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RESEARCH ARTICLE

Emerging new role of NFAT5 in inducible nitric oxide synthase in response to hypoxia in mouse embryonic fibroblast cells

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Serman Y, Fuentealba RA, Pasten C, Rocco J, Ko BCB, Carrión F, Irarrázabal CE. Emerging new role of NFAT5 in inducible nitric oxide synthase in response to hypoxia in mouse embryonic fibroblast cells. *Am J Physiol Cell Physiol* 317: C31–C38, 2019. First published May 8, 2019; doi:10.1152/ajpcell.00054.2019.—We previously described the protective role of the nuclear factor of activated T cells 5 (NFAT5) during hypoxia. Alternatively, inducible nitric oxide synthase (iNOS) is also induced by hypoxia. Some evidence indicates that NFAT5 is essential for the expression of iNOS in Toll-like receptor-stimulated macrophages and that iNOS inhibition increases NFAT5 expression in renal ischemia-reperfusion. Here we studied potential NFAT5 target genes stimulated by hypoxia in mouse embryonic fibroblast (MEF) cells. We used three types of MEF cells associated with NFAT5 gene: NFAT5 wild type (MEF-NFAT5$^{+/+}$), NFAT5 knockout (MEF-NFAT5$^{-/-}$), and NFAT5 dominant-negative (MEF-NFAT5$^{\Delta/\Delta}$) cells. MEF cells were exposed to 21% or 1% O_2 in a time course curve of 48 h. We found that, in MEF-NFAT5$^{+/+}$ cells exposed to 1% O_2, NFAT5 was upregulated and translocated into the nuclei, and its transactivation domain activity was induced, concomitant with iNOS, aquaporin 1 (AQP-1), and urea transporter 1 (UTA-1) upregulation. Interestingly, in MEF-NFAT5$^{-/-}$ or MEF-NFAT5$^{\Delta/\Delta}$ cells, the basal levels of iNOS and AQP-1 expression were strongly downregulated, but not for UTA-1. The upregulation of AQP-1, UTA-1, and iNOS by hypoxia was blocked in both NFAT5-mutated cells. The iNOS induction by hypoxia was recovered in MEF-NFAT5$^{-/-}$ MEF cells, when recombinant NFAT5 protein expression was reconstituted, but not in MEF-NFAT5$^{\Delta/\Delta}$ cells, confirming the dominant-negative effect of MEF-NFAT5$^{\Delta/\Delta}$ cells. We did not see the rescue effect on AQP-1 expression. This work provides novel and relevant information about the signaling pathway of NFAT5 during responses to oxygen depletion in mammalian cells and suggests that the expression of iNOS induced by hypoxia is dependent on NFAT5.

AQP-1; hypoxia; iNOS; NFAT5; UTA-1

INTRODUCTION

Hypoxia is a condition in which insufficient levels of oxygen are supplied to one or more tissues in the body. Molecular oxygen serves as the final electron acceptor in oxidative phosphorylation, and hence low oxygen supply increases the risk for generating reactive oxygen species (ROS), resulting in cell dysfunction and ultimately cell death (33). In this context, the nuclear factor of activated T cells 5 (NFAT5), a master regulator of the osmoprotective program, plays a protective role against hypoxia [independent of hepatocyte-inducible factor (HIF)-1α], but the signaling pathway is still under revision (28, 37).

NFAT5 belongs to the Rel family of transcriptional activators, which includes NF-κB (29). When cells are immersed in hypertonic media, NFAT5 is translocated to the nuclei (7, 39), where it binds to osmotic response elements on target genes, increasing the activity of the transactivation domain (TAD) (3, 9). The activation of NFAT5 induces the intracellular accumulation of organic osmolytes and water transport, via induction of osmoregulatory target genes such as aldose reductase (AR) (21), urea transporters (UTA-1) (14), the betaine-GABA transporter (BGT1) (15), and membrane transporters of water [aquaporins (AQPs)] (4, 23). In addition, NFAT5 mRNA and protein levels are increased by high NaCl, which confers an additional level of regulation by hypertonicity (6, 18, 19, 37). Besides hypertonic stimulation, NFAT5 is also necessary for isotonic Toll-like receptor (TLR)-4 downstream expression of inflammatory target genes like IL-1β, IL-6, TNF-α, and inducible nitric oxide synthase (iNOS) (5, 16, 17, 24).

Previously, we showed that NFAT5 is induced by hypoxic stimulation (0.1–2.5% O_2), increasing its nuclear translocation and transcriptional activity. Hypoxia and hypertonicity effects on NFAT5 induction are additive, and activation of NFAT5 during hypoxia is independent of the HIF-1α pathway (36). Importantly, silencing of NFAT5 during hypoxia increased cleaved caspase-3 levels, suggesting that NFAT5 prevented the apoptosis induced by hypoxia (34a, 37).

Endogenous nitric oxide (NO) is synthesized by three isoforms of nitric oxide-synthesizing enzymes (NOS). The NOS use L-arginine to metabolize it to L-citrulline and NO. iNOS enzyme is calcium independent, and its expression is induced by several cytokines and immune regulators, ultimately by binding of transcription factors such as NF-κB to its proximal promoter (1).

Several studies have shown that hypoxia induces the expression of iNOS, which leads to the rapid production of NO (6, 10, 25, 36). In turn, NO combines with superoxide anion to

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generate peroxynitrite, a highly toxic reactive nitrogen species that causes lipid peroxidation, nitration of tyrosine residues, activation of caspase-3, apoptosis, and cell death (26, 27, 40). There are some studies where a relationship between NFAT5 and iNOS has been suggested: induction of iNOS by lipopolysaccharide (LPS) causes impairment of renal NFAT5 function because of an increase in NFAT5 *S*-nitrosylation, which causes a reduction of NFAT5 target gene expression in the renal medulla (ClC-K1, UTA-1, and AQP2) and a consecutive sepsis-induced urinary concentration defect (22), indicating that iNOS affects the NFAT5 activity during sepsis. Another study provided evidence that NFAT5 is essential for the expression of iNOS in TLR-stimulated macrophages (4, 15). Recently, we have described in a mouse model of renal ischemia and reperfusion that pharmacological inhibition of iNOS [N^6-(1-iminoethyl)lysine, hydrochloride] increased the NFAT5 signaling pathway in the kidney (32). Although this evidence points toward functional reciprocity between these two signaling pathways, the interaction of NFAT5 and iNOS signaling in the setting of hypoxia remains completely controversial.

In this research, we investigated the role of NFAT5 on some potential NFAT5 target genes in mouse embryonic fibroblast (MEF) cells that expressed normal, mutated, or deleted NFAT5 gene. We found that protein expression, nuclear localization, and transactivating activity of NFAT5 was induced by hypoxia in MEF-NFAT5$^{+/+}$ cells, concomitantly with upregulation of iNOS, AQP-1, and UTA-1 protein expression. The induction by hypoxia of all these genes was impaired when functional NFAT5 was altered (MEF-NFAT5$^{-/-}$ and MEF-NFAT5$^{\Delta/\Delta}$ cells). Transient transfection of a recombinant version of NFAT5 in MEF-NFAT5$^{-/-}$ cells recovered iNOS upregulation during hypoxia, but not AQP-1. As was expected, the mutated NFAT5 cells (dominant negative for NFAT5) blocked the iNOS upregulation in rescue experiments. These findings provide new insight into the role of NFAT5 during hypoxia-induced iNOS expression.

MATERIALS AND METHODS

Cell lines. Wild-type mouse embryonic fibroblasts (MEF-NFAT5$^{+/+}$), MEF knockout for NFAT5 (MEF-NFAT5$^{-/-}$) (28), and a dominant-negative form of NFAT5 (MEF-NFAT5$^{\Delta/\Delta}$) cells (10) were used. The dominant-negative of NFAT5 version contains a deletion of 254–380 residues of the protein, inhibiting NFAT5 function by forming dimers with wild-type protein that are incapable of binding DNA in a sequence-specific manner (29). The MEF cells are an immortalized cell line, and the experiments were conducted between passage 10 and 20. These cells were a gift from Dr. Maurice Burg's laboratory (National Heart, Lung, and Blood Institute).

Cell culture treatment. Cell lines were all maintained in high-glucose DMEM media (Sigma) supplemented with 2 mM L-glutamine (HyClone), penicillin-streptomycin (Gibco), and 10% of fetal bovine serum (Sigma). Cells were incubated at 37°C in a 5% CO_2 atmosphere.

Hypoxia in cell culture. The hypoxia condition was obtained using a Heracell 150i CO_2 incubator (Thermos) coupled to a Drager-X-am Multi-Gas Detector system set at 1% O_2, 5% CO_2, and 37°C (36). A time course ranging from 4 to 48 h was performed. Hypertonic medium (500 mosmol/kgH_2O) was adjusted by adding NaCl (100 mM) to the culture medium (38). As a positive control for iNOS expression, incubation for 24 h with 500 ng/ml LPS and 25 ng/ml IFNγ was used.

Real-time PCR. An RNeasy mini kit (Qiagen) was used for total RNA extraction according to the manufacturer's instructions. After verification of RNA integrity by agarose gel electrophoresis, 1 μg of RNA was reverse transcribed using a reverse transcription system (MaxyGene thermal cycler; AxyGene). The amplicons were detected by fluorescence detection by Real Time (Rotor-Gene Q; Qiagen). The results were normalized to the 18S gene.

Primers used were as follows: NFAT5 forward 5′-CCTTCAGCAGTCTCCAGTTTA-3′, NFAT5 reverse 5′-GCTCCAGTTTCTTCTCCATCTC-3′, iNOS forward 5′-TCCTGCCTCATGCCATTGAGTT-3′, iNOS reverse 5′-GCCTGGCCAGATGTTCCTCTATTT-3′, AQP-1 forward 5′-TCTGCCCTAGGCTTCAATTACCCA-3′, AQP-1 reverse 5′-CCAGAGTAGCGATGCTCAAACCAA-3′, UTA forward 5′-GCTTACATGCAGCCATAGGGTCAA-3′, UTA reverse 5′-AAAGGCCCAGGTAGATCGCTCCAA-3′, 18S forward 5′-CTTCTCTTTCCGCCAACCCC-3′, and 18S reverse 5′-CGACACCTCTCTTATCCGCT-3′.

Luciferase assay. The binary GAL4 reporter system has been previously described (9). To assess the transactivation activity of the NFAT5-TAD, cells were cotransfected with the GAL4 reporter (pFR-Luc) and GAL4 DNA-binding domains (dbd)-548–1531, which contains the recombinant TAD of NFAT5. After transfection (24 h), cells were switched to hypoxia or high NaCl (500 mosmol/kg NaCl added), and luciferase activity was measured 16 h later using the Luciferase Assay System kit (Promega) and a Synergy 2 multidetector microplate reader (Biotek).

Cell lysate preparation and Western blot analysis of MEF cells. Adherent cells were lysed with cold PBS (1% Triton X-100) supplemented with protease inhibitor cocktail (Roche) and 1 mM PMSF (Sigma). Lysates were centrifuged at 10,000 *g*, 10 min at 4°C. Cleared lysates were determined using the BCA Protein Assay kit (Pierce). The pellet was maintained at −80°C for additional analysis. The nuclear and cytoplasmic fractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to supplier instructions. Equal amounts of soluble proteins were separated by SDS-PAGE and transferred to a PVDF or nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer-PBS (1:4) and overnight incubated at 4°C with primary antibodies in Odyssey blocking buffer. The next day, membranes were incubated 2 h with a fluorophore-conjugated secondary antibody (Invitrogen). Proteins were detected by Infra-Red fluorescence using the Odyssey CLx system (Li-Cor). The intensities of the resulting bands were quantitated by infrared fluorescence signal (Image Studio Lite version 5.25; Li-Cor). The antibodies used were rabbit anti-NFAT5 (PA1–023; Thermos), mouse anti-NFAT5 (sc-398171; Santa Cruz), mouse anti-V5 (Invitrogen), mouse anti-iNOS (no. 610431; BD Bioscience), rabbit anti-AQP-1 (ab168387; Abcam), rabbit anti-UTA-1 (AB156589; Abcam), anti-BGT1 (SC-241911; Santa Cruz), or rabbit anti-β-actin (5316; Sigma) antibodies.

Plasmids. The human NFAT5 recombinant protein was obtained from cDNA clone KIAA0827 (30), and it was a gift from Dr. Maurice Burg's Laboratory. In brief, the construct containing NFAT5 amino acids 1–1,531 of clone KIAA0827 was again cloned into expression vector pcDNA6V5-His (Invitrogen) to generate 1–1531V5-His (13).

The binary GAL4 reporter system has been described (9). In brief, plasmid pFR-Luc (Stratagene) contains the yeast GAL4-binding site (upstream activating sequence) upstream of a minimal promoter and the *Photinus pyralis* luciferase gene. Expression plasmid pFA-CMV (Stratagene) contains a sequence coding for the yeast GAL4 DNA-binding domain. A fusion protein was generated by in-frame insertion of the sequence coding for amino acids 548–1531 of clone KIAA0827 into pFA-CMV to generate GAL4dbd-548 –1531. GAL4dbd contains no transactivation domain but expresses the GAL4dbd (pFC2-dbd; Stratagene).

Cell transfection. Transient transfection of MEF cells with Lipofectamine 3000 (Invitrogen) was done following the manufacturer's instructions. The transfection efficiency was evaluated by green fluorescent protein expression in a fluorescence microscope (530 nm). Cells were fixed at room temperature for 20 min using paraformal-

dehyde diluted to 4% in PBS (Sigma), followed by four PBS washes and a final PBS incubation until imaged. Cells were registered and counted using a fluorescence-inverted microscope (Eclipse TE2000; Nikon), and image processing and analysis were performed with NIS Elements 4.0 software and ImageJ software (National Institutes of Health). Routinely, transfection efficiencies were around 50%.

Statistical analysis. Data are expressed as averages ± SE. Values from different groups were assessed with one-way ANOVA test for multiple comparisons and the Bonferroni or Dunnett method or in some cases with Mann-Whitney and *t*-test. The significance level was $P \leq 0.05$, 0.01, or 0.001.

RESULTS

Hypoxia induced NFAT5 expression in MEF cells. A time course of hypoxia on NFAT5 expression was measured by qRT-PCR and Western blot (Fig. 1, *A* and *B*). In MEF-NFAT5$^{+/+}$ cells, the levels of NFAT5 mRNA increased 3.8 times at 4 h of hypoxia (Fig. 1*A*), returning it to baseline values at 16 h after hypoxia incubation. During basal conditions (21% O_2), detectable levels of NFAT5 protein were found (Fig. 1*B*). Compared with 21% of O_2, hypoxia (1% O_2) produced a significant increase of NFAT5 protein levels (40%) at 4 h of hypoxia, and it was sustained toward 24 h (Fig. 1*B*). Furthermore, hypoxia-induced NFAT5 transactivation activity was higher than 21% O_2. Interestingly, the positive control of transactivation activity (16 h hypertonicity) was significantly higher than hypoxia (Fig. 1*C*). In addition, 30 min of hypoxia induced NFAT5 translocation into the nucleus compared with 21% of O_2 in MEF-NFAT5$^{+/+}$ cells (Fig. 1*D*).

Hypoxia induced iNOS expression in MEF cells. Next, we studied the iNOS expression in MEF-NFAT5$^{+/+}$ cells. As shown in Fig. 2*A*, during basal conditions (21% O_2), the iNOS expression was scarcely detected in this cell type by Western blotting. The iNOS mRNA increased 3.9 times at 4 h of hypoxia, and it was sustained in time points analyzed. At 4 h of hypoxia, iNOS protein levels were significantly induced, reaching a plateau of expression from 8 h of hypoxia (3-fold over 21% O_2 levels) (Fig. 2*B*). According to these results, hypoxia induced iNOS expression (4–48 h) in MEF-NFAT5$^{+/+}$ cells.

Potential NFAT5 target genes activated by hypoxia. By osmotic stress, the induction of AQP-1, UTA-1, AR, and BGT1 is under transcriptional control of NFAT5. Here we studied if these genes are induced by hypoxia in MEF-NFAT5$^{+/+}$ cells. We observed that protein levels of AQP-1 and UTA-1 were significantly induced at 16 h of hypoxia (Fig. 2, *D* and *F*), but not AR or BGT1 (data not shown). The mRNA levels of AQP-1 were increased by 4 h of hypoxia, returning to basal levels at 16 h. Surprisingly, a second peak was detected at 24 h of hypoxia (Fig. 2*C*). The protein abundance of AQP-1 increased from 16 h, reaching a peak at 24 h of hypoxia. UTA-1 mRNA levels were not affected by hypoxia (Fig. 2*E*). However, we noted a significant increase of protein level at 16

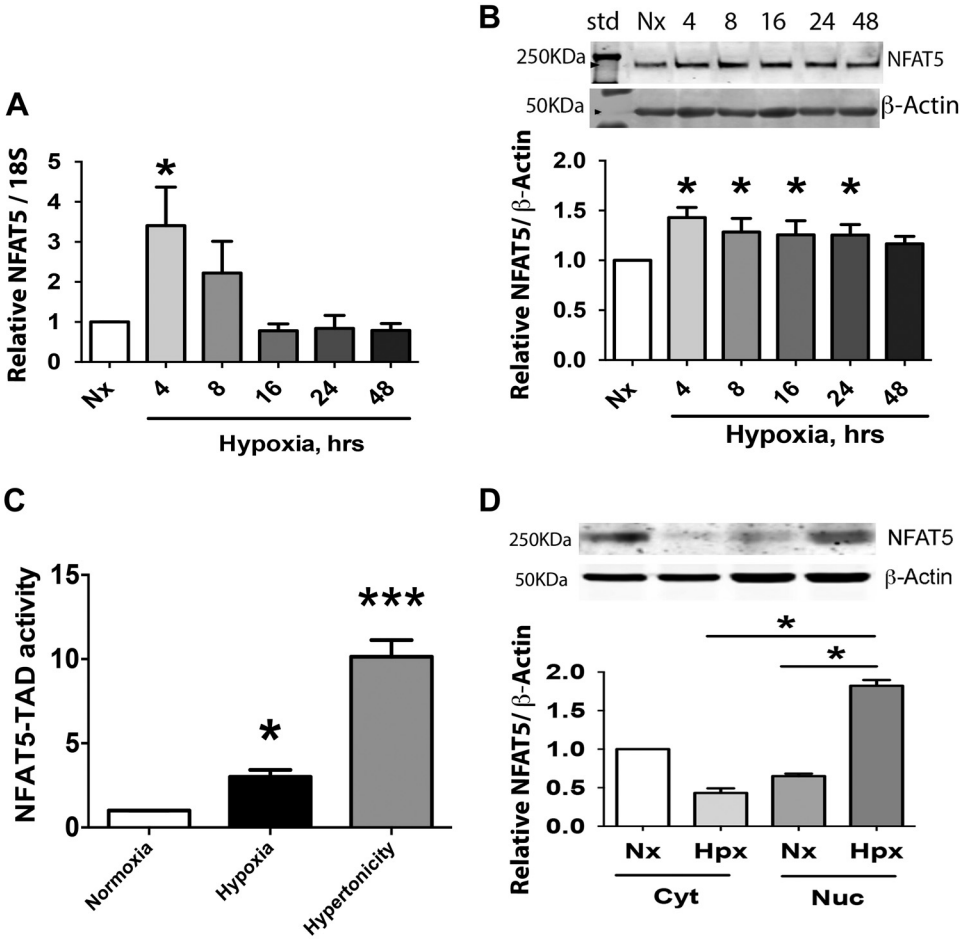

Fig. 1. Hypoxia induced upregulation of the mRNA, protein abundance, and nuclear translocation of nuclear factor of activated T cells 5 (NFAT5) in mouse embryonic fibroblast (MEF)-NFAT5$^{+/+}$ cells. Wild-type MEF cells were incubated in 21% O_2 conditions (Nx), or time-course curve of hypoxia (4–48 h, 1% O_2). *A*: relative NFAT5 mRNA was determined by qRT-PCR. *B*: relative NFAT5 protein was determined by Western blotting. *A* and *B* data were analyzed by Mann-Whitney and *t*-test. *C*: MEF cells were cotransfected with pFR-LUC plus pFA-NFAT5-TAD. Data were analyzed by one-way ANOVA and Bonferroni test. *D*: NFAT5 abundance was determined by Western blotting in nuclear (nuc) and cytoplasmic (cyt) fractions of the cells. Means ± SE; $n = 7$; $^*P \leq 0.05$ and $^{***}P \leq 0.001$ compared with 21% O_2 condition [hypoxia (Hyp)]. Data were analyzed by ANOVA and Dunnett's test. A representative image is shown in the *top* of *B* and *D*. The mRNA and protein abundance were normalized by 18S and β-actin, respectively.

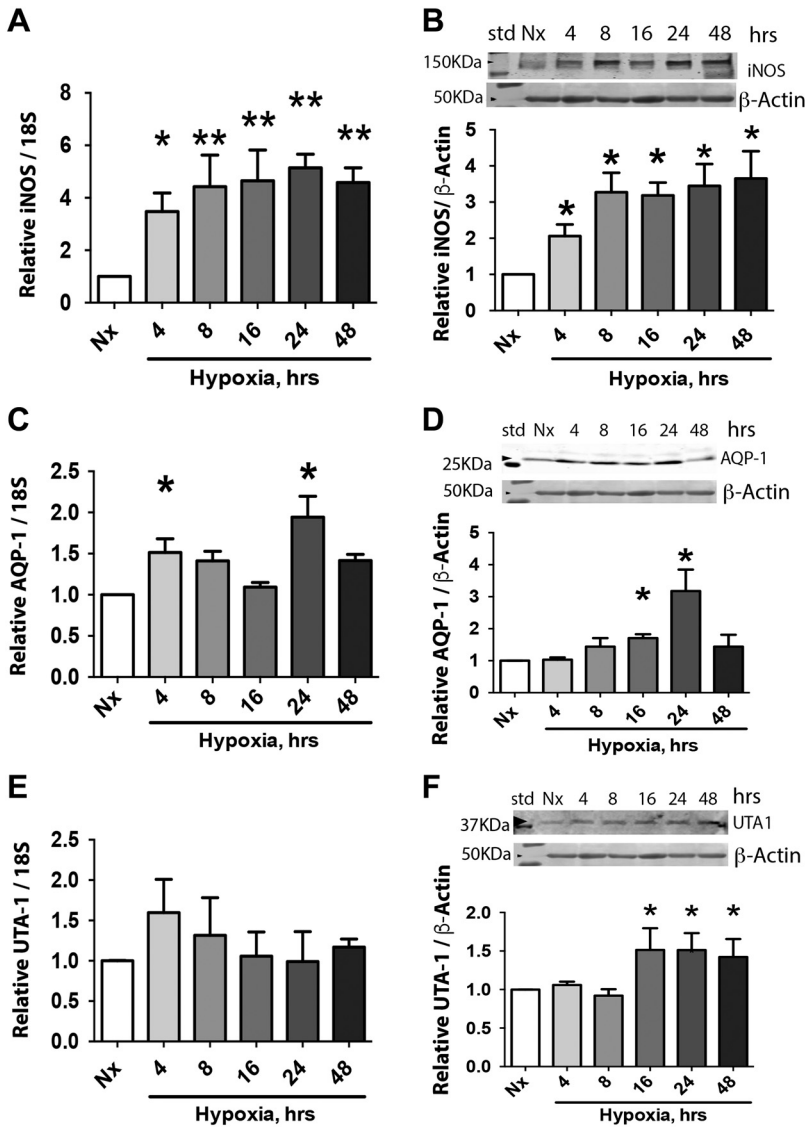

Fig. 2. Hypoxia increased the mRNA and protein levels of inducible nitric oxide synthase (iNOS), aquaporin 1 (AQP-1), and urea transporter 1 (UTA-1) in mouse embryonic fibroblast (MEF) cells-nuclear factor of activated T cells 5 (NFAT5$^{+/+}$). Cells were incubated in 21% O_2 (Nx) or time-course curve of hypoxia (4–48 h, 1% O_2). Gene expression was studied by qRT-PCR and protein abundance by Western blotting. *A*: relative mRNA expression of iNOS. *B*: relative protein expression of iNOS. *C*: relative mRNA levels of AQP-1. *A–C*: data were analyzed by ANOVA and Dunnett's test. *D*: relative protein expression of AQP-1. *E*: relative mRNA expression of UTA-1. *F*: relative protein expression of UTA-1. Means ± SE; $n = 7$; *$P \leq 0.05$ and **$P \leq 0.01$ compared with 21% O_2 condition. *D* and *F*: data were analyzed by *t*-test. The mRNA and protein abundance were normalized by 18S and β-actin, respectively. A representative image is shown in the *top* of each panel.

h of hypoxia (Fig. 2*F*). These results showed that, in MEF-NFAT5$^{+/+}$ cells, the hypoxia produced upregulation of iNOS, AQP-1, and UTA-1.

AQP-1, UTA-1, and iNOS upregulation associated with hypoxia was impaired in MEF-NFAT5$^{-/-}$ cells. To understand the role of NFAT5 on iNOS, AQP-1, and UTA-1 induction by hypoxia, we use MEF-NFAT5$^{-/-}$ cells. We confirmed that MEF-NFAT5$^{-/-}$ cells did not express NFAT5 protein (Fig. 3*A*). Interestingly, we noted that protein expression of the iNOS and AQP-1 condition was strongly downregulated in MEF-NFAT5$^{-/-}$ cells in both kinds of oxygen conditions (Fig. 3, *B* and *C*). However, for UTA-1, we observed only a deficient effect on protein upregulation produced by hypoxia, keeping the basal expression of the protein (Fig. 3*D*).

AQP-1 and iNOS upregulation observed by hypoxia was impaired in MEF-NFAT5$^{\Delta/\Delta}$ cells. Additionally, we explored MEF-NFAT5$^{\Delta/\Delta}$ cells. This NFAT5 allele encodes a mutant protein containing an internal deletion of amino acid residues encoded by exons 6 and 7 (254–380 residues), and mutated NFAT5 did not have the ability of DNA binding. Both constitutive and hypertonicity-induced expression of wild-type NFAT5 expression was significantly reduced in MEF-NFAT5$^{\Delta/\Delta}$ cells (21). Remarkably, our results showed that hypoxia- and hypertonicity-induced iNOS expression was strongly eliminated in MEF-NFAT5$^{\Delta/\Delta}$ cells (Fig. 4). Notably, the expression of AQP-1 was also lost in all the conditions tested (21% O_2, 1% O_2, or hypertonicity) in MEF-NFAT5$^{\Delta/\Delta}$ cells. These results strongly suggested that DNA binding of NFAT5 in the *AQP-1* and *Nos2* (iNOS) promoter is also necessary for hypoxia stimulation of AQP-1 and iNOS. Collectively, our results demonstrated that the induction of AQP-1 and iNOS by hypoxia stimulation was dependent on the wild-type NFAT5 expression.

Restoration of NFAT5 expression in MEF-NFAT5$^{-/-}$ cells produced a rescue of iNOS upregulation during hypoxia. To improve evidence of the role of NFAT5 role in the iNOS upregulation by hypoxia, we performed rescue experiments in MEF-NFAT5$^{-/-}$ cells by transient transfection with a recombinant protein of NFAT5. The results showed that MEF-

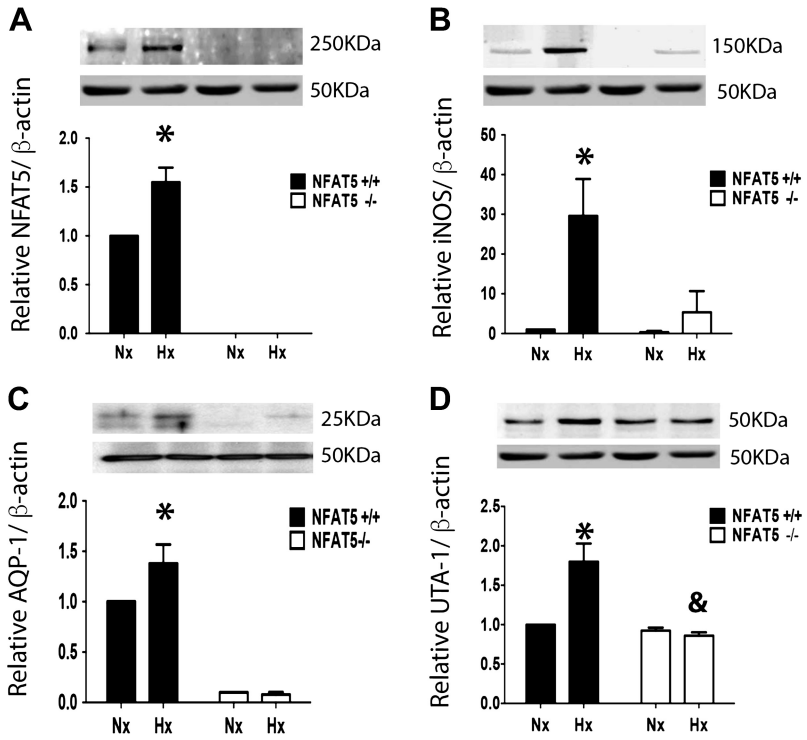

Fig. 3. Nuclear factor of activated T cells 5 (NFAT5) wild type was required for the hypoxia-stimulated upregulation of inducible nitric oxide synthase (iNOS), aquaporin 1 (AQP-1), and urea transporter 1 (UTA-1) expression. Mouse embryonic fibroblast (MEF)-NFAT5$^{+/+}$ and MEF-NFAT5$^{-/-}$ cells were incubated under 21% O_2 (Nx) or hypoxia (1% of O_2) conditions. Relative protein levels were determined by Western blotting. *A*: relative protein levels of NFAT5. *B*: relative protein levels of iNOS. *C*: relative protein levels of AQP-1. *D*: relative protein levels of UTA-1. Means ± SE; $n = 7$; $^{*}P \leq 0.05$ relative to 21% O and $^{\&}P \leq 0.05$ relative to hypoxia. Data were analyzed by Mann-Whitney *t*-test. A representative image is shown in the *top* of each panel. Protein abundance was normalized by β-actin.

NFAT5$^{-/-}$ cells reconstituted with NFAT5 recovered the iNOS upregulation by hypoxia compared with cells transfected with empty vector (Fig. 5*A*). The protein level of iNOS significantly was increased (3-fold) when NFAT5 wild type was reconstituted in MEF cells during hypoxia (Fig. 5*B*). These findings suggested that iNOS upregulation by hypoxia in MEF cells was dependent of NFAT5.

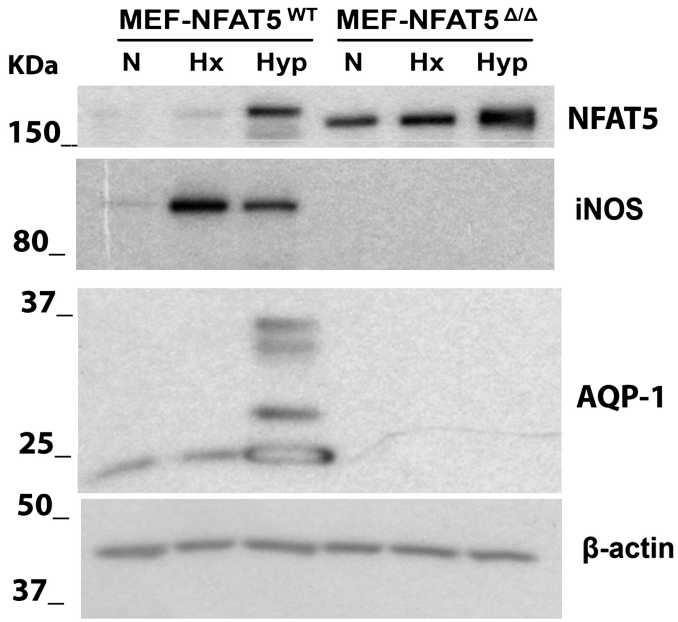

Fig. 4. Mutant nuclear factor of activated T cells 5 (NFAT5) protein blunts inducible nitric oxide synthase (iNOS) and aquaporin 1 (AQP-1) responsiveness to hypoxia and hypertonicity. Mouse embryonic fibroblast (MEF)-NFAT5$^{+/+}$ and MEF-NFAT5$^{\Delta/\Delta}$ cells were subjected to 21% O_2 (N), hypoxia (Hx, 1% O_2), or hypertonic (Hyp, 500 mosmol/kgH_2O) conditions for 24 h. Proteins were studied by Western blotting. The image is representative of 5 independent experiments.

iNOS induction by hypoxia was impaired in MEF-NFAT5$^{\Delta/\Delta}$ cells. Finally, we performed rescue experiments in MEF-NFAT5$^{\Delta/\Delta}$ cells by transient transfection of a recombinant NFAT5. As shown in Fig. 6, recombinant NFAT5 was expressed in MEF-NFAT5$^{\Delta/\Delta}$ cells. As was speculated, recombinant transfection of the wild-type version of NFAT5 did not recover the iNOS expression, probably because of the effect of the dominant inhibitory effect of NFAT5$^{\Delta/\Delta}$. This striking observation suggests that iNOS expression by hypoxia depends on intact NFAT5 heterodimers.

DISCUSSION

NFAT5 is a master regulator of cellular osmoadaptive responses to hypertonic stress (4). Moreover, NFAT5 has been implicated in diverse processes, such as organ development (28), immune response (10), and blood pressure control (24, 35). In addition, NFAT5 is considered a novel protective factor against hypoxia independent of HIF-1α (36), preventing cell death (34a) and playing a role in the urine concentration mechanism (23).

Previous results of our group showed that anoxia/hypoxia (0–2.5% oxygen) induced NFAT5 protein abundance and transcriptional activity in cell culture. Moreover, knock down (small-interfering RNA) of the NFAT5 expression causes an important increase in apoptosis and necrosis after 8 h of hypoxia, suggesting that NFAT5 has a protective role against hypoxia (36). By another way, silencing NFAT5 increased renal damage and the number of TdT-dUTP nick end labeling-positive cells in a model of renal ischemia-reperfusion injury in mice (11). Therefore, NFAT5 can be considered as a pivotal transcription factor and integrator of multiple adverse stimuli

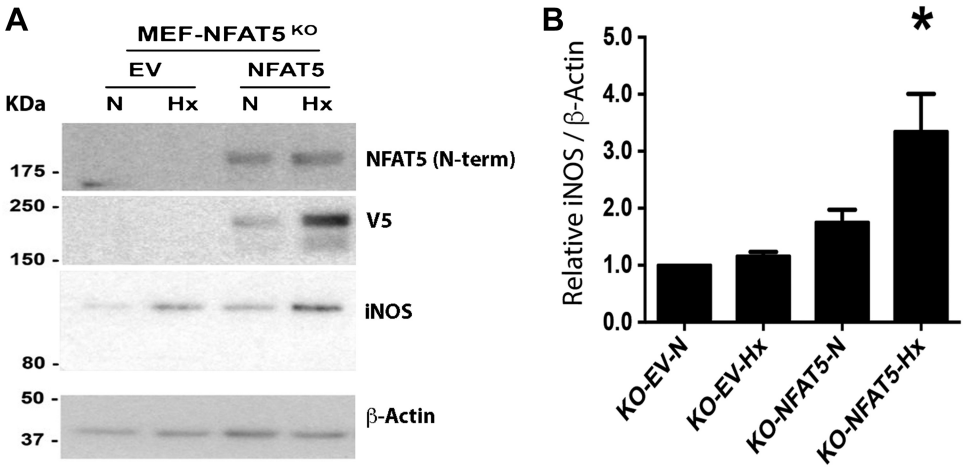

Fig. 5. Transient transfection of nuclear factor of activated T cells 5 (NFAT5) in mouse embryonic fibroblast (MEF)-NFAT5$^{-/-}$ cells recovered the inducible nitric oxide synthase (iNOS) induction by hypoxia. MEF-NFAT5$^{-/-}$ cells were transfected with recombinant NFAT5 or corresponding empty vector (EV). Cells were recovered for 16 h and then subjected to 21% O_2 (N) or hypoxia (Hx, 1% O_2) conditions for 24 h. *A*: representative Western blot analysis showing NFAT5, iNOS, V5, and β-actin detection. *B*: analysis of densitometry was conducted for iNOS detection normalized with β-actin obtained from 5 independent experiments. Means ± SE; *$P \leq 0.05$, respect of 21% O_2 with empty vector (KO-EV-N). Data were analyzed by *t*-test.

produced by stress. Here we hypothesized that NFAT5 acts as a protective transcription factor in an environment exposed to low oxygen concentration by inducing the expression of a specific gene program in response to hypoxia.

We found that, under hypoxia conditions, iNOS, AQP-1, and UTA-1 protein expression was also increased in MEF-NFAT5$^{+/+}$ cells. However, neither AR nor BGT1 (data not shown) levels changed upon stimulation, suggesting that not all of the genes described to be under control of NFAT5 are activated in the hypoxia condition. Regardless of the mechanism, our results are in agreement with previous results showing that AR is not induced by hypoxia (8).

On the other hand, iNOS enzyme is independent of intracellular calcium concentration, and it has been described that iNOS could exert a negative regulatory role in NFAT5-mediated signaling in the renal medulla during sepsis (22). Additionally, in a renal model of ischemia-reperfusion, we also found that iNOS inhibition increased NFAT5 expression (33). Interestingly, evidence has been provided to suggest that NFAT5 is essential for the expression of iNOS in TLR-stimulated macrophages (5). The present work provides evidence about the relationship between NFAT5 and iNOS in a setting of hypoxia.

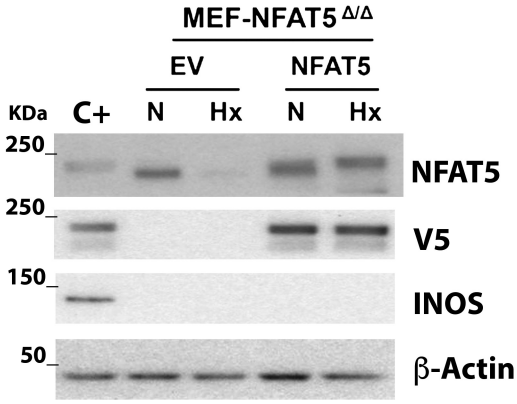

Fig. 6. A dominant-negative form of nuclear factor of activated T cells 5 (NFAT5) impaired the rescue of inducible nitric oxide synthase (iNOS) expression by NFTA5-wild-type during hypoxia. Mouse embryonic fibroblast (MEF)-NFAT5$^{\Delta/\Delta}$ cells were transfected with recombinant NFAT5 or corresponding empty vector (EV). Cells were recovered for 16 h and then subjected to 21% O_2 (N) or hypoxia (Hx, 1% O_2) conditions for 24 h. C+, MEF-NFAT5$^{+/+}$ cells exposed to hypoxia and transfected with recombinant NFAT5. A representative Western blot image of NFAT5, V5, iNOS, and β-actin detection from 5 independent experiments is shown.

In the present study, we found that both NFAT5 and iNOS were induced shortly after hypoxia in MEF cells, starting 4 h after stimulation. Moreover, the transactivation activity and nuclear translocation of NFAT5 were also increased during hypoxia, confirming our previous results in HEK 293 and rat primary inner medullary connecting duct cells (36). The mRNA of NFAT5 upregulation by hypoxia could be a result of mRNA stabilization and/or transcription activation. Interestingly, in the MEF-NFAT5$^{-/-}$ and MEF-NFAT5$^{\Delta/\Delta}$ cells, we did not find upregulation of iNOS or AQP-1 expression by hypoxia. These results clearly suggested that NFAT5 controls the gene expression of iNOS or AQP-1 in this type of cells. To explore this interaction in depth, we conducted rescue experiments in the NFAT5-deficient cell line by restoring its expression using a recombinant protein of NFAT5 (isoform C). In MEF-NFAT5$^{-/-}$ cells, iNOS protein abundance was recovered when NFAT5 was reconstituted, but not in MEF-NFAT5$^{\Delta/\Delta}$ cells. As mentioned in MATERIALS AND METHODS, the mutated NFAT5 (deletion of 254–380 residues) protein expressed in MEF-NFAT5$^{\Delta/\Delta}$ cells has a dominant-negative effect on NFAT5 function by forming dimers with wild-type protein, inhibiting the binding to DNA (28). Interestingly, in agreement with our findings, it has been described in macrophages that NFAT5 is essential for the expression of iNOS by LPS stimulation. Chromatin immunoprecipitation experiments had shown that NFAT5 is recruited to the iNOS promoter after stimulation with LPS (5). Some antecedents on the literature indicate that other members of the NFAT family can regulate iNOS expression. For example, in cardiomyocytes, calcineurins regulate iNOS expression through the NFATc1 isoform (31), and NFATc3 regulates expression of iNOS in macrophages stimulated with LPS (34). In addition, NFATc3 has been shown to be activated by hypoxia in smooth muscle (3). It was recently published that hypoxia induces pulmonary fibroblast proliferation through NFAT signaling (34a); thus, we do not eliminate the possibility that NFATc isoforms could be activated by hypoxia and upregulate NFAT5. Analysis of 15,000 bp upstream to the initiation site of the human NFAT5 gene (chromosome 16, NC_000016.10) is possible to find three potential dbd for the NFAT consensus site. A similar dbd

sequence for NFATc was observed in the promoter region of the murine version of NFAT5.

It was previously described that NFAT5 mutant mice display marked decrement in the expression of AQP-1 in the inner medulla. Therefore, NFAT5 is necessary for the regulation of AQP-1 expression in the inner medulla of the kidney under hypertonic conditions (24). We also observed the absence of AQP-1 expression in MEF-NFAT5$^{-/-}$ and MEF-NFAT5$^{\Delta/\Delta}$ cells. However, we did not see that transient transfection of NFAT5 in MEF-NFAT5$^{-/-}$ cells recovered the expression of AQP-1. Five isoforms of NFAT5 have been described (a-e), and we use the canonical isoform c in the recombinant NFAT5 protein for rescue experiments. We speculate that other isoforms of NFAT5 might be involved in the AQP-1 upregulation by hypoxia.

The UTA-1 expression in MEF-NFAT5$^{+/+}$ cells was upregulated at 16 h after hypoxia, probably because hypoxia also induces arginase-2, increasing the levels of ornithine and urea. Arginase-2 is induced in hypoxia and constitutes a protective mechanism for the deleterious effects of iNOS activity (20). Thus, we speculate that UTA-1 upregulation was a secondary response to urea overproduction by hypoxia (2). We observed that, in MEF-NFAT5$^{-/-}$ cells, UTA-1 is expressed in the basal form, but hypoxia-UTA-1 induction was lost, suggesting that NFAT5 participates in the induction of UTA-1 during hypoxia and in MEF cells.

In conclusion, our results support previous findings showing the NFAT5 activation by hypoxia. We found that NFAT5 was activated by hypoxia in MEF cells (induction of protein expression, nuclear localization, and transactivation activity). We also observed iNOS, AQP-1, and UTA-1 protein upregulation by hypoxia. The upregulation of iNOS and AQP-1 by hypoxia was blocked in MEF-NFAT5$^{-/-}$ and MEF-NFAT5$^{\Delta/\Delta}$ cells. Transient transfection of the recombinant version of NFAT5 in MEF-NFAT5$^{-/-}$ cells recovered the iNOS upregulation observed during hypoxia, but not for AQP-1. A dominant-negative version of NFAT5 blocked the iNOS upregulation rescue (MEF-NFAT5$^{\Delta/\Delta}$). These data provide novel information to understand the role of isoform c of NFAT5 during hypoxia activation of iNOS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.E.I. conceived and designed research; Y.S., R.A.F., B.C.K., and F.C. performed experiments; Y.S., R.A.F., and C.E.I. analyzed data; Y.S., R.A.F., and C.E.I. interpreted results of experiments; Y.S., R.A.F., J.R., and C.E.I. prepared figures; C.P., J.R., and C.E.I. drafted manuscript; C.P. and C.E.I. edited and revised manuscript; C.E.I. approved final version of manuscript.

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