Insulin Stabilizes Microvascular Endothelial Barrier Function via Phosphatidylinositol 3-Kinase/Akt-Mediated Rac1 Activation

Dursun Gündüz, Johannes Thom, Imran Hussain, Diego Lopez, Frauke V. Härtel, Ali Erdogan, Mathias Grebe, Daniel Sedding, Hans Michael Piper, Harald Tillmanns, Thomas Noll, Muhammad Aslam

Objective—Insulin is a key regulator of metabolism, but it also confers protective effects on the cardiovascular system. Here, we analyze the mechanism by which insulin stabilizes endothelial barrier function.

Methods and Results—Insulin reduced basal and antagonized tumor necrosis factor-α–induced macromolecule permeability of rat coronary microvascular endothelial monolayers. It also abolished reperfusion-induced vascular leakage in isolated-perfused rat hearts. Insulin induced dephosphorylation of the regulatory myosin light chains, as well as translocation of actin and VE-cadherin to cell borders, indicating a reduction in contractile activation and stabilization of cell adhesion structures. These protective effects were blocked by genistein or HNMP(AM)₃, a pan-tyrosine-kinase or specific insulin-receptor-kinase inhibitor, respectively. Insulin stimulated the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and NO production, and it activated Rac1. Inhibition of PI3K/Akt abrogated Rac1 activation and insulin-induced barrier protection, whereas inhibition of the endothelial nitric oxide synthase/guanylylcyclase pathway partially inhibited them. Inhibition of Rac1 abrogated the assembly of actin at cell borders. Accordingly, it abolished the protective effect of insulin on barrier function of the cultured endothelial monolayer, as well as the intact coronary system of ischemic-reperfused hearts.

Conclusion—Insulin stabilizes endothelial barrier via inactivation of the endothelial contractile machinery and enhancement of cell-cell adhesions. These effects are mediated via PI3K/Akt- and NO/cGMP-induced Rac1 activation. Insulin reduced basal and antagonized tumor necrosis factor-α–induced hyperpermeability of rat coronary microvascular endothelial monolayers. It also abolished reperfusion-induced vascular leakage in isolated-perfused rat hearts. These insulin effects were partially blocked by endothelial nitric oxide synthase inhibitors and abolished by PI3K and Rac1 inhibitors. In conclusion, insulin mediates these effects via PI3K/Akt- and NO/cGMP-induced Rac1 activation. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: capillary permeability | coronary circulation | endothelium | nitric oxide | vascular biology

Insulin is an essential hormone of metabolic homeostasis. Recent clinical findings show that intensive insulin therapy has vasoprotective effects under inflammatory conditions and reduces major cardiovascular events in diabetics. These effects of insulin seem to be independent of its metabolic effects on endothelial cells.

Vascular endothelial cells forming the inner lining of all vessels play an important role in the regulation of vascular homeostasis. They provide a semiselective barrier for water, solutes, macromolecules, and blood-borne components and are also involved in regulating the trafficking of blood cells across the vessel wall. This function can be altered by a variety of diverse circulating vasoactive inflammatory mediators and hormones such as insulin. It has recently been demonstrated that endothelial cells of macrovascular origin express insulin receptors. Binding of insulin to the receptors induces nitric oxide production via phosphatidylinositol 3-kinase (PI3K)-mediated activation of endothelial nitric oxide synthase (eNOS) and causes relaxation of the smooth muscles. Recently, it has been shown that insulin reduces mesenteric venular albumin leakage on systemic insulin administration in rats. However, the underlying signaling mechanism of this effect is largely unknown.

The integrity of the endothelial barrier is highly dependent on the endothelial actomyosin-based contractile machinery.
and actin cytoskeleton-mediated adherens junctions consisting of VE-cadherin, which, together with other actin binding proteins, seals the adjoining cells and thereby limits the passage of macromolecules across the microvasculature. The activation of endothelial contractile machinery is precisely regulated by phosphorylation state of the regulatory myosin light chains (MLC). MLC phosphorylation increases actin-myosin interaction, cell contraction and barrier failure, whereas reduction in MLC phosphorylation causes stabilization of endothelial barrier.

The members of the Rho family of GTPases are key regulators of endothelial barrier function, controlling the endothelial contractile machinery, assembly of actin cytoskeleton, and cell adhesion structures. It is well established that the Rho GTPase Rac1 is required for the stability of endothelial adherens junctions and barrier function. Similarly, activation of Rac1 strengthens VE-cadherin-based cell adhesions and reduces the macromolecule permeability of endothelial monolayers. Rac1 activity is regulated by guanine nucleotide exchange factors Tiam1 and TrioN. The activation of Rac1 can be inhibited by the specific pharmacological inhibitor NSC23766, which specifically inhibits Rac1 guanine nucleotide exchange factors Tiam1 and TrioN.

The aim of the present study was to analyze whether insulin can enhance the barrier function of coronary microvascular endothelial cells. Special emphasis was laid on the molecular mechanism by which insulin may accomplish this barrier protection.

Materials and Methods

The sources of reagents are listed in the supplemental Materials and Methods section, available online at http://atvb.ahajournals.org.

Endothelial Monolayer Permeability

Isolation and culture of rat coronary microvascular endothelial cells was performed as described previously. Immunoprecipitation was performed by labeling protein-G-coated magnetic beads with anti-insulin-receptor β-subunit antibody followed by Western blotting. Densitometric analyses of Western blots were performed using the Quantity One image analyzer software (Bio-Rad). The procedure is described in detail in the online data supplement.

Immunofluorescence Microscopy

The endothelial monolayers cultured on glass coverslips were examined as described previously using a Zeiss LSM-510M inverted microscope. Data are given as means±SD of 3 to 5 experiments using independent cell preparations. The comparison between means of two groups was performed by 1-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test. Changes in parameters within the same group were assessed by multiple ANOVA analyses. Probability (P) values of less than 0.05 were considered significant.

Results

Effect of Insulin on Insulin Receptors and Endothelial Monolayer Permeability

The presence of insulin receptors in rat coronary microvascular endothelial cells was confirmed by immunoprecipitation with an antibody targeting the β-subunit of the insulin receptor (Figure 1A). Insulin induced an increase in tyrosine phosphorylation of the β-subunit, as shown by reprobe of the Western blot membrane with an anti-phosphotyrosine antibody.

The unstimulated endothelial monolayers showed a constant albumin permeability of 4.9±0.2×10⁻⁶ cm/s. The addition of insulin caused a prompt reduction in permeability. It dropped within 10 minutes and remained at that lower level for the rest of the period of observations. This reduction was concentration dependent (Figure 1B and 1C); it was significant as early as 0.001 IU/mL (equivalent to ≈5 nmol/L), half-maximal at 0.01 IU/mL, and at its maximum at 1 IU/mL. Therefore, this concentration was used for all further experiments.

To analyze whether insulin can also protect endothelial cells against agonist-induced hyperpermeability, cells were challenged by tumor necrosis factor-α (TNFα). Exposure of endothelial cells to TNFα (100 ng/mL) for 30 minutes led to a marked increase in macromolecule permeability, which was reversed by 1 IU/mL insulin (Figure 1D).

In the next step, the involvement of insulin receptors in the barrier protective effects of insulin was analyzed. Insulin receptors are receptor-tyrosine-kinases; therefore, genistein, a pan-tyrosine-kinase inhibitor, or HNMP(AM)₃, a highly specific insulin-receptor-kinase inhibitor, was applied to block insulin receptors. Preincubation of the cells for 30 minutes with genistein (10 μmol/L) significantly attenuated the insulin effect on macromolecule permeability (Figure 2A), whereas HNMP(AM)₃ (10 μmol/L) completely abolished it (Figure 2B).

Effect of Insulin on the PI3K/Akt Pathway and Macromolecule Permeability

Insulin mediates most of its effects in endothelial cells via activation of the PI3K/Akt pathway. In line with previous reports, insulin induced a prompt increase in Akt phosphorylation in coronary microvascular endothelial cells in a concentration-dependent manner (Figure 3A). Akt phosphorylation reached its maximum within 1 minute and was sustained over the whole period of observation (Figure 3B). Maneuvers that inhibit insulin-induced receptor-kinase activation, such as genistein and HNMP(AM)₃, reduced or abolished Akt phosphorylation, respectively (Figure 3C). Likewise, wortmannin (1 μmol/L), a PI3K inhibitor, completely abolished insulin-induced Akt phosphorylation. In line with this, wortmannin also completely abrogated the insulin-mediated reduction in macromolecule permeability (Figure 3D). Similar results were obtained with LY294002 (data not shown).
Role of Insulin-Induced NO Production in Insulin-Mediated Barrier Stabilization

In line with previous reports, insulin induced NO production in a concentration-dependent manner in coronary microvascular endothelial cells (Figure 4A); its effect was at its maximum at 0.1 IU/mL and was completely abolished by preincubation with the competitive eNOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) (100 μmol/L) for 30 minutes. Preincubation of endothelial monolayers with L-NAME, or with L-NNA (100 μmol/L) for 30 minutes attenuated but did not abolish the insulin effect on permeability (Figure 4B). Because NO mediates many of its actions via activation of soluble guanylate cyclase (sGC) and generation of cGMP, endothelial monolayers were preincubated with ODQ (100 μmol/L) or NS2028 (100 μmol/L), 2 specific sGC inhibitors, for 30 minutes. ODQ and NS2028 attenuated the insulin effect on macromolecule permeability to a similar extent as the eNOS inhibitors (Figure 4C).

Effect of Insulin on Contractile Machinery and VE-Cadherin-Mediated Adherens Junctions

The endothelial barrier is regulated by 2 principal mechanisms: the actomyosin-based endothelial contractile machinery and VE-cadherin-dependent adherens junctions. Here, the effect of insulin on both elements was tested. Exposure of endothelial cells to insulin caused a reduction in MLC phosphorylation, which was maximum as early as 0.1 IU/mL (Figure 5A). The effect on MLC dephosphorylation was rather delayed and was significant after 10 minutes (Figure 5B).

Under control conditions, VE-cadherin was located at the borders of adjacent cells (Figure 5C and 5D). However, VE-cadherin staining is already enhanced at that site 5 minutes after the addition of insulin (1 IU/mL), indicating that the strengthening effect of insulin on cell-cell adhesion structures precedes inactivation of the contractile machinery. In line with the data on macromolecule permeability, translocation of VE-cadherin to cell-cell junctions was abolished by preincubation with insulin-receptor-kinase inhibitor HNMP(AM)3 (10 μmol/L) and the PI3K inhibitor wortmannin (1 μmol/L) for 30 minutes. Likewise, insulin-induced translocation of VE-cadherin is only partially inhibited by the NOS inhibitor L-NAME.

Effect of Insulin on Rac1 Activation and Reorganization of Actin Cytoskeleton and Barrier Stabilization

Insulin (1 IU/mL) induced a 3-fold increase in Rac1 activation after 10 minutes (Figure 6A). This insulin effect was abolished by insulin-receptor-kinase inhibitor HNMP(AM)3 (10 μmol/L) and the PI3K inhibitor wortmannin (1 μmol/L), whereas the inhibitors of NOS and sGC, L-NAME (100 μmol/L) and ODQ (100 μmol/L), respectively, led only to partial inhibition of insulin-induced Rac1 activation. This corresponds to a partial inhibitory effect of L-NAME and ODQ on insulin-mediated barrier stabilization.
It is well established that Rac1 strengthens adherens junctions via enhancement of the peripheral actin cytoskeleton, accompanied by reduction of F-actin stress fibers. Therefore, the effect of insulin on actin rearrangement was analyzed in early-confluent coronary microvascular endothelial monolayers. Under these conditions, the cells characteristically do not develop a prominent band of peripheral actin but still exhibit F-actin stress fibers running across the cell. Thus, the maneuver inducing actin rearrangement can be easily

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**Figure 2.** Effect of insulin receptor inhibition on insulin-mediated reduction in macromolecule permeability. A, Endothelial monolayers were exposed to insulin (1 IU/mL), genistein (10 μmol/L), a pan-tyrosine-kinase inhibitor, insulin plus genistein, or vehicle (control) as indicated. B, Endothelial monolayers were exposed to insulin (1 IU/mL), HNMP(AM)₃ (HNMP; 10 μmol/L), a specific insulin-receptor-kinase inhibitor, insulin plus HNMP, or vehicle (control) as indicated. Data are mean±SD of 5 experiments with independent cell preparations. *P<0.05 versus control; #P<0.05 versus insulin alone.

**Figure 3.** Effects of insulin on Akt phosphorylation and its role in insulin-mediated reduction in permeability. A, Representative Western blots of Akt phosphorylation (P–Akt). Cells were exposed to different concentrations of insulin for 10 minutes. B, Representative Western blots of P–Akt. Cells were exposed to insulin (1 IU/mL) for different time intervals as indicated. C, Representative Western blots of P–Akt. Cells were exposed to insulin (1 IU/mL) for 10 minutes or to genistein (Gen; 10 μmol/L), HNMP(AM)₃ (HNMP; 10 μmol/L), or wortmannin (1 μmol/L; Wort) for 30 minutes with or without insulin for 10 minutes. D, Effect of insulin (1 IU/mL), wortmannin (1 μmol/L), and wortmannin plus insulin or vehicle (control) on macromolecule permeability. Data are mean±SD of 5 experiments with independent cell preparations. *P<0.05 versus control; #P<0.05 versus insulin alone.
detected. As shown in Figure 6B, insulin induced formation of peripheral actin band and reduction in stress fibers after 5 minutes of exposure. Preincubation of the monolayers with the insulin-receptor-kinase inhibitor HNMP(AM)₃ (10 μmol/L), the Rac1 inhibitor NSC23766 (100 μmol/L),¹⁷ and the PI3K inhibitor wortmannin (1 μmol/L) for 30 minutes abolished peripheral actin band formation, whereas the NOS inhibitor L-NAME only weakly antagonized insulin-induced actin reorganization. The role of Rac1 activation in insulin-mediated barrier stabilization was proven by using the Rac1 inhibitor NSC23766. Preincubation of endothelial cells with NSC23766 abolished the insulin effect on endothelial barrier (Figure 6C).

A final series of experiments was performed in a well-established isolated perfused rat heart model¹⁹ to verify the barrier protective effects of insulin in an intact coronary system. Myocardial water content was determined as an index for capillary leakage. Under control conditions, the myocardial water content of the normoxic perfused rat hearts was 455 mL/100 g dry weight after 90 minutes (Figure 6D). Exposure of the hearts to ischemia for 60 minutes followed by 30 minutes of reperfusion caused an increase in myocardial water content to 554 mL/100 g dry weight. In contrast, addition of 0.1 IU/mL insulin, a concentration only one-tenth that applied in the cell culture model, during first 10 minutes of reperfusion abolished the reperfusion-induced increase in myocardial water content. In one set of experiments, 50 μmol/L NSC23766 was added 20 minutes before the onset and during the first 10 minutes of reperfusion. This maneuver abrogated the protective effect of insulin on reperfusion-induced increase in water content.

Discussion

It is well established that intensive insulin therapy reduces vascular complications of the coronary system and other vascular provinces because of its antiatherosclerotic and antiinflammatory effects.¹⁻³ Hyperpermeability of macro- and microvasculature is the hallmark of these disease states. In the present study, we show for the first time that insulin stabilizes the coronary microvascular endothelial barrier function in an in vitro model of coronary microvascular endothelial cells as well as in the intact coronary vascular system of isolated-perfused rat heart. Insulin not only reduced the basal permeability of endothelial monolayers but also abolished hyperpermeability induced by the inflammatory mediator TNFα.

Immunoprecipitation experiments revealed the presence of insulin receptors and phosphorylation of the β-subunit of the receptor on exposure to insulin. Inhibition of the insulin receptor-tyrosine-kinase abrogated the barrier-enhancing effect of insulin, demonstrating a receptor-

Figure 4. Role of NO pathway in insulin-mediated reduction of macromolecule permeability. A, Effect of insulin on NO production in endothelial cells. Cells were loaded with DAF2-FM and treated with different concentrations of insulin or L-NAME (100 μmol/L) plus insulin (1.0 IU/mL) for 10 minutes, and then DAF-2 triazole production, which is directly related to NO levels, was measured with a fluorescence plate reader. B, Effect of NOS inhibitors L-NAME or L-NNA on insulin-mediated reduction in macromolecule permeability. Endothelial monolayers were exposed to insulin (1 IU/mL), L-NAME (100 μmol/L), L-NNA (100 μmol/L), insulin plus L-NAME, insulin plus L-NNA, or vehicle (control). C, Effect of sGC inhibitors ODQ or NS2028 on insulin-mediated reduction in endothelial permeability. Endothelial monolayers were exposed to insulin (1 IU/mL), ODQ (100 μmol/L), ODQ (100 μmol/L), insulin plus ODQ, insulin plus NS2028, or vehicle (control). Data are mean ± SD of 5 experiments with independent cell preparations. *P < 0.05 versus control; §P < 0.05 versus insulin alone.
mediated effect. In line with the findings of the present study, Sasaki et al.6 have shown that insulin could reduce mesentery venular leakage in rats. However, the molecular mechanism of this insulin effect on endothelial barrier has not been clear until now.

In line with previous reports,5 we show activation of the PI3K/Akt pathway in coronary microvascular endothelial cells, as demonstrated by Akt phosphorylation. The Akt phosphorylation is rapid and is sustained over longer periods of time, similar to its effect on barrier function. Moreover, we demonstrate that this Akt phosphorylation is via receptor-mediated activation of PI3K. Accordingly, inhibition of PI3K abolished insulin-mediated reduction in permeability, further supporting the notion that insulin strengthens the endothelial barrier via receptor-mediated activation of PI3K/Akt pathway.

Furthermore, we show that insulin induces NO production in endothelial cells under study and inhibition of eNOS with specific inhibitors not only blocked insulin-induced NO production but also attenuated the insulin-mediated barrier stabilization. Accordingly, inhibition of cGMP/phosphoglycerate kinase pathway by specific sGC inhibitors attenuated insulin-mediated barrier protection to a similar extent as eNOS inhibitors, confirming that this is a cGMP/phosphoglycerate kinase effect. The role of NO in the control of the endothelial barrier is controversial.22 The NO effect may differ depending on the stimulus, experimental conditions, vascular bed, time window, and local concentration of NO. A number of studies in isolated perfused microvessels,23 as well as in eNOS knockout mice,24,25 show that hyperpermeability induced by growth factors or inflammatory mediators could be attenuated with eNOS inhibitors, supporting the concept that NO triggers this increase in permeability. We have previously demonstrated that vascular endothelial growth factor has a biphasic effect on endothelial permeability.26 The initial transient barrier protective effect is NO dependent, and inhibition of eNOS abolishes this initial reduction in permeability, suggesting that the NO effect is context dependent and can be influenced by another repertoire of signaling, activated simultaneously by growth factors or inflammatory mediators. Similarly, several other studies have elegantly demonstrated that NO donors and cGMP analogs can antag-

Figure 5. Effect of insulin on MLC phosphorylation (P–MLC) and localization of VE-cadherin in endothelial cells. A, Time-dependent effect of insulin on P–MLC. Top: Representative Western blots of MLC phosphorylation. Cells were exposed to insulin (1.0 IU/mL) for indicated time points. Bottom: Densitometric analysis of Western blots of MLC phosphorylation. B, Concentration-dependent effect of insulin on P–MLC. Top: Representative Western blots of MLC phosphorylation. Endothelial monolayers were exposed to different concentration of insulin for 10 minutes. Bottom: Densitometric analysis of Western blots of MLC phosphorylation. C, Effect of insulin on VE-cadherin localization. Representative immunofluorescence images of endothelial monolayers exposed to vehicle (control [C]), insulin (Ins; 1 IU/mL), insulin plus HNMP(AM)3 (HNMP; 10 μmol/L), insulin plus Wortmannin (Wort; 1 μmol/L), or insulin plus L-NAME (100 μmol/L) for 5 minutes. Arrows denote VE-cadherin localized at cell borders (scale bar=20 μm; shown is a representative immuno-staining of 3 experiments with independent cell preparations). D, Quantification of the immunofluorescence staining of VE-cadherin determined by image analysis. Data are mean±SD; n= 10 cells per endothelial monolayer of 3 independent experiments. *P<0.05 versus C; #P<0.05 versus insulin alone.
Onize agonist-induced hyperpermeability.27,28 Likewise, it has been shown that even basal NO is required for the maintenance of vascular integrity.29 In line with this, Predescu et al, using eNOS knockout mice, have demonstrated that basal activity of eNOS is indeed required for endothelial adherens junction maintenance independent of vascular bed.30 Accordingly, the present study demonstrates that insulin, in part, mediates its barrier protective effect via the NO/cGMP pathway.

Insulin reduced the state of contractile activation and strengthened adherens junctions in coronary endothelial cells, which explains the mechanistic aspects of this barrier stabilization. Insulin stimulates NO production and reduces MLC phosphorylation, the latter of which is rather delayed and was observed after 10 minutes. The maximum effect on both parameters was obtained at 0.1 IU/mL, a concentration one-tenth that observed for the maximal effect on permeability. Presently, the detailed mechanism of insulin-induced contractile inactivation is still elusive. It has previously been shown that in isolated vessels, a maneuver activating cGMP/phosphoglycerate kinase signaling led to dephosphorylation of MLC via activation of MLC phosphatase.31 Therefore, our data suggest that insulin reduces MLC phosphorylation via NO/cGMP-mediated pathway.

Insulin-induced stabilization of adherens junctions is mediated via translocation of VE-cadherin, a major component of endothelial adherens junctions, to cell-cell junctions. This translocation was abrogated by inhibition of insulin receptor-
tyrosine-kinase PI3K and weakly attenuated by inhibition of NOS, suggesting that insulin mediates its effect on endothelial adherens junctions via both the PI3K/Akt and the NO pathway.

Rac1, a member of the Rho family of GTPases, is known to regulate assembly of peripheral actin and stimulates the formation of adherens junctions. The results of the present study clearly show that insulin activates Rac1 via PI3K/Akt and also in part via the NO/cGMP pathway. This could be mediated via activation of the Rac1-specific guanine nucleotide exchange factors Tiam1 and TrioN, because a specific inhibitor of these guanine nucleotide exchange factors, NSC23766, abolished the insulin effect. Inhibition of PI3K abolished Rac1 activation and reorganization of the peripheral actin cytoskeleton, whereas inhibition of either NOS or sGC led only to partial inhibition of Rac1 and a weak antagonistic effect on peripheral actin. In line with our findings, 2 reports on fibroblasts show that activation of either PI3K/Akt or NO/cGMP can induce Rac1 activation.

Inhibition of Rac1 with a specific inhibitor, NSC23766, abolished the insulin-mediated barrier stabilization effect. Remarkably, the effect of inhibition of Rac1 was much more effective than inhibition of NOS or sGC, indicating that Rac1 is a central signaling element of the insulin-mediated barrier stabilization beyond NO/cGMP pathway. Furthermore, the inability of eNOS and sGC inhibitors to block the initial effect of insulin on permeability and the delayed effect of insulin on MLC phosphorylation suggest that NO signaling does not contribute to the acute but rather to sustained effect of insulin.

The barrier protective effect of insulin is further supported by our data obtained in an isolated, saline-perfused rat heart model. In this model, insulin caused a strong reduction of endothelial permeability. This barrier stabilizing effect is dependent on PI3K/Akt, NO/cGMP, and Rac1, which play a decisive role in insulin-mediated actin cytoskeleton rearrangement and stabilization of cell adhesion. The proposed signal transduction pathways suggested by the data of the present study are illustrated in Figure 6E.

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Disclosures

None.

References

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Materials and Methods

**Materials:** HRP-conjugated anti-mouse IgG and rabbit IgG antibodies were from Amersham Biosciences (Heidelberg, Germany); anti-phospho-tyrosine antibody (mouse monoclonal IgG) and 96-well ELISA plates were from Becton Dickinson GmbH (Heidelberg, Germany); anti-insulin-receptor antibody (clone 29B4, mouse monoclonal IgG), benzonase, genistein, HNMP(AM)₃ [Hydroxy-2-naphthalenylmethylphosphonic acid tris acetoxymethyl ester], an inhibitor of insulin receptor (IR) tyrosine kinase activity, ¹ L-NAME, L-NNA, LY294002, and wortmannin were from Calbiochem (Darmstadt, Germany); anti-phospho-Akt (Ser473) and anti-phospho-MLC (rabbit polyclonal) antibodies were from Cell Signaling (Danvers, USA); Rac1 pulldown assay kit and anti-Rac1 antibody (rabbit polyclonal) were from Cytoskeleton (Denver, USA); DAF-FM diacetate [4-amino-5-methylamino- 2′, 7′-difluorofluorescein diacetate], Protein G magnetic beads and Alexa Flour 488 conjugated secondary (anti rabbit IgG) antibody were from Invitrogen (Karlsruhe, Germany); M199 medium was from Dianova (Hamburg, Germany); fetal calf serum [FCS] and neonatal calf serum [NCS] were from PAA (Pasching, Austria); ECL solution was from Pierce (Rockford, USA); Complete® [protease inhibitor cocktail] was from Roche (Mannheim, Germany); anti-actin antibody (clone C4, mouse IgG), insulin solution (recombinant from yeast, human) [290 IU/ml in 25 mM HEPES], anti-pan-cadherin antibody (rabbit polyclonal), phalloidin-TRITC, and anti-vinculin antibody (clone hVIN-1, mouse IgG) were from Sigma (Steinheim, Germany); NSC23706 [N6-2-[[4-(Diethylamino)-1-methylbutyl]amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinoline diamine trihydrochloride], NS-2028 [8-bromo-4H-2,5-dioxao-3,9b-diaza-cyclopenta[a]naphthalen-1-one], and ODQ [1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one], were from Tocris bioscience (Bristol, UK); Costar Transwell®
Supplement Material

Polycarbonate membrane filters (24-mm round) were from Vitaris (Baar, Germany). All other chemicals were of the best available quality, usually analytical grade.

Cell culture: The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Microvascular coronary endothelial cells were isolated from 200–250 g male Wistar rats and cultured as previously described.²⁻⁴

Experimental protocols: The basal medium used in incubations was modified Tyrode's solution (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.0 CaCl₂, and 30.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES]; pH 7.4, 37° C). Agents were added as indicated. Stock solution of insulin was in 25 mM HEPES and stock solutions of L-NAME and L-NNA were prepared immediately before use with basal medium. Stock solutions of genistein, HNMP(AM)₃, and wortmannin were prepared with dimethyl sulfoxide (DMSO). Appropriate volumes of these solutions were added to the cells yielding final solvent concentrations < 0.1% (vol/vol). The same final concentrations of HEPES, DMSO, or basal medium were included in all respective control experiments.

In a set of pilot experiments concentration-response relationships were determined to find the optimal effective concentration. Unless otherwise stated the following agents were applied in their optimal effective concentrations: genistein (10 µM), insulin (1 IU/ml), HNMP(AM)₃ (10 µM), L-NAME (100 µM), L-NNA (100 µM) and wortmannin (1 µM).

Rac1 Pulldown Assay: Activity of Rac1 was measured with a pulldown Rac1 activation assay biochem kit according to the manufacturer’s protocol.
Macromolecule permeability measurement: Rat coronary microvascular endothelial monolayer macromolecule permeability was measured as described previously. 3,5,6

Myocardial water content: Hearts from 250-g male Wistar rats were mounted immediately after isolation on a Langendorff perfusion system in a temperature-controlled chamber (37 °C), as previously described 3 with some modifications. Hearts were perfused with Krebs-Henseleit buffer (composition in mM: 140.0 NaCl, 24.0 NaHCO3, 2.7 KCl, 0.4 KH2PO4, 1.0 MgSO4, 1.8 CaCl2, 5.0 glucose, pH 7.4) for 30 minutes (10 ml/min) before each experiment and then exposed to one of the following protocols: (1) Normoxic conditions for 90 minutes, (2) 60 minutes of hypoxia followed by 30 minutes of reperfusion, (3) 60 minutes of hypoxia followed by 30 of reperfusion with 0.1 IU/ml insulin, during first 10 minutes of reperfusion, (4) 60 minutes of hypoxia followed by 30 minutes of reperfusion in which Rac1 inhibitor, NSC23766 was added to the perfusion medium during last 20 min of hypoxia and first 10 minutes of reperfusion with insulin (during first 10 minutes of reperfusion only). The normoxic perfusion (10 ml/min) was with Krebs-Henseleit buffer gassed with 95% O2 [vol/vol]/5% CO2 [vol/vol]), the chamber was flushed with humidified air, and hypoxic perfusion with Krebs-Henseleit buffer with humidified 95% N2 [vol/vol]/5% CO2 [vol/vol]). At the end of each experiment, wet weight and after 24 h, dry weight of the perfused rat hearts were measured.

Immunoprecipitation: immunoprecipitation was carried out as described previously. 7 Briefly, confluent endothelial monolayers grown in 10-cm dishes were stimulated as indicated in the text. Cells were lysed for 10 min on ice (composition of lysis buffer: 1% (vol./vol.) Triton X-100, 0.5% (vol./vol.) Nonidet P-40, 150 mM NaCl, 1 mM EDTA,
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1 mM EGTA, 1 mM Na-orthovanadate, 0.5 mM PMSF, protease inhibitor Complete™, 50 mM Tris/HCl pH 7.4). Lysates were cleared by centrifugation (1000 × g, 5 min, 4 °C). The supernatant was incubated with insulin receptor (IR)-specific antibody pre-immobilized on magnetic protein-G beads overnight at 4 °C. After that beads were washed three times with PBS (pH 7.4) containing 0.1% (vol./vol.) Tween 20. The beads were collected and bound proteins were eluted in Laemmli sample buffer 8, 9 for Western blot analysis using pY20 antibody.

Nitric oxide (NO) assay: Intracellular NO was measured using the NO-specific fluorescence probe DAF-FM diacetate. The cell permeable DAF-FM diacetate diffuses freely across the cell membrane and once within the cells it is deacetylated by endogenous esterases, resulting in the formation of DAF-FM. DAF-FM reacts with the NO oxidation product N$_2$O$_3$ and generates the highly fluorescent DAF-FM triazole, which was detected with a “Infinite® 200” fluorescent plate reader (Tecan, Austria) using excitation wavelength of 495 nm and emission wavelength of 515 nM ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 515$ nm).

The confluent cultures of coronary microvascular endothelial cells in 96-well plates were washed twice with PBS and incubated with 5 μM DAF-FM diacetate in DMEM for 30 min, washed again and incubated with HBSS for 30 min to allow time for DAF-FM diacetate de-esterification. After 30 min incubation, cells were exposed to insulin or vehicle and DAF-FM triazole fluorescence was measured. The results were corrected by subtracting the non-specific fluorescence detected in wells that had not been treated with DAF-FM diacetate or which did not contain cells. Four measurements per treatment group were performed on cells from an individual culture and averaged to yield one value. Experiments were done on 3 individual cultures.
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*Immunofluorescence.* Confluent endothelial monolayers were rinsed three times with PBS (pH 7.4), fixed with 100% methanol for 20 min at -20°C or 4% paraformaldehyde (PFA) for 20 min at room temperature, and washed again thrice with PBS and permeabilized with 0.2% Triton X-100 for 20 min. The cells were incubated with an anti-pan-cadherin antibody (1:100) overnight at 4°C. Afterward, the coverslips were washed thrice with PBS and incubated with FITC-conjugated anti-rabbit IgG (1:200) for 1h at 37°C. The coverslips were finally mounted on glass slides with a drop of buffered glycerol (pH 8.5).

*Confocal microscopy and image analysis.* Confocal images were obtained by laser scanning microscopy (LSM 510; Zeiss, Jena, Germany). Fluorophores were excited using He-Ne (545 nm) and argon (492 nm) lasers. Image acquisition and analyses were carried out using software provided with the confocal microscope. For quantification of VE-cadherin distribution, we quantified fluorescence in a zone that included only the cell junction using the line feature of the image analysis software to trace the cell junction along its contours. Fluorescence data obtained reflects analyses on 10 cells per experiment from three experiments.

*Statistical analysis.* Data are given as means ± S.D. of 3–5 experiments using independent cell preparations. The comparison of means between groups was performed by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc test. Changes in parameters within the same group were assessed by multiple ANOVA analysis. Probability (P) values of less than 0.05 were considered significant.
Supplement Material
Reference List


