A Protein Kinase Inhibitor in Brown Adipose Tissue of Developing Rats

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(Received 7 September 1973)

A heat-stable protein was extracted from brown adipose tissue of infant rats that inhibited both purified bovine heart protein kinase and a crude preparation of protein kinase from brown fat. It did not act as an adenosine triphosphatase nor did it exert its effect by proteolytic action. Inhibition of protein kinase was affected in both the presence and the absence of cyclic AMP. Most of the inhibitory activity was present in the 100000g supernatant fraction of tissue homogenates. Inhibitory activity was highest perinatally and it declined 10 days after birth. It is suggested that the protein kinase inhibitor may play a role in regulating cyclic AMP actions.

A protein in rabbit skeletal muscle that inhibits the cyclic AMP-dependent activation of phosphorylase *b* kinase has been described by Walsh *et al.* (1971*a*). The factor was shown to exert its action on cyclic AMP-dependent protein kinase (Walsh *et al.*, 1971*a,b*). The inhibitor protein has been purified from rabbit skeletal muscle (Walsh *et al.*, 1971*b*), and Ashby & Walsh (1972, 1973) have examined its mechanism of action in detail. The inhibitor was found to block the activity of either holoenzyme (measured in the presence of cyclic AMP) or isolated catalytic subunit of protein kinase. Ashby & Walsh (1973) demonstrated that the inhibitor interacts with cyclic AMP-dependent protein kinase after cyclic nucleotide-promoted dissociation of the catalytic subunit from the holoenzyme. Donnelly *et al.* (1973*a,b*) described a similar heat-stable protein from lobster tail muscle which modulates the activity of cyclic AMP-dependent protein kinase from several tissues. They reported that the modulator protein altered the substrate specificity of both cyclic GMP- and cyclic AMP-dependent protein kinases, increasing the phosphorylation of some protein substrates and decreasing that of others.

We have previously described cyclic AMP-dependent protein kinase activity in interscapular brown adipose tissue of rats and have examined changes during development (Skala *et al.*, 1972*a*). Activity of the cyclic AMP-dependent enzyme at different stages of development correlated with the rate of glycogenolysis, lipolysis and also with the rate of tissue proliferation and differentiation. During these studies it became apparent (Skala *et al.*, 1972*a,b*) that an active inhibitor of protein kinase was present in brown fat of neonatal rats. We have now investigated this inhibitory factor in more detail, and have examined changes occurring during development.

Experimental

Materials

Animals. Wistar Albino rats aged 1–45 days and fetuses weighing 3.5–5g were used. Litters were decreased to eight animals on the first day after delivery. The young were separated from the mother animal on the 30th postnatal day. Animals were fed *ad libitum* on Purina Chow pellet diet (Ralston, St. Louis, Mo., U.S.A.).

Tissue preparation. Pregnant rats were anaesthetized with ether and fetuses were delivered by resection. Animals of all ages were decapitated and interscapular brown adipose tissue was rapidly removed, trimmed of extraneous tissue and placed in beakers immersed in ice. Pooled samples of tissue (from 8 to 40 animals depending on the age) were then homogenized in 4mM-EDTA, pH 7.2 (2ml/g of tissue).

Preparation of extracts containing inhibitor protein. Tissue homogenates in 4mM-EDTA were centrifuged at 100000g for 60min in an IEC B-50 ultracentrifuge at 4°C. The supernatant fluid was filtered through cheesecloth to remove floating lipid. The clear fluid was then heated at 70°C for 10min, cooled to 4°C and re-centrifuged. In some experiments the resulting supernatant fraction was dialysed against 50 vol. of 4mM-EDTA for 18h at 4°C (the external medium was changed four times). The dialysed solution was stored at –25°C.

Preparation of extracts containing protein kinase. Tissue homogenates in 4mM-EDTA were centrifuged at 20000g for 20min at 4°C with the centrifuge described above. After filtration through cheesecloth, the clear fluid was used immediately for protein kinase determinations.

Purification of bovine heart protein kinase. The enzyme from this source was purified by the procedure of Kuo *et al.* (1970). Preparations were stored at -80°C ; solutions were thawed, appropriately diluted with water and used immediately.

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (35 Ci/mmol) was purchased from ICN, Irvine, Calif., U.S.A. Other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Methods

Inhibitor assay. Protein kinase inhibitor activity was assayed by using purified bovine heart protein kinase. Inhibitory activity was expressed as the percentage decrease in protein kinase activity in the presence of the inhibitor. In rabbit skeletal muscle, the degree of inhibition depends not only on the amount of inhibitor but also on the amount of protein kinase (Ashby & Walsh, 1972). This was also true of the inhibitor from brown fat. Care was therefore taken to use an amount of protein kinase that catalysed the incorporation of approx. 5 pmol of ^{32}P into histone/min, in both the presence and the absence of cyclic AMP. Although EDTA inhibits protein kinase, the amount carried into the assay from the tissue extracts was not sufficient to interfere with the assay.

Assay of protein kinase. Protein kinase (ATP-protein phosphotransferase, EC 2.7.1.37) was assayed by the method of Kuo *et al.* (1970) as described by Skala *et al.* (1972a). Calf thymus histone (Sigma, Type II A), 200 μg /assay mixture (final vol. 0.2 ml), was the substrate.

Protein. This was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma, Fraction V) as standard.

Results

Protein kinase inhibitory activity in brown-fat preparations can be determined reliably only in the absence of endogenous protein kinase. Studies were performed to determine if the two activities could be separated on the basis of different subcellular localizations. Protein kinase activity was associated primarily with particulate fractions in rat brain (Maeno *et al.*, 1971). Kuo & Greengard (1969) used 20000g supernatant fractions for determination of protein kinase activity in a variety of tissues. In interscapular brown fat of newborn rats cyclic AMP-independent protein kinase possessed similar specific activities in all subcellular fractions, whereas the cyclic AMP-dependent activity was highest in the 100000g supernatant fluid. Distribution studies revealed that 42 and 81 % of cyclic AMP-independent and cyclic AMP-dependent activities respectively were present in this supernatant fraction. The intracellular

distribution of protein kinase inhibitory activity in brown adipose tissue was also examined. Highest activity was present in the 100000g supernatant fraction; 15% was present in the 12000g pellet and 41% appeared in the pellet when homogenates were centrifuged at 100000g for 1 hr. Thus the intracellular distribution of protein kinase and the inhibitor in adipose tissue was similar and no effective separation could be achieved by differential centrifugation.

Protein kinase inhibitor in skeletal muscle can be freed from the kinase by heating the tissue extracts at 60°C ; the inhibitor is stable at this temperature, whereas protein kinase is largely destroyed (Walsh *et al.*, 1971a,b). Similarly, protein kinase in brown-fat extracts was completely destroyed by heating extracts at 70°C for 10 min and this procedure was used for obtaining inhibitor preparations free of protein kinase. Obtaining brown-fat protein kinase preparations free of the inhibitor was more difficult. However, when 100000g supernatant fluid was diluted so that not more than 15 μg of protein was present in the assay, reaction velocity was a linear function of time and of protein content (Skala *et al.*, 1972a); although inhibitory protein was still present it did not interfere with the assay under these conditions.

Heat-treated brown-fat preparations were examined for their ability to inhibit bovine heart protein kinase in the presence and the absence of 5 μM -cyclic AMP (Fig. 1). Concentration-dependent inhibition was observed under both conditions. Inhibition was more pronounced on the cyclic AMP-independent activity. However, four times as much protein kinase was used to measure cyclic AMP-independent activity (to ensure reaction velocities similar to those of the cyclic AMP-dependent enzyme) and this might account for the higher degree of inhibition. Tissue extracts prepared from foetal and newborn animals appeared to be more potent than those from the adult (Fig. 1).

It was considered that the inhibitory action of these crude brown-fat extracts might arise from an endogenous metabolite or ion rather than a protein. Several experiments were performed to define the nature of the inhibitory activity more closely. Samples of heat-treated preparations were exhaustively dialysed against 4 mM-EDTA (50 vol., changed four times during 18 h) at 4°C , and then assayed for inhibitory activity along with undialysed controls. Both preparations retained identical inhibitory activity when assayed in both the presence and the absence of cyclic AMP. Hence inhibitory activity seemed to reside in a macromolecule. The heat-stability of the factor and its sensitivity to trypsin were also examined. Several 100000g supernatant fractions from 2-day-old rats were boiled for 3 min and the supernatant fluids were assayed by using ox heart protein kinase. Such preparations were as active as control preparations (Table 1). Hence the inhibitor possessed

Fig. 1. Effect of heat-treated brown-adipose-tissue extracts on bovine heart protein kinase

Protein kinase was assayed (a) in the absence of cyclic AMP (5.25 μ g of protein) and (b) in the presence of 5 μ M-cyclic AMP (1.3 μ g of protein). Heat-treated extracts were prepared from foetal ($\circ$), newborn ($\bullet$) and 45-day-old ($\square$) rats as indicated.

Table 1. Effect of heat treatment and trypsin on protein kinase inhibitor in brown adipose tissue

Bovine heart protein kinase (1.3 μ g of protein) was assayed in the presence of 5 μ M-cyclic AMP. A dialysed 100000g supernatant fraction of brown adipose tissue from 2-day-old rats was the source of the inhibitor. Preincubation with trypsin (50 μ g/100 μ g of inhibitor protein) was carried out for 60 min at 30°C and terminated by the addition of soya-bean trypsin inhibitor (150 μ g/100 μ g of inhibitor protein). Each value is the mean $\pm$ S.E.M. of eight determinations.

Treatment	Inhibition of protein kinase (%)
Control (heated at 70°C for 10 min)	40.75 $\pm$ 2.96
Heated at 100°C for 3 min	45.25 $\pm$ 3.25
Preincubated with trypsin	6.43 $\pm$ 3.01

similar heat-stability to the lobster tail modulator protein described by Donnelly *et al.* (1973a). Several preparations were incubated with trypsin (50 μ g/100 μ g of extract protein) for 1 h at 30°C. Proteolytic activity was terminated by the addition of soya-bean trypsin inhibitor (150 μ g/100 μ g of extract protein). Table 1 shows that this treatment abolished the inhibitory activity towards the protein kinase. A

Fig. 2. Effect of incubation of bovine heart protein kinase with brown-adipose-tissue extract before assay for inhibitory action

Bovine heart protein kinase (1.3 μ g of protein) was incubated with a heat-treated dialysed brown-fat extract from newborn rats (75 μ g of protein) for 5–46 min at 37°C. The inhibitor assay was then performed as usual after addition of substrates. Activity is expressed as pmol of 32 P incorporated into histone in 5 min. $\circ$, No inhibitor; $\bullet$, plus inhibitor.

control composed of trypsin and trypsin inhibitor alone, in amounts identical with that added to the extracts, did not affect protein kinase activity.

The possibility was considered that the inhibitor might possess proteolytic activity that could destroy protein kinase. To test this, bovine heart protein kinase was incubated in the presence of a heat-treated brown-fat preparation from newborn rats for 5–46 min before addition of substrate (histone and ATP). Substrate was then added and the assay was completed as usual. An amount of inhibitory protein was chosen that produced half-maximal inhibition. The results are shown in Fig. 2. Protein kinase activity in the presence of inhibitory protein remained constant regardless of the time of incubation before assay. Protein kinase in the absence of inhibitor showed a slight decline with time (25% after 46 min). This could be due to a stabilizing effect of inhibitor protein on protein kinase. At any rate, this experiment ruled out proteolysis as a mechanism of inhibitor action.

It was not feasible to purify protein kinase from brown adipose tissue because of the small amounts of tissue available. Nevertheless, it was necessary to establish that the inhibitory protein actually inhibited brown-adipose-tissue protein kinase. Fig. 1 shows that brown fat of 45-day-old rats possessed less inhibitory activity than tissue from perinatal animals. Hence inhibitor was prepared from newborn rats and a 20000g supernatant fraction from 45-day-old animals was used as source of protein kinase. As shown in Fig. 3, the cyclic AMP-dependent and -independent activities were both clearly inhibited,

Fig. 3. Effect of brown-adipose-tissue inhibitor on brown-adipose-tissue protein kinase

A 20000g supernatant fraction (20 μ g of protein) from brown adipose tissue of 45-day-old rats was the source of protein kinase. Heat-treated, dialysed (○) and non-dialysed (●) inhibitory protein was prepared from tissue of newborn animals. (a) Cyclic AMP (5 μ M) present in the assay; (b) no cyclic AMP present.

The above studies established that the protein kinase inhibitory activity in brown adipose tissue was due to a non-diffusible, heat-stable, trypsin-sensitive, non-proteolytic molecule and was thus similar to the skeletal-muscle inhibitor protein (Walsh *et al.*, 1971a,b). The effect of varying the ATP concentration on the inhibitor action was examined. It was revealed that the inhibitor interacts non-competitively with respect to ATP. This is also similar to the findings of Walsh *et al.* (1971b) for the skeletal-muscle inhibitor.

Protein kinase in brown adipose tissue has been shown to undergo pronounced developmental changes (Skala *et al.*, 1972a,b). During these earlier experiments it seemed that inhibitory activity was most pronounced in the tissue of newborn animals. Inhibitor activity was prepared from pooled samples of brown adipose tissue of rats of five different ages and tested for activity against cyclic AMP-dependent protein kinase from ox heart (Fig. 4). The highest specific activity was exhibited by preparations from late-foetal and newborn rats. Inhibitory activities of preparations from 11–15- and 33–35-day-old rats were similar and significantly lower than those of perinatal animals. Preparations from 3–5-day-old rats possessed intermediate inhibitory activity. It

Fig. 4. Developmental changes in brown-fat protein kinase inhibitor activity

Bovine heart protein kinase (1.3 μ g of protein) was used and assays were performed in the presence of 5 μ M-cyclic AMP. Heat-treated and dialysed extracts of brown adipose tissue of rats of various ages indicated [▲, foetuses (–1 to –3 days); ■, 1–3 days old; ○, 3–5 days old; □, 11–15 days old; ●, 33–35 days old] were prepared simultaneously as described in the text.

seems therefore that the high specific inhibitory activity from perinatal animals decreases considerably during the first 10 days after birth. The total yield of the heat-stable protein in the high-speed supernatant fractions per unit fresh weight of the tissue did not vary with age; nevertheless only further purification and characterization will determine whether the observed higher specific inhibitory activity in the perinatal period is due to higher amounts of the same protein or whether the properties of the protein change during development.

Discussion

This study establishes the presence of protein kinase inhibitory activity in brown adipose tissue of the rat. The inhibitor is a heat-stable protein localized primarily in the 'particle-free' supernatant. It inhibits protein kinase prepared from bovine heart and rat brown adipose tissue in both the presence and the absence of cyclic AMP. Its relative activity is highest perinatally, when also the cyclic AMP concentration, protein kinase activity (Skala *et al.*,

1972a), the rate of tissue maturation (Barnard & Skala, 1970; Skala *et al.*, 1970) and the tissue functional capacity (Barnard *et al.*, 1970; Skala & Lindberg, 1970; Hahn & Skala, 1972a,b) are at their highest values.

Cyclic AMP-dependent protein kinase activates glycogenolysis by activating phosphorylase *b* kinase, which in turn catalyses the conversion of phosphorylase *b* into phosphorylase *a* (see Robison *et al.*, 1971). Protein kinase also catalyses the phosphorylation and activation of hormone-sensitive lipase in white adipose tissue (Huttenen *et al.*, 1970; Khoo *et al.*, 1972). In addition it may be involved in regulating transcriptional and translational processes. The developmental pattern of cyclic AMP concentrations and of cyclic AMP-dependent protein kinase at different stages of perinatal and early postnatal development correlated well with these processes in brown adipose tissue (Skala *et al.*, 1972a). The fact that protein kinase inhibitor follows a similar development pattern would lend support to the suggestion of Ashby & Walsh (1973) that this protein may play a role in regulating cyclic AMP function.

This work was supported by M.R.C. (Canada) grants no. MA 3713 to P. H. and no. MT 68-1691 to G. I. D. J. P. S. is an M.R.C. Fellow and P. H. is an M.R.C. Associate.

References

- Ashby, C. D. & Walsh, D. A. (1972) *J. Biol. Chem.* **247**, 6637-6642
- Ashby, C. D. & Walsh, D. A. (1973) *J. Biol. Chem.* **248**, 1255-1261
- Barnard, T. & Skala, J. (1970) in *Brown Adipose Tissue* (Lindberg, O., ed.), pp. 33-72, Elsevier, New York and Amsterdam
- Barnard, T., Skala, J. & Lindberg, O. (1970) *Comp. Biochem. Physiol.* **33**, 499-508
- Donnelly, T. E., Jr., Kuo, J. F., Reyes, P. L., Liu, Y.-P. & Greengard, P. (1973a) *J. Biol. Chem.* **248**, 190-198
- Donnelly, T. E., Jr., Kuo, J. F., Miyamoto, E. & Greengard, P. (1973b) *J. Biol. Chem.* **248**, 199-203
- Hahn, P. & Skala, J. (1972a) *Comp. Biochem. Physiol.* **41B**, 147-156
- Hahn, P. & Skala, J. (1972b) *Biochem. J.* **127**, 107-111
- Huttenen, J. K., Steinberg, D. & Mayer, S. E. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1350-1356
- Khoo, J. C., Fong, W. W. & Steinberg, D. (1972) *Biochem. Biophys. Res. Commun.* **49**, 407-413
- Kuo, J. F. & Greengard, P. (1969) *Proc. Nat. Acad. Sci. U.S.A.* **64**, 1349-1355
- Kuo, J. F., Krueger, B. K., Sanes, J. R. & Greengard, P. (1970) *Biochim. Biophys. Acta.* **212**, 79-91
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Maeno, H., Johnson, E. M. & Greengard, P. (1971) *J. Biol. Chem.* **246**, 134-142
- Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP*, Academic Press, New York
- Skala, J. & Lindberg, O. (1970) *Int. J. Biochem.* **1**, 513-522
- Skala, J., Barnard, T., & Lindberg, O. (1970) *Comp. Biochem. Physiol.* **33**, 509-528
- Skala, J., Novak, E., Hahn, P. & Drummond, G. I. (1972a) *Int. J. Biochem.* **3**, 229-242
- Skala, J., Novak, E. & Hahn, P. (1972b) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **31**, 224
- Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S. & Krebs, E. G. (1971a) *J. Biol. Chem.* **246**, 1968-1976
- Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. & Krebs, E. G. (1971b) *J. Biol. Chem.* **246**, 1977-1985