 doi:10.20944/preprints202206.0085.v1

Article

Facilitation of Insulin Effects by Ranolazine in Astrocytes in Primary Culture

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Abstract: Ranolazine (Rn) is a drug used to treat persistent chronic coronary ischemia. It has also been shown to have therapeutic benefits on the central nervous system and an anti-diabetic effect by lowering blood glucose levels and however, no effects of Rn on cellular sensitivity to insulin (Ins) have been demonstrated yet. The present study aimed to investigate the permissive effects of Rn on the actions of Ins in astrocytes in primary culture. Ins at 10^{-8} M, Rn (10^{-6} M) and Ins+Rn (10^{-8} M and 10^{-6} M respectively) were added to astrocytes during 24 h. In comparison to control cells, Rn and/or Ins caused modifications in cell viability and proliferation. p-AKT, p-ERK, p-eNOS, Mn-SOD, COX-2, and the anti-inflammatory protein COX-2 were all upregulated by ins. On the contrary, no significant changes were found in the protein expression of Cu/Zn-SOD, NF-κB and IκB. The presence of Rn produced an increase in p-ERK protein and a significant decrease in COX-2 protein expression. Furthermore, Rn significantly increased the effects of Ins on the expression of p-AKT, p-eNOS, p-ERK, Mn-SOD, and PPAR-γ. On the other hand, Rn+Ins produced a significant decrease in COX-2 expression. In conclusion, Rn facilitated the effects of insulin on the p-AKT, p-eNOS, p-ERK, Mn-SOD and PPAR-γ, signaling pathways, as well as on the anti-inflammatory and antioxidant effects of the hormone.

Keywords: Ranolazine; Insulin; astrocytes; inflammation; antioxidants

1. Introduction

Astrocytes are the most abundant cells in the central nervous system (CNS) and perform a variety of functions, including structural support, blood-brain barrier integrity, and the development of important protective roles (1). They take part also in immunological responses and in the reparative processes that occur at different stages of neuroinflammation (2).

Astrocytes secrete both neurotrophic and inflammatory cytokines, and express receptors for mediators like IL-1β, and TNF-α, among others (3,4). Glucose absorption and storage are two of insulin's most essential effects (5). Insulin crosses the blood-brain barrier acting on astrocytes and, indirectly, on neurons (6). The brain expresses insulin receptors (IR) on neurons, microglia, and astrocytes. Its effects include metabolic functions and neuronal survival after trauma or during neurodegeneration (7). In fact, these effects are due to anti-inflammatory insulin action. At 10^{-8} M, insulin inhibits inducible nitric oxide synthase (iNOS) expression and NFκB level increase in astrocytes induced by LPS (8). Furthermore, insulin increased the vitality of rat and human astrocytes (9,10). Insulin is generally degraded in lysosomes within cells [6), although there is evidence of the presence of the insulin-degrading enzyme (IDE) in different types of cells, including astrocytes (11). In addition, IDE degrades other peptides such as a beta-amyloid peptide, which is involved in the pathogenesis of Alzheimer's disease (12).

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In clinical practice, ranolazine (Rn) is used to treat refractory chronic stable angina (13,14). Data from patients indicate that ranolazine preserves myocardial blood flow during ischemic insults (15). Human studies back up the idea that ranolazine can help improve coronary blood flow by lowering the mechanical consequences of ischemia contracture, enhancing endothelial function, or both (16,17). At therapeutic concentrations, Rn inhibits the late inward sodium current (I(NaL)) (18) reducing tissue damage caused by intracellular sodium and calcium overload that is associated with myocardial ischemia (19,20,21). I(NaL) amplitude is increased in many pathological situations, such as myocardial ischemia and oxidative stress (22,23,24). In addition to its antianginal effects, Rn acts as an anti-inflammatory agent reducing asymmetric dimethylarginine and C-reactive protein plasma levels and promoting the endothelial release of vasodilator mediators in patients with ischemic coronary disease (25). Furthermore, metabolic effects, such as the lowering of hemoglobin A1c (HbA1c) in patients with ischemic heart disease and diabetes (26,27,28), or the improvement of insulin secretion and β-cell survival in diabetic mice (29) have already been described. Moreover, several studies evaluated the effects of Rn on the nervous system (30,31,32). They suggested that these effects would be also mediated by late INa or inwardly rectifying K^+ current (33).

Therefore, the objective of this study is to evaluate the effects of insulin on astrocytes in primary culture and the facilitating actions of ranolazine on the sensitivity of astrocytes to insulin. It is intended to evaluate the effects of insulin and ranolazine on cell viability, as well as on anti-inflammatory and antioxidant mechanisms and processes.

2. Results

2.1. Cell Viability

The role of Rn, Ins or Ins+Rn on cell viability were studied using MTT conversion assay. Figure 1 shows that incubation with Rn, Ins or Ins+Rn, produced significant increase compared with control astrocytes (Figure 1) (Rn 28%, Ins 27% and Ins+Rn 72%). Furthermore, Ins+Rn produced an increase in viability compared to Ins about 25%.

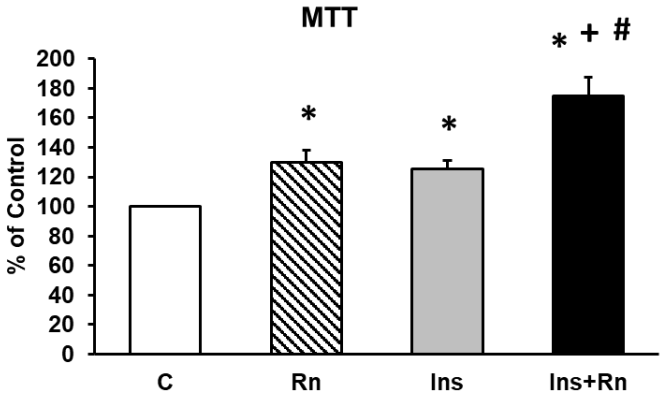

Figure 1. Effect of Ins and Rn on astrocytes viability. Cell viability was determined by MTT assay in cells treated for 24 h. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-6} M+10^{-8} M). Data are mean ± SD of four independent experiments (four different rats). *p < 0.05 vs. control. +p < 0.05 vs. Rn. #p < 0.05 vs. Ins.

Figure 2 shows that Ins or Ins+Rn increased astrocytes number compared to control cells.

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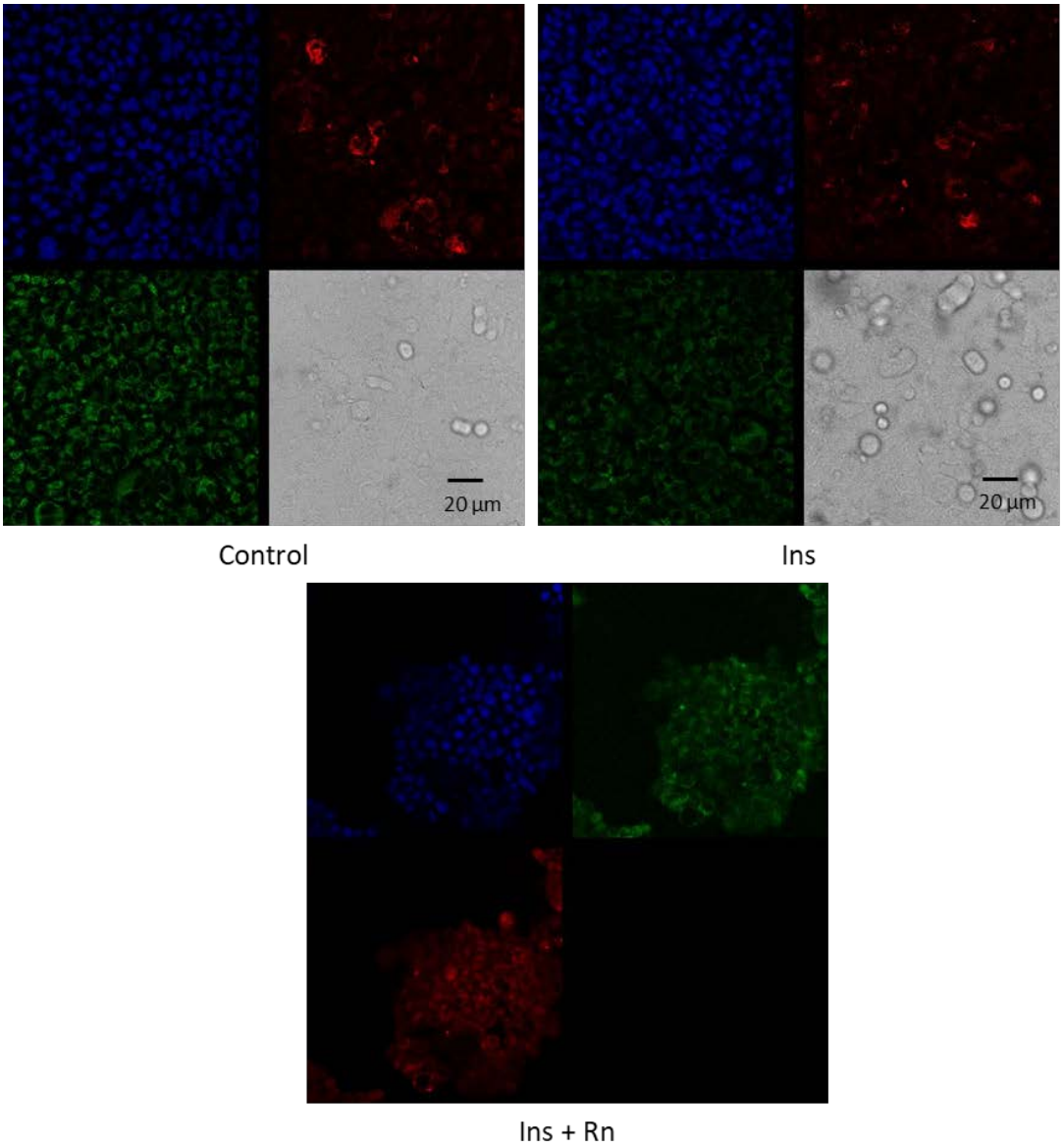

Figure 2. Effect of Ins and Ins + Rn on astrocytes in primary culture. Cells were isolated and seeded at 7 x 10^4 cells/35 mm dish for 5 days. Currently, cells were incubated without (control, C), with Ins (10^{-8} M) or with Ins+Rn (10^{-8}+10^{-6} M) for 24 h. Fluorescence products used were: Mitotracker (250 nM) to stain mitochondria, Lysotracker (250 nM) to stain lysosomes and Hoechst 33342 (2 µg ml^{-1}) to stain nuclei. Contrast images are added. Bar represents 20 µm.

2.2. Cell proliferation

Trypan blue exclusion assay was used to count the living cells and monitor cell proliferation. Astrocytes were isolated and seeded at 7x10^4 cells/35 mm dish. After 5 days of culture, cells were incubated without (control, C) or with Rn (10^{-6} M), Ins (10^{-8} M), or with Ins+Rn (10^{-8} and 10^{-6} M) for 24 h. In control conditions proliferation was 0.85%, with Rn 30.31%, with Ins 29.18%% and with Ins+Rn 33.91%, demonstrating significant differences (Table 1).

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	Seeding cells (x10⁴/35 mm dish)	5 days of culture	24 h Treatment	% Proliferation
Control	7	12.86 ± 0.32	12.97 ± 0.24	0.85
Rn	7	12.87 ± 0.25	16.77 ± 0.35	30.31*
Ins	7	12.85± 0.23	16.60 ± 0.37	29.18*
Ins+Rn	7	12.88 ± 0.26	17.25 ± 0.35	33.91*#

Table 1. Effect of Ins and Rn on astrocytes proliferation. Cell proliferation and counting living cells. Astrocytes were isolated and seeded at 7 x 10^4 cells/35 mm dish for 5 days. Currently, cells were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-8}+10^{-6} M) for 24 h. Trypan blue exclusion was used to count the living cells and monitor cell proliferation. Data are mean ± SD of four independent experiments (four different rats). *p < 0.05 vs. control.

2.3. Protein expression of p-AKT

Figure 3 shows that Rn (10^{-6} M), Ins (10^{-8} M) and Ins+Rn (10^{-8} M and 10^{-6} M) produced significant differences in p-AKT compared to control cells. In fact, Ins increased the expression of p-AKT by 43.3% compared to the control and Ins+Rn increased by 87.2% compared to the control. Furthermore, Rn did not produce significant changes compared to control cells. In addition, the joint effect of Ins+Rn increased the expression of p-AKT with respect to Ins by 31.6% (Figure 3).

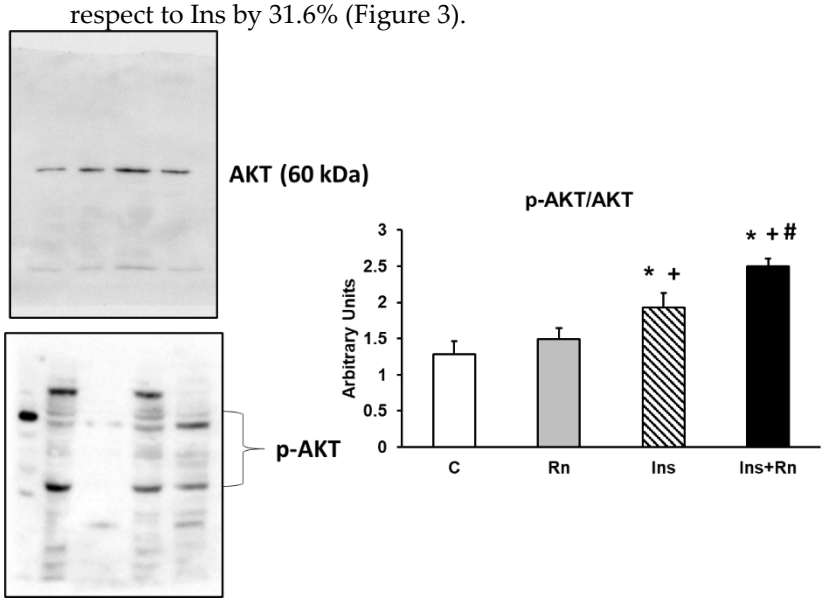

Figure 3. Effect of Ins and Rn on p-AKT and AKT protein expression. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-8} M+10^{-6} M) for 24 h and collected to determine p-AKT and AKT protein expressions by Western blot. A representative immunoblot is shown in the panel. Data are mean ± SD of four independent experiments (four different rats). *p < 0.05 vs. control. +p < 0.05 vs. Rn. #p < 0.05 vs. Ins.

2.4. Expression of p-eNOS protein

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We determined the expression of p-eNOS in astrocytes in primary culture. The presence of Rn did not produce any significant differences respect to control cells. Ins increased the expression of p-eNOS protein compared to control cells (32.25%). Ins+Rn significantly increased the expression of p-eNOS compared to the control (74.1%). Furthermore, the joint effect of Ins+Rn significantly increased (30.6%) the expression of p-eNOS with respect to the Ins group (Figure 4).

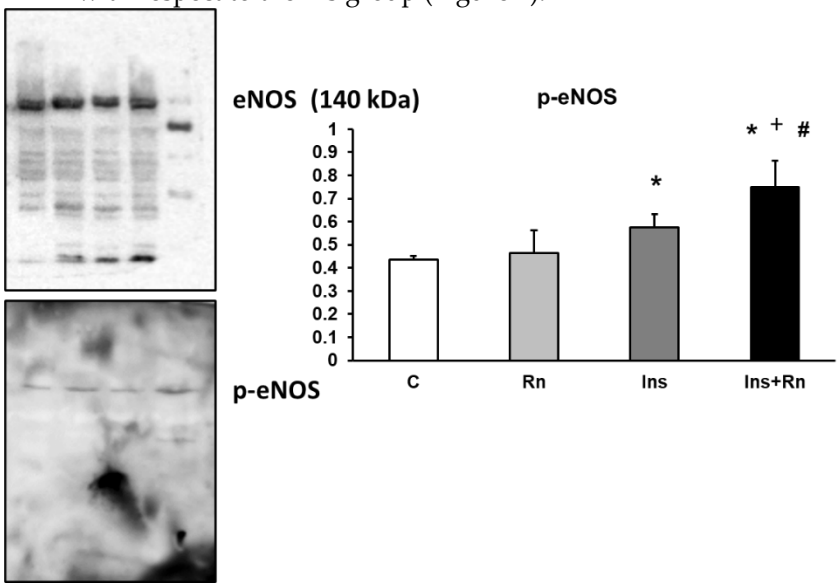

Figure 4. Effect of Ins and Rn on p-eNOS and eNos protein expression. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-8} M+10^{-6} M) for 24 h and collected to determine p-eNOS and eNOS protein expressions by Western blot. A representative immunoblot is shown in the panel. Data are mean ± SD of four independent *experiments (four different rats). *p < 0.05 vs. control. +p < 0.05 vs. Rn. #p < 0.05 vs. Ins.*

2.5. p-ERK protein expression

We determined p-ERK protein expression in astrocytes in primary culture. After addition of Rn or Ins, a significant increase in p-ERK protein expression was detected compared to control astrocytes (22.8% and 33.2%). The incubation with Ins+Rn significantly increased p-ERK expression compared to control cells (60.1%) and respect to Rn or Ins treated cells (29.7 and 21.4% respectively) (Figure 5).

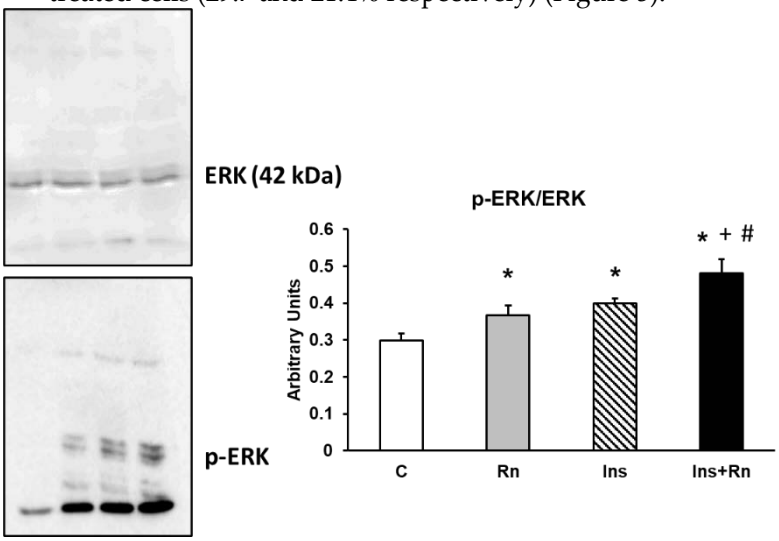

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Figure 5. Effect of Ins and Rn on p-ERK and ERK protein expression. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-8} M+10^{-6} M) for 24 h and collected to determine p-ERK and ERK protein expressions by Western blot. A representative immunoblot is shown in the panel. Data are mean ± SD of four independent experiments (four different rats). *p < 0.05 vs. control. +p < 0.05 vs. Rn. #p < 0.05 vs. Ins.

2.6. COX-2 protein expression

We detected a significant decrease after addition of Rn (10^{-6} M) and an increase of COX-2 protein expression after addition of Ins (10^{-8} M) compared with control values (15.2% and 20.1% respectively). Furthermore, the presence of Ins+Rn decreased COX-2 expression (18.1%) respect to control astrocytes and 48.8% respect to astrocytes treated with Ins, showing no differences respect to Rn addition (Figure 6).

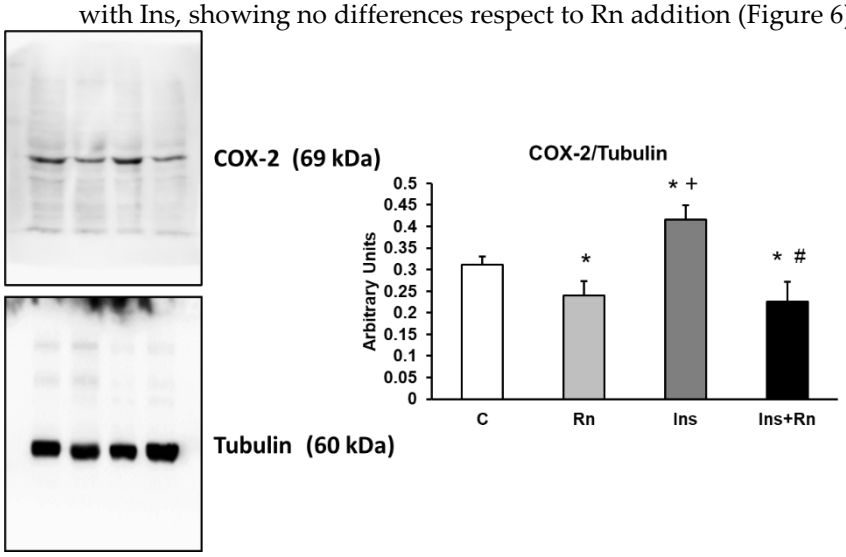

Figure 6. Effect of Ins and Rn on COX-2 protein expression. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-8} M+10^{-6} M) for 24 h and collected to determine COX-2 protein expression by Western blot. A representative immunoblot is shown in the panel. Data are mean ± SD of four independent experiments (four different rats). *p < 0.05 vs. control. +p < 0.05 vs. Rn. #p < 0.05 vs. Ins.

2.7. Expression of Cu/Zn-SOD and Mn-SOD proteins

In astrocytes, Rn, Ins or Ins+Rn did not produced changes in Cu/Zn-SOD (Figure 7A) protein expression compared to control cells (Figure 7A). Expression of Mn-SOD was determined and showed in Figure 7B. Addition of Ins significantly increased protein expression compared to control astrocytes (51.2%). Incubation with Ins+Rn significantly increased Mn-SOD protein expression compared to control (59.1%), Rn (58.9%) and 16.4% respect to Ins treated astrocytes (Figure 7B).

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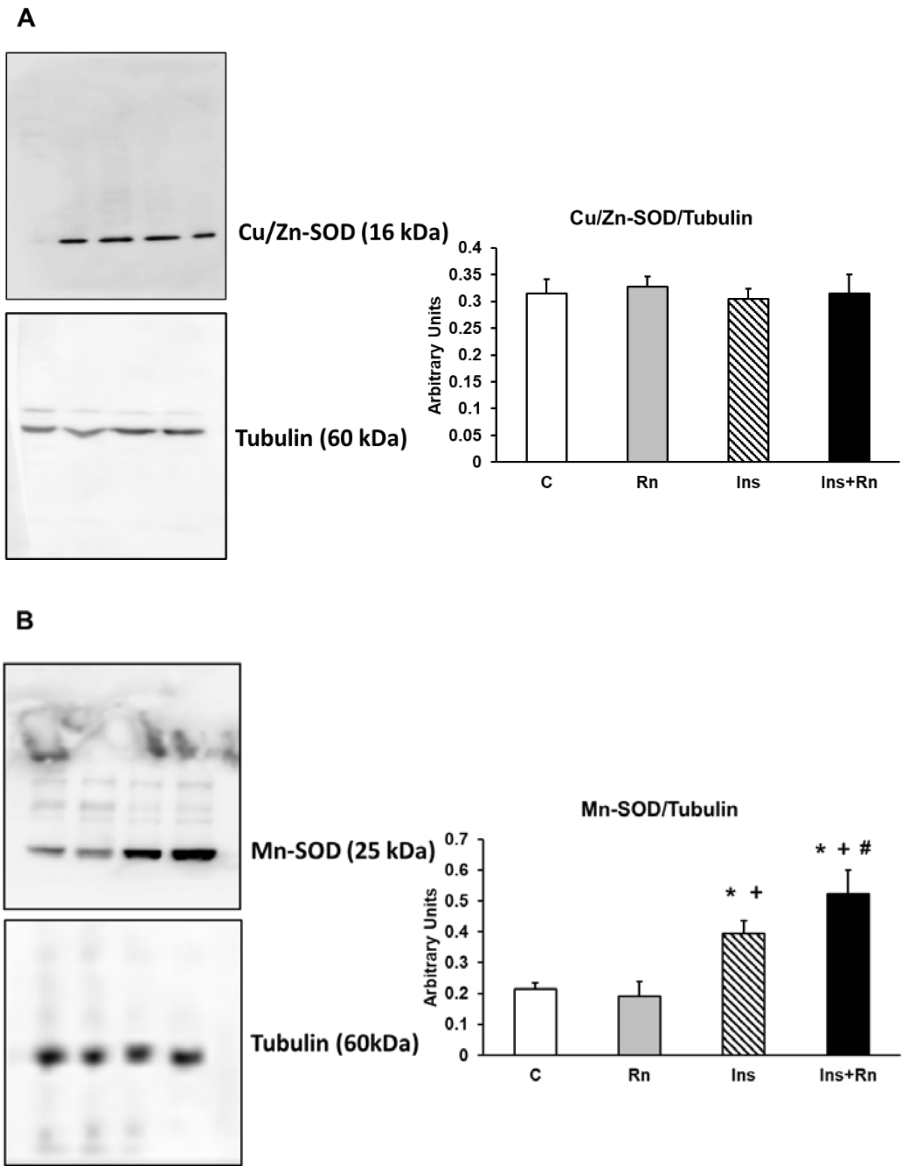

Figure 7. Effect of Ins and Rn on Cu/Zn-SOD and Mn-SOD protein expression. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-8} M+10^{-6} M) for 24 h and collected to determine Cu/Zn-SOD (Figure 6A) and Mn-SOD (Figure 6B) protein expression by Western blot. A representative immunoblot is shown in the panel. Data are mean ± SD of four independent experiments (four different rats). *p < 0.05 vs. control. +p < 0.05 vs. Rn. #p < 0.05 vs. Ins.

2.8. NF-κB and IκB expression

NF-κB is a transcription factor that regulates positively gene expression of pro-inflammatory proteins. Figure 8A shows that Rn (10^{-6} M), Ins (10^{-8} M) and Ins+Rn (10^{-8} M and 10^{-6} M) did not produce significant differences compared to control cells. On the other hand, IκB is one member of a family of cellular proteins that inhibit the NF-κB transcription factor. Figure 8B shows that Rn, Ins and Ins+Rn did not induce significant differences in IκB protein expression compared to control cells.

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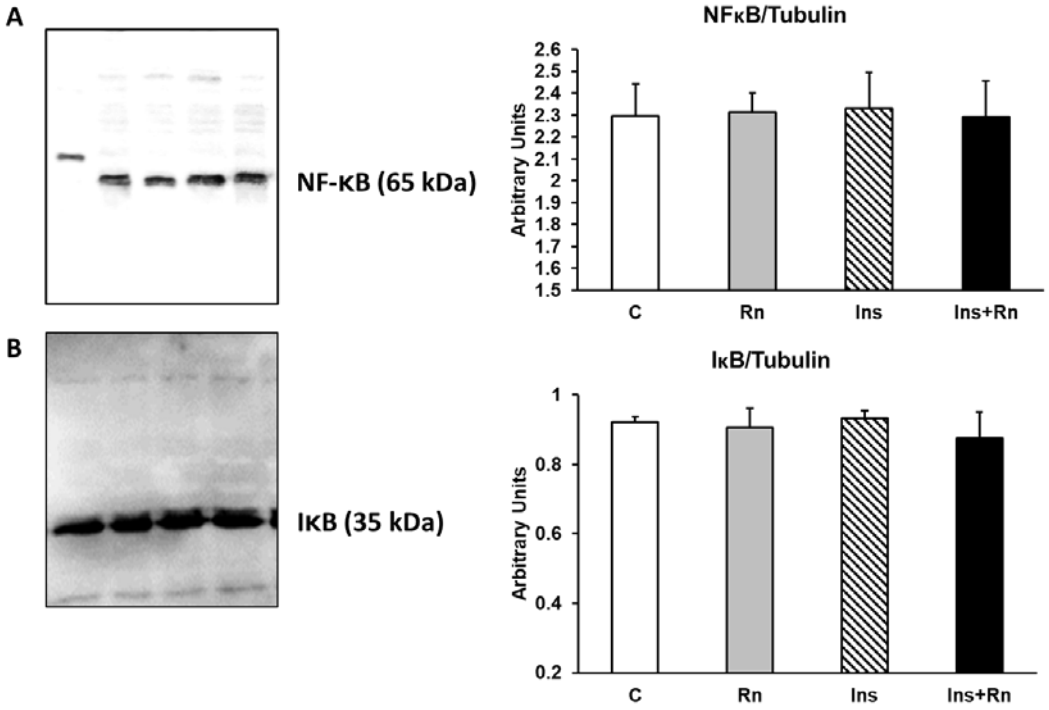

Figure 8. Effect of Ins and Rn on NFκB and IκB protein expression. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M) or with Ins+Rn (10^{-8} M+10^{-6} M) for 24 h and collected to determine NFκB (Figure 7A) and IκB (Figure 7B) protein expression by Western blot. A representative immunoblot is shown in the panel. Data are mean ± SD of four independent experiments (four different rats).

2.9. PPAR-γ expression

PPARs family negatively regulates gene expression of pro-inflammatory proteins. Figure 9 shows PPAR-γ expression in astrocytes in primary culture. Ins increased PPAR-γ expression compared to control astrocytes (46.8%). Furthermore, incubation with Ins+Rn increased PPAR-γ protein expression compared to control astrocytes (74.6%) and 18.4% with respect to Ins treated cells (Figure 9).

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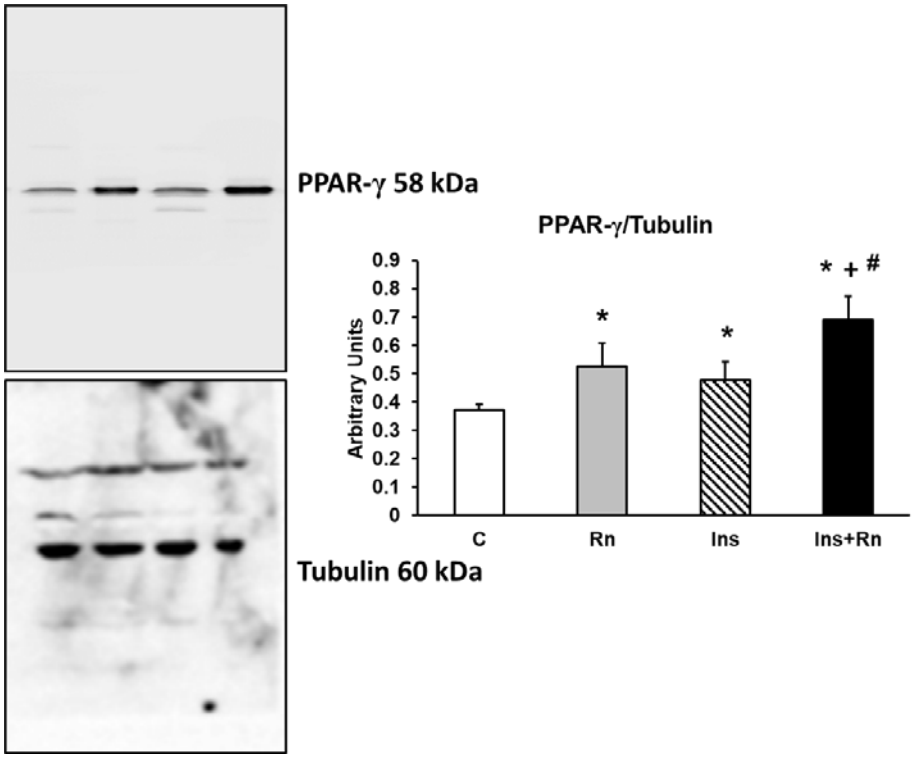

Figure 9. Effect of Ins and Rn on PPAR-γ protein expression. Astrocytes were incubated without (control, C), with Rn (10^{-6} M), with Ins (10^{-8} M), or with Ins+Rn (10^{-8} M+10^{-6} M) for 24 h and collected to determine PPAR-γ protein expression by Western blot. A representative immunoblot is shown in the top panel. Data are mean ± SD of four independent experiments (four different rats). *p < 0.05 vs. control. +p < 0.05 vs. Rn. #p < 0.05 vs. Ins.

3. Discussion

The main findings of this research are that Ins enhanced both cell viability and proliferation. Moreover, Ins increases p-AKT, p-eNOS, p-ERK, Mn-SOD, COX-2 and PPAR-γ protein expression in astrocytes in primary culture. Furthermore, Rn potentiated insulin-induced effects at doses similar to those seen in individuals treated with this medication. On the contrary, the expression of Cu/Zn-SOD, NF-κB and IκB after Rn, Ins or Ins+Rn addition did not produce any alterations in astrocytes in the primary culture. The inclusion of Rn in the culture also resulted in a decrease in COX-2 protein expression.

Astrocytes are glial cells that perform a variety of functions in the brain, including structural and metabolic support for the cell brain, maintenance of the blood-brain barrier (36), glutathione synthesis and neuroprotective actions against oxidative stress and inflammation (2,37). Astrocytes play a fundamental role in neuronal protection through variety of mechanisms, the most notable of which is mitochondrial biogenesis, which allows them to shield neurons against inflammatory and oxidative processes (38).

Furthermore, astrocytes play roles in neuroendocrine, regulation of energy balance and metabolism control by responding to the different hormonal stimuli (39,40). Glucose uptake by astrocytes is an insulin-dependent process (41). Astrocytes and microglia express insulin receptor isoforms as well as insulin receptor substrate (IRS)-1 and IRS-2 (42).

In our experiments, we found that Ins boosted the expression of p-AKT and p-eNOS. Functional studies with glial cells demonstrated that Ins activates PI3K and AKT (43). Furthermore, AKT promotes NO production by mediating eNOS activation (44). Insulin treatment of hippocampal CA1 cells improves memory and spatial learning. The synthesis of endogenous NO seems to be involved in these effects, since they are inhibited by L-

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NAME, a blocker of NO synthesis (45,46). Insulin resistance appears to be implicated in cognitive decline in patients with type II diabetes and Alzheimer's disease. In addition, there is evidence that D1D and T2D patients show a higher frequency of depression, anxiety, cognitive impairment, and dementia (47,48).

A decrease in insulin release and/or a reduction in its sensitivity, is a risk factors in both Alzheimer's disease (AD) (49,50) and Parkinson's disease (PD) (51). Downregulation in PI3K/AKT pathway is characteristic of insulin resistance (52). Cognitive decline is associated with serine phosphorylation of IRS1 and co-localized with neurofibrillary tangles (53), decreasing insulin actions (54) by changes in PI3K signaling pathway [55]. Furthermore, Rn causes a protective effect against cognitive decline in T2DM patients (56).

Insulin binding to its receptor activates the MAPK and ERK signalling pathways in addition to the AKT/eNOS pathway. ERK controls cell proliferation, mitogenesis, and differentiation, and the production of endothelin 1 (57). Moreover, in the brain insulin plays a key role in the direct regulation of ERK, which is involved in maintaining the type of memory involved in Alzheimer's disease (58). Our results show that insulin increases the expression of p-ERK, coinciding with the data presented by these authors.

Insulin inhibits the production of reactive oxygen species and iNOS expression when the cells are exposed to pro-inflammatory agents (59). Furthermore, at low concentrations, insulin shows pro-inflammatory actions (42). However, in our experiments, insulin does not show pro-inflammatory effects since there is no variation in the expression of NFκB and IκB and, on the other hand, it produces an overexpression of PPAR-γ. In diabetic patients and in animals with insulin resistance, PPARγ improves both glucose tolerance and cellular insulin sensitivity (60,61,62). On the other hand, insulin induces anti-inflammatory effects mediated by PPARγ, and PI3K/Akt/Rac-1 signaling pathways (63). In cardiovascular cells, activation of PPARγ inhibits the effects of angiotensin II and acts as an antioxidant and anti-inflammatory (64). The use of PPARγ antagonists in neurodegenerative diseases associated with inflammatory processes has recently been proposed (65).

In our study, we observed that insulin causes an increase in the expression of COX-2. Insulin reduced amyloidogenesis and COX-2-mediated neuroinflammation in astrocytes treated with streptozotocin, which are hallmarks of Alzheimer's disease (1). On the contrary, intracerebral insulin administration decreased the expression of the inflammatory factor COX-2 in rats treated with streptozotocin (66).

In our experiments, insulin increased the expression of Mn-SOD and did not produce changes in Cu/Zn-SOD protein expression. In cardiomyocytes, the absence of insulin has been related to an increase in free radicals due to a decrease in SOD activity (67). Insulin improves cognitive impairment in Wistar rats by reducing brain oxidative stress and increasing antioxidant systems like SOD, catalase, and GSH (68). Insulin resistance can be reversed with Mn-SOD mimetics or Mn-SOD overexpression Insulin resistance can be reversed with Mn-SOD mimetics or overexpression (69). In diabetic rats, insulin has been shown to protect against oxidative stress and inhibit apoptosis induced by H_2O_2, intracellular ROS, and increases superoxide dismutase, catalase, and glutathione peroxidase activity (70).

Ranolazine improves ATP production and O2 consumption by stimulating glucose oxidation and decreasing fatty acid oxidation (71). In type II diabetic patients, RN has been shown to offer a variety of effects, including lowering blood glucose and glycosylated haemoglobin levels, promoting insulin release, and decreasing glucagon synthesis, therefore improving pre- and postprandial blood glucose (72,73,74) and decreasing glucagon synthesis, thus improving pre- and postprandial blood glucose (75). Rn reduced the pro-inflammatory profile and improved learning and long-term memory in a Wistar rat model of type II diabetes. Rn may be useful in addressing cognitive deterioration in type 2 diabetes in this way (56). Its clinical use is especially interesting in patients with type II diabetes and coronary ischemia (27,76) and, in fact, Rn has been proposed as the first treatment for type II diabetes (74). Rn does not modify the AKT pathway, or the kinases involved in glucose uptake (77). In our experiments, Rn enhanced the effects of insulin on

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AKT and eNOS, increasing the expression of p-AKT and p-eNOS, indicating that this effect is probably due to a facilitation of insulin action.

The Rn improved insulin resistance in non-diabetic patients with coronary heart disease, reducing the HOMA-IR index with better results than that obtained with treatment with beta-blockers or calcium-channel blockers (78). However, there is no direct evidence of the effects of Rn that increase cellular sensitivity to insulin. The data from our study seem to indicate a facilitating effect of Rn on the sensitivity of astrocytes to insulin.

Ranolazine interacts with different isoforms of the neuronal Nav channel (79), such as those involved in altered neuronal excitability in different forms of epilepsy, migraine, or neuropathic pain (80,81), which would allow its clinical use (80,30). Moreover, Rn has recently been shown to improve diabetic neuropathy in rats (82). Together, the cardioprotective and neuroprotective effects of Rn are related to its anti-inflammatory and antioxidant actions (4,83).

Ranolazine enhances the effects of insulin in primary culture astrocytes by boosting the expression of anti-inflammatory mediators like PPAR-γ and reducing the production of pro-inflammatory mediators like COX-2. Furthermore, ranolazine increased the action of insulin on the Mn-SOD antioxidant enzyme, as well as components of the AKT-eNOS and ERK signalling pathways (Figure 10).

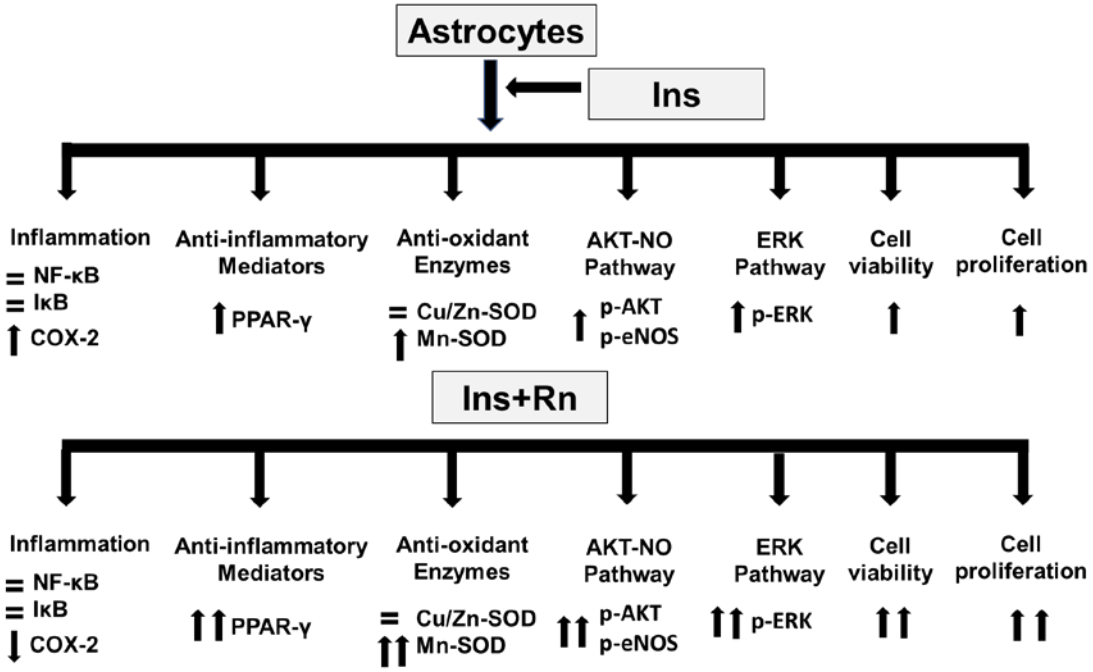

Figure 10. Changes after Ins and Rn to astrocytes in primary culture. Rn facilitates the effects of insulin increasing cell viability and proliferation, the expression of anti-inflammatory mediators, such as PPAR-γ, and inhibiting that of pro-inflammatory mediators, such as COX-2. Furthermore, Rn potentiated the effect of insulin on the expression of antioxidant enzyme (Mn-SOD), the components of the AKT-eNOS pathway and the ERK signaling pathway.

4. Materials and Methods

4.1. Materials

3-(4,5-dimethyl-2-thiazolyl)-2,5-dipheniyl-2H tetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gibco Invitrogen Corporation, Barcelona, Spain). Ranolazine (Rn) and Insulin (Ins) were obtained from Sigma-

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Aldrich biotechnology and dissolved in Krebs solution to the proper final concentration 10^{-6} M and 10^{-8} M respectively. Western Blot Chemiluminescent Detection System (ECL) was from Amersham (Amersham Biosciences, Barcelona, Spain). Antibodies: polyclonal anti-manganese superoxide dismutase (anti-MnSOD) (1:250), monoclonal anti-NF-κB) (1:250), monoclonal anti-IκB (1:250), polyclonal anti-PPAR-γ (1:300), monoclonal anti-COX-2 (1:500), monoclonal anti-Cu/Zn-SOD (1:500), monoclonal anti-AKT (1:500), monoclonal anti-p-AKT (1:500), monoclonal anti-e-NOS (1:250), monoclonal anti-ERK (1:500), monoclonal anti-p-ERK (1:500) and monoclonal anti-tubulin (1:3000) antibodies (Sigma Aldrich, Madrid, Spain) were used. All other reagents were of analytical or culture-grade purity.

4.2. Primary culture of cortical astrocytes

All animals were handled according to the rules established by the bioethics committee of the School of Medicine, University of Valencia, Spain. Cerebral cortical astrocytes were isolated from rat fetuses of 21 days gestation. Fetuses were obtained by cesarean section and decapitated. Cerebral cortices were removed and cut into 1 mm cubes and triturated 10-15 times through a Pasteur pipette. After centrifugation at 1000 rpm for 5 min the pellet was resuspended in DMEM containing 20% fetal bovine serum (FBS), supplemented with L-glutamine (1%), HEPES (10 mM), fungizone (1%), and antibiotics (1%). Cells were plated on T75 culture flask. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C and allowed to grow to confluence and used at 15-20 days in vitro. After one week of culture, the FBS content was reduced to 10%, and the medium was changed twice a week. The purity of astrocytes was assessed by immunofluorescence using anti-glial fibrillary acidic protein (anti-GFAP, astrocyte marker: Sigma-Aldrich, Madrid, Spain), anti-CD-68 (microglial marker: Serotec, Kidlington, UK), anti-myelin basic protein (oligodendroglial marker; Sigma-Aldrich, Madrid, Spain) and anti-microtubule-associated protein 2 (anti-MAP2, neuronal marker; Sigma-Aldrich, Madrid, Spain). The astrocytes were found to be at least 99% glial fibrillary acidic protein positive. No cells were found to express CD-68, myelin basic protein or microtubule-associated protein-2. For all the experiments we used toxin-free sterile culture materials.

4.3. MTT assay

Cell viability of the cultures was determined by the MTT assay (34). Astrocytes were plated in 96 well cultures. Rn, Ins or Ins+Rn were added to wells for 24h. After cell treatments, the medium was removed and the cortical cells were incubated with red free medium and MTT solution [0.5 mg/ml, prepared in phosphate buffer saline (PBS) solution] for 4 h at 37ºC. Finally, the medium was removed, and formazan particles were dissolved in dimethyl sulfoxide (DMSO). Cell viability, defined as the relative amount of MTT reduction was determined by spectrophotometry at 570 nm.

4.4. Trypan Blue Assay

Trypan blue exclusion assay was used to count the living cells and monitor cell proliferation. Astrocytes were isolated and seeded at $7x10^4$ cells/35 mm dish. After 5 days of culture, cells were incubated without (control, C), with Rn (10^{-6}M), Ins (10^{-8}), or with Ins+Rn (10^{-8} + 10^{-6} M) for 24 h. 1.5% trypan blue solution was applied to astrocyte cultures at room temperature for 3 min.

4.5. Western blot analysis

Cultured cells were treated with lysis buffer and then mechanically degraded to release the proteins. Protein concentration was determined using modified Lowry method (35). Loading buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 0.5% (v/v) 2-mercaptoethanol, 1% bromophenol blue and 19% glycerol) was added to protein sample and heated for 5 min at 95ºC. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose

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membranes in a humid environment using a transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). Membranes were blocked with 5% milk in TBS (0.05% Tween-20) and were incubated with primary antibodies overnight at 4ºC. Membranes were washed 3 times with wash buffer TBS-T (TBS, 0.2% Tween-20) and were incubated with a secondary anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technologies Danvers, MA) antibody conjugated to the enzyme horseradish peroxidase (HRP) for 1 h. Membranes were washed three times and proteins were detected using the ECL method as specified by the manufacturer. Autoradiography signals were assessed using digital image system ImageQuant LAS 4000 (GE Healthcare).

4.6. Statistical methods

Values are expressed as mean ± S.D. Differences between groups were assessed using t-test (Student´s test) and by one-way analysis of variance (ANOVA) with the program GraphPad Prism. Statistical significance was accepted at $p \leq 0.05$. Data sets in which F was significant were examined by a modified t-test.

5. Conclusions

Ranolazine enhances the effects of insulin in primary culture astrocytes by boosting the expression of anti-inflammatory mediators like PPAR-γ and reducing the production of pro-inflammatory mediators like COX-2. Furthermore, ranolazine increased the action of insulin on the Mn-SOD antioxidant enzyme, as well as components of the AKT-eNOS and ERK signalling pathways (Figure 10).

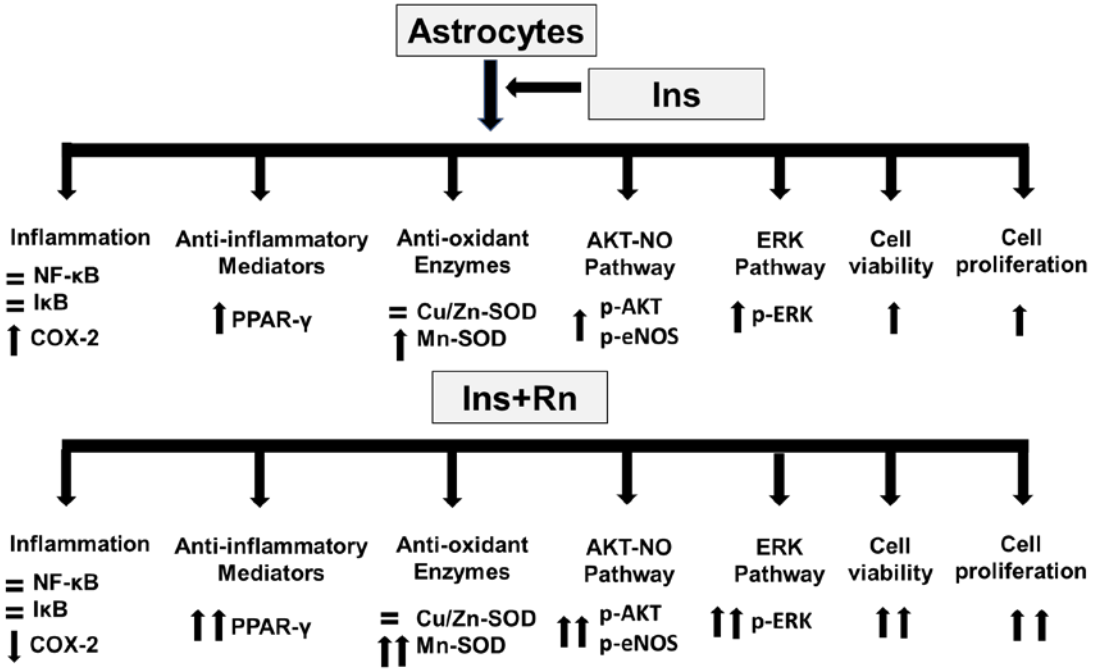

Figure 10. Changes after Ins and Rn to astrocytes in primary culture. Rn facilitates the effects of insulin increasing cell viability and proliferation, the expression of anti-inflammatory mediators, such as PPAR-γ, and inhibiting that of pro-inflammatory mediators, such as COX-2. Furthermore, Rn potentiated the effect of insulin on the expression of antioxidant enzyme (Mn-SOD), the components of the AKT-eNOS pathway and the ERK signaling pathway.

Abbreviations: Ins: insulin; Rn, ranolazine; AKT, protein kinase B; p-AKT, phosphor-protein kinase B; eNOS, endothelial nitric oxide synthase; p-eNOS, phosphor-endothelial nitric oxide synthase; ERK, extracellular regulated kinase; p-ERK, phospho-extracellular regulated kinase; COX-2, cyclooxygenase 2; Cu/Zn-SOD, Cu/Zn-superoxide dismutase; Mn-SOD, Mn-superoxide dismutase; NF-κB, nuclear factor-kappa B; IκB, an inhibitor of nuclear factor-kappa B; PPAR-γ, peroxisome proliferator activated receptor γ.

Acknowledgments: We thank Mrs. Pilar Ribera for her help in the laboratory.

Funding: Financial support from the University of Valencia. (Specific Key 20180268).

Authors' contributions: AJ, IC and JCC did most of the experiments and interpreted the data. CA performed the statistical analysis. JMV and EO performed cell viability experiments. SKS helps with English. SLV and MA conceived of and designed the study, collected data, interpreted the data, and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate: All animal procedures were carried out in accordance with the European legislation on the use and care of laboratory animals (CEE 86/609). Experimental research on mice was performed with the approval of the ethics committee on animal research of the University of Valencia (Spain) and all participants provided written informed consent. All procedures were performed in accordance with the 1964 Helsinki declaration and its later amendments.

Competing Interests: The authors have declared that no competing interest exists.

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