Role of HIF-1α in proton-mediated CXCR4 down-regulation in endothelial cells

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Aims
Acidification is associated with a variety of pathological and physiological conditions. In the present study, we aimed at investigating whether acidic pH may regulate endothelial cell (EC) functions via the chemokine receptor CXCR4, a key modulator of EC biological activities.

Methods and results
Exposure of ECs to acidic pH reversibly inhibited mRNA and protein CXCR4 expression, CXCL12/stromal cell-derived factor (SDF)-1-driven EC chemotaxis in vitro, and CXCR4 expression and activation in vivo in a mouse model. Further, CXCR4 signalling impaired acidosis-induced rescue from apoptosis in ECs. The inhibition of CXCR4 expression occurred transcriptionally and was hypoxia-inducible factor (HIF)-1α-dependent as demonstrated by both HIF-1α and HIF-1α dominant negative overexpression, by HIF-1α silencing, and by targeted mutation of the −29 to −25 hypoxia response element (HRE) in the −357/−59 CXCR4 promoter fragment. Moreover, chromatin immunoprecipitation (ChIP) analysis showed endogenous HIF-1α binding to the CXCR4 promoter that was enhanced by acidification.

Conclusion
The results of the present study identify CXCR4 as a key player in the EC response to acidic pH and show, for the first time, that HRE may function not only as an effector of hypoxia, but also as an acidosis response element, and raise the possibility that this may constitute a more general mechanism of transcriptional regulation at acidic pH.

Keywords
Chemokine • HIF-1 • Acidosis

1. Introduction
Endothelial cells (ECs) are exposed to an acidic pH in a variety of pathological and physiological conditions including ischaemia, diabetic ketoacidosis, respiratory failure, renal failure, wound healing, and haemodynamic shear stress. Therefore, in some conditions, acidosis is associated with hypoxia, e.g. in ischaemia, while in other instances EC acidification occurs under normoxic conditions, e.g. in renal failure and in response to haemodynamic shear stress.1 Acidification that follows hypoxia may constitute an effector of hypoxia-mediated cell functions,2 in addition, acidosis may exert biological activities by itself. For example, von Hippel-Lindau sub-nuclear foci distribution, loss of activity, and increase in hypoxia-inducible factor (HIF) levels and activity are induced in normoxic acidosis.3 Prior studies from our laboratory have shown that acidification per se, in normoxia, has profound effects on ECs.4,5 A decrease in EC pH induces a marked increase in cytosolic Ca2+ and enhances CD18-mediated neutrophil adhesion despite a decrease in ICAM-1.6 Further, acidic pH inhibits EC proliferation, chemotaxis, and differentiation and protects ECs from cellular apoptosis, in part, via the Axl molecule.5 Acidosis attenuates endotoxin-induced nuclear factor-kappa B (NFkB) activation in pulmonary artery ECs and induces cyclooxygenase-2 expression.7 Moreover, acidosis affects the expression of target genes9 such as vascular endothelial growth factor (VEGF),4 matrix metalloproteinase (MMP)-1, and IL-8.10 At present, the mechanisms of proton sensing are poorly characterized, although has been suggested a role for AP-1 and NFkB.10 The SDF-1/CXCR4 axis has been shown to play a pivotal role in EC function; it regulates

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2. Methods

2.1 Cells

Human umbilical vein ECs (HUVECs) (Clonetics, LONZA, Switzerland) were grown in complete EBM-2 medium (LONZA).

2.2 Acidification and hypoxic protocols

Culture dishes were placed in air tight modular incubator chambers (Forma Scientific) as previously described; infused for 20 min, with 5%CO2/95% air, 20%CO2/80% air, and 25%CO2/75% air to achieve a buffer pH of 7.4 ± 0.02, 7.0 ± 0.05, and 6.6 ± 0.05, respectively. Chambers were sealed and placed at 37°C in an incubator for the duration of the experiments. Hypoxic conditions were achieved by culturing cells in a sealed chamber after flushing with 1%O2/5%CO2/94%N2. Hypoxia plus acidosis was achieved by culturing cells with 1%O2/5%CO2/94%N2, 1%O2/5%CO2/94%N2, and 1%O2/5%CO2/94%N2.

2.3 mRNA expression

qRT–PCR. Total RNA was converted to cDNA by reverse transcription. mRNA expression levels were calculated by Comparative ct Method (Applied Biosystem, CA, USA). Normalization was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels, and mRNA expression determined by the 2^–ct ct method. CXCR4, HIF-1α, Glut-1, VE-cadherin, Tie-2, and CXCR2 primer sequences are shown in Supplementary material, Methods.

2.4 Protein expression

Flow cytometry. CXCR4 surface receptor expression was analysed by flow cytometry as previously described. Western blot analysis. HUVECs were adenovirus-infected for 24 h and HIF-1α protein was analysed by WB analysis as specified in Supplementary material, Methods.

2.5 Chemotaxis assay

HUVEC chemotaxis to either SDF-1 (10–100 ng/mL) or 2.5–10% FCS was evaluated in 48-well Boyden’s chambers as previously described. Briefly, HUVECs were exposed to pH 7.0 or pH 7.4 for 16 h in serum-free medium. Cells were then incubated for 4 h at 37°C at pH 7.4 and the number of migrating cells was counted at ×40 magnification. Migration index was calculated as described in Supplementary material, Methods.

2.6 Cell death quantification

siRNA transfected, shRNA-infected cells, or uninfected cells were serum-starved and either exposed to pH 7.4 or pH 7.0 for 24 h; nucleosome-bound DNA fragments were then measured using a cell death ELISA detection kit (Roche).

2.7 In vivo experiments

Three-month-old Swiss CD1 male mice were rendered acidic by NH4Cl treatment. Average blood pH was 7.5 ± 0.2 in control mice, and pH 7.3 ± 0.2 in acidic mice P = 0.017. Aorta sections were isolated from normo (n = 5) and acidic (n = 4) mice. CXCR4 and phospho-CXCR4 (kind gift of Dr J.B. Rubin, Washington University School of Medicine, St Louis, MO, USA) expression were analysed by immunohistochemistry as described in Supplementary material, Methods.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the study was approved by the Institutional Animal Care and Use Committee.

2.8 Transfection and luciferase assay

Cells were co-transfected for 24 h with CXCR4 promoter-pGL2 reporter and Renilla plasmids. pGL-CXCR4 (−2600 to +1) and pGL-CXCR4 (−357 to +59) plasmids were generous gifts from Dr A. Sica (Istituto Humanitas, Rozzano, Italy), and Prof. H. Moriuchi (Nagasaki University School of Medicine, Nagasaki, Japan), respectively. After 24 h in hypoxia, in acidosis, or hypoxia plus acidosis, luciferase activity was measured. Dominant negative HIF-1α construct was a kind gift of Prof. M. Kobayashi, Hokkaido University Graduate School of Medicine, Sapporo, Japan. Site-directed mutagenesis was performed using the pGL-CXCR4 (−357 to +59) construct by replacing TA with GC in the GCCGTGT motif by using Quick Change Site Directed mutagenesis kit (Strategene, CA, USA).

2.9 Adenovirus vector construction

A recombinant adenovirus vector expressing human HIF-1α was generated using the Ad-Easy™ system as previously described. Full-length HIF-1α cDNA was kindly provided by Dr Max Gassmann (Institute for Veterinary Physiology, University of Zurich).

2.11 HIF-1α silencing by lentivirus

HIF-1α MISSION shRNA clones (Sigma) were constructed within the lentivirus plasmid vector pLKO.1-Puro. Vectors were grown and purified, and lentiviral supernatants were produced, according to manufacturer’s instructions. shRNA control is MISSION non-target control vector.

2.12 Chromatin immunoprecipitation assay (ChIP)

ECs, subjected either to pH 7.4 or pH 7.0, or to pH 7.0 vs. hypoxia, were fixed and cross linked; chromatin was then sheared by sonication and incubated with anti-HIF-1α or control antibody O/N at 4°C to be immunoprecipitated. Pellets were then subjected to 35 cycles of PCR amplification with primers encompassing the −29 to −25 HRE site in the human CXCR4 promoter.

Primer sequences are shown in Supplementary material, Methods.

2.13 Statistical analysis

Statistical evaluation was done with GraphPad Prism software version 4.0 (GraphPad, San Diego, CA, USA). Data are expressed as mean ± SD. Statistical significance was assessed by t-test or by univariate ANOVA followed by the Bonferroni test for multiple comparisons. A value of p ≤ 0.05 was considered statistically significant.
3. Results

3.1 Acidosis inhibits CXCR4 mRNA and protein expression

The effect of acidosis on CXCR4 expression was examined in HUVECs exposed to either pH 7.4, pH 7.0, or pH 6.6 for 24 h. Acidification strongly inhibited CXCR4 mRNA levels, at both pH 7.0 and pH 6.6, as assessed by real-time PCR (Figure 1A). In all subsequent experiments, the effect of acidification was examined only at pH 7.0, because this pH level is found in many pathophysiologic conditions and it has been shown to have profound effect on EC functions.4 CXCR4 mRNA levels were inhibited at 4–48 h exposure to pH 7.0 (Figure 1B).

We next determined whether acidic pH also reduced CXCR4 protein levels. HUVECs were cultured either at pH 7.4 or 7.0 for 12 and 24 h and then examined by FACS analysis for CXCR4 expression. A statistically significant (P < 0.05) decrease in CXCR4 mean fluorescence intensity both at 12 h (59.2 ± 6.8 vs. 38.3 ± 5) (Figure 1C), and at 24 h (52.5 ± 4.4 vs. 41.5 ± 1.1) (data not shown) time point was observed. In additional experiments, we determined whether the inhibition of CXCR4 expression by acidosis was permanent or transient. HUVECs were first incubated at pH 7.4 or pH 7.0 for 16 h; medium was then replaced, and cells were incubated at pH 7.4 up to 24 h. We found that acidosis-mediated CXCR4 downregulation is reversible both at the mRNA (not shown) and protein level.

Figure 1  Acidic pH inhibits CXCR4 mRNA and protein expression in HUVECs. (A) qRT–PCR analysis of CXCR4 mRNA levels in HUVECs following acidification. *P < 0.05 vs. pH 7.4 (n = 3). (B) CXCR4 mRNA expression, measured by qRT–PCR, following 1–48 h of acidification (n = 5). *P < 0.05 vs. pH 7.4. (C) Flow cytometric analysis of surface CXCR4 expression in HUVECs after 12 h at pH 7.4 (black line histogram) or pH 7.0 (gray line histogram). Control isotype is also shown (dotted line histogram). The figure shows a representative experiment out of five. (D) Cell surface CXCR4 protein expression of HUVECs cultured at pH 7.4 (black) or pH 7.0 (grey) for 16 h and subsequently exposed to normal pH for 24 h (dotted). Chemotaxis in response to (E): 10–100 ng/mL SDF-1 and (F): 2.5–10% FCS (n = 4). *P < 0.05 vs. pH 7.4 (—). Migration index description: see Supplementary methods.
level (Figure 1D). We next investigated the functional significance of CXCR4 down-regulation induced by acidic pH. HUVECs were kept either at pH 7.0 or pH 7.4 for 16 h and analysed for their ability to migrate in response to SDF-1. SDF-1-driven chemotaxis, at 10–100 ng/mL, was inhibited by acidification (Figure 1E). In contrast, migration elicited by 2.5 to 10% FCS, used as a control, was not affected (Figure 1F). CXCR4 regulation at pH 7.0 was specific; in fact, no cytotoxic effect of acidosis was observed (see Supplementary material online, Figure S1A). Furthermore, expression of other cell surface molecules, such as VE-cadherin and CXCR2, is unaffected by 24 h acidosis while Tie-2 is upregulated, thus indicating that the effect of acidosis on CXCR4 expression is specific (see Supplementary material online, Figure S1B–D).

Additionally, we demonstrated that inhibition was endothelium-specific as CXCR4 expression decrease at pH 7.0 was also found in Bovine Aortic Endothelial Cells (BAECs) (see Supplementary material online, Figure S2A), but not in Hela (see Supplementary material online, Figure S2B) and Jurkat cells (see Supplementary material online, Figure S2C).

### 3.2 CXCR4 expression and phosphorylation are inhibited by acidosis in vivo

In these experiments, it was examined whether acidosis may also modulate CXCR4 expression and activation in vivo. To this end, 3-month-old CD1 mice were made acidotic by 7 day NH₄Cl treatment. We then analysed CXCR4 expression in aortas isolated from control (Figure 2A) vs. acidotic (Figure 2B) mice by immunohistochemistry and found a decrease in CXCR4 expression in ECs of acidotic vs. control mice, i.e. 34.9 ± 2.8 vs. 8.4 ± 7.7%, respectively (Figure 2C). Further, we analysed the expression of phosphorylated CXCR4 in control (Figure 2D) vs. acidotic mice (Figure 2E). The anti-CXCR4 antibody that was utilized recognized Ser 339

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/86/2/293/369849)
phosphorylated CXCR4 that reflects a receptor-activated form. Phosphorylated CXCR4 expression was present in 27.3 ± 2.3% aortic ECs in control mice vs. 9 ± 3.6% ECs in acidotic mice (Figure 2F).

### 3.3 Acidosis induces transcriptional inhibition of CXCR4 expression in ECs

As a first step to elucidate the molecular mechanisms of CXCR4 repression by acidosis, we analyzed the promoter activity in HUVECs transfected with plasmid constructs expressing the full-length 2.6 Kb (Figure 3A), or the −357/+59 CXCR4 promoter fragment (Figure 3B), linked to the firefly luciferase (Luc) reporter gene. The −357/+59 fragment was chosen as it exhibits significant transcriptional activity in ECs and it is a known target of transcriptional regulation. As shown in Figure 3A and B, acidification reduced luciferase activity of both constructs (grey bars), when compared with cells cultured at pH 7.4 (black bars). The region spanning −357/+59 of the CXCR4 promoter conferred a more significant decrease of luciferase activity when compared with the full-length promoter, thus indicating that repressive sequences involved in acidosis-induced CXCR4 down-regulation were contained in this promoter fragment. As hypoxia is known, from previous studies, to induce a positive rather than negative regulation of CXCR4 expression, via a HRE located at −1.3 kb, we also tested, as a control, the activity of the CXCR4 promoter fragment in hypoxia and confirmed an increase of such activity, in the 2.6 Kb promoter fragment by 2.2 ± 0.1-fold (Figure 3A) and, to a lesser extent, hypoxia also induced −357 bp CXCR4 luciferase activity by 1.6 ± 0.12-fold (Figure 3B). We next examined whether CXCR4 regulation may also occur at the post-transcriptional level, but this was not the case as CXCR4 transcripts were even more stable at pH 7.0 than at pH 7.4 in HUVECs treated with the transcription inhibitor Actinomycin D for up to 4 h (Figure 3C).

### 3.4 HIF-1α is a negative regulator of CXCR4 expression in acidosis

HIF-1α is known to mediate both transcriptional activation or repression in different promoters, and is known to regulate CXCR4 expression levels in hypoxia. Further, HIF-1 is both induced and activated by acidosis in some reports. Therefore, we investigated whether HIF-1α may be involved in CXCR4 expression regulation by acidosis. We found that, also in our experimental conditions, i.e. in ECs at pH 7.0, HIF-1α was both induced and activated (see Supplementary material online, Figure S3A–C).

We next performed experiments aimed at assessing whether HIF-1α had an effect on CXCR4 expression and/or function in acidosis. We first induced a forced overexpression of HIF-1α in HUVECs by infecting cells with a HIF-1α adenovirus vector and cultured them under physiologic or acidic pH for 24 h. CXCR4 mRNA levels, after 24 h at pH 7.0, showed 3.2 ± 1.9-fold down-regulation vs. pH 7.4 in AdCMV.Null-infected cells (P < 0.05), and 9.5 ± 2.2-fold down-regulation vs. pH 7.4 in AdCMV.HIF-1α-infected cells (Figure 4A) (P < 0.05). Therefore, HIF-1α overexpression markedly enhanced acidosis ability to inhibit CXCR4 mRNA expression. Further, silencing HIF-1α by shRNA interference totally prevented CXCR4 down-regulation at pH 7.0 (Figure 4B).

### 3.5 HIF-1α induces transcriptional repression of the CXCR4 promoter in acidosis

We next tested the effect of a dominant-negative (DN) HIF-1α expression construct on −357/+59 CXCR4 promoter activity. Interestingly, DN HIF-1α construct markedly inhibited the −357/+59 CXCR4 promoter down-regulation at pH 7.0 (Figure 4A). Further, shRNA silencing of HIF-1α yielded similar results (Figure 5B). We therefore looked for hypoxia response elements (HRE) in the −357/+59 promoter

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**Figure 3** Transcriptional inhibition of CXCR4 expression on ECs by acidification. Luciferase activity of −2.6 kb to +1 (A) and −357 to +59 (B) CXCR4 promoter constructs transiently transfected in HUVECs cultured at pH 7.4 (black) or pH 7.0 (grey) or at pH 7.4 in hypoxia (white) (n = 3) *P < 0.05 vs. pH 7.4.CXCR4 expression levels in Actinomycin D (Act)-D-treated HUVECs cultured at pH 7.4 (left panel) or at pH 7.0 (right panel) for 1, 2, and 4 h, assessed by qRT-PCR (n = 3).
construct and identified a canonical site, i.e. GCGTG located at position -29 to -25 relative to the transcription start site (Figure 5C).

We next determined whether such HRE may be involved in acidosis-induced CXCR4 repression in HUVECs by introducing a targeted mutation at position -29 to -25 of the CXCR4 promoter, and showed a complete inhibition of repression in transfectants containing the targeted mutation (Figure 5D and E), thus indicating that such HRE was crucial for acidosis responsiveness. In contrast, hypoxia-induced activation of the -357 CXCR4 promoter fragment was not modulated by -29 to -25 HRE mutation (Figure 5E). Previous studies had shown that hypoxia responsiveness is due to a different HRE, located at -1.3 kb.

As hypoxia and acidosis exert a double effect on CXCR4 expression, we next assessed whether hypoxic stimulation or acidotic inhibition of CXCR4 expression prevails and found that acidosis-induced CXCR4 down-regulation prevails over the effect of hypoxia in terms of luciferase activity (Figure 5D) and mRNA expression at 48 h (Figure 5F, dotted vs. solid line).

In order to demonstrate whether endogenous binding of HIF-1α on CXCR4 promoter occurred at pH 7.4 and pH 7.0, we performed a chromatin immunoprecipitation (ChIP) assay using primers encompassing the -29 to -25 site and adjacent flanking regions. As shown in Figure 5G, endogenous HIF-1α was bound to CXCR4 promoter in anti-HIF-1α/chromatin immunoprecipitates. Further, acidosis increased the amount of HIF-1α bound to CXCR4 promoter. ChIP assay was also performed under hypoxic conditions using primers encompassing the -29 to -25 site and adjacent flanking regions (Figure 5H and I) and no endogenous binding was found; further suggesting that such HRE is specific for acidosis but not for hypoxia responsiveness.

3.6 Acidosis inhibits CXCR4-mediated EC functions

We have previously shown that CXCR4 overexpression promotes EC apoptosis, further enhanced by SDF-1 treatment. We therefore hypothesized that acidosis-induced CXCR4 down-regulation, in addition to a role of Axl, may contribute to acidification ability to inhibit cell death in ECs. To test this hypothesis, we selectively silenced endogenous CXCR4 (see Supplementary material online, Figure S4A) and found a marked increase in acidosis-mediated rescue from serum starvation-induced apoptosis in respect to control (Figure 6A). Additionally, SDF-1 treatment of serum-starved HUVECs brought to a small, yet statistically significant, impairment of apoptosis rescue, which was abrogated by SDF-1 inactivation (Figure 6B). Moreover, when CXCR4-overexpressing HUVECs (see Supplementary material online, Figure S4B) were serum-deprived and exposed to pH 7.0 for 24 h, the strong anti-apoptotic effect exerted by acidosis on ECs was partially inhibited (see Supplementary material online, Figure S4C). Further, the inhibition of HIF-1α by shRNAs reverted the protective effect of pH 7.0 on ECs (Figure 6C). Thus, the HIF-1-dependent decrease of CXCR4 expression at pH 7.0 may play an important role in EC survival during acidification.

4. Discussion

Due to the important role of CXCR4 in the response to ischaemia, our study aimed at investigating whether acidic pH per se, independently from hypoxia, may regulate CXCR4 expression and function and at getting insights into the molecular mechanisms leading to such regulation. Further, it was assessed whether the CXCR4

Figure 4 HIF-1α is a negative regulator of CXCR4 expression in acidosis. (A) Western blot analysis showing HIF-1α protein levels in AdCMV.HIF-1α vs. AdCMV.Null infected cells (left panel). Shown is a representative experiment out of two. CXCR4 mRNA expression, evaluated by qRT–PCR, of HIF-1α-expressing cells vs. control cells cultured at pH 7.4 vs. 7.0 for 24 h (right panel). *P < 0.05 vs. pH 7.4 (Null); **P < 0.05 (n = 5). (B) HIF-1α mRNA levels after HIF-1α shRNA silencing of HUVECs (left panel) (n = 3). CXCR4 mRNA expression, assessed by qRT–PCR, of HIF-1α – silenced vs. control cells (right panel). *P < 0.05 vs. pH 7.4 (CTRL shRNA) (n = 3).
receptor may be responsible, at least in part, of acidosis-induced functional effects in ECs. It is noteworthy that a decrease in pH well within the range examined in the present study has been reported both in physiologic conditions such as physical exercise and haemodynamic shear stress as well as in pathological states including cardiac ischaemia. The effect of pH variation on chemokine receptor expression and function has been ill defined. In the present study, we found transcriptional repression of CXCR4 by acidic pH, and that, functionally, such down-regulation affected SDF-1-directed EC migration and apoptosis. Interestingly, in a mouse model of acidosis, we showed a marked inhibition of CXCR4 expression and activation of aortic ECs. Acidosis-mediated CXCR4 down-regulation occurs in ECs, i.e. HUVECs and BAECs, where acidosis induces several functional effects including rescue from apoptosis. We therefore analysed the molecular mechanisms leading to CXCR4 down-regulation by acidosis. The −357/+59 CXCR4 promoter fragment, important for the regulatory activity of YY1 and c-Myc and human herpesvirus 6 (HHV6), was characterized as showed a marked inhibition of activity in acidic conditions and was examined for binding sites that may constitute putative acidosis-responsive elements. Even though no bona fide consensus sequence for transcriptional response to acidosis is known, prior studies showed that NFkB is an important site for acidosis responsiveness in the metalloproteinase (MMP)-9 promoter and both NFkB and AP-1 sites are involved in the regulation of IL-8 expression by acidosis, but neither of these sites were present in the −357/+59 CXCR4 promoter fragment. As HIF-1α

Figure 5 HIF-1α transcriptionally represses the CXCR4 promoter. (A) Dominant negative (DN) HIF-1α overexpression inhibited acidosis-induced decrease of −357 CXCR4 promoter activity (3.5 ± 0.6 vs. 1.8 ± 0.3-fold decrease, *P < 0.05) (n = 5). (B) HIF-1α silencing by shRNA inhibited acidosis-induced decrease of −357 CXCR4 promoter activity (3.16 ± 0.47 vs. 1.66 ± 0.74-fold down-regulation, *P < 0.05) (n = 3). (C) Schematic representation of transcription binding sites present in the −357 CXCR4 promoter construct. HRE consensus sequence is present at −29 to −25 bp. (D and E) −29 to −25 HRE mutation abrogates CXCR4 promoter down-regulation by acidosis, but not the response to hypoxia. *P < 0.05 vs. pH 7.4 (n = 5). (F) At 48 h, exposure to pH 7.0 plus hypoxia impairs hypoxia ability to upregulate CXCR4, as assessed by qRT–PCR, *P < 0.05 vs. hypoxia (n = 5). ChIP assay performed with primers encompassing the CXCR4 promoter −29 to −25 HRE at pH 7.0 vs. 7.4 (G), and at pH 7.0 vs. hypoxia (H and I). Control Ab (IgG) or anti-HIF-1α Ab were used to immunoprecipitate associated chromatin. Shown is a representative experiment out of four (G) and out of two (H and I).
was shown to be induced and activated by acidosis in some studies, and to be a CXCR4 transcriptional regulator, we looked for HRE sites in the CXCR4 promoter fragment. We found a newly identified canonical HRE consensus at position to and evaluated whether HIF-1α might be involved in acidosis-induced CXCR4 inhibition. We obtained evidence on the role of HIF-1α in such regulation by using gain- and loss-of-function studies. Furthermore, a targeted mutation of the HRE showed that such site acts as an acidosis response element as it affects the response to acidosis. At last, ChIP analysis demonstrated HIF-1α binding to CXCR4 promoter and that HIF-1α binding increased in acidosis. No binding occurred to this site, instead, in hypoxic conditions. HIF-1α not only can act as a transcriptional activator, but may also exert a repressive regulation of a number of promoters, including α-fetoprotein and E-cadherin. In the latter case, HIF-1α has an indirect effect on such promoter by inducing TCF3, ZFHX1A and B transcriptional repressors and not by directly binding HRE sequences. We cannot exclude that HIF-1α may exert its repressive activity not only through −29/−25, but also via other HREs or by interacting with other factors binding non-HRE sites. Therefore, transcriptional inhibition of CXCR4 by HIF-1α, at acidic pH, can occur directly and, possibly, also indirectly. More work will be necessary to define the nature of HIF-mediated repression. Further, as the stability and activity of HIF-1α can be regulated by post-translational modification, such as hydroxylation, ubiquitination, acetylation, or phosphorylation, it is possible that acidosis may affect HIF-1α activity by inducing such protein modifications. HRE has been described as a hypoxia response element in several promoters including VEGF, Flk-1 receptor, Endothelin-1, and CXCR4. It is noteworthy that hypoxia induces a HIF-1α-dependent CXCR4 up-regulation and increases chemotaxis to SDF-1 in different cell types.

We here report, for the first time, the role of HIF-1α as an acidosis response factor, in the CXCR4 promoter, via a HRE site, located at position −29 to −25. This finding opens the possibility that this may represent a more general mechanism of HIF-1α/HRE-mediated acidosis response and, thus, other promoters might be regulated by acidosis via HRE sites. Therefore, HIF-1α differentially regulates the same promoter, i.e. CXCR4, inducing either up- or down-regulation, in hypoxia and acidosis, respectively, both conditions that follow ischaemia. Such regulation occurs via distinct HRE sites in the CXCR4 promoter, i.e. −1.3 kb in hypoxia and −29 to −25 in acidosis. It is possible that the mild, yet statistically significant, increase of CXCR4 promoter activity we observe in hypoxia is HRE-independent, as such site does not bind HIF-1α in hypoxia. This finding raises the question of which is the biological significance of the opposite coordinated regulation of a key inducer of angiogenesis, i.e. CXCR4, in almost concomitantly-occurring conditions, i.e. hypoxia and acidosis, in which a number of angiogenic factors are upregulated. Further, our data show that, when cells are subjected to both acidosis and hypoxia, acidosis-induced CXCR4 down-regulation prevails over...
the effect of hypoxia at 48 h. Interestingly, acidosis is known to partially inhibit hypoxia’s ability to enhance expression of discrete HIF-responsive genes, e.g. VEGF and erythropoietin.39 Our data show that acidosis is able to impair the up-regulation, exerted by hypoxia, of another HIF-responsive gene, i.e. CXCR4.

As in our current studies, we have shown, in acidosis, an impaired SDF-1-directed chemotaxis, a role of SDF-1/CXCR4 in acidosis-induced rescue from apoptosis, and a strong inhibition of CXCR4 expression and activation in vitro and of aortic ECs in vivo. We hypothesize that acidosis may prepare/program ECs to the neoangiogenic response to ischaemia also by blunting the angiogenic response via inhibition of CXCR4 expression and function.

Further studies will be needed to address whether HIF-1α may be a player not only in the regulation of CXCR4 expression, but may be regarded as a more general regulator of gene expression by acidic pH.

Supplementary material
Supplementary Material is available at Cardiovascular Research Online.

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