

Title	Ethanolamine is a novel STAT-3 dependent cardioprotective agent
Authors	Kelly-Laubscher, Roisin;Lamont, Kim T.;Somers, Sarin;Hacking, Damian;Lacerda, Lydia;Thomas, Paul;Opie, Lionel H.;Lecour, Sandrine
Publication date	2010-10-12
Original Citation	Kelly, R. F., Lamont, K. T., Somers, S., Hacking, D., Lacerda, L., Thomas, P., Opie, L. H. and Lecour, S. (2010) 'Ethanolamine is a novel STAT-3 dependent cardioprotective agent', Basic Research in Cardiology, 105 (6), pp. 763-770. doi: 10.1007/s00395-010-0125-0
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1007/s00395-010-0125-0
Rights	© Springer-Verlag 2010. This is a post-peer-review, pre-copyedit version of an article published in Basic Research in Cardiology. The final authenticated version is available online at: <a href="http://dx.doi.org/10.1007/s00395-010-0125-0">http://dx.doi.org/10.1007/s00395-010-0125-0</a> - <a href="https://link.springer.com/article/10.1007/s12012-015-9355-6">https://link.springer.com/article/10.1007/s12012-015-9355-6</a>
Download date	2025-01-21 04:43:18
Item downloaded from	<a href="https://hdl.handle.net/10468/12482">https://hdl.handle.net/10468/12482</a>



# UCC

**University College Cork, Ireland**  
Coláiste na hOllscoile Corcaigh

*This version of the article has been accepted for publication, after peer review and is subject to Springer Natures Accepted Manuscript terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: <http://dx.doi.org/10.1007/s00395-010-0125-0>.*

Title: Ethanolamine is a novel STAT-3 dependent cardioprotective agent.

Article Type: Original Contribution

Keywords: ethanolamine, sphingosine-1-phosphate, ischaemia-reperfusion, cardioprotection, STAT-3

Corresponding Author: Roisin Kelly, PhD

Corresponding Author's Institution:

First Author: Roisin Kelly, PhD

Order of Authors: Roisin Kelly, PhD; Kim T Lamont, MSc; Sarin Somers, MSc; Damian Hacking, BSc; Lydia Lacerda, MSc; Paul Thomas, PhD; Lionel H Opie, MD PhD; Sandrine Lecour, PhD

**Abstract:** Ethanolamine is a biogenic amine found naturally in the body as part of membrane lipids and as a metabolite of the cardioprotective substances, sphingosine-1-phosphate (S1P) and anandamide. In the brain, ethanolamine, formed from the breakdown of anandamide protects against ischaemic apoptosis. However, the effects of ethanolamine in the heart are unknown. Signal transducer and activator of transcription 3 (STAT-3) is a critical prosurvival factor in ischaemia/reperfusion (I/R) injury. Therefore, we investigated whether ethanolamine protects the heart via activation of STAT-3. **Methods:** Isolated hearts from wildtype or cardiomyocyte specific STAT-3 knockout (K/O) mice were pretreated with ethanolamine (Etn) (0.3 mmol/L) before an I/R insult. In vivo rat hearts were subjected to 30 min ischaemia/2h reperfusion in the presence or absence of 5mg/kg S1P and/or the FAAH inhibitor, URB597. Infarct size was measured at the end of each protocol by triphenyltetrazolium chloride staining. **Results:** Pre-treatment with ethanolamine decreased infarct size in isolated mouse or rat hearts subjected to I/R but this infarct sparing effect was lost in cardiomyocyte specific STAT-3 deficient mice. Pre-treatment with ethanolamine increased nuclear phosphorylated STAT-3 [control;  $0.75 \pm 0.08$  vs. Etn;  $1.50 \pm 0.09$  arbitrary units;  $p < 0.05$ ]. **Conclusion** Our findings suggest a novel cardioprotective role for ethanolamine against I/R injury via activation of STAT-3.

**Ethanolamine is a novel STAT-3 dependent cardioprotective agent.**

Roisin F Kelly<sup>1</sup>, Kim T Lamont<sup>1</sup>, Sarin Somers<sup>1</sup>, Damian Hacking<sup>1</sup>, Lydia Lacerda<sup>1</sup>, Paul Thomas<sup>2</sup>, Lionel H Opie<sup>1</sup>, Sandrine Lecour<sup>1</sup>.

1. Hatter Cardiovascular Research Institute, Department of Medicine, Faculty of Health Sciences, University of Cape Town, South Africa, 2. Division of Kinesiology & Health, University of Wyoming, USA.

Corresponding Author: Roisin Kelly Tel: 0027 214047707 Fax: 0027 214478789  
email: [R.Kelly@uct.ac.za](mailto:R.Kelly@uct.ac.za)

**Abstract:** Ethanolamine is a biogenic amine found naturally in the body as part of membrane lipids and as a metabolite of the cardioprotective substances, sphingosine-1-phosphate (S1P) and anandamide. In the brain, ethanolamine, formed from the breakdown of anandamide protects against ischaemic apoptosis. However, the effects of ethanolamine in the heart are unknown. Signal transducer and activator of transcription 3 (STAT-3) is a critical prosurvival factor in ischaemia/reperfusion (I/R) injury. Therefore, we investigated whether ethanolamine protects the heart via activation of STAT-3. **Methods:** Isolated hearts from wildtype or cardiomyocyte specific STAT-3 knockout (K/O) mice were pretreated with ethanolamine (Etn) (0.3 mmol/L) before an I/R insult. *In vivo* rat hearts were subjected to 30 min ischaemia/2h reperfusion in the presence or absence of 5mg/kg S1P and/or the FAAH inhibitor, URB597. Infarct size was measured at the end of each protocol by triphenyltetrazolium chloride staining. **Results:** Pre-treatment with ethanolamine decreased infarct size in isolated mouse or rat hearts subjected to I/R but this infarct sparing effect was lost in cardiomyocyte specific STAT-3 deficient mice. Pre-treatment with ethanolamine increased nuclear phosphorylated STAT-3 [control;  $0.75 \pm 0.08$  vs. Etn;  $1.50 \pm 0.09$  arbitrary units;  $p < 0.05$ ]. **Conclusion:** Our findings suggest a novel cardioprotective role for ethanolamine against I/R injury via activation of STAT-3.

**Keywords:** ethanolamine, sphingosine-1-phosphate, ischaemia-reperfusion, cardioprotection, STAT-3

## Introduction

Exogenous ethanolamine can be obtained from many foods and beverages including wine [16], milk [20], and grapes [4]. Stored in the body as the membrane lipid, phosphatidylethanolamine, ethanolamine is also found in the body as a product of metabolism of many cardioprotective molecules such as the endogenous cannabinoid anandamide, and the sphingolipid metabolite sphingosine-1-phosphate (S1P). In the brain, ethanolamine formed from the breakdown of anandamide in the presence of fatty acid amide hydrolase (FAAH) can protect against ischaemia induced apoptosis [36]. Both S1P and anandamide can protect against ischaemia-reperfusion (I/R) [22, 33, 48, 56, 57] but the cardioprotective effect of ethanolamine has not been explored.

Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT-3), as part of the “Survivor activating factor enhancement” (SAFE) pathway, are critical protective molecules that protect against I/R injury [1, 2, 8, 27, 31, 63]. STAT-3 is activated by ischaemic pre- and postconditioning [1, 8, 25, 51] and by various pharmacological agents such as adenosine,[51] opioids,[7] erythropoietin [46], tumor necrosis factor alpha [23, 29, 55] and insulin [6].

Therefore, the main hypothesis investigated in this study was that ethanolamine may confer cardioprotection against I/R. Furthermore, we proposed that this protective effect is mediated via the activation of the JAK/STAT-3 pathway. The protective range of exogenous ethanolamine was determined in an isolated rat heart model of I/R injury. Subsequently, the role of STAT-3 in ethanolamine mediated cardioprotection was studied in cardiomyocyte specific STAT-3 knockout (K/O) mice subjected to an I/R insult ex vivo.

**Materials and Methods:****Ethical approval:**

All experimental procedures were carried out with the approval of the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town. All protocols were carried out in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes for Health (NIH Publication No. 85 (23), revised 1996). Male Wistar (200-350g) rats, wildtype and cardiomyocyte specific STAT-3 deficient mice (14-16 weeks) were bred and obtained from the University of Cape Town Animal Unit.

**Isolated rat heart model**

Rats were anaesthetised with sodium pentobarbital (50 mg/kg i.p.) and heparinised (500 IU i.v.). Hearts were rapidly excised and perfused retrogradely by the Langendorff technique as previously described.[30] All hearts were subjected to 30 min of regional ischaemia by occlusion of the left coronary artery and 120 min of reperfusion as described previously.[28] Hearts were pretreated with either 0, 0.1, 0.3, 1, or 5 mmol/L ethanolamine for 15 min, followed by a 10 min washout period before the ischaemia.

In another experiment, hearts were pretreated with 0.3 mmol/L ethanolamine for 15 min followed by a 10 min washout period before the ischaemia. The JAK-STAT-3 inhibitor, AG490 (100 nmol/L),[29] was given for 15 min: 3 min before, concomitantly with the ethanolamine (Etn +AG490 group) and 5 min after.

Hemodynamic parameters were assessed throughout the experiment and included heart rate (HR), left ventricular end diastolic pressure (LVEDP) and coronary flow (CF). For measurement of infarct size, the coronary artery was reoccluded at the end of the reperfusion period and a solution of 2.5% Evans blue was perfused to delineate the area at risk. Hearts were then frozen and cut into slices, incubated in sodium phosphate buffer containing 1% w/v

TTC for 15 min to visualise the unstained infarct region. Infarct and risk zone areas were determined with planimetry and infarct size was expressed as a percentage of the risk zone.

Two different strains of rats were used in these studies due to insufficient numbers of Wistar rats at the time of the experiment. However, appropriate control groups were performed for each strain.

Preparation of hearts for Western Blots: In isolated rat hearts, the left ventricular tissue from control and ethanolamine (with or without AG490) pretreated hearts was excised before the regional ischaemic insult, freeze clamped in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Nuclear and cytosolic proteins were extracted as previously described.[29]

### **Isolated STAT-3<sup>-/-</sup> knockout heart model**

Cardiomyocyte specific STAT-3 knockout mice and wildtype littermate control mice were anaesthetised (sodium pentobarbitone, 60mg/kg i.p.) and heparinised (25 IU i.p.). Once an adequate level of anaesthesia was achieved, the chest was opened, the heart rapidly removed, placed in ice cold ( $4^{\circ}\text{C}$ ) Krebs-Henseleit buffer and the aorta was cannulated. Hearts were then perfused with a modified Krebs-Henseleit buffer using the Langendorff system as previously described.[52] After a 20 min stabilisation period, hearts were subjected to 35 min global ischaemia followed by 45 min reperfusion. Hearts were pretreated with 0.3 mmol/L ethanolamine for 7 min and 15 min, respectively, followed by a 10 minute washout period before global ischaemia. At the end of the experimental protocol, the infarct size was assessed by triphenyltetrazolium chloride (TTC) staining. Infarct size was determined with planimetry.[52]

### **In vivo coronary artery ligation in the rat**

Rats were anaesthetised with sodium pentobarbital (60 mg/kg i.p.), intubated and ventilated with room air (2.5 ml/stroke) at a rate of 70 strokes per minute. Rats were placed on a custom-made heating block to maintain body temperature throughout the surgical procedure. Depth of anaesthesia was monitored by assessing the pedal withdrawal reflex and monitoring heart rate. Maintenance doses of anaesthetic (6mg/kg i.p.) were administered as required. A left thoracotomy was performed and the left anterior descending coronary artery was ligated as previously described[5]. Control rats were subjected to a period of 30 min of ischaemia followed by 2 h of reperfusion. In the S1P group, a bolus dose of 5 mg/kg S1P i.v. was injected 30 min prior to the I/R protocol [56]. In a third group, a single dose of URB597 (0.3 mg/kg i.p.) was administered 90 min before the I/R protocol [21] with (S1P+URB group) or without (URB group) S1P pretreatment. After 2 h of reperfusion, the coronary artery was reoccluded with the suture that had been left in place and staining was carried out with patent blue and 1% wt/vol triphenyltetrazolium chloride as previously described. [5] Infarct and risk-zone areas were determined by planimetry, and infarct size was expressed as a percentage of the risk zone.

### **Western Blot analysis**

Phosphorylated states of STAT-3 (phospho-STAT-3 Tyr 705) and total levels of STAT-3 were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with antibodies from Cell Signaling Technology as previously described [55]. Equal loading was verified with Ponceau staining and levels of phosphorylated proteins were normalized to their total protein



levels performed in the same samples and under the same conditions but on a separate membrane. Relative densitometry was determined with use of a computerized software package.

**Statistical analysis.**

Data are presented as mean $\pm$ SEM. Comparisons between multiple groups were performed by 1-way ANOVA followed by the Dunnett's post hoc test (Graph Pad Instat). A value of  $p < 0.05$  was considered statistically significant.

## Results

### **Ethanolamine protects against ischaemic-reperfusion injury**

In the isolated rat heart model, I/R in untreated hearts resulted in an infarct size of  $75 \pm 2\%$  (Fig. 1a). Pre-treatment with ethanolamine at a concentration of 0.1 mmol/L and 0.3 mmol/L decreased infarct size after an I/R insult [Etn (0.1 mmol/L)  $57 \pm 4\%$ , Etn (0.3 mmol/L)  $58 \pm 3\%$ ,  $P < 0.05$  vs. Control] whereas protection was lost at higher concentrations [Etn (1 mmol/L)  $63 \pm 9\%$ , Etn (5 mmol/L)  $76 \pm 6\%$ , ns vs. control]. Area at risk did not differ significantly between the groups [Etn (0mmol/L)  $44 \pm 3\%$ , Etn (0.1 mmol/L)  $46 \pm 4\%$ , Etn (0.3 mmol/L)  $38 \pm 7\%$ , Etn (1 mmol/L)  $50 \pm 4\%$ , Etn (5 mmol/L)  $47 \pm 11\%$ ].

### **Inhibition of STAT-3 activation abrogates protection induced by ethanolamine**

In the isolated rat heart model, control hearts subjected to an I/R insult had an infarct of  $40 \pm 3\%$  (Fig 3). Pretreatment with ethanolamine (0.3 mmol/L) reduced infarct size ( $20 \pm 3\%$  vs. ischaemic control,  $P < 0.01$ ,  $n=6$ ). To investigate the role of STAT-3 in ethanolamine induced cardioprotection, we administered an inhibitor of the JAK/STAT-3 pathway, AG490 [29]. Perfusion of AG490 abolished the cardioprotective effect of ethanolamine ( $34 \pm 3\%$  vs. ischaemic control,  $P < 0.05$ ) (Fig 3).

### **Ethanolamine mediated cardioprotection was inhibited in the STAT-3 knockout mice**

Preliminary experiments conducted in **C57B6** mice demonstrated that control hearts subjected to 35 min global ischaemia and 45 min reperfusion presented a similar infarct ( $47.75 \pm 2.56\%$ ) compared with hearts subjected to 35 min global ischaemia and 2 hrs of reperfusion ( $42.20 \pm 1.83\%$ ). Therefore, we reduced the duration of reperfusion to 45 min for further

experiments. Wildtype STAT-3 mice had an infarct size of  $33 \pm 3\%$  (Fig 2). Pretreatment with ethanolamine (0.3 mmol/L) resulted in a significant reduction of the infarct size to  $15 \pm 3\%$  ( $P < 0.05$  vs. wild type Control). Ischaemic control hearts from cardiomyocyte deficient STAT-3 mice had an infarct size of  $30 \pm 3\%$ . The infarct sparing effect observed with ethanolamine pretreatment in the wildtype mice was absent in knockout hearts ( $29 \pm 4\%$ ,  $P =$  n.s vs. control group) (Fig 2).

### **Ethanolamine induced an increase in phosphorylated STAT-3 in the nucleus**

Western blot analysis of tissue isolated from Langendorff perfused rat hearts revealed a decrease in cytosolic tyrosine phosphorylated /total STAT-3 after ethanolamine pre-treatment (control;  $0.87 \pm 0.19$  vs. Etn;  $0.28 \pm 0.12$  arbitrary units) (Fig 4a). There was no significant change in total STAT-3 in the cytosolic fraction amongst the different groups [Control  $5.3 \pm 0.6$ , Etn  $4.5 \pm 0.3$ , Etn + AG490  $4.0 \pm 0.7$ , AG490  $6.4 \pm 2.7$ , arbitrary units].

Ethanolamine pre-treatment was associated with an increase of nuclear tyrosine phosphorylated/total STAT-3 (Control;  $0.75 \pm 0.08$  vs. Etn;  $1.50 \pm 0.01$  arbitrary units,  $P < 0.001$ ) (Fig 4b). This increase in phosphorylated/total STAT-3 was inhibited by AG490 ( $0.65 \pm 0.39$  arbitrary units,  $P < 0.001$  vs ethanolamine). There was no significant change in total STAT-3 in the nuclear fraction function to the treatment [Control  $4.0 \pm 2.6$ , Etn  $3.3 \pm 2.3$ , Etn + AG490  $2.0 \pm 1.3$ , AG490  $0.9 \pm 0.2$ ].

A trend for an increase in STAT-3 serine phosphorylation was observed in both the nucleus [Control  $0.59 \pm 0.10$ , Etn  $2.09 \pm 0.66$ ,  $p =$  n.s.] and the cytoplasm [Control  $0.25 \pm 0.09$ , Etn  $1.03 \pm 0.54$ ,  $p =$  n.s.].

### **S1P induced cardioprotection is inhibited by the FAAH inhibitor URB597**

To ensure that exogenous S1P could protect the heart from ischaemia reperfusion injury *in vivo*, rats were pretreated with a single injection of S1P (5 mg/kg i.v.) (Fig 5). Ischaemic control hearts had an infarct size of  $61\pm 3\%$  (n=7) (calculated as a percentage of the risk zone). Although the area at risk did not differ among the various groups (Control  $41\pm 4\%$ , S1P  $45\pm 6\%$ , S1P +URB  $44\pm 5\%$ , URB  $50\pm 5\%$ ), pretreatment with S1P (5 mg/kg, i.p.) reduced infarct size ( $43\pm 4\%$ , n=6) compared with the ischaemic control group ( $P<0.01$ ). This protection was abolished by pretreatment of the rats with URB597 ( $62\pm 2\%$  (n=6),  $P<0.01$  vs. S1P). Pretreatment with URB597 alone had no effect on infarct size ( $59\pm 2\%$  (n=4), n.s. vs. Control) (Fig. 5).

## **Discussion**

Using different models and animal species, our novel data demonstrate that ethanolamine, a component of wine and food products, can mediate cardioprotection against an I/R insult.

Of note, ethanolamine protected against two different types of ischaemia/ reperfusion injury, each of which has different clinical implications; 1) Regional ischaemia/ reperfusion mimics a complete thrombotic coronary artery occlusion, and 2) Global ischaemia/ reperfusion mimics ischaemia/reperfusion caused clinically during cardiac arrest followed by restoration of circulation[15]. Furthermore, we have recognised STAT-3 as a downstream mediator of ethanolamine induced cardioprotection. The main data leading to these conclusions are as follows: 1) Ethanolamine induced cardioprotection against I/R injury; 2) the protective effect of ethanolamine was lost in cardiomyocyte specific STAT-3 knockout mice or in the presence of the JAK-STAT-3 inhibitor, AG490, in isolated rat hearts.

### ***Ethanolamine confers cardioprotection against ischaemia-reperfusion injury***

The ability of ethanolamine to protect the heart from ischemia may give novel insights into the cardioprotective characteristics of wine and certain diets. Ingestion of two to three glasses of red wine a day is thought to be cardioprotective[42]. Therefore, it is noteworthy that ethanolamine, at a concentration of 0.3 mmol/L (the concentration found in red wine), was cardioprotective[16]. We must acknowledge, however, that the concentration in the blood after this glass of wine would at most be inversely proportional to the blood volume of the subject. This reservation does not however nullify the possible relevance of our finding to human cardioprotection because there are other sources of ethanolamine in the diet, such as milk[20], balsamic vinegar [41] and egg yolks[47]. Furthermore, soyabean proteins in the diet increase hepatic concentrations of free ethanolamine. These increases in free ethanolamine are thought

to influence plasma cholesterol levels [54]. Therefore, epidemiological links between an ethanolamine rich diet and coronary heart disease in humans would be worth exploring.

Endogenous ethanolamine is stored as membrane phospholipids such as phosphatidylethanolamine, plasmalogenethanolamine and plasmalogenethanolamine. In the heart, plasmalogens are the major form of ethanolamine phospholipid[62]. The vinyl ether of plasmalogens scavenges oxygen radicals[35]. Cells lacking plasmalogens have increased sensitivity to chemical hypoxia induced by actinomycin A or cyanide compared to wild types[64]. However, it has previously been shown that exogenous ethanolamine has no effect on membrane phospholipid concentration[45]. Interestingly, exogenous ethanolamine can affect the mitochondria of the cell. Ethanolamine, given at concentrations between 0 mM and 5 mM induced mild uncoupling of mitochondria whereas 10mM ethanolamine completely inhibited mitochondrial respiration[40]. Ischemic preconditioning confers cardioprotection as a result of mild uncoupling of the mitochondria [34, 49] and in our experimental protocol, ethanolamine was used as a preconditioning mimetic at a concentration leading to mild uncoupling. Therefore, it may be possible that the protective effect of ethanolamine may result of mild uncoupling of the mitochondria.

### ***Ethanolamine can activate the JAK/ STAT-3 pathway***

Multiple protective signalling pathways have been identified in the heart[19] and recent studies have highlighted the cardioprotective importance of STAT-3 activated in the SAFE pathway, an alternative signalling path to the well described reperfusion injury salvage kinase (RISK) pathway known to involve the kinases Akt and extracellular regulated kinase [9-11, 18, 27, 31, 50]. The present study establishes that the transcription factor STAT-3 acts as a downstream mediator of ethanolamine induced cardioprotection. Ethanolamine caused a decrease of phosphorylated STAT-3 in the cytosol concomitant with an increase of

phosphorylated STAT-3 in the nucleus. STAT-3 is known to translocate to the nucleus, where it acts as a transcription factor. However, the results of transcription induced by ethanolamine are unlikely to produce the protective effects seen in such short term experiments, therefore suggesting that STAT-3 is acting as a signalling molecule in the nucleus or other organelles in the cell rather than as a transcription factor. Recent evidence suggests that phosphorylated STAT-3 can also translocate to the mitochondria and affect cellular respiration and metabolism [60]. As mitochondria play a key role in the protection achieved by many cardioprotective agents and techniques [12-14, 17, 19, 24, 26, 34, 39, 49, 61] future experiments will aim at elucidating whether the cardioprotective effect of ethanolamine is due to phosphorylation of STAT-3 within the nucleus or within the mitochondria.

#### ***Endogenous Ethanolamine and possible link with S1P***

Using various experimental models, the cardioprotective effect of S1P alone, or as part of the high density lipoproteins, is now well established [38, 48, 56, 58]. S1P protects against I/R if given before the insult or during the reperfusion phase. It is known that S1P is metabolised to phosphoethanolamine [53] and incorporated into the lipid membrane as phosphatidylethanolamine. Recent evidence suggests that phosphatidylethanolamine can lead to the production of the endogenous cannabinoid anandamide [43, 59]. FAAH is a membrane bound serine hydrolase responsible for the hydrolysis of anandamide to ethanolamine [37]. Our present findings raise the possibility that ethanolamine may be involved in S1P induced cardioprotection. Although the specificity of the inhibitor URB597 for FAAH is supported by in vitro and in vivo studies, [3, 21, 32, 44] these results need confirmation using FAAH knockout mice.

#### ***Conclusion***

Our findings suggest a novel cardioprotective role for ethanolamine, a natural biogenic amine found in various food products, against I/R injury via STAT-3. Moreover, we speculate that SIP-induced cardioprotection is mediated by production of endogenous ethanolamine.



**Acknowledgements**

This work was supported in part by the National Research Foundation of South Africa, the Inter-University Cape Heart Group of the South African Medical Research Council and the Servier Heart Failure Project. Dr RF Kelly was supported by the Claude Leon Foundation and A/Prof S Lecour was partly supported by the Medical Research Council Career Award.

## Figure Legends:

### Fig 1: Dose-dependent cardioprotective effect of ethanolamine (Etn)

- a) Pretreatment with ethanolamine in isolated Langendorff perfused Long Evans rat hearts subjected to 30 min ischaemia and 2 hours reperfusion decreased infarct size in a dose-dependent manner. \*  $P < 0.05$  vs. Control group with no ethanolamine.
- b) Similar results were observed in isolated L-cell fibroblasts subjected to a simulated ischaemic insult. \*\*  $P < 0.01$  vs. Control group with no ethanolamine.

### Fig 2: The cardioprotective effect of ethanolamine is abolished in cardiomyocyte specific STAT-3 knockout mice subjected to ischaemia/reperfusion.

In isolated mouse hearts, ethanolamine failed to protect the cardiac specific STAT-3 deficient mice against an I/R insult. \*\*\*  $P < 0.001$  vs. wild type control (n=6 per group). WT= wildtype, KO=knockout. STAT-3= Signal transducer and activator of transcription-3, Etn = ethanolamine

### Fig 3: Ethanolamine confers protection via STAT-3 in the isolated perfused rat heart

Addition of the STAT-3 inhibitor AG490 (100nmol/L) with ethanolamine, abolished the infarct sparing effect of ethanolamine in isolated rat hearts (n>6). \*\*\*  $P < 0.001$  vs control. Etn = ethanolamine, AG = AG490.

### Fig 4: Ethanolamine decreased phosphorylation of cytosolic STAT-3

- a) Representative Western Blots demonstrating decreased cytosolic levels of phospho-STAT-3/Total STAT-3 after 15 min of ethanolamine pre-treatment in isolated rat Wistar hearts. \*\*\*  $P < 0.001$  vs control. Etn = ethanolamine

- b) Representative Western Blots demonstrating increased nuclear levels of phospho-STAT-3/Total STAT-3 after 15 min of ethanolamine pre-treatment in isolated rat hearts. \*\*\*  $P < 0.001$  vs control. Etn = ethanolamine

**Fig 5: Sphingosine-1 phosphate (S1P) induced cardioprotection is abolished by URB597  
in vivo**

S1P (5mg/kg i.v.) given 30 min prior to I/R in the in vivo rat model of myocardial infarction decreased infarct size (\*  $P < 0.01$  vs. Control,  $n \geq 6$ ). However co-treatment with URB597 abolished this protective effect (#  $P < 0.01$  vs. S1P+URB,  $n=6$ ). Pretreatment with URB597 alone had no effect on infarct size (n.s. vs. Control)

**Conflict of Interest**

None declared

**References:**

1. Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, Schulz R (2008) Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. *Circ Res* 102:131-135
2. Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, Schulz R (2008) The myocardial JAK/STAT pathway: from protection to failure. *Pharmacol Ther* 120:172-185
3. Clapper JR, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2006) The fatty-acid amide hydrolase inhibitor URB597 does not affect triacylglycerol hydrolysis in rat tissues. *Pharmacol Res* 54:341-344
4. Del Prete V, Costantini A, Cecchini F, Morassut M, Garcia-Moruno E (2009) Occurrence of biogenic amines in wine: The role of grapes. *Food Chem* 112:474-481
5. Deuchar GA, Opie LH, Lecour S (2007) TNFalpha is required to confer protection in an in vivo model of classical ischaemic preconditioning. *Life Sci* 80:1686-1691
6. Fuglestad BN, Suleman N, Tiron C, Kanhema T, Lacerda L, Andreasen TV, Sack MN, Jonassen AK, Mjos OD, Opie LH, Lecour S (2008) Signal transducer and activator of transcription 3 is involved in the cardioprotective signalling pathway activated by insulin therapy at reperfusion. *Basic Res Cardiol* 103:444-453
7. Gross ER, Hsu AK, Gross GJ (2006) The JAK/STAT pathway is essential for opioid-induced cardioprotection: JAK2 as a mediator of STAT3, Akt, and GSK-3 beta. *Am J Physiol Heart Circ Physiol* 291:H827-34
8. Hattori R, Maulik N, Otani H, Zhu L, Cordis G, Engelman RM, Siddiqui MA, Das DK (2001) Role of STAT3 in ischemic preconditioning. *J Mol Cell Cardiol* 33:1929-1936
9. Hausenloy DJ, Yellon DM (2007) Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail Rev* 12:217-234
10. Hausenloy DJ, Tsang A, Yellon DM (2005) The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. *Trends Cardiovasc Med* 15:69-75
11. Hausenloy DJ, Yellon DM (2004) New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc Res* 61:448-460
12. Hausenloy DJ, Yellon DM, Mani-Babu S, Duchon MR (2004) Preconditioning protects by inhibiting the mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol* 287:H841-9
13. Hausenloy DJ, Duchon MR, Yellon DM (2003) Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. *Cardiovasc Res* 60:617-625

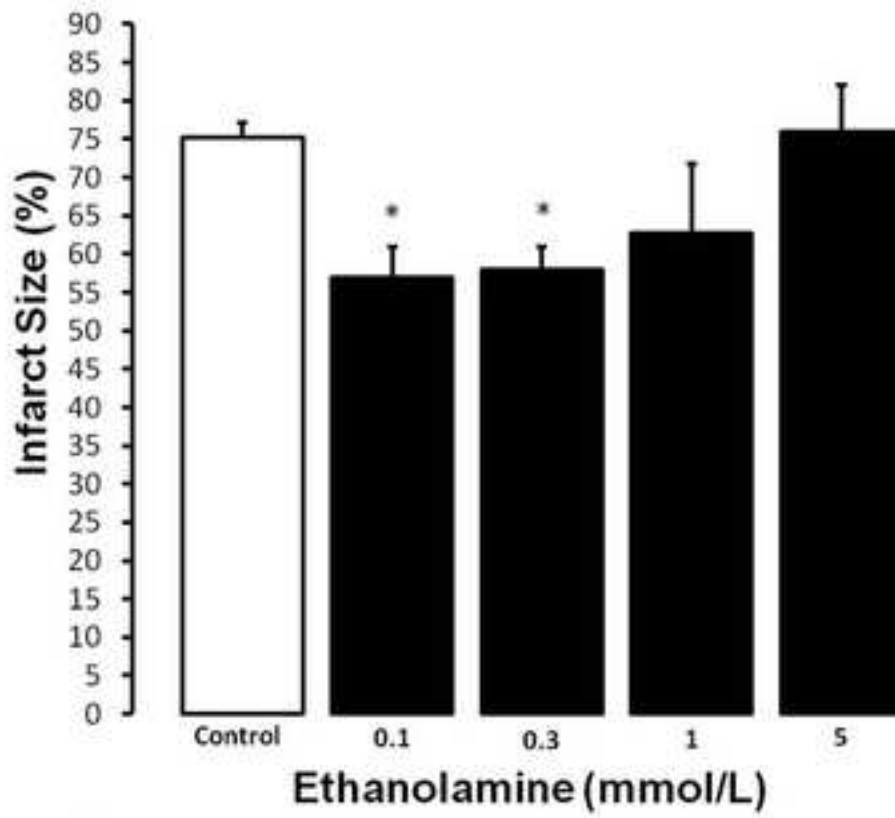
14. Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM (2002) Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning?. *Cardiovasc Res* 55:534-543
15. Hausenloy DJ, Baxter GF, Bell R, Botker HE:D,S.M., Downey J, Heusch G, Kitakaze M, Lecour S, Mentzer R, Mocanu MM, Ovize M, Schulz R, Shannon R, Walker M, Walkinshaw G, Yellon DM ((In Press)) Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. *Basic Res Cardiol*
16. Hernandez-Borges J, D'Orazio G, Aturki Z, Fanali S (2007) Nano-liquid chromatography analysis of dansylated biogenic amines in wines. *J Chromatogr A* 1147:192-199
17. Heusch G, Boengler K, Schulz R (2010) Inhibition of mitochondrial permeability transition pore opening: the Holy Grail of cardioprotection. *Basic Res Cardiol* 105:151-154
18. Heusch G (2009) No risk, no ... cardioprotection? A critical perspective. *Cardiovasc Res* 84:173-175
19. Heusch G, Boengler K, Schulz R (2008) Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation* 118:1915-1919
20. Jenson G (1995) Handbook of Milk composition, pp 375-376. Academic Press, San Diego
21. Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* 9:76-81
22. Keul P, Sattler K, Levkau B (2007) HDL and its sphingosine-1-phosphate content in cardioprotection. *Heart Fail Rev* 12:301-306
23. Kleinbongard P, Heusch G, Schulz R (2010) TNFalpha in atherosclerosis, myocardial ischemia/reperfusion and heart failure. *Pharmacol Ther* 127:295-314
24. Lacerda L, McCarthy J, Mungly SF, Lynn EG, Sack MN, Opie LH, Lecour S (2010) TNFalpha protects cardiac mitochondria independently of its cell surface receptors. *Basic Res Cardiol*
25. Lacerda L, Somers S, Opie LH, Lecour S (2009) Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. *Cardiovasc Res* 84:201-208
26. Lacerda L, Smith RM, Opie L, Lecour S (2006) TNFalpha-induced cytoprotection requires the production of free radicals within mitochondria in C2C12 myotubes. *Life Sci* 79:2194-2201
27. Lecour S (2009) Multiple protective pathways against reperfusion injury: a SAFE path without Aktion?. *J Mol Cell Cardiol* 46:607-609
28. Lecour S, Rochette L, Opie L (2005) Free radicals trigger TNF alpha-induced cardioprotection. *Cardiovasc Res* 65:239-243

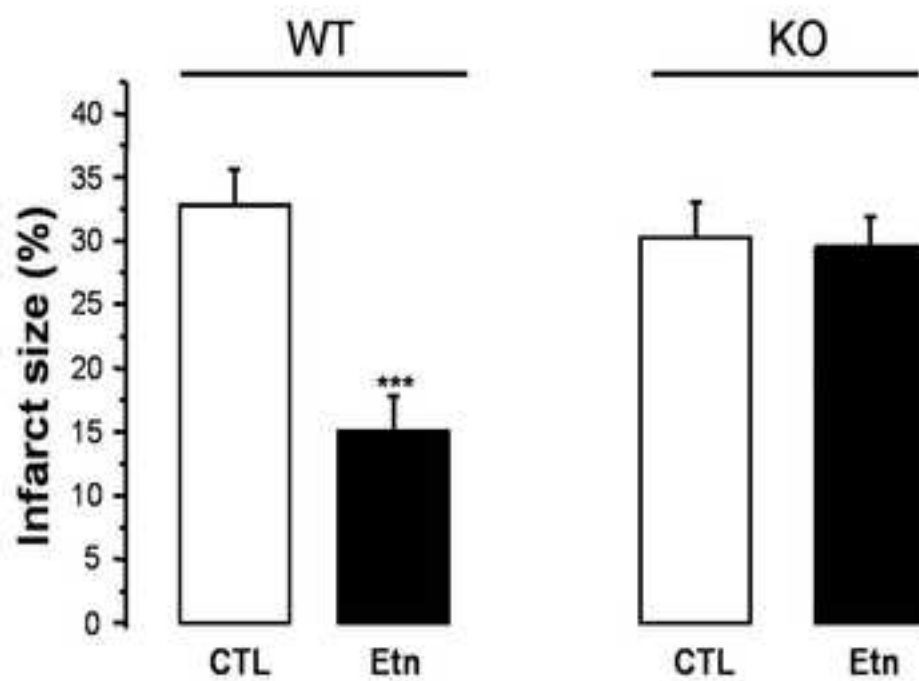
29. Lecour S, Suleman N, Deuchar GA, Somers S, Lacerda L, Huisamen B, Opie LH (2005) Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). *Circulation* 112:3911-3918
30. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN (2002) Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. *J Mol Cell Cardiol* 34:509-518
31. Lecour S (2009) Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway?. *J Mol Cell Cardiol* 47:32-40
32. Lichtman AH, Leung D, Shelton CC, Saghatelian A, Hardouin C, Boger DL, Cravatt BF (2004) Reversible inhibitors of fatty acid amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. *J Pharmacol Exp Ther* 311:441-448
33. Lim SY, Davidson SM, Yellon DM, Smith CC (2009) The cannabinoid CB1 receptor antagonist, rimonabant, protects against acute myocardial infarction. *Basic Res Cardiol* 104:781-792
34. Lim SY, Davidson SM, Hausenloy DJ, Yellon DM (2007) Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. *Cardiovasc Res* 75:530-535
35. Maeba R, Maeda T, Kinoshita M, Takao K, Takenaka H, Kusano J, Yoshimura N, Takeoka Y, Yasuda D, Okazaki T, Teramoto T (2007) Plasmalogens in human serum positively correlate with high-density lipoprotein and decrease with aging. *J Atheroscler Thromb* 14:12-18
36. Matas D, Juknat A, Pietr M, Klin Y, Vogel Z (2007) Anandamide protects from low serum-induced apoptosis via its degradation to ethanolamine. *J Biol Chem* 282:7885-7892
37. McKinney MK, Cravatt BF (2005) Structure and function of fatty acid amide hydrolase. *Annu Rev Biochem* 74:411-432
38. Means CK, Xiao CY, Li Z, Zhang T, Omens JH, Ishii I, Chun J, Brown JH (2007) Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 292:H2944-51
39. Minners J, Lacerda L, McCarthy J, Meiring JJ, Yellon DM, Sack MN (2001) Ischemic and pharmacological preconditioning in Girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Res* 89:787-792
40. Modica-Napolitano JS, Renshaw PF (2004) Ethanolamine and phosphoethanolamine inhibit mitochondrial function in vitro: implications for mitochondrial dysfunction hypothesis in depression and bipolar disorder. *Biol Psychiatry* 55:273-277
41. Montevicchi G, Masino F, Chinnici F, Antonelli A (2010) Occurrence and evolution of amino acids during grape must cooking. *Food Chem* 121:69-77

42. Opie LH, Lecour S (2007) The red wine hypothesis: from concepts to protective signalling molecules. *Eur Heart J* 28:1683-1693
43. Petersen G, Pedersen AH, Pickering DS, Begtrup M, Hansen HS (2009) Effect of synthetic and natural phospholipids on N-acylphosphatidylethanolamine-hydrolyzing phospholipase D activity. *Chem Phys Lipids* 162:53-61
44. Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR, Dasse O, Monaghan EP, Parrott JA, Putman D (2006) Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Rev* 12:21-38
45. Post JA, Bijvelt JJ, Verkleij AJ (1995) Phosphatidylethanolamine and sarcolemmal damage during ischemia or metabolic inhibition of heart myocytes. *Am J Physiol* 268:H773-80
46. Rafiee P, Shi Y, Su J, Pritchard KA, Jr., Tweddell JS, Baker JE (2005) Erythropoietin protects the infant heart against ischemia-reperfusion injury by triggering multiple signaling pathways. *Basic Res Cardiol* 100:187-197
47. Ramos B, Pinho O, Ferreira IMPLVO (2009) Changes of yolk biogenic amine concentrations during storage of shell hen eggs. *Food Chem* 116:340-344
48. Sattler K, Levkau B (2009) Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. *Cardiovasc Res* 82:201-211
49. Shanmuganathan S, Hausenloy DJ, Duchon MR, Yellon DM (2005) Mitochondrial permeability transition pore as a target for cardioprotection in the human heart. *Am J Physiol Heart Circ Physiol* 289:H237-42
50. Skyschally A, van Caster P, Boengler K, Gres P, Musiolik J, Schilawa D, Schulz R, Heusch G (2009) Ischemic postconditioning in pigs: no causal role for RISK activation. *Circ Res* 104:15-18
51. Smith RM, Suleman N, Lacerda L, Opie LH, Akira S, Chien KR, Sack MN (2004) Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. *Cardiovasc Res* 63:611-616
52. Smith RM, Suleman N, McCarthy J, Sack MN (2002) Classic ischemic but not pharmacologic preconditioning is abrogated following genetic ablation of the TNFalpha gene. *Cardiovasc Res* 55:553-560
53. Spiegel S, Milstien S (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 4:397-407
54. Sugiyama K, Kanamori H, Akachi T, Yamakawa A (1996) Amino acid composition of dietary proteins affects plasma cholesterol concentration through alteration of hepatic phospholipid metabolism in rats fed a cholesterol-free diet. *J Nutr Biochem* 7:40-48

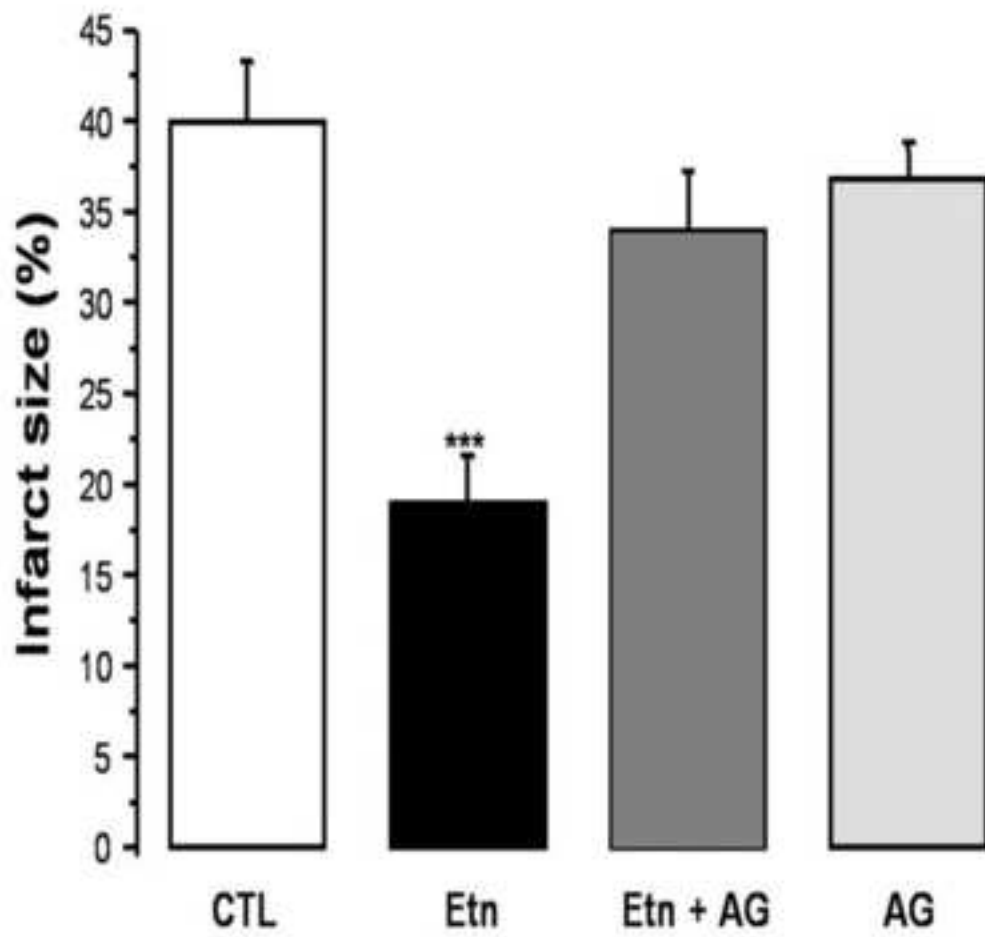


55. Suleman N, Somers S, Smith R, Opie LH, Lecour SC (2008) Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning. *Cardiovasc Res* 79:127-133
56. Theilmeier G, Schmidt C, Herrmann J, Keul P, Schafers M, Herrgott I, Mersmann J, Larmann J, Hermann S, Stypmann J, Schober O, Hildebrand R, Schulz R, Heusch G, Haude M, von Wnuck Lipinski K, Herzog C, Schmitz M, Erbel R, Chun J, Levkau B (2006) High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation* 114:1403-1409
57. Underdown NJ, Hiley CR, Ford WR (2005) Anandamide reduces infarct size in rat isolated hearts subjected to ischaemia-reperfusion by a novel cannabinoid mechanism. *Br J Pharmacol* 146:809-816
58. Vessey DA, Li L, Kelley M, Zhang J, Karliner JS (2008) Sphingosine can pre- and post-condition heart and utilizes a different mechanism from sphingosine 1-phosphate. *J Biochem Mol Toxicol* 22:113-118
59. Wang J, Okamoto Y, Tsuboi K, Ueda N (2008) The stimulatory effect of phosphatidylethanolamine on N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD). *Neuropharmacology* 54:8-15
60. Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnfsky EJ, Larner AC (2009) Function of mitochondrial Stat3 in cellular respiration. *Science* 323:793-797
61. Williams SD, Gottlieb RA (2002) Inhibition of mitochondrial calcium-independent phospholipase A2 (iPLA2) attenuates mitochondrial phospholipid loss and is cardioprotective. *Biochem J* 362:23-32
62. Xu FY, O K, Choy PC (1997) Biosynthesis of plasmenylethanolamine (1-O-alk-1'-enyl-2-acyl-sn-glycero-3-phosphoethanolamine) in the guinea pig heart. *J Lipid Res* 38:670-679
63. Xuan YT, Guo Y, Han H, Zhu Y, Bolli R (2001) An essential role of the JAK-STAT pathway in ischemic preconditioning. *Proc Natl Acad Sci U S A* 98:9050-9055
64. Zoeller RA, Lake AC, Nagan N, Gaposchkin DP, Legner MA, Lieberthal W (1999) Plasmalogens as endogenous antioxidants: somatic cell mutants reveal the importance of the vinyl ether. *Biochem J* 338 ( Pt 3):769-776



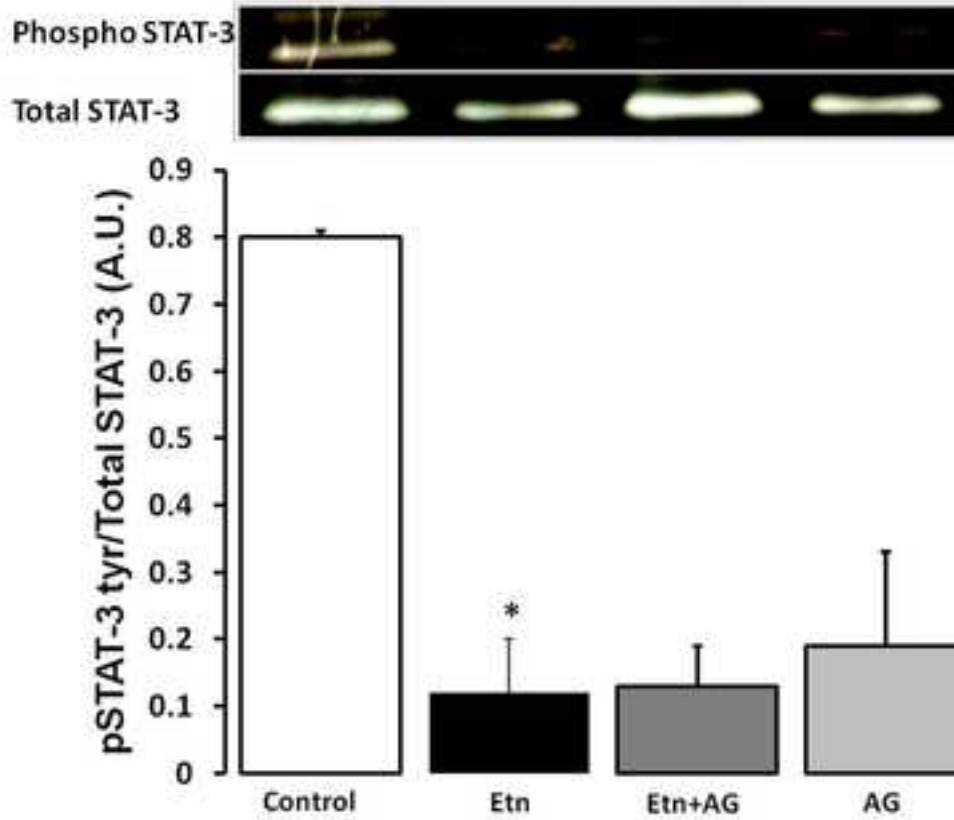


Kelly et al, Figure 2

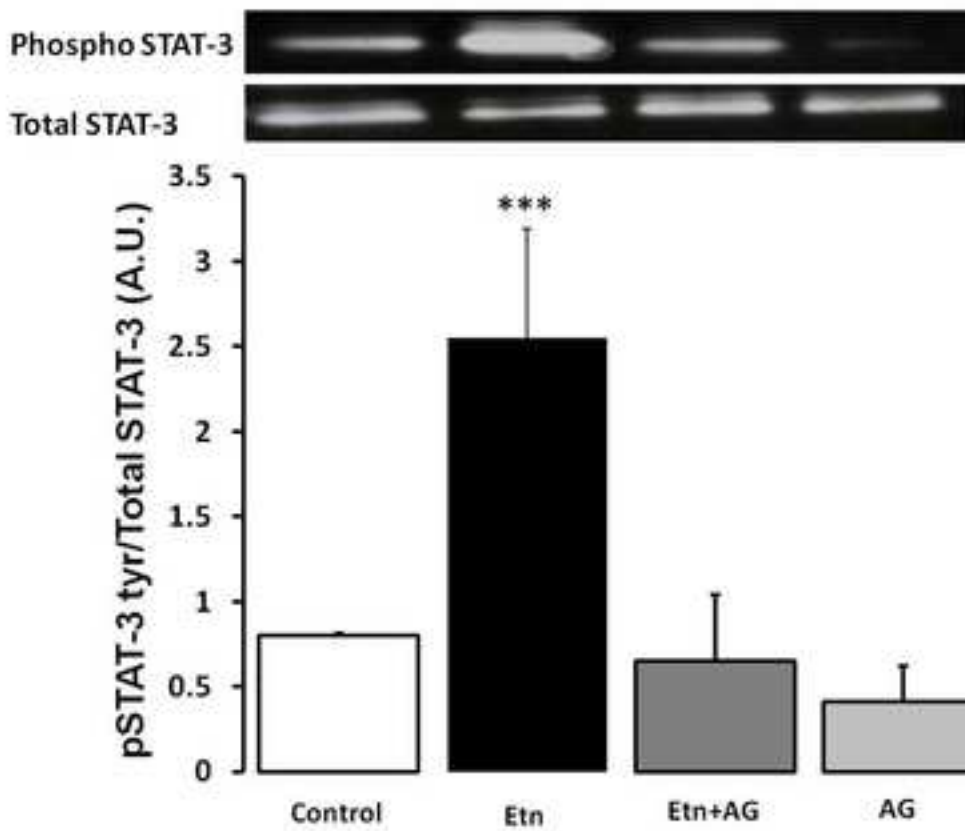


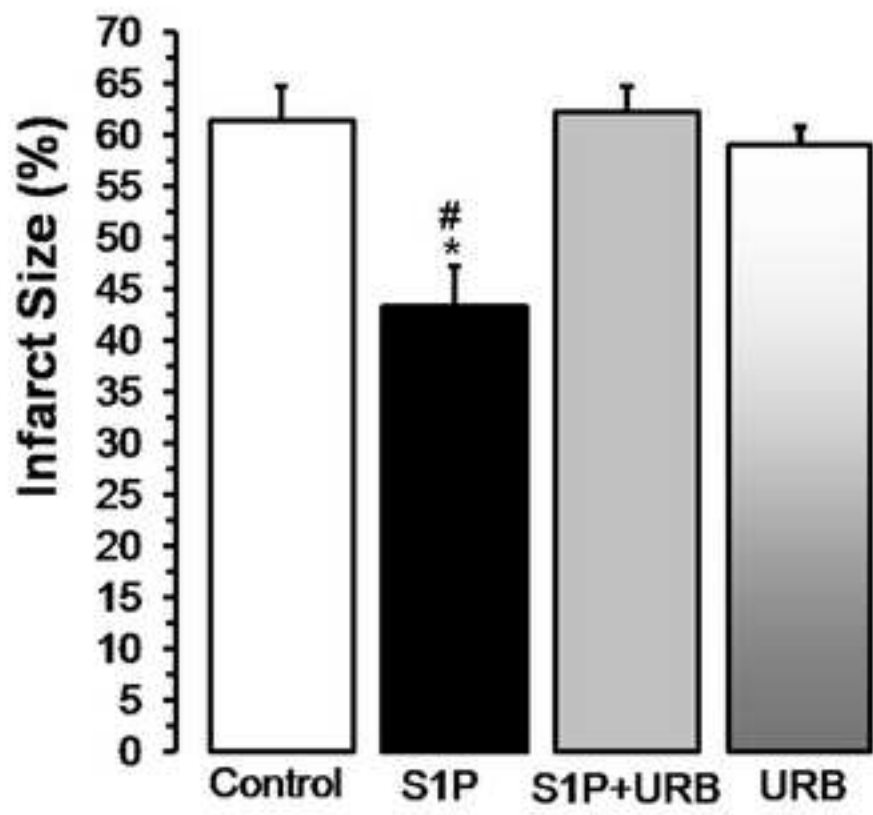
Kelly et al, Figure 3

a)



b)





Kelly et al, Figure 5

