Factors contributing to pressure overload-induced immediate early gene expression in adult rat hearts in vivo

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Ogino, Kazuhide, Bolin Cai, Anguo Gu, Takushi Kohmoto, Noriyoshi Yamamoto, and Daniel Burkhoff. Factors contributing to pressure overload-induced immediate early gene expression in adult rat hearts in vivo. *Am. J. Physiol.* 277 (*Heart Circ. Physiol.* 46): H380–H387, 1999.—We determined the contributions of angiotensin II type 1 receptor (AT_1) stimulation, adrenergic stimulation, and autonomic activation to pressure overload-induced c-*fos* expression in the adult rat heart in vivo. c-*fos* expression was increased in pressure-overloaded hearts created by aortic banding compared with sham-operated rats (458 ± 100% vs. sham, $P < 0.05$). GR-138950, a selective AT_1 antagonist, did not blunt this expression (banding vs. banding + GR-138950: 458 ± 100% vs. 500 ± 125%, not significant). Atropine and hexamethonium partially decreased c-*fos* expression (banding vs. banding + atropine/hexamethonium: 700 ± 67% vs. 400 ± 67%, $P < 0.05$). Phentolamine had no significant effect on c-*fos* expression; however, propranolol inhibited the expression (banding vs. banding + propranolol: 492 ± 108% vs. 154 ± 15%, $P < 0.05$). The inhibition by propranolol was independent of the decreases in heart rate. Thus factors contributing to pressure overload-induced c-*fos* expression in adult rat hearts in vivo are different from those in neonatal myocytes in vitro undergoing stretch.

angiotensin; autonomic nervous system; signal transduction; stress

INCREASED HEMODYNAMIC LOAD imposed on the heart results in rapid induction of genes associated with protein synthesis and the development of hypertrophy (7). Whereas hypertrophy contributes beneficially to several aspects of myocardial adaptation to cardiac disease states, in advanced stages it is also associated with significant cardiac morbidity and mortality (22). Thus understanding the factors that regulate myocyte growth in response to altered mechanical load is of primary importance.

Among the molecular events triggered by acute pressure overload in intact hearts is the induction of immediate early genes (IEGs) such as c-*myc*, c-*jun*, and c-*fos* (14, 20), the gene products of which are in turn involved in regulation of the expression of other genes. To investigate the mechanisms responsible for this induction, investigators have utilized cell culture systems in which changes in mechanical load can be imposed reproducibly under well-controlled conditions (19, 31). In addition, myocytes and cardiac fibroblasts can be studied separately to identify their individual responses to load. These studies have revealed that, as in intact hearts, IEGs are induced rapidly after mechanical stretch in both myocytes and fibroblasts and that these are associated with rapid hypertrophy (myocytes) or cell division (fibroblasts). One group of investigators suggested that these events are mediated by stretch-induced release of preformed ANG II, which acts as an autocrine-paracrine substance specifically via an ANG II type 1 (AT_1)-receptor-dependent pathway (30, 32). However, the mediators of IEG induction following imposition of acute pressure overload in adult hearts in vivo have not been established. Results of several recent studies suggest that AT_1-mediated mechanisms may not be involved. First, whereas pressure overload induces IEGs in isolated perfused rat hearts, direct administration of ANG II to these hearts does not induce IEG expression (34). Second, recent studies of cultured neonatal myocytes suggest that stretch-induced IEG expression may not be entirely mediated by AT_1 stimulation (18, 41). Finally, the increase in c-*fos* and c-*myc* mRNA levels due to increased systolic load in isolated adult rat hearts was not blocked by losartan (38).

In in vivo models of myocardial pressure overload, autonomic nervous system activation may occur in response to aortic banding because aortic pressure (and carotid artery pressure) may decrease distal to the stenosis. Results of several studies suggest that α-adrenergic stimulation may lead to IEG induction via pathways involving protein kinase C (PKC), diacylglycerol (26), and mitogen-activated protein kinase (MAPK) (42). On the other hand, it is controversial whether β-adrenergic stimulation leads to increased IEG expression. Thus it is unknown whether IEG induction following proximal aortic banding in vivo is due to direct effects of the increased stress, which may be mediated by AT_1-associated pathways, or whether it is mediated indirectly by neurohormonal factors liberated by autonomic nervous system activation (6, 10, 27). Furthermore, the intracellular signaling pathways that may be primarily involved in IEG induction have not been established for the heart in vivo.

Therefore, because of the primary importance of understanding the mechanisms of mechanochemotransduction, this study was undertaken to determine the contributions of AT_1 stimulation, adrenergic stimulation (α- and/or β-adrenergic pathways) and the relative importance of autonomic activation and increased pressure per se to IEG activation in the adult rat heart.

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MATERIALS AND METHODS

Surgical procedures. A total of 87 Wistar rats (3 mo old, 350–400 g) were used for the main portions of this study. Rats were anesthetized with chloral hydrate (400 mg/kg ip). Surgical procedures for producing pressure overload were similar to those used previously (37). Briefly, the rats were intubated and respiration was controlled by a rodent ventilator (model 683, Harvard Apparatus, South Natick, MA) with room air. The left thorax was opened to expose the ascending aorta and aortic arch, which were carefully dissected free from the surrounding tissues, and a suture (3-0 silk) was drawn under the ascending aorta. An 18-gauge needle was placed alongside the ascending aorta and tied tightly together with the suture. The needle was then removed. The thorax was closed, and the lungs were inflated with positive end-expiratory pressure. For rats assigned to a sham-operated group, the identical procedure was performed (including placement of the suture around the aorta) except that the final step (tying of the suture) was not performed.

In preliminary studies, we constricted the aortic arch between the brachiocephalic artery and left carotid artery and measured proximal arterial pressure through the brachiocephalic artery using MIKRO-TIP catheter pressure transducers (SPR-524, Millar Instruments, Houston, TX).

Experimental protocols. To evaluate the effect of the involvement of AT_1 receptor in IEG induction, rats were divided into three groups. In the first group of rats (n = 9), pressure overload was created by aortic banding to increase left ventricular systolic pressure by ~60 mmHg for 90 min. This was performed 1 h after a 0.5-ml intravenous bolus injection of saline, which served as a control for the bolus injection of AT_1 antagonist performed in the second group. In the second group (n = 8), the same degree of pressure overload was induced 1 h after administration of GR-138950, a selective AT_1 blocker (12). The dose of GR-138950 (4 mg/kg) was twice that required in an additional series of five Wistar rats to abolish hemodynamic responses to a 0.4-μg bolus injection of ANG II. Finally, a third group (n = 9) consisted of sham-operated animals that underwent the same surgery but without aortic banding or administration of the AT_1 antagonist.

To elucidate the effect of the activation of autonomic nervous system due to aortic banding, three additional groups of rats were studied. In the first group (n = 6), pressure overload was created by aortic banding for 90 min as mentioned above, starting 30 min after the bolus injection of saline (0.5 ml, saline control). In the second group (n = 6), the same degree of pressure overload was induced 30 min after administration of atropine (1 mg/kg) and hexamethonium (15 mg/kg). Finally, a third group (n = 6) consisted of sham-operated animals that underwent the same surgery but without aortic banding or administration of the atropine and hexamethonium. In preliminary studies, it was demonstrated that pharmacological blockade could be achieved by atropine (1 mg/kg) and hexamethonium (15 mg/kg) as confirmed by the demonstration that acetylcholine (2 μg/kg)-induced changes in blood pressure and heart rate were abolished for at least 120 min.

To elucidate the involvement of α-adrenergic receptor- and β-adrenergic receptor-related pathways, an additional four groups of rats were studied. In the first group (n = 5), pressure overload was created by aortic banding for 90 min as detailed above 10 min after the bolus injection of saline (0.5 ml, saline control). In the second and third groups (n = 5 each), the same degree of pressure overload was induced 10 min after the administration of an α-adrenergic receptor blocker (phentolamine, 2 mg/kg) or a β-adrenergic receptor blocker (propranolol, 1 mg/kg), respectively. Finally, a fourth group (n = 5) consisted of sham-operated animals that underwent the same surgery but without aortic banding or administration of phentolamine or propranolol. In preliminary studies, it was demonstrated that α-adrenergic blockade was achieved with phentolamine (2 mg/kg) by demonstrating a lack of a hemodynamic response to intravenous phenylephrine (5 μg/kg). Similarly, it was demonstrated that β-blockade was achieved with propranolol (1 mg/kg) by demonstrating a lack of a hemodynamic response to intravenous isoproterenol (2 μg/kg). Furthermore, it was shown in preliminary studies in an additional three rats that neither phentolamine (2 mg/kg) nor propranolol (1 mg/kg) when administered alone affected blood pressure during the 90-min period after their administration. Whereas heart rate was not affected by phentolamine, it was decreased by propranolol. To eliminate potential effects of heart rate on IEG expression, another three groups of rats were studied in which pacing (330 beats/min via electrodes placed on the right atrium) was employed in combination with β-blockade. The first group of these rats (n = 5) was treated with propranolol (1 mg/kg) and paced 10 min before and during aortic banding. In the second group (n = 5), the same surgery was performed including pacing lead placement and propranolol (1 mg/kg) administration; however, these rats were not paced. Finally, a third group (n = 5) consisted of sham-operated animals that underwent the same surgery but without aortic banding, pacing, or administration of the propranolol.

In situ hybridization. In situ hybridization was used to confirm that IEG expression was being induced in cardiac myocytes. Hearts were fixed in Formalin and embedded in paraffin. The sections were dewaxed, rehydrated, and then digested with proteinase K. After acetylation of acetic anhydride, the sections were prehybridized for 2 h at 37°C in a solution containing 50% formamide, 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 1× Denhardt's, 10% dextran sulfate, 0.1% SDS, 4 mM EDTA, 250 μg/ml yeast tRNA, and salmon testis DNA. Hybridization was then performed overnight at 42°C with the addition of c-*fos* RNA probe labeled by in vitro transcription of linearized DNA with digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN). After stringent wash, the signals were detected by a standard immunoalkaline phosphatase reaction using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as a substrate (Boehringer Mannheim) and counterstained with ethyl green.

RNA preparation and analysis. Total RNA was extracted from the left ventricular myocardium of each heart studied. This was performed using a single-step RNA extraction protocol employing acid guanidium thiocyanate-phenol-chloroform (4) with minor modifications. RNA (40 μg, quantified by absorbance at 260 nm) was electrophoresed on a 1% agarose-15% formaldehyde gel in a 1× MOPS (Fisher Scientific, NJ) buffer at 35 V overnight. The gels were transferred to nitrocellulose membranes (NitroPure, MSI, Westboro, MA) in 10× SSPE (1.8 M NaCl, 0.1 M sodium phosphate, 0.01 mM EDTA, pH 7.7). These membranes were baked in a vacuum oven at 80°C for 1 h until dried and prehybridized at 42°C for 6 h in a prehybridization mix (50% formamide, 4× SSC, 1× Denhardt's, 0.1% SDS, 1 mM EDTA, and 0.125 mg/ml of denatured salmon testes DNA). These blots were hybridized overnight with a mouse c-*fos* cDNA and *egr*-1 (generous gifts from Dr. Mark H. Soonpaa, Indiana University and Dr. Vikas P. Sukhatme, Harvard Medical School, respectively) labeled with [^{32}P]dCTP (800 Ci/mmol, Amersham, Arlington Heights, IL) to a specific activity of 1×10^6 cpm/g of cDNA using a

multiprimer DNA-labeling system (Amersham). The blots were washed twice in 2× SSC at room temperature for 15 min, three times in 0.1× SSC/0.1% SDS at 68°C for 15 min, and exposed to X-OMAT AR film at −70°C with an intensifying screen for 48–72 h. Human β-actin cDNA probe of 1.1 kb *Pst* I fragment was used in a hybridization procedure. To ensure equal loading, hybridization was also performed with either human β-actin cDNA probe of 1.1 kb *Pst* I fragment or GAPDH cDNA probe of 1.3 kb. Relative amounts of c-*fos* mRNA were quantified by laser densitometry (Molecular Dynamics) in the linear response range of the X-ray films. The values obtained for c-*fos* band intensities in each experiment were normalized to the corresponding band intensity of the β-actin or GAPDH signal to correct for the differences in loading and/or transfer.

PKC and PKA assay. To assess PKC activity, rat hearts were minced and homogenized with buffer (20 mM Tris·HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA-Na, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). The homogenate was centrifuged at 100,000 *g* for another 45 min using an airfuge (Beckman airfuge, 30 A-100 rotor). The pellet was resuspended in buffer (20 mM Tris·HCl, pH 7.5, 0.3% Triton X-100, 2 mM EGTA, 2 mM EDTA-Na_2, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin), incubated on ice for 30 min, and then centrifuged at 100,000 *g* for 45 min. The supernatant was ready for assay. The PKC assay used was the Pierce Colorimetric PKC Assay Kit (catalog number 29540, SpinZyme Format, Pierce, IL). The assay was performed according to instructions provided with the kit. Before we performed the assay, the protein concentration of each sample was measured by the Bradford method using Bio-Rad protein assay reagents (catalog number 500–0006, Bio-Rad). The PKC activity was expressed as units per milligram of protein, with one unit defined as the enzyme activity that catalyzes the transfer of 1 nmol of phosphate to histone H1 in 1 min at 30°C.

To assess protein kinase A (PKA) activity, the hearts were minced and homogenized with ice-cold buffer (10 mM potassium phosphate, 1 mM EDTA-Na_2, 0.1% Triton X-100, 0.1 dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 10 mM sodium fluoride). The homogenate was centrifuged at 20,000 *g* for 40 min at 4°C. PKA activity of the supernatant was determined using a Pierce Colorimetric PKA Assay Kit (catalog number 29500, SpinZyme format, Pierce). The assay was performed according to instructions provided with the kit. The protein concentration of each sample was measured by the Bradford method using Bio-Rad protein assay reagents (catalog number 500–0006, Bio-Rad). PKA activity was expressed as units per milligram of protein, with one unit of activity defined as the amount of enzyme required to catalyze the transfer of 1 pmol of phosphate to casein in 1 min (in an assay buffer of 40 mM Tris·HCl, pH 7.40, 20 mM magnesium acetate, 0.2 ATP, and 30,000 cpm/l [^{32}P]ATP).

Statistical analysis. All data are presented as means ± SE. The statistical significance of differences in hemodynamic data among groups was assessed by ANOVA with individual differences assessed using a Fisher's multiple range test. In all cases, $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of aortic banding on hemodynamic parameters. Systolic and mean aortic pressures in the proximal aorta averaged 105.8 ± 14.5 and 87.9 ± 13.8 mmHg, respectively, in the sham-operated animals. In addition, systolic and mean aortic pressures averaged 152.8 ± 18.3 and 111.9 ± 15.9 mmHg, respectively, in all of the animals that underwent 90 min of aortic banding without any form of pharmacological blockade pretreatment (all of the control groups, $n = 25$), and averaged 145.0 ± 17.4 and 100.6 ± 18.5 mmHg, respectively, in the groups of banded animals that were pretreated with the various pharmacological blockers ($n = 34$). In addition we evaluated whether each blocker used in this study affected hemodynamics (Table 1). None of the blockers had an effect on aortic pressure. Although propranolol decreased heart rate, it was not statistically significant (ANOVA; $P = 0.07$). As mentioned above, there is no statistically significant difference between blood pressure responses to aortic banding in the control and actively pretreated groups of animals regardless of the blocker used. Thus systolic and mean pressures were significantly higher in banded animals, and the increase was comparable in all groups studied.

c-fos expression in situ hybridization. Myocardial samples of hearts subjected to pressure overload were hybridized with digoxigenin-UTP-labeled antisense c-*fos* probe. c-*fos* expression was extensively and clearly derived from myocytes (Fig. 1*A*, arrows). It was also observed in cells within the perivascular space. The same tissue was hybridized with the sense probe as the negative control for nonspecific staining (Fig. 1*B*). Also, the myocardium of sham-operated hearts failed to exhibit nuclear staining (Fig. 1, *C* and *D*).

c-*fos* expression in the pressure-overloaded hearts is not inhibited by AT_1 blocker (Fig. 2). As shown in the Northern blots of Fig. 2, c-*fos* expression increased after 90 min of pressure overload compared with the low level of expression noted in sham-operated animals. Quantitative analysis revealed that average c-*fos* band intensities normalized by β-actin band intensities were over fourfold greater in banded than in sham-operated animals (4.58 ± 1.00-fold vs. sham, $P < 0.05$). c-*fos* expression also increased in GR-138950 (AT_1 blocker)-pretreated animals, and this increase was of a similar magnitude as that in the nontreated, aortic-banded animals. There was no statistically significant differ-

Table 1. *Hemodynamic effects of pharmacological interventions*

	Sham ($n = 4$)	GR-138950 ($n = 5$)	HM + Atropine ($n = 5$)	Phentolamine ($n = 4$)	Propranolol ($n = 4$)
Heart rate, beats/min	251.5 ± 21.1	241.2 ± 26.5	232.6 ± 7.1	235.5 ± 10.8	178.5 ± 22.0
Systolic aortic pressure, mmHg	105.8 ± 4.5	93.8 ± 6.7	95.7 ± 5.0	94.0 ± 3.9	96.0 ± 4.1
Mean aortic pressure, mmHg	82.3 ± 6.8	72.0 ± 7.3	68.3 ± 2.9	73.5 ± 6.0	71.0 ± 6.9

Values are means ± SE; *n*, number of rats. HM, hexamethonium.

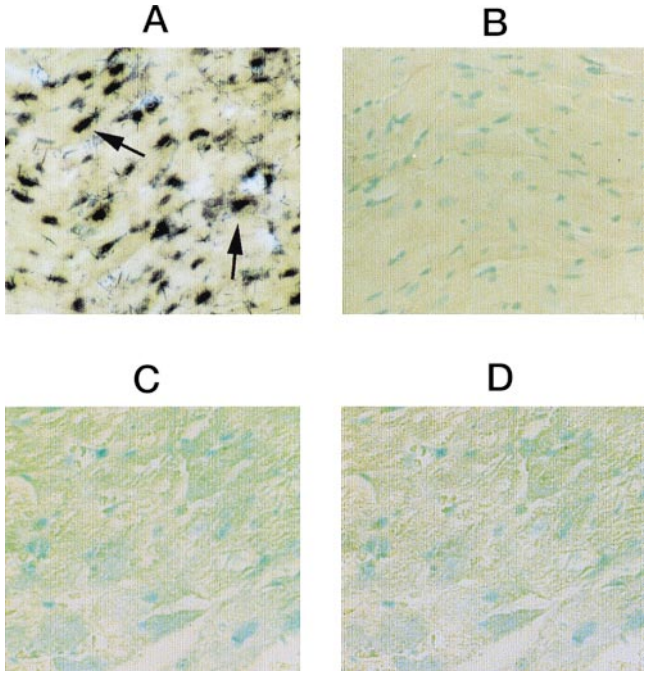

Fig. 1. c-*fos* expression in situ hybridization. c-*fos* expression was extensively and clearly derived from myocytes (*A*, arrows). Same tissue was hybridized with the sense probe as negative control for nonspecific staining (*B*). Hearts samples of sham-operated animals were hybridized with the antisense and sense probe (*C* and *D*, respectively).

ence between c-*fos* expression in these two groups (4.58 ± 1.00-fold and 5.00 ± 1.25-fold vs. sham, respectively). To corroborate that these findings were not limited to a single AT_1 blocker, we tested the effect of losartan (10 mg/kg iv) on c-*fos* expression induced by aortic banding. There was no difference in c-*fos* expression in banded animals treated with losartan and control animals, which were banded but did not receive losartan (n = 3, each group, data not shown). Thus pressure overload-induced c-*fos* expression in adult rat hearts in vivo is not dependent on ANG II stimulation of AT_1 receptors.

Pressure-overload c-*fos* expression is partially inhibited by autonomic blockade (Fig. 3). c-*fos* expression was increased in the pressure-overloaded hearts following autonomic blockade with hexamethonium plus atropine compared with sham-operated animals: 4.00 ± 0.67-fold vs. sham group ($P < 0.05$). However, c-*fos* expression in the control banded group was 7.00 ± 0.67-fold greater, which was significantly greater than the banding plus blockade group ($P < 0.05$). Thus c-*fos* expression was partially inhibited by blockade of autonomic nervous system.

Pressure overload-induced c-*fos* expression is not inhibited by the α-adrenergic receptor blocker but was inhibited by the β-adrenergic receptor blocker (Fig. 4). To test which arm of the autonomic pathway was involved in aortic banding-induced c-*fos* expression, studies were performed individually with α- and β-blockers. Phentolamine had no significant effect on c-*fos* expression (banding and banding + phentolamine vs. sham: 4.92 ± 1.08-fold in control banded hearts and 4.15 ± 0.92-fold in hearts treated with phentolamine; P = not significant). However, propranolol almost completely inhibited c-*fos* expression due to aortic banding (1.54 ± 0.15-fold vs. sham-operated animals; P = not significant). Thus aortic banding-induced c-*fos* expression in adult rat hearts in vivo appears to be independent of α-adrenergic receptor stimulation but does appear to depend on β-adrenergic pathway-related mechanisms.

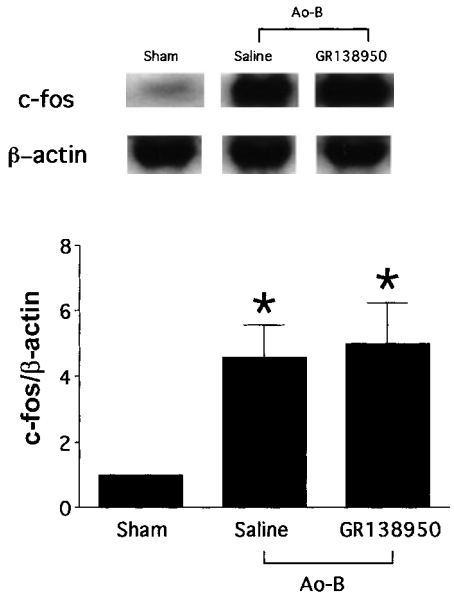

Fig. 2. c-*fos* expression in pressure-overloaded hearts is not inhibited by AT_1 blocker. *Top*: representative autoradiograph. *Bottom*: intensity of each band on autoradiogram quantified by densitometric scanning, and c-*fos* expressions normalized by β-actin. Ao-B, aortic banding. *$P < 0.05$ vs. sham.

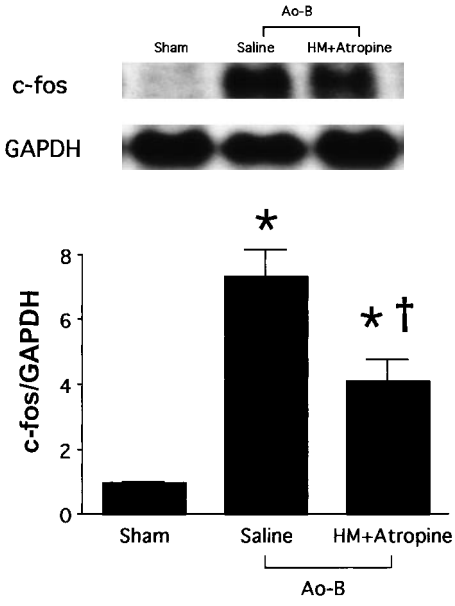

Fig. 3. Pressure-overload c-*fos* expression is partially inhibited by autonomic blockade. *Top*: representative autoradiograph. *Bottom*: intensity of each band on autoradiogram quantified by densitometric scanning, and c-*fos* expressions normalized by GAPDH. Ao-B, aortic banding; HM, hexamethonium. *$P < 0.05$ vs. sham. †$P < 0.05$ vs. Ao-B alone (saline).

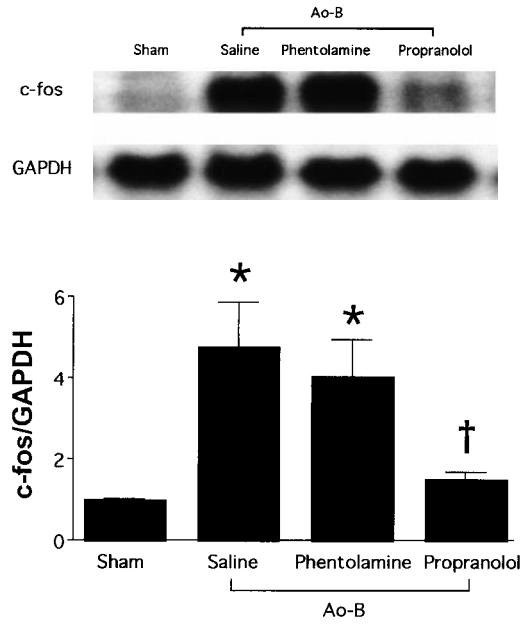

Fig. 4. Pressure overload-induced c-*fos* expression is not inhibited by α-adrenergic receptor blocker but was inhibited by β-adrenergic receptor blocker. *Top*: representative autoradiograph. *Bottom*: intensity of each band on autoradiogram quantified by densitometric scanning, and c-*fos* expressions normalized by GAPDH. $*P < 0.05$ vs. sham. $\dagger P < 0.05$ vs. Ao-B alone.

The inhibition of c-*fos* expression by β-blocker is not dependent on the decrease in heart rate (Fig. 5). To test whether the elimination of c-*fos* induction following β-blockade was not due to the reduction in heart rate observed with propranolol, rat hearts were paced at 330 beats/min. Propranolol inhibited c-*fos* expression in the pressure-overloaded hearts even when heart rate was similar to that of sham and banding groups ($P <$ 0.05). Thus β-blocker-mediated inhibition of c-*fos* expression following aortic banding is independent of the effects on heart rate.

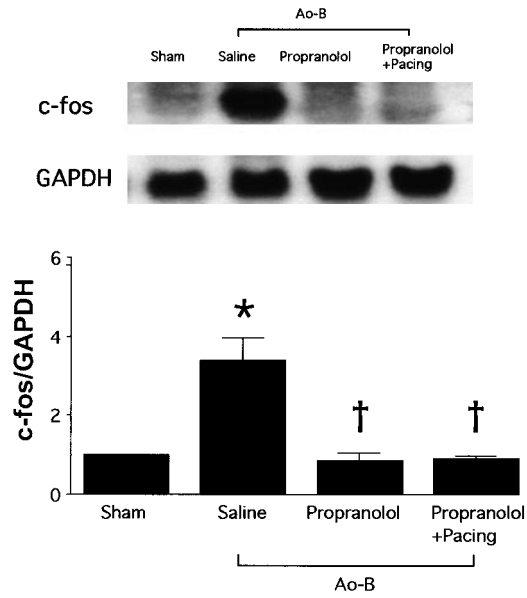

Fig. 5. Inhibition of c-*fos* expression by β-blocker is not dependent on decrease in heart rate. *Top*: representative autoradiograph. *Bottom*: intensity of each band on autoradiogram quantified by densitometric scanning, and c-*fos* expressions normalized by GAPDH. $*P < 0.05$ vs. sham. $\dagger P < 0.05$ vs. Ao-B alone.

Finally, to confirm that the effect of propranolol on c-*fos* expression is specific for the β-receptor, we evaluated whether or not phenylephrine-induced c-*fos* expression is inhibited by propranolol. Systolic and mean arterial pressure increased significantly immediately after phenylephrine administration (from 89.7 ± 19.7 and 71.0 ± 10.6 mmHg, respectively, to 165.9 ± 19.1 and 145.6 ± 15.6 mmHg, respectively); however, they returned to baseline values 30 min after injections. Phenylephrine (2.5 mg/kg iv) induced significant c-*fos* expression (3.65 ± 0.11-fold vs. sham), and the pretreatment of propranolol (1 mg/kg iv) did not inhibit the c-*fos* expression (3.57 ± 0.22-fold vs. sham). Additionally, propranolol did not affect the c-*fos* expression in sham-operated animals (data not shown). Thus the inhibition of c-*fos* expression by propranolol is specific for the β-receptor and does not interfere with the effects of α-adrenergic stimulation. In addition, we confirmed that PKA and PKC activation was caused by aortic banding and that it was inhibited by propranolol. Aortic banding resulted in an increase in PKA and PKC activity (126.5 ± 10.4% and 119.4 ± 20.2% compared with sham, respectively), which was associated with a fourfold increase in c-*fos* expression compared with sham-operated animals. Propranolol inhibited the rise in PKA and PKC activity (116.1 ± 6.6% and 96.9 ± 20.2%, respectively, vs. sham, $P < 0.05$) and, as noted above, c-*fos* expression was decreased significantly compared with control aortic-banded animals. Thus acute pressure overload activated PKA and PKC, and the inhibition of β-adrenergic receptor inhibited this activation.

DISCUSSION

Many workload-responsive IEGs have been identified in cardiac cells both in vivo and in vitro, and their expression has been hypothesized to play an important role in the hypertrophic cardiac adaptation to increased mechanical stress (21). The recent identification of increased levels of Fos and Myc proteins within myocyte nuclei in the setting of pressure overload has provided further support for the proposed link among mechanical stress, altered gene expression, and rapid production of transcription factors that may be involved in transcription of other gene products (28, 33, 36); our finding from in situ hybridization showing increased c-*fos* message within myocytes during pressure overload is complementary to these previous observations. However, the mechanisms involved in this important form of gene regulation have not been elucidated, particularly for the adult heart in vivo. In the present study, several important findings that lead to new insights have been obtained.

First, we showed that central autonomic blockade partially blunted IEG expression. This implies that baroreflex activation known to occur following proximal aortic banding (due to a reduction in pressure distal to the site of banding) with subsequent adrenergic stimulation accounts for some, but not all, of the

phenomena. This implies that autonomic activation is partially responsible for c-*fos* expression but that pressure overload per se also contributes.

Second, ANG II does not appear to be involved with banding-induced IEG expression in vivo. Although pressure overload-induced IEG expression was first identified in rat hearts in vivo (14, 20), investigators turned to studies of cardiac myocytes in culture, because in many respects these preparations more readily lend themselves to probing the underlying molecular mechanisms (19, 30). Initial studies revealed that stretch-induced release of preformed ANG II acting via AT_1 receptors plays a central role as an autocrine-paracrine factor underlying the stretch-induced increase in IEG expression in neonatal myocytes and fibroblasts (29, 30, 32). However, results of more recent studies suggest that even in neonatal cardiac myocytes, AT_1 blockade did not inhibit stretch-induced c-*fos* expression or myocyte hypertrophy completely (18, 41); in adult cells, which appear to lack ANG II receptors (25), the phenomenon is even less ANG II dependent. On the other hand, whereas in the intact hearts, angiotensin II failed to stimulate c-*fos* and c-*jun* mRNA expression, ANG II induced c-*fos* expression in primary cultured myocytes, and it was greater in neonatal rat ventricular myocytes than in adult cardiac myocytes (34). ANG II promotes adult rat myocyte growth through the activation of AT_1 receptors (23). Thus whether or not adult cardiac myocytes possess AT_1 receptor seems undetermined.

Thienelt et al. (38) reported that AT_1-receptor blockade with losartan did not block the effects of elevated systolic wall stress on protooncogene induction or new cardiac protein synthesis in isolated crystalloid-perfused hearts. Recently, Harada et al. (11) demonstrated that AT_1-mediated ANG II signaling is not essential for the development of pressure overload-induced cardiac hypertrophy using AT_{1a}-receptor knockout mice. Thus the physiological relevance of ANG II as an autocrine-paracrine substance involved in the myocardial response to hemodynamic stress in the adult heart in vivo has been questioned. The results of the present study, obtained in an established in vivo model of IEG induction by pressure overload (14, 20) showing that AT_1 blockade has no detectable effect on c-*fos* expression, definitively indicate that this pathway is not involved in this aspect of mechano-chemotransduction for the rat heart in vivo.

In neonatal rat myocardial cells in vitro, α- and β-adrenergic stimulation each induce the expression of IEGs such as c-*fos* and c-*jun* (1, 13). One group of investigators has recently reported that α-adrenergic (and not AT_1)-mediated pathways are relevant to the activation of IEGs in the intact adult heart in vitro (8). On the other hand, at least one group has advocated that cAMP may serve as a mediator of mechanotransduction (40). This latter line of thinking would implicate members of the β-adrenergic pathway, including adenylate cyclase, cAMP, and PKA as being involved (15). Yet, previous studies have failed to specifically confirm that stress-mediated alterations in IEG expression are mediated via alterations in cAMP (17, 24). Thus, even for in vitro experimental models of increased stress, the relative importance of α- and β-adrenergic pathways has not been fully established. In contrast to previous studies in vitro, α-adrenergic blockade did not play any detectable role in vivo in the present study. Also in contrast to previous studies in other experimental models, β-adrenergic receptor blockade by propranolol almost completely abolished IEG induction following aortic banding; these effects of β-blockade were not due to changes in blood pressure or heart rate, indicating that the suppression of IEG expression is due to the direct actions of propranolol rather than to secondary effects of this β-blocker (9).

There are several potential limitations of the present study. No specific data are available to address the mechanisms by which propranolol so completely blocks c-*fos* induction in response to pressure overload. One possibility is that propranolol, at the doses used, is not acting as a specific β-blocker but could have membrane effects that influence functioning of several elements of membrane signaling cascades. However, as noted in RESULTS, continuous injection of propranolol did not inhibit phenylephrine-induced c-*fos* expression in vivo, which indicates that propranolol does not have a cross talk with α-adrenergic receptor. On the other hand, propranolol suppressed the activation of both PKA and PKC due to aortic banding. The reason for this is uncertain. We must acknowledge the possibility that the assay for PKC was not completely specific and that other factors are influencing the assay, potentially leading to erroneous conclusions regarding the mechanisms underlying the clear-cut phenomena that we have observed. Recently, it has been reported that isoproterenol and cAMP significantly activated raf-1 kinase and MAPK (39, 43). In addition, activated PKA phosphorylates the β-adrenergic receptor leading to the activation of MAPK (5). These results further support the concept that β-adrenergic blockade could inhibit c-*fos* expression by inhibiting MAPK through both PKA and PKC mechanisms.

We have, at several points, commented on some important similarities and some important differences between the results that we have obtained in rat hearts in vivo and results that have been obtained previously in isolated (generally neonatal cell or isolated crystalloid perfused heart) in vitro preparations. The physical forces experienced by myocytes due to ventricular pressure overload in vivo may not be comparable to those experienced by stretched myocytes in vitro. The experimental conditions (temperature, degree of cellular oxygenation) may be significantly different. Whereas hearts maintain rhythmic contractions, isolated myocytes are generally quiescent and remain so during the interval from isolation to experimentation (which may be several days). Cardiac myocytes on culture dishes interact with extracellular matrix through integrins and normal matrix-to-cell connections, which may directly relay external physical forces to the nuclear membrane (16). It has been demonstrated that in vitro culture conditions may greatly affect adrenergic responsiveness (2, 3). Finally, there is evidence that the

molecular responsiveness to both mechanical stimulation and ANG II are significantly blunted in adult compared with neonatal and less mature rat myocardium (35, 37). Similarly, there are several differences between isolated perfused whole hearts and hearts in vivo. Myocardial perfusion with an oxygenated crystalloid solution instead of blood may alter extracellular and outer membrane properties compared with normal perfusion with whole blood. Differences in temperature and heart rate between these two types of preparations may exert other influences. On the other hand, as with all studies performed in intact animals, we are faced with several complexities and unknown factors that can confound interpretations (e.g., the presence of baroreflexes) and that introduce limitations on the conclusions that may be drawn from the observations.

In conclusion, complete sympathetic blockade of the autonomic reflexes partially inhibited c-*fos* expression in response to pressure overload. β-Blockade with propranolol abolished the phenomenon. Neither α-receptor nor AT_1-receptor blockade affected the phenomenon, eliminating activation of these receptors as being involved in vivo, findings that are different from those found in vitro. Thus, whereas the fundamental questions of mechanism of action have not been identified, several possibilities have been raised. For example, stress-mediated activation of enzymes (e.g., phospholipase C, adenylate cyclase, and other G protein-mediated enzymes) may be able to be activated by either mechanical forces transmitted by the membrane or secondarily via either specific "mechano-receptors" or other commonly identified proteins associated with the membrane. As with any complex biological process, parallel pathways may be in action to activate genes during periods of stress.

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