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THROMBOPOIETIN RECEPTOR AGONISTS PROTECT HUMAN CARDIAC MYOCYTES FROM INJURY BY ACTIVATION OF CELL SURVIVAL PATHWAYS

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Running title: Cardioprotection by eltrombopag

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Non standard abbreviations

AG-490, (*E*)-2-Cyano-3-(3,4-dihydrophenyl)-*N*-(phenylmethyl)-2-propenamide;

SB203580, 4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl) phenyl]-1*H*-imidazol-4-yl] pyridine;

PD98059, 2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one;

PP1, 1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine;

c-Mpl, the thrombopoietin receptor;

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,

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DMSO, Dimethyl sulfoxide;

TUNEL, terminal deoxynucleotidyl transferase mediated nick-end labeling;

Hoechst 33342, 2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1*H*-benzimidazole trihydrochloride;

PBS, phosphate buffered saline;

DEVD-*p*NA, acetyl-Asp-Glu- Val-Asp *para*-nitroanilide;

LEHD, 7-amino-4-trifluoromethylcoumarin;

IED, acetyl-IETD-7-amido-4-methylcoumarin;

pNA, para nitro anilide;

TBST, Tris Buffered Saline with Tween 20;

BSA, bovine serum albumin;

MAPK, mitogen activated protein kinase;

HRP, horse radish peroxidase;

ANOVA, analysis of variance;

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase;

JAK-STAT, Janus kinase- signal transducer and activator of transcription;

JAK-2, Janus kinase-2;

PI_3-K, Phosphatidylinositol-3 kinase;

IGF-1, Insulin-like growth factor 1;

Src, Proto-oncogene tyrosine-protein kinase;

K_{ATP}, ATP-dependent potassium

NSTEMI, non-ST segment elevation myocardial infarction;

STEMI, ST segment elevation myocardial infarction.

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 physiological 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% N_2/5%CO_2) 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 an 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 pro-survival pathways; inhibition of JAK-2, src kinase, Akt/PI_3 kinase, p44/42 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 long-lasting benefit through activation of pro-survival 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, 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 physiological 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 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 (i) the presence of the receptor for thrombopoietin in human cardiac myocytes, (ii) the ability of eltrombopag and thrombopoietin to protect human cardiac myocytes against injury produced by hypoxia/reoxygenation, and (iii) 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 (Ca# C-12810), plated in a T-25 flask following arrival and cultured in an incubator at 37^{o}C in room air with 5% CO_2 present. Cells were grown in a monolayer to 80% confluence and then sub cultured using "Ready-to-use" Myocyte Growth Medium, (20% serum), (PromoCell Ca# C-22070) and then transferred to a 96 well plate at a density of 7500 cells per plate for 1 - 2 days prior to study. The experiments with cardiomyocytes were performed at passage three to seven. Myotubes were not formed. The human cardiac myocytes express markers of early stage differentiation such as GATA-4, sarcomeric alpha-actin and slow myosin heavy chain, and act more like progenitor cells with capacity for proliferation.

Hypoxia/reoxygenation studies

To conduct the hypoxia/reoxygenation studies, cells were placed 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 (5mM). This medium did not contain glucose. Cells were then subjected to hypoxia (95% nitrogen/5% CO_2) at 37^{o}C. Cells were reoxygenated by replacing the nitrogen/5% CO_2 gas mixture with 5% CO_2 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 hypoxia and 16 hours 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 Dickenson, 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 (1ng/ml), (Leinco Technologies, Inc. Ca # T155), Eltrombopag (3μM) (GlaxoSmithKline), or saline for 30 minutes prior to and during 5 hours of hypoxia.

Pharmacologic inhibitor studies

Human cardiac myocytes were treated with inhibitors of JAK-2 (AG-490, 10 μM), src tyrosine kinase (PP1, 20μM), Akt (Wortmannin, 100 nM), p44/42 MAPK (PD98059, 10 μM) or p38 MAPK (SB203580, 10 μM) for 30 minutes prior to and during 5 hours of hypoxia.

Measurement of myocyte injury

Cell viability

Necrotic cell death was determined by the MTT assay as described in the manufacturer's protocol (Cat. No. V-13154; 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 DMSO to the medium, followed by incubation for 10 min 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 percentage of control.

TUNEL measurements

The 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-dUTP catalyzed by terminal deoxynucleotidyltransferase. Procedures were followed according to the commercially available kit (ApoAlert) from Clontech.

Hoechst Staining

Cells were stained with 1 µl of Hoechst 33342 (5 mg/ml; Cat. No. V-13244, Molecular Probes) in 1 ml basal medium and incubated for 30 min. Stained cells were washed twice with PBS (Sigma, 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 CHAPS and 5 mM dithiothreitol. After the cytosolic fraction was taken by centrifugation at 12,000 *x g* for 30 min, caspase activity as a measure of late apoptotic cell death was measured in the supernatant using DEVD-*p*NA (acetyl-Asp-Glu- Val-Asp *para*-nitroanilide), acetyl-LEHD-*p*NA, and acetyl-IETD-*p*NA as substrates. The absorbance at 405 nm of the released pNA was monitored in a spectrophotometer and quantitated by using *p*NA as standard. Caspase-3 activity was expressed as nmoles pnitroanilde/mg protein.

Western analysis

Cardiac myocytes were washed with ice-cold PBS, and the cells manually detached and spun down for 5 minutes at 3,000-5,000 x *g*. Treatment media was aspirated, the cells were washed briefly with 1x PBS and re-pelleted. Cells were then resuspended in 160µl of chilled Lysis buffer, transferred to an Eppendorf tube, vortex mixed and cells incubated on ice for 15 minutes, pipetting every 5 minutes. Cells were then sonicated briefly on ice 3 times. Ten µl of the cell lysate was removed and used for spectrophotometry and the remainder was stored at -80^{o}C. The BioRad Bradford Assay (Ca # 500-0006) was used to quantify protein. Ten-50 µl of protein was loaded per gel. The protein sample to be analyzed was diluted 1:1 with Laemmli sample buffer (50µl of BME to 950 µl sample buffer), (BioRad Ca#161-0737) boiled for 10 minutes followed by an incubation on ice until cool and run out on a 10% Tris-HCl gel (BioRad Mini-PROTEAN TGX Gel Ca# 456-1034), at 165V for 30-60 minutes at room temperature. Prior to transfer, the membrane was immersed in 1x Transfer buffer for 20 minutes.

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Protein was transferred to a nitrocellulose membrane (BioRad Nitrocellulose/Filter Paper Sandwiched, 0.45um, Ca# 162-0235), at 100V for 1 hour at 4^{o}C. The membrane was washed in 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% BSA/TBST overnight at 4^{o}C. Antibodies used were purified mouse anti-Human c-Mpl (CD110) (1:1000 dilution) (BD Pharmingen Ca# 562137), phosphorylated (Ser 473) Akt (Cat# 4051) and non-phosphorylated Akt (Cat# 4691) (1:1000 dilution) (Cell signaling), phosphorylated (Thr202/Tyr 204) p44/42 MAPK (Ca# 4370) and non-phosphorylated p44/42 MAPK (Ca# 4695) (1:1000 dilution) (Cell Signaling), phosphorylated (Thr180/Tyr183) p38 MAPK (Ca# 9837) and non-phosphorylated p38 MAPK (Ca# 8690) (1:1000 dilution) (Cell Signaling), The membrane was washed again with TBST and incubated with secondary antibody (BioRad goat-anti-mouse IgG HRP-conjugated Ca# 170-6516) for 1 hour at room temperature, washed with TBST and incubated with Amersham ECL Prime Western Blotting Detection Reagent, (Ca# RPN 2232).

Statistical analysis

All values are expressed as the mean ± standard deviation from at least six or more samples in each experiment. Comparisons between controls and treatments were analyzed by ANOVA, followed by Tukey's test when permitted. Values for $P < 0.05$ were considered 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. GAPDH 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 (Figure 1). The level of thrombopoietin receptor expression did not diminish following 30 minutes of treatment with either thrombopoietin (1 ng/ml) or eltrombopag (3μM). The size of the protein detected was 55 kDa (Figure 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 prior to 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 (Figure 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 prior to 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 (Figure 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 morphological changes such as nuclear condensation and pyknosis (nuclear fragmentation characteristic of apoptosis). Typical nuclei stained with Hoechst 33342 are shown in Figure 3. Hypoxia and reoxygenation increased DNA condensation considerably in human cardiac myocytes. We observed pyknotic nuclei after hypoxia and reoxygenation. Thrombopoietin (1 ng/ml) (Figure 3A) and Eltrombopag (3 μM) (Figure 3 B) 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 confer 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 (3μM) or thrombopoietin (1 ng/ml) prior to 5 hours hypoxia and 16 hours 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 following hypoxia and reoxygenation as measured by caspase-3 activity (Figure 4A). Myocyte injury was present after hypoxia and reoxygenation, manifest as a decrease in cell viability when compared with control (100%) to 52 $\pm$ 3% (Figure 4B). Eltrombopag and thrombopoietin increased cell viability to 64 $\pm$ 4% and 70 $\pm$ 3%, respectively. The extent of protection conferred by eltrombopag and thrombopoietin as measured my 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.

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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 pharmacological 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 prior to 5 hours hypoxia and 16 hours 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 prior to hypoxia/reoxygenation increased cell viability compared with untreated controls (Figure 5A). The cardio protective effect of both thrombopoietin and eltrombopag was abolished in the presence of AG-490 (10 μM) (Figure 5A).

Role of src kinase in cardioprotection by thrombopoietin and eltrombopag

Among the large number of tyrosine kinases, we focused on the Src 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 prior to hypoxia, and then during 5 hours hypoxia with eltrombopag or thrombopoietin prior to reoxygenation. PP1 was not present during reoxygenation. PP1 abolished the cardio protective effect of eltrombopag or thrombopoietin (Figure 5B).

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Role of PI_3-K/Akt in cardioprotection by thrombopoietin and eltrombopag

The PI_3K/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, the growth factor IGF-I can block apoptosis by activation of PI_3K/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 involved activation of PI_3K/Akt.

Following 30 minute treatment with saline, thrombopoietin (1ng/ml) or eltrombopag (3μM) 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 (Figure 6A). Thrombopoietin and eltrombopag did not alter 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 prior to 5 hours hypoxia and 16 hours reoxygenation. The PI_3/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 prior to hypoxia/reoxygenation increased cell viability compared with untreated controls (Figure 6B). The cardio protective effect of both thrombopoietin and eltrombopag was abolished in the presence of the PI_3K/Akt inhibitor Wortmannin (100 nM) (Figure 6B). Cardioprotection of human cardiac myocytes by eltrombopag and thrombopoietin involves the activation of PI_3K/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 signal transducer and activator of transcription (STAT) (Chung et al., 1997; Decker and Kovarik, 2000; Wierenga et al., 2003) and are required for phosphorylation of STAT3 on Serine-727 in non-cardiac cells (Chung et al., 1997; Decker and Kovarik, 2000). We determined whether p44/42 MAPK is activated by thrombopoietin or eltrombopag. Following 30 minute treatment with saline, thrombopoietin (1ng/ml) or eltrombopag (3μM), cell lysates from human cardiac myocytes were prepared. Cell lysates were probed with specific antibodies against phosphorylated and non-phosphorylated p44/42 MAPK. Thrombopoietin and eltrombopag phosphorylated p44/42 MAPK (Figure 7A). Thrombopoietin and eltrombopag did not alter 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 $\pm$ 7% following 5 hours of hypoxia and 16 hours of reoxygenation (Figure 7B). Thrombopoietin or eltrombopag treatment prior to hypoxia/reoxygenation increased cell viability compared with untreated controls (Figure 7B). The cardio protective effect of both thrombopoietin and eltrombopag was abolished in the presence of the p44/42 MAPK inhibitor PD98059 (10 μM) (Figure 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 (Figure 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 NH_2-terminal protein kinases /stress-activated protein kinases. Ischemic preconditioning, the gold standard of cardioprotection, involves p38 MAPK as a downstream signaling molecule.

The present study was designed to investigate whether, similar to ischemic preconditioning, thrombopoietin and eltrombopag preconditioning also involved p38 MAPK signaling. Following 30 minute treatment with saline, thrombopoietin (1ng/ml) or eltrombopag (3uM) cell lysates from human cardiac myocytes were prepared. Cell lysates were probed with specific antibodies against phosphorylated and non-phosphorylated p38 MAPK. Thrombopoietin and eltrombopag phosphorylated p38

MAPK (Figure 8A). Thrombopoietin and eltrombopag did not alter 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 prior to 5 hours hypoxia and 16 hours 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 prior to hypoxia/reoxygenation increased cell viability compared with untreated controls (Figure 8B). The cardio protective effect of both thrombopoietin and eltrombopag was abolished in the presence of the p38 MAPK inhibitor SB203580 (10 μM) (Figure 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 pro-survival pathways. Our findings suggest the same components of the pro-survival pathways downstream from the thrombopoietin receptor are activated

following 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 cardio protective 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 prior to 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.

We identified 6 targets that mediate the action of thrombopoietin receptor agonists: JAK-2, src kinase, PI_3K/Akt, p44/42 MAPK, p38 MAPK and caspase-3. Binding of thrombopoietin receptor agonists to their receptor activates multiple signaling pathways. Blockade of one pathway prevents protection conferred by either thrombopoietin or eltrombopag suggesting the survival pathways are connected. All pathways appear to be important for cardioprotection in that 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 figure 9. Cross-talk between the PI_3K/Rac/Akt and Raf/MEK/p42/44 MAPK pathways is important in cell growth, differentiation, survival and death. However the existence of cross-talk between these pathways in human cardiac myocytes following 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 following a hypoxic or ischemic insult. We previously showed that thrombopoietin activates the K_{ATP} channel, which when opened increase the resistance of the heart to ischemia. Delayed protection in a human cardiomyocyte-derived cell line involves p38 MAPK and the opening of K_{ATP} channels (Carroll and Yellon, 2000). p44/42 MAPK is known to open the K_{ATP} channels (Zhang et al., 2014). We suggest the signaling pathways activated by thrombopoietin receptor agonists converge on K_{ATP} channels in order to manifest their cardioprotective effects.

Completely characterized and validated antibodies are valuable tools for both basic and clinical research as they can be utilized 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 non-specific 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 and 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 blotting (Catalog number 562137).

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 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 sub-threshold level of ADP. In contrast, pre-incubation with thrombopoietin alone induced phosphorylation of Akt in washed platelet preparations, and co-incubation 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 MI, whereas those without ST elevation may evolve to develop a NSTEMI. 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 instable angina pectoris and then evolve to NSTEMI or subsequently or directly to STEMI.

Myocardial ischemia of varying severity and duration is common to all acute coronary syndromes, with and without reperfusion. Therefore, an agent that protects ischemic 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 cardio protection, and may offer long-lasting benefit through preservation of viable myocardium during ischemia.

Conclusion

Eltrombopag confers immediate cardioprotection by activating multiple signaling pathways, which could open a window of therapeutic opportunity, where a single treatment with eltrombopag prior to 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.

Acknowledgments

This manuscript is dedicated 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

Abbott C, Huang G, Ellison AR, Chen C, Arora T, Szilvassy SJ and Wei P (2010) Mouse monoclonal antibodies against human c-Mpl and characterization for flow cytometry applications. *Hybridoma (Larchmt)* **29**:103-113.

Baker JE, Su J, Hsu A, Shi Y, Zhao M, Strande JL, Fu X, Xu H, Eis A, Komorowski R, Jensen ES, Tweddell JS, Rafiee P and Gross GJ (2008) Human thrombopoietin reduces myocardial infarct size, apoptosis, and stunning following ischaemia/reperfusion in rats. *Cardiovasc Res* **77**:44-53.

Brar BK, Stephanou A, Knight R and Latchman DS (2002) Activation of protein kinase B/Akt by urocortin is essential for its ability to protect cardiac cells against hypoxia/reoxygenation-induced cell death. *J Mol Cell Cardiol* **34**:483-492.

Carroll R and Yellon DM (2000) Delayed cardioprotection in a human cardiomyocyte-derived cell line: the role of adenosine, p38MAP kinase and mitochondrial KATP. *Basic Res Cardiol* **95**:243-249.

Chung J, Uchida E, Grammer TC and Blenis J (1997) STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol* **17**:6508-6516.

Coers J, Ranft C and Skoda RC (2004) A truncated isoform of c-Mpl with an essential C-terminal peptide targets the full-length receptor for degradation. *J Biol Chem* **279**:36397-36404.

Decker T and Kovarik P (2000) Serine phosphorylation of STATs. *Oncogene* **19**:2628-2637.

Dhanasekaran A, Gruenloh SK, Buonaccorsi JN, Zhang R, Gross GJ, Falck JR, Patel PK, Jacobs ER and Medhora M (2008) Multiple antiapoptotic targets of the PI3K/Akt survival pathway are activated by epoxyeicosatrienoic acids to protect cardiomyocytes from hypoxia/anoxia. *Am J Phsyiol Heart Circ Physiol* **294**:H724-735.

El Jamali A, Freund C, Rechner C, Scheidereit C, Dietz R and Bergmann MW (2004) Reoxygenation after severe hypoxia induces cardiomyocyte hypertrophy in vitro: activation of CREB downstream of GSK3beta. *Faseb J* **18**:1096-1098.

Erhardt JA, Erickson-Miller CL, Aivado M, Abboud M, Pillarisetti K and Toomey JR (2009) Comparative analyses of the small molecule thrombopoietin receptor agonist eltrombopag and thrombopoietin on in vitro platelet function. *Exp Hematol* **37**:1030-1037.

Erickson-Miller CL, Delorme E, Tian SS, Hopson CB, Landis AJ, Valoret EI, Sellers TS, Rosen J, Miller SG, Luengo JI, Duffy KJ and Jenkins JM (2009) Preclinical activity of eltrombopag (SB-497115), an oral, nonpeptide thrombopoietin receptor agonist. *Stem Cells* **27**:424-430.

Germack R and Dickenson JM (2005) Adenosine triggers preconditioning through MEK/ERK1/2 signalling pathway during hypoxia/reoxygenation in neonatal rat cardiomyocytes. *J Mol Cell Cardiol* **39**:429-442.

Kiladjian JJ, Elkassar N, Hetet G, Briere J, Grandchamp B and Gardin C (1997) Study of the thrombopoitin receptor in essential thrombocythemia. *Leukemia* **11**:1821-1826.

Li J, Sabath DF and Kuter DJ (2000) Cloning and functional characterization of a novel c-mpl variant expressed in human CD34 cells and platelets. *Cytokine* **12**:835-844.

Vigon I, Mornon JP, Cocault L, Mitjavila MT, Tambourin P, Gisselbrecht S and Souyri M (1992) Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. *Proc Natl Acad Sci USA* **89**:5640-5644.

Wierenga AT, Vogelzang I, Eggen BJ and Vellenga E (2003) Erythropoietin-induced serine 727 phosphorylation of STAT3 in erythroid cells is mediated by a MEK-, ERK-, and MSK1-dependent pathway. *Exp Hematol* **31**:398-405.

Zhang DM, Chai Y, Erickson JR, Brown JH, Bers DM and Lin YF (2014) Intracellular signalling mechanism responsible for modulation of sarcolemmal ATP-sensitive potassium channels by nitric oxide in ventricular cardiomyocytes. *J Physiol* **592**:971-990.

Zhou J, Li J, Rosenbaum DM and Barone FC (2011) Thrombopoietin protects the brain and improves sensorimotor functions: reduction of stroke-induced MMP-9 upregulation and blood-brain barrier injury. *J Cereb Blood Flow Metab* **31**:924-933.

Footnotes

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Figure legends

Figure 1. The receptor for thrombopoietin is present in human cardiac myocytes. C = untreated. T= thrombopoietin treated. E = eltrombopag treated. Human erythroleukemia cells were used as a positive control. Data are representative of three analyses per sample.

Figure 2. Cardiac myocyte protection by thrombopoietin and eltrombopag is concentration-dependent. A. Thrombopoietin (Tpo). B. Eltrombopag (Elt). Data are means ± SD, n=6/group. Solid column = normoxic control. Open column = hypoxia/reoxygenation (Hyp) control. Hatched column = hypoxia/reoxygenation plus thrombopoietin or eltrombopag. * $P < 0.01$ vs. Hyp control.

Figure 3. Thrombopoietin and eltrombopag protect against apoptosis. A. Thrombopoietin (Tpo) (1 ng/ml) blocks fragmentation of nuclei induced by 5 hours hypoxia and 16 hours reoxygenation (HR). Nuclei were stained with Hoechst 33342 and imaged. B. Eltrombopag (Elt) (3μM) blocks fragmentation of nuclei induced by 5 hours hypoxia and 16 hours reoxygenation (HR). Nuclei were stained with Hoechst 33342 and imaged. * $P <0.001$ vs. control. † $P <0.001$ compared with HR.

Figure 4. Eltrombopag (Elt) and thrombopoietin (Tpo) confer equivalent protection of human cardiac myocytes against injury following hypoxia/reoxygenation (Hyp). A. Caspase activation. B. Cell viability. Data are mean ± SD, n=10/group. * $P <0.05$, vs. normoxic control. † $P <0.05$, vs. Hyp.

Figure 5. A. Inhibition of JAK-2 abolishes protection by thrombopoietin and eltrombopag against injury from hypoxia/reoxygenation. B. Inhibition of src tyrosine

kinase abolishes protection by thrombopoietin and eltrombopag against injury from hypoxia/reoxygenation. Data are mean ± SD, n=6/group. * $P < 0.05$, vs. normoxic control. † $P < 0.05$, thrombopoietin receptor ligand vs. thrombopoietin receptor ligand plus AG-490 (AG) or PP1. Nor = normoxia, Hyp = hypoxia/reoxygenation, Tpo = thrombopoietin, Elt = eltrombopag.

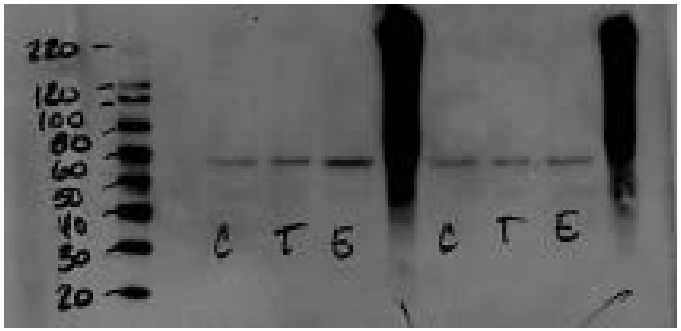
Figure 6. A. Western analysis showing phosphorylation of Akt by thrombopoietin (Tpo) and eltrombopag (Elt) compared with untreated control (Ctl). B. Inhibition of Akt/PI_3K abolishes protection by thrombopoietin (Tpo) and eltrombopag (Elt) against injury from hypoxia/reoxygenation. Data are mean ± SD, n=6/group. * $P < 0.05$, vs. normoxic control (Ctl). † $P < 0.05$, thrombopoietin receptor ligand vs. thrombopoietin receptor ligand plus Wortmannin (Wrt).

Figure 7. A. Western analysis showing phosphorylation of p44/42 MAPK by thrombopoietin (Tpo) and eltrombopag (Elt) compared with untreated control (Ctl). B. Inhibition of p44/42 MAPK abolishes protection by Tpo and Elt against injury from hypoxia/reoxygenation. Data are mean ± SD, n=6/group. * $P < 0.05$, vs. normoxic control. † $P < 0.05$, thrombopoietin receptor ligand vs. thrombopoietin receptor ligand plus PD 98059 (PD).

Figure 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 abolishes protection by Tpo and Elt against injury from hypoxia/reoxygenation. Data are mean ± SD, n=6/group. * $P < 0.05$, vs. normoxic control. † $P < 0.05$, thrombopoietin receptor ligand vs. thrombopoietin receptor ligand plus SB 203580 (SB).

Figure 9. Model for thrombopoietin receptor agonist activation of survival pathways and inhibition of apoptosis in human cardiac myocytes. We have reported that treatment with a thrombopoietin receptor agonist activates 1) JAK-2, 2) src kinase, 3) PI_3K/Akt, 4) p44/42 MAPK, *5*) p38 MAPK and 6) inhibits caspase-3. Each of these events protects the human cardiac myocyte against injury from hypoxia/reoxygenation. These targets align in a signaling cascade as illustrated in the schematic. It should be noted that the results do not implicate absence of other pro survival or apoptotic pathways that may also be targeted by thrombopoietin receptor agonists.

Figure 1



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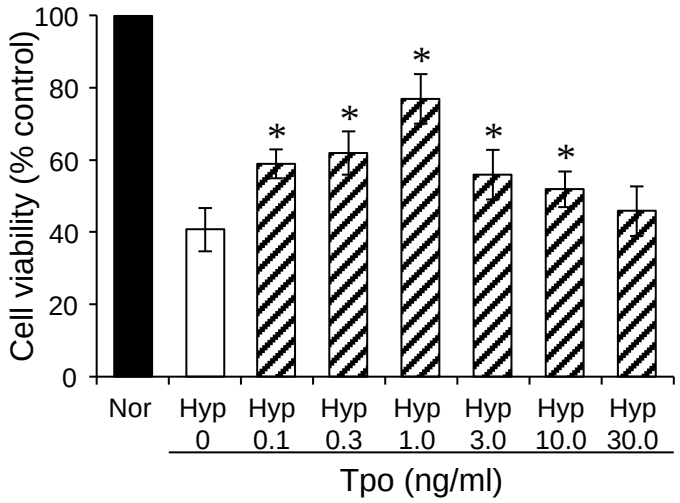

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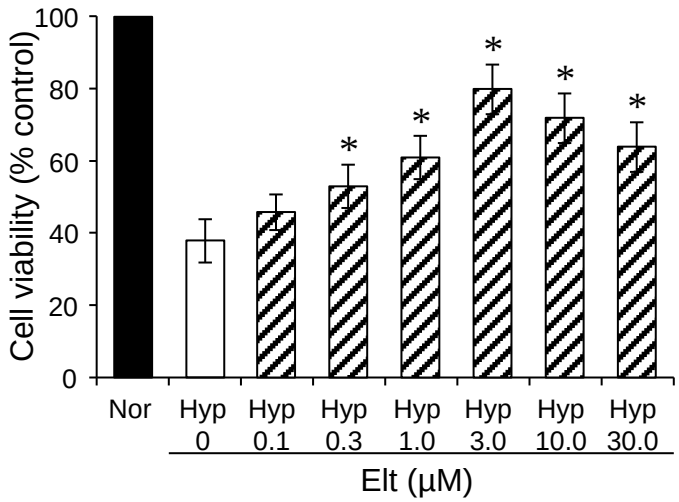

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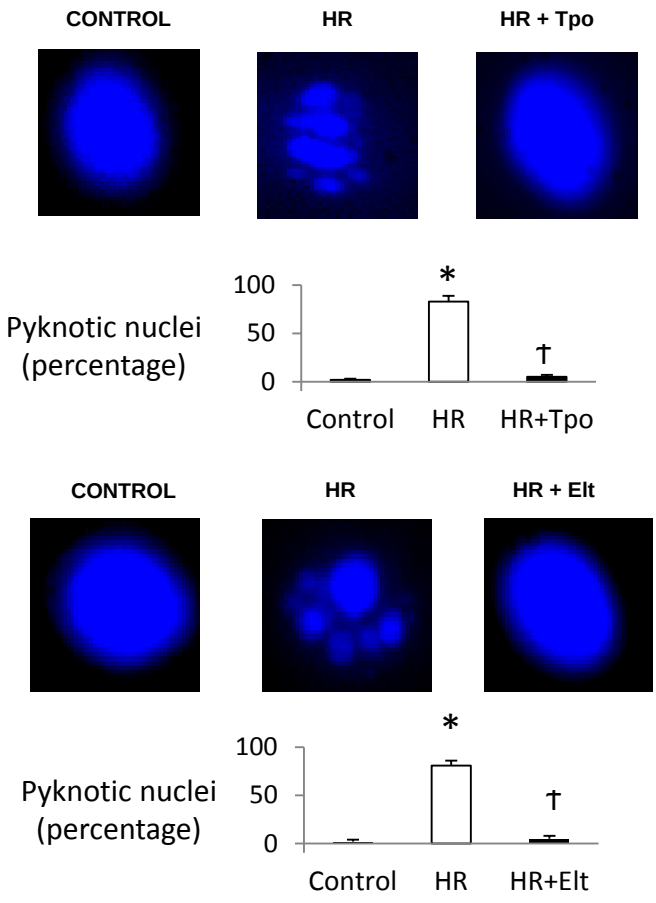
CONTROL
HR
HR + Tpo
Pyknotic nuclei (percentage)
100
50
0
*
†
Control
HR
HR+Tpo
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Pyknotic nuclei (percentage)
100
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Control
HR
HR+Elt

Figure 4A

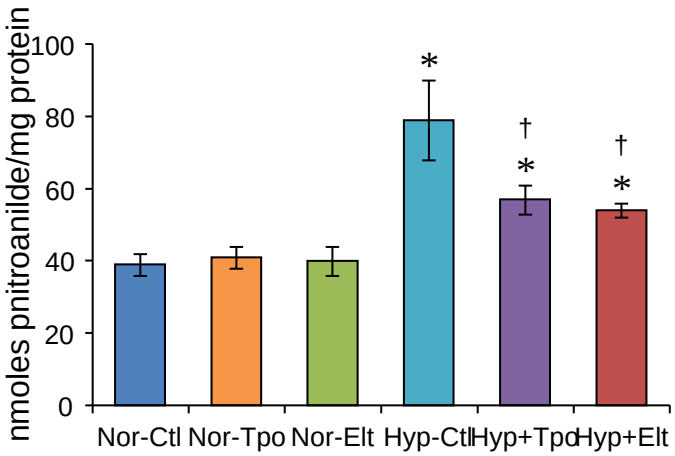
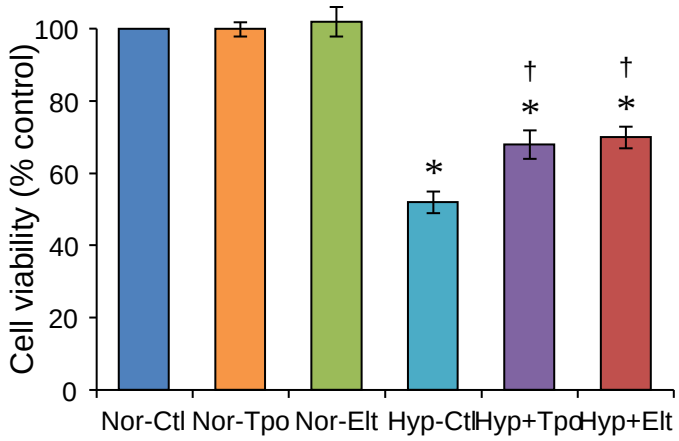


Figure 4B



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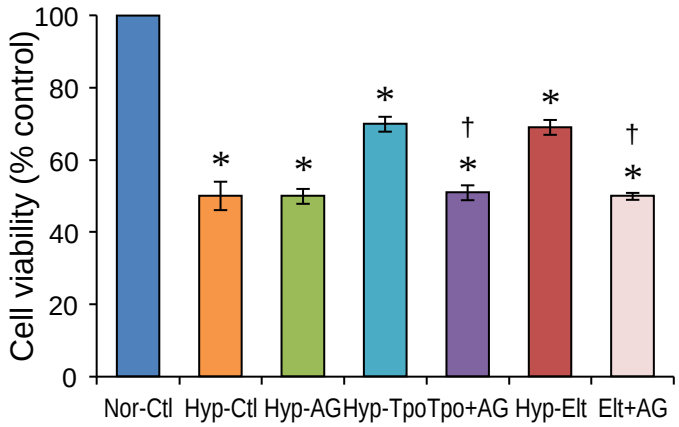

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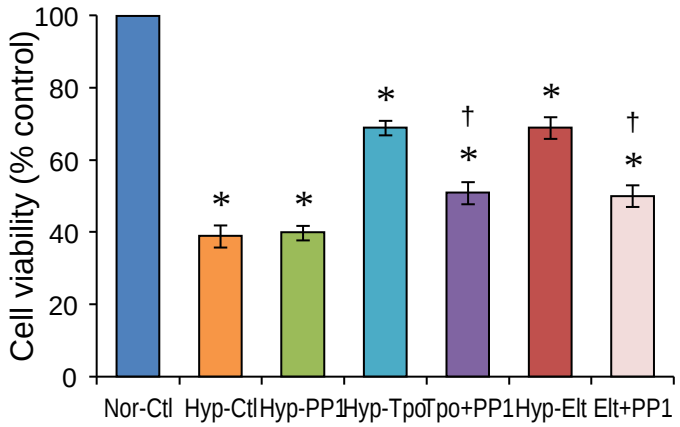

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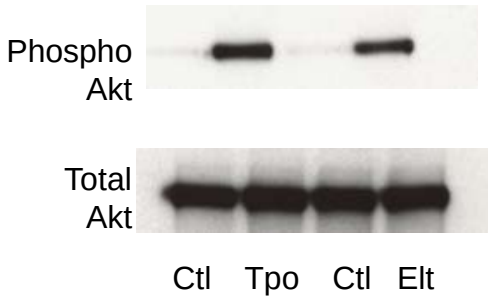

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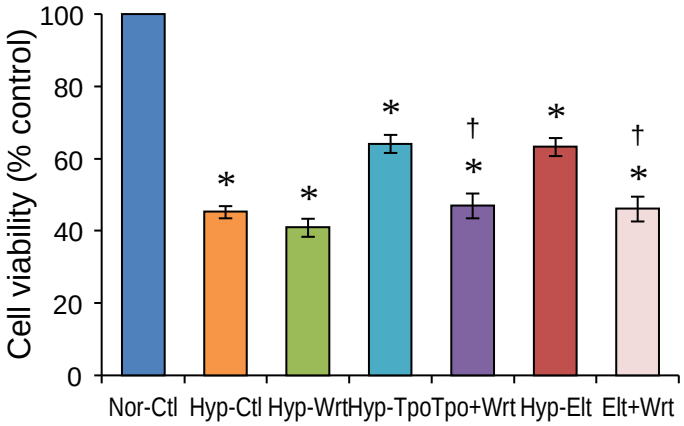

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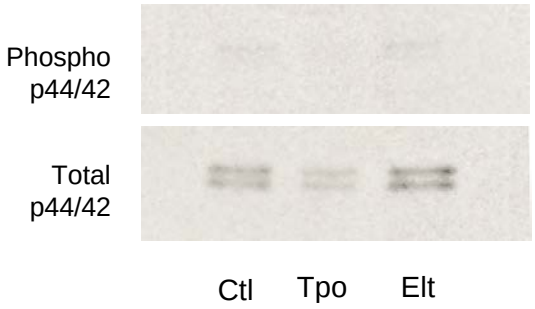

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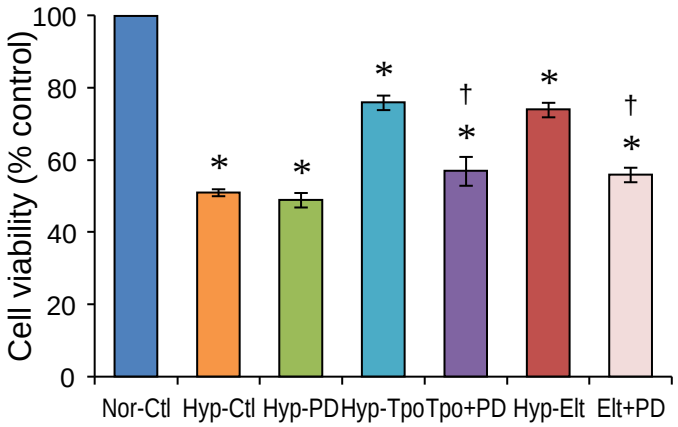

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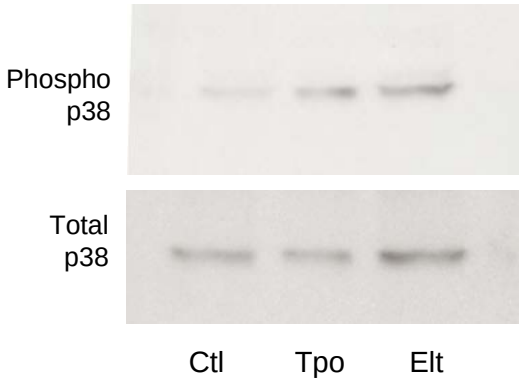

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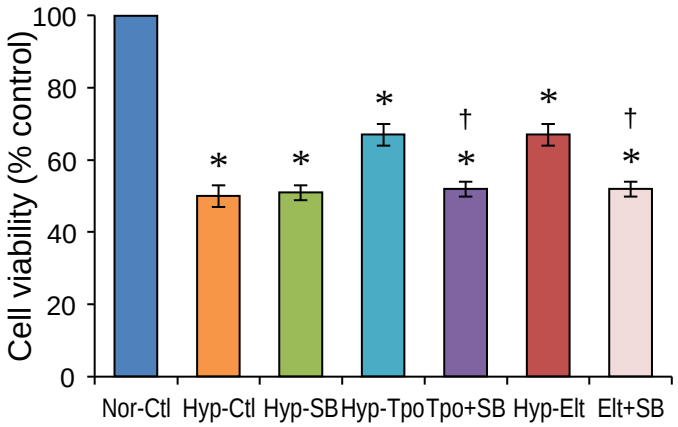

Figure 9