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THE STIMULATORY EFFECT OF PLATELET HOMOGENATE ON PROLYL HYDROXYLASE ACTIVITY IN L-929 CELLS 

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We have found that the addition of platelet homogenate to confluent cultures of L-929 cells increases 2-3 times the activity of prolyl hydroxylase in these cells. Furthermore, it was found that the platelet homogenate potentiates the effect of ferrous ions and ascorbic acid, which are known activators of prolyl hydroxylase. The effect of the platelet homogenate is diminished by cycloheximide.

It seems probable that some products present in the platelet homogenate may promote biosynthesis of the enzyme or they stimulate glycolysis and accumulation of lactic acid, an activator of the hydroxylase.

Hydroxyproline, one of the main amino acid constituents of collagen, is a product of posttranslational modification of propyl residues incorporated into protocollagen. Formation of hydroxyproline is catalysed by prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) which requires ferrous ions, α-ketoglutarate, ascorbate and atmospheric oxygen (for review see: Cardinale & Udenfriend, 1974; Kivirikko & Myllylä, 1980).

It was found that platelet homogenate stimulates biosynthesis of collagen in cell cultures (Bańkowski & Dąbrowski, 1980; Bańkowski et al., 1980). The mechanism of this phenomenon is still unknown.

In the present study we have found that the platelet homogenate stimulates the activity of prolyl hydroxylase in cultured L-929 cells.

MATERIALS AND METHODS

Nutritional medium and reagents. Eagle’s minimal essential medium (MEM), bovine serum and trypsin were products of the Serum and Vaccine Factory (Lublin, Poland). [5-3H]Proline (spec. act. 1.85 - 3.70×10¹⁰Bq/nmol) was purchased from

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Amersham (Bucks., England). PCS-scintillation fluid was supplied by Amersham-
Searle (Arlington Heights, U.S.A.). Other reagents of analytical grade were from
POCh (Gliwice, Poland).

**Blood platelet preparation.** Platelets were isolated from bovine citrated blood by
differential centrifugation (Kay et al., 1977) and then submitted to sonication (20 kH,
4×15 s). The resulting preparation is further referred to as the platelet homogenate.

**Protocollagen preparation.** Protocollagen, labelled with [5-3H]proline, used as
a substrate for the assay of prolyl hydroxylase activity, was isolated from L-929
cells treated with α,α'-dipyrpydyl as described by Hutton et al. (1965). The substrate
(spec. act. 1 - 1.5×10^5 cpm/mg protein) was lyophilized and stored at —20°C.

**Protein determination.** Protein was determined by the method of Lowry et al.
(1951). Bovine serum albumin was used as a standard.

**Cell cultures.** Studies were performed on mouse L-929 cells obtained from the
Department of Tumour Immunology, L. Hirszfeld Institute of Immunology and
Experimental Therapy (Wrocław). The cells were grown in Legroux culture flasks
in 20 ml of MEM containing 10% of heat-denatured bovine serum for 48 h, then
they were submitted to the following experiments.

**Experiment 1.** The serum-containing medium was removed and replaced with
the same volume of fresh MEM, then the cells were submitted to incubation at
37°C. After 12 h the medium was removed and replaced with 20 ml of MEM or
MEM supplemented with the platelet homogenate to a final concentration of 0.1 mg
protein/ml, in the control and investigated cultures, respectively. Both cultures were
incubated at 37°C for 3, 6, 12 and 24 h.

**Experiment 2.** The serum-containing medium was replaced with the same volume
of fresh MEM supplemented with ascorbic acid (0.05 mg/ml) and ferrous ammonium
sulphate (final concentration 2.5×10^-7 M), and submitted to incubation at 37°C.
After 12 h this medium was removed and replaced with 20 ml of the same medium
alone (control culture) or supplemented with the platelet homogenate (final concen-
tration 0.1 mg protein/ml). Both cultures were incubated at 37°C for 3, 6, 12 and
24 h.

**Experiment 3.** The cell cultures were prepared as described in experiments 1
and 2 except that cycloheximide was added to the culture media (25 μg/ml) before
the addition of the platelet homogenate. Simultaneously some control cultures were
supplemented with this substance.

After incubation the cell cultures were cooled in an ice-water mixture. The media
were removed and cell layers were washed with 0.05 M-Tris/HCl containing 0.11 M-
NaCl, pH 7.4 (Peterkofsky et al., 1980). The cells were collected from the bottom
of culture flasks with a rubber stick and suspended in the same solution. The cell
suspension was centrifuged (1000 g, 5 min). The supernatant was discarded, and
the sedimented cells were washed once again in the same buffer. The cells collected
from four flasks were suspended in 1 ml of 0.05 μM-Tris/HCl containing 0.11 μM-NaCl,
pH 7.4 and disrupted by sonication (20 kHz, 2 × 15 s), then prolyl hydroxylase
activity was determined.

Prolyl hydroxylase assay. The enzyme activity was determined by the method of
Hutton et al. (1966). The sample contained in a volume of 1 ml: protocollagen as
a substrate (100 000 cpm), homogenate of L-929 cells (0.3 mg of protein) as a source
of enzyme, 0.2 mg of catalase, 0.1 mM ferrous ammonium sulphate, 0.1 mM-sodium
ascorbate, 0.1 mM-α-ketoglutarate and 0.05 mM-Tris/HCl, pH 7.4. The sample was incu-
bated at 30°C for 30 min. The reaction was terminated by the addition of 1 ml of
12 m-HCl and the material was hydrolysed at 110°C for 16 h (Jimenez et al., 1973).
Radioactive hydroxyproline was determined by the method of Blumenkrantz & Aas-
boe-Hansen (1975). The activity of prolyl hydroxylase was expressed in cpm of
radioactive hydroxyproline formed in the above system.

RESULTS

It was observed that hydroxyproline formation was proportional with the time
of incubation up to 60 min and with the amount of L-929 cell homogenate used up
to 0.6 mg of protein. Since the stimulatory effect of the platelet homogenate on
collagen biosynthesis in L-929 cells is the greatest at the protein concentration of
0.1 mg per 1 ml of culture medium (Bańkowski & Dąbrowski, 1980; Bańkowski
et al., 1980), the same concentration was used in studies on prolyl hydroxylase
activity.

L-929 cells cultured for 48 h and then incubated for 12 h in MEM showed low
prolyl hydroxylase activity. During further incubation in fresh MEM for 3, 6, 12
and 24 h a significant increase in enzyme activity was observed (Fig. 1, open bars).
In the presence of the platelet homogenate about 2-3 times higher activity of the
investigated enzyme was found (Fig. 1, dotted bars).

In the cell cultures treated with ascorbic acid and ferrous ammonium sulphate
as activators of prolyl hydroxylase (Prockop, 1971; Stassen et al., 1973; Levene
et al., 1974) the activity of this enzyme was higher than in the cells cultured without
any addition (Fig. 2, open bars). Addition of the platelet homogenate to such cultures
potentiated the effect of ascorbate and ferrous ions. The activity of prolyl hydroxy-
lase in the cells pretreated with ascorbate plus ferrous ammonium sulphate and
then incubated with the platelet homogenate (Fig. 2, dotted bars) appeared to be
distinctly higher in comparison to the cells incubated in the medium without the
activators (cf Fig. 1).

On the other hand we have found that cycloheximide added to the incubation
medium diminishes the stimulatory effect of the platelet homogenate (Fig. 3). It is
worth noting that despite the action of cycloheximide the activity of prolyl hydroxy-
lase was higher in cells treated with the platelet homogenate than in control cells.
Fig. 1. Effect of platelet homogenate on the activity of prolyl hydroxylase in L-929 cells. Mean values from 4 experiments ± S.D. are given (cpm of [5-³H]hydroxyproline/mg of L-929 cell protein). □, control cultures; ■, platelet-treated cultures.

Fig. 2. Effect of platelet homogenate on the activity of prolyl hydroxylase in L-929 cells incubated with ferrous ammonium sulphate and ascorbic acid. Mean values from 4 experiments ± S.D. are given (cpm of [5-³H]hydroxyproline/mg of L-929 cell protein). □, control cultures; ■ platelet-treated cultures.
Fig. 3. Effect of cycloheximide on prolyl hydroxylase activity in L-929 cells treated with the platelet homogenate. Mean values from 4 experiments ± S.D. are given (cpm of [5-3H]hydroxyproline/mg of L-929 cell protein). A, Cultures incubated in the medium without ferrous ammonium sulphate and ascorbic acid; B, cultures incubated in the medium supplemented with ferrous ammonium sulphate and ascorbic acid. □, control cultures; ■, platelet-treated cultures; ■■ platelet-treated cultures in the presence of cycloheximide.

DISCUSSION

It is known that prolyl hydroxylase is synthesized as an inactive precursor and then is converted into an active form. Ferrous ions, ascorbate and lactate are commonly known activators of this enzyme (Comstock & Udenfriend, 1970; Stassen et al., 1973; Levene et al., 1974; Kuttan, 1981).

From the results presented in this paper it can be concluded that blood platelets contain a factor (factors) which is able to enhance the prolyl hydroxylase activity in L-929 cells by a mechanism not yet understood. We have reported (Tomasiak et al., 1982) that the platelet homogenate enhances glycolysis in cultured L-929 cells and increases the accumulation of lactate in the culture medium. It seems possible that the platelet homogenate stimulates prolyl hydroxylase activity by two independent mechanisms: (i) it promotes biosynthesis of the enzyme (this effect is abolished by cycloheximide); and (ii) it enhances glycolysis which results in accumulation of lactic acid, an activator of the proenzyme. The stimulatory effect of platelet homogenate is not eliminated by cycloheximide.

It is commonly known that blood serum stimulates many physiological functions of cells cultured in vitro (Hershko et al., 1971; Cunningham & Pardee, 1972; Hoffmann et al., 1972; Wolf & Lipton, 1973). Serum contains some components which are released from platelets during blood coagulation (Scher et al., 1979). It is supposed that these products may also stimulate cell proliferation, and collagen biosynthesis.
The nature of the collagen biosynthesis-enhancing factor(s) present in the platelet homogenate is not yet known. It seems to be a high-molecular-weight, termolabile, trypsin-sensitive substance (Z. Galewska and E. Bańkowski, unpublished).

REFERENCES


STYMULACJA AKTYWNOŚCI HYDROKSYLAZY PROLIŁOWEJ
W KOMÓRKACH L-929 PRZEZ HOMOGENAT KRWINEK PŁYTKOWYCH

Streszczenie

Stwierdzono, że dodanie homogenatu płytkowego do dojrzanych hodowli komórek L-929 zwiększa 2-3 razy aktywność hydroksylazy proliłowej w tych komórkach. Ponadto stwierdzono, że homogenat krwinek płytkowych zwiększa efekty wywierane przez znane aktywatory hydroksylazy proliłowej, takie jak jony żelazowe i kwas askorbinowy. Cykloheksoinid znosi częściowo stymulację wywołaną przez homogenat płytkowy.

Przypuszcza się, że homogenat krwinek płytkowych może bezpośrednio stymulować biosyntezę hydroksylazy proliłowej, bądź działać pośrednio - poprzez stymulację glikozy i akumulację kwasu mlekowego, znanego aktywatora tego enzymu.

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