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Authors	Kelly-Laubscher, Roisin;Lamont, Kim T.;Somers, Sarin;Hacking, Damian;Lacerda, Lydia;Thomas, Paul;Opie, Lionel H.;Lecour, Sandrine
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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±0.08 vs. Etn; 1.50 ±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[1], Kim T Lamont[1], Sarin Somers[1], Damian Hacking[1], Lydia Lacerda[1], Paul Thomas[2], Lionel H Opie[1], Sandrine Lecour[1].

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

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±0.08 vs. Etn; 1.50 ±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^{0}C. Nuclear and cytosolic proteins were extracted as previously described.[29]

Isolated STAT-3 $^{-/-}$ 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^{o}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±SEM. Comparisons between multiple groups were performed by 1-way ANOVA followed by the Dunnets 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 ± 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±4%, Etn (0.3 mmol/L) 58±3%, $P<0.05$ vs. Control] whereas protection was lost at higher concentrations [Etn (1 mmol/L) 63±9%, Etn (5 mmol/L) 76±6%, ns vs. control]. Area at risk did not differ significantly between the groups [Etn (0mmol/L) 44±3%, Etn (0.1 mmol/L) 46±4%, Etn (0.3 mmol/L) 38±7%, Etn (1 mmol/L) 50±4%, Etn (5 mmol/L) 47±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 ± 3% (Fig 3). Pretreatment with ethanolamine (0.3 mmol/L) reduced infarct size (20 ± 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 ± 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±2.56 %) compared with hearts subjected to 35 min global ischaemia and 2 hrs of reperfusion (42.20±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 ± 3% (Fig 2). Pretreatment with ethanolamine (0.3 mmol/L) resulted in a significant reduction of the infarct size to 15 ± 3 % ($P < 0.05$ vs. wild type Control). Ischaemic control hearts from cardiomyocyte deficient STAT-3 mice had an infarct size of 30 ± 3 %. The infarct sparing effect observed with ethanolamine pretreatment in the wildtype mice was absent in knockout hearts (29 ± 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±0.19 vs. Etn; 0.28 ±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±0.6, Etn 4.5±0.3, Etn + AG490 4.0±0.7, AG490 6.4±2.7, arbitrary units].

Ethanolamine pre-treatment was associated with an increase of nuclear tyrosine phosphorylated/total STAT-3 (Control; 0.75±0.08 vs. Etn; 1.50 ±0.01 arbitrary units, $P<0.001$) (Fig 4b). This increase in phosphorylated/total STAT-3 was inhibited by AG490 (0.65±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±2.6, Etn 3.3±2.3, Etn + AG490 2.0±1.3, AG490 0.9±0.2].

A trend for an increase in STAT-3 serine phosphorylation was observed in both the nucleus [Control 0.59±0.10, Etn 2.09±0.66, p=n.s.] and the cytoplasm [Control 0.25 ±0.09, Etn 1.03±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±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±4%, S1P 45±6%, S1P +URB 44±5%, URB 50±5%), pretreatment with S1P (5 mg/kg, i.p.) reduced infarct size (43±4%, n=6) compared with the ischaemic control group ($P<0.01$). This protection was abolished by pretreatment of the rats with URB597 (62±2 %(n=6), P<0.01 vs. S1P). Pretreatment with URB597 alone had no effect on infarct size (59±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, plasmanylethanolamine and plasmenylethanolamine. 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 S1P-induced cardioprotection is mediated by production of endogenous ethanolamine.

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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 phoshpate (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≥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

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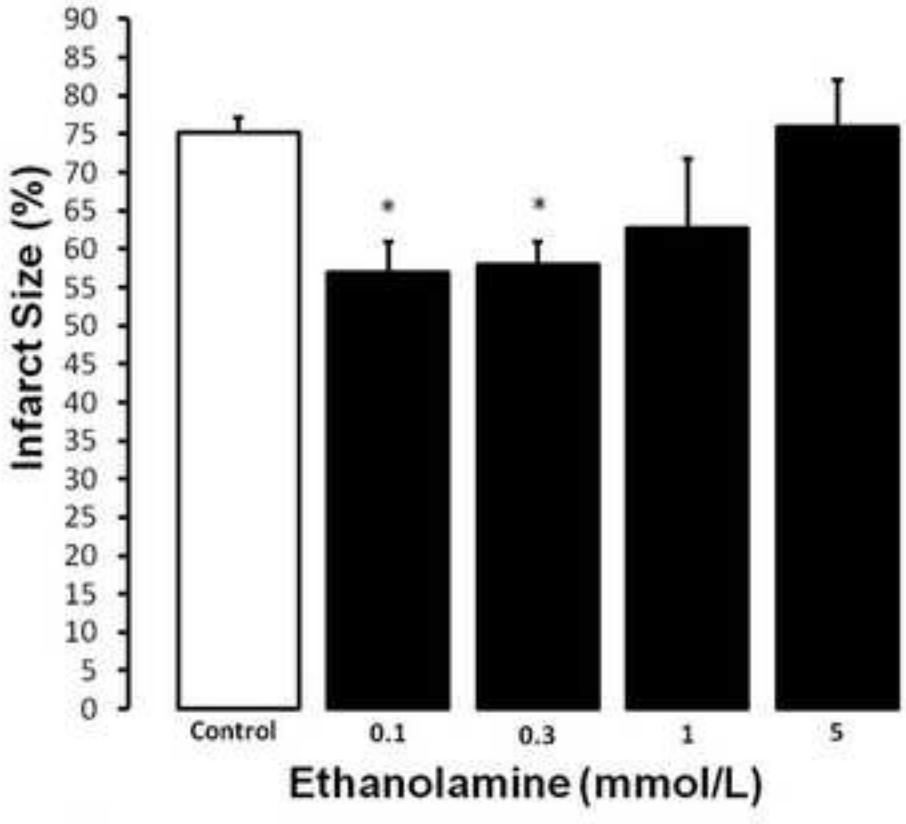
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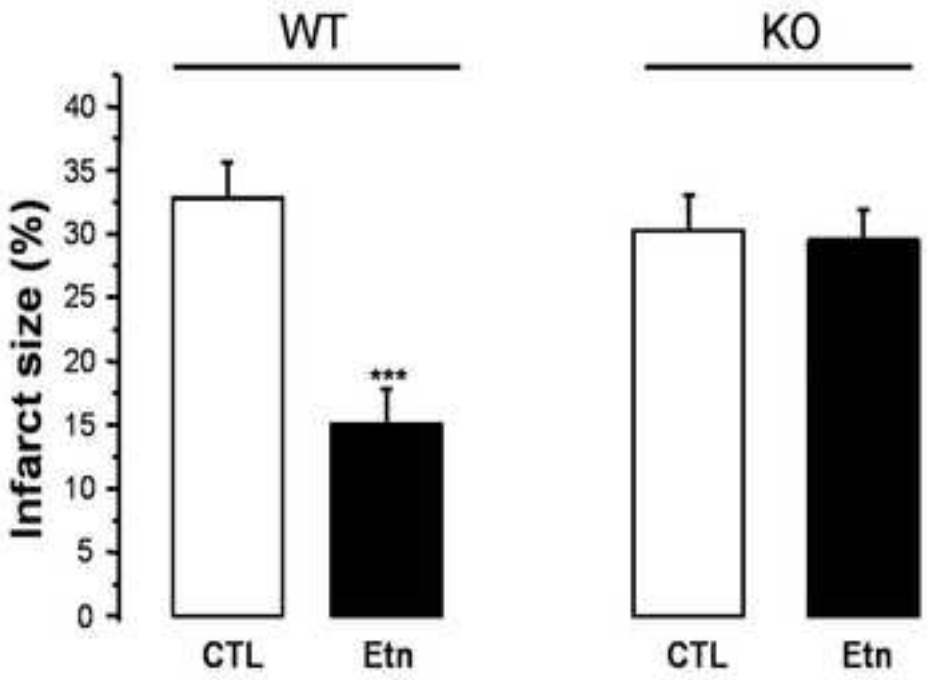
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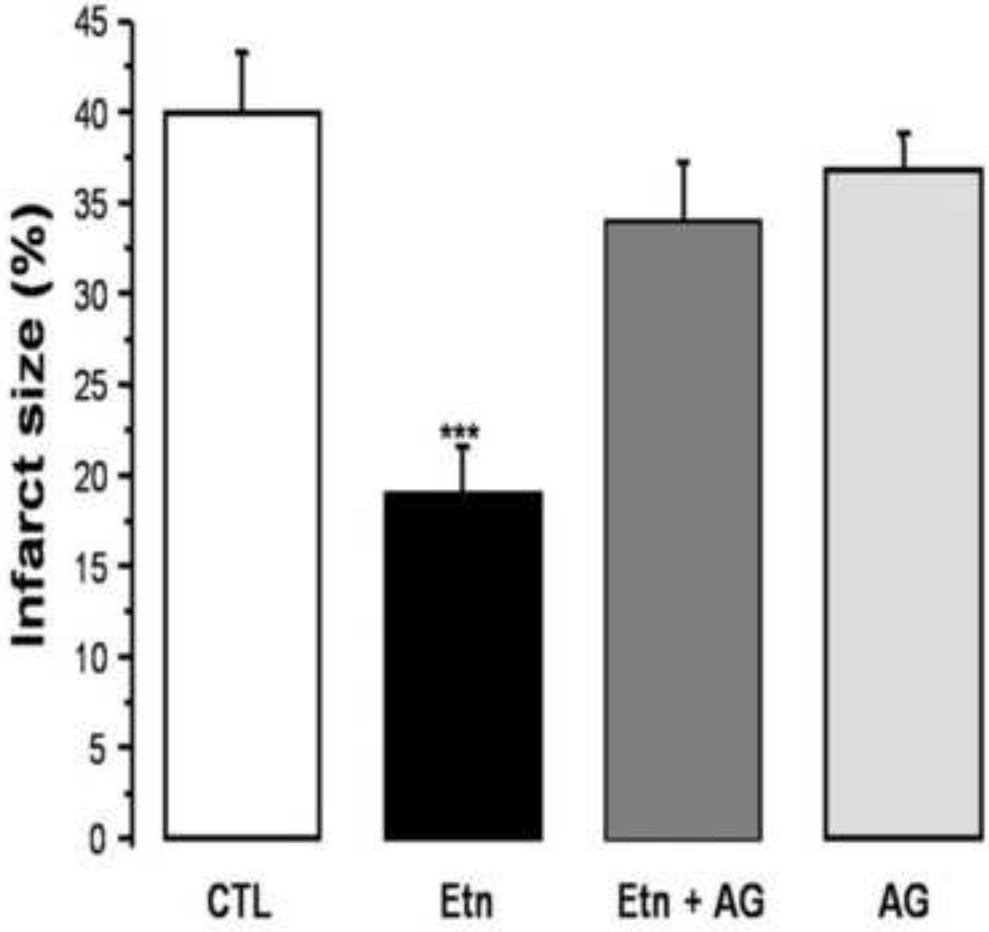
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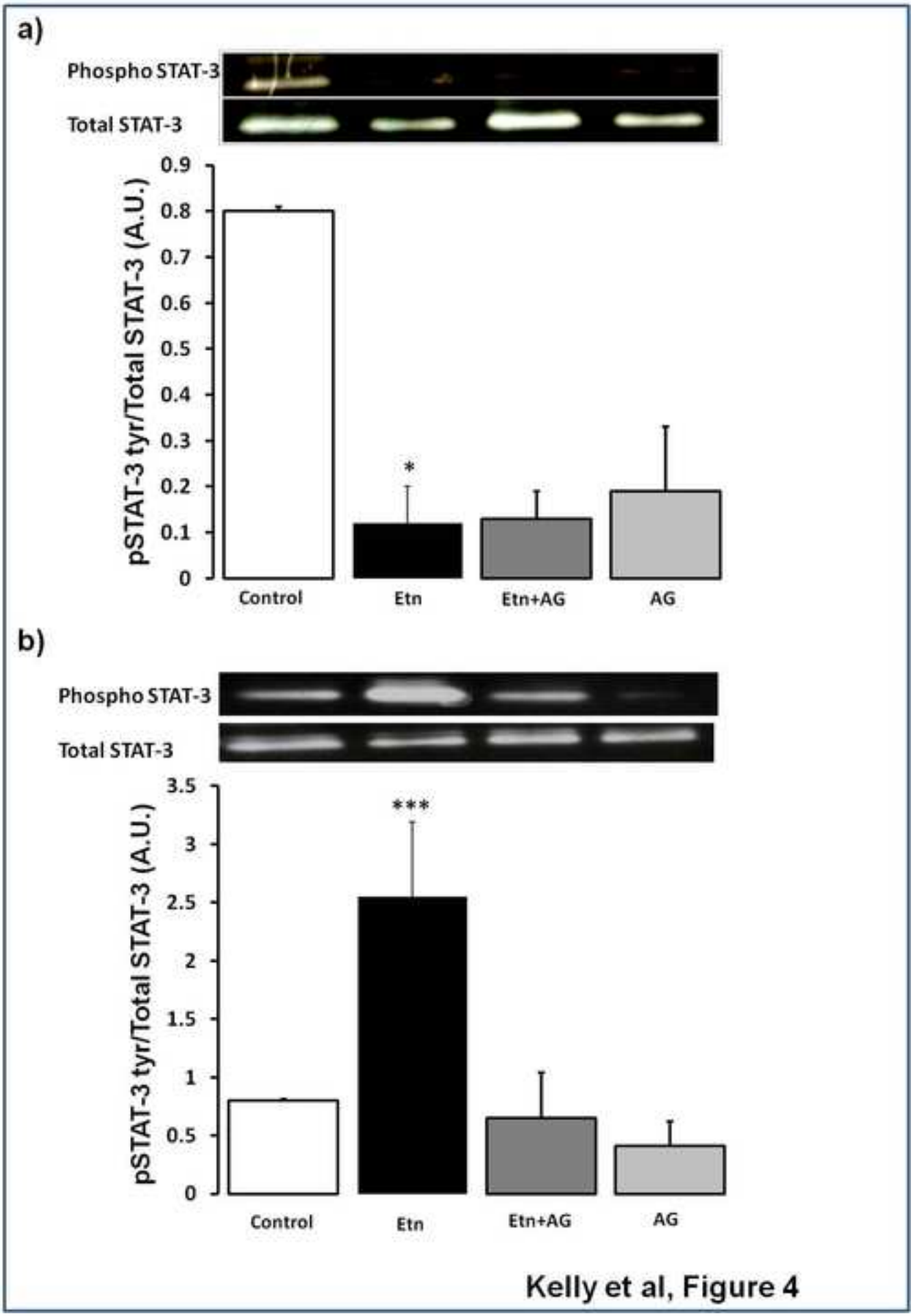
Kelly et al. Figure 1



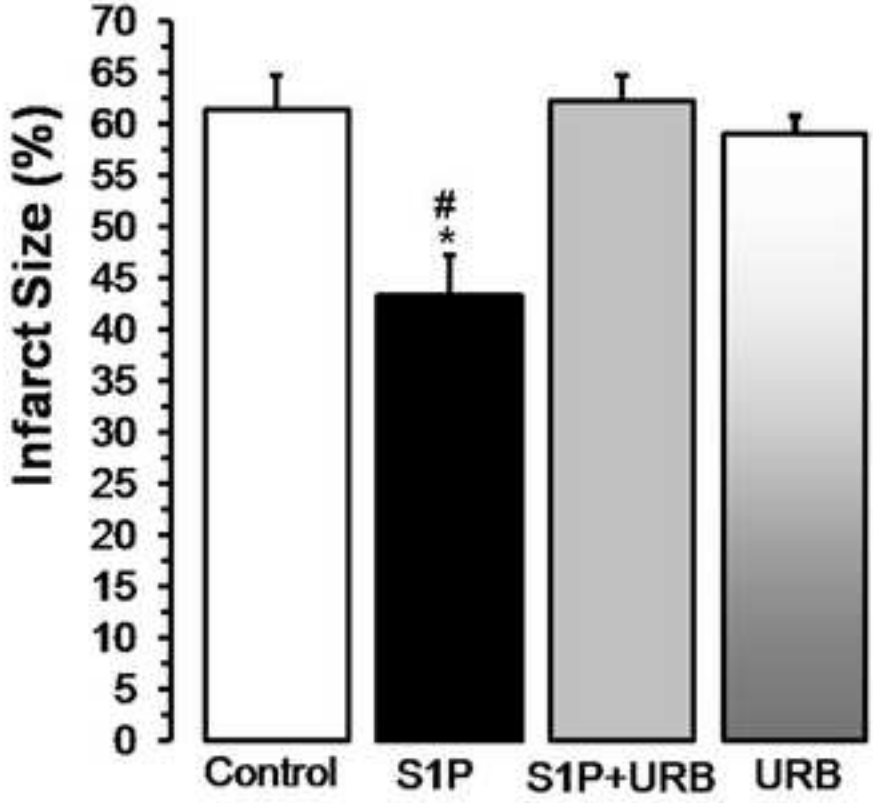
Kelly et al, Figure 2



Kelly et al, Figure 3



Kelly et al, Figure 4



Kelly et al, Figure 5