TRIB3 R84 Variant Is Associated With Impaired Insulin-Mediated Nitric Oxide Production in Human Endothelial Cells

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Background—In the endothelium, insulin promotes nitric oxide (NO) production, through the insulin receptor/IRS-1/PI3-Kinase/Akt/eNOS signaling pathway. An inhibitor of insulin action, TRIB3, has recently been identified which affects insulin action by binding to and inhibiting Akt phosphorylation. We have recently described a Q84R gain-of-function polymorphism of TRIB3 with the R84 variant being associated with insulin resistance and an earlier age at myocardial infarction.

Methods and Results—To investigate the TRIB3 R84 variant impact on endothelial insulin action, we cultured human umbilical vein endothelial cells (HUVECs) naturally carrying different TRIB3 genotypes (QQ-, QR-, or RR-HUVECs). TRIB3 inhibitory activity on insulin-stimulated Akt phosphorylation and the amount of protein which was communoprecipitable with Akt were significantly greater in QR- and RR- as compared to QQ- HUVECs. After insulin stimulation, Akt and eNOS activation as well as NO production were markedly decreased in QR- and RR- as compared to QQ-HUVECs. TRIB3 molecular modeling analysis provided insights into the structural changes related to the polymorphisms potentially determining differences in protein-protein interaction with Akt.

Conclusions—Our data demonstrate that the TRIB3 R84 variant impairs insulin signaling and NO production in human endothelial cells. This finding provides a plausible biological background for the deleterious role of TRIB3 R84 on genetic susceptibility to coronary artery disease. (Arterioscler Thromb Vasc Biol. 2008;28:1355-1360)

Key Words: insulin signaling • endothelium • nitric oxide synthase • genetics • HUVEC

Endothelial dysfunction represents one of the earliest steps in atherosclerosis development, and it is often associated with insulin resistance-related features, such as diabetes, obesity, and hypertension.1,2 Endothelium is an insulin target tissue: in endothelial cells, insulin activating a signaling pathway involving insulin receptor (IR), insulin receptor substrate-1 (IRS-1), phosphatydilinositol 3-kinase (PI 3-kinase), and Akt, leading to endothelial nitric oxide synthase (eNOS) activation, nitric oxide (NO) synthesis, and vasodilation.3,4 Up to Akt activation, this signaling pathway is virtually identical to the pathway mediating insulin-stimulated glucose uptake in tissues implicated in glucose regulation.5 Therefore, molecular defects in this upstream pathway are likely to affect not only insulin-stimulated glucose uptake in typical target tissues but also insulin-stimulated endothelial NO synthesis, thus contributing to both altered glucose homeostasis and endothelial dysfunction.

Several inhibitors of insulin signaling have been recently proposed as possible determinants of insulin resistance.6,7 Among them is TRIB3, a mammalian tribbles homolog also known as TRB3/NIPK, which affects insulin signaling and action by binding to and inhibiting Akt phosphorylation.6,8 We have recently described a TRIB3 missense polymorphism consisting in a substitution of a Glutamine (Q) with an Arginine (R) at position 84 (Q84R), which plays a role in insulin resistance both in vitro and in vivo.8 Transfection of human HepG2 hepatoma cell line with either Q84 or R84 TRIB3 full-length cDNAs revealed that the R84 variant was more effective than the Q84 in reducing insulin mediated Ser473 Akt phosphorylation.8 In the same study it was reported that, in whites, the R84 variant was associated with insulin resistance–related phenotypes including, most importantly in
this specific context, early myocardial infarction.9 Taken together, these data support the idea that, as compared to the wild-type Q84, the R84 variant is more effective in impairing insulin signaling at the Akt level in several target tissues, thus possibly modulating genetic susceptibility to insulin-resistance related clinical outcomes, including endothelial dysfunction. To test this hypothesis, we used as a model primary human umbilical vein endothelial cells (HUVECs) naturally carrying the 3 possible TRIB3 Q84R genotypes (either QQ or QR or RR). These cells represent an in vitro model uniquely suited to determine whether in the endothelium the R84 variant impairs insulin ability to stimulate eNOS activity and NO production, and thus it may cause endothelial insulin resistance. We also carried out a TRIB3 structure modeling analysis to assess how the presence of an arginine in position 84 could affect TRIB3 molecular structure and thus the interaction between TRIB3 and the Akt PH domain.

Methods

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Cell Culture

Umbilical cords were obtained from randomly selected healthy mothers delivering at the Pescara Town Hospital who had signed a written consent form. Umbilical cord arteries were screened for the polymorphism of interest as described below. Primary HUVECs were obtained and cultured as previously described.9

Genotyping of the Q84R Polymorphism

Genomic DNA was isolated from umbilical cord arteries using the Wizard Genomic DNA Purification kit (Promega, Milan, Italy) according to manufacturer’s protocol. Genotyping was performed by restriction fragment length polymorphism (RFLP) method, as previously reported.8

Modeling of TRIB3 Structure

TRIB3 structure (aa 61 to 338) was modeled by the program MODELLER8 using as a template the amino acid region 5 to 292 of the calcium/calmodulin activated kinase II structure (PDB code 2bdw, chain A) according to the alignment shown in the Online Data Supplements.

Docking of the PH Domain of Akt on TRIB3

Docking of the PH domain of human Akt1 (PDB code 1ung, aa 1 to 117) on the TRIB3 model was made by Hex (version 4.5) using steric and electrostatic correlation to order 25. The best 500 docking results were analyzed to identify the native-like docking using as a criterion the search of the TRIB3/PH complex with the highest residue conservation at the protein/protein binding interface.13

Molecular Dynamics Simulation of TRIB3 Q84 and R84 Variants

The TRIB3 model was used as the starting structure for molecular dynamics (MD) simulations of the Q84 variant of TRIB3. The model of the R84 variant was generated after replacement of glutamine 84 with an arginine and energy minimization of this residue. Distinct MD simulations of wild-type and mutant TRIB3 were performed in explicit solvent at ionic strength of 0.05 mol/L (generated by introduction of sodium and chloride ions to neutralize the protein net charge), under periodic boundary conditions using NAMD (v2.6)12 using the CHARMM 27 parameter set. The two molecular systems were initially minimized for 500 steps (1 fs per step) and equilibrated for 500 steps (allowing movements of water molecules and salt ions and keeping the protein atoms fixed). Afterward, the two systems were minimized for further 500 steps allowing all atoms to move. Subsequently, MD simulation were carried out for 500 000 steps (500 ps) maintaining the temperature at 310 K.

Statistical Analysis

Results are expressed as mean±SD of at least 3 different experiments. Differences were assessed by Student t test and by 2-way ANOVA test. Significance was defined as P<0.05. All analyses were performed using SPSS software program Version 12.0 for Windows.

Results

Impact of TRIB3 R84 Variant on Insulin-Stimulated Tyrosine Phosphorylation of IR and IRS-1, and IRS-1 Association With PI3-Kinase p85 Regulatory Subunit

We tested the impact of TRIB3 R84 variant on insulin-stimulated tyrosine phosphorylation of IR and IRS-1 (supplemental Figure I). Both basal and insulin-stimulated tyrosine phosphorylation of IR and IRS-1 were similar in QQ-, QR-, and RR-HUVECs (supplemental Figure IA and IB). Similarly, no differences in insulin-stimulated binding of IR to IRS-1 were observed in HUVECs carrying the 3 different genotypes (supplemental Figure IC). Finally, no differences were observed in HUVECs carrying the 3 different genotypes in the association of tyrosine-phosphorylated IRS-1 with the PI3-kinase p85 regulatory subunit, an essential step in promoting downstream signaling (supplemental Figure ID).

Impact of TRIB3 R84 Variant on Acute Insulin-Stimulated Akt Phosphorylation

Insulin-induced Akt phosphorylation at both Thr308 and Ser473 was markedly impaired in QQ-, QR-, and RR-HUVECs (supplemental Figure I) with no differences in Akt expression (Figure 1). There is evidence that TRIB3 may block Akt activation by direct protein-protein interaction.6 To test whether an altered protein–protein interaction may underlie the different ability of the 2 variants to affect insulin-stimulated Akt phosphorylation, cell lysates were immunoprecipitated with anti-Akt antibody and then immunoblotted with anti-TRIB3 peptide antibody (supplemental Figure II). The amount of TRIB3 coimmunoprecipitated with Akt was greater in QR- and RR-HUVECs as compared to that observed in QQ-HUVECs (supplemental Figure IIA), thus suggesting that the more efficient Akt inhibition observed in cells carrying the TRIB3 R84 variant is indeed attributable to a stronger protein–protein interaction. It is worth noting that also in the basal state the amount of TRIB3 coimmunoprecipitated with Akt was marginally greater in QR and RR than in QQ-HUVECs, although this difference did not reach statistical significance. Similar results were observed when cell lysates were immunoprecipitated with anti-TRIB3 peptide antibody followed by immunoblotting with anti-Akt antibody (data not shown). No difference in TRIB3 cellular protein content was observed in HUVECs carrying the three different genotypes (supplemental Figure IIB).

Impact of TRIB3 R84 Variant on Acute Insulin-Stimulated eNOS Phosphorylation

Insulin stimulates eNOS activity and subsequent NO production via Akt activation.13,14 eNOS activity is regulated by
phosphorylation at multiple sites, 2 of which, Ser1177 and Thr495, are the best characterized. Ser1177 is rapidly phosphorylated by Akt in response to insulin, resulting in increased eNOS activity and NO production. By contrast, Thr495 is constitutively phosphorylated in endothelial cells, and phosphorylation at this site is thought to be a negative regulator of enzyme activity.15,16 Indeed, insulin stimulation decreases eNOS phosphorylation at Thr495. Insulin-induced stimulation of Ser1177 eNOS phosphorylation was markedly impaired in QR- and RR- as compared to QQ-HUVECs (Figure 2A). On the other hand, insulin-induced reduction in Thr495 eNOS phosphorylation was markedly impaired in QR-, RR-, as compared to QQ-HUVECs, although at basal state eNOS showed a similar phosphorylation at Thr495 in cell lines carrying the 3 different genotypes (Figure 2A). There is evidence that protein phosphatase 1 (PP1) is involved in Thr495 eNOS dephosphorylation via a PI 3-kinase/Akt-dependent pathway.15–18 Therefore, we tested the impact of TRIB3 R84 variant on insulin-stimulated PP1 activation. Insulin-induced PP1 activation was markedly impaired in QR- and RR- as compared to QQ-HUVECs (Figure 2B).

Impact of TRIB3 R84 Variant on Acute Insulin-Stimulated Glycogen Synthase Kinase-3 Phosphorylation
To further confirm that the TRIB3 R84 variant specifically affects Akt substrates, we determined the impact of this variant on acute insulin-stimulated glycogen synthase kinase-3 (GSK-3) phosphorylation. GSK-3 is the first Akt substrate identified and has been implicated in the regulation of glycogen synthesis through a mechanism involving its phosphorylation and inactivation by Akt.19,20 As shown in Figure 3, insulin-stimulated phosphorylation of Ser1018 of GSK-3 was markedly impaired in QR- and RR-HUVECs as compared to QQ-HUVECs.

Impact of TRIB3 R84 Variant on Insulin-Stimulated eNOS Activity
To assess the impact of TRIB3 R84 variant on eNOS activity we monitored the insulin-stimulated conversion of L-[3H]arginine into L-[3H]citrulline. As shown in Figure 4A, basal eNOS activity was not different in HUVECs carrying the 3 different genotypes. However, on insulin stimulation, NO synthesis was increased ∼3-fold above baseline in QQ- but not in QR- and RR-HUVECs where insulin had almost no effect at all on NO synthesis (Figure 4A). To assess the efficiency of calcium-dependent eNOS activation in HUVECs carrying the 3 different genotypes, cells were also exposed to ionomycin, an agent that induces NO production via mobilization of intracellular Ca2+. HUVECs carrying the 3 different genotypes exhibited a comparable increase in NO production in response to ionomycin (Figure 4A). Preincubation with the NOS inhibitor L-NAME before insulin or ionomycin stimulation abolished insulin-stimulated eNOS activity in HUVECs carrying the QQ genotype and induced a significant inhibition in ionomicyn-stimulated NOS activity in all cell groups (Figure 4A).

Impact of TRIB3 R84 Variant on Insulin-Stimulated Intracellular cGMP Levels
Since increased NO synthesis does not necessarily translate into increased NO bioavailability, we measured intracellular cGMP levels, which ought to reflect the action of bioavailable NO. As shown in Figure 4B, basal cGMP levels were not different in HUVECs carrying the 3 different genotypes. However, on insulin stimulation, intracellular cGMP levels were increased ∼2-fold above baseline in QQ- but not in QR- and RR-HUVECs, where insulin had almost no effect at all (Figure 4B). HUVECs carrying the 3 different genotypes showed a comparable increase in cGMP in response to ionomycin (Figure 4B). Preincubation with the NOS inhibitor L-NAME abolished insulin-induced increase of cGMP levels in HUVECs carrying the QQ genotype (Figure 4B).

Molecular Modeling
To identify the regions of TRIB3 involved in the interaction with Akt and to establish a possible direct role for the R84 variant in the observed alteration of Akt regulation, various structures representing the complex formed by TRIB3 and the PH domain of Akt were generated with a computational docking procedure. The best docking solution was retained (Figure 5) after filtering.
out improbable complexes on the basis of a residue conservation score measured at each protein-protein interface. Because the topology of interaction in this complex indicated the absence of a direct interaction between residue 84 of TRIB3 and the PH domain of Akt, this information alone was not sufficient to explain the observed difference of interaction with Akt exhibited by the 2 TRIB3 variants. Therefore, we searched structural differences that could arise in TRIB3 depending on the specific amino acid occupying position 84. In particular, we expected that the presence of a neutral glutamine or of a positively charged arginine at position 84 could result in distinct intramolecular salt-bridge patterns in the 2 TRIB3 variants. To evaluate this possibility, MD simulations of the Q84 and R84 variants of TRIB3 were carried out. Indeed, significant differences in the structures of the 2 TRIB3 variants were observed: in the Q84 variant, Glu 93 was stably engaged in the formation of a salt-bridge with Arg 139, whereas in the R84 variant, Arg 84 competed with Arg 139 in the interaction with Glu 93, and, importantly, effects of such structural changes were relayed also to the region predicted to interact with the PH domain of Akt (Figure 5).

Discussion

Endothelial dysfunction is an early event in atherosclerosis and contributes to cardiovascular diseases.1,2,21 Insulin resistance, a hallmark of metabolic disorders such as type 2 diabetes mellitus and obesity, is often associated to endothelial dysfunction and it is thought to be a risk factor for cardiovascular diseases.22 Although several evidences suggest that genetic factors could contribute to both endothelial dysfunction and insulin resistance,23–25 the genetic bases of these 2 interrelated disorders have not yet been identified. Insulin has important vascular actions, including, among others, stimulation of endothelial NO production, which leads to vasodilation and increased blood flow.3,4 These effects appear to be mediated via the activation of a signaling pathway involving IR/IRS-1/PI3-kinase/Akt/eNOS.

TRIB3 is a mammalian tribbles homolog which binds and inhibits Akt and whose overexpression promotes glucose output from mice liver.6 Although transgenic mice knock-out for TRIB3 appear to be free of major defects in glucose regulation,26 TRIB3 overexpression has been indicated as a possible mechanism for ethanol induced impaired insulin signaling in mice liver.27 Furthermore, TRIB3 is likely to be involved in the decreased insulin action induced by fasting7 and in the FoxO1 regulation of Akt phosphorylation.28 We have recently reported that the TRIB3 R84 variant inhibits insulin-mediated Akt activation in vitro more than the common Q84 variant, and it is associated with in vivo insulin resistance and early myocardial infarction.8 Because of this observations, and because endothelium has emerged as a key insulin target tissue, aim of the present study was to investigate whether the TRIB3 R84 variant was associated with impaired insulin signaling and action in the endothelium. To address this question we: (1) investigated insulin signaling and insulin effects on eNOS activity and NO synthesis in HUVECs naturally carrying the variant of interest, and (2) developed a model of TRIB3 structure to envision the structural changes brought about by the R84 variant and how these could possibly affect Akt binding.

As to the experiments in endothelial cells naturally carrying the variant, insulin-stimulated Akt and eNOS activation was impaired in QR- and RR-, as compared to QQ-HUVECs. This defect was not attributable to alterations in upstream insulin signaling involving the IR/IRS-1/PI 3-kinase pathway because no differences among QQ-, QR-, and RR-HUVECs were observed in IR and IRS-1 tyrosine phosphorylation or in insulin-stimulated association of tyrosine-phosphorylated IRS-1 with the PI 3-kinase p85 regulatory subunit. Rather, the reduced insulin-stimulated Akt activation appears attributable to the observed reduced stimulation of Akt phosphorylation at both Thr56 and Ser773 residues, which is likely a consequence of increased TRIB3-Akt physical interaction. As to the molecular explanation for this increased TRIB3-Akt interaction, it is known that the PH domain of Akt interacts with TRIB3.27 As a matter of fact, our molecular modeling study showed that the Q84 and R84 variants of TRIB3 are characterized by a different pattern in their intramolecular salt-bridges: this causes structural differences in the TRIB3 region which was predicted to interact with the PH domain of Akt.

[References]


[Figure 2. Impact of TRIB3 R84 variant on eNOS phosphorylation. Ser\(^177\) (A, middle panel), Thr\(^945\) (A, lower panel), and total (A, upper panel) eNOS levels and PP1 activity (B) in insulin-stimulated QQ, QR, and RR HUVECs. Each bar represents the mean±SD of 3 independent experiments (*\(P<0.05\); **\(P<0.01\); ***\(P<0.001\)).

[Figure 3. Impact of TRIB3 R84 variant on GSK-3 phosphorylation. Ser\(^21\) (lower panel) and total (upper panel) GSK-3 levels in insulin-stimulated QQ, QR, and RR HUVECs. Each bar represents the mean±SD of 3 independent experiments, representative experiments are shown (*\(P<0.05\) **\(P<0.01\)).]
These results suggest that, indeed, the 2 TRIB3 variants interact differently with Akt, thus providing a structural rationale for their different ability to inhibit insulin-mediated Akt activation observed in our experimental model. In keeping with this scenario is the observation that Ser921 phosphorylation of a key Akt substrate, GSK-3, an important regulator of the glycogen synthesis process, was markedly impaired in QR- and RR- as compared to QQ-HUVECs. Although entirely speculative, it is certainly possible that the tighter TRIB3/Akt interaction brought about the R84 variant might result in impaired Akt phosphorylation also in response to stimuli other than insulin (e.g., VEGF, PDGF, IGF-1).

A limitation of the study is that model structure for physical interaction between TRIB3 and Akt is related to Akt1 isoform, and we used an anti-Akt antibody which did not discriminate between Akt isoforms. Although Akt1 almost completely accounts for Akt-dependent effects in endothelial cells, results obtained in the present study might not be extensible to the other 2 isoforms of this enzyme.

Insulin-mediated Akt activation is also involved in the regulation of eNOS activity by a coordinated enzyme phosphorylation on Ser and Thr residues. Insulin, via Akt, stimulates Ser1177 phosphorylation, resulting in an increased electron flux through the reductase domain and, as a consequence, an enhanced eNOS activity. On the other hand, eNOS Thr495 constitutive phosphorylation appears to down-regulate eNOS activity. HUVECs expressing the TRIB3 R84 variant were characterized by impaired insulin modulation of eNOS Ser1177 phosphorylation and Thr495 dephosphorylation and by an almost complete impairment of insulin ability to stimulate eNOS activity, a likely consequence of the increased inhibitory activity of the R84 variant on insulin-stimulated Akt phosphorylation. It ought to be pointed out that eNOS dephosphorylation at Thr495 is thought to occur via insulin-mediated activation of the PP1 phosphatase by a PI3-kinase/Akt–dependent pathway. Interestingly, we observed that ability of insulin to stimulate PP1 activity was impaired in QR- and RR-, as compared to QQ-HUVECs, thus providing a molecular link for the altered Thr495 eNOS dephosphorylation observed in cells expressing the R84 variant. Finally, the different ability of insulin to regulate eNOS phosphorylation and dephosphorylation in cells with different TRIB3 genotypes translated indeed in a different ability of insulin to stimulate eNOS activity and thus NO synthesis and to elicit a biological response to NO. As a
matter of fact, intracellular cGMP levels, a marker of NO bioavailability, increased in response to insulin stimulus in wild-type cells whereas no change at all was observed in cells carrying the QR- or the RR genotypes.

Taken together with our previous report, these data strongly reinforce the hypothesis that the Q84R amino acid variation induces a TRIB3 conformational change which makes the R84 variant a more effective Akt inhibitor and thus a major player in the modulation of insulin sensitivity in several target tissues among which, of utmost importance in our specific context, the endothelium. Although obtained in venous endothelial cells, a model which does not necessarily resemble that of coronary endothelial cells, present in vitro data provide a biological plausibility of our earlier in vivo findings showing that individuals carrying the TRIB3 R84 variant are at increased risk of early myocardial infarction.

In conclusion, the present data demonstrate that the TRIB3 R84 variant impairs in vitro insulin signaling in human endothelial cells, thus suggesting it might contribute to genetic susceptibility to endothelial dysfunction and cardiovascular disease. If further clinical prospective studies were to confirm this, TRIB3 genotyping might serve the crucial function of helping the identification of at risk individuals at whom to target specific preventive and therapeutic strategies.

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**Disclosures**

None.

**References**


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EXPANDED MATERIALS AND METHODS

Materials
M199 endothelial growth medium, Fetal Calf Serum (FCS), glutamine, phosphate buffered saline (PBS) and 0.05% trypsin/0.02% EDTA were purchased from Mascia Brunelli (Milan, Italy) and tissue-culture disposables from Hiwaki Glass (Tokio, Japan). Anti-phosphotyrosine, anti-IRS-1, anti-p85 subunit of PI 3-kinase, anti-Thr^{495}-eNOS antibodies were from Upstate Biotechnology (Lake Placid, NY, USA); anti-IR, anti-Tyr^{972}-IR from Biosource (Camarillo, CA, USA), anti-eNOS and anti-Ser^{1177}-eNOS from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Akt, anti-Ser^{473}-Akt and anti-Thr^{308}-Akt antibodies were from Cell Signaling Technology (Beverly, MA, USA). L-(3H)-arginine was purchased from PerkinElmer Italia S.p.a. (Milan, Italy). N-nitro L-arginine methyl ester (L-NAME), Tris/HCl, KF, EDTA, Dowex AGWX8-200 were from Sigma Chemicals (St. Louis, MO, USA). The anti-TRIB3 peptide antibody was generated by immunizing rabbits with a peptide corresponding to residues 14-30 of human TRIB3.

Cell cultures
HUVEC were grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium supplemented with 20% FCS, 10µg/ml heparin, and 50µg/ml ECGF. In all experiments cells were used between the 3rd and 5th passage.

Insulin signaling studies on HUVEC
HUVEC were cultured for 18 hours in serum-deprived medium and incubated for 15 minutes with or without insulin (100 nmol/L). Cell lysates were then subjected to either immunoprecipitation with the appropriate antibody or to direct immunoblotting. Total cell lysates or immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the required primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies. Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. To normalize for protein levels, the blots were stripped and reprobed with primary antibodies against the total unphosphorylated form of the appropriate protein.
Assay of protein phosphatase 1 (PP1) activity

PP1 activity was assayed using ProFluor™Ser/Thr Phosphatase assay (Promega, WI), according to manufacturer’s instructions.

Nitric Oxide Synthase activity

eNOS activity was determined by measuring the conversion of L-(3H)-arginine into L-(3H)-citrulline as described by Pandolfi et al. 1. In selected experiments, L-NAME (1 mmol/L) was added 40 minutes before L-(3H)-arginine.

Intracellular cGMP level

Intracellular cGMP levels were evaluated by EIA procedure according to the instructions provided by the supplier (Kit Biotrak, Amersham Pharmacia Biotech). In selected experiments, L-NAME (1 mmol/L) was added 40 minutes before stimulation.

Modeling of TRIB3 structure.

Alignment of TRIB3 (Swiss-Prot entry Q96RU7, amino acids 61-338) with the sequence of the template structure (PDB entry 2bdw-A, corresponding to calcium/calmodulin-dependent protein kinase II, amino acids 5-292).
REFERENCES

Figure I

A  WB: IR
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WB: pY972
WB: IRS-1

B  WB: IRS-1
   IP: IRS-1
WB: pY

C  WB: IRS-1
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Figure I. Impact of *TRIB3* R84 variant on insulin-stimulated tyrosine phosphorylation of IR and IRS-1, and IRS-1 association with p85 and IR.

IR (A) and IRS-1 (B) tyrosine phosphorylation (lower panels) and total levels (upper panels) in insulin stimulated QQ, QR and RR HUVECs. Co-precipitation of IRS-1 (lower panels) with IR (C) and p85 (D), total IRS-1 levels in immunoprecipitates were assessed as control (upper panels). Each bar represents the mean±SD of 3 independent experiments, representative experiments are shown.
Figure II. Impact of *TRIB3* R84 variant on *TRIB3* association with Akt.
Co-precipitation of Akt with *TRIB3* (A, lower panel), Akt levels (A, upper panel), tubulin and *TRIB3* levels (B) in QQ, QR and RR HUVECs. Each bar represents the mean±SD of 3 experiments, representative experiments are shown (* p<0.02, ** p<0.01).