The G protein coupled receptor kinase 2 plays an essential role in beta-adrenergic receptor-induced insulin resistance

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Aims Insulin (Ins) resistance (IRES) associates to increased cardiovascular risk as observed in metabolic syndrome. Chronic stimulation of β-adrenergic receptors (βAR) due to exaggerated sympathetic nervous system activity is involved in the pathogenesis of IRES. The cellular levels of G protein coupled receptor kinase 2 (GRK2) increase during chronic βAR stimulation, leading to βAR desensitization. We tested the hypothesis that GRK2 plays a role in βAR-induced IRES.

Methods and results We evaluated Ins-induced glucose uptake and signalling responses in vitro in cell overexpressing the β2AR, the GRK2, or the catalytically dead mutant GRK2-DN. In a model of increased adrenergic activity, IRES and elevated cellular GRK2 levels, the spontaneously hypertensive rats (SHR) we performed the intravenous glucose tolerance test load. To inhibit GRK2, we synthesized a peptide based on the catalytical sequence of GRK2 conjugated with the antennapedia internalization sequence (Ant-124). Ins in human kidney embryonic (HEK-293) cells causes rapid accumulation of GRK2, tyrosine phosphorylation of Ins receptor substrate 1 (IRS1) and induces glucose uptake. In the same cell type, transgenic β2AR overexpression causes GRK2 accumulation associated with significant deficit of IRS1 activation and glucose uptake by Ins. Similarly, transgenic GRK2 overexpression prevents Ins-induced tyrosine phosphorylation of IRS1 and glucose uptake, whereas GRK2-DN ameliorates glucose extraction. By immunoprecipitation, GRK2 binds IRS1 but not the Ins receptor in an Ins-dependent fashion, which is lost in HEK-GRK2 cells. Ant-124 improves Ins-induced glucose uptake in HEK-293 and HEK-GRK2 cells, but does not prevent GRK2/IRS1 interaction. In SHR, Ant-124 infusion for 30 days ameliorates IRES and IRS1 tyrosine phosphorylation.

Conclusion Our results suggest that GRK2 mediates adrenergic IRES and that inhibition of GRK2 activity leads to increased Ins sensitivity both in cells and in animal model of IRES.

1. Introduction

The sympathetic nervous system is a fine regulator of several metabolic pathways, by controlling lipolysis, lipoprotein metabolism, and glucose homeostasis on adipose tissue, liver and skeletal muscle through the β-adrenergic receptors (βARs).1–3 Its ability to modulate metabolic responses to insulin (Ins) together with the pivotal role in the control of vascular tone make it appear as a major determinant for insulin (Ins) together with the pivotal role in the control of liver and skeletal muscle through the metabolism, and glucose homeostasis on adipose tissue, metabolic pathways, by controlling lipolysis, lipoprotein metabolism. Indeed, epidemiological studies have shown the association of β2AR gene variants with obesity, type 2 diabetes, and hypertriglyceridemia.9–11 In fact, at the present time, very little is known about the molecular mechanisms involved in β2AR-induced IRES. Interestingly, though, a molecule that is intimately related to βAR signalling and desensitization, the G protein coupled receptor kinase 2 (GRK2) has also been implicated in regulation of the Ins receptor signalling leading to IRES.12–14 According to the canonical pathway, GRK2 induces desensitization by phosphorylating the agonist-activated βARs.15 GRK2 exerts inhibitory effects on Ins signaling, through mechanisms that are still disputed. In animal model of diabetes, GRK2 inhibition through small peptides designed on its catalytic domain restores glucose tolerance and Ins sensitivity.17 This result suggests
that GRK2 phosphorylates a not yet defined substrate to induce impaired glucose tolerance. It is interesting to note that GRK2 is ubiquitously expressed in mammalian tissues, and in conditions characterized by chronic adrenergic activation, such as human congestive heart failure.19–21

Figure 1
Insulin mediated glucose uptake in HEK-293 cells. (A) HEK-293 were stimulated with Ins 100 nM for 30 min. L6 cells served as positive control. Plasma membrane fractions were subjected to SDS-PAGE using anti-Glut1 or anti-Glut4 antibodies. HEK-293 express Glut1 (upper panel) but not Glut4 (middle panel). Data from immunoblot were quantified and corrected by Gapd densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 59.08; *P < 0.05 vs. L6; Bonferroni post hoc test. (B) HEK-293 cells were stimulated with Ins at different time points, ranging from 10 min to 24 h in the presence or absence of the βAR agonist ISO (10 nM for 12 h) and the rate of glucose uptake was determined by 3H-2-DG. The graphs show the mean ± SEM of three independent experiments. F = 34.31; *P < 0.05 vs. Ins 100 nM; #P < 0.05 vs. control; Bonferroni post hoc test. (C) We selected two clones of HEK-293 cells with stable overexpression of the β2AR gene (HEK-β2AR). Cells were stimulated with Ins (100 nM for 30 min) in the presence or absence of chronic ISO pre-treatment (12 h). Graphs show the mean ± SEM of three independent experiments. F = 66.26; *P < 0.05 vs. HEK-293 Ins; Bonferroni post hoc test.

Figure 2
Ins and ISO affect GRK2 expression levels. (A) HEK-293 cells were exposed to ISO 10 nM for indicated time points. Total cell lysates were analysed by western blotting for GRK2 with anti-GRK2 antibody. Immunoblots were quantified by densitometry. GRK2 was corrected by actin densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 45.58; *P < 0.05 vs. HEK-293 0 h; Bonferroni post hoc test. (B) HEK-293 cells were exposed to Ins for indicated time points. Cellular GRK2 and actin content were evaluated. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 14.51; *P < 0.05 vs. HEK-293 0 h; Bonferroni post hoc test. (C) Cells were stimulated with Ins for 30 min in the presence or absence of ISO pretreatment (12 h) and GRK2 was evaluated by western blot. Immunoblots were quantified by densitometry and GRK2 was corrected by actin. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 2.258; *P < 0.05 vs. HEK-293 0 h; Bonferroni post hoc test.
Figure 3 GRK2 overexpression induces IRS. 

(A) Cellular GRK2 content was evaluated in HEK-293, HEK-β2AR, HEK-GRK2-WT, and HEK-GRK2-DN. Immunoblots were quantified by densitometry and GRK2 levels were corrected by actin densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. *F = 37.15, P < 0.05 ANOVA; *P < 0.05 vs. HEK-293; Bonferroni post hoc test.

(B) HEK-293, HEK-GRK2-WT, and HEK-GRK2-DN cells were stimulated with Ins 100 nM for 30 min. Data from immunoblots were quantified by densitometric analysis. GRK2 levels were corrected by actin densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. *F = 66.83, P < 0.05 ANOVA; *P < 0.05 vs. HEK-293; #P < 0.05 vs. HEK-293 Ins; Bonferroni post hoc test.

(C) Ins-induced (30 min) glucose uptake was determined in HEK-293, HEK-GRK2WT, and HEK-GRK2-DN. Graphs show the mean ± SEM of three independent experiments. *F = 56.06; P < 0.05 ANOVA; *P < 0.05 vs. HEK-293; Bonferroni post hoc test.
and hypertension, it is upregulated through chronic activation of βAR.

All the above considerations support the hypothesis that GRK2 participates in the onset of IRES associated with βAR chronic activation. In the present study, we tested this hypothesis in cellular models of enhanced βAR activation and GRK2 accumulation through stable overexpression of β2AR or GRK2 in human kidney embryonic (HEK-293) cultured cells. We show that β2AR overexpression impairs Ins signalling cascade in a GRK2-dependent manner. We found a regulatory network between Ins and GRK2 protein levels in which GRK2 kinase activity is causally related to IRS1 degradation and IRES. Finally, in an animal model of increased sympathetic activity and IRES chronic GRK2 inhibition results in amelioration of glucose tolerance.

2. Methods

2.1 GRK2 cloning, cell culture, western blot, and 2-deoxy glucose uptake analysis

For cell culture, HEK-293 cells were used, since they are easily transfectable, and present Ins-induced glucose extraction. The βAR agonist isoproterenol (ISO) was used to induce βAR activation. The human GRK2 (GRK2-WT) gene was cloned from cDNA obtained from peripheral blood lymphocyte of a voluntary donor. Written informed consent was obtained from the donor, according to the
principles of the Declaration of Helsinki and approved by the Ethics Committee of our Institution. Kinase dead mutant of GRK2 (GRK2-DN) is a kind gift of Dr Walter J. Koch. 2-Deoxy glucose uptake (2-DG) was assayed by radiometric analysis on cell extracts. For overlay assay, GRK2 and IRS1 purified proteins were subjected to SDS/PAGE and blotted on nitrocellulose, which was then incubated with either GRK2 or IRS1 purified protein in binding buffer and immunodetected with specific antibodies. Ant-124, Ant-107, and scramble peptides were synthesized loading the synthesizer with the pre-assembled peptide–resin. Conjugates were cleaved from the resin by treatment with trifluoroacetic acid (70%) and purified by preparative RP-HPLC (Lichrospher® 100 C18, 10 μm).

For phosphorylation assay, cytosolic extracts or purified GRK2 were incubated with rhodopsin or IRS1 in binding buffer plus [32P]-γ-ATP; reactions were subjected to PAGE, and the gel dried and subjected to autoradiography. Two peptide inhibitors, Ant-124 and Ant-107, were synthesized as indicated in Supplementary material online, Methods.

2.2 In vivo study and statistical analysis

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication 85-23, rev. 1996) and approved by the Ethics Committee of the Federico II University. Experiments were carried on 12-week-old male normotensive Wistar Kyoto (WKY, n = 6) or spontaneously hypertensive SHR rats (n = 12). Intravenous glucose tolerance test (IVGTT) was performed after 4 week of treatment with Ant-124.

All values are presented as mean ± SEM. One-way and two-way ANOVA were performed as appropriated to compare the different parameters with controls or among the different groups. A significance level of P < 0.05 was assumed for all statistical evaluations. Statistics were computed with GraphPad Prism software. Extended details of experimental procedures are provided in Supplementary material online, Pages S1–S5.

3. Results

3.1 Ins-induced glucose uptake in HEK-293 cells

To confirm the presence of an Ins sensitive glucose transport in HEK-293 cells, we verified the expression of glucose transporters GLUT1 but not GLUT4 (Figure 1A). As shown in Figure 1B, Ins induces a significant time-dependent increase in 2-DG uptake which is maximal at 30 min and then exhibited a progressive decrease. Figure 1B also shows that ISO pretreatment (10 nM for 12 h), significantly inhibits Ins dependent 2-DG uptake, indicating that chronic βAR stimulation induces IRES. To investigate the role of β2AR in IRES, we selected a clone of HEK-293 cells with stable overexpression of the β2AR gene (HEK-β2AR) which also presents impaired Ins-induced glucose uptake (Figure 1C).

3.2 Ins increases GRK2 protein levels

We then confirmed that chronic βAR stimulation by ISO increases GRK2 levels (Figure 2A). We also found that Ins significantly increased in a time-dependent manner the...
Figure 6  GRK2 inhibition improves glucose homeostasis. (A) HEK-293 and HEK-GRK2 cells were pretreated with Ant-124 for 30 min. Subsequently, cells were stimulated with Ins (30 min) and 2-DG uptake was measured. Graphs show the mean ± SEM of six independent experiments. HEK-293: F = 12.76; P < 0.05 ANOVA; * P < 0.05, Bonferroni post hoc test. HEK-GRK2: F = 14.85; # P < 0.05, Bonferroni post hoc test. (B) HEK-293 and HEK-GRK2 cells were pretreated with Ant-124 for 30 min, then stimulated with Ins (100 nM for 30 min). Total cell lysates were immunoprecipitated using anti-IRS1 antibody, and subjected to western blot using anti-GRK2 and anti-IRS1 antibodies. Data from immunoblots was quantified by densitometric analysis. GRK2 levels were corrected by IRS1 densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 38.99; P < 0.05 ANOVA; * P < 0.05 vs. HEK-293; # P < 0.05 vs. HEK-293 Ant-124; P < 0.05 vs. HEK-GRK2 Ant-124; Bonferroni post hoc test. (C) HEK-293 and HEK-GRK2 cells were pretreated with Ant-124 for 30 min, then stimulated with Ins (100 nM for 30 min). Total cell lysates were immunoprecipitated using anti-IRS1 antibody and subjected to western blot using anti-P-Tyr and anti-IRS1 antibodies. Data from immunoblots was quantified by densitometric analysis. P-Tyr levels were corrected by IRS1 densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 66.13; P < 0.05 ANOVA; * P < 0.05 vs. HEK-293; # P < 0.05 vs. HEK-293 Ant-124; P < 0.05 vs. HEK-GRK2 Ant-124; Bonferroni post hoc test. (D) To investigate the effect of Ant-124 treatment on GRK activity in SHR, we assessed rod outer segment 32P incorporation after exposure to 32P g-ATP and 100 μg of cytosolic protein from skeletal muscle extract from SHR-I, SHR-C, and WKY rats. Incorporated 32P
cellular contents of GRK2 (Figure 2B). HEK-β2AR cells express higher GRK2 levels, and in these cells, ISO and Ins fail to affect its levels (Figure 2C).

### 3.3 GRK2 overexpression induces IRES

To explore the role of GRK2 in Ins signalling apart from β2-AR stimulation, we have selected HEK-293 cells with a severe impairment in IRES uptake (HEK-GRK2-DN). To be consistent with the previous results, we selected clones of HEK-GRK2-WT and HEK-GRK2-DN with levels of overexpression similar to that observed in HEK-β2-AR cells (Figure 3A). In both clones, GRK2 levels were not affected by Ins (Figure 3B). HEK-GRK2-WT presents a severe impairment in Ins 2-DG uptake (Figure 3C). Interestingly, the overexpression of GRK2-DN enhances glucose extraction in HEK cells (Figure 3C).

### 3.4 GRK2 inhibits Ins-induced activation of IRS1

We then investigated the crosstalk between GRK2 and Ins receptor β subunit (β-IR) or IRS1. As presented in detail in Supplementary material online, β-IR did not interact with GRK2, but GRK2 overexpression altered tyrosine phosphorylation of β-IR and down regulated it (see Supplementary material online, Page S6 and Figure S1).

Ins induced a rapid tyrosine phosphorylation of IRS1 in HEK-293 cells, but it was inhibited in HEK-GRK2-WT cells (Figure 4A). Interestingly, HEK-GRK2-DN cells presented a significant increase of tyrosine phosphorylation of IRS1 (Figure 4A). We then explored the interaction of GRK2 and IRS1. The cells were treated with Ins at different time points and IRS1 was immunoprecipitated from whole extracts to visualize the associated GRK2 by western blot. In HEK-293 cells, endogenous GRK2 and IRS1 interaction was detected at low levels in the basal state but was markedly enhanced after Ins exposure in a time-dependent manner (Figure 4B). In the un-stimulated HEK-β2-AR and HEK-GRK2-WT cells, there was a significantly higher amount of GRK2 associated with IRS1 in resting cells, and this interaction was not further increased by Ins stimulation (Figure 4C and D). Similar results were obtained in HEK-GRK2-DN cells (data not shown).

### 3.5 Direct interaction of GRK2 and IRS1 by purified proteins

The data shown in Figure 4 suggests that GRK2 is part of an intracellular complex including IRS1. Therefore, we explored the ability of purified GRK2 to bind purified IRS1 in an in vitro set-up. Figure 5 shows the results of the overlay assay using purified IRS1 and GRK2; we observed the interaction between the two proteins using each purified protein either as probe or proband (Figure 5A). Next, to investigate whether this association resulted in IRS1 phosphorylation by GRK2, we performed an in vitro 32P labelling assay with purified proteins. Competing purified IRS1 with the β-IR immunoprecipitated from Ins-stimulated HEK-293 cells, we obtained a reference for molecular weight and phosphorylation of IRS1. IRS1 was also challenged with GRK2, showing the ability of the kinase to phosphorylate IRS1. In order to verify the role of intact GRK2 catalytic activity, we synthesized two peptides, Ant-124 and Ant-107, partially modifying previously described GRK2 inhibitors (Supplementary material online, Page S4). We show that peptide inhibitors pretreatment abrogates GRK2 induced IRS1 phosphorylation (Figure 5B). Moreover, to test the hypothesis of a direct interaction between IRS1 and GRK2, we performed an in vitro binding assay with purified proteins in the presence or absence of Ant-124. Our results demonstrated that the interaction between IRS1 and GRK2 cannot be broken by either one of the inhibitors (Figure 5C).

### 3.6 GRK2 inhibition improves glucose homeostasis

In order to verify the biological relevance of GRK2 kinase activity in Ins signalling, we inhibited GRK2 in cells by incubation with Ant-124. This manoeuvre ameliorated in a dose-dependent manner glucose uptake in HEK-293 cells (Figure 6A). As expected, in HEK-GRK2 cells glucose uptake was impaired and Ant-124 corrected this alteration in a dose-dependent manner (Figure 6A). The exposure to Ant-124 was not accompanied by inhibition of endogenous association of IRS1 to GRK2 (Figure 6B), but Ant-124 enhanced Ins-induced IRS1 tyrosine phosphorylation (Figure 6C). Similar results were replicated using the second peptide inhibitor Ant-107 (see Supplementary material online, Page S6 and Figure S2).

### 3.7 In vivo results

#### 3.7.1 Animal characteristics

To determine the role of GRK2 on Ins signalling, we tested the effects of chronic administration of Ant-124 in SHR rats (SHR-I n = 6) compared with scramble treated SHR (SHR-C n = 6), whereas age-matched WKY were used as normotensive control (n = 6). As expected, in SHR-C, mean arterial pressure (MAP) was higher than in WKY (182 ± 16 vs. 116 ± 20 mmHg, P < 0.05). Moreover, we found that SHR-I presented lower albeit not normal blood pressure (MAP: 135 ± 6 mmHg, P < 0.05 vs. SHR-C). This result was accompanied by no differences in heart rate, body weight (data not shown), fasting blood glucose (WKY: 108 ± 8.5; SHR-C: 88.6 ± 8.2, SHR-I: 104 ± 9.5 mg/dL), and Ins (WKY: 0.28 ± 0.03; SHR-C: 0.28 ± 0.04, SHR-I: 0.44 ± 0.08 ng/dL) levels between groups.

In ROS was visualized by autoradiography and quantified by densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 130.2, P < 0.05 ANOVA; *P < 0.05 vs. WKY; Bonferroni post hoc test. (E) To examine the association between GRK2 and IRS1 in skeletal muscle from SHR after IVGTT, total cell lysate from SHR-I, SHR-C, and WKY samples were immunoprecipitated using anti-IR51 antibody and subjected to western blotting with anti-GRK2 or anti-IRS1. GRK2 levels were corrected by IRS1 densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 11.67, P < 0.05 ANOVA; *P < 0.05 vs. WKY; Bonferroni post hoc test. (F) To assess IRS1 tyrosine phosphorylation in skeletal muscle after IVGTT, total cell lysate from SHR-I, SHR-C, and WKY samples were immunoprecipitated using anti-IRS1 antibody and subjected to western blotting with anti-P-Tyr or anti-IRS1 antibodies. P-Tyr levels were corrected by IRS1 densitometry. Each data point in the graph represents the mean ± SEM of three independent experiments. F = 68, P < 0.05 ANOVA; *P < 0.05 vs. WKY; **P < 0.05 vs. SHR IVGTT; Bonferroni post hoc test.
3.7.2 Intravenous glucose tolerance test
Glucose load caused a similar peak in blood glucose levels between groups (WKY: 258 ± 45; SHR-C: 192 ± 48, SHR-I: 227 ± 39 mg/dL). In response to glucose challenge, Ins levels increased by 159 ± 13% in WKY. In SHR-C, the increase was significantly greater 515 ± 109% (P < 0.03). Ant-124 treatment in SHR ameliorated the Ins response, which was increased by 274 ± 32%, and therefore intermediate between SHR-C (P < 0.03) and WKY (P < 0.05). To better correlate the insulin release to the increase in blood glucose, we calculated the product of plasma glucose and Ins concentration during GTT, which is an index of whole body IRES. According to this parameter, we found that SHR-C were more Ins resistant than WKY (136 ± 15 vs. 78 ± 6) and that ANT-124 ameliorated their IRES (76 ± 18, P < 0.03 vs. SHR-C).

3.7.3 Muscle biochemistry
In SHR, the skeletal muscle content of GRK2 was higher than that observed in normotensive WKY rats (Supplementary material online, Figure S3 and Page S11). IVGTT caused an accumulation of GRK2 in WKY but not in SHR. Treatment with Ant-124 did not change the muscle content of GRK2 in SHR (Supplementary material online, Figure S3 and Page S11). To explore the effects of GRK2 inhibition on tissue metabolism, we assayed the interaction of GRK2 and IRS1 in the skeletal muscle of WKY and SHR. This latter showed increased GRK2 activity which was inhibited by chronic infusion of Ant-124 (Figure 6D). In WKY muscle as in HEK-293 cells, GRK2 and IRS1 interacted and such interaction was induced by IVGTT (Figure 6E). In SHR-C and SHR-I, basal interaction of GRK2 and IRS1 was already maximal and not induced by IVGTT (Figure 6E).

IVGTT increased pTyr of IRS1, which was attenuated in SHR-C, but not in SHR-I (Figure 6F).

4. Discussion
Alterations of glucose homeostasis and IRES are often associated with pathological conditions characterized by excessive activation of sympathoadrenal nervous and sustained stimulation of βARs. The occurrence of an unfavourable metabolism worsen the prognosis of cardiovascular diseases; therefore, the identification of the underlying mechanisms is pivotal in the therapeutic perspective of finding the specific treatment. Indeed, it is known that chronic catecholamine treatment inhibits Ins mediated glucose uptake, but, presently, the mechanism by which βAR activation induces IRS is largely unknown. Much data have recently demonstrated a complex interplay between βAR and Ins signalling, suggesting that proteins involved in βAR signalling can also participate in the Ins cascade. In particular, growing evidence supports the notion that GRK2 beside desensitizing the βAR is also a negative regulator of Ins metabolic signals, although the mechanism through which the kinase exerts its inhibitory effect is still controversial. In particular, Usui et al. showed that GRK2 negatively regulates Ins-stimulated glucose transport in 3T3L1 by binding to Gαq/11. They stated that inhibition is independent of GRK2 kinase activity and did not affect Tyr phosphorylation of the Ins receptor; these authors also proposed that chronic endothelin1 stimulation, but not Ins, induces GRK2 kinase activity and may cause IRES by enhancing IRS1 serine phosphorylation via a not yet defined other kinase. Anis et al., on the contrary, have demonstrated that inhibitory peptides of GRK2 kinase activity in animal models of type 2 diabetes increase Ins sensitivity and improve glucose homeostasis. Recently, the relationship between GRK2 expression levels and Ins-dependent and -independent glucagon synthesis in liver cells has been demonstrated. In this model, Ins induces translocation of GRK2 from cytosol to the plasma membrane and depletion of cellular GRK2 reduces IRS1 serine phosphorylation and increases IRS1 tyrosine phosphorylation, although the authors did not identify the kinase responsible for IRS1 phosphorylation.

Our study produces an advancement of current knowledge, by clearly defining the role of GRK2 in βAR-induced IRES. First of all, we demonstrate that GRK2 is relevant to Ins signalling in cells. Although HEK-293 cells do not express GLUT4, they are widely used to study Ins signalling. Ins induces an increase of GRK2 expression levels and causes GRK2–IRS1 association in a time-dependent manner. A similar rapid increase in GRK2 cellular levels was described by Salcedo et al., in response to the Ins cognate hormone IGF-1. These authors have shown that GRK2 protein accumulation is induced by IGF-1 via inhibition of GRK2 ubiquitination by MDM2 thus preventing proteasome degradation. In our hands, the GRK2 accumulation after Ins is within the same time lapse of that induced by IGF-1. The similarities between the Ins and IGF-1 signalling make us believe that, also in our system, GRK2 accumulation is due to inhibition of GRK2 degradation, although we did not perform ubiquitination studies.

A second important new acquisition of our study is that GRK2 induces a direct phosphorylation of IRS1 in serum/threonine and inhibits IRS1 tyrosine phosphorylation. This mechanism provides a feedback that allows for the termination of the Ins receptor signalling, a negative regulation that is similar to that mediated by GRK2 on G protein coupled receptors. Indeed, stable GRK2 overexpression affects Ins action indicating a direct correlation between GRK2 expression and IRES.

Thirdly, we demonstrate that chronic activation of βAR signalling through ISO or the marked overexpression of β2ARs causes the development of IRES through an increase of GRK2 expression levels. GRK2 inhibition through peptides can ameliorate Ins response in this experimental model.

We describe a modified version of the GRK2 inhibiting peptides previously published by Anis et al., which enclose in the carboxyl terminus the amino acid sequence from the antennapedia protein, which allows for intracellular localization. This structural modification is aimed to force tissue extraction of the peptide and is well tolerated by rats in vivo. Interestingly, in SHRs, chronic treatment with Ant-124 not only leads to an amelioration of the glucose homeostasis and IRS1 tyrosine phosphorylation, but also to the reduction of the blood pressure levels. Amelioration of IRES is not secondary to improved haemodynamics, since the favourable metabolic effects of the peptide are also seen in vitro, where there are no haemodynamic changes. On the contrary, our results do not clarify whether the amelioration of the glucose handling by the hypertensive rats causes the reduction of blood pressure. It has been demonstrated that excessive vascular GRK2 may impaire vasorelaxation, thus leading to increase peripheral resistance and
blood pressure, but it has still to be demonstrated that GRK2 inhibition ameliorates vasorelaxation. This is the object of ongoing investigation.

The relevance of our finding to pathophysiological conditions is associated to the observation that elevated GRK2 levels are found in both humans and animal models of spontaneous hypertension. This latter is a very well-defined IRES condition, and therefore the observation that GRK2 is increased in this condition proposes this molecule as a possible underlying molecular mechanism.

Growing evidence indicates that GRK2 phosphorylates non-receptor substrates and also interacts with a variety of signalling proteins such as PI3K, Gq, and GTP; so it is likely that GRK2 function in cells goes beyond receptor desensitization. Giving its role in the set-up of sympathetic induced IRES, we propose GRK2 as a possible target for treating IRES in all those conditions in which this metabolic disorder occurs in association with exaggerated sympathetic tone.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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