Thrombopoietin Receptor Agonists Protect Human Cardiac Myocytes from Injury by Activation of Cell Survival Pathways

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ABSTRACT

Thrombopoietin confers immediate protection against injury caused by ischemia/reperfusion in the rat heart. Eltrombopag is a small molecule agonist of the thrombopoietin receptor, the physiologic target of thrombopoietin. However, the ability of eltrombopag and thrombopoietin to protect human cardiac myocytes against injury and the mechanisms underlying myocyte protection are not known. Human cardiac myocytes (n = 6–10/group) were treated with eltrombopag (0.1–30.0 μM) or thrombopoietin (0.1–30.0 ng/ml) and then subjected to 5 hours of hypoxia (95% N2/5% CO2) and 16 hours of reoxygenation to determine their ability to confer resistance to myocardial injury. The thrombopoietin receptor c-Mpl was detected in unstimulated human cardiac myocytes by Western blotting. Eltrombopag and thrombopoietin confer immediate protection to human cardiac myocytes against injury from hypoxia/reoxygenation by decreasing necrotic and apoptotic cell death in a concentration-dependent manner, with optimal concentration of 3 μM for eltrombopag and 1.0 ng/ml for thrombopoietin. The extent of protection conferred with eltrombopag is equivalent to that of thrombopoietin. Eltrombopag and thrombopoietin activate multiple prosurvival pathways; inhibition of Janus kinase-2, proto-oncogene tyrosine-protein kinase, protein kinase B/phosphatidylinositol-3kinase, p44/42 mitogen-activated protein kinase (MAPK), and p38 MAPK abolished cardiac myocyte protection by eltrombopag and thrombopoietin. Eltrombopag and thrombopoietin may represent important and potent agents for immediately and substantially increasing protection of human cardiac myocytes, and may offer a long-lasting benefit through activation of prosurvival pathways during ischemia.

Introduction

Protection of the heart against injury from acute ischemia remains a challenge for emergency physicians and cardiologists because there are no current therapies that have been proven to directly protect the heart against the deleterious effects of ischemia in humans. Despite major advances in the care of patients with acute coronary syndrome over the past three decades, the rates of early morbidity and mortality associated with this condition remain unacceptably high.

Thrombopoietin is a clinically available cytokine used to stimulate platelet production and correct thrombocytopenia in patients with chronic immune thrombocytopenic purpura who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy. Recent studies have shown that thrombopoietin directly protects the heart (Baker et al., 2008) and brain (Zhou et al., 2011) against injury from ischemia/reperfusion. The decrease in injury is manifest at a dose that does not increase platelet count or hematocrit (Baker et al., 2008). The thrombopoietin receptor is functionally expressed in rat cardiac myocytes, a nonhematopoietic cell (Baker et al., 2008). Cell survival pathways downstream from the thrombopoietin receptor activated by thrombopoietin inhibit apoptotic and necrotic cell death associated with injury from myocardial ischemia/reperfusion both in vivo and in vitro in the rat heart (Baker et al., 2008).

Eltrombopag olamine, the bis-monoethanolamine salt form of eltrombopag, is a small-molecule agonist of the thrombopoietin receptor, the physiologic target of thrombopoietin. Eltrombopag was developed for the treatment of disorders associated with thrombocytopenia, including hematology/oncology-related thrombocytopenia, chronic liver disease–related thrombocytopenia and hepatitis C–related thrombocytopenia. These findings, coupled

ABBREVIATIONS: Akt, protein kinase B; AG-490, (E)-2-cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-propenamide; Hoechst 33342, 2-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5-dimethylimidazole trihydrochloride; Jak2, Janus kinase-2; KATP, ATP-dependent potassium; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PD98059, 2-(2-amino-3-methoxyphenyl)-4-(1H-pyrazol-4-yl)-3-(3,4-dihydroxyphenyl)propionic acid; p38 MAPK, 3-(4-Fluorophenyl)-2-(4-methyl-1-piperazinyl)-1-(3-(4-methylphenoxy)ethyl)imidazo[4,5-f]quinoline; PI3K, phosphatidylinositol-3 kinase; PP1, 1-(1,1-dimethylethyl)-3-(phenylmethyl)-2-propenamide; PPARgamma, peroxisome proliferator-activated receptor gamma; PKC, protein kinase C; PP2, 4-(2-fluoro-3-methylphenoxy)-2-(3,4-difluorophenyl)-1-phenylethylamine; PRI-121, 2-[3-(1H-imidazol-4-yl)phenyl]-1-(4-fluorophenyl)ethanol; STAT, signal transducer and activator of transcription; TUNEL, terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling.
with supporting clinical efficacy data, suggest that eltrombopag is an active thrombopoietin receptor agonist that functions in a similar manner to endogenous thrombopoietin. However, the ability of eltrombopag and thrombopoietin to protect human cardiac myocytes against ischemic and hypoxic injury and the mechanisms underlying myocyte protection are not known. Furthermore, the expression of a functional thrombopoietin receptor in the human heart has not been shown. This information is critical to support the evaluation of thrombopoietin receptor agonists in human clinical studies in the setting of myocardial infarction.

We hypothesized that thrombopoietin receptor agonists would protect human cardiac myocytes against injury. We selected an in vitro human cardiac myocyte model of hypoxia/reoxygenation to determine the efficacy of eltrombopag and thrombopoietin to confer resistance to myocardial injury. Necrosis and apoptosis were measured as indices of protection against cardiac myocyte injury. Pharmacologic probes were used to assess the role of individual cell survival proteins in protection against myocyte injury.

The objectives of the present study were to determine 1) the presence of the receptor for thrombopoietin in human cardiac myocytes, 2) the ability of eltrombopag and thrombopoietin to protect human cardiac myocytes against injury produced by hypoxia/reoxygenation, and 3) the signal transduction pathways activated by eltrombopag and thrombopoietin responsible for enhancing cardiac myocyte survival.

Materials and Methods

Human Cardiac Myocytes

Human cardiac myocytes isolated from the ventricles of the adult heart were purchased from PromoCell (Heidelberg, Germany), plated in a T-25 flask after arrival, and cultured in an incubator at 37°C in room air with 5% CO₂ present. Cells were grown in a monolayer to 80% confluence and then subcultured using Ready-to-Use Myocyte Growth Medium (20% serum; PromoCell) and then transferred to a 96-well plate at a density of 7500 cells per plate for 1 to 2 days before the study. The experiments with cardiomyocytes were performed at passages three to seven. Myotubes were not formed. The human cardiac myocytes express markers of early-stage differentiation, such as α-actin, and slow myosin heavy chain, and they act more like progenitor cells with capacity for proliferation.

Hypoxia/Reoxygenation Studies

To conduct the hypoxia/reoxygenation studies, we placed the cells in Dulbecco’s modified Eagle’s medium supplemented with serum consisting of 17% medium 199, 10% horse serum, 5% horse serum, 5% fetal bovine serum, and 0.5% penicillin-streptomycin in 20 mM HEPES (pH 7.2) buffer. To this was added deoxyglucose (5 mM). This medium did not contain glucose. Cells were then subjected to hypoxia (95% nitrogen/5% CO₂) at 37°C. Cells were reoxygenated by replacing the nitrogen/5% CO₂ gas mixture with 5% CO₂ in room air for 16 hours. The duration of hypoxia and reoxygenation used in the current study is comparable with our previous study (Dhanasekaran et al., 2008). In this previous study, rat neonatal myocytes were subjected to 8 hours of hypoxia and 16 hours of reoxygenation. Extended durations of hypoxia are typically needed to produce injury in cardiac myocytes (Brar et al., 2002; El Jamali et al., 2004; Germack and Dickerson, 2005). Extended durations of reoxygenation are used to ensure cardiac myocytes undergo apoptosis. In rodents and humans, from 5 to 30% of cardiac myocytes undergo apoptosis within 16 hours of reperfusion (Dhanasekaran et al., 2008).

Thrombopoietin Receptor Ligand Studies

Human cardiac myocytes were treated with thrombopoietin (1 ng/ml; Leinco Technologies, St. Louis, MO), eltrombopag (3 µM; GlaxoSmithKline, Brentford, UK), or saline for 30 minutes before and during 5 hours of hypoxia.

Pharmacologic Inhibitor Studies

Human cardiac myocytes were treated with inhibitors of Janus kinase-2 (JAK-2) (AG-490 [6E2-2-3yano-5(3,4-dihydroxyphenyl)-N-(phenethyl)-2-propanenamide], 10 µM), Src (proto-oncogene tyrosine-protein kinase (PPI) [1-(1,1-dimethylhexyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine], 20 µM), protein kinase B (Akt) (Wortmannin, 100 nM), p44/42 mitogen-activated protein kinase (MAPK) (PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzo[4,5]pyridazine-4-one], 10 µM), or p38 MAPK (SB203580 [4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl] pyridine], 10 µM) for 30 minutes before and during 5 hours of hypoxia.

Measurement of Myocyte Injury

Cell Viability. Necrotic cell death was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described in the manufacturer’s protocol (Molecular Probes, Eugene, OR). Briefly, the cells were incubated for 3 hours in phenol red-free medium containing 0.5% of the yellow mitochondrial dye MTT”. The amount of blue formazan dye generated from MTT” was proportional to the number of live cells. The MTT” reaction was terminated by the addition of dimethylsulfoxide to the medium, followed by incubation for 10 minutes at 37°C. The absorbance was read at 540 nm in a spectrophotometer. The values of the reaction were obtained after the subtraction of matched blanks, and the optical densities of the controls were taken as 100% for comparisons with values for other samples. The readings for the test cells were expressed as the percentage of the control.

TUNEL Measurements. The terminal deoxynucleotidyl transferasemediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) assay was used for microscopic detection of apoptosis. This assay was based on labeling of 3′-free hydroxyl ends of the fragmented DNA with fluorescein-diUTP catalyzed by terminal deoxynucleotidyltransferase. Procedures were followed according to the commercially available kit (ApoAlert) from Clontech (Mountain View, CA).

 Hoechst Staining. Cells were stained with 1 µl Hoechst 33342 [2′,4′(4-ethoxyphenyl)-5′(4-methyl-piperazinyl)-2′,5′-bi-1H-benzimidazole trihydrochloride] (5 mg/ml; Molecular Probes) in 1 ml of basal medium and incubated for 30 minutes. Stained cells were washed twice with phosphate-buffered saline (PBS)/Sigma-Aldrich, St. Louis, MO) and imaged under a fluorescent microscope (excitation, 350 nm; emission, 460 nm).

Caspase Activity. Cells were lysed with 50 mM HEPES buffer, pH 7.4, containing 5 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, and 5 mM dithiothreitol. After the cytosolic fraction was taken by centrifugation at 12,000g for 30 minutes, caspase activity as a measure of late apoptotic cell death was measured in the supernatant using DEVD-pNA (acetyl-Asp-Glu-Val-Asp-p-nitro anilide), acetyl-LEHD-pNA (acetyl-7-amino-3-fluoromethylcoumarin p-nitro anilide), and acetyl-IETD-pNA (acetyl-Ile-Glu-Thr-Asp-p-nitro anilide) as substrates. The absorbance at 405 nm of the released pNA was monitored in a spectrophotometer and quantitated by using pNA as standard. Caspase-3 activity was expressed as nanomoles pNA per milligram of protein.

Western Blot Analysis

Cardiac myocytes were washed with ice-cold PBS, and the cells were manually detached and spun down for 5 minutes at 3000–5000g. The treatment media was aspirated, and the cells were washed briefly with 1× PBS and resuspended. The cells were then resuspended in 160 µl of chilled Lysis buffer, transferred to an Eppendorf tube, vortex mixed, and incubated on ice for 15 minutes, pipetting every 5 minutes.
The cells were then sonicated briefly on ice 3 times. We removed 10 μl of the cell lysate for spectrophotometry, and the remainder was stored at −80°C. The Bradford Assay (Bio-Rad Laboratories, Hercules, CA) was used to quantify protein. We loaded 10–50 μl of protein per gel. The protein sample to be analyzed was diluted 1:1 with Laemmli sample buffer (50 μl of β-mercaptoethanol to 550 μl sample buffer), (Bio-Rad Laboratories), boiled for 10 minutes, incubated on ice until cool, and run out on a 10% Tris-HCl gel (Mini-Protean TGX Gel; Bio-Rad Laboratories) at 165 V for 30–60 minutes at room temperature.

Before transfer, the membrane was immersed in 1× transfer buffer for 20 minutes. The protein was transferred to a nitrocellulose membrane (Nitrocellulose/Filter Paper Sandwiched, 0.45 μm; Bio-Rad Laboratories) at 100 V for 1 hour at 4°C. The membrane was washed in Tris-buffered saline with Tween 20 (TBST) to remove any residual transfer buffer and blocked in 5% milk/TBST for 1 hour at room temperature. The membrane was then washed 3 times for 5 minutes with TBST, and incubated in primary antibody and 5% bovine serum albumin/TBST overnight at 4°C.

The antibodies used were purified mouse anti-human thrombopoietin receptor c-Mpl (CD110, 1:1000 dilution; BD Biosciences/Pharmingen, San Jose, CA); phosphorylated (Ser473) Akt and nonphosphorylated Akt (1:1000 dilution; Cell Signaling Technologies); phosphorylated (Thr202/Tyr204) p44/42 MAPK and nonphosphorylated p44/42 MAPK (1:1000 dilution; Cell Signaling Technologies, Beverly, MA); and phosphorylated (Thr180/Tyr183) p38 MAPK and nonphosphorylated p38 MAPK (1:1000 dilution; Cell Signaling Technologies); phosphorylated (Thr202/Tyr204) p38 MAPK and nonphosphorylated p38 MAPK (1:1000 dilution; Cell Signaling Technologies). The membrane was washed again with TBST and incubated with secondary antibody (goat-anti-mouse IgG horseradish peroxidase–conjugated; Bio-Rad Laboratories) for 1 hour at room temperature, washed with TBST, and incubated with Prime Western Blotting Detection Reagent (Amersham Biosciences/GE Healthcare, Little Chalfont, UK).

**Statistical Analysis**

All values are expressed as the mean ± S.D. from at least six or more samples in each experiment. Comparisons between controls and treatments were analyzed by analysis of variance, followed by Tukey’s test when permitted. *P* < 0.05 was considered statistically significant.

**Results**

**The Receptor for Thrombopoietin (c-Mpl) Is Present in Human Cardiac Myocytes**

An antibody specific to the human thrombopoietin receptor extracellular domain was used as a tool to detect protein levels of c-Mpl in human cardiac myocytes by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control to confirm equal protein loading. Human erythroleukemia cells were used as a positive control. The thrombopoietin receptor c-Mpl was detected in unstimulated human cardiac myocytes (Fig. 1). The level of thrombopoietin receptor expression did not diminish after 30 minutes of treatment with either thrombopoietin (1 ng/ml) or eltrombopag (3 μM). The size of the protein detected was 55 kDa (Fig. 1).

**Cardiac Myocyte Protection by Thrombopoietin Is Concentration Dependent**

Isolated human cardiac myocytes were treated with thrombopoietin at a concentration of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 ng/ml before 5 hours of hypoxia and 16 hours of reoxygenation. Each study was repeated a minimum of six times. Myocytes maintained in an aerobic environment were subjected to an equivalent duration of pretreatment, hypoxia, and reoxygenation to act as time-matched controls. Myocytes maintained in culture under aerobic conditions for 21 hours were 100% viable as measured by MTT staining, indicating the viability and stability of the human cardiac myocyte model. Thrombopoietin increased cell viability in a bell-shaped concentration-dependent manner (Fig. 2A). The optimal concentration of thrombopoietin that afforded maximal protection was 1.0 ng/ml.

**Cardiac Myocyte Protection by Eltrombopag Is Concentration Dependent**

Isolated human cardiac myocytes were treated with eltrombopag at a concentration of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 μM before 5 hours of hypoxia and 16 hours of reoxygenation. Each study was repeated a minimum of six times. Thrombopoietin increased cell viability in a bell-shaped concentration-dependent manner (Fig. 2B). The optimal concentration of thrombopoietin that afforded maximal protection was 3.0 μM.

**Eltrombopag and Thrombopoietin Protect against Apoptosis in Human Cardiac Myocytes**

The DNA stain Hoechst 33342 was used to determine the extent of morphologic changes such as nuclear condensation and pyknosis (nuclear fragmentation characteristic of apoptosis). Typical nuclei stained with Hoechst 33342 are shown in Fig. 3. Hypoxia and reoxygenation increased DNA condensation considerably in human cardiac myocytes. We observed pyknotic nuclei after hypoxia and reoxygenation. Thrombopoietin (1 ng/ml) (Fig. 3A) and Eltrombopag (3 μM) (Fig. 3B) protected against DNA fragmentation as manifested by the appearance of abnormal nuclei. TUNEL measurements of decreased apoptosis with thrombopoietin and Eltrombopag confirmed the results with Hoechst 33342 (results not shown). There were no spontaneous contractions observed in the myocytes.

**Eltrombopag and Thrombopoietin Confér Equivalent Protection**

In a separate study, we determined whether thrombopoietin and eltrombopag protect human heart cells against injury to an equivalent amount. Human cardiac myocytes were pretreated with eltrombopag (3 μM) or thrombopoietin (1 ng/ml) before 5 hours of hypoxia and 16 hours of reoxygenation. In preliminary studies, these levels of thrombopoietin receptor agonist were found to confer maximal protection against injury from hypoxia/reoxygenation. Myocytes were then harvested and
analyzed for viability using caspase-3 activation and MTT staining.

Caspase-3 activation was increased after hypoxia/reoxygenation. Eltrombopag and thrombopoietin decreased apoptosis after hypoxia and reoxygenation as measured by caspase-3 activity (Fig. 4A). Myocyte injury was present after hypoxia and reoxygenation, manifested as a decrease in cell viability when compared with control (100%) to 52% ± 3% (Fig. 4B). Eltrombopag and thrombopoietin increased cell viability to 64% ± 4% and 70% ± 3%, respectively. The extent of protection conferred by eltrombopag and thrombopoietin as measured by caspase-3 activity and MTT was equivalent. Additive effects were not observed when the optimal concentrations of thrombopoietin (1.0 ng/ml) and eltrombopag (3 μM) were combined.

**Mechanism Studies**

**Role of JAK-2 in Cardioprotection by Thrombopoietin and Eltrombopag.** Janus kinases are a family of intracellular nonreceptor tyrosine kinases that mediate cytokine signal transduction via the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway. JAK-2 has been reported to be activated by high oxidative stress. AG-490 is a selective inhibitor of JAK-2. Previous findings have shown that pharmacologic inhibition by AG-490 of JAKs abolishes the acute cardioprotection achieved by ischemic preconditioning.

Human cardiac myocytes were treated with either thrombopoietin or eltrombopag in the presence or absence of AG-490 (10 μM) for 30 minutes before 5 hours of hypoxia and 16 hours of reoxygenation. The JAK-2 inhibitor and both thrombopoietin receptor ligands were present throughout the hypoxic period. During the reoxygenation period, the inhibitor and the ligands were not added. Thrombopoietin or eltrombopag treatment before hypoxia/reoxygenation increased cell viability compared with untreated controls (Fig. 5A). The cardioprotective effect of both thrombopoietin and eltrombopag was abolished in the presence of AG-490 (10 μM) (Fig. 5A).

**Role of Src Kinase in Cardioprotection by Thrombopoietin and Eltrombopag.** Among the large number of tyrosine kinases, we focused on the Src kinase family as a candidate for the protein tyrosine kinase member responsible for triggering or mediating cardioprotection by eltrombopag and thrombopoietin. Src tyrosine kinase acts as a membrane-attached molecular switch that links a variety of cues to crucial intracellular signaling pathways. Src tyrosine kinase has been implicated in mechanisms of cell survival and death, which are regulated by complex signal transduction processes. Thus, Src tyrosine kinase activation may represent a key element in mediation of myocardial injury or protection.
associated with ischemia and reperfusion. Myocardial ischemia represents a powerful stimulus for activation of the Src family of tyrosine kinases. It has been shown that hypoxia causes rapid activation of the Src family of tyrosine kinases in cultured rat cardiac myocytes.

Rapid activation of the Src family of tyrosine kinases after ischemia has also been documented in the isolated guinea pig heart. Because the Src family of tyrosine kinases is activated by stimulation of G protein–coupled receptors, an increase in intracellular Ca$^{2+}$, oxidative stress, and enhanced nitric oxide synthesis, all of which can be elicited by ischemic preconditioning challenges, we anticipated that eltrombopag and thrombopoietin could function as a trigger for the activation of the Src family of tyrosine kinases.

We determined the role of Src in eltrombopag- and thrombopoietin-mediated cardioprotection. Human cardiac myocytes were treated with PP1 (20 μM), a selective inhibitor of Src tyrosine kinase that has been shown to prevent phosphorylation and activation of STAT, for 30 minutes alone before hypoxia, and then during 5 hours of hypoxia with eltrombopag or thrombopoietin before reoxygenation. PP1 was not present during reoxygenation. PP1 abolished the cardioprotective effect of eltrombopag or thrombopoietin (Fig. 5B).

**Role of Phosphatidylinositol-3 Kinase/Akt in Cardioprotection by Thrombopoietin and Eltrombopag.** The phosphatidylinositol-3 kinase (PI3K)/Akt signal transduction pathway is known to play an important role in modulating apoptosis. This is evident in studies that show a decreased apoptosis by increasing Akt activity. For example, insulin-like growth factor 1 can block apoptosis by activation of PI3K/Akt.

The underlying mechanism of action for eltrombopag appears distinct from thrombopoietin in platelets (Erhardt et al., 2009), with thrombopoietin but not eltrombopag activating Akt. We determined whether cardioprotection of human cardiac myocytes by eltrombopag or thrombopoietin is involved in activation of PI3K/Akt.

After 30 minutes of treatment with saline, thrombopoietin (1 ng/ml) or eltrombopag (3 μM) cell lysates from human cardiac myocytes were prepared. Cell lysates were probed with specific antibodies against phosphorylated and non-phosphorylated Akt. Thrombopoietin and eltrombopag phosphorylated Akt (Fig. 6A). Thrombopoietin and eltrombopag did not alter the total Akt levels.

Human cardiac myocytes were treated with either thrombopoietin or eltrombopag in the presence or absence of Wortmannin (100 nM) for 30 minutes before 5 hours of hypoxia and 16 hours of reoxygenation. The PI3K/Akt inhibitor and both thrombopoietin receptor ligands were present throughout the hypoxic period. During the reoxygenation period, the inhibitor and the ligands were not added. Thrombopoietin or eltrombopag treatment before hypoxia/reoxygenation increased cell viability compared with untreated controls (Fig. 6B). The cardioprotective effect of both thrombopoietin and eltrombopag was abolished in the presence of the PI3K/Akt inhibitor Wortmannin (100 nM) (Fig. 6B). Cardioprotection of human cardiac myocytes by eltrombopag and thrombopoietin involves the activation of PI3K/Akt.
Role of p44/42 MAPK Kinase in Cardioprotection by Thrombopoietin and Eltrombopag. Our previous studies in the rat heart with thrombopoietin demonstrated activation of numerous signal transduction pathways, including the mitogen-activated p44/42 protein kinase/extracellular signal-regulated kinase (Baker et al., 2008). The serine-threonine kinases p44/42 MAPKs have been reported to be involved in serine phosphorylation of STAT (Chung et al., 1997; Decker and Kovarik, 2000; Wierenga et al., 2003) and are required for phosphorylation of STAT3 on serine 727 in noncardiac cells (Chung et al., 1997; Decker and Kovarik, 2000). We determined whether p44/42 MAPK is activated by thrombopoietin or eltrombopag. After 30 minutes of treatment with saline, thrombopoietin (1 ng/ml) or eltrombopag (3 μM), cell lysates from human cardiac myocytes were prepared. Cell lysates were probed with specific antibodies against phosphorylated and nonphosphorylated p44/42 MAPK. Thrombopoietin and eltrombopag phosphorylated p44/42 MAPK (Fig. 7A). Thrombopoietin and eltrombopag did not alter the total p44/42 MAPK levels.

We determined whether p44/42 MAPK plays a role in the mechanism underlying cardioprotection by thrombopoietin and eltrombopag. Hypoxia/reoxygenation decreased cell viability from 100% in cardiac myocytes maintained under normoxic conditions to 60% ± 7% after 5 hours of hypoxia and 16 hours of reoxygenation (Fig. 7B). Thrombopoietin or eltrombopag treatment before hypoxia/reoxygenation increased the cells’ viability compared with untreated controls (Fig. 7B). The cardioprotective effect of both thrombopoietin and eltrombopag was abolished in the presence of the p44/42 MAPK inhibitor PD98059 (10 μM) (Fig. 7B). PD98059 is a potent and selective cell-permeable inhibitor of p44/42 MAPK. It selectively blocks the activation of p44/42 MAPK, thereby inhibiting the phosphorylation and the activation of this kinase. In human cardiac myocytes treated with PD98059 alone, hypoxia/reoxygenation-induced cell death was no different compared with hypoxia/reoxygenation alone (Fig. 7B). PD90859 had no effect on cell viability in human cardiac myocytes maintained under aerobic conditions (data not shown).

Role of p38 MAPK in Cardioprotection by Thrombopoietin and Eltrombopag. The MAPKs are a family of serine-threonine protein kinases that are activated in response to a variety of stimuli, such as growth factors and cellular stresses. The three major MAPK signaling pathways that have been identified in mammalian cells involve extracellular signal-regulated protein kinases (p44/42 MAPK), p38 MAPK, and c-Jun NH2-terminal protein kinases/stress-activated protein kinases. Ischemic preconditioning, the gold standard of cardioprotection, involves p38 MAPK as a downstream signaling molecule.

Our present study was designed to investigate whether, similar to ischemic preconditioning, thrombopoietin and eltrombopag preconditioning also involve p38 MAPK signaling. After 30 minutes of treatment with saline, thrombopoietin (1 ng/ml) or eltrombopag (3 μM) cell lysates from human cardiac myocytes were prepared. Cell lysates were probed with specific antibodies against phosphorylated and nonphosphorylated p38 MAPK. Thrombopoietin and eltrombopag phosphorylated p38 MAPK (Fig. 8A). Thrombopoietin and eltrombopag did not alter the total p38 MAPK levels.

Human cardiac myocytes were treated with either thrombopoietin or eltrombopag in the presence or absence of SB203580 (10 μM) for 30 minutes before 5 hours of hypoxia and 16 hours of reoxygenation. The p38 MAPK inhibitor and both thrombopoietin receptor ligands were present throughout the hypoxic period. During the reoxygenation period, the inhibitor and the ligands were not added. Thrombopoietin or eltrombopag treatment before hypoxia/reoxygenation increased cell viability compared with untreated controls (Fig. 8B). The cardioprotective effect of both thrombopoietin and eltrombopag was abolished in the presence of the p38 MAPK inhibitor SB203580 (10 μM) (Fig. 8B).
Discussion

Eltrombopag and thrombopoietin confer immediate protection to human cardiac myocytes against injury from hypoxia/reoxygenation. This is the first description of the protective actions of eltrombopag on human cardiac myocytes. We investigated four different end points to determine that thrombopoietin receptor agonists inhibit cell death/apoptosis in isolated human cardiac myocytes: cell viability by MTT, apoptosis by TUNEL, measurements, nuclear fragmentation by Hoechst staining, and activation of the late apoptotic protease caspase-3.

Eltrombopag and thrombopoietin confer protection to human cardiac myocytes to an equivalent extent. Eltrombopag and thrombopoietin activate multiple prosurvival pathways. Our findings suggest the same components of the prosurvival pathways downstream from the thrombopoietin receptor are activated after treatment of human cardiac myocytes with either eltrombopag or thrombopoietin. The optimal concentration of 3 μM for eltrombopag and 1.0 ng/ml for thrombopoietin needed to confer protection in human cardiac myocytes was lower than needed to activate downstream signaling molecules in human platelets (Erhardt et al., 2009; Erickson-Miller et al., 2009). All pathways appear to be important for cardioprotection in that inhibition of any one pathway is sufficient to block the cardioprotective effect of eltrombopag and thrombopoietin. These findings support the presence of a functional thrombopoietin receptor in human cardiac myocytes that is activated upon binding of a thrombopoietin receptor agonist to its receptor to increase myocyte survival during hypoxia.

Eltrombopag confers immediate protection to cardiac myocytes, which could open a window of therapeutic opportunity where a single treatment with eltrombopag before or during an ischemic cardiac episode (such as cardiac surgery, cardiac transplantation, or acute coronary syndromes) may offer immediate and substantial protection as well as a long-lasting benefit through preservation of viable myocardium during the ischemia/reperfusion period through activation of cell survival pathways.

We identified six targets that mediate the action of thrombopoietin receptor agonists: JAK-2, Src kinase, Plk5/Erk1, p42/p44 MAPK, p38 MAPK, and caspase-3. Binding of thrombopoietin receptor agonists to their receptor activates multiple signaling pathways. Blockade of one pathway prevents the protection conferred by either thrombopoietin or eltrombopag, suggesting that the survival pathways are connected. All pathways appear to be important for cardioprotection because inhibition of any one pathway is sufficient to block the cardioprotective effect of thrombopoietin and eltrombopag.

A model for thrombopoietin receptor agonist activation of survival pathways in human cardiac myocytes is proposed in Fig. 9. Cross-talk between the PI3K/Rac/Akt and Raf/MEK/mAPK pathways is important in cell growth, differentiation, survival, and death. However, the existence of cross-talk between these pathways in human cardiac myocytes after treatment with a thrombopoietin receptor agonist has not been shown in human cardiac myocytes until now. These multiple signaling pathways may converge on a common set of targets, resulting in cell survival after a hypoxic or ischemic insult. We previously showed that thrombopoietin activates the ATP-dependent potassium (KATP) channel, which when opened increases the resistance of the heart to ischemia. Delayed protection in a human cardiomyocyte-derived cell line involves p38 MAPK and the opening of KATP channels (Carroll and Yellon, 2000). The KATP channels are known to open to p44/42 MAPK (Zhang et al., 2014). We suggest that the signaling pathways activated by thrombopoietin receptor agonists converge on KATP channels to manifest their cardioprotective effects.

Completely characterized and validated antibodies are valuable tools for both basic and clinical research as they can be used in various applications to define the expression patterns and functional properties of their target proteins. However, the quality of such antibodies from different sources varies; uniformly adopted standards for their selectivity and specificity are lacking. Although c-Mpl was discovered almost 20 years ago and several antibodies have been generated against this receptor, details of the characterization of only two such anti-c-Mpl monoclonal antibodies have been described. Both of these, monoclonal antibodies M1 and BAH-1, were reported to be suitable for flow cytometry (Abbott et al., 2010). The M1 monoclonal antibody was generated by immunizing mice with recombinant human c-Mpl protein AA 84-231. The M1 monoclonal antibody exhibited some nonspecific staining of c-Mpl–containing cells and is no longer available.

To address the need for an antibody directed against the thrombopoietin receptor, several new anti-human c-Mpl monoclonal antibodies have been developed; they have been shown to be specific for human c-Mpl and are suitable for use in flow cytometry (Abbott et al., 2010). Two of these monoclonal antibodies, 1.6 and 1.75, exhibited good sensitivity and bound specifically to various cells that express human c-Mpl. BD Biosciences have now produced an antibody specific to human c-Mpl extracellular domain that is suitable for Western blot analysis.

Using this recently developed antibody directed to the entire extracellular domain of the thrombopoietin receptor, we have shown this receptor is present in human cardiac

**Fig. 8.** (A) Western analysis of phosphorylation of p38 MAPK by thrombopoietin (Tpo) and eltrombopag (Elt) compared with untreated control (Ctl). (B) Inhibition of p38 MAPK abolished protection by Tpo and Elt against injury from hypoxia/reoxygenation. Data are mean ± S.D. *P* = 0.05, versus normoxic (Nor) control; †*P* < 0.05, thrombopoietin receptor ligand versus thrombopoietin receptor ligand plus SB203580 (SB).
myocytes. Four isoforms of the thrombopoietin receptor mRNA have been identified, all arising from alternative splicing of RNA (Vigon et al., 1992; Kiladjian et al., 1997; Li et al., 2000; Coers et al., 2004). Based on the size of the protein detected, it appears to be a truncated version of the thrombopoietin receptor. This truncated version of thrombopoietin receptor in human cardiac myocytes appears functionally viable, as pharmacologic blockade of cell survival proteins downstream from the thrombopoietin receptor limits the ability of thrombopoietin receptor agonists to decrease myocyte necrosis and apoptosis.

Thrombopoietin is the main cytokine involved in regulation of megakaryopoiesis and platelet production, and is the endogenous ligand for the thrombopoietin receptor. In platelets, eltrombopag binds with the human thrombopoietin receptor and initiates signaling cascades similar but not identical to that of endogenous thrombopoietin, inducing proliferation and differentiation of megakaryocytes from bone marrow progenitor cells. At the signaling level, no Akt phosphorylation, an index of protein activation, occurred in platelets treated with eltrombopag in the presence or absence of a subthreshold level of ADP. In contrast, preincubation with thrombopoietin alone induced phosphorylation of Akt in washed platelet preparations, and coinubcation with ADP synergized with thrombopoietin to increase Akt phosphorylation (Erhardt et al., 2009). Our findings suggest eltrombopag and thrombopoietin both activate Akt in human cardiac myocytes, and that this activation is needed for cardiac myocyte protection. This finding is in contrast with previous studies in human platelets where thrombopoietin but not eltrombopag activated Akt (Erhardt et al., 2009).

The precipitating event for acute coronary syndrome (including acute myocardial infarction and unstable angina pectoris) is usually partial or complete coronary artery occlusion due to a combination of platelet aggregates, thrombus formation, and vasospasm. The resulting decrease in coronary flow causes myocardial ischemia, with the severity dependent on the degree of coronary occlusion and the extent of collateral coronary circulation. The patient typically presents with characteristic ischemic symptoms of chest pain and/or pressure, often accompanied by diaphoresis, nausea, and dyspnea. Initial ECG changes may consist of ST elevation, ST depression, and/or T wave inversion. Typically, but not always, untreated ST elevation progresses to a Q wave myocardial infarction, whereas those without ST elevation may evolve to develop a non-ST segment elevation myocardial infarction.

If the ischemia is relatively short-lived and no necrosis occurs, then the episode is categorized as unstable angina pectoris. If ischemia is severe and sustained, after about 20 minutes, myocardial necrosis begins at the subendocardial layer and progresses transmurally. Although the time course varies, complete infarction of the severely ischemic region is usually complete by 6 hours. In a small number of patients, reperfusion may still salvage tissue after 6–12 hours of ischemia. An important dynamic feature of acute coronary syndrome is that a patient may begin with an episode of unstable angina pectoris and then evolve to non-ST segment elevation myocardial infarction or subsequently or directly to ST segment elevation myocardial infarction.

Myocardial ischemia of varying severity and duration is common to all acute coronary syndromes, with and without reperfusion. Therefore, an agent that protects ischamic myocardium from necrosis should increase survival for all forms of acute coronary syndrome. Pharmacologic preconditioning against injury from ischemia/reperfusion with eltrombopag may offer a practical way of harnessing the molecular mechanisms responsible for increased cardioprotection. In patients experiencing symptoms of a disease state, such as a myocardial infarction, or those who are about to undergo cardiac surgery or cardiac transplantation, eltrombopag may be administered to substantially and immediately decrease ischemia-induced injury to the heart. Thus, eltrombopag may represent an important and potent agent for immediately and substantially increasing cardioprotection, and may offer long-lasting benefit through preservation of viable myocardium during ischemia.

**Conclusion.** Ertrombopag confers immediate cardioprotection by activating multiple signaling pathways, which could open a window of therapeutic opportunity where a single treatment with eltrombopag before or during an ischemic cardiac episode (such as cardiac surgery, cardiac transplantation, or acute coronary syndromes) may offer immediate and substantial protection as well as long-lasting benefit through preservation of viable myocardium during the ischemia/reperfusion period through activation of cell survival pathways. All pathways appear to be important for cardioprotection in that inhibition of any one pathway is sufficient to block the cardioprotective effect of eltrombopag.

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The authors dedicate this manuscript to Professor David Hearse on the occasion of his 70th birthday.

**Authorship Contributions**

- **Participated in research design:** Baker, Dhanasekaran, Gross.
- **Conducted experiments:** Su, Koprowski, Dhanasekaran.
- **Performed data analysis:** Baker, Su, Dhanasekaran.
- **Wrote or contributed to the writing of the manuscript:** Baker, Aufderheide, Gross.
References


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