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EGLN1 Inhibition and Rerouting of α -Ketoglutarate Suffice for Remote Ischemic Protection

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SUMMARY

Ischemic preconditioning is the phenomenon whereby brief periods of sublethal ischemia protect against a subsequent, more prolonged, ischemic insult. In remote ischemic preconditioning (RIPC), ischemia to one organ protects others organs at a distance. We created mouse models to ask if inhibition of the alpha-ketoglutarate (α KG)-dependent dioxygenase EglN1, which senses

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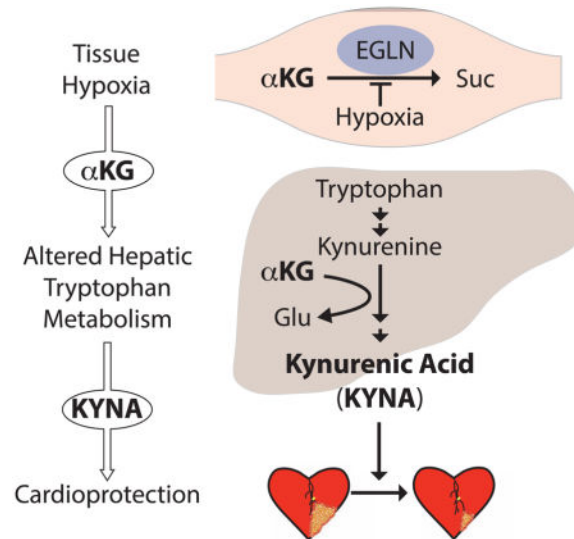
AUTHOR CONTRIBUTIONS

B.A.O., S.M.D. and J.W. did the metabolism experiments and GC-MS. J.M. and A.H.B. did the mouse physiology experiments. A.W. designed parabiosis experiments, and C.M. did the parabiosis surgeries. W.J.G. performed computational analysis. E.A.H. and A.K. supplied U-¹³C-dimethyl-alpha-ketoglutarate. K.A.P. and C.B.C. performed and analyzed LC-MS analyses. B.A.O., J.M., M.G.V.H. and W.G.K. designed the experiments. B.A.O. and W.G.K. wrote the manuscript.

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oxygen and regulates the HIF transcription factor, could suffice to mediate local and remote ischemic preconditioning. Using somatic gene deletion and a pharmacological inhibitor, we found that inhibiting EglN1 systemically or in skeletal muscles protects mice against myocardial ischemia-reperfusion (I/R) injury. Parabiosis experiments confirmed that RIPC in this latter model was mediated by a secreted factor. EglN1 loss causes accumulation of circulating α KG, which drives hepatic production and secretion of kynurenic acid (KYNA) that is necessary and sufficient to mediate cardiac ischemic protection in this setting.

Graphical Abstract



INTRODUCTION

Brief periods of sublethal ischemia can protect tissues from a subsequent, more severe, ischemia-reperfusion (I/R) insult. This phenomenon of 'ischemic preconditioning' was first observed in experimental models of myocardial infarction (MI) (Murry et al., 1986) and later in coronary heart disease patients (Kloner et al., 1995). Patients who have angina (ischemic cardiac chest pain) within 48 hours before a MI have better outcomes than patients who do not experience preceding angina. Subsequent studies with animals showed that ischemia in one coronary artery territory could protect myocardium perfused by another coronary artery (Przyklenk et al., 1993), and that coronary effluent from an ischemic heart can protect a naive acceptor heart *ex vivo* (Dickson et al., 1999). Remarkably, ischemia to a non-cardiac organ also protects the heart at a distance (Gho et al., 1996). Some, but not all, human clinical trials showed that inducing arm ischemia improved outcomes after coronary artery interventions associated with iatrogenic cardiac ischemia (Davies et al., 2013) (Hausenloy et al., 2015; Meybohm et al., 2015; Thielmann et al., 2013). Many RIPC mechanisms have been proposed, including both humoral and neural mechanisms (Przyklenk, 2013).

The HIF transcription factor, which consists of a labile α subunit and a stable β subunit, accumulates during hypoxia and activates genes whose products promote cellular survival

under ischemic conditions. The HIF α subunit is regulated through prolyl hydroxylation by α -ketoglutarate (α KG) dependent-dioxygenases known as EGLNs (also called PHDs). Of the 3 EGLN paralogs, EGLN1 is the primary regulator of HIF α (Kaelin and Ratcliffe, 2008). Hydroxylated HIF α is bound by the von Hippel Lindau (VHL) tumor suppressor protein, which marks HIF α for degradation. EGLNs require O₂, and HIF α hydroxylation is thus impaired when O₂ is limited, allowing HIF α accumulation. In sum, EGLNs act as 'O₂ sensors' in metazoans and coordinate cellular responses that promote adaptation to hypoxia and ischemia (Kaelin and Ratcliffe, 2008).

Cardiac-specific *Egln1* inactivation during late embryogenesis protects adult mice from MI after permanent coronary artery occlusion (Hölscher et al., 2011). Similarly, mice homozygous for a hypomorphic *Egln1* allele have less myocardial damage after I/R than *Egln1* *+/+* mice (Hyvärinen et al., 2010). Conversely, both local and remote preconditioning are attenuated in *Hif1 α* *+/-* mice (Cai et al., 2007; 2013). Collectively, these results support that HIF protects the heart during acute MI. However, chronic manipulation of HIF causes adaptations, such as increased angiogenesis and decreased mitochondria, that might be irrelevant to therapies aimed at acutely modulating the HIF response in patients with acute myocardial ischemia and impending MI (Huang et al., 2008). Moreover, a recent report challenged the conclusion that HIF1 α is required for RIPC (Kalakech et al., 2013).

Others have acutely inactivated EglN at the time of experimental MI. The pharmacological prolyl hydroxylase inhibitors, FG0041, GSK360, and 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetate (ICA) have been shown to be cardioprotective in rodents (Bao et al., 2010; Nwogu et al., 2001; Vogler et al., 2015). However, these drugs might inhibit other α -ketoglutarate (α KG)-dependent dioxygenases in addition to the EglNs. Indeed FG0041 was initially tested in this setting because it inhibits the collagen prolyl hydroxylases and only later shown to inhibit the EglNs (Nwogu et al., 2001). Several groups reported ischemic cardioprotection in mice after intraperitoneal (i.p.) (Natarajan et al., 2006) or intraventricular (Eckle et al., 2008) injection of naked *Egln1* siRNAs or intramyocardial injection of a plasmid encoding an *Egln1* shRNA (Huang et al., 2008). Although these interventions reportedly induced HIF, the bioavailability of siRNAs and plasmids delivered in this way is suspect. Moreover, it was later revealed that the *Egln1* siRNA used in one of these studies targeted a collagen prolyl hydroxylase rather than *Egln1*, raising questions about specificity (Natarajan et al., 2006). Finally, a recent study reported that intramuscular injection of an adenovirus encoding HIF1 α acutely protected the heart at a distance (Cai et al., 2013).

RESULTS

Chronic *Egln1* Inactivation in Cardiomyocytes Protects Against I/R Injury

We used genetic and pharmacological tools to probe the role of *Egln1* and HIF in local and remote ischemic preconditioning. Adult mice in which *Egln1* has been deleted in the heart at ~E12.5 experience less myocardial damage after permanent occlusion of the left anterior descending (LAD) coronary artery than do control mice, implying that *Egln1* plays a cardiac-intrinsic role in cardioprotection (Hölscher et al., 2011). To ask whether this is also true in an ischemia-reperfusion (I/R) model, which more closely mimics myocardial injury during clinical MI, we crossed mice with a conditional (floxed or "F") *Egln1* allele with a

mouse strain expressing Cre recombinase under the control of the cardiac-specific alpha myosin heavy chain (MHC) promoter (Agah et al., 1997). We confirmed inactivation of *Egln1* in the heart, but not skeletal muscle, liver and kidney, in *Egln1^{F/F};MHC-Cre* mice (Figure S1A). As expected, heart-specific *Egln1* inactivation induced HIF target genes (Figure S1B). Moreover, cardiac-specific *Egln1* inactivation was cardioprotective in mice subjected to cardiac I/R injury (Figure S1C) and in isolated hearts in a Langendorff model of global myocardial ischemia, as reflected by a faster recovery (lowering) of end-diastolic pressures compared to control mice (Figure S1D). Therefore chronic *Egln1* inactivation confers cardiac-intrinsic protection against both permanent and transient cardiac ischemia.

Acute Systemic *Egln1* Inactivation Protects the Heart Against I/R Injury

We next tested an *Egln* inhibitor, FG-4497 (Fibrogen; (Laitala et al., 2012; Robinson et al., 2008)), in the same cardiac I/R injury models. We first confirmed that FG-4497 induced HIF1 α in HL-1 cardiomyocytes (Figure 1A) and inhibited *Egln* *in vivo* as determined by imaging mice that ubiquitously express a HIF1 α -luciferase fusion reporter (Safran et al., 2006). (Figure 1B). Notably, FG-4497 stabilized HIF1 α and induced HIF target genes in the heart (Figure 1C and 1D). Pretreatment with FG-4497 decreased cardiac injury after I/R in the Langendorff model (Figure 1E) and decreased MI size in mice subjected to LAD occlusion/release *in vivo* by ~30% (Figure 1F). Importantly from a clinical perspective, FG-4497 treatment at the time of reperfusion also substantially reduced infarct sizes (Figure 1G).

Next we used *Egln1^{F/F};Cre^{ER}* mice that ubiquitously express a tamoxifen (TAM)-activatable Cre^{ER} fusion protein (Minamishima et al., 2008). Giving such mice TAM for three days significantly decreased *Egln1* mRNA levels in the heart, kidney and skeletal muscle compared to TAM-treated *Egln1^{+/+};Cre^{ER}* mice (Figure 2A), and induced HIF1 α protein (Figure 1C) and HIF-responsive mRNAs in the heart (Figure 2B). Acute, systemic, inactivation of *Egln1* decreased myocardial injury in the Langendorff I/R model (Figure 2C) and decreased MI size in mice subjected to cardiac I/R *in vivo* compared to TAM-treated *Egln1^{+/+};Cre^{ER}* control mice (Figure 2D). Consistent with *Egln1* being the relevant target of FG-4497, the effects of the combined *Egln1* gene deletion and pharmacologic *Egln* inhibition were subadditive (Figure 2E). Therefore acute systemic *Egln1* inactivation protects against cardiac I/R injury.

Skeletal Muscle *Egln1* Deletion Confers Remote Cardiac Ischemic Protection

Protection against cardiac I/R injury after systemic *Egln1* loss could reflect a cell-autonomous effect in cardiomyocytes, a non-cell autonomous “remote” effect on the heart, or both. To explore non-cell autonomous effects, we made mice in which *Egln1* could be conditionally inactivated specifically in skeletal muscle. *Egln1^{F/F}* and *Egln1^{+/+}* mice were crossed with mice expressing a TAM-dependent Cre^{ER} transgene driven by a skeletal muscle-specific promoter (human alpha actin; *HSA-Cre-ER^{T2}*) (Schuler et al., 2005). *Egln1^{F/F};HSA-Cre-ER^{T2}* and *Egln1^{+/+};HSA-Cre-ER^{T2}* mice were given TAM or vehicle for five days and sacrificed 5 days later. *Egln1* was recombined in multiple skeletal muscle groups of TAM-treated *Egln1^{F/F};HSA-Cre-ER^{T2}* mice, but not in other tissues tested, such as the heart and liver (Figure S2A). *Egln1* deletion decreased skeletal muscle *Egln1* mRNA

and protein levels (Figure 3A and 3B). Importantly, hearts from TAM-treated *Egln1^{F/F};HSA-Cre-ER^{T2}* mice did not exhibit decreased *Egln1* mRNA (Figure 3A), decreased EglN1 protein (Figure S2B), increased HIF1 α protein (Figure S2C), or increased HIF-responsive mRNAs (Figure S2D and S2E).

We next asked whether *Egln1* deletion in the skeletal muscle can protect the heart at a distance. TAM-treated *Egln1^{F/F};HSA-Cre-ER^{T2}* and *Egln1^{+/+};HSA-Cre-ER^{T2}* mice were subjected to *in vivo* cardiac I/R injury. Deletion of *Egln1* in the skeletal muscle reduced MI size after cardiac I/R injury by ~40% (Figure 3C). HIF induces nitric oxide synthase in certain cell types (Coulet et al., 2003), which might protect the heart by increasing nitric oxide production and decreasing afterload. We did not, however, detect differences in blood pressures between TAM-treated *Egln1^{F/F};HSA-Cre-ER^{T2}* and *Egln1^{+/+};HSA-Cre-ER^{T2}* mice (Figure S2F), nor alterations of blood nitrite levels (Figure S2G) (Coulet et al., 2003). These data show that *Egln1* inactivation in skeletal muscles confers remote ischemic cardioprotection.

Remote Ischemic Protection by Skeletal Muscle *Egln1* Gene Deletion is Mediated by a Humoral Factor

It is debated whether an intact peripheral nervous system, a soluble humoral factor, or both are required for RIPC (Przyklenk, 2013). To ask whether a humoral factor mediates RIPC in mice with skeletal muscle-specific *Egln1* deletion, we performed a parabiosis experiment. Parabiosis pairings were made between wild-type (WT) mice (“recipient” partner) and either *Egln1^{F/F};HSA-Cre-ER^{T2}* or *Egln1^{+/+};HSA-Cre-ER^{T2}* mice (“donor” partner). The donor mice were given TAM for 5 days, and 5 days later the recipient mice were subjected to cardiac I/R injury. Evan’s blue dye injected 2 days later confirmed shared circulation in both types of pairings (Figure 3E). Genotyping confirmed *Egln1* deletion specifically in the skeletal muscle of TAM-treated *Egln1^{F/F};HSA-Cre-ER^{T2}* donor mice (Figure 3D). Recipient parabiosis partners of *Egln1^{F/F};HSA-Cre-ER^{T2}* mice had ~30% smaller MIs than did recipient partners of *Egln1^{+/+};HSA-Cre-ER^{T2}* mice (Figure 3F). These data argue that a humoral factor mediates RIPC after *Egln1* deletion in skeletal muscle.

Egln1 Inactivation Alters Circulating Tryptophan Metabolites

To look for circulating cardioprotective factors that were upregulated after skeletal muscle deletion of *Egln1*, we profiled serum cytokines in TAM-treated *Egln1^{F/F};HSA-Cre-ER^{T2}* and *Egln1^{+/+};HSA-Cre-ER^{T2}* mice. None of the 144 cytokines profiled were upregulated >10% after *Egln1* loss in skeletal muscle (Figure S3A). In particular, we did not detect changes in the levels of IL-10 and EPO, which have previously been implicated in RIPC (Cai et al., 2013; 2003) (Figure S3B). In addition, pretreatment with a JAK1/2 inhibitor (Ruxolitinib), which blocks signaling by many cytokine receptors, did not abrogate RIPC in our model (Figure S3C). We concluded that the humoral mediator of RIPC in our model was not one of the cytokines examined or a cytokine that signals through JAK1/2.

Next, we analyzed gene expression profiles in skeletal muscles from TAM-treated *Egln1^{F/F};HSA-Cre-ER^{T2}* and *Egln1^{+/+};HSA-Cre-ER^{T2}* mice, and compared differentially regulated genes to a published list of genes predicted to encode secreted proteins (Wu et al.,

2010). 41 genes were differentially expressed in the skeletal muscles from TAM-treated *Egln1^{F/F};HSA-Cre-ERT²* ($q < 0.1$), 7 of which encode proteins predicted to be secreted (Natarajan et al., 2006; Wu et al., 2010). Real-time PCR confirmed up-regulation of 2 of these 7 mRNAs, *PLAC9* and *LYPD1* (Figure S3D). Relatively little is known of the biologic function of *PLAC9* and neither gene, to our knowledge, is known to encode circulating polypeptides.

We next looked for a potential small molecule mediator(s) of cardioprotection. Blood was collected from TAM-treated *Egln1^{F/F};HSA-Cre-ERT²* and *Egln1^{+/+};HSA-Cre-ERT²* mice, and serum and plasma metabolites were profiled by LC-MS. Among the metabolites with statistically significant differences were various tryptophan (TRP)-related metabolites (Figure 4A). To narrow the list of possible mediators of remote cardiac I/R protection, we did an orthogonal experiment by profiling selected serum metabolites in WT mice given FG-4497 or vehicle (Figure 4B). We hypothesized that the cardioprotective mediator(s) would be induced by both genetic and pharmacological *Egln1* inhibition, that systemic *Egln1* inhibition might alter levels of the metabolite(s) of interest to an even greater extent than skeletal muscle deletion of *Egln1*, and that the cardioprotective metabolite(s) would be induced rapidly by FG-4497 since FG-4497 protected hearts even when administered *after* ischemic injury (Figure 1G). Strikingly, we observed significant changes in circulating TRP metabolites 10 minutes after FG-4497 treatment (Figure 4B).

The Tryptophan Metabolite Kynurenic Acid Mediates Remote Ischemic Protection after *Egln1* Inactivation

We focused on the TRP metabolite kynurenic acid (KYNA) as a possible cardioprotective agent because KYNA is tissue protective in models of cerebral and renal injury (Germano et al., 1987; Pundir et al., 2013; Zwilling et al., 2011). KYNA production from TRP is regulated by a rate-limiting pyrolyase TRP dioxygenase (TDO) in the liver or indoleamine 2,3 dioxygenase (IDO) in peripheral tissues, as well as by α KG-dependent transamination performed by kynurenine transaminases (KAT; Figure 4C) (Agudelo et al., 2014). We confirmed that the mass spectrometry ion counts we obtained for serum KYNA and α KG in mice were in the linear ranges for their respective assays and consistent with serum concentrations of $\sim 5 \mu\text{M}$ and $\sim 50 \mu\text{M}$, respectively in untreated WT mice (Figure S3E–F).

To ask whether KYNA was needed for cardiac I/R protection after skeletal muscle *Egln1* deletion, TAM-treated *Egln1^{F/F};HSA-Cre-ERT²* and *Egln1^{+/+};HSA-Cre-ERT²* controls were given 1-methyl tryptophan (1MT), a reversible inhibitor of both TDO and IDO, before cardiac I/R injury. 1-MT partially abrogated the protection caused by skeletal muscle deletion of *Egln1*, but did not affect MI sizes in control animals (Figure 4D). Conversely, pretreatment of WT mice with KYNA (Andin  et al., 1988) or the KYNA mimetic L689,560 decreased MI size compared to vehicle-treated mice (Figure 4E and 4F). Cardioprotection by KYNA appeared to be heart-autonomous, as addition of KYNA to the perfusate of isolated hearts improved ischemia tolerance in Langendorff assays (Figure 4G). These data argue that KYNA is necessary and sufficient for remote cardiac ischemic protection following *Egln1* inhibition.

Egln1 Inhibition Generates KYNA via Altered Systemic α KG Metabolism

KYNA was not induced by inactivating Egln1 in various cell culture models (Figure S4A) and mRNAs linked to TRP metabolism were not induced in skeletal muscle after inactivating Egln1 (Figure S4B), suggesting that the increased KYNA observed after Egln1 inactivation in skeletal muscle *in vivo* was indirect and non-cell autonomous. In order to determine the organ(s) producing KYNA in our model, mice were infused with U-¹³C-TRP and then given FG-4497 (Figure S4C). Newly formed U-¹³C-KYNA was detected in the serum, liver, kidneys, and lung, but not in skeletal muscle (Figure 5A). We concluded that KYNA is made by a tissue other than muscle after Egln1 inactivation in skeletal muscle.

Hepatic KYNA production was intriguing because the TRP metabolites n-methylnicotinamide (NMN) and niacinamide (NAM), which can be made only by the liver and the kidney, were among the serum metabolites downregulated by skeletal muscle *Egln1* loss (Figure 4A). Moreover, our U-¹³C-TRP studies confirmed conversion of TRP to NMN and NAM in the liver and not the kidney (data not shown). Up-regulation of circulating KYNA combined with down-regulation of NMN and NAM suggested altered hepatic TRP metabolism after Egln inhibition (Figure 4C).

How might targeting Egln—via systemic FG-4497 administration or skeletal muscle deletion of *Egln1*—regulate hepatic TRP metabolism? We found no differences in mRNA levels for KYNA pathway enzymes in the liver (Figure S4D) of TAM-treated *Egln1^{FF};HSA-Cre-ER^{T2}* mice compared to control mice. Moreover, serum KYNA was increased 10 minutes after systemic FG-4497 administration (Figure 4B). These two observations suggested that control of KYNA by Egln1 in this model did not involve transcriptional changes in the liver.

KYNA production is regulated by KATs (Agudelo et al., 2014) which, like Egln1, require α KG as a co-substrate. α KG accumulates under hypoxic conditions and after pVHL loss (with consequent HIF stabilization) (Metallo et al., 2012; Wise et al., 2011), suggesting that α KG might link Egln1 inactivation to KYNA production. Indeed, α KG levels were elevated in the serum (Figure 4A), skeletal muscle, and liver in TAM-treated *Egln1^{FF};HSA-Cre-ER^{T2}* mice compared to control mice (Figure 5B) and in the livers of mice given FG-4497 compared to vehicle (Figure 5C). Systemic administration of α KG, like FG-4497, rapidly (10 minutes) increased circulating and hepatic KYNA levels (Figure 5D). Importantly, systemic α KG administration also protected hearts subjected to I/R injury *in vivo* (Figure 5E) and *ex vivo* in the Langendorff model (Figure 5F). In notable contrast to KYNA, however, α KG did not provide I/R protection when added directly to the perfusate in the Langendorff model (Figure 5F), consistent with the idea that α KG protects *in vivo* by increasing hepatic KYNA production.

HIF-independent Regulation of α KG Metabolism by Egln1

In cell culture models, hypoxia and FG-4497 both increased α KG levels (Figure 6A–B). This was true whether α KG levels were normalized to cell number, or to the dioxygenase product succinate (α KG/Suc ratio) or to the transamination partner glutamate (α KG/Glu ratio). FG-4497 had minimal effect on α KG in cells *Egln1*^{–/–} cells, supporting an on-target

drug effect (Figure S5A). The rapid induction of α KG (Figure 5C) and KYNA (Figure 4B) after EglN1 inactivation *in vivo* suggested that the accumulation of α KG in this setting does not require the canonical EglN1 target HIF α and transcriptional induction of HIF target genes. Consistent with this view, FG-4497 and hypoxia increased the α KG/Glu ratio in Hepa-1c1c7 cells that lack the requisite HIF α transcriptional partner ARNT (Figure 6C) and, accordingly, cannot activate a HIF-responsive luciferase reporter after EglN inhibition (Figure 6D) despite HIF1 α stabilization (Figure 6E). Taken together, our data suggest that EglN inhibition in skeletal muscle—in a manner independent of HIF transcriptional activity—increases circulating and hepatic α KG, which drives the production of the cardioprotective metabolite KYNA.

We hypothesized that EglN1 inhibition increases α KG levels as a direct or indirect consequence of altered decarboxylation of its co-substrate, α KG. EglN1 is highly active, with a maximum rate of α KG decarboxylation of 45 mol/mol EglN1/min (Hirsilä et al., 2005). We estimate that 1 gram of skeletal muscle protein contains approximately 6.11 picomoles of EglN1, which based on the above rate, could decarboxylate 275 picomoles α KG /minute (Figure S5B–F). As a point of reference, this flux is ~0.5% of the skeletal muscle glycolytic rate (Kummitha et al., 2014).

To assess this predicted high rate of flux further, we traced the conversion of esterified, ^{13}C - α KG to succinate over time in cells. ^{13}C - α KG was rapidly converted to ^{13}C -succinate in both WT (Figure 6F) and Cytochrome B mutant 143B cybrid cells (Figure S5G–H). This conversion was enhanced by ectopic expression of WT, but not catalytically-defective, EGLN1 (Ladroue et al., 2008), and was decreased by 10-minute pretreatment with FG-4497. These effects of manipulating EGLN1 were not due to indirect changes in O_2 consumption because Cytochrome B mutant cells lack an intact electron chain and are respiration defective (Sullivan et al., 2015). These experiments yielded an estimated rate of α KG decarboxylation by EGLN1 of ~200 picomoles/min/g of tissue (Figure S5I). The actual rate might be higher in tissues with high metabolic rates such as the heart, muscle and liver. Collectively, these findings support a high rate of EGLN1-dependent conversion of α KG to succinate and implicate EGLN1 in the direct control of central carbon metabolism.

DISCUSSION

Cardiovascular diseases such as MI and stroke are the leading cause of death worldwide. Our findings confirm and extend earlier claims that chronic EglN1 inactivation, as well as chronic HIF stabilization, protects the heart against I/R injury (Cai et al., 2007; Hyvärinen et al., 2010) and permanent ligation injury (Bao et al., 2010; Nwogu et al., 2001). However, prolonged HIF activation can ultimately cause deleterious cardiac effects, culminating in dilated cardiomyopathy (Bekeredjian et al., 2010; Huang et al., 2004; Moslehi et al., 2010). Moreover, these preclinical models of chronic HIF activation do not necessarily address the utility of acutely inactivating EglN1 in the pre or peri-infarct setting. Using both a conditional *EglN1* allele and a small molecule EglN1 inhibitor we found that acute EglN1 inactivation protects the heart against I/R injury. Importantly, the use of a pharmacological inhibitor, in contrast to the genetic model, allowed us to model drug treatment at the time of injury, where we again observed significant protection. Nonetheless, EglN1 inhibition might

ultimately be most effective when used prophylactically, such as in the setting of unstable angina or elective cardiac surgery, which are associated with a high risk of an ischemic insult.

The cardioprotective effects observed after acute, systemic, EglN1 inactivation are likely to involve both cardiomyocyte-intrinsic HIF-dependent effects as well as remote effects. Since EglN1 is ubiquitously expressed and would predictably be inhibited by tissue ischemia, such remote effects could potentially underlie RIPC. Indeed, we found that acute, genetic ablation of *EglN1* in skeletal muscles protects the heart at a distance.

We discovered that acute EglN1 inhibition leads to rapid, HIF-independent, systemic induction of α KG, which drives hepatic transamination of the TRP metabolite kynurenine (KYN) to produce KYNA, presumably by stimulating one of several known α KG-dependent KATs. Notably, perivenous hepatocytes are able to utilize circulating α KG to support transamination reactions (Stoll and Hüssinger, 1989). KYNA was previously shown to be tissue protective in models of cerebral and renal ischemia (Andiné et al., 1988; Germano et al., 1987; Pundir et al., 2013). We observed that KYNA is cardioprotective both *in vivo* and *ex vivo*, and is both necessary and sufficient for remote cardiac protection after skeletal muscle inactivation of EglN1. Although KYNA is critical in our model, additional humoral and neural factors might also contribute to RIPC.

Notably, KYNA is elevated in survivors of cardiac arrest—a condition of systemic ischemia—in both animal models and in humans (Ristagno et al., 2013). Also, the KYNA precursor, KYN, is induced in hypoxic cells as part of a ‘catabolic signature’ of hypoxia (Frezza et al., 2011). Additionally the downstream kynurenine pathway metabolite anthranilate is one of the few metabolites elevated for sustained periods after MI, suggesting changes in TRP metabolism during cardiac ischemia (Lewis et al., 2008). These observations underscore a potential role of TRP metabolism in the response to ischemia.

Our data suggest that acute inactivation of EglN1, which utilizes α KG as a co-substrate, causes the rapid accumulation of α KG. The rate of α KG decarboxylation by EglN1 is predicted to be high because of the rapid turnover of its hydroxylation targets, the HIF α proteins. Biochemical experiments also suggest that the EglNs decarboxylate α KG in an uncoupled reaction in the absence of a polypeptide substrate so long as adequate reducing equivalents are available (Hirsilä et al., 2005), although the relative importance of coupled and uncoupled α KG decarboxylation by the EglNs *in vivo* is unknown. Nonetheless, our experiments with ^{13}C - α KG are consistent with a very high rate of α KG metabolism by EglN1. Although it is difficult to predict changes in metabolite pool sizes from changes in flux rates, the rapid induction of α KG after EglN1 inhibition is likely due, at least in part, to the abrupt decrease in α KG utilization by EglN1. Notably, acutely inhibiting an α KG flux of nanomoles per minute on a whole mouse basis could theoretically alter serum α KG concentrations in the physiological (μM) range. It is also possible that EglN1 has a non-canonical substrate that acutely influences α KG pool sizes, or that changes in EglN1 metabolic flux indirectly alter other processes that ultimately impact α KG pool sizes. Finally, decreased α KG uptake by tissues after EglN1 loss might contribute to redistribution of α KG *in vivo*.

KYNA is an agonist for the aryl hydrocarbon receptor, where it can affect transcription, and a ligand for various receptors including NMDA receptors, neuronal cholinergic $\alpha 7$ nicotine receptors, and the orphan G-coupled receptor GPR35. Interestingly, GPR35 is a HIF target that is induced during cardiac remodeling (Ronkainen et al., 2014). Clearly additional studies are required to determine how KYNA protects the heart.

The mediator(s) of RIPC have been sought for more than 20 years, in hopes they could be used to treat cardiovascular diseases. Two recent large, randomized, studies, however, did not find a benefit of RIPC in patients undergoing cardiac surgery (Hausenloy et al., 2015; Meybohm et al., 2015). Our findings suggest that the efficacy of RIPC could be influenced by many variables including duration and magnitude of ischemia, skeletal muscle mass, hepatic function, and concurrent medications, including drugs metabolized in the liver, such as anesthetics. With respect to the latter, these two studies required and allowed, respectively, the use of propofol, which has been suggested to block RIPC (Kottenberg et al., 2012; 2014). Direct administration of KYNA might be a more robust way to protect tissues such as the heart than past attempts to induce RIPC with controlled regional ischemia.

EXPERIMENTAL PROCEDURES

Materials

FG-4497 was obtained from FibroGen, Inc. (San Francisco, CA, USA), and dosed at 50 mg/kg for mouse studies and 30 μ M concentration for *in vitro* studies. 5 mg/ml 1-methyl-DL-tryptophan (1-MT; Sigma-Aldrich, St. Louis, MO, USA) was added to the drinking water of mice.

Cell Culture

Immortalized mouse embryonic fibroblasts (MEFs) were made from *Egln1*^{+/+} and *Egln1*^{-/-} littermates and maintained in pyruvate-free DMEM containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S). Hepa-1c1c7 mouse hepatoma cells (ATCC, Manassas, VA, USA) were grown in MEM (Corning Mediatech, Manassas, VA, USA) supplemented with 10% FBS and 1% P/S. 143B cybrid cells (Sullivan et al., 2015) were grown in DMEM supplemented with 10% FBS, 1% P/S, 1 mM sodium pyruvate, and 0.1 mg/ml uridine.

Retroviruses

A Flag-tagged WT human *EGLN1* cDNA (Lorenzo et al., 2014) was subcloned into a pLenti vector with a CMV promoter. The H374R mutation (c.1121A→G) was made by site-directed mutagenesis. The 3XHRE luciferase reporter (Yan et al., 2007) was shuttled into a promoterless lentivirus. Lentiviruses were made by co-transfection (TransIT, Mirus Bio LLC, Madison, WI, USA) of 293TL cells with expression vectors along with the packaging constructs. Cells were infected by centrifugation in the presence of viral supernatants and polybrene.

Mice

The *Egln1*^{F/F}, α MHC-Cre, Cre^{ER}, HSA-Cre-ER^{T2} and ODD-Luc mice were previously described (Minamishima et al., 2008; Moslehi et al., 2010; Safran et al., 2006; Schuler et al., 2005). *Egln1* was deleted by treating age- and sex-matched *Egln1*^{F/F} mice harboring a TAM-regulated Cre with TAM 1 mg/dose i.p.. For studies in HSA-Cre-ER^{T2} mice, TAM was given daily for 5 days and mice were studied 5 days later. For studies in Cre^{ER} mice, three days of TAM were given and mice were analyzed on day 4 (Minamishima et al., 2008).

In Vivo Bioluminescence Imaging

In vivo bioluminescence imaging was done as previously described (Safran et al., 2006). Mice were given FG-4497 (i.p.) at the stated doses 2 hours before D-luciferin (150 mg/kg, i.p.).

Ischemia-Reperfusion Injury

8–12 week old mice were anesthetized, intubated and ventilated. Left thoractomy was performed, and the left anterior descending (LAD) artery was ligated with a 6.0 silk suture. Ischemia was confirmed by myocardial blanching and electrocardiographic (ECG) evidence of injury. Five minutes into ischemia, 50 μ L of fluorescent microspheres (10 μ M FluoSpheres, Molecular Probes, Eugene, OR, USA) was injected into the left ventricular cavity. The LAD ligature was released 30 minutes later and reperfusion confirmed visually by ECG. Overall survival was 70–80% at 24 hours. Mice were terminally anesthetized 24 hours after ischemia with ketamine/xylazine followed by cervical dislocation, hearts were harvested, and the ventricles were sectioned from apex to base in 2 mm sections. Sections were incubated in 2% (wt/vol) triphenyltetrazolium (TTC, Sigma) in phosphate-buffered saline at 25°C for 30 minutes. Infarct size and area at risk (AAR) was quantified from light and fluorescent micrographs of myocardial sections using Adobe Photoshop. Percent MI was calculated as the infarcted area divided by the AAR. Assessment of I/R injury in the Langendorff Model was as described (Liao et al., 2012).

Parabiosis surgeries were done as per published protocols (Wright et al., 2001). Ten days after the parabiosis operation, a subset (n=2) of parabiosed mice were used to confirm the presence of cross-circulation of blood by Evan's Blue dye injection. One mouse in each joined pair was injected with 100 μ L of Evan's Blue into the retro-orbital venous plexus. Peripheral blood was collected before and 2 hours after injection. Cardiac I/R injury was performed as described above. The “recipient” partner (mouse undergoing I/R) was anesthetized and intubated per protocol while “donor” partners was anesthetized using ketamine at 80 mg/kg IP for the duration of the surgery.

In Vivo Delivery of Stable Isotope Tracers

Conscious, unrestrained mice were infused with 0.19 mg/kg/minute U-¹³C-TRP (Cambridge Isotopes Laboratories, Inc., Tewksbury, MA, USA) in normal saline via a central venous catheter. At 30-minute intervals, 20 μ L of blood was sampled from an arterial catheter and 10 μ L aliquot of plasma was snap-frozen in liquid N₂. After 90 min, a 50 mg/kg bolus of FG-4497 was dosed over 4 minutes. Mice were sacrificed by sodium pentobarbital (120 mg/kg), and tissues collected.

Metabolite Extraction and Mass Spectrometry

For metabolic analyses organs were quickly dissected from euthanized mice. ~50 mg tissue sections were clamped between liquid nitrogen-cooled flat forceps, weighed, and ground. Metabolites were extracted in methanol:water:chloroform (6:3:4) by vortexing for 10 minutes at 4°C followed by centrifugation at 10,000 x *g* for 10 minutes at 4°C. Aliquots of aqueous fractions, in proportion to tissue exact mass, were dried in a refrigerated centrifuge. For cell culture experiments, sample extraction were done as described (Fendt et al., 2013).

For gas chromatography mass spectrometry (GCMS) analyses, dried metabolites were derivatized and analyzed as described (Fendt et al., 2013). Reported are the dominant ions for derivatives of alpha-ketoglutarate (α KG, *m/z* 346), Succinate (Suc, *m/z* 289), and Glutamate (Glu, *m/z* 432). ¹³C mass isotopes were quantified and corrected for natural mass isotope abundances. For liquid chromatography mass spectrometry (LC-MS) analysis of tissue metabolites, dried metabolites were resuspended in acetonitrile:methanol:formic acid (75:25:0.5 v:v:v) and samples were analyzed in positive and negative ion mode using via hydrophilic interaction liquid chromatography (HILIC) MS analyses (Avanesov et al., 2014; Townsend et al., 2013; Wang et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Inhibition of EglN1 locally or at a distance protects the heart against I/R injury
- Remote ischemic protection after EglN1 loss is mediated by a humoral factor
- Diversion of the EglN co-substrate α KG stimulates hepatic kynurenic acid production

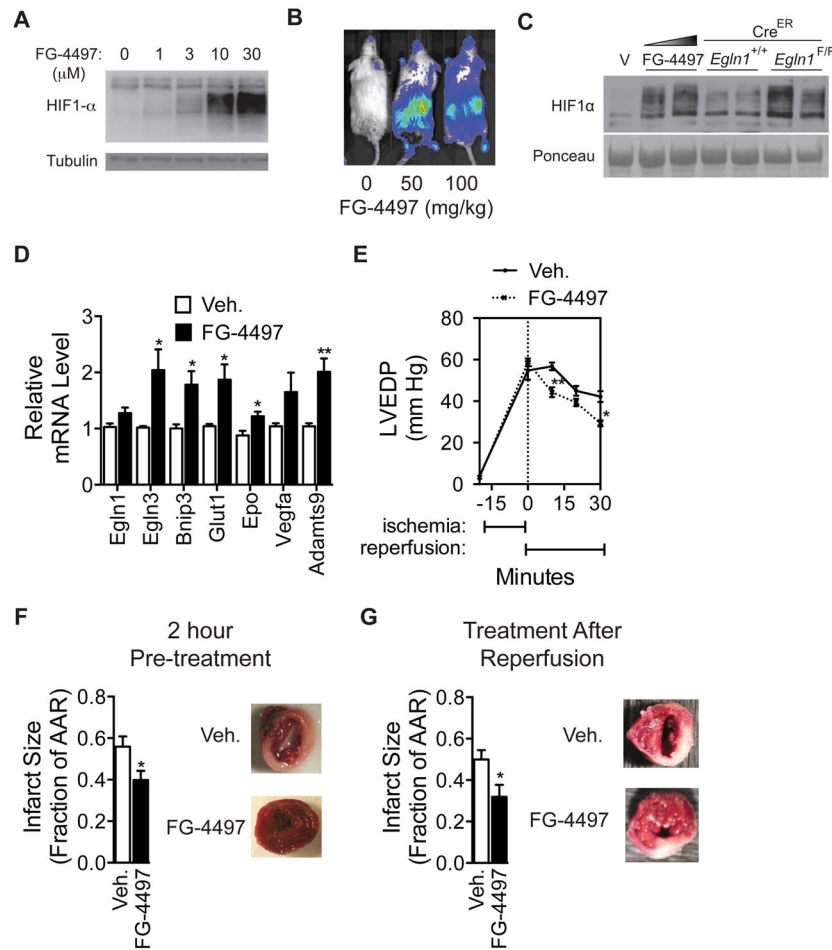


Figure 1. Acute Systemic Inhibition of Egn Protects Against Cardiac I/R Injury

(A) Representative immunoblots of HL-1 cardiomyocytes treated with FG-4497 for 6 hours.

(B) Bioluminescent images of representative mice expressing a HIF-luciferase fusion protein 3 hours after being given intravenous (i.v.) FG-4497.

(C) HIF1 α protein levels in the hearts of mice given 50 or 100 mg/kg FG-4497 i.v., as indicated by the triangle, or after systemic *Egn1* genetic deletion. *Egn1*^{F/F}; Cre-ER and *Egn1*^{+/+}; Cre-ER mice were given TAM for 5 days before sacrifice.

(D) Real-time PCR assays of mRNAs in the heart 3 hours after i.p. administration of 50 mg/kg FG-4497 (n=5) or vehicle (n=4). Data shown are mean fold changes \pm SEM. *p<0.05, **p<0.01, Student's *t*-test, FG-4497 versus vehicle (Veh.).

(E) LVEDP in Langendorff assays of global I/R injury. Mice were given 50 mg/kg FG-4497 i.v. or Veh. 2 hours before cardiectomy. Data shown are mean pressures \pm SEM, n = 8 in each group. *p<0.05, **p<0.01, Student's *t*-test.

(F) MI size after cardiac I/R injury in mice given 50 mg/kg FG-4497 i.p. or Veh. two hours before *in vivo* cardiac I/R injury. Data are normalized to the Area-at-Risk (AAR). Data shown are means \pm SEM, n = 8 FG-4497 and n = 7 vehicle. *p<0.05, Student's *t*-test. Representative photographs of TTC-stained hearts are shown.

(G) MI size relative to AAR in mice given 50 mg/kg FG-4497 i.p. or Veh. at the time of LAD reperfusion. Data shown are means \pm SEM, n = 6 FG-4497 and n = 5 vehicle. *p<0.05, Student's *t*-test. Representative photographs of TTC-stained hearts are shown.

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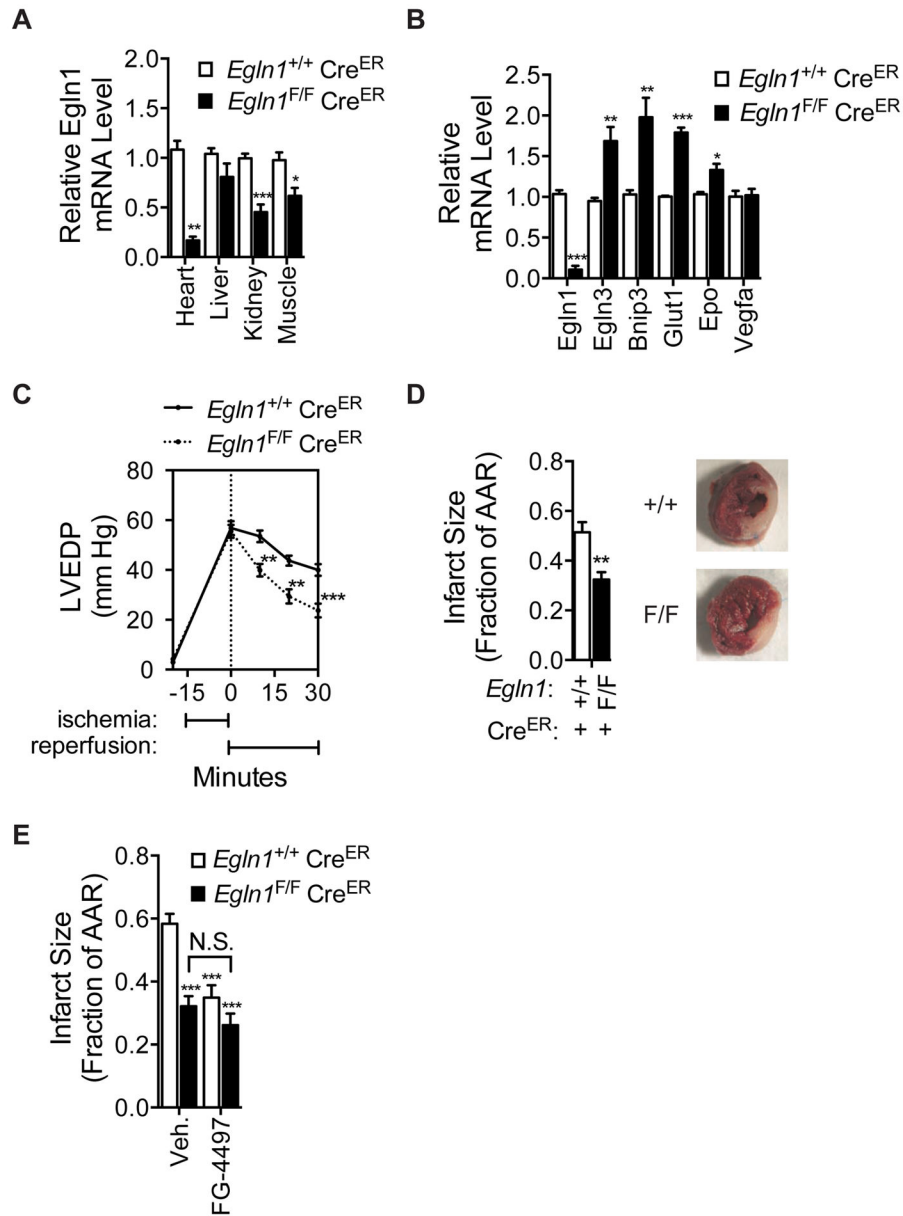


Figure 2. Acute Systemic Deletion of *EglN1* Protects Against Cardiac I/R Injury

(A) Real-time PCR assays of *EglN1* mRNA in tissues from *EglN1*^{F/F}; Cre^{ER} and *EglN1*^{+/+}; Cre^{ER} mice. Mice were given TAM for 3 days and sacrificed 3 days later. Data shown are mean fold changes \pm SEM, $n = 4$ in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t -test, difference in mRNA levels between *EglN1*^{F/F}; Cre^{ER} and *EglN1*^{+/+}; Cre^{ER} mice.

(B) Real-time PCR assays of select HIF-responsive mRNAs in the hearts from TAM-treated *EglN1*^{F/F}; Cre^{ER} and *EglN1*^{+/+}; Cre^{ER} mice. Data shown are mean fold changes \pm SEM, $n = 4$ in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t -test, difference in mRNA levels between *EglN1*^{F/F}; Cre^{ER} and *EglN1*^{+/+}; Cre^{ER} mice.

(C) LVEDP in Langendorff assays of global I/R injury. Mice were given TAM as in (A) before cardiectomy. Data shown are mean pressures \pm SEM, $n = 6$ in each group. ** $p < 0.01$, *** $p < 0.001$, Student's t -test.

(D) MI size relative to AAR after *in vivo* cardiac I/R injury in TAM-treated *Egln1^{F/F}; Cre^{ER}* and *Egln1^{+/+}; Cre^{ER}* mice. Data shown are means \pm SEM, n = 10 mice per group. **p<0.01, Student's *t*-test.

(E) MI size after cardiac I/R injury in TAM-treated *Egln1^{F/F}; Cre^{ER}* and *Egln1^{+/+}; Cre^{ER}* mice, pre-treated with 50 mg/kg FG-4497 i.v. or Veh. two hours before cardiac I/R injury. Data shown are means \pm SEM, n = 8 FG-4497 and n = 7 vehicle groups. ***p<0.001, Student's *t*-test, differences MI sizes compared to Veh.-treated *Egln1^{+/+}; Cre^{ER}* mice. See Figure S1.

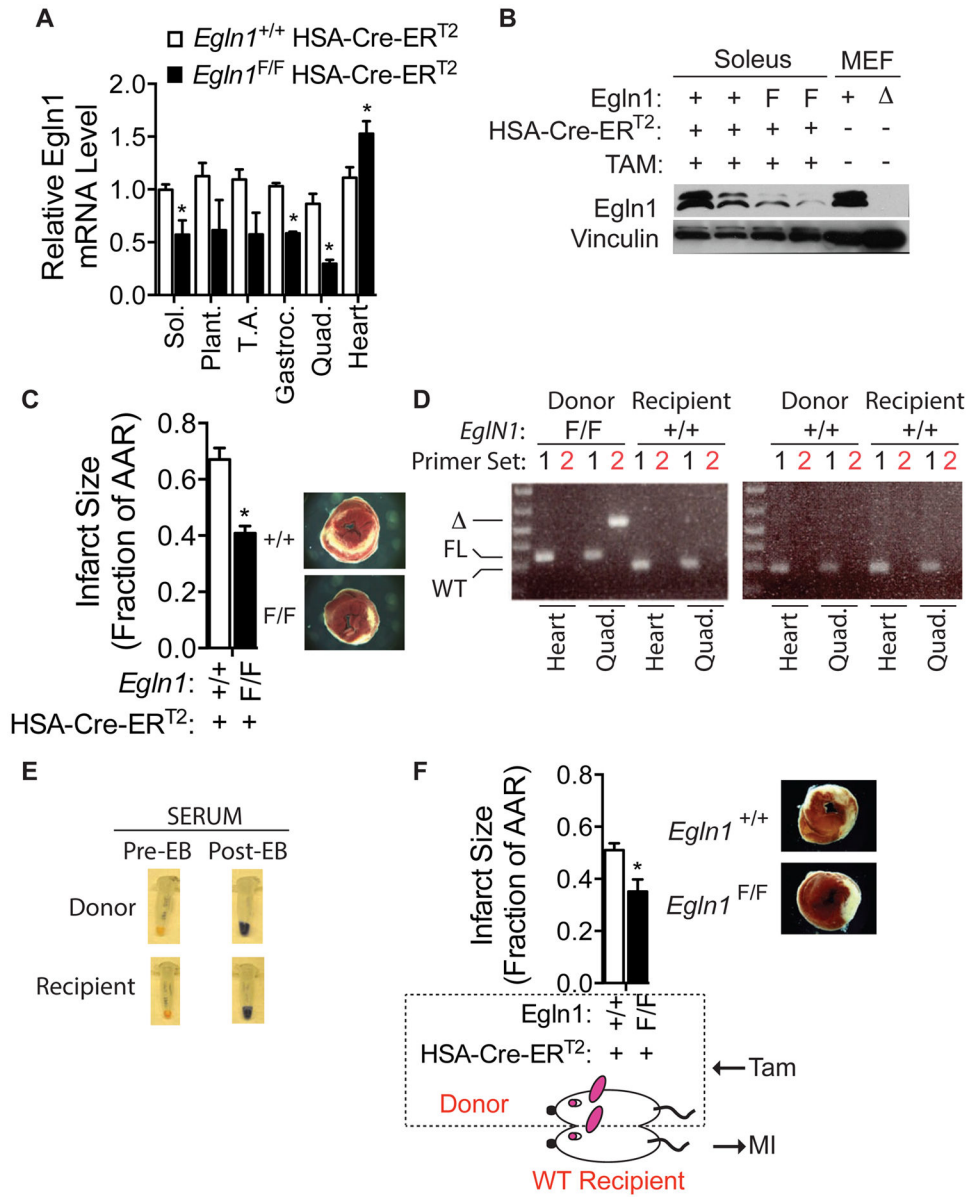


Figure 3. Skeletal Muscle *EglN1* Deletion Confers Remote Cardioprotection via a Circulating Factor

(A) Real-time PCR assays of *EglN1* mRNA levels in indicated tissues from TAM-treated *EglN1*^{F/F}; HSA-Cre-ER^{T2} and *EglN1*^{+/+}; HSA-Cre-ER^{T2} mice. Abbreviations: soleus (Sol.), plantaris (Plant.), tibialis anterior (T.A.), gastrocnemius (Gastroc.), quadriceps (Quad.). Data shown are mean fold changes ± SEM. *p<0.05, Student's *t*-test, differences in mRNA levels in *EglN1*^{F/F}; HSA-Cre-ER^{T2} mice compared to *EglN1*^{+/+}; HSA-Cre-ER^{T2} mice.

(B) Immunoblots of extracts from Sol. muscle of TAM-treated *EglN1*^{F/F}; HSA-Cre-ER^{T2} and *EglN1*^{+/+}; HSA-Cre-ER^{T2} mice. *EglN1* WT (+) and knockout (Δ) MEFs were included for comparison.

(C) MI size after cardiac I/R injury in TAM-treated *EglN1*^{F/F}; HSA-Cre-ER^{T2} and *EglN1*^{+/+}; HSA-Cre-ER^{T2} mice. Data shown are means ± SEM, n = 6 mice per group. *p<0.05, Student's *t*-test. Representative photographs of TTC-stained hearts are shown.

(D) PCR genotyping of the heart and quadriceps (Quad) of ‘donor’ and ‘recipient’ parabiosis mice. Donor mice were TAM-treated *Egln1*^{F/F}; HSA-Cre-ER^{T2} and *Egln1*^{+/+}; HSA-Cre-ER^{T2} mice, as indicated. *Egln1* primer set 1 amplifies WT and floxed (FL) alleles, and primer set 2 amplifies the deleted allele ().

(E) Representative serum samples from parabiosis ‘donor’ and ‘recipient’ mice before and 2 hours after i.v. injection of Evan’s blue (EB) dye.

(F) MI size after cardiac I/R injury in WT mice that were surgically conjoined to either a TAM-treated *Egln1*^{F/F}; HSA-Cre-ER^{T2} mouse or a TAM-treated *Egln1*^{+/+}; HSA-Cre-ER^{T2} mouse. WT ‘recipient’ mice were subjected to cardiac I/R and MI size quantified 24 hours later. Data shown are means ± SEM, n = 6 *Egln1*^{F/F}; HSA-Cre-ER^{T2} and n = 8 *Egln1*^{+/+}; HSA-Cre-ER^{T2} mice. *p<0.05, Student’s *t*-test, difference in MI size in WT mice conjoined to *Egln1*^{F/F}; HSA-Cre-ER^{T2} mice vs. WT mice conjoined to *Egln1*^{+/+}; HSA-Cre-ER^{T2} mice. Representative photographs of TTC-stained hearts are shown.

See also Figure S2.

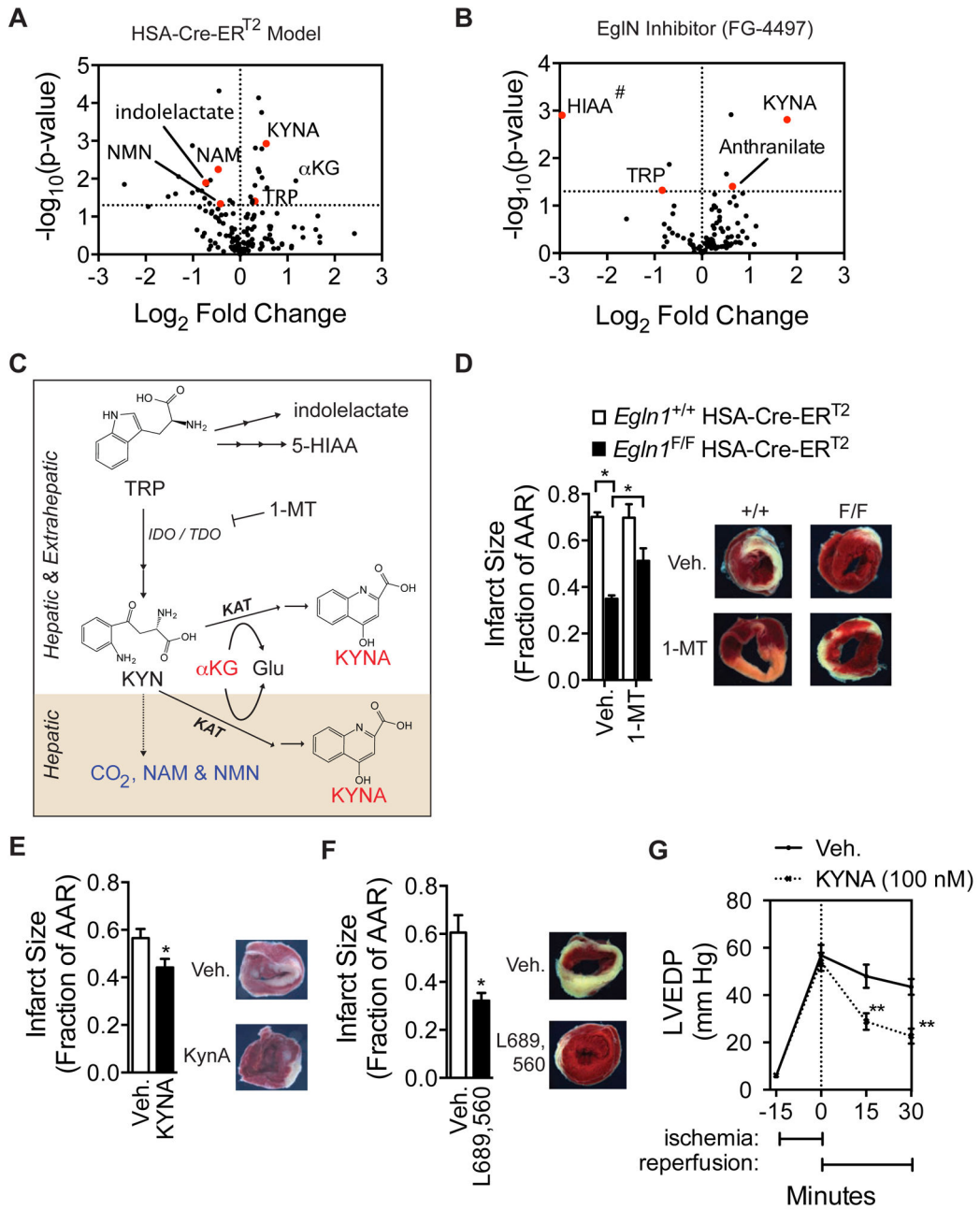


Figure 4. The TRP Metabolite Kynurenic Acid is Necessary and Sufficient for Remote Cardiac I/R Protection

(A) Volcano plot depicting fold change and statistical significance in ion counts for serum metabolites in TAM-treated *Egln1^{F/F}*, HSA-Cre-ER^{T2} mice compared to *Egln1^{+/+}*, HSA-Cre-ER^{T2} mice. Abbreviations: KYNA – kynurenic acid; αKG – alpha-ketoglutarate; TRP – tryptophan; NAM – niacinamide; NMN – n-methyl-nicotinamide. Metabolites above the dotted line are those that differ with $p < 0.05$ (Student’s *t*-test, $n=6$). TRP metabolites are shown in red.

(B) Volcano plot depicting fold change and statistical significance of serum metabolites in mice given FG-4497 compared to Veh-treated animals. Labeled are the TRP metabolites

KYNA, Anthranilate, and 5-hydroxyindoleacetic acid (HIAA). Metabolites above the dotted line are those that differ with $p < 0.05$ (Student's t -test, $n=3$). #HIAA was undetectable in serum of mice given FG-4497. TRP metabolites are shown in red.

(C) Diagram of TRP metabolism, including the rate-limiting pyrolyase IDO, present in peripheral tissues, TDO, present in the liver, the IDO/TDO inhibitor 1-methyltryptophan (1-MT), and the regulated kynurenine (KYN) aminotransferases (KAT). The direct product of KYN transamination by KATs, 4-(2-aminophenyl)-2,3-dioxobutanoate (not shown), is non-enzymatically dehydrated to produce KYNA.

(D) MI size after cardiac I/R injury in TAM-treated *Egln1^{F/F}*; HSA-Cre-ER^{T2} and *Egln1^{+/+}*; HSA-Cre-ER^{T2} mice given the IDO/TDO inhibitor 1-methyltryptophan (1-MT) or vehicle (Veh.) for 3 days before I/R. Data shown are means \pm SEM, $n = 5$ *Egln1^{F/F}*; HSA-Cre-ER^{T2} vehicle, $n = 5$ *Egln1^{F/F}*; HSA-Cre-ER^{T2} 1-MT, $n = 3$ *Egln1^{+/+}*; HSA-Cre-ER^{T2} vehicle, $n = 4$ *Egln1^{+/+}*; HSA-Cre-ER^{T2} 1-MT. * $p < 0.05$, Student's t -test, for designated comparisons.

(E) MI size after cardiac I/R injury in WT mice pretreated with 100 ng KYNA or Veh. i.p. 2-hr before and 2 hr after *in vivo* cardiac I/R. Data shown are means \pm SEM, $n = 16$ vehicle, $n=14$ KYNA. * $p < 0.05$, Student's t -test.

(F) MI size after cardiac I/R injury in WT mice given 40 mg/kg KYNA mimetic L689,560 or Veh. i.p. immediately before I/R injury. Data shown are means \pm SEM, $n = 3$ per group. * $p < 0.05$, Student's t -test.

(G) LVEDP in Langendorff assays after global I/R injury, with or without 100 nM KYNA present in the perfusate. Data shown are mean pressures \pm SEM, $n = 10$ KYNA group and $n = 9$ control group. ** $p < 0.01$, Student's t -test.

See also Figure S3.

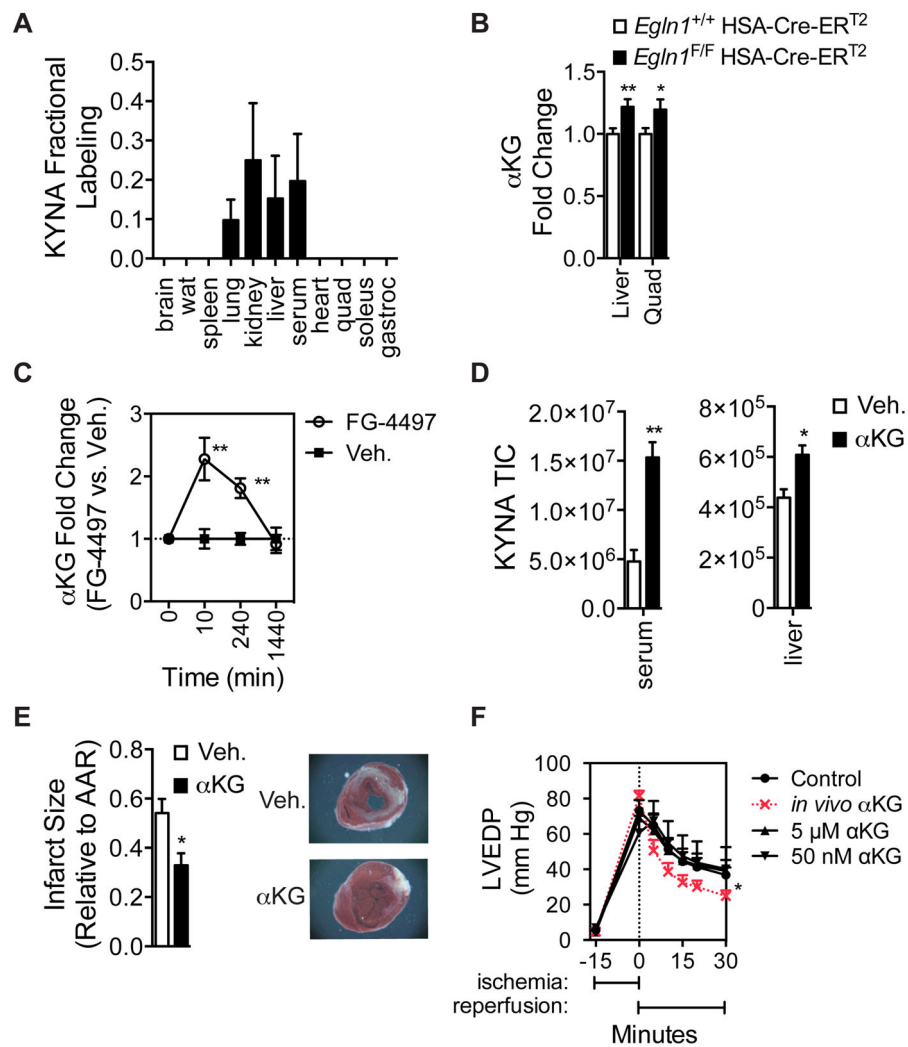


Figure 5. Altered Systemic αKG Metabolism in *EglN1*^{F/F} HSA-Cre-ER^{T2} Mice Leads to Cardioprotection Through KYNA

(A) Fractional labeling of U-¹³C-KYNA in tissues. WT mice were infused with U-¹³C-TRP until serum isotopic equilibrium was reached and then given 50 mg/kg FG-4497 i.v. Tissues were harvest 30 minutes later. Shown are mean enrichment ± SEM, n = 2.

(B) αKG levels in liver and quadriceps (Quad) of TAM-treated *EglN1*^{F/F}; HSA-Cre-ER^{T2} and *EglN1*^{+/+}; HSA-Cre-ER^{T2} mice. Shown are mean fold change ± SEM, n = 23, **p<0.01, *p<0.05, Student's *t*-test, comparison between *EglN1*^{F/F}; HSA-Cre-ER^{T2} and *EglN1*^{+/+}; HSA-Cre-ER^{T2} mice.

(C) Hepatic αKG levels in WT mice given 50 mg/kg FG-4497 i.v. or Veh. Shown are mean fold change ± SEM, n = 5. **p<0.01 Student's *t*-test.

(D) KYNA levels in serum and liver of mice given 1 mg/kg αKG or Veh. i.p. Shown are mean total ion counts (TIC) quantified by LC-MS, ± SEM, n = 3. *p<0.05, **p<0.01 Student's *t*-test.

(E) MI size in WT mice treated as in (D) 2 hours before cardiac I/R injury. Data shown are means \pm SEM, n = 4 vehicle, n=6 α KG. *p<0.05, Student's *t*-test. Representative photographs of TTC-stained hearts are shown.

(F) LVEDP in Langendorff assays after global I/R injury with or without α KG present in the perfusate at the indicated concentrations. One group of mice was pretreated with 1 mg/kg α KG i.p. 45 minutes before sacrifice. Data shown are mean pressures \pm SEM. *p<0.05, Student's *t*-test, for differences at 30 minutes compared to control.

See also Figure S4.

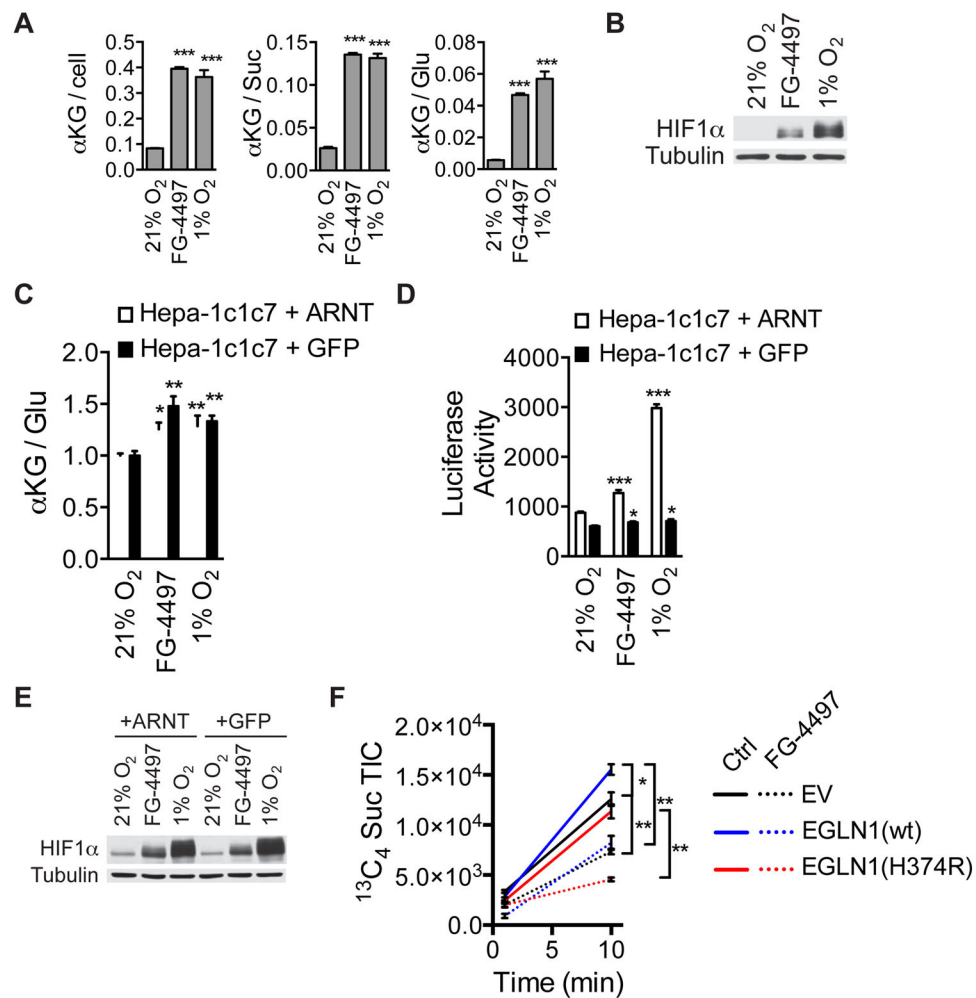


Figure 6. EglN Activity Regulates α KG Levels in a HIF-independent Manner

(A) α KG levels in WT MEFs given FG-4497 or cultured under 1% O₂ conditions for 24 hours. Data are normalized to internal standard and cell number, succinate TIC (Suc), or glutamate TIC (Glu). *** p <0.001, Student's t -test, differences compared to untreated MEFs grown in 21% O₂. Shown are mean \pm SEM, n = 3.

(B) Immunoblots of WT MEFs treated as in (A).

(C) α KG / Glutamate ratios of Hepa-1c1c7 ARNT^{-/-} cells stably expressing exogenous ARNT or GFP and treated with FG-4497 or 1% O₂ for 24 hours. * p <0.05, ** p <0.01, Student's t -test, differences compared to untreated MEFs grown in 21% O₂. Shown are mean \pm SEM, n = 8.

(D) Firefly luciferase activity in Hepa-1c1c7 expressing exogenous ARNT or GFP, as in (C), and harboring a HIF-responsive firefly luciferase reporter. Cells were treated with FG-4497 or 1% O₂ for 24 hours as indicated. Shown are mean \pm SEM, n = 8. * p <0.05, *** p <0.001, Student's t -test, differences relative to untreated cells grown in 21% O₂.

(E) Immunoblots of cells used in (C) and (D).

(F) Total ion counts of $^{13}\text{C}_4$ -succinate (Suc) in WT 143B cells at indicated time points after addition of U- ^{13}C -dimethyl- α KG. Shown are mean \pm SEM, n = 4. *p<0.05 and **p<0.01, Student's *t*-test, comparisons designated.
See also Figure S5.

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