal

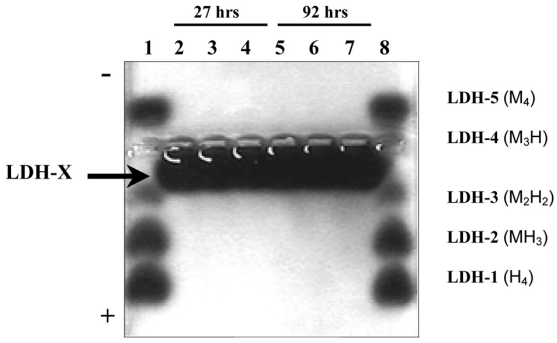

Fig. 9. Agarose gel electrophoretic pattern of LDH of r-CHO cells in MEM-α supplemented with 40 mM sodium chloride (lanes 3 and 6) or 40 mM sodium lactate (lanes 4 and 7). No supplement including sodium chloride and sodium lactate was added in the isoenzyme-determination of control (lanes 2 and 5). Cells were cultured for 27 (lanes 2, 3, and 4) and 92 (lanes 5, 6, and 7) h, respectively. Cells were inoculated at 1×10^5 cells/mL in 100 mm dishes. Lactate dehydrogenase isoenzymes were separated by electrophoresis on 1% agarose gel. A marker (lanes 1 and 8) containing 5 human lactate dehydrogenase isoenzymes was used to aid in the identification of sample LDH isoenzymes separated by electrophoresis.

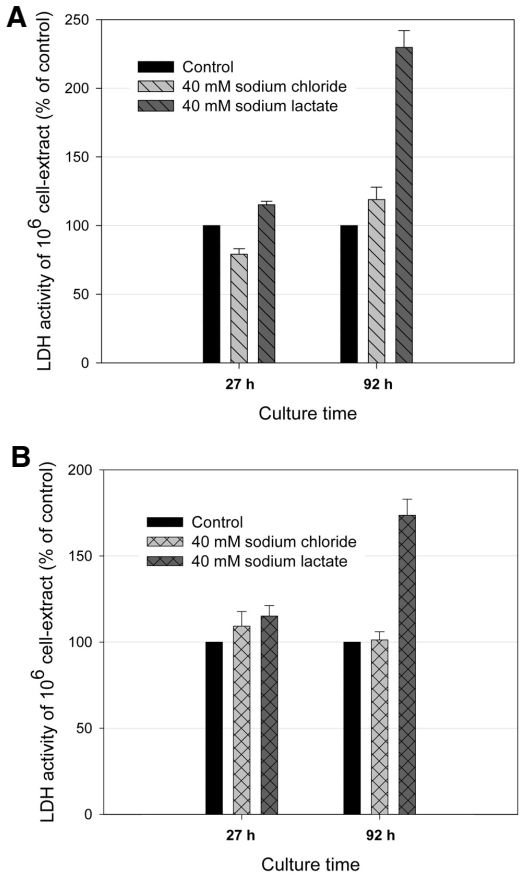

Fig. 10. Lactate dehydrogenase (LDH) activity based on the oxidation of lactate to pyruvate (A) and the reduction of pyruvate to lactate (B) in r-CHO cells. LDH activity was measured after 27 and 92 h. Cells were inoculated at 1×10^5 cells/mL in 100 mm dishes and cultured in MEM-α supplemented with 40 mM sodium chloride or 40 mM sodium lactate. No supplement including sodium chloride and sodium lactate was added in the control experiment.

muscle, and heart. Heart and muscle mitochondria were noted by the prevalence of both LDH-1 (H_4) and LDH-5 (M_4), while liver mitochondria were distinguished by the presence of LDH-5 (M_4). Taking these reports together, it is likely that the ability to convert lactate to pyruvate is not necessarily governed by only LDH isoenzyme pattern. Therefore, we expected that r-CHO cells would not only reduce pyruvate but also oxidize lactate well despite LDH isoenzyme type of r-CHO cells presented between the LDH-3 and LDH-4 types.

Figs. 10A or 10B shows LDH activities based on the oxidation of lactate to pyruvate or the reduction of pyruvate to lactate, respectively. LDH activities in Fig. 10 were obtained to a similar pattern regardless of contrary reactions. It is shown in Figs. 10A and 10B that LDH activity of r-CHO cells added 40 mM sodium lactate is higher than that in both the control and the 40 mM sodium chloride addition. However, the difference of LDH activities at 27 h-culture time is much smaller than that at 92 h-culture time. One of differences between 27 and 92 h-culture time is the remained concentration of glucose that is a major carbon source in culture medium. About 4 mM of glucose remained at 27 h-culture time, while glucose was completely exhausted at 92 h-culture time (data not shown). This indicates that the noticeable enhancement of LDH activity by the added lactate does not occur in the presence of glucose, but the added lactate can influence LDH activity greatly when glucose is almost consumed. The addition of 40 mM sodium chloride, which had same osmolarity as 40 mM sodium lactate, did not enhance LDH activity like the addition of lactate. The result in sodium chloride addition was preferably close to the control. The results above prove indirectly that the results obtained by addition of sodium lactate differ from others in many respects because of lactate effect in itself rather than only osmolarity effect. The measured activity *in vitro* of LDH enzyme, which was obtained by the addition of 40 mM sodium lactate, was high in the assay based on the reduction of pyruvate (Fig. 10B) as well as in the assay based on the oxidation of lactate (Fig. 10A). Then, why did lactate production decrease in the culture with sodium lactate? The reason might be attributed to the difference between *in vitro* LDH assay condition and *in vivo* cell culture condition. The concentration of pyruvate used in LDH assay, based on the reduction of pyruvate, was 22.7 mM and there was no addition of lactate. However, the used culture medium contained 1 mM pyruvate and 40 mM sodium lactate. In other words, when lactate concentration was low (nearly zero), LDH in r-

CHO cells would mainly catalyze the reduction of pyruvate. However, on the condition that high concentration (40 mM in this study) of lactate was added, LDH was likely to catalyze the oxidation of lactate at a considerable rate, since enzyme activity could be varied despite the same enzyme when the environment of enzyme reaction changed. Therefore, a considerable amount of lactate accumulation (about 10 mM) was in the control experiment contained only 1 mM pyruvate without the addition of lactate, and only 5 mM of lactate accumulation was observed in the case of the addition of 40 mM sodium lactate (data not shown). From the results above, we also explained why specific glucose uptake rate decreased by the addition of 40 mM sodium lactate. LDH of r-CHO cells is activated by the addition of 40 mM sodium lactate in our study. This activated LDH catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of NAD to NADH, which will lead to a higher accumulation of NADH than in the control. The somewhat increased concentration of NADH will lower the specific consumption rate of glucose in our study, since glycolysis is inhibited at the presence of excess NADH.

CONCLUSION

We suggest that the main reason for results obtained in our study is a series of alterations of metabolism by the lactate addition. Sodium lactate added with 40 mM lets a LDH enzyme catalyze the oxidation of lactate to pyruvate at a relatively high rate, so the accumulation of NADH is increased. The uptake rate of glucose becomes slow because of the NADH accumulation. The decreased consumption rate of glucose causes a high-energy yield and a small amount of lactate accumulation, and then the acidification of a culture medium is suppressed. In addition, the pyruvate produced by the oxidation of lactate is used for production of energy and formation of various amino acids. Consequently, EPO production is enhanced at 40 mM lactate addition, since r-CHO cells can synthesize more proteins through enough amino acids and energy. By using this approach, the cells can be maintained viable for a longer period with good growth. In result, a higher product concentration can be anticipated.

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