Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 507-512

ALTERATIONS IN PURINE NUCLEOTIDE METABOLISM DURING MUSCLE DIFFERENTIATION IN VITRO

Esther Zoref-Shani[a], Asher Shainberg[b] and Oded Sperling[a,c]

[a]Department of Chemical Pathology,
Tel Aviv University Medical School, Tel Hashomer,

[b]Department of Life Sciences, Bar-Ilan University, Ramat Gan and

[c]Department of Clinical Biochemistry, Beilinson Medical Center,
Petah-Tikva, Israel

Received July 12, 1983

Summary: Pathways of purine nucleotide metabolism affecting the availability of ATP in the muscle tissue were studied in differentiating rat muscle cultures. The rate of de novo purine nucleotide synthesis and of AMP deamination were found to increase markedly with cell differentiation, but the rate of IMP dephosphorylation was similarly low in both myoblasts and contracting fibers. The above differentiation-associated alterations in purine nucleotide metabolism conform with the greater need for ATP as a source of energy in the contracting myotubes.

The study of cultured muscle fibers as a model for muscle tissue in vivo, extended considerably during the last decade. Recently we demonstrated that the spontaneously contracting, nondividing multinucleated fibers (myotubes) of cultured rat muscle resemble muscle tissue in vivo also concerning the role of purine nucleotide metabolism in muscle work (1). In comparison to other cell cultures, such as human skin fibroblasts (2), the contracting fibers were characterized by relatively enhanced rates of de novo purine nucleotide synthesis and of AMP deamination whereas by a relatively low rate of IMP dephosphorylation. These alterations in purine nucleotide metabolism which are in accordance with increased availability of purine precursors for fast restoration of the ATP pool, conform with the physiological requirements characteristic to the muscle tissue for mechanical contractions, restoration of electrochemical gradients (like nerve cells) and synthesis of contractile proteins.

In the present communication, we show that the acquisition by the contracting myotubes of two of the above properties, the enhanced rates of purine synthesis and of AMP deamination, is differentiation-associated.

0006-291X/83 $1.50

MATERIALS AND METHODS

Muscle cell cultures

Myoblasts were obtained by trypsinization of extensor digitorum longus muscle from the hindlimb of 20-day-old rat embryos (3). Primary muscle cultures were established with 3.10^5 cells/ml in collagen-coated 90 mm dishes (Nunc) and fibroblasts were eliminated by preplating. The cultures were grown in high glucose dulbecco's modified Eagle's medium, supplemented with 10% horse serum and 2% chick embryo extract, under a humid 90% air/10% CO_2 atmosphere, but 20 hr, and again 1 hr before the experiments, the medium was changed to Eagle's minimum essential medium, supplemented with 10% nondialyzed fetal calf serum.

Purine synthesis

The rate of de novo purine nucleotide synthesis was gauged by the rate of (^{14}C)formate incorporation into total purines produced, as described before (1). Following incubation, the purine compounds were hydrolyzed in 1N perchloric acid and the purine bases precipitated as silver salts, extracted in 0.1 M HCl and counted. For the study of the distribution of label between IMP, and adenine nucleotides, the purines extracted in HCl were chromatographed on microcrystalline cellulose thin-layer plates with butanol/methanol/H_2O/25% NH_4OH (60:20:20:1,v/v) as solvent. The spots of adenine and hypoxanthine were identified under ultraviolet light, scraped off and counted.

Incorporation of labelled purine bases

The incorporation of labelled purine bases into cellular purine compounds and the distribution of label among the various purine derivatives was measured as described before (4).

Enzyme activities

The cultured cells were harvested by trypsinization and the cells washed thrice with 0.9% NaCl. For the assay of IMP nucleotidase the cells were suspended in 10mM Tris-HCl pH 7.4 and subjected 3 times to freezing and thawing. The cell lysates were centrifuged at 40000 g for 15 min at 4°C and the supernatant dialyzed at 4°C against 1mM Tris-HCl pH 7.4. The incubation mixture contained in a total volume of 100 ul, 55 mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 0.5 mM (8-^{14}C)IMP (1.6 mCi/mmole; Amersham International) and lysate containing up to 100 ug protein. The incubation was carried for 10 min at 37°C and the reaction arrested by addition of 20 ul 15% PCA. The product inosine was separated from the substrate IMP by thin-layer chromatography on microcrystalline cellulose, as described above. For the assay of AMP deaminase, the washed cells were suspended in distilled water, lyzed by freezing and thawing 3 times and centrifuged at 40000 g for 15 min at 4°C. The reaction mixture contained in a total volume of 100ul, 30mM TRA-HCl, pH 6.8, 0.3 M KCl, 0.4 mM (2-^{3}H)AMP (25mCi/mmole; Amersham International) and lysate containing up to 25 ug protein. The reaction was carried for 10 min at 37°C and arrested by subjecting the tubes to 100°C for 2 min. IMP was separated from AMP by thin layer chromatography on microcrystalline cellulose, utilizing as solvent saturated $(NH_4)_2SO_4$ / 0.1M phosphate buffer pH 6.0/isopropanol (79:19:2 v/v). The reactions for both enzymes were linear with time and enzyme protein.

RESULTS AND DISCUSSION

In a typical experiment, the cultured myoblasts multiplied up to about 50 to 52 hr after plating, at which time the cultures entered a

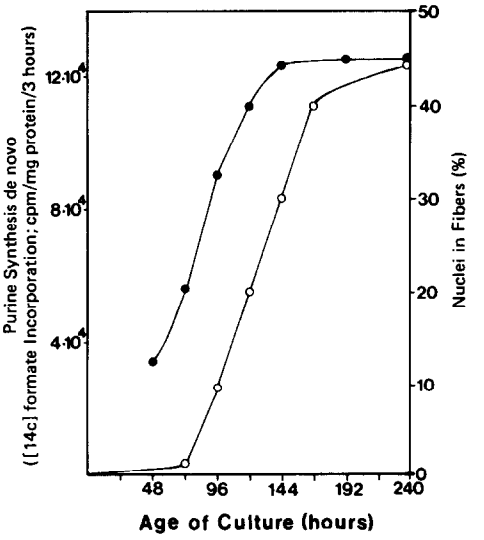

Fig. 1. The effect of muscle differentiation on the rate of de novo purine nucleotide synthesis; ●——● de novo purine synthesis; ○——○ % of nuclei within the multinucleated muscle fibers.

period of rapid cell fusion, resulting in formation of a network of rapidly growing multinucleated fibers (myotubes). Spontaneous contractions could be observed about one day later, the contractions and cross striation increasing during the following days. A representative experiment of the rate of fusion of the cells is given in Fig. 1. The differentiation of the proliferating mononucleated myoblasts into the contracting nondividing multinucleated fibers was shown to be associated with many distinct biochemical alterations, including cessation of DNA synthesis (5), marked decrease of RNA synthesis (6), intense synthesis of the contractile proteins and large change in activity of many other enzymes (7). In the present study we observed that also pathways of purine nucleotide metabolism, which are characteristic to the contracting muscle fibers, develop gradually with the differentiation process.

The rate of de novo purine nucleotide synthesis in the intact muscle cultures, gauged by the rate of incorporation of precursor (^{14}C)formate into total purines produced, increased rapidly with the age of the cultures, reaching plateau at about 144 hr following plating (Fig. 1). The maximal rate of purine synthesis in the muscle fibers was almost 4-fold that measured at the myoblast stage of the cultures, at 48 hr after plating. The rapid increase in the rate of de novo purine nucleotide synthesis with differentiation was parallel to the rate of fusion of the myoblasts to form myotubes (Fig. 1), i.e., in inverse proportion to the decrease in the rate of cell multiplication. As mentioned above, the muscle differentiation process is associated with cessation of DNA synthesis and with a marked decrease

Table 1. Activity of AMP deaminase and of IMP nucleotidase in cell cultures.

Cell Source	AMP deaminase (cpm/mg protein/min)	IMP nucleotidase (nmoles/mg protein/hr)
Human skin fibroblasts	10082 (6)[c]	2153 (4)
Mononucleated dividing myoblasts[a]	7029 (7)	73 (6)
Multinucleated contracting fibers[b]	71991 (11)	81 (4)

[a]48 hr after plating.
[b]190-240 hr after plating
[c]numbers in parentheses represent number of experiments.

in RNA synthesis, but with increased consumption of ATP for both, contractions and restoration of electrochemical gradients. Thus, the increased rate of purine synthesis in the contracting fibers should be taken to reflect mainly the greater need for precursors of ATP as a source of energy. The mechanism underlying the increased rate of purine synthesis in the contracting fibers is not yet clarified. Preliminary experiments in our laboratory suggest however that this alteration is not associated with increased availability of phosphoribosylpyrophosphate, a rate-limiting substrate for purine synthesis in the muscle tissue (1). Probably, the enhancement of this pathway reflects increased activity of phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14), catalyzing the rate-limiting step in the pathway of de novo purine nucleotide synthesis.

The differentiation of the myoblasts into contracting myotubes was also found to be associated with a marked increase in the activity of AMP deaminase (EC 3.5.4.6). This was demonstrated by both, direct assay of this enzyme in cell extracts (a 10-fold increase; Table 1), as well as by gauging the activity of the enzyme in the intact tissue. The latter was done by measuring the distribution of radioactivity among the various nucleotides, following incubation with the labelled precursors (^{14}C) formate (de novo IMP synthesis) and (^{14}C) adenine (salvage AMP synthesis). The increased activity of AMP deaminase in the myotubes, in comparison to the myoblasts, was reflected in the intact tissue in a greater flow from AMP to IMP, as indicated by the finding that in the myoblasts the incorporated radioactivity from both precursors accumulated mainly in adenine nucleotides whereas in the contracting myotubes it accumulated mainly in IMP (Fig. 2 and Table 2). As can be seen from the data, following incubation with precursor (^{14}C) formate , the ratio of labelling between adenine nucleotides and IMP decreased gradually from 9.16 at the myoblast stage to 0.56 at the myotubes stage (Fig. 2). Similarly, following incorporation of (^{14}C) adenine,

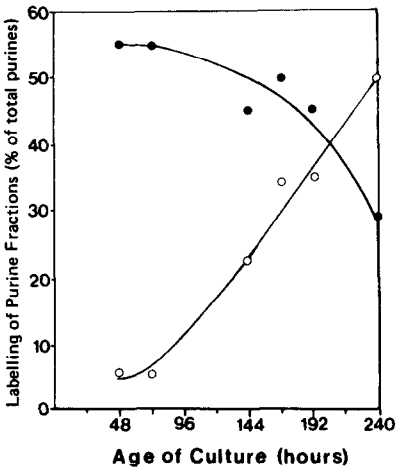

Fig. 2. The effect of cell differentiation on the distribution of labelling from precursor (^{14}C)formate between IMP and adenine nucleotides; ●——● radioactivity in adenine nucleotides; O——O radioactivity in IMP, in % of total radioactivity in intracellular purines.

the above ratio decreased from 6 at the myoblast stage to 0.5 at the fibers stage (Table 2). The increased activity of AMP deaminase in the muscle fibers conforms with the roles of the purine nucleotide cycle (AMP deamination and IMP reamination) in the conservation of purine nucleotides and production of ATP in the working muscle in vivo. It was suggested that muscle AMP formed from ATP consumption during work is deaminated to IMP, which being non-diffusable accumulates in the muscle tissue to be reaminated at rest back to AMP. These interconversions between AMP and IMP allow fast restoration of the depleted ATP pool (8,9). In addition, the purine nucleotide cycle produces

Table 2. Distribution of label from (^{14}C) adenine among intracellular purine derivatives in cell cultures.

Cell source	Distribution of label among purine derivatives (% of total intracellular)		
	Adenine	hypoxanthine	guanine
Human skin fibroblasts	88.7[c]	8.4	2.7
Mononucleated dividing myoblasts[a]	78.2	13.6	8.2
Multinucleated contracting fibers[b]	34.4	60.0	5.6

[a] 48 hr after plating
[b] 190-240 hr after plating
[c] Numbers represent averages of 3 to 5 experiments

fumarate, which is utilized for ATP production through the activity of the citric acid cycle. Ammonia, another product of the cycle, buffers the increase in H^+ associated with lactate formation. The combination of high activity of AMP deaminase and of low activity of IMP nucleotidase, demonstrated in the present study in the muscle fibers, is essential for the operation of the purine nucleotide cycle. The high deaminase activity directs AMP metabolism to IMP, rather than to the formation of the diffusable adenosine, whereas the low nucleotidase activity avoids dephosphorylation of IMP to the diffusable inosine, conserving IMP for reamination to AMP.

Unlike the activity of AMP deaminase, which exhibited a marked alteration with muscle differentiation, the activity of IMP nucleotidase was similarly low in both the myoblast and contracting myotubes. However, in contrast to the myotubes, the low nucleotidase activity in the myoblasts was not associated with IMP accumulation. This is probably the result of the lower rate of ATP degradation to AMP and the lower rate of AMP deamination in these cells.

ACKNOWLEDGEMENT

We thank Mrs. T. Zinman and Mrs. H. Oelsner, for their excellent technical assistance.

REFERENCES

1. Zoref-Shani, E., Shainberg, A., and Sperling, O. (1982) Biochim. Biophys. Acta 716, 324-330.
2. Zoref-Shani, E., and Sperling, O. (1980) Biochim. Biophys. Acta 607, 503-511.
3. Yaffe, D. (1973) in Tissue Culture Methods and Applications (kruse, P.F. Jr, and Patterson, M.K., Jr., Eds), pp. 106-114, Academic Press, New York.
4. Zoref, E., Sivan, O., and Sperling, O. (1978) Biochim. Biophys. Acta 521, 452-458.
5. Okazaki, K., and Holtzer, H. (1966) Proc.Nat.Acad.Sci. (USA), 56, 1484-1490.
6. Yaffe, D., and Fuchs, S. (1967) Develop. Biol. 15, 33-50.
7. Shainberg, A., Yagil, G., and Yaffe, D. (1971) Develop. Biol. 25, 1-29.
8. Sahlin, K., Palmskog, G., and Hultman, E. (1978) Pflüger Archiv (European Journal of Physiology) 374, 193-198.
9. Sabina, R.L., Swain, I.L., Patten, B.M., Ashijawa, T., O'brien, W.E., and Holmes, E.W. (1980) J. Clin. Invest. 66, 1419-1423.