Hypoxia Enhances Ecto-5′-Nucleotidase Activity and Cell Surface Expression in Endothelial Cells

Role of Membrane Lipids

S. Ledoux, I. Runembert, K. Koumanov, J.B. Michel, G. Trugnan, G. Friedlander

Abstract—Extracellular adenosine production by the glycosyl-phosphatidyl-inositol-anchored Ecto-5′-Nucleotidase plays an important role in the defense against hypoxia, particularly in the intravascular space. The present study was designed in order to elucidate the mechanisms underlying hypoxia-induced stimulation of Ecto-5′-Nucleotidase in endothelial cells. For this purpose, aortic endothelial cells (SVARECs) were submitted to hypoxic gas mixture. Hypoxia (0% O_2 for 18 hours) induced a 2-fold increase of Ecto-5′-Nucleotidase activity (V_{max} 19.78±0.53 versus 8.82±1.12 nmol/mg protein per min), whereas mRNA abundance and total amount of the protein were unmodified. By contrast, hypoxia enhanced cell surface expression of Ecto-5′-Nucleotidase, as evidenced both by biotinylation and immunostaining. This effect was accompanied by a decrease of Ecto-5′-Nucleotidase endocytosis, without modification of Ecto-5′-Nucleotidase association with detergent-resistant membranes. Finally, whereas cholesterol content was unmodified, hypoxia induced a time-dependent increase of saturated fatty acids in SVARECs, which was reversed by reoxygenation, in parallel to Ecto-5′-Nucleotidase stimulation. Incubation of normoxic cells with palmitic acid enhanced Ecto-5′-Nucleotidase activity and cell surface expression. In conclusion, hypoxia enhances cell surface expression of Ecto-5′-Nucleotidase in endothelial cells. This effect could be supported by a decrease of Ecto-5′-Nucleotidase endocytosis through modification of plasma membrane fatty acid composition. **(*Circ Res*. 2003;92:●●●-●●●.)**

Key Words: hypoxia ■ Ecto-5′-Nucleotidase ■ membrane lipids

The primary function of the glycosyl-phosphatidyl-inositol (GPI)–anchored enzyme Ecto-5′-Nucleotidase (Ecto-5′-Nu) is to catalyze the dephosphorylation of 5′-AMP in the extracellular space.[1,2] This enzyme is then implicated, with other ecto-nucleotidases, in situations characterized by ATP hypercatabolism such as hypoxia. Indeed, Ecto-5′-Nu is necessary for nucleotide salvage pathway[1,3] because AMP, produced from ATP released in the extracellular medium, must be converted into adenosine before reuptake. Moreover, adenosine, the product of Ecto-5′-Nu, plays a crucial role in adaptation to hypoxia. This nucleoside has been implicated in the whole body respiratory and circulatory response to hypoxia[4] and can protect organs against hypoxic lesions, as demonstrated particularly for myocardial preconditioning.[5,6]

At the endothelial level, adenosine is one of the main regulators of endothelial function. We have previously shown that stimulation of Ecto-5′-Nu activity in endothelial cells by statins inhibited platelet aggregation.[7] The increase of adenosine production by endothelial Ecto-5′-Nu under hypoxia could also be instrumental in the regulation of vasomotoricity, vascular permeability, and interaction with circulating cells.[8,9]

Stimulation of Ecto-5′-Nu activity has been reported in situations of tissular hypoxia like anemia,[10] myocardial ischemia,[5,6] or cerebral ischemia.[11] Although the mechanisms whereby hypoxia enhances Ecto-5′-Nu in endothelium have not been elucidated, some hypotheses can be put forward. Most of the proteins upregulated by hypoxia are transcriptionally regulated by hypoxia-inducible factor (HIF),[12] and a HIF responsive sequence has been described in the Ecto-5′-Nu promoter.[13] However, other pathways of Ecto-5′-Nu stimulation have been described. Indeed, one group has suggested that the increase of Ecto-5′-Nu activity in myocardial ischemia is mediated by protein kinase C.[5,6] We have previously shown that statins enhance Ecto-5′-Nu activity through modulation of Rho-GTPases in endothelial cells.[7] Finally, modifications of lipid composition of cell membranes such as decrease of cholesterol level or alterations of phospholipid and fatty acid content have been described under hypoxia[14,15] and could modulate membrane sorting of GPI-anchored proteins.[16–18]

The aim of this study was therefore to elucidate the mechanisms of hypoxia-induced stimulation of Ecto-5′-Nu activity in endothelial cells. To address these questions, we

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have studied the activity and expression of Ecto-5′-Nu in aortic endothelial cells incubated with hypoxic gas mixture. The results show that hypoxia enhances cell surface expression of endothelial Ecto-5′-Nu and suggest that this effect could result from modification of fatty acids membrane composition.

Materials and Methods

Materials

Gas mixtures were purchased from Air Products; culture products were from Gibco BRL (France); [^{14}C]5′-AMP and ^{125}I-labeled goat anti-rabbit antibody were from Amersham (Amersham, UK); EZ-link NHS-SS-Biotine was from Pierce. Other products were from Sigma.

Endothelial Cells

SV 40 transfected aortic rat endothelial cells (SVARECs) have been previously characterized.[19] Aortic rat primary endothelial cells were obtained as previously described.[20] Palmitic acid supplementation was performed as previously described[21]: a 100 mmol/L solution of palmitic acid in ethanol was vigorously mixed with newborn calf serum (1 to 10:100 v/v) and then dissolved in Dulbecco's modified Eagle's medium (10:100 v/v). This medium was replaced daily for prolonged incubations. The activity of Ecto-5′-Nu was determined on intact cells with [^{14}C]5′-AMP as a substrate, as previously described.[22] All experiments were performed on confluent cells.

Hypoxia

Exposure to hypoxia was performed as previously described[23] in a airtight incubator kept at 37°C for 3, 6, 12, or 18 hours, with an hypoxic gas mixture (0% or 5% O_2; 5% CO_2 in N_2), whereas control normoxic cells were placed in a 21% O_2, 5% CO_2, 74% N_2 incubator for the same periods of time. For hypoxia-reoxygenation experiments, cells were exposed to 0% O_2 for 18 hours and then placed in normoxia for 1, 3, 6, or 24 additional hours. LDH release, measured as previously described[22] in order to estimate cell toxicity, was not modified after exposure to hypoxia (data not shown).

RNase Protection Assay

RNase protection assay was performed with riboprobes for Ecto-5′-Nu and β-actin as previously described.[23] The ratio of radioactivity for 5′-Nu to the radioactivity for β-actin was calculated for each sample, because actin mRNA abundance was unaffected by hypoxia.[23]

Cell Surface Biotinylation

Biotinylation of cell surface, previously used to study Ecto-5′-Nu expression to plasma membrane,[24] was performed with EZ-link NHS-SS-Biotine. The biotinylated proteins were separated by incubation of the cell lysate with streptavidin agarose beads.

Isolation of Detergent-Resistant Membranes (DRMs)

Isolation of DRMs was performed by flotation on a sucrose gradient as previously described.[7,24] Twelve fractions of 1 mL were collected from top to bottom of the sucrose gradient. In some experiments, fractions 4 to 7 were pooled to quantify Ecto-5′-Nu expression in whole DRM.

Western Blot

Western blot analyses of the samples were performed as previously described.[22] Ecto-5′-Nu contents were determined with polyclonal anti-5′-Nu antibody (1:5000), or monoclonal anti-5′-Nu antibody (1:200) for biotinylated samples (kind gifts of B. Kaissling). Actin expression in total cell membrane samples was used as an internal standard because the protein level was unaffected by hypoxia.[23] Caveolin and α-subunit of Gs protein (Gsα) contents in DRM were determined with polyclonal anti-caveolin antibody (1:5000, Transduction laboratories) and polyclonal anti-Gsα antibody (1:200, Santa Cruz Biotechnology).

Immunostaining of Ecto-5′-Nu

Nonpermeabilized fixed cells were incubated overnight at 4°C with polyclonal anti-5′-Nu antibody (1:1000), and then incubated for 30 minutes at 37°C with FITC-labeled goat anti rabbit antibody for confocal fluorescence microscopy studies, or with ^{125}I-labeled goat anti-rabbit antibody for quantification of Ecto-5′-Nu cell surface expression.[7] For studies of endocytosis, nonfixed cells were incubated for 1 hour at 4°C with the anti-5′-Nu antibody, rinsed three times, and then fixed (for T_0) or incubated for 18 hours at 37°C, before being fixed and labeled with the ^{125}I-secondary antibody.[7]

Lipid Analysis With Gas Chromatography–Mass Spectrometry (GC-MS)

Cell lipids were extracted according to Bligh and Dyer[25] and separated by thin-layer chromatography on silica gel plates. To analyze fatty acid composition of different phospholipids, the corresponding spot was saponified with 0.5 N methanolic KOH and methylated with boron trifluoride in 14% methanol. The cellular nonesterified cholesterol was assayed in the form of trimethylsilyl ether prepared with N,O-bis (trimethylsilyl) trifluoroacetamide–10% trimethylchlorosilane. Fatty acid and cholesterol were assayed by gas chromatography [7,26]

Data Presentation

Results were presented as mean±SEM. Statistical analyses were performed by unpaired *t* test, or by analysis of variance (ANOVA) when appropriate.

Results

Effect of Hypoxia on Ecto-5′-Nu Activity

Ecto-5′-Nu activity measured in control conditions (21% O_2) in SVARECs was 2.25±0.37 nmol/mg protein per min after a 5 minutes incubation with 50 μmol/L 5′-AMP. Hypoxia enhanced Ecto-5′-Nu activity: this effect was observed after incubation for 18 hours with 5% O_2, but reached statistical significance only with 0% O_2 (Figure 1). Stimulation of Ecto-5′-Nu by hypoxia appeared between 6 and 12 hours of incubation with 0% O_2 and was maximal at 18 hours (Figure 2) as previously reported in epithelial cells.[13] For the subsequent experiments, we chose an 18-hour incubation period with 0% O_2 gas mixture, which induced a 80% decrease of oxygen tension in the culture medium.[23] After reoxygenation, Ecto-5′-Nu activity decreased progressively to control values at 24 hours (Figure 2). The decrease of enzyme activity became significant after 3 hours of normoxia. Determination of 5′-Nu activity with various concentrations of 5′-AMP allowed us to show (Figure 3A) that hypoxia induced a 2-fold increase of the V_{max} value of the enzyme, without modification of its apparent affinity (K_m).

In order to ascertain that the effect of hypoxia on Ecto-5′-Nu was not restricted to SVARECs, we evaluated the effect of hypoxia on Ecto-5′-Nu in primary cultures of rat aortic endothelial cells. Ecto-5′-Nu activity measured in control conditions (21% O_2) was 5.69±2.28 nmol/mg protein per min after a 5-minute incubation with 50 μmol/L 5′-AMP. Hypoxia (0% O_2 for 18 hours) induced a stimulation of Ecto-5′-Nu activity in the same range as that in SVARECs (226±44.85% of control, n=4; $P<0.05$).

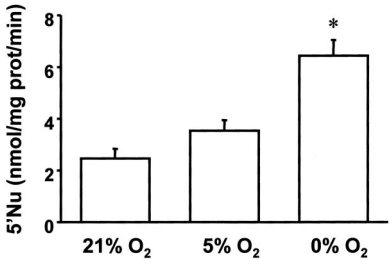

Figure 1. Effect of oxygen content on Ecto-5′-Nu activity. SVARECs were incubated for 18 hours with gas mixtures containing 21%, 5%, or 0% O_2. Ecto-5′-Nu activity was measured by incubating the cells with 50 μmol/L 5′-AMP for 5 minutes. Results are expressed as mean±SEM of 3 different experiments. *$P<0.05$ vs control.

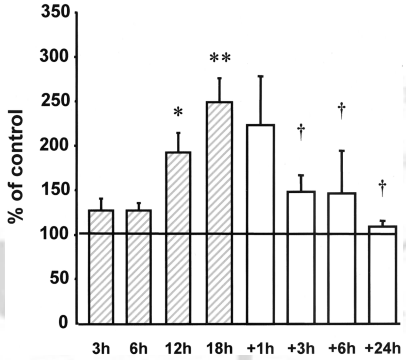

Figure 2. Effect of time of hypoxia and reoxygenation on Ecto-5′-Nu activity. Effect of time was studied after incubation of SVARECs for 3 hours to 18 hours with a 0% O_2 gas mixture (hatched bars). For reoxygenation studies, the 0% O_2 gas mixture was replaced after 18 hours by a 21% O_2 gas mixture for an additional incubation of 1 to 24 hours (open bars). Ecto-5′-Nu activity was measured with 50 μmol/L 5′-AMP for 5 minutes. Results are expressed as percent of controls (maintained in a 21% O_2 gas mixture during the same times of incubation) and are mean±SEM of 3 different experiments. *$P<0.05$, **$P<0.01$ vs control, †$P<0.05$ vs hypoxia.

A

	CT	HX	p
Vmax (nmol/mg prot/min)	8.82 ± 1.12	19.78 ± 0.53	< 0.01
Km (μM)	73.87 ± 18.17	90.68 ± 26.97	ns

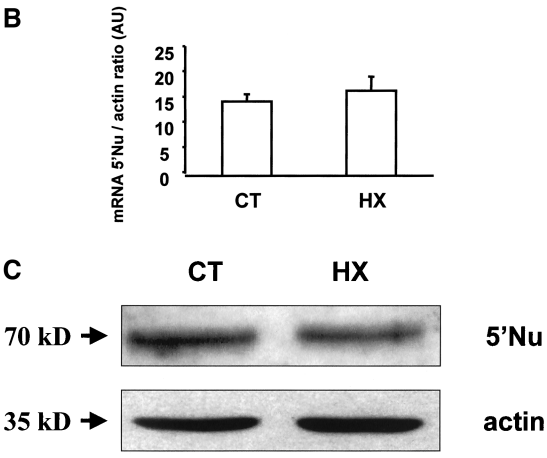

Figure 3. Effect of hypoxia on enzymatic parameters and synthesis of Ecto-5′-Nu. SVARECs were preincubated for 18 hours with a 0% O_2 gas mixture (HX) or a 21% O_2 gas mixture (CT) and then incubated for 5 minutes with different concentrations of 5′-AMP between 7.5 and 1000 μmol/L. V_{max} and K_m values were calculated after Eddie-Hoftee transformation (n=3) (A). Ecto-5′Nu and β-actin mRNA were quantified by RNAse protection assay. Results are expressed as arbitrary units (AU) of Ecto-5′-Nu/actin mRNA ratio and are mean±SEM of 3 different experiments (B). Western blot analysis was performed in total cell membrane samples and labeled with polyclonal anti-5′-Nu antibody (70 kDa) and monoclonal anti-actin antibody (35 kDa) (C, representative picture of 3 experiments).

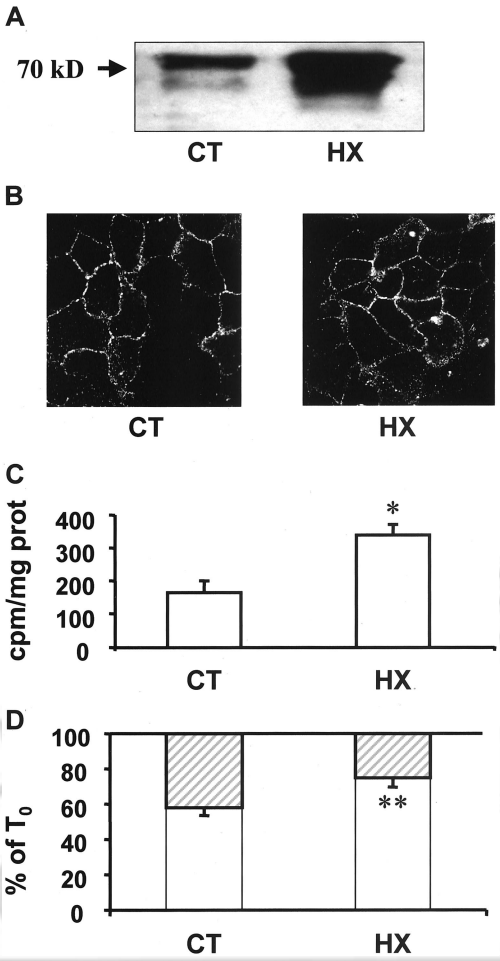

Figure 4. Plasma membrane expression of Ecto-5′Nu. After 18 hours of preincubation with 21% O_2 (CT) or 0% O_2 (HX), samples of cell surface proteins isolated by biotinylation in SVARECs were labeled with monoclonal anti-5′Nu antibody (A, representative pictures of 3 experiments). Fixed cells were incubated with polyclonal anti-5′-Nu antibody, and then stained with a FITC-conjugated secondary antibody for immunofluorescence study (B) or with a ^{123}I labeled anti-rabbit antibody for immunoquantification of Ecto-5′-Nu (C). Nonfixed cells were incubated with anti-5′-Nu antibody for 1 hour and then fixed (T_0) or submitted to a 18 hours incubation with 21% O_2 (CT) or 0% O_2 (HX): endocytosis (hatched part of the columns) was estimated as Ecto-5′-Nu staining at T_0 minus the staining which persists after the 18-hour incubation (open part of the columns) (D). Results are mean±SEM of 3 different experiments. *$P<0.05$, **$P<0.01$ vs control.

Effect of Hypoxia on Ecto-5′-Nu Protein Synthesis

To evaluate whether enhancement of Ecto-5′-Nu activity by hypoxia resulted from an increased protein synthesis, we quantified the level of Ecto-5′-Nu mRNA by RNase protection assay in SVARECs. As shown in Figure 3B, Ecto-5′-Nu/actin mRNA ratio was not modified by hypoxia. Along the same line, quantification of Ecto-5′-Nu expression by Western blot analysis in total membrane fractions (Figure 3C) did not show any significant difference in protein level between hypoxic and control conditions (Ecto-5′-Nu/actin ratio: 0.507±0.126 versus 0.522±0.125 for hypoxia and control, respectively, n=3; P=NS).

Effect of Hypoxia on Ecto-5′-Nu Expression at the Plasma Membrane

Two different approaches were used to quantify the cell surface expression of Ecto-5′-Nu in SVARECs. Biotinylation has been previously described as a reliable method to assess Ecto-5′-Nu expression to plasma membrane.[24] As shown in Figure 4A, Western blot analysis of cell surface proteins isolated by biotinylation with monoclonal anti-5′-Nu antibody demonstrated an increase of Ecto-5′-Nu protein level under hypoxia. The accessory additional band revealed by this method seems related to enzyme modifications induced by biotinylation rather than to antibody specificity, because a similar result with a distinct antibody was reported by Strohmeier et al.[24]

This increase of Ecto-5′-Nu cell surface expression was confirmed by immunoquantification of Ecto-5′-Nu. As shown in Figure 4B, staining of Ecto-5′-Nu with polyclonal antibody was located exclusively on the plasma membrane. Quantification of the fixation of this anti-5′-Nu antibody after the period of hypoxia with a ^{125}I-labeled secondary antibody showed a 2-fold increase of Ecto-5′-Nu membrane expression (Figure 4C), in accordance with the 2-fold increase of the V_{max} value. The amount of labeled Ecto-5′-Nu that disappeared after 18 hours of incubation as compared with T_0 was lower in hypoxic than in control condition in SVARECs

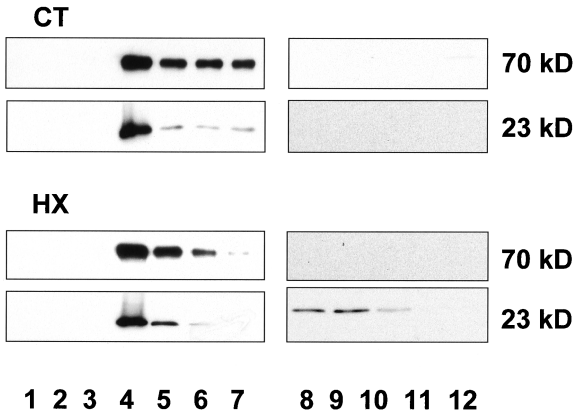

Figure 5. Ecto-5′-Nu expression in sucrose gradient fractions. After incubation of SVARECs with 21% O_2 (CT) or 0% O_2 (HX) for 18 hours, Western blot analysis of each sucrose gradient fraction was performed with polyclonal anti-5′-Nu antibody (70 kDa) and polyclonal anti-caveolin antibody (23 kDa). Representative picture of 3 experiments.

(Figure 4D), suggesting that hypoxia induced a decrease of endocytosis.

Effect of Hypoxia on Ecto-5′-Nu Localization in DRMs

Because GPI-anchored proteins are mainly located in DRMs,[17,27] we tested whether hypoxia affected Ecto-5′-Nu content in DRMs, assessed in SVARECs by Western blot after separation by sucrose gradient. Ecto-5′-Nu was located in fractions 4 to 7, which correspond to DRM fractions.[27] Hypoxia did not significantly affect Ecto-5′-Nu repartition in sucrose gradient fractions (Figure 5). Furthermore, Ecto-5′-Nu/caveolin ratio in whole DRMs was not significantly modified by hypoxia (1.05±0.04 versus 0.90±0.11 for hypoxia and control, respectively). By contrast, Gsα/caveolin ratio was increased (1.93±0.13 versus 0.69±0.11 for hypoxia and control, respectively, n=3; $P<0.01$)

Effect of Hypoxia on Membrane Lipid Composition

Because hypoxia was reported to affect lipid membrane contents[14,15] and because lipidic composition is critical for membrane expression of GPI-anchored proteins,[16,17] we evaluated the effect of hypoxia on cholesterol, phospholipid, and fatty acid composition in SVARECs. As shown in the Table, nonesterified cholesterol level was not affected significantly by hypoxia. Similarly, the total amount of phospholipids was not significantly modified. By contrast, hypoxia affected dramatically the fatty acid composition of membranes: saturated fatty acids increased almost 2-fold, whereas monounsaturated fatty acids decreased significantly. These lipids modifications were reversed at 3 hours of reoxygenation, whereas Ecto-5′-Nu activity was almost returned to control value (Table). As a consequence, the saturated over unsaturated fatty acid ratio was increased by hypoxia (0.52±0.11 versus 0.24±0.04 for hypoxia and control, respectively; $P<0.05$) and returned to control value after reoxygenation (0.28±0.02 at 3 hours). In order to further delineate which fatty acids are affected, the amount of various fatty acids was quantified by GC-MS. As shown in the Table, palmitic acid (C16:0) content significantly increased after hypoxia, whereas the increase of stearic acid (C18:0) did not reach significance.

Effect of Hypoxia on Membrane Lipid Composition

	Control	Hypoxia	Reoxygenation
Cholesterol, nmol/mg protein	77.40±11.39	85.42±10.57	ND
Phospholipids, nmol/mg protein	450.22±65.18	381.21±90.75	ND
Fatty acids, percent of total			
Saturated	19.03±2.70	30.62±4.31*	21.80±0.19
Monounsaturated	79.03±2.98	67.73±4.34*	76.11±0.08
Polyunsaturated	1.95±0.87	1.65±0.31	2.02±0.07
C16:0	12.57±2.03	21.06±2.27*	16.57±0.18
C16:1	8.79±1.68	6.81±0.53	10.18±0.47
C18:0	6.46±0.84	10.56±2.04	5.23±0.15
C18:1	70.24±4.62	60.92±4.32	65.93±0.55
C18:2	1.57±0.80	1.31±0.19	1.58±0.07
C20:4	0.29±0.04	0.26±0.14	0.31±0.01
C20:5	0.12±0.02	0.10±0.04	0.13±0.01

SVARECs were preincubated for 18 hours with a 0% O_2 gas mixture (hypoxia) or a 21% O_2 gas mixture (control) before the lipids were extracted and measured by GC-MS (n=3). For reoxygenation studies, the 0% O_2 gas mixture was replaced after 18 hours by a 21% O_2 gas mixture for an additional 3 hours of incubation. Results are mean±SEM of 3 to 6 different experiments. *$P<0.05$ vs control. ND indicates not done.

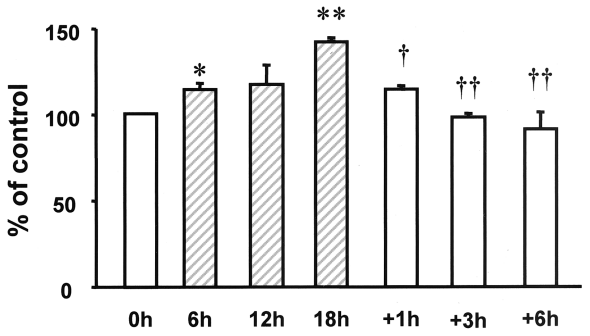

Figure 6. Time course of palmitic acid modification under hypoxia. SVARECs were incubated for 6 hours to 18 hours with a 0% O_2 gas mixture before the lipids were extracted and palmitic acid measured by GC-MS. For reoxygenation studies, the 0% O_2 gas mixture was replaced after 18 hours by a 21% O_2 gas mixture for an additional incubation of 1 hour to 6 hours. Results are expressed as percent of controls (maintained in a 21% O_2 gas mixture during the same times of incubation) and are mean±SEM of 3 different experiments. *$P<0.05$, **$P<0.01$ vs control; †$P<0.05$, ††$P<0.01$ vs hypoxia.

Role of Palmitic Acid in Hypoxic Stimulation of Ecto-5′-Nu

Because the effect of hypoxia was particularly prominent on C16 fatty acids, we asked whether modifications of palmitic acid content are implicated in hypoxic stimulation of Ecto-5′-Nu. First, we studied the time course of palmitic acid modifications under hypoxia and reoxygenation, and we have shown that the increase of palmitic acid content preceded the increase of Ecto-5′-Nu activity under hypoxia and was promptly reversed by reoxygenation (Figure 6). We then studied the effect of addition of palmitic acid in the culture medium. Incubation of normoxic cells with 1 mmol/L palmitic acid for 18 hours increased Ecto-5′-Nu activity (Figure 7A) and membrane expression assessed by immunostaining (Figure 7B) and biotinylation (Figure 7C). Palmitic acid also enhanced the amount of labeled Ecto-5′-Nu, which persists at the cell surface after 18 hours of incubation (Figure 7D), suggesting a decrease of endocytosis, as shown for hypoxia. Supplementation with 100 μmol/L palmitic acid for 5 days, which induced a similar increase of Ecto-5′-Nu activity (142±9% of control; $P<0.05$) and membrane expression (149±5% of control; $P<0.01$), did not modify Ecto-5′-Nu/caveolin ratio in DRM (0.79±0.22 versus 0.93±0.09 for palmitic acid and control, respectively), as observed under hypoxia.

Preincubation with 100 μmol/L 2-bromopalmitate, an inhibitor of palmitoyl-transferases,[28] 24 hours before hypoxic incubation, did not modify Ecto-5′-Nu activity in normoxic cells (1.10±0.17 versus 1.20±0.11 pmol/mg protein per min for normoxic cells supplemented or not with 2-bromopalmitate respectively, n=3), and did not reverse the effect of hypoxia on Ecto-5′-Nu activity (2.92±0.19 versus 2.84±0.18 pmol/mg protein per min for hypoxic cells supplemented or not with 2-bromopalmitate respectively, n=3).

Discussion

In the present study, we have shown that hypoxia enhanced Ecto-5′-Nu activity and cell surface expression in endothelial cells. This effect was accompanied by a decrease of Ecto-5′-Nu endocytosis, whereas synthesis of the enzyme was unaffected by hypoxia. Saturated fatty acids were increased under hypoxia, and incubation of normoxic cells with palmitic acid mimicked the effect of hypoxia.

Physiological implication of Ecto-5′-Nu in adaptation to hypoxia is supported by numerous publications showing that adenosine metabolism is shifted toward extracellular production under hypoxia,[29] and that adenosine produced extracellularly by Ecto-5′-Nu contributes to adaptation to hypoxia,[1,11,30] notably in the cardiovascular system.[3,5,31] However, few studies concerned the regulation of Ecto-5′-Nu activity by hypoxia,[6,13,29,31] and none has focused on the endothelium.

Ecto-5′-Nu activity was enhanced by hypoxia in SVARECs. A nonspecific toxic effect was ruled out by absence of LDH release by hypoxic cells together with the quick reversion of Ecto-5′-Nu activation in normoxia. Moreover, this effect could not be related to SV40 transfection because it was reproduced in endothelial cells grown in primary culture.

The hypoxia-inducible factor (HIF) has been implicated in hypoxic stimulation of Ecto-5′-Nu in intestinal cells.[13] An increase of Ecto-5′-Nu mRNA and protein was also reported in hypoxic neuronal cells.[29,30] Although stimulation of Ecto-5′-Nu activity by hypoxia in SVARECs resulted from an increase of the V_{max} without modification of its affinity, the finding that Ecto-5′-Nu mRNA and protein levels were not increased under hypoxia strongly argue against a direct implication of HIF in our system. The discrepancy between our results and those previously mentioned could be related to the diversity and tissue specificity of the mechanisms of hypoxic regulation, as reported for many hypoxia inducible proteins.[12,32]

Protein kinase C has been implicated in the regulation of Ecto-5′-Nu.[2,33] Minamino et al[5,6] have studied Ecto-5′-Nu activity in dog coronary arteries and cardiomyocytes under hypoxia. They showed that activation of Ecto-5′-Nu by hypoxia in their model was abolished by inhibitors of protein kinase C. These reports contrast with our observation that modulation of protein kinase C in SVARECs did not modify Ecto-5′-Nu activity, neither in normoxic nor in hypoxic conditions (see online Figure 1, available in the online data supplement at http://www.circresaha.org). This could again suggest that regulation of Ecto-5′-Nu is tissue-specific or that the isoform of protein kinase C implicated in Ecto-5′-Nu

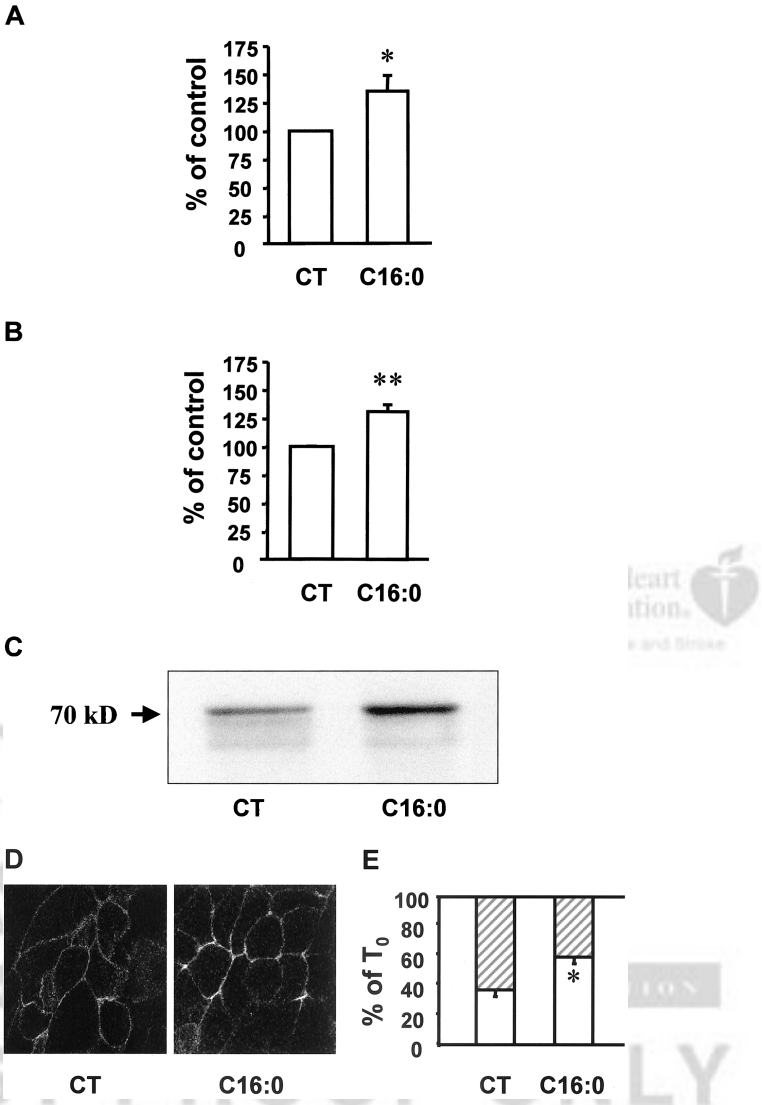

Figure 7. Effect of palmitic acid on Ecto-5′-Nu activity and membrane expression. SVARECs were supplemented with 1 mmol/L palmitic acid (C16:0) and added in the culture medium for 18 hours. Ecto-5′-Nu activity was measured on confluent cells incubated with 50 μmol/L 5′-AMP for 5 minutes (A). Samples of cell surface proteins isolated by biotinylation were labeled with monoclonal anti-5′Nu antibody (C). For immunoquantification of Ecto-5′-Nu, fixed cells were incubated with polyclonal anti-5′-Nu antibody, and then stained with a ^{123}I-labeled (B) or a FITC-labeled (D) anti-rabbit antibody. Nonfixed cells were incubated with anti-5′-Nu antibody for 1 hour and then fixed (T_0) or submitted to an 18-hour incubation without (CT) or with 1 mmol/L palmitic acid (C16:0): endocytosis (hatched part of the columns) was estimated as Ecto-5′-Nu staining at T_0 minus the staining, which persists after the 18-hour incubation (open part of the columns) (E). Results are mean±SEM of 3 different experiments. *$P<0.05$, **$P<0.01$ vs control.

regulation was not active in these cultured cells. However, our results ruled out an implication of protein kinase C in the effect of hypoxia in SVARECs.

Cell surface expression of Ecto-5′-Nu, assessed by two different methods in SVARECs, was increased by hypoxia. It has been previously shown that only 50% of total cellular pool of Ecto-5′-Nu is expressed at the cell surface in basal conditions, and that the enzyme permanently cycled between cell surface and intracellular compartments.[34] In our study, the amount of cell surface labeled Ecto-5′-Nu that disappeared after 18 hours of incubation was lower in hypoxic than in normoxic conditions, suggesting that the enhancement of cell surface exposure of Ecto-5′-Nu under hypoxia was supported by a decrease of endocytosis.

We have previously shown that lovastatin enhanced Ecto-5′-Nu cell surface expression through an inhibition of isoprenylation of Rho-GTPases, which are implicated in actin polymerization.[7] However, whereas CNF, an activator of Rho-GTPases, reversed the effect of lovastatin, this compound did not modify the stimulation of Ecto-5′-Nu by hypoxia. Furthermore, hypoxia did not reproduce the decrease of F-actin content induced by lovastatin in SVARECs, and actin organization (assessed by fluorescent microscopy) was not significantly altered in hypoxic cells (see online Figure 2). This result is consistent with previous reports of an activation rather than an inhibition of Rho-GTPase and actin polymerization under hypoxia.[35,36]

Lipid composition of cell membranes has been shown to play a critical role in the sorting of GPI-anchored proteins.[16,17] A decrease of cholesterol-enhanced cell surface expression of GPI-anchored proteins in several cell types including endothelial cells[17] and a 40% decrease of cellular cholesterol content has been evidenced in aortic endothelial cells after incubation with 0% O_2 gas mixture for 24 hours.[14] A decrease of sphingomyelin content under hypoxia has also been reported,[37] but the effect of sphingomyelin on the sorting of GPI-anchored proteins is controversial.[18,38] In contrast with these studies, nonesterified cholesterol content, measured by GC-MS, was not modified in hypoxic SVARECs. Furthermore, addition of 50 μg/mL LDL-cholesterol 24 hours before the 18-hour period of hypoxia did not reverse the increase of Ecto-5′-Nu membrane expression. Along the same line, the absence of significant effect of sphingomyelinase on Ecto-5′-Nu activity ruled out a prominent role of sphingomyelin content in Ecto-5′-Nu hypoxic stimulation (see online Figure 3).

We show here that hypoxia modified fatty acid composition in SVARECs. The proportion of saturated fatty acids was increased, as previously observed in hypoxic brain,[15] and this effect was particularly significant on C16 fatty acids. The kinetics of palmitic acid modifications under hypoxia were consistent with a involvement of these changes in Ecto-5′-Nu stimulation. This possible role is reinforced by the observation that addition of palmitic acid in the culture medium partially reproduced the effect of hypoxia on the activity and expression of the enzyme.

It was hypothesized[17,39] that lipid modifications could enhance cell surface expression of GPI-anchored proteins through modulation of their association with membrane microdomains, the detergent-resistant membranes (DRMs). Association of GPI-anchored proteins with DRMs under hypoxia has never been studied to our knowledge, but one group[27] has studied the repartition of GPI-anchored proteins in sucrose gradient fractions after supplementation with saturated or unsaturated fatty acids. Whereas association of acylated cytoplasmic proteins to DRMs was influenced by fatty acid saturation, no modification of GPI-anchored proteins repartition was observed. In agreement with this observation, we did not observe significant modifications of Ecto-5′-Nu association with sucrose gradient fractions during hypoxia or palmitic acid supplementation, whereas we observed a increase of the ratio between the raft-associated Gsα subunit, which is palmitoylated,[40] and caveolin. The effect of hypoxia was not reversed by 2-bromopalmitate, an inhibitor of palmitoyltransferase, which suggests that the effect of hypoxia on Ecto-5′-Nu was unrelated to modifications of protein palmitoylation. These results suggest that enhancement of Ecto-5′-Nu cell surface expression under hypoxia is not related to changes in its association with rafts, but rather to the redistribution of the enzyme from an intracellular pool to a plasma membrane pool.

It has been reported[41] that dietary polyunsaturated fatty acid deficiency in piglet intestinal brush border membrane simultaneously increased saturated over unsaturated fatty acid ratio, membrane viscosity, and Ecto-5′-Nu activity. Our results are then consistent with the hypothesis that the decrease of Ecto-5′-Nu endocytosis observed under hypoxia is related to a decrease of cell membrane fluidity by saturated fatty acids. The physiological implication of our findings is reinforced by the observation that exogenous administration of palmitic acid in endothelial cells also increases Ecto-ATP Diphosphohydrolase,[42] which hydrolyzes ATP and ADP before the action of Ecto-5′-Nu, and is also implicated in hypoxic adaptation.[13,30]

In conclusion, we show that hypoxia enhances Ecto-5′-Nu activity in endothelial cells. This effect is underlain by an original mechanism, which does not involve HIF-mediated transcriptional activation. Stimulation of endothelial Ecto-5′-Nu by hypoxia results from enhancement of cell surface expression of the enzyme, which could be related to a decrease of the enzyme endocytosis through modifications of membrane composition in fatty acids. The physiological significance of this Ecto-5′-Nu stimulation is supported by many previous studies that have shown evidence of the critical role of extracellular adenosine in adaptive responses to hypoxia.

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