UNIVERSITAT DE BARCELONA

The possible link between high glucose-induced PKCβ expression and the appearance of GLP-1 resistance in endothelial cells

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RESULTS

1. PI3K/AKT signalling is disrupted in HUVEC cells exposed to chronic high glucose

As it was explained in the *Introduction* Section, apart from the well-documented incretin effect of GLP-1, its role in the cardiovascular system also arouses interest. This may include a direct action on the endothelium, where the presence of specific receptors for GLP-1 has been demonstrated (Okerson and Chilton 2012; Saraiva and Sposito 2014).

Activation of the GLP-1R canonical signalling triggers at least 2 downstream pathways: on one hand it generates the second messenger cAMP and this results in activation of PKA, which in turns phosphorylates ERK1/2, and on the other hand it indirectly activates PI3K inducing AKT phosphorylation (Drucker 2006).

Firstly, we wonder to test GLP-1 signalling cascade in HUVEC cells, in order to assess if it was disrupted due to sustained high glucose, as we speculated. To this aim, we cultured HUVEC cells during 21 days without passaging the cells and changing the media each 48h. Two different glucose conditions were used for our experiments: normal glucose concentration (5mmol/L, NG) or high glucose concentration (25mmol/L, HG). Before harvesting, cells were acutely treated during 1 hour with 50nM GLP-1. As detailed in *Materials and methods* Section, whole cell lysates were prepared and the expression of total AKT and its phosphorylated form at Ser473 residue was assessed by western blot.

HUVEC cells exposed to normal glucose concentrations normally responded to GLP-1 treatment, initiating its canonical signalling cascade, which was confirmed by AKT phosphorylation (Figure 23). However, when HUVECs were maintained for 21 days under high glucose concentrations, acute GLP-1 treatment had no effect on AKT phosphorylation (Figure 23).

This data is possibly indicating that the pathway was disrupted by chronic HG condition.

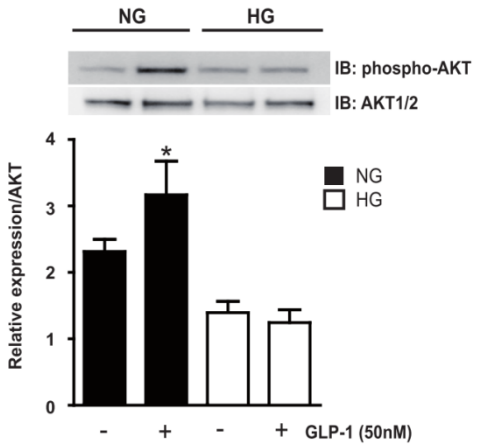


Figure 23: GLP-1 canonical signal disruption. Whole cell lysates from HUVECs, cultured during 21 days under NG or HG and treated with 50nM GLP-1, as indicated at the end of the experiment, were prepared. The expression of active (phosphorylated) or total AKT was assessed by western blot. The panels show representative images of different independent experiments. Densitometric values were normalized to total AKT. *p<0.05 vs NG. Bars represent mean±SEM for six independent experiments.

In order to further insight in the mechanism described above, we specifically blocked GLP-1 signalling cascade using the chemical inhibitor for PI3K, Wortmannin. For this purpose, we cultured HUVEC cells during 21 days under NG. At the end of the experiment, cells were treated with 1µM Wortmannin overnight and/or 50nM GLP-1 during 1 hour before their harvesting. As it was expected, under the condition of normal glucose concentration, the activation of AKT, analysed by its phosphorylation in Ser473 residue and normally induced by PI3K action after the binding of GLP-1 to its receptor, was abrogated when Wortmannin was added to the medium. So, Wortmannin was inhibiting PI3K, blocking its capacity to phosphorylate AKT (Figure 24).

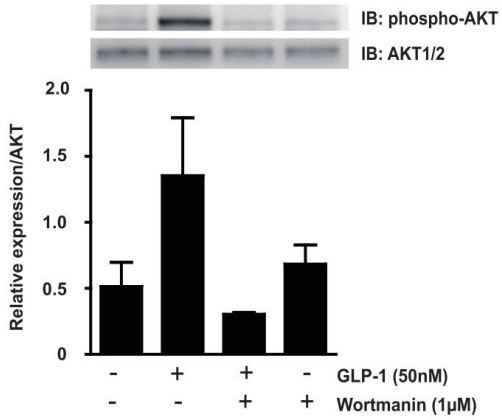


Figure 24: GLP-1 canonical signal disruption. Whole cell lysates from HUVECs, cultured during 21 days only under NG and treated with 50nM GLP-1 and/or 1μM Wortmannin, as indicated at the end of the experiment, were prepared. The expression of active (phosphorylated) or total AKT was assessed by western blot. The panels show representative images of different independent experiments. Densitometric values were normalized to total AKT. Bars represent mean±SEM for six independent experiments.

With the aim to establish if GLP-1 acts through PI3K/AKT arm in HUVECs, the expression of different GLP-1 target genes was assessed. As it was described by Oeseburg et al. (Oeseburg et al. 2010), GLP-1 was able to induce gene expression of the antioxidant enzymes Nqo-1 and Hmox-1. Moreover, in pancreatic cell line (D'Amico et al. 2005) also Bcl-2 expression was induced by GLP-1 administration, as well as it was observed in HUVEC cells (Yi Zhan, Hui-lin Sun, Hong Chen, Hua Zhang and Zhen Zhang 2012). We confirmed this data in our *in vitro* model of 21 days of normal glucose condition culture of HUVEC cells. GLP-1 induced the mRNA expression of Hmox-1, Nqo-1 and Bcl-2, but when we inhibited PI3K by the addition of Wortmannin, no induction of studied transcripts was observed (Figure 25).

This data confirmed that also in our cellular model of endothelial cells exposed to chronic NG for 21 days, GLP-1 acts via PI3K and AKT.

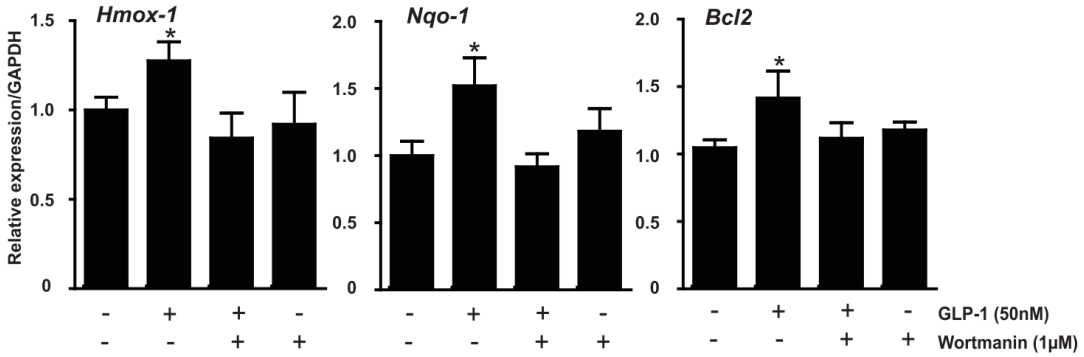


Figure 25: GLP-1 canonical signal disruption. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG conditions. Cells were treated with 50nM of GLP-1 and/or 1μM Wortmannin, as indicated at the end of the experiment, and mRNAs encoding for the indicated genes, Hmox-1 – Nqo-1 – Bcl2, were assessed by qRT-PCR and expressed relative to GAPDH. *p<0.05 vs NG. Bars represent mean±SEM for six independent experiments.

2. PKA signalling is also affected in HUVEC cells due to prolonged exposure to high glucose

The first consequence of the bind of GLP-1 to its receptor is the activation of adenylate cyclase and the consequent production of cAMP. As described in the *Introduction* Section, cAMP mediates its stimulatory actions activating PKA (Ussher and Drucker 2012).

So, once established the effects of GLP-1 on PI3K/AKT signalling in HUVECs exposed to chronic high glucose, the following step was to study the effects on the other pathway activated by the binding of GLP-1 to its receptor: the PKA signalling.

The serine/threonine kinase PKA consists of a regulatory (R) subunit dimer bind to two catalytic (C) subunits (Søberg et al. 2013). Several variants of the R and C subunits have been identified in human cells: R1α (PRKAR1A), R1β (PRKAR1B), R2α (PRKAR2A) and R2β (PRKAR2B) as regulatory subunits and Cα (PRKACA) and Cβ (PRKACB) as catalytic subunits (Taskén et al. 1997).

As far as we know, no previous studies check the status and the expression of different PKA subunits in HUVECs. Thus, with the aim of expanding the study on the GLP-1R signalling, gene expression levels of the different PKA catalytic and regulatory subunits were assessed in order to study how GLP-1 could modulate this kinase activity. In parallel we would assess how hyperglycaemia could regulate them in HUVEC cells.

To this objective we firstly checked if all the existing PKA subunits were expressed in HUVEC cells under normal glucose conditions. As shown in Figure 26, the catalytic subunits PRKACA and PRKACB as well as the regulatory subunits PRKAR1A, PRKAR1B, PRKAR2A and PRKAR2B, were all present at different levels in our cellular model. Among them, the PRKAR1A resulted to be the most expressed PKA subunit in HUVECs, while the others were expressed at similar levels (Figure 26).

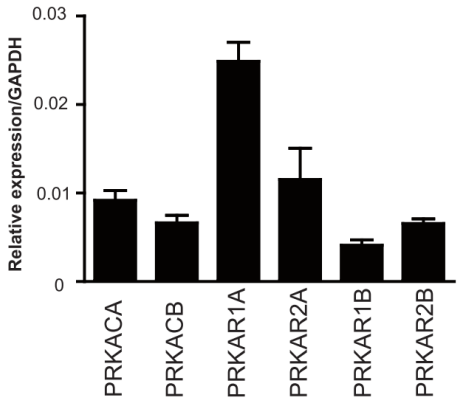


Figure 26: PKA subunits expression in HUVECs under NG. Total cellular RNA was isolated from HUVECs cultured during 21 days under NG conditions. mRNAs encoding for the indicated PKA subunits were assessed by qRT-PCR and expressed relative to GADPH. Bars represent mean±SEM for six independent experiments.

It has been recently described in pancreatic cultured β-cells that prolonged hyperglycaemia diminishes PKA activity by increasing mRNA and protein levels of some of its subunits, and the most important changes are observed in PRKAR1A protein levels (Rajan et al. 2015). On the basis of these results, we presumed that GLP-1 could exert its positive functions on endothelium by activating PKA and in turn by decreasing its transcriptional levels. When we acutely treated HUVECs maintained for 21 days in normal glucose conditions with GLP-1, we could observe that the only regulated PKA catalytic subunit was PRKACA, which resulted to be significantly decreased under the normoglycaemic state (Figure 27). As concern as the regulatory subunits, GLP-1 addition to HUVEC cells exposed to normal glucose levels showed a statistical significant reduction in the gene expression of PRKAR1B and PRKAR2A subunits (Figure 27).

Next step was to assess how these subunit transcripts were affected by hyperglycaemia in HUVECs. According to the studies of Rajan and coworkers (Rajan et al. 2015), we expected that chronic high glucose could decrease PKA activity, increasing its mRNA levels also in our cellular model. 21 days exposure of HUVECs to high glucose condition showed that the catalytic subunit PRKACB and the regulatory subunits PRKAR1A, PRKAR1B and PRKAR2B were increased by hyperglycaemia, whereas the other ones were unaffected. Contrary to what happened in the normal glucose state, GLP-1 treatment was not able to decrease PKA subunits gene expression levels under high glucose conditions in any of the studied PKA subunits (Figure 27).

As far as we know, this is the first study describing this effect in HUVEC cells.

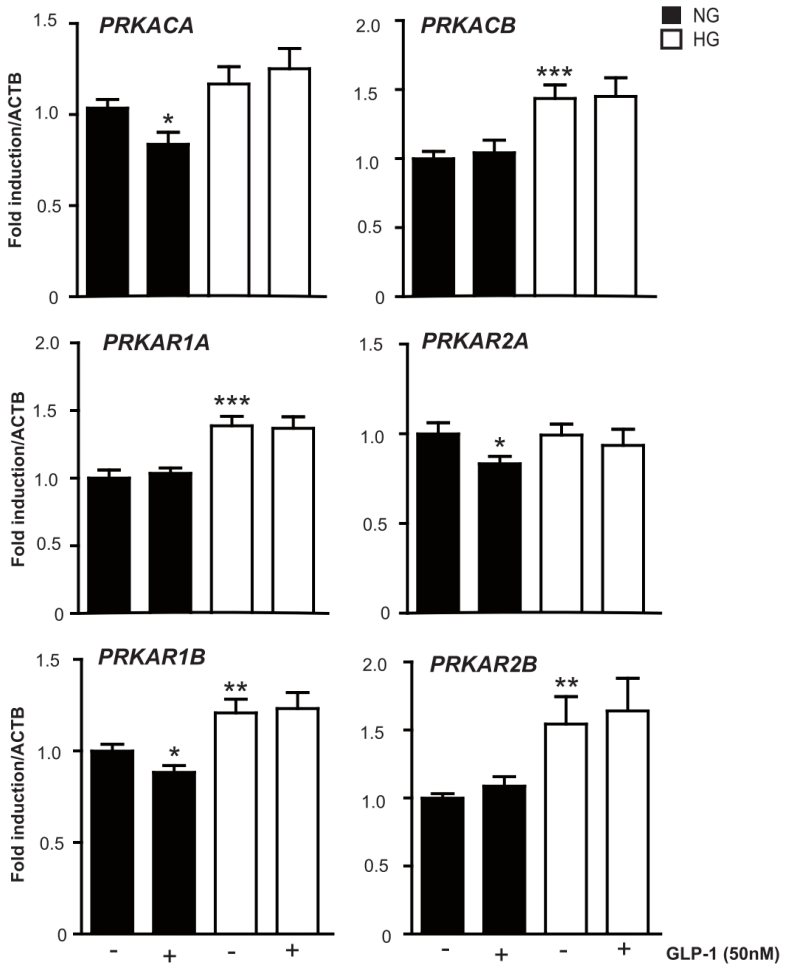


Figure 27: GLP-1 effects on PKA subunits expression in HUVECs cultured under HG. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM of GLP-1 1 hour before cell harvesting. mRNAs encoding for the indicated PKA subunits were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05, **p<0.01 and ***p<0.001 vs NG. Bars represent mean±SEM for six independent experiments.

3. GLP-1-induced antioxidant response in HUVECs is disrupted after high glucose exposure

It has been shown that GLP-1 improves endothelial function in diabetes (Ceriello et al. 2011; Nyström et al. 2004), however the mechanisms underlying the GLP-1 protective effects have not yet been fully elucidated.

As detailed in the *Introduction* Section, the substantial portion of deleterious effects of hyperglycaemia and diabetes results from oxidative stress and redox unbalance caused by ROS overproduction (Giacco and Brownlee 2010). ROS are produced by both enzymatic and non-enzymatic reactions catalyzed by different molecules, among them the NAD(P)H oxidase (Gorrini, Harris, and Mak 2013), and they play a crucial role in the normal physiology of the cells. In a healthy state, when ROS production exceeds, the system of antioxidant enzymes is activated in order to reduce their amount. So in a healthy condition, redox homeostasis is achieved by a constant tight regulation of both ROS-producing pathways, for example NAD(P)H oxidase, and ROS-detoxifying pathways, governed for example by NRF2 and its target genes Hmox-1 and Nqo-1 (Gorrini, Harris, and Mak 2013). However, when overproduction of ROS cannot be counteracted, it may result in impaired homeostasis and this leads to the generation of oxidative stress (West 2000).

In order to investigate the role of GLP-1 in ROS-producing and ROS-detoxifying pathways in our *in vitro* model of HUVECs exposed to chronic high glucose, we firstly examined the expression of one of the most important ROS inducers, the NAD(P)H oxidase (Gorrini, Harris, and Mak 2013).

We tested the different NAD(P)H oxidase subunits: the membrane-bound subunits, p22-^{phox}, Nox2 and Nox4, and the cytoplasmic subunits, p67-^{phox} and p47-^{phox}. Among them, only Nox4, p22-^{phox} and p47-^{phox} transcripts were detected in HUVEC cells and particularly we focused our attention in Nox4 and p22-^{phox} because we observed that they were up-regulated by chronic hyperglycaemia.

Afterwards, using our model of HUVECs maintained under NG or HG during 21 days, we treated them with 50nM of GLP-1 during 1 hour before their harvesting, and we assessed mRNAs expression. Unexpectedly, we observed that gene expression levels of Nox4 as well as p22-^{phox} were induced by GLP-1 under the condition of NG. On the other hand, in HUVECs exposed to sustained high glucose, Nox4 and p22-^{phox} gene expression levels were increased; it is noteworthy that, in the same condition of chronic HG, GLP-1 administration was not able to exert any effect on gene expression in any of them (Figure 28), unless we expected a decrease. This result reinforced our hypothesis of an endothelial GLP-1 resistance under the condition of chronic high glucose.

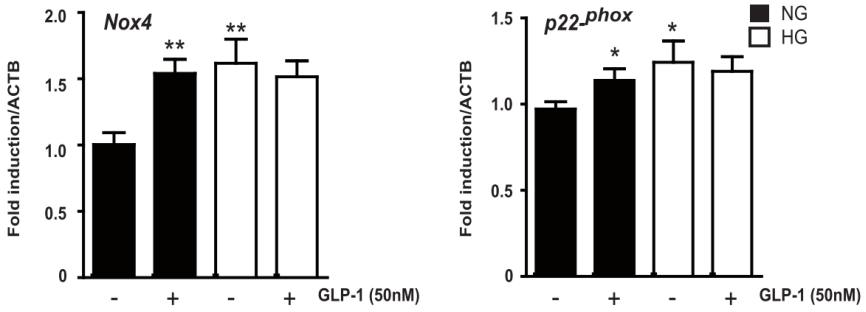


Figure 28: GLP-1 effects on the ROS producer NAD(P)H oxidase in HUVECs cultured under HG. HUVECs were maintained under NG or HG conditions during 21 days. Before harvesting, HUVECs were treated with 50nM GLP-1 during 1 hour. Total cellular RNA was isolated from HUVECs and mRNAs encoding for Nox4 and p22-^{phox} genes were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05 and **p<0.01 vs NG. Bars represent mean±SEM for six independent experiments.

In parallel, we wonder to assess the effects of GLP-1 on ROS production in HUVEC cells exposed to chronic high glucose.

Significant reductions in ROS levels have been demonstrated with GLP-1 agonist-based therapies (Chaudhuri et al. 2012). Particularly recent studies have shown that GLP-1 agonists reduce the extent of oxidative stress by reducing ROS levels in H9c2 myocytes and in human monocytes (Chang et al. 2014).

As detailed in *Materials and Methods* Section, the fluorescent probe H_2DCFDA was used to measure the intracellular generation of ROS in our cellular model. Briefly, HUVECs were grown during 21 days under NG or HG conditions and, at the end of the experiment, cells were treated with 50nM of GLP-1 during 1 hour. After GLP-1 treatment, cells were treated with H_2DCFDA for 30 minutes at 37°C and the fluorescence intensity of H_2DCFDA was kinetically measured at an excitation and emission wavelength of 485nm and 530nm, respectively. As it was expected, we observed a significant increase in ROS generation when HUVEC cells were exposed to chronic high glucose. However, we were not able to see any effect of GLP-1 in neither NG nor HG conditions (Figure 29).

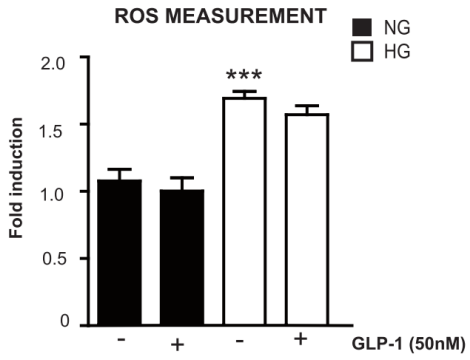


Figure 29: GLP-1 effects on ROS levels in HG. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1, as indicated at the end of the experiment. ROS production was stained by 20µM H_2DCFDA for 30 minutes and its oxidation product (DCF) fluorescence indicated ROS formation. ***p<0.001 vs NG. Bars represent mean±SEM for six independent experiments.

A major mechanism in the cellular defence system against oxidative stress and ROS overproduction is the activation of the NRF2-ARE signalling pathway, which is considered the master regulator of intracellular antioxidant response (Brewer et al. 2011). NRF2 is critical for defence against oxidative stress in diabetes (Gorrini, Harris, and Mak 2013; He and Ma 2012). Once activated, it is translocated into the nucleus, where is able to bind the ARE elements situated in the regulatory regions of its target genes, whose protein products are involved in the detoxification and elimination of ROS through conjugative reactions. This leads to the transcription of these genes, finally contributing to enhance cellular antioxidant capacity (Nguyen, Nioi, and Pickett 2009).

We assessed the activation status of NRF2 by studying nuclear translocation of this transcription factor. Firstly, we performed an experiment of immunoblotting of NRF2 protein nuclear and cytosolic fractions (Figure 30A). Again, we cultured HUVECs during 21 days under the two different glucose concentrations: NG or HG. Before harvesting, cells were acutely treated with 50nM GLP-1 during 1 hour. Nuclear and cytoplasm fractioning was performed as detailed in the *Materials and Methods* Section, followed by immunoblotting of all mentioned experimental conditions, in order to evaluate NRF2 protein cellular localization. α-tubulin (TUBA) was used as an internal control for cytoplasm fraction and laminin-β1 (LMNB1) for nuclear fraction.

Under the condition of NG the proportion of NRF2 protein was equal between nucleus and cytoplasm. When GLP-1 was added to the medium this proportional localization was unbalanced and the major amount of the protein was located into the nucleus, indicating NRF2 activation. Under the condition of HG we observed a significant increase of NRF2 nuclear proportion compared to the same proportion in the control NG and also related with the cytoplasm proportion of NRF2 in HG. So, we could say that hyperglycaemia induced a translocation of NRF2 protein in the nucleus, indicating that in this unhealthy condition NRF2 was activated initiating the cellular defence antioxidant cascade. Strikingly, under chronic hyperglycaemia, GLP-1 was not able to enhance NRF2 activation (Figure 30A). This result was indicating, once again, that a resistance of GLP-1 antioxidant actions could be happening.

In order to complete the study of NRF2 cellular localization, we also used the technique of immunocytochemistry (Figure 30B). By imaging, we confirmed the results obtained by western blot. In the top images of the panel, we could confirm that after GLP-1 treatment an increase in the nuclear fraction of NRF2 was observed for the condition of NG, however this effect was not observed under HG culturing (bottom images), indicating that GLP-1 enhanced NRF2 activation under the condition of NG but not in HG (Figure 30B). If we compare left images of the panel, where the conditions of NG and HG without GLP-1 treatment are reproduced, we could also identify an increase of NRF2 total amount as well as nuclear fraction, reproducing what we observed by immunoblot.

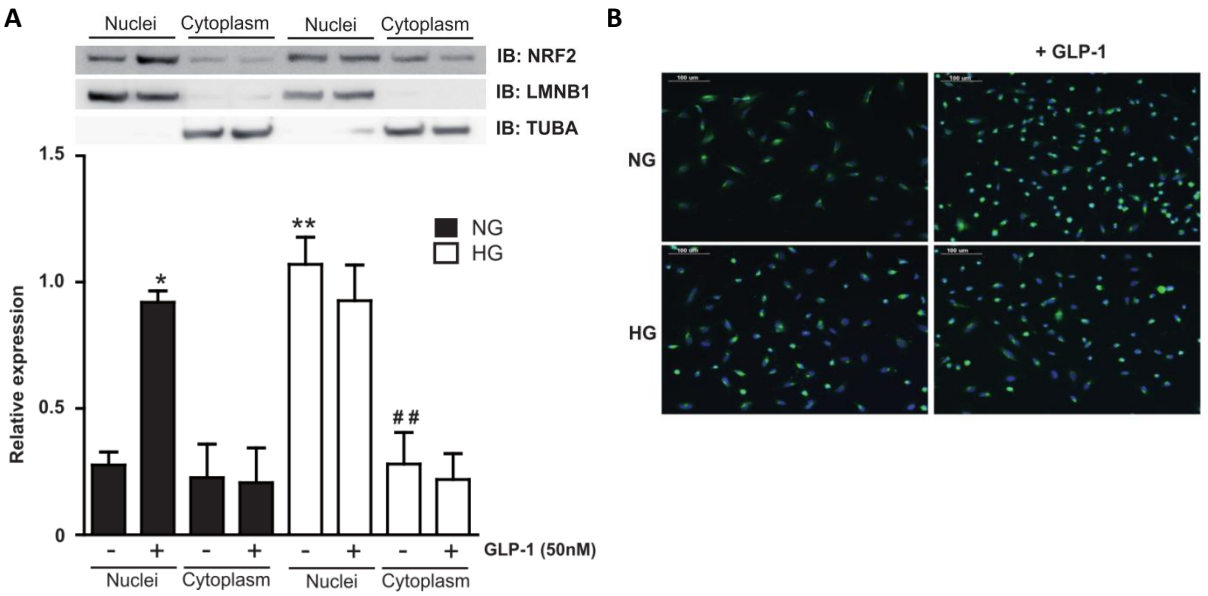


Figure 30: GLP-1 effects on the master regulator of antioxidant response NRF2 in HUVECs cultured under HG. HUVECs were maintained under NG or HG during 21 days. Before harvesting HUVECs were treated with 50nM GLP-1 during 1 hour.
(A) Nuclear and cytoplasm fractioning was performed to evaluate NRF2 protein localization by immunoblot under all mentioned experimental conditions (30µg of protein loaded in each case). TUBA was used as an internal control for cytoplasm fraction and LMNB1 for nuclear fraction. The upper panel shows a representative blot. The lower panel shows the densitometric analysis of western blots. *p<0.05 and **p<0.01 vs NG nuclear fraction and ##p<0.01 vs HG nuclear fraction. Bars represent mean±SEM for four independent experiments.
(B) Immunocytochemistry using an antibody specific for NRF2 (green) to study cellular localization of this protein. Hoechst staining was used to observe the cellular nucleus. Scale bar of 100µM.

As described before, the activation of NRF2 leads to its translocation into the nucleus, where it binds the ARE elements and induces the transcription of some genes involved in ROS detoxification: Hmox-1 and Nqo-1 (Nguyen et al., 2009; Oeseburg et al., 2010).

HUVECs maintained under NG or HG during 21 days, were treated with 50nM of GLP-1 during the last 1 hour. As detailed in *Materials and Methods* Section, after GLP-1 treatment, total cellular RNA was isolated from HUVECs and mRNAs encoding for Hmox-1 and Nqo-1 genes were assessed by qRT-PCR and expressed relative to GADPH.

The effects of GLP-1 under NG and HG on the activation status of NRF2 perfectly matched with the transcriptional activation of the studied ARE-linked genes (Figure 31). We observed that the gene expression levels of both Hmox-1 and Nqo-1 were induced by GLP-1 under normal glucose condition. In HUVECs exposed to sustained high glucose, Hmox-1 and Nqo-1 gene expression levels were also increased. Both results indicated that there was an activation of NRF2-ARE pathway as detoxifying defence mechanism of the cells. However, when GLP-1 was added under the hyperglycaemic conditions, it had no effect in any of the studied transcripts (Figure 31). In other words, no effect on the antioxidant cascade regulated by NRF2 was observed after GLP-1 addition under HG, although a clear increase in Hmox-1 and Nqo-1 was detected due to HG *per se* as a cellular defence mechanism.

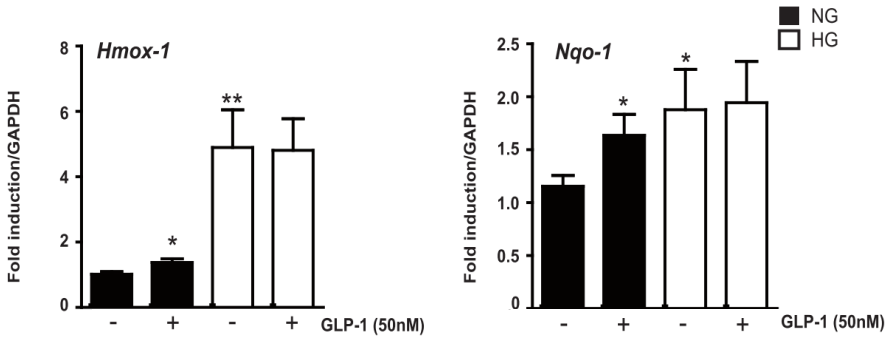


Figure 31: GLP-1 resistance on antioxidant response in HUVECs cultured under HG. HUVECs were maintained under NG or HG during 21 days. Before harvesting, HUVECs were treated with 50nM GLP-1 during 1 hour. Total cellular RNA was isolated from HUVECs and mRNAs encoding for the indicated genes, Hmox-1 and Nqo-1, were assessed by qRT-PCR and expressed relative to GAPDH. *p<0.05 and **p<0.01 vs NG. Bars represent mean±SEM for four independent experiments.

NRF2 controls not only HMOX-1 and NQO-1 which are related to ROS detoxification process, but also other antioxidants pathways (Gorrini, Harris, and Mak 2013). One of them is the pathway involved in thioredoxin (TRX) production, regeneration and utilization, which is regulated by thioredoxin reductase (TXNRD) and thioredoxin integrating protein (TXNIP) (Gorrini, Harris, and Mak 2013).

As detailed in the *Introduction* Section, cytosolic TRX-1 has a redox-regulatory activity, playing a role in controlling the signalling pathways that are involved in ROS production in endothelium (Haendeler et al. 2004). TRX-1 acts reducing hydrogen peroxide via peroxiredoxin (PRX) and oxidized TRX-1 is reduced by TXNRD-1. Moreover, it regulates proliferation and apoptosis signalling pathways by inhibiting ASK-1 (Saitoh et al. 1998). TXNIP inhibits TRX-1 activity when the enzyme is present in its reduced form (Nishiyama et al. 1999).

In diabetes, TXNIP is persistently elevated due to hyperglycaemia and oxidative stress (Parikh et al. 2007; Schulze et al. 2004). TXNIP has been implicated in the pathophysiology of T2DM through several mechanisms, including: the promotion of β-cell apoptosis by regulating ASK-1 in the mitochondria (Saxena, Chen, and Shalev 2010), the inhibition of glucose uptake in fat tissue and skeletal muscle due to an increase in insulin resistance (Parikh et al. 2007), the stimulation of glucose production in the liver (Chutkow et al. 2008) and the regulation of adipogenesis (Chutkow et al. 2010) and fatty acid utilization (Oka et al. 2006). In vascular endothelium it has been demonstrated that in human aortic endothelial cells TXNIP expression, induced by the FOXO1 transcription factor in a glucose-dependent manner, is correlated to increased ROS production (Li and Kong 2009).

As observed for Hmox-1 and Nqo-1, Txnrd gene expression levels were increased when HUVECs where treated with 50nM GLP-1 under the condition of NG. Moreover, as it was described by Parikh and coworkers (Parikh et al. 2007), also in our *in vitro* model of HUVEC cells high glucose induced the expression of Txnip gene as an indicator of cell damage (Figure 32). In parallel, an increase in Txnrd gene expression was observed due to prolonged hyperglycaemia exposure, a result that fits with Nrf2 activation. However, although a decrease of Txnip and an enhancement of Txnrd were expected, once again any effect on neither Txnip nor Txnrd transcripts was observed after GLP-1 addition under HG condition (Figure 32).

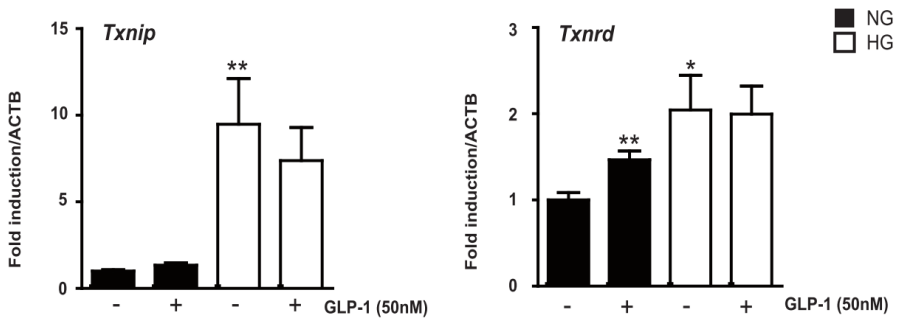


Figure 32: GLP-1 resistance on antioxidant response in HUVECs cultured under HG. HUVECs were maintained under NG or HG during 21 days. Before harvesting, HUVECs were treated with 50nM GLP-1 during 1 hour. Total cellular RNA was isolated from HUVECs and mRNAs encoding for the indicated genes, Txnip and Txnrd, were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05 and **p<0.01 vs NG. Bars represent mean±SEM for four independent experiments.

4. The antioxidant modulators of FOXO family are diversely regulated by GLP-1 in HUVEC cells exposed to chronic high glucose

Apart from the positive actions of NRF2, other sensors and modulators of redox homeostasis exist in the cells, and they play a crucial role in regulating the expression of antioxidant genes (Gorrini, Harris, and Mak 2013; Klotz et al. 2015). One of these is the FOXO family of transcription factors: although primarily known as inducers of cell cycle arrest, they also have a significant impact in preventing or reducing oxidative stress by counteracting ROS production (Gorrini, Harris, and Mak 2013).

With the objective to further insight in the antioxidant defence mechanism of HUVEC cells, we extended the panel of analysed genes to FOXO family. Firstly, we tested the expression of mRNA transcripts of the ubiquitously expressed family members, Foxo1, Foxo3a and Foxo4, in our cellular model of HUVECs. As shown in Figure 33 and accordingly to other reports (Potente et al. 2005), after 21 days exposure of endothelial cells to normal glucose conditions, all these transcription factors were expressed at different levels, resulting Foxo1 the most expressed one.

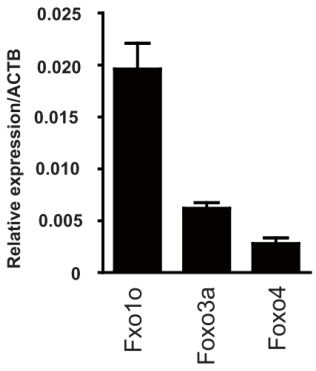


Figure 33: Gene expression of the antioxidant family of Foxos in HUVECs. Total cellular RNA was isolated from HUVECs cultured during 21 days under NG. mRNAs encoding for the indicated transcription factors were assessed by qRT-PCR and expressed relative to ACTB. Bars represent mean±SEM for six independent experiments.

Following 1 hour treatment with GLP-1 under NG state, all of them were differently regulated: Foxo1 and Foxo4 were transcriptionally induced, whereas Foxo3a was downregulated (Figure 34). When HUVECs where exposed to chronic high glucose conditions, we observed an increase in gene expression levels of Foxo3a and Foxo4, whereas Foxo1, the most expressed transcription factor in our cellular model of endothelial cells, resulted downregulated (Figure 34).

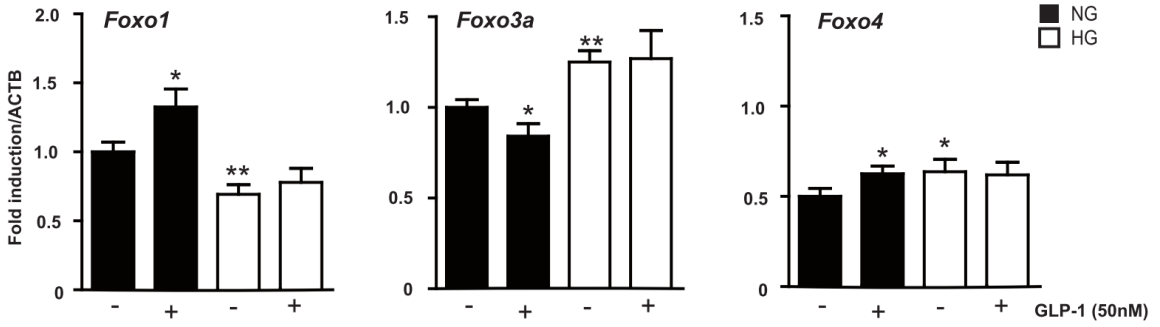


Figure 34: GLP-1 effects on the antioxidant family of Foxos in HUVECs under HG. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM GLP-1 during 1 hour before cell harvesting. Total cellular RNA was isolated and mRNAs encoding for the indicated transcription factors, Foxo1 – Foxo3a – Foxo4, were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05 and **p<0.01 vs NG. Bars represent mean±SEM for six independent experiments.

As detailed in the *Introduction* Section, FOXO transcription factors exert their antioxidant effects in different manners and one of these is the regulation of the scavengers superoxide dismutases and catalase. SODs appear to counteract antioxidant detoxification by catalysing the dismutation of the superoxide radical O_{2-} into hydrogen peroxide H_2O_2 that in turn is reduced to H_2O by CAT (Li and Shah 2004).

Hence, in parallel, we wonder to test the effects of GLP-1 in HUVEC cells on the mRNA expression of the scavengers: Sod1, Sod2 and Cat. Under normal glucose conditions, GLP-1 treatment did not exert effects in any of the antioxidant scavengers modulated by FOXOs. When HUVEC cells were exposed to 21 days of chronic high glucose, these scavengers resulted diversely regulated by hyperglycaemia: both Sod1 and Sod2 increased in their gene expression levels, as expected, whereas Cat resulted not to be affected by HG (Figure 35). Once again, GLP-1 treatment under high glucose conditions was not able to exert any positive effect in the regulation of mRNA transcripts of the scavengers modulated by FOXO transcription factors, although a GLP-1 effect was expected.

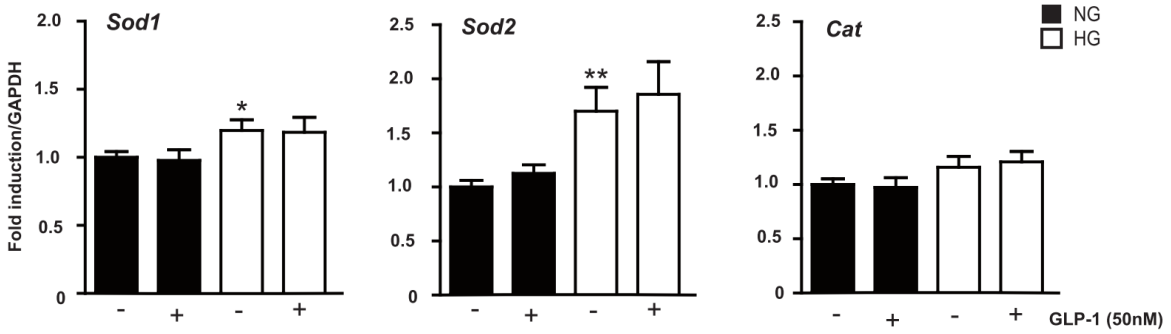


Figure 35: GLP-1 effects on the scavengers modulated by Foxos in HUVECs under HG. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM GLP-1 during 1 hour before cell harvesting. Total cellular RNA was isolated and mRNAs encoding for the indicated transcription factors, Sod1 – Sod2 – Cat, were assessed by qRT-PCR and expressed relative to GADPH. *p<0.05 and **p<0.01 vs NG. Bars represent mean±SEM for six independent experiments.

5. Pro-proliferative and anti-apoptotic GLP-1 properties are lost in HUVEC cells cultured in chronic high glucose conditions

GLP-1 has well-known pro-proliferative and anti-apoptotic properties (Drucker 2003). These positive effects have predominantly been demonstrated in diabetic rodents, islet cell lines and purified rat β-cells (Farilla et al. 2002; Y. Li et al. 2003; Wang and Brubaker 2002). For example, it has been shown that GLP-1-treated islets exhibit a progressive increase in the levels of the anti-apoptotic protein BCL-2 and a decrease in the pro-apoptotic active Caspase 3, contributing to survival properties of the incretin hormone in cultured islets (Farilla et al. 2003). Intriguingly, GLP-1 receptor activation also enhances survival in diverse cellular and animal models of neuronal toxicity, and abrogation of GLP-1 receptor function in mice is associated with an increased neurotoxicity (During et al. 2003; Perry et al. 2002).

In order to assess the pro-survival capacities of GLP-1 in the HUVEC cells exposed to sustained HG conditions, gene expression levels of several markers of cell proliferation and apoptosis were analysed. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG conditions with or without 1 hour GLP-1 treatment. After retrotranscription to cDNA, the mRNAs encoding for Cdkn1a (also known as p21), Cdkn1b (also known as p27), p53, Bcl-2, Bax, and Caspase 3 were assessed by qRT-PCR.

Cdkn1a, a member of the cyclin-dependent kinase (cdk) inhibitors family together with Cdkn1b, plays important functions in cell cycle regulation. Cdkn1a is a downstream target of p53, it is up-regulated following the persistent generation of ROS and functions as one of the major determinants of the cellular response to stress (Vitiello et al. 2009). Cdkn1a also has pro-survival functions in response to oxidative stress by inhibiting the activation of Caspase 3, Caspase9 and other pro-apoptotic factors, thus preventing cell death (Nakamura, Arai, and Fukuchi 2004). Some of the mediators of Cdkn1a protection system against hyperglycaemia-induced cell death belong to the Bcl-2 family and, among them, the anti-apoptotic Bcl-2 and the pro-apoptotic Bax genes (Wu and O'Reilly 2011).

In our experiments on HUVECs we observed that gene expression levels of the anti-apoptotic Bcl-2 were increased by GLP-1 under normal glucose conditions (Figure 36A), while no other tested genes were affected. Contrary to what happened in NG state, under HG conditions GLP-1 was not able to hamper the Bcl-2 decrease (Figure 36A). By the other hand, regarding the hyperglycaemia-induced negative effects on cell survival, the exposure of HUVECs to sustained high glucose concentrations affected the expression of all the studied transcripts, except for p53 (see Figure 38). Particularly, as we expected, the expression of Bcl-2 was decreased in a significant statistical manner respect with the control NG and in an opposite manner the expression of the pro-apoptotic marker gene Bax was increased (Figure 36A) indicating a misbalance of the Bcl-2/Bax ratio (Figure 36B) that conceptually fitted with an increase in apoptosis.

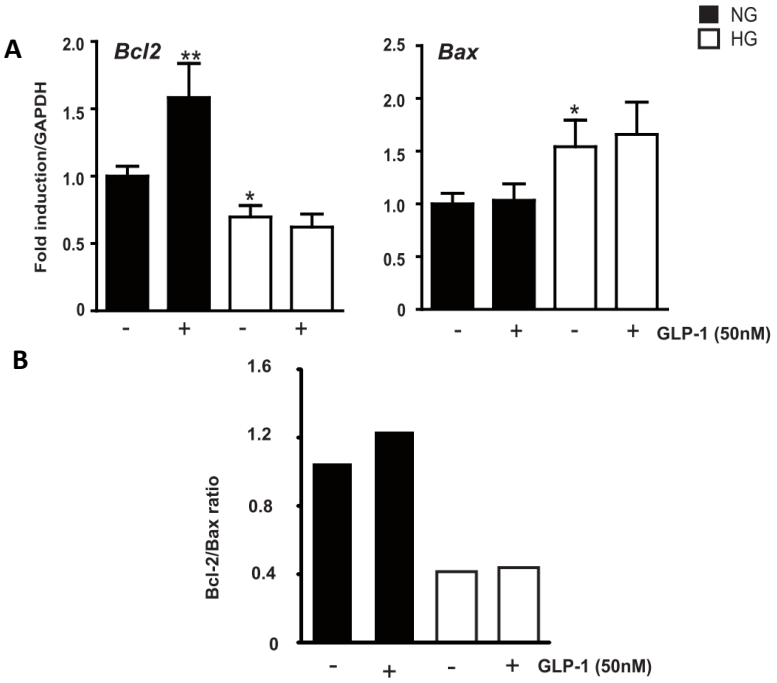


Figure 36: GLP-1 effects on the anti-apoptotic Bcl-2 and the pro-apoptotic Bax gene activation in HUVECs under HG. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG conditions. Cells were treated with 50nM of GLP-1 1 hour before cell harvesting. (A) mRNAs encoding for the indicated genes, Bcl-2 and Bax, were assessed by qRT-PCR and expressed relative to GAPDH. *p<0.05, **p<0.01 vs NG. Bars represent mean±SEM for six independent experiments. (B) The last panel shows the Bcl-2/Bax ratio in HUVECs. Data are expressed as % of control.

To further insight the levels of cell death in our cellular model, both transcript and protein levels of Caspase 3 were assessed. Although no effect on Caspase 3 mRNA expression level was observed after GLP-1 treatment in the two conditions of NG and HG, an increase was observed due to high glucose *per se* (Figure 37A). This was in accordance with the negative effects on proliferation induced by hyperglycaemia. By the other hand, GLP-1 administration was able to reduce protein expression levels of cleaved Caspase 3 only under NG condition but not in the state of chronic HG exposure. Moreover, as observed for gene expression, Caspase 3 protein levels also were increased under high glucose conditions (Figure 37B).

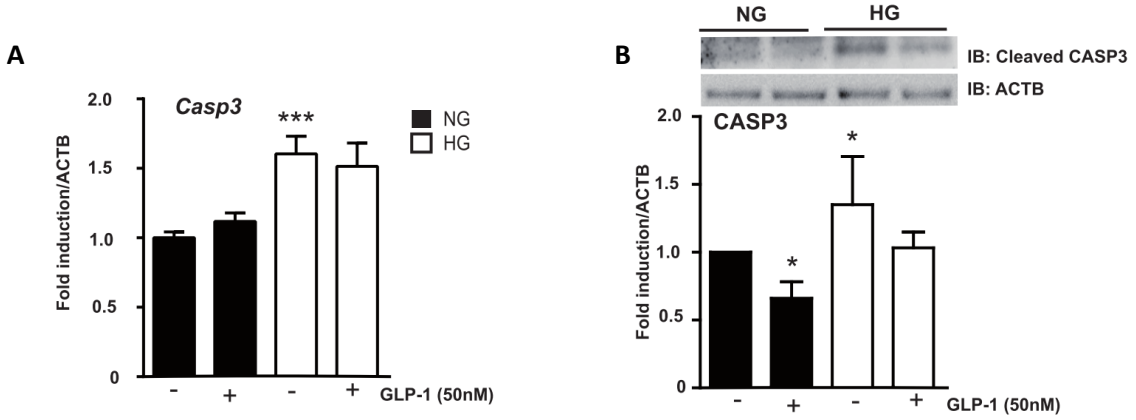


Figure 37: GLP-1 effects on gene expression and protein levels of Caspase 3 in HUVECs under HG. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM GLP-1 during 1 hour before cell harvesting. (A) mRNA encoding for Caspase 3 was assessed by qRT-PCR and expressed relative to ACTB. (B) Protein expression of Caspase 3 was assessed by western blot. The upper panel shows a representative image of different independent experiments. Densitometric values were normalized to ACTB and represented relative to the control cells (NG), normalized to 1. *p<0.05, ***p<0.001 vs NG. Bars represent mean±SEM for six independent experiments.

The expression of the cell cycle related genes Cdkn1a, Cdkn1b and p53 was also studied (Figure 38). While any effect of p53 expression was observed in any of the studied conditions, the expression of Cdkn1a and Cdkn1b transcripts was upregulated under high glucose conditions. However, GLP-1 was not able to exert any effect on the expression of these genes in both NG and HG conditions (Figure 38).

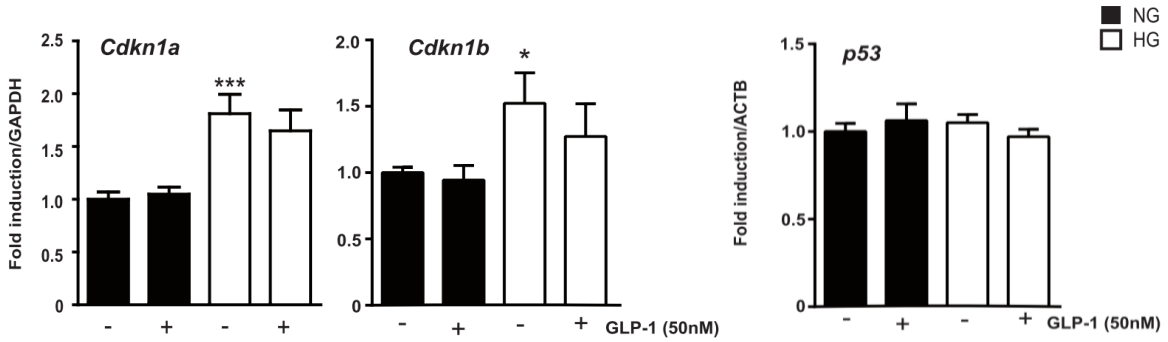


Figure 38: GLP-1 effects on the gene expression of the cell cycle related genes Cdkn1a, Cdkn1b and p53 in HUVECs under HG. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG conditions. Cells were treated with 50nM of GLP-1 1 hour before cell harvesting and mRNAs encoding for the indicated genes, Cdkn1a - Cdkn1b - p53, were assessed by qRT-PCR and expressed relative to GAPDH or ACTB. *p<0.05, ***p<0.001 vs NG. Bars represent mean±SEM for six independent experiments.

In an attempt to assess the effects of chronic high glucose exposure on the functional proliferative capacity of HUVECs, a 5-bromodeoxyuridine (BrdU) Incorporation Assay was performed. We could not observe any significant variation in the proliferation ratio of cells exposed for 21 days to sustained high glucose, although a reduction was expected. Moreover GLP-1 had no capacity to induce any effect on proliferation by itself, neither in NG nor HG in a significant manner, albeit a tendency of GLP-1 to increase proliferation rates in NG was observed (Figure 39A).

In parallel, we also performed an MTT Assay to also analyse the viability of HUVECs cultured under the mentioned conditions. As we expected, a significant reduction in the cell viability of cells cultured in HG was observed. However, this effect was not accompanied by a reduction in cell proliferation. In accordance to the data observed in proliferation gene expression analysis, GLP-1 added in NG had the capacity to induce an increase in HUVECs cell viability, and once again no effect was observed in HG (Figure 39B).

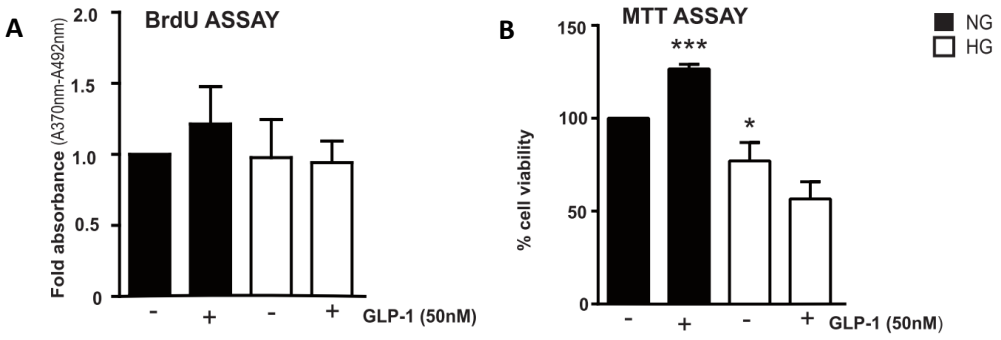


Figure 39: GLP-1 proliferative capacity in HUVECs under HG. HUVECs were exposed for 21 days to NG or HG. Proliferation was examined by measuring BrdU incorporation (A) and by MTT Assay (B). Cells were pre-treated with or without 50nM GLP-1 before BrdU overnight incubation or before MTT 30 minutes incubation. *p<0.05, ***p<0.001 vs NG. Bars represent mean±SEM for four independent experiments.

6. GLP-1 is not able to counteract hyperglycaemia-induced damage on ER function and UPR mechanism in HUVEC cells

The pathological role of ER stress in the pathogenesis of diabetes and hyperglycaemia is increasingly recognized (Cnop, Foufelle, and Velloso 2012). Diabetes induces ER stress in many organs such as the pancreas, liver (Malhi and Kaufman 2011), heart (Li et al. 2010), nervous system (Ozcan et al. 2009), adipose tissue (Tsutsumi et al. 2011) and kidney. Inducers of ER stress that trigger accumulation of unfolded ER proteins include altered nutrient availability, free fatty acids, cytokines, perturbations in calcium transits, oxidative stress, and hypoxia (Cunard and Sharma 2011).

The ability of cells to respond to perturbations in ER function is critical for their survival, and chronic or unresolved ER stress can lead to apoptosis (Gorrini, Harris, and Mak 2013; Tabas and Ron 2011). As detailed in the *Introduction* Section, ER stress is modulated by the Unfolded Protein Response that includes three upstream signalling proteins, PERK, IRE1α and ATF6, which, once activated, begin a cascade of corrective actions in order to re-establish the ER homeostasis (Gorrini, Harris, and Mak 2013).

We assessed the expression of ER stress response-related transcripts under NG and HG conditions. As reported before (Schisano et al. 2012), we confirmed that, when HUVECs were cultured in the high glucose state, the expression of some ER stress markers and UPR was increased (Figure 40). Particularly, we observed an increase in the gene expression of the chaperon Bip and this result was in accordance with an increased UPR activity, which has the objective to reduce the number of unfolded proteins by increasing the concentration of chaperones in the ER lumen (Mori 2009).

We also checked the gene expression of the markers of the three different signalling cascades described for UPR: Perk, Ire1a and Atf6 in HUVECs cultured in NG and HG conditions.

Related to the PERK pathway, we assessed the mRNA expression of Perk, Atf4, Chop, Bim and Erol1a. Acute treatment of GLP-1 under normal glucose condition did not affect mRNA levels of the studied genes Perk, Atf4, Bim and Erol1a. The only exception was Chop, for which an unexpected increase in its gene expression levels after GLP-1 addition was observed. Furthermore, as expected, all these transcripts were increased under sustained HG and this result was in accordance with published data based on the demonstration of hyperglycaemia-induced damage in ER homeostasis (Li et al. 2010; Malhi and Kaufman 2011; Ozcan et al. 2009; Tsutsumi et al. 2011). However, when HUVECs exposed to high glucose were treated with GLP-1, the incretin hormone once more was not able to induce any effect and counteract the negative consequences of sustained HG (Figure 40).

As concern as IRE1α pathway, we tested the gene expression of Ire1α, Traf2 and Xbp in its two isoforms spliced and unspliced. As observed for the PERK pathway, under NG state we did not observe any effect due to GLP-1 treatment in mRNA levels of all the studied genes. Again, as expected, 21 days of high glucose exposure induced an increase in ER stress markers related also with IRE1α pathway, although the response of these genes to HG state was more moderate in general. Particularly Traf2 transcript levels, contrary to what happened for Ire1α and Xbpsp, slightly increased but not in a statistical manner ($p = 0.08$). Once again, GLP-1 was not able to exert any effect in the expression of these genes when cells were exposes to HG (Figure 40).

Finally, related to the ATF6 pathway, we evaluated gene expression levels of the ER stress sensor Atf6. As in generally observed for the other transcripts, administration of GLP-1 under normal glucose condition did not affect Atf6 mRNA levels. Contrary to what happened for the other two signalling cascades, sustained high glucose exposure did not induce an increase in gene expression

levels of Atf6, and once again GLP-1 treatment under HG conditions was not able to exert any effect (Figure 40).

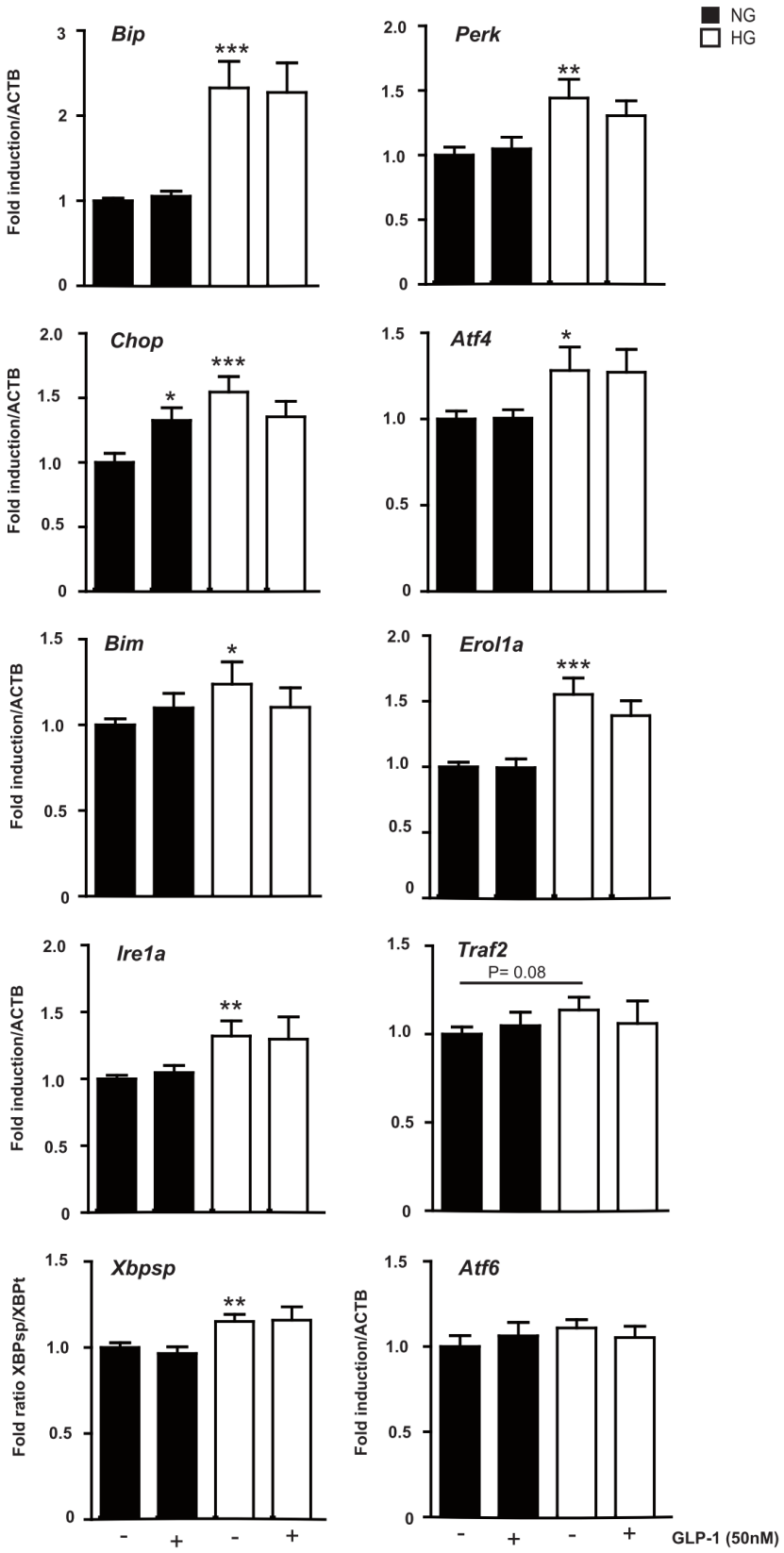


Figure 40: GLP-1 effects on ER stress and UPR markers in HUVECs under HG. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG. Cells were treated with 50nM GLP-1 1 hour before cell harvesting and mRNAs encoding for the indicated genes were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05, **p<0.01 and ***p<0.001 vs NG. Bars represent mean±SEM for six independent experiments.

7. A possible link between GLP-1R and PKCβ in HUVECs exposed to high glucose

Hyperglycaemia is a crucial etiological factor for the development of macrovascular and microvascular complications in diabetes (Giacco and Brownlee 2010; Inoguchi et al. 1994). As exposed in the *Introduction* Section, among the multiple hypotheses that have been proposed to link the adverse effects of hyperglycaemia with vascular complications in diabetes (Giacco and Brownlee 2010), the activation of the DAG-PKC pathway has an important role (Nishizuka 1992).

The activity of PKC has been reported to regulate permeability, contractility, hormone receptor turnover and proliferation of vascular endothelium (Craven, Davidson, and DeRubertis 1990; Shiba et al. 1993). All of these properties have been shown to be abnormal in diabetes (Inoguchi et al. 1994). As detailed in the *Introduction* Section, high glucose concentrations as well as non-esterified fatty acids cause increased DAG concentrations and this is associated with the endothelial activation of some PKC isoforms, including the PKCβ1 and -β2, in several tissues and cell culture systems, for example in renal glomeruli from diabetic animals (Craven and DeRubertis 1989) and in cultured aortic endothelial cells and vascular smooth muscle cells (Inoguchi et al. 1994).

With the aim to assess the effects of hyperglycaemia on PKC pathway in our *in vitro* model of HUVECs treated during 21 days with sustained high glucose concentrations, we assessed PKC protein levels and we could observe an increase of PKC isoform β1 related to the control NG state (Figure 41).

In a work conducted on human differentiated muscle satellite cells, Green et al. observed that the gene expression of GLP-1R was decreased in high glucose conditions (Green et al. 2012). Also Mima and coworkers observed that GLP1-R gene expression levels as well as protein levels were decreased in diabetic mice compared with non-diabetic mice (Mima et al. 2012). Also in HUVEC cells, we could observe that 21 days chronic high glucose concentrations induced a decrease of GLP-1 protein levels (Figure 41).

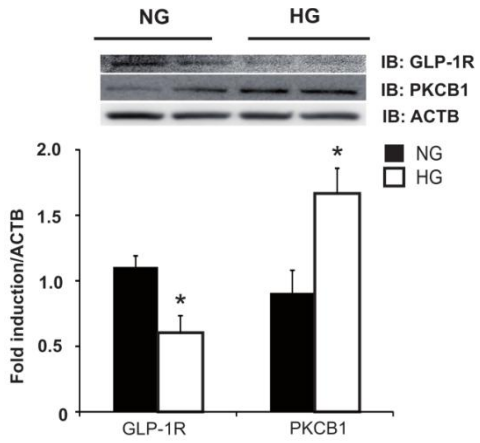


Figure 41: A possible link between GLP-1R and PKCβ in HUVECs exposed to HG. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1, as indicated at the end of the experiment. Whole cell lysates were prepared and the expression of GLP-1R and PKCβ1 was assessed by western blot. The panels show representative images of different independent experiments. Densitometric values were normalized to ACTB and represented relative to the control cells (NG), normalized to 1. *p<0.05 vs NG. Bars represent mean±SEM from six to eight independent experiments.

In the same study conducted by Mima et al., in order to insight the mechanisms of this reduction, the effects of PKCβ activation on GLP-1R expression in glomerular endothelial cells (RGECs) were analysed (Mima et al. 2012). By activating PKCβ with the phorbol ester PMA, Mima and coworkers observed that protein expression of GLP-1R was decreased without any alteration of its mRNA levels. Moreover, they found that the administration of Ruboxistaurin, the specific inhibitor of PKCβ, reversed the inhibitory effects of PMA, increasing GLP-1R protein levels. Thus, they demonstrated that PKCβ activation induced by hyperglycaemia can inhibit GLP-1 protective effects on endothelium by reducing its receptors in RGECs.

According to Mima et al. (Mima et al. 2012), we proposed that the increased levels of PKCβ, due to high glucose concentrations, were involved in GLP-1R decreased levels, which, in turn, lead to the loose of GLP-1 protective actions in HUVEC cells. So we hypothesized that blocking PKCβ we could recover GLP-1R levels and consequently GLP-1 actions.

To this aim, the specific inhibitor of the PKCβ isoform, Ruboxistaurin, was used. First of all, we wonder to confirm the correct inhibition of PKCβ, so gene expression in parallel to protein levels of the isoform β1 were assessed (Figures 42 - 43). As detailed in *Materials and Methods* Section, HUVECs were cultured during 21 days under HG and, before their harvesting, they were treated during 1 hour with or without 0.5μM Ruboxistaurin.

As expected, the levels of PKCβ transcript decreased when its inhibitor was added under HG condition (Figure 42), as well as it happened with PKCβ protein levels (in the panel below in the Figure 43 a representative image was reported).

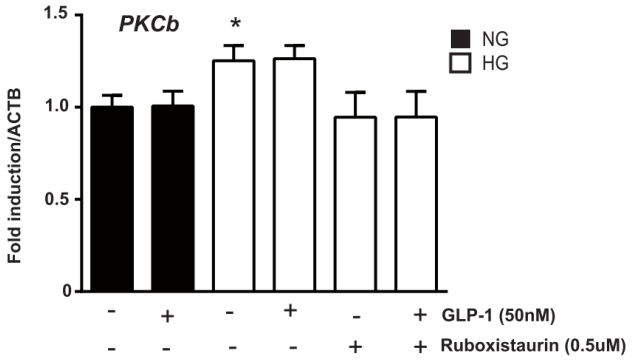


Figure 42: GLP-1 effects on PKCβ gene expression in HUVECs under HG. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1 and/or 0.5μM Ruboxistaurin, as indicated at the end of the experiment. Total cellular RNA was isolated and mRNA encoding for PKCβ was assessed by qRT-PCR and expressed relative to ACTB. *p<0.05 vs NG. Bars represent mean±SEM from six to eight independent experiments.

Once PKCβ inhibition with Ruboxistaurin was confirmed, next step was to assess if protein levels of GLP-1R were modulated by PKCβ under high glucose conditions, as we speculated. Noteworthy, we could observe that when HUVEC cells were exposed to prolonged high glucose concentrations, GLP-1R levels slightly increased after Ruboxistaurin administration, thus indicating a possible molecular connection between GLP-1 and PKCβ also in our cellular model. Interestingly, the addition of GLP-1 in combination with Ruboxistaurin in HG enhanced the effects of the inhibitor *per se*, compared to the condition in which HUVECs were only treated with GLP-1 (Figure 43).

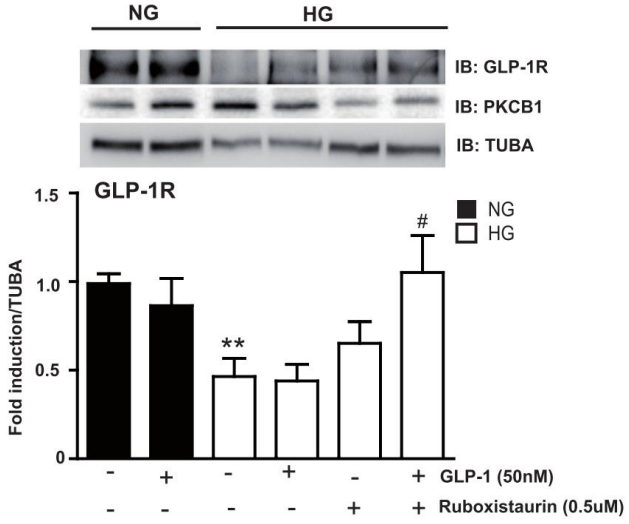


Figure 43: A possible link between GLP-1R and PKCβ in HUVECs exposed to HG. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1 and/or 0.5μM Ruboxistaurin, as indicated at the end of the experiment. Whole cell lysates were prepared and the expression of GLP-1R and PKCβ1 was assessed by western blot. The panels show representative images of different independent experiments. Densitometric values were normalized to TUBA and represented relative to the control cells (NG), normalized to 1. **p<0.01 vs NG and #p<0.05 vs HG. Bars represent mean±SEM from six to eight independent experiments.

8. GLP-1R trafficking is affected by hyperglycaemia and recovered inhibiting PKCβ

GLP-1R levels depend on a continuous trafficking in the cell, as it has been described for other GPCRs family members (Drake, Shenoy, and Lefkowitz 2006). After the binding with GLP-1 or one of its analogues, the receptor is internalized and undergoes to a counterbalance between recycling and degradation: GLP-1R may be either recycled to the plasma membrane or sorted to endosome pathway for its degradation (Drake, Shenoy et al. 2006). G protein receptor kinases (GRKs) and β-arrestin proteins have an active role on this process. Cessation of the signalling pathway occurs via recruitment of these modulatory proteins, the β-arrestins, to the cytoplasmic surface of the receptor, a process that is enhanced by the receptor phosphorylation that is promoted by GRKs (Lefkowitz and Shenoy 2005). β-arrestin binding physically prevents receptor-Gs interaction, leading to desensitization of receptor-mediated activation of Gs. β-arrestin binding further promotes the subsequent cytosol to membrane translocation of clathrin and adaptor protein AP-2, resulting in receptor endocytosis in clathrin-coated vesicles (Shenoy et al. 2006).

On the basis of these observations, we wonder asses if GLP-1R trafficking in our *in vitro* cellular model was affected under hyperglycaemia conditions.

With the aim to insight the GLP-1R signalling under hyperglycaemia in the endothelium, we assessed the gene expression of the two isoforms of β-arrestins, both expressed in HUVECs. Exposure of HUVECs to 21 days HG revealed that the mRNA levels of β-arrestin isoform 1 (Barr1) was significantly reduced, while the expression of β-arrestin isoform 2 (Barr2) was not affected by chronic hyperglycaemia (Figure 44). Intriguingly, the expression of Barr1 was recovered when PKCβ was specifically inhibited with Ruboxistaurin, indicating a possible role of PKCβ in the regulation of GLP-1R trafficking (Esseltine, Ribeiro, and Ferguson 2012).

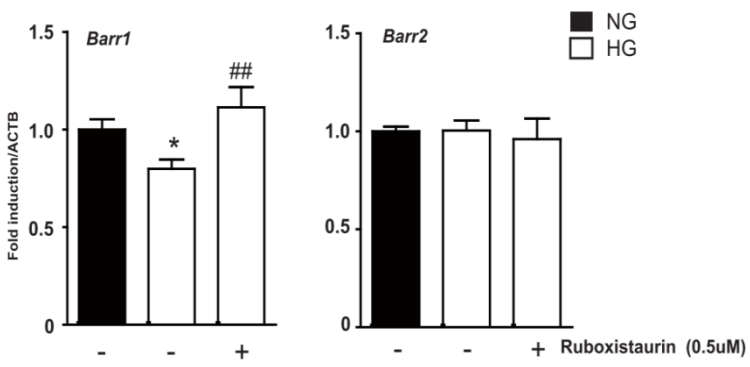


Figure 44: Gene expression of Barr1 was recovered after PKC inhibition on HUVECs under HG. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG. Cells were treated with 0.5μM Ruboxistaurin 1 hour before cell harvesting. After RNA extraction, mRNAs encoding for the indicated genes, Barr1 and Barr2, were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05 vs NG and ##p<0.01 vs HG. Bars represent mean±SEM for six independent experiments.

In order to confirm the disruption of GLP-1R trafficking under hyperglycaemia, we assessed by immunocytochemistry its protein localization in HUVECs. We observed that after GLP-1 addition under normal glucose conditions, it seems that there was an internalization of the receptor with a like-perinuclear disposition (Figure 45). Such receptor expression localization was lost when HUVECs were exposed for 21 days to chronic high glucose and the administration of GLP-1 in this condition was not able to recover the receptor distribution observed in the normal condition. However, further studies need to be addressed, for example with the usage of local-specific markers that could help us to confirm this data.

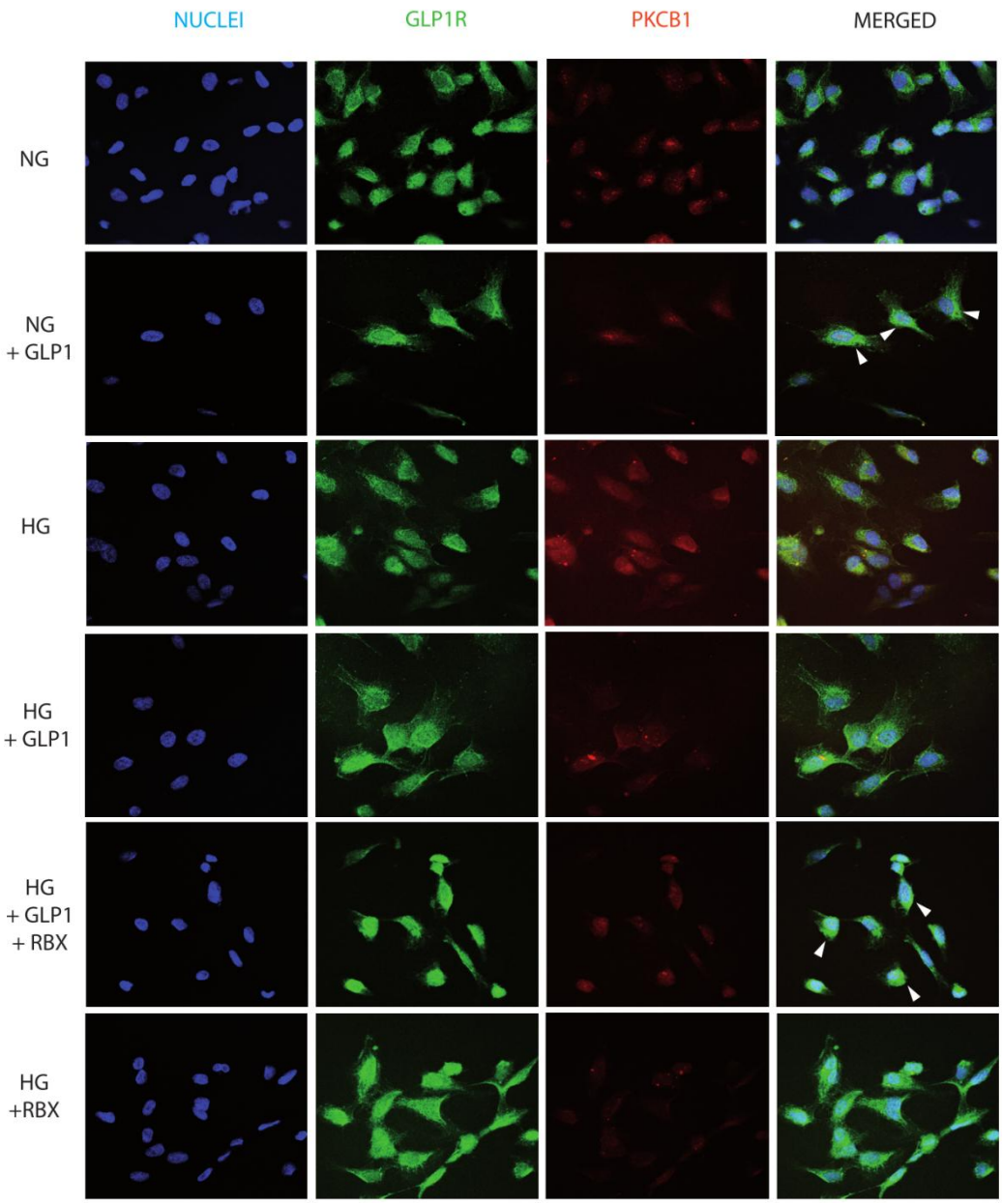


Figure 45: GLP-1 effects on localization of GLP-1R in HUVECs under HG. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1 and/or 0.5µM Ruboxistaurin, as indicated. At the end of the experiment, the nucleus was immunolabeled with Hoechst (blue fluorescence), GLP-1R was immunolabeled with FITC (green fluorescence) and PKCβ1 was immunolabeled with TRITC (red fluorescence). Slides were observed under the confocal microscope using identical parameters (scale bar=100 µm).

As it was expected, in accordance with this phenomenon, we had observed that the levels of GLP-1R were lower, while PKCβ levels were higher in HUVECs exposed to 21 days HG, as described above (see Figure 41). It is noteworthy that, when we administrated the PKCβ inhibitor, apart from a qualitative increase in GLP-1R levels and an expected decrease in PKCβ levels, in this condition GLP-1 addition could promote a perinuclear localization of its receptor recovering what we observed under NG (Figure 45).

9. Blockage of high glucose induced-PKCβ activation partially recovers the GLP-1 protective actions in HUVECs

GLP-1R signalling was completely disrupted when HUVEC cells were exposed to prolonged high glucose conditions. As consequence, GLP-1-induced antioxidant response was inhibited as well as GLP-1 pro-survival properties and its positive effects on ER function where abolished due to HG. As Mima et al. observed for glomerular endothelial cells (Mima et al. 2012), we hypothesized that the loose of all protective actions of this incretin hormone on HUVEC cells could be due to hypergycaemia-induced PKCβ activation.

To further investigate the role of PKCβ in the GLP-1R signalling pathway and to study if the positive expected actions of this incretin were recovered by blocking PKCβ activation, we analysed all the studied pathways related to oxidative stress, ROS production, antioxidant defence system, proliferation, apoptosis and ER stress, including in our experiments the specific inhibitor of PKCβ, Ruboxistaurin. To this aim, as detailed in the *Materials and Methods* Section, HUVECs were cultured for 21 days under NG or HG and, during the last day, they were treated for 1 hour with or without 50nM GLP-1 and/or 0.5μM Ruboxistaurin. Total cellular RNA was isolated and mRNAs encoding for the different genes were assessed by qRT-PCR.

In general, we could observe that the addition of GLP-1 in combination with Ruboxistaurin under high glucose conditions enhanced the effects of the inhibitor *per se*, although the recovery of GLP-1 actions was not a global effect, as not all studied genes and pathways had the expected positive response after PKCβ inhibition.

9.1. GLP-1 signalling is recovered when PKCβ is specifically blocked

Regarding the downstream pathway involving cAMP and PKA activation, we observed that the mRNA transcripts of catalytic subunit PRKACB and the regulatory subunits PRKAR1A, PRKAR1B and PRKAR2B were increased by hyperglycaemia. As we exposed in the second paragraph of this Section, we observed that GLP-1 addition to HUVECs exposed to normal glucose levels decreased in a significant statistical manner the gene expression levels of PRKACA, PRKAR1B and PRKAR2A subunits, whereas the incretin hormone was not able to promote any effect under high glucose conditions (see Figure 27).

However, when GLP-1 was added in combination with Ruboxistaurin in HG, we observed a significant decrease in mRNA transcripts of PRKACA, PRKAR1A and PRKAR1B, and a moderate but not significant reduction in PRKACB (Figure 46). This decrease in some of the different catalytic and regulatory subunits of PKA was observed also with the addition of the PKCβ inhibitor alone, but we could demonstrate that only in the experimental condition in which Ruboxistaurin was used in combination with GLP-1, the decrease in the gene expression levels of these PKA subunits was statistically significant.

Regarding the regulatory subunit PRKAR2A, we could not observe any change in its mRNA transcript in both the experimental conditions of Ruboxistaurin alone and Ruboxistaurin + GLP-1. Finally, for the R subunit PRKAR2B, GLP-1 administration could not enhance the decrease in gene expression than the PKCβ inhibitor did *per se* (Figure 46).

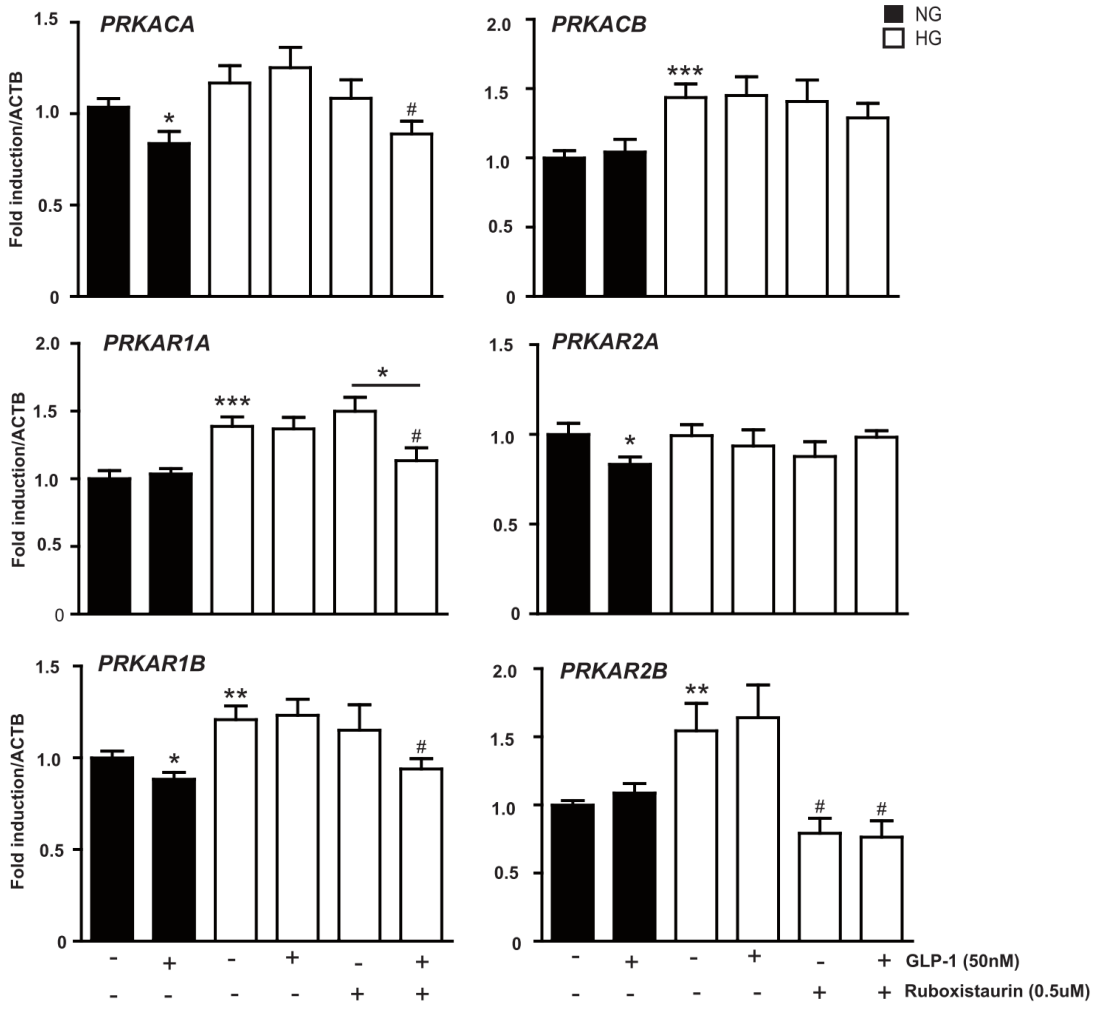
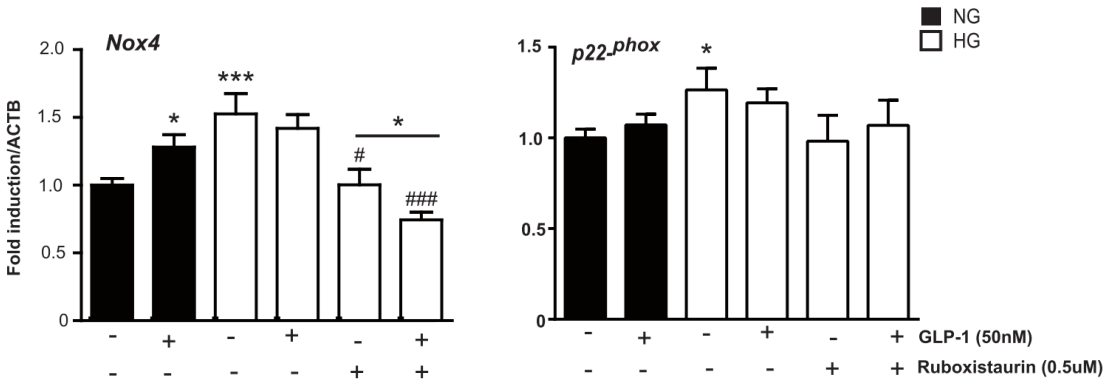


Figure 46: PKA subunits gene expression was differently modulated by GLP-1 and Ruboxistaurin in HUVECs under HG. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM of GLP-1 and/or 0.5µM Ruboxistaurin 1 hour before cell harvesting. After mRNA extraction, PKA subunits transcripts were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05, **p<0.01 and ***p<0.001 vs NG and #p<0.05 vs HG. Bars represent mean±SEM for six independent experiments.

9.2. GLP-1 antioxidant properties are recovered when PKCβ is blocked

As concern as the ROS inducer NAD(P)H oxidase, we confirmed that gene expression levels of the membrane-bound subunit Nox4 were increased under chronic hyperglycaemia, as expected, and also unexpectedly after GLP-1 treatment under NG condition, while the incretin hormone was not able to produce any effect HG state. Moreover we could observe that mRNA transcript was reduced when, under chronic high glucose, PKCβ was inhibited with Ruboxistaurin and this reduction was significantly enhanced when GLP-1 was added together with Ruboxistaurin (Figure 47).

On the contrary, regarding the other membrane-bound subunit p22-^{phox}, which gene expression was incremented under high glucose conditions, but did not respond to GLP-1 action in both states of NG and HG, we did not observe any change after treatment with Ruboxistaurin with or without GLP-1: in details, Ruboxistaurin *per se* induced a slight reduction of p22-^{phox} mRNA transcript although it was not statistical significant, and GLP-1 was not able to enhance such decrease (Figure 47).



Figures 47: Gene expression levels of NAD(P)H subunits were partially recovered after GLP-1 plus Ruboxistaurin treatment in HUVECs under HG. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1 and/or 0.5μM Ruboxistaurin, as indicated at the end of the experiment. Total cellular RNA was isolated from cells and mRNAs encoding for the indicated genes, Nox4 and p22-^{phox} were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05 and ***p<0.001 vs NG, #p<0.05 and ###p<0.001 vs HG. Bars represent mean±SEM from six to eight independent experiments.

Strikingly, ROS production, which was increased due to chronic high glucose, was reduced only with the simultaneous treatment of GLP-1 and Ruboxistaurin (Figure 48). In other terms, GLP-1 or Ruboxistaurin alone were not able to counteract ROS increase under HG state, and the incretin hormone could exert its positive effects only when PKCβ increase due to hyperglycaemia was inhibited.

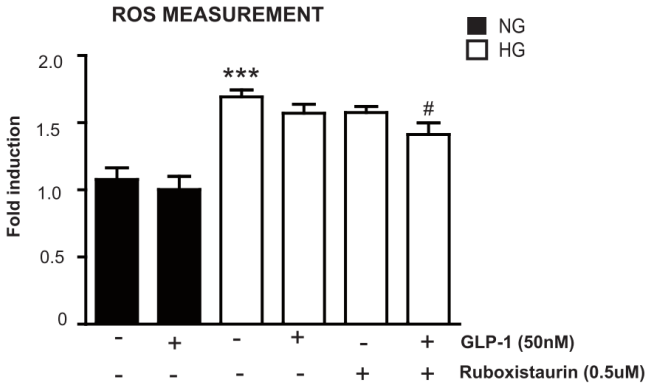


Figure 48: ROS levels in HG were decreased after GLP-1 plus Ruboxistaurin treatment. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1 and/or 0.5μM Ruboxistaurin as indicated at the end of the experiment. ROS production was stained by 20μM H_2DCFDA for 30 minutes and its oxidation product (DCF) fluorescence indicated ROS formation. ***p<0.001 vs NG and #<0.05 vs HG. Bars represent mean±SEM from six to eight independent experiments.

On the other hand, regarding the antioxidant defence system, we studied the expression levels of the ARE-linked genes activated by NRF2 action. These target genes also recovered the GLP-1 effects in HUVECs cultured under HG. Particularly, the expression of Hmox-1 was clearly decreased in the presence of PKCβ inhibitor alone; anyhow, GLP-1 treatment in combination with Ruboxistaurin was able to induce the gene expression of Hmox-1. Although, contrary to our expectations, such increase in Hmox-1 was not so important to recover and improve the positive GLP-1 effects in HUVECs exposed to sustained high glucose, as indeed observed for the NG state, it resulted statistically significant respect to the condition in which the cells where treated under HG conditions with the inhibitor alone (Figure 49).

The same recovery was not observed for the Nqo-1 gene expression. mRNA transcript was induced after GLP-1 treatment under NG condition, but had no response to incretin action under HG state. Also we could observe that gene expression levels of this second Nrf2 target were slightly reduced when, under chronic high glucose, PKCβ was inhibited with Ruboxistaurin but not in a

significant statistical manner, and this reduction was not enhanced when GLP-1 was added together with Ruboxistaurin (Figure 49).

The thioredoxin-related genes, Txnip and Txnrd, are other ARE-linked genes activated by NRF2 pathway. Their gene expression was increased in presence of HG, and GLP-1 treatment did not induce any effect in both conditions of NG and HG. Interestingly, their expression was significantly reduced when GLP-1 was added in combination with the PKCβ inhibitor and this reduction reached levels higher that Ruboxistaurin did *per se* (Figure 49). Particularly, administration of PKCβ inhibitor under chronic high glucose reduced Txnip gene expression levels but not in a significant statistical manner, and only the addition of GLP-1 together with Ruboxistaurin could decrease its mRNA transcript at lower levels that the inhibitor did *per se*, resulting statistically significant compared to the condition in which HUVECs were only exposed to sustained HG (Figure 49).

Regarding the effects of GLP-1 and/or Ruboxistaurin on the gene expression levels of Txnrd, surprisingly, we could observe that this gene was significantly reduced when under chronic high glucose PKCβ was inhibited with Ruboxistaurin, and this reduction was enhanced when GLP-1 was added together with Ruboxistaurin (Figure 49).

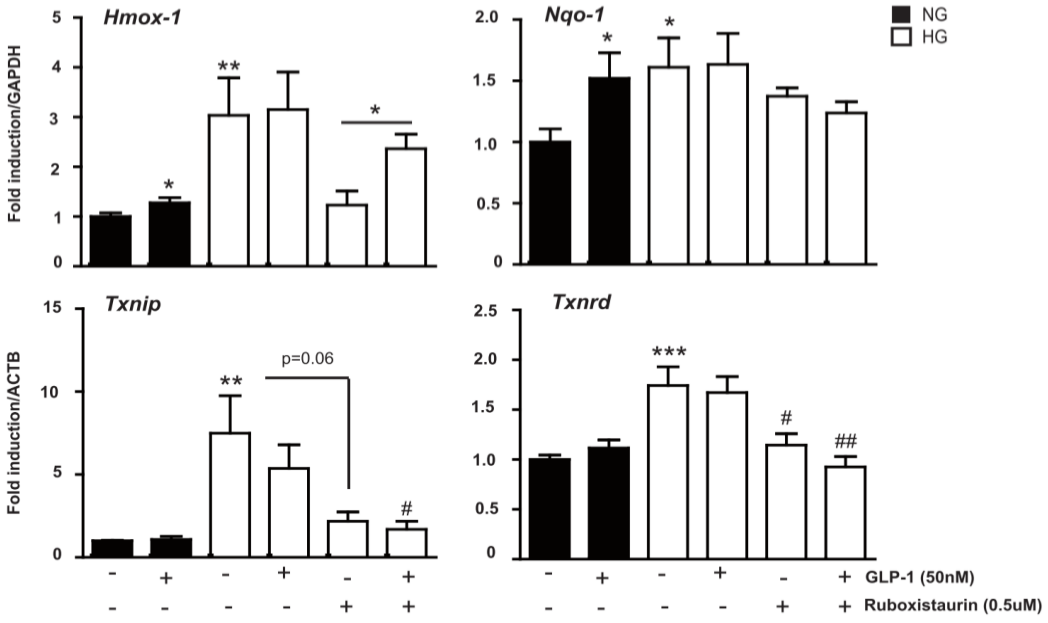


Figure 49: PKCβ increased levels in HG is responsible of GLP-1 antioxidant properties lose. HUVECs were cultured during 21 days under NG or HG and were treated with or without 50nM GLP-1 and/or 0.5µM Ruboxistaurin, as indicated at the end of the experiment. Total cellular RNA was isolated from cells and mRNAs encoding for the indicated genes were assessed by qRT-PCR and expressed relative to GAPDH or ACTB. *p<0.05, **p<0.01 and ***p<0.001 vs NG, #p<0.05 and ##p<0.01 vs HG. Bars represent mean±SEM from six to eight independent experiments.

As detailed in the fourth Section of the *Results*, Foxo transcription factors were differently regulated by GLP-1 under NG: while Foxo1 and Foxo4 were transcriptionally induced, Foxo3a was downregulated. However any effect of GLP-1 was observed in HG (see Figure 34).

Under HG condition, when we added PKCβ alone, we observed an increase in gene expression of Foxo1 and Foxo3a transcripts, whereas it did not exert any effect on Foxo4 mRNA regulation. When GLP-1 was added to Ruboxistaurin in HG, we could not observe any effect of this incretin hormone on Foxo1 and Foxo3a, whereas the combination of GLP-1 with Ruboxistaurin could increase in a significant statistical manner Foxo4 gene expression levels, despite any effect was observed by GLP-1 or Ruboxistaurin added alone (Figure 50).

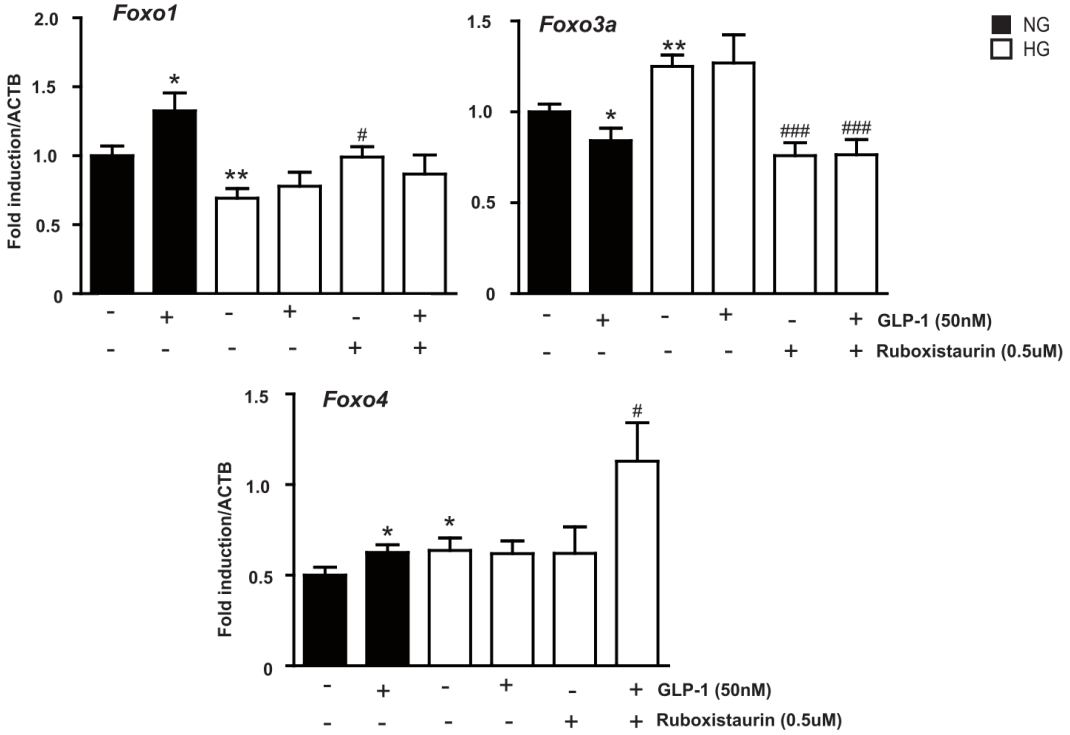


Figure 50: GLP-1 effects on Foxo gene expression after PKCβ inhibition in HUVECs under HG. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM of GLP-1 and/or 0.5μM Ruboxistaurin 1 hour before cell harvesting. mRNAs encoding for the indicated genes were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05 and **p<0.01 vs NG, #p<0.05 and ###p<0.001 vs HG. Bars represent mean±SEM for six independent experiments.

In parallel, we tested mRNA expression of the scavengers regulated by Foxos: Sod1, Sod2 and Cat. None of them were regulated by GLP-1 under NG. Also, both Sod1 and Sod2 were increased by hyperglycaemia, as expected, and GLP-1 was not able to exert any positive effect on the regulation of these genes under high glucose conditions (see Figure 35).

In this case, when PKCβ inhibitor was added alone, any effect was observed, however when PKCβ was blocked and GLP-1 was added under HG state, the expression of Sod1 and Cat was significantly increased, respect to the effects observed for Ruboxistaurin treatment alone (Figure 51). Such increase followed the same Foxo4 expression profile, suggesting a possible regulation of Sod1 and Cat by this FOXO family member. No effect was observed related to regulation of Sod2 gene expression levels in both conditions in which GLP-1 and Ruboxistaurin were administrated alone or in combination under HG conditions (Figure 51).

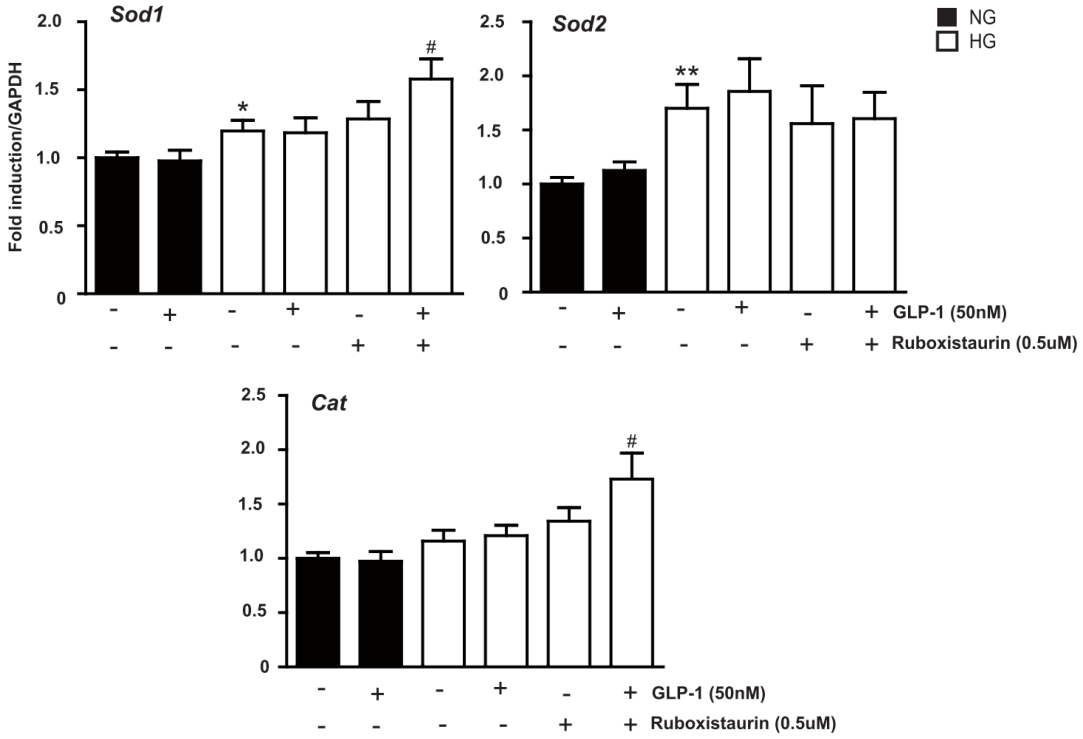


Figure 51: GLP-1 effects on gene expression of the scavengers regulated by Foxos after PKCβ inhibition in HUVECs under HG. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM of GLP-1 and/or 0.5μM Ruboxistaurin 1 hour before cell harvesting. mRNAs encoding for the indicated genes were assessed by qRT-PCR and expressed relative to GADPH. *p<0.05 and **p<0.01 vs NG, #p<0.05 vs HG. Bars represent mean±SEM for six independent experiments.

9.3. GLP-1 proliferative capacities were recovered after PKCβ blockage

As concern as the pro-survival properties, we demonstrated that GLP-1 was not able to counteract the negative effects of sustained high glucose exposure (see Figures 36 – 37 – 38 – 39). On the other hand, under HG conditions, GLP-1 was able to recover its proliferative capacities when PKCβ increase was blocked.

In the case of the anti-apoptotic Bcl-2 mRNA transcript, we observed an increase in its expression when Ruboxistaurin was added to HG medium, but only when GLP-1 was added in combination with the inhibitor, a statistical significant increase in Bcl-2 gene expression levels was reached (Figure 52). Contrary, GLP-1 was able to reduce the pro-apoptotic Bax mRNA expression when HUVECs exposed to high glucose conditions were treated with Ruboxistaurin. The inhibitor *per se* slightly decreased Bax gene expression, but it did not exert any statistically significant effect (Figure 52).

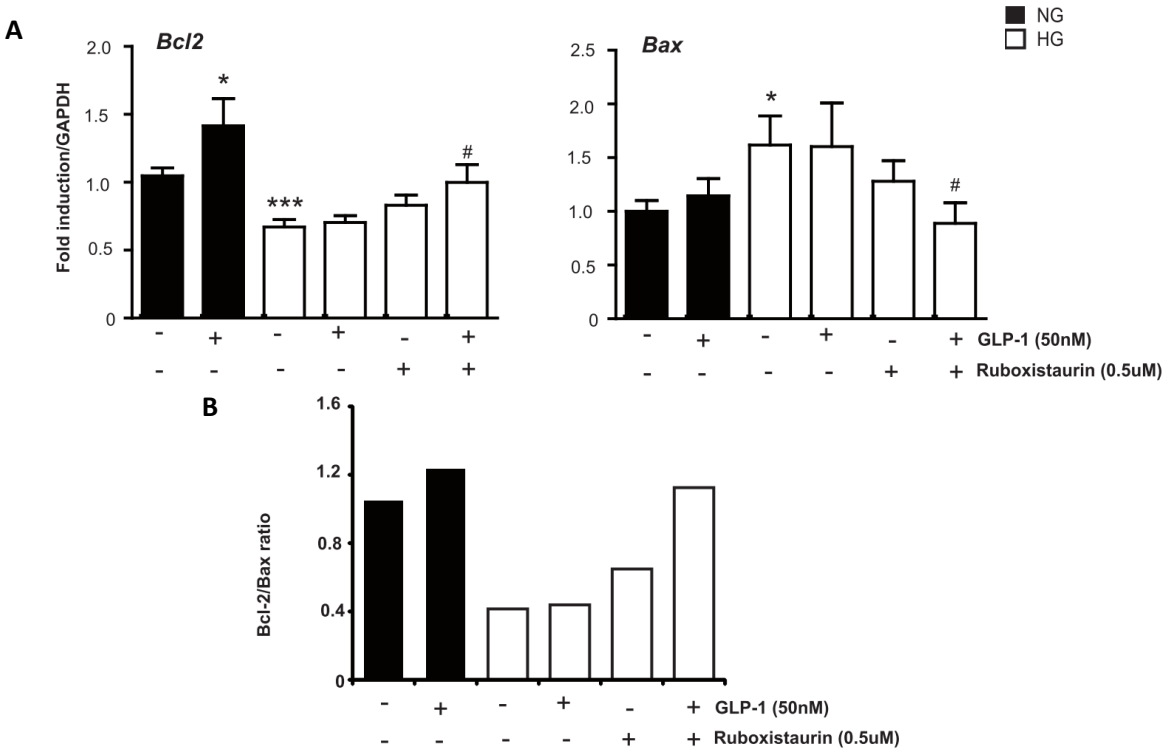


Figure 52: The anti-apoptotic Bcl-2 and the pro-apoptotic Bax gene expression was recovered after PKCβ inhibition in HUVECs under HG. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG conditions. Cells were treated with 50nM of GLP-1 and/or 0.5μM Ruboxistaurin 1 hour before cell harvesting and (A) mRNAs encoding for the indicated genes, Bcl-2 and Bax, were assessed by qRT-PCR and expressed relative to GAPDH. (B)The last panel shows the Bcl-2/Bax ratio in HUVECs. Data are expressed as % of control. *p<0.05, **p<0.01 vs NG and #p<0.05 vs HG. Bars represent mean±SEM for six independent experiments.

Accordingly to these results, the expression of Cdkn1a was also significantly reduced after the administration of GLP-1 plus Ruboxistaurin under HG state (Figure 53), while Ruboxistaurin *per se* was not able to revert the negative phenotype induced by hyperglycaemia, indicating at least a decrease in pro-apoptotic phenotype of HUVECs and an increase in proliferation when PKCβ activity was inhibited. As concern as Cdkn1b, the administration of GLP-1 plus Ruboxistaurin did not enhance the decrease that Ruboxistaurin did *per se* when HUVECs were exposed to chronic high glucose (Figure 53).

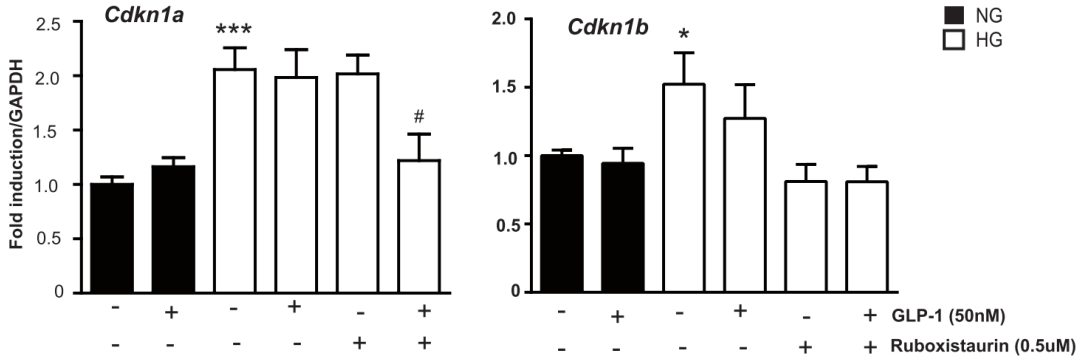


Figure 53: The gene expression of the cell cycle related gene Cdkn1a was recovered after PKCβ inhibition in HUVECs under HG. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG conditions. Cells were treated with 50nM of GLP-1 and/or 0.5μM Ruboxistaurin 1 hour before cell harvesting and mRNAs encoding for the indicated genes, Cdkn1a and Cdkn1b, were assessed by qRT-PCR and expressed relative to GAPDH. *p<0.05, ***p<0.001 vs NG and #p<0.05 vs HG. Bars represent mean±SEM for six independent experiments.

Even if any effect of GLP-1 was observed in the expression of p53 neither in NG or HG, and HG also did not exert any increase in p53 gene expression, as we on the contrary expected, when GLP-1 was added in combination with Ruboxistaurin in the condition of high glucose, a decrease in p53 transcript levels was observed (Figure 54).

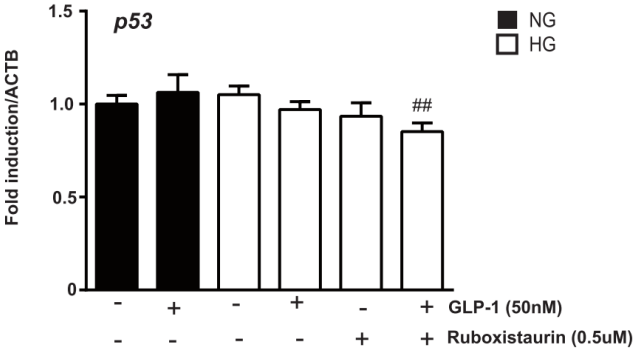


Figure 54: GLP-1 effects on the gene expression of the cell cycle related gene p53 after PKCβ inhibition in HUVECs under HG. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG conditions. Cells were treated with 50nM of GLP-1 and/or 0.5μM Ruboxistaurin 1 hour before cell harvesting and mRNA encoding for p53 was assessed by qRT-PCR and expressed relative to ACTB. ##p<0.01 vs HG. Bars represent mean±SEM for six independent experiments.

In order to assess if GLP-1 was able to reduce Caspase 3 levels in HG when PKCβ induction due to hyperglycaemia was blocked, we checked mRNA and protein expression in presence of Ruboxistaurin. As it was previously described, Caspase 3 mRNA was increased in HUVECs cultured under HG conditions, but any effect of GLP-1 could be observed even with the addition of Ruboxistaurin in HG (Figure 55A). Regarding to Caspase 3 protein levels, while GLP-1 was able to reduce its levels under NG in a significant manner, this reduction observed in HG was not statistically significant and the levels of this pro-apoptotic protein did not decrease when Ruboxistaurin alone was added to the medium. According with the results of gene expression of Bcl-2, Bax and Cdkn1a, as expected, Caspase 3 protein levels were reduced in a significant statistical manner only when HUVEC cells were treated with GLP-1 in combination of the PKCβ inhibitor (Figure 55B).

Thus, GLP-1 *per se* was not able to reverse the negative phenotype induced by hyperglycaemia in HUVEC cells, but when PKCβ expression induced by HG was inhibited with the administration of Ruboxistaurin, we could observe a decrease in pro-apoptotic phenotype and an increase in proliferative capacities.

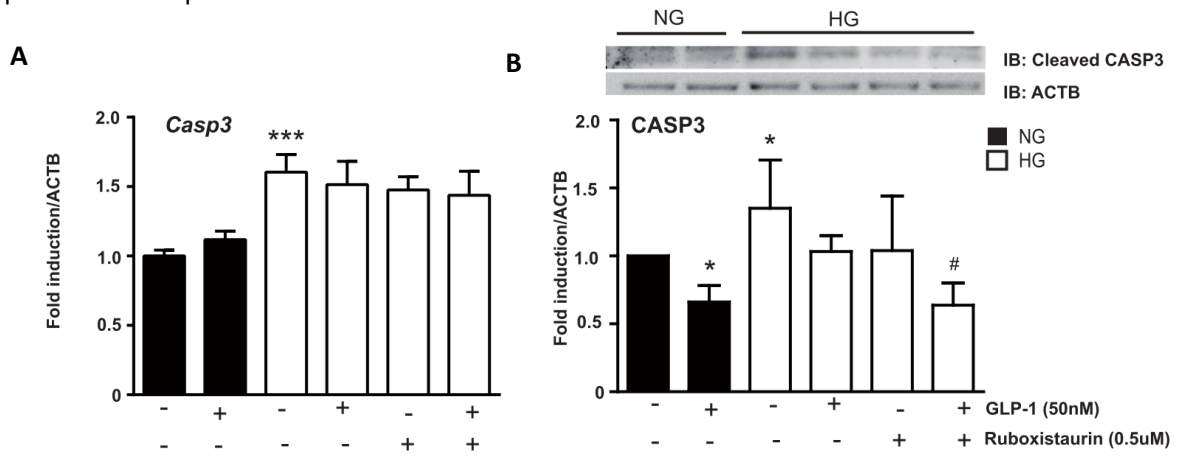


Figure 55: GLP-1 effects on gene expression and protein levels of Caspase 3 in HUVECs under HG treated with Ruboxistaurin. HUVECs, maintained 21 days on culture under NG or HG, were treated with 50nM GLP-1 and/or 0.5μM Ruboxistaurin during 1 hour before cell harvesting. (A) Total cellular RNA was isolated and mRNA encoding for Caspase 3 was assessed by qRT-PCR and expressed relative to ACTB. (B) As for RNA, also proteins levels were analysed. Whole cell lysates were prepared and the expression of Caspase 3 was assessed by western blot. The upper panel shows a representative image of different independent experiments. Densitometric values were normalized to ACTB and represented relative to the control cells (NG), normalized to 1. *p<0.05, ***p<0.001 vs NG and #p<0.05 vs HG. Bars represent mean±SEM for six independent experiments.

These results about gene expression and protein levels on proliferation and apoptosis were confirmed by analysing functional proliferative capacity and cell viability of HUVECs with the BrdU incorporation Assay and the MTT Assay, respectively. Regarding to the BrdU Assay, despite any effect of GLP-1 alone was observed, when we blocked PKCβ under high glucose, we could observe a statistically significant increase in the ratio of proliferating cells that was due to GLP-1 actions, as confirmed by the fact that the PKCβ inhibitor alone was not able to increase this ratio (Figure 56A). Regarding to the cell viability and the MTT Assay, although Ruboxistaurin was added to the media, any effect could be observed with the administration of GLP-1 under high glucose conditions (Figure 56B).

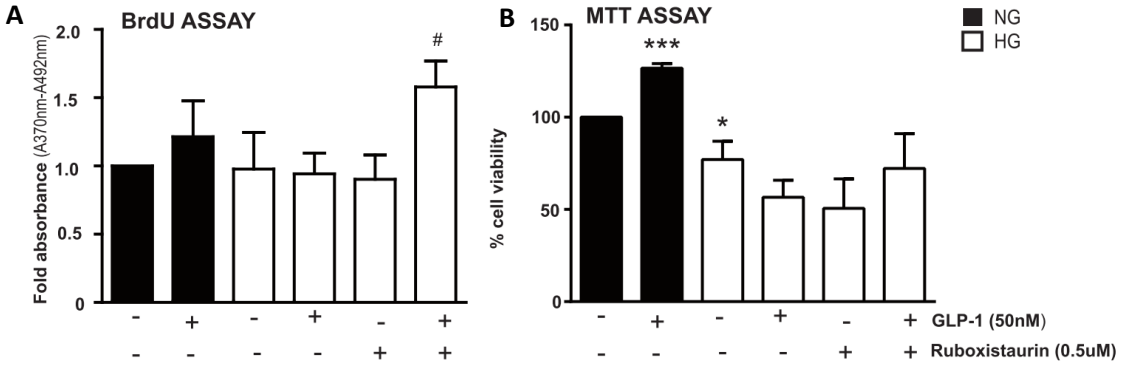


Figure 56: GLP-1 proliferative capacity was recovered in HUVECs under HG treated with Ruboxistaurin. HUVECs were exposed for 21 days to NG or HG. Proliferation was examined by measuring BrdU incorporation (A) and by MTT Assay (B). Cells were pre-treated with or without 50nM GLP-1 and/or 0.5μM Ruboxistaurin before BrdU overnight incubation or before MTT 30 minutes incubation. *p<0.05, ***p<0.001 vs NG and #p<0.05 vs HG. Bars represent mean±SEM for four independent experiments.

9.4. HG-induced ER stress was counteracted by GLP-1 after PKCβ was blocked

Regarding the endoplasmic reticulum function, as demonstrated before (Schisano et al. 2012), we confirmed that when HUVECs were cultured during 21 days under high glucose conditions, the expression of ER stress markers was increased in general for all the studied genes of the three signalling cascades described for the UPR. Moreover, as detailed in the paragraph 6 of this Section, we observed that GLP-1 treatment alone did not exert any effects on gene expression under HG state, being unable to counteract the negative consequences of sustained HG (see Figure 40).

When PKCβ was inhibited using Ruboxistaurin treatment under the condition of high glucose, the gene expression levels of these ER stress markers were in general decreased. In this state the expected GLP-1 positive effects on ER function were recovered, enhancing the action of Ruboxistaurin (Figure 57).

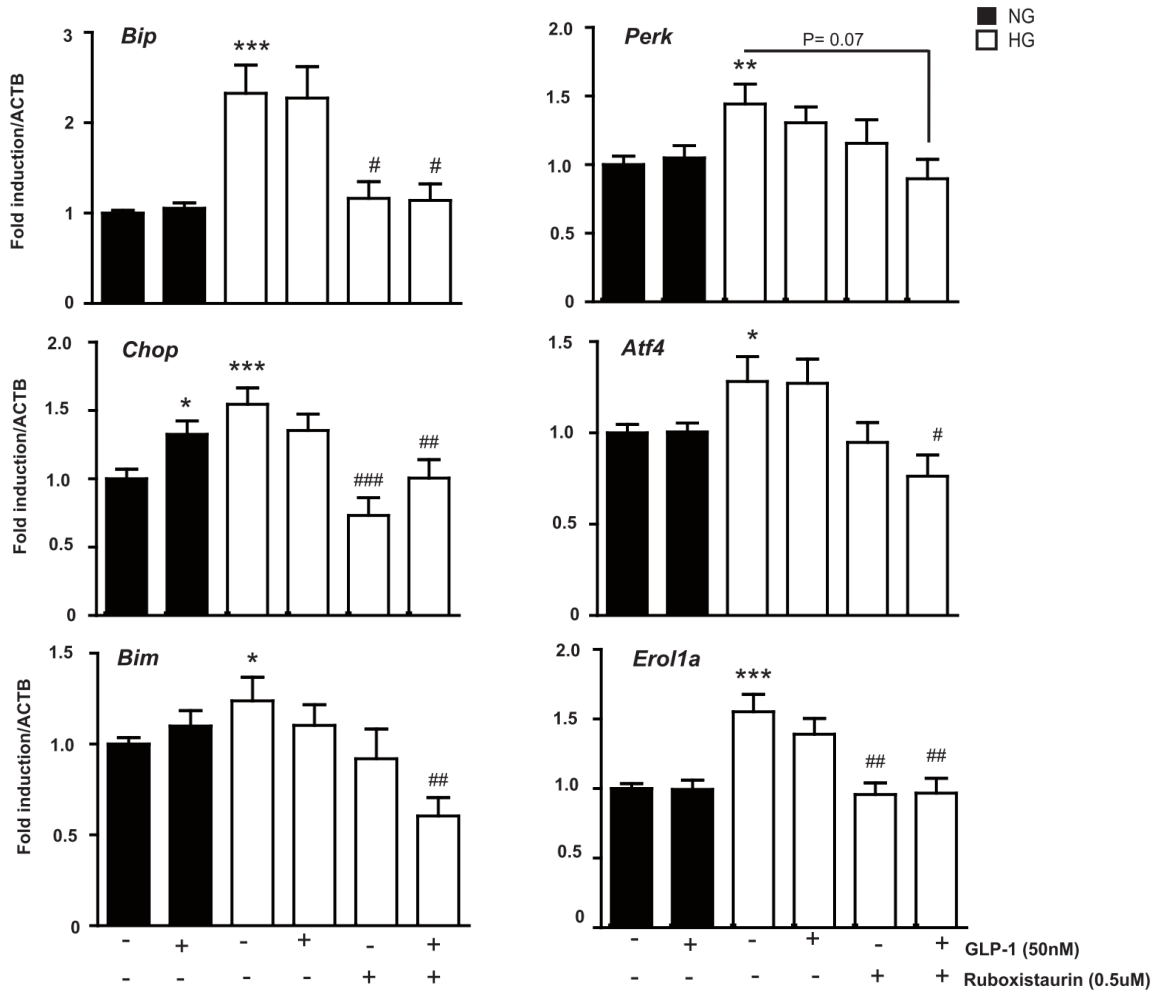
Specifically, first of all we observed a decrease in the gene expression of the chaperon Bip when HUVEC cells cultured under high glucose conditions were exposed to Ruboxistaurin. In this case the addition of GLP-1 did not enhance the positive effect exerted by the PCKβ inhibitor *per se* on our *in vitro* cellular model (Figure 57).

Related to the PERK pathway, inhibition of PKCβ under high glucose condition allowed a decrease in gene expression levels of Perk, Atf4 and Bim, but this reduction was not statistically significant. Noteworthy, administration of Ruboxistaurin in combination with GLP-1 in the same high glucose condition could induce a significant decrease in the mRNA of Atf4 and Bim, and nearly to be

significant in the case of Perk (p=0.07) (Figure 57). As it happened in the case of Bip, Chop and Erol1a expression levels were significantly decreased by Ruboxistaurin *per se*, and the addition of GLP-1 did not enhance such decrease. Moreover an unexpected increase in Chop transcript levels was observed after Ruboxistaurin plus GLP-1 addition (Figure 57).

As concern as IRE1α pathway, we tested the gene expression levels of Ire1α, Traf2 and Xbp. As observed for the PERK pathway, under HG state, treatment with Ruboxistaurn *per se* could exert some positive effects on ER function, by decreasing in a significant statistical manner the gene expression levels of Ire1α and Traf2. When HUVECs were exposed to high glucose conditions followed by the combined treatment of Ruboxistaurin and GLP-1, we do not observe any additional positive effect correlated to incretin administration on gene expression levels of Ire1α and Traf2, while for mRNA transcript of the other studied gene Xbp-sp we observed a significant decrease that was not reached in the condition in which HUVECs were treated with the PCKβ inhibitor alone (Figure 57).

Finally, regarding to the ATF6 pathway, as it was generally observed for the other studied transcripts, only the inhibition of PKCβ in combination with GLP-1 addition in the high glucose condition could induce a significant decrease in mRNA levels of Atf6 (Figure 57).



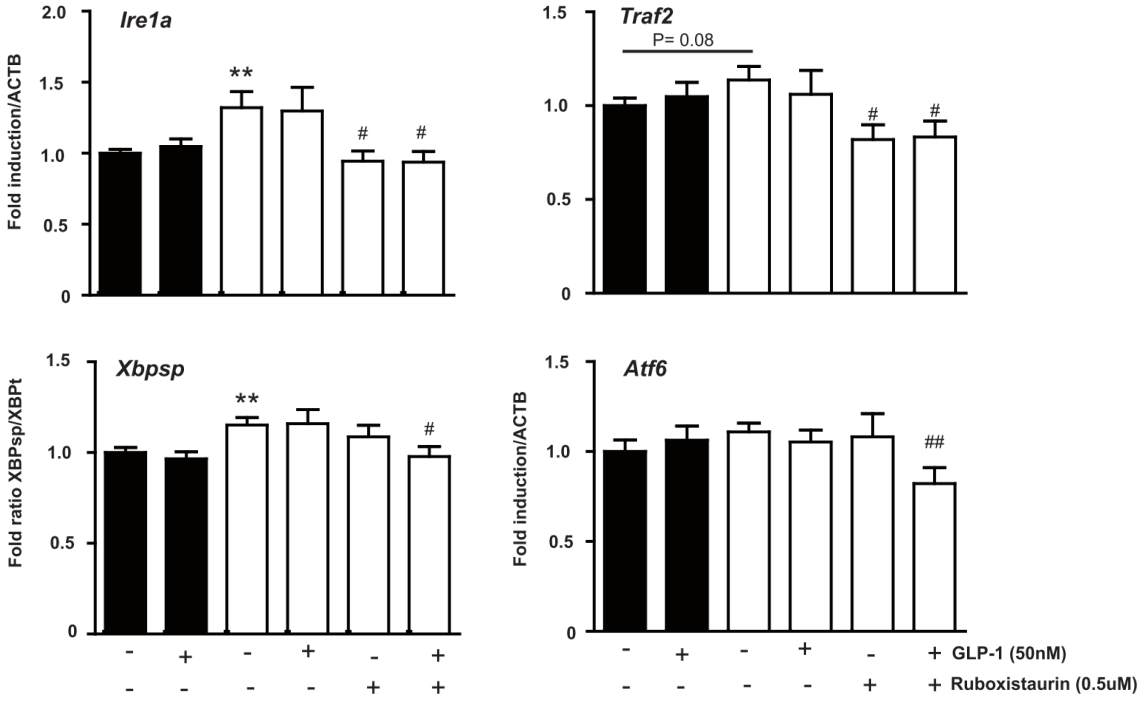


Figure 57: ER stress induced by HG is ameliorated by GLP-1 only in the presence of PKCβ inhibitor. Total cellular RNA was isolated from HUVECs after 21 days on culture under NG or HG. Cells were treated with 50nM of GLP-1 and/or 0.5μM Ruboxistaurn 1 hour before cell harvesting and mRNAs encoding for the indicated genes were assessed by qRT-PCR and expressed relative to ACTB. *p<0.05, **p<0.01 and ***p<0.001 vs NG and #p<0.05, ##p<0.01and ###p<0.001 vs HG. Bars represent mean±SEM for six independent experiments.

DISCUSSION

Type 2 diabetes mellitus is a global epidemic, with significant social and economic consequences both for individuals and overall public health (Smith 2007). Patients with T2DM have almost twice the risk of cardiovascular complications of non-diabetic individuals after adjustment for other cardiovascular risk factors (Resnick and Howard 2002; Snell-Bergeon and Wadwa 2012).

Tight glycemic control, which has the aim to maintain a HbA1c concentration of 7% or lower, is recommended for diabetic patients in order to minimize the risk of long-term vascular complications (American Diabetes Association 2014). So, with the objective to reduce HbA1C, current diabetes guidelines (Inzucchi et al. 2012) suggest antihyperglycaemic treatment as basic therapy approach for T2DM subjects. However, little is still known about the relative and specific effects of the various commercially used antihyperglycaemic therapies on clinical cardiovascular outcomes (Selvin et al. 2008). For example, for the newer glucose lowering agents, including GLP-1 agonists and DPP-4 inhibitors, a number of cardiovascular outcomes trials are currently in progress involving thousands of high-risk patients (Ferrannini and DeFronzo 2015); the problem is that although they are all designed as safety trials, they need lots of investigation to insight in the reversibility of CVD risk in diabetes (Ferrannini and DeFronzo 2015). However, a recent trial with the SGLT-2 inhibitor Empaglifozin, has demonstrated a positive action on cardiovascular complications (Zinman et al. 2015). Thus, all these concerns are indicating that further studies need to be addressed to improve our knowledge on the pathogenesis of cardiovascular complications of T2DM and how the new therapeutic drugs, in particular those that target gut-derived incretin hormones (Capuano et al. 2013), could ameliorate diabetic phenotype.

The incretin hormone GLP-1, endogenously secreted by the intestinal L-cells in response to food intake, inhibits gastric emptying, decreases glucagon secretion in a glucose-dependent manner and reduces appetite; these actions contribute to improve glycemic control (Drucker 2006; Ussher and Drucker 2012). Most of the studies regarding GLP-1 have been conducted in β-cells, where the incretin hormone has important pancreatic actions such as: stimulation of insulin production, increase in proliferation and reduction of apoptosis (Ussher & Drucker, 2012).

However, GLP-1 also exerts extra-pancreatic effects at different organs, including: lung, kidney, central and peripheral nervous system, lymphocytes, blood vessels, heart (Bullock, Heller, and Habener 1996; Thorens et al. 1993) and endothelium (Ishibashi et al. 2010), where its receptors have been widely demonstrated to be expressed (Okerson and Chilton 2012; Saraiva and Sposito 2014). However, these tissue-specific extra-pancreatic functions need to be further studied. For example, at vascular level, it has been observed that GLP-1 exerts functions as a potent vasodilator and it has been associated with the improvement of endothelial function in animal models as well as in T2DM patients (Nyström et al., 2004). However the mechanisms underlying these GLP-1 protective effects have not yet been fully elucidated.

To further insight in the molecular mechanisms involved in GLP-1 protective actions, specifically in the endothelium exposed to high glucose, the aim of this study was to decipher if GLP-1 acute treatment was able to counteract chronic high glucose-induced damage in cultured endothelial cells.

1. Effects of hyperglycaemia on GLP-1R signalling pathway

All forms of diabetes are characterized by chronic hyperglycaemia (Brownlee 2001). Prolonged exposure to high glucose causes an overproduction of reactive oxygen species and this is now recognized as the major factor in the pathogenesis and progression not only of diabetes *per se*, but also of its micro- and macrovascular complications (Xu and Zou 2009). Hyperglycaemia induces a large number of cellular alterations (Giacco and Brownlee 2010) that potentially accelerate the pathological consequences in the vasculature of diabetic subjects.

Regarding to GLP-1 related therapies, it has been shown that administration of the GLP-1R agonist Liraglutide to young *db/db* mice with only moderate hyperglycaemia provides more robust pancreatic β-cell responses than in older more hyperglycaemic mice (Shao et al. 2014), Moreover, chronic hyperglycaemia is shown to downregulate β-cell responses to another GLP-1R agonist, Exendin-4, with reduced insulin secretion, decreased cAMP response and impaired CREB phosphorylation (Baggio, Kim, and Drucker 2004). These results are indicating that prolonged hyperglycaemic state may be a contributing factor to the diminished efficacy of GLP-1 agonists in T2DM. Consistent with these observations, intensive insulin therapy to normalize glucose levels preceding GLP-1R agonist administration improves the insulin secretory response in individuals with type 2 diabetes (Højberg et al. 2008), whereas the disruption of glucose homeostasis through the induction of insulin resistance diminishes the potentiating effects of GLP-1 on insulin secretion in human subjects (Hansen et al. 2012).

Understanding the mechanisms by which poorly controlled glucose diminishes GLP-1R signalling, increases the potential for developing strategies to improve the effectiveness of GLP-1R targeting therapies.

In this study, with the objective to assess how GLP-1R signalling is affected by chronic hyperglycaemia in HUVEC cells, we evaluated its downstream pathways. GLP-1 initiates its signalling through the binding with its receptor GLP-1R, which belongs to the G-protein coupled receptors family (Drucker 2006). Although the GLP-1 signalling cascade is very complex as different pathways can be initiated, activation of the GLP-1R can trigger at least two downstream pathways: (1) generation of the second messenger cAMP followed by activation of PKA and (2) the indirect activation of epidermal growth factor receptor followed by PI3K and AKT signaling.

Using our experimental model, we observed the disruption of both GLP-1 signalling pathways due to chronic hyperglycaemic conditions. For one hand, GLP-1 was not able to activate the PI3K/AKT signalling cascade in high glucose cultured endothelial cells, and this result was confirmed by the inability of GLP-1 in phosphorylating AKT. By the other hand, we observed an increase in some catalytic and regulatory subunits of PKA due to hyperglycaemia, which indicated a decrease in PKA activity (Rajan et al. 2015).

To our knowledge, apart from the report of Rajan et al. conducted in the pancreatic β-cells (Rajan et al. 2015), no other studies have been performed in endothelial cells regarding transcriptional regulation of PKA subunits by high glucose conditions. So in this study we described for the first time the transcriptional regulation of PKA subunits by GLP-1 and hyperglycaemia in the endothelium.

It has been shown in pancreatic β-cells that GLP-1 reduces apoptosis, stimulates survival and proliferation, and increases insulin secretion (Buteau et al. 2003; Buteau, Spatz, and Accili 2006).

These effects have primarily been ascribed to the activation of downstream cAMP/PKA and PI3K/AKT signalling pathways in these cells (Buteau, Spatz, and Accili 2006; Yusta et al. 2006). Also in neuronal cells it has been described that GLP-1 prevents apoptosis through the activation of PI3K/AKT pathway (Kimura et al. 2009). Moreover, the well-known cardioprotective effects of GLP-1 after ischemia/reperfusion have been demonstrated to be abolished by inhibiting cAMP and PI3K signalling (Bose et al. 2005). These results obtained in different organs and tissues indicate that the protective effects of GLP-1 generally involve multiple pathways.

Particularly, the PI3K/AKT pathway has been demonstrated to participate in the activation of some target genes: the antioxidant genes Nqo-1 and Hmox-1 and the anti-apoptotic gene Bcl-2 (Oeseburg et al. 2010). The expression of these genes has been demonstrated to be induced by GLP-1 in pancreatic cell lines (D'Amico et al. 2005) as well as in endothelial cells (Yi Zhan, Hui-lin Sun, Hong Chen, Hua Zhang & Zhen Zhang, 2012).

As the PI3K/AKT arm is considered a general survival pathway (Oeseburg et al., 2010), we tested whether it could be involved in the protective effects of GLP-1 also in our *in vitro* endothelial model. We confirmed this data in our experimental model of 21 days NG culture of HUVECs: GLP-1 induced the mRNA expression of Hmox-1, Nqo-1 and Bcl-2, and when PI3K/AKT pathway was chemically disrupted by Wortmannin, which specifically inhibits PI3K, we could not observe induction of the indicated transcripts by GLP-1. Thus, our data indicated that also in HUVEC cells the PI3K/AKT signalling is activated by GLP-1 in normal glucose conditions and this activation is required for GLP-1 protective effects.

2. GLP-1 Resistance and the role of hyperglycaemia-induced PKC activation

In T2DM, a marked reduction in the insulinotropic activity of the other incretin hormone GIP has been clearly demonstrated as well as it has been postulated that the reduced expression of GIPR in β-cells might contribute to this phenomenon (Holst, Gromada, and Nauck 1997). This has been observed also in an animal model of type 2 diabetes, where both the reduced number and activity of GIPR have been described (Lynn et al. 2001). However, contradicting information has been published regarding the insulinotropic response to GLP-1 in patients with type 2 diabetes. It has been demonstrated that GLP-1 was active in patients with type 2 diabetes (Holst, Gromada, and Nauck 1997), in contrast to the reduced or even lost insulinotropic action of GIP. However, in other cohorts, the insulinotropic response to GLP-1 was impaired in patients with T2DM (Kjems et al. 2003).

Many reports describe a decrease in GLP-1R expression in different experimental models due to HG. It was found that GLP-1R mRNA expression was three-fold less in islets isolated from diabetes-prone BioBreeding rats (Valverde et al. 2004). The expression of both GLP-1R and GIPR was found significantly decreased in islets of 90% pancreatectomized (Px) hyperglycemic rats, and the perfused islets isolated from these hyperglycemic Px rats showed reduced insulin responses to GLP-1 and GIP (Xu et al. 2007), indicating that chronic hyperglycemia may be involved in the regulation of GLP-1R and GIPR expression. Also using the *in vitro* model of pancreatic INS-1 β-cells, it has been observed that high glucose downregulated mRNA and protein expressions of both GIPR and GLP-1R in a dose dependent-manner (Pan et al. 2009), and this result might contribute to explain the decreased actions of GIP and GLP-1 observed in T2DM. In the same work, Pan and coworkers (Pan et al. 2009) also found that the downregulation occurred with a statistical difference after 6 h (GIPR) and 12 h (GLP-1R) at high glucose concentrations. In conclusion, glucose downregulated the gene expression

of GPCR family members – GLP-1R and GIPR – in the rat β-cell line INS-1. Both GLP-1R and GIPR strongly participate in metabolic and growth functions of β-cells, so the fact that glucose concentration regulated mRNA and protein expressions of these receptors indicated a new and independent mechanism of glucose-induced change of β-cell function.

In parallel this result was reproduced in extra-pancreatic models. Two independent groups working in different models and cell types have confirmed the high glucose-induced reduction of GLP-1 receptor expression: Mima A. et al. showed a reduction of GLP-1 receptor expression on renal cortex protein extracts of diabetic mice (Mima et al. 2012); Green C.J. et al. showed a reduction on GLP-1 receptor expression in fully differentiated human myocytes cultured under chronic high glucose levels (Green et al. 2012).

In summary, recent evidences claim a decreased GLP-1 action that could be explained with a hyperglycaemia-induced reduction in its receptor, not only in pancreas, muscle or kidney, but also in other cellular types. In particular, as referred to endothelium, it has been postulated that in T2DM subjects, hyperglycaemia induces an endothelial "GLP-1 resistance", which could be restored improving glycemic control (Ceriello et al. 2011; Herzberg-Schäfer et al. 2012).

Two mechanisms have been suggested to explain this resistance to GLP-1 action in diabetes: the generation of oxidative stress by hyperglycaemia (Ceriello et al. 2011) and the activation of PKCβ, induced by hyperglycemia, able to reduce the expression of GLP-1 receptors (Mima et al. 2012). Nevertheless, these two proposed mechanisms could be convincingly correlated, as it is well known that PKCβ is activated by free radicals overproduction (Quagliaro et al. 2003).

Regarding the first hypothesis, it has been suggested that hyperglycaemia might induce such a GLP-1 endothelial resistance, mainly through the generation of an oxidative stress environment (Ceriello et al. 2011). This hypothesis has been confirmed by *in vivo* studies showing that GLP-1 action can be improved by an antioxidant, vitamin C (Ceriello, Novials, Ortega, Canivell, La Sala, et al. 2013; Ceriello, Novials, Ortega, Canivell, Pujadas, et al. 2013). Another study based on the use of Mediterranean diet with olive oil, not only confirmed this finding, but had a significant clinical impact. Mediterranean diet resulted to prevent the acute hyperglycemia effect on endothelial function, inflammation and oxidative stress, and also resulted to improve the positive actions of GLP-1, having a favorable effect on the management of T2DM and the prevention of the associated cardiovascular complications (Ceriello et al. 2014). While chronic administration of vitamin C may not be a definitive solution (Frei, Birlouez-Aragon, and Lykkesfeldt 2012), the evidence that the Mediterranean diet improves GLP-1 action on both insulin secretion and endothelial dysfunction in diabetes might shed new light on the daily management of this disease.

The second mechanism proposed to explain the endothelial resistance to the GLP-1 actions in diabetes is the activation of PKCβ, induced by hyperglycaemia, which is able to reduce the expression of GLP-1R (Mima et al. 2012). Activation of β-isoform of PKC by hyperglycaemia can cause glomerular endothelial dysfunction and reduce eNOS activation partially owing to inhibition of insulin action on glomerular endothelial cells (Mima et al. 2011; Naruse et al. 2006).

Firstly, we confirmed that HUVECs express GLP-1R protein, as others reported (Ding & Zhang, 2012; Ishibashi et al., 2010; Oeseburg et al., 2010). Moreover we observed a reduction on GLP-1R protein expression when the cells were exposed to sustained high glucose. In parallel, we could corroborate a significant increase in PKCβ protein and mRNA levels in the same condition of HG, which correlated with the opposite results about the expression of GLP-1R.

The role of PKCβ induced by HG in the regulation of GLP-1R (Mima et al. 2012) was also confirmed in our study: when PKCβ was specifically inhibited by Ruboxistaurin, GLP-1R levels and signalling were restored.

An interesting hypothesis that has recently emerged to explain the disrupted GLP-1 signalling, regards the effects of hyperglycaemia on GLP-1R trafficking. It has been reported that GLP-1R, as other GPCR family members, is internalized and then recycled to the membrane or degraded via proteasoma (Roed et al. 2014; Syme, Zhang, and Bisello 2006). It has been described the existence of additional proteins involved in this process, the GPCR kinases (GRKs) and β-arrestins, having a role in the process of GPCRs trafficking (Drake, Shenoy, and Lefkowitz 2006). Both GRKs and β-arrestins were firstly identified as proteins involved in the receptor desensitization process, having an active role in the endocytosis and internalization of the receptor after ligand binding. Interestingly, PKC has also been related with GPCRs intracellular trafficking (Esseltine, Ribeiro, and Ferguson 2012). So it is tempting to hypothesize that HG-induced PKCβ is affecting GLP-1R recycling to the membrane, probably leading to an increase in its degradation and to the consequent decrease of GLP-1R intracellular levels.

As initial step to corroborate this hypothesis, we assessed β-arrestins expression in HUVECs and how they were affected due to HG exposure. In our cellular model exposed to chronic high glucose, we observed that the mRNA expression of the β-arrestin 1, but not β-arrestin 2, was decreased under HG condition. Strikingly, when PKCβ-activation was inhibited with the addition of its specific inhibitor Ruboxistaurin under the condition of hyperglycaemia, the expression of β-arrestin 1 was recovered. In accordance with these results, it has been published that in pancreatic β-cells the same isoform 1 of β-arrestin is able to bind to GLP-1R, playing an important role in GLP-1 signalling (Portha, Tourrel-Cuzin, and Movassat 2011).

In parallel to these observations, we could note that GLP-1 binding to its receptor under the condition of NG resulted in a “perinuclear concentration” of the receptor, a localization that was lost when cells were exposed to chronic HG. Moreover, the administration of GLP-1 in the condition of hyperglycaemia could not restore the receptor localization observed in the NG state. However, when HG-induced PKCβ was blocked with Ruboxistaurin, GLP-1 was able to act recovering this perinuclear localization.

Obviously, further studies need to be addressed in order to confirm these observations, although our results suggest that GLP-1R trafficking is affected by hyperglycaemia due to an increase in PKCβ levels, but with a non-defined mechanism.

3. Effects of hyperglycaemia and GLP-1 on antioxidant response

NAD(P)H oxidase family of enzymes (NOXs) has been implicated as the major source of ROS generation in the vasculature in response to high glucose (Cave et al. 2006). The catalytic core of each NAD(P)H oxidase comprises a Nox subunit, 7 members of which have been identified (Nox1-5, Duox-1 and -2). These transmembrane proteins facilitate the transfer of an electron from NAD(P)H within the cell cytosol, via coordinated heme groups to reduce molecular oxygen O_2 to superoxide O_2^- on the opposite side of the membrane (Lambeth 2004). Despite the apparent similarity in the biochemical function of the different isoforms, the diverse family members display distinct quantitative and qualitative cellular expression patterns, suggesting distinct functions (Bedard and Krause 2007). Moreover, the activity of the different Nox isoforms is subject to very different modes

of regulation. For example, Nox1 and Nox2 are acutely regulated by posttranslational mechanisms such as the phosphorylation of regulatory subunits induced by the activities of agonists (Brewer et al. 2011); by contrast the activity of Nox4 is constitutive and does not require agonist stimulation or the association of regulatory proteins other than p22-^{phox} (Ambasta et al. 2004).

In this study we examined the mRNA expression of the different NAD(P)H oxidase subunits, and we focused our attention on Nox4 and p22-^{phox}. Unexpectedly, the expression of both Nox4 and p22-^{phox} transcripts was induced by GLP-1 treatment in HUVEC cells exposed to normal glucose condition, however it is noteworthy that no increase in ROS production was observed after GLP-1 addition, as well as no increase in apoptotic phenotype was described. These results are also in accordance with the idea that under physiological conditions, ROS production via NAD(P)H oxidase is eliminated efficiently by antioxidant defense systems, while excessive activation of NAD(P)H oxidase leads to oxidative stress, mitochondrial dysfunction and impaired antioxidant gene expression (Tsai et al. 2013).

In HUVECs exposed to HG, Nox4 and p22-^{phox} gene expression levels were increased, accordingly to what was reported before regarding how high glucose is one of the major causes of Noxs activation and ROS production (Cave et al. 2006). However, in the condition of HG, although a decrease of Nox4 and p22-^{phox} transcripts was observed with the addition of GLP-1, no statistically significant effect in any of them was observed. The expression of Nox4 was reduced when PKCβ was inhibited, as expected, and this reduction was significantly enhanced only when GLP-1 was added in combination with Ruboxistaurin.

These results confirm that GLP-1 cannot exert its positive effects in a hyperglycaemic environment due to the increased activation of PKCβ.

There are lots of evidences suggesting that ROS generated by Nox4 play an important role in many different cell types to determine cell fate and function, such as in vascular smooth muscle cells (Clempus et al. 2007), cardiac fibroblast and myofibroblasts (Cucoranu et al. 2005), preadypocites (Schröder et al. 2009) and pulmonary artery smooth muscle cells (Sturrock et al. 2007; Sturrock et al. 2006). Nox4 produces ROS constitutively and consequently its activity reflects its level of expression (Serrander et al. 2007). So, significant changes in intracellular Nox4 mRNA and protein levels are implicated in the regulation of cellular redox homeostasis.

Consistent with the studies of Serrander et al. (Serrander et al. 2007) and in parallel with the results observed for the gene expression of the studied NAD(P)H subunits, we observed that ROS production was increased under the condition of high glucose and it was reduced in a statistical manner only when GLP-1 was added to HUVEC cells in combination with the PKCβ inhibitor.

It has been shown that ROS overproduction damages pancreatic β-cells and reduce insulin excretion (Mukai et al. 2011). Additionally, ROS impair insulin sensitivity in peripheral tissues and are directly or indirectly associated with the multistage process of atherogenesis (Rains and Jain 2011). Significant reductions in ROS levels have been demonstrated with GLP-1 agonist-based therapies (Chaudhuri et al. 2012). In particular, recent studies have shown that GLP-1 agonists reduce the extent of oxidative stress by reducing ROS levels in H9c2 myocytes and in human monocytes (Chang et al. 2014) or by decreasing NAD(P)H oxidase activity (Ojima et al. 2012).

Following these observations, we demonstrated that also in our *in vitro* model of HUVEC cells, the hyperglycaemia-induced ROS overproduction in endothelium could be reverted by GLP-1 treatment, but only when PKCβ activation was blocked.

The specific molecular mechanisms which underlie the Nox4-dependent cellular functions are largely unknown. In particular, the targets of ROS oxidation, generated by Nox4, need to be elucidated. It has been demonstrated the ability of Nox4-generated ROS to activate the NRF2-dependent pathway in cardiomyocytes *in vivo* (Brewer et al. 2011). NRF2 is a positive regulator of a plethora of genes involved in antioxidant and xenobiotic defence. These include the phase II detoxifying enzymes, such as GSTs and NQO-1, and the antioxidant enzymes such as TXNRD1 and HMOX-1 (Osburn and Kensler 2008).

There are several reports describing distinct mechanisms of NRF2 activation, most of which appear to be redox-mediated (Yamamoto et al. 2008). *In vitro* and *in vivo*, the expression of antioxidant and detoxifying enzymes regulated by NRF2 has been shown to be induced by a variety of chemical agents which act as electrophiles, including diphenols, quinones, isothiocyanates, arsenicals and heavy metals (Friling et al. 1990). Moreover, both NRF2 itself and KEAP1, which acts as a negative regulator of NRF2 activity, are susceptible to oxidative thiol modifications of reactive cysteines which regulate their functions (Yamamoto et al. 2008), and are therefore potential targets of NAD(P)H oxidase-generated ROS. In addition, phoshorylation of NRF2 by PKC has been shown to facilitate the dissociation of NRF2 from KEAP1, a process which may also be redox regulated (Huang, Nguyen, and Pickett 2002).

As regarding the beneficial effects of GLP-1, it has been suggested that this incretin hormone could exert its protective vascular actions inducing intracellular expression of the antioxidant enzymes HMOX-1 and NQO-1 (Oeseburg et al., 2010). The expression of the antioxidant genes Hmox-1 and Nqo-1 requires the activation of the transcription factor NRF2 and the subsequent stimulation of AKT (Pecorelli et al. 2013). This is one of the most important cellular mechanisms regulating the expression of phase II detoxifying enzymes (Osburn and Kensler 2008). The activation of NRF2 is associated to its translocation into the nucleus, the heterodimerization with the regulatory MAF protein, and the binding to the ARE sequence in the promoter of several target genes (Pecorelli et al. 2013). So, this binding is associated to the induction of the expression of a great number of enzymes involved in cell detoxification and antioxidant defence (Kensler et al., 2007; Kim et al., 2010; Motohashi and Yamamoto, 2004; Taguchi et al., 2011; Zhang, 2006).

Our results showed that in normal glucose conditions, GLP-1 added at pharmacological concentrations was able to initiate the antioxidant response in HUVECs through nuclear translocation of NRF2. We investigated the expression of the oxidative stress defence gene Hmox-1 and we could show here that, also in our *in vitro* model of endothelial cells, its expression was significantly induced by GLP-1. This extends a previous observation in which *in vivo* Liraglutide treatment results in Hmox-1 induction and cytoprotection of cardiac tissue (Noyan-Ashraf et al. 2009). Another oxidative defence gene, Nqo-1, a second potential downstream target of the GLP-1-induced NRF2/ARE pathway, was also induced in endothelial cells in our study.

So, these results confirmed that GLP-1 could activate oxidative stress defence pathways in endothelial cells cultured in normal glucose concentrations.

It has been observed that in the presence of oxidative stress stimulus, induced by sustained high glucose, there is an accumulation of NRF2 into the nucleus, which upregulates the expression of its targets (Kobayashi and Yamamoto 2006; Kobayashi et al. 2009; Tsai et al. 2013). In our cellular model we were not able to see an enhancement of NRF2 activation when GLP-1 was added alone in hyperglycaemia. Only when PKCβ activation was specifically blocked, GLP-1 became capable to exert its effects under HG. This observation could be explained once again by a receptor resistance phenomenon.

4. Effects of hyperglycaemia and GLP-1 on FOXOs and scavengers

Apart from the well-known antioxidant actions of NRF2, other scavengers function counteracting ROS production, such as the transcription factors of the forkhead O family. FOXOs are important regulators of the cellular stress response and promote the antioxidant defense (Accili and Arden 2004). For one hand, FOXOs stimulate the transcription of genes coding for antioxidant proteins located in different subcellular compartments, such as the cytoplasmic SOD1 (Marinkovic et al. 2007), the mitochondrial SOD2 (Kops et al. 2002) and the peroxisomal CAT (Nemoto and Finkel 2002). On the other hand, ROS *per se*, as well as other stressful stimuli that elicit the formation of ROS, may modulate FOXOs activity at multiple levels, including their posttranslational modifications such as phosphorylation and acetylation, interaction with coregulators, alterations in their subcellular localization, protein synthesis and stability (Calnan and Brunet 2008; Zhao, Wang, and Zhu 2011).

FOXO proteins, in particular FOXO1, are highly expressed in the major insulin sensitive tissues as well as in the pancreatic β-cells (Klotz et al. 2015). Hyperactivation of FOXOs has been reported to be associated with hallmarks of overt diabetes such as hyperglycaemia, hypertriglyceridemia, insulin resistance and an impaired compensatory increase in β-cell mass as well as with diabetic cardiovascular complications (Altomonte et al. 2004; Gross, Wan, and Birnbaum 2009; Nakae et al. 2002; Wang, Zhou, and Graves 2014). For example, diabetic complications such as retinopathy and impaired fracture healing have been linked to elevated FoxO1a transcriptional activity under hyperglycaemic conditions (Alblowi et al. 2009; Behl et al. 2009; Wang, Zhou, and Graves 2014).

However, FOXOs also have beneficial effects in diabetes, as FOXO-dependent transcription of antioxidant enzymes may counteract oxidative stress-induced cellular damage (Gorrini, Harris, and Mak 2013; Gross, Wan, and Birnbaum 2009). For example, FOXO1-mediated induction of NeuroD and MafA has been shown to protect pancreatic β-cells against glucose toxicity and cell failure (Kitamura et al. 2005). Moreover, mice with a triple knockdown of Foxos (Foxo1a, Foxo3a and Foxo4) in pancreatic β-cells developed a MODY-like phenotype characterized by an insulin secretory defect due to impaired ATP generation after glucose stimulation and preferential use of lipids as nutrient source instead of glucose (Kim-Muller et al. 2014).

Among the FOXO proteins that are present in humans, FOXO1a, FOXO3a, FOXO4 and FOXO6, all are widely expressed in diverse tissues (Monsalve and Olmos 2011), including FOXO6, which has frequently been said to be primarily found in brain, but has now been shown to be ubiquitously expressed (Kim et al. 2011).

As it has been described before (Potente et al. 2005), we corroborated that all these transcription factors were expressed at different levels also in HUVECs, being Foxo1 the most expressed one. Furthermore, we observed that they were differentially regulated by GLP-1: while Foxo1 and Foxo4 were transcriptionally induced, Foxo3a was downregulated. The effects of hyperglycaemia on Foxos transcripts were also diverse: while Foxo3a and Foxo4 were increased, Foxo1 was decreased. Again, as it happened with Nrf2 target genes, Foxo4 was only regulated by GLP-1 in HG when PKCβ was blocked.

In parallel, we tested mRNA expression of the scavengers: Sod1, Sod2 and Cat. We observed that GLP-1 was not able to regulate any of them under normal glucose conditions; however both Sod1 and Sod2 were increased by hyperglycaemia, as expected. Once again, a GLP-1 resistance phenomenon was observed in the HG condition, as the incretin hormone could not exert any effect

on these transcripts if PKCβ activation was not blocked with Ruboxistaurin. In this condition the expression of Sod1 and Cat increased, following the same Foxo4 gene expression profile. We could speculate that a specific regulation of Sod1 and Cat by Foxo4 in HUVECs exists, however further studies need to be addressed to confirm this hypothesis.

From our knowledge, this is the first time that a regulation of Sods and Cat by GLP-1 is described in ECs. Accordingly, these results perfectly matched with the reduction in ROS production observed when Ruboxistaurin plus GLP-1 were added to the HG medium.

FOXOs transcriptional activities are also promoted by the protein kinase PERK. PERK has been reported to directly act on FOXOs, phosphorylating them and, as consequence, increasing their activity, in response to ER stress (Zhang et al. 2013). As well as for FOXOs, ER stress also induces NRF2 nuclear translocation in a PERK-dependent manner (Cullinan et al. 2003). These finding are in accordance with our results: inhibition of PKCβ under high glucose condition allowed GLP-1 to decrease gene expression levels of Perk. This data perfectly matched with the expression levels of Foxos and Nrf2 targets in the same culture conditions.

Given the role of FOXOs and NRF2 in counteracting oxidative stress, these findings suggest a cytoprotective role of PERK in response to GLP-1 action in the presence of ER stress. The identification of FOXOs and NRF2 as PERK substrates gives strong support to the hypothesis that PERK signalling initiates two independent programs of gene and protein expression. As detailed in the *Introduction* Section, by directly phosphorylating by one hand eIF2 (Harding, Zhang, and Ron 1999) and, by the other hand, Nrf2 (Cullinan et al. 2003) and Foxos (Zhang et al. 2013), PERK signalling regulates many aspects of the overall cellular response to ER stress. The phosphorylation of eIF2 results in the attenuation of protein translation (Harding, Zhang, and Ron 1999) and in the subsequent cell cycle arrest (Brewer and Diehl 2000), giving to the cells the opportunity to pause, to conserve energy and, in conjunction with Ire1 and ATF6-dependent transcriptional activity, to return the protein folding status in the ER to homeostatic conditions. Nrf2/Foxos phosphorylation promotes the transcription of genes whose protein products result in redox homeostasis (Cullinan and Diehl 2006).

5. Effects of hyperglycaemia and GLP-1 on proliferation/apoptosis

Proliferative dysfunction of endothelial cells is believed to contribute to premature development of atherosclerosis (Giacco and Brownlee 2010).

It is well documented that cell proliferation is controlled by the sequential formation, activation and inactivation of a series of cell cycle regulators that include the cyclins and the cyclin-dependent kinases (Cdks), which are referred as positive regulators of the cell cycle (Vitiello et al. 2009). Cyclin D1 levels rise in G1 phase and remain elevated until mitosis. Cdk 4, 5 and 6 complexes mainly belong to the cyclin D family and function during the G0/G1-phases of the cell cycle (Bicknell, Surry, and Brooks 2003). A number of negative regulators also exist, including the Cdk inhibitors (CDKIs) p21/Cdkn1a and p27/Cdkn1b, that have a region involved in cyclin binding and kinase inhibitory function (Pines 1997).

Several previous studies have revealed that GLP-1 may promote proliferation and differentiation in pancreatic β-cells and may suppress cell apoptosis by activating the GLP-1R signalling pathway (Drucker 2003; Portha, Tourrel-Cuzin, and Movassat 2011). Interestingly, it has been recently

described the capacity of GLP-1 to induce cell cycle progression by regulating the expression of several components of the complex survival process in cultured β-cells, as it was the case for the Cdk inhibitor Cdkn1a (Zhang et al. 2014). In this study Zhang and collaborators observed that seven days of GLP-1 treatment at the concentration of 100 nmol/l enhanced INS-1 cells viability, decreasing the accumulation of cells in the G0/G1 phase and notably increasing their ratio in the G2/M phase (Z. Zhang et al. 2014). Moreover, they demonstrated that cells intervened with constant and intermittent high glucose exhibited a decreased expression of cyclin D1 and Skp2, and increased levels of Cdkn1a and Cdkn1b; also in these conditions the cell cycle was blocked mainly in the G0/G1 phase, which traduced decreased cell viability. The administration of GLP-1 reversed this effect, leading to an upregulation of cyclin D1 and Skp2 levels, and a downregulation of Cdk inhibitors expression (Z. Zhang et al. 2014).

Cyclin D1 induction by mitogens is dependent on downstream pathways of GLP-1R and the overexpression of cyclin D1 induces β-cell proliferation in rat and human pancreatic islets (Bernal-Mizrachi et al. 2014). There are several studies demonstrating that a reduction of endogenous expression of Cdkn1a and Cdkn1b, mediated by an upregulation of Skp2 in response to incretin signalling, is required for β-cell mass expansion (Frouin et al. 2002; Kossatz et al. 2004) or proliferation (Tschen et al. 2011).

In the present study we confirmed how also in HUVEC cells exposed to sustained HG for 21 days there was an alteration in the expression of different survival markers. Particularly, we analysed the expression of both cell cycle inhibitors Cdkn1a and Cdkn1b, and we observed that they were significantly upregulated under high glucose conditions, which was traduced in a decrease in cell viability. Although only Cdkn1a was reduced after the administration of GLP-1 plus Ruboxistaurin in the same HG state, it was enough to induce an increase in HUVECs viability, unless not in a statistical manner. Once again we demonstrated that acute treatment of 1 hour GLP-1 alone in our cellular model was not sufficient to reverse the pathological phenotype induced by the sustained hyperglycaemia, and that the blockage of the PKCβ activation was needed for GLP-1 positive actions.

Several previous studies have revealed that GLP-1 mediated mitogenic signalling is cAMP dependent in pancreatic β-cells (Frödin et al. 1995; List and Habener 2004). The activation of cAMP/PKA following the binding of GLP-1 to its receptor stimulates the induction of genes such as c-fos, c-Jun and Jun-D that are involved in cell proliferation, and, as a result, the pathway regulates cell cycle associated cyclin expression (Susini et al. 1998).

Further studies need to be addressed to the possibility that also in HUVEC cells GLP-1 may activate early genes involved in cell survival in a cAMP/PKA dependent manner, in order to insight the possible mechanism underlying the promotion of cell proliferation in HUVECs by GLP-1.

It is well accepted that members of the Bcl-2 family represent central regulators of cell death. The pro-apoptotic protein Bax is essential for permeabilization of the mitochondrial outer membrane, leading to cytochrome c release and subsequently to the activation of caspases (Chipuk and Green 2008). The anti-apoptotic protein Bcl-2 inhibits this process by blocking the translocation of Bax upstream of mitochondria and thus reducing the activity of caspases (Chipuk and Green 2008). Caspase-9 and -3, the initiator caspase and the executor caspase respectively, both amplify the pro-apoptotic signal and result in apoptosis (Marsden et al. 2002). Caspases appear to be important for the progression of apoptotic cell death. After delivery of death signals to cells in culture, Bax protein moves to the mitochondria and other membrane sites and triggers a catastrophic change of

mitochondrial function, with the subsequent release of cytochrome c. Cytochrome c is necessary for Caspase 9 activation (Oeseburg et al., 2010). Caspase 9 can function as an initiator of the caspase cascade when mitochondrial dysfunction is the primary event in apoptosis, whereas it serves to amplify the apoptotic signaling of other initiator caspases under conditions in which disruption of mitochondria is a late event (Méndez et al. 2010). Cytochrome c and Apaf-1 cooperatively activate initiator caspase-9 that triggers caspase cascade and activates Caspase 3. Caspase 3 in turn can amplify the signal by cleaving initiator-caspases and leading to apoptosis by cleaving key intracellular targets.

Here, we showed that chronic exposure to hyperglycaemia for 21 days significantly decreased the gene expression of the anti-apoptotic Bcl-2, increased the gene expression of the pro-apoptotic Bax, activated Caspase 3 at mRNA and protein levels and subsequently induced the apoptosis of HUVECs. All these activities induced by hyperglycaemia could not be attenuated in the presence of acute GLP-1 alone. GLP-1 was able to restore the expression of Bcl-2, as well as to reduce the expression of Bax and the protein amount of Caspase3 only in the presence of Ruboxistaurin.

The anti-apoptotic actions of GLP-1 via alteration of the Bcl-2 family proteins - caspase protease pathway may be effective not only in endothelial cells, but also in other cell types. For example, it has been demonstrated that GLP-1 or one of its receptor agonists upregulate Bcl-2 and inhibit Bax expression in cholangiocytes (Marzioni et al. 2009) and neuronal cells (Qin et al. 2008). In addition, GLP-1 prevents Bax/Bcl-2 protein ratio increase, cytochrome c release and Caspase 3 activity increase induced by staurosporine in cardiomyocytes (Ravassa et al. 2011). Moreover, GLP-1 induces Bcl-2 upregulation (Natalicchio et al. 2010), Bad inactivation (Quoyer et al. 2010) and Caspase 3 activity reduction (Tews et al. 2009) in pancreatic β-cells.

Here, we confirmed and expanded these data demonstrating that GLP-1 prevents the reduction of the Bcl-2/Bax ratio and reverses the increase in Caspase 3 activity induced by sustained exposure to high glucose in HUVECs.

These data are consistent with the previous investigations about the hyperglycaemia-induced damage in survival and proliferation of other cell types and may account for the possible mechanism underlying the promotion of cell survival by GLP-1 in cultured endothelial cells. The results in the present study indicate that GLP-1R activation in HUVECs could exert cytoprotective effects and ameliorate the endothelial phenotype in terms of proliferation and viability only when hyperglycaemia-induced PKCβ activation is blocked by Ruboxistaurin.

6. Effects of hyperglycaemia and GLP-1 on ER function

The ER is a dynamic tubular network that participates, among its various actions, in the maturation and the proper folding of proteins (Hetz 2012). Perturbations of ER function initiate a series of complementary adaptive mechanisms which together are known as the Unfolded Protein Response. The UPR is a set of three signalling pathways, PERK, IRE and ATF6, which are activated with the objective to resolve the initial cause of ER stress (Tabas and Ron 2011) by inhibition of protein synthesis, protein refolding and clearance of mis-folded proteins. While acute activation of the UPR is cytoprotective, prolonged activation of the UPR initiates a proapoptotic pathway (Tabas and Ron 2011).

The effect of hyperglycaemia-induced ER dysfunction has become a focus of increasing research. It has been proposed as one of the causes of beta cell loss of function (Fonseca et al. 2009), and damage in other tissues, such as the adipose tissue (Alhusaini et al. 2010; van der Kallen et al. 2009; Xu, Spinas, and Niessen 2010), the liver (van der Kallen et al. 2009) and the cardiovascular system (Bakker et al. 2009; McAlpine, Bowes, and Werstuck 2010).

As far as we know, it has not been yet described a GLP-1 direct effect in ameliorating ER stress, however several papers describe the actions of GLP-1-derived drugs in different experimental contexts. For example, Exendin-4 is able to reduce several induced-ER stress markers in different *in vitro* and *in vivo* models (Lee et al. 2014; Younce, Burmeister, and Ayala 2013); Liraglutide ameliorates ER stress in different contexts (Liu et al. 2013; Shimoda et al. 2011; L. Zhao et al. 2013) and also in HUVEC cells exposed to sustained HG (Schisano et al. 2012). Another study showed that 1 week treatment with the GLP-1R agonist Liraglutide induces the UPR in the hearts of mice fed with a high-fat diet for 32 weeks (Noyan-Ashraf et al. 2013).

It has been proposed that GLP-1 could restore high glucose-ER stress induction and enhance the activation of UPR in endothelial cells. We tested this hypothesis and we demonstrated that GLP-1R signalling activation attenuated the expression of most of the ER stress markers studied in response to sustained high glucose in HUVEC cells, however this reduction was not statistically significant. This data suggested again that GLP1 alone was not sufficient to counteract the negative effects of hyperglycaemia on ER function. Particularly, high glucose activated the three arms of the UPR, and GLP-1 treatment in the same condition slightly attenuated this activation, but not in a significant statistical manner. Only when GLP-1 was used in combination with Ruboxistaurin, the pattern of UPR gene expression in HUVEC cells cultured under high glucose was restored to basal condition. Chronic high glucose increased Perk, Chop, Atf4, Bim and Erol1a transcripts as well as Ire1α, Traf2 and Xbpsp, and this pattern of gene expression was not prevented by GLP-1 alone, but only when the incretin hormone was used in combination with Ruboxistaurin we observed a general reversion of the phenotype.

These data are in line with a previous study in pancreatic β-cells where GLP-1R activation reduced eIF2α phosphorylation as a consequence of an increased expression of Perk, Chop and other ER stress marker (Yusta et al., 2006). Moreover our results are consistent with previous studies demonstrating that, the activation of GLP-1R in HUVECs through the agonist Liraglutide can prevent high glucose-induced protein expression of the UPR markers Bip and Ire1α (Schisano et al. 2012).

CONCLUSIONS

This study, conducted on an *in vitro* model system of primary HUVECs, adds molecular insights and concerns about the GLP-1 actions in the endothelium and supports the observation that GLP-1 could directly attenuate endothelial hyperglycaemia-induced damage.

In particular, these are the main conclusions of the present study:

(i) Hyperglycaemia causes a reduction in the protein levels of GLP-1R in HUVECs, producing an impairment of the GLP-1 signalling, due to an increase of PKCβ. GLP-1R protein levels are downregulated in endothelial cells maintained in a sustained hyperglycaemic state and this observation nicely correlates with an increase in PKCβ protein and mRNA levels. This effect causes an impairment of GLP-1 canonical signalling, which is corroborated by the inability of GLP-1 to phosphorylate AKT and also by an increase in PKA subunits mRNA levels, indicating a decrease in PKA activity (Rajan et al. 2015). This last effect is counteracted by GLP-1 only when PKCβ is blocked by Ruboxistaurin under hyperglycaemia, indicating a recovery of the GLP-1R pathway.

(ii) Hyperglycaemia causes endothelial resistance to GLP-1 antioxidant actions, which are recovered by specifically blockage of PKCβ. GLP-1 added at pharmacological concentrations initiates the antioxidant cascade in HUVECs in normal glucose conditions through different pathways: (1) the nuclear translocation of NRF2, which, in turn, triggers the transcriptional activation of Nrf2 targets Hmox-1 and Nqo-1, (2) the expression of Foxo transcription factors, which consequently activates the scavengers Sod1, Sod2 and Cat. However, in the presence of oxidative stress stimulus, induced by hyperglycaemia, GLP-1 is able to enhance this activation only in presence of the PKCβ inhibitor, Ruboxistaurin.

(iii) The apoptotic phenotype of HUVECs caused by hyperglycaemia is recovered by GLP-1 only in the presence of Ruboxistaurin. Under hyperglycaemic conditions there is an alteration in the expression of different proliferative and/or apoptotic markers, such as Bcl-2, Bax, Caspase 3 and Cdkn1a. The phenomenon of endothelial resistance to GLP-1 positive actions has been observed also for the proliferative capacities of HUVECs, as GLP-1 is able to restore the expression of the studied markers under chronic high glucose only when PKCβ activation is blocked by the presence of its inhibitor Ruboxistaurin.

(iv) Hyperglycaemia leads to the disruption of the ER stress response in HUVECs and GLP-1 counteracts this effect only in the presence of PKCβ specific inhibitor. Under the condition of sustained high glucose, there is a maladapted response of several genes involved in the Unfolded Protein Response, like Bip, Perk, Ire1α or Atf6 among others. Novelty, as no other reports describe the direct capacity of GLP-1 to reverse this effect, we demonstrate that GLP-1, only in the presence of Ruboxistaurin, could ameliorate ER stress in HUVEC cells. These results also are in accordance with the idea of endothelial resistance to GLP-1 beneficial actions.

As final conclusion, this study supports the concept that under hyperglycaemic conditions an endothelial resistance of GLP-1 antioxidant, proliferative and ER stress recovery actions exists. This effect is explained by a downregulation of GLP-1R expression which could, probably, be related to the PKCβ overexpression.

These findings can have important clinical repercussions for the management of diabetic patients. They also provide further evidence for the potential cardiovascular effects of GLP-1 and could serve as a starting point to further investigate the protective mechanisms and to explore GLP-1 treatment options to delay hyperglycaemia-related endothelial damage.

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ANNEX 1:
List of SYBR Green primers and TaqMan probes

ANNEX 1

List of primers used in qRT-PCR with SYBR Green®.

Gene	*Forward sequence*	*Reverse sequence*
Bip	GACCTGGGGACCACCTACTC	TCAGGAGTGAAGGCGACAT
Perk	TGTCGCCAATGGGATAGTGACGAA	ATCCGGCTCTCGTTTCCATGTCT
Atf4	GGGTTCTCCAGCGACAAGGCTAAG	ACAGGGCATCCAAGTCGAACTC
Atf6	ATGTCTCCCCTTTCCTTATATGGT	AGGCTTGGGCTGAATTGAA
Chop	AGGGAGAACCAGGAAACGGAAACA	CCTGCTTGAGCCGTTCATTCTCT
Bim	AGGCCTTCAACCACTATCTCAGT	ATTCAAAAATACCCTCCTTGCAT
Erol1a	ATTCTTGTTTGGCCTCCTGG	AGTAACCACTAACCTGGCAGAAG
Actinβ	CAGCCATGTACGTTGCTATCCAGG	GGTCCAGACGCAGGATGGCATG
PKCβ	ATGACCAAACACCCAGGCAA	CATGGATGCAACTTGGCAAT
GLP-1R	GGCTACGCACTCTCCTTCTC	AGCCGGATAATGAGCCAGT
β arrestin-1	ACAGCTCAGTACAAGTGCCC	GCTAGAGGCCAAGTTCGTGT
β arrestin-2	AGAAGTCGAGCCCTAACTGC	CACTTTGCGGTCCTTCAGGT
Nox4	AGGGCCAGAGTATCACTACCTCCAC	TGATCCTCGGAGGTAAGCCAAG
Txnrd	ACGTTACTTGGGCATCCCTG	AGAAATCCAGCGCACTCCAA
Txnip	GCAGTGCAAACAGACTTCGG	TCACCTGTTGGCTGGTCTTC
p22^{-phox}	TGGTGCCTACTCCATTGTGG	GTACTTCTGTCCCCAGCGCT
p47^{-phox}	ATGAAAGCAAAGCGAGGCTG	CGACGTATGGCTCACCTGC
p67^{-phox}	CTGGTGCCCCTTTCAGAAGA	AAGCCTTGGTCACCCACTGT
Xbp1	CGCTGAGGAGGAAACTGAA	CACTTGCTGTTCCAGCTCACTCAT
Xbp-^{sp}	CCGCAGCAGGTGCAGG	GAGTCAATACCGCCAGAATCCA
GADPH	TCCTCTGACTTCAACAGCGACACC	TCTCTCTTCCTCTTGTGCTCTTGC
p53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
Cdkn1b	TCTGAGGACACGCATTTGGT	GAAGAATCGTCGGTTGCAGG
Caspase 3	TGGAGGCCGACTTCTTGTATG	ATGGCACAAAGCGACTGGA
TRAF2	GGTACTGCTCCTTCTGCCTG	AAGGCCGAACTGCTTTCTAAAATA
Ire1a	CCGGCCTCGGGATTTTTGG	TTTGATTGAGCCTGTCCTC
PRKACA	TGAGCAAAGGCTACAACAAGG	CAAGTCAGAGCTGAAGTGGGAA
PRKACB	TTCCTTGTTCGACTGGAGTATG	GAGCTGCATAGAACCGTGC
PRKAR1A	CAGTGCAGTTTGAAGATGGGC	GGCAGCACGAGGACGATT
PRKAR2A	AGTCTGGCGAAGTGAGCATC	CAGGGCAAGCTCTCCAAAGT
PRKAR1B	GACTGTATCGTCCACCTCTGC	GGAGTCCGACTGTGAGTTTGA
PRKAR2B	ACAAGGCGTGCCTCAGTATG	CAGCAGGATGTCTTTGCAAGC
Foxo3	TCACGCACCAATTCTAACGC	CACGGCTTGCTTACTGAAGG
Sod2	GGCCTACGTGAACAACCTGA	CAGGACGTTATCTTGCTGGG

List of TaqMan® probes (Applied Biosystems) used in qRT-PCR.

Gene	*Reference*
Bax	Hs00180269_m1
Bcl-2	Hs00608023_m1
Cdkn1a	Hs00355782_m1
Foxo1	Hs01054576_m1
Foxo4	Hs00936217_g1
Sod1	Hs00533490_m1
Nrf2	Hs00975961_g1
Nqo-1	Hs00168547_m1
Cat	Hs00156308_m1
Hmox-1	Hs01110250_m1