Oxytocin signalling involved in cardiac protection against ischemia-reperfusion

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Abstract

The oxytocin (OT) treatment protects heart against ischemia. Here we investigated heart-derived H9c2 cells in simulated ischemia-reperfusion (I-R) experiments in order to examine the mechanism of OT-induced cardioprotection.
I-R was induced in an anoxic chamber for 2 hours and followed by 2 h of reperfusion. In comparison to normoxia, I-R resulted in decrease of formazan production by H9c2 cells to 63.5 ± 1.7% (MTT assay, assessing cell metabolic activity) and enhanced apoptosis from 1.7 ± 0.3% to 2.8 ± 0.4% (Tunel test, detecting DNA fragmentation). Using these assays we found that treatment with OT (1 to 500 nM) exerted dose-dependent protection during I-R, especially when OT was added at the time of reperfusion. This involved OT receptor (OTR) because the cells exposed to I-R and treated with specific OTR siRNA responded to OT by enhanced apoptosis. The OT stimulation causes intracellular signaling involving Pi3K, Akt and eNOS, known as the canonical factors of signalosomes. Confocal microscopy demonstrated in OT-treated cells, the phosphorylated Akt co-localized with the mitochondrial marker Cox IV. In addition, the NOS dissociation from caveolin-3 and eNOS phosphorylation was accompanied by increased production of NO. The increased cell viability induced by OT was abolished by the co-treatment of the cells with a PKG inhibitor, KT-5823. Furthermore, stimulation with OT resulted in enhanced release of atrial natriuretic peptide. Using the CM-H_2DCFDA probe we have also observed the paradox that OT treatment stimulates moderate levels of reactive oxygen species (ROS) production in cells whereas inhibits excess of ROS produced as a consequence of ischemia evoked by I-R.

The OTR protected H9c2 cells against I-R, especially if activated at the onset of reperfusion. The OTR-transduced signals include pro-survival kinases, such as Akt and PKG. These kinases translocate to the mitochondria, where they act in a localized signalosome involving activation of ROS in a positive feedback loop.

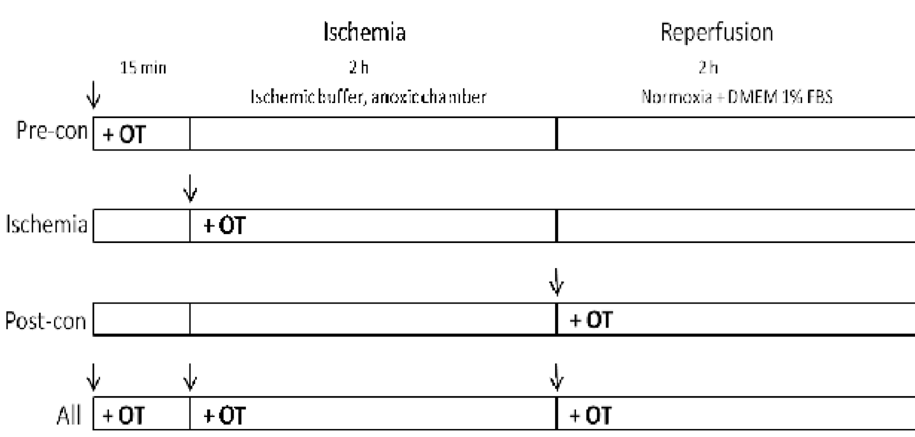
Background

- Oxytocin (OT) is a nonapeptide mainly produced by the hypothalamus and stored in the neurohypophysis.
- OT acts via its receptor both centrally as a neuromodulator and peripherally as a hormone, released by the neurohypophysis into the circulation.
- OT is involved in the regulation of energy metabolism and in the activation of cardio-protective mechanisms.
- The accumulated evidence indicates that local OT action in the heart is important for the protection against ischemic stress.
- OT has repeatedly been demonstrated to enhance myocardial recovery following experimental I-R *in vivo* as well as in the isolated, perfused heart.
- Mitochondria are effectors of both I-R injury and cardioprotection exerted by OT.
- We hypothesized that OT treatment directly protects cardiomyocyte viability through intracellular signalling that targets the mitochondria and prevents cell death.

Objective

To determine whether OT could directly protect isolated cardiomyocytes from damage induced during the acute phase of simulated I-R *in vitro* and to identify the key intracellular processes involved.

Materials & Methods

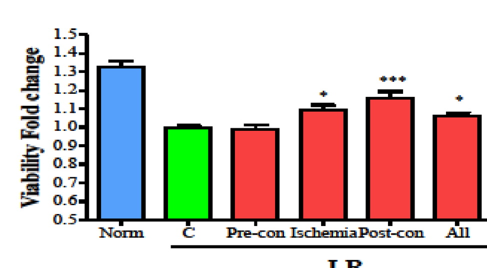


Schematic representation of ischemia – reperfusion protocol

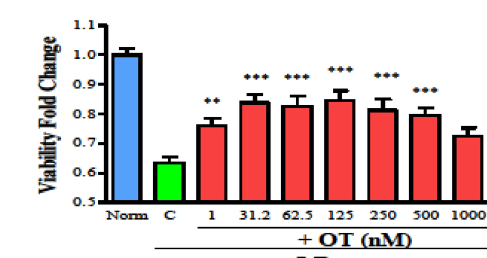
- **Rat ventricular myoblasts (H9c2 cells)** were obtained from the American Type Culture Collection .
- **MTT-test**. Cell viability was measured by the mitochondrial reduction of the tetrazolium salt MTS [3-(3,5-dimethylthialzol-2-yl)-5-(3 carboxymethoxypheny)-2-(4-sulphophenyl)-2H-tetrazolium] into a formazan product.
- **Apoptosis** was evaluated by TUNEL test.
- **Intracellular ROS production** was measured with with the CM-H_2DCFDA probe.
- **Nitrites** in medium and lysate of H9c2 cells were measured by the modified Griess method.
- **Proteins** were measured by Western blot and localized in the cells by immunofluorescence and confocal microscopy.

Results

Treatment with oxytocin directly protects H9c2 cell survival and metabolic activity in simulated ischemia-reperfusion (I-R)

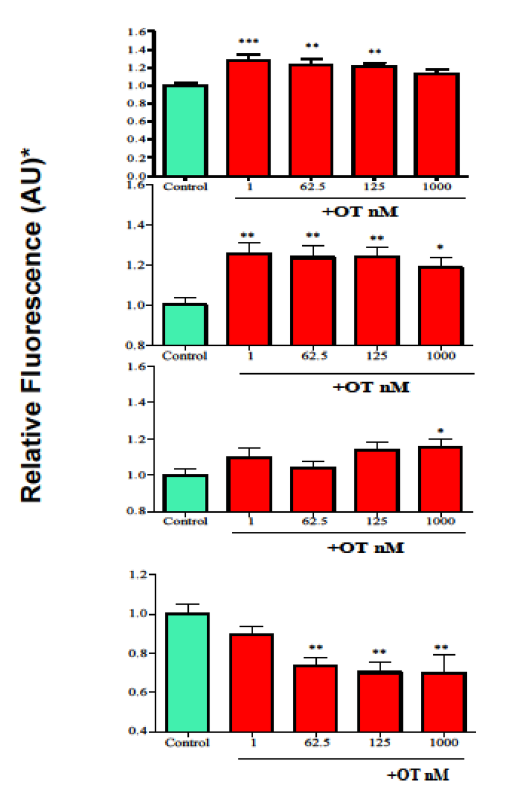


Effect of 1 nM OT on cell viability in a MTT test when administered either before ischemia (Pre-con), during ischemia (Ischemia), at reperfusion (Post-con), or at all three points



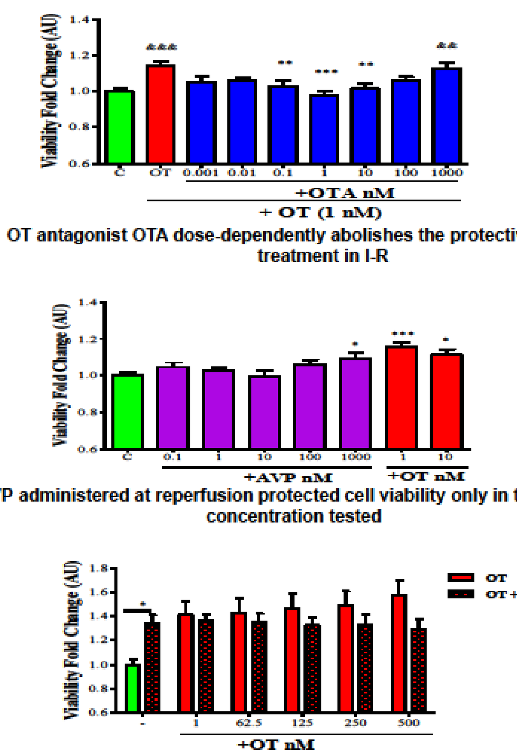
H9c2 cell viability after 2 hours of simulated I-R in the presence of different concentrations of OT added at reperfusion

OT induces a short-lived ROS burst in normoxic conditions and prevents ROS formation following I-R



*Intracellular ROS production measured with the CM-H_2DCFDA probe

Effect of OT and arginine-vasopressin (AVP) agonists and antagonists on MTT cell viability test in simulated I-R

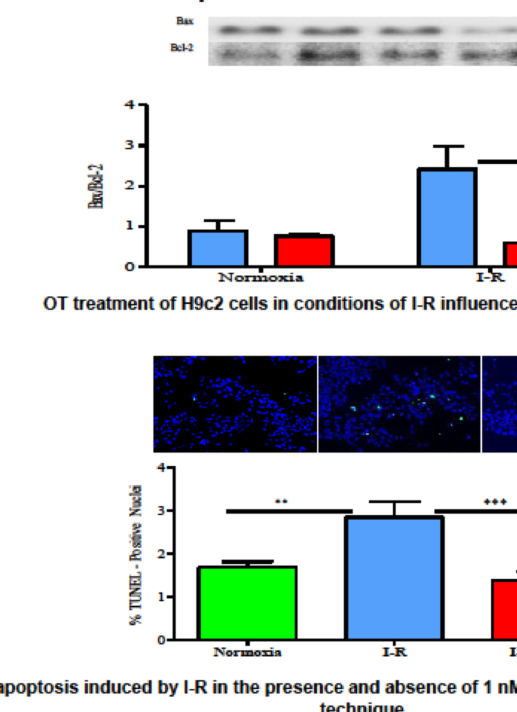


OT antagonist OTA dose-dependently abolishes the protective effect of OT treatment in I-R

AVP administered at reperfusion protected cell viability only in the highest concentration tested

In conditions of I-R, the vasopressin receptor V1a and V2 antagonist, Conivaptan (50nM), protects cell viability (MTT test) in absence of OT

Cell apoptosis induced by I-R is inhibited by OT in the presence of functional OTR

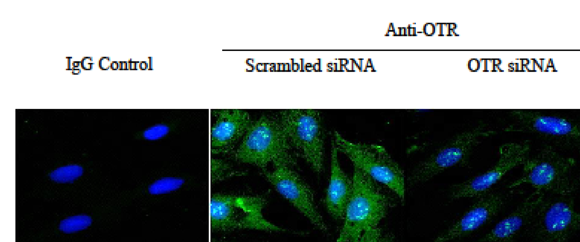


OT treatment of H9c2 cells in conditions of I-R influences the Bax/Bcl-2 ratio

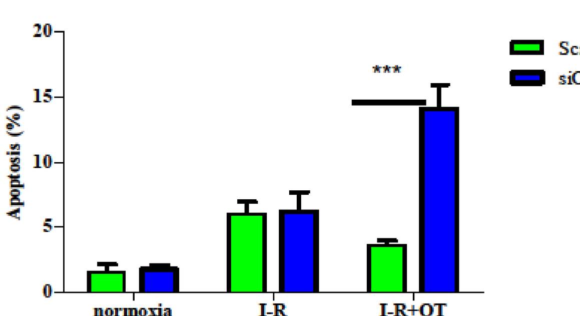
Cell apoptosis induced by I-R in the presence and absence of 1 nM OT and stained by the Tunel technique

Results

OT induces apoptosis in H9c2 cells deficient in OTR

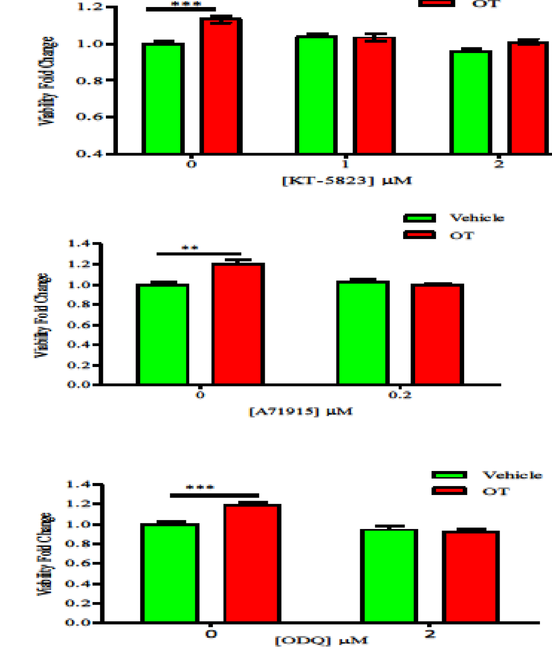


siRNA knockdown of OTR in H9c2 cells reduces OTR expression detected by specific immunofluorescence

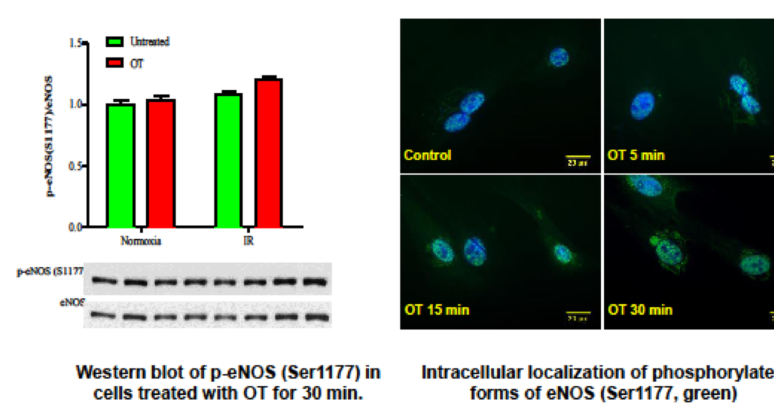


Cells transfected with OTR siRNA respond to OT treatment in I-R with increase of apoptotic cells

Experiments with specific inhibitors disclosed protein kinase G and guanylyl cyclases as OT mediators of cell protection in simulated I-R

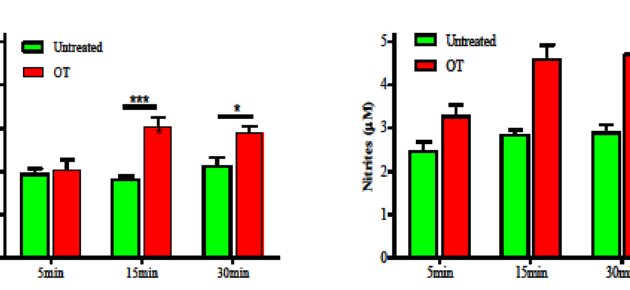


Analysis of phosphorylated eNOS and NO release in H9c2 cells treated with OT



Western blot of p-eNOS (Ser1177) in cells treated with OT for 30 min.

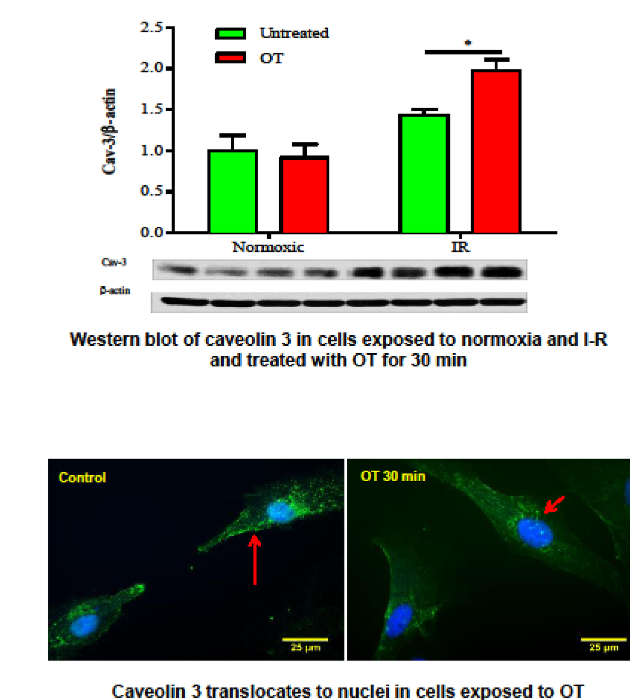
Intracellular localization of phosphorylated forms of eNOS (Ser1177, green)



Extracellular production of nitrites in H9c2 cells in the presence or absence of 62.5 nM of OT

Intracellular production of nitrites in H9c2 cells in the presence or absence of 62.5 nM of OT

Analysis of caveolin 3 in H9c2 cells treated with OT

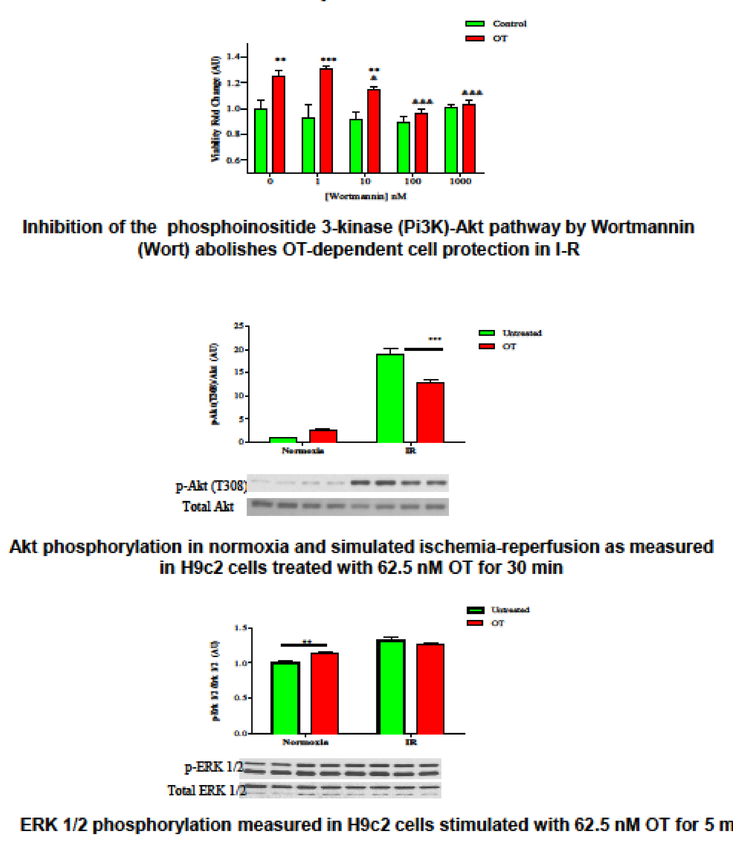


Western blot of caveolin 3 in cells exposed to normoxia and I-R and treated with OT for 30 min

Caveolin 3 translocates to nuclei in cells exposed to OT

Results

Pi3k-Akt-PKG signalling is involved in OT–induced cell protection

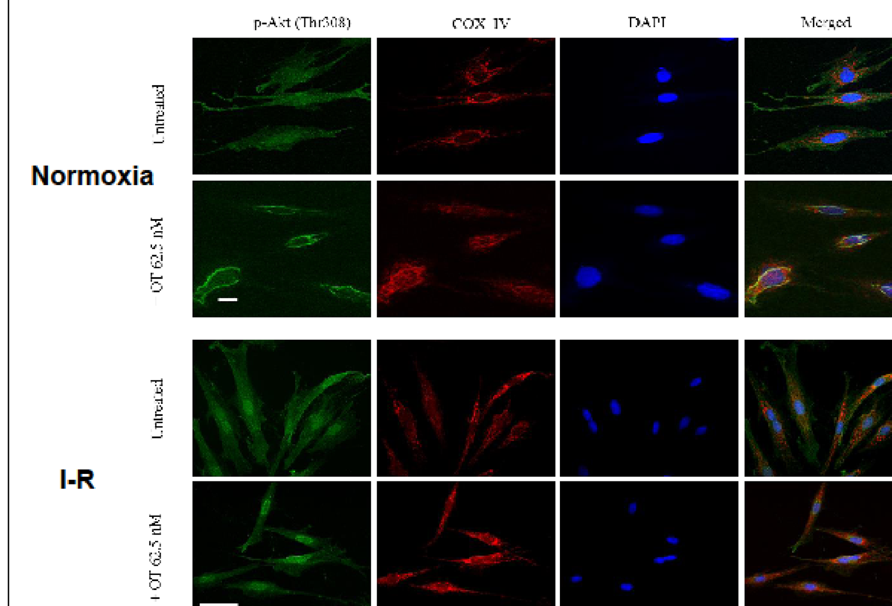


Inhibition of the phosphoinositide 3-kinase (Pi3K)-Akt pathway by Wortmannin (Wort) abolishes OT-dependent cell protection in I-R

Akt phosphorylation in normoxia and simulated ischemia-reperfusion as measured in H9c2 cells treated with 62.5 nM OT for 30 min

ERK 1/2 phosphorylation measured in H9c2 cells stimulated with 62.5 nM OT for 5 min

OT treatment causes Akt phosphorylation and its co-localization with mitochondrial marker COX IV in a structure within or around cells' nuclei



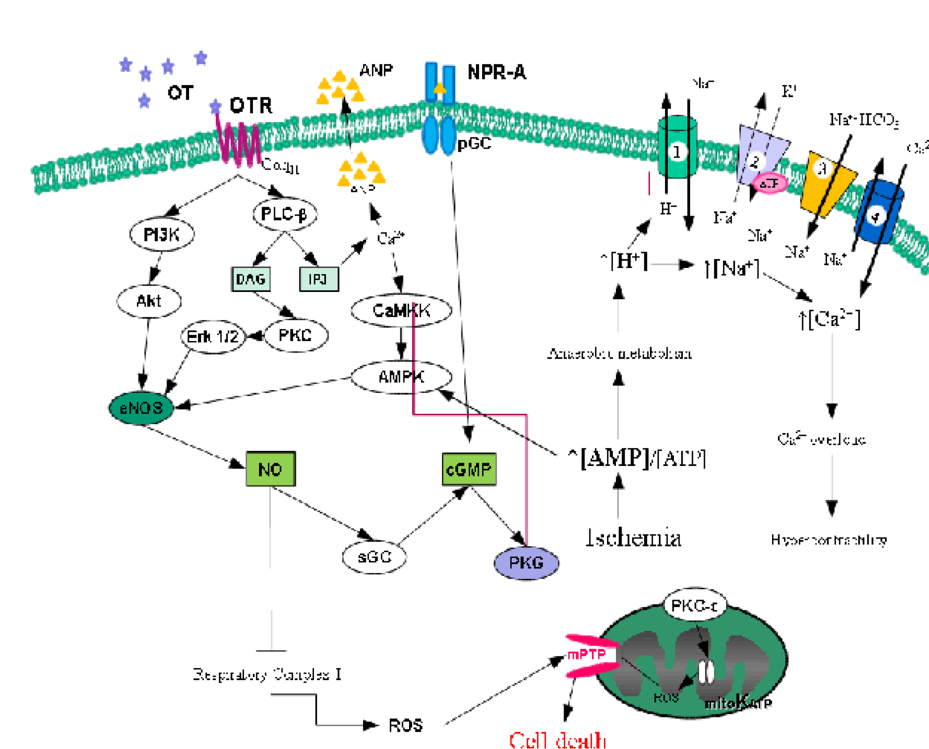
Confocal micrographs of H9c2 cells probed with p-Akt antibody (T308, green) and antibody for mitochondrial complex IV (Cox IV, red). The nuclei are stained blue with DAPI.

Cells were stimulated with 62.5 nM OT for 30 min in normoxia or for 30 min at reperfusion in conditions od I-R.

Highlights

- We have demonstrated that treatment with oxytocin (OT) prevents the lethal reperfusion injury of H9c2 cardiomyoblasts.
- OT-mediated cardioprotective signals are transferred from the cell surface to mitochondria and nuclei as part of multimolecular complexes containing pro-survival kinases.
- We propose that OT evokes mitochondrial reactive oxygen species generation in H9c2 cardiomyoblasts via Ca^{+2}-dependent mechanism, leading to activation of ERK1/2, Pi3K/Akt, and eNOS with rapid NO release.

Mechanisms?



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