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Adrenergic agents, but not triiodo-L-thyronine induce c-*fos* and c-*myc* expression in the rat heart

R. D. Hannan and A. K. West

Department of Biochemistry, University of Tasmania, Australia

Summary: We have examined the expression of two nuclear-acting oncogenes, c-*fos* and c-*myc* in the rat heart following administration of hormones implicated in the development of cardiac hypertrophy. A single injection of norepinephrine (2.5 μg/kg to 2.5 mg/kg) led to transient increases in the levels of both c-*fos* and c-*myc* mRNA. The response was sequential: elevated levels of c-*fos* mRNA were first observed 15 min after treatment and peaked at 1 h whilst c-*myc* mRNA levels increased 30 min after treatment and peaked at 2 h. The response of both cellular oncogenes to norepinephrine was reduced significantly by α blockade but β blockade was less effective. Administration of triiodo-L-thyronine (0.25 mg/kg), a level known to promote cardiac hypertrophy, did not produce elevated levels of c-*fos* or c-*myc* mRNA. In an initial study, it was possible to demonstrate induction of c-*fos* and c-*myc* in rat hearts perfused in vitro with medium containing 2×10^{-7} M norepinephrine. These results provide support for the notion that c-*fos* and c-*myc* expression may play a transducing role in the development of adrenergic-mediated, but not thyroid hormone-mediated cardiac hypertrophy.

Key words: c-*myc* expression; c-*fos* expression; adrenergic agents; triiodo-L-thyronine; cardiac hypertrophy

Introduction

Synthesis of the structural proteins, enzymes and other components required for tissue growth depends on a coordinated pattern of gene expression. This genetic program must be activated and regulated by the extracellular factors, such as hormones and physical stimuli responsible for promoting growth. However, whilst considerable progress has been made in describing the intracellular signalling pathways which mediate the actions of, for example, adrenergic agents (5, 15, 32), little is known about the mechanism or sites at which such pathways interact with the transcription apparatus. This situation is further complicated by the fact that most tissues grow as a consequence of the effects of multiple factors which may activate parallel or divergent signalling pathways. One important model of tissue growth is cardiac hypertrophy, in which myocytes contribute to the growth of the heart by increasing in size rather than by cell division (35). Whilst both haemodynamic and humoral components are involved in the hypertrophic response, a major regulator of myocyte growth has been shown to be adrenergic agents, both in vivo (11, 19, 36) and in cultured myocytes in vitro (16, 22, 28) and Simpson's group have further characterized this to be an α_1-adrenergic response (29). One consequence of adrenergic-mediated hypertrophy of myocytes in the adult heart is that quantitative and qualitative shifts in protein synthesis occur (13, 17), for example, causing resynthesis of foetal isoforms of myosin and other contractile proteins (4, 30). However, such changes may not be due to the primary actions of adrenergic-responsive pathways, leading to the notion that expression of other genes is involved at an earlier point

in the process. One group of candidate genes which have been implicated in the early response to a wide variety of events, including mitogenesis, cell division and differentiation is the nuclear-acting protooncogenes, including c-*fos* and c-*myc* (18). These genes are expressed rapidly following the application of the appropriate stimulus (27) and the protooncogene products are localized to the cell nucleus where they may interact with other genes (9, 26). We have examined the ability of the hypertrophic hormone norepinephrine and its synthetic agonists and antagonists to modulate cardiac c-*fos* and c-*myc* expression and, as a comparison, the actions of triiodo-L-thyronine (T_3) were also investigated. We report that adrenergic agents, but not T_3 were able to elevate levels of c-*fos* and c-*myc* RNA in the rat heart, and that these responses can be demonstrated in the isolated, perfused heart.

Methods

Animals and treatments

All animals used were male hooded Wistar rats (180–200 g), approximately 9–11 weeks old. For experiments examining the effect of hypertrophic hormones in vivo, the animals received a single injection i.p. containing the agent dissolved in vehicle. Norepinephrine, isoproterenol and phenylephrine were prepared in 0.9 % (w/v) saline with 0.1 % (w/v) ascorbic acid whilst triiodo-L-thyronine was dissolved in phosphate buffered saline (pH 11.0). All preparations were made fresh each day. Where used, adrenergic antagonists phenoxybenzamine, phentolamine or propranolol were injected twice, 1 h and again 10 min before norepinephrine administration. These compounds were also prepared in 0.9 % (w/v) saline with 0.1 % (w/v) ascorbic acid. At the appropriate time after administration of the agents, the rats were killed by cervical dislocation and the hearts quickly removed and frozen in liquid nitrogen to await RNA extraction.

Langendorff perfusion (non-recirculating) of isolated hearts was carried out in a similar manner to that described by Williamson (34). Rats were anaesthetized with an i.p. injection of 0.4 ml sodium pentobarbitone, 0.2 ml heparin and 0.4 ml of 0.9 % saline. When unconscious, hearts were removed, cannulated via the ascending aorta and perfused at constant pressure equivalent to 80 cm H_2O to give a flow rate of 5–8 ml/min. The perfusate was modified Krebs-Henseleit buffer containing 1.27 mM $CaCl_2$, 0.05 mM EDTA, 2 mM glucose and 2 mM pyruvate, equilibrated against 95 % O_2/5 % CO_2. All components of the perfusion apparatus were maintained at 37 °C. The hearts were perfused for 15 min to allow equilibration and then a norepinephrine solution was introduced into the perfusion buffer. At the conclusion of the perfusion, the hearts were frozen in liquid nitrogen until RNA extraction.

RNA extraction and northern blots

Total RNA was extracted according to the method of Chomczynski and Sacchi (6) with slight modification. 1 gram of frozen tissue was finely ground under liquid nitrogen and transferred to a sterile plastic tube containing 10 ml of homogenizing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol) and given two bursts at high speed with an Ultra-Turrax homogenizer. To the homogenate was added sequentially 1 ml of 2 M sodium acetate pH 4.0, 10 ml phenol (saturated with 10 mM Tris-HCl pH 7.6, 1 mM EDTA) and 2 ml of chloroform: isoamyl alcohol mixture (49:1), with thorough mixing after each addition. The final mixture was shaken vigorously for 4 min then centrifuged in glass tubes at 10 000 g for 20 min at 4 °C. The upper aqueous phase was mixed with 10 ml of isopropyl alcohol in a fresh tube and cooled at −20 °C for 1 h. Total RNA was pelleted by centrifugation at 15 000 g for 10 min at 4 °C, drained and redissolved in 1.5 ml 1 mM EDTA pH 7.4. The RNA was reprecipitated by addition of 2 ml 4.5 M sodium acetate pH 6.0 and incubated at −20 °C for 1 h following centrifugations, as before. The RNA pellet was washed in 70 % ethanol, dried under vacuum for 10 min and dissolved at 65 °C in 0.5 % SDS.

20–50 μg of total RNA was denatured by addition of formamide to 50 % (final) and heated to 65 °C for 10 min. Following electrophoresis through a 1.2 % denaturing agarose gel (20), the RNA was blotted on Zetaprobe (BioRad) membranes (33). The quantity of RNA in each track was verified by including ethidium bromide in the agarose gel and observing RNA fluorescence before and after transfer. In

addition, all filters were hybridized to a control probe, rat β-tubulin. Oncogene probes were labelled to a specific activity of 10^8 cpm/μg using random priming (10) and hybridized as described previously (33). Probes used were a 2 kb *Hind*III fragment containing exon 2 of human c-*myc*, a 1.06 kb *Pst*-I fragment from v-*fos* and a 1.5 kb *Pst*-I fragment of the rat β-tubulin gene. All cloned probes were kindly supplied by Dr. R. Crawford (Howard Florey Institute). Experiments were repeated at least three times and representative autoradiograms are shown.

Results

Effect of adrenergic agents on protooncogene expression in vivo

Total RNA was extracted from rat hearts and analyzed for c-*fos* and c-*myc* mRNA by northern blotting. Basal levels of both protooncogene mRNAs could be detected in the hearts of both untreated and saline-injected animals, although this required over-exposure of the appropriate autoradiographs (Fig. 1, Lanes 1, 2 and unpublished results). A single injection of norepinephrine (2.5 mg/kg) however, greatly elevated both c-*fos* and c-*myc* mRNA levels, with c-*fos* transcripts increasing 15 min after injection to a peak at 1 h and returning to basal levels by 6 h. Elevated c-*myc* mRNA was observed at 30 min after injection of norepinephrine, peaked at 2 h and returned to basal levels after 6 h (Fig. 1, Lanes 3–10). Thus, the increase in mRNA levels of these two genes in response to norepinephrine was both transient and sequential. Similar, but lesser increases of c-*fos* and c-*myc* mRNA were observed following single doses of norepinephrine at doses as low as

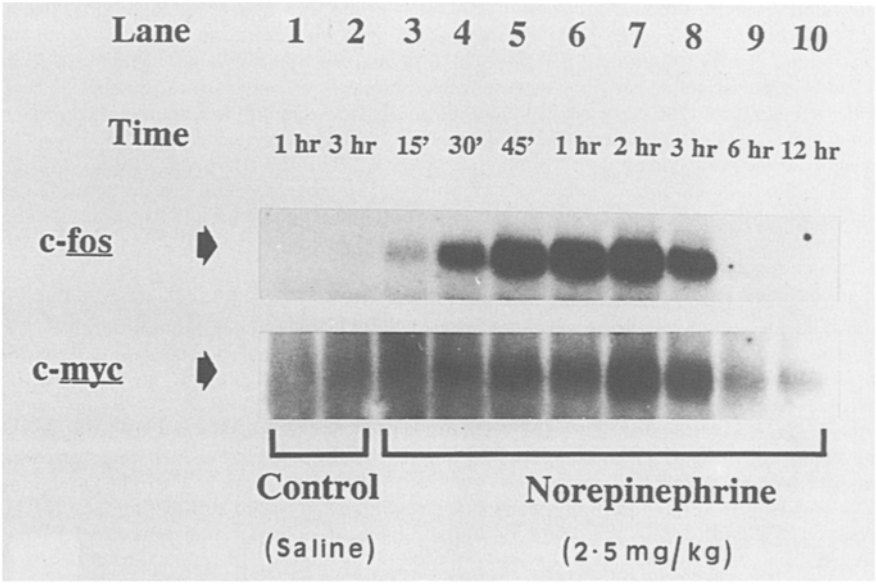

Fig. 1. Cardiac expression of c-*fos* and c-*myc* in response to a single injection of norepinephrine. Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9% saline (Lanes 1 + 2) or norepinephrine (2.5 mg/kg, Lanes 3–10). After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to c-*fos* (upper tracks) and c-*myc* (lower tracks).

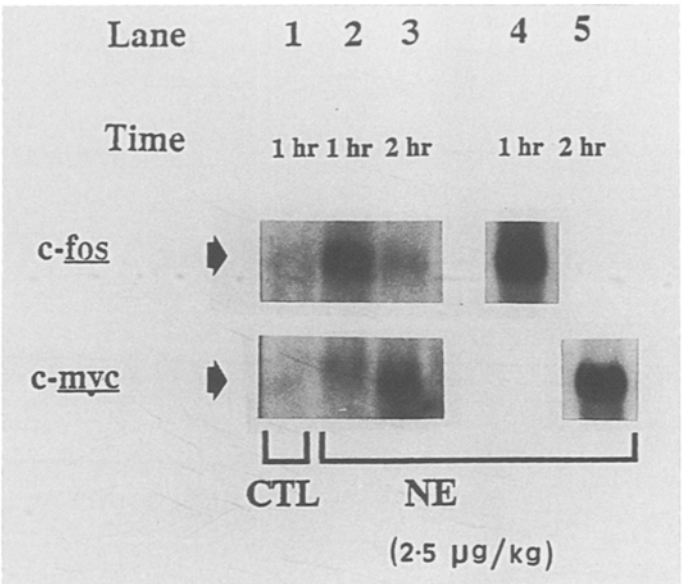

Fig. 2. Cardiac expression of c-*fos* and c-*myc* in response to a single low dose injection of norepinephrine.
Total RNA was extracted from rat hearts removed at the times indicated following an i.p. injection of 0.9% saline (Lane 1) or norepinephrine (2.5 µg/kg, Lanes 2 + 3) and 50 µg was hybridized to c-*fos* (upper tracks) or c-*myc* (lower tracks). In addition, 7 µg of poly(A)$^+$ RNA was isolated from the 1 h and 2 h total RNA samples for norepinephrine-treated rats and hybridized to c-*fos* (Lane 4) and c-*myc* (Lane 5) probes respectively.

2.5 µg/kg, indicating that this response is not restricted to extremely high doses of hormone (Fig. 2).

In an attempt to determine which component of norepinephrine action was responsible for the change in protooncogene levels, rats were treated with an α-adrenergic agonist, phenylephrine (2.5 mg/kg) or a β-adrenergic agonist, isoproterenol (2.5 mg/kg). Both agents caused an increase in c-*fos* and c-*myc* mRNA which was similar in magnitude to that due to norepinephrine (Figs. 3 and 4). The slight cross-over affinity of phenylephrine for β-adrenergic receptors was not responsible for the observed inductions since pretreatment of rats with a selective β-blocker propranolol, prior to phenylephrine administration did not alter c-*fos* and c-*myc* expression levels (results not shown). Interestingly, the time required for c-*myc* mRNA to reach maximal levels appeared to be dependent on which agent was administered, peaking at 1 h, 2 h and 3 h following treatment of rats with phenylephrine, norepinephrine and isoproterenol respectively. The timing of maximal c-*fos* expression was not similarly affected. These findings were extended by examining protooncogene expression in hearts from rats exposed to selective α or β blockade prior to norepinephrine administration. Pretreatment with propranolol (50 mg/kg), a synthetic β-antagonist, produced similar induction of c-*fos* to norepinephrine alone, with maximal expression at 1 h. It was noted again, however, that the time of maximal c-*myc* expression was altered in response to a specific component of adrenergic action; in this case prior blockade with propranolol resulted in greater c-*myc* mRNA levels at 1 h compared to 3 h (Fig. 5, Lanes 5,

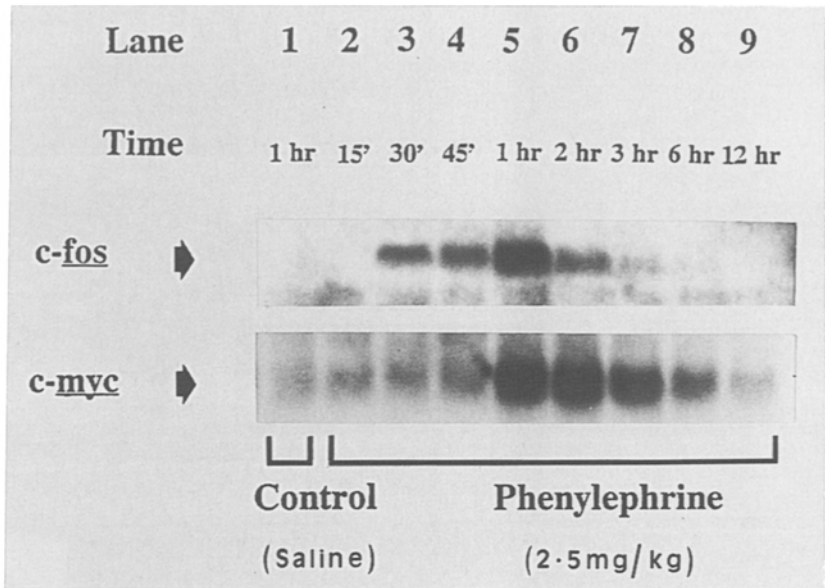

Fig. 3. Cardiac expression of c-*fos* and c-*myc* in response to a single injection of phenylephrine. Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9 % saline (Lane 1) or phenylephrine (2.5 mg/kg, Lanes 2–9). c-*fos* and c-*myc* transcripts were analysed as described previously.

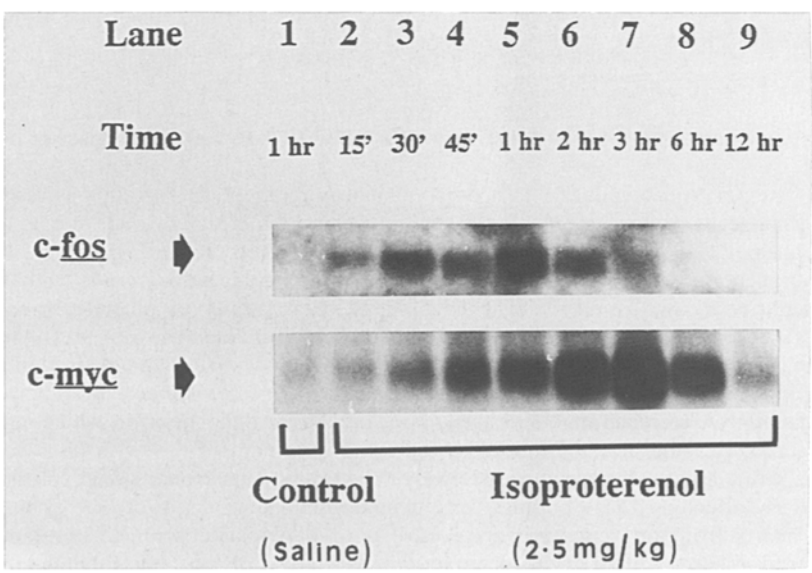

Fig. 4. Cardiac expression of c-*fos* and c-*myc* in response to a single injection of isoproterenol. Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9 % saline (Lane 1) or isoproterenol (2.5 mg/kg, Lanes 2–9), c-*fos* and c-*myc* transcripts were analysed as described previously.

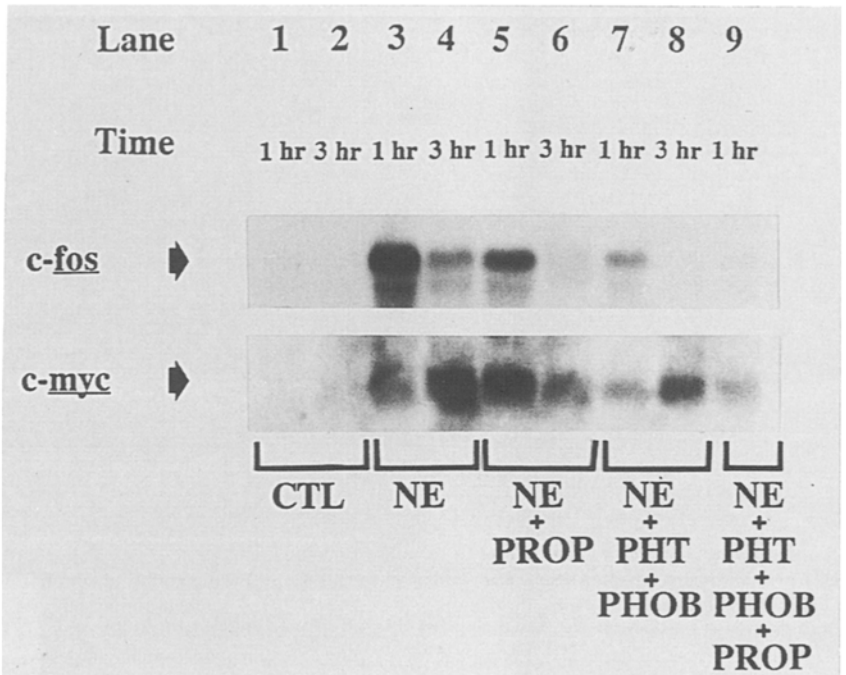

Fig. 5. Cardiac expression of c-*fos* and c-*myc* in response to α- or β-adrenergic blockade prior to NE administration.
Total RNA was extracted from rat hearts removed 1 h and 3 h following an i.p. injection of 0.9 % saline (CTL, Lanes 1 + 2) or norepinephrine (2.5 mg/kg). The β-antagonist propranolol (PROP, 50 mg/kg) or α-antagonists phentolamine (PHT, 25 mg/kg) and phenoxybenzamine (PHOB, 25 mg/kg), or a combination of both α- and β-antagonists were given twice, 1 h and 10 min prior to norepinephrine administration. c-*fos* and c-*myc* transcripts were analysed as described previously.

6). Pretreatment of rats with a combination of the α-adrenergic blockers phentolamine (25 mg/kg) and phenoxybenzamine (25 mg/kg) significantly lowered, although did not abolish, both c-*fos* and c-*myc* expression, suggesting that norepinephrine is acting predominately through the α-adrenergic receptors. Blockade with both α and β adrenergic antagonists before norepinephrine administration prevented almost all induction of c-*fos* and c-*myc* (Fig. 5, Lane 9; 3 h result not shown), indicating that the hormone acts via adrenergic receptors.

Effect of triiodo-L-thyronine on protooncogene expression

Rats were given a single dose of T_3 (0.25 mg/kg) and hearts were subsequently removed for analysis. No change in c-*fos* and c-*myc* expression compared to control experiments, was observed up to 12 h following hormone administration (Fig. 6). We (unpublished results) and others (7) have shown that continued administration at this level of T_3 promotes statistically significant cardiac hypertrophy.

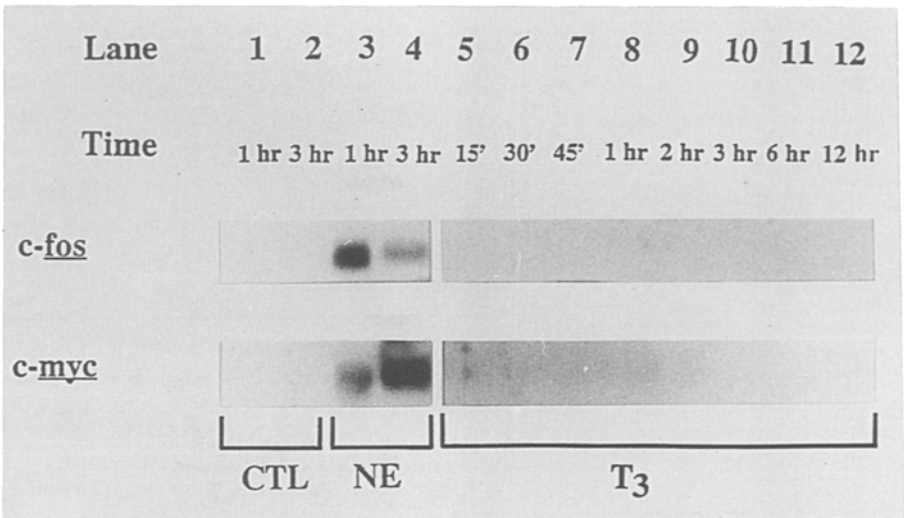

Fig. 6. Cardiac expression of c-*fos* and c-*myc* in response to a single injection of triiodo-L-thyronine. Total RNA was extracted from rat hearts removed at various times following an i.p. injection of vehicle (Lanes 1 and 2) or triiodo-L-thyronine (0.25 mg/kg, T_3, Lanes 5–12). c-*fos* and c-*myc* transcripts were analysed as described previously. As a positive control, cardiac RNA obtained from rats treated i.p. 1 h or 3 h previously with norepinephrine (2.5 mg/kg, NE, Lanes 3 + 4) has been included.

Induction of protooncogenes in the isolated perfused heart

Hearts were rapidly removed from anaesthetized rats and perfused with Krebs-Henseleit buffer for 1 h or 3 h. This process alone caused a significant elevation of c-*fos* and c-*myc* mRNA levels compared to those in hearts removed from animals and frozen immediately prior to RNA isolation (Fig. 7). Inclusion of norepinephrine (2×10^{-7} M) in the perfusate additionally elevated both c-*myc* and c-*fos* expression, reaching levels similar to that found in rat hearts following injection of norepinephrine (2.5 mg/kg) in vivo.

Discussion

Protooncogene products, like those encoded by *myc* and *fos* and which are localized to the nucleus, are thought to play key roles in linking extracellular signals with terminal patterns of gene expression. In support of this idea, it has been shown in many model systems that c-*fos* (8, 14, 24, 25) and c-*myc* (21, 24) expression is modulated during establishment of conditions which promote growth and differentiation. The growth of the adult heart is particularly interesting since cardiac myocytes grow by hypertrophy without concomitant cell division (35). We have asked whether administration of hormonal agents which promote cardiac hypertrophy will influence the expression of protooncogenes in the heart and, as a first step, have examined c-*fos* and c-*myc* mRNA levels following administration of adrenergic agents or thyroid hormone. A single injection of the non-selective adrenergic agent norepinephrine produced a significant increase in c-*fos* and c-*myc* mRNA levels in the rat heart and this response could be seen at doses ranging from 2.5 μg/kg up to 2.5 mg/kg. We chose to further characterize this response using the higher dose of adrenergic agents since it was qualitatively similar to that produced by doses in the upper phsyiological range

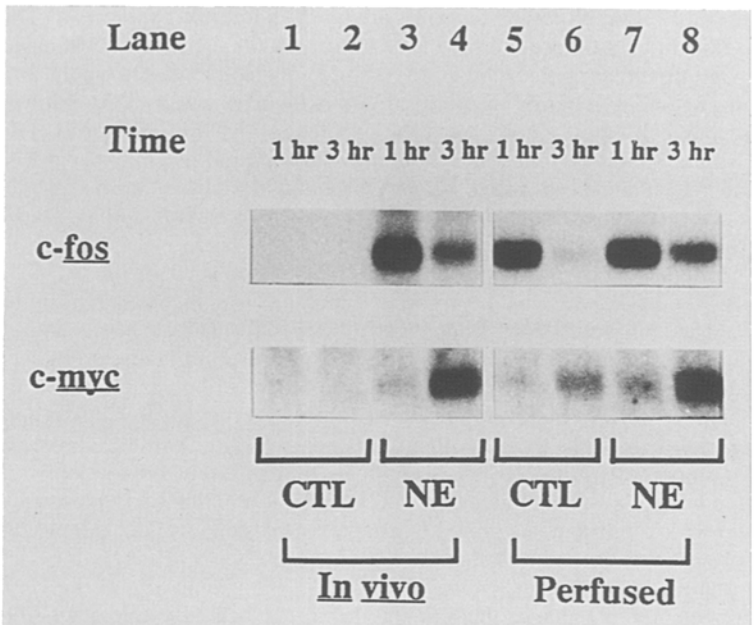

Fig. 7. Cardiac expression of c-*fos* and c-*myc* in rat hearts perfused in vitro with buffer containing norepinephrine.
Total RNA was extracted from rat hearts perfused in vitro for 1 h or 3 h with either Krebs buffer alone (perfused CTL, Lanes 5 + 6) or with buffer containing 2×10^{-7} M norepinephrine (perfused NE, Lanes 7 + 8). c-*fos* and c-*myc* transcripts were analysed as described previously. As a comparison, cardiac RNA obtained from rats treated i.p. 1 h or 3 h previously with 0.9 % saline (in vivo CTL, Lanes 1 + 2) or with norepinephrine (2.5 mg/kg, in vivo NE, Lanes 3 + 4) has been included.

(2.5 μg/kg), and also because others have shown that significant cardiac hypertrophy can result following single daily injections of adrenergic agents in the range 2.5–5 mg/kg (7). Elevation of both c-*fos* and c-*myc* mRNA following α-adrenergic stimulation was greater than that induced by β-adrenergic agents, as shown by blockade of the α and β components of norepinephrine respectively. This is consistent with reports that both hypertrophy and c-*myc* expression can be induced in cultured rat cardiomyocytes by an α_1-adrenergic mechanism (29, 31). However, we also observed increased levels of protooncogene mRNAs following β-adrenergic stimulation. Since c-*myc* levels in cultured cardiomyocytes do not respond to β-adrenergic agents (29), the rise in c-*myc* mRNA observed in the heart following in vivo treatment could represent induction of mRNA in myocytes by a secondary mechanism, or alternatively indicate that gene induction is occurring in a non-myocyte cell type. Elevation of cardiac c-*fos* levels in response to in vivo administration of isoproterenol has been reported previously (3), but it is not known whether this is a cardiomyocyte-specific response.

Interestingly, the time-course of c-*myc*, but not c-*fos* expression appeared to be dependent on which component of adrenergic action was administered: maximal levels of c-*myc* mRNA were observed between 1 h and 2 h following α-adrenergic treatment whilst β-adrenergic agents caused peak levels at 3 h. The maximal c-*myc* response to norepinephrine

occurred midway between these two points, at about 2 h following treatment. This differential response might be attributed to the distinct intracellular signalling mechanisms activated by the α- and β-adrenergic receptors (5, 15, 32), or alternatively might be a further indication that the β-adrenergic response of c-*myc* involves a secondary pathway perhaps involving several cell types. Another possibility is that c-*myc* transcription is elevated by α-adrenergic stimulation, an event which would produce a rapid rise in c-*myc* mRNAs, whilst β-adrenergic agents increase c-*myc* mRNA accumulation by reducing its degradation. Regulation of c-*myc* expression in other systems is known to include both transcriptional and posttranscriptional mechanisms (1, 18) and resolution of these possibilities will require a detailed examination of c-*myc* transcript synthesis and turnover.

This study reports elevation of protooncogene mRNAs in the heart in response to adrenergic agents, but since RNA samples were obtained from whole hearts, it is not possible to draw conclusions about the cellular localization of this response. Since it has been shown in cultured cardiomyocytes that c-*myc* is induced by α-adrenergic agents, this cell-type must contribute at least partly to the overall response. However, it is possible that other cell types, for example endothelial cells and smooth muscle cells associated with the cardiac vasculature may also be important. One possible scenario is that different populations of cells have specific responses to both haemodynamic and hormonal initiators of hypertrophy, and that they can interact to produce tissue growth in a coordinated manner. For example, Moalic et al. (23) have observed c-*fos* and c-*myc* expression in response to phenylephrine in rat aorta, suggesting that an analogous response might occur in the vasculature of the heart. For this reason, investigation of gene expression in the intact heart complements studies using monocultures of cardiac myocytes and other cell-types. We are currently localizing gene expression in response to adrenergic agents to specific cardiac cell types using hybridization histochemistry on sections of whole hearts removed from experimental animals.

c-*fos* and c-*myc* mRNA levels remained at basal levels following a single dose of triiodo-L-thyronine (T_3, 0.25 mg/kg) even though we have demonstrated that this treatment will produce significant cardiac hypertrophy within 24 h (unpublished results). Daily administration of T_3 at 2, 20 and 200 μg/kg doses for 1 week was also unsuccessful in elevating protooncogene levels (S Ragg, AK West, RD Hannan, unpublished results). These experiments suggest that the qualitative differences between thyroid hormone- and adrenergic hormone-induced hypertrophy may involve differential protooncogene expression. It will be interesting to examine c-*fos* and c-*myc* mRNA levels during development of cardiac hypertrophy induced by exercise training, a physiological state which produces hypertrophy with some similarities to that caused by high levels of thyroid hormone.

Cardiac hypertrophy is a complex process which is initiated and promoted by both haemodynamic and hormonal factors, but resolution of these can be difficult in animal models. For this reason, we have examined c-*fos* and c-*myc* mRNA levels in rat hearts perfused in vitro by the Langendorff preparation (34). In this system, the effect of norepinephrine on protooncogene expression can be examined whilst haemodynamic parameters such as coronary perfusion pressure and flow rate are kept constant. Interestingly, perfusion of the heart for 1 h or 3 h with Krebs buffer alone is sufficient to elevate both c-*fos* and c-*myc* levels, which is consistent with suggestions that the *fos* gene product, at least, is involved in the amelioration of cellular stress (2, 12). When norepinephrine (2×10^{-7} M) was included in the perfusion buffer, both c-*fos* and c-*myc* mRNA levels were significantly elevated above the buffer-alone controls. These results indicate that the response of c-*fos* and c-*myc* to adrenergic agents is a direct effect of the hormone and is independent of haemodynamic factors. These preliminary observations establish in vitro perfusion as a useful alternative model to investigate the interaction between hormonal factors and cardiac gene expression.

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Authors' address:

R. D. Hannan, Department of Biochemistry, University of Tasmania, GPO Box 252C, Hobart 7001, Tasmania, Australia