Vol. 94, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 16, 1980 Pages 901-908

Effect of Fluorine Containing Compounds on the Activity of Glycolytic Enzymes in Rat Hepatocytes

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Received February 6,1980

SUMMARY

Inhibition of three glycolytic enzymes by NaF and Na_2PO_3F* in isolated rat hepatocytes has been demonstrated. The data indicate that incubation of hepatocytes with NaF or MFP and subsequent removal of NaF and MFP results in a significant inhibition of enolase (E.C. 4.2.1.11), phosphoglucomutase (E.C. 2.7.5.1.), and pyruvate kinase (E.C. 2.7.1.40). It is suggested that the fluorine compound enters the hepatocyte, becomes bound to the enzyme (phosphoglucomutase and enolase) and inhibits its activity. The inhibition of pyruvate kinase may be due to a cAMP dependent phosphorylation of the enzyme.

Sodium fluoride is known to inhibit the activity of many enzymes in vitro including enolase and phosphoglucomutase (1,2). Kanapka and Hamilton (3) demonstrated that addition of 2.4 mM NaF to Streptococcus salivarius resulted in an increase in 2-phosphoglycerate and a decrease in phosphoenolpyruvate and pyruvate suggesting an inhibition of enolase. Shearer and Suttie (4) made similar observations in liver isolated from rats injected with NaF. Guminska and Sterkowicz (5,6) reported an inhibition of both enolase and pyruvate kinase activity in human erythrocytes and Ehrlich ascites tumor cells by NaF. Najjar (7) demonstrated that the activity of muscle phosphoglucomutase in vitro was also inhibited by NaF. We have previously reported (8) that when isolated rat hepatocytes were incubated with either NaF or MFP, the intracellular concentration of 3-phosphoglycerate and 2-phosphoglycerate increased dramatically relative to the level of phosphoenol pyruvate and pyruvate. These reports (3-5,8), however, provide no information on whether one or both of these enzyme activities are inhibited by fluoride in the cell.

* Abbreviations: MFP, Na_2PO_3F; cAMP, 3'5'cyclic AMP; FDP, fructose-1, 6-diphosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; 2PG, 2-phosphoglycerate, 3-PG, 3-phosphoglycerate; DTT, dithiothritol.

0006-291X/80/110901-08$01.00/0

The purpose of this investigation was to determine if these enzymes (phosphoglucomutase, enolase, and pyruvate kinase) were inhibited by fluoride in situ. To investigate this, hepatocytes were incubated in the presence of NaF or MFP and the enzyme activity was assayed in a broken cell fraction that was devoid of extracellular fluoride.

METHODS

Animals and diet. Male Wistar rats (200-240 gm) were used throughout the study. The rats were fed ad libitum with a fluoride deficient diet and distilled water for a week prior to their use for hepatocyte preparation (9).

Preparation of hepatocytes and incubations. Preparation of hepatocytes and the composition of incubation medium was described previously (9). Hepatocytes were separated from the medium in separation tubes after a 60 min incubation with a desired concentration of NaF, MFP or glucagon (8). The hepatocytes were removed with a pasteur pipette from the bulb of the separation tube and used for the enzyme assays. This procedure removed over 95% of the extracellular fluoride and concentrated the cells in a small volume. The calculated fluoride concentration in the enzyme assay was between 5 and 10 µM. This concentration of NaF or MFP is not sufficient to inhibit purified preparations phosphoglucomutase, enolase, and pyruvate kinase in vitro.

Enzyme assays. The activity of enolase (10) and phosphoglucomutase (11) was assayed spectrophotometrically in Triton X-100 disrupted hepatocytes. The assay of pyruvate kinase was adapted from Blair et al. (12) and Ochs and Harris (13). A typical assay contained 136 mM Tris-HCl, pH 7.5, 55 mM KCl, 0.1 mM $MgSO_4$, 2.5 mM ADP, 0.16 mM NADH, 3.2 units of lactic dehydrogenase, phosphoenol pyruvate (1.33 mM or 6.6 mM), and 20 mg Triton X-100.

Preparation of $(NH_4)_2SO_4$ fraction of hepatocyte supernatant. After incubation, the hepatocytes were homogenized for 60 seconds using a Polytron homogenizer. The cell homogenate was centrifuged for 45 min at 12,000 x g. Saturated $(NH_4)_2SO_4$ was added to the supernatant to a final saturation of 60%. After 30 min at 0°C the mixture was centrifuged for 5 min at 12,000 x g. The pellet was suspended in 1.5 ml of 20 mM potassium phosphate pH 7.2, 30% glycerol, 0.1 mM EDTA, 10 mM NaF, and 0.1 mM DTI as described by Pilkis et al. (14).

RESULTS

Effect of NaF, MFP, and glucagon on enolase activity. In an earlier report (8), we speculated that NaF and MFP decreased enolase activity in intact hepatocytes. To determine if the cross-over data was a reflection of a decreased enzyme activity, we assayed enolase activity in disrupted hepatocytes after the removal of media fluoride. Prior to assaying the enzyme activity, the hepatocytes were preincubated with NaF, MFP, or glucagon, and sedimented through NaCl in separation tubes. This procedure

TABLE I
Effect of NaF, MFP, and Glucagon on Enolase Activity in Hepatocytes

Additions	Enolase Activity µmoles/g wet wt/min
None	11.2 ± 2.0
NaF (10 mM)	3.4 ± 1.3*
MFP (20 mM)	3.8 ± 2.5*
Glucagon (100 nM)	10.6 ± 1.7

Results are expressed as the mean ± S.E.M. of 6 different hepatocyte preparations. After a 60 minute incubation, the hepatocytes were separated from the medium by centrifugation (500 rpm for 1 min) in separation tubes. An aliquot of the hepatocyte suspension collected from the bulb of the separation tube was used for enzyme assay. The concentration of fluoride (NaF or MFP) in the assay cuvette was calculated to be between 5 to 10 µM.

* $P < 0.01$

removes over 95% of the media fluoride. The estimated NaF or MFP concentration in the enzyme assay was between 5-10 µM. The data in Table I indicate that under these conditions, NaF or MFP resulted in a 65 - 70% inhibition of enolase activity. The *in situ* inhibition of enolase observed here is consistent with the cross-over between 2PG and Pyr reported earlier (8).

Effect of NaF, MFP, and glucagon on pyruvate kinase activity. Our earlier report (8) indicated that when hepatocytes were incubated with either NaF, MFP or glucagon the PEP/Pyr ratio was increased 3-4 fold over the control. From this observation it was apparent that the activity of liver pyruvate kinase might be sensitive to fluoride. In the present study the effect of NaF and MFP on rat liver pyruvate kinase activity was investigated. Glucagon was used as a control since it is known to inhibit the activity of L-type liver pyruvate kinase by a cAMP dependent phosphorylation (14,15). Pyruvate kinase activity was assayed at a 1.33 mM and 6.6 mM PEP (12,13). The data in Table II are presented as the ratio of pyruvate kinase activity obtained at 1.33 mM to that at 6.6 mM PEP. A decrease in the ratio is taken as an inhibitory effect on pyruvate kinase activity. The data in

TABLE II
Effect of NaF, MFP and Glucagon on Pyruvate Kinase Activity in Hepatocytes

Addition	Pyruvate Kinase Ratio (1.33/6.66 mM PEP) (a)	(b)	(c)
None	0.14 ± 0.02	0.23 ± 0.05	0.17 ± 0.03
NaF (10 mM)	0.09 ± 0.02*	0.22 ± 0.06	0.08 ± 0.01*
MFP (20 mM)	0.09 ± 0.04*	0.25 ± 0.06	0.10 ± 0.01*
Glucagon (100 nM)	0.08 ± 0.02*	0.30 ± 0.12	0.08 ± 0.01*

Results are expressed as the mean ± S.E.M. of 5 different hepatocyte preparations.

(a) After a 60 minute incubation, the hepatocytes were separated from the medium in separation tubes, as described under Experimental Methods.

(b) The hepatocytes were homogenized for 1 minute in a Polytron homogenizer prior to their incubation.

(c) After a 60 minute incubation with NaF, MFP, or glucagon, the cell suspension was homogenized for 60 seconds in a Polytron homogenizer and centrifuged for 45 minutes at 12,000 x g. The supernatant was treated with $(NH_4)_2SO_4$ as described under Experimental Methods.

* $P < 0.05$

Table II (column a) clearly demonstrate that the pyruvate kinase ratio was significantly reduced in the presence of NaF (36%), MFP (31%) and glucagon (43%). When a homogenate of hepatocytes was incubated for 60 min with the above compounds, pyruvate kinase activity ratios were not altered (Table II, column b). NaF or MFP were found to have no effect on the activity of purified rabbit muscle pyruvate kinase activity (data not shown).

Effect of NaF, MFP and glucagon on the activity of partially purified pyruvate kinase activity. Pyruvate kinase is an allosteric enzyme and its activity is sensitive to the presence of various metabolites e.g., FDP, ATP, alanine, etc. (15). It has been shown (14) that pyruvate kinase from glucagon pretreated hepatocytes remains in a less active form after partial purification with $(NH_4)_2SO_4$. To investigate this possibility in our system, the hepatocytes were incubated with NaF, MFP or glucagon and the supernatant was partially purified with $(NH_4)_2SO_4$. The data presented in Table II

TABLE III
Effect of NaF, MFP, and Glucagon on Phosphoglucomutase Activity in Hepatocytes

Addition	Phosphoglucomutase Activity umol/g wet wt/min
None	26.4 ± 3.0
NaF (10 mM)	18.4 ± 2.1*
MFP (20 mM)	19.6 ± 3.1*
Glucagon (100 nM)	30.0 ± 4.0

Results are expressed as mean ± S.E.M. of 6 different hepatocyte preparations. The hepatocytes were treated as described in the legend of Table I.

* $P < 0.05$.

(column c) demonstrate that pyruvate kinase activity ratio (after partial purification) was significantly reduced in the presence of NaF (53%), MFP (41%), and glucagon (53%).

Effect of NaF, MFP and glucagon on phosphoglucomutase activity. The results presented in Table III illustrate the inhibition of phosphoglucomutase by fluoride in rat hepatocytes. As in the case of the inhibition of enolase, phosphoglucomutase activity was measured after the removal of the extracellular fluoride. A 31% and 26% inhibition of the enzyme activity was observed in hepatocytes pretreated with NaF and MFP respectively. Therefore it appears that the intracellular fluoride may have been responsible for the inhibition of phosphoglucomutase. Glucagon, as expected, had no effect on phosphoglucomutase activity.

DISCUSSION

The results presented above demonstrate that the activity of enolase in rat hepatocytes is inhibited by both NaF and MFP (Table I). We reported earlier (8) that when rat hepatocytes were exposed to either NaF or MFP the ratio of 2PG/PEP increased 3-4 fold over that of the control, indicating an inhibition of enolase. Several investigators (16,17) have reported that MFP has no effect on the activity of muscle enolase. A satisfactory explanation for the difference in the reported lack of inhibition of enolase by MFP and

the inhibition observed here is not offered at this time. It should be noted, however, that the crossover plots of metabolites (8) and the measurement of enzyme activity in the presence of MFP reported here support the idea that MFP may be inhibiting enolase activity in hepatocytes. Warburg and Christian (2) proposed that a dissociable complex of Mg^{++}, phosphate, and fluoride may be responsible for enolase inhibition. Later Cimasoni (17) proposed that fluoride bound directly to the enzyme in the presence of phosphate and behaved like a competitive inhibitor. Recently Kashket and Bunick (18) showed that fluoride was bound to enolase both in the presence and absence of phosphate. The enzyme was found to bind 2.2 atoms of fluoride per mole of enzyme in the presence of phosphate with a dissociation constant of 2×10^{-5}M. The data of this study (Table I) show that fluoride could be binding to the enzyme *in situ* and reducing its activity by the same mechanism as has been shown *in vitro* (17,18). This suggestion is based on the fact that in the present investigation extracellular fluoride was removed prior to the assay of enolase. This step reduced the fluoride concentration in the assay cuvette to between 5-10 μM which is not sufficient to inhibit the enzyme *in vitro*. It is concluded that intracellular fluoride binds to the enzyme (enolase) and it remains bound to the enzyme upon disruption of the hepatocyte.

The crossover data reported earlier demonstrate that the activity of liver pyruvate kinase may also be affected by NaF and MFP (8). The results of this report indicate that the activity of pyruvate kinase is inhibited by NaF and MFP (Table II). When the hepatocyte homogenate was incubated with either NaF, MFP, or glucagon no decrease of pyruvate kinase ratio was observed (Table II). These data show that for the inhibition of pyruvate kinase by NaF, MFP and glucagon, intact hepatocytes are needed. Pilkis *et al*. (14) demonstrated that if the supernatant from hepatocytes are treated with ammonium sulphate after preincubation with glucagon, to partially purify the pyruvate kinase and to remove low molecular weight substances

such as ATP, FDP, etc., the enzyme activity was still reduced compared to the control. The data presented in Table II show similar results for hepatocytes preincubated with NaF, MFP, or glucagon. Glucagon inhibits the activity of liver (L-type) pyruvate kinase by catalyzing a cAMP dependent phosphorylation of the enzyme (15). A similar mechanism can be speculated for NaF and MFP dependent inhibition of pyruvate kinase for the following reasons: (a) the activity of purified rabbit muscle pyruvate kinase is not affected by NaF and MFP; (b) activity of pyruvate kinase remained unchanged when a broken cell preparation was incubated with these compounds (Table II); (c) incubation of hepatocytes with either NaF or MFP results in a 2-4 fold increase in cAMP (9,19); and (d) hepatocyte pyruvate kinase activity ratio remained decreased after partial purification (Table II). To the best of our knowledge this is the first time the inhibition of rat liver pyruvate kinase activity by fluoride has been reported. Another possible explanation of these results is that intracellular NaF or MFP may inhibit some phosphoprotein phosphatases and result in an increase of the phosphorylated pyruvate kinase which is less active. The above explanation is based on the observation that fluoride inhibits phosphoprotein phosphatase in vitro (20,21).

Najjar (7) reported that the activity of purified phosphoglucomutase is inhibited by NaF. The results presented here show that the activity of hepatocyte phosphoglucomutase is significantly inhibited by preincubation with NaF and MFP but not glucagon (Table III). Phosphoglucomutase, like enolase, requires Mg^{++} ions for its activity.

ACKNOWLEDGEMENT

This research was supported in part by the Grace M. Showalter Trust and NIDR grant 04387.

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