ELSEVIER

European Journal of Pharmacology 390 (2000) 237–244

ejp

www.elsevier.nl/locate/ejphar

Troglitazone does not initiate hypertrophy but can sensitise cardiomyocytes to growth effects of serum

David Bell *, Barbara J. McDermott

Department of Therapeutics and Pharmacology, Centre for Cardiovascular Research, School of Medicine, The Queen's University of Belfast, Whitla Medical Building, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK

Received 29 April 1999; received in revised form 20 December 1999; accepted 24 December 1999

Abstract

Chronic administration of troglitazone might predispose to cardiac hypertrophy. The aims of the study were to determine if troglitazone could (i) initiate a trophic response directly in ventricular cardiomyocytes and (ii) modify responses to other trophic stimuli. After 24 h, troglitazone (10 nM–10 μM) (i) did not increase cellular protein mass and decreased incorporation of [^{14}C]phenylalanine, a marker of protein synthesis, (ii) interacted with serum (10% v/v) and insulin-like growth factor-1 (10 nM) to produce small trophic responses, (iii) increased cellular protein mass but not protein synthesis with insulin (1 unit/ml). Troglitazone (1 μM) attenuated responses to phorbol-12-myristate-13-acetate (PMA) (100 nM), and noradrenaline (5 μM) and endothelin-1 (100 nM), which also activate protein kinase C. In summary, troglitazone does not initiate cardiomyocyte growth directly in vitro, and can inhibit protein kinase C-mediated growth mechanisms. However, the interaction of troglitazone with serum growth factors may contribute modestly to the development of hypertrophy. As troglitazone produced a moderate hypertrophic effect per se in re-differentiated cardiomyocytes, it may directly increase the severity of established hypertrophy.

Keywords: Cardiac hypertrophy; Diabetes; Hyperglycaemia; Peroxisome proliferator-activated receptor γ

1. Introduction

The thiazolidinedione, troglitazone, is a novel antiglycaemic agent. Although long-term administration of troglitazone is not associated with cardiac mass increase or functional impairment in Type II diabetic patients (Ghazzi et al., 1997) or healthy Cynomolgus monkeys (Mayfield et al., 1993), evidence that chronic administration of thiazolidinediones is associated with development of cardiac hypertrophy in experimental animal models in vivo (Stephens et al., 1995; Ghazzi et al., 1997) has generated a level of concern regarding cardiac safety. In view of the pathophysiological complications in heart function caused by cardiac hypertrophy and because the target population for these antidiabetic agents is at particular risk from the development of cardiovascular disease, it is necessary to assess this hypertrophic response.

Systemic haemodynamic effects have been attributed to troglitazone (Ghazzi et al., 1997; Hopkins, 1997). It is difficult to distinguish the hypertrophic effects that occur as a consequence of increased mechanical loading of cardiomyocytes (due to the systemic haemodynamic effects of a given drug) from the direct effects of that drug on the cardiomyocytes themselves. For this reason, the use of in vitro cellular models is warranted, in which the influence of mechanical loading is eliminated, to determine whether troglitazone elicits a trophic effect directly in cardiomyocytes. There is preliminary evidence that troglitazone initiates a trophic response in neonatal rat cardiomyocytes (Stephens et al., 1995). However, significant differences exist between cardiomyocytes isolated from neonatal and adult mammals (Schluter et al., 1995). It is more appropriate that experimental cell models employed in vitro use as a source cardiac tissue obtained from adult mammals since the clinical problems associated with myocardial hypertrophy are related to the adult state. Isolated adult mammalian ventricular cardiomyocytes, maintained in short-term serum-free culture, provide an appropriate experimental model in which to investigate the direct influence of novel

* Corresponding author. Tel.: +44-1232-335770; fax: +44-1232-438346.

E-mail address: d.bell@qub.ac.uk (D. Bell).

0014-2999/00/$ - see front matter
PII: S0014-2999(99)00932-2

drugs on the initiation of hypertrophic growth of the myocardium. At a cellular level, myocardial hypertrophy is based on the total mass and/or size, not number, of cells since adult cardiomyocytes do not undergo cell division (Jacobson and Piper, 1986; Vliegen et al., 1990). Increased mass is achieved either by increased synthesis of de novo protein or by reduced degradation of existing protein (reviewed in Schluter et al.,1995). Re-differentiated cardiomyocytes, obtained after a long-term culture in the presence of serum, provide a model of relevance to exacerbation or maintenance of established cardiac hypertrophy of the compromised heart (Pinson et al.,1993).

Such cellular models have been applied successfully in the investigation of the hypertrophic effects of a number of (patho)physiological stimuli, including α-adrenoceptor agonists and endothelin-1 (reviewed in Schluter et al., 1995). These agonists bind to cell surface receptors coupled to the activation of protein kinase C. In contrast, insulin and insulin-like growth factor-1 stimulate hypertrophic responses after binding to receptors that regulate the activation of tyrosine kinase. Troglitazone binds to a nuclear receptor, peroxisome proliferator-activated receptor, specifically the γ subtype (Lehmann et al., 1995). However, it is uncertain that this subtype is expressed sufficiently in cardiomyocytes (Braissant et al., 1996) to be associated with a significant trophic response.

Troglitazone antagonises phorbol-ester mediated activation of protein kinase C in rat ventricular cardiomyocytes in vitro (Bahr et al., 1996) and might attenuate the hypertrophic effects elicited by agonists coupled to the activation of protein kinase C by an action independent of the growth promoting effects of the drug. The ability of troglitazone to improve insulin sensitivity (Kellerer et al., 1994; Saltiel and Olefsky, 1996) and regulate the cellular uptake and metabolism of glucose (Bahr et al., 1996) has important implications for the heart. Glucose is a physiological regulator of protein kinase C activation (Draznin et al., 1988) and alterations in membrane-associated protein kinase C activity would have a marked influence upon responses to those stimuli whose effects are mediated by this enzyme. In addition, troglitazone increases selectively the potency and maximal activity for insulin- and insulin-like growth factor 1-induced protein synthesis in foetal cardiomyocytes in vitro (Stephens et al., 1995). It is possible that the insulins and troglitazone could act synergistically in the development of cardiomyocyte hypertrophy.

The purpose of the study was, therefore, to determine whether troglitazone (i) initiated a trophic response directly in freshly isolated adult ventricular cardiomyocytes or enhanced the underlying trophic response observed in re-differentiated cardiomyocytes, (ii) sensitised cardiomyocytes to the trophic effects of a general growth stimulus, serum, and, specifically, to the effects of insulin and insulin-like growth factor-1, (iii) elicited per se a more marked trophic response in the presence of elevated glucose concentration, (iv) attenuated protein kinase C-mediated cardiomyocyte growth.

2. Materials and methods

2.1. Materials

Troglitazone was obtained as a gift from SmithKline Beecham Pharmaceuticals (Herts., UK). L-Noradrenaline hydrochloride, isoprenaline hydrochloride, phorbol-12-myristate-13-acetate (PMA), L-carnitine, creatinine, taurine, cytosine-β-D-arabinofuranoside, bovine serum albumin, trypsin, DNA (sodium salt, from calf thymus) and assay kits for the quantification of microprotein were obtained from Sigma (Poole, Dorset, UK). Insulin (porcine, highly purified) was obtained from Novo Nordisk Wellcome (England) and insulin-like growth factor-1 (human) from Peninsula Laboratories (Europe). Endothelin-1 was purchased from Bachem (CA, USA). Bisbenzamide was supplied by Riedel-de-Haen (Germany). Collagenase was purchased from Serva Feinbiochemica (Heidelberg, Germany). L-U-[^{14}C]phenylalanine was obtained from Amersham International (Buckinghamshire, UK). Medium M199 (glutamine free with Earle's salts), foetal calf serum and penicillin (5000 IU)/streptomycin (5 mg/ml) were purchased from Gibco (UK). Plastic Petri dishes were supplied by Falcon (Becton-Dickinson, UK). Liquid scintillation fluid was obtained from Zinsser Analytic (Berkshire, UK). All other chemicals used were of analytical grade and purchased from BDH Chemicals (UK).

Serum-free 'creatinine–carnitine–taurine' (CCT) medium for the culture of cardiomyocytes consisted of modified glutamine-free Medium M199 supplemented with Earle's salts, creatinine (5 mM), L-carnitine (2 mM), taurine (5 mM), ascorbic acid (100 μM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Medium was also supplemented with cytosine-β-D-arabinofuranoside (10 μM) to prevent growth of non-myocytes. The composition of the calcium-free Krebs–Ringer solution used in the isolation of cardiomyocytes was as follows: NaCl (110 mM); KCl (2.6 mM); $NaHCO_3$ (25 mM); $MgSO_4$ (1.2 mM); KH_2PO_4 (1.2 mM); glucose (11 mM). This solution was gassed with 95% O_2/5% CO_2 and maintained at a pH of 7.4°C at 37°C. The composition of the phosphate-buffered saline was as follows: NaCl (137 mM); KH_2PO_4 (1.5 mM); Na_2PO_4 (1.0 mM), pH 7.4. The composition of DNA assay solution was NaCl (1.985 M), Na_2HPO_4 (25 mM), at pH 7.4. Bisbenzamide was dissolved in water (0.2 mg/ml). This stock solution was diluted 1:200 with DNA assay solution to give a working concentration of 1 μg/ml. Solutions of bisbenzamide are very sensitive to light, and were, therefore, kept in the dark prior to use. The stock solution was stable for 6 months in the dark at 4°C. Dilute solutions were prepared daily.

2.2. Isolation and culture of cardiomyocytes

Ventricular cardiomyocytes were isolated as described previously (Pinson et al., 1993). Briefly, two 12-week-old male Sprague–Dawley rats were subjected to deep isoflurane anaesthesia and their hearts excised. The excised hearts were perfused using a Langendorff apparatus with Ca^{2+}-free Krebs Ringer solution containing collagenase (0.4 mg/ml) until they became flaccid. The two hearts were chopped finely and the mince was pooled and agitated gently in the same medium to dissociate individual cells. The resulting cell suspension was filtered to remove undigested material and the cells were sedimented at 500 rpm for 3 min. Ca^{2+} tolerance of the cells was restored gently by resuspending the sediment in Krebs–Ringer solution containing a progressively higher concentration of Ca^{2+} to a final concentration of 1 mM. The cell suspension (3–4 ml) was then layered gently onto a 4% w/v albumin solution (12.5 ml), contained in a tube of length 20 cm and internal diameter 1 cm, in order to sediment viable cardiomyocytes and effectively remove non-muscle cells and cell debris. The resultant sediment was resuspended in serum-free CCT medium. Cells derived from the two hearts were pooled, mixed thoroughly and resuspended at a concentration of 1.5×10^5 viable cardiomyocytes/ml. Aliquots (1 ml) were pipetted gently onto Petri dishes (35 mm diameter), which had been pre-incubated for 2 h with foetal calf serum (4% v/v) in M199. After 1 h, viable cardiomyocytes had attached to the surface of the dish. The dishes were washed with fresh CCT medium to remove non-attached cells and cell debris and the attached cells, employed as an experimental model for investigation of the initiation of cardiomyocyte hypertrophy, were incubated at 37°C for 24 h in CCT medium (1 ml) containing the appropriate concentrations of drugs as specified in the experimental protocols. Under all experimental conditions, cardiomyocytes remained mechanically quiescent.

For establishment of cultures of re-differentiated cardiomyocytes, used as a source of hypertrophied cardiomyocytes for investigation of the exacerbation or maintenance of an established hypertrophic response by the drug, cells were first incubated for 6 days in the presence of foetal calf serum (20% v/v). The dishes were washed with fresh CCT medium to remove non-attached cells and cell debris and the attached cells were subsequently incubated at 37°C for 24 h, in the absence of serum, in CCT medium (1 ml) containing the appropriate concentrations of various drugs as specified in the experimental protocols.

2.3. Incorporation of L-U-[^{14}C]phenylalanine and total mass of cellular protein and total content of cellular DNA

The extent of de novo synthesis of protein in the cell cultures was estimated by measuring uptake of radiolabelled amino acid into cellular protein. The cells were exposed for 24 h to L-U-[^{14}C]phenylalanine (0.1 μCi/ml culture medium). Incorporation of radioactivity into the acid-insoluble cell fraction was determined. At the end of the chosen period of incubation, experiments were terminated by removal of the supernatant medium from the dishes. The attached cells were washed with an aliquot (1 ml) of ice-cold phosphate-buffered saline, prior to the addition of an aliquot (1 ml) of ice-cold trichloroacetic acid (10% w/v). After an overnight storage at 4°C, the acid containing the intracellular precursor pool was removed from the dishes and the attached cells were washed with an aliquot (1 ml) of phosphate-buffered saline. The precipitate remaining on the culture dishes was dissolved in an aliquot (1 ml) of NaOH (0.1 M)/sodium dodecyl sulphate (0.01% w/v) by overnight incubation at 37°C. In these samples, concentration of protein was determined by the colorimetric method of Lowry, the concentration of DNA in the neutralized sample was determined by a spectrophotometric method in which bisbenzamide dye was incorporated into DNA (Mullan et al., 1997), and the radioactivity was counted. The ratio of protein to DNA per dish served as the parameter of cell mass and the ratio of L-U-[^{14}C]phenylalanine incorporated to DNA per dish served as a measure of de novo synthesis of protein.

2.4. Data analysis

In each experiment, the total population of cells contained in culture plates was obtained from a pooled suspension prepared from the two hearts. Under each condition (in the absence/presence of drug at various concentrations, with or without stimuli), the average value measured in three culture plates was calculated for each parameter ([^{14}C]phenylalanine incorporation or protein/DNA content). Replicate data were obtained for n preparations ($3 < n < 8$) and the mean value ± S.E.M. was calculated. Because of inter-assay variability of the basal values between experiments, which was 29% (c.v.) for measurement of incorporation of [^{14}C]phenylalanine ($n = 46$) and 33% (c.v.) for total protein mass ($n = 40$), data were calculated as the percentage of differences from the respective basal values. Data were analysed statistically using a one- or two-factor repeated-measures analysis of variance (SPSS-PC, version 8.0). In the experiments to examine drug concentration–response relationships in the presence of different stimuli, average tests of significance for within-subjects effects (concentration and the concentration–stimulus interaction) were adjusted if necessary to accommodate lack of constant variance using the Huynh–Feldt epsilon. If $P < 0.05$ for the overall effect of concentration under a particular condition, differences between the mean values at a particular concentration (x_1) and at baseline (x_0) were tested by calculation of the t-statistic as $(x_1 - x_0)/\sqrt{\text{residual mean square}\,(2/n)}$. In experiments in which a single concentration of drug was applied, differences from the basal condition were analysed using the paired Student's t-test.

3. Results

3.1. Cell viability

Troglitazone, at the maximum concentration tested (10 μM), did not exert any deleterious effects upon the viability of cardiomyocytes maintained in short-term (24 h) culture: 68.5 ± 2.9% ($n = 6$) and 69.2 ± 1.6% ($n = 6$) of the cells were viable after 24 h in the absence and presence, respectively, of troglitazone (10 μM).

3.2. pH considerations

The pH of media maintained for 24 h in the absence of cardiomyocytes was 7.38 ± 0.01 ($n = 3$). Medium became significantly more alkaline (pH = 7.45 ± 0.01, $n = 3$) in the presence of serum (10% v/v). These values were not influenced by the presence of troglitazone (10 μM). The pH of basal medium maintained for 24 h in the presence of cardiomyocytes was 7.34 ± 0.01 ($n = 3$). These untreated cultures also became significantly more alkaline (pH = 7.43 ± 0.01, $n = 3$) in the presence of serum (10% v/v). In the absence of serum, cardiomyocyte cultures became significantly more acidic (pH = 7.11 ± 0.11, $n = 3$) in the presence of troglitazone (10 μM). This enhanced acidity due to the presence of troglitazone was not observed in cultures with serum (pH = 7.48 ± 0.02, $n = 3$).

3.3. Effects of troglitazone in the absence and presence of serum

Troglitazone (10 nM–10 μM) did not exert any hypertrophic effect per se on cardiomyocytes maintained in culture for 24 h since the incorporation of [^{14}C]phenylalanine into cellular protein (Fig. 1a) was not increased above the basal value (841.7 ± 60.4 dpm/μg DNA, $n = 6$). Indeed, significant decreases were observed in the presence of troglitazone at concentrations ≥ 1 μM. Increases in total mass of cellular protein, at maximum 15% above the basal value of 45.7 ± 1.9 μg/μg DNA ($n = 6$), were not significant (Fig. 1b).

Foetal calf serum (10% v/v) increased the incorporation of [^{14}C]phenylalanine and the total mass of cellular protein by 33% and 13%, respectively, above the basal values (493.3 ± 34.3 dpm/μg DNA and 40.5 ± 6.7 μg/μg DNA, $n = 6$) (Fig 1a). A significant interaction between troglitazone (10 nM–10 μM) and serum was detected ($P < 0.001$), and under this condition, the drug elicited small trophic effects, since increases in the incorporation of [^{14}C]phenylalanine into cellular protein were observed above that produced by serum per se at all concentrations tested, and significantly at 33 μM (Fig. 1a). Furthermore, the marked attenuation of the incorporation of [^{14}C]phenylalanine into cellular protein observed at higher concentrations of troglitazone alone was abolished by the presence of serum. Troglitazone also increased the total mass of cellular protein above that elicited by serum per se particularly at lower concentrations, but effects were additive and there was no evidence of synergism (Fig.1b).

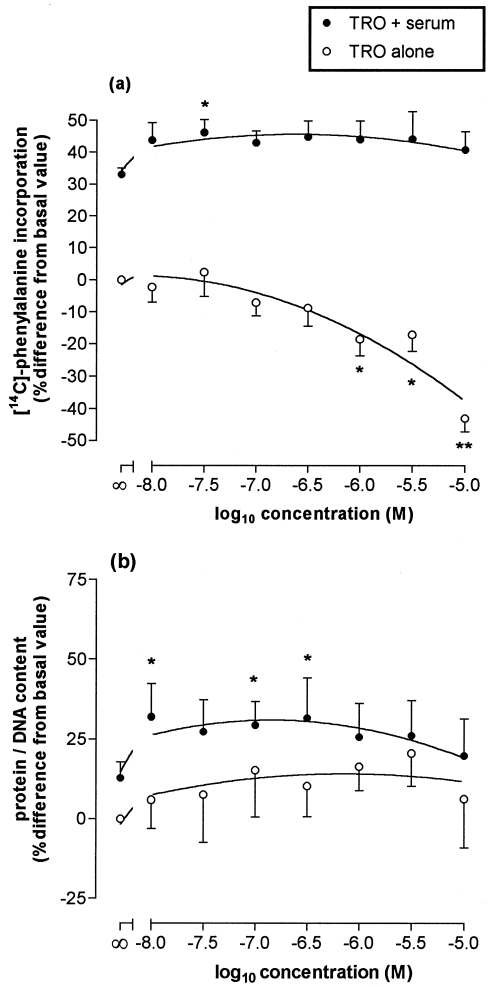

Fig 1. Effect of troglitazone (10 nM–10 μM) alone or in the presence of serum (10% v/v) on (a) incorporation of [^{14}C]phenylalanine into de novo cellular protein and (b) total mass of cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are the means ± S.E.M. of six experiments. Significant variation between responses elicited with and without troglitazone ($^{*}P < 0.05$, $^{**}P < 0.005$).

3.4. Interaction of troglitazone with insulin-like growth factor-1 and insulin

Insulin-like growth factor-1 (10 nM) increased the incorporation of [^{14}C]phenylalanine and the total mass of cellular protein by 26% and 8%, respectively, above the basal values (511.4 ± 34.3 dpm/μg DNA and 43.6 ± 2.9 μg/μg DNA, $n = 6$) (Fig. 2a). A significant interaction between troglitazone (10 nM–1 μM) and insulin-like growth factor-1 was observed ($P < 0.05$), and at lower concentrations, the drug produced small increases in the

incorporation of [^{14}C]phenylalanine into cellular protein above that elicited by insulin-like growth factor-1 per se (Fig. 2a). This response, however, was significantly attenuated in the presence of troglitazone (10 μM). Troglitazone tended to increase the total protein mass above that elicited by insulin-like growth factor-1 per se at all concentrations tested, but effects were additive and there was no evidence of synergism (Fig. 2b).

Insulin (1 unit/ml) increased the incorporation of [^{14}C]phenylalanine and the total mass of cellular protein by 57% and 7%, respectively, above the basal values (681.8 ± 72.7 dpm/μg DNA and 45.7 ± 3.2 μg/μg DNA, n = 6) (Fig. 3). Troglitazone (10 nM–10 μM) did not exert a trophic effect in the presence of insulin in that incorporation of [^{14}C]phenylalanine into cellular protein was not increased above that elicited by insulin per se, and there was no interaction observed (Fig. 3a). However, troglitazone increased the total protein mass above that stimulated by insulin at all concentrations tested, and at lower concentrations effects were additive, not synergistic (Fig. 3b).

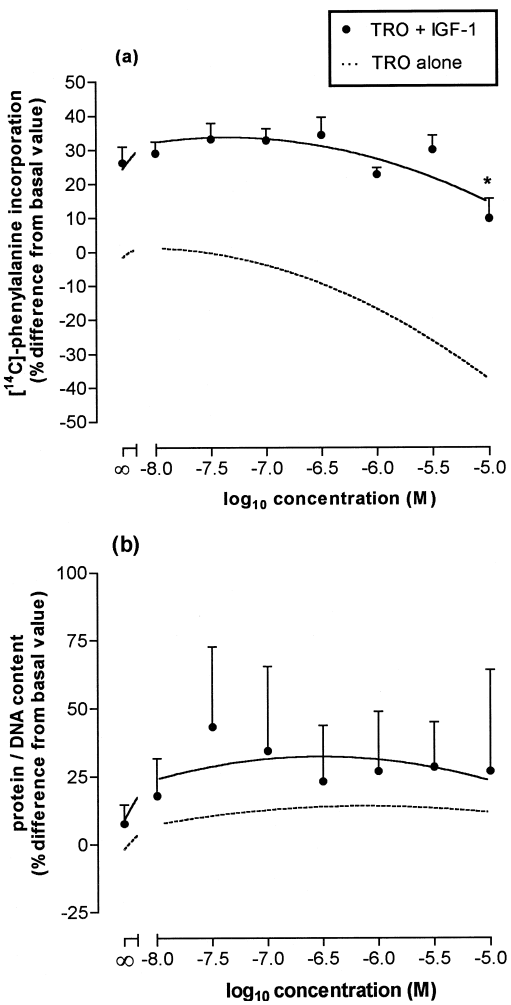

Fig 2. Effect of troglitazone (10 nM–10 μM) alone or in the presence of IGF-1 (10 nM) on (a) incorporation of [^{14}C]phenylalanine into de novo cellular protein and (b) total mass of cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are the means ± S.E.M. of six experiments. Significant variation between responses elicited with and without troglitazone (*$P < 0.01$).

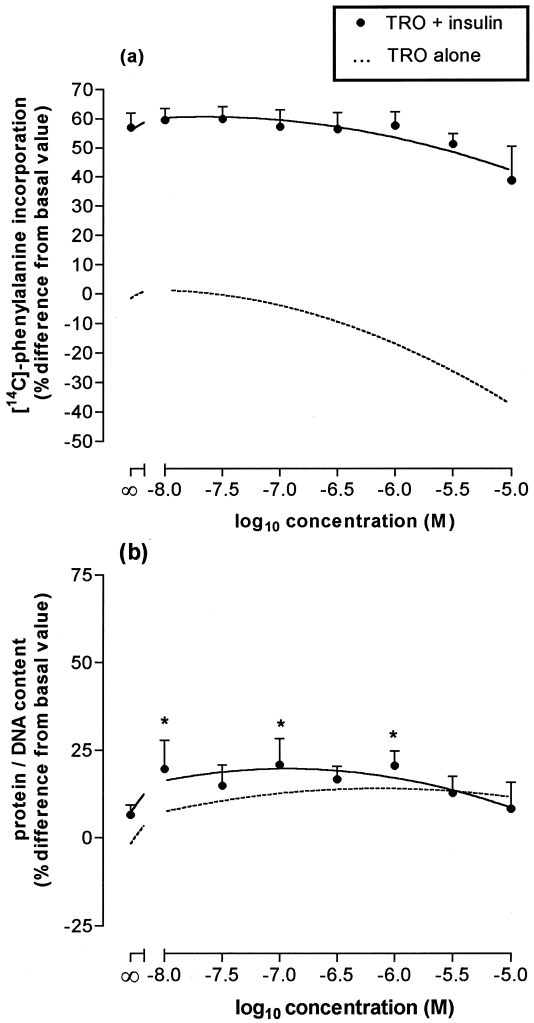

Fig 3. Effect of troglitazone (10 nM–10 μM) alone or in the presence of insulin (1 unit/ml) on (a) incorporation of [^{14}C]phenylalanine into de novo cellular protein and (b) total mass of cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are the means ± S.E.M. of six experiments. Significant variation between responses elicited with and without troglitazone (*$P < 0.01$).

3.5. Interaction of troglitazone with activators of protein kinase C

The activators of protein kinase C, noradrenaline (5 μM), phorbol ester (100 nM) and endothelin-1 (100 nM), produced marked trophic effects as evidenced by increased incorporation of [^{14}C]phenylalanine by 23%, 29% and 18%, respectively, above the basal value (603.5 ± 35.7 dpm/μg DNA, $n = 7$) (Fig. 4a) and increased the total mass of cellular protein by 30%, 20% and 12% above the basal value (57.3 ± 7.0 μg/μg DNA, $n = 7$) (Fig. 4b). Increases in de novo protein synthesis in response to

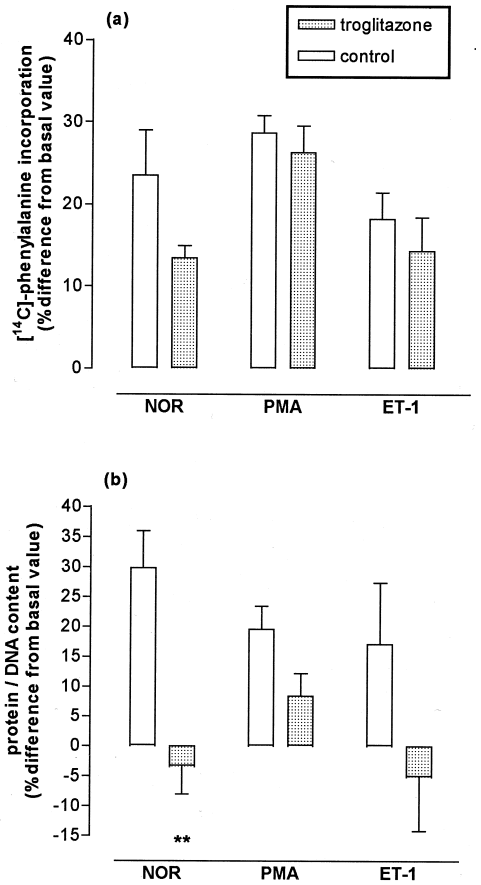

Fig 4. Interaction of troglitazone with activators of protein kinase C: (a) incorporation of [^{14}C]phenylalanine into de novo cellular protein (dpm/μg DNA); (b) total mass of cellular protein (μg / μg DNA); of cardiomyocytes maintained in short-term (24 h) serum-free primary culture in the presence of noradrenaline (5 μM) (NOR), phorbol-12 myristate-13 acetate (100 nM) (PMA) and endothelin-1 (100 nM) (ET-1), in the absence (control) and presence of troglitazone (1 μM). Because of effects of this concentration of drug on basal values, data obtained in the presence of troglitazone were adjusted accordingly and are the mean values ± S.E.M. of seven experiments. Significant variation between paired data ($^{*}P < 0.05$, $^{**}P < 0.005$).

noradrenaline, endothelin-1 and phorbol ester were not significantly affected by troglitazone (1 μM) (Fig. 4a). However, the drug attenuated markedly the increases in mass of cellular protein, such that responses to noradrenaline and endothelin-1 were abolished (Fig. 4b).

3.6. Interaction of troglitazone with elevated glucose concentration

Elevated glucose concentration (25 mM) per se did not influence the amount of [^{14}C]phenylalanine incorporated into cellular protein either in the absence or presence of insulin (1 unit/ml), above the basal value obtained in the presence of 5.5 mM glucose (700.1 ± 70.4 dpm/μg DNA (Fig. 5a). Troglitazone (1 μM) did not influence the amount of [^{14}C]phenylalanine incorporated under basal conditions, or in the presence of either insulin or elevated glucose alone, but tended to increase (by 7%) the response to combined insulin and elevated glucose. In contrast, troglitazone abolished the increases in mass of cellular protein (from a basal value of 75.3 ± 5.6 dpm/μg DNA) elicited by these stimuli alone or in combination (Fig. 5b).

3.7. Effects of troglitazone on re-differentiated cardiomyocytes

Phorbol ester (100 nM) and isoprenaline (1 μM), produced marked stimulation of the already hypertrophied cells as evidenced by increased incorporation of [^{14}C]phenylalanine, each by 26% above the basal value (1110.2 ± 108.1 dpm/μg DNA, $n = 7$) (Fig. 6a) and increased the total mass of cellular protein by 9.6% and 7.3%, respectively, above basal value (43.55 ± 3.5 μg/μg DNA, $n = 7$) (Fig. 6b). Troglitazone also increased the total mass of cellular protein and incorporation of [^{14}C]-

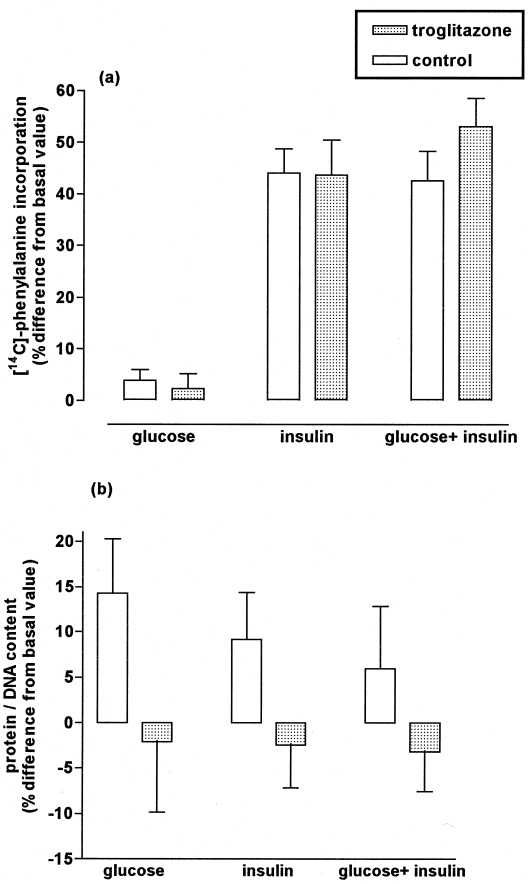

Fig 5. Interaction of troglitazone with elevated glucose concentration: (a) incorporation of [^{14}C]phenylalanine into de novo cellular protein; (b) total mass of cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture in the presence of elevated glucose concentration (25 μM), insulin (1 unit/ml), and glucose (25 mM) in combination with insulin (1 unit/ml), in the absence (control) and presence of troglitazone (1 μM). Data are expressed as differences from basal value obtained in the absence and presence, respectively, of troglitazone and are the means ± S.E.M of seven experiments.

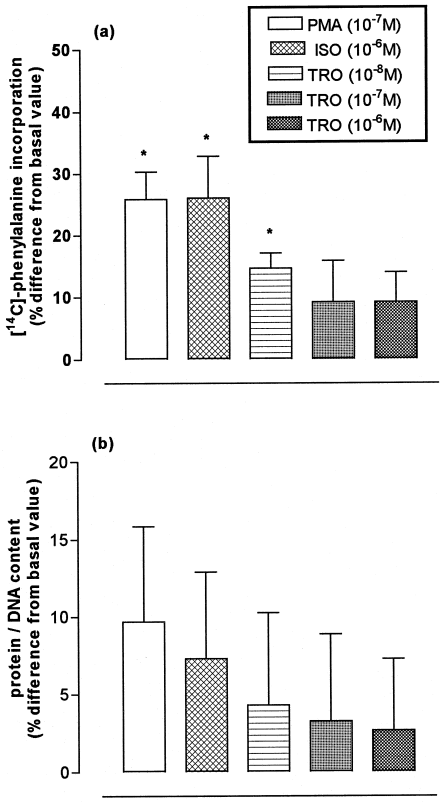

Fig 6. Effect of troglitazone (10 nM–1 μM) on: (a) incorporation of [^{14}C]phenylalanine into de novo cellular protein; (b) total mass of cellular protein of redifferentiated cardiomyocytes. Phorbol-12-myristate-13 acetate (100 nM) (PMA) and isoprenaline (1 μM) (ISO) were included as positive controls. Data are the means ± S.E.M. of seven experiments. Significant variation from basal value ($^*P < 0.05$).

phenylalanine, significantly at the lowest concentration, in re-differentiated cardiomyocytes.

4. Discussion

Troglitazone per se did not initiate a hypertrophic effect directly in cardiomyocytes since neither the total mass of cellular protein nor the incorporation of [^{14}C]phenylalanine, a marker of de novo protein synthesis, were elevated markedly above basal values when cells were maintained in short-term (24 h) serum-free primary culture. The marked attenuation of de novo protein synthesis observed in response to higher concentrations of troglitazone may be an indication of its cytotoxicity. This could not be attributed, however, to the cell-killing effects of the drug at micromolar concentrations, since the viability of the cell population was not reduced relative to that of cells incubated in the absence of troglitazone for a similar period of time. Alternatively, the underlying cause may be explained by the observation that the supernatant media from cultures maintained in the presence of troglitazone (10 μM) became more acidic compared to control media. Since troglitazone did not influence the pH of media in the absence of cells, it is possible that cellular metabolism or drug degradation may produce acidic products. A resultant fall in pH of the extracellular environment of the cells might result in an attenuation of cellular metabolism, manifest as reduced synthesis of cellular protein. Alternatively, troglitazone itself, or one of its metabolites or breakdown products, could be directly inhibitory at micromolar concentrations.

The finding that troglitazone acted synergistically with serum to increase protein synthesis in cardiomyocytes indicates that the thiazolidinedione can sensitise the cells to the effect of serum or, alternatively, that the presence of serum can prime the cells to the hypertrophic effect of troglitazone. The conclusion that the latter is true is strengthened by data obtained in re-differentiated cardiomyocytes, a cellular model in which hypertrophy is already established (Pinson et al., 1993). Troglitazone per se elicited a more marked trophic response in these cells than in freshly isolated cells. These data indicate that the re-differentiated cells had become serum-independent for sensitivity to thiazolidinediones.

The cellular mechanism by which troglitazone elicits a trophic response is unknown. The thiazolidinediones do bind to nuclear receptors, particularly peroxisome proliferator-activated receptor γ (Lehmann et al., 1995), and it might be that the trophic response to the drug would be initiated by binding to a nuclear receptor, in a manner similar to thyroid or steroid hormone-induced cardiomyocyte hypertrophy (Nishiyama et al., 1997). It is uncertain that such receptors, and, in particular, the γ subtype, are expressed sufficiently abundantly in rat cardiomyocytes (Braissant et al., 1996) to mediate the response. It is possible, however, that factors present in serum may upregulate the expression of peroxisome proliferator-activated receptors and that the upregulated process might become constitutive in re-differentiated cells. It is unclear which factor(s) present in the serum might prime cardiomyocytes to the hypertrophic effect of troglitazone. Catecholamines, insulin-like growth factor-1 and endothelin-1 have each been implicated in hypertrophic cardiomyocyte growth (Schluter et al., 1995) and elevated serum concentration of each of these factors correlates with the severity of myocardial hypertrophy in vivo (Furlanetto et al., 1977; Nakashima et al., 1984; Morgan and Baker, 1991; Battistini et al., 1993). In this study, the interaction between troglitazone and insulin-like growth factor-1, being smaller than obtained with serum, indicates that insulin-like growth factor-1 may be one of a number of factors present in serum that can sensitise cardiomyocytes to the action of troglitazone. However, there was no evidence for synergism of the drug with insulin, noradrenaline or endothelin-1.

The finding that troglitazone caused a marginal potentiation of the trophic response to insulin and glucose in combination indicates that troglitazone may sensitise the

cardiomyocytes to insulin-stimulated glucose transport possibly via upregulation of transporter proteins (Bahr et al., 1996) and, thereby, achieve an increase in de novo protein synthesis. However, the marked attenuation by troglitazone of increases in protein mass elicited in response to elevated glucose or insulin either alone or in combination, indicate that complex interactions occur between cellular protein synthesis and metabolism under these conditions.

Troglitazone has been shown to antagonise phorbol-ester mediated activation of protein kinase C in rat ventricular cardiomyocytes in vitro (Bahr et al., 1996) and so the drug might be expected to attenuate the trophic responses to stimuli coupled to the activation of protein kinase C by an action independent of any growth promoting effects of troglitazone per se. Accordingly, in the present study, it was found that troglitazone attenuated the increase in protein mass initiated in freshly isolated cardiomyocytes by phorbol ester. Furthermore, responses to noradrenaline, endothelin-1 and glucose, a physiological regulator of protein kinase C, were abolished by troglitazone.

In conclusion, this investigation has demonstrated that troglitazone exerts no direct effect to promote growth in freshly isolated rat cardiomyocytes. Evidence is provided, however, for indirect effects of the drug, which can either attenuate (through interaction with protein kinase C mechanisms) or promote the actions of other hypertrophic factors, including IGF-1, in serum. The finding of serum-dependent increases in de novo protein synthesis after treatment with 10 nM–10 μM concentrations of troglitazone may have relevance to clinical treatment, as the maximum plasma concentrations of this drug at the highest doses given are about 7.0 μM (Ghazzi et al., 1997). It is possible, therefore, that the interaction of troglitazone with serum growth factors may contribute to cardiac remodelling. However, the effects of the drug were small, even in re-differentiated cells, so troglitazone may make only a minimal contribution to initiating ventricular hypertrophy in at-risk individuals with diabetes or to increasing severity in cases of established disease.

References

Bahr, M., Spelleken, M., Bock, M., von Holtey, M., Kiehn, R., Eckel, J., 1996. Acute and chronic effects of troglitazone (CS-045) on isolated rat ventricular cardiomyocytes. Diabetologia 39, 766–774.

Battistini, B., D'Orleans-Juste, P., Sirois, P., 1993. Endothelins: circulating plasma levels and presence in other biologic fluids. Lab. Invest. 68, 600–617.

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., Wahli, W., 1996. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, β and γ in the adult rat. Endocrinology 137, 354–366.

Draznin, B., Leitner, J.W., Sussman, K.E., Sherman, N.A., 1988. Insulin and glucose modulate protein kinase C activity in rat adipocytes. Biochem. Biophys. Res. Commun. 156, 570–575.

Furlanetto, R.V., Underwood, L.E., Van Wyk, J.J., 1977. Estimation of somatomedin-C levels in normal and patients with pituitary disease by radioimmunoassay. J. Clin. Invest. 60, 648–657.

The Troglitazone Study Group, Ghazzi, M.N., Perez, J.E., Antonucci, T.K., Driscoll, J.H., Haung, S.M., Faja, B.W., Whitcomb, R.W., 1997. Cardiac and glycaemic benefits of troglitazone treatment in NIDDM. Diabetes 46, 433–439.

Hopkins, K.D., 1997. Heart mass not altered by troglitazone. The Lancet 349, 781.

Jacobson, S.L., Piper, H.M., 1986. Cell cultures of adult cardiomyocytes as models of the myocardium. J. Mol. Cell. Cardiol. 18, 439–448.

Kellerer, M., Kroder, G., Tippmer, S., 1994. Troglitazone prevents glucose-induced insulin resistance of insulin receptor in rat-1 fibroblasts. Diabetes 43, 447–453.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Wilson, T.M., 1995

Mayfield, R., McLean, D., Crook, D., Buist, D.P., Gopinath, C., 1993. CS-045 toxicity to the cynomolgus monkeys by repeated oral administration for 52 weeks. J. Clin. Ther. Med. 9 (Suppl. 3), 317–341.

Morgan, H.E., Baker, K.M., 1991. Cardiac hypertrophy: mechanical, neural and endocrine dependence. Circulation 83, 13–25.

Mullan, D.M., Bell, D., Kelso, E.J., McDermott, B.J., 1997. Involvement of endothelin (ET)-A and ET-B receptors in the hypertrophic effects of ET-1 in rabbit ventricular cardiomyocytes. J. Cardiovasc. Pharmacol. 29, 350–359.

Nakashima, Y., Fouad, F.M., Tarazi, R.C., 1984. Regression of left ventricular hypertrophy from systemic hypertension by enalapril. Am. J.Cardiol. 53, 1044–1049.

Nishiyama, A., Kambe, F., Kamiya, K., Yamaguchi, S., Murata, Y., Seo, H., Toyama, J., 1997. Effects of thyroid and glucocorticoid hormones on Kv1.5 potassium channel gene expression in the rat left ventricle. Biochem. Biophys. Res. Commun. 237, 521–526.

Pinson, A., Schluter, K.D., Zhou, X.J., Schwartz, P., Kessler-Icekson, G., Piper, H.M., 1993. Alpha and Beta adrenergic stimulation in cultured adult ventricular cardiomyocytes. J. Mol. Cell. Cardiol. 25, 477–490.

Saltiel, A.R., Olefsky, J.M., 1996. Thiazolidinediones in the treatment of insulin resistance and Type II diabetes. Diabetes 45, 1661–1669.

Schluter, K.D., Millar, B.C., McDermott, B.J., Piper, H.M., 1995. Regulation of protein synthesis and degradation in adult ventricular cardiomyocytes. Am. J. Physiol. 38, C1347–C1355.

Stephens, T.W., Bergman, J.A., Bue-Valleskey, J.M., DiMarchi, R.D., Slieker, L.J., Tinsley, F.C., Williams, G.D., 1995. Thiazolidinedione induced cardiac biochemical changes and increased IGF-1 action on cardiomyocytes. Diabetologia 38, A200.

Vliegen, H.W., Bruschke, A.V.G., Van der Laarse, A., 1990. Different response of cellular DNA content to cardiac hypertrophy in human and rat heart myocytes. Comp. Biochem. Physiol. 95, 109–114.