Abstract—Myocardial infarction, stroke, and venous thromboembolism are characterized by oxygen deprivation. In hypoxia, biological responses are activated that evoke tissue damage. Rapid activation of early growth response-1 in hypoxia upregulates fundamental inflammatory and prothrombogenic stress genes. We probed the mechanisms mediating regulation of early growth response-1 and demonstrate that hypoxia stimulates brisk generation of advanced glycation end products (AGEs) by endothelial cells. Via AGE interaction with their chief signaling receptor, RAGE, membrane translocation of protein kinase C-βII occurs, provoking phosphorylation of c-Jun NH2-terminal kinase and increased transcription of early growth response-1 and its downstream target genes. These findings identify RAGE as a master regulator of tissue stress elicited by hypoxia and highlight this receptor as a central therapeutic target to suppress the tissue injury–provoking effects of oxygen deprivation. (Circ Res. 2008;102:905-913.)

Key Words: cardiovascular ■ endothelial cells ■ hypoxia ■ RAGE ■ signal transduction

Understanding the central biochemical and molecular mechanisms that evoke maladaptive tissue responses in oxygen deprivation is essential to design pathway-specific therapeutic agents to limit tissue injury in diverse organs such as the heart, peripheral vasculature, brain and venous circulation. The immediate early gene, Early Growth Response-1 (Egr-1), is integral to the biological response to hypoxia,1–5 because its upregulation mediates increased expression of inflammatory and prothrombogenic genes. Previous studies elucidated a critical and an upstream role for protein kinase (PK)C, specifically the βII isoform, in regulation of Egr-1 to provoke a pathological response to acute hypoxia, ischemia/reperfusion injury, or acute vascular injury.3–6–8 Ligand engagement of the receptor for AGEs (advanced glycation end products) (RAGE) triggers activation of cell signaling pathways and expression of genes linked to vascular and inflammatory cell dysfunction in both acute and chronic stresses.9–16 Here, we tested the hypothesis that RAGE-dependent signaling regulates the biological response to acute hypoxia via PKCβII and upregulation of Egr-1.

Materials and Methods

Induction of Hypoxia in Mice

RAGE-null (RAGE−/−)7,16 and RAGE+/+ mice (in C57BL6 background, n=5/time point per group) were subjected to hypoxia as described previously19 and according to protocols approved by Institutional Animal Care and Use Committee at Columbia University. To block AGE production in vivo, RAGE−/− mice were pretreated with the AGE inhibitor aminoguanidine (AG) (100 mg/kg) for 1 hour by IP injection before they were exposed to hypoxia.20

Induction of Hypoxia in the Primary Cultured Murine Aortic Endothelial Cells and Human Aortic Endothelial Cells

Three lines of RAGE−/− and RAGE+/+ murine aortic endothelial cells (MAECs) were established from 3 individual mouse aortas as described.21 Human aortic endothelial cells (HAECs) were purchased from LONZA. When cells reached 70% to 80% confluence, they were serum-starved for 24 hours and then transferred to an In Vivo 400 hypoxic workstation (Biotrace, Cincinnati, Ohio), followed by replacing hypoxic medium. Cells were preincubated with soluble (s)RAGE (25 μg/mL), anti-AGE IgG (15 μg/mL), anti-RAGE IgG (15 μg/mL), or AG (50 and 200 μmol/L) for 2 hours22 or the PKCβ inhibitor LY379196 (30 nmol/L), c-Jun NH2-terminal kinase (JNK) inhibitor SP600125 (20 μmol/L), or p38 inhibitor SB203580 (20 μmol/L) for 45 minutes and then subjected to hypoxia.

Small Interference RNA to Knockdown RAGE and JNK

Small interference (si)RNA duplexes against human RAGE were purchased from Ambion. The JNK siRNA was synthesized by Qiagen.23,24 siRNA duplexes against RAGE or JNK were electroporated into HAECs or MAECs using the Nucleofector device (Amaza). To control for off-target effects of siRNA, a separate well of endothelial cells (ECs) was electroporated with a scramble siRNA as a negative control. After 48 hours, cells were subjected to hypoxia, followed by RNA isolation using TRIzol (Invitrogen).
Real-Time Quantitative PCR and Northern Blot Analysis

Total RNA was extracted from heart or cells using TRIzol reagent (Invitrogen, Rockville, Md). The real-time PCR analysis for Egr-1 and 18S rRNA was performed as described previously. For Northern blot, analysis of mouse JE/MCP-1 or 18S RNA was performed as described. Western blotting

Membrane protein fractions, nuclear extracts and total proteins were prepared from heart and cells and were subjected to Western blotting with rabbit anti–Egr-1 or Sp1 IgG (1:1000; Santa Cruz Biotechnology Inc). To detect membrane protein translocation, blots were incubated with the primary antibody,27 at a concentration of 30 μg of protein/lane. To detect JNK, blots were preincubated with anti–Egr-1 IgG (1:1000; Santa Cruz Biotechnology Inc) and then incubated with a biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories Inc, Burlingame, Calif), followed by incubation with Texas Red–avidin D. The sections were blocked with avidin/biotin blocking solution. The sections were incubated with a goat polyclonal antimouse platelet endothelial cell adhesion molecule-1 (CD31) antibody (1:50; Santa Cruz Biotechnology Inc), and incubated with a biotinylated rabbit anti-goat IgG (1:200; Vector Laboratories Inc, Burlingame, Calif), followed by incubation with fluorescein–avidin D. A similar procedure was performed on the adjacent section with a nonimmune IgG, and then nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The signals of individual and merged images for antigen detection were performed using a LaserSharp 2000 scanning confocal microscope with epifluorescent illumination (Bio-Rad Laboratories Inc), followed by fluorescein–avidin D. A similar procedure was performed with Texas Red–avidin D.

Immunofluorescence

Sections from formalin-fixed, paraffin-embedded heart tissues of mice were deparaffinized and stained with a rabbit polyclonal anti-mouse Egr-1 antibody (1:30; Santa Cruz Biotechnology Inc) and then incubated with a biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories Inc, Burlingame, Calif), followed by incubation with Texas Red–avidin D. The sections were blocked with avidin/biotin blocking solution. The sections were incubated with a goat polyclonal antimouse platelet endothelial cell adhesion molecule-1 (CD31) antibody (1:50; Santa Cruz Biotechnology Inc), and incubated with a biotinylated rabbit anti-goat IgG (1:200; Vector Laboratories Inc, Burlingame, Calif), followed by incubation with fluorescein–avidin D. A similar procedure was performed on the adjacent section with a nonimmune IgG, and then nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The signals of individual and merged images for antigen detection were performed using a LaserSharp 2000 scanning confocal microscope with epifluorescent illumination (Bio-Rad Laboratories Inc), followed by fluorescein–avidin D. A similar procedure was performed on the adjacent section with a nonimmune IgG, and then nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The signals of individual and merged images for antigen detection were performed using a LaserSharp 2000 scanning confocal microscope with epifluorescent illumination (Bio-Rad Laboratories Inc), followed by fluorescein–avidin D. A similar procedure was performed on the adjacent section with a nonimmune IgG, and then nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The signals of individual and merged images for antigen detection were performed using a LaserSharp 2000 scanning confocal microscope with epifluorescent illumination (Bio-Rad Laboratories Inc), followed by fluorescein–avidin D. A similar procedure was performed on the adjacent section with a nonimmune IgG, and then nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The signals of individual and merged images for antigen detection were performed using a LaserSharp 2000 scanning confocal microscope with epifluorescent illumination (Bio-Rad Laboratories Inc), followed by fluorescein–avidin D. A similar procedure was performed on the adjacent section with a nonimmune IgG, and then nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The signals of individual and merged images for antigen detection were performed using a LaserSharp 2000 scanning confocal microscope with epifluorescent illumination (Bio-Rad Laboratories Inc), followed by fluorescein–avidin D.
Results

Expression of Egr-1 in Hypoxic Heart: Effect of RAGE Deletion

To identify the specific biochemical and molecular mechanisms by which blockade or genetic deletion of RAGE mitigates the adverse impact of ischemia/reperfusion injury, we addressed the specific biological impact of RAGE in hypoxia and focused on the potential role of Egr-1.1,5 RAGE+/+ mice were exposed to hypoxia (oxygen, 6%) or normoxia. Real-time PCR analysis of RNA from hearts subjected to oxygen deprivation. Compared with hearts from RAGE+/+ mice, Egr-1 mRNA transcripts were significantly lower in RAGE−/− mouse hearts after 30 minutes hypoxia (Figure 1b; P<0.0001). Immunoblotting of nuclear extracts from hypoxic hearts confirmed increased Egr-1 nuclear protein in RAGE+/+ mice hearts after 45 minutes of hypoxia, compared with normoxic controls (Figure 1c; P<0.0001); this was significantly blunted in RAGE−/− mouse hearts (Figure 1c; P<0.0001). Electrophoretic mobility shift analysis of nuclear extracts from RAGE+/+ hearts, but not RAGE−/− hearts, demonstrated an Egr-1 gel shift band with an intensity that increased strongly in hypoxia (Figure 1d; P<0.0001). Supershift assays showed that the hypoxia-induced Egr-1 DNA-binding band in RAGE+/+ hearts was diminished in the presence of Egr-1 antibody (Figure 1e).

Because Egr-1 activates downstream mediators of inflammation such as JE/MCP-1 in hypoxia, we tested the effect of RAGE deletion.5 Northern blotting demonstrated that RAGE+/+ mice displayed highly significant, ∼12-fold up-regulation of JE/MCP-1 transcripts in heart tissue after 4 hours of hypoxia (Figure 1f; P<0.0001). In contrast, transcripts for JE/MCP-1 in the hearts of RAGE−/− did not differ in hypoxia versus normoxia (Figure 1f).

To determine the principal cell type(s) expressing Egr-1 in the heart subjected to global hypoxia, we performed immunohistochemistry. In hypoxic hearts from RAGE+/+ mice, immunofluorescence experiments using anti–Egr-1 and anti–phosphorylated JNK (P-JNK) antibody to PKCα, PKCβ, PKCe, and PKCδ (a and d), as well as β-actin (a and c), was performed on membrane protein from the heart (25 μg protein/lane), e through f, Immunoblotting with specific antibody to PKCβII (a and c), PKCα, PKCβ, PKCe, and PKCδ (a and d), was performed on membrane protein from the heart (25 μg protein/lane) from the hearts. Representative bands are shown, and the mean±SE is shown. In c, d, and f, animals were exposed to hypoxia for 15 minutes. At least 5 mice per group.

Membrane Translocation of PKCβII and Activation of JNK in Hypoxic Heart: Effect of RAGE Deletion

In view of previous findings identifying rapid recruitment of PKCβII to upregulation of Egr-1 in the response to hypoxia,3,6 we tested the impact of deletion of RAGE. An increase of PKCβII in RAGE+/+ hearts associated with the membrane fraction was initiated at 10 minutes of hypoxia (Figure 2a; P<0.0005) reached an apparent maximum after 15 minutes (Figure 2a; P<0.0001) and remained significantly activated up to 30 minutes of hypoxia (Figure 2a; P<0.0005) compared with baseline (Figure 2a). In contrast, immunoblotting with antibodies specific for 4 other PKC isoforms, including PKCα, PKCβI, PKCδ, and PKCe (Figure 2b), revealed no
In view of roles for PKCβII and RAGE and downstream mitogen-activated protein kinases in environmental stress, we investigated the specific impact of RAGE on JNK activation in hypoxia. Immunoblotting of extracts from hypoxic hearts of RAGE+/− mice displayed an ~5-fold increase in intensity of phospho-JNK, with peak activation at 15 minutes exposure to hypoxia, compared with extracts from normoxic hearts (Figure 2e and 2f). In comparison, RAGE−/− mice hearts displayed a significantly lower increase in phosphorylation of JNK in hypoxia compared with tissue from hypoxic RAGE+/+ hearts; P<0.0001 (Figure 2f).

**Probing the Mechanisms Regulating Egr-1 in Hypoxia: Effect of RAGE Deletion in Primary MAECs**

Our findings demonstrated that global hypoxia upregulated Egr-1 in the murine heart, particularly in ECs. To probe the specific RAGE-dependent pathways mediating these responses, MAECs were studied. MAECs isolated from RAGE+/+ and RAGE−/− mice were subjected to hypoxia (O2, 0.5%) using an In Vivo 400 hypoxic workstation. Real-time PCR analysis revealed that in RAGE+/+ MAECs, Egr-1 mRNA was increased ~20-fold at 15 minutes of hypoxia versus normoxia (Figure 3a and 3b; P<0.0001). In contrast, RAGE−/− MAECs subjected to hypoxia for the same time showed only ~3.8-fold increase in Egr-1 transcripts compared with normoxia (Figure 3b). Immunoblotting of nuclear extracts in RAGE+/+ MAECs displayed ~6-fold increase in Egr-1 nuclear protein at 30 minutes of hypoxia compared with normoxia (Figure 3c; P<0.0001). In contrast, RAGE−/− MAECs showed an immunoreactive band of very low intensity after exposure to hypoxia, versus the robust response in RAGE+/+ MAECs (Figure 3c; P<0.0001). Electrophoretic mobility shift analysis of nuclear extracts using 32P-labeled consensus Egr-1 oligonucleotide probe for Egr-1 demonstrated a gel shift band with an intensity that increased strongly in response to 30 minutes of hypoxia in RAGE+/+ MAECs (Figure 3d; P<0.0001) but not RAGE−/− MAECs (Figure 3d). In addition, a supershift band shown in nuclear extracts from hypoxic RAGE+/+ MAECs confirmed that hypoxia-induced Egr-1 DNA protein binding was affected by Egr-1 antibody (Figure 3e).

**Membrane Translocation of PKCβII and Activation of JNK in Primary MAECs After Hypoxia: Effect of RAGE Deletion**

We next traced the role of RAGE on PKCβII translocation and JNK activation in primary ECs. When MAECs from RAGE+/+ mice were subjected to hypoxia, PKCβII membrane translocation was significantly increased at 15, 30, and 60 minutes of hypoxia versus normoxia (Figure 4a; P<0.05). However, 4 other PKC isoforms, PKCα, PKCβI, PKCδ, and PKCe (Figure 4b), revealed no changes in membrane translocation over this time course. When MAECs from RAGE−/− mice were subjected to hypoxia, there were no changes in membrane protein after 15 minutes of hypoxia by Western blotting using antibodies for detection of PKCβII (Figure 4c),

change in RAGE−/− mice after exposure to 10, 15, or 30 minutes of hypoxia versus baseline. RAGE was necessary for rapid membrane translocation of PKCβII, because RAGE−/− mouse hearts displayed an immunoreactive band of PKCβII in the membrane fraction of very low intensity after exposure to hypoxia (Figure 2c; P<0.005). Compared with baseline, PKC isoforms PKCα, PKCβII, PKCδ, and PKCe failed to reveal increased membrane translocation resulting from 15 minutes in RAGE+/+ or RAGE−/− mice (Figure 2d) or 10 or 30 minutes (data not shown) of hypoxia. Thus, over the same time course, during which hypoxia activated Egr-1, selective membrane translocation of PKCβII, via RAGE, was observed.

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PKCα, PKCβI, PKCδ, and PKCe (Figure 4d) compared with baseline.

Next, we determined whether deletion of RAGE affected phosphorylation of JNK in hypoxic MAECs. Immunoblotting of extracts from hypoxic MAECs retrieved from RAGE+/+ mice revealed increased phosphorylation of JNK in hypoxia and was sustained up to 2 hours (≈6-fold) versus normoxic MAECs (Figure 4e and 4f; P<0.005). In contrast, RAGE−/− MAECs failed to display increased phosphorylation of JNK after 15 minutes of hypoxia (Figure 4f; P<0.005).

Mechanisms by Which RAGE Signaling Upregulates Egr-1 in MAECs

We next sought to establish the specific signals elicited in hypoxia that rapidly evoked RAGE-dependent signaling. We hypothesized that hypoxia generated RAGE ligands and first focused on assessment of AGEs. We measured these species in MAEC supernatants in normoxia or after oxygen deprivation. Interestingly, increased AGE-immunoreactive epitopes were detected in supernatants of RAGE+/+ MAECs by ELISA, beginning at 10 minutes of hypoxia, achieving significance at 15 minutes of hypoxia (Figure 5a; P<0.05) and persisting through 60 minutes of hypoxia versus normoxia control (Figure 5a; P<0.0001). Because RAGE is a multiligand receptor, we investigated whether hypoxia induced release of S100 and HMGB1. Neither S100 nor HMGB1 was detectable by Western blotting in normoxic and hypoxic RAGE+/+ MAEC supernatants, even after they were concentrated 20-fold using Amicon Ultra Centrifugal Filter Devices (data not shown).

To directly implicate AGE-RAGE in the downstream events leading to upregulation of Egr-1 in hypoxia, we used specific reagents to block AGE, RAGE, and their interaction in WT ECs exposed to hypoxia. Treatment of ECs with anti-AGE IgG, anti-RAGE IgG or sRAGE, the extracellular ligand-binding domain of RAGE, blocked hypoxia-mediated membrane translocation of PKCβII (Figure 5b), suppressed phosphorylation of JNK (Figure 5c), and upregulation of Egr-1 nuclear protein (Figure 5d) and mRNA (Figure 5e). In addition, hypoxia-stimulated phosphorylation of JNK was blocked by an inhibitor of PKCβ (LY379196) (Figure 5c), and induction of Egr-1 nuclear protein and mRNA was suppressed by inhibitors of PKCβ (LY379196) and JNK (SP600125) (Figure 5d and 5e), but not by an inhibitor of p38 mitogen-activated protein kinase (SB203580) (Figure 5d and 5e). Furthermore, introduction of siRNA to knock-down JNK expression blunted the effect of hypoxia on upregulation of Egr-1; P<0.0001, but had no effect on expression of 18S rRNA (Figure 5e). Introduction of scrambled siRNA had no effect of hypoxia on upregulation of Egr-1 in mouse ECs (Figure 5e).
Effect of the AGE Inhibitor AG on RAGE-Dependent Upregulation of Egr-1: In Vivo and In Vitro Analyses

In view of the striking effects of anti-AGE IgG and sRAGE on suppression of RAGE-dependent Egr-1 upregulation in hypoxia, we next directly tested the effects of the AGE inhibitor AG in vivo and in vitro. First, pretreatment of RAGE+/−/− mice with AG (100 mg/kg) for 1 hour by intraperitoneal injection before global hypoxia resulted in a 45% reduction in upregulation of Egr-1 transcripts in the heart (Figure 6a; P<0.0001). Second, in vitro, increased expression of Egr-1 transcripts in hypoxic RAGE+/−/− MAECs was significantly reduced by 45 to 55% when those MAECs were preincubated with AG (200 and 50 μmol/L) for 2 hour, compared with that in the absence of AG (Figure 6b; P<0.0001).

RAGE Regulates Upregulation of Egr-1 in HAECs

A critical test of these concepts was to address the potential roles for RAGE in regulation of Egr-1 in HAECs in hypoxia. First, to determine whether HAECs released AGEs into supernatants in hypoxia, we next directly tested the effects of the AGE inhibitor AG in vivo and in vitro. First, pretreatment of RAGE+/−/− MAECs were serum-starved for 24 hours and subjected to hypoxia. Supernatants were subjected to ELISA for detection of AGE epitopes. In Figure 6a, RAGE+/−/− MAECs were serum-starved for 24 hours and preincubated with or without sRAGE (25 μg/mL), anti-AGE IgG (15 μg/mL), or anti-RAGE IgG (15 μg/mL) for 2 hours or PKCβ inhibitor LY379196 (30 nmol/L), JNK inhibitor SP600125 (20 μmol/L), or p38 inhibitor SB203580 (20 μmol/L) for 45 minutes and then subjected to hypoxia for 15 minutes (b, c, and e) or 30 minutes (d). Membrane protein, total protein, total RNA, and nuclear protein were prepared from MAECs. Immunoblotting with antibodies to PKCβII and β-actin (b), phospho-JNK (P-JNK) or total JNK (T-JNK) (c), or Egr-1 or Sp1 (d) was performed. Total RNA was subjected to real-time PCR analysis of Egr-1 expression. MAECs were subjected to the introduction of siRNA-JNK to reduce Egr-1 transcripts by hypoxia using scramble siRNA as a negative control. All experiments were repeated more than 3 times; representative bands are shown, and the mean±SE is shown.
suppressed hypoxia-mediated upregulation of Egr-1 transcripts compared with scramble siRNA (Figure 7b).

Discussion

Previous studies established key roles for Egr-1 in upregulation of inflammatory and thrombosis-provoking genes in oxygen deprivation; here, we show for the first time that regulation of Egr-1 in hypoxia is mediated by RAGE. RAGE-dependent membrane translocation of PKCβII and consequent activation of JNK signaling in the heart and in endothelial cells subjected to hypoxia directly impact on regulation of Egr-1 (Figure 8). Studies in RAGE−/− mice hearts subjected to global hypoxia revealed marked reduction in hypoxia-stimulated increases in Egr-1 transcripts compared with RAGE+/+ mice. In isolated murine ECs, deletion of RAGE, or, in RAGE+/− ECs, blockade of AGE-RAGE highlighted central roles for this axis in Egr-1 regulation. Similar regulatory roles for RAGE in hypoxia were observed in HAECs.

The observation that hypoxia stimulates rapid production and cellular release of AGEs by murine and human endothelial cells suggests novel mechanisms by which PKCβII may be recruited. Importantly, non-AGE ligands S100 and non-AGE ligands S100 and HMGB1 were not detected in MAEC supernatants over the same time course in which RAGE-dependent mechanisms regulated Egr-1 in hypoxia. We conclude that within the time course of hypoxia in which upregulation of Egr-1 occurred, only the PKCβII isoform, and not other “classic” PKC isoforms (α and β1) or “novel” isoforms (δ and ε) of PKC, were found to be translocated to the membrane in the intact heart or in primary endothelial cells, at least in part by AGE-dependent signaling, via RAGE.28 Thus, pathways distinct from diacylglycerol selectively recruit PKCβII in hypoxic stress in vivo and in vitro.28 Disturbances in oxidation states are potent activators of JNK signaling.29 The direct impact of hypoxia, as well as the secondary generation of oxidative stress species via AGE-RAGE interaction,15,16 may stimulate and sustain activation of JNK signaling in hypoxia. In vivo, our studies revealed that hypoxia stimulated rapid activation of JNK in the heart; in isolated primary endothelial cells, in vitro–applied hypoxia stimulated activation of JNK signaling through at least the first 60 minutes. These findings are consistent with very recent studies that have revealed specific pathogenic roles for JNK signaling in ischemia/reperfusion injury. Administration of specific small-molecule or peptide-based JNK inhibitors to animals resulted in significant attenuation of injury induced by ischemia and reperfusion in the heart and brain.30–33 Furthermore, mice deficient in the JNK3 isoform revealed decreased apoptosis in the hippocampus after ischemic brain injury.34 Our data identify RAGE as the key upstream regulator of JNK activation in hypoxia.

It is important to note that cellular fate consequent to activation of JNK may be diverse, from upregulation of

Figure 6. AGE inhibitor AG blocked hypoxia-induced expression of Egr-1. a, RAGE+/+ mice were exposed to hypoxia (H) (6% oxygen) or normoxia (N) for 30 minutes after they were pretreated with the AG (100 mg/kg) for 1 hour by IP injection or in the absence of AG. Total RNA was isolated from the heart and subjected to real-time PCR analysis of Egr-1 expression. At least 5 mice per group. b, RAGE+/− MAECs were serum-starved for 24 hours and then subjected to hypoxia or normoxia for 15 minutes after they were preincubated or not with AG (50 and 200 μmol/L) for 2 hours. Total RNA was isolated from MAECs and subjected to real-time PCR analysis of Egr-1 expression. ly379196 (30 nmol/L) or JNK inhibitor SP600125 (20 μmol/L) for 45 minutes and then subjected to hypoxia (H) for 15 minutes. HAECs were subjected to introduction of siRNA-RAGE1, siRNA-RAGE2, or siRNA-JNK to reduce Egr-1 transcripts by 50–60%.

Figure 7. RAGE regulates hypoxia-mediated induction of Egr-1 in HAECs. a, HAECs were serum-starved for 24 hours and subjected to hypoxia. Supernatants were harvested, concentrated, and subjected to ELISA for detection of AGE epitopes. b, HAECs were serum-starved for 24 hours and preincubated with or without sRAGE (25 μg/mL), anti-AGE IgG (15 μg/mL), or anti-RAGE IgG (15 μg/mL) for 2 hours or the PKCβ inhibitor LY379916 (30 nmol/L) or JNK inhibitor SP600125 (20 μmol/L) for 45 minutes and then subjected to hypoxia (H) for 15 minutes. HAECs were subjected to introduction of siRNA-RAGE1, siRNA-RAGE2, or siRNA-JNK to reduce Egr-1 transcripts by hypoxia using scramble siRNA as a negative control. Total RNA was isolated from HAECs for analysis of Egr-1 expression by real-time PCR. All experiments were repeated more than 3 times; representative data and means ± SE are shown.
Inflammatory pathways to activation of cell death programs. In the present studies, we illustrated that expression of a downstream target gene of Egr-1, monocyte chemoattractant protein (MCP)-1, was significantly reduced in RAGE+/-H11002 mouse hearts retrieved after induction of hypoxia. MCP-1 critically contributes to vascular dysfunction via its mediation of monocyte-endothelial binding and consequent signaling and amplification of proinflammatory mechanisms. Thus, although hypoxia sparks key mechanisms in tissue damage via immediate activation of Egr-1, the downstream consequences of this factor amplify perturbation in hypoxia-stressed tissues. Here, we have linked these early and later consequences of hypoxia to RAGE.

In conclusion, we show for the first time that RAGE regulates hypoxia-stimulated membrane translocation of PKCβII and consequent activation of JNK signaling, processes that regulate Egr-1 transcripts and protein in the intact heart and in endothelial cells. RAGE-dependent orchestration of vascular perturbation in oxygen deprivation identifies this molecule as a master regulator of inflammatory stress triggered by hypoxia.

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Oxygen Deprivation Triggers Upregulation of Early Growth Response-1 by the Receptor for Advanced Glycation End Products

Jong Sun Chang, Thoralf Wendt, Wu Qu, Linghua Kong, Yu Shan Zou, Ann Marie Schmidt and Shi-Fang Yan

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Supplemental Figure S. Immunohistochemistry. (a-d) Immunofluorescence double stained heart sections after 2 h of hypoxia with anti-Egr-1 IgG (red) (a) and anti-CD31 IgG (green) (b). The merged image of a (Egr-1) and b (CD31) is shown in (c). No immunofluorescence staining with non-immune IgG was found, but nuclei were stained with DAPI (blue) (d). Marker bar=50 µm. N= at least 5 mice per group.