Cross-Talk Between PKA and Akt Protects Endothelial Cells From Apoptosis in the Late Ischemic Preconditioning

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Objective—The aim of this study was to explore the molecular mechanisms involved in late preconditioning-induced cell protection in endothelial cells.

Methods and Results—Preconditioning (PC) was induced by exposing bovine aortic endothelial cells (BAECs) to 3 cycles of 15 minutes of hypoxia followed by 15 minutes of reoxygenation. A 12-hour period of hypoxia induced cell death in 60% of BAECs (48±5% apoptosis, 12±4% necrosis). Early and late PC decreased hypoxia-induced apoptotic (25±5% and 28±4%, respectively) and necrotic (6±3%, and 8±2%, respectively) cell death. Consistently, hypoxia-induced caspase-3 cleavage was reduced by PC. Pretreatment with H89 (protein kinase A [PKA] inhibitor), LY294002 (phosphatidylinositol-3-kinase [PI3K] inhibitor), and N-acetyl-cysteine (antioxidant) abrogated late PC-induced cell protection, whereas inhibition of protein kinase C by Go6983, and of nitric oxide synthesis by L-NAME,1400W and bovine eNOS siRNA did not. In addition, in early and late PC, PKA physically interacted with the phosphorylated form of Akt, suggesting that PKA is required for Akt phosphorylation. Expression of PKA and Akt dominant negative mutants inhibited ischemic late PC-induced protection, indicating that these kinases play a key role in late PC-mediated cell protection.

Conclusions—Late ischemic PC protects BAECs against hypoxia through PKA- and PI3K-dependent activation of Akt.

Key Words: hypoxia ■ phosphatidylinositol-3-kinase ■ caspase-3 ■ nitric oxide ■ cell protection

Ischemic preconditioning (PC) is a physiological phenomenon in which nonsustained, repetitive, sublethal ischemic stimulation enhances tolerance to a subsequent prolonged ischemic stress. PC has great pathophysiological relevance, because it confers protection against ischemia-induced cell death to those organs that are composed of terminally differentiated cells, like the brain and heart.

The molecular mechanisms and mediators that account for PC have been extensively investigated, and it has emerged that nitric oxide (NO) plays a key role in this phenomenon in both the brain and heart. Endothelium represents one of the principal sources of NO. Although there is considerable evidence that the protective effects of both early and late PC extend to endothelial cells, the molecular mechanisms of NO generation in endothelial cells during late PC (LPC) have been poorly investigated. In recent years it has been demonstrated that Akt, a serine/threonine kinase, plays a pivotal role in the genesis of NO in endothelial cells by inducing endothelial nitric oxide synthase (eNOS) phosphorylation at serine1177 (ser1177), leading to an increase in eNOS activity. Interestingly, Akt is activated by several ligand-receptor systems previously shown to be protective against cell death, such as insulin-,

Thus, Akt activation results in a powerful protective effect after transient ischemia in the heart and brain, which probably reflects its ability to inhibit cell death. Moreover, it has been reported that, in cultured endothelial cells, antiapoptotic effects induced by fluid shear stress and ischemic PC are mediated by an Akt-dependent mechanism.

Therefore, it is possible to hypothesize that ischemic PC activates Akt. If this is the case, Akt-phosphorylation/activation could directly contribute to PC-induced cytoprotection by promoting cell survival and stimulating NO synthesis.

We planned this study to explore the molecular mechanisms involved in LPC-induced cell protection through a model of ischemic PC in cultured endothelial cells, and, in particular, to investigate the specific roles of Akt and NO.

Materials and Methods
Detailed methods can be found in the supplemental material (available online at http://atvb.ahajournals.org).

Cell Culture and Hypoxia
Bovine aortic endothelial cells (BAECs) were subjected, after overnight starvation, to preconditioning or hypoxia.

Estimation of NO Production
NO production was assessed by both conversion of L-arginine into L-citrulline and GRIESS reaction.
Annexin V Staining
Endothelial cells grown on coverslips were stained with Annexin-V–Fluorescein and Propidium Iodide, according to manufacturer’s instructions. Apoptotic and necrotic cell death were assessed as described in the supplemental material.

Protein Kinase A Activity Assay
The protein kinase A (PKA) activity was assessed by a specific radioactive assay, as described in the supplemental material.

Immunoblotting and Immunoprecipitation
Cells were grown in 100-mm plates. At the end of the stimulation period, the medium was removed and the cells processed, as described in the supplemental material.

Transient Transfection
Plasmids encoding epitope-tagged dominant negative PKA (generous gift of Drs Michael S. Deal and Susan S. Taylor, University of California, San Diego) and Akt (HA-Akt [K179M]) were expressed in BAECs by transient transfection, as previously reported.12 eNOS expression was silenced by bovine endothelial NOS-short interfering RNA (beNOS-siRNA).

Statistical Analysis
Data are given as mean±SEM. Statistical analyses were performed using analysis of variance. The post test comparison was performed by the method of Tukey. A $\chi^2$ test was used for categorical variables. Significance was accepted at $P<0.05$ levels.

Results
Preconditioning Protects Endothelial Cells From Hypoxia-Induced Cell Death
We performed a pilot experiment to evaluate the rate of cell death after different periods of hypoxia (supplemental Figure I and Table I). Because 12 hours represented the time point at which hypoxia induced nearly 60% cell death, mainly attributable to apoptosis, we tested whether brief periods of hypoxia were able to reduce hypoxia-induced cell mortality. Three cycles of 15 minutes of hypoxia followed by 15 minutes of reoxygenation (Figure 1A) reduced the cell death induced by 12 hours of hypoxia. A decrease in apoptotic cell death was mainly responsible for this phenomenon (Figure 1B). The observed cytoprotective effect had a biphasic trend, with early and late windows detectable immediately and 24 hours after PC was applied (supplemental Table II). In particular, apoptotic cell death was reduced in “early” and “late” preconditioned cells to 25±5% and 28±4%, respectively, compared to 48±5% in nonpreconditioned cells. Apoptosis was further explored by analysis of caspase-3 cleavage, which plays a key role in regulation of the cellular suicide cascade.13 This analysis confirmed the biphasic trend of PC-induced cytoprotection (Figure 1C), with early and late windows characterized by reduction of hypoxia-induced caspase-3 cleavage.

These results show that in endothelial cells, brief periods of repeated hypoxia followed by reoxygenation reduce hypoxia-induced apoptotic cell death with 2 windows of protection (early and late), indicating that our experimental conditions resemble the PC phenomenon.

Preconditioning Induces NO Synthesis in Endothelial Cells
Because NO is believed to be a key mediator of PC, we measured NO production by citrulline accumulation and by

Figure 1. A, Preconditioning (PC) protocol (H indicates hypoxia; R, reoxygenation). B, Cells were subjected to hypoxia after PC, as indicated. Apoptotic and necrotic cell death were assessed. C, Cells were subjected to hypoxia after PC as indicated. Caspase-3 cleavage was assessed by immunoblotting.

Figure 2. Cells were subjected to preconditioning (PC). Citrulline production (A) and nitrite accumulation (B) were assessed. Phosphorylation of endothelial nitric oxide synthase (eNOS; C) and expression of inducible nitric oxide synthase (iNOS; D) were assessed by immunoblotting.
same biphasic trend (Figure 2B). Analysis of enzymes accounting for NO production showed that both eNOS and inducible nitric oxide synthase (iNOS) were involved in preconditioning-induced NO synthesis. In particular, phosphorylation/activation of eNOS at serine1177 (ser1177) and expression of iNOS were detected immediately and 24 hours after PC (Figure 2C and 2D).

These data indicate that, in endothelial cells, PC induces NO production, eNOS activation, and iNOS expression.

PKA and PI3K Mediate NO Synthesis in the Late Preconditioning

Because the LPC confers a longer window of cytoprotection, we focused our interest on the molecular pathways that regulate LPC-induced NO production. Furthermore, in other models, it has been demonstrated that several mechanisms account for NO production in LPC, including activation of protein kinase A (PKA),14 phosphatidyl-inositol-3-kinase (PI3K),5 and protein kinase C (PKC)15 and production of reactive oxygen species (ROS).16 To examine which molecular pathway accounts for LPC-induced NO production in endothelial cells, we measured LPC-induced NO production in the presence of PKA-, PI3K-, PKC-, and iNOS-inhibitors, nonspecific NOS inhibition and an antioxidant (N-acetyl-cysteine, NAC). Inhibition of iNOS by 1400W (20 μmol/L, 15 minutes pretreatment) significantly blunted PC-induced NO production, whereas administration of NO synthesis inhibitor N,N-Nitro-L-arginine methyl ester (L-NAME; 1 mmol/L, 15 minutes pretreatment), alone or in combination with 1400W, abrogated it completely (Figure 3A and 3B). Consistently, LPC-induced phosphorylation/activation of eNOS was inhibited by H89, LY294002, and NAC (supplemental Figure IVA). In addition, LPC-induced iNOS expression was inhibited by pretreatment with H89 and NAC, and was significantly blunted by LY294002 (supplemental Figure IVB).

Next, because we found that NAC inhibited LPC-induced NO synthesis, we checked whether PC induced ROS production. ROS generation was assessed by evaluation of protein nitration.17 LPC induced an increase in nitrite protein content (3.2-fold versus control), which was significantly smaller than those detected at 1, 6, and 12 hours after PC application (6.1-fold versus control; Figure 3C). Pretreatment of late preconditioned BAECs with NAC abrogated formation of nitrated proteins (Figure 3D).

Together, these data indicate that PKA, PI3K, and ROS play a pivotal role in NO production during the late phase of PC.

Preconditioning Activates Both PKA and Akt

Because we found that LPC-induced NO production was mediated by PKA- and PI3K-dependent pathways, we asked whether LPC activates these kinases.

PKA activation was assayed by phosphorylation of kemptide (Kp), which is a synthetic substrate of PKA. Both early and late PC induced phosphorylation of Kp. In particular, Kp phosphorylation increased by 3.8-fold in LPC, compared to control (supplemental Figure V).

Next, we explored Akt, the serine/threonine kinase downstream of PI3K that promotes NO synthesis through the activation of eNOS.6 LPC resulted in phosphorylation/activation of Akt at both threonine308 (thr308; 3.2-fold versus control, P<0.001; Figure 4A) and serine473 (ser473; 5.8-fold versus control, P<0.001; Figure 4B).

To confirm that PC-induced PKA and Akt activation account for NO production through phosphorylation/activation of eNOS, we evaluated the physical interaction between...
PKA, Akt, and phospho-eNOS (ser1177) during PC. For this purpose, cell lysates from different time points after PC induction were subjected to immunoprecipitation with antibodies against the PKA catalytic subunit and Akt. These samples were then blotted with an antiphospho-eNOS (ser1177) antibody. Early PC induced a complex between PKA and phospho-eNOS, whereas LPC induced a complex between Akt and phospho-eNOS (Figure 4C and 4D).

Together these experiments indicate that PC activates PKA and Akt, which, in turn, physically interact with and phosphorylate eNOS. In particular, PKA is mainly involved in eNOS phosphorylation during the early phase of PC, whereas Akt accounts for eNOS phosphorylation in the late phase of PC.

**PKA and PI3K/Akt Mediate Cell Protection During Late PC**

Next, we explored which mechanism accounts for LPC-induced cytoprotection. For this purpose, we evaluated hypoxia-induced cell death and caspase-3 cleavage in LPC in the presence and absence of PKA-, PI3K-, and PKC-inhibitors, nonspecific inhibition of NO production and ROS scavenger. Hypoxia-induced apoptotic and necrotic cell death (supplemental Figure VIA and supplemental Table IV), as well as caspase-3 cleavage, were reduced by LPC (Figure 5A). This phenomenon was unaffected by inhibition of PKC (Go6983) and of NO production (L-NAME +1400W), but was abolished by inhibition of PKA (H89), PI3K (LY294002), and ROS activity (NAC). Interstingly, hypoxia-induced caspase-3 cleavage detected during PKA and PI3K inhibition was dramatically increased compared to that induced by hypoxia alone. To further rule out the contribution of iNOS and eNOS in LPC-induced cell protection, we evaluated cell death and caspase-3 cleavage in LPC after treatment with 1400W (supplemental Figure VIB) and after silencing eNOS expression by beNOS-siRNA transfection (Figure 5B). The efficacy of beNOS-siRNA transfection in BAECs was assessed by measurement of both eNOS protein expression and by enzymatic activity. Transfected cells showed a reduction in both eNOS expression (Figure 5B) and nitrite production (supplemental Figure XIA). Neither 1400W nor beNOS-siRNA treatment affected the cytoprotective effect of LPC, assessed by measurement of apoptotic and necrotic cell death and by caspase-3 cleavage.

Finally, to explore whether PKA and PI3K are required for LPC-induced cell protection, we assessed the effect of LPC on hypoxia-induced cell death and caspase-3 cleavage in cells with transient expression of dominant negative mutants of PKA (DN PKA) and Akt (DN Akt), which is the serine/threonine kinase that regulates several PI3K-dependent biological activities including cell survival. Transfection of plasmid harboring either DN PKA or DN Akt inhibited cell protection conferred by LPC. In fact, both plasmids abolished the inhibition of hypoxia-induced apoptotic and necrotic cell death (supplemental Figure VIIA and supplemental Table V) and caspase-3 cleavage induced by LPC (Figure 5C and 5D). The efficacy of DN PKA and DN Akt transfection in BAECs was assessed by immunoblotting (supplemental Figure VII B and VIIC). Evaluation of Bad phosphorylation, which confers cell protection against apoptosis, showed that LPC induces phosphorylation of serine112 and serine136, consesus sites for PKA and Akt, respectively (supplemental Figure VICE).

Together these data indicate that, in endothelial cells, the LPC-induced cytoprotective effect requires a PKA- and PI3K/Akt-dependent pathway.

**PKA and PI3K Mediate PC-Induced Akt Activation**

Because both PKA- and PI3K/Akt-dependent pathways account for the LPC-induced cytoprotective effect, we explored the cross-talk between these 2 pathways. First, we analyzed the mechanism of LPC-induced Akt activation. For this purpose, LPC-induced Akt phosphorylation was assessed in the presence of H89, LY294002, Go6983, L-NAME, 1400W, and NAC. LPC-induced Akt phosphorylation, at thr308 and ser473, was abrogated by pretreatment with H89 and NAC, but was only blunted by LY294002 (supplemental Figure XA and XB), suggesting that PKA plays a critical role in Akt activation during LPC. Therefore, we asked whether PKA and Akt physically interact during PC. For this purpose, lysates from cells subjected to PC were immunoprecipitated with antibodies against the PKA catalytic subunit and then blotted with antiphospho-Akt (thr308/ser473) antibodies. Both early and late PC induced an immunocomplex between PKA and phospho-Akt. (Figure 6A and 6B). To verify that Akt is a substrate of PKA during PC, we immunoprecipitated cell lysates using an anti-Akt antibody and blotted with an antibody recognizing PKA substrates. PC increased the recognition of Akt by anti-PKA phospho-substrate antibody, confirming that Akt is phosphorylated by PKA during PC (Figure 6C).

These results indicate that both PKA and PI3K are required for LPC-induced Akt activation and that Akt is a substrate of PKA.

**Discussion**

The present study focused on the molecular mechanisms that account for the PC-induced cytoprotective effect in endothelial cells. Our data appear to contrast with the reports showing that NO production is required for PC-induced cytoprotec-
Several factors can account for these discrepancies. First, our experimental setting was completely different from that used by other authors, because we used cell culture, whereas Xuan et al.\(^1\) evaluated infarct size in transgenic mice and Laude et al.\(^2\) analyzed coronary vasorelaxation in pre-conditioned rat hearts. Moreover, we exclusively explored the molecular mechanisms involved in cell survival, whereas these authors investigated different functional aspects of PC. These controversial data highlight the need to specifically define the mechanisms that account for the different biological components of PC.

In this study, we found that PC induces activation of Akt, which in turn accounts for the PC-induced cytoprotection. This mechanism allows for speculation that PC-induced NO production, rather than being a mediator of cell survival, is an epiphenomenon of Akt activation. However, we do not exclude the possibility that in a more complex biological system, like in vivo experimental models, NO may play a critical role in PC-induced cytoprotection. In addition, it should be noted that our experimental setting excluded the contribution of inflammation to the endothelial response. This could explain the absence of an NO protective role in PC-induced endothelial protection.

Our data show that, in endothelial cells, the PC-induced cytoprotective effect is mediated by PKA- and PI3K-dependent activation of Akt. A substantial body of evidence indicates that PI3K-dependent Akt activation accounts for a cytoprotective effect in different cell types and in response to several stress stimuli. In contrast, it has been demonstrated that PKA activation, rather than leading to a survival signal, promotes apoptosis in different cell types, including endothelial cells.\(^2\) Akt can be activated by distinct mechanisms, including wortmannin-sensitive and -insensitive pathways, and it has been reported that Akt can be activated by a PKA-dependent mechanism in endothelial cells.\(^2\) In the present study, we found that Akt activation in LPC requires PKA, that PKA forms a physical complex with Akt, and, more interestingly, that Akt acts as a substrate of PKA. This suggests that, in endothelial cells, the PC-induced cytoprotective effect is mediated by the cross-talk between PKA and Akt, which directly interact. Thus, PKA does not exclusively act as a proapoptotic kinase, but can also account for a cytoprotective effect through its downstream target.

We found that LPC-induced phosphorylation of Akt was blunted, but not entirely inhibited, by pretreatment with LY294002, suggesting that, in endothelial cells, PI3K plays a partial role in PC-induced cytoprotection. Although this was an unexpected result, it is reasonable to speculate that the differential relevance of PKA and PI3K in promoting cell protection depends on the characteristics of the survival stimuli. For instance, PI3K-dependent mechanisms play a pivotal role in insulin-\(^2\) or IGF-1–induced cytoprotection. On the other hand, PKA-dependent mechanisms account for some biological responses evoked by hypoxia.\(^2\)

We also noted that NAC abrogates LPC-induced protection in BAECS, suggesting an important role of ROS in this phenomenon. Consistently, protein nitration peaked between early and late PC, when the protective effect is abolished. These data highlight the need to clarify the role of ROS.
production. In particular, low doses of ROS, by reducing thiolic residues in transmembrane receptors, induce activation of downstream protection pathways,27 whereas high doses of ROS, by affecting the tertiary structure of these receptors, inhibit activation of protective mechanisms.27 Finally, at high concentrations, ROS react with NO, inducing generation of peroxynitrite which uncouples eNOS function.28

We found that expression of beNOS-siRNA significantly reduced LPC-induced nitrite production. Although this would appear to be inconsistent with the finding that, during LPC, iNOS mainly accounts for nitrite production, there is, in fact, no contradiction, because it has been demonstrated that nuclear translocation of eNOS is required for iNOS expression.29 Consistent with this observation, we found that LPC induces nuclear translocation of eNOS and that beNOS-siRNA transfection inhibits LPC-induced iNOS expression (supplemental Figure XI).

In conclusion, our data indicate that, in endothelial cells, the protective effect of LPC against hypoxic injury is mediated by PKA-, PI3K-dependent Akt activation and ROS generation.

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Disclosures

None.

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SUPPLEMENT MATERIAL

The Cross-talk between PKA and Akt Protects Endothelial Cells from Apoptosis in the Late Ischemic Preconditioning.

Materials and Methods

Chemicals and Reagents

H89, LY294002, Go6983, Nω-Nitro-L-arginine methyl ester (L-NAME), 1400W, N-Acetyl-Cysteine, N-naphtyl-ethylene-diamine dihydrochloride, sulphanilamide, sodium nitrite solution, phosphoric acid, kemptide and NOC-12 were purchased from Sigma Aldrich Corporation (St. Louis, MO; USA). H85 was obtained from D. Western Therapeutics Institute, Inc. (Nishiki, Naka-ku, Nagoyashi; Japan).

Cell Culture

Bovine aortic endothelial cells (BAECs) were purchased from Lonza Biologics Inc (Portsmouth, USA), cultured in DMEM medium (Lonza Biologics Inc; Portsmouth, USA) supplemented with 10% fetal bovine serum (Lonza Biologics Inc; Portsmouth, USA), 1% penicillin-streptomycin (Lonza Biologics Inc; Portsmouth, USA) and 1% glutamine (Lonza Biologics Inc; Portsmouth, USA) and maintained at 37°C in 5% CO2. BAECs were used at passages 3-7.

Hypoxia

Hypoxia was induced by incubation of cells in a medium previously saturated for 10 min at 1 atm with 95% N2 and 5% CO2 mixture, containing (mM) concentrations of 116 NaCl, 5.4 KCl, 0.8 MgSO4, 26.2 NaHCO3, 1 NaH2PO4, 1.8 CaCl2, 0.01 glycine and 0.001 (% w/v) phenol red, and placement in an anaerobic chamber (hypoxia chamber) filled with the same gas mixture and heated to 37°C. The pH, PO2 and PCO2 of the resulting
medium was 7.36±0.2, 45.3±1.2 mmHg, and 35.3±0.8 mmHg and 7.32±0.9, 32.6±1.1 and 37.9±2.1 mmHg, before and at the end of hypoxia, respectively.¹

Estimation of Nitric Oxide (NO) Production

Conversion assay of L-arginine into L-citrulline (Stratagene Agilent Technologies, La Jolla, USA) - Cells were plated on 100 mm dishes and serum-starved overnight. The next day, cells were stimulated as described and homogenized in 25 mmol/L Tris HCl, pH 7.4, 1 mmol/L EDTA, and 1 mmol/L EGTA. The pellet was collected after centrifugation and 20 µg of protein were incubated in 25 mmol/L Tris HCl, 3 µmol/L tetrahydrobiopterin, 1 µmol/L flavin adenine dinucleotide, 1 µmol/L flavin adenine mononucleotide, 25 µmol/L NADPH, 10 µmol/L CaCl₂, and 2 nCi/µL of [³H] arginine (Bcs Biotech SpA; ITALY) for 60 minutes at 37°C. The reaction was stopped with an equal volume of 50 mmol/L HEPES and 5 mmol/L EDTA and chromatographed on Dowex AG50WX-8 columns. Flow-throughs were counted by liquid scintillation. Citrulline production was measured in picomoles per minute per milligram protein and expressed as unitary changes from basal values.²

GRIESS reaction - Nitrite accumulation was measured in the culture medium by GRIESS reaction.³ Briefly, cells were plated on six-well plates and serum-starved overnight. The next day, cells were stimulated as described, and the culture medium was removed and centrifuged at 1000 rpm to remove dead cells. The supernatant was subsequently challenged with a 1 : 1 mixed solution of α-naphthyl-ethylenediamine (stock solution 0.1% w/v in H₂O) and sulphanilamide (stock solution 1% w/v, in 5% H₃PO₄) for 10 min and kept under dark conditions at 23°C. Nitrite production was recorded with a Perkin-Elmer LS 55 luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, England) by monitoring absorbance at 550 nm. A NaNO₂ standard curve was used to quantify the nitrite production obtained during the experiments.
Apoptosis and Necrosis Detection

Cells undergoing early apoptosis were stained with Annexin V (AV), but not with Propidium Iodide (PI) (Annexin-V- FLUOS Staining Kit, Roche), whereas necrotic cells or cells in late apoptosis stages were stained with both AV and PI. The percentage of apoptotic and necrotic cells was calculated by dividing the number of AV-positive/PI-negative cells and AV-positive/PI-positive cells by the total number of nuclei detected with DAPI staining, respectively.1

Protein Kinase A (PKA) Activity Assay

The activity of PKA was tested in a cell-free reaction system using the synthetic peptide kemptide (Kp) as a specific substrate. The assay was conducted as previously described.4 BAECs were lysed in a buffer containing 50 mM β-glycerophosphate, 1 mM NaF 1.5 mM EGTA, 1% NONIDET P-40, 1 mM EDTA, 0.1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM DTT. Phosphorylation was carried out in a final volume of 50 µl containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂ and 1 µg Kp. Phosphorylation was initiated by the addition of 10 µM [γ-32P] ATP (6 Ci/mmol; Bcs Biotech SpA; ITALY). The reaction was allowed to proceed for 20 min at room temperature arrested by the addition of sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol) and boiled for 3 min at 95 ºC. The samples were subjected to 10% SDS–PAGE and autoradiography. Phosphorylation of Kp by the catalytic subunit of PKA (Sigma Aldrich Corporation, St. Louis, MO; USA) served as a positive control.

Immunoblotting and Immunoprecipitation

Cells washed twice with ice-cold Ca²⁺/Mg²⁺-free Dulbecco PBS and lysed with buffer containing 50 mM HEPES (pH 7.6), 1mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 50 mM β-glycerophosphate, 1 mM vanadate, 10 mM sodium fluoride, 30 mM
sodiumpyrophosphate, 2 mM dithiothreitol, 1 mM AEBSF were used for detection of Akt phosphorylation by anti-phospho-Akt (serine473 and threonine308) antibodies (Cell Signaling Technology). Cells lysed with buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% deoxycholic acid, 1% NP-40 (IGEPAL CA-630), 0.1% SDS, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 0.5 mM PMSF, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin were used for other immunoblottings and immunoprecipitations. Immunoblottings and immunoprecipititates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane and assessed with primary antibodies. We used the following primary antibodies: anti-phospho-endothelial nitric oxide synthase (eNOS) serine1177 (ser1177), anti-phospho-Bad serine112 and serine136 (ser112 and ser136), anti-cleaved caspase-3, anti-nitro-tyrosine, anti-caspase-3, anti-Bad (Cell Signaling Technology); anti-inducible nitric oxide synthase (iNOS), anti-actin, anti-Akt, anti-PKA catalytic subunit (Santa Cruz Technology); anti-eNOS (BD Transduction Laboratories™; San Jose, CA USA); anti-HA (Covance, Princeton, New Jersey).

Horseradish peroxidase-conjugated (Cell Signaling and Santa Cruz Technology) antibodies were used as secondary antibody. The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Transient Transfection**

Bovine endothelial NOS-short interfering RNA (beNOS-siRNA) was generated from the bovine eNOS sequence found 1824 bp after the start codon (GAGTTACAAGATCCGCTTC; EUROGENTEC S.A., Belgium).^5^ BAECs were plated at a density of 1×10^6^ per well in six-well plates. Twenty-four hours after plating, the medium was changed to Dulbecco's modified Eagle's medium/Ham's F-12 medium without supplement. Transfections were carried out using 10 μl/ml of LipofectAMINE (Life Technologies) in 1 ml/well Dulbecco's modified Eagle's
medium. Four hours after transfection, the culture medium was changed to the culture medium supplemented with serum. Forty-eight hours after transfection, BAECs were serum-starved overnight and stimulated as described.

**Preparation of Nuclear Extracts**

Nuclear proteins were extracted as described. Cells were washed and scraped with Tris-buffered saline. After centrifugation, cells were resuspended in 200 µl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM Na3VO4, 1 mM dithiothreitol, 0.5 mM AEBSF) by gentle pipetting and allowed to swell on ice for 15 min. Cells were lysed by adding 12.5 µl of 10% Nonidet P-40 and vortexing vigorously for 10 s, followed by centrifugation (12,000 rpm) for 30 s. The supernatant was saved as the cytosolic fraction. Pellets were resuspended in 50 µl of ice-cold buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM Na3VO4, 1 mM dithiothreitol, 0.5 mM AEBSF) and rocked for 15 min. The samples were centrifuged at 14,000 rpm for 5 min. The supernatant was saved as the nuclear fraction. The entire procedure was carried out at 4 °C. The samples were stored at -85 °C.

**Results**

*Preconditioning (PC) Protects Endothelial Cells from Hypoxia-induced Cell Death*

First, we tested the possibility of producing an in vitro model of PC in endothelial cells. For this purpose, we analyzed the rate of cell death following different periods of hypoxia. Hypoxia induced a time-dependent increase in cell death by both apoptosis and necrosis (Figure I A). In particular, after 1, 6, 12 and 24 hours of hypoxia, the rate of cell death (apoptotic + necrotic) was 28%, 58%, 81% and 94%, respectively (Table I). Apoptosis was mainly detectable after short term hypoxia, whereas prolonged hypoxia
induced both apoptosis and necrosis. Apoptosis was confirmed by cleavage of caspase-3 (Figure I B).

Next, to ensure that our PC protocol did not itself induce apoptosis, we assessed caspase-3 cleavage and pro-caspase-3 expression in endothelial cells at various time points (0, 1, 6, 12, 24, 48 hours) following PC application in the absence of hypoxia. PC alone did not increase caspase-3 cleavage, nor did it affect the protein expression of pro-caspase (Figure I C).

Chemical Inhibition of PKA and Reactive Oxygen Species (ROS) Scavenging

We performed dose-response curves for H89 (PKA inhibitor) and N-acetyl-cysteine (anti-oxidant, NAC) to evaluate the concentrations required to inhibit late PC (LPC)-induced caspase-3 cleavage. H89 at a concentration of 10 μM (30 minutes pretreatment) affected LPC-induced inhibition of hypoxia-induced caspase 3-cleavage, but not at concentrations of 0.1 μM and 1 μM (Figure II A). Higher concentrations of H89 (100 μM) resulted in cell toxicity. Therefore, 10 μM H89 was considered adequate for inhibition of PKA-mediated caspase-3 cleavage. Furthermore, to test whether H89 specifically inhibits PKA, we assessed hypoxia-induced caspase-3 cleavage following LPC in the presence and absence of H85 (a closely related ineffective control molecule). H89 (10 μM, 30 minutes pretreatment) affected LPC-induced inhibition of hypoxia-induced caspase 3-cleavage, whereas an equal concentration of H85 did not (Figure II B), indicating the specificity of H89 as inhibitor of PKA.

LPC-induced inhibition of hypoxia-induced caspase 3-cleavage was assessed in the presence of graded concentrations of NAC (100 μM, 1 mM, 10 mM). NAC at a concentration of 10 mM (30 minutes pretreatment) affected LPC-induced inhibition of hypoxia-induced caspase 3-cleavage, whereas lower concentrations did not. Higher concentrations of NAC (100 mM, 30 minutes pretreatment) resulted in toxicity. This finding
indicates that, in our experimental setting, NAC at a concentration of 10 mM is adequate for inhibition of ROS activities in LPC (Figure II C).

PKA Inhibition Alone Affects Early PC (EPC)-Induced Protection

To confirm the hypothesis that the PKA-pathway, rather than the phosphatidylinositol-3-kinase (PI3K)-pathway, is primarily responsible for EPC-induced cell protection, we assessed cell death by AV/PI staining and caspase-3 cleavage in early preconditioned BAECs, in the presence and absence of PKA and PI3K inhibitors (H89 and LY294002, respectively) (Figure III A). H89 (10 μM, 30 minutes pretreatment) abrogated EPC-induced cell protection, causing an increase in apoptosis and necrotic cell death (Table III), whereas LY294002 (30 μM, 30 minutes pretreatment) did not have this effect. Consistently, EPC-induced inhibition of caspase-3 cleavage (Figure III B) was abrogated by H89. Moreover, EPC-induced Akt phosphorylation was inhibited by H89 rather than LY294002 (Figure III C), suggesting that activation of Akt in EPC is a PKA-dependent phenomenon.

These results indicate that PKA, rather than PI3K, is critically involved in EPC-induced cell protection.

PKA and PI3K/Akt Mediate Cell Protection during LPC through Bad Phosphorylation

To clarify which mechanism accounts for LPC-induced cytoprotection, we evaluated hypoxia-induced apoptotic and necrotic cell death in LPC in the presence and absence of PKA, PI3K and protein kinase C (PKC) inhibitors, selective antagonism of inducible NOS, non-specific inhibition of NO production and ROS scavenger.

Apoptotic and necrotic cell death (Figure VI A) induced by 12 hours of hypoxia were reduced by LPC. This phenomenon was abolished by inhibition of PKA and PI3K and by
ROS neutralization, obtained with H89, LY294002 and NAC, respectively (Table IV). In contrast, the cytoprotective effect of LPC was not affected by inhibition of PKC (Go6983; 1 μM, 60 minutes pretreatment), selective antagonism of iNOS (1400W; 20 μM, 15 minutes pretreatment), or non-specific inhibition of NO synthesis (L-NAME [1 mM, 15 minutes pretreatment] + 1400W).

Furthermore, to elucidate a possible mechanism that accounts for LPC-induced cell protection, we assessed Bad phosphorylation in late preconditioned cells. Bad acts as an anti-apoptotic molecule when phosphorylated at ser112 and ser136. LPC induced phosphorylation of both ser112 and ser136. Interestingly, these are the consensus sites for phosphorylation by PKA and Akt, respectively (Figure VI C).

Together these data indicate that LPC-induced cytoprotection is a PKA- and PI3K-dependent phenomenon.

**Transient Expression of Dominant Negative Mutants of PKA and Akt Abrogated LPC-Induced Cytoprotection**

To confirm the key role of PKA and Akt in LPC-induced cell protection, epitope tagged dominant negative mutants of PKA (DN PKA) and Akt (DN Akt) were transiently expressed in BAECs. Apoptotic and necrotic cell death (Figure VII A) induced by 12 hours of hypoxia were reduced by LPC. This phenomenon was abolished by expression of either DN PKA or DN Akt (Table V). Furthermore, we explored whether suppression of eNOS by beNOS-siRNA affects the cytoprotective effect of LPC. The cytoprotective effect of LPC was unaffected by inhibition of eNOS expression (Figure VII A and Table V).

The efficacy of DN PKA and DN Akt transfection in endothelial cells was assessed by immunoblotting. HA expression was detectable only in cells transfected with epitope tagged DN PKA (Figure VII B) and DN Akt (Figure VII C). Similarly, the expression of PKA
(Figure VII B) and Akt (Figure VII C) was increased in cells transfected with DN PKA and DN Akt compared to those with pcDNA3.

Together, these data indicate that LPC-induced cell protection is a PKA- and Akt-dependent phenomenon and does not require eNOS protein expression.

**NO-donor (NOC-12) Does Not Confer Protection from Hypoxia-Induced Apoptosis in BAECs**

To explore whether NO plays a role in LPC-induced cytoprotection, independently from eNOS and iNOS activity, we evaluated the cytoprotective effect of administration of an NO long-term donor (NOC-12) in preconditioned cells exposed to hypoxia. As expected, EPC and LPC significantly reduced hypoxia induced caspase-3 cleavage. The cytoprotective effect of PC was partially lost after 1 hour and undetectable 48 hours after PC. Compared to control, NOC-12 administration increased nitrite accumulation in culture media by 6.4 fold, and did not reduce the caspase-3 cleavage observed in cells exposed to 12 hours of hypoxia at 1 hour and 48 hours after PC (Figure VIII).

These results indicate that NO release from NO donors does not induce cytoprotection.

**LPC Does Not Induce Interaction between PKA, Akt and Inducible NOS (iNOS)**

To evaluate whether PKA and Akt interact with iNOS during PC, we immunoprecipitated lysates from cells at different times following PC application with PKA and Akt antibodies. Then, immunoprecipitates were subjected to SDS-PAGE, transferred to a PVDF membrane and assessed with anti-iNOS antibody. No interaction between PKA, Akt and iNOS was observed in LPC (Figure IX A and IX B).

These data exclude any physical interaction between iNOS and PKA or Akt in LPC.
Nuclear Translocation of eNOS Is Required for LPC-Induced iNOS Expression

It has been demonstrated that iNOS gene expression can be regulated by nuclearization of eNOS. Therefore, we explored whether this transcriptional mechanism also accounts for LPC-induced iNOS expression in BAECs. For this purpose, we first evaluated the time-course of changes in subcellular localization of eNOS. Both nuclear and cytoplasmic fractions were prepared and immunoblot analyses performed. Under control conditions, eNOS was totally localized in the cytosolic fraction. However, LPC increased nuclear expression of eNOS (Figure XI B). Consistently, immunoblot analyses of the cytosolic fraction showed a progressive decrease in eNOS after PC was applied (Figure XI C). Next, we examined whether suppression of eNOS synthesis by transfection with beNOS-siRNA affects LPC-induced iNOS expression. For this purpose, BAECs were transfected with beNOS-siRNA (20 μg), then subjected to LPC. beNOS-siRNA tranfection resulted in inhibition of LPC-induced iNOS expression (Figure XI D).

These data indicate that, in BAECs, LPC induces nuclear translocation of eNOS, and LPC-induced iNOS expression requires an eNOS-dependent transcriptional pathway.
Bibliography


Figure I

A

Control Hypoxia 1h Hypoxia 6h Hypoxia 12h Hypoxia 24h

B

C

Cleaved Caspase-3

Actin

Cleaved Caspase-3

Actin

Cleaved Caspase-3

Actin

Control PC PC PC PC PC PC
+ + + + + +
1h 6h 12h 24h 48h Nox Nox Nox Nox Nox Nox
Figure II

A

B

C
Figure III

A

Control Hypoxia 12h EPC H89 LY
+ + +
Hypoxia 12h EPC EPC
+ +
Hypoxia 12h Hypoxia 12h

B

Cleaved Caspase-3
Unitary Changes

Actin

Control Hypoxia 12h EPC H89 LY
+ + +
Hypoxia 12h EPC EPC
+ +
Hypoxia 12h Hypoxia 12h

C

p-Akt (ser473)
Unitary Changes

Akt

Control Hypoxia 12h EPC H89 LY
+ + +
Hypoxia 12h EPC EPC
+ +
Hypoxia 12h Hypoxia 12h
Figure IV

A

Unitary Changes (p-eNOS ser1177)

p-eNOS (ser1177)

Control LPC H89 + LPC NAC + LPC Go6983 + LPC LY + LPC L-NAME + LPC 1400W + LPC L-NAME + LPC

B

Unitary Changes (iNOS)

iNOS

Control LPC H89 + LPC LY + LPC Go6983 L-NAME 1400W L-NAME NAC + LPC + 1400W + LPC + LPC
Figure V

Unitary Changes (Kemptide Phosphorylation)

Control  PC  PC  PC  PC  PC  PC
+ + + + + +
1h 6h 12h 24h 48h
Nox Nox Nox Nox Nox

*p §* §* §
Figure VI

A

Control  Hypoxia 12h  LPC  +  Hypoxia 12h  H89  +  Hypoxia 12h  LY  +  Hypoxia 12h  Go6983  +  Hypoxia 12h  1400W  +  Hypoxia 12h  L-NAME+1400W  +  Hypoxia 12h  NAC  +  Hypoxia 12h

B

C

[Diagrams and graphs showing experimental results]
Figure VII

A

Control  Hypoxia 12h  LPC  +  Hypoxia 12h  DN PKA  +  LPC  +  Hypoxia 12h  DN Akt  +  EPC  +  Hypoxia 12h  beNOS-siRNA  +  LPC  +  Hypoxia 12h

B

HA
PKA
Actin
pcDNA3  DN PKA

C

HA
Akt
Actin
pcDNA3  DN Akt
Figure VIII

Unitary Changes (Cleaved Caspase-3)

Cleaved Caspase-3

Actin

Control Hypoxia 12h EPC LPC PC PC PC PC
+ + + + + +
1h 1h 48h 48h 1h 1h
Nox Nox Nox Nox Nox Nox

Hypoxia 12h NOC-12 +Hypoxia 12h

* §
Figure IX

A

IP: Anti-PKA Catalytic Subunit  
WB: Anti-iNOS

Unitary Changes (iNOS)

PKA Catalytic Subunit

Control  PC  PC  PC  PC  PC  PC
+  1h  Nox  +  24h  Nox
+  6h  Nox  +  12h  Nox
+  1h  Nox

B

IP: Anti-Akt  
WB: Anti-iNOS

Unitary Changes (iNOS)

Akt

Control  PC  PC  PC  PC  PC  PC
+  1h  Nox  +  6h  Nox  +  12h  Nox  +  24h  Nox

Figure X

A

Unitary Changes (pAkt (thr308))

Act

Control LPC H89 + LPC NAC + LPC Go6983 + LPC L-NAME + LPC 1400W + LPC

p-Akt (thr308)

B

Unitary Changes (pAkt (ser473))

Act

Control LPC H89 + LPC LY + LPC Go6983 + LPC L-NAME + LPC 1400W + LPC L-NAME + LPC 1400W + LPC NAC + LPC
Figure XI

A

Scrambled RNA
beNOS-siRNA

Control Hypoxia 12h

Scrambled RNA + - + -
beNOS-siRNA - + - +

B

eNOS
Histone H3

Control PC PC PC PC PC PC
+ 1h 6h 12h 24h 48h Nox

C

eNOS
Actin

Control PC PC PC PC PC PC
+ 1h 6h 12h 24h 48h Nox

D

iNOS
eNOS
Actin

Control LPC
Scrambled RNA + - + -
beNOS-siRNA - + - +
**FIGURE LEGENDS**

**Figure I.** (A) Cells were subjected to hypoxia, as indicated. Apoptosis was assessed by Annexin V (green) and necrosis by Propidium Iodide (red) staining, and nuclei were stained with DAPI (blue). The figure shown is representative of six independent experiments. (B) Cells were subjected to hypoxia, as indicated. Caspase-3 cleavage was assessed by immunoblotting. (C) Pro-caspase-3 expression in endothelial cells at various time points (0, 1, 6, 12, 24, 48 hours) after PC application was evaluated by immunoblotting. (Nox: normoxia). Bar graphs represent densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage and protein expression over that in control cells, of four independent experiments.

* p<0,001 vs control.

**Figure II.** (A) Late preconditioned BAECs were subjected to a dose-response curve with H89 and hypoxia-induced caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of four independent experiments. (B) Late preconditioned BAECs were pretreated with H85 and hypoxia-induced caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of three independent experiments. (C) Late preconditioned BAECs were subjected to a dose-response curve with N-acetyl-cysteine (NAC) and hypoxia-induced caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of four independent experiments. LPC: late preconditioning.

* p<0,001 vs control; § p<0,001 vs LPC.
**Figure III.** (A) Early preconditioned cells were subjected to hypoxia, in the presence and absence of H89 and LY294002. Apoptosis was assessed by Annexin V (green) and necrosis by Propidium Iodide (red) staining, and nuclei were stained with DAPI (blue). The figure shown is representative of five independent experiments. Early preconditioned BAECs were exposed to hypoxia. Caspase-3 cleavage (B) and Akt phosphorylation at serine473 (ser473) (C) were assessed in the presence and absence of H89 and LY294002. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage and phosphorylation over that in control cells, of three independent experiments. EPC: early preconditioning; LY: LY294002.

* p<0,001 vs control; § p<0,001 vs hypoxia 12 h.

**Figure IV.** Cells were subjected to late preconditioning (LPC) in the presence and absence of different inhibitors, as reported in the text. (A) Phosphorylation of endothelial nitric oxide synthase (eNOS) and (B) expression of inducible nitric oxide synthase (iNOS) were assessed. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein phosphorylation and expression over that in control cells, of three independent experiments.

* p<0,001 vs control; † p<0,001 vs H89, LY and NAC.

**Figure V.** Preconditioning (PC) induced phosphorylation of kemptide. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein phosphorylation over that in control cells, of three independent experiments. (Nox: normoxia).

* p<0,001 vs control; § p<0,001 vs PC + 6h Nox.
**Figure VI.** (A) Late preconditioned cells were subjected to hypoxia in the presence and absence of specific inhibitors, as described in the text. Apoptosis was assessed by Annexin V (green) and necrosis by Propidium Iodide (red) staining, and nuclei were stained with DAPI (blue). The figure shown is representative of four independent experiments. (B) Cells were treated with an inhibitor of inducible nitric oxide synthase (iNOS; 1400W), then subjected to hypoxia in the presence of late preconditioning (LPC). Caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of four independent experiments. (C) Bad phosphorylation at serine112/serine136 (ser112/ser136) was assessed by immunoblotting in BAECs exposed to hypoxia in the presence and absence of LPC. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein phosphorylation over that in control cells, of three independent experiments. White bars represent phosphorylation at ser112; black bars indicate phosphorylation at ser136. LPC: late preconditioning; LY: LY294002.

* p<0.001 vs control; § p<0.001 vs hypoxia 12 h.

**Figure VII.** (A) BAECs were transfected with expression vectors encoding for hemagglutinin (HA) tagged dominant negative protein kinase A (DN PKA) and Akt (DN Akt) mutants, and with bovine endothelial nitric oxide synthase-short interfering RNA (beNOS-siRNA). They were then treated as described in text. Apoptosis was assessed by Annexin V (green) and necrosis by Propidium Iodide (red) staining, and nuclei were stained with DAPI (blue). The figure shown is representative of three independent experiments. Efficiency of transfection was determined for both DN PKA (B) and DN Akt (C) by evaluation of HA, and by PKA and Akt protein expression, assessed by immunoblotting.
**Figure VIII.** Hypoxia-induced caspase-3 cleavage was assessed by immunoblotting in both early and late preconditioned cells 1 hour and 48 hours after PC application. Preconditioned cells at 1 hour and 48 hours after PC application were also treated with a nitric oxide long-term donor (NOC-12, 0.1 mM), as described in the text. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of four independent experiments. Nox: Normoxia; EPC: early preconditioning; LPC: late preconditioning.

* p<0,001 vs control; § p<0,01 vs EPC and LPC.

**Figure IX.** Cells were harvested at the indicated time-points after PC, then immunoprecipitated with an antibodies recognizing the protein kinase A (PKA) catalytic subunit (A) and Akt (B), and immunoblotted with inducible nitric oxide synthase (iNOS) antibody. (Nox: normoxia). Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein co-immunoprecipitation over that in control cells, of four independent experiments.

**Figure X.** Cells were subjected to late preconditioning (LPC) in the presence or absence of inhibitors, as described. Phosphorylation of Akt at both threonine308 (thr308) (A) and serine473 (ser473) (B) was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein phosphorylation over that in control cells, of three independent experiments.

* p<0,001 vs control; † p<0,001 vs H89, LY and NAC; # p<0,001 vs H89.

**Figure XI.** (A) BAECs were transfected with bovine endothelial nitric oxide synthase-short interfering RNA (beNOS-siRNA, 20 μg), then subjected to late
preconditioning (LPC). Nitrite production was detected by GRIESS reaction. Cells were harvested at the indicated time-points after preconditioning (PC). Nuclear (50 µg) (B) and cytosolic (50 µg) (C) fractions were subjected to immunoblot analyses using anti-eNOS antibody. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase/decrease in protein translocation over that in control cells, of three independent experiments. (D) BAECs were transfected with beNOS-siRNA, then subjected to 12 hours of hypoxia in the presence and absence of LPC. Protein expression of eNOS and inducible nitric oxide synthase (iNOS) was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein expression over that in control cells, of three independent experiments.

* p<0.001 vs control.
SUPPLEMENT FIGURE LEGENDS: details for figure legends in manuscript

Figure 1. (A) Preconditioning (PC) protocol. (H: Hypoxia; R: Reoxygenation). (B) Cells were subjected to hypoxia after PC, as indicated. Apoptosis was assessed using Annexin V (green) and necrosis using Propidium Iodide (red) (PI) staining; nuclei were stained with DAPI (blue). The rates of apoptosis and necrosis were calculated by dividing the number of Annexin-V-positive/PI-negative cells and annexin-V-positive/PI-positive cells respectively by the total number of nuclei detected with DAPI staining. The percentage of apoptotic and necrotic cell death was calculated from six independent experiments (Nox: normoxia). (C) Cells were subjected to hypoxia after PC as indicated. Caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of four independent experiments. *p<0.001 vs control.

Figure 2. Cells were subjected to preconditioning (PC). Citrulline production (A) and nitrite accumulation (B) were assessed at different times following PC (Nox: normoxia). Bar graph shows the mean±SEM, expressed as fold increase in citrulline production and nitrite accumulation over that in control cells, of four independent experiments. (C) Phosphorylation of endothelial nitric oxide synthase (eNOS) and (D) expression of inducible nitric oxide synthase (iNOS) were assessed at different times following PC. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein phosphorylation or expression over that in control cells, of four independent experiments. ser1177: serine1177.

* p<0.001 vs control; § p<0.001 vs PC + 6h Nox.

Figure 3. Cells were subjected to late preconditioning (LPC) in the presence and absence of different inhibitors, as reported in the text. Citrulline production (A) and nitrite
accumulation (B) were assessed. Bar graph shows the mean±SEM of five independent experiments. (C) BAECs were subjected to preconditioning (PC) and harvested at different time points. Protein extracts were blotted with an anti-nitrotyrosine antibody to evaluate protein nitration. (D) Pretreatment of late preconditioned cells with NAC caused a reduction in protein nitration compared to late preconditioned control cells. (Nox: normoxia). Bar graph represents densitometric analysis of six major bands, mean±SEM, expressed as fold increase in protein nitration over that in control cells, of three independent experiments. LY: LY294002; L-NAME: Nω-Nitro-L-arginine methyl ester; NAC: N-acetyl-cysteine.

* p<0.001 vs control; † p<0.001 vs H89, LY and NAC; § p<0.001 vs PC and PC+24h Nox.

**Figure 4.** Preconditioning (PC) induced phosphorylation of Akt (A, B). Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein phosphorylation over that in control cells, of three independent experiments. (C, D) Cells were harvested at the indicated times after PC, then immunoprecipitated with an antibody recognizing the catalytic subunit of protein kinase A (PKA) and Akt and immunoblotted with anti-phospho-eNOS antibody. (Nox: normoxia). Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein co-immunoprecipitation over that in control cells, of four independent experiments.

* p<0.001 vs control; § p<0.001 vs PC + 6h Nox.

**Figure 5.** (A) Cells were treated as indicated, then subjected to hypoxia in the absence or presence of late preconditioning (LPC). Caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of four independent
experiments. (B) BAECs were transfected with bovine endothelial nitric oxide synthase-short interfering RNA (beNOS-siRNA, 20 μg), then subjected to hypoxia in the absence or presence of LPC. Caspase-3 cleavage was assessed by immunoblotting. eNOS protein expression was detected in transfected cells by immunoblotting. (C) Cells were transfected with expression vector encoding for dominant negative mutant of protein kinase A (DN PKA; 6 μg) or pcDNA3 (6 μg) and subjected to hypoxia in the presence or absence of LPC. Caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of five independent experiments. (D) Cells were transfected with expression vector encoding for dominant negative mutant of Akt (DN Akt; 6 μg) or pcDNA3 (6 μg), then subjected to hypoxia in the presence or absence of LPC. Caspase-3 cleavage was assessed by immunoblotting. Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein cleavage over that in control cells, of five independent experiments. LY: LY294002; L-NAME: Nω-Nitro-L-arginine methyl ester; NAC: N-acetyl-cysteine.

* p<0.05 vs control; § p<0.001 vs Hypoxia 12h.

Figure 6. (A, B) Cells were harvested at the indicated times after PC, then immunoprecipitated with an antibody recognizing the catalytic subunit of protein kinase A (PKA) and immunoblotted with anti-phospho-Akt antibodies. (C) Cells were harvested at the indicated times after PC, then immunoprecipitated with an antibody recognizing Akt and immunoblotted with anti-phospho-PKA substrate antibody. (Nox: normoxia). Bar graph represents densitometric analysis, mean±SEM, expressed as fold increase in protein co-immunoprecipitation over that in control cells, of three independent experiments.

* p<0.001 vs control; § p<0.001 vs PC + 6h Nox.
**TABLE I**

Rate of apoptotic (Annexin V) and necrotic (Annexin V + Propidium Iodide) cell death following different periods of hypoxia.

<table>
<thead>
<tr>
<th></th>
<th>Annexin V</th>
<th>Annexin V + Propidium Iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7±2%</td>
<td>2±1%</td>
</tr>
<tr>
<td>Hypoxia 1 h</td>
<td>23±8%*</td>
<td>5±2%</td>
</tr>
<tr>
<td>Hypoxia 6 h</td>
<td>46±11%*</td>
<td>12±6%*</td>
</tr>
<tr>
<td>Hypoxia 12 h</td>
<td>54±11%*</td>
<td>27±6%*</td>
</tr>
<tr>
<td>Hypoxia 24 h</td>
<td>21±8%*</td>
<td>73±5%*</td>
</tr>
</tbody>
</table>

* p<0.05 vs control. The rate of apoptotic and necrotic cell death was calculated by dividing the number of Annexin-V-positive/PI-negative cells and annexin-V-positive/PI-positive cells by the total number of nuclei detected with DAPI staining, respectively. The rate of apoptotic and necrotic cell death was calculated from six independent experiments.
TABLE II

Rate of apoptotic (Annexin V) and necrotic (Annexin V + Propidium Iodide) cell death following 12 hours of hypoxia preceded by preconditioning (PC).

<table>
<thead>
<tr>
<th></th>
<th>Annexin V</th>
<th>Annexin V + Propidium Iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6±3%</td>
<td>3±1%</td>
</tr>
<tr>
<td>Hypoxia 12 h</td>
<td>48±5%*</td>
<td>12±4%*</td>
</tr>
<tr>
<td>PC + Hypoxia 12 h</td>
<td>25±5%*§</td>
<td>6±3%§</td>
</tr>
<tr>
<td>PC + 6 h Nox + Hypoxia 12 h</td>
<td>55±9%*</td>
<td>18±6%*</td>
</tr>
<tr>
<td>PC + 24 h Nox + Hypoxia 12 h</td>
<td>28±4%*§</td>
<td>8±2%§</td>
</tr>
</tbody>
</table>

* p<0,05 vs control; § p<0,05 vs Hypoxia 12h. (Nox: normoxia). The rate of apoptotic and necrotic cell death was calculated by dividing the number of Annexin-V-positive/PI-negative cells and Annexin-V-positive/PI-positive cells by the total number of nuclei detected with DAPI staining, respectively. The rate of apoptotic and necrotic cell death was calculated from six independent experiments.
**TABLE III**

Rate of apoptotic (Annexin V) and necrotic (Annexin V + Propidium Iodide) cell death following 12 hours of hypoxia preceded by early preconditioning in the presence or absence of inhibitors of protein kinase A (H89) and phosphatidyl-inositol-3-kinase (LY294002).

<table>
<thead>
<tr>
<th></th>
<th>Annexin V</th>
<th>Annexin V + Propidium Iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5±3%</td>
<td>2±1%</td>
</tr>
<tr>
<td>Hypoxia 12 h</td>
<td>47±4%*</td>
<td>13±3%*</td>
</tr>
<tr>
<td>EPC + Hypoxia 12 h</td>
<td>28±3%*§</td>
<td>5±3%§</td>
</tr>
<tr>
<td>H89 + EPC + Hypoxia 12 h</td>
<td>60±6%*‡</td>
<td>23±6%*‡</td>
</tr>
<tr>
<td>LY294002 + EPC + Hypoxia 12 h</td>
<td>27±4%*§</td>
<td>6±2%§</td>
</tr>
</tbody>
</table>

* p<0.05 vs control; § p<0.05 vs hypoxia 12 h ‡ p<0.05 vs EPC + hypoxia 12 h. The rate of apoptotic and necrotic cell death was calculated by dividing the number of Annexin-V-positive/PI-negative cells and annexin-V-positive/PI-positive cells by the total number of nuclei detected with DAPI staining, respectively. The rate of apoptotic and necrotic cell death was calculated from five independent experiments. EPC: early preconditioning.
TABLE IV

Rate of apoptotic (Annexin V) and necrotic (Annexin V + Propidium Iodide) cell death following 12 hours of hypoxia preceded by late preconditioning in the presence or absence of reactive oxygen species scavenger (NAC), inhibitors of protein kinase A (H89), phosphatidyl-inositol-3-kinase (LY294002), protein kinase C (Go6983), and inducible nitric oxide synthase (1400W), and non-specific inhibition of nitric oxide synthesis (L-NAME + 1400W).

<table>
<thead>
<tr>
<th></th>
<th>Annexin V</th>
<th>Annexin V + Propidium Iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8±4%</td>
<td>4±2%</td>
</tr>
<tr>
<td>Hypoxia 12 h</td>
<td>51±6%*</td>
<td>16±3%*</td>
</tr>
<tr>
<td>LPC + Hypoxia 12 h</td>
<td>29±4%*§</td>
<td>9±4%§</td>
</tr>
<tr>
<td>H89 + LPC + Hypoxia 12 h</td>
<td>61±5%‡</td>
<td>25±6%‡</td>
</tr>
<tr>
<td>LY294002 + LPC + Hypoxia 12 h</td>
<td>58±4%‡</td>
<td>24±3%‡</td>
</tr>
<tr>
<td>Go6983 + LPC+ Hypoxia 12 h</td>
<td>34±6%§</td>
<td>9±2§</td>
</tr>
<tr>
<td>1400W + LPC + Hypoxia 12 h</td>
<td>35±3%§</td>
<td>6±2§</td>
</tr>
<tr>
<td>L-NAME + 1400W + LPC Hypoxia 12 h</td>
<td>33±7%§</td>
<td>8±4§</td>
</tr>
<tr>
<td>NAC + LPC +Hypoxia 12 h</td>
<td>57±4%‡</td>
<td>23±4%‡</td>
</tr>
</tbody>
</table>

* p<0.05 vs control; § p<0.05 vs hypoxia 12 h; ‡ p<0.05 vs LPC + hypoxia 12 h. The rate of apoptotic and necrotic cell death was calculated by dividing the number of Annexin-V-positive/PI-negative cells and annexin-V-positive/PI-positive cells by the total number of nuclei detected with DAPI staining, respectively. The rate of apoptotic and necrotic cell death was calculated from four independent experiments. LPC: Late preconditioning, NAC: N-acetyl-cysteine, L-NAME: Nω-Nitro-L-arginine methyl ester.
TABLE V

Rate of apoptotic (Annexin V) and necrotic (Annexin V + Propidium Iodide) cell death following 12 hours of hypoxia preceded by late preconditioning in the presence or absence of transfection with dominant negative mutants of PKA (DN PKA) and Akt (DN Akt), and with bovine endothelial nitric oxide synthase-short interfering RNA (beNOS-siRNA).

<table>
<thead>
<tr>
<th></th>
<th>Annexin V</th>
<th>Annexin V + Propidium Iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7±1%</td>
<td>3±1%</td>
</tr>
<tr>
<td>Hypoxia 12 h</td>
<td>49±2%*</td>
<td>15±2%*</td>
</tr>
<tr>
<td>LPC + hypoxia 12 h</td>
<td>27±2%*§</td>
<td>6±1%§</td>
</tr>
<tr>
<td>DN PKA + LPC + Hypoxia 12h</td>
<td>60±5%‡</td>
<td>23±6%‡</td>
</tr>
<tr>
<td>DN Akt + LPC + Hypoxia 12h</td>
<td>59±4%‡</td>
<td>24±2%‡</td>
</tr>
<tr>
<td>beNOS-siRNA + LPC + Hypoxia 12 h</td>
<td>25±2%§</td>
<td>6±2§</td>
</tr>
</tbody>
</table>

* p<0.05 vs control, § p<0.05 vs hypoxia 12 h, ‡ p<0.05 vs LPC + hypoxia 12 h. The rate of apoptotic and necrotic cell death was calculated by dividing the number of Annexin-V-positive/PI-negative cells and annexin-V-positive/PI-positive cells by the total number of nuclei detected with DAPI staining, respectively. The rate of apoptotic and necrotic cell death was calculated from three independent experiments. LPC: Late preconditioning.