Propofol Mediates RISK-SAFE Cross-Talk in H9c2 Myofibroblasts

by

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Abstract

Intro: Propofol, an IV anesthetic that activates cardioprotective signaling mechanisms against I/R injury. It activates the PI3K/Akt branch of the RISK pathway to increase Bcl-2 and, therefore the anti-apoptotic potential of cardiomyocytes. SAFE pathway is another branch of cardioprotective signaling. Dual activation of the RISK and SAFE pathways may induce cross-talk. NFκB pathway is also known to be involved in TNFα induced cardioprotection. It is unknown if the SAFE pathway is activated under propofol stimulation and if propofol stimulation activates cross-talk with the RISK pathway. The exact mechanism for increased Bcl-2 under propofol stimulation is also unknown. NFκB pathway may also be activated in response to propofol stimulation.

Methodology: H9c2 cells were serum-starved for 48 hours for all experiments. The effects of propofol on the SAFE pathway, namely Stat3 activation were investigated in a time-course experiment. Inhibitors, specific to the RISK (wortmannin and API-2) and SAFE (AG490 and stattic) pathways were used to identify mechanisms of cross-talk. Cells were inhibited for 30 minutes followed by 10 or 30 minutes (for phosphorylation studies) or 24 hours (for inhibitor Bcl-2 studies) stimulation with propofol. DMSO was used as a vehicle control. RNAi was used to knockdown Stat3. Effect of propofol on IκBα degradation was investigated in a time-course experiment with TNFα as the positive control. Western blot analysis was used to gauge protein phosphorylation and levels. Immunofluorescence was used to observe nuclear localization of NFκB in response to propofol stimulation.

Results: Propofol activated the SAFE pathway through increased phosphorylation of Stat3 at both Tyr705 and Ser727 residues. Propofol also activated cross-talk between the RISK and

SAFE pathways. Propofol modulation showed a trend towards increased Bcl-2. Formation of Bcl-2 was blocked by PI3K, Jak2, and Stat3 inhibition. Inhibition was relieved after addition of propofol. Stat3 knockdown was counteracted by an increase in NFκB (p65). Propofol increased degradation of IκBα but did not increase NFκB nuclear localization, which instead, showed peri-nuclear accumulation.

Conclusion: Propofol activates the SAFE pathway and stimulates its cross-talk with the RISK pathway. Propofol modulation of Bcl-2 utilizes RISK and SAFE pathways. Propofol increases IκBα degradation but not NFκB nuclear localization.

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List of Abbreviations or Symbols:

ACBP	**aortocoronary bypass**
SV	**Saphenous vein**
I/R	**ischemia/reperfusion**
RI	**reperfusion injury**
LVEF	**left ventricular ejection fraction**
ROS	**reactive oxygen species**
H_2O_2	**hydrogen peroxide**
$O2^{\cdot -}$	**superoxide radical**
$OH^{\cdot -}$	**hydroxyl radical**
$ONOO^-$	**peroxynitrite**
SOD	**superoxide dismutase**
GSHPx	**glutathione peroxidase**
ET-1	**endothelin-1**
GIK	**glucose-insulin-potassium**
IIT	**intensive insulin therapy**
IP	**ischemic preconditioning**

ICU	**intensive care unit**
TNFα	**tumor necrosis factor α**
PI3K	**phosphoinositide 3-kinase**
AKT	**AKT**
MAPK	**mitogen-activated protein kinase**
ERK	**extracellular signal-regulated kinase**
BHT	**butylated hydroxytoluene**
MDA	**malondialdehyde**
NO	**nitric oxide**
Bcl-2	**B-cell lymphoma 2**
Bax	**Bcl-2-associated X protein**
HUVEC	**human umbilical vein endothelial cell**
eNOS	**endothelial nitric oxide synthase**
RISK	**reperfusion injury salvage kinase**
HO-1	**heme oxygenase-1**
SAFE	**survivor activating factor enhancement**
mTOR	**mammalian target of rapamycin**

JAK2	**janus kinase 2**
STAT3	**signal transducer and activator of transcription 3**
mTORC2	**mammalian target of rapamycin complex 2**
PIP2	**phosphatidylinositol 4,5-bisphosphate**
PIP3	**phosphatidylinositol 3,4,5-triphosphate**
PDPK1	**3-phosphoinositide dependent protein kinase-1**
SH2	**src homology 2**
ETC	**electron transport chain**
mPTP	**mitochondrial permeability transition pore**
PTEN	**phosphatase and tensin homologue on chromosome 10**
ΔΨm	**resting mitochondrial membrane potential**
APAF 1	**apoptotic protease activating factor 1**
MOMP	**mitochondrial outer membrane pore**
CREB	**cAMP response element-binding**
NFκB	**Nuclear factor kappa-light-chain-enhancer of activated B cells**
PKCζ	**protein kinase C ζ**
IκBα	**nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α**

DMEM	**Dulbecco’s modified Eagle's medium**
EGF	**endothelial growth factor**
PBS	**phosphate buffered saline**
BSA	**bovine serum albumin**
FBS	**fetal bovine serum**
NGS	**normal goat serum**
siRNA	**small interfering RNA**
JNK	**c-Jun NH2 kinase**
LPS	**lipopolysaccharide**
CDK1	**cyclin-dependent kinase 1**

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I would like to first thank my **parents** who first exposed me to science, and since that initial spark I hope I have carried on well on my own. They also gave me freedom to develop myself and mark my own path for which I thank them very much. The rest of my **family** has been of great comfort and a good support to fall back on. I am very grateful for having them in my life.

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I will always try to abide by a few rules, some of those are:

"If you can't explain it simply, you don't understand it well enough." **Albert Einstein**.

"Never give up, never surrender." **Galaxy Quest (1999)**.

"Let's Do This!" **Animal.**

1. Introduction

1.1 Ischemic Heart Disease and Treatments

1.1.1 Ischemic Heart Disease and the Need for ACBP Surgery

Ischemic heart disease is the leading cause of mortality globally[1] and accounts for approximately 7.25 million deaths (12.8%) annually. Risk factors for ischemic heart disease include hyperlipidemia[2], hyperinsulinemia[3], diabetes[2], smoking[4], and obesity[5] among others. Surgical treatment for ischemic heart disease caused by atherosclerosis includes revascularization of the distal artery through aortocoronary bypass (ACBP) surgery. The surgical aim is to bypass atherosclerotic blockages and restore blood flow to the myocardium using the patient's leg vein or internal mammary artery for "bypass grafts".

Fully, one third of patients presenting for ACBP grafting suffer from diabetes mellitus. Patients with diabetes are also 24% more likely to be re-admitted post-operatively for adverse cardiac events, including angina, heart failure, and re-infarction[6]. Saphenous vein (SV) conduits from diabetic patients, which are regularly used as grafts for ACBP surgery, show impaired vasorelaxation and intimal degenaration[7]. Current pharmacological therapies to reduce mortality and adverse outcomes have shown to be insufficient for the diabetic population.

1.1.2 ACBP Surgery and Inherent Dangers

ACBP surgery prevents the progression of risk of myocardial infarction that may occur due to progression of atherosclerosis. The decision to pursue surgical management is taken when pharmacological management of the ischemic disease is deemed insufficient or

inadequate to protect against this risk. However, there are inherent dangers associated with ischemia-reperfusion which accompanies this surgery. Collectively, ischemia/reperfusion (I/R) injury may result in permanent loss of terminally differentiated cardiomyocytes. Interestingly, approximately 25% of deaths following ACBP surgery can be attributed to reperfusion injury[8] (RI) alone. RI is clinically characterized by an increased incidence of sudden ventricular arrhythmias[9], ventricular dysfunction or failure (myocardial stunning)[9]secodnary to no-reflow phenomenon[9] or vascular reperfusion injury[10], and lethal reperfusion injury[9]. Of the four, lethal reperfusion injury causes irreversible cell death in the form of an infarct. Cell death from reperfusion injury is apoptotic[11] and is clinically correlated with decreased left ventricular ejection fraction (LVEF). Lethal reperfusion injury causes irreversible cell death in the form of an infarct.

The cause of lethal reperfusion injury is complex and may be attributed to the "*oxygen paradox*". The re-introduction of oxygen during reperfusion of the myocardium leads to paradoxic, enzymatic driven, generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot -}$), and the hydroxyl radical ($OH^{\cdot -}$) that contribute to cardiomyocyte damage. Formation of ROS also stimulates production of other destructive molecules such as, peroxynitrite ($ONOO^-$)[12] during reperfusion. Peroxynitrite can have deleterious effects on the reperfused myocardium[13]. ROS mediated oxidative stress can be counteracted by overexpression of anti-oxidant enzymes such as, superoxide dismutase (SOD) or glutathione peroxidase (GSHPx), which show beneficial effects in rat hearts exposed to I/R injury[14] and mice hearts following myocardial infarction[15], respectively. Injury results when there is an imbalance in favor of generation of radicals to confer cell damage and mediate IRI. Treatments and interventions designed to lower or attenuate RI

have focused on prevention or reversal, but have proven inadequate in the diabetic heart. Given the poorer post-operative outcomes that diabetics suffer from ACBP surgery, treatments to lower RI are of paramount importance to this population.

1.1.3 Specific Challenges in Diabetes

Diabetes provides a challenging framework in ACBP patients in that it is associated with significantly higher risk of post-operative complications and mortality[16]. Several factors may be attributed to this increased risk including, significantly high levels of the vasoconstrictor endothelin-1 (ET-1) at the coronary sinus after ACBP surgery[17], impaired vasorelaxative properties of the vein graft[7], and high levels of serum adhesion molecules[18] which may accommodate infiltration of inflammatory response cells. Past attempts to improve patient outcome post-operatively have not shown encouraging results. Such interventions included administration of steroids to lower heightened inflammatory responses[19] and glucose-insulin-potassium (GIK) infusion therapy[20,21]. Peri-operative infusion of insulin for better glucose control and to reduce hyperglycemia has also undercome questioning due to reported incidences of increased non-fatal cardiac events[22] and increased mortality after intensive insulin therapy (IIT)[23].

As mentioned above, generation of ROS during reperfusion can contribute to reperfusion injury. Scavenging of excess ROS can be a cardioprotective strategy. However, antioxidant therapy, such as through administration of vitamin E, was insufficient in protecting non-diabetic patients from cardiovascular disease[24] and failed to reduce myocardial injury after ACBP surgery[25]. Therefore, it may be postulated that anti-oxidant defenses may be insufficient at protecting diabetics from ROS mediated damage. To date,

there are no clinical trials that measure post-operative efficacy of anti-oxidant treatment in diabetics undergoing ACBP surgery.

1.1.4 Treatments against Reperfusion Injury: Ischemic Preconditioning

The reduction of myocardial IRI is a therapeutic goal and stimulated the search for therapeutic modalities to alleviate the risks. The most potent form of preventive therapy was developed by Murry *et al.* in 1986 and termed ischemic preconditioning[26]. Utilizing an experimental model of myocardial infarction caused by using circumflex artery occlusion Murry *et al.* found that short intervals of ischemia and reperfusion significantly lowered infarct size in the dog myocardium. The process was termed preconditioning as it occurred prior to the ischemic episode: the occlusion of the circumflex artery.

Ischemic preconditioning (IP) on non-diabetic patients undergoing ACBP surgery shows statistically significant post-operative improvement including lowered incidences of ventricular arrhythmias[27], decreased myocardial necrosis as measured by mean serum troponin T levels[28], decreased myocardial stunning[29], and shorter intensive care unit (ICU) stays[30].

1.1.5 Ischemic Preconditioning of the Diabetic Myocardium

As previously mentioned, diabetic myocardium provides significant, multi-factorial challenges in patient recovery from ACBP surgery compared to non-diabetic patients. To date no clinical trials are available investigating the protective effects of IP during ACBP surgery on diabetic patients. Research in animal models has shown that diabetes abolishes the protective effects of IP[31–33] on I/R injury. This is discussed in greater detail in sections 1.3.2 and 1.3.3.

1.2 Novel Therapies for Preconditioning: Pharmacological Preconditioning

Novel strategies to induce preconditioning have shown promise; more specifically, administration of pharmacological agents prior to the ischemic episode - adequately termed pharmacological preconditioning. Various pharmacological compounds such as, adenosine[34,35], tumor necrosis factor (TNF) α[36], resveratrol[37], and, interestingly, anesthetics such as sevoflurane[38] and propofol[39] have been shown to protect the myocardium to a similar extent as IPC in animal models.

Pharmacological preconditioning may be easier and perhaps safer to administer than classical preconditioning. Myocardium of patients undergoing bypass surgery is already under levels of ischemia due to the narrowing of the coronary arteries. Further ischemic episodes, such as through IP, may further exacerbate the stressed myocardium and confer cardiac damage. Pharmacological preconditioning typically involves application of protective agents via inhalation or intravenous routes of administration without risk of further disruption of coronary artery flow.

1.2.1 Pharmacological Preconditioning through Anesthetic Drugs

As previously mentioned, anesthetic drugs can be used to precondition the heart. Cason *et al.*[40] showed that volatile anesthetic isoflurane can protect rabbits against simulated I/R injury. Isoflurane preconditioning decreased infarct size suffered through I/R injury and this effect was comparative to IP. Other volatile anesthetics, including sevoflurane and desflurane [41], have been shown to mimic pre-conditioning. However, the diabetic myocardium is resistant to pharmacological conditioning by volatile anesthetics[42–44,]. Resistance to pharmacological conditioning may be due to the effects of hyperlipidemia, hyperglycemia, underlying condition of the diabetic state, or patient medications like

glyburide, a K_{ATP} channel blocker through which volatile anesthetics mediate cardioprotection and/or the effect of the diabetic condition on cellular pathways such as, hyperglycemia as indicated by Kehl *et al*[45]. Inability of volatile conditioning to confer cardioprotection can be attributed to the impairment of the phosphoinositide 3-kinase (PI3K)/AKT[46] and mitogen-activated protein kinase kinase (MAPKK)/extracellular signal-regulated kinase (ERK)1/2[46] signaling pathways, which seem to characteristic of the diabetic heart. Therefore, a need for new therapeutic approaches to protect the diabetic myocardium against I/R injury currently exists.

1.2.2 Intravenous Anesthetic Propofol may Confer Preconditioning

Propofol (2,6-diisopropylphenol) is an IV anesthetic with cardioprotective properties[47,48] believed to be based on its antioxidant scavenging mechanism of action[49]. Mussachio *et al.* first compared propofol's anti-oxidant capabilities to the anti-oxidant butylated hydroxytoluene (BHT)[50]. Both compounds share structural similarity including a phenolic group. Mussachio *et al.* showed that propofol reduced malondialdehyde (MDA) concentration and this decrease was comparative to BHT. MDA is a product of lipid peroxidation and therefore propofol could be cytoprotective by attenuating lipid peroxidation under oxidative stress. Further studies compared propofol to the potent anti-oxidant α-tocopherol (vitamin E)[51]. Similar to propofol, vitamin E contains a phenol group that is responsible for its anti-oxidant activity. Murphy *et al*., using electron spin resonance spectroscopy, showed that propofol's anti-oxidant mechanism was similar to that of vitamin E[52], namely propofol attenuated lipid peroxidation by scavenging lipid peroxide radicals and therefore prevented peroxide propagation and peroxide mediated cell damage. Subsequent studies implicated that propofol may be able to replace vitamin E in clinical situations where

lipid peroxidation conferred damage[51]. Propofol was also shown to significantly reduce 15-F_{2t}-isoprostane generation, a marker of lipid peroxidation during ischemia, and preserve left ventricular function after I/R injury in mature rat hearts[53].

Kokita *et al.*[48] investigated whether propofol's anti-oxidant properties could also be expanded to the heart to counter oxidative damage. Propofol attenuated H_2O_2 mediated oxidative damage and decreased MDA production in isolated rat hearts. This effect was dose-dependent. Ansley *et al.* expanded on previous *in vitro* studies and showed that high dose propofol increased red blood cell antioxidant capability in patients undergoing cardiopulmonary bypass surgery[49]. This effect was greater than that of isoflurane. Propofol's cytoprotective properties increased antioxidant capability and decreased lipid peroxidation of the myocardium against I/R injury in rat hearts[54]. Propofol also conferred dose-dependent anti-apoptotic activity under TNFα mediated apoptosis in human umbilical vein endothelial cell (HUVEC) cells[55], with high dose propofol conferring the greatest protection. Anti-apoptotic potential against TNFα was related to an increase in nitric oxide (NO) production and increased B-cell lymphoma 2 **(**Bcl-2) and decreased Bcl-2-associated X protein (Bax) production.

The anti-apoptotic and cardioprotective actions of propofol may not be solely due to its anti-oxidant properties alone. Propofol enhances cellular protection against apoptotic and oxidative stimuli through increased protein expressions of Bcl-2[55,56] and endothelial nitric oxide synthase (eNOS)[57], respectively. We have shown that propofol also activates the PI3K/Akt branch of the reperfusion injury salvage kinase (RISK) pathway, in a time dependent manner, to avert apoptotic death from oxidative stress through H_2O_2[56]. Propofol also increases levels of heme oxygenase-1 (HO-1)[58] in newborn rat cardiomyocytes, which

activates PI3K/Akt signaling[59]. Taken together, propofol may act via a novel mechanism, namely the induction of pro-survival cell signaling, to confer cardioprotection.

The protective effects of high dose propofol in type 2 diabetic patients undergoing ACBP surgery are currently being investigated under the PRO-TECT II trial[60]. Results from this study will be interesting compared with clinical trials that showed the anti-oxidant vitamin E failed to protect myocardium from I/R injury[25].

1.3 Cardioprotection through preconditioning involves activation of pro-survival signaling pathways

1.3.1 Role of Signal Induction in Cardioprotection

Cellular protective mechanisms activated under pre-conditioning include two major pathways, the Reperfusion Injury Salvage Kinase[61] (RISK) and the Survivor Activating Factor Enhancement[62] (SAFE). Components of the RISK pathway include activation of the PI3K/AKT, ERK1/2, and mammalian target of rapamycin (mTOR) kinases. The research of this thesis focuses on detailed examination of the PI3K/Akt branch of the RISK pathway. Components of the SAFE pathway include activation of the janus kinase (JAK) 2/ signal transducer and activator of transcription (STAT) 3 signaling pathway. Various conditioning methods activate one or both pathways, as shown in **Table 1.1**.

Table 1.1 Effect of Ischemic or Pharmacologic conditioning on RISK-SAFE pathway activation.

	RISK	**SAFE**
Ischemic pre-conditioning	yes[63]	yes[64]
Ischemic post-conditioning	yes[65]	yes[66]
Pharmacological Pre-Conditioning		
TNFα	no[36]	yes[36]
Adenosine	yes[67]	yes[68]
IV anesthetics		
Propofol	yes[56]	?
Volatile anesthetics		
Isoflurane	yes[69]	yes[70]
Desflurane	yes[41]	

1.3.2 RISK and SAFE pathways

Conditioning stimulus activates pro-survival signaling of the RISK and SAFE pathways. Activation of the RISK pathway includes phosphorylation and activation of AKT at both Serine 473 and Threonine 308 residues. As seen in **figure 1**, membrane bound AKT is initially phosphorylated at Ser473 by a multi-protein complex called, mammalian target of rapamycin complex (mTORC) 2. Subsequent steps in AKT phosphorylation occur after ligand-receptor interaction leads to the activation of PI3K, that in turn, phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 acts as a docking site for PI3K and 3-phosphoinositide dependent protein kinase-1 (PDPK1 aka PDK1), both of which bind PIP3 via their pleckstrin homology

domains. Subsequent phosphorylation of AKT then occurs at the Thr308 residue by the constitutively active PDK1. Fully phosphorylated AKT is involved in activating various cell proliferation and cell survival pathways and is therefore an important target for conditioning therapy. Studies abolishing AKT phosphorylation through various pharmacological inhibitors[63] (e.g. wortmannin or LY290042) or studies that alternatively over-express constitutively activated AKT in animal models[71] have highlighted the importance of AKT activation/activity in protection against I/R injury. AKT serves as a focal point for protection through the RISK pathway.

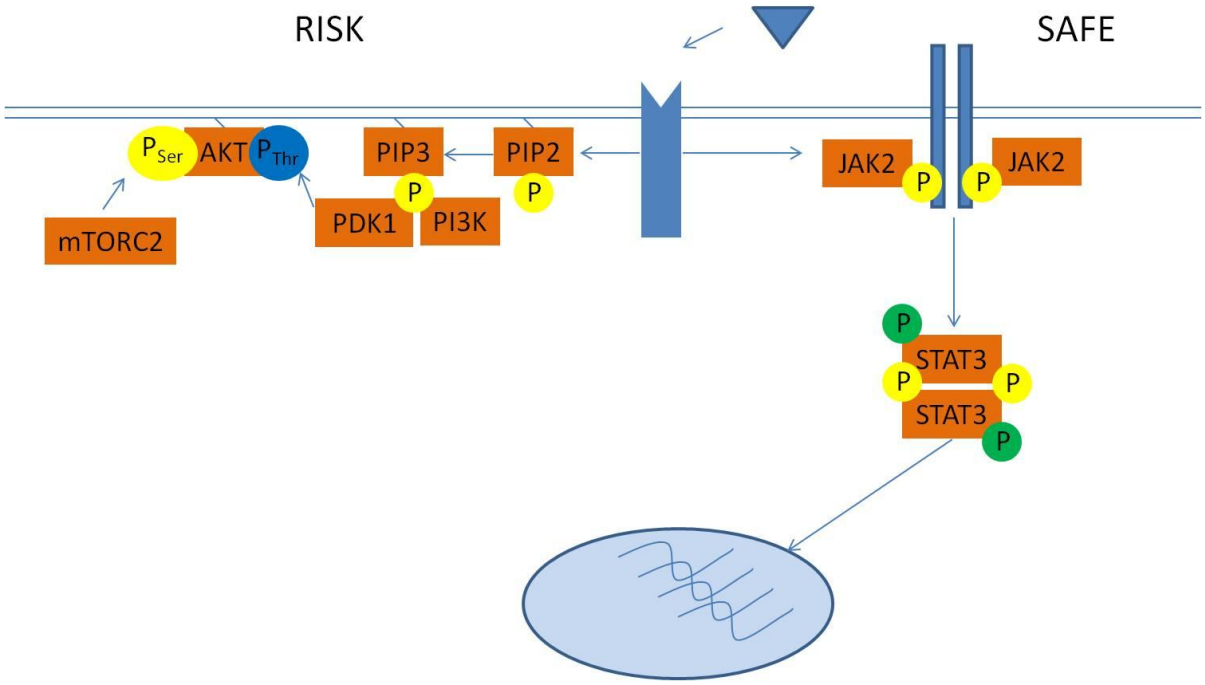


Figure 1. Activation of RISK and SAFE pathways. Activation of the RISK pathway involves phosphorylation of AKT by mTORC2 at Serine 473 and by PDK1 at Threonine 308. Signaling is initiated at the receptor and involves activation of PI3K which phosphorylates PIP2 to generate PIP3. PIP3 acts as an anchor for PDK1, which binds PIP3 via its pleckstrin homology domain. Activation of the SAFE pathway involves ligand mediated activation of a receptor tyrosine kinase. Auto-trans-phosphorylation of tyrosine residues of the cytoplasmic domain of the receptor allows JAK2 binding. JAK2 then further phosphorylates tyrosine residues on the receptor, which allows other proteins with src homology 2 (SH2) domains such as, STAT3 to bind. JAK2 then phosphorylates STAT3 at the Tyrosine 705 residues. Phosphorylated STAT3 dimerizes and translocates into the nucleus to activate transcription of target genes. STAT3 can also be phosphorylated at Serine727.

SAFE pathway is another branch of protective cell signaling activated in the cardiomyocyte during conditioning. Activation of the SAFE pathway (**figure 1**) results in phosphorylation of the signal transducer and activator of transcription (STAT) 3 transcription factor. STAT3 is activated through phosphorylation of its Tyrosine705 residue by Janus kinase (JAK) 2. Conditional cardiac specific knockout of STAT3[68] or inhibition of JAK2 by AG490[64] abolishes conditioning mediated cardioprotection. Pre-conditioning is also compromised in aged mice[72] as they have lower levels of cardiac STAT3. Recently it was shown that mitochondrial localized STAT3 phosphorylated at the serine727 residue played an important role in the electron transport chain (ETC) as a component of complex I and II[73]. Mitochondrial localized Ser727-STAT3 may also play a role in prevention of the mitochondrial permeability transition pore (mPTP) opening[74]; a mitochondrial pore between the intermembrane space and the matrix of the mitochondria. However, the exact mechanism of protection remains unknown.

Components of both RISK and SAFE pathways are activated through kinase interactions at the membrane. Both pathways play a protective role in cell survival during I/R injury. Therefore, there may be a requisite for cross-talk between the two pathways. As seen in **Table 1** this may indeed be the case with certain conditioning stimuli such as through, classical IP or adenosine. Another example of RISK-SAFE cross-talk may be found in the paper published by Drenger *et al.* where the authors showed that diabetic mice conditioned with sevoflurane showed impaired cardioprotection and STAT3 phosphorylation after treatment with PI3K inhibitor wortmannin[43]. Pathway cross-talk adds an element of complexity to an already complex picture of cardioprotective cell signaling. Cross-talk highlights the redundancy between signaling pathways that may guarantee cell survival

against a variety of cell stressors. The mechanism of cross-talk between RISK and SAFE pathways during conditioning has not been fully elucidated.

1.3.3 Diabetes Abolishes Cardioprotection through inhibition of RISK and SAFE pathways

Activation of the RISK and SAFE pathways in non-diabetic animals provides the necessary cardioprotection against I/R injury in animal models. However, in diabetes these pathways are not activated[75,76] in the same manner as wild-type/non-diabetic animals. Therefore protective mechanisms such as IP are not available to confer cardioprotection of the ischemic reperfused diabetic myocardium.

Diabetes confers an inhibition on the RISK pathway which increases resistance to preconditioning stimuli. Phosphorylation of AKT is impaired in animal diabetic models. This effect can be due to increased phosphatase and tensin homologue on chromosome 10 (PTEN) expression. We have recently showed that atrial tissue biopsies of diabetic patients contain increased expression of PTEN, the main phosphatase to AKT phosphorylation[77]. PTEN dephosphorylates PIP3 and, therefore, prevents membrane anchoring of PDK1. This action prevents PDK1-AKT interaction and thus reduces Thr308-AKT phosphorylation. Studies in diabetic animal models show impaired AKT phosphorylation[78]. However, chronic activation of AKT is detrimental and facilitates I/R injury[79].

In diabetic models, conditioning is unable to elevate STAT3 phosphorylation and therefore unable to utilize its protective effects[46]. Decreased STAT3 transcriptional activity may result in downregulation of anti-apoptotic and other cytoprotective proteins. Inhibition of STAT3 at Ser727 may also mediate formation of mPTP and exacerbate RI. Further

research needs to be done on animal models to delineate the effect of diabetes on the SAFE pathway during preconditioning.

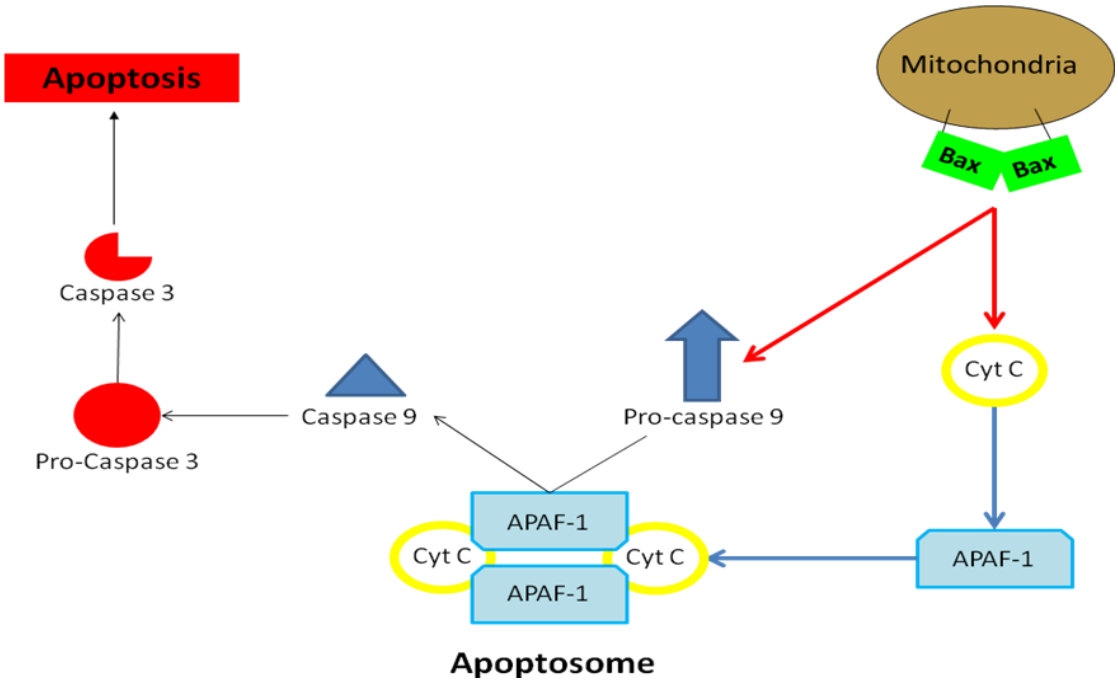
1.4 Reperfusion Injury stimuli aggregate at the Mitochondria to initiate Apoptosis

Mitochondria are the focal point of one of the three main pathways of apoptosis. mPTP is the initial event that occurs during mitochondrial mediated apoptosis. It is characterized by a drop in the resting mitochondrial membrane potential ($\Delta\Psi_m$) across the inner mitochondrial membrane. The drop in membrane potential occurs when protons escape from the intermembrane space to the matrix through the mPTP. In addition, damage to the ETC proteins due to IRI leads to escape of unpaired electrons from the ETC. These escaped electrons then react with O_2 to produce $O_2^{\cdot -}$, which can cause cellular damage, as well as, propagate formation of other ROS species. mPTP is often accelerated in diabetes, due to greater free-radical production and lower anti-oxidant availability and capabilities characteristic of the disease. Uncontrolled mPTP can drive cardiomyocyte apoptosis under RI. Inhibition of mPTP with cyclosporine A[80,81] can alleviate loss of cardiomyocytes.

During the next phase of the mitochondrial mediated apoptosis, apoptotic stimuli promote BCL-2 associated X protein (BAX) oligomerization and pore formation on the outer mitochondrial membrane. The presence of a pore allows intermembrane mitochondrial proteins such as, cytochrome c and pro-caspase 9 to translocate to the cytosol. Cytochrome c interacts with apoptotic protease activating factor (APAF) 1 in the cytosol to form the apoptosome. The apoptosome proceeds to cleave pro-caspase-9 to caspase-9 which cleaves pro-caspase 3 to form caspase-3. Caspase 3 then begins cleavage of cellular proteins which initiates cellular apoptosis. See **figure 2A** for details.

BAX oligomerization forms the mitochondrial outer membrane pore (MOMP) and plays a critical role in apoptosis. B-cell lymphoma (BCL) 2 is an anti-apoptotic protein that binds monomeric BAX and prevents BAX oligomerization and formation of the MOMP, thereby averting apoptosis, as shown in **figure 2B**. Several studies have highlighted the importance of BCL-2 in averting mitochondrial mediated apoptosis[82,83]. We have also shown that propofol mediated up-regulation of BCL-2 was effective in averting H_2O_2 mediated apoptosis[56].

A)



B)

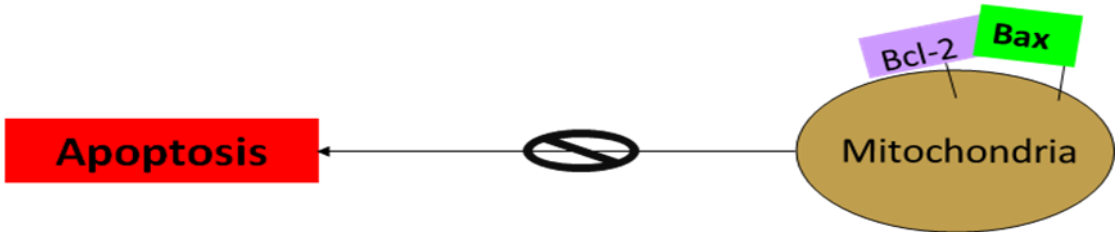


Figure 2. Mitochondrial mediated Apoptosis. A) I/R injury can lead to permeabilization of the outer mitochondrial membrane by BAX oligomerization to form the MOMP pore. Cytochrome c and pro-caspase-9 escape through this pore into the cytosol. Cytochrome c combines with APAF-1 to form the apoptosome that cleaves pro-caspase-9 into caspase-9. Caspase-9 cleaves cytosolic pro-caspase-3 into caspase-3, which initiates protein degradation and apoptosis. **B)** BCL-2 can prevent BAX oligomerization on the outer mitochondrial membrane by forming a complex with monomeric BAX. Prevention of BAX oligomerization therefore prevents cellular apoptosis.

1.4.1 Conditioning Stimuli Prevent Mitochondrial mediated Apoptosis

Cardiomyocyte apoptosis during RI is mitochondrial in origin. Several studies have shown that attenuation of the mPTP prevents apoptosis [81,84]. Indeed various preconditioning methods work through inhibition of mPTP by preventing $\Delta\Psi_m$ disruption, ROS production, and cytochrome c release in the rat heart[85].

Pharmacological conditioning is not reproduced in the diabetic myocardium with volatile anesthetics[86]. This is characterized by the inability to activate the RISK pathway of cardioprotection in the diabetic myocardium. This implies that mPTP may not be prevented in the diabetic myocardium with the use of volatile preconditioning. Interestingly, pharmacological preconditioning by propofol attenuated apoptosis by inhibiting mPTP [47,56]. Therefore, propofol may be able to prevent cardiac damage where volatile anesthetics cannot.

1.4.2 Transcriptional Increase of BCL-2

Increase in transcription of BCL-2 to prevent apoptosis is a key element of cardioprotection that is activated by both ischemic[64] and pharmacologic pre-conditioning[87]. Several transcription factors are known to be anti-apoptotic by increasing BCL-2 transcription, for example, cAMP response element binding-protein (CREB)[88,37] and STAT3[52]. Transcriptional increase of BCL-2 to counteract BAX oligomerization on the outer mitochondrial membrane is a cardioprotective strategy employed by cell signaling pathways to increase cell survival.

Nuclear factor kappa-light-chain-enhancer of activated B cells **(**NFκB) is another transcription factor involved in cardioprotection. It is activated under inflammatory stimuli such as through TNFα to transcribe pro-inflammatory genes, but has also been shown to

increase transcription of cell survival genes such as, BCL-2[89]. Therefore, its role as a protective element in the cell is controversial. In connection, Wickley *et al.* found that propofol activated protein kinase C (PKC) ζ and increased its nuclear localization in rat ventricular cardiomyocytes[90]. PKCζ is known to increase NFκB activity and may also do so under propofol stimulation. Xu *et al.* demonstrated that attenuation of NFκB activity prevented propofol mediated increase in HO-1 expression[58]. Interestingly, Lecour *et al.* have shown that low dose TNFα can be used to induce pre-conditioning[36]. Therefore, NFκB may play a role in propofol mediated preconditioning by increasing transcription of anti-apoptotic proteins, such as BCL-2.

2. Rationale, Hypothesis, and Specific Aims

Rationale

1) Propofol's cytoprotective properties are independent of its anti-oxidant mechanism. It is shown to activate the RISK pathway of cardioprotection. The SAFE pathway is also involved in cardioprotection. Propofol may be able to activate the SAFE pathway.
2) Stat3 plays a critical role in pre-/post-conditioning. Activated Stat3 also increases Bcl-2 transcription. Propofol mediated increase in Bcl-2 has been shown to be both PI3K/Akt dependent and independent. Cross-talk between the RISK and SAFE pathways to modulate Bcl-2 may be a mechanism of propofol mediated cell signaling.
3) NFκB is a transcription factor also known to increase Bcl-2 transcription. NFκB activity can be directly attributed to TNFα stimulation and low dose TNFα can be used as a pre-conditioning agent. Propofol may increase Bcl-2 transcription through the NFκB pathway.

Hypothesis:

Propofol modulates the activation and cross-talk between the RISK-SAFE pathways to modulate Bcl-2. Propofol mediated cell signaling mediates conditioning through the NFκB pathway to confer cytoprotection.

Aims:

1) To determine whether propofol increases Stat3 phosphorylation.

2) To determine if and how RISK and SAFE pathways interact upon stimulation by propofol.

3) To determine the contributions of RISK and SAFE pathways on Bcl-2 production.

4) To determine the role of propofol in the NFκB pathway.

3. Materials and Methods

3.1 Cell culture and reagents

H9c2 cells were obtained from ATCC. Cells were grown under standard conditions of 95% air/5% CO_2 at 37^{o}C in a cell culture incubator. Cells were fed with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were passaged at 70-80% confluency. For all experiments, cells were plated on 6-well plates and serum starved for 48 hours with DMEM containing 0.5% FBS. Cells were approximately 80% confluent upon experimentation, this prevented inhibition of signaling due to overpopulation and contact-mediated signaling inhibition attributed to cell cycle growth arrest. Concentrations of the inhibitors in our experiments were derived from past research AG490[91], stattic[92], and wortmannin[56]. We did not find sufficient information on API-2 and therefore conducted dose dependent experiments (data not shown). AG490 was purchased from Cayman and was diluted to a working concentration of 100µM. Stattic was purchased from Tocris and was diluted to a working concentration of 20µM. Wortmannin was purchased from Sigma Aldrich and was diluted to a working concentration of 500nM. API-2 was purchased from Tocris and was diluted to a working concentration of 10µM. Endothelial growth factor (EGF) was purchased from Sigma Aldrich and was diluted to a working concentration of 200ng/mL. TNFα was purchased from Invitrogen/Gibco and was diluted to a working concentration of 50ng/mL. DMSO was purchased from Sigma Aldrich and was diluted to compromise 0.1% of a solution e.g., as a vehicle.

3.2 Western Blot

Upon completion of the experiment cells were washed with ice-cold sterile phosphate buffered saline (PBS) and lysed with either MOSLB buffer (for detection of phosphorylated proteins) or RIPA buffer with protease inhibitors and PMSF (for Bcl-2 detection). Cell lysate then underwent 3 rounds of freeze-thaw and spun at 13,000 rpm for 15 minutes. Cell lysates were then separated from cell debris pellet. Cell lysates were then vortexed and protein concentration was measured using the Bradford protein assay. Each well was loaded with 20 µg of protein; remainder lysate was stored at -20^{o}C. To detect phosphorylated proteins samples were run on 10% acrylamide gels. To detect Bcl-2, samples were run on 12% acrylamide gels. Gels were run at 120V at room temperature. After completion, gels were transferred onto a 0.2µm nitrocellulose membrane. Gels were transferred at 100V at 4^{o}C for 1 hour. Following transfer, membranes were blocked with 5% bovine serum albumin (BSA) (for detection of phosphorylated proteins) or 5% milk (for detection of Bcl-2) with TBS-T for 1 hour at room temperature. Membranes were then probed for primary antibodies at a concentration of 1:1000 in 2.5% BSA or milk overnight at 4^{o}C. After primary antibody incubation, membranes were washed 3X10 minutes with TBST-T to remove excess antibody and then probed with secondary antibody at a concentration of 1:10,000 in 2.5% BSA or milk for 1 hour at room temperature. Following incubation membranes were again washed for 3X10 minutes and developed under a western blot imager using the Femto electrochemiluminescence (ECL) substrate. Primary antibodies mouse anti-Tyr705-Stat3, rabbit anti-Ser727-Stat3, rabbit anti-total-Stat3, rabbit anti-Thr308-Akt, rabbit anti-Ser473-Akt, rabbit anti-total-Akt, and rabbit anti-IκBα were purchased from Cell Signaling. Primary antibodies rabbit anti-p65 and mouse anti-β-actin and secondary antibodies anti-rabbit and

anti-mouse were purchased from ABM. Primary antibody rabbit anti-Bcl-2 was also purchased from Santa Cruz Biotechnology Inc and Cell Signaling. β-actin antibody was used at a concentration of 1:10,000. FluorChem and ImageJ software were used for densitometric analysis. Densitometric data is presented as relative to β-actin, unless otherwise indicated. Data was then normalized to DMSO control. Western blot development was done in Dr. Ujendra Kumar and Dr. Harley Kurata's labs. We are grateful for their continued assistance during this project.

3.3 Immunofluorescence

H9c2 cells were plated on glass coverslips coated overnight at $4^{o}C$ with poly-D-lysine at a density of $2X10^{5}$ cells/mL in a 24 well plate. H9c2 cells were then serum starved for 48 hours. Following treatment, cells were washed with ice-cold PBS and fixed in 4% paraformaldehyde for 15 minutes. Fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 15 minutes. Cells were blocked in 5% normal goat serum (NGS) in PBS for 1 hour. Primary antibody was diluted at 1:300 in 2.5% goat serum in PBS and incubated with permeabilized cells over night. The next day cells were washed 3X5minutes with PBS under gentle agitation. Secondary antibody was diluted at 1:200 in 2.5% goat serum in PBS and incubated at room temperature for 1 hour. Cells were again washed for 3X5 minutes with PBS under gentle agitation. DAPI stain was applied at a concentration of 50 nM for 5 minutes. Cells were then washed 1X5 minutes. Coverslips containing stained cells were adhered to glass slides using Fluoromount-G from Southern Biotech. Primary antibody, rabbit-anti p65 was purchased from ABM technologies. Secondary antibody, Alexa Flour-568 goat-anti rabbit was purchased from Invitrogen.

Immunofluorescence was detected using the 40X oil based objective on an Olympus fluorescence microscope. Images were captured using the Q capture Pro 6.0 software. IFC slides were visualized using Dr. Ujendra Kumar's fluorescence microscope.

3.4 siRNA Transfection

H9c2 cells were plated in 0.5% FBS serum starvation medium for 48 hours followed by overnight incubation with 33nM small interfering RNA (siRNA)-Lipofectamine 2000 complexes. Media was changed the next day and experiments were carried out.

siScramble 5'-CUUCCUCUCUUUCUCUCCCUUGUGA-3' was used as control. siRNA sequence for siSTAT3 was 5'-ACAUAGAAGCUAGGACUA-3'. siRNA was purchased from integrated DNA technologies. Lipofectamine 2000 mediated transfection was carried out as per merchant's instructions. Lipofectamine 2000 was purchased from Invitrogen.

3.5 Statistics

Microsoft Excel was used for calculations. GraphPad Prism was used for statistical analysis. One way ANOVA with Tukey's test was used to evaluate significance between more than two groups. Student's t-test was used to evaluate significance between two groups. A $p<0.05$ was seen as statistically significant. Data is presented as μ+SD.

Results from phosphorylation experiments (**figures 4 and 5**) were considered as biologically relevant if they occurred 2 out of 3 times. A representative blot and its densitometrical analysis are presented.

4. Results

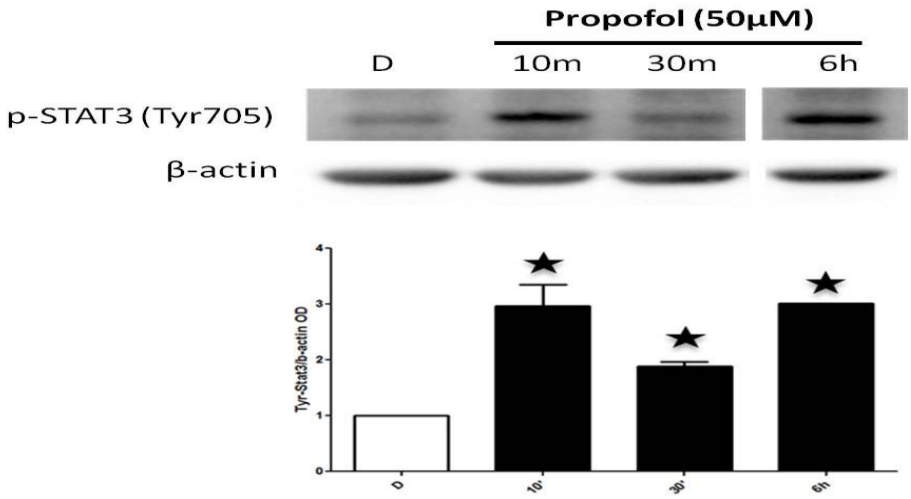
4.1 Propofol Interaction with the RISK and SAFE Pathways

4.1.1 Propofol stimulates phosphorylation of Stat3

Propofol significantly increased phosphorylation at Tyr705 at 10m, 30m, and 6h (**figure 3A**) relative to DMSO. Propofol also significantly increased phosphorylation at Ser727 at 10m and 30m but not 6h (**figure 3B**).

From these observations (**figure 3A and B)**, as well as, from our previous publication[56] where propofol significantly increased phosphorylation of Akt at 30m we chose a propofol stimulation time period of 10m, as well as, 30m for further phosphorylation experiments.

A)



B)

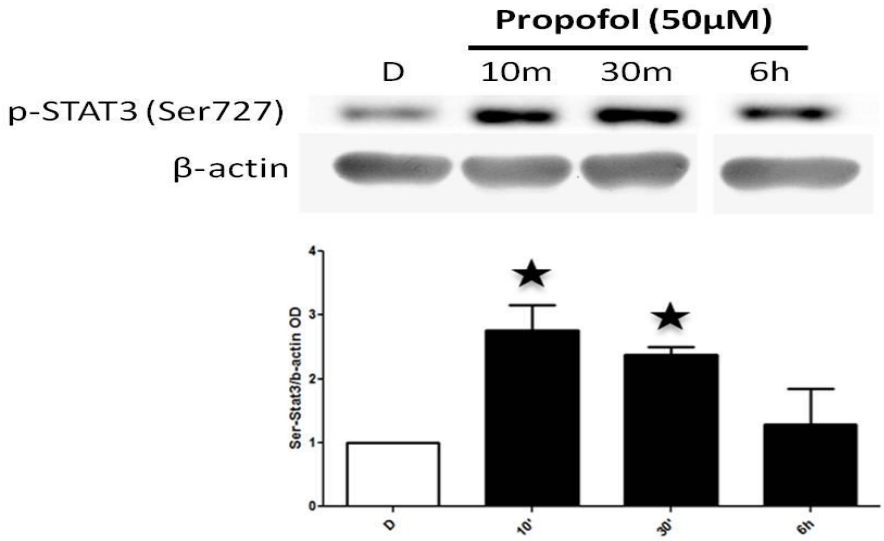


Figure 3. Propofol stimulates Stat3 phosphorylation. H9c2 cells were serum-starved in 0.5% FBS in DMEM for 48 hours and stimulated with propofol (50µM) for 10m, 30m, and 6h. β-actin was detected as loading control. **A)** Representative blot for Tyr705-Stat3 phosphorylation and densitometric analysis. **B)** Representative blot for Ser727-Stat3 phosphorylation and densitometric analysis. *$p<0.05$ vs. D. n=2. Data for analysis provided by Dr. Baohua Wang and Marijana Pavlovich.

4.1.2 Inhibition of RISK and SAFE Pathways Show Cross-Talk: 10 minute propofol stimulation

To distinguish cross-talk between RISK and SAFE pathways under propofol stimulation, pathway specific inhibitors were used. Cross-over pathway inhibition was treated as indicative of cross-talk. For example, phosphorylation changes in RISK pathway following inhibition of SAFE pathway were considered indicative of cross-talk. To explore the temporal effect on pathway activation, cells were stimulated with DMSO or propofol for 10 minutes or 30 minutes (next section).

Changes in Stat3 phosphorylation indicated SAFE pathway activity and inhibition. Similarly, changes in Akt phosphorylation indicated RISK pathway activity and inhibition.

We discuss the 10 minute stimulation (**figure 4**) changes first followed by 30 minute stimulation (**figure 5**) changes.

Western blots from phosphorylation inhibition experiments are representative of two out of three independent experiments. The most representative blot and its densitometry result are presented (**figures 4 and 5)**. Results are considered significant when an increase or decrease was seen as occurring at least two out of three times.

Ser727-Akt

Propofol did not stimulate an increase in phosphorylation when compared to the DMSO control group (**figure 4A**). Inhibition from SAFE pathway inhibitors did not show a decrease in phosphorylation. Phosphorylation of the Ser727 residue decreased with RISK pathway inhibitors, alone or in combination with propofol (W, A2, PW, and PA2).

Thr308-Akt

Propofol did not stimulate an increase in phosphorylation when compared to the DMSO control group (**figure 4B**). Inhibition from SAFE pathway inhibitor AG490 did not decrease phosphorylation (AG and PAG). However, stattic, which directly inhibits Stat3, decreased phosphorylation without and with propofol (St and PSt). Phosphorylation of the Thr308-Akt residue was decreased with RISK pathway inhibitors alone or in combination with propofol (W, A2, PW, and PA2). No significant changes were observed with t-Akt**, figure 4C**.

Tyr705-Stat3

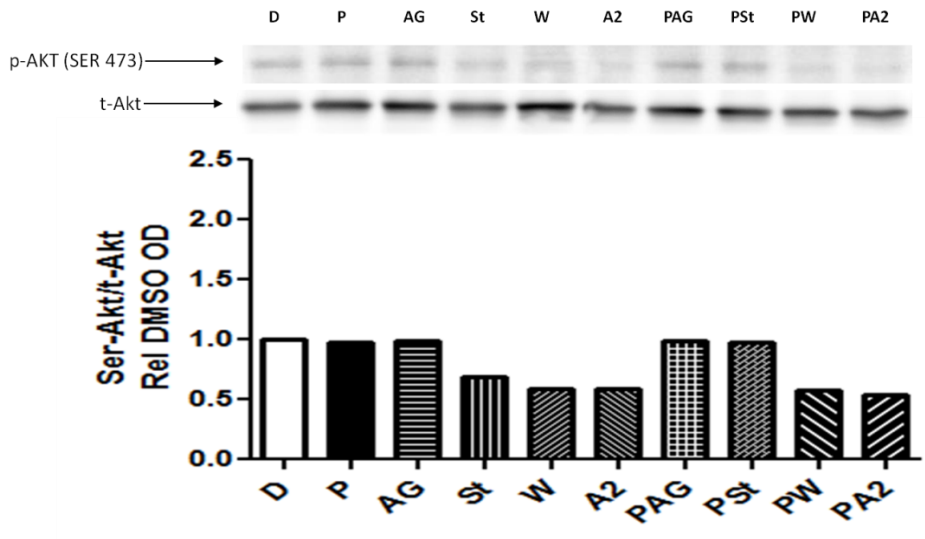
Propofol did not stimulate an increase in phosphorylation when compared to the DMSO control group (**figure 4D**). SAFE pathway inhibitor AG490 did not decrease phosphorylation with or without propofol (AG and PAG). SAFE pathway inhibitor stattic lowered phosphorylation in absence of propofol (St). Addition of propofol (PSt) increased phosphorylation to levels that were still below DMSO control. Phosphorylation of the Tyr705 residue decreased with the RISK pathway inhibitor wortmannin, alone and in combination with propofol (W and PW). RISK pathway inhibitor API-2, however, did not decrease phosphorylation (A2 and PA2).

Ser727-Stat3

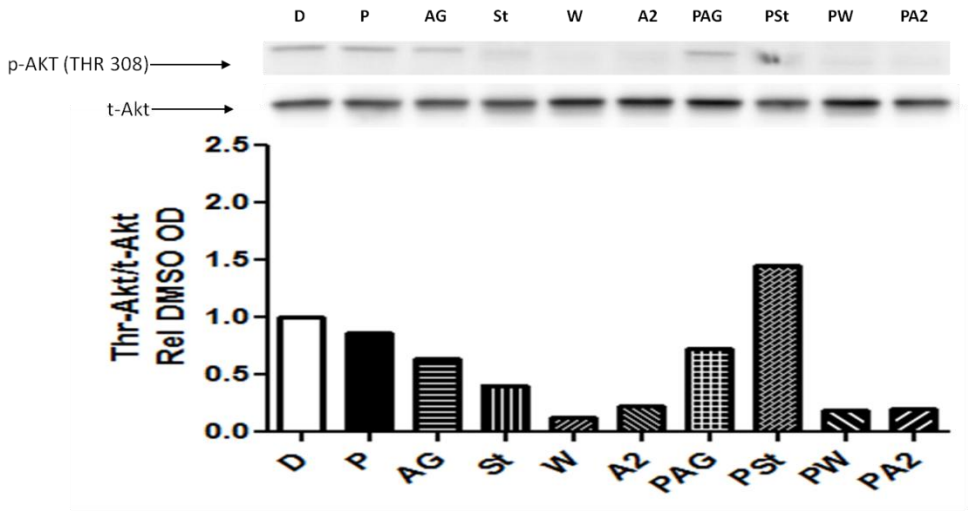
Propofol did not stimulate an increase in phosphorylation when compared to the DMSO control group (**figure 4E**). Phosphorylation of the Ser727 residue did not decrease with either SAFE or RISK pathway inhibitors alone or in combination with propofol (PAG, PSt, PW, and PA2). As seen in **figure 4F**, no significant changes were observed with t-Stat3.

Results for the above experiments are summarized in **figure 4G**.

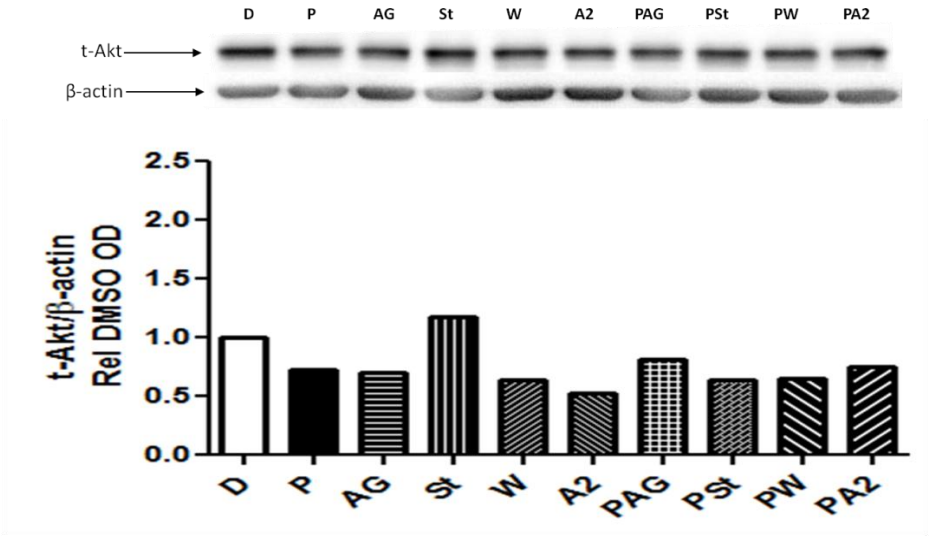
A)



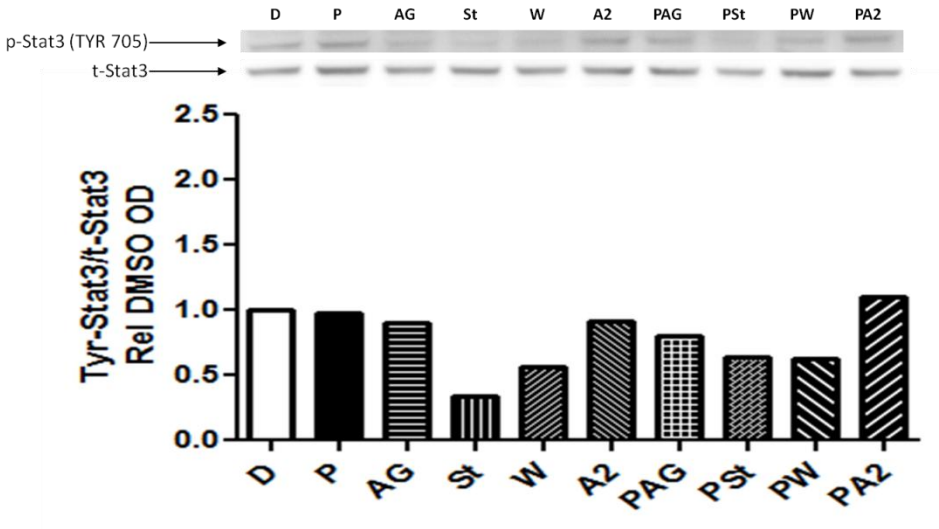
B)



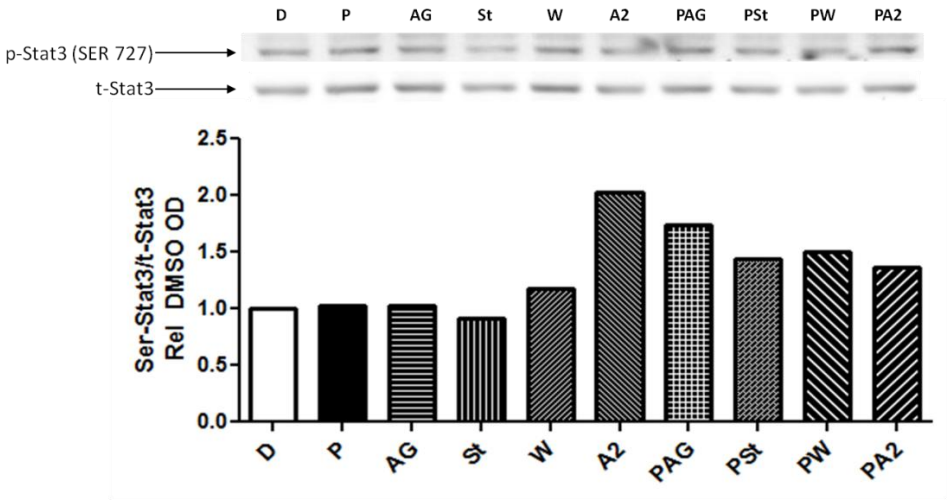
C)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
t-Akt
β-actin
t-Akt/β-actin
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

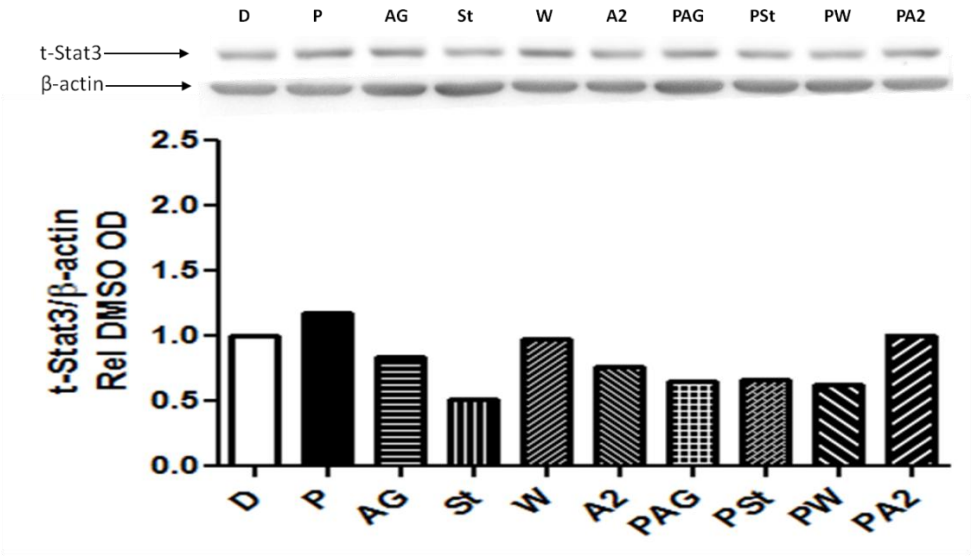
D)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
p-Stat3 (TYR 705)
t-Stat3
Tyr-Stat3/t-Stat3
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

E)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
p-Stat3 (SER 727)
t-Stat3
Ser-Stat3/t-Stat3
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

F)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
t-Stat3
β-actin
t-Stat3/β-actin
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

G)

10 minute	D	P	AG	St	W	A2	PAG	PSt	PW	PA2
Ser473-Akt	-	-	-	-	↓	↓	-	-	↓	↓
Thr308-Akt	-	-	-	↓	↓	↓	-	↓	↓	↓
Tyr705-Stat3	-	-	-	↓	↓	-	-	↓	↓	-
Ser727-Stat3	-	-	-	-	-	-	-	-	-	-

Figure 4. Inhibition of RISK and SAFE pathways reveals propofol mediated cross-talk: 10 minute propofol stimulation. H9c2 cells were serum-starved for 48h. Cells were then inhibited with RISK or SAFE pathway inhibitors for 30 minutes followed by **10 minutes** of stimulation by 50µM propofol (P) (groups PAG, PSt, PW, PA2). DMSO (D) was used as vehicle control. Representative blot and corresponding densitometrical analysis are shown. Inhibitors were used at following concentrations: AG (AG490, 100µM), St (stattic, 20µM), W (wortmannin, 500nM), and A2 (API-2, 10µM). Groups with inhibitors plus propofol are shown as following: PAG (AG490 + propofol), PSt (stattic + propofol), PW (wortmannin + propofol), and PA2 (API-2 + propofol). **A)** Phosphorylation of Ser473-Akt and densitometric analysis. **B)** Phosphorylation of Thr308-Akt and densitometric analysis. C**)** t-Akt and densitometric analysis. **D)** Phosphorylation of Tyr705-Stat3 and densitometric analysis. **E)** Phosphorylation of Ser727-Stat3 and densitometric analysis. **F)** t-Stat3 and densitometric analysis. **G)** Experimental conclusions. (-) depicts no change. ↓ depicts decrease in phosphorylation when compared to P. The presented blot represents the typical results in at least two out of three independent trials. Representative blot and its densitometry data are presented. n=3.

4.1.3 Inhibition of RISK and SAFE Pathways Show Cross-Talk: 30 minute propofol stimulation

Ser473-Akt

The phosphorylation of Ser 473 Akt at 30 min increased in response to propofol when compared to the DMSO control group (**figure 5A**). Inhibition from SAFE pathway inhibitors decreased phosphorylation, alone or in combination with propofol (AG, St, PAG, and PSt). Phosphorylation of the Ser473 residue decreased with RISK pathway inhibitors, alone or in combination with propofol (W, A2, PW, and PA2).

Thr308-Akt

The phosphorylation of Ser 473 Akt at 30 min increased in response to propofol when compared to the DMSO control group (**figure 5B**). Inhibition from SAFE pathway inhibitors lowered phosphorylation alone or in combination with propofol (AG, St, PAG, and PSt). Phosphorylation of the Thr308 residue was decrease with RISK pathway inhibitors alone or in combination with propofol (W, A2, PW, and PA2). As seen in **figure 5C**, no significant changes were observed with t-Akt.

Tyr705-Stat3

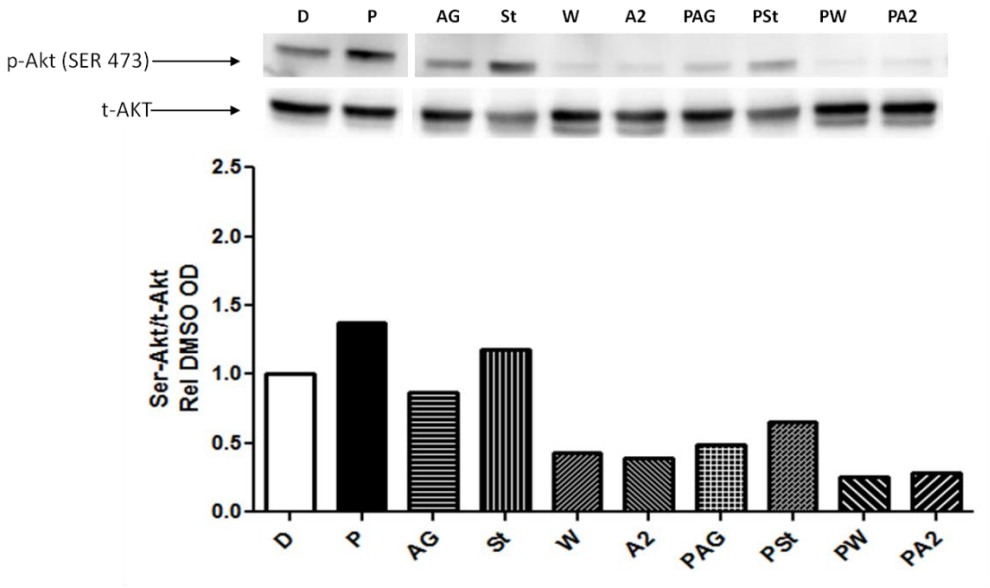
The phosphorylation of Ser 473 Akt at 30 min increased in response to propofol when compared to the DMSO control group (**figure 5D**). Inhibition from SAFE pathway inhibitors lowered phosphorylation only in combination with propofol (PAG and PSt). Phosphorylation of the Tyr705 residue decreased with RISK pathway inhibitors in combination with propofol (PW and PA2). This effect was greater than with RISK inhibitors alone (W and A2).

Ser727-Stat3

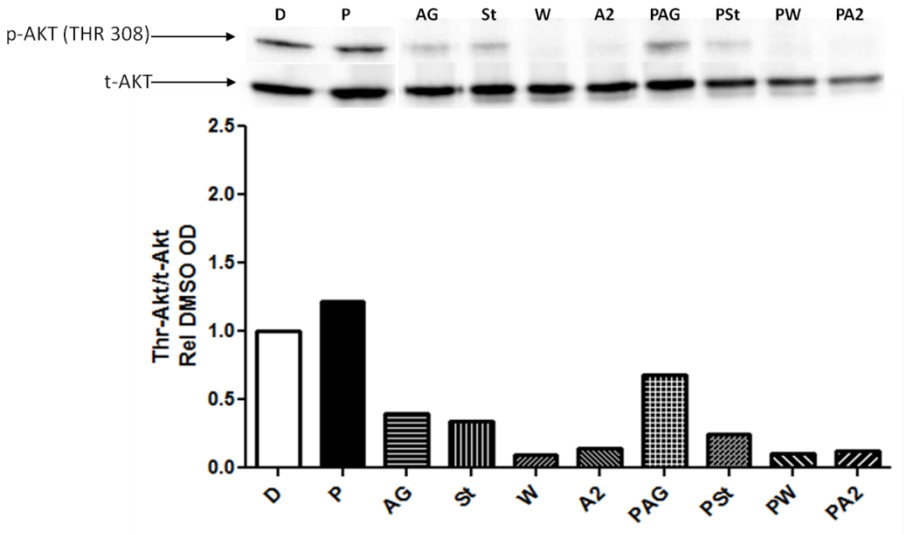
The phosphorylation of Ser 473 Akt at 30 min increased in response to propofol when compared to the DMSO control group (**figure 5E**). Phosphorylation of the Ser727 residue decreased with RISK pathway inhibitors alone and in combination with propofol (W, A2, PW, and PA2). Inhibition from SAFE pathway inhibitors did not decrease phosphorylation alone or in combination with propofol (AG, St, PAG, and PSt). As seen in **figure 5F**, no significant changes were observed with t-Stat3.

Results for the above experiments are summarized in **figure 5G**.

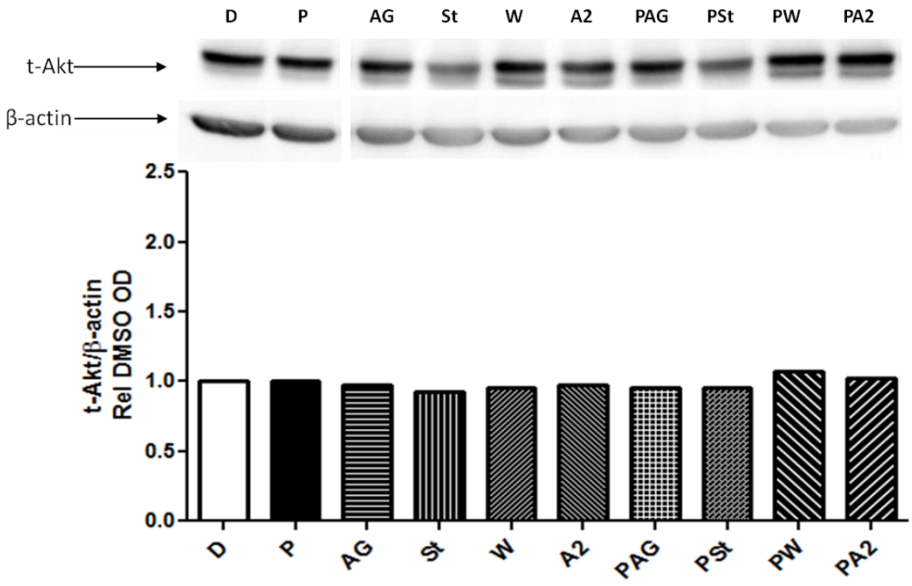
A)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
p-Akt (SER 473)
t-AKT
Ser-Akt/t-Akt
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

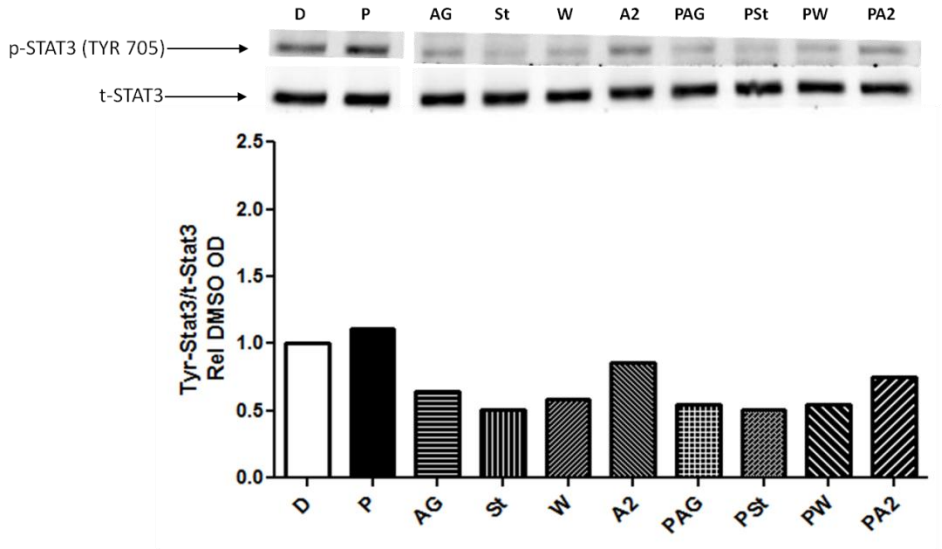
B)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
p-AKT (THR 308)
t-AKT
Thr-Akt/t-Akt
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

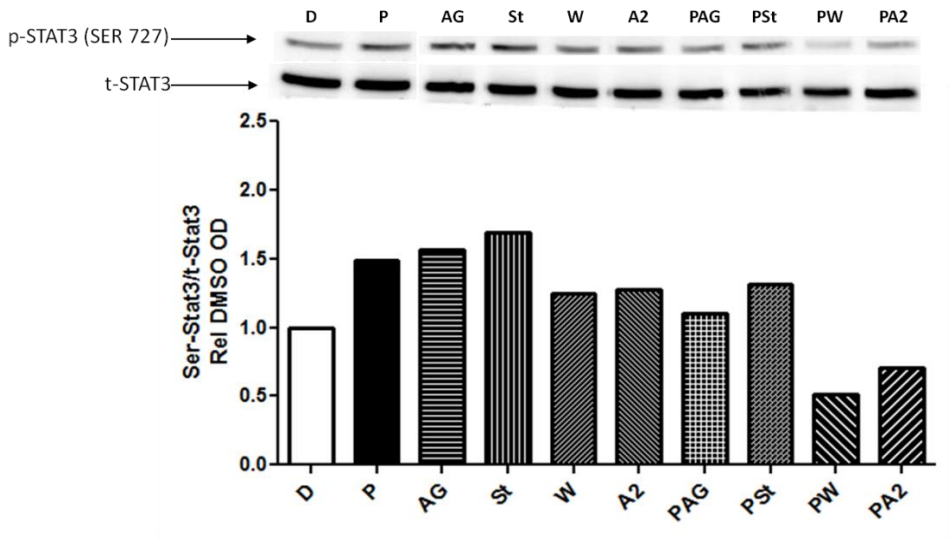
C)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
t-Akt
β-actin
t-Akt/β-actin
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

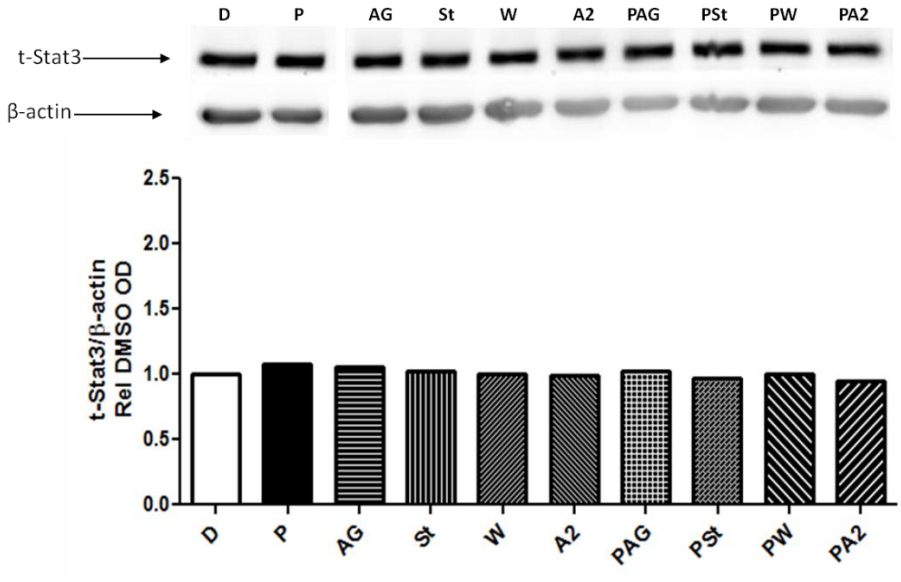
D)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
p-STAT3 (TYR 705)
t-STAT3
Tyr-Stat3/t-Stat3
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

E)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
p-STAT3 (SER 727)
t-STAT3
Ser-Stat3/t-Stat3
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

F)


D
P
AG
St
W
A2
PAG
PSt
PW
PA2
t-Stat3
β-actin
t-Stat3/β-actin
Rel DMSO OD
2.5
2.0
1.5
1.0
0.5
0.0

G)

30 minute	D	P	AG	St	W	A2	PAG	PSt	PW	PA2
Ser473-Akt	-	↑	↓	↓	↓	↓	↓	↓	↓	↓
Thr308-Akt	-	↑	↓	↓	↓	↓	↓	↓	↓	↓
Tyr705-Stat3	-	↑	-	-	↓	↓	↓	↓	↓	↓
Ser727-Stat3	-	↑	-	-	↓	↓	-	-	↓	↓

Figure 5. Inhibition of RISK and SAFE pathways reveals propofol mediated cross-talk: 30 minute propofol stimulation. H9c2 cells were serum-starved for 48h. Cells were then inhibited with RISK or SAFE pathway inhibitors for 30 minutes followed by **30 minutes** of stimulation by 50μM propofol (P) (groups PAG, PSt, PW, PA2). DMSO (D) was used as vehicle control. Representative blot and corresponding densitometrical analysis are shown. **A)** Phosphorylation of Ser473-Akt and densitometric analysis. **B)** Phosphorylation of Thr308-Akt and densitometric analysis. C**)** t-Akt and densitometric analysis. **D)** Phosphorylation of Tyr705-Stat3 and densitometric analysis. **E)** Phosphorylation of Ser727-Stat3 and densitometric analysis. **F)** t-Stat3 and densitometric analysis. **G)** Table of experimental conclusions. (-) depicts no change. ↑ depicts increase in phosphorylation compared to D. ↓ depicts decrease in phosphorylation when compared to P. The presented blot represents the typical results in at least two out of three independent trials. Representative blot and its densitometry data are presented. n=3.

4.1.4 Components of RISK and SAFE pathways play a role towards total cellular level of Bcl-2

We previously demonstrated that propofol increased Bcl-2 production in serum-starved, peroxide stressed, H9c2 cells and that this increase was not attenuated at the transcriptional level with the PI3K inhibitor wortmannin[56]. To see if and how RISK and SAFE pathways

interact to increase Bcl-2 levels, we pre-incubated cells with RISK and SAFE pathway specific inhibitors followed by propofol stimulation for 24 hours.

There was a trend towards increased Bcl-2 protein in response to propofol when compared to DMSO (**figure 6**).

There was a trend towards decreased Bcl-2 protein expression in response to SAFE pathway inhibitor, AG490 (AG). This trend was absent when followed by the application of propofol. (PAG) (**figure 6**). Inhibition of Stat3 with stattic alone (St) significantly reduced Bcl-2 compared to propofol. This inhibition was not present upon addition of propofol (PSt) (**figure 6**).

There was a trend towards decreased Bcl-2 protein expression in response to inhibition of the RISK pathway with wortmannin (W) that did not occur after addition of propofol (PW) (**figure 6**). RISK pathway inhibition with API-2 did not attenuate Bcl-2 production alone (A2) or in the presence of propofol (PA2) (**figure 6**).

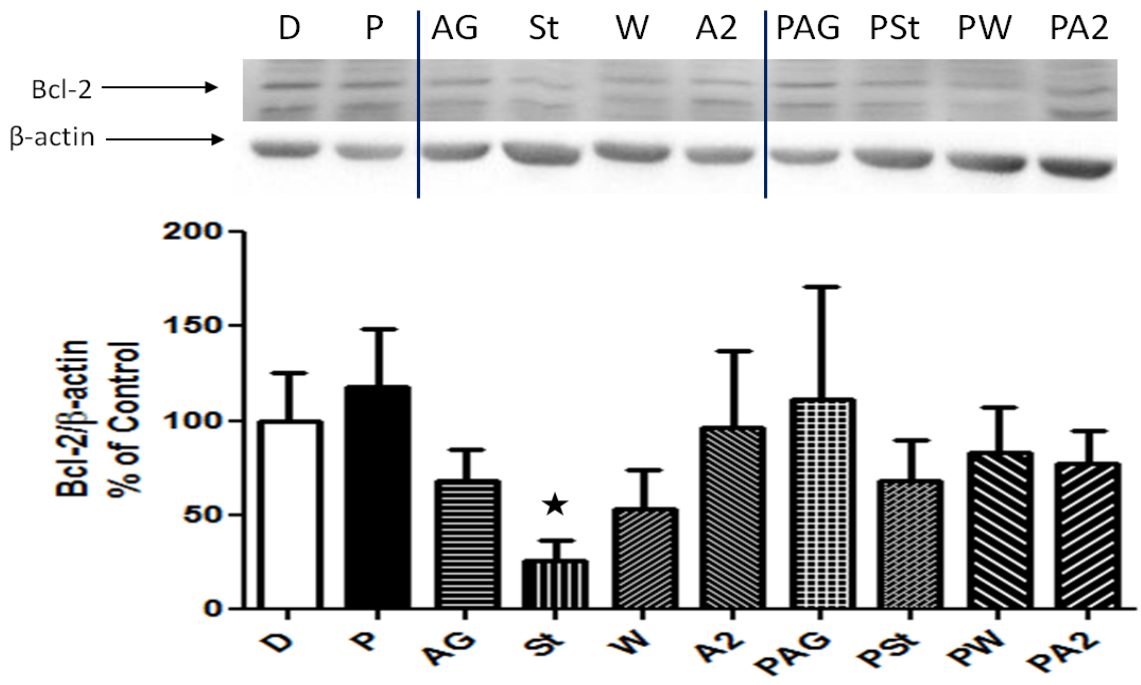


Figure 6. RISK and SAFE pathway inhibition attenuates Bcl-2 production. 48 hour serum-starved H9c2 cells were serum-starved for 48h followed by inhibition with RISK or SAFE pathway inhibitors for 30 minutes and treated with propofol for 24h. Representative western blot is presented. A lane representing EGF between P and AG was cropped. Loss of the lane does not compromise the blot. Densitometric values for Bcl-2 were normalized to β-actin. Densitometric data is presented as normalized to DMSO control (D). *p<0.05 vs. P. n=3-5.

4.1.5 Stat3 knockdown does not decrease Bcl-2 but increases NFκB (p65)

To further investigate the inhibitory effect of Stat3 inhibition on Bcl-2 production we used siRNA to knockdown Stat3 in H9c2 cells. No statistical analysis was done on the degree of siRNA mediated knockdown. Experiments with t-Stat3 knockdown of greater than 70% were considered for further analysis.

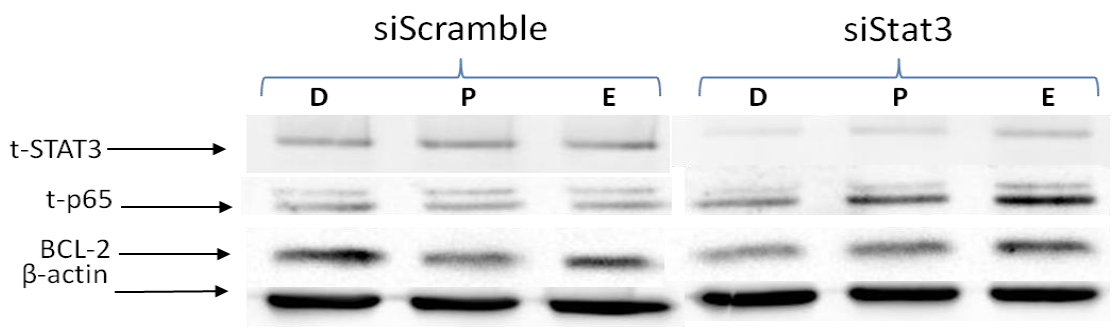
Following overnight incubation, siRNA treated cells were treated with DMSO, propofol, or EGF (200ng/mL) for 1.5h based on results from our previous study. at 1h[56]. Bcl-2 levels did not significantly differ between groups, **figure 7B**.

Stat3 knockdown did not affect Bcl-2 levels. This could be explained by residual Stat3 is not sufficient to increase Bcl-2 transcription or the actions of another transcription factor that may elaborate Bcl-2 in the absence of STAT3. We chose not to investigate the first possibility because overnight 33nM siRNA treatment had a high lethality rate in the cells. Addition of more siRNA and lipofectamine 2000 would increase cell death and leave too few cells to experiment on. We therefore decided to investigate for alternative transcription factors that may be activated in response to propofol stimulation. Our immediate focus was on the possible role of NFκB.

Lecour *et al*. had previously shown that TNFα may be a preconditioning mimetic[36]. TNFα traditionally activates the transcription factor NFκB, which has been previously shown to interact with Stat3[93,94]. In addition, Wickley *et al.*[90] have previously shown that propofol increases PKCζ nuclear translocation in rat cardiomyocytes. PKCζ was also postulated to activate NFκB. We therefore investigated for evidence of NFκB modulation by first measuring for its p65 subunit in our model.

As seen in **figure 7A**, Stat3 knockdown increased p65 levels. Although, these levels were significantly higher in H9c2 cells treated with EGF (**figure 7C**) there is a general trend towards higher p65 levels in cells with Stat3 knockdown.

A)



B) **C)**

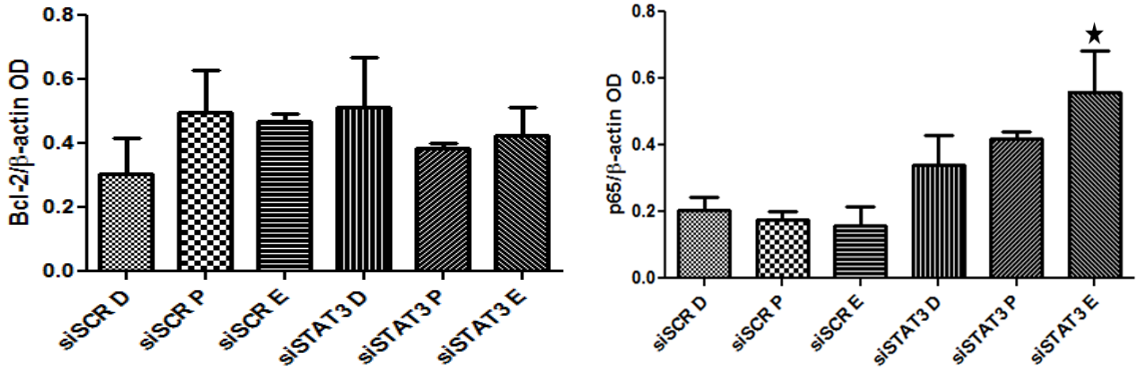


Figure 7. STAT3 knockdown increases p65. 48 hour serum-starved H9c2 cells were transfected with 33nM control siScramble or 33nM siStat3 overnight. Cells were then stimulated with DMSO, propofol, or EGF for 1.5 hours to induce Bcl-2 production. **A)** Representative western blot. **B)** Densitometric analysis for Bcl-2/β-actin. **C)** Densitometric analysis for p65/β-actin. * $p<0.05$ vs. siSCR E. n=2.

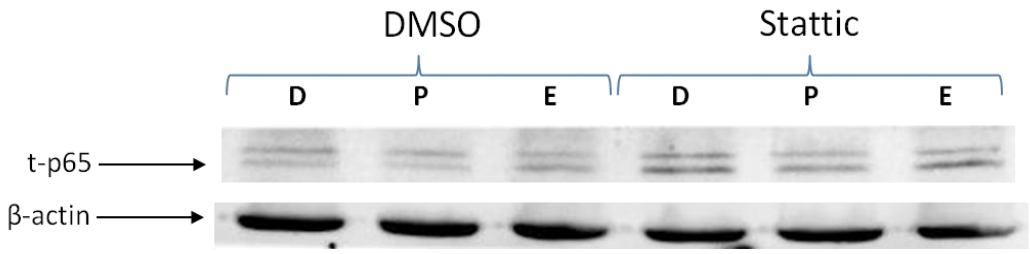
4.1.6 Stat3 activity inhibition increases NFκB (p65)

Knockdown of Stat3 should reduce overall Stat3 activity in the cell similar to Stat3 inhibition by stattic. To check if stattic inhibition of Stat3 activity also resulted in increased p65 protein

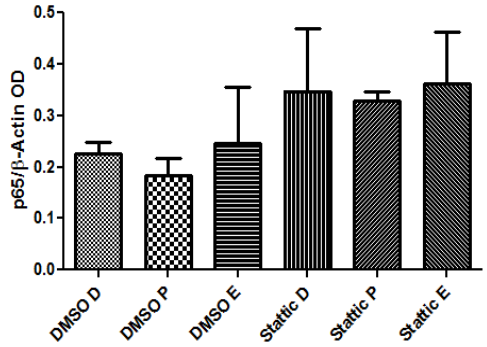
levels, cells were inhibited with stattic or DMSO control and then stimulated with vehicle control DMSO or propofol or EGF. This experimental set-up was similar as that in **figure 7**.

Stat3 activity inhibition by stattic did not increase p65 levels within the 6 individual groups (**figure 8A and B**). However there was a significant increase when data from the three groups (D, P, and E) was pooled together and DMSO was compared to stattic only (**figure 8C**).

A)



B)



C)

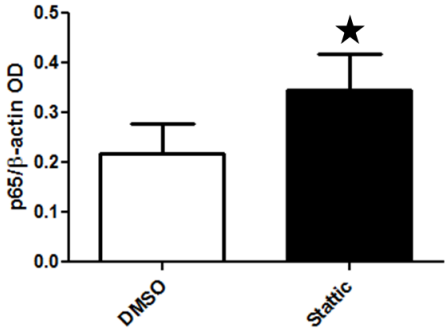


Figure 8. STAT3 inhibition may increase p65. 48 hour serum-starved H9c2 cells were treated with stattic or DMSO for 30 minutes and then treated with DMSO or propofol or EGF for 1.5 hours. Cells were then lysed and total proteins were analyzed through western blot. **A)** Representative western blot. **B)** Densitometric analysis of p65/ β-actin. **C)** Densitometric analysis of pooled DMSO vs. Stattic. * $p<0.05$ vs. DMSO. n=2.

4.2 Propofol Signaling in the NFκB pathway

To further elaborate the contribution of the NFκB pathway, we conducted experiments to characterize IκBα degradation in response to propofol.

4.2.1 Propofol increases IκBα degradation

Time-course treatment of serum-starved cells with propofol showed significant increase in IκBα degradation at 2h (**figure 9**). Propofol mediated IκBα degradation at 2h was comparable to the positive control for IκBα degradation, TNFα.

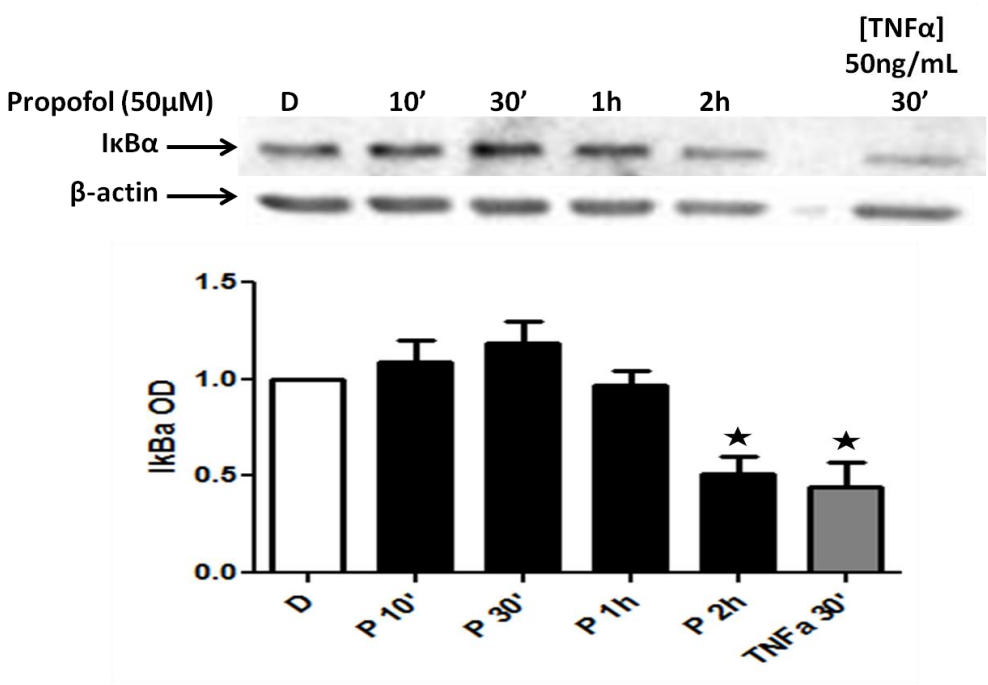
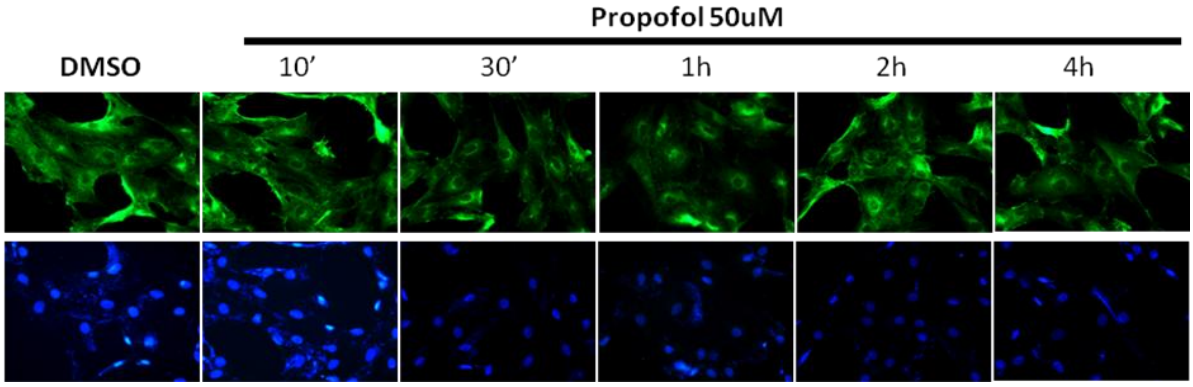


Figure 9. Propofol increases IκBα degradation. Serum-starved H9c2 cells were treated with 50μM propofol for 10m, 30m, 1h, and 2h. DMSO was used as vehicle control. TNFα at 50ng/mL for 30m was used as positive control. Representative western blot. Densitometric analysis of IκBα bands. Results are shown normalized to DMSO. * $p<0.05$ vs. DMSO. n=3.

4.2.2 Propofol does not increase NFκB (p65) nuclear translocation

To assess NFκB (p65) nuclear translocation, serum starved H9c2 cells were treated with 50μM propofol for 10m, 30m, 1h, 2h, and 4h and stained for p65 and DAPI, a nuclear specific dye. As seen in **figure 10A**, relative to DMSO control propofol mediated IκBα degradation did not increase nuclear translocation of p65 at up-to 4 hours of propofol exposure. However, there was increased peri-nuclear staining of p65 (arrows in **figure 10C**) relative to DMSO control (**figure 10B**).

A)



B) **C)**

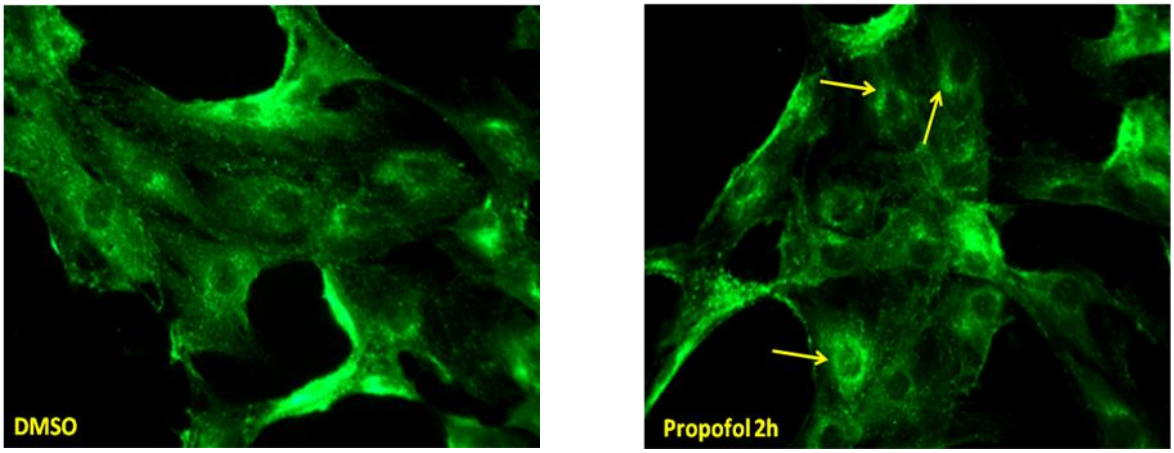


Figure 10. Propofol does not increase p65 nuclear translocation. Serum-starved H9c2 cells were treated with propofol for 10m, 30m, 1h, 2h, and 4h. Cells were stained for p65 (green) and DAPI (blue). **A)** Time course of H9c2 cells treated with 50μM propofol. DMSO was used as vehicle control. **B)** Enlarged picture of DMSO control. **C)** Enlarged picture of cells treated with propofol for 2h. Yellow arrows indicate cells with increased peri-nuclear p65 staining (n =3).

5. Discussion

5.1 Propofol Interaction with the RISK and SAFE Pathways

5.1.1 Propofol Stimulates Phosphorylation of Stat3

Our results demonstrate that 50μM propofol increased Tyr705-Stat3 phosphorylation at 10m, 30m, and 6h and Ser727-Stat3 phosphorylation at 10 and 30 min. Phosphorylation of Stat3 at Tyr705 activates its dimerization, nuclear translocation, and transcriptional activity of target genes. Phosphorylation at Tyr705 is also shown to increase cell survival by increasing transcription of anti-apoptotic proteins such as Bcl-2[95]. Therefore, propofol mediated activity of Stat3 increases anti-apoptotic potential of the cell.

Phosphorylation at Ser727 was shown to increase mitochondrial localization and participation in the electron transport chain (ETC), this effect was independent of Tyr705 phosphorylation[73]. Stat3 localized at the mitochondria was also found to play an important role in protecting rats from I/R injury through prevention of mPTP[74]. Although this effect was not attributed to phosphorylation at Ser727 alone, it may be hypothesized that mitochondrial localized Ser727-Stat3 plays an important role in cardioprotection through prevention of mPTP. Phosphorylation of Ser727 by propofol may mediate similar type of protection in patients undergoing bypass surgery. Activation of both residues in bypass patients may prove to be a mechanism through which propofol mediates cardioprotection.

5.1.2 Inhibition of RISK and SAFE Pathways Show Cross-Talk

5.1.2.1 Effects on Akt

A phosphorylation/inhibition event occurring in at least two out of three independent experiments was considered biologically relevant. Due to high the degree of variability in densitometry results, statistical significance could not be established by ANOVA. We therefore decided on the current observational method of evaluating immunoblot data without densitometry and/or statistical analysis , which we believe correctly represents our results and is supported by examples in the literature[96,97].

Ser473-Akt: SAFE pathway

Phosphorylation by propofol at Ser473-Akt did not occur at 10 minutes but at 30 minutes after stimulation from propofol. Of interest, SAFE pathway inhibitors only showed effects on phosphorylation after 30 minutes of propofol stimulation (**figure 5A and B**, PAG and PSt). This timeframe coincides with propofol phosphorylation of Ser473-Akt.

AG490 belongs to the tryphostin family of tyrosine kinase inhibitors. Inhibition occurs through AG490 binding to the SH2 domain of Jak2, which prevents interaction between Jak2 and the activated receptor's cytoplasmic domain[98]. This disruption prevents Jak2 auto-phosphorylation and subsequent downstream phosphorylation of target proteins by Jak2, such as Stat3.

Stattic binds the SH2 domain on Stat3 and prevents phosphorylation, activation, and dimerization to inhibit Stat3[92].

Inhibition of propofol mediated phosphorylation of Ser473-Akt from both SAFE inhibitors implies that Jak2 alone and/or Jak2 activated Stat3 participate in phosphorylation of Akt at Ser473. Jak2 is a tyrosine kinase that binds to ligand activated receptors' cytoplasmic domains via its SH2 domain. Jak2 then auto-phosphorylates itself, the cytoplasmic domain of the receptor, and eventually Stat3. It could be possible that Jak2 also phosphorylates PI3K which then activates mTORC2 to phosphorylate Akt. Mechanistic studies have shown that Jak2 lies upstream of PI3K and increases its activation and affects subsequent downstream activation of Akt[96]. The role of Stat3 may be that of an adaptor protein that encourages interaction between the involved proteins. Whether the Stat3 involved in this process requires phosphorylation is not known. Our results show that propofol increased Stat3 phosphorylation at both Tyr705 and Ser727 as early as 10 minutes and up to 30 minutes (**figure 3**). Therefore, fully phosphorylated Stat3 may indeed also function as an adaptor protein.

Ser473-Akt: RISK pathway

Our results demonstrate that inhibition of Ser473-Akt phosphorylation by RISK pathway inhibitors occurred at 10 and 30 minutes following the application of propofol (**figure 4A and 5A**, PW and PA2).

Wortmannin is a potent inhibitor of the membrane kinase PI3K and inhibits by covalently inactivating the kinase activity of the active subunit of PI3K, p110[99]. API-2 is a direct inhibitor of Akt phosphorylation and activity. It inhibits Akt membrane translocation[100] by binding to the pleckstrin homology domain on Akt and preventing interaction with PI3K and subsequent activation by PDK1. Phosphorylation of Akt at Ser473 is through the cytosolic

complex, mTORC2 which has been shown to be activated under stimulation from PI3K in the presence of amino acids[101]. We have previously shown that the RISK pathway is involved in propofol mediated phosphorylation of Akt at the Ser473 residue. Propofol signaling may activate PI3K to increase mTORC2 complex assembly for phosphorylation of Akt at Ser473.

Thr308-Akt: SAFE pathway

Inhibition through the SAFE pathway inhibitor, AG490, showed marked inhibition of phosphorylation of Thr308-Akt at 30 minutes following the application of propofol (**figure 5B**, PAG). As previously discussed, inhibition of propofol mediated phosphorylation at Ser473-Akt by AG490 also occurred after 30 minutes in our model. The similar timelines of Akt inhibition may imply that Jak2 is mechanistically involved in the in phosphorylation of both Akt residues in response to propofol, perhaps through its actions on PI3K.

Inhibition of phosphorylation at Thr308-Akt by stattic, an inhibitor of Stat3 phosphorylation[74], was present at both 10 and 30 minute time points (**figure 4B and 5B**, PSt). Therefore, Stat3 may be involved in the events that are involved in early phosphorylation at both Ser473 and Thr308 residues of Akt. Indeed, Stat3 was also phosphorylated at both Ser727 and Tyr705 residues at 10m post propofol stimulation (**figure 3**), therefore we can postulate that fully activated Stat3 may be involved in phosphorylation of Thr308-Akt.

Thr308-Akt: RISK pathway

Propofol increased phosphorylation of Akt at Thr308 after 30 minutes of stimulation to the same degree as Ser473-Akt. Thr308-Akt phosphorylation is a PI3K/PDK1 dependent event

that occurs at the membrane. PI3K recruits the constitutively active kinase PDK1 to increase Thr308-Akt phosphorylation. In our experiments, both RISK pathway inhibitors decreased Thr308-Akt phosphorylation, either alone or in presence of propofol, at both 10 and 30 minutes (**figure 4B and 5B**, PW and PA2). These results were similar to that at Ser473-Akt.

Akt cross-talk: conclusion

Our results show that the transcription factor Stat3 may be involved in both the mTORC2 and PI3K/PDK1 dependent processes of Ser473-Akt and Thr308-Akt phosphorylation, respectively.

Stat3 actions on Akt phosphorylation are not novel. Indeed, it has been shown that inhibition of Stat3 can negatively affect Akt phosphorylation[102]. Therefore, activation of Stat3 compliments Akt phosphorylation and activation. Stat3 is a transcription factor that displays several non-canonical roles, such as participation in the ETC. Therefore, Stat3 may also play a novel role in membrane sequestration or translocation of either Akt or PDK1. Alternatively, Stat3 may also play a role of an adaptor protein and increase interactions between Akt and the PI3K/PDK1 complex. Stat3 is activated by Jak2 at Tyr705 and so inhibition of Jak2 by AG490 will prevent Stat3 activation and downstream actions. Alternatively, Jak2 may also play a more direct role in Akt phosphorylation. Indeed, several reports show that Jak2 lies upstream of PI3K[96]. Inhibition of Jak2 and therefore of PI3K may also explain our findings at Thr308-Akt.

5.1.2.2 Effects on Stat3

Tyr705-Stat3: SAFE pathway

Phosphorylation at Tyr705-Stat3 was increased after 30 minutes of propofol stimulation. Based on our findings, the effect of SAFE pathway inhibitors on propofol mediated phosphorylation of Tyr705-Stat3 (**figure 4D and 5D**, PAG and PSt) was most evident at 30 minutes than at 10 minutes in our model. In the case of AG490, its inhibitory effects were only evident at the 30 min time point of our experiment (**figure 5D**, PAG). An increased time for inhibition by AG490 may therefore be required to affect Tyr705-Stat3 phosphorylation.

Similarly, stattic mediated inhibition was relatively greater after 30 minutes (**figure 5D**, PSt), when compared to the inhibition at 10 min in cells treated with propofol (**figure 4D**, PSt). Our results for both SAFE pathway inhibitors may imply that they bind more preferentially at longer time points. In both time groups AG490 and stattic was initially added 30 minutes prior to co-addition of propofol. Therefore, in cells stimulated by propofol for 10 or 30 minutes, inhibitors were present for a total of 40 or 60 minutes, respectively. A longer presence in the cell may give the inhibitors extra time to bind a more preferential binding site on their respective targets and therefore form a stronger inhibition.

Tyr705-Stat3: RISK pathway

A decrease in phosphorylation in response to the RISK pathway inhibitor wortmannin at both 10 and 30 minutes in cells treated with propofol (**figure 4D and 5D**, PW) was observed. These results imply that PI3K mediates phosphorylation of Tyr705-Stat3. However, literature states that Stat3 is phosphorylated directly by Jak2 and that Jak2 lies upstream of PI3K[96].

Similar studies have also found that wortmannin does indeed lower Stat3 phosphorylation[96]. Lin *et al.* have thus suggested that Jak2 and PI3K may work together to activate Stat3[96].

The Akt inhibitor, API-2, also decreased phosphorylation of Tyr705-Stat3 after 30 minutes of propofol stimulation (**figure 4D and 5D**, PA2). Stat3 phosphorylation at Tyr705 drives the canonical function of Stat3 as a transcription factor through increased nuclear localization and transcriptional activity. Our results in this report also show that API-2 lowered Akt phosphorylation at both residues (**figure 4A&B and 5A&B**, PA2) and therefore it may be possible that fully phosphorylated Akt is responsible for activation of Stat3.

Akt is a central kinase responsible for phosphorylation of many proteins to increase cell survival. As mentioned above, activation of both Akt and Stat3 may be complimentary. Our results are also in accordance with Tu *et al.* who showed that expression of a dominant negative Akt or inhibition of PI3K with LY294002 decreased Stat3 phosphorylation at both Tyr705 and Ser727 residues in response to oncostatinM, a cytokine of the Stat3 activating IL-6 family[103].

Ser727-Stat3: SAFE pathway

AG490 and stattic did not decrease propofol induced phosphorylation of Stat3 at Ser727 (**figure 4E and 5E**, PAG). (**figure 4E and 5E**, PSt). This is in contradiction with past reports[74,92] and may be a finding specific to H9c2 cells. We have previously mentioned in this report that both Akt and Stat3 are complimentary to each other in terms of activation. Our results with the SAFE pathway inhibitors, however, imply that Akt-Stat3 cross-talk may involve non-Jak2 dependent mechanisms of activation at Ser727-Stat3.

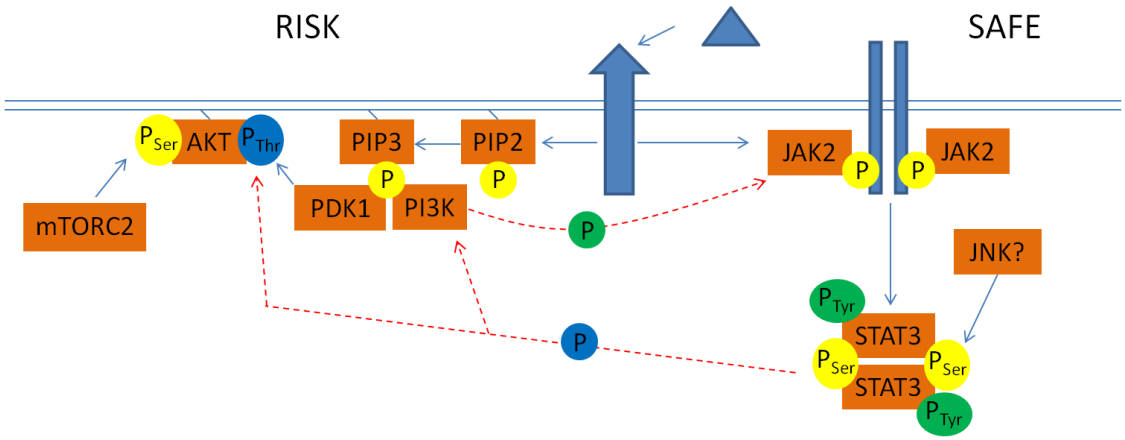
Ser727-Stat3: RISK pathway

Phosphorylation and inhibition of phosphorylation showed temporal effects on the Ser727 residue of Stat3 (**figure 4E and 5E**). Propofol increased phosphorylation only after 30 minutes of stimulation and similarly both RISK pathway inhibitors showed inhibition at that time point (**figure 5E**, PW and PA2). These results imply that phosphorylation of Ser727-Stat3 occurs through a pathway that requires a longer stimulation and utilizes the PI3K/Akt signaling pathway.

Liu *et al.* have shown that Ser727-Stat3 phosphorylation occurs in response to Arsenic (As^{3+}) at both a higher concentration or at a longer period of stimulation than Tyr705-Stat3[102]. Arsenic induced activation occurred through the c-Jun NH2 kinase (Jnk) pathway. Knockdown of Jnk disrupted Stat3 mediated transcription of IL-6 in response to As^{3+}. The migrational activity of the cell was similarly lowered after knockdown of either Jnk or Stat3. Jnk is a protein kinase that may either directly phosphorylate Ser727-Stat3 or activate another kinase that may then phosphorylate Ser727-Stat3. According to literature Ser727-Stat3 phosphorylation may not occur under Jak2 but rather several different kinases such as, Erk1/2[104], cyclin-dependent kinase (Cdk) 1[105], or mTor[106]. This may explain why we did not observe a decrease in propofol induced phosphorylation under AG490 inhibition of Stat3 at Ser727 (**figure 4E and 5E**, PAG)

The results of our studies on propofol mediated cross-talk study between RISK and SAFE signaling pathways and their interactions at 10 and 30 min are summarized in (**figure 11A**) and (**figure 11B**), respectively.

A)



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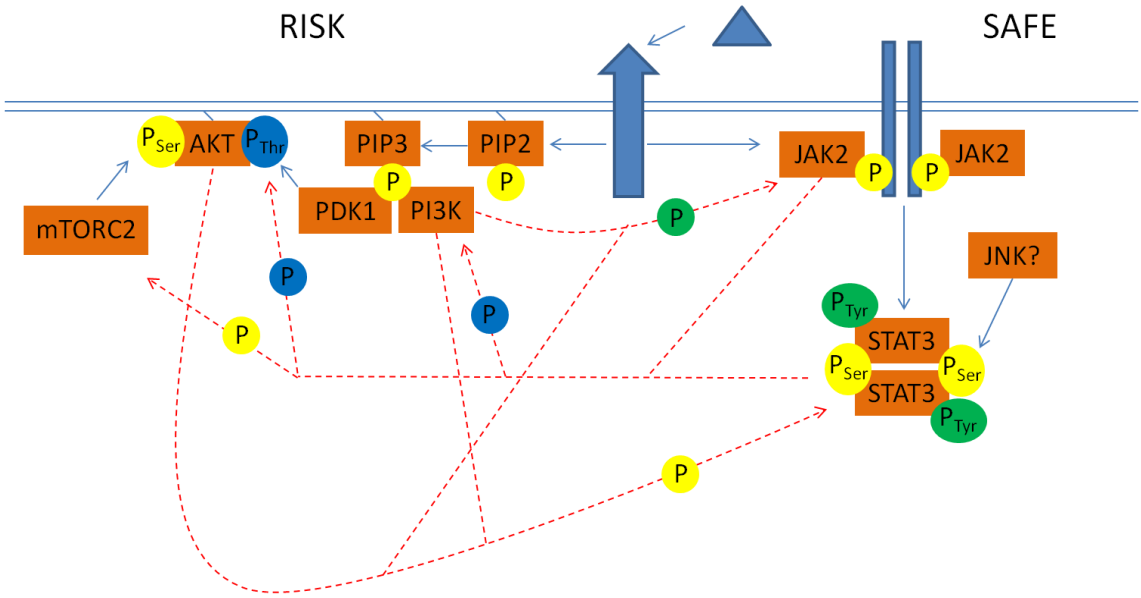


Figure 11. Propofol mediated cross-talk between the RISK-SAFE pathways. Results of cross-talk from **figures 4 and 5** are summarized above. **A)** 10 minutes post-propofol stimulation. **B)** 30 minutes post-propofol stimulation. Blue arrows indicate known mechanisms of interaction/activation. Red dotted arrows indicate cross-talk mechanisms suggested by our results.

5.1.3 Components of RISK and SAFE Pathways Play a Role in Bcl-2 Levels

We previously published that propofol increased both Bcl-2 transcription and translation, but only protein translation was RISK pathway dependent[56]. Our current results extend the findings of that initial study by identifying a role for both the SAFE pathway and the RISK pathway in Bcl-2 production. However, we only observed a trend towards increased Bcl-2 after 24h stimulation by propofol which failed to reach statistical significance. Our results of the experiment, however, may be attributed to basal Bcl-2 levels and the disruption, by RISK or SAFE pathway inhibitors, on formation of new Bcl-2. Any differences upon addition of propofol may be attributed to either DMSO or propofol.

The half-life of Bcl-2 mRNA and protein is 2-3h[107] and 20h[108], respectively. In our experimental protocol, H9c2 cells were inhibited for 24h, in the presence or absence of propofol. Therefore, pathways involved in Bcl-2 mRNA/protein synthesis and therefore formation of new Bcl-2 were blocked for greater than the half-life of Bcl-2 protein. Assuming normal cellular degradation of Bcl-2 according to its half-life, our experimental design should allow us to observe which pathways may be involved in Bcl-2 formation. These results may be specific to H9c2 cells under serum-starvation stress.

Our results indicate that inhibition of Jak2, Stat3, and PI3K by AG490, stattic, and wortmannin resulted in lower Bcl-2 in serum-starved H9c2 cells (**figure 6**, AG, St, and W), respectively. These results indicate that basal production of Bcl-2 may be both Jak2/Stat3 and PI3K dependent. However, only inhibition from stattic was statistically significant and inhibition from AG490 and wortmannin showed a trend towards decreased Bcl-2.

The inhibition by AG490, stattic, and wortmannin seen in our experiments was offset, at least in part, in response to propofol (**figure 6**). These findings suggest that propofol can overcome the limitations of either SAFE pathway and/or PI3K inhibition of Bcl-2 production.

Bcl-2: SAFE pathway

AG490 showed a decreased trend towards inhibition of Bcl-2 production, which was offset by addition of propofol (**figure 6**, AG and PAG). Inhibition of Jak2 by AG490 translates into Stat3 inhibition. Therefore, AG490 inhibition may lead to decreased Bcl-2. Indeed, other reports support this view[95,96]. Addition of propofol may activate other pathways to modulate Bcl-2, such as through a Jak2-independent PI3K-dependent mechanism.

Stattic inhibition of Bcl-2 production was offset by propofol (**figure 6**, PSt). These results indicate that Stat3 may be a major factor in the formation of Bcl-2 in H9c2 cells. Previous reports have shown that Stat3 transcribes for Bcl-2 mRNA[95]. Inhibition of Stat3 may prevent the formation of new Bcl-2 through inhibition of Bcl-2 transcription. Stat3 may, therefore, be critically involved in the basal production of Bcl-2. However, propofol may be able to overcome stattic inhibition of Stat3 or activate alternative pathways that may include PI3K to increase Bcl-2. These pathways may not be activated under basal conditions and possibly only by modulators such as, propofol.

Bcl-2: RISK pathway

Wortmannin based inhibition of propofol mediated Bcl-2 production is in accordance with our previous results[56]. However, the inhibition in Bcl-2 production was not significantly greater than that from the Wortmannin alone, (i.e. PW vs. W). This indicates that PI3K may be directly involved in propofol modulation of Bcl-2.

Surprisingly, Akt inhibition by API-2 did not significantly affect or show a trend affecting Bcl-2 production alone or in conjunction with propofol (**figure 6**, A2 and PA2). These results suggest that Akt may not play a role in Bcl-2 formation.

Previous studies have indicated that Akt activation increases Bcl-2 production[109]. Akt is a major pro-survival kinase in many cells and its disruption can lead to activation of cell death mechanisms such as, apoptosis. As a major kinase, Akt may increase phosphorylation and activity of one or several transcription factors such as, CREB[109], which can transcribe for Bcl-2 downstream. Our results are in contradiction with the previous reports where Akt was shown to be a critical mediator of Bcl-2. However, it may be possible that PI3K mediated inhibition of propofol mediated Bcl-2 production may not include Akt. PI3K may instead activate other kinases that affect transcription factors or activate transcription factors directly.

Our results imply that the RISK-SAFE cross-talk can occur at the level of Akt-Stat3 to mediate basal transcription of Bcl-2 by Stat3. Indeed, this view is supported by the literature [110].

5.1.4 Knockdown of Stat3 does not decrease Bcl-2 and increases p65

To further explore the role of Stat3 in propofol mediated increase in Bcl-2 we used specific siRNA to knockdown Stat3. There was no significant effect on Bcl-2 levels in any groups compared to control siScramble (**figure 7A, B**). These results suggest the possibility that another transcription factor that may be activated by propofol to increase Bcl-2 transcription. Alternatively, the time course for DMSO, propofol, or EGF treatment was 1.5 hours, which may be too short for significant increases in Bcl-2 production to be detected in our model.

We chose to investigate the transcription factor NFκB, more specifically its subunit p65, because of its potential role in TNFα[36] mediated pre-conditioning and possible activation under propofol stimulation through the kinase, PKCζ[90]. Interestingly, Stat3 knockdown was counteracted by a significant increase in p65 levels (**figure 7A, C**). This effect was observed throughout siSTAT3 but was significantly increased only under treatment with EGF. Increased p65 during Stat3 knockdown may highlight an underlying redundancy between the two transcription factors in that p65 may be able to take over functions particular to Stat3, such as Bcl-2 transcription. Indeed, an increase in p65 after Stat3 knockdown may be responsible for lack of Bcl-2 decrease (**figure 7A, C**).

Interaction between NFκB-Stat3 is not a novel concept. Both transcription factors work in both negating and supporting manners depending on cell type and conditions; Stat3 can be a dominant-negative repressor of NFκB[93] or promote p65 nuclear retention and p65 mediated production of IL-6[94] in cancer cells. Increased p65 after Stat3 knockdown may imply that each transcription factor may compensate for the loss of the other. These results may highlight a redundancy that may exist between the two factors. Future experiments should further explore the relationship between Stat3 and p65, specifically the effect on Stat3 levels following p65 knockdown.

5.1.5 Stat3 activity inhibition increases NFκB (p65)

Stat3 knockdown indicates a loss of total protein, which indicates a loss of Stat3 activity. This is similar to the effect of Stat3 inhibition by stattic. Using a similar experimental setup as the Stat3 knockdown experiment, we blocked Stat3 activity with stattic and looked for changes in p65 protein levels (**figure 8A**). There was a trend towards increased p65 in cells

treated with stattic that did not reach significance (**figure 8B**). However, when a cumulative comparison between DMSO vs. stattic was made there was a significant increase in p65 under Stat3 inhibition (**figure 8C**).Our results indicate that loss of Stat3 activity rather than the Stat3 protein may be the stimulus to induce a compensating increase in p65. Additionally, as seen in **figure 5E**, stattic inhibited propofol mediated phosphorylation at only Tyr705-Stat3. Therefore, it may be possible that decreased phosphorylation at Tyr705 may be solely responsible for increased p65.

5.1.6 Summary of Cross-Talk Results

Propofol induced activation and cross-talk between RISK-SAFE pathways involve phosphorylation of RISK-SAFE components. Our work showed that propofol mediated-cross-talk affected phosphorylation of residues on both Akt and Stat3. Our work also indicated that RISK-SAFE cross-talk played a role in production of Bcl-2. Stat3 knockdown, however, did not lead to decreased Bcl-2 levels in response to propofol. These results indicated that a novel transcription factor may be involved in propofol mediated Bcl-2 transcription. We found that p65 was up-regulated in response to Stat3 knockdown or loss of activity. This novel finding may be a cell mechanism to counteract loss of Stat3 function or a relief from p65 transcription inhibition by Stat3. Increased p65 may have also been responsible for the consistent levels of Bcl-2 protein after Stat3 knockdown.

Our results may explain the mechanism through which propofol induces cardioprotection in the diabetic myocardium against RI. Identification of molecular mechanisms of protection can be used for design of better patient care. Similarly, identification of protective

mechanisms activated by propofol may make propofol a prototype for better drug design for protection against RI.

5.2 Propofol Signaling in the NFκB pathway

5.2.1 Propofol increases IκBα degradation

To further investigate the role of propofol in activating the NFκB pathway we observed the levels of the NFκB repressor IκBα during a time course of propofol exposure. IκBα is a cytoplasmic repressor of NFκB dimers. Upon NFκB activating stimuli, such as inflammatory stimuli from TNFα, IκBα is ubiquitinated and degraded by the ATP-dependent 26S proteasome. This leaves the free NFκB dimer to translocate into the nucleus and bind to target genes to activate transcription.

There was significant degradation of IκBα at 2 hours post propofol exposure (**figure 9A, B**) in serum-starved H9c2 cells. The levels were comparable to TNFα, which was the positive control for IκBα degradation and subsequent NFκB activation.

Our results indicate that propofol may increase NFκB activity and nuclear translocation by increasing IκBα degradation.

5.2.2 Propofol does not increase NFκB (p65) nuclear translocation

Degradation of IκBα occurred at 2 hours following propofol stimulation **(figure 9A, B)**. Liberated NFκB may translocate into the nucleus to activate Bcl-2 transcription under propofol stimulation. Propofol mediated activation of NFκB may imply a novel mechanism through which propofol can assert cardioprotection. Therefore, propofol may mimic pre-conditioning in a similar manner to TNFα[36].

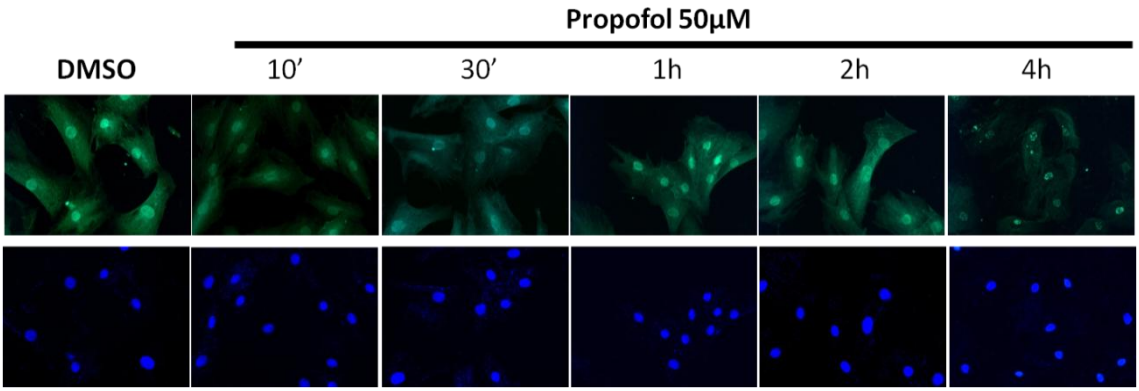
To investigate whether propofol stimulates NFκB nuclear translocation we stimulated serum-starved H9c2 cells with propofol for 10m, 30m, 1h, 2h, and 4h. We did not observe nuclear translocation in any time groups (**figure 10A**). However, there was an increase in peri-nuclear staining in propofol treated cells (**figure 10B, C**). These results indicate that liberated p65 is able to transit to the nucleus but unable to translocate into the nucleus.

Failure of NFκB to localize to the nucleus under propofol stimulation may be explained in several ways. It is possible that liberated NFκB requires phosphorylation to be fully active for nuclear translocation. Indeed Hall *et al.* found that lipopolysaccharide (LPS) mediated nuclear translocation of p65 and subsequent TNFα transcription was reliant on Ser536-p65 phosphorylation[111]. Secondly, the activation of p65 may be mediated through PKCζ, which was shown to be activated under propofol stimulation in rat cardiomyocytes[90]. It may be possible that propofol does not activate PKCζ and therefore p65 in H9c2 cells.

We did not check for p65 phosphorylation in our current study. It may be possible that propofol mediated actions on the NFκB pathway do not fully activate p65. Another possible explanation may be that we did not properly permeabilize the H9c2 cells. Therefore, primary antibody against p65 would not have stained for p65 in the nucleus. However, our previous staining for t-Stat3 showed marked nuclear staining (**figure 12**), which means our negative data regarding p65 nuclear localization may not be due to this experimental error. Interestingly, Stat3 knockdown or inhibition of activity did not significantly lower Bcl-2. We originally thought p65 may be able to compensate for the loss of Stat3 activity and therefore prevent a drop in Bcl-2 levels. However, propofol did not increase p65 nuclear translocation. This may imply that Bcl-2 is being transcribed by other transcription factor(s).

Finally, of interest, we noticed that the time point of p65 peri-nuclear localization (2-4 hours) was strikingly similar to that for our preliminary experimental results on t-Stat3 nuclear accumulation **(**4 hours, **figure 12 A, B)**. Stat3 was present in the nucleus at 4 hours post-stimulation from propofol and present as punctate dots. It may be possible that p65 acts as a chaperone for nuclear transport of Stat3 after propofol stimulation. Stat3 nuclear transport is managed through the importin proteins[112,113]. Activation of p65 under propofol stimulation may increase formation of a complex with importin proteins to increase Stat3 nuclear transport.

A)



B)

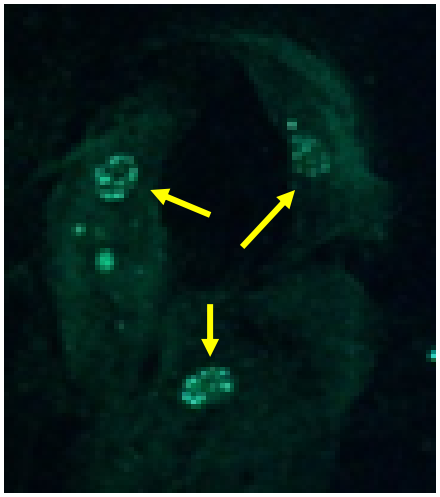


Figure 12. Propofol may increase Stat3 nuclear accumulation. Serum-starved H9c2 cells were treated with propofol for 10m, 30m, 1h, 2h, and 4h. Cells were stained for t-Stat3 (green) and DAPI (blue). **A)** Time course of H9c2 cells treated with 50µM propofol. DMSO was used as vehicle control. **B)** Enlarged and cropped picture of cells treated with propofol for 4 hours. Yellow arrows highlight cells with nuclear localized Stat3. Figure shown is representative of 1 experiment.

Our results for the thesis are summarized below in **figure 12.**

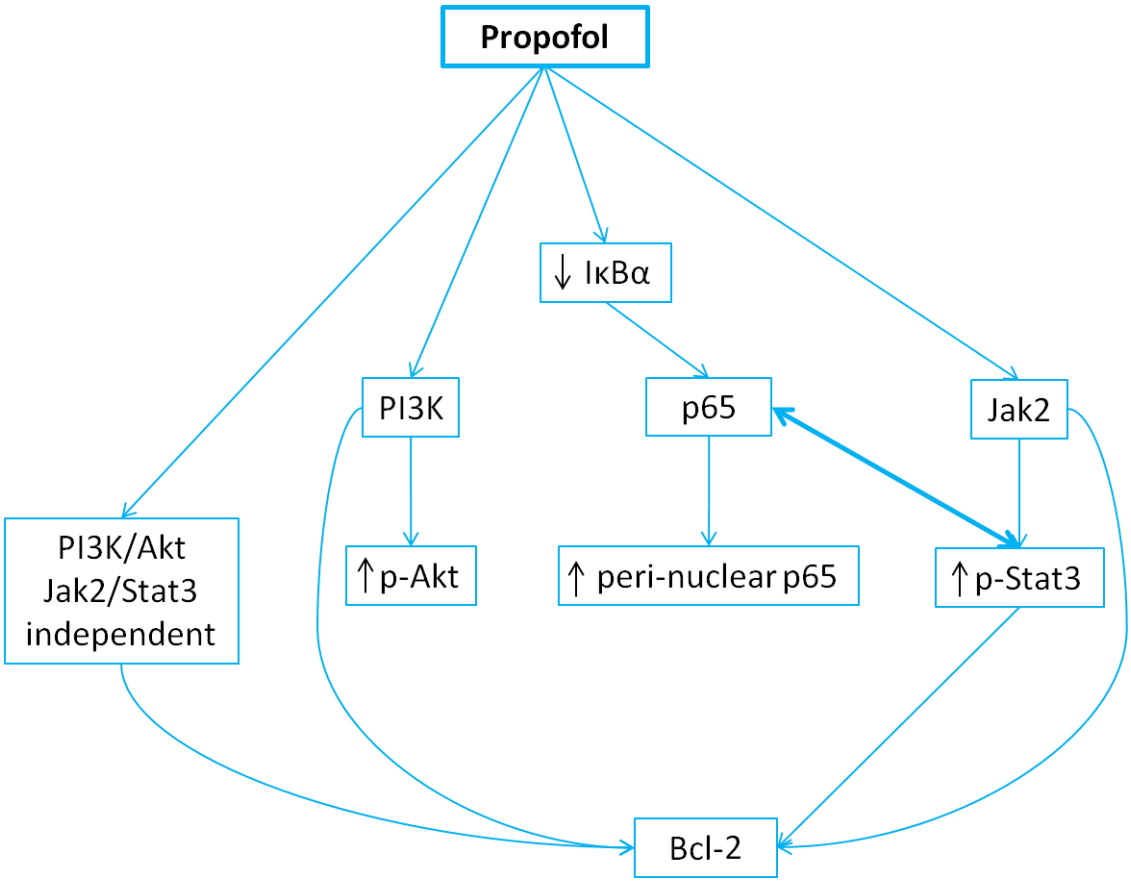


Figure 13. Summary. Propofol mediated phosphorylation occurs at both Thr308 and Ser473 and Tyr705 and Ser727 of Akt and Stat3, respectively. Activation of PI3K and Jak2/Stat3, but not Akt is responsible for propofol modulation of Bcl-2. Loss of Stat3 or its activity is counteracted by increased levels of p65. Propofol increases IκBα degradation and p65 peri-nuclear translocation. Propofol may also activate PI3K/Akt- and Jak2/Stat3-independent pathways to modulate Bcl-2.

6. Conclusion

6.1 General Discussion

Diabetes presents a challenging scenario where traditional conditioning methods against I/R injury are ineffective. The work from our lab is focused on finding a therapeutic alternative. We present that propofol may be that alternative. Our work in this report has focused on uncovering the molecular signaling mechanisms in an embryonic rat cardiac cell line model that may provide evidence that could translate how propofol may protect the diabetic heart from I/R injury.

Cardioprotective signaling events are anti-apoptotic in nature and can be stimulated through exogenous compounds, including volatile anesthetics. Cardioprotection through pharmacological preconditioning can be advantageous. Volatile and IV anesthetics both present cardioprotective properties vital to protection of the myocardium from I/R injury. However, volatile anesthetics do not protect the diabetic heart against I/R injury and therefore their use in this patient population may not be beneficial.

Our work has shown that propofol is cytoprotective against oxidative stress. Previous work has shown that propofol has cytoprotective properties in addition to its role as an anti-oxidant. Propofol also initiates cell signaling and further downstream cytoprotective effects. These properties present a novel mechanism of action distinct from propofol's well acknowledged anti-oxidant properties.

Our current work shows that propofol activates both RISK and SAFE pathways. This activation operates through a cross-talk mechanism that may be unique to propofol stimulation. Our work has shown that both RISK and SAFE pathways are involved in Bcl-2

production in serum-starved H9c2 cells. In addition, our work also demonstrates that propofol may act via the NFκB pathway but does not identify if propofol signaling leads to increased NFκB activity. We have also identified a novel interaction between Stat3 and NFκB. Stat3 knockdown stimulated an increase in NFκB protein. Similar results were seen in response to decreased Stat3 activity. This may indicate a novel mechanism of redundancy through which H9c2 cells counteract the loss of Stat3 protein or its activity. Or it may represent relief of transcriptional block by Stat3 protein on p65 gene. Our results suggest at the possibility that a loss in activity rather than protein may be responsible for the concomitant increase in p65. The role propofol may play in this cellular event remains to be elucidated.

Our work also highlights that propofol, secondary to its role of an anti-oxidant, can induce complex cardioprotective cell signaling. This is a novel and evolving feature of cardioprotective research on propofol. Anti-apoptotic features of propofol may highlight activation of other signaling pathways as well. Propofol may activate cell signaling via receptor based interactions at the lipid membrane. Further research into propofol's mode of action may look into mechanism of receptor activation.

6.2 Research Limitations

In **figure 4B,** our densitometry graph shows a high value at lane PSt. This is due to the dark artifact that produced a high signal. The artifact was leftover residue from the SDS-Gel left stuck after the transfer. Therefore, this result should be interpreted in caution. Our conclusion that PSt did not show increase in phosphorylation was supported in our other trials and therefore resulted in the said conclusion.

Past experiments from our laboratory on HUVEC cells have shown that propofol does not increase Bcl-2 levels unless in the presence of a stress stimuli such as, TNFα[55]. We also observed increased modulation of Bcl-2 under propofol in H9c2 cells that were stressed with H_2O_2[56]. However, increased Bcl-2 modulation under propofol was also seen in serum-starved non-stressed H9c2 cells. An absence of stress stimuli in our experimental setup may have prevented a significant effect on Bcl-2 modulation under RISK/SAFE pathway inhibition.

We did not measure the levels of Bax in our experiments. Bcl-2 binds to Bax in a 1:1 ratio to disrupt formation of the MOMP. Therefore, several papers on apoptosis display a Bax/Bcl-2 ratio to highlight the apoptotic potential of the cells in an experiment. We may include measurement of Bax in our future experiments.

Our experiments were not designed to delineate the order in which specific components of the RISK and SAFE pathways interplay during cross-talk. Such an experiment would need to utilize a combination of double inhibitors. Our experiments also do not address the status of PI3K or Jak2 from the RISK and SAFE pathways. Current western blot data was too dirty and unable to clearly answer those questions. Future studies should study the activation of PI3K and Jak2. PI3K activation occurs when the p85 regulatory subunit is phosphorylated by a G-protein coupled receptor or a tyrosine kinase receptor. Phosphorylation of p85 causes a conformational change that leads to release of the catalytic p110 subunit. Phosphorylated p85 can act as an indirect indication for the activation of the catalytic p110 subunit of PI3K. Jak2 activation is measured by the phosphorylation of any of its multiple tyrosine residues that are phosphorylated upon activation. Incorporation of PI3K and Jak2 data will give a more complete picture of the RISK-SAFE cross-talk.

One of our limitations in this work may be that we relied on the use of inhibitors to investigate signaling mechanisms. Inhibitors may not be expected to have single targets; therefore, our results may be attributed to cross-over inhibition rather than cross-talk. It can be stated that we did not uncover cross-talk but rather our inhibitors inhibited other pathways. To counteract, literature shows that components of both RISK and SAFE pathways have been shown to interact and mediate cross-talk. We searched the literature to look for highly specific inhibitors for Stat3 and Akt and chose stattic and API-2, respectively. We believe the use of these novel specific inhibitors strengthens our experimental approach.

Inhibitors used in our experiments may have intrinsic effects on the production of Bcl-2. Wortmannin[87,96] and AG490[96] have not been shown to modulate Bcl-2 levels. We could not find examples on effects of API-2 and stattic on basal Bcl-2 levels. Although API-1 inhibits the central kinase Akt, signaling pathway redundancy may exist through which to increase Bcl-2. However, we expect a decrease with stattic because it inhibits the transcription factor that codes for basal levels of Bcl-2. This is seen in our results (**figure 6**). We also show, however, stattic based inhibition was prevented in part by propofol, although not conclusively, in our model. . DMSO is considered inert and not shown to modulate Bcl-2[114]. Therefore, we believe the effects seen with propofol alone (labeled as P) or with inhibitors (labeled as PW, PA2, PAG, and PSt) do not represent confounding effects from DMSO.

Inhibition of Akt and Stat3 was offset by propofol (**figure 5 and 6**). Stimulation of the inhibited proteins can represent replace-/displacement of the inhibitor from the target protein by propofol or activation by propofol of an alternative pathway to modulate Bcl-2. Our current work cannot answer which possible explanation is correct.

Our experiments were done on serum-starved (1% FBS) H9c2 cells. Serum-starvation was done to lower the phosphorylation levels of proteins in the H9c2 cells. This would allow us to detect changes in phosphorylation as induced through propofol stimulation. Without serum-starvation we would not detect such changes. Serum-starvation was originally proposed to synchronize a culture of cells to the G0 phase, (quiescent arrest in growth cycle) thereby, allowing the researchers to create a point from which to re-start the cell cycle, for example, though addition of serum. Serum-starvation was thus a tool to study the cell-cycle. Serum-starvation to view phosphorylation from novel agents is a common practice[115,116]. We do not expect serum-starvation to affect our results or that serum-starvation may result in the observed results.

Current molecular research utilizes genetic manipulation, such as protein overexpression (dominant negative vs. constitutively active), siRNA mediated knockdown, and single amino acid substitution. This methodology was unavailable to incorporate into our experimental approach. Future use of genetic manipulation techniques can only enhance the work done from our lab.

Another limitation of our work is that our experiments did not involve animals or isolated hearts and therefore our results cannot yet apply to cardiac I/R injury protection. However, work done on H9c2 cells is translatable to experiments utilizing working heart models or animals[46]. A benefit to using a cell line to delineate mechanisms is that the findings are not confounded by interactions between different cells or tissues. Therefore, findings in H9c2 cells can be attributed to events occurring in cardiomyocytes. Vice versa, our experiments cannot take into account contributions from different cell types and tissues

and the net outcome of propofol treatment under I/R injury in an animal model. However, previous literature presents evidence that propofol is beneficial in an *in vivo* setting[39,117].

Our experiments were done in high glucose (25mM) DMEM medium. Therefore, our work may apply to the diabetic setting characterized by hyperglycemia. Cells raised in high glucose medium showed translatable experimental results to an *in vivo* model of diabetes in rats[46]. As mentioned previously, hyperglycemia inhibits volatile preconditioning. Our current work shows that propofol activates pro-survival pathways under hyperglycemic conditions. Therefore, our findings may be clinically translatable to diabetic patients.

6.3 Future Directions

Future work on propofol mediated cardiac signaling should investigate the role in other forms of cell injury. For example, autophagy, ER stress, and necrosis. Cell injury pathways share common components and interactions and therefore propofol is likely to have an effect on these. As stated previously, work on NFκB pathway is of importance and can provide another pathway through which propofol interacts and affords cardioprotection. Our lab is situated to move into incorporating organelle fractionation. Ser727-Stat3 was recently found to be localized in the mitochondria. It would be interesting to see if propofol mediated phosphorylation at Ser727-Stat3 promotes mitochondrial localization and if this is another mechanism through which propofol mediates protection against mPTP.

PTEN, a cell membrane phosphatase, is the main regulator of Akt phosphorylation and activity. PTEN dephosphorylates PI3K at the membrane and prevents downstream Akt phosphorylation. Inactivation of PTEN is pro-survival as it increases Akt phosphorylation of targets that promote cell survival. One of these targets is the transcription factor p65[118]. Akt

mediated activation of p65 results in its increased transcriptional activity. Our data shows that propofol activates Stat3. However, in the case of Stat3 knockdown, our data also shows that p65 levels can rise to perhaps compensate for the loss in Stat3. Propofol also activates the PI3K/Akt pathway. Therefore, consideration should be given to the role of p65 under propofol stimulation. Of increased importance will be to consider the interaction between propofol and p65 in the diabetic heart where PTEN expression is significantly increased.

Identification of the receptor through which propofol initiates its cytoprotective signaling can be of great importance as it will allow identification of a receptor that mediates cardioprotective signaling. Such information can be used to design drugs in the future that are cytoprotective against cardiac damage.

6.4 Clinical Relevance

Our current work may aid further research into strategies aimed at preventing IRI in patients. IRI is not a phenomenon of the heart. For example, organ transplants may also incur IRI. Propofol may also be of benefit in this clinical setting.

Pathways activated by propofol may form a signalosome of protection against IRI. Drugs that activate a similar cell mechanism of protection may also be vital in the clinical setting of preventing IRI. Therefore, activation of a "propofol signalosome" may be used as a screening tool for future discovery of protective drugs. Similarly, the chemical structure of propofol may be used a base for future drug design in the treatment of IRI and related injuries. Such drugs may be of critical importance in clinical settings with low treatment options against IRI such as, diabetes.

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