Thrombin receptor (ThR) plays a significant role in myocyte contractility and hypertrophy. Heart myocyte ischemic damage, caused by insufficient blood supply, is the leading cause of heart infarction. Here we demonstrate that when primary myocyte cultures are subjected to hypoxic stress, ThR mRNA levels are reduced markedly. This takes place also in vivo in a model of ischemic pig heart, exhibiting reduced levels of ThR compared with normal heart sections. Prior activation of ThR however, by either thrombin receptor-activating peptide (TRAP) or by α-thrombin resulted in full protection of ThR mRNA levels under hypoxia. The effect appeared specific to ThR because the addition of TRAP did not affect the hypoxic damage as shown by the levels of lactic dehydrogenase release and up-regulated GLUT-1, a glucose transporter gene. This protection effect took place not only in primary myocytes but also in NIH3T3 fibroblasts. ThR protection occurs via specific cell signaling events because activation of the receptor by TRAP, following interruption of the signaling cascade by calphostin C, a protein kinase C inhibitor, resulted in loss of ThR mRNA protection. Because Ras and Src are part of the ThR signaling cascade, the introduction of either dominant ras or src oncogenes to NIH3T3 murine fibroblasts gave rise to similar protection of ThR mRNA levels under hypoxic conditions without the exogenous addition of TRAP. Likewise, ThR mRNA protection was obtained after transfection with proto-oncogene ras.

The 95-kDa protein Vav undergoes tyrosine phosphorylation after ThR activation, serving thus as part of the receptor machinery cascade. We therefore conclude that the initiation of the signaling cascades either exogenously by TRAP or within the cell via src or ras, as well as via vav oncogene interconnecting G-binding protein to the tyrosine kinase pathway, ultimately results in ThR protection under hypoxia. We present hereby, a novel concept of activated receptors, which under minimal oxygen tension protect their otherwise decaying mRNA. Maintaining the level of ThR that plays an active role in normal myocyte function may provide a significant repair mechanism in ischemic tissue, assisting in the regaining of normal myocyte functions.

Insufficient blood supply after primary coronary obstruction

Protection of Thrombin Receptor Expression under Hypoxia*

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1 The abbreviations used are: Thr, thrombin receptor; PAR, protease-activated receptor; SH, Src homology domain; PH, pleckstrin homology; LDH, lactate dehydrogenase; TRAP, thrombin receptor-activating peptide; PKC, protein kinase C; UTR, untranslated region.

2 The abbreviations used are: Thr, thrombin receptor; PAR, protease-activated receptor; SH, Src homology domain; PH, pleckstrin homology; LDH, lactate dehydrogenase; TRAP, thrombin receptor-activating peptide; PKC, protein kinase C; UTR, untranslated region.
activation of ThR activates Src and Fyn tyrosine kinases as agonist synthetic peptides of the receptor cause the tyrosine phosphorylation effect (26). This suggests that nonreceptor tyrosine kinases of the Src family may represent a novel effector system linking G-protein-coupled receptors to activated Ras and the mitogen-activated protein kinase cascade downstream (26). It is unlikely that Src-related kinases interact directly with Ras. Rather, one or more kinases or adapter proteins are presumed to be located between the Src kinases and Ras. Ras activation is an important early event in growth promoting signal transduction by G-protein-coupled receptors (27, 28). ThR activation is accompanied by the accumulation of Ras in its GTP-bound form. Furthermore, expression of a dominant negative form of Ras has been shown to inhibit thrombin-stimulated gene induction and DNA synthesis reinitiation in astrocytoma cells (29) as well as proliferation of vascular smooth muscle cells (30).

ThR activation leads to phosphorylation of an immediate upstream cellular target, the Vav protein (31). Vav protein plays a key role as a cytoplasmic signal transducer. This proto-oncogene encodes for a product that contains different modular domains known to function in tyrosine signal transduction events such as: PH, SH2, and SH3. Some of these domains were shown to be involved in protein-protein interactions. An additional domain that is characteristic of the guanine nucleotide exchange factors is also present in Vav (32, 34). Therefore, Vav could potentially serve to link tyrosine signaling to the G-binding family by specific interactions with different proteins and through its activity as a GDP/GTP exchange factor (33).

In this paper we propose a novel myocardial ThR regulation under hypoxia. We show that after hypoxia, the otherwise decaying ThR mRNA is maintained at its normal level upon initiation of the signaling cascade regardless of whether activated exogenously or in an “inside-on” manner. Maintaining the level of ThR, a cell surface receptor that takes an active part in normal myocyte function, may provide a significant repair mechanism for ischemic tissue, assisting in regaining normal myocyte function. Whether protection of ThR mRNA results from an induced stability or from an induced rate of transcription remains to be determined.

EXPERIMENTAL PROCEDURES

Cells—Neonatal rat ventricular heart muscle cells were prepared and plated as described previously (35). Briefly, ventricles of 1-day-old rats were minced and treated with trypsin, and the combined fractions were resuspended in growth medium into a sterile flask (Nunc; Nuclon Delta Herlev, Denmark) through a sterile mesh to exclude explants. The pooled cells were suspended in growth medium to a density of 9 × 10⁵ to 1 × 10⁶ cells/ml and seeded into a 35-mm-diameter Petri dish (Falcon 3001; Falcon Labware, Oxnard, CA). This concentration yielded, after 24–36 h, a near confluent layer of beating heart cells at a final density of about 2 × 10⁵ cells. Cultures were kept at 37 °C in an atmosphere of 5% CO₂ and 95% air. Experiments were performed at 5 days of culture when more than 80% of the cells exhibited myocardial cells.

The NIH3T3 murine fibroblasts included NIH3T3 cells that express the vav proto-oncogene (K62, 35); NIH3T3 cells that express the vavSH3 mutant protein P832L2;² NIH3T3 cells that express the vavSH2 mutant R695L (34); NIH3T3 cells that express the ras oncogene (H-rasLys12, 32), and NIH3T3 cells transfected with v-src. Single transformed foci were used in these experiments. These cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum. Tissue culture media were supplemented with penicillin (50 units/ml) and streptomycin (50 μg/ml) (Life Technologies, Inc.), and the cells were dissociated with a 0.05% trypsin, 0.02% EDTA, 0.01M sodium phosphate, pH 7.4, solution and subcultured at a split ratio of 1:5.

RNA Isolation and Northern Blot Analysis—RNA was prepared using TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer’s instructions. The RNA (20 μg total RNA) was separated by electrophoresis through a 1.1% agarose gel containing 2 μl formaldehyde, transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech), and hybridized either to cDNA probes or to polynucleotide chains radiolabeled by random primer extension with [α-32P]dCTP (37) for 24 h at 42 °C. The membrane was washed twice for 30 min at room temperature with 2× SSC containing 2% SDS and 15 min at 50 °C with 0.1× SSC containing 0.1% SDS. The blots were exposed for 2–4 days at −70 °C, and the relative amounts of mRNA transcripts were analyzed by laser densitometry, using an Ultrascan XL enhanced laser densitometer and normalized relative to internal β-actin controls.

Hypoxia—Hypoxic conditions may be induced in cultures incubated with oxygen- and glucose-deprived medium preequilibrated with 95% N₂ and 5% CO₂. A special incubation device is used in which the gas mixture of 95% N₂ and 5% CO₂ is saturated with water (38). Prior to subjecting the cells to hypoxia, the medium was replaced by fresh Ham’s F-10 serum-free medium preequilibrated for 30 min with 95% N₂ and 5% CO₂ and incubated for 2–4 h in a special incubation device. Alternatively, after replacement of medium deficient in glucose and pyruvate, tissue culture plates were subjected to hypoxia in anaerobic jars (BBLGASPAC, Anerobic Systems, San Francisco) at 37 °C in a humidified atmosphere with 5% CO₂ and less than 0.2% oxygen for 12 h, as demonstrated by relevant catalyzer.

Lactate Dehydrogenase (LDH) Activity—Cell damage under hypoxic conditions was measured by LDH assay of the medium using a diagnostic kit according the manufacturer’s instructions (Sigma). Total LDH activity was determined in culture medium plus LDH released from the cells after treatment with 0.5% Triton X-100. All results are expressed as mean ± S.E. of triplicate samples.

Western Blotting—Cells were dissolved in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors (5 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) for 30 min at 4 °C. After centrifugation at 10,000 × g for 20 min at 4 °C, the supernatants were transferred, and the protein content was measured. Lysates (50 μg) were loaded and resolved in 10% SDS-polyacrylamide gel electrophoresis followed by transfer to Immobilon-P membrane (Millipore, MA). Membranes were blocked and probed with anti-ThR antibodies (1:4000) in 1% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.05% Tween 20. After washes, blots were incubated with the appropriate secondary antibodies and conjugated to horseradish peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) reagent using luminol and p-iodanisocyanurate (Sigma).

Antibodies—Anti-Vav antibodies were raised in rabbits against a specific peptide of Vav, residues 528–541 (38). Anti-phosphotyrosine antibodies (PY-20, Signal Transduction, Lexington KY).

Ischemia Induction in Pig Myocardium—Ischemia was induced as described previously (39). Briefly, pigs (15–30 kg, of either sex) were anesthetized, and thoracotomy was performed. The pericardium was opened, and the left anterior descending coronary artery was carefully dissected. Using an arterial bulldog clamp the artery was occluded for 2 min followed by 20 min of reperfusion. The artery was then reocluded for 3, 5, and 7 min, each occlusion being followed by 20 min of reperfusion. Thereafter, repeated occlusions of 10 min each were performed, each followed by a 20-min reperfusion, for a total period of 6 h. A 5-mm-thick slice was cut from the left ventricle distal to the coronary occlusion site, and the slice was divided into circumferential wedges. Each wedge was subdivided into an endocardial and epicardial segment. Tissues were immediately frozen in liquid nitrogen, and RNA was extracted and analyzed as described above.

RESULTS

ThR Transcript Level Is Reduced under Hypoxia in a Reversible Manner—Primary cultures of neonatal rat ventricular myocytes were subjected to hypoxic conditions (0% oxygen, 20 torr), and the level of ThR transcripts was compared with the level of transcripts under normal oxygen conditions (21% oxygen). As shown in Fig. 1, mRNA levels of ThR decreased mark-

² E. Landau, R. Tirosh, A. Pinson, S. Banai, S. Even-Ram, M. Mazo, S. Katzav, and R. Bar-Shavit, unpublished data.
Regulation of Thrombin Receptor under Hypoxia

Fig. 1. Panel A, ThR transcript level is reduced under hypoxia. RNA was isolated from rat ventricular myocytes before (lane a) and after various periods of hypoxia (lanes b–d). Cells were subjected to hypoxia for 1 h (lane b), 2 and 4 h (lanes b and d, respectively). The level of ThR mRNA was compared with normal (lane a) and hypoxic (lanes b and c) conditions. RNAs were size fractionated on 1.1% formaldehyde-agarose gels after loading 20 μg of total RNA on each lane. The blots were probed with α²P-labeled full-length rat ThR cDNA and α²P-labeled α-actin. Laser densitometry was used to quantitate the intensity of the bands relative to control bands of α-actin. Panel B, ThR transcript level is reduced in a reversible manner. Total RNA was isolated from rat ventricular myocytes under normal (lane a) and hypoxic conditions (lane b). Myocytes that were subjected to hypoxia were then reexposed to normal oxygen levels (21%, at 37 °C, 18 h) (lane c). Panel C, expression of ThR mRNA in a porcine myocardium in ischemic and normal tissues. Induction of myocardial ischemia was carried out as described under “Experimental Procedures.” RNA was isolated from nonischemic (lane a), partially ischemic (lane b), and fully ischemic tissue (lanes c and d). Northern blot analysis following hybridization with α²P-labeled ThR cDNA was carried out (upper section). The profile of total RNA is shown in the lower section.

edly, in a time-dependent manner (Fig. 1A, lanes b–d), with complete disappearance after 4 h of hypoxia (Fig. 1A, lane d compared with lane a). The effect observed is specific because, during this time frame, the total RNA level was not affected, as shown by the β-actin RNA (Fig. 1A, lower section). The decrease in mRNA levels is reversible because cells that were subjected to 4 h of hypoxia (Fig. 1B, lane b) and then reexposed to normal oxygen levels (i.e. 18 h at 21% oxygen) regained their normal ThR transcript levels (Fig. 1B, lane c compared with lane a). In an in vivo experiment, myocardial ischemia in pig hearts was induced by repeated 2–10-min left anterior descending coronary artery occlusion, separated by 20 min of reperfusion (39). Slices of heart were retrieved after 6 h of intermittent ischemia, and RNA was isolated. As shown in Fig. 1C, the levels of ThR mRNA were analyzed in either nonischemic myocardial tissue (Fig. 1C, lane a), partially ischemic tissue (lane b), or fully ischemic myocardium (lanes c and d). Complete disappearance of ThR was observed in the ischemic myocardium (lanes c and d) compared with normal levels in the nonischemic healthy myocardium (lane a). The specific reduced levels of ThR in the ischemic pig model in vivo thus adequately confirm the data obtained for ThR mRNA under hypoxic conditions.

Activation of ThR Protects Its mRNA Levels under Hypoxia—Because thrombin is generated rapidly at sites of vascular injury or heart muscle ischemia, we have examined the effect of receptor activation on the ThR transcript levels under hypoxia. For this purpose, rat ventricular myocytes were subjected to serum-free conditions, and either the receptor internal ligand TRAP (thrombin receptor-activating peptide, Fig. 2A) or α-thrombin (5 × 10⁻⁸ M, 48 h, data not shown) was added. When TRAP was added to the cells (at concentrations of either 1 μM or 20 μM) and then subjected to hypoxia, the ThR mRNA levels remained unaltered (Fig. 2A, lanes g, h, and j), whereas the level of ThR mRNA disappeared completely under hypoxia (Fig. 2A, lanes b and c). TRAP, at these concentrations, did not affect the normal level of ThR mRNA (Fig. 2A, lanes f and i). When an irrelevant ligand (i.e. basic fibroblast growth factor, 100 ng) was added to the cells prior to subjecting them to hypoxia no effect was observed, regardless of whether they were maintained under normal (Fig. 2A, lane d) or hypoxic (Fig. 2A, lane e) conditions. The effects on ThR mRNA levels were specific because no effect was observed on the total RNA level (as observed for 18 S, lower band in the figure). Furthermore, the addition of TRAP did not affect the stressed hypoxic condition, as observed by the released LDH, an enzyme normally present in granules of the endoplasmic reticulum and released upon hypoxic stress to the medium (Fig. 2B). When measured in the medium under normal conditions, regardless of whether the cells were activated by TRAP (1 or 20 μM, Fig. 2B, lanes b and c, respectively), no release of LDH was obtained (Fig. 2B, lanes a–c). Under hypoxic stress, however, significant amounts of LDH were released into the medium also in the presence of TRAP (1 or 20 μM, Fig. 2B, lanes e and f, respectively). Similar data were obtained when α-thrombin was used to activate the cells (data not shown). It appeared that at least 48 h of activation by TRAP is required to obtain the full protection as shown in Fig. 2C, lane e. Shorter periods of activation such as 6 h (lane c) or 12 h (lane d) were not sufficient to achieve normal ThR levels under hypoxia. The protection effect was not specific only to primary cultures of neonatal rat ventricular myocytes but was also observed in NIH3T3 fibroblasts that were subjected to hypoxia following prior treatment of TRAP (Fig. 3, lanes D and F, 1 μM and 20 μM, respectively). No induction of ThR mRNA was observed under normal conditions after the addition of TRAP (Fig. 3, lanes C and E, 1 μM and 20 μM, respectively) compared with the normal TR transcript level (Fig. 3, lane A) or hypoxic conditions (Fig. 3, lane B). Activation of ThR did not affect hypoxic conditions as also observed by the induced levels of GLUT-1 transcript, a glucose-regulated protein that has been characterized as a glucose transporter and which is known to be up-regulated under hypoxia (Fig. 3, lanes B and D, and F). The protection of ThR mRNA level is specific because no induction in a housekeeping control gene (α-actin) level was observed (Fig. 3, bottom panel).

Protection of Activated ThR Transcripts under Hypoxia Is Interrupted by a Protein Kinase C (PKC) Inhibitor—Activation of ThR initiates a cell signaling cascade involving the G-protein system, PKC activation, and members of the tyrosine kinase family such as Src and mitogen-activated protein kinase downstream. The fact that activated receptors are protected suggests that initiation of cell signaling relays a message that ultimately leads to the protection of ThR from decaying under
Cells were either activated by the addition of 1 μM ligand (regulated gene, under normal (Middle panel) or from cell cultures subjected to hypoxia. TRAP either at 1 μM (lanes b, c, e, g, and j) either in the absence (lanes b, c, and e) or presence of 1 μM (lanes g and h) or 20 μM TRAP (lane j). An irrelevant ligand (i.e. basic fibroblast growth factor, 100 nM) was added under normal (lane d) and hypoxic (lane e) conditions. The level of ThR mRNA was compared with normal (lane a) and hypoxic (lanes b and c) conditions. RNAs were size fractionated on 1.1% formaldehyde-agarose gels after loading 20 μg of total RNA on each lane. The blots were probed with 32P-labeled full-length rat ThR cDNA and 32P-labeled α-actin. Laser densitometry was used to quantitate the intensity of the bands relative to control bands of α-actin. Panel B, released LDH levels under normal and hypoxic conditions. LDH levels were monitored in the medium of normal (bars a–c) and hypoxic (bars d–f) myocytes. TRAP at 1 and 20 μM (bars b, c and e, f, respectively) were added for 48 h. LDH levels were monitored at 340 nm. Panel C, ThR transcript level is reduced in a reversible manner. Total RNA was isolated from rat ventricular myocytes from normal (lane a) and hypoxic (lanes b–f) conditions. TRAP at 20 μM was added either for 6 h (lane c), 12 and 48 h (lanes d and e) before subjecting the cells to hypoxia. Myocytes that were subjected to hypoxia were then reexposed to normal oxygen levels (21%, at 37 °C, 18 h) (lane f).

**Fig. 3.** Northern blot analysis of NIH3T3 fibroblasts under normal and hypoxic conditions. NIH 3T3 mRNA are shown under normal (lanes A, C, and E) and hypoxic (lanes B, D, and F) conditions. Cells were either activated by the addition of 1 μM TRAP (lanes C and D), 20 μM TRAP (lanes E and F), or were not treated (lanes A and B). The top panel shows levels. The blots were probed with 32P-labeled full-length rat ThR cDNA. Middle panel, level of GLUT-1, a hypoxia-regulated gene, under normal (lanes A, C, and E) and hypoxic (lanes B, D, and F) conditions. TRAP activation was at 1 μM (for 48 h) (lanes C and D) or 20 μM (lanes E and F). The blots were probed with 32P-labeled GLUT-1 cDNA. Bottom panel, 32P-labeled α-actin for control housekeeping gene.

Hypoxic conditions. We have therefore analyzed whether interruption of the signaling cascade will inhibit the protective effect. To do this, we have added calphostin C, a PKC inhibitor, during the activation of ThR with TRAP. Both calphostin C (1 nM and 100 nM) and TRAP added under normal conditions did not affect the ThR transcript level (Fig. 4A, lanes f and h). Neither did calphostin C (i.e. 100 nM) alone (Fig. 4A, lane j). TRAP-activated ThR, however, no longer showed the protection obtained under hypoxia of the activated receptor in the presence of calphostin C (Fig. 4A, lanes g and i). In the presence of calphostin C (Fig. 4A, lanes g and i), however, TRAP-activated ThR no longer showed the protection of the activated receptor obtained under hypoxia. TRAP alone under hypoxia protects the levels of ThR (Fig. 4A, lane e) compared with normal conditions (Fig. 4A, lanes a and d) or hypoxic ones (Fig. 4A, lanes b and c). The interruption in the ThR-protected transcript levels in the presence of calphostin C was specific and did not result in changes in RNA levels (Fig. 4A, lower section). Likewise, the addition of calphostin C in the presence or absence of TRAP did not affect the stressed hypoxic conditions as was observed by released LDH activity (Fig. 4B). Similar data were obtained by using two other PKC inhibitors: a bisindolylmaleimide GF109203X, a potent membrane-permeable and specific inhibitor; and the Go 6976 inhibitor (specific for Ca2+-dependent PKCα and PKCβI isozyme; Boehringer Mannheim) In the presence of GF109203X, a dose-dependent loss of ThR protection after receptor activation was obtained, whereas in the presence of Go 6976, a somewhat lesser extent of loss was obtained (data not shown). The concentration range of the GF109203X inhibitor used (25–100 nM) effectively inhibited the PKC-dependent phosphorylation (as previously shown by Albert and Ford (40) of the substrate myelin basic peptide (EKRPSSQRKYL), determined by the colorimetric PKC assay (signal transduction) (data not shown).

**Initiation of Signaling Inside-on Protects ThR mRNA Levels Under Hypoxia**—For this purpose, we have utilized NIH3T3 cells transfected with dominant ras or src oncogenes compared with NIH3T3 mock transfectants. It is important to point out that the myocyte system used by us refers to primary cultures, which are resistant to transformation. Therefore, it was impos-
sible to transfet these cells stably with cDNAs for signaling molecules. To be able to follow the signal transduction processes after ThR activation, we had to use another cel line. When NIH3T3 fibroblasts were subjected to hypoxia, the same pattern of decreasing levels in ThR mRNA after hypoxic stress was observed. Complete abolishment, however, was obtained after 8 h of hypoxia compared with 4 h of hypoxia in cardiac cells (data not shown). NIH3T3 overexpressing either src or ras oncogenes exhibit “on” signals in the cells, similar to exogenously TRAP-activated ThR. We have subjected these cells to hypoxia and analyzed the levels of ThR (Fig. 5, top panel) and GLUT-1 mRNA levels (Fig. 5) compared with the housekeeping gene α-actin levels (Fig. 5, bottom panel). As one can see, Fig. 5 indicates that although in the mock transfecteds ThR mRNA level was not protected under hypoxia (lane B), this is not the case in either Ras (lane D) or Src (lane F) transfected cells, which exhibit unaltered ThR mRNA compared with normal ThR transcript levels (lanes A, C, and E). Similarly, transfection of NIH3T3 cells with either Ras or Src did not affect the stressed hypoxic conditions as shown by the induced level of GLUT-1 (lanes B, D, and F, middle panel) indicative of the stressed conditions. The RNA levels remained unchanged under the various conditions (bottom panel, for α-actin levels, as a control housekeeping gene). Thus, we conclude that when the signaling cascade is “turned on” as part of the ThR relay signaling events, the level of ThR mRNA is protected under hypoxia.

Introduction of Vav Proto-oncogene Protects ThR mRNA under Hypoxia—vav could potentially serve as a link between the tyrosine kinase signaling pathway of activated receptors and the G-protein family. This linkage could be mediated by specific interactions either with various adapter proteins or through a GDP/GTP exchange factor. The Vav proto-oncogene comprises part of the ThR activation pathway as demonstrated via the phosphorylation of the 95-kDa Vav protein after either α-thrombin or TRAP stimulation (Fig. 6, upper panel). NIH3T3 mouse fibroblast cells transfected either with proto-oncogene vav (i.e. K62 cell line, transfected with vav proto-oncogene inserted into a mammalian expression vector pSK115), a mutant version of vav oncogene, R695L, in which arginine 695 of the vav-SH2 domain was replaced by leucine (33) or a mutant defective in SH3 domain of vav (PS632L; proline replaced by leucine) was used in our experimental system. The vav oncogene mutants failed to show any phosphorylation after activation with TRAP compared with proto-vav-induced phosphorylation of the Vav protein, although the level of the protein was not altered (data not shown) (Fig. 6, lower panel).

Altogether our data demonstrate that cells transfected with the vav proto-oncogene also remained unaltered under hypoxic (Fig. 7, lanes E–J, top panel) conditions. This was shown to be true for either transfected vav proto-oncogene (Fig. 7, lane F) or for the SH2 or SH3 mutants of the oncogene (Fig. 7, lanes H and J). The level of ThR transcripts was compared with non-transfected NIH3T3 cells (Fig. 7, lanes A and B) and mock transfected cells (Fig. 7, lanes C and D) as well as normal conditions (Fig. 7, lanes A, C, E, G, and I). The level of hypoxia
FIG. 6. ThR activation induces Vav phosphorylation. NIH3T3 cells transfected to express the vav oncogene as described (32–34) were immunoprecipitated using anti-vav antibodies (kindly supplied by Prof. S. Katzav from the Department of Experimental Medicine, The Hebrew University Medical School, Jerusalem) and detected for the level of phosphorylation by anti P-Tyr antibodies (i.e. PY-20, Transduction Laboratories, Lexington, KY). The cells were briefly induced to activate ThR (lanes B–D, F, and G). The mutant vav, defective in the SH2 domain, however, failed to show any phosphorylation. The blots were stripped further and reacted with anti-vav antibodies, demonstrating similar levels of vav protein in both normal and SH2-defective mutant. Lane A, control; lanes B and C, TRAP activation for 5 and 30 min, respectively; lane D, α-thrombin stimulation for 30 min; lanes E–G, SH2 mutant of vav oncogene; lane E, control; lane F, TRAP stimulation for 5 min; lane G, α-thrombin stimulation for 30 min.

FIG. 7. Top panel, protection of ThR expression under hypoxia of either proto-vav or vav oncogene mutants in NIH3T3 fibroblasts. Northern blot analysis of ThR under normal (lanes A, C, E, G, and I) and hypoxia (lanes B, D, F, H, and J) conditions was performed. Lanes A and B, NIH3T3; lanes C and D, mock transfectants; lanes E and F, proto vav transfected; lanes G and H, oncogene mutants in SH3; lanes I and J, SH2 defects (lanes I and J). Hybridization with 32p-labeled ThR cDNA. Middle panel, GLUT-1 levels under hypoxia of either proto vav or vav oncogene mutants in NIH3T3 fibroblasts. ThR under normal (lanes A, C, E, G, and I) and hypoxia (lanes B, D, F, H, and J) conditions were analyzed. Hybridization with 32p-labeled GLUT-1 cDNA. NIH3T3 cells and mutants are as in the top panel. Bottom panel, hybridization with 32p-labeled α-actin for control.

in the cells was monitored by GLUT-1 expression (Fig. 7, lanes A, D, F, H, and J) compared with the RNA levels applied (Fig. 7, lanes A–J, bottom panel).

DISCUSSION

Functional impairment of blood vessels leading to insufficient blood supply to the heart may often result in reduced oxygen and glucose levels leading ultimately to tissue ischemia. Ischemic episodes, whether acute or chronic, may have different outcomes in the tissue with respect to oxygen deficit, which, when it reaches a critical level, affects the basic myocardial functions that are essential for normal heart myocyte maintenance. Hypoxic and ischemic stress cause a series of well-documented changes in the myocardial tissue, including increased anaerobic glycolysis, loss of contractility, changes in lipid and fatty acid metabolism, and eventually irreversible membrane damage and cell death (1–7). ThR plays an active role in heart contractility and atrial natriuretic factor secretion (15), therefore affecting part of the normal essential myocardial function. It was thus of great interest to study the controlled expression of ThR following hypoxia and ischemia. Our major findings indicate that the mRNA of ThR decays under hypoxic conditions in myocytes as well as in other cell types such as fibroblasts. Interestingly, however, if the receptor is preactivated and the signaling cascade initiated, full protection of ThR mRNA levels is achieved and maintained at levels that are similar to normal conditions, even under hypoxia. Full protection of ThR mRNA was observed 48 h after activation. This phenomenon might be the result of events that take place either at the transcriptional or post-transcriptional regulation. Post-transcriptional regulation involves the binding of either preexisting or newly synthesized proteins to the RNA for its protection. Instability elements reside usually within the 3′-untranslated region (3′-UTR) of mRNA. Indeed, analysis of the 3′-UTR of ThR gene reveals abundant distribution of pentamer and larger sequences such as ATTAAAA, immediately upstream of the stop codon translated region. ThR possesses a rather large 3′-UTR spanning of nucleotide 1510–3480 base pairs. Therefore, preactivation of the receptor may lead to the synthesis of proteins that specifically binds to the 3′-UTR and ultimately leads to RNA protection under hypoxia. This process, which involves protein synthesis and the appropriate complex formation with the 3′-UTR, may require a period of 48 h. Both transcription and post-transcriptional regulation of ThR protection under hypoxia are under current investigation.

The earliest signaling event that we have analyzed is the PKC involvement as demonstrated by inhibition of the calphostin C, a PKC inhibitor, and a set of two other inhibitors at a concentration range that effectively inhibits PKC function. In parallel the Src kinase activation following ThR preactivation has been demonstrated. One of the possible explanations is that the signaling cascade leads eventually to the up-regulation or synthesis of a factors that bind to regulatory motifs within the ThR promoter. Among the regulatory motifs found in the promoter are a GATA motif, potential cis-acting DNA elements including SPI binding sites (19, 41), and AP-2-like elements as well as some sequences of the Ets family of transcription factors have been identified. In addition, there is a hexanucleotide CCCACG sequence motif shown to be the binding site for hypoxia-induced factor, which is primarily responsible for up-regulated gene levels under hypoxia (6, 7). Which of the regulatory elements are involved in the control of ThR levels under hypoxia is not yet known. In addition to, or as part of the factors that influence the promoter, the degradation rate of many eukaryotic mRNAs appears to be regulated through binding of factors to a sequence important for mRNA stabilization located mainly at the 3′-UTR. Both options are now under investigation.

The regulation under hypoxia of a number of oxygen-regulated and glucose-regulated protein genes has been described. Of these, much knowledge was gained by studying the molecular mechanism of vascular endothelial growth factor and GLUT-1 genes, which are known to have essential biological functions (4, 5, 8–10). The increase in the steady-state levels of erythropoietin mRNAs is caused primarily by an increased transcription rate and only in part by mRNA stabilization (6, 7). As for the regulation of vascular endothelial growth factor and GLUT-1, both possess an unstable mRNA that is stabilized by hypoxia and hypoglycemia in a protein synthesis-dependent manner (8–10). We now present evidence showing that upon activation of ThR and initiation of the signaling cascade, the otherwise decaying ThR mRNA is maintained at near normal levels. This protection of ThR mRNA takes place regardless of whether signaling activation is mediated exogenously via the addition of TRAP or inside-on by the introduction of genes that take part in the ThR signaling cascade (for example, src, vav, or vav). It has been demonstrated previously in various cell systems that src, vav, and ras are involved in ThR signaling.
cascade for example in platelets, fibroblasts, and astrocytes (31, 26, 44–46). Therefore, it appears that they provide a rather significant avenues common for the signaling pathway elicited by ThR activation in many cell types. We have utilized NIH3T3 fibroblasts stably transfected with either ras, src, or vav in order to demonstrate their involvement in ThR signaling pathway and their effects on ThR mRNA protection under hypoxia. In light of the major similar signaling pathways in myocytes and fibroblasts we chose to investigate ThR mRNA under hypoxia in NIH3T3 fibroblasts. ThR couples to different G-proteins and activates the nonreceptor tyrosine kinasesSrc and Fyn (19). Thrombin has also been shown to induce Tyr phosphorylation of the adapter protein Shc, which is then recruited to Grb2 (42). It has been reported that a dominant negative Shc that is deficient in Grb2 binding suppresses thrombin-stimulated activation of p44 mitogen-activated protein kinase and cell growth, indicating the importance of Shc in this pathway. In CCL-39 fibroblasts, thrombin activates p21ras in a manner that is inhibited by pertussis toxin and by the Tyr kinase inhibitor genistein, suggesting that involvement of Ras involves G-protein and requires activation of protein kinases (28). Although the exact mechanism by which ThR couples to Ras is still unclear, it is likely that Src and Fyn activate Ras through the adapter protein Shc complexed with Grb2 and the SOS Ras exchange factor (44). It is still unknown whether this activation involves the βγ subunit of Go. Introduction of both Src or Ras is an important part of the ThR signaling pathway. In their oncogenic version, the signaling pathway is constantly on, leading to protection of the decaying ThR mRNA under hypoxia. The fact that either induction of the signaling cascade or activation of the receptor yields similar results, namely the protection of the mRNA under hypoxia, shows that signaling is indeed significant in maintaining the ThR mRNA. In addition, Vav, which activates GTP-binding proteins and is part of the signaling cascade, has a similar protective effect. Mutant versions of Vav either in the SH2 and SH3 also resulted in the protection of ThR mRNA, indicating that Vav may activate another set of proteins leading to protection although not through the phosphorylated sites. Indeed, several SH2 Vav mutants were shown to be defective in their tyrosine phosphorylation properties, yet they still maintain their high transforming potential (34). One possibility is that its activity as a GTP exchange factor is retained in the SH2 vav mutants or alternatively, it can bind proteins through different domains that do not participate in ThR mRNA protection.

In cardiac myocytes ThR activation induces hypertrophy and augments the expression of atrial natriuretic factor through a mechanism that appears to involve both PKC and protein kinases (15). How thrombin can come into direct contact with myocardial cells and influence the contractile function of the heart is another issue. Evidence presented by Goldstein et al. (47) has shown that acute ischemia induced by thrombotic coronary occlusions resulted in a higher incidence of malignant ventricular arrhythmias than does nonthrombogenic balloon occlusion, despite equivalent amounts of jeopardized myocardium. This implies that thrombin, or factors produced during coronary thrombosis, directly influence myocytes in the ischemic regions of the heart. In addition, thrombin stimulation of hypoxic myocytes shows exaggerated phosphoinositide hydrolysis and enhanced inositol trisphosphate accumulation (43, 47). Whether this may result in ThR mRNA protection under hypoxia and further translate to induced myocardial contractility, hypertrophy, and atrial natriuretic factor secretion remains to be determined.

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12. Xu, W.-F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., and Pouyssegur, J., or src, or ras, or vav, or in order to demonstrate their involvement in ThR signaling pathway and their effects on ThR mRNA protection under hypoxia. In light of the major similar signaling pathways in myocytes and fibroblasts we chose to investigate ThR mRNA under hypoxia in NIH3T3 fibroblasts. ThR couples to different G-proteins and activates the nonreceptor tyrosine kinases Src and Fyn (19). Thrombin has also been shown to induce Tyr phosphorylation of the adapter protein Shc, which is then recruited to Grb2 (42). It has been reported that a dominant negative Shc that is deficient in Grb2 binding suppresses thrombin-stimulated activation of p44 mitogen-activated protein kinase and cell growth, indicating the importance of Shc in this pathway. In CCL-39 fibroblasts, thrombin activates p21ras in a manner that is inhibited by pertussis toxin and by the Tyr kinase inhibitor genistein, suggesting that involvement of Ras involves G-protein and requires activation of protein kinases (28). Although the exact mechanism by which ThR couples to Ras is still unclear, it is likely that Src and Fyn activate Ras through the adapter protein Shc complexed with Grb2 and the SOS Ras exchange factor (44). It is still unknown whether this activation involves the βγ subunit of Go. Introduction of both Src or Ras is an important part of the ThR signaling pathway. In their oncogenic version, the signaling pathway is constantly on, leading to protection of the decaying ThR mRNA under hypoxia. The fact that either induction of the signaling cascade or activation of the receptor yields similar results, namely the protection of the mRNA under hypoxia, shows that signaling is indeed significant in maintaining the ThR mRNA. In addition, Vav, which activates GTP-binding proteins and is part of the signaling cascade, has a similar protective effect. Mutant versions of Vav either in the SH2 and SH3 also resulted in the protection of ThR mRNA, indicating that Vav may activate another set of proteins leading to protection although not through the phosphorylated sites. Indeed, several SH2 Vav mutants were shown to be defective in their tyrosine phosphorylation properties, yet they still maintain their high transforming potential (34). One possibility is that its activity as a GTP exchange factor is retained in the SH2 vav mutants or alternatively, it can bind proteins through different domains that do not participate in ThR mRNA protection.

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