Microscopic analysis of enzyme activity, mitochondrial distribution and hydrogen peroxide in two-cell rat embryos

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A developmental block is induced by phosphate in rat embryos at the late two-cell stage. The present study was designed to examine the energy metabolism of rat two-cell blocked and non-blocked embryos. Enzyme activity was measured in individual embryos by histochemical techniques. The activities of malate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase, pyruvate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, and phosphorylase did not differ among non-blocked and blocked embryos. However, the activity of succinate dehydrogenase was significantly decreased in blocked embryos compared with non-blocked embryos. In blocked embryos, cytochrome oxidase activity was distributed homogeneously, but was located at the perinuclear region in non-blocked embryos. Active mitochondrial organization was visualized using the fluorescent probe rhodamine 123 and laser scanning confocal microscopy. In both non-blocked and blocked embryos, mitochondria were distributed homogeneously. The concentration of H_2O_2 measured fluorometrically in embryos cultured without phosphate did not change significantly during the culture period, but decreased in embryos cultured with phosphate. The timing corresponded to the occurrence of the two-cell block. In summary, these results suggest that the developmental block in rat two-cell embryos is induced by disturbance of mitochondrial energy metabolism.

Introduction

Mammalian embryos cultured *in vitro* tend to show developmental arrest at a stage characteristic to each species. Such a 'developmental block' was reported in rat embryos at the two-cell to four-cell stage (Folstad *et al.*, 1969; Mayer and Fritz, 1974) and occurred in the presence of inorganic phosphate (P_i) (Miyoshi *et al.*, 1994; Matsumoto and Sugawara, 1995). At the early two-cell stage, rat embryos are not affected by exposure to a medium containing P_i (Matsumoto and Sugawara, 1995), but development beyond the four-cell stage is decreased at 18 h and inhibited at 21 h of culture (Matsumoto and Sugawara, 1995).

A study of energy metabolism may help to explain the reasons for suboptimal development because the energy substrate composition of culture media is critical for embryos to achieve developmental competence (Chatot *et al.*, 1989; McKiernan *et al.*, 1991; Lawitts and Biggers, 1992; Conaghan *et al.*, 1993). In hamsters, embryos cultured in medium devoid of glucose and inorganic phosphate (Glu/P_i) are able to overcome the two-cell block (Schini and Bavister, 1988). It has been suggested that the presence of Glu/P_i stimulates glycolysis and suppresses oxidative phosphorylation (Koobs, 1972) in this species by altering the balance between cytosolic and mitochondrial metabolism (Seshagiri and Bavister, 1991). Moreover, it is possible that Glu/P_i directly damages the mitochondria, making embryos dependent on glycolytic activity, which may be

inadequate for complete development *in vitro* (Barnett and Bavister, 1996a). Indeed, the organization of active mitochondria is disturbed in blocked mouse (Muggleton-Harris and Brown, 1988) and hamster embryos (Barnett and Bavister, 1996b).

There is now considerable evidence that oxygen free radical-induced cellular dysfunction contributes to developmental blocks (Noda *et al.*, 1989; Nasr-Esfahani *et al.*, 1990; Goto *et al.*, 1992; Umaoka *et al.*, 1992; Li and Foote, 1993; Natsuyama *et al.*, 1993). For example, Nasr-Esfahani *et al.* (1990) reported that intracellular hydrogen peroxide (H_2O_2) concentrations are higher in two-cell mouse embryos cultured *in vitro* than *in vivo*.

Therefore, the energy metabolism of two-cell, non-blocked and blocked rat embryos was examined. Enzyme activities were measured in individual embryos using histochemical techniques. Active mitochondrial organization was detected using the fluorescent probe rhodamine 123 and laser scanning confocal microscopy. Intracellular H_2O_2 concentrations in two-cell rat embryos were quantified by a fluorometric method.

Materials and Methods

Animals

Wistar rats housed with exposure to light between 06:00 h and 18:00 h, bred and maintained in our laboratory, were used. The stage of the oestrous cycle was monitored by examination of vaginal smears. At pro-oestrus, females were placed with

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males for mating, and the next morning was designated as day 1 of pregnancy if a vaginal plug or spermatozoa in the vaginal smear were detected.

Culture media

The basal culture medium used in this study was hamster embryo culture medium-1 (HECM-1) (Schini and Bavister, 1988) with modifications (mHECM-1) and containing 5.56 mmol glucose l^{-1} , 4 μg phenol red ml^{-1} , 100 iu penicillin ml^{-1} , 0.1 mg streptomycin ml^{-1} and 0.5 mg polyvinyl alcohol ml^{-1} , and lacked phosphate ($P_i(-)$). The medium induced to two-cell block contained 1.19 mmol KH_2PO_4 l^{-1} ($P_i(+)$).

Embryo collection

Early two-cell embryos were collected at 11:00 h on day 2 by flushing with mHECM-1. Embryos were washed three times with mHECM-1 and then placed in the medium in which they were to be cultured ($P_i(-)$ or $P_i(+)$). All embryos were cultured in 100 μl drops under a layer of mineral oil in a humidified atmosphere of 5% CO_2 in air at 37.5°C.

Histochemical characteristics of enzyme activities

The distributions and activities of enzymes in non-blocked and blocked embryos were measured histochemically, after culture for 20–24 h in $P_i(-)$ or $P_i(+)$ medium. Malate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase and glutamate dehydrogenase activities (dependent on NAD or NADP type) were detected by the method of Barka and Anderson (1963). Pyruvate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphatase, phosphorylase, cytochrome oxidase and succinate dehydrogenase activities were detected by the method of Ferguson (1966), Himmelhoch-Karnovsky (1961), Wachstein and Meisel (1956), Takeuchi (1962), Burstone (1962) and Nachlas *et al.* (1957), respectively. For controls, embryos were incubated in a medium devoid of substrate. Stained embryos were placed in the centre of four Vaseline spots on a glass slide. A cover slip was then placed carefully onto the Vaseline spots and pressed gently to anchor the embryos between the cover slip and the slide. Mounted embryos were observed under a light microscope (Olympus, BH-2). All assessments of enzyme staining and intensity were made by the same investigator to eliminate observer variation. The intensity of the enzyme activity was judged by the amount of deposited reaction products using the criteria of Niimura and Ishida (1976, 1978) and estimated on a three-point scale: strong, weak and not detectable.

Distribution of active mitochondria

Embryos were stained with Rhodamine 123 and imaged with confocal laser-scanning microscopy. Rhodamine 123

(Molecular Probes, Eugene, OR) stock solution (10 mg ml^{-1} ; dissolved in methanol) was stored at $-40^\circ C$. Embryos were cultured for 24 h and then stained for 15 min in 10 μg Rhodamine 123 ml^{-1} in mHECM-1 at 37.5°C under 5% CO_2 in air. Confocal images were obtained using a Biorad MRC-1024 laser scanning microscope, mounted on an Axioplan Zeiss microscope.

Measurement of H_2O_2 in embryos

The fluorometric method of Nasr-Esfahani *et al.* (1990) and Goto *et al.* (1993) with modifications was used to measure the concentration of H_2O_2 in embryos. A 1 mmol l^{-1} stock solution of 2',7'-dichlorodihydrofluorescein diacetate (DCH-DFA; Molecular Probes) was prepared in acetone before the start of each experiment and kept in the dark. The stock solution was diluted in mHECM-1 to the experimental concentration (10 μmol l^{-1}). At intervals of 6 h, embryos were incubated for 30 min in 10 μmol DCHDFA l^{-1} at 37.5°C under 5% CO_2 in air. After reaction, embryos were washed in mHECM-1 to remove traces of dye and placed in small chambers containing mHECM-1 and covered with a coverslip. The fluorescence emissions of single embryos were measured immediately with λ_{ex} : 460 nm and λ_{em} : 530 nm, using a microscopic fluorometer using CAM-220 (Japan Spectroscopic Co., Tokyo, Japan).

Statistical analysis

Histochemical characteristics of enzyme activities were analysed using the Mann–Whitney U test. The concentrations of H_2O_2 were analysed by ANOVA. Differences in group means were detected by Fisher's protected least significant difference.

Results

Histochemical characteristics of enzyme activities

In both non-blocked and blocked embryos, there were strong activities of malate dehydrogenase, isocitrate dehydrogenase and lactate dehydrogenase, and weak activities of glutamate dehydrogenase (dependent on NAD or NADP type). Pyruvate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphatase could not be detected. Weak activity of phosphorylase was detected in both non-blocked (94%) and blocked (94%) embryos. Strong and weak activity of glucose-6-phosphate dehydrogenase was detected in non-blocked (strong and weak: 42 and 58%, respectively) and in blocked (strong and weak: 40 and 60%, respectively) embryos. The overall activity of these enzymes did not differ between non-blocked and blocked embryos (Figs 1 and 2; Table 1). However, succinate dehydrogenase and cytochrome oxidase activities differed between non-blocked and blocked embryos. The activity of succinate dehydrogenase

Fig. 1. Histochemical characteristics of enzyme activities in developmentally non-blocked (a,c,e,g,i) and blocked (b,d,f,h,j) rat embryos. (a,b) Strong activity of malate dehydrogenase. (c,d) Strong activity of isocitrate dehydrogenase. (e,f) Strong activity of lactate dehydrogenase. (g,h) Activity of pyruvate dehydrogenase not detectable. (i,j) Activity of glyceraldehyde-3-phosphate dehydrogenase not detectable. Scale bar represents 30 μm .

Table 1. Histochemical characteristics of enzyme activities in non-blocked and blocked rat two-cell embryos

Enzyme	Non-blocked embryos					Blocked embryos				
	<i>n</i>	ND	Weak	Strong		<i>n</i>	ND	Weak	Strong	
Malate dehydrogenase	26	0 (0)	0 (0)	26 (100)		23	0 (0)	0 (0)	23 (100)	
Isocitrate dehydrogenase	30	0 (0)	0 (0)	30 (100)		29	0 (0)	0 (0)	29 (100)	
Lactate dehydrogenase	31	0 (0)	0 (0)	31 (100)		32	0 (0)	0 (0)	32 (100)	
Pyruvate dehydrogenase	27	27 (100)	0 (0)	0 (0)		21	21 (100)	0 (0)	0 (0)	
Glyceraldehyde-3-phosphate dehydrogenase	31	31 (100)	0 (0)	0 (0)		29	29 (100)	0 (0)	0 (0)	
Glutamate dehydrogenase (NAD type)	33	0 (0)	33 (100)	0 (0)		34	0 (0)	34 (100)	0 (0)	
Glutamate dehydrogenase (NADP type)	29	0 (0)	29 (100)	0 (0)		32	0 (0)	32 (100)	0 (0)	
Glucose-6-phosphate dehydrogenase	36	0 (0)	21 (58)	15 (42)		37	0 (0)	22 (60)	15 (40)	
Glucose-6-phosphatase	26	26 (100)	0 (0)	0 (0)		32	32 (100)	0 (0)	0 (0)	
Phosphorylase	32	30 (94)	2 (6)	0 (0)		32	30 (94)	2 (6)	0 (0)	
Succinate dehydrogenase*	50	26 (52)	7 (14)	17 (34)		51	44 (86)	1 (2)	6 (12)	
Cytochrome oxidase	47	0 (0)	0 (0)	47 (100) [†]		38	0 (0)	0 (0)	38 (100)	

Numbers in parentheses show percentages.

ND, not detectable.

*Significant difference between non-blocked and blocked embryos ($P < 0.05$).

[†]Enzyme activities were distributed in the perinuclear regions.

in blocked embryos (strong and weak: 12 and 2%, respectively) was significantly decreased relative to non-blocked embryos (strong and weak: 34 and 14%, respectively) (Fig. 3; Table 1; $P < 0.05$). The activity of cytochrome oxidase was strong in both types of embryo (Fig. 4; Table 1). While strong activity for cytochrome oxidase was located at the perinuclear region in non-blocked embryos, it was distributed homogeneously in blocked embryos.

Distribution of active mitochondria

Active mitochondrial organization was imaged using the fluorescent probe Rhodamine 123 and laser scanning confocal microscopy (Fig. 5). Data were collected from 49 non-blocked embryos and 43 blocked embryos. Mitochondrial distribution was homogeneous in non-blocked embryos, cultured in $P_i(-)$. In blocked embryos, mitochondrial distribution was similarly distributed homogeneously. Fluorescence intensity did not differ between non-blocked and blocked embryos.

Concentrations of H_2O_2 in embryos

The data for H_2O_2 concentration are relative to the value for culture in $P_i(-)$ at 0 h. The concentration of H_2O_2 in the embryos incubated in $P_i(-)$ did not change significantly with time in culture, but those grown in $P_i(+)$ showed a gradual significant decrease over the culture period for more than 18 h (Fig. 6; $P < 0.05$).

Discussion

Energy metabolism in non-blocking and blocking two-cell rat embryos has been examined. In a previous study, when early

two-cell embryos were cultured in medium containing P_i , development beyond the four-cell stage was decreased at 18 h and inhibited at 21 h after cultivation (Matsumoto and Sugawara, 1995) whereas the majority of embryos cultured in $P_i(-)$ cleaved at 24–30 h after cultivation. Therefore, non-blocking and blocking two-cell embryos were collected after culture for 20–24 h and subjected to histochemical analysis of enzyme activity and mitochondrial distribution. Succinate dehydrogenase and cytochrome oxidase, which are present in mitochondria, showed different activities in non-blocking and blocking embryos; however, the other enzymes in mitochondria: malate dehydrogenase, isocitrate dehydrogenase, pyruvate dehydrogenase and glutamate dehydrogenase, were unchanged. Moreover, no differences were demonstrated for the enzymes of other metabolic pathways: lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (glycolysis), glucose-6-phosphatase and phosphorylase (glycogen metabolism) and glucose-6-phosphate dehydrogenase (pentose phosphate pathway). These results indicate that the developmental block in rat two-cell embryos is disturbed by specific enzyme activities and a specific pathway in energy metabolism in mitochondria.

Rhodamine 123, a fluorescent probe that signals the electrical potential across the inner mitochondrial membrane, was used to measure 'active' mitochondrial distribution. Mitochondria were distributed homogeneously in both non-blocked and blocked rat two-cell embryos. In mice, Muggleton-Harris and Brown (1988) found that mitochondria in normally cleaving two-cell embryos were distributed homogeneously throughout the cytoplasm of blastomeres during interphase, with specific mitochondrial translocations to the mitotic spindle being observed during cleavage. In developmentally arrested two-cell

Fig. 2. Histochemical characteristics of enzyme activities in developmentally non-blocked (a,c,e,g,i) and blocked (b,d,f,h,j) rat embryos. (a,b) Weak activity of glutamate dehydrogenase (NAD type). (c,d) Weak activity of glutamate dehydrogenase (NADP type). (e,f) Strong activity of glucose-6-phosphate dehydrogenase. (g,h) Activity of glucose-6-phosphatase not detectable. (i,j) Activity of phosphorylase not detectable. Scale bar represents 30 μ m.

Fig. 3. Histochemical characteristics of succinate dehydrogenase activity in developmentally non-blocked (a,c,e) and blocked (b,d,f) rat embryos. (a,b) Strong, (c,d) weak and (e,f) not detectable activities. Scale bar represents 30 μm .

mouse embryos, the mitochondria became progressively aggregated and localized in the perinuclear region and an area of cytocortex immediately adjacent to the plasma membrane (Muggleton-Harris and Brown, 1988). In non-blocked two-cell hamster embryos, mitochondria were clustered in the perinuclear region (Barnett and Bavister, 1996b). In developmentally blocked two-cell hamster embryos, mitochondria showed no perinuclear clustering and were much more homogeneously distributed (Barnett and Bavister, 1996b). However, blocked two-cell rat embryos showed no differences in mitochondrial distribution, suggesting that such embryos have the same electrical potential across the inner mitochondrial membrane as do non-blocked embryos.

The concentration of H_2O_2 in the embryos cultured in $\text{P}_i(-)$ did not significantly change during the culture period, but gradually and significantly decreased in embryos cultured in $\text{P}_i(+)$ for more than 18 h. This timing coincided with the developmental block in two-cell rat embryos (Matsumoto and Sugawara, 1995). At the early two-cell stage, rat embryos were not affected by exposure to a medium containing P_i , but in embryos cultured in medium containing P_i , the development decreased at 18 h and was inhibited at 21 h after cultivation (Matsumoto and Sugawara, 1995). The decrease in H_2O_2 concentration could reflect some restricted function of the oxygen activation system, because the respiratory chain in mitochondria produces the reactive oxygen species (H_2O_2).

Fig. 4. Histochemical characteristics of cytochrome oxidase activity in developmentally non-blocked (a) and blocked (b) rat embryos. Activities were strong in non-blocked and blocked embryos. (a) In non-blocked embryos, cytochrome oxidase activity was strong in the perinuclear region. (b) In blocked embryos, strong activity was distributed homogeneously. Scale bar represents 30 μm .

Fig. 5. Active mitochondrial organization imaged by the fluorescent probe Rhodamine 123. Mitochondrial distribution was homogeneous in both developmentally non-blocked (a) and blocked (b) rat embryos. Fluorescence intensity did not differ between non-blocked and blocked embryos. Scale bar represents 30 μm .

(Boveris and Chance, 1973; Loschen *et al.*, 1974; Boveris and Cadenas, 1975; Rich and Borner, 1978).

In mouse embryos, there is evidence that oxygen free radical-induced cellular dysfunction contributes to developmental block and there is a strong association between increasing H_2O_2 concentration and the arrest (Noda *et al.*, 1989; Nasr-Esfahani *et al.*, 1990; Goto *et al.*, 1992; Umaoka *et al.*, 1992; Natsuyama *et al.*, 1993). In rats, the rate of blastocyst formation of pronuclear embryos cultured in HECM-1 under a low oxygen concentration (5%) was significantly greater than the value obtained under a standard oxygen concentration (20%) (Kishi *et al.*, 1991). However, when cultured in 20% oxygen, a large proportion of rat one-cell embryos can develop to the blastocyst stage in a chemically defined medium supplemented with amino acids and with the osmolality adjusted to about 246 mmol (Miyoshi *et al.*, 1995). Therefore, oxygen radical formation may not be a major cause of developmental block in the early rat embryo.

The mechanism of the developmental block in early mammalian embryos remains one of the most intriguing aspects of embryo metabolism (Bavister, 1995; Barnett and Bavister,

1996a). The most likely biochemical explanation for the observed glucose/ P_i inhibition of hamster embryo development is inadequate energy production owing to the inhibition of mitochondrial oxidative metabolism (Seshagiri and Bavister, 1991; Barnett and Bavister, 1996a). In medium containing glucose/ P_i , P_i stimulates cellular glycolysis by activating three key glycolytic enzymes (hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase) and possibly by relieving inhibition of hexokinase by glucose-6-phosphate (Wu, 1965). Enhanced glycolysis results in the inhibition of mitochondrial respiration, a classic phenomenon first described in 1929 and known as the 'Crabtree effect' (Crabtree, 1929; Koobs, 1972). Although the activities of glyceraldehyde-3-phosphate dehydrogenase and other enzymes in glycolysis did not differ between non-blocked and blocked rat embryos, respiratory metabolism in blocked embryos was disturbed, as reflected by a reduced H_2O_2 production and an altered cytochrome oxidase activity. These results may indicate that the developmental block observed in rats is caused by the 'Crabtree effect'.

Fig. 6. Changes in production of H_2O_2 in two-cell rat embryos incubated in the absence (■) and presence (□) of inorganic phosphate. Results are means $\pm$ SEM using a total of 39–62 embryos. Bars with different letters are significantly different ($P < 0.05$).

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