Insensitivity of cardiac phosphofructokinase to adrenergic activation in Zucker rats

A post-receptor defect

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1. The apparent insensitivity of phosphofructokinase to activation by adrenaline in hearts from genetically obese Zucker (*fa/fa*) rats [Patten, Filsell & Clark (1982) *Metab. Clin. Exp.* **31**, 1137–1141] was examined in detail. 2. Perfusion of hearts from obese (*fa/fa*) animals with medium containing 1 nM–100 μ M-adrenaline for 4 min did not significantly activate phosphofructokinase, but fully activated glycogen phosphorylase. 3. Activation of phosphofructokinase occurred in hearts from lean (*Fa/?*) animals, with half-maximal activation at 10 nM-adrenaline. 4. Binding characteristics of cardiac α_1 -adrenergic receptors were comparable between lean and obese animals. 5. Properties of phosphofructokinase from obese and lean hearts were similar. 6. High extracellular Ca^{2+} concentration, which activated phosphofructokinase in hearts from lean animals and phosphorylase in all hearts, failed to activate phosphofructokinase in hearts from obese rats. 7. The data indicate that the failure of adrenaline to activate phosphofructokinase in hearts from obese (*fa/fa*) rats results from a post-receptor defect in the as-yet unidentified Ca^{2+} -dependent phosphofructokinase-activating reactions.

Phosphofructokinase plays a central role in the control of energy supply in the heart. The catalytic activity of the isolated purified muscle enzyme is regulated by numerous effectors (Bloxham & Lardy, 1973; Ramaiah, 1976), which have provided the basis for proposals concerning its regulation *in vivo*, including heart. In contrast, our studies over the last 3 years suggest that adrenergic control of this enzyme in rat heart involves a mechanism independent of changes in the concentration of intracellular effectors (for review, see Clark & Patten, 1984). In addition, short-term control of the enzyme appears to be mediated by adrenaline via an α -adrenergic receptor mechanism (Clark & Patten, 1981a) independent of cyclic AMP (Clark & Patten, 1981a), protein phosphorylation (Patten *et al.*, 1981; Clark *et al.*, 1982) and the β -adrenergic receptor-mediated activation of glycogen phosphorylase (Clark & Patten, 1981a). The mechanism of activation requires Ca^{2+} (Patten *et al.*, 1982b) and activation may be achieved by increased perfusion concentrations of Ca^{2+} (Patten & Clark, 1983). However, in hearts from genetically obese (*fa/fa*) rats, activation of phosphofructokinase by perfusion with medium

containing 10 μ M-adrenaline does not occur, even though normal activation of phosphorylase was noted (Patten *et al.*, 1982a). Thus in the present study a detailed examination of the above apparent insensitivity was made.

Experimental

Animals

Male and female lean (*Fa/?*) and obese (*fa/fa*) Zucker rats, approx. 100 days old, were used and maintained *ad libitum* on the Division's 'colony' high-carbohydrate diet. Details for this diet are given elsewhere (Patten *et al.*, 1982a). Comparable obesity was induced in Hooded Wistar rats by feeding with a high-fat diet (Patten *et al.*, 1982a).

Perfusions

Langendorff perfusion of rat hearts with glucose-free medium was conducted in a non-recirculating manner as described previously (Clark & Patten, 1981a). Hearts were pre-perfused for 8–10 min with medium containing 3 mM- CaCl_2 and 0.5 mM-EDTA. After this time, media containing

different concentrations of adrenaline or CaCl_2 were introduced, as described in the Figures. Unless indicated otherwise, hearts were exposed to the different agonists etc. for 4 min.

Assays

Experimental details for extract preparation (Clark & Patten, 1981a, b) and for the determination of phosphofructokinase and phosphorylase activity ratios were as given previously (Clark & Patten, 1981a, b). The activity ratio of phosphofructokinase is defined as the activity at 1 mM-ATP and 10 μM -hexose 6-phosphate/activity at 0.1 mM-ATP and 10 μM -hexose 6-phosphate (Clark & Patten, 1981a). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. The methods for preparing membranes and for conducting binding studies were as described by Williams & Lefkowitz (1978). All determinations were made in duplicate.

Materials

Adrenaline bitartrate and noradrenaline hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [^3H]Prazosin hydrochloride was obtained from New England Nuclear, Boston, MA, U.S.A. Each agent was freshly prepared as a concentrated solution in 0.9% NaCl (saline). Controls involved the addition of saline.

Results and discussion

In a previous study from this laboratory (Patten *et al.*, 1982a) it was reported that adrenaline-mediated activation of cardiac phosphofructokinase was defective in mature genetically obese Zucker rats. In Fig. 1 the dose-response relationships for adrenaline-mediated effects on both phosphofructokinase and phosphorylase are shown. Younger rats than previously were used in this study (Patten *et al.*, 1982a), and thus the activity ratio for phosphofructokinase was initially lower. Activation of phosphofructokinase occurred in hearts from lean (*Fa/?*) animals, with a half-maximal activation at 10 nM-adrenaline. Activation was attributable to an increase in the activity at 1 mM-ATP, as previously noted (Patten *et al.*, 1982a). Adrenaline (10 nM) also produced half-maximal activation of cardiac phosphofructokinase in our colony Hooded Wistar rats (Patten *et al.*, 1982b). However, no significant activation of phosphofructokinase occurred in hearts from obese (*fa/fa*) animals, regardless of the concentration of adrenaline used (Fig. 1a). In addition, it was also evident that perfusion for longer periods with 10 μM -adrenaline (10 min) did not lead to an activation of phosphofructokinase (results not shown).

Fig. 1. Effect of adrenaline concentration on the activity ratios of phosphofructokinase and phosphorylase in hearts from lean (○) and obese (●) Zucker rats

Hearts were pre-perfused for 8 min as described in the Experimental section. After this time the concentrations shown of adrenaline were added. The hearts were freeze-clamped after 4 min. Supernatants (8000g, 30s) were prepared and assayed for phosphofructokinase (a) at 0.1 mM-ATP and 0.01 mM-hexose 6-phosphate and at 1 mM-ATP and 0.01 mM-hexose 6-phosphate. The ratio of the two activities is shown. Phosphorylase activity ratio, $a/a+b$ (b), was determined on supernatants after passage through small columns of Sephadex G-25. Methods are given in the Experimental section. Where appropriate, means $\pm$ S.E.M. have been calculated for the number of hearts shown at each concentration of adrenaline.

Fig. 1(b) shows that identical activation of phosphorylase occurred for lean (*Fa/?*) and obese (*fa/fa*) animals for all concentrations of adrenaline. Half-maximal activation was noted at 0.3 μM -adrenaline and was thus similar to dose-response data that we have previously reported for the Hooded Wistar rat (Patten *et al.*, 1982b). It is noteworthy that the half-maximal activation of phosphorylase occurred at a concentration of adrenaline that was 30–50 times that required to produce similar activation of phosphofructokinase in lean (*Fa/?*) and our colony animals (Patten *et al.*,

Fig. 2. Binding of $[^3\text{H}]$ prazosin to heart membranes from obese (*fa/fa*; ●) and lean (*Fa/?*; ○) rats

Heart membranes were pooled from four hearts and incubated with the concentrations of $[^3\text{H}]$ prazosin shown. Means of duplicate determination were calculated. Scatchard analysis of the data is given in (b).

1982b). A possible explanation for the requirement of high concentrations of adrenaline to activate phosphorylase may relate to an inhibitory effect that α -receptors exert on the β -adrenergic mechanisms [e.g. α_2 inhibitory effects on adenylate cyclase in hamster fat-cells (McMahon & Schimmel, 1982) and rat parotid (Oron *et al.*, 1980) and indirect data for rat heart (Watanabe *et al.*, 1977)]. The data of Fig. 1 suggest that the mechanism involved in modulating the β -adrenergic control of phosphorylase is not defective in the hearts of Zucker obese (*fa/fa*) rats.

Failure of phosphofructokinase to respond to adrenaline may result from a number of defects, including receptor and post-receptor changes.

Fig. 3. Effect of noradrenaline on binding of $[^3\text{H}]$ prazosin to heart membranes from obese (*fa/fa*; ●) and lean (*Fa/?*; ○) rats

Heart membranes were pooled from four hearts and incubated with $0.15 \text{ nM } [^3\text{H}]$ prazosin and the concentrations of noradrenaline shown. Means of duplicate determinations are given.

Adrenaline is considered to bind to both α - and β -adrenergic receptors. Activation of phosphorylase is clearly functioning (Fig. 1b); thus β -adrenergic and, to a lesser extent, α -adrenergic receptor mechanisms (Ramachandran *et al.*, 1982) controlling this enzyme were assumed to be unchanged as a consequence of the genetic determinants for obesity. Activation of phosphofructokinase can be mediated by both α - and β -adrenergic receptors (Patten *et al.*, 1982b). However, adrenaline mediates the activation of this enzyme predominantly via α -adrenergic receptors that are probably α_1 in subtype (Clark & Patten, 1984). Thus experiments were conducted with sarcolemma preparations from hearts of obese (*fa/fa*) and lean (*Fa/?*) rats to assess the binding properties of the α_1 -receptors.

Fig. 2 shows that binding of the α_1 -adrenergic antagonist $[^3\text{H}]$ prazosin differed between the two preparations. Scatchard analysis indicated that membranes from obese (*fa/fa*) animals possessed approx. 20% more binding sites than did those from lean (*Fa/?*) animals. Thus the B_{max} values for obese and lean animals were 79 and 65 fmol/mg of protein respectively; K_D values also differed, and were 0.10 and 0.17 nM respectively. However, in neither case did the characteristics of receptors from hearts of obese animals indicate a potential for diminished α -adrenergic response. In addition there was no significant difference between the two preparations in the ability of noradrenaline to displace $0.15 \text{ nM } [^3\text{H}]$ prazosin (Fig. 3). Thus the binding data indicated that the failure to respond to adrenaline did not result from altered α -

Fig. 4. Properties of extract phosphofructokinases from hearts of obese and lean rats

Hearts were perfused for a total of 12 min as described in the Experimental section and freeze-clamped. Supernatants (8000g, 30s) were prepared and the effects of ATP (a), fructose 1,6-bisphosphate (b), citrate (c) and AMP (d) concentration on the activity of extract phosphofructokinase from hearts of obese (●) and lean (○) rats compared. The concentration of hexose 6-phosphate was $10 \mu\text{M}$. The concentration of ATP was 0.1 mM in (c) and 1 mM in (b) and (d).

adrenergic receptor populations. If analogies between insulin and adrenergic receptor properties exist, it would have been expected that membrane preparations from hearts of the obese (*fa/fa*) animals would contain significantly fewer receptors than the lean counterparts. Instead the binding properties favoured greater sensitivity to adrenaline, with only a marginal difference from the properties of heart preparations from Hooded Wistar rats (Patten & Clark, 1983). These findings indicate a defect distal to the receptors.

Two approaches were used to assess post-receptor differences between hearts of lean (*Fa/?*) and obese (*fa/fa*) rats. In the first of these, the properties of extract phosphofructokinase from hearts of obese and lean animals were compared. Fig. 4(a) shows that the heart extract phosphofructokinase from lean (*Fa/?*) and obese (*fa/fa*) animals used in the present study (100 days of age) responded similarly to increasing concentrations of ATP from $1 \mu\text{M}$ to 3 mM . For heart extracts from older animals the ATP dose-response curves differed markedly (results not shown); the difference was most evident at ATP concentrations greater than 0.1 mM . Thus at 1 mM -ATP the

activities were 0.2 and $0.4 \text{ nmol/min per mg}$ of protein from hearts of obese and lean rats respectively. Since only marginal differences in activity were apparent at 0.1 mM -ATP, the activity ratios for heart phosphofructokinase from obese and lean older animals were approx. 0.2 and 0.4 respectively. As discussed in our previous study (Patten *et al.*, 1982a), this appears to result from an age-dependent decrease in the sensitivity of phosphofructokinase from hearts of lean rats to inhibitory concentrations of ATP (e.g. 1 mM).

There was no significant difference between the response to fructose 1,6-bisphosphate (Fig. 4b) or citrate (Fig. 4c) for the phosphofructokinase activities. Although less sensitive to low concentrations of AMP (Fig. 4d), the enzyme from hearts of obese (*fa/fa*) rats was activated to the same extent as that from lean animals at the maximal concentration of AMP. In additional experiments (results not shown), reactivity of the two extract phosphofructokinases with goat anti-(rabbit skeletal-muscle phosphofructokinase) serum was examined. Identical titres were observed for each; 3 mg of antibody completely inactivated the phosphofructokinase activity in $100 \mu\text{l}$ of each 1:5

Fig. 5. Effect of perfusate Ca^{2+} concentration on the activity ratios of phosphofructokinase (a) and phosphorylase (b) in hearts of obese and lean rats

Hearts were pre-perfused for 8 min as described in the Experimental section. After this time the perfusion medium was changed to one containing 0.5 mM-EDTA and the concentrations of Ca^{2+} shown. The hearts from lean (open symbols) and obese rats (closed symbols) were freeze-clamped after 4 min. Supernatants (8000g, 30s) were prepared and assayed for phosphofructokinase at 0.1 mM-ATP and 0.01 mM-hexose 6-phosphate (not shown) and at 1 mM-ATP and 0.01 mM-hexose 6-phosphate (Δ , $\blacktriangle$). The ratio of the two activities is also shown ($\circ$, $\bullet$). Phosphorylase activity ratio, $a/a+b$ ($\square$, $\blacksquare$), was determined on supernatants after passage through small columns of Sephadex G-25. Methods are given in the Experimental section. The numbers of hearts are shown in parentheses. Where appropriate, standard errors were calculated and are represented as bars.

heart extract. Moreover, the dissociated subunits from the immunoprecipitates were indistinguishable when run on sodium dodecyl sulphate/polyacrylamide disc-gel electrophoresis. Overall, these findings suggest that the inability of adrenaline to activate phosphofructokinase of hearts of obese (*fa/fa*) rats does not result from a readily discernible difference in the properties of the target enzyme.

The second approach used to assess post-

receptor differences between hearts of lean (*Fa/?*) and obese (*fa/fa*) rats involved the addition of high concentrations of Ca^{2+} . In these experiments hearts from obese and lean animals were perfused with medium containing various concentrations of Ca^{2+} , ranging from 3 to 15 mM (Fig. 5). For Hooded Wistar rats, perfusate Ca^{2+} concentrations exceeding 3 mM activated cardiac phosphofructokinase (Patten & Clark, 1983). Fig. 5(a) shows that a similar response resulted with the lean (*Fa/?*) Zucker rats; half-maximal activation occurred at approx. 7 mM- CaCl_2 . The overall increase in activity ratio of phosphofructokinase was from 0.22 at 3 mM- CaCl_2 to 0.50 at 15 mM- CaCl_2 . The hearts from the obese Zucker (*fa/fa*) rats showed markedly less response to Ca^{2+} (Fig. 5a); the activity ratio increased from 0.20 at 3 mM- CaCl_2 to only 0.27 at 15 mM- CaCl_2 .

Fig. 5(b) shows the effect of increasing perfusate Ca^{2+} concentration on the activity ratio of cardiac phosphorylase. Essentially the responses of hearts from lean (*Fa/?*) and obese (*fa/fa*) rats were identical, and the activity ratio increased from 0.2 to 0.4 over the range of CaCl_2 from 3 to 15 mM. A similar extent of activation of cardiac phosphorylase achieved by increasing the perfusate Ca^{2+} concentration from 3 to 15 mM implied that a similar myoplasmic Ca^{2+} concentration was attained in hearts of both lean and obese rats. Thus defective entry mechanisms (e.g. Ca^{2+} channels) for the inward movement of Ca^{2+} would appear unlikely.

The possibility that the obese state acted to inhibit Ca^{2+} -dependent activating mechanisms was also explored. Hearts from diet-induced obese rats were perfused with high concentrations of CaCl_2 . The activity ratio of phosphofructokinase increased from 0.21 ± 0.04 at 3 mM- CaCl_2 (six hearts) to 0.72 ± 0.01 for the range 7–12 mM- CaCl_2 (four hearts). Thus a marked activation of phosphofructokinase occurred, even though these hearts were from animals of similar obesity to the Zucker (*fa/fa*) animals of Fig. 5.

To summarize, we have previously proposed that α - and β -adrenergic mechanisms exist for the activation of phosphofructokinase in rat heart (Patten *et al.*, 1982b). Both adrenergic mechanisms are Ca^{2+} -dependent and are inhibited by removal of Ca^{2+} from the perfusion medium or by Ca^{2+} -channel-blocking agents (e.g. nifedipine) (Patten & Clark, 1983). The present work consolidates a previous publication from this laboratory (Patten *et al.*, 1982a) that phosphofructokinase was not activated by adrenaline in hearts from genetically obese (*fa/fa*) rats. It is now shown that the defect occurs over a wide range of adrenaline concentrations, and also that it is not the result of a slowed time response to the hormone. Binding studies

show no alteration in α_1 -adrenergic binding with obesity. Ca^{2+} entry appears to be normal, as comparable contractile responses (results not shown) and activation of phosphorylase occur. Finally, since the properties of phosphofructokinase from obese (*fa/fa*) and lean (*Fa/?*) hearts appear to be similar, the defect would appear to reside in the as-yet unidentified Ca^{2+} -dependent phosphofructokinase-activating reactions.

It remains to be seen whether this apparent defect at the post-receptor level relates to the loss of insulin responsiveness for glucose uptake and metabolism noted for heart and skeletal muscles of this genetically obese rat (Crettaz *et al.*, 1981).

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